Pregnancy swimming causes short- and long-term neuroprotection against hypoxia–ischemia in very immature rats

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BACKGROUND: Hypoxia–ischemia (HI) is a major cause of neurological damage in preterm newborn. Swimming during pregnancy alters the offspring's brain development. We tested the effects of swimming during pregnancy in the very immature rat brain.

METHODS: Female Wistar rats ($n = 12$) were assigned to the sedentary (SE, $n = 6$) or the swimming (SW, $n = 6$) group. From gestational day 0 (GD0) to GD21 the rats in the SW group were made to swim for 20 min/day. HI on postnatal day (PND) 3 rats caused sensorimotor and cognitive impairments. Animals were distributed into SE sham (SESH), sedentary HIP3 (SEHI), swimming sham (SWSH), and swimming HIP3 (SWHI) groups. At PND4 and PND5, Na^+/K^+-ATPase activity and brain-derived neurotrophic factor (BDNF) levels were assessed. During lactation and adulthood, neurological reflexes, sensorimotor, anxiety-related, and cognitive evaluations were made, followed by histological assessment at PND60.

RESULTS: At early stages, swimming caused an increase in hippocampal BDNF levels and in the maintenance of Na^+/K^+-ATPase function in the SWHI group. The SWHI group showed smaller lesions and the preservation of white matter tracts. SEHI animals showed a delay in reflex maturation, which was reverted in the SWHI group. HIP3 induced spatial memory deficits and hypomyelination in SEHI rats, which was reverted in the SWHI group.

CONCLUSION: Swimming during pregnancy neuroprotected the brains against HI in very immature neonatal rats.

Neonatal hypoxia–ischemia (HI) is a common cause of neonatal morbidity and mortality, leading to permanent brain damage and chronic disabilities in 50% of the survivors born prematurely (1), and the behavioral and memory deficits later in life are a significant health problem (2). In this population, the cerebral white matter is preferentially damaged (3), causing hypomyelination (1). In addition, there is a decrease in the volume of cerebral gray matter, without cortical or striatal neuronal loss (4). Treatment options are urgently required to prevent the brain damage and associated memory deficits observed.

Hypoxic–ischemic brain injury evolves over time, and different mechanisms operate during different phases of injury, including ionic-pump failure, excitatory toxicity, and inflammation, which lead to neuronal and non-neuronal cell death within the first 12–36 h after the initial insult, and persist (5). Na^+/K^+-ATPase is an enzyme responsible for the establishment of cell membrane electrochemical gradients and the maintenance of resting membrane potential in neurons. Na^+/K^+-ATPase activity is inhibited in various neuropathological conditions, including epilepsy (6), neurodegeneration (7), cerebral ischemia (8), and HI (9). Performed at postnatal day 3 (HIP3), the Levine-Rice HI model was adapted to access the impairments observed in animals with central nervous system development similar to that seen in extremely premature human babies (24–28 weeks) (10). HIP3 induces cell loss in white matter areas (such as the corpus callosum), in the parietal cortex (11), and can lead to ventriculomegaly and hypomyelination (12). It has been shown that HIP3 causes feeding-behavior disorders and growing impairments (13), spatial and aversive memory impairments (9,12), and sensorimotor deficits (11).

Physical exercise is a non-pharmacological intervention that can be used to counteract risk factors for prematurity, such as obesity, hypertension, type II diabetes, and increases in cholesterol and triglyceride levels. Physical activity during pregnancy can reduce complications to both the mother and the fetus through the improvement of the intrauterine environment, which may have long-lasting effects on the future health of the offspring (14). Because of its benefits on the mother–fetus environment, swimming is a widely recommended exercise during pregnancy (15). Experimental studies have shown that maternal exercise before and/or
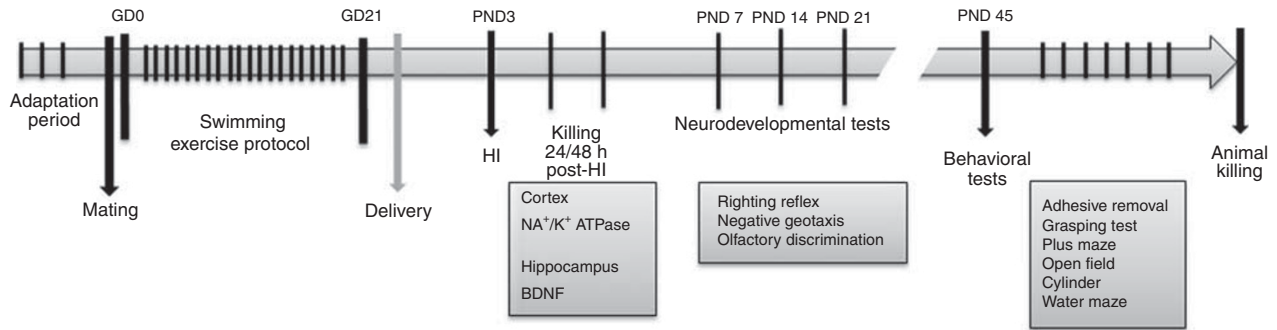
Figure 1. Timeline of experiments. Schematic overview of the experimental design.

during gestation has effects on the offsprings' central nervous system, leading to an increase in cell proliferation and hippocampal neurogenesis (16,17), in the activity of the offsprings' antioxidant system (18), and in the availability of growth factors such as BDNF (19). BDNF is considered the main cognitive enhancer released after maternal swimming (16,20) and can directly protect injured neurons from apoptosis by inhibiting the activation of caspases, an important mechanism of cell death (21) inducing neuroprotection after HI (22).

Considering the impact of physical exercise on the development of the offsprings' central nervous system and the possible modulation in the brain response following a perinatal injury, the present study aimed to investigate the effects of swimming during pregnancy on the damage caused by HIP3 during lactation and adult periods through evaluation of the development of the neurological reflex and sensorimotor and cognitive testing. In order to determine the possible mechanisms responsible for the protective effects of swimming, cortical Na^+/K^+-ATPase activity and hippocampal BDNF levels were assessed 24 h after injury.

METHODS

This study was conducted in accordance with the guidelines laid down by the National Institute of Health (Guide for the Care and Use of Laboratory Animals and the US Public Health Service Policy on Human Care and Use of Laboratory Animals; NIH publication No. 80-23, revised 1996), the Directive 2010/63/EU, and also directives of CONCEA—Brazilian law no. 11.794. The procedures were approved by the Institutional Ethical Committee on Animal Use—Protocol number 27842. All efforts were made to minimize animal suffering as well as reduce the number of animals used.

Animals

Wistar rats were received from the colony of our Department and housed under standard conditions (22 ± 2 °C, 12 h light/dark cycle). Gestational day (GD0) was considered through the observation of spermatozoids in the vaginal smear. Pregnant rats were divided into SE and SW groups. The experimental schedule is presented in **Figure 1**.

Swimming Exercise Protocol

The swimming apparatus consisted of a circular acrylic tank (200 cm × 50 cm) filled with water at 32 ± 1 °C. The water temperature was the same as that used in previous reports, which had shown no effect on corticosteroid levels after the training sessions (20). One week prior to mating, the rats were trained in the tank to avoid the

stress due to novelty. The swimming training protocol consisted of 20-min daily sessions of free swimming from the first to the twenty-first day of pregnancy. SE pregnant females were exposed to the same apparatus (tank filled with 1-cm-high water) and allowed to walk freely for 20 min. After the last training session, each pregnant rat was housed in a clean standard cage until delivery.

Cerebral HI (HIP3)

The HIP3 protocol used in this study was previously described (23). Three-day-old rat pups of both sexes (~50% of each sex) were chosen from the litters, anesthetized with halothane, and subjected to a midline longitudinal neck incision. The right common carotid artery was identified, isolated, and permanently occluded with a 4.0 surgical silk thread in the HI animals. After a 15-min recovery period, the animals were returned to their mothers for another 2 h. Hypoxia was induced, exposing pups to 8% of O_2 balanced in 92% of N_2 at 37 °C for 120 min. Sham-operated rats were submitted to anesthesia and neck incision; however, they had no carotid ligature or hypoxia. After the surgical procedure, the rats were assigned to the following four experimental groups: SESH, SEHI, SWSH, and SWHI. Male and female pups from different litters were used for biochemical analysis ($n = 6$–8 animals per group, killed 24 or 48 h after HI). Behavioral evaluation during lactation ($n = 11$–20 per group) and adulthood ($n = 7$–11 per group) and samples from the animals used for the adult tests were processed for histology.

Biochemical Analysis

In order to test possible molecular mechanisms involved in the neuroprotection caused by maternal swimming, we evaluated the activities of Na^+/K^+-ATPase (as a membrane damage and neuronal excitability marker) and BDNF levels (as increased levels of this neurotrophin is one of the most cited mechanisms attributed to the benefits of physical exercise). We assessed these markers at 24 and 48 h after HIP3, as the literature shows that the events occurred because of primary and secondary injuries that decreased after 72 h (ref. 5).

Tissue Preparation

The animals were killed by decapitation 24 and 48 h after HIP3. The right and left parietal cortices and hippocampi were quickly dissected out, placed in liquid nitrogen, and stored at − 80 °C until biochemical assays were run.

Na^+/K^+-ATPase Activity Assay

Briefly, parietal cortices were homogenized in 10 volumes (1:10, w/v) of 0.32 mM sucrose solution containing 5.0 mM HEPES and 1.0 mM EDTA, pH 7.5. Homogenates were centrifuged at 1,000g for 10 min, and supernatants were removed for the assay. Sample protein amounts were normalized to 1 mg/ml by Bradford method before the enzymatic assay. Na^+/K^+-ATPase activity was determined in 40 mM Tris-HCl, pH 7.4, containing 5 mM $MgCl_2$, 80 mM NaCl, and 20 mM KCl (in a final volume of 200 µl). After 10 min of pre-

incubation at 37 °C, ATPase reactions were started by the addition of ATP for a final concentration of 3 mM, and the samples were incubated for 20 min (at 37 °C). All samples were assayed twice under the same conditions; however, one sample was incubated with 1 mM ouabain (Na^+/K^+-ATPase inhibitor) and the other with water. The reaction was stopped by adding 20% trichloroacetic acid to the reaction. Na^+/K^+-ATPase activity was calculated as the difference between the two assays as previously described (8). A curve with purified Pi was performed in order to determine enzyme-specific activity, which was expressed as nmol Pi released per min/mg of protein. All assays were performed in 96-well plates and the wavelength used to determine Pi concentration was 630 nm.

Analysis of BDNF Immunocontent
The mature BDNF protein was assessed in the hippocampus using the E-Max ELISA kit (Promega, Madison, WI, USA) according to the manufacturer's recommendations. Briefly, hippocampi were individually homogenized (1:10 w/v) in lysis buffer containing 137 mM NaCl, 20 mM Tris-HCl (pH 8.0), Igepal (1%), glycerol (10%), 1 mM phenylmethanesulfonyl fluoride, 0.5 mM sodium vanadate, 0.1 mM EDTA, and 0.1 mM EGTA and centrifuged for 3 min at 1,4000 r.p.m. at 4 °C. The supernatant was diluted (1:5 v/v) in sample buffer and incubated in 96-well flat-bottomed plates previously coated with anti-BDNF monoclonal antibody and blocked with block and sample buffer. After sample incubation, the plates were incubated with polyclonal anti-human antibody for 2 h and with horseradish peroxidase for 1 h. The color reaction with tetramethylbenzidine was quantified in a plate reader at 450 nm. The standard BDNF curve, ranging from 0 to 500 pg/ml, was included in each plate.

Protein Quantification
As previously described, protein was measured by Lowry's method, modified by Peterson, using bovine serum albumin as standard.

Behavioral Assessment
Behavioral testing was performed during different stages of rat development: during lactation the neurological reflexes (righting, negative geotaxis, and olfactory discrimination) were assessed and from PND45 (at an early adult age) the sensorimotor and cognitive tests were conducted.

Neurological Reflex Testing
Righting reflex.   Pups were placed in the supine position and the time needed, in seconds, to turn over to the prone position and place all four paws on the surface was recorded, to a maximum of 15 s.

Negative geotaxis.   This task tests an automatic and stimulus-bound orientation movement. Animals were placed head down in the middle of an inclined 30-cm board (angle of 30°) and latency to make a 180° turn was recorded, with a ceiling of 60 s.

Olfactory discrimination.   This task was performed in an acrylic box ($20 \times 40 \times 20$ cm). Soiled bedding from the home cage was placed at one side and the same quantity of clean bedding was put on the contralateral side of the box. Each animal was gently placed in the middle of the cage. Rats with intact olfactory discrimination ability show a preference for the familiar bedding. The latency to reach the home bedding was recorded in a maximal time of 180 s.

Adult Behavioral Testing
Starting at 45 days of age, the animals were subjected to the plus maze, open field, adhesive removal, grasping strength, and cylinder tests. All animals performed the tasks in the above cited order; the apparatuses were cleaned with 70% ethanol solution after each animal testing. Spatial memory was assessed in the Morris water maze test.

Plus Maze
The elevated plus maze is an apparatus with two open arms (50×10 cm), surrounded by an edge of 0.5 cm, and two closed arms ($50 \times 10 \times 15$ cm); the central area measures 10 cm^2. The maze was elevated to a height of 70 cm. Each rat was placed at the center of the apparatus facing one enclosed arm. The test was video-recorded during 5 min and analyzed using ANY-Maze software (Wood Dale, IL, USA). The total time spent on each arm was recorded. An entry was defined as the placement of four paws on an arm.

Open Field
This apparatus consisted of a circular wooden chamber (100 cm diameter × 30 cm high wall) with a floor divided into 21 fields. Using ANY-Maze software, the open field exploration of each rat was video-recorded for 5 min; the number of crossings was considered indicative of spontaneous motor activity.

Adhesive Removal
Gently, an adhesive tape (1 cm × 1 cm) was attached to the left or the right forepaw's palm. The animal performed the task in a clean transparent box (20 cm × 40 cm × 20 cm) until the adhesive tape was completely removed from the forepaw. Randomly, half of the animals performed the adhesive tape removal on the right forepaw first, and others on the left forepaw. Five minutes later, the other paw was tested. During the test, the left forepaw was assessed first and the time taken by the animal to remove the adhesive tape from each paw was recorded. The maximum time for each paw test was 120 s. The difference between the first and the second exposure to the adhesive was calculated and expressed as mean ± SEM.

Grasping Strength
The grasping strength assessed with a digital force gauge instrument (TEC-04422 Instrutherm DD-500, São Paulo, SP, Brazil) was used as an indicator of forepaw sensorimotor function. A handle rod was attached to the instrument and the body of the rat was held up and its forepaws were placed on the handle rod, allowing it to grip. The animal was gently and securely pulled away in the horizontal direction by its tail until it had released the rod. The force produced was recorded (expressed in kg). Each animal performed three trials, and the mean value was calculated. The same procedure was used to establish the force for the four paws; however, the animal was placed on a grid with all paws above the surface before being pulled away.

Cylinder Test
The animals were placed inside a Plexiglas cylinder (20 cm diameter × 40 cm height) located on a glass tabletop and videotaped from below with an angled mirror. Spontaneous ipsilateral and contralateral forelimb wall contacts on the cylinder wall were recorded for 4 min. The equation (ipsilateral contacts/ipsilateral +contralateral) × 100 was used to calculate the percentage of asymmetrical use of forelimbs (9).

Morris Water Maze
Reference and working memory was tested for two different spatial memory protocols in the Morris water maze task.

Reference Memory
During the reference memory protocol testing, the platform remained in the same location throughout the training period (5 consecutive days). The animals were placed in the pool facing the wall in one of the start positions, designated as N, S, W, or E. All rats performed four trials a day, with a 10-min intertrial interval, and every starting position was used in a different order each day. The latency to find the platform during each trial was measured. A 60-s probe test (without the platform) was performed on the sixth day, and parameters such as latency to cross the platform zone, time spent in the platform quadrant, time spent in the opposite platform

Table 1. Cortical Na$^+$/K$^+$-ATPase activity and hippocampal BDNF levels at 24 and 48 h after HIP3 on the pups' brain

	SESH; right/left	SEHI; right/left	SWSH; right/left	SWHI; right/left
Na$^+$/K$^+$-ATPase				
24 h	74.25 ± 12.39/86.96 ± 9.33	48.47 ± 13.85/55.54 ± 9.33[a,b]	80.39 ± 13.85/81.05 ± 10.22	127.24 ± 11.31[a,c]/91.73 ± 9.33[c]
48 h	66.9 ± 8.87/70.54 ± 10.14	93.11 ± 9.72/99.26 ± 9.26	130.40 ± 15.37/118.62 ± 13.10	94.93 ± 8.87[c]/118.62 ± 9.26[d]
BDNF				
24 h	3.56 ± 0.59/3.99 ± .47	3.21 ± 0.64/2.53 ± 0.47	3.88 ± 0.72/4.57 ± 0.57	4.81 ± 0.64[b]/4.34 ± 0.51[d]
48 h	4.68 ± 0.72/5.72 ± 0.64	5.23 ± 0.72/4.57 ± 0.58	4.71 ± 1.02/5.25 ± 0.72	3.14 ± 0.79/4.92 ± 0.64

HI, hypoxia–ischemia; SEHI, sedentary HIP3; SESH, sedentary sham; SWHI, swimming HIP3; SWSH, swimming sham.
Results are expressed as mean ± SEM for four to six animals in each group.
[a]Different from sham group.
[b]Different from SWHI.
[c]Interaction between HI and gestational swimming (two-way ANOVA followed by Duncan's *post hoc* test).
[d]Effect of gestational swimming.

quadrant, and total distance travelled were assessed using ANY-Maze software (24).

Working Memory
The pool was set to obtain novel platform positions in non-standard locations each day (e.g., in the center of the pool). The rats performed four trials/day, with a maximum 5 min of intertrial interval from different platform positions on each of 4 consecutive days. Four starting points were used in a distinct order each day. Significant differences between groups were not expected during the acquisition trial because the platform location was unknown by the animals. Working memory was assessed by the mean latencies of acquisition (trial 1) and test (trial 4) in the four sessions. There is no probe trial in working memory assessment (modified from (ref. 9)).

Histological Injury Assessment
Following behavioral testing (at PND60), the animals were anesthetized with thiopental sodium (100 mg/kg) and lidocaine (5 mg/kg). Transcardiac perfusion was performed with saline solution, followed by 4% formaldehyde through the left cardiac ventricle. Their brains were removed from the skull, stored in the same fixative solution, and cryoprotected (sucrose 30% solution) before being sectioned. Serial 30-μm-thick slices were used. One in every fifth section was mounted on gelatin-coated slides and stained with cresyl violet (Sigma-Aldrich, St. Louis, MO). Images were captured and the cross-sectional area of each hemisphere (from +1.70 mm to −3.3 mm) and specific structures (hemisphere and hippocampus) were measured using the software ImageJ-NIH (National Institute of Health, USA) (9). The Cavalieri method was used to calculate the volume of brain structures, and the results are expressed as mean volume ± SEM (mm^3). The thickness of the corpus callosum was measured in straight rows positioned midline and above CA1 in the ipsilateral and contralateral hemispheres at −3.14 mm from the bregma level (n = 4–7 per group). Data are expressed in μm.

Immunofluorescence
The slices were washed three times in phosphate-buffered saline (PBS), and membranes were permeabilized in 0.25% PBS–Triton X. Sections were then blocked with 1% albumin for 30 min. The primary antibodies used were as follows: anti-GFAP (mouse IgG, 1:200, Sigma-Aldrich) to identify astrocytes; anti-MBP (rabbit IgG, 1:100, Abcam) to identify oligodendrocytes; and anti-NeuN (rabbit IgG, 1:200) to identify neurons. The procedure was carried out in 1% albumin in PBS–Triton X at 4 °C for 24 h. Following PBS washes the sections were incubated with secondary antibody anti-mouse Alexa 488 (1:500, Molecular Probes, Invitrogen, Waltham, MA) and

secondary antibody anti-rabbit Alexa 555 (1:500, Molecular Probes, Invitrogen). The slices were covered in aqueous mounting medium (FluorSave, Calbiochem, Germany) and coverslipped. Staining intensities were assessed in the slices containing the dorsal hippocampus (−3.60 mm from Bregma). The intensities for GFAP and NeuN (double-labeled) were quantified in the CA1 area. MBP intensity was quantified bilaterally in the corpus callosum area to determine the white matter loss; all analyses were carried out using high-magnification images (×20). Regularly, some sections were treated without primary antibodies to confirm specificity. One region of interest per hemisphere was determined (4,000 μ^2) to assess the staining intensities (n = 3–5 animals per group). The captured images were analyzed using the software ImageJ-NIH (National Institute of Health, USA), and the integrated density value per unit of area was obtained. Data are reported as mean integrated density/μm^2.

Data Analysis
All statistical analyses were performed using SPSS 21.0 for Windows (SPSS, Chicago, IL). On the basis of our previous data using the HIP3 model, we calculated the number of adult animals necessary for our main finding (the behavioral analysis; n = 15) (20). The calculation of the number of animals considered a power of 80% and P < 0.05. Parametric data were analyzed by one- or two-way ANOVAs, as indicated. Data are expressed as mean ± SE. *Post hoc* analyses for main effects and interactions were run by Duncan's *post hoc* test. *T*-tests were used to compare differences between hemispheres in the biochemical and histological measurements; significance was accepted whenever P < 0.05.

RESULTS

Biochemical Analysis
As shown in **Table 1**, at PND4 (24 h after HIP3), two-way ANOVA revealed an interaction between HIP3 and swimming and its consequent effect on Na$^+$/K$^+$-ATPase activity in the ipsilateral (F(1,18) = 7.925, P = 0.013) and contralateral (F(1,22) = 4.84, P = 0.04) hemispheres: HI caused a decrease in Na$^+$/K$^+$-ATPase activity in the SE HIP3 (SEHI) group, which was prevented by maternal swimming. At 48 h, the effect of an interaction between HIP3 and swimming was observed in Na$^+$/K$^+$-ATPase activity in the ipsilateral hemisphere (F(1,18) = 7.78, P = 0.014), in which swimming HIP3 (SWHI) animals presented increased enzyme activity. There was an effect of swimming in the uninjured hemisphere (left)
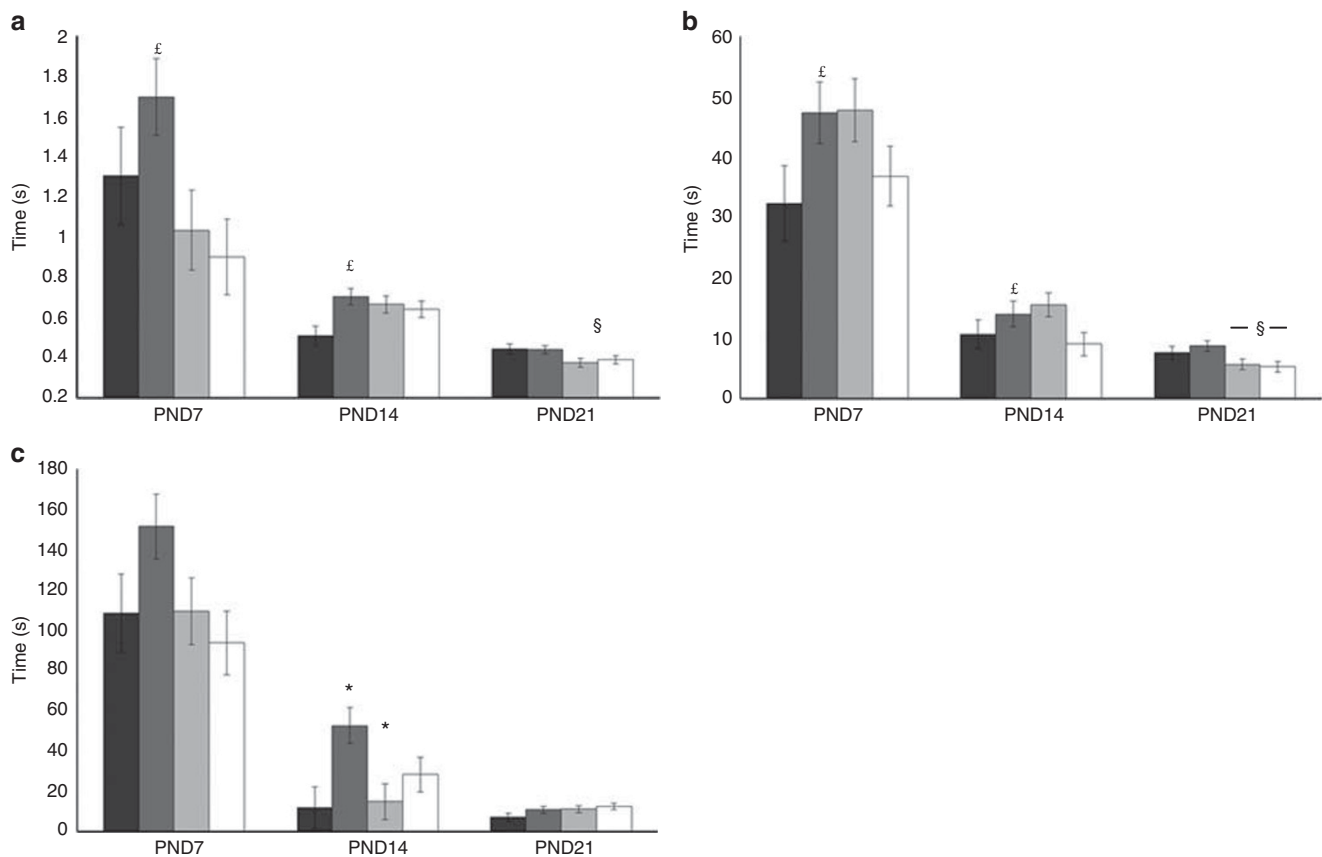
Figure 2. Effect of maternal swimming exercise on neurodevelopmental tests. Effect of maternal swimming exercise on (**a**) righting reflex, (**b**) negative geotaxis, and (**c**) olfactory discrimination during the lactation period. Results are expressed as mean ± SEM for 11–20 animals/group. *Difference from respective sham group. §Effect of gestational swimming. £Interaction between HIP3 and gestational swimming (two-way ANOVA followed by Duncan's *post hoc* test). Groups: dark gray represents SESH; gray represents SEHI; light gray represents SWSH; white represents SWHI. SEHI, sedentary HIP3; SESH, sedentary sham; SWHI, swimming HIP3; SWSH, swimming sham.

(F(1,19) = 10.01, $P = 0.006$), which saw SW animals present an increase in Na$^+$/K$^+$-ATPase activity. *T*-tests revealed no intragroup differences between ipsilateral and contralateral hemispheres at 24 and 48 h.

Table 1 shows that swimming caused an increase in BDNF in the SW groups in the left (contralateral to the lesion) hemisphere (F(1,20) = 5.45, $P < 0.05$).

Behavioral Analysis

Neurological reflex testing. The tested neurological reflexes showed that SEHI animals had a delay in development compared with control, SESH pups. Allied to this, the SEHI group presented worse performance in the olfactory discrimination task (Figure 2a–c). The righting reflex was improved in the SWHI group compared with that in the SEHI group, and an interaction between HIP3 and swimming was observed at PND7 and PND14 (F(1,64) = 7.94, $P = 0.007$; F (1,66) = 9.57, $P = 0.003$, respectively), and an effect of swimming was observed at PND21 (F(1,66) = 9.50, $P = 0.003$; Figure 2a). The same pattern was observed in the negative geotaxis evaluation: SEHI animals presented increased latencies to perform the task compared with the SESH group, and the

SWHI animals performed better compared with the SEHI animals at PND7 (F(1,68) = 5.86, $P = 0.018$), PND14 (F(1,65) = 5.38, $P = 0.024$), and PND21 (F(1,67) = 10.99, $P = 0.02$). Performance on olfactory discrimination was poorer in the HIP3 animals (SE and SW; no statistical significance) at PND7 (F(1,67) = 3.60, $P = 0.06$). ANOVA evidenced an effect of HIP3 at PND14 (F(1,67) = 7.66, $P = 0.007$) in which SEHI animals took longer to approach home bedding compared with swimming sham (SWSH) and SWHI animals. There was no statistical difference at PND21.

Behavioral analysis at adulthood. Reference and working memory were assessed by two different Morris water maze protocols. As shown in the left panel of Figure 3, repeated-measures two-way ANOVA indicated a significant effect of groups (F(1,67) = 7.66, $P < 0.05$) and days of training (F(1,67) = 7.66, $P < 0.05$); SWHI showed decreased escape latencies from the third day of training, as observed in Figure 3a. Duncan's *post hoc* test showed a significant difference between SEHI and SESH groups on days 3 (F(1,67) = 7.66, $P < 0.05$), 4(F(1,67) = 7.66, $P < 0.05$), and 5 (F(1,67) = 7.66, $P < 0.05$) of training. In the probe trial, rat pups swam for 60 s in the pool in which they received the training
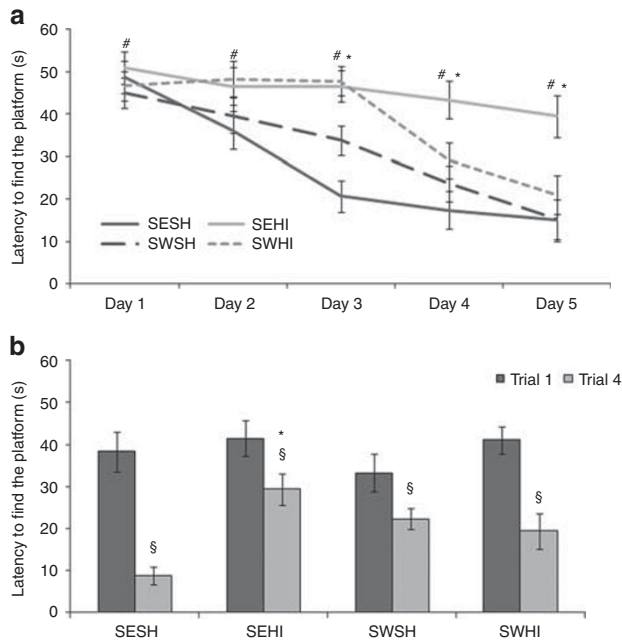
Figure 3. Effect of swimming during pregnancy on spatial learning in the Morris water maze (**a**) reference and (**b**) working memory tests. (**a**) Reference memory: latency to find the platform during the training sessions. (**b**) Working memory: means of the first (left) and fourth (right bars) trials of the four sessions. Data are expressed as the mean ± SEM. Results were analyzed by repeated-measures two-way ANOVA followed by Duncan's *post hoc* test. Data are expressed as mean ± SE ($n = 9–11$ animals per group). Values were considered significant when $P < 0.05$. (**a**) #Repeated-measures ANOVA: effect of training days. *Difference from respective sham group. (**b**) *Difference from respective sham group. £paired *t*-test: difference between trials 1 and 4. (___) SESH, (___) SEHI, (- - - -) SWSH, and (– – – –) SWHI. SEHI, sedentary HIP3; SESH, sedentary sham; SWHI, swimming HIP3; SWSH, swimming sham.

with the escape platform removed, and no differences were detected. In the working memory test (**Figure 4**), as expected, there was no difference between groups in the acquisition trial (trial 1, left bars). *T*-tests revealed that all groups decreased latencies to find the platform during the training days. In the fourth trial (test trial, right bars), ANOVA revealed an effect of HIP3 ($F_{(1,33)} = 23.96$, $P < 0.05$), in which HI animals presented increased latencies to find the platform compared with sham rats.

Table 2 shows the sensorimotor (adhesive removal and grasping tests), anxiety (plus maze), exploratory activity (open field), and motor coordination (cylinder test) evaluations. The tests showed no obvious sensorimotor, increased activity, or anxiety levels in HIP3 animals tested at adulthood.

Histological analysis. As depicted in **Table 3**, the two-way ANOVA showed that HIP3 caused no significant tissue loss in the ipsilateral and contralateral hemispheres at PND60. There was no hippocampal volume reduction in HIP3 animals (SE nor SW). However, the corpus callosum thickness was affected by HIP3 on the ipsilateral ($F_{(1,21)} = 5.99$, $P < 0.05$) and contralateral ($F_{(1,21)} = 7.44$, $P < 0.05$) hemispheres, with SEHI animals presenting a significantly thinner corpus callosum as

compared with the SESH group, confirming the hypomyelination caused by the injury (6,7). This effect was not observed in SWHI rats, confirming the tissue protection provided by swimming. In addition, the myelin basic protein (MBP) immunostaining evidenced a significant decrease in the right hemisphere in the SEHI group, which was not observed in the SWHI group ($F_{(1, 16)} = 8.33$, $P < 0.05$; **Figure 4**). There was an interaction between treatment and lesion, as seen on NeuN imunolabeling, in the CA1 region: the protein was decreased in the SEHI group and was restored to sham levels in the SWHI group ($F_{(1, 12)} = 5.43$, $P < 0.05$). No significant alterations among groups were observed in the glial fibrillary acidic protein (GFAP) immunocontent.

DISCUSSION

Our results add new data to the study of HI using very immature animals, as well as the gestational period as a therapeutic window. Gestational swimming prevented the biochemical impairments induced by HIP3 on Na^+/K^+-ATPase activity and increased the hippocampal BDNF levels in the acute phase of injury. In addition, the neuroprotective effects of swimming were long-lasting, acting on offsprings' development and reverting the delays on reflex maturation, improving the performance in spatial-learning tasks, and reducing the histological damage induced by the model.

Motor coordination tests at early stages of life serve as a useful tool for observing differences between normal and hypoxic–ischemic rat pups (24,25). Following HIP3, cortical somatosensory regions are the most vulnerable regions to injury (10), and thus some delay in neurological maturation can be expected. To our knowledge, this is the first study to evaluate neurological development in rats following HIP3. Confirming previous reports showing a delay in reflex maturation after injury using the HI model at PND7 (12,25), HIP3 caused a delay in righting reflex and in negative geotaxis testing in the SEHI group, which was reverted in the SWHI group (**Figure 2a,b**). Ten *et al.* (26) showed that HI mice had worsened performance in righting reflex, cliff aversion, and geotaxis reflexes at early stages (1 and 24 h) after HI; however, the age of injury (PND7) and the period of testing (PND8) in their study were distinct from those used here. The performance in the olfactory discrimination test was worse in the SEHI group compared with that in the SESH group and was preserved in the SWHI group, confirming the neuroprotection provided by pregnancy swimming on reflex maturation. The behavioral tests performed at adult age (from PND45) showed that strength (grasping test), sensitivity (adhesive removal), and locomotor activity (open field test) were similar between sham groups in the HIP3 rats evidencing a spontaneous recovery. Barth and Stanfield (27) suggested that the preservation of corticospinal projections from the contralateral hemisphere was responsible for the absence of alterations in motor activity in the lesioned rats. These results are in agreement with previous reports from our research group and others showing that motor deficits are more pronounced when HI is performed at PND7, in
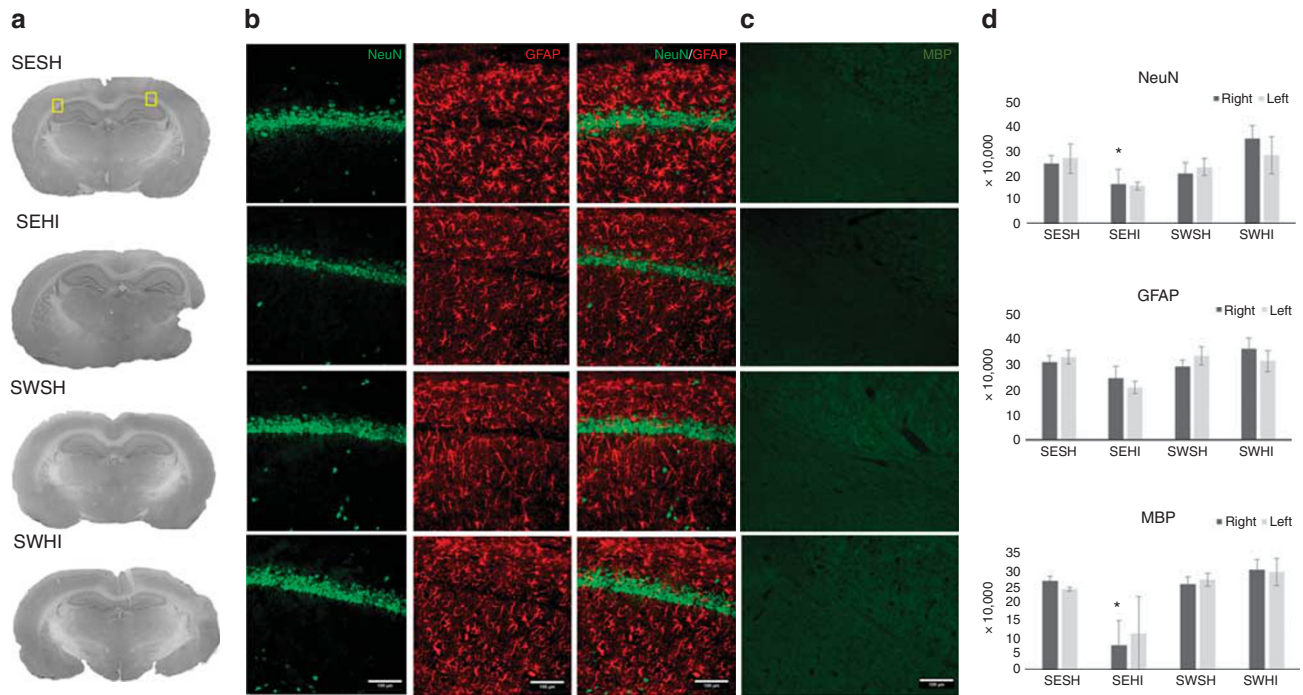
Figure 4. Histological effects of maternal swimming following HIP3 at PND60. (**a**) Representative images of the four groups at the hippocampal level (−3.60 mm from Bregma) using cresyl violet staining (×10 magnification). The yellow square represents the region of interest assessed in the immunofluorescence analysis. (**b**) Representative images of the double labeling of neurons (NeuN) and astrocytes (GFAP) in the right hippocampus-CA1. (**c**) Representative MBP immunostaining in the right corpus callosum. (**d**) Protein quantification at the bregma level (−3.60 mm) at CA1 for GFAP, NeuN, and MBP. Scale bars in the immunofluorescence analysis = 100 μm. *SESH vs. SEHI group ($P < 0.05$).

Table 2. Behavioral analysis at adult age

	SESH	SEHI	SWSH	SWHI
Plus maze				
Open time (s)	76.9 ± 18.5	88.7 ± 16.3	76.5 ± 16.6	89.0 ± 13.19
Closed time (s)	147.0 ± 15.08	132. ± 12.89	137.0 ± 12.9	131.0 ± 11.8
Ratio (open/closed)	0.64 ± .021	0.86 ± 0.29	0.70 ± 0.20	0.78 ± 0.16
Open field				
Number of crossings	101.1 ± 10.5	128.1 ± 13.9	120.8 ± 8.18	128.3 ± 8.7
Adhesive removal				
Time (s)	18.2 ± 10.9	26.8 ± 7.3	23.8 ± 8.9	29.8 ± 11.6
Grasping test (N)				
Forepaws	247.4 ± 18.7	210.4 ± 13.0	250.4 ± 19.2	260.5 ± 19.6
Four paws	337.4 ± 25.0	334.7 ± 24.3	332.4 ± 21.4	369.7 ± 43.3
Cylinder				
Use of paws (%)	47.3 ± 3.20	47.1 ± 2.13	46.4 ± 2.28	45.6 ± 2.84

HI, hypoxia–ischemia; SEHI, sedentary HIP3; SESH, sedentary sham; SWHI, swimming HIP3; SWSH, swimming sham.
Adhesive removal, grasping, and cylinder tests were used for assessing sensorimotor function.
Elevated plus maze for assessing the anxiety levels and the open field test for assessing the exploratory activity of the rats.
Data are expressed as mean ± SEM ($n = 7$–11). The results were analyzed by two-way ANOVA followed by Duncan's *post hoc* test. Significance was accepted when $P < 0.05$. No differences were observed.

Table 3. Effects of gestational swimming on cresyl violet-stained brain hemispheres and hippocampus volumes and corpus callosum thickness at adult age at PND60

	SESH	SEHI	SWSH	SWHI
Hemisphere (mm^3)				
Right	851.08 ± 145.05	740.17 ± 162.17	714.47 ± 132.41	721.09 ± 122.59
Left	852.45 ± 114.21	936.60 ± 127.69	729.60 ± 104.26	804.24 ± 96.52
Hippocampus (mm^3)				
Right	23.69 ± 4.41	16.91 ± 4.98	24.12 ± 4.46	14.30 ± 4.07
Left	22.48 ± 4.11	22.48 ± 4.60	22.53 ± 4.11	14.31 ± 3.75
Corpus callosum (µm)				
Right	241.73 ± 27.03	154.44 ± 30.22[a]	250.23 ± 24.67	208.52 ± 22.84
Midline	617.71 ± 58.87	440.27 ± 65.81	587.20 ± 53.74	533.92 ± 49.75
Left	237.23 ± 21.11	176.94 ± 23.60[a]	251.02 ± 19.27	199.03 ± 17.84

SEHI, sedentary HIP3; SESH, sedentary sham; SWHI, swimming HIP3; SWSH, swimming sham.
Mean ± SEM of absolute values. Data were analyzed by two-way ANOVA followed by Duncan's *post hoc* test. Values were considered significant when $P < 0.05$.
[a]SEHI vs. SESH.

which structures related to the motricity are more severely affected (9).

HIP3 animals showed clear deficits in reference (Figure 3a) as well as in working (Figure 3b) memory in the Morris water maze test. SWHI animals showed a similar learning curve performance compared with sham groups, evidencing the neuroprotection induced by swimming. As previously reported by our research group (9) and others (13,28), HIP3 brain injury did not result in gross cerebral hemisphere atrophy or cystic infarcts that can be correlated to cognitive impairment (Table 3). Huang *et al.* (12) observed a decrease in myelination (evidenced by the reduction in the thickness of the corpus callosum) after HIP3 and indicate this reduction as the cause of the learning deficits observed in their study. In agreement, we observed a decrease in corpus callosum thickness (Table 3) and a decrease in the immunostaining for MBP (Figure 4) in the SEHI group, which were not observed in the SWHI group. This could reflect the preservation in myelination afforded by swimming and can be responsible for the cognitive function maintenance observed. In addition, gestational swimming was able to reduce the cell loss observed in the CA1 region in the ipsilateral hemisphere (Figure 4). The SEHI group showed a decrease in NeuN immunofluorescence in the hippocampus, commonly reported in the model (3). The astrocytic reaction observed in the HI model was not evident in our study, which can reflect the milder nature of the HIP3 lesion as previously reported by our research group (29) and by others and reflects the fact that, in the immature white matter, astrocyte response to diffuse injury is developmentally regulated, being evident in the earlier stages of injury but not at later time points (30).

The literature shows that Na$^+$/K$^+$-ATPase activity is decreased during cerebral ischemia in adults and after HI in rats (9) and directly affects neurotransmitter signaling, neural activity, as well as the whole animal behavior (31). In agreement with previous results reported by our research group (24), we observed a decrease in Na$^+$/K$^+$-ATPase activity in SEHI animals (24 h), returning to control levels 48 h after injury in both ipsilateral and contralateral cortices (Table 1). In contrast, SWHI animals presented an increase in the activity of this enzyme (24 and 48 h), as compared with controls (in both hemispheres). The energetic deficiency induced by HI and the failure of Na$^+$/K$^+$-ATPase function lead to ionic imbalance, membrane depolarization, and excessive release of excitatory amino acids (especially glutamate), causing overstimulation of the NMDA receptors and excitotoxicity, cell swelling, and necrotic cell death (1). We suggest that swimming could exert neuroprotection by preserving Na$^+$/K$^+$-ATPase function (Table 1) and by blocking, or at least decreasing, the activation of the NMDA receptors and consequent necrosis. Such a mechanism and its neuroprotective effects were demonstrated by Veldhuis *et al.* (32) in a model of ouabain-mediated excitotoxicity (a Na$^+$/K$^+$-ATPase inhibitor). They showed that after NMDA receptor blockade before the injection of ouabain (using the specific antagonist MK-801) the Na$^+$/Na$^+$-ATPase functioning was preserved and cell death was reduced. Physical exercise stimulates metabolic programming in the central nervous system by increasing glucose uptake, oxidative capacity, and electron transport chain activity (18). There are a few studies showing that the effects of swimming during pregnancy could induce tissue adaptations that increase cell survival under oxygen deficiency conditions, possibly preventing the decline of brain ATP (33). Furthermore, physical exercise can be considered a stressor during pregnancy, imposing a challenge on the mother and the fetus (20). If we assume that gestational swimming could induce a certain degree of preconditioning, the literature shows that in the

brain ischemic preconditioning increases the activity of the Na/K-ATPase, protecting neurons from an experimental ischemia (34,35).

The vast majority of studies evaluating the effects of swimming during pregnancy on the offspring attributed the neuroprotective effects to BDNF, which is widely expressed in the rodent and the human brain and is abundant in the hippocampus and has a major role in the cognitive functioning (16,20). In agreement with the literature, we observed an increase in BDNF levels 24 h after injury in the hippocampus in the SWHI group, which could help explain the preservation of memory at adult age. In agreement, Gomes da Silva *et al.* (36) also reported enhanced levels of BDNF and hippocampal cells in adult rats after exercise during pregnancy. It is generally accepted that BDNF has a putative role in brain plasticity, enhancing neuronal survival, migration, and differentiation, and supporting neurogenesis in neonatal HI models, and that the upregulation in the protein that is observed in the early periods after injury (24–96 h) gives rise to neuroprotective effects and can be attributed to a reduction in inflammatory levels (37,38). Another possibility is that BDNF acts directly on the lesion site, protecting cells against the effects of HIP3 through a decrease in apoptosis, as reported by Han *et al.* (22). Recently, maternal swimming was tested in a model of HI at PND7 and it was found that swimming was unable to revert the biochemical and cognitive impairments during adulthood (39). This could be attributed to the fact that lesion severity (as well the cells and structures lesioned) is different after HIP7 and HIP3 (24) and the neuroprotective effects of swimming occur in the early stages after injury and may decrease with time.

In summary, this study shows that maternal exercise during pregnancy causes short- and long-term neuroprotection after hypoxic–ischemic injury in very immature rats. Maternal swimming prevented the loss of cellular homeostasis as well as prevented sensorimotor deficits and cognitive impairment, preserving brain structures from the lesion induced by HIP3. We hope to contribute to further comprehension of the mechanisms by which maternal swimming improves brain functions and induces long-term neuroprotection.

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No writing assistance was utilized in the preparation of the manuscript.

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