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Effects of early malnutrition, isolation and seizures on memory and spatial learning in the developing rat

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ABSTRACT

Purpose: In this study we evaluated the effects of undernourishment and seizures on memory and spatial learning in a model of developing brain.
Experimental procedures: Male Wistar rat pups were allocated to one of six experimental groups: nourished control (NC), nourished recurrent seizures (NRS), nourished status epilepticus (NSE), undernourished control (UC), undernourished recurrent seizures (URS) or undernourished status epilepticus (USE). The UC, URS and USE groups were maintained on a starvation regimen from postnatal day 2 (P2) to postnatal day 15 (P15). URS and NRS groups suffered three daily Flurothyl-induced seizures from P2 to P4. The USE and NSE groups suffered a status epilepticus (SE) on P15. Beginning on P21 all groups were trained in the Morris water maze. At P30 the animals were sacrificed and their brains weighed.
Results: Our data indicate that early undernourishment does not alter seizure susceptibility at P15, but diminishes body and brain weight ($p < 0.001$), whereas seizures diminish body ($p < 0.001$) but not brain weight ($p = 0.972$). In the Morris water probe test we have observed that undernourished rats spent less time in the target quadrant than nourished animals ($p < 0.001$). Also, rats submitted to recurrent seizures and rats submitted to status epilepticus spent less time in the target quadrant than seizure-free animals ($p = 0.001$). There was a significant interaction between undernourishment and seizure ($p = 0.013$).
Discussion: Our findings show that undernourishment and seizures have an additive detrimental effect on body and brain weight as well as on spatial memory.
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Undernourishment is responsible for serious physiological and morphological changes in the developing central nervous system (Huang et al., 2003; Hoffmann et al., 2004). In rodents, the adverse effects of poor nutritional status include changes in neurotransmitter release, decrease in brain size and altered neuroplasticity (Morgane et al., 2002; Rotta et al., 2003). Early malnutrition, during the prenatal and lactation periods, affects spatial memory in rats (Jordan et al., 1981; Huang et al., 2003).

Undernourishment is not a direct cause of epilepsy (Palencia et al., 1996), but in previous studies it is has been reported that it might reduce the seizure-induction threshold (Stern et al., 1974; Palencia et al., 1996). Several reports using animal models have suggested that epilepsy and undernourishment are related (Taber et al., 1980; Bronzino et al., 1986, 1990; Gietzen et al., 1996; Palencia et al., 1996). However, a cause–effect relationship between them has not yet been established. Indeed, few experimental studies have evaluated undernourishment and seizures in relation to memory deficit and none show a direct interaction between these conditions and cognition (Huang et al., 2003; Akman et al., 2004).

Considering the epidemiological importance of early and prenatal malnutrition and seizures on cognitive aspects, we decided to analyze whether undernourishment and seizures interact to modify their functional effects in the rat's developing brain. In this experiment we evaluate the effect of nutritional status on seizure susceptibility, brain and body weight as well as on spatial learning and memory following early recurrent seizures and induction of status epilepticus. To provoke early recurrent seizures and status epilepticus , we used Flurothyl [bis(2,2,2-trifluoroethyl)ether] (99% min.), a volatile convulsive agent that rapidly stimulates the central nervous system (CNS), inducing generalized seizures (Truitt et al., 1960; Velísková et al., 1996). To properly evaluate spatial learning and memory, we used the Morris water maze, which has often been used in the validation of rodent models for neurocognitive disorders.

1. Experimental procedures

The experiments were conducted under conditions approved by the Scientific and Research Ethics Committees of the Pontificia Universidade Católica do Rio Grande do Sul (PUCRS) regarding animal welfare. Twelve (12) pregnant female Wistar rats from our breeding colony were maintained on a 12-h dark–light cycle with food and water freely available. After delivery, each dam with the litter was housed individually. Each litter was culled to 10 pups. The day of birth was counted as P0. All

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animals were weaned at P21. The entire litters were assigned to specific groups. The litters were randomly allocated in to six groups, using only male pups (NC: nourished control) ($n = 12$); NRS: nourished recurrent seizures ($n = 12$); NSE: nourished status epilepticus ($n = 12$); UC: undernourished control ($n = 12$); URS: undernourished recurrent seizures ($n = 17$); USE: undernourished status epilepticus ($n = 12$).

1.1. Flurothyl-induced seizures

To provoke early recurrent seizures and status epilepticus, we used Flurothyl [bis(2,2,2-trifluoroethyl)ether] (99% min.), a volatile convulsive agent that rapidly stimulates the central nervous system (CNS), inducing generalized seizures (Truitt et al., 1960; Velísková et al., 1996). Animals were challenged with Flurothyl (20 μL/min constant flow rate) in an air-tight chamber (9.38 L) to provoke two different models of seizures: early recurrent seizures and status epilepticus. Latency to seizure was considered the time from first exposure to Flurothyl until the onset of the first seizure. Animals from the NRS and URS groups were submitted to early recurrent seizures-3 exposures of Flurothyl per day (1 h inter-exposure interval) from P2 to P4. Each time, the animals were exposed to the constant flow rate until the appearance of the first clonic seizure. For all animals, the exposure to Flurothyl took place immediately after separation from their mother. Immediately after that, the animals were returned to their home cages. Rats from the NSE and USE groups were submitted to status epilepticus by exposure of Flurothyl at P15. The animals were exposed to the constant flow rate for 20 min, after which they spent 10 more minutes inside the drug filled chamber. In all the SE groups, the time of the first clonic seizure was recorded in order to calculate the seizure threshold. However, while in the NRS and URS groups the animals were removed from the chamber right after the seizure, in the NSE and USE groups the animals continued to receive Flurothyl for 20 min despite the seizures. Control animals were exposed to the same procedure except that water was used instead of. After the experiment, the animals were returned to their original litters.

1.2. Undernourishment paradigm

The undernourishment paradigm consisted of limiting the offspring's access to nutrition by removing the dams from the cage starting at P2. The deprivation period was increased by 2 h for 6 consecutive days, from 2 h on P2 to 12 h on P7. The deprivation period remained at 12 h/day for the next 8 days (P8 to P15). This method of food deprivation has been successfully used before, despite the neonatal isolation stress (Nunes et al., 2000). During deprivation, pups remained in a light heated cage, with room temperature maintained at 34 °C (measured with a thermometer placed in the room). After the deprivation period, the pups were housed with their respective dams. Age-matched control rats remained with their dams. Body weights were measured daily, first thing in the morning, before any intervention, from P2 to P30.

1.3. Training in the spatial version of the Morris water maze

To properly evaluate spatial learning and memory, we used the Morris water maze, which has often been used in the validation of rodent models for neurocognitive disorders (Rossato et al., 2006). The observer was blind to the protocol.

Before the experiment, we carried out a pilot study with 10 well nourished pups at 20 days of life and all of them were able to learn how to find the hidden platform.

The experimental groups were trained in the spatial version of the Morris water maze from P21 to P25. The maze consisted of a black circular pool (100-cm in diameter) conceptually divided into 4 equal imaginary quadrants for the purpose of data analysis. The water temperature was 24 °C. One and a half cm beneath the surface of the water and hidden from the rat's view was a black circular platform (8 cm in diameter). It had a rough surface, which allowed rats to climb onto it easily. The water maze was located in a well-lit white room with several posters and other distal visual stimuli hanging on the walls to provide spatial cues. A curtain separated the water maze room from the room where the computer was setup and where the animals were temporarily housed during the behavioral sessions. Training in the hidden platform (spatial) version of the Morris water maze was carried out during 5 consecutive days, as previously described (Rossato et al., 2006). On each day, rats received 4 consecutive training trials during which the hidden platform was kept in a constant location. A different starting location was used in each trial, which consisted of a swim followed by a 60-s platform sit. Any rat that did not find the platform within 60 s was guided to it by the experimenter. The inter-trial interval was 30 s. During the inter-trial interval, rats were carefully dried with a towel by the experimenter.

Memory retention was evaluated in a 60-s probe trial carried out in the absence of the escape platform 24 h after the last training session. Data (latency to reach the platform and time spent in each quadrant) were measured by a single person with a chronometer and analyzed using one-way or multi-way ANOVA followed by post hoc tests, as appropriate.

At P30 animals were sacrificed with a lethal dose of thiopental sodium (0.3 mL/100 g, i.p.). The brains were sectioned immediately below the cerebellum and weighed on a digital scale.

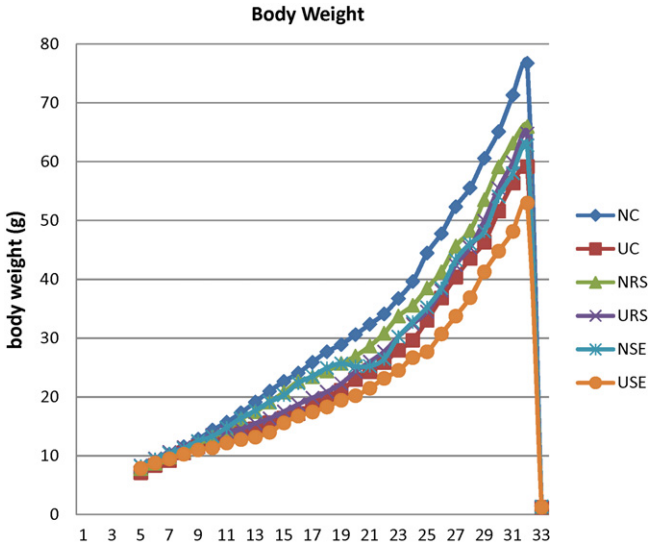

Fig. 1. Comparison of growth curves among nourished total (NT), nourished control (NC), nourished recurrent seizures (NRS), nourished status epilepticus (NSE) undernourished total (UT), undernourished control (UC), undernourished recurrent seizures (URS) and undernourished status epilepticus (USE) groups, between P2 and P29, in Wistar rats.

1.4. Statistical analysis

The data from the seizure susceptibility experiments were analyzed with the aid of the Student's *t*-test. Two-way ANOVA was used to analyze body and brain weights. Two- and three-way ANOVA were employed to analyze spatial memory retention and acquisition, respectively. Values are expressed as mean ± SEM. Statistical significance was defined as $p < 0.05$ for all tests.

2. Results

2.1. Body weight

Fig. 1 shows the growth curve of all groups between P2 and P29.

Fig. 2 shows that the mean body weight gain between P2 and P15 was lower in undernourished (UC, URS and USE groups; 11.9 ± 0.2 g) than in nourished animals (NC, NRS and NSE groups; 17.8 ± 0.2 g; $F_{1;71} = 362.26$, $p < 0.001$). Likewise, body weight gain was lower in rats submitted to recurrent seizures from P2 to P4 (URS and NRS groups; 14.6 ± 0.3 g) or to status epilepticus at P15 (USE and NSE groups; 13.6 ± 0.3 g) than in control animals (UC and NC groups; 16.3 ± 0.3 g; $F_{2;71} = 25.25$, $p < 0.001$). Two-way ANOVA analysis indicated an interaction between undernourishment and seizures ($F_{2;71} = 10.70$, $p < 0.001$).

2.2. Brain weight

Fig. 3 shows that the brain weight at P30 was lower in undernourished (UC, URS and USE groups; 1.26 ± 0.20 g) than in nourished animals (NC, NRS and NSE groups; 1.38 ± 0.20 g; $F_{1;71} = 53.63$, $p < 0.001$). However, there was no difference in brain weight between the control animals (NC and UC groups; 1.32 ± 0.14 g) and the rats submitted to either recurrent seizures from P2 to P4 (NRS and URS groups; 1.32 ± 0.13 g), or to status epilepticus at P15 (NSE and USE groups; 1.32 ± 0.14 g; $F_{2;71} = 0.02$, $p = 0.972$). Two-way ANOVA analysis indicated an interaction between undernourishment and seizures ($F_{2;71} = 9.85$, $p < 0.001$).

2.3. Seizure susceptibility

Among the animals who suffered recurrent seizures there was no noticeable change in onset time over the days or the individual

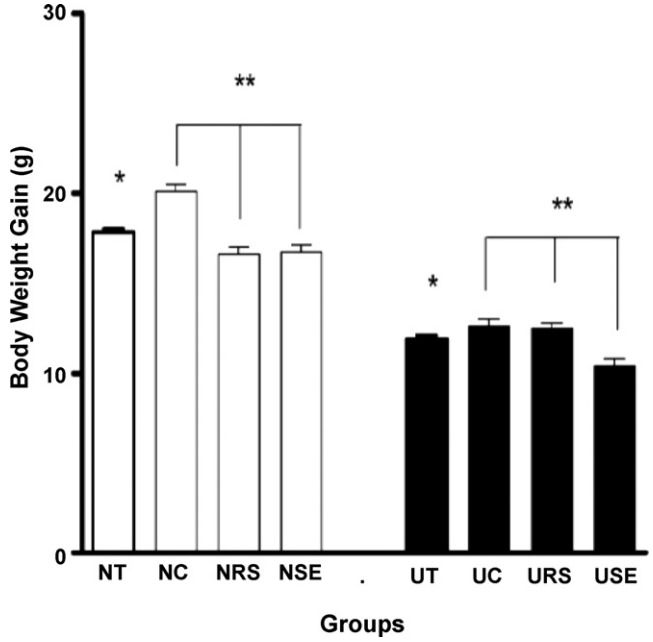

Fig. 2. Comparison of body weight gain among nourished total (NT), nourished control (NC), nourished recurrent seizures (NRS), nourished status epilepticus (NSE) undernourished total (UT), undernourished control (UC), undernourished recurrent seizures (URS) and undernourished status epilepticus (USE) groups, between P2 and P15, in Wistar rats. Effect of undernourishment on body weight gain ($^{*}p_{[\text{undernourishment}]} < 0.001$). Effect of seizure on body weight gain ($^{**}p_{[\text{seizure}]} < 0.001$). Interaction undernourishment × seizure ($p_{[\text{undernourishment} \times \text{seizure}]} < 0.001$).

episodes of Flurothyl exposure between the nourished and undernourished groups.

Student's *t*-test analysis revealed no difference in the mean time to seizure onset between the NSE (7.45 ± 0.37 min) and the USE (7.88 ± 0.41 min) groups on P15.

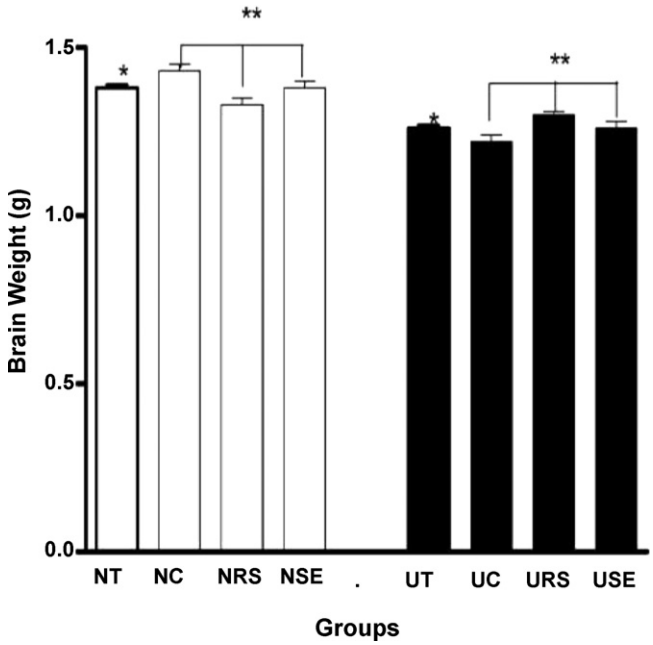

Fig. 3. Comparison of brain weight among nourished total (NT), nourished control (NC), nourished recurrent seizures (NRS), nourished status epilepticus (NSE) undernourished total (UT), undernourished control (UC), undernourished recurrent seizures (URS) and undernourished status epilepticus (USE) groups, in P30, in Wistar rats. Effect of undernourishment on brain weight ($^{*}p_{[\text{undernourishment}]} < 0.001$). Effect of seizure on brain weight ($^{**}p_{[\text{seizure}]} = 0.972$). Interaction undernourishment × seizure ($p_{[\text{undernourishment} \times \text{seizure}]} < 0.001$).

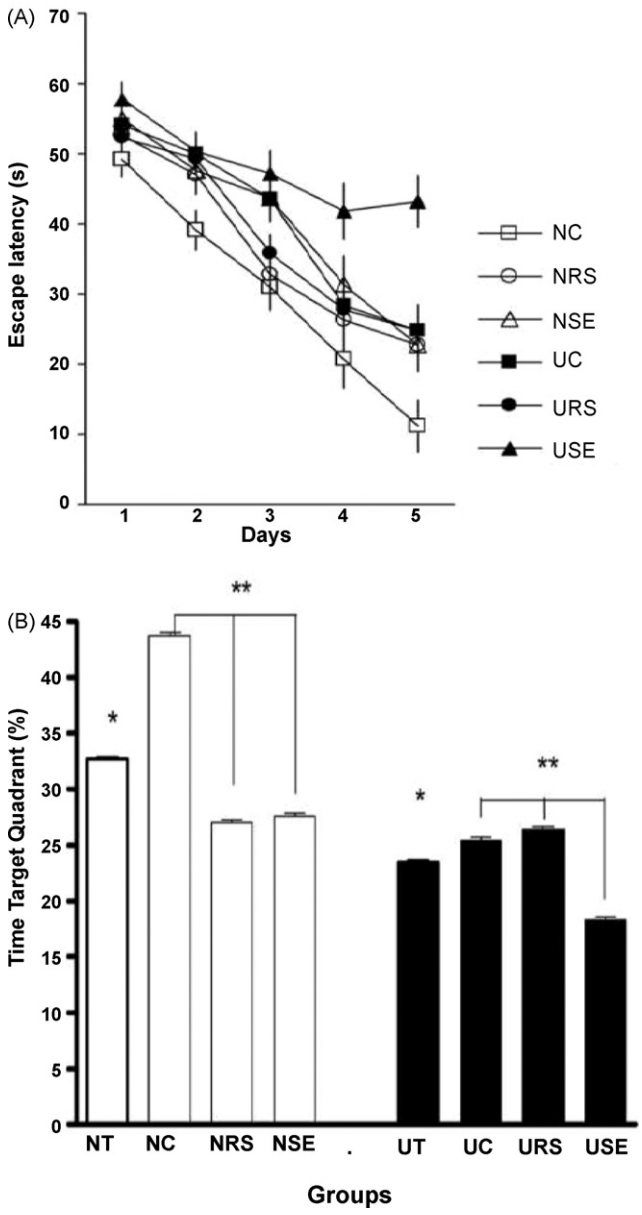

Fig. 4. Comparison of the performance in the MWM task among nourished total (NT), nourished control (NC), nourished recurrent seizures (NRS), nourished status epilepticus (NSE) undernourished total (UT), undernourished control (UC), undernourished recurrent seizures (URS) and undernourished status epilepticus (USE) groups. (A) Acquisition test. The mean escape latencies to the hidden platform in MWM as a function of day. Effect of undernourishment on escape latency ($p_{[\text{undernourishment}]} < 0.001$). Effect of seizure on escape latency ($p_{[\text{seizure}]} < 0.001$). Interaction undernourishment × seizure ($p_{[\text{undernourishment} \times \text{seizure}]} < 0.096$). (B) Probe Test. The percentage of time spent in the target quadrant where the platform was located during the acquisition test were NT 0.00132.7 ± 1.7%; NC 43.7 ± 2.9%; NRS 27.0 ± 2.9%; NSE 27.6 ± 2.9; UT 23.4 ± 1.7; UC 25.4 ± 3.2; URS 26.4 ± 2.4; USE 18.3 ± 2.9%. Effect of undernourishment on the percentage of time spent in the target quadrant ($^{*}p_{[\text{undernourishment}]} < 0.001$). Effect of seizure on the percentage of time spent in the target quadrant ($^{**}p_{[\text{seizure}]} = 0.001$). Interaction undernourishment × seizure ($p_{[\text{undernourishment} \times \text{seizure}]} = 0.013$).

2.4. Morris water maze

Fig. 4(A) shows the mean escape latencies to the hidden platform during the 5 days of training in the Morris water maze. Multi-factorial ANOVA analysis indicates that the escape latency decreased in all experimental groups as training progressed ($F_{3.5;247.3} = 116.5$, $p < 0.001$). Nevertheless, this reduction was more evident in nourished than in undernourished animals ($F_{1;71} = 14.0$,

$p < 0.001$) and in seizure-free animals than in animals submitted to recurrent seizures or to a single status epilepticus ($F_{2;71} = 9.6$, $p < 0.001$). The analysis indicated no interaction between undernourishment and seizures ($F_{2;71} = 2.42$, $p = 0.096$). As can be seen in Fig. 4(B), undernourished rats spent less time in the target quadrant (UC, URS and USE groups; $23.4 \pm 1.7\%$) than nourished animals (NC, NRS and NSE groups; $32.7 \pm 1.7\%$) ($F_{1;69} = 15.33$, $p < 0.001$) during a probe test performed on P26 (i.e. 1 day after the last Morris water maze training session). In the same way, rats submitted to recurrent seizures (URS and NRS groups; $26.7 \pm 1.9\%$) and rats submitted to status epilepticus (USE and NSE groups; $23.0 \pm 2.0\%$) spent less time in the target quadrant than seizure-free animals (UC and NC groups; $34.5 \pm 2.1\%$) ($F_{2;69} = 7.57$, $p = 0.001$). There was a significant interaction between undernourishment and seizure ($F_{2;69} = 4.62$, $p = 0.013$).

3. Discussion

In this report we present evidence showing that undernourishment from postnatal day 2 (P2) to 15 (P15) reduces body and brain weight gain and impairs spatial memory without affecting the seizure threshold in the Flurothyl model. We also found that undernourishment and seizures have an additive detrimental effect on body weight and learning. Interestingly this effect was not observed in brain weight, as malnourished animals submitted to status or recurrent or both types of seizures had brain weights higher than undernourished rats not submitted to seizures.

It has been shown that animals undernourished "in utero" or during the lactation period are more susceptible to the development of seizures in adulthood (Taber et al., 1980; Bronzino et al., 1986, 1990; Gietzen et al., 1996; Palencia et al., 1996). Although Flurothyl is a reliable agent to elicit convulsions, our findings indicated that nourished and undernourished rats were equally susceptible to Flurothyl-induced status epilepticus. This discrepancy was also reported in previous studies employing a similar model (Nunes et al., 2000, 2002). We decided to use Flurothyl in our experiment because, in contrast to other convulsant agents, it does not cause spontaneous seizures, something that could complicate the interpretation of behavioral experiments in the Morris water maze.

We chose to use two different seizure models because we expected to simulate the human condition, as early recurrent seizures may mimic human premature neonatal seizures (Holmes, 1997; Holmes et al., 1998). Status epilepticus was elicited at an older age, as we intended to evaluate its effect on the brain beyond the neonatal period. We did not try to compare the effects of both seizure models on the animals' behavior, but instead, we tried to analyze the effects of both kinds of seizures, separately, on memory acquisition and retention

We found no difference in brain weight, on P30, among animals who suffered seizures. In contrast to the suggestion (Wasterlain, 1976) that it is not malnutrition, but rather the occurrence of repeated seizures during critical developmental periods that causes a reduction in brain weight, among the undernourished rats, those that suffered seizures presented higher brain weight than those that did not. However, the brain weight of undernourished rats was still lower than that of nourished animals. Brain edema secondary to the deleterious association between undernourishment and seizures might account for these results; however this factor was not controlled at the time the rats were sacrificed. In fact, several studies have indicated that seizures and nutritional deficits are responsible for neuronal and brain edema (Carlton and Kelly, 1969; Seitelberger et al., 1990; Andrew and MacVicar, 1994; Fountain, 2000; Chan et al., 2004).

There was an interaction between undernourishment and seizures in relation to spatial memory retention on P26. Reports showed that undernourishment and epilepsy impair the memory in experimental animals (Jordan et al., 1981; Huang et al., 2003; Majak and Pitkänen, 2004).

From P2 to P15, as expected, undernourished rats gained less body weight than nourished rats. These data prove that the undernourishment paradigm employed is as effective in reducing animal body weight as a short period of food deprivation (14 days) (Nunes et al., 2002). A factor that may complicate the interpretation of our results is that maternal deprivation may have other effects besides food restriction, in particular those induced by stress (Huang et al., 2002; Genest et al., 2004), although this method has been successfully used before (Nunes et al., 2000, 2002). Another possible complication for the interpretation of our results concerns neonatal handling. However, to minimize this confounding factor we did not handle the pups while they were separated from the mothers.

On P30, undernourished rats presented lower brain weight than nourished animals. Clinical reports indicate that when an organism suffers a nutritional insult, the brain is protected to the detriment of other body organs (Hales and Barker, 1992). However, several reports indicate that nutritional restriction during gestation, or soon after birth, affects the growth of different areas of the brain, causing cerebral asymmetry, altered cortical responses and low brain weight (Soto-Moyano et al., 1993; Nunes et al., 2002; Feoli et al., 2006).

From P21 to P25 (i.e. during the 5 days of training in the Morris water maze), there was a progressive reduction in platform latency among all animals. That means that all animals learned over the duration of the training period, because the more the rats learn the less time they take to reach the escape platform. Escape latency reduction was more evident among nourished than undernourished rats. The same occurred among seizure-free animals when compared to animals that suffered recurrent seizures or status epilepticus. There was no interaction between undernourishment and seizures during the acquisition tests. On P26, during the Morris water maze probe test, undernourished rats spent less time in the target quadrant than nourished animals. Likewise, rats that suffered recurrent seizures or status epilepticus spent less time in the target quadrant than control animals. As mentioned above, there was an interaction between undernourishment and seizures in the percentage of time spent in the target quadrant. The Morris water maze is a test of hippocampus-dependent spatial memory (Morris, 1984). The testing procedure used during the 5 days of locating the hidden platform provides a measure of spatial reference memory, while the probe test, on P26, is a measure of the strength of spatial learning.

Several reports show cognitive and behavioral disturbances as well as learning and memory impairment after undernourishment or epilepsy (Akman et al., 2004; Huang et al., 2003; Hoffmann et al., 2004; Majak and Pitkänen, 2004). We found an interaction between undernourishment and seizures in relation to cognitive injury in the rat. This interaction suggests that a combination of these two conditions worsens memory retention.

It is important to remember that the authors cannot exclude the possibility that the observed effects are in part a consequence of stress and increased level of cortisol.

To conclude, our findings show that undernourishment and seizures have an additive detrimental effect on body and brain weight as well as on spatial learning and memory processing.

Conflicts of interest

We confirm that the authors as well as the co-authors have no conflicts of interest to disclose.

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We confirm that we have read the Journal's position on issues concerning ethical publication and affirm that this report is consistent with those guidelines.

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