Psychology & Neuroscience
ISSN: 1984-3054
landeira@puc-rio.br
Pontifícia Universidade Católica do Rio de Janeiro
Brasil

de Oliveira Soares, Roberto; de Oliveira, Luiz Marcellino; Sousa Almeida, Sebastião
Effects of environmental stimulation during different periods of central nervous system development in malnourished rats subjected to the elevated plus maze
Psychology & Neuroscience, vol. 7, núm. 4, 2014, pp. 521-529
Pontifícia Universidade Católica do Rio de Janeiro
Rio de Janeiro, Brasil

PSYCHOLOGY & NEUROSCIENCE

Psychology & Neuroscience, 2014, 7, 4, 521-529
DOI: 10.3922/j.psns.2014.4.11

Effects of environmental stimulation during different periods of central nervous system development in malnourished rats subjected to the elevated plus maze

Roberto de Oliveira Soares, Luiz Marcellino de Oliveira*, and Sebastião Sousa Almeida

Universidade de São Paulo, Ribeirão Preto, SP, Brazil

*In memoriam

Abstract

We compared the effects of environmental enrichment (EE) and tactile stimulation (handling [H]) during a critical period of development of the central nervous system (CNS) on behavior in malnourished (M; 6% protein) and control (C; 16% protein) rats in the elevated plus maze (EPM) on postnatal day 36 (P36) and P37. The stimulation occurred during two different periods of development: P8-21 (lactation period) and P22-35 (post-lactation period). The results showed higher exploration of the open arms of the EPM in the M group compared with the C group. Malnourished and stimulated rats presented lower exploration of the open arms compared with malnourished and non-stimulated rats and similar exploration of the open arms compared with control non-stimulated animals. The biochemical analysis showed that rats that were exposed to H during the post-lactation period, regardless of diet, had higher plasma corticosterone levels compared with non-stimulated rats and rats subjected to EE. Environmental enrichment during the lactation period appeared to protect animals from the effects of malnutrition on risk assessment behavior in the EPM. The type of stimulation may alter the stress response in the EPM during this critical period of CNS development. **Keywords:** protein malnutrition, critical period of CNS development, environmental enrichment, elevated plus maze, stress response.

Received 30 April 2014; received in revised form 16 September 2014; accepted 17 September 2014. Available online 16 December 204.

Introduction

Protein malnutrition in newborns and children during the most vulnerable stages of cerebral development compromises several maturational events, resulting in morphological, neurochemical, behavioral, and cognitive impairments (Levitsky & Barnes, 1972; Almeida, Tonkiss, & Galler, 1996; Morgane, Mokler, & Galler, 2002; Pereira-da-Silva, Cabral-Filho, & de-Oliveira, 2009). Neonatal protein malnutrition delays the maturation of sensory systems and impairs motor patterns, leading to significant depression of somatosensory, auditory, and visual stimulation that may be fundamental for long-term social behavioral performance (Soriano, Regalado, Torrero, & Salas, 2006; Zhou, Nagarajan, Mossop, & Merzenich, 2008).

Roberto de Oliveira Soares, Faculdade de Medicina de Ribeirão Preto, Departamento de Cirurgia e Anatomia, Universidade de São Paulo. Luiz Marcellino de Oliveira (in memoriam) and Sebastião Sousa Almeida, Faculdade de Filosofia Ciências e Letras de Ribeirão Preto, Psicologia, Universidade de São Paulo. Correspondence regarding this article should be directed to: Roberto de Oliveira Soares, Laboratório de Neuroanatomia, Departamento de Cirurgia e Anatomia, Faculdade de Medicina de Ribeirão Preto, Avenida dos Bandeirantes, 3900, Ribeirão Preto, São Paulo, 14040-901, Brazil. E-mail: robertosoares@usp.br

Prenatal and postnatal protein deprivation causes significant changes in neurogenesis, reflected by the postnatal development of hippocampal granule cells (King et al., 2004). Granados-Rojas, Larriva-Sahd, Cintra, Gutiérrez-Ospina, Rondán, and Díaz-Cintra (2002) reported a significant reduction of the number of synaptic contacts in malnourished rats. Many studies have demonstrated the long-lasting effects of early malnutrition on brain structures such as the hippocampal formation (Morgane et al., 2002; Cintra et al., 1997; Kehoe, Mallinson, Bronzino, & McCormick, 2001).

Moreover, protein malnutrition during the postnatal period can alter the levels of plasma corticosterone (CORT; Adlard & Smart, 1971; Sampaio, Oliveira, Almeida, Marchini, Antunes-Rodrigues, & Elias, 2008), suggesting modification of the adrenocorticotropin response to stress. Postnatal malnutrition can decrease glucocorticoid receptors in the hippocampus (Kehoe et al., 2001; Lister et al., 2005), thus altering the sensitivity of the hypothalamic-pituitary-adrenal axis (HPA) and consequently affecting the stress response of the organism (Sampaio et al., 2008).

Anxiety responses are also caused by neonatal malnutrition. Rats that were subjected to a low protein diet and exposed to the elevated plus maze (EPM) exhibited an increase in the number of entries into and time spent in the extremities of the open arms of the

maze (Almeida, Garcia, & de Oliveira, 1993; Françolin-Silva, da Silva Hernandes, Fukuda, Valadares, & Almeida, 2006; Soares, Aliveira, Marchini, Antunes-Rodrigues, & Elias, 2013), demonstrating the effects of protein malnutrition on risk assessment behavior and a decrease in the anxiety response.

Neither the behavioral alterations nor biochemical impairments in the CNS caused by protein malnutrition can be completely reversed by nutritional rehabilitation. Some studies have shown that environmental stimulation can have a protective effect against the damage caused by protein malnutrition (Riul, Carvalho, Almeida, De-Oliveira, & Almeida, 1999; Pereira-da-Silva et al., 2009; Soares et al., 2013). However, considering the critical period of development when postnatal cerebral plasticity occurs is important, characterized by large-scale changes in response selectivity induced by exposure to environmental stimuli (Carughi, Carpenter, & Diamond, 1989; Will, Galani, Kelche, & Rosenzweig, 2004).

One type of environmental stimulation is handling, which can alter the sensitivity of the HPA axis and concentration of glucocorticoid receptors when performed during the first weeks of life (Liu et al., 1997). Another type of environmental stimulation is environmental enrichment (EE). According to some studies, this type of stimulation can alter the levels of some neurotransmitters (Wood, Buse, Wellman, & Rebec, 2005) and cause morphologic alterations, such as increased dendritic branching in the CNS, among other effects (Diamond, Krech, & Rosenzweig, 1964; Rosenzweig, Love, & Bennett, 1968; Rosenzweig & Bennett, 1996; Will et al., 2004). Environmental stimulation can modulate behavioral responses and the development of the sensory-motor system (Cancedda, Putignano, Sale, Viegi, Berardi, & Maffei, 1989; Will et al., 2004). It can also attenuate the possible losses caused by malnutrition in some structures of the CNS.

Animals that are exposed to an enriched environment exhibit significant increases in mean cortical thickness and dendritic branching compared with animals that are rehabilitated in standard environments (Carughi et al., 1989). With regard to the thickness of the occipital cortex and dendritic branching, the environmental effect experienced by rehabilitated animals was significantly greater compared with well-nourished animals. Previous studies showed that daily exposure to a sensory-enriched environment, where the newborn may give and receive sensorimotor stimulation, increased brain weight (Soriano et al., 2006). Artola et al. (2006) demonstrated long-lasting changes in the induction of subsequent synaptic plasticity in the hippocampal CA1 region in rats that were exposed to EE.

Rats that were exposed to environmental stimulation presented an increase in exploration of the open arms of the EPM (Santucci, Daud, Almeida, & de Oliveira, 1994; McIntosh, Anisman, & Merali, 1999). Behavioral experiences can modulate the induction of synaptic plasticity. This modulation can persist for a prolonged period of time after the behavioral experience.

No studies of which we are aware have reported the simultaneous biochemical and behavioral effects of handling and EE in well-nourished (control) and malnourished rats during CNS development. The aim of the present study was to investigate the effects of early protein malnutrition and environmental stimulation (handling and EE) on behavior in the EPM and CORT levels during a critical period of CNS development. Rats were separately evaluated from postnatal day 8 (P8) to P21 (lactation) and from P22 to P35 (post-lactation).

Methods

Animals

Male Wistar rats from the animal colony of the Ribeirão Preto Campus of the University of São Paulo (USP) were used. Each litter was culled to six male and two female pups on the day of birth. The dams and pups were housed in transparent plastic cages (40 x 25 x 20 cm). After the lactation period, the mothers and female pups were discarded, and the male pups were placed in individual polypropylene cages (30 x 19 x 18 cm). The animals were randomly divided into two groups: control (C; diet that contained 16% protein) and malnourished (M; diet that contained 6% protein). The diets were prepared according to the proportion of nutrients recommended by the American Institute of Nutrition and Association of Official Agriculture Chemists with the addition of methionine and choline as described by Cambraia, Vannucchi, and De-Oliveira (1997). Briefly, the diet that was given to the M group consisted of 6% protein (casein), 79.8% corn starch, 8% lipids (corn oil), 5% salt mixture, 1% vitamin mixture, .2% choline, and methionine (2 g/kg casein). The diet that was given to the C group consisted of 16% protein (casein), 69.8% corn starch, 8%, lipids (corn oil), 5% salt mixture, 1% vitamin mixture, .2% choline, and methionine (2 g/kg casein). The animals were given the experimental diet from birth until P35. During the lactation period, the diet was offered only to the mother. In the post-lactation period, it was offered to each rat individually.

The animals were maintained on a 12 h/12 h light/dark cycle (lights on at 6:00 AM) with a controlled temperature (23-25ºC) and free access to water and food throughout the experiment. The behavioral tests were conducted during the light period. The experiments were performed in compliance with the recommendations of the Brazilian Society of Neuroscience and Behavior (SBNeC), which are based on the United States National Institutes of Health Guide for the Care and Use of Laboratory Animals. The rats in the C group (16% protein) and M group (6% protein) were further distributed into six subgroups: handling (CH and MH groups), environmental enrichment (CEE and MEE groups), and no stimulation (CN and MN groups). All of the litters were weighed on P1, P7, P14, and P21 during the lactation period (i.e., from 0 to 21 days of age). After the lactation period, the animals were individually

weighed on P28 and P35. Sixty-four animals were used, eight in each group. All of the animals were tested in the EPM. Only six of the animals from each group were used for plasma CORT measurements. The experimental design and formation of the groups are presented in Table 1. The present study was approved by the Ethics Committee on Animal Use of the USP campus of Ribeirão Preto (2007.1.262.53.4).

Apparatus

The rats were exposed to EE from P8 to P21 and from P22 to P35. Environmental enrichment from P8 to P21 consisted of a transparent plastic cage (40 × 25 × 20 cm) that contained activity wheels, tunnels, plastic toys (with different shapes and textures), marbles, objects that emitted sounds (e.g., rattles), mirrors, and pieces of wood (EA1). Each litter that was assigned to the EE group was placed in the enriched environment without their mothers for 1 h per day. The litters were then returned to their mothers in their respective home cages. From P22 to P35, a cage (40 × 60 × 90 cm) with three floors that were connected by ramps contained an activity wheel, plastic toys, rubber balls, wooden objects with different shapes, textures, and colors, objects that could emit sound when touched by the animals, and mirrors on the lateral walls (EA2), as described by Soares et al. (2013).

The EPM was made of wood and consisted of two open arms (50 cm × 10 cm) opposite each other, crossed by two closed arms (50 cm × 10 cm × 40 cm) with an open top. The maze was elevated 50 cm from the floor. Fluorescent ceiling lights (two 60 W bulbs) provided the only illumination in the experimental room.

Procedure

Environmental stimulation was only performed during either the lactation period or post-lactation period. Two types of environmental stimulation were performed: H and EE. Handling was performed from P8 to P21, which consisted of removing the pups from their nursery boxes and subjecting them to individual tactile stimulation (handling) for 3 min. The stimulation consisted of holding the pup with one hand and sliding the thumb of the other hand on the back of the animal in the cephalo-caudal direction for 3 min. Afterward, these rats were returned to their litter and subjected to sonorous stimulation (3 kHz, 70 dB) for 3 min at regular intervals of 24 s. From P22 to P35, the animals were housed in individual cages and subjected to the same handling procedure described above, with the exception that after tactile stimulation these animals were placed in collective cages for sonorous stimulation. These animals were then returned to their individual cages.

As described previously (Soares et al., 2013), EE was also performed from P8 to P21. This procedure consisted of removing the pups from their nursery boxes and placing them in EA1 for 1 h per day. Afterward, the animals were returned to their original cages. From P22 to P35, the animals were housed in individual cages, with the exception of 1 h per day when seven to 10 animals from the different groups were placed in EA2. The objects used for EE during both periods were changed weekly to ensure an effect of novelty.

The animals in all of the nutrition conditions were subjected to only one type of environmental stimulation (H or EE) during a single period of life (lactation or post-lactation).

Table 1. Experimental design and formation of groups according to nutrition, environmental stimulation (enrichment and handling), and period of life (lactation and post-lactation).

Diet	Environmental stimulation				Group
	Enrichment during lactation period	Handling during lactation period	Enrichment during post-lactation period	Handling during post-lactation period	
Control	N	N	N	N	CNN
	E	N	N	N	CEN
	N	H	N	N	CHN
	N	N	E	N	CNE
	N	N	T	H	CNH
Malnourished	N	N	N	N	MNN
	E	N	N	N	MEN
	N	H	N	N	MHN
	N	N	E	N	MNE
	N	N	T	H	MNH

C, control; M, malnourished; N; not stimulated; E, enriched environment; H, handling.

Behavioral testing

On P36, the animals were subjected to the EPM for 5 min. The experimental sessions were recorded with a video camera above the EPM apparatus. The recordings were analyzed using X-Plot-Rat 2005 software, version 1.1.0, which was developed in the Laboratory of Exploratory Behavior of the Faculty of Philosophy, Sciences and Literature of Ribeirão Preto (USP; available at http://scotty.ffclrp.usp.br; accessed September 4, 2014). The animals were individually placed in the center of the maze, facing a closed arm, and allowed to explore the apparatus for 5 min. The following behaviors were analyzed: number of entries into the closed arms, percentage of entries into and time spent on the open arms, the number of rearings, the number of head-dips (protected and unprotected), and the number of stretch/attend postures (protected and unprotected), as described by Cruz, Frei, and Graeff (1994). Activity was considered protected when the behavior occurred in the closed arms or center of the maze and considered unprotected when the behavior occurred in the open arms.

Biochemical analyses

On P37, between 8:00 AM and 9:30 AM, the rats were decapitated. The brains were removed, and blood was collected for biochemical processing. The plasma and hypothalamus (obtained after dissection of the brain) were frozen at -70ºC to conserve them until the biochemical analyses were performed.

The levels of CORT were obtained using a radioimmunoassay method as described by Elias, Elias, Castro, Antunes-Rodrigues, and Moreira (2004). Anti-CORT antibody was provided by Dr. José Gilberto Vieira (Federal University of São Paulo, Brazil), and 1,2,6,7-3H corticosterone was purchased from GE Healthcare Life Sciences. The assay sensitivity and intra- and inter-assay coefficients of variation were .4 µg/dl, 5.1%, and 8.4%, respectively.

Statistical analysis

The corporal weight data (diet × day of life × stimulation) and behavioral data (diet × stimulation) were analyzed using a three-way and two-way analysis of variance (ANOVA). The biochemical data were analyzed using a two-way ANOVA (diet × stimulation). When appropriate, *post hoc* comparisons were conducted using the Newman-Keuls test. The level of significance was $p < .05$.

Results

Body weight

The weight of the animals during lactation and post-lactation is presented in Figure 1. The ANOVA showed that malnourished rats weighed less on P8 ($F_{3,42} = 51.13$, $p < .001$), and the weight difference was maintained until the end of the lactation period ($F_{1,18} = 752.84$, $p < .001$). During the post-lactation period, the weight of malnourished rats remained lower compared with control rats ($F_{1,88} = 1734.34$, $p < .001$).

Behavioral measures

The ANOVA showed that the diet condition had a significant effect on the behavioral measures in the EPM in Trial 1. Malnourished rats presented a higher percentage of entries into the open arms ($F_{1,70} = 12.50$, $p < .001$) and a higher percentage of time spent on the open arms ($F_{1,70} = 28.31$, $p < .001$) compared with the control rats. Environmental stimulation also showed a significant main effect. Animals that were stimulated during the lactation period ($F_{1.} = 13.52$, $p < .001$) and post-lactation period ($F_{1.38} = 41.16$, $p < .001$) exhibited a higher percentage of open-arm entries compared with control animals. The same effect was observed for the percentage of open-arm time in rats that were stimulated during lactation ($F_{1,37} = 11.83$, $p < .01$) and post-lactation ($F_{1,38} = 28.83$, $p < .001$). The ANOVA revealed an interaction between nutrition and environmental stimulation. The MHN and MEN groups presented a lower percentage of open-arm entries ($F_{2,37} = 5.31$, $p < .01$) and open-arm time ($F_{2,37} = 3.78$, $p < .05$) compared with the MNN group. The MEN group presented a similar percentage of open-arm entries (11.37% ± 5.93%) and open-arm time (12.43% ± 4.9%) compared with the CNN group (14% ± 2.44% and 12.93% ± 1.3%, respectively; Figure 2).

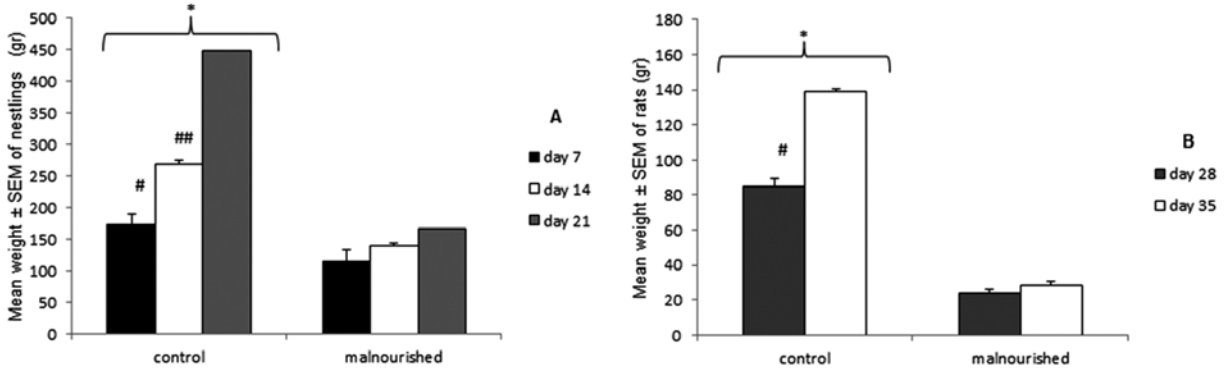

Figure 1. (A) Mean weight ± SEM of control and malnourished pups at 7, 14, and 21 days of life. (B) Mean weight ± SEM of rats under the same feeding conditions at 28 and 35 days of life. *$p < .05$, compared with malnourished groups; #$p < .05$, compared with other days; ##$p < .05$, compared with other days.

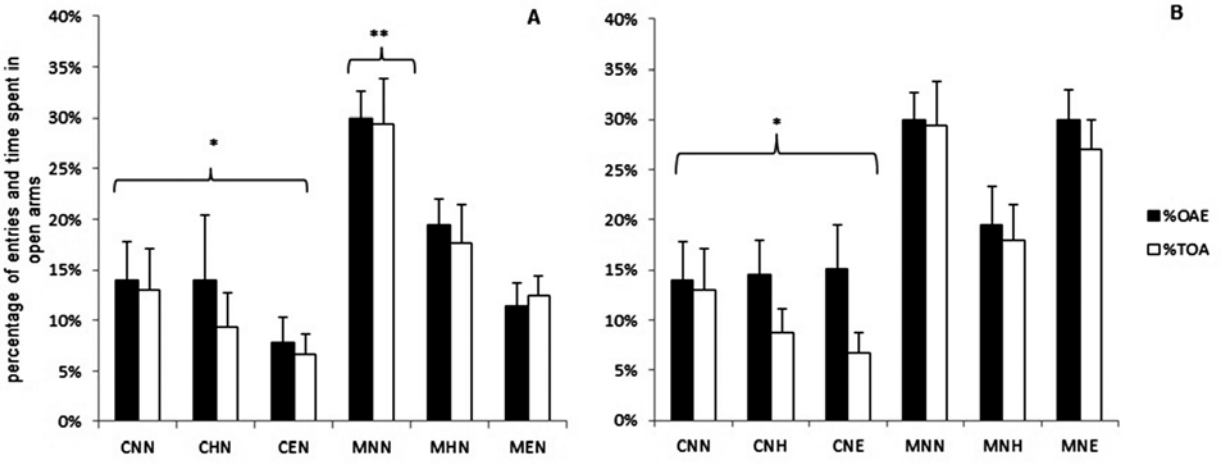

Figure 2. Percentage of open-arm entries (% EOA; mean ± SEM) and percentage of time spent on the open arms (% TOA; mean ± SEM) in the EPM. (A) Groups subjected to stimulation from P8 to P21. (B) Groups subjected to stimulation from P21 to P35. *$p < .05$, compared with malnourished groups; **$p < .05$, compared with all other groups. CNN, control, not stimulated; CHN, control, handling during lactation period; CEN, control, environmental enrichment during lactation period; CNH, control, handling during post-lactation period; CNE, control, environmental enrichment during post-lactation period; MNN, malnourished, not stimulated; MHN, malnourished, handling during lactation period; MEN, malnourished, environmental enrichment during lactation period; MNH, malnourished, handling during post-lactation period; MNE, malnourished, environmental enrichment during post-lactation period. The data are expressed as mean ± standard error of the mean.

With regard to the number of closed-arm entries in Trial 1 in the EPM, the ANOVA revealed a significant effect of nutrition. Malnourished rats presented more closed-arm entries ($F_{1,70} = 8.42$, $p < .01$) compared with well-nourished rats. With regard to environmental stimulation, the ANOVA revealed that non-stimulated rats presented a lower number of closed-arm entries compared with stimulated rats, independent of the stimulation performed ($F_{2,37} = 7.99$, $p < .001$). With regard to stimulation, the MNN group presented fewer closed-arm entries compared with the MNH and MNE groups ($F_{2,38} = 4.71$, $p < .05$), and the CNE group presented more closed-arm entries compared with the CNN group ($F_{2,38} = 7.08$, $p < .01$; Figure 3).

Figure 4 shows the number of head-dips in the open arms (unprotected) during Trial 1 in the EPM. The ANOVA revealed a significant effect of nutrition. Malnourished rats presented more unprotected head-dips ($F_{1,70} = 10.97$, $p < .01$) in the EPM compared with control rats. The interaction between nutrition and environmental stimulation was also significant. The MEN group presented fewer unprotected head-dips compared with the MNN group ($F_{2,37} = 3.61$, $p < .01$) and a similar number of head-dips (7 ± 1.32) compared with the CNN group (6.75 ± 2.55). The ANOVA also showed a significant effect of diet on stretch/attend behavior ($F_{1,70} = 40.96$, $p < .01$).

The ANOVA showed that diet also had a significant effect on rearing behavior in Trial 1 in the EPM.

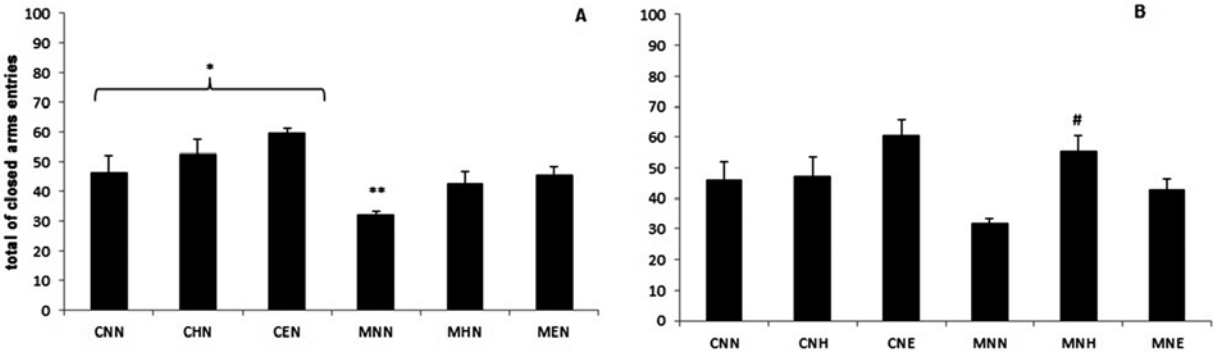

Figure 3. Total number of entries into the closed arms (mean ± SEM). (A) Groups subjected to stimulation from P8 to P21. (B) Groups subjected to stimulation from P21 to P35. *$p < .05$, compared with malnourished groups; #$p < .05$, compared with other days and other groups in the same diet condition; **$p < .05$, compared with other days and other groups in the same diet condition. CNN, control, not stimulated; CHN, control, handling during lactation period; CEN, control, environmental enrichment during lactation period; CNH, control, handling during post-lactation period; CNE, control, environmental enrichment during post-lactation period; MNN, malnourished, not stimulated; MHN, malnourished, handling during lactation period; MEN, malnourished, environmental enrichment during lactation period; MNH, malnourished, handling during post-lactation period; MNE, malnourished, environmental enrichment during post-lactation period. The data are expressed as mean ± standard error of the mean.

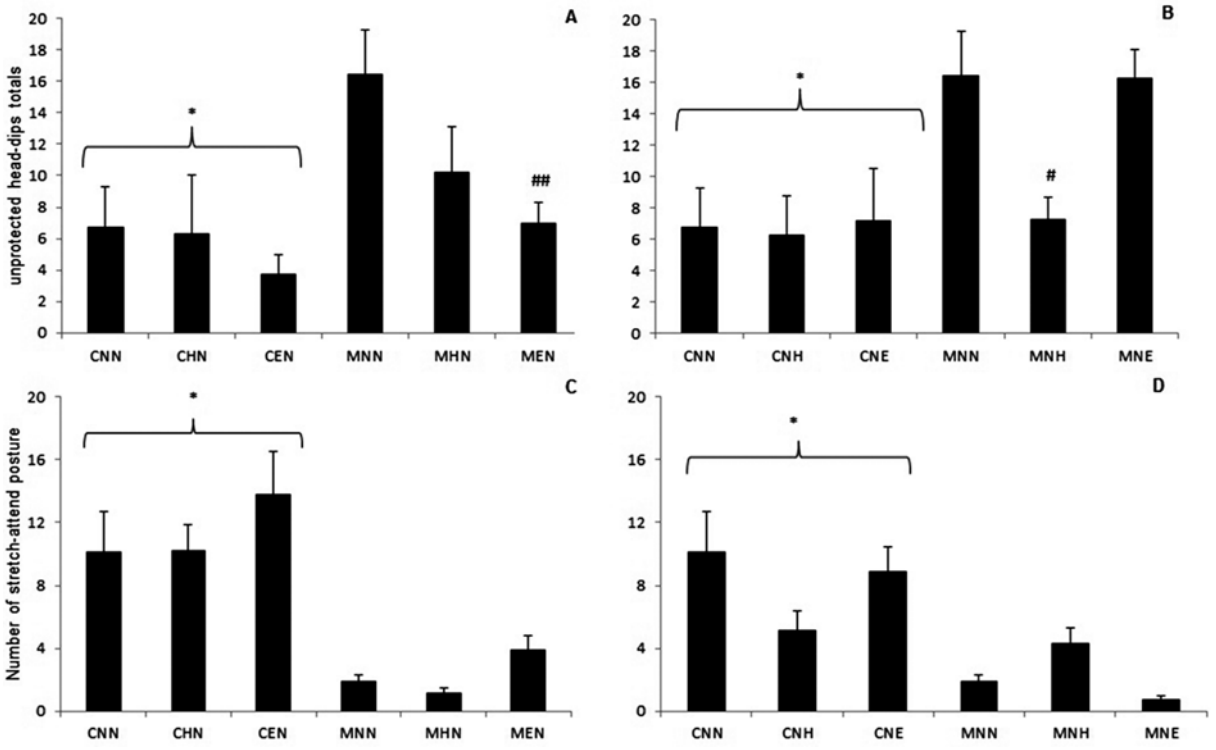

Figure 4. Risk-assessment behaviors assessed by the numbers of head-dips on the open arms (unprotected) and stretch/attend posture in the EPM (mean ± SEM). (A, C) Groups subjected to stimulation from P8 to P21. (B, D) Groups subjected to stimulation from P21 to P35. $^{*}p < .05$, compared with malnourished groups; $^{\#}p < .05$, compared with other days and other groups in the same diet condition; $^{\#\#}p < .05$, compared with MNN group. CNN, control, not stimulated; CHN, control, handling during lactation period; CEN, control, environmental enrichment during lactation period; CNH, control, handling during post-lactation period; CNE, control, environmental enrichment during post-lactation period; MNN, malnourished, not stimulated; MHN, malnourished, handling during lactation period; MEN, malnourished, environmental enrichment during lactation period; MNH, malnourished, handling during post-lactation period; MNE, malnourished, environmental enrichment during post-lactation period. The data are expressed as mean ± standard error of the mean.

Malnourished rats presented a lower number of rearings compared with the control group ($F_{1,70} = 10.35$, $p < .01$). With regard to environmental stimulation, the MHN group presented a lower number of rearings compared with the MNN and MEN groups ($F_{2,37} = 5.14$, $p < .015$).

Plasma corticosterone analysis

The ANOVA revealed a significant effect of environmental stimulation on plasma CORT levels. Rats that were subjected to handling presented a higher level of CORT compared with non-stimulated and EE rats ($F_{2,42} = 14.38$, $p < .001$; Table 2).

Discussion

A low-protein diet decreased body weight in rat dams that were subjected to protein malnutrition during the lactation period, which is consistent with previous studies (Almeida et al., 1993; Rocinholi, Almeida, & de Oliveira, 1997; Cabral & Almeida, 2008). The decrease in body weight in pups subjected to protein malnourishment beginning in the first week of life can be considered a consequence of protein deficiency from maternal milk, in which the dams were fed a low-protein diet (6% protein) that caused physical impairments in pup development (Cambraia et al., 1997; Rocinholi et al., 1997; Françolin-Silva et al., 2006). The present study also showed that protein malnutrition, if continued into the post-lactation period, can exacerbate the decrease in body weight in rats subjected to protein malnutrition. Additionally, malnutrition can cause a series of physical, behavioral, and neurochemical alterations (Françolin-Silva et al., 2006; Zhou et al., 2008).

The higher percentage of open-arm entries in the EPM and the increase in the time spent on the open arms in malnourished rats compared with well-nourished rats are already well-documented in the literature and interpreted as lower anxiety and/or higher impulsivity caused by the malnutrition procedure itself (Almeida et al., 1993; Françolin-Silva et al., 2006; Soares et al., 2013). These results suggest that early nutritional deficits can change rats' responsiveness to the EPM later in adult life. These results cannot be attributed to changes in locomotor or exploratory activity produced by malnutrition (Françolin-Silva et al., 2006). This anxiety response may be attributable to a possible effect of protein malnutrition imposed during the neonatal period or the maturation of such brain structures as the septum, hippocampus, and amygdala, which are directly involved in behavioral inhibition (McNaughton & Gray, 2000). Thus, malnutrition may impair neonatal risk assessment, reflected by an

Table 2. Plasma corticosterone in µg/dl (mean ± SEM), collected on P37 in the groups subjected to stimulation from P8 to P21 and from P22 to P35.

Lactation stimulation				Post-lactation stimulation			
Group	**Mean**		**SEM**	**Group**	**Mean**		**SEM**
CNN	6.45	±	.86	CNN	6.45	±	.86
CHN	6.79	±	1.67	CNH	11.56*	±	1.08
CEN	5.12	±	.72	CNE	5.80	±	1.47
MNN	7.43	±	1.06	MNN	7.43	±	1.06
MHN	6.14	±	1.26	MNH	10.24#	±	1.08
MEN	8.39	±	1.59	MNE	5.67	±	.52

#$p < .05$, compared with other days and other groups in the same diet condition; **$p < .05$, compared with other days and other groups in the same diet condition. CNN, control, not stimulated; CHN, control, handling during lactation period; CEN, control, environmental enrichment during lactation period; CNH, control, handling during post-lactation period; CNE, control, environmental enrichment during post-lactation period; MNN, malnourished, not stimulated; MHN, malnourished, handling during lactation period; MEN, malnourished, environmental enrichment during lactation period; MNH, malnourished, handling during post-lactation period; MNE, malnourished, environmental enrichment during post-lactation period.

increase in the probability of unprotected head-dips in more naturalistic models of behavior, such as the EPM (Hernandes & Almeida, 2003).

Moreover, environmental stimulation performed during a critical period of development was shown to exert a protective effect on the development of brain plasticity (Liu et al., 1997; Cancceda et al., 2004; Will et al., 2004; Artola et al., 2006) and animal behavior (Rosenzweig & Bennett, 1996; Will et al., 2004). Environmental stimulation was more effective when performed during the lactation period. According to Morgane et al. (2002), this is the period when brain growth peaks and granular cells differentiate in the hippocampus, occipital cortex, and olfactory bulb. Environmental enrichment performed during the lactation period may have been more effective because malnourished rats exhibited the same performance in the EPM as control rats, demonstrating that EE had a protective effect on the development of brain structures in the limbic system, even in animals that were subjected to a low-protein diet. The protective effect of EE was shown to be more effective than handling. In the present study, the malnourished rats that were subjected to EE exhibited the same pattern of anxiety-like behavior as the control rats in the EPM.

Previous studies showed that EE caused morphological and biochemical alterations, mainly in the hippocampus, amygdala, septum, and occipital cortex (Diamond et al., 1964; Rosenzweig et al., 1968; Peña, Prunell, Rotllant, Armario, & Escorihuela, 2009). Continuous exposure to EE is also known to alter behavioral responses in rats in the EPM (Will et al., 2004; Peña, Prunell, Dimitsantos, Nadal, & Escorihuela, 2006; Soares et al., 2013). The effects of EE during the first 21 days of life showed that this type of stimulation had protective effects against impairments caused by protein malnutrition in the development of such brain structures as the hippocampus. The effects of EE in malnourished rats, performed during a critical period of brain development, may be the result of greater activation of mechanisms associated with brain plasticity (Van Praag, Kempermannn, & Gage, 2000; Johansson & Belichenko, 2002).

Even in the absence of any observable changes in baseline synaptic transmission, EE may alter the ability to induce synaptic plasticity (Johansson & Belichenko, 2002; Nithianantharajah, Levis, & Murphy, 2004). Studies have demonstrated that rats that are subjected to EE show a greater degree of long-term potentiation in the CA1 area of the hippocampus and changes in the dentate gyrus compared with rats that live in standard conditions (Artola et al., 2004).

The protective effect of EE may also be the result of epigenetic processes (Baroncelli, Braschi, Spolidoro, Begenisic, Sale, & Maffei, 2010). Learning and memory processes may involve epigenetic changes, producing lasting effects on hippocampal plasticity (Covic, Karaca, & Lie, 2010). Environmental enrichment can induce epigenetic changes in the function of chromatin by modulating histone acetylation (Baroncelli et al., 2010), interfering directly with mRNA synthesis and consequently interfering with protein synthesis (Covic et al., 2010). The levels of CORT may be altered by protein malnutrition (Sampaio et al., 2008), and such alterations can modify the adrenocortical response to stress, demonstrating the influence of early nutrition on the HPA axis response to stress (Kehoe et al., 2001). Moreover, handling during the lactation period in rats, regardless of nutrition status, can increase CORT levels and consequently increase stress. This mechanism reinforces the theory that prior experiences alter the activity of the HPA axis.

In summary, the present data showed that malnourished animals exposed themselves more frequently to risky situations compared with well-nourished rats. Environmental enrichment, when

performed only during the lactation period, was shown to protect against malnutrition-induced risk assessment behavior in the EPM. The data also showed that tactile stimulation, when performed only during the post-lactation period, regardless of the diet condition, elevated plasma CORT levels, suggesting that this type of stimulation may alter the stress response in the EPM.

Acknowledgements

The authors express their gratitude to Dalmo Presta Nicola (in memoriam), a technician in the Laboratory of Nutrition and Behavior of the University of São Paulo, for assistance with animal care and the behavioral tests, Maria Valci Aparecida dos Santos, a technician in the Laboratory of Neuroendocrinology of the University of São Paulo, for assistance with the corticosterone analysis, and Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) for financial support (grant no. 2007/51380-7).

References

Adlard, B. P. F., & Smart, J. L. (1972). Adrenocortical function in rats subjected to nutritional deprivation in early life. *Journal of Endocrinology*, *54*(1), 99-105.

Almeida, S. S., Garcia, R. A., & de Oliveira, L. M. (1993). Effects of early protein malnutrition and repeated testing upon locomotor and exploratory behaviors in the elevated plus-maze. *Physiology and Behavior*, *54*(4), 749-752.

Almeida, S. S., Tonkiss, J., & Galler, J. R. (1996). Malnutrition and reactivity to drugs acting in the central nervous system. *Neuroscience and Biobehavioral Reviews*, *20*(3), 389-402.

Artola, A., von Frijtag, J. C., Fermont, P. C., Gispen, W. H., Schrama, L. H., Kamal, A., & Spruijt, B. M. (2006). Long-lasting modulation of the induction of LTD and LTP in rat hippocampal CA1 by behavioural stress and environmental enrichment. *European Journal of Neuroscience*, *23*(1), 261-272.

Baroncelli, L., Braschi, C., Spolidoro, M., Begenisic, T., Sale, A., & Maffei, L. (2010). Nurturing brain plasticity: impact of environmental enrichment. *Cell Death and Differentiation*, *17*(7), 1092-1103.

Cabral, A., & Almeida, S. S. (2008). Effects of tactile stimulation and underwater trauma on the behavior of protein-malnourished rats in the elevated plus-maze test. *Psychology & Neuroscience*, *1*(1), 67-72.

Cambraia, R. P. B., Vannucchi, H., & De Oliveira, L. M. (1997). Food intake and weight of lactating rats maintained on different protein-calorie diets, and pup growth. *Brazilian Journal of Medical and Biological Research*, *30*(8), 985-988.

Cancedda, L., Putignano, E., Sale, A., Viegi, A., Berardi, N., & Maffei, L. (2004). Acceleration of visual system development by environmental enrichment. *Journal of Neuroscience*, *24*(20), 4840-4848.

Carughi, A., Carpenter, K. J., & Diamond, M. C. (1989). Effect of environmental enrichment during nutritional rehabilitation on body growth, blood parameters and cerebral cortical development of rats. *Journal of Nutrition*, *119*(12), 2005-2016.

Cintra, L., Granados, L., Aguilar, A., Kemper, T., DeBassio, W., Galler, J., ... Díaz-Cintra, S. (1997). Effects of prenatal protein malnutrition on mossy fibers of the hippocampal formation in rats of four age groups. *Hippocampus*, *7*(2), 184-191.

Covic, M., Karaca, E., & Lie, D. C. (2010). Epigenetic regulation of neurogenesis in the adult hippocampus. *Heredity*, *105*(1), 122-134.

Cruz, A. P. M., Frei, F., & Graeff, F. G. (1994). Ethopharmacological analysis of rat behavior on the elevated plus-maze. *Pharmacology Biochemistry and Behavior*, *49*(1), 171-176.

Diamond, M. C., Krech, D., & Rosenzweig, M. R. (1964). The effects of an enriched environment on the histology of the rat cerebral cortex. *Journal of Comparative Neurology*, *123*(1), 111-119.

Elias, P. C., Elias, L. L., Castro, M., Antunes-Rodrigues, J., & Moreira, A. C. (2004). Hypothalamic-pituitary-adrenal axis up-regulation in rats submitted to pituitary stalk compression. *Journal of Endocrinology*, *180*(2), 297-302.

Françolin-Silva, A. L., da Silva Hernandes, A., Fukuda, M. T. H., Valadares, C. T., & Almeida, S. S. (2006). Anxiolytic-like effects of short-term postnatal protein malnutrition in the elevated plus-maze test. *Behavioural Brain Research*, *173*(2), 310-314.

Granados-Rojas, L., Larriva-Sahd, J., Cintra, L., Gutiérrez-Ospina, G., Rondán, A., & Díaz-Cintra, S. (2002). Prenatal protein malnutrition decreases mossy fibers-CA3 thorny excrescences asymmetrical synapses in adult rats. *Brain Research*, *933*(2), 164-171.

Hernandes, A. S., & Almeida, S. S. (2003). Postnatal protein malnutrition affects inhibitory avoidance and risk assessment behaviors in two models of anxiety in rats. *Nutritional Neuroscience*, *6*(4), 213-219.

Johansson, B. B., & Belichenko, P. V. (2002). Neuronal plasticity and dendritic spines: effect of environmental enrichment on intact and postischemic rat brain. *Journal of Cerebral Blood Flow and Metabolism*, *22*(1), 89-96.

Kehoe, P., Mallinson, K., Bronzino, J., & McCormick, C. M. (2001). Effects of prenatal protein malnutrition and neonatal stress on CNS responsiveness. *Developmental Brain Research*, *132*(1), 23-31.

King, R. S., DeBassio, W. A., Kemper, T. L., Rosene, D. L., Tonkiss, J., Galler, J. R., & Blatt, G. J. (2004). Effects of prenatal protein malnutrition and acute postnatal stress on granule cell genesis in the fascia dentata of neonatal and juvenile rats. *Developmental Brain Research*, *150*(1), 9-15.

Levitsky, D. A., & Barnes, R. H. (1972). Nutritional and environmental interactions in the behavioral development of the rat: long-term effects. *Science*, *176*(4030), 68-71.

Lister, J. P., Blatt, G. J., DeBassio, W. A., Kemper, T. L., Tonkiss, J., Galler, J. R., & Rosene, D. L. (2005). Effect of prenatal protein malnutrition on numbers of neurons in the principal cell layers of the adult rat hippocampal formation. *Hippocampus*, *15*(3), 393-403.

Liu, D., Diorio, J., Tannenbaum, B., Caldji, C., Francis, D., Freedman, A., ... Meaney, M. J. (1997). Maternal care, hippocampal glucocorticoid receptors, and hypothalamic-pituitary-adrenal responses to stress. *Science*, *277*(5332), 1659-1662.

McIntosh, J., Anisman, H., & Merali, Z. (1999). Short- and long-periods of neonatal maternal separation differentially affect anxiety and feeding in adult rats: gender-dependent effects. *Developmental Brain Research*, *113*(1), 97-106.

McNaughton, N. & Gray, J. A. (2000). Anxiolytic action on the behavioural inhibition system implies multiple types of arousal contribute to anxiety. *Journal of Affective Disorders*, *61*(3), 161-176.

Morgane, P. J., Mokler, D.J., & Galler, J.R. (2002). Effects of prenatal protein malnutrition on the hippocampal formation. *Neuroscience and Biobehavioral Reviews*, *26*(4), 471-483.

Nithianantharajah, J., Levis, H., & Murphy, M. (2004). Environmental enrichment results in cortical and subcortical changes in levels of synaptophysin and PSD-95 proteins. *Neurobiology of Learning and Memory*, *81*(3), 200-210.

Peña, Y., Prunell, M., Dimitsantos, V., Nadal, R., & Escorihuela, R. M. (2006). Environmental enrichment effects in social investigation in rats are gender dependent. *Behavioural Brain Research*, *174*(1), 181-187.

Peña, Y., Prunell, M., Rotllant, D., Armario, A., & Escorihuela, R. M. (2009). Enduring effects of environmental enrichment from weaning to adulthood on pituitary-adrenal function, pre-pulse inhibition and learning in male and female rats. *Psychoneuroendocrinology*, *34*(9), 1390-1404.

Pereira-da-Silva, M. S., Cabral-Filho, J. E., & de-Oliveira, L. M. (2009). Effect of early malnutrition and environmental stimulation in the performance of rats in the elevated plus maze. *Behavioural Brain Research*, *205*(1), 286-289.

Riul, T. R., Carvalho, A. F., Almeida, P. S., De-Oliveira, L. M., & Almeida, S. S. (1999). Ethological analysis of mother-pup interactions and other behavioral reactions in rats: effects of malnutrition and tactile stimulation of the pups. *Brazilian Journal of Medical and Biological Research*, *32*(8), 975-983.

Rocinholi, L. F., Almeida, S. S., & de Oliveira, L. M. (1997). Response threshold to aversive stimuli in stimulated early protein-malnourished rats. *Brazilian Journal of Medical and Biological Research*, *30*, 407-413.

Rosenzweig, M. R., & Bennett, E. L. (1996). Psychobiology of plasticity: effects of training and experience on brain and behavior. *Behavioural Brain Research*, *78*(1), 57-65.

Rosenzweig, M. R., Love, W., & Bennett, E. L. (1968). Effects of a few hours a day of enriched experience on brain chemistry and brain weights. *Physiology and Behavior*, *3*(6), 819-825.

Sampaio, V. D. F., Oliveira, L. M. D., Almeida, S. S., Marchini, J. S., Antunes-Rodrigues, J., & Elias, L. L. K. (2008). The effects of early protein malnutrition and environmental stimulation on behavioral and biochemical parameters in rats. *Psychology & Neuroscience*, *1*(2), 109-119.

Santucci, L. B., Daud, M. M., Almeida, S. S., & de Oliveira, L. M. (1994). Effects of early protein malnutrition and environmental stimulation upon the reactivity to diazepam in two animal models of anxiety. *Pharmacology Biochemistry and Behavior*, *49*(2), 393-398.

Soares, R. O., Oliveira, L. M., Marchini, J. S., Antunes-Rodrigues, J., Elias, L. L., & Almeida, S. S. (2013). Effects of early protein malnutrition and environmental stimulation on behavioral and biochemical parameters in rats submitted to the elevated plus-maze test. *Nutritional Neuroscience*, *16*(3), 104-112.

Soriano, O., Regalado, M., Torrero, C., & Salas, M. (2006). Contributions of undernutrition and handling to huddling development of rats. *Physiology and Behavior*, *89*(4), 543-551.

Van Praag, H., Kempermann, G., & Gage, F. H. (2000). Neural consequences of enviromental enrichment. *Nature Reviews Neuroscience*, *1*(3), 191-198.

Will, B., Galani, R., Kelche, C., & Rosenzweig, M. R. (2004). Recovery from brain injury in animals: relative efficacy of environmental enrichment, physical exercise or formal training (1990–2002). *Progress in Neurobiology*, *72*(3), 167-182.

Wood, D.A., Buse, J. E., Wellman, C. L., & Rebec, G. V. (2005). Differential environmental exposure alters NMDA but not AMPA receptor subunit expression in nucleus accumbens core and shell. *Brain Research*, *1042*(2), 176-183.

Zhou, X., Nagarajan, N., Mossop, B. J., & Merzenich, M. M. (2008). Influences of un-modulated acoustic inputs on functional maturation and critical-period plasticity of the primary auditory cortex. *Neuroscience*, *154*(1), 390-396.