Early environmental enrichment rescues memory impairments provoked by mild neonatal hypoxia-ischemia in adolescent mice.

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Abbreviations

BDNF, brain-derived neurotrophic factor; CEUA, ethics committee on animal use; CeMBE, center for experimental biological models; EE, environmental enrichment; HI, Hypoxia-ischemia; ICLAS, international council for laboratory animal science; NOR, novel object recognition; NR3C1, glucocorticoid receptor; PFA, paraformaldehyde; SC, standard cage.

Abstract

Hypoxia-ischemia (HI) is a consequence of a lack of oxygen and glucose support to the developing brain, which causes several neurodevelopmental impairments. Environmental enrichment (EE) is considered an option to recover the alterations observed in rodents exposed to HI. The aim of this study was to investigate the impact of early EE on memory, hippocampal volume and brain-derived neurotrophic factor (*Bbnf*) and glucocorticoid receptor (*Nr3c1*) gene expression of mice exposed to HI. At P10, pups underwent right carotid artery permanent occlusion followed by 35 minutes of 8% O_2 hypoxic environment. Starting at P11, animals were reared in EE or in standard cage (SC-HI or SC-SHAM) conditions until behavioral testing (P45). SHAM pups did not undergo carotid ligation and hypoxic exposure. Memory performance was assessed in the Y-maze, Novel object recognition, and Barnes maze. Animals were then sacrificed for analysis of hippocampal volume and *Bdnf* and *Nr3c1* gene expression. We observed that animals exposed to HI performed worse in all three tests compared to SHAM animals. Furthermore, HI animals exposed to EE did not differ from SHAM animals in all tasks. Moreover, HI decreased hippocampal volume, while animals reared in early EE were not different compared to SHAM animals. Animals exposed to HI also showed upregulated hippocampal *Bdnf* expression compared to SHAM animals. We conclude that early EE from P11 to P45 proved to be effective in recovering memory impairments and hippocampal volume loss elicited by HI. Nevertheless, *Bdnf* expression was not associated with the improvements in memory performance observed in animals exposed to EE after a hypoxic-ischemic event.

Keywords: Hypoxia-Ischemia; Environmental Enrichment; Memory; Hippocampus; BDNF.

1. Introduction

Hypoxia-Ischemia (HI) during early development is of the main causes for brain damage in newborns (Kurinczuk, White-Koning, & Badawi, 2010). Worldwide it is estimated that HI occurs in about 1.5 per 1000 live births, and it is the fifth leading cause of death in children under the age of five (Cerio, Lara-Celador, Alvarez, & Hilario, 2013; Gonzalez & Miller, 2006). This condition is a consequence of a lack of oxygen and glucose support to the brain, which leads to long-term neurodevelopmental disabilities, such as cerebral palsy, epilepsy and cognitive impairments (Durán-Carabali et al., 2018; Rojas et al., 2013). Learning and memory deficits are commonly observed, since the hippocampus is one of the most vulnerable regions affected by the limited supply of oxygen due to its high rate of activity (Almli et al., 2000; Macri et al., 2010). However, the damage extent is highly dependent on the severity of the insult, which tends to promote heterogeneity between patients (Pin, Eldridge, & Galea, 2009). Moreover, there is no cure for this condition, so understanding the effectiveness of possible therapeutic approaches is a key factor for possible behavioral and cognitive recovery.

To better comprehend the characteristics of a neonatal hypoxic-ischemic insult, Rice-Vanucci (1981) developed a model in rodents to replicate some of the characteristics observed in humans, such as white matter damage, neuronal loss and cognitive impairments (Martinez-Biarge et al., 2012; Rocha-Ferreira & Hristova, 2016). Mainly performed in rats, this protocol was later adapted to be used in mice (Ditelberg, Sheldon, Epstein, & Ferriero, 1996). It consists in a permanent unilateral ligation of the carotid artery combined with exposure to a hypoxic environment (8% oxygen) (Rice et al., 1981). Neonatal HI model has already been shown to induce long-term spatial memory (de Paula et al., 2009), working memory (Pereira et al., 2007) and inhibitory memory (Young, Kolonich, Woods, & Yagel, 1986) impairments at adulthood. Histological analysis from previous studies have shown that these behavioral impairments could be related to an atrophy observed in the hippocampal area and to white matter damage (Forbes et al., 2020; Kirino, Tamura, & Sano, 1985).

During early life development, brain is considered to be at its peak level of plasticity, for both positive and negative stimuli (Dawson, 2008; Kolb & Gibb, 2011). Studies have shown that neurogenesis, axonal and dendritic density and overall volumetric changes in the rodent brain mainly occur during the first weeks of development (Semple, Blomgren, Gimlin, Ferriero, & Noble-Haeusslein, 2013), so this period is considered one of the main therapeutic windows for intervention (Ismail, Fatemi, & Johnston, 2017). A wide range of drugs have been tested over the last years, but until now there is no consistent evidence of the efficacy of a specific drug in patients or rodents exposed to HI injury (Fan, Kavelaars,

Heijnen, Groenendaal, & van Bel, 2010; Perrone, Stazzoni, Tataranno, & Buonocore, 2012). For this reason, environmental modification and behavioral stimulation are being tested as possible strategies to reduce the long-term impairments provoked by neonatal HI.

As a potential non-pharmacological intervention, environmental enrichment (EE) has shown interesting long-term benefits to the adult brain (Griva et al., 2017; Toth, Kregel, Leon, & Musch, 2011). EE is a stimulation paradigm, which consists on increasing the amount of physical activity, social/object interaction, and cognitive stimulation, factors that rodents are not normally exposed in a regular cage environment (Bailoo et al., 2018). To generate these conditions, animals are kept in larger cages, which usually have running wheels, platforms, toys and tunnels (Hutchinson, Avery, & Vandewoude, 2005). Studies have shown that animals housed in EE conditions can show behavioral and cognitive alterations, such as learning and memory improvements (Birch, McGarry, & Kelly, 2013) and decreased anxiety-like behavior (Benaroya-Milshtein et al., 2004). Regarding benefits at cellular and molecular level, previous studies reported that EE exposure significantly increased synaptic density (Stuart et al., 2017), neurogenesis (Speisman et al., 2013), and dendritic branching (Bindu et al., 2007). It has been proposed that brain-derived neurotrophic factor (*Bdnf*) is associated with several benefits provoked by EE exposure due to its neuroprotective role in order to modulate neuronal survival rates (Chen, Xiong, Tong, & Mao, 2013; Dandi et al., 2018; Rossi et al., 2006). Furthermore, EE is believed to alter the expression of glucocorticoid receptor (*Nr3c1*), which is known to influence cognitive performance (Roozendaal, 2002; Zanca et al., 2015). Previous studies have discussed that a more severe HI damage may reach a "point of no return" for behavioral and structural recovery (Allen & Brandon, 2011; Ten & Starkov, 2012), but it is believed that this environmental intervention could improve the long-term deleterious effects of a mild to moderate hypoxic-ischemic events.

Even though some studies have reported the impact of EE on rodents exposed to HI, there is still limited evidences on the effects of EE on memory performance immediately after animals are exposed to a mild hypoxic-ischemic event. There are multiple studies that investigated the effects of HI exposure in rats (Durán-Carabali et al., 2018; Durán-Carabali et al., 2019) and C57BL/6 mice (Muntsant, Shrivastava, Recasens, & Giménez-Llort, 2019; Wolf et al., 2016). As a novelty factor, our study aims to investigate the impact of HI and EE on BALB/c mice, which is considered a vulnerable strain for stress related impairments, and also not commonly utilized for evaluating cognitive performance. Since early development stage is known to be a critical window for recovery, it is important to understand the long-term effects of early EE intervention on cognitive impairments provoked by HI and its relation with *Bdnf*

and *Nr3c1* expression. Furthermore, the vast majority of the literature focus on the impact of HI on spatial memory, but further understanding on how HI and EE can alter different memory subsets is also necessary in order to push the field forward. To do this, the study sought to investigate i) the impact of an early EE intervention in male BALB/c mice exposed to neonatal HI on working, short-term and spatial memory, ii) hippocampal volume, and iii) expression of *Bdnf* and Nr3c1 in the hippocampus.

2. Experimental Procedures

2.1 Animals

This study was performed with male BALB/c mice. Male and female animals used for breeding were obtained from the Center for Experimental Biological Models (CeMBE) at Pontifical Catholic University of Rio Grande do Sul (PUCRS, Brazil). Breeding procedures involved housing two females with one male per cage for 72 hours. After this period, the male was removed from the cage, and two weeks later the females were individually housed and inspected daily until the presence of pups was confirmed. The day of birth was designated as postnatal day 0 (P0). Litters were then culled to 5-8 pups per litter, and were randomly assigned to the following groups: hypoxia-ischemia standard cage (HI-SC), hypoxia-ischemia environmental enrichment (HI-EE), SHAM standard cage (SHAM-SC), SHAM environmental enrichment (SHAM-EE). Only two animals per litter were assigned to each experimental group to avoid potential litter effects (Lazic & Essioux, 2013). For this experiment, 7 litters for the HI-SC group, 6 litters for the HI-EE group, 7 litters for the SHAM-SC group and 6 litters for the SHAM-EE group were utilized. At P21, males were weaned, and group housed (3 animals per cage for SC and 6-7 for EE group). Mice were briefly weighed on P2, P21 and P45. Pre-weaning (P2 and P21), animals were weighed as a litter and the result was divided by the number of animals in the litter. Considering the circadian rhythm of rodents, all behavioral experiments were performed late afternoon. The experimental design and timeline are summarized in Figure 1 (Created with BioRender).

All animals were housed in standard Plexiglas mouse cages, under 12 h/12 h light–dark cycle (lights on at 7:00 a.m.) with mouse chow and water available *ad libitum.* The animal facility room had controlled air circulation (temperature 21 ± 1°C, humidity 55% ± 5%). All experiments were approved by the Ethics Committee on Animal Use (CEUA) of PUCRS and were conducted in accordance with the Guide for the Care and Use of Laboratory Animals and International Council for Laboratory Animal Science (ICLAS).

2.2 Hypoxia-Ischemia

The HI protocol was performed using an adapted version of the Rice-Vannucci model for mice (Kim et al., 2017). On P10, pups were anesthetized (isoflurane 4-5% for induction and 1.5% for maintenance), and through a ventral neck incision the right common carotid was identified, isolated and permanently occluded using a surgical silk thread. The carotid occlusion procedure took no more than five minutes per animal. 15 minutes after the surgery, the pups were returned to their dams for two hours. After the recovery, pups were allocated in a hypoxic chamber (8% oxygen and 92% nitrogen) for 35 minutes. A thermal pad (32 ± 3°C) was placed under the hypoxic chamber to control the body temperature of the pups to avoid any stress induced by hypothermia. Following the hypoxic environment exposure, pups were returned to their home cages. For the following 72h the staff at the vivarium checked the animals for any possible occurrences. Mortality rate for this procedure was less than 10%. SHAM animals were submitted to the same procedures and time away from the dam, but without the carotid occlusion and hypoxic atmosphere exposure.

2.3 Environmental Enrichment

Due to the higher degree of plasticity during early development, we opted to implement the EE protocol one day after the hypoxia-ischemia damage to optimize the window for intervention (Oberman & Pascual-Leone, 2013). At P11, pups from the EE groups (HI-EE and SHAM-EE) were allocated with their respective dams in a new cage. The enriched environment consisted of a rat Plexiglas cage with ramps, a running wheel and several toys with different shapes, textures and sizes. The rat cage used for EE had a floor area of 901 cm2 and overall dimension of 395 x 346 x 213 mm (W x D x H), while the mouse cage had a floor area of 501 cm2 and overall dimension of 391 x 199 x 160 mm. During weekly cage cleaning the objects in the cage were changed to maintain the novelty factor. Animals were weaned at P21 and remained in the enriched cages until the behavioral tests were performed (P45). Animals that were not exposed to EE remained in standard cages throughout the whole experiment.

2.4 Y-Maze

Working memory was evaluated at P45 in the Y-maze apparatus (Viola et al., 2019). The apparatus consisted of three identical arms (30 cm x 5 cm x 10 cm). The test is based on the natural tendency of rodents to explore novel environments compared to previously known areas. This task is performed in two phases, named sample and test phases. In the sample phase, animals were only allowed to explore two

arms of the apparatus, start and familiar arm. The third arm, referred as novel arm, was blocked with a removable wall. In the sample phase, animals were placed in the start arm and were allowed to explore both open arms (start and familiar) for 5 minutes. Animals were then removed from the Y-maze and had an interval of 1 minute until the test phase started. For the test phase, animals were again allocated in the start arm and could freely explore all three arms of the apparatus for 2 min. After the test, animals were removed from the apparatus and returned to the home-cage. Between each trial the apparatus was cleaned with 70% ethanol. A working memory index for the test phase was calculated using the following formula: [Time spent in novel arm / 120] x 100. The number of entries in the novel arm was also recorded. All analyses were performed using Any-Maze software version 4.9 (ANY-Maze, Inc., Greensburg, PA).

2.5 Novel Object Recognition

The Novel object recognition (NOR) task was performed in an open field arena (33 x 33 cm) to investigate short-term recognition memory (Antunes & Biala, 2012). One day before the task, animals were allowed to habituate to the apparatus for 10 min. During the habituation phase, the total distance travelled was accounted as a measure for locomotor activity. For the training phase, two identical objects were placed in opposite quadrants of the arena. Animals were allowed to explore the objects for 5 min. Testing phase started after 5 min of retention. In the testing phase, animals were reintroduced to the same arena, but one of the objects was replaced. The novel object was similar in size, but different in shape and texture. Animals were then allowed to explore both objects for another 5 min. Test was recorded for further analysis. Object exploration was defined by actively touching with the head, sniffing, or pawing the objects. If an animal climbed on top of the objects it was not considered exploratory behavior (Antunes & Biala, 2012). The apparatus and the objects were cleaned with 70% ethanol between each trial and animal to avoid any olfactory clue. A recognition index was used to evaluate the time exploring the new object in relation to the familiar object using the following formula: [Time exploring in novel object / (time exploring the old + time exploring the novel object)] x 100. All analyses were performed using Any-Maze software version 4.9 (ANY-Maze, Inc., Greensburg, PA).

2.6 Barnes Maze

Mice were trained in the Barnes maze to evaluate spatial learning and memory. The test is based on the assumption that when a rodent is placed in an aversive environment (open and brightly field), it can learn and memorize the location of a safe spot (escape box located below the platform). The advantage

of this less stressful protocol compared to other well-known spatial learning and memory tasks (Morris water maze or Radial arm maze) is that the animals are not exposed to water or need to be food or water restricted (Gawel, Gibula, Marszalek-Grabska, Filarowska, & Kotlinska, 2019). The apparatus consisted of a circular grey platform (92 cm in diameter) with 20 circular holes (5 cm in diameter) evenly spaced (7.5 cm between each holes) along the periphery of the platform. Furthermore, the apparatus was elevated 95 cm in relation to the floor. Extra-maze cues with different shapes (circles, triangles and squares) and colors (blue, green and black) were mounted in the walls around the maze. The aversive stimulus used was a bright light (400-lx) positioned directly above the maze to evenly illuminate the platform and motivate animals to search the escape box. A dark escape box was positioned below one of the holes and had a ramp to facilitate the entrance of the animals, which was an additional motivation since rodents tend to prefer dark environments.

Animals performed two days of habituation (10 min per day) to the apparatus. During the habituation phase the bright light above the maze was not switched on. On the second day of habituation the animals were guided to the escape hole after 8 min of exploration and had 2 min to familiarize with the escape box. During habituation, the escape box was placed in a different hole of the apparatus, to minimize possible influences in the training phase. After habituation, animals performed 4 days of training, which consisted of 2 trials per day with a max time of 3 min per trial. After locating and entering the escape box, the animal was allowed to remain inside the box for 60 seconds for each trial. If after 3 min the animal did not locate the escape box, it was gently guided to the box by the experimenter, and it was allowed to remain for 60 seconds inside the box. The inter-trial interval was 15 min for each animal. A single trial probe phase was performed 24h after the last training trial, in which the escape box was removed, and animals had 3 min to locate the hole. When animals located the hole, the probe trial was over. The latency to reach the escape hole, the number of errors (interaction with incorrect holes) before reaching the escape hole, and the search strategy were measured. Search strategy was classified in three different possibilities: random, serial and spatial. Random strategy was defined as crossing the center of the maze in a random searching behavior. Serial strategy was characterized as searching multiple holes in a sequence, and without crossing the center of the maze more than one time. Spatial strategy was defined as performing a direct movement towards the escape hole. Between each trial the apparatus was cleaned with 70% ethanol. A single experimenter blinded to the experimental groups remained inside the room to handle the animals and record the latency and number of errors.

2.7 Histological Analysis

Thirty minutes after behavioral testing, 5-6 animals from each group were randomly selected for histological analysis. Animals were anesthetized via i.p. using a cocktail of ketamine (100mg/kg) and xylazine (10mg/kg) and transcardially perfused with 0.9% saline solution followed by 4% paraformaldehyde (PFA). The brains were extracted from the skull, maintained in 4% PFA for 24 hours, and cryoprotected in sucrose (30%) for three days. Brains were then sectioned in a cryostat (Leica) in 40-µm thickness slices. Slices were allocated in gelatin-coated slides and then stained with hematoxylin and eosin. Images were captured using a digital camera coupled to a Nikon microscope. The hippocampus was identified using the mice brain atlas (Paxinos & Franklin, 2019), and delineated using NIH-ImageJ software. The hippocampal area of four slides was used to implement the Cavalieri method for volumetric measurements. The volume ratio was later calculated using the formula: ipsilateral volume (mm3) / contralateral volume (mm3).

2.8 Gene Expression Analysis

Animals were euthanized thirty minutes after behavioral testing for gene expression analysis. Whole brains were immediately removed, and the hippocampus was dissected by free-hand technique. Samples were then frozen on dry ice and stored at -80 °C until molecular analysis was performed. For all groups, gene expression analysis was performed exclusively in the hippocampus ipsilateral to the carotid occlusion. Total RNA was isolated from 6-9 randomly selected samples using QIAzol (Qiagen; Hilden, Germany) and reconstituted in 20µL of RNase-free water. NanoDrop spectrophotometer (Thermo Fisher; Waltham, USA) was used to measure RNA concentration. Reverse transcription was performed with High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems - Foster City, USA) using a total of 500 ng of RNA per sample. miScript SYBR Green PCR (Qiagen; Hilden, Germany) reactions were performed in duplicates for each sample using a Rotor Gene Real-Time PCR machine (Qiagen; Hilden, Germany). The following IDT primers were design and used in this study: *Bdnf* exon IX (Forward: GCAGCTGGAGTGGATCAGTAA Reverse: CATTCACGCTCTCCACAGTCC); *Nr3c1*: (Forward: GGACCACCTCCCAAACTCTG, Reverse: ATTGTGCTGTCCTTCCACTG); *Pgk* (Forward: TGCACGCTTCAAAAGCGCACG, Reverse: AAGTCCACCCTCATCACGACCC). ΔΔCt method was used to calculate the fold change relative expression, using SHAM-SC group as reference. *Pgk* Ct values were used as endogenous control for mRNA analysis. To verify primer specificities, melting curve analyses and agarose gels were performed.

2.9 Statistical Analysis

Statistical analyses were performed with SPSS software v.25.0 (SPSS, Chicago, IL, USA) and GraphPad Prism 8 (GraphPad Software Inc., La Jolla, CA, USA). Two-way ANOVAs were conducted to analyze group differences with lesion (HI x SHAM) and rearing (EE x SC) as factors (Y-maze, NOR and Barnes Maze probe trial). For the Barnes Maze training phase (Day 1-4), data was analyzed using repeated measures ANOVA. Tukey's post-hoc tests were conducted to identify the specific effects in pairwise comparison. Pearson's correlation was used to evaluate association between behavioral and hippocampal volume data. Normality of data distribution was analyzed for all variables, using the Shapiro-Wilk Test of Normality, and homogeneity of variances was tested via Levene's Test of Equality of Error Variances. All data are presented as group mean ± standard error of the mean (SEM) with individual subjects represented with dots, and statistical significance was considered as a p-value < 0.05.

3. Results

3.1 Body weight control

Animals were weighed at P2, with no statistically significant differences between groups (Fig. 2A). At P21 a lesion effect was observed, where animals that underwent HI showed a reduction in body weight independent of the rearing condition (EE or SC) [$F(1,24) = 10.22$, $p = 0.003$; Fig. 2B]. When animals reached adolescence (P45), this reduction was no longer statistically significant, and no differences among groups were observed (Fig. 2C).

3.2 Environmental Enrichment improves memory performance of animals exposed to hypoxia-ischemia in different memory paradigms

3.2.1 Y-maze task

Regarding the performance in the Y-maze task, we identified a lesion effect, in which animals exposed to HI showed a decrease in working memory index when compared to SHAM animals [$F(1,45) = 5.903$, $p = 0.019$; Fig. 3A]. Furthermore, a significant lesion x rearing interaction was observed [$F(1,45) = 6.675$, $p = 0.013$; Fig. 3A]. *Post-hoc* analysis indicated that HI-SC animals had a reduction in working memory index compared to SHAM-SC, SHAM-EE and HI-EE groups ($p = 0.003$; $p = 0.031$; $p = 0.028$, respectively).

In relation to the number of entries in the novel arm of the Y-maze, a lesion effect was also observed, in which HI animals entered fewer times in the novel arm compared to SHAM condition [F

(1,45) = 6.762, p = 0.012; Fig. 3B]. Also, a statistically significant lesion x rearing interaction was identified [F (1,45) = 8.489, p = 0.005; Fig. 3B]. *Post-hoc* analysis showed that HI-SC animals entered fewer times in the novel arm compared to SHAM-SC, SHAM-EE and HI-EE groups (p = 0.001; p = 0.039; p = 0.026, respectively).

3.2.2 Novel object recognition task

When assessing the recognition index for the NOR task, we observed that animals exposed to HI performed significantly worse compared to SHAM animals [lesion effect: F (1,45) = 6.980, p = 0.011; Fig. 3C]. Animals reared in EE condition performed better when compared to animals reared in SC condition independent of being exposed to HI or SHAM surgery [rearing effect: F (1,45) = 7.195, p = 0.01; Fig. 3C]. Furthermore, we found a significant lesion x rearing interaction effect [F (1,45) = 4.553, p = 0.038; Fig. 3C]. *Post-hoc* analysis revealed that HI-SC group had a significant reduction in the recognition index compared to SHAM-SC, SHAM-EE and HI-EE groups (p = 0.005; p = 0.002; p = 0.008, respectively).

Locomotor activity was analyzed based on the distance covered in the habituation phase of the NOR task, and no statistically significant differences was observed among groups (Fig. 3D).

3.2.3 Barnes Maze

Training Phase (Days 1 to 4)

Regarding the latency to find the escape hole, a repeated measures ANOVA indicated a significant lesion x rearing interaction [F (1,45) = 4.415, p = 0.041; Fig. 4A]. *Post-hoc* analysis showed that on the first and second day of training there were no statistically significant differences between groups. On the third day, HI-SC animals presented increased latency to find the escape hole when compared to SHAM-SC and SHAM-EE (p = 0.007; p = 0.009, respectively); HI-EE animals did not differ from SHAM groups. On fourth day, the same pattern was observed, in which HI-SC animals took more time to find the escape hole when compared to SHAM-SC and SHAM-EE (p = 0.027; p = 0.045, respectively). HI-EE group was not different from SHAM animals.

In relation to the number of errors prior to locating the escape hole, a repeated measures ANOVA showed a significant lesion effect, in which HI animals committed more errors compared to SHAM animals independent of the rearing condition [F (1,45) = 6.362, p = 0.015; Fig. 4B]. Moreover, a significant lesion x rearing interaction was detected [F (1,45) = 5.955, p = 0.019; Fig. 4B]. *Post-hoc* analysis revealed that on the first and second days, HI-SC animals had more errors compared to SHAM-SC group (p = 0.003; 0.024, respectively). On the third day, HI-SC animals committed more errors before

locating the escape hole compared to SHAM-SC, SHAM-EE and HI-EE groups ($p = 0.009$; 0.023; 0.037, respectively). On the last day of training, HI-SC animals also had more errors compared to SHAM-SC, SHAM-EE and HI-EE groups ($p = 0.008$; 0.045; 0.009, respectively).

Probe Trial (Day 5)

When analyzing the latency to locate the escape hole during the probe trial, a two-way ANOVA showed a significant rearing effect, in which animals that underwent EE, reached the hole significantly faster when compared to SC groups independent of being exposed to HI or SHAM surgery [$F(1,45) = 6.199$, $p = 0.016$; Fig. 4C]. Furthermore, we observed a significant lesion x rearing interaction [$F(1,45) = 5.994$, $p = 0.018$; Fig. 4C]. *Post-hoc* analysis indicated that HI-SC animals took significantly more time to locate the escape hole when compared to SHAM-SC, SHAM-EE and HI-EE groups ($p = 0.012$; $p = 0.013$; $p = 0.006$, respectively). In relation to the number of errors during the probe trial, we observed that animals exposed to HI surgery had significantly more errors when compared to SHAM animals [$F(1,45) = 9.434$, $p = 0.003$; Fig. 4D]. Moreover, animals that underwent EE committed fewer errors in the probe trial when compared to SC animals independent of HI or SHAM surgery [$F(1,45) = 7.865$, $p = 0.007$; Fig. 4D]. We also observed a significant lesion x rearing interaction [$F(1,45) = 5.478$, $p = 0.023$; Fig. 4D]. *Post-hoc* analysis revealed that animals from the HI-SC group presented more errors until finding the escape hole when compared to SHAM-SC, SHAM-EE and HI-EE groups ($p = 0.001$; $p < 0.001$; $p = 0.004$, respectively). Regarding search strategy utilized to locate the escape hole in the probe trial, we observed that random strategy was used 15% of the time for SHAM-SC group, 8% for SHAM-EE and 9% for HI-EE animals, while animals exposed to HI without EE utilized a random strategy 46% of the time. Serial strategy was used similarly between all groups (SHAM-SC: 54%, HI-SC: 46%, SHAM-EE: 58% and HI-EE: 55%). The spatial strategy was used 31% of the time for the SHAM-SC animals, 34% for SHAM-EE and 36% for HI-EE, but HI-SC group utilized this direct strategy only 8% of the time (Fig. 4F).

3.3 Hippocampal volume loss due to hypoxia-ischemia injury is altered by environmental enrichment

Regarding structural hippocampal alterations, we observed that animals exposed to HI had a significant hippocampal volume reduction when compared to SHAM animals independent of the rearing condition [lesion effect: $F(1,18) = 48.31$, $p < 0.001$; Fig. 5B]. Animals exposed to EE showed a significant increase in hippocampal volume when compared to animals reared in SC condition [rearing effect: $F(1,18) = 10.42$, $p = 0.004$; Fig. 5B]. Furthermore, a significant lesion x rearing interaction effect was observed [$F(1,18) = 11.81$, $p = 0.002$; Fig. 5B]. *Post-hoc* analysis indicated that HI-SC animals had a

reduction hippocampal volume when compared to SHAM-SC, SHAM-EE and HI-EE groups ($p < 0.001$; $p < 0.001$; $p < 0.001$, respectively). Finally, Pearson's correlation analysis revealed a positive association between hippocampal volume and performance in the Y-Maze ($r = 0.64$, $p = 0.001$; Fig 5C) and NOR ($r = 0.47$, $p = 0.025$; Fig 5D) tasks. A negative correlation was observed regarding hippocampal volume and latency to locate the scape hole in the Barnes maze probe trial ($r = - 0.58$, $p = 0.004$; Fig 5E).

3.4 Bdnf and Nr3c1 gene expression are not related with the positive effects of environmental enrichment on memory performance

When analyzing *Bdnf* mRNA levels, we observed that animals exposed to HI had increased expression compared to SHAM animals independent of being reared in SC or EE [lesion effect: $F(1,26) = 21.18$, $p < 0.001$; Fig. 6A]. Overall, there was no effect of environmental enrichment exposure on the expression of BDNF. Regarding *Nr3c1* mRNA levels, no statistically significant differences among groups was observed (Fig. 6B).

4. Discussion

In this study, animals were reared in an early EE condition as a possible treatment for recovering the memory impairments and hippocampal volume loss provoked by neonatal HI. The main findings from the present study are: (1) Animals that underwent HI under standard conditions (HI-SC) performed significantly worse in the Y-maze and NOR, compared to all other groups, suggesting that EE recovered the memory impairments provoked by HI. (2) HI-SC animals showed increased latency and had more errors in the Barnes maze, while animals reared in HI-EE condition did not differ from SHAM animals. Moreover, HI-SC animals were the only group to mostly use a random search strategy to locate the escape hole. (3) Hippocampal volume was reduced in animals exposed to HI, however, HI animals exposed to EE showed preserved hippocampal volume. Also, hippocampal volume was correlated with the performance in all memory tasks. (4) *Bdnf* mRNA levels were increased in animals exposed to HI, but both *Bdnf* and *Nr3c1* gene expression were not related with memory benefits observed after early EE intervention.

Consistent with previous literature showing that neonatal HI can lead to long-term working memory (Arteni, Salgueiro, Torres, Achaval, & Netto, 2003), recognition memory (Domnick et al., 2015), and spatial memory deficits (Balduini, De Angelis, Mazzoni, & Cimino, 2000), we showed that after ischemia followed by 35 minutes of hypoxia, animals have long-term memory deficits. It is well known

that the duration of the hypoxic event can directly impact the long-term histological damage and behavioral impairment observed in the animals (McAuliffe, Miles, & Vorhees, 2006). Here we add new data showing that a short hypoxic period (35 min) could indeed lead to significant impairments on three different subsets of memory. van der Kooij et al. (2010) has shown that mice exposed to 45 minutes of hypoxia performed worse in a motor task, and showed impairments in a hippocampal-dependent memory task when compared to SHAM animals. Furthermore, we observed a decrease in hippocampal volume ipsilateral to the injury in HI animals. Previous data has demonstrated that a short hypoxic period was sufficient to promote alterations in hippocampal and thalamus volume (Ten et al., 2004), which corroborates with our findings. van der Kooij et al. (2010) showed that 75 minutes of hypoxia decreased the hippocampal volume of mice when compared to animals that underwent 45 min of hypoxic environment. On the other hand, Pereira et al. (2008) reported that rats exposed to 90 minutes of hypoxia continued to present hippocampal volume alterations even after being reared in enriched environment conditions. These studies support the hypothesis that the duration of exposure to the hypoxic environment is highly correlated with the long-term damages and the possibility for recovery. A plausible hypothesis is that when the excitotoxicity damage provoked by the hypoxic ischemic insult exceeds its recoverable limits, a "point of no return" is reached, and certain treatments may not be effective (Allen & Brandon, 2011; Ten & Starkov, 2012).

This assumption can be understood in light of multiple compensatory mechanisms involved, with resilience demonstrated in small/shorter-lasting hypoxic events. Indeed, the processes in HI is a continuum depending on size and severity, initially with energy metabolites depleted, anoxic depolarization of cells, cytotoxic edema (Gunn & Bennet, 2009), and extracellular accumulation of excitatory amino acids with subsequent excessive depolarization, synthesis of Nitric Oxide and Reactive Oxygen formation (Bolaños & Almeida, 1999; Garry, Ezra, Rowland, Westbrook, & Pattinson, 2015; Tan et al., 1996). Interestingly, many neurons may initially partially recover with transient restoration of mitochondrial function and cerebral oxidative metabolism after renewed oxygenation (Azzopardi et al., 1989; Bennet, Roelfsema, Pathipati, Quaedackers, & Gunn, 2006). Mitochondria appears to play a central role in determining the fate of cells subjected to hypoxia-ischemia (Gilland, Puka-Sundvall, Hillered, & Hagberg, 1998; Ten & Starkov, 2012). Mitochondria are major buffers of intracellular calcium ions and can become overloaded by cytoplasmic calcium increase secondary to opening of N-methyl-D-aspartate and voltage-dependent calcium channels. Interestingly, arachidonic acid, metabolized to eicosanoids can potently induce vasoconstriction, brain edema formation, and blood cell aggregation (Bazan, 1989; Hsu et al., 1989).

Arachidonic acid metabolism may therefore elicit a delayed period of tissue hypoperfusion worsening the original ischemic insult. Depending on the buffer capacity of the tissue, a progressive failure of oxidative metabolism, seizures, secondary cytotoxic edema and ultimately cell death may occur.

Early postnatal period is considered to be the first intervention window for several neuropathological conditions since brain is at its peak level of plasticity (Andersen, 2003). Due to the fact that EE has been shown to increase neurogenesis (Speisman et al., 2013), dendritic spine density (Rojas et al., 2013) and synaptogenesis (Birch et al., 2013), some studies report that neurological interventions should be implemented during early development in order to fully take advantage from this opportunity (Cioni, Inguaggiato, & Sgandurra, 2016; Herskind, Greisen, & Nielsen, 2015). Here we showed that early EE intervention rescued working and short-term memory impairments provoked by a mild HI event. These structural and functional alterations provoked by an early stimulus can be associated with the concept of brain reserve, which explains that an increased number of neurons and synapses may allow the brain to absorb a higher degree of injury before cognitive function is affected (Stern, Barnes, Grady, Jones, & Raz, 2019). Furthermore, EE may also influence the model of cognitive reserve, which indicates that an improved cognitive function is essential for an individual to actively cope with a disease or brain damage (Stern et al., 2019). Pereira et al. (2007) has shown that EE exposure after weaning has recovered working memory deficits observed in rats exposed to HI. Nevertheless, in the same study animals exposed to HI continue to show decreased hippocampal volume after EE intervention. On the other hand, we showed that HI animals that underwent early EE did not present alterations in hippocampal volume compared to SHAM animals. Moreover, we observed that hippocampal volume was positively correlated with the performance in Y-Maze and NOR tasks, which indicates that a decrease in hippocampal volume is indeed involved in the performance of those tasks. Recently, Durán-Carabali et al. (2018) indicated that rats reared in early EE after HI did not show hippocampal volume alterations when compared to SHAM animals, which corroborates with the findings reported in our study. Furthermore, Schuch et al. (2016) showed that early EE positively affects neurobehavioral milestones during development, such as, eye opening, eyelid reflex, ear unfolding and ear twitch reflex in animals exposed to HI. Conversely, in a previous study exposing males and females to early EE, only female HI rats showed improvements on working and reference memory performance after EE stimulation (Pereira et al., 2008).

Spatial memory testing in the Barnes maze revealed significant impairments in animals exposed to HI protocol; such deficits were fully restored after early EE intervention. HI animals reared in early EE condition showed no statistically significant alteration in latency and number of errors when compared to

SHAM animals. It is important to consider that the locomotor activity among all groups was not different, which supports the hypothesis that indeed those differences were related to learning and memory processes. Similar results regarding the impact of EE on spatial memory performance tested in the Morris water maze have been shown in the past years. Griva et al. (2017) reported that animals exposed to post-weaning EE recovered from the spatial memory deficits provoked by neonatal HI. In addition, other studies also found similar results, reporting the positive effects of EE on spatial memory performance of animals that underwent a hypoxic-ischemic event (Arteni et al., 2010; Durán-Carabali et al., 2017; Pereira et al., 2007). It appears that the improvements on spatial memory following EE exposure tested in the water maze have been well documented, but as far as we know this is the first study that investigated the spatial memory performance of HI animals exposed to an early EE protocol in the Barnes maze test. Barnes maze is considered to be a less stressful version of the Morris water maze since it does not involve placing the animals in the water, which could be an additional stress factor, and interfere with how the animals explore the apparatus (Gawel et al., 2019; Harrison, Hosseini, & McDonald, 2009). Moreover, we showed that animals exposed to HI and reared under standard cage conditions used the random strategy 46% of the time in the probe trial of Barnes maze, while no other group used this strategy more than 15%. This data indicates that not only these animals took longer and made more mistakes in order to complete the task, but also had difficulties in learning a more efficient strategy, such as, using serial or spatial ones to locate the target hole. This finding functionally supports the morphological findings also reported here, as only animals with a normal hippocampal volume could perform the task. We showed that the latency to locate the scape hole in the probe trial was negatively correlated with hippocampal volume, so animals with decrease volume took longer to finish the task. Although it has not been previously reported in our model, the results are in accordance with findings from other paradigms modulating hippocampal volume and function. For example, several studies show that chronic immobilization stress or chronic corticosterone negatively influence the hippocampal CA1 and CA3 morphology, and also impairs memory performance in the Barnes maze or Morris Water Maze (Bodnoff et al., 1995; Kleen, Sitomer, Killeen, & Conrad, 2006; Luine, Martinez, Villegas, Magariños, & McEwen, 1996; McLaughlin, Gomez, Baran, & Conrad, 2007; Sandi et al., 2003).

In this study, we observed that *Bdnf* mRNA levels were upregulated in animals exposed to HI. This result could be explained by the neuroprotective function associated with BDNF expression in the brain (Chen et al., 2013). It has been shown that BDNF is critical for neuronal survival trough an anti-apoptotic effect (Liu, Ma, Feng, Ma, & Hu, 2007), so it is possible to hypothesize that a spike in *Bdnf*

levels occurred after HI as an attempt to reduce brain damages. Past evidence has shown that intracerebroventricular BDNF infusion prior to hypoxia-ischemia blocked the expression of caspase-3 in the hippocampus of rats (Han et al., 2000). It is important to accentuate that caspase-3 is a crucial mediator of programmed cell death. Moreover, an increase in glutamate concentration following a depletion in glucose and oxygen supply is tightly linked with the long-term brain damages provoked by a hypoxic-ischemic event (Nakajima & Kohsaka, 2004; Rumajogee, Bregman, Miller, Yager, & Fehlings, 2016). BDNF secretion has been shown to inhibit the neurotoxic effects provoked by elevated glutamate levels, which may also indicate a tentative to reduce the long-term impairments observed in our study (Akaike, Katsuki, Kume, & Maeda, 1999). Even though it has been shown in the literature that BDNF levels are altered after EE exposure (Gobbo & O'Mara, 2004; Sun et al., 2010), we did not find this result. Bdnf and Nr3c1 levels were not related with memory improvements observed after EE intervention. Similar to our results, Pereira et al. (2009) has shown that rats exposed to HI and reared in EE showed no alteration in hippocampus BDNF protein levels when compared to SHAM animals.

Certain limitations should be considered when interpreting the current study. This study was only performed in males. We acknowledge the importance to investigate sex-differences since it has been shown that there are sex-related differences in learning and memory processes in healthy and injured animals (Jonasson, 2005; Pereira et al., 2008). Once the mechanisms related to EE intervention are better understood, future studies should seek to understand physiopathological sex-differences of the HI model. Moreover, sample size was not calculated prior to the study, and the same cohort of animals was used for the three behavioral tests. We opted to use the same animals in order to reduce the number of animals utilized (Jonasson, 2005), as recommended by the 3Rs (Replacement, Reduction and Refinement) principle (Flecknell, 2002). We also performed the tests from less to most stressful, so they have minimum impact on the animals.

In conclusion, EE stimulation during early development proved to be an effective strategy to recover working, short-term and spatial memory impairments provoked by neonatal HI. Furthermore, early intervention may be important in order to prevent the hippocampal volume loss following a hypoxic-ischemic event. We believe that the present study adds to the current HI literature, and reinforces the importance of an early intervention for the recovery of brain injuries. Moreover, it is possible that BDNF exerted an attempt to reduce the brain damage following the hypoxic ischemic event, but the expression of this neurotrophin was not related with memory improvements observed following early EE intervention. Nevertheless, we reinforce that future studies should be performed in order to fully

comprehend the mechanisms behind how early EE can improve cognitive performance of animals exposed to neonatal HI.

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Conflicts of Interest

Dr. Wegener report having received lecture/consultancy fees from H. Lundbeck A/S, Servier SA, Astra Zeneca AB, Eli Lilly A/S, Sun Pharma Pty Ltd, Pfizer Inc, Shire A/S, HB Pharma A/S, Arla Foods A.m.b.A., Alkermes Inc, and Mundipharma International Ltd. All other authors declare no conflict of interest.

Figure Captions

Figure 1

Experimental design and study outcomes.

Figure 2

Body weight analysis of SHAM and HI animals reared in EE or SC conditions; A) postnatal day 2; B) postnatal day 21; C) postnatal day 45. Results are expressed as mean ± SEM; n = 7 litters for each group on P2 and P21 and 11-13 animals on P45. * $p < 0.05$ (two-way ANOVA). & represents interaction effect.

Figure 3

Y-maze and Novel object recognition performance of SHAM and HI animals reared in EE or SC conditions. A) working memory index based on the time spent in the novel arm of the apparatus; B) number of entries in the novel arm during the Y-maze test; C) recognition index based on the time spent exploring the novel object; D) locomotor activity in the open field arena. Results are expressed as mean ± SEM; n = 11-13 per group. * $p < 0.05$ (two-way ANOVA); & represents interaction effect.

Figure 4

Performance in the Barnes maze test; A) latency in each day of the training phase to locate the scape box; B) number of errors in each day of the training phase prior to locating the scape box; C) latency to locate the hole where the scape box was in the probe trial; D) number of errors prior locating the hole where the scape box was in the probe trial; E) schematic draft of the possible search strategies utilized; F) percentage of search strategy performed in the probe trial for each group. Data are expressed as mean ± SEM; n = 11-13 per group. Repeated measures ANOVA was utilized for the training phase and two-way ANOVA was used for the probe trial. * $p < 0.05$; & represents interaction effect.

Figure 5

Hippocampal volume analysis of SHAM and HI animals reared in EE and SC conditions and correlational analysis. (A) Representative images of coronal brain sections; (B) Ipsilateral / contralateral ratio of the hippocampal volume; (C) positive correlation between Y-Maze working memory index and hippocampal volume; (D) positive correlation between NOR recognition memory index and hippocampal volume; (E)

negative correlation between Barnes maze latency in the probe trial and hippocampal volume. Results are expressed as mean ± SEM; n 5-6 per group. * $p < 0.05$ (two-way ANOVA); & represents interaction effect.

Figure 6

Hippocampal gene expression analysis of SHAM and HI animals reared in EE and SC conditions. (A) *Bdnf* gene expression; (B) *Nr3c1* gene expression. Results are expressed as mean ± SEM; n 6-9 per group. * $p < 0.05$ (two-way ANOVA); & represents interaction effect.

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