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Long-lasting effects of perinatal asphyxia on exploration, memory and incentive downshift

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ABSTRACT

Perinatal asphyxia remains as one of the most important causes of death and disability in children, without an effective treatment. Moreover, little is known about the long-lasting behavioral consequences of asphyxia at birth. Therefore, the main aim of the present study was to investigate the motor, emotional and cognitive functions of adult asphyctic rats. Experimental subjects consisted of rats born vaginally (CTL), by cesarean section (C+), or by cesarean section following 19 min of asphyxia (PA). At three months of age, animals were examined in a behavioral test battery including elevated plus maze, open field, Morris water maze, and an incentive downshift procedure. Results indicated that groups did not differ in anxiety-related behaviors, although a large variability was observed in the asphyctic group and therefore, the results are not completely conclusive. In addition, PA and C+ rats showed a deficit in exploration of new environments, but to a much lesser extent in the latter group. Spatial reference and working memory impairments were also found in PA rats. Finally, when animals were downshifted from a 32% to a 4% sucrose solution, an attenuated suppression of consummatory behavior was observed in PA rats. These results confirmed and extended those reported previously about the behavioral alterations associated with acute asphyxia around birth.

1. Introduction

Perinatal asphyxia is a worldwide health problem which results from a lack of oxygen supply to the fetus or newborn during a certain period of time (Adcock and Papile, 2008).The most common childbirth complications associated with perinatal asphyxia are compression of the umbilical cord, abruption of the placenta, abnormal uterine contractions, and failure to begin breathing (de Haan et al., 2006). The estimated incidence is 1/1000 live births, being five- to ten-fold higher in less developed countries (McGuire, 2006). Perinatal asphyxia is associated not only with a high mortality rate but also with neurological and psychiatric sequelae such as cerebral palsy, mental retardation, epilepsy, hearing loss, visual impairment (Borg, 1997; Crofts et al., 1998; Hill, 1991; Hill and Volpe, 1981; Younkin, 1992), hyperactivity (van Handel et al., 2007), schizophrenia (Cannon et al., 2002; Lewis and Murray, 1987) and neurodegenerative disorders (Weitzdoerfer et al., 2004).

Some of the areas of the central nervous systems most affected by a perinatal hypoxia-ischemia episode are the basal ganglia, the hippocampus, the cerebral cortex and the cerebellum (Vannucci, 1990; Berger and Garnier, 1999). These areas are well known to be implicated in motor, emotional, memory and learning processes, which makes frequently finding neurologic and psychiatric problems following perinatal asphyxia something to be expected.

Abbreviations: CTL, rats born by vaginal delivery; C+, rats born by cesarean section; PA, perinatally asphyxiated rats; EPM, elevated plus maze; OF, open field; MWM, Morris water maze.

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Up to now, there is not an established treatment for perinatal asphyxia although experimental data and clinical trials have shown that hypothermia is able to reduce death, ameliorate brain damage, and improves neurological outcomes associated with asphyxia around birth (Azzopardi et al., 2009; Capani et al., 1997, 2003, 2009; Cebral and Loidl, 2011; Engidawork et al., 2001; Hoeger et al., 2006; Shankaran et al., 2005). However, the effects of hypothermia therapy on the long-lasting neurological and psychiatric consequences of perinatal asphyxia remain unknown (Azzopardi et al., 2009).

We and others have extensively employed a modified version of the perinatal asphyxia murine model originally developed by Bjelke et al. (1991) (Boksa and El-Khodor, 2003; Brake et al., 2000; Capani et al., 1997, 2001, 2003, 2009; Cebral et al., 2006; Chen et al., 1995; Morales et al., 2010; Saraceno et al., 2010; Strackx et al., 2010; Wakuda et al., 2008; Weitzdoerfer et al., 2004). The model exhibits some remarkable advantages such as: (a) asphyxia is produced at the time of delivery reproducing more accurately some clinical situations, i.e. when umbilical cord circulation is altered (Capani et al., 2009); (b) acidosis, hypercapnia and hypoxia are present in the whole body, mimicking global asphyxia which is the most common type (Lubec et al., 1997; Loidl et al., 2000; Strackx et al., 2010); (c) it is not invasive, avoiding the confounding effects of surgical procedures; (d) the fact that hypoxia is produced in the whole body, and therefore affecting both cerebral hemispheres and deep brain structures, makes the model specially suitable for behavioral studies, since rats, like humans, have lateralized brain functions (Arteni et al., 2010; Bradshaw, 1991).

Despite the usefulness of the model and the fact that it is very difficult to study the adulthood consequences of perinatal asphyxia in humans, there are few studies addressing behavioral features in adult asphyctic rats (Hoeger et al., 2000). Moreover, since the behavioral outcomes vary according to the severity of the insult and the stage of the development at which the animal is tested (Strackx et al., 2010), it is not unusual to find that the results obtained in one or more studies are not replicated in others. In view of these inconclusive findings, the aim of this work is to study the motor, emotional and cognitive consequences in adult rats that had undergone a moderate-to-severe asphyxia at birth. To this purpose, we evaluated exploration, general activity, and anxiety-like behaviors in the open field and elevated plus maze tests, spatial reference and working memory in the Morris water maze test, and the behavioral responses to an unexpected reward downshift.

2. Experimental procedures

2.1. Animals

Subjects consisted of 45 pregnant *Sprague Dawley* rats obtained from the School of Veterinary Sciences' central *vivarium* at the Universidad de Buenos Aires. Pregnant rats arrived one week prior to delivery to our local *vivarium* in order to acclimate to the new environment. All animals were housed in individual cages and maintained in a temperature- (21 ± 2 °C) and humidity- (65 ± 5%) controlled environment on a 12-h light/dark cycle (lights on at 6 a.m.). Animals had *ad libitum* access to food (Purina chow) and tap water.

2.2. Induction of cesarean section and perinatal asphyxia

Rat pups were subjected to acute asphyxia immediately after birth by cesarean section using procedures modified from Bjelke et al. (1991) and previously described by our laboratory (Capani et al., 2009; Saraceno et al., 2010). At expected day of delivery (E22), pregnant rats were individually observed and when no more than two pups were delivered, the dam was immediately euthanized by decapitation and the uterus horns were rapidly isolated through an abdominal incision. Next, one of the uterus horns was rapidly opened, pups were removed, the amniotic fluid was cleaned, and the umbilical cord was ligated (cesarean section or C-section procedure). The other uterus horn was placed in a water bath at 37 °C for 19 min (moderate to severe perinatal asphyxia) (Fig. 1). Immediately after the time of asphyxia elapsed, the same procedures applied for the C-section were followed, but before ligation of the umbilical cord took place, pups were stimulated to breathe by performing tactile intermittent stimulation with pieces of medical wipes for a few minutes until regular breathing was established. This was unnecessary for pups born by C-section since they started breathing spontaneously. Pups born vaginally (control group, CTL), by C-section (cesarean section group, C+) or by C-section plus acute asphyxia (perinatal asphyxia group, PA) were left approximately for 1 h under a heating lump in order to allow the asphyxiated pups improve their physiological conditions. Next, all pups were given to surrogate mothers which had delivered normally within the last 24 h. The different groups of pups were marked and mixed with the surrogate mothers' normal litters. We maintained litters of 10–12 pups with each surrogate mother. Only rats that were vaginally delivered by dams subjected to C-section procedure were used. Rats were weaned at 21 days of age and housed in groups of 3–4 rats per cage throughout the experiment. Only male pups were used for behavioral studies. All procedures involving animals were approved by the Institutional Animal Care and Use Committee at the University of Buenos Aires (School of Medicine) and conducted according to the principles of the Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23, revised 1996). All efforts were made to reduce the number of animals used and to minimize suffering.

2.3. Behavioral experiments

2.3.1. General procedures

All animals were randomly assigned to two experimental cohorts. One cohort of animals (set 1, $n = 36$) was employed for evaluation of anxiety, exploration/locomotion, and spatial reference and working memory. Another cohort (set 2, $n = 33$) was used for the incentive downshift protocol. Two days prior to the elevated plus maze test, all animals were handled once a day for 5 min and weighed. Behavioral procedures were carried out between 7:00 a.m. and 5:00 p.m. in two experimental rooms. White noise was provided throughout testing. Testing order of the groups was counterbalanced to avoid the confounding effect of time of the day at which animals were tested. All training/testing sessions were recorded (JVC Everio GZ-HD620 or Sony DCR-SR47 Handycam with Carl Zeiss optics) and later analyzed using a computerized video-tracking system (Ethovision XT, version 5, Noldus Information Technology, Wageningen, The Netherlands) or the ethological observation software JWatcher V1.0.

2.3.2. Elevated plus maze

The elevated plus maze (EPM) was validated by Pellow et al. (1985) to assess anxiety-relative behaviors. The apparatus consisted of a black melamine central square platform (11 cm × 11 cm) from which four black melamine arms radiate (50 cm × 11 cm) separated by 90° from each other. Two of the arms are called protected or closed because they have a wall (40 cm in height) all around its perimeter but not in the entrance and the other two arms are called unprotected or open arms because they do not have any wall but with raised edges (0.25 cm) around its perimeter. The maze was elevated one meter from the floor by five legs, one below at the end of each arm and one below the central square platform. The light intensity in the open arms was 85–90 lux. At 90 days of age each rat was placed onto the central platform facing an open arm and allowed to freely explore the maze for 5 min. After each session the apparatus was cleaned with 70% ethanol and dried. An arm entry was counted when rat introduced its four paws into an arm. Dependent variables were: total distance moved, number of closed arm entries, percentage of open arm entries, percentage of time spent in open arms and percentage of the distance moved in the open arms (calculated as: [open arm entries/total entries × 100], [time spent in open arms/300 × 100) and [distance moved in the open arms/total distance moved × 100]). It is important to note that although "Total distance moved" is a more accurate measure than other classical parameters like "Total arm entries", it is not an uncontaminated index of locomotion activity since it includes the distance moved in the open arms. For this reason, we also analyzed the "Number of closed arm entries" which could be used as an uncontaminated index of locomotor activity since in previous factorial analysis showed to load highly on "Locomotion factor" and did not load on the "Anxiety factor" (Rodgers and Johnson, 1995).

2.3.3. Open field

The open field (OF) is a widely used test to evaluate general activity and anxiety-related behaviors in rodents (Walsh and Cummins, 1976). The apparatus was made of black melamine and consisted of a square (60 cm × 60 cm) surrounded by high walls (40 cm in height). The central area was arbitrarily defined as a square of 30 cm × 30 cm and it was drawn over the image of the OF in the video-tracking system. A rat was considered to be into the central area when its four paws were on it. Arena was uniformly and indirectly illuminated by four spiral compact fluorescent lamp in each corner facing the walls. Light intensity in the center of the OF was 70 lux. All animals were evaluated 2–5 days later EPM session took place. Each rat was placed individually in the center of the maze and its behavior was analyzed for 30 min. Between sessions, the apparatus was cleaned with 70% ethanol and dried. Dependent variables were: total distance moved, number of rearings, ratio central over total distance moved (calculated as: [distance moved in the central area/total distance moved × 100]), central area frequency (number of entries into the central area), and central area duration (time spent in central area). In an attempt to obtain more complete information about the behavioral patterns displayed by animals in this test, all the mentioned dependent variables were also measured at 5-min time bins. A significantly increased time spent freezing when rats are exposed to OF is a sign of anxiety (Walsh and Cummins, 1976). Since a significant effect on exploration was found (Section 3.3) in the first 10 min of the OF session, and this effect could be

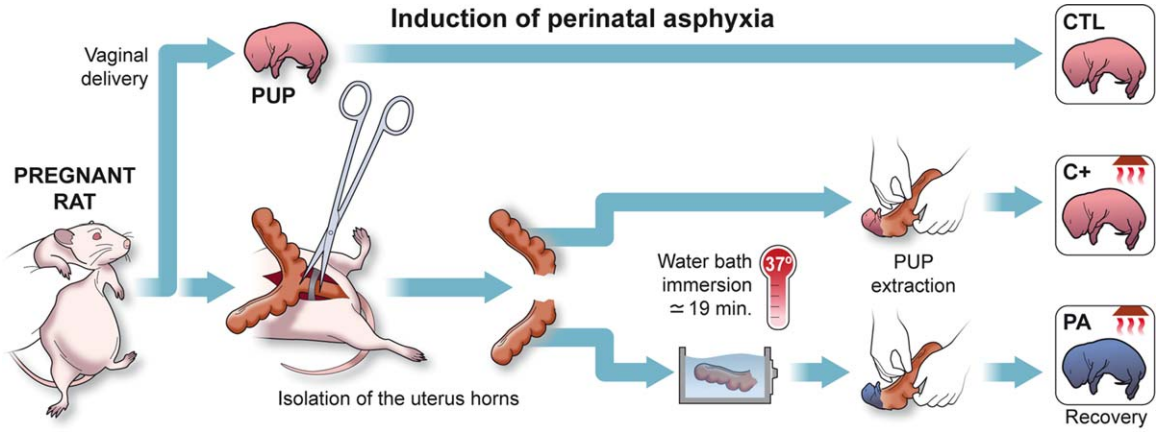

Fig. 1. Schematic illustration of the procedures performed in the murine model of perinatal asphyxia. Dam rats that delivered no more than two pups (vaginally delivered controls, CTL) were hysterectomized, one of the uterus horns was opened and pups removed (pups born by cesarean section, C+), and the other uterus horn was immersed in a water bath at 37 °C during 19 min (pups born by cesarean section plus asphyxia, PA). Rat pups were left to recover under a heating lamp and given to foster mothers. This experimental model reproduces clinical situations, such as when umbilical cord circulation is altered triggering brain damage in the central nervous system that has long-lasting effects on behavior.

ascribed to an increased time spent freezing, we measured freezing duration during time bins 1 and 2. A blind evaluation of freezing behavior was carried out by two trained observers using the JWatcher V1.0 (The probability of agreement between observers was 0.89). Freezing behavior was operationally defined as "total absence of body and head movement" (Carlini et al., 2002).

2.3.4. Morris water maze

2.3.4.1. Apparatus. The Morris water maze (MWM) was developed to assess spatial learning and memory processes (Morris, 1981; Morris et al., 1982). The apparatus consisted of a circular galvanized steel pool (180 cm in diameter by 60 cm in height), painted black, and filled with water to a height of 40 cm. A circular transparent platform (10 cm in diameter) was placed 2 cm beneath the water surface (hidden escape platform). The pool was divided into four imaginary quadrants (A, B, C, and D) and the platform was placed in the center of one of them, 35 cm from the pool edge. The pool was mounted 50 cm above the floor, located in the center of an experimental room with multiple extra-maze visual geometric cues hanging on the wall. Indirect illumination was provided by four spiral compact fluorescent lamp in each corner facing the walls. The water temperature was kept at 22 ± 1 °C. Variables registered were: latency to find the hidden escape platform, distance swam to the platform, swimming speed and time spent in each quadrant. In the acquisition phase of the reference memory task, data were averaged across trials for each test day. In the working memory task, data were averaged across all trials in order to stabilize the mean (Vorhees and Williams, 2006).

2.3.4.2. Spatial learning and reference memory task. We used procedures extensively described previously (Miranda et al., 2006; Rubio et al., 2002) with some modifications. Briefly, one day before the first acquisition session of the reference memory task, all rats (100 days old) were given a habituation session that consisted of four trials when rats were allowed to swim freely for 90 s without the escape platform. During the habituation session, the pool was surrounding by a black curtain in order to hide the extra-maze cues. Next, the acquisition phase of the task was conducted over four consecutive days with four trials per day. At the beginning of each trial, rats were gently released into the pool from one of the four starting positions according to four quadrants. Rats were able to escape from the water using the hidden escape platform that was kept in the same location throughout the four sessions of the acquisition phase. A trial was finished when the animal found the escape platform or when 120 s had elapsed, whichever occurred first. If rat failed to find the platform, the experimenter guided to it by hand. Rats remained on the platform for 15 s and immediately the following trial began (Vorhees and Williams, 2006). In each session, the four starting position were used and the order of the sequence was changed pseudo-randomly between days. 24 h after the last trial of the acquisition phase, reference memory was assessed with a probe trial in which the escape platform was removed from the pool and rats were released from a new starting position not used during the acquisition phase. Time spent in each quadrant was recorded. When sessions finished rats were dried and returned to their home cage in the colony room.

2.3.4.3. Spatial working memory task. Two days after the probe trial, rats were submitted to a working memory task in the MWM. Procedures to assess spatial working memory were similar to those used for reference memory with the following modifications: only one daily session was given, each consisting of two identical trials (sample and retention), for five consecutive days; between sample and retention trials a 30 s inter-trial interval was introduced, during which the rat remained in its transport cage; starting points and location of the platform were pseudo-randomly varied for each rat throughout the 5 days but fixed within a single session; starting points and platform were never been at the same quadrant; neither the location of the platform nor the starting points were the same as from the previous day. For more details see Santín et al. (1999) and Vorhees and Williams (2006). To solve this task during retention trials, rats have to hold the information about the location of the platform during acquisition trials easily available, being useless the information from previous days.

2.3.5. Incentive downshift

The protocol was similar to others used before with modifications added (Kamenetzky et al., 2009; Ruetti et al., 2009). Ten days before the induction of incentive downshift, rats were transferred to individual cages with water freely available. The daily amount of food was gradually reduced until their weights were lowered to ≈85% of individual *ad libitum* weights. Body weight reached was kept constant throughout the entire experiment. Training was conducted in four stainless steel cages (44 cm × 29 cm × 19 cm). A tray filled with sawdust bedding was placed on the floor to collect feces and urine. Spouts attached to graduated burettes containing the sucrose solution were placed into the chamber through a 1.5 cm hole located in the front panel of the cage.

At 92 days old, CTL ($n = 11$), C+ ($n = 11$) and PA ($n = 11$) rats were submitted to a daily 5-min trial throughout 14 days, in which free-access to a 32% sucrose solution was available (1–10 trial, pre-shift trials) and to a 4% sucrose solution (11–14 trial, post-shift trials). The 5-min duration of each trial was counted from the first lick. Sucrose solutions were prepared by mixing 32 g of sucrose for every 100 ml of total solution of tap water. During the course of the experiment, rats were fed daily at least 20 min after the training trial. The dependent variable recorded in all trials was consumption. First and second post-shift trials were video recorded and a blind evaluation was conducted in these trials to measure spout contact, locomotion and rearing duration using the ethological observation software JWatcher V1.0. Due to technical problems, one video from the first pre-shift trial was lost, so in trial 11 spout contact, locomotion and rearing duration were measured in only 10 CTL rats. However, the amount of consumption in this trial was available for all animals.

2.4. Statistical analyses

The results were expressed as the means ± SEM. Independent *t*-tests and paired *t*-tests were conducted. Also, one-way ANOVAs and mixed ANOVAs (with Group as between-subject factor and Bin, Day or Trial as within-subject factors) followed by Tukey HSD post hoc comparisons were carried out. If assumption of normality and/or homoscedasticity was violated, Kruskal–Wallis or Mann–Whitney test was used. Bonferroni correction was applied if necessary. When assumption of sphericity was not met, degrees of freedom were corrected by Greenhouse–Geisser. A probability was considered to be significant at 5% or less. Two-tailed probabilities were always reported. Statistical analyses were performed using the SSPS 15.0 for windows (SPSS Inc., Chicago, IL, USA).

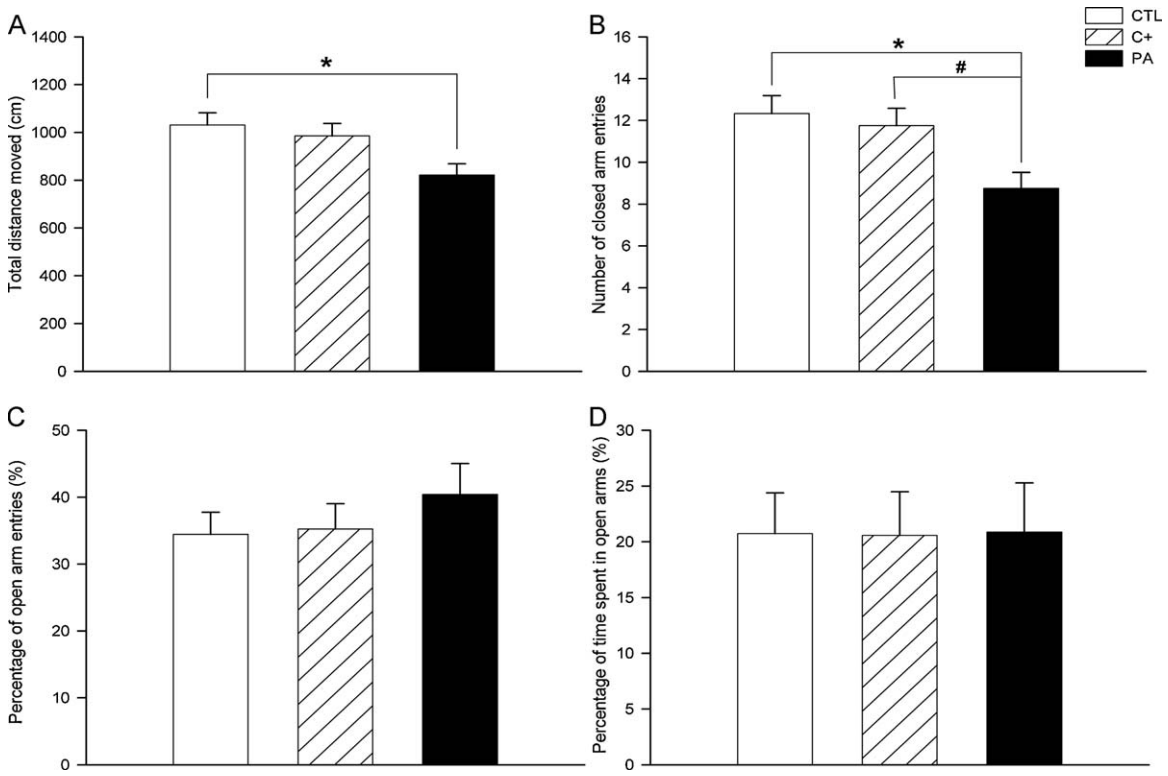

Fig. 2. The performance of the different groups in the Elevated Plus Maze. Upper panels show levels of horizontal locomotor activity measured by the total distance moved (a) and by the number of closed arm entries (b). Below panels show anxiety levels measured by the percentage of open arm entries (c) and by the percentage of time spent in open arms (d). Experimental groups: Vaginal delivery rats (CTL, $n = 12$), rats born by cesarean section (C+, $n = 12$) and rats born by cesarean section + asphyxia (PA, $n = 12$). Bars and error bars represent mean + SEM. $^{*}p < 0.05$ for CTL vs. PA; $^{\#}p < 0.05$ for C+ vs. PA.

3. Results

3.1. Mortality and body weights

Mortality rate was approximately 30% in male pups that had undergone 19 min of asphyxia. This outcome is similar to that reported by Loidl et al. (2000). Mortality was not observed among male pups born vaginally or by cesarean section (100% of survival). Mean group weights one/two days before starting the behavioral procedures did not differ between groups ($F_{(2,66)} = 1.56$, p = n.s.).

3.2. Elevated plus maze

When total distance moved by rats was analyzed, a significant main effect of group was found ($F_{(2,33)} = 4.77$, $p = 0.015$). Post hoc analyses revealed that PA rats moved a significantly less distance than CTL rats ($p = 0.016$, Fig. 2a) and a strong tendency of PA rats to move less than C+ rats although it did not reach a significant level ($p = 0.07$) was also observed. The distance moved by CTL rats did not differ from that observed in C+ rats (p = n.s.). For the number of closed arm entries, the main effect of group was also significant ($F_{(2,33)} = 5.52$, $p = 0.009$). Post hoc multiple comparisons revealed that PA rats made significantly fewer entries into the closed arms than CTL and C+ rats ($p = 0.011$ and $p = 0.037$, respectively, Fig. 2b), being no difference between CTL and C+ rats (p = n.s.). Neither for the percentage of open arm entries nor for the percentage of time spent in open arms were found differences between the groups ($F < 1$ for both cases, Fig. 2c and d). Also, experimental groups did not differ in the percentage of the distance moved in the open arms ($F_{(2,33)} < 1$, p = n.s., Supplementary Fig. 1).

3.3. Open field

For total distance moved in the 30-min OF session, data showed a significant main effect of group ($F_{(2,33)} = 4.80$, $p = 0.015$). Post hoc multiple comparisons confirmed that PA rats moved less distance than CTL rats did ($p = 0.01$, Fig. 3a). The total distance moved by C+ rats was not significantly different from CTL and PA rats (p = n.s. for both comparisons, Fig. 3a). Neither for the number of rearings (Fig. 3b), nor for the ratio central over total distance moved (Fig. 4a), nor for central area duration (Fig. 4b), nor for central area frequency (Supplementary Fig. 2a) in the 30-min OF session significant main effects of group were found ($F_{(2,33)} = 1.36$, p = n.s.; $H = 1.64$, $d.f. = 2$, p = n.s.; $H = 3.66$, $d.f. = 2$, p = n.s.; and $H = 3.33$, $d.f. = 2$, p = n.s., respectively). When total distance moved was reanalyzed in 5-min bins, mixed ANOVA revealed a significant main effect of bin ($F_{(3.84,126.87)} = 88.18$, $p < 0.001$) and a significant bin × group interaction ($F_{(7.69,126.87)} = 3.36$, $p = 0.002$). One-way ANOVAs for each bin showed a significant main effect of group in the first bin ($F_{(2,33)} = 15.55$, $p < 0.001$) and a strong tendency in the second bin ($F_{(2,33)} = 3.02$, $p = 0.063$). Post hoc analysis for the first bin revealed that PA rats displayed significantly lower levels of horizontal locomotor activity than CTL and C+ rats did ($p < 0.001$ and $p = 0.023$, respectively, Fig. 3c). C+ rats showed an intermediate level of horizontal locomotor activity, being significantly higher than that of PA rats, as stated in previous sentence, and significantly lower than that of CTL rats ($p = 0.023$, Fig. 3c). In the second bin, post hoc multiple comparisons showed that PA rats continued showing a significantly reduced level of horizontal locomotor activity in comparison to CTL rats ($p = 0.05$, Fig. 3c), while C+ rats did not differ from CTL and PA rats (p = n.s. for both comparisons). Number of rearings was also reanalyzed in 5-min bins, showing a significant

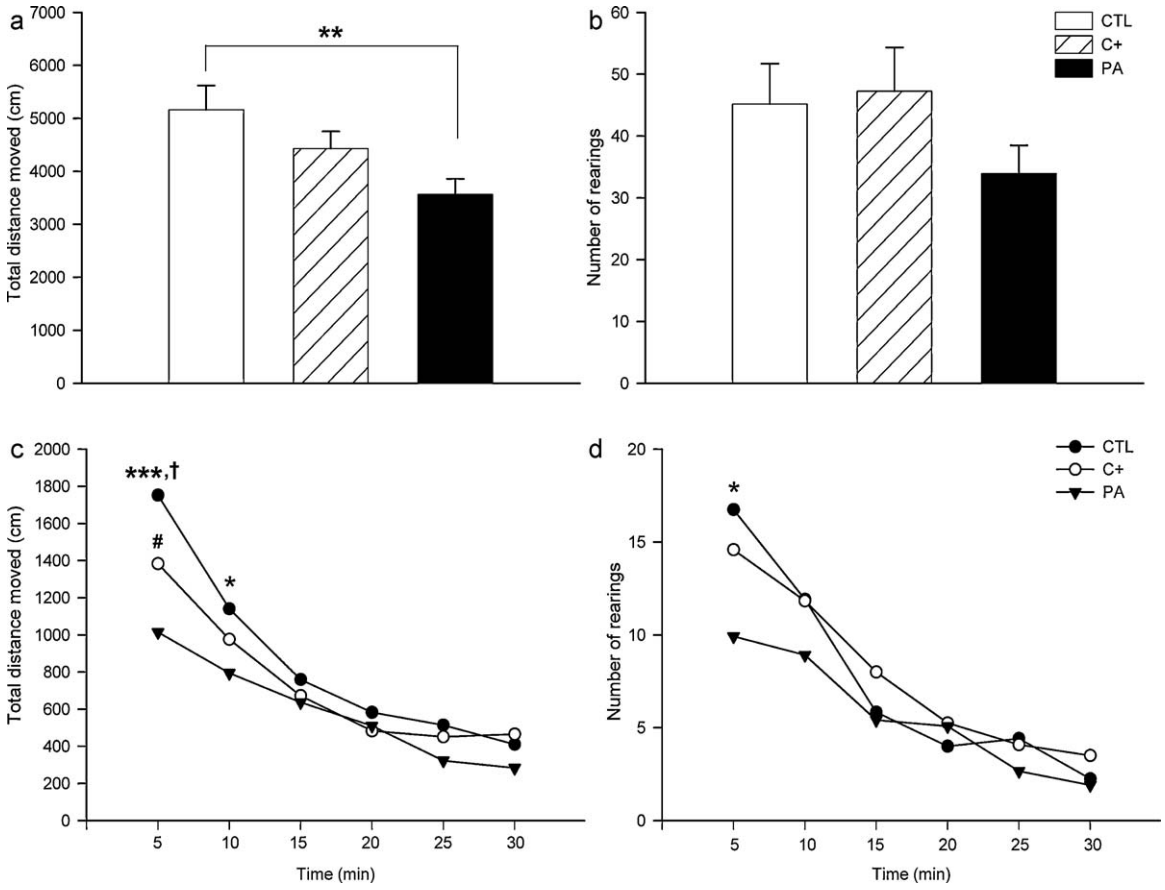

Fig. 3. Exploratory activity in open field test. Upper panels show the total horizontal locomotor activity (a) and the total number of rearing behaviors (b) displayed by experimental groups in the 30-min open field session. Below panels show horizontal locomotor activity and total number of rearing behaviors collected in 5-min bins. Experimental groups: Vaginal delivery rats (CTL, $n = 12$), rats born by cesarean section (C+, $n = 12$) and rats born by cesarean section + asphyxia (PA, $n = 12$). Data are expressed as mean + SEM, except in below panels (c and d) where SEM values were omitted for clarity. $^{*}p \leq 0.05$ for CTL vs. PA; $^{**}p = 0.01$ for CTL vs. PA; $^{***}p < 0.001$ for CTL vs. PA; $^{\#}p < 0.05$ for C+ vs. PA; $^{\dagger}p < 0.05$ for CTL vs. C+.

main effect of bin ($F_{(3.66,120,93)} = 53.53$, $p < 0.001$) and a strong tendency for the bin × group interaction ($F_{(7.33,120,93)} = 1.94$, $p = 0.066$). Only the one-way ANOVA for the first bin revealed to be significant ($F_{(2,33)} = 3.28$, $p = 0.05$), showing the post hoc tests that PA rats displayed a significantly less number of rearings in comparison to CTL rats ($p = 0.044$, Fig. 3d). C+ rats did not have a statistically different number of rearings relative to CTL and PA rats (p = n.s. for both comparisons, Fig. 3d). This reduced exploratory activity could not be ascribed to a increased time spent freezing (freezing duration), since groups did not differ in the time spent freezing neither during the first 5-min-time bin nor during the second 5-min time bin ($F_{(2,33)} < 1$, p = n.s.; $F_{(2,33)} < 1$, p = n.s, respectively, Supplementary Fig. 3a and b).

Finally, when variables "ratio central over total distance moved", "central area duration" (time spent in the central area), and "central area frequency" (number of entries into the central area), that measure anxiety levels in the OF, were analyzed in 5-min-time bins, no differences were found between groups in any time bin

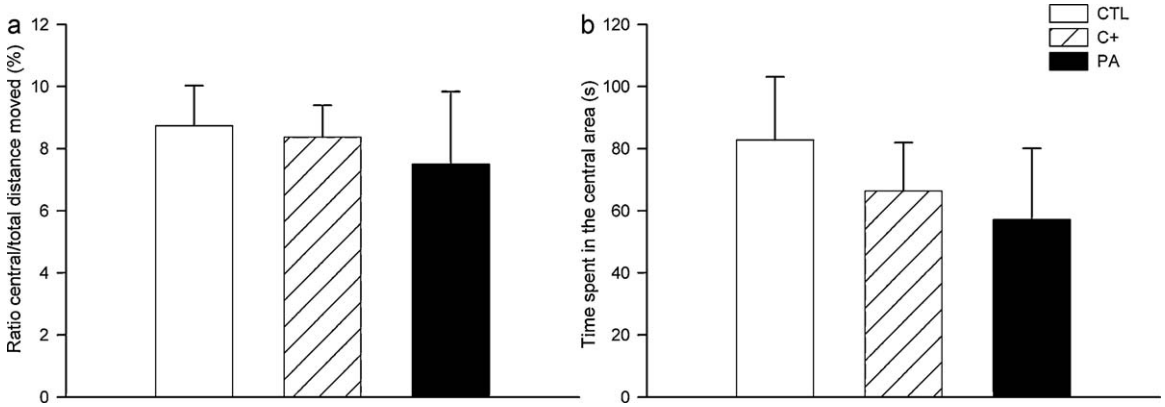

Fig. 4. Anxiety levels in the open field test. Panels show the ratio central over total distance moved (a) and the time spent in the central area ("central area duration") (b) averaged over the whole 30 min duration of the open field session. Experimental groups: Vaginal delivery rats (CTL, $n = 12$), rats born by cesarean section (C+, $n = 12$) and rats born by cesarean section + asphyxia (PA, $n = 12$). Data are expressed as mean +SEM.

for any variable (Variable "ratio central distance over total distance moved", bin 1: $H = 2.78$, p = n.s.; bin 2: $H = 0.68$, p = n.s.; bin 3: $H = 1.79$, p = n.s.; bin 4: $H = 1.87$, p = n.s.; bin 5: $H = 2.72$, p = n.s.; bin 6: $H = 1.31$, p = n.s.; Variable "central area duration", bin 1: $H = 2.73$, p = n.s.; bin 2: $H = 5.28$, p = n.s.; bin 3: $H = 2.00$, p = n.s.; bin 4: $H = 2.13$, p = n.s.; bin 5: $H = 4.58$, p = n.s.; bin 6: $H = 2.60$, p = n.s. Variable "central area frequency", bin 1: $H = 1.24$, p = n.s.; bin 2: $H = 1.95$, p = n.s.; bin 3: $H = 1.30$, p = n.s.; bin 4: $H = 0.10$, p = n.s.; bin 5: $H = 1.44$, p = n.s.; bin 6: $H = 0.14$, p = n.s., Supplementary Fig. 2b).

3.4. Spatial reference memory task

When latencies to reach the hidden escape platform were analyzed, the main effect of day and the interaction day × group revealed to be significant ($F_{(1.58,52.16)} = 151.83$, $p < 0.001$ and $F_{(3.16,52.16)} = 10.12$, $p < 0.001$, respectively). This indicates that not all groups improved their performance across days in the same manner. One-way ANOVAs revealed that the main effect of group factor was significant in the first and third day of acquisition of the task ($F_{(2,33)} = 5.84$, $p = 0.007$ and $F_{(2,33)} = 5.55$; $p = 0.008$, respectively). Post hoc multiple comparisons showed that during the first and third day PA rats spent significantly longer time to reach the hidden platform than CTL and C+ rats did (day 1: $p = 0.023$ for CTL vs. PA and $p = 0.011$ for C+ vs. PA; day 3: $p = 0.016$ for CTL vs. PA and $p = 0.02$ for C+ vs. PA, Fig. 5a). No differences were found between latencies of CTL and C+ rats in any day of acquisition (p = n.s. for all comparisons, Fig. 5a). The same pattern of results was observed when path lengths were analyzed, being both the main factor of day and the interaction day × group significant ($F_{(2,66.30)} = 257.99$, $p < 0.001$ and $F_{(4.02,66.30)} = 14.05$, $p < 0.001$, respectively). The main effect of group factor was also significant in the first and third day of acquisition, as it was revealed by one-way ANOVAs ($F_{(2,33)} = 5.89$, $p = 0.006$ and $F_{(2,33)} = 6.62$; $p = 0.004$, respectively). During the first and third day of acquisition, PA rats had significantly longer path lengths than CTL and C+ rats did (day 1: $p = 0.01$ for CTL vs. PA and $p = 0.024$ for C+ vs. PA; day 3: $p = 0.008$ for CTL vs. PA and $p = 0.011$ for C+ vs. PA, Fig. 5b). The path lengths of the latter two groups did not differ from each other in any day of the acquisition phase (p = n.s. for all comparisons, Fig. 5b). It is important to note that these results could not be attributable to confounding factors such as underlying sensorimotor deficits or differences in motivation to escape water, since one-way ANOVAs showed that swimming speed was not statistically different between the groups in any day (day 1: $F < 1$; day 2: $F_{(2,33)} = 1.75$, p = n.s.; day 3: $F < 1$; day 4: $F < 1$). During the probe trial, CTL and C+ rats spent a significantly longer time in the target quadrant than it is expected by chance (Mann–Whitney tests: $U = 0$, $p < 0.001$ and $U = 0$, $p < 0.001$, Fig. 5c) suggesting that they still remembered the location of the platform 24 h after the last acquisition trial. On the contrary, time spent by PA rats in the target quadrant did not differ from chance ($U = 72$, p = n.s., Fig. 5c). Thus, the bad performance shown by PA rats during acquisition and probe trial demonstrates that spatial learning and reference memory deficits are associated with perinatal asphyxia.

3.5. Spatial working memory

Analyses of the classical parameters, i.e. latency to reach the hidden escape platform and path lengths, showed the same pattern of results. In both cases, mixed ANOVA showed a significant main effect of the type of trial (sample and retention) (Latency: $F_{(1,33)} = 56.11$, $p < 0.001$; Path length: $F_{(1,33)} = 41.18$, $p < 0.001$) and a significant type of trial × group interaction (Latency: $F_{(2,33)} = 5.73$, $p = 0.007$; Path length: $F_{(2,33)} = 5.69$, $p = 0.008$). To investigate the source of the interaction, paired t-tests were conducted. Results

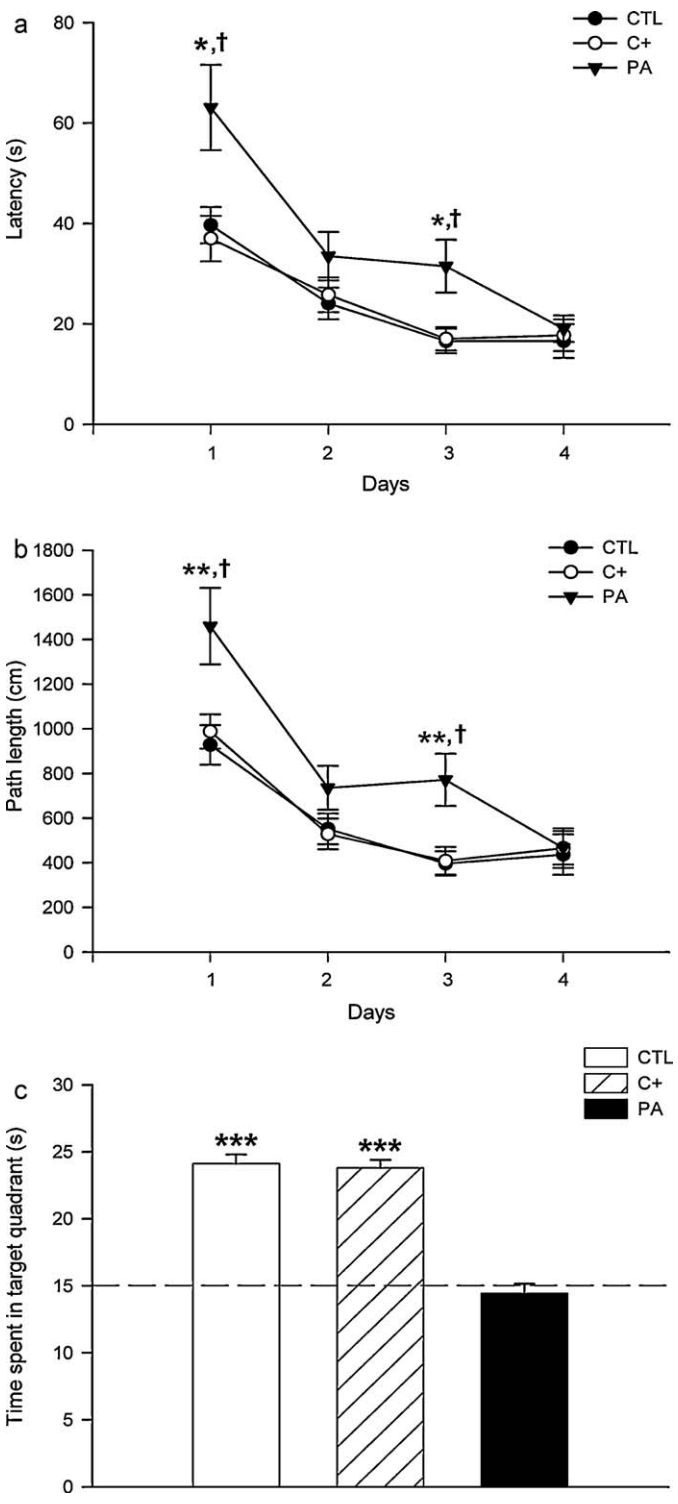

Fig. 5. Spatial reference memory in the Morris water maze. Latencies (a) and distances swam (b) to reach the hidden escape platform across the four days of the acquisition phase (spatial learning). For each training day, data were averaged across the four trials. Time spent (c) by rats in the quadrant where platform was located in acquisition sessions during the probe trial. The dashed line indicates the time expected by chance for rats to spend in any one of the four quadrants of the water maze during the 60 s probe trial. Experimental groups: Vaginal delivery rats (CTL, $n = 12$), rats born by cesarean section (C+, $n = 12$) and rats born by cesarean section + asphyxia (PA, $n = 12$). Data are expressed as mean ± SEM. $^{*}p < 0.05$ for CTL vs. PA; $^{**}p \leq 0.01$ for CTL vs. PA; $^{\dagger}p < 0.05$ for C+ vs. PA; $^{***}p < 0.001$ vs. time expected by chance.

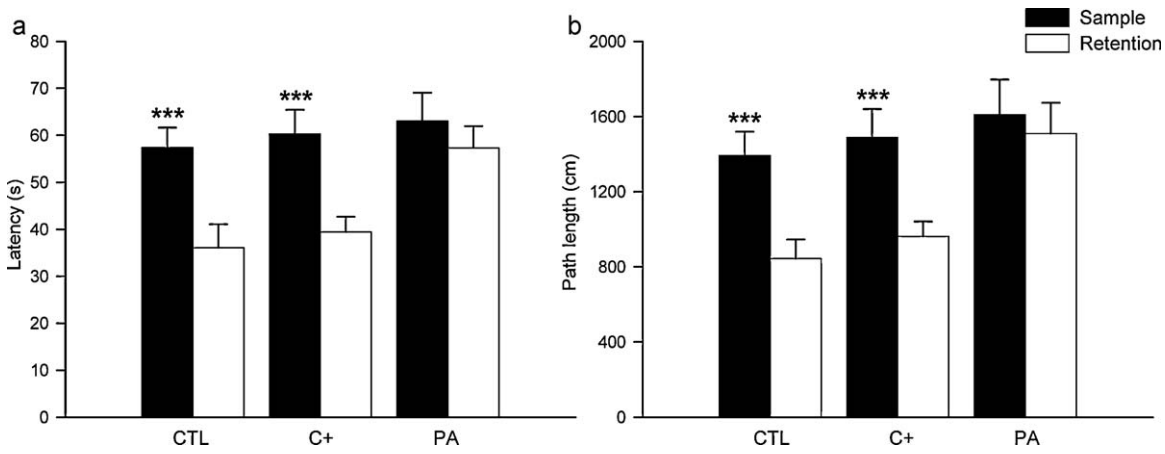

Fig. 6. Spatial working memory in the Morris water maze. Averaged latencies (a) and distance swam (b) to reach the hidden escape platform. Rats received two trials per day (sample and retention) for five consecutive days and location of the escape platform was held constant within days but varied across days. To reduce variability latencies and path lengths for each type of trial were averaged across days. Experimental groups: Vaginal delivery rats (CTL, $n = 12$), rats born by cesarean section (C+, $n = 12$) and rats born by cesarean section + asphyxia (PA, $n = 12$). Data are expressed as mean +SEM. $^{***}p < 0.001$ vs. retention trial.

revealed that during retention trials, both CTL and C+ rats displayed significantly shorter mean latency and path length in comparison to sample trials (Latency: $t = 5.19$, *d.f.* = 11, $p < 0.001$ for CTL rats; $t = 5.74$, *d.f.* = 11, $p < 0.001$ for C+ rats; Path lengths: $t = 5.71$, *d.f.* = 11, $p < 0.001$ for CTL rats; $t = 4.46$, *d.f.* = 11, $p = 0.001$ for C+ rats, Fig. 6a and b). This was not the case for PA rats which show no significant differences between sample and retention trials neither for the mean latency nor for the mean path length ($t = 1.74$, *d.f.* = 11, p = n.s.; $t = 0.98$, *d.f.* = 11, p = n.s., respectively, Fig. 6a and b). Analysis of the swimming speed by a mixed ANOVA showed that neither the main effect of type of trial nor the type of trial × group interaction were significant ($F < 1$ for both cases). Thus, PA rats were not able to remember the location of the platform in the sample trial, as efficiently as CTL and C+ rats did. This reveals a deficit in spatial working memory that is associated with perinatal asphyxia.

3.6. Incentive downshift

A mixed ANOVA with Group (CTL, C+ or PA) as between-subject factor and Trial (1–10) as within-subject factor indicated that during the pre-shift phase all groups increased their consumption of the 32% sucrose solution since the main effect of Trial was significant ($F_{(4.12,123.57)} = 29.56$, $p < 0.001$). Group × Trial interaction was not significant ($F_{(8.24,123.57)} = 0.508$, p = n.s.), indicating that no differences in the levels of consumption were found between experimental groups (Fig. 7a) and therefore differences in the post-shift phase could not be attributable to a more marked preference for sucrose solution by any particular group.

To analyze the effect of the surprising reduction in sucrose concentration (32%–4%) on different groups, amount of sucrose solution intake during the last pre-shift trial (10) was compared with those measured in post-shift trials (11–14) using paired *t*-tests corrected by Bonferroni method. During the first post-shift trial (11) a significant reduction in consumption was observed in all groups (CTL: $t = 21.67$; C+: $t = 23.24$; PA: $t = 5.36$; *d.f.* = 10 and $p < 0.01$ for all cases) (Fig. 7a). From the second to the fourth post-shift trials (12–14), quantities consumed of 4% sucrose solution by PA rats were not statistically different from that consumed during the last pre-shift trial (p = n.s. for all comparisons). On the contrary, from the second to the fourth post-shift trials, CTL and C+ rats still displayed a significant reduction of consumption in comparison to the last pre-shift trial (Trial 10 vs. 12: $t = 8.36$ (CTL), $t = 8.92$ (C+); Trial 10 vs. 13: $t = 7.08$ (CTL), $t = 6.62$ (C+); Trial 10 vs. 14: $t = 4.77$ (CTL), $t = 4.46$ (C+); *d.f.* = 10 and $p < 0.01$ for all cases, Fig. 7a). In the first post-shift trial (11), we conducted a one-way ANOVA to compare the amount of consumption of the 4% sucrose solution between groups ($F_{(2,30)} = 5.81$, $p = 0.007$). Post hoc comparisons showed that PA rats consumed a higher amount of the 4% sucrose solution than CTL ($p < 0.01$) and C+ rats ($p < 0.05$).

For the assessment of the behaviors displayed by groups during the first and second post-shit trials (11 and 12), one-way ANOVAs followed by Tukey HSD post hoc comparisons were conducted. For both trials, the main effect of group was significant for the time spent in contact with the spout (Trial 11: $F_{(2,29)} = 10.24$, $p < 0.01$; Trial 12: $F_{(2,30)} = 11.71$, $p < 0.01$), rearing duration (Trial 11: $F_{(2,29)} = 4.76$, $p = 0.02$; Trial 12: $F_{(2,30)} = 12.62$, $p < 0.01$) and locomotion (Trial 11: $F_{(2,29)} = 6.14$, $p < 0.01$; Trial 12: $F_{(2,30)} = 7.85$, $p < 0.01$). Post hoc analyses revealed that, during both post-shift trials, PA rats spent significantly more time in contact with spout than CTL and C+ rats did ($p < 0.01$ for all comparisons, Fig. 7b and c). Regarding rearing and locomotion, PA rats spent significantly less time engaged in those behaviors than CTL and C+ rats did in both post-shift trials ($p < 0.05$ for all comparisons, Fig. 7b and c).

4. Discussion

4.1. Anxiety-related behaviors

Based on the results obtained in the OF and EPM we could conclude that experimental groups did not differ in anxiety-related behaviors, consistent with studies by Boksa et al. (1998) and Strackx et al. (2010). Although, it is important to note that a large variability was observed in variables such as "ratio central over total distance moved" (Fig. 4a), "central area duration" (Fig. 4b) and "central area frequency" (Supplementary Fig. 2). Morales et al. (2010) and Hoeger et al. (2000) using the same model of perinatal asphyxia, showed some differences with our observations. Morales et al. (2010) found an enhanced anxiety in rats that had undergone 20 min of asphyxia at birth. The duration of asphyxia is a critical factor in the model used in these studies, since survival rate is drastically reduced and CNS damage increases with the duration of asphyxia (Capani et al., 1997, 2009; Loidl et al., 2000). It could be possible that 20 min of asphyxia, but not 19 min, produce enough damage to disclose anxiety-related behaviors in spite of large inter-individual differences. Another possibility is that rats that had undergone 20 min of asphyxia at birth show more homogeneous behaviors in anxiety tests than rats with 19 min of perinatal asphyxia. Regarding the study by Hoeger et al. (2000), who found a reduction of anx-

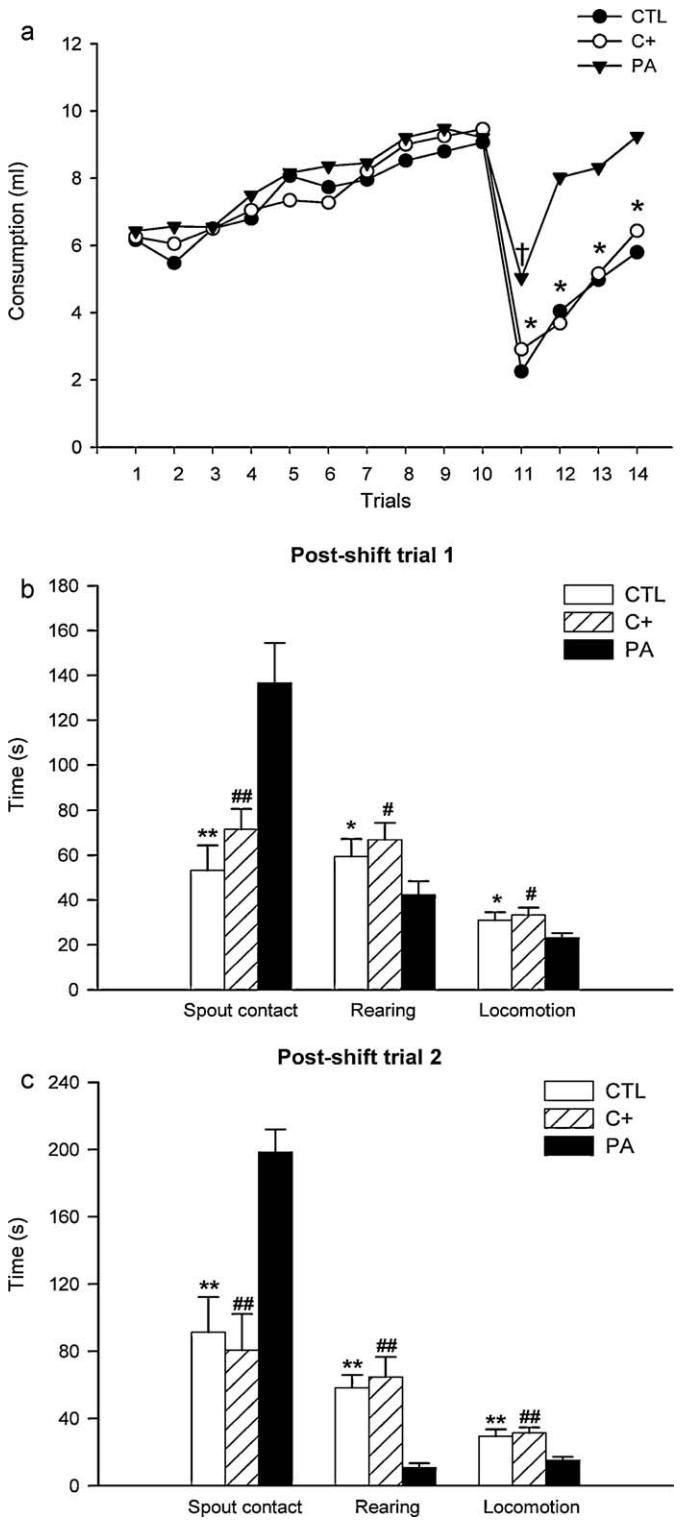

Fig. 7. Incentive downshift protocol. (a) Consumption of a 32% sucrose solution from trial 1 to 10 (pre-shift trials). In trial 11 (first post-shift trial), rats were exposed to an unexpected downshift from 32% to 4% sucrose solution. From trial 12 to 14 (second to fourth post-shift trials) rats continued receiving the devaluated reward (4% sucrose solution). (b and c) Mean duration engaged by rats in different behaviors during first and second post-shift trial. Experimental groups: Vaginal delivery rats (CTL, $n = 10$–11 see text), rats born by cesarean section (C+, $n = 11$) and rats born by cesarean section + asphyxia (PA, $n = 11$). (a) Data are expressed as mean. SEMs were omitted for clarity. $^{*}p < 0.01$ vs. consumption in trial 10 for CTL and C+ rats, † indicate both: $p < 0.01$ consumption in trial 10 vs. 11 for PA rats, and $p \leq 0.05$ consumption of PA rats vs. consumption of CTL and C+ rats during trial 11. (b and c) Data are expressed as mean +SEM. $^{*}p < 0.05$ and $^{**}p < 0.01$ for CTL vs. PA rats; $^{\#}p < 0.05$ $^{\#\#}p < 0.01$ for C+ vs. PA rats.

iety in PA rats exposed to EPM, it is important to note that only female animals were used. It has been reported that female rats show less anxiety in the EPM (Johnston and File, 1991), and also that the behavior of both sexes in this task is controlled by different factors, so results obtained with females may not be comparable with those obtained with males (Fernandes et al., 1999). Moreover, perinatal asphyxia has been found to differentially affect both sexes (Loidl et al., 2000). Summarizing, our results does not support the hypothesis that 19 min of perinatal asphyxia is associated with anxiety-related behaviors at adulthood, although the evidence is not completely conclusive. Since contradictory results have also been reported further studies are needed to clarify this issue.

4.2. Locomotor activity and novelty exploration

The reduction in horizontal and vertical locomotor activity, displayed by PA rats, is in accordance to many other studies (Chen et al., 1995; Loidl et al., 2000; Hoeger et al., 2006; Strackx et al., 2010; Van de Berg et al., 2003). The fact that the diminished locomotor activity in OF took place during the first 10 min and also that the number of rearings was only significantly reduced in the first 5 min allows us to hypothesize that rather than a motor impairment, PA rats displayed less motivation/curiosity to explore novel environments as it was also suggested by Strackx et al. (2010). When normal rats are exposed to novel environments, an enhancement in both horizontal and vertical locomotor response can be seen (novelty exploration), although important individual differences exist in this kind of behavioral response (Piazza et al., 1989). PA rats would seem to have a deficit in novelty exploration response. To a much lesser extent, C+ rats would also seem to show this kind of deficit, since they displayed a significantly less horizontal locomotor activity during the first 5 min of exposition to OF.

4.3. Spatial reference and working memory impairments

It is well known that the performance in spatial tests, such as reference and working memory tasks in the Morris Water Maze, is disrupted after hippocampal damage (Cassel et al., 1998). One of the most affected cerebral areas following perinatal asphyxia is the hippocampus (Kohlhauser et al., 1999; Morales et al., 2010; Saraceno et al., 2010) and, therefore, we hypothesized that PA rats would display spatial memory deficits. Our results provide support to this hypothesis. During the acquisition phase of the reference memory task, PA rats showed an impairment in spatial learning, with longer escape latencies and path lengths in the first and third day of training. In the most complete study about spatial learning in asphyctic rats, Boksa et al. (1995) also found deficits in spatial learning in 4-month-old rats that had undergone 10–20 min of asphyxia. However, Boksa et al. (1995) did not find differences in performance between CTL, C+ and PA rats, during the probe trial. In this study, we showed a reference memory deficit since PA rats did not search for the hidden escape platform, during the probe trial, an amount of time different from that expected by chance. Several methodological differences could account for this discrepancy. For instance, Boksa et al. (1995) submitted animals to eight acquisition sessions, while we used only four. When more acquisition sessions are employed, ceiling effects could mask deficits in reference memory. Also, the Morris water maze used in the present study had a longer diameter than that used by Boksa et al. (1995) (180 cm vs. 136 cm). Performance in the Morris water maze showed to be sensitive to variations in the diameter of the apparatus (Vorhees and Williams, 2006). In addition, we assessed reference memory 24 h after the last acquisition trial, while Boksa et al. (1995) assessed it after the second trial of the eighth session. So, it is possible that the apparatus and the procedures used in the present study to

assess reference memory were more sensitive to detect differences between experimental groups.

We also found that PA rats were unable to solve a spatial working memory task as efficiently as control and cesarean section rats did. As far as we know, this is the first time spatial working memory impairment is reported in this animal model. Considering the results in the reference memory task, we could ascribe the deficit in this test to its spatial component. Although this hypothesis could not be ruled out, it is important to note that adult rats that had undergone severe perinatal asphyxia also showed a disrupted performance in the novel object recognition task, which also assess working memory but it does not require the spatial memory component (Simola et al., 2008; Strackx et al., 2010). The ability to solve working memory tasks has been related to dopaminergic neurotransmission in the prefrontal cortex (Sawaguchi and Goldman-Rakic, 1991; Seamans et al., 1998; Simon et al., 1980) and it has also been demonstrated that perinatal asphyxia can produce long-lasting changes in dopaminergic function (Boksa and El-Khodor, 2003). Interestingly, Brake et al. (2000) reported a hyporesponsiveness of the dopaminergic neurotransmission in the right medial prefrontal cortex (mPFC), when adult asphyctic rats were submitted to a once-daily stress protocol. Exposure to the water maze implies a certain level of stress, and therefore, it could be hypothesized that the alteration of the stress-induced dopaminergic transmission in the right mPFC could be associated with the poor performance in the spatial working memory task. This hypothesis remains to be tested by further studies.

Additionally, it is important to note that despite PA anc C+ rats showed diminished horizontal locomotor activity in EPM and OF, no differences in swimming speed were found in the Morris water maze and thus, spatial deficits could not be attributable to differences in swimming abilities and/or motivation to solve the task. This is not surprising, since it has been showed that land-based locomotor reductions did not affect swimming speed (Vorhees and Williams, 2006). Finally, the deficits found in spatial tasks seem to be specifically associated to the acute asphyxia at birth because C+ rats, like CTL rats, showed normal performance in both tests.

4.4. Attenuated behavioral response to incentive downshift

The main finding of this test was that PA rats did not reject the devaluated reward to the same extent as CTL and C+ rats did, when they were downshifted from a 32% to a 4% sucrose solution. The analyses of the behaviors displayed by experimental groups during post-shift trial 1 and 2 (Fig. 7a and b) confirmed the results obtained by measurement of sucrose solution intake. For instance, PA rats spent significantly more time in contact with the spout, which is in accordance with their higher consumption of the 4% sucrose solution. The reduction in rearing and locomotion is expected because these behaviors are somewhat incompatible with the increased time in spout contact. The enhanced rearing and locomotor activity of CTL and C+ rats could be interpreted as a searching for the missing 32% solution (Flaherty, 1996).

Based on many experimental findings, it has been proposed that a complex interplay between emotional and cognitive processes could account for the exaggerated reduction of intake after surprising incentive downshift (Flaherty, 1996; Papini, 2003). For instance, unexpected downshift from 32% to 4% sucrose activates the HPA axis (Pecoraro et al., 2009) and elevates corticosterone levels (Mitchell and Flaherty, 1998). Moreover, corticosterone administration after the first post-shift trial enhanced the exaggerated suppression of intake that takes place after the incentive downshift (Bentosela et al., 2006; Ruetti et al., 2009) and anxiolytic treatment reduced the behavioral response to the devalued reward (Flaherty et al., 1986; Mustaca et al., 2000). Taking into account our data, we could not ascribe the attenuated behavioral response to incentive downshift to reduced anxiety levels, since we were able to find group differences neither in the EPM nor in the OF with regard to this variable (see Section 4.1 for discussion about this issue). However, it is important to note that we did not measure anxiety levels after the animals were exposed to a potentially stressful situation, such as an unexpected devaluation in reward value. Boksa et al. (1996) found a diminished corticosterone secretion after restrain stress in rats subjected to mild perinatal asphyxia (10 min and 15 min of anoxia). In contrast, Strackx et al. (2010) found no differences, relative to control animals, neither at behavioral level nor in corticosterone response, when adult rats that had undergone 19 min of asphyxia were exposed to stressful conditions (forced swim test and restrain stress). It has been proposed that when changes in the quality or quantity of a reward occur, the memory of the pre-shift reward is reactivated and compared with the current downshifted reward, triggering an approach-avoidance conflict that finally leads the animal to reject the new reward (Amsel, 1992). In this and other studies mentioned above, different kinds of memory and learning deficits were found, therefore we could hypothesize that some of the cognitive processes required to compare the pre- and post-shift rewards are disrupted in PA animals, not even allowing that the approach-avoidance conflict triggers. If this happens, since animals are food deprived, they will not reject the downshifted reward. However, it is worth mentioning that rejection of the 4% sucrose solution was detected in PA rats in the first post-shift trial, although to a much lesser extent compared with CTL and C+ rats. Additionally, from second to fourth post-shift trials the amount of 4% sucrose solution consumed by PA rats did not statistically differ from the amount of 32% sucrose solution consumed in the last pre-shift trial. Other experimental studies must be conducted to establish which specific processes underlie the attenuated behavioral response to incentive downshift displayed by PA rats.

5. Conclusions

The main findings of the present study are that 3-month-old male rats that had undergone a moderate to severe (19 min) asphyxia during cesarean section at birth showed reduced exploration when faced to a novel environment, spatial reference and working memory deficits and an attenuated behavioral response to incentive downshift. In addition, animals born by cesarean section displayed a mild deficit in exploration. These results confirmed and extend those previously reported about the long-lasting behavioral consequences of perinatal asphyxia.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ijdevneu.2011.05.002.

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