Original Article

Effects of Protein Malnutrition on Newly-Weaned Rats: Effects on Behavior, Brain Catecholamines and Nucleic Acids

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ABSTRACT

Newly weaned rats were exposed to a low protein die (5% casein) during 6 or 12 weeks. Control animals were fed with 20 % casein diet for the same period. A third group was first exposed to 5 % casein diet and recovered with laboratory Purine chow during the second period of 6 weeks (Recovered group). One day before the sacrifice all groups were submitted to behavioral test (open field and passive avoidance test). After the sacrifice the total brain weight was evaluated and the brain was sectioned into the telencephalon, brain stem, hypothalamus and cerebellum. The concentrations of Norepinephrine (NE), 5-hydroxytryptamine (5-HT) nucleic acids (DNA and RNA) were determined in each region. The behavioral studies showed that malnourished rats were more active in the open field and showed a higher inhibitory avoidance. The brains of malnourished showed higher NE in the brain stem (6 weeks) and hypothalamus (12 weeks and recovered group); higher of 5-HT in the telencephalon and hypothalamus (6 weeks); lower of DNA in all brain regions of malnourished and recovered animals; lower of RNA in all brain regions (6 weeks). Therefore the protein calorie malnutrition during early post-weaning period leads to behavioral changes and significant brain changes even in the rats that were recovered from early malnutrition.

Key Words: low-protein diet, post-weaning malnutrition, locomotor activity, passive avoidance, brain weight, norepinephrine, 5-hydroxytryptamine, and nucleic acids.

INTRODUCTION

Protein-calorie malnutrition causes several changes in the central nervous system. In all mammalian species, brain growth and follow the sigmoidal curve[37], whose rapid ascending segment is described as the "rapid brain growth" period, or critical or vulnerable phase of developing brain[15,62]. During this phase, any possible adverse effect on brain growth and development is more deleterious. Post-weaning malnutrition has much less or even no effect on brain growth[52,40,41,42].

In rats, rapid brain growth is demonstrated by the progressive increase of deoxyribonucleic acid (DNA) during lactation, followed by virtual cessation of DNA synthesis during the weaning

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period[71] In rats, early protein-calorie malnutrition, specially during lactation, causes significant disturbances in the central nervous system, with serious nucleic acid deficiency, even after the animals are exposed to adequate diets[56,14,38,13,32]. Protein-Calorie malnutrition also causes disturbances to the brain biogenic amines. All evidences indicates that brain norepinephrine and 5-hydroxytrypotamine are strictly related to learning, aggressive behavior, reaction to stress and other behavioral changes[39]. However, contra-dictory results have been reported about the levels of brain biogenic amines after protein-calorie malnutrition, showing or increases[67,35,23,44] or decreases in the brain noradrenaline and 5-hydroxytryptamine levels[54,55,9,24,35]. After the animals returned to adequate diets the concentration of norepineprhine levels tend to return to normal levels[34]. Behavioral studies have consistently demonstrated an intensified emotional reactivity in animals exposed to malnutrition early in live, even after nutritional rehabilitation[29]. This alteration in the emotional states is documented by delayed adaptation to a new environment[57] with higher locomotor activity in the open field[59] and by a lower response threshold to electric shock[60,22,16,5]; increased passive avoidance[50,3,4] or spontaneous fighting behavior[31]. The intensified reaction to electric shock has been clearly shown in several species, such as rats[30,63], mice[28], pigs[8] and monkeys[73]. It has also been shown that malnutrition early in life increases the number of errors and latency in a more complex learning or maze test[26] and also increases the rate of responding for food in operant situation[52]. Most investigators introduce malnutrition during lactation period, in an attempt to interfere with a brain development and growth during the critical or vulnerable period. However, the techniques available for malnutrition during lactation period are potentially harmful to the mother: nipple removal[11] or increased number of pups per litter[48,1]. Obviously, all these techniques or reduced about of died[7] produce strong changes in the mother-pup interacttion[36] an may result in other behavioral changes in the litter which are not related to reduced protein levels in the diet per se but rather to the technique used to produce malnutrition[61]. The objective of the present study was to evaluate if the protein-calorie malnutrition caused by offering free amounts of a low-protein died immediately after the vulnerable phase of the brain growth and maturation causes any changes in the brain and behavioral measures such as brain nucleic acid and biogenic amines levels and animal reaction in the locomotor and passive avoidance test.

MATERIAL AND METHODS

Animals

Sixty male albino rats from the local breeding colony, weighing 42.55 ± 5.3g, were transferred soon after weaning to the animal house of the Department of Pathology. The rats were individually housed in wire cage, kept in the same environment, with no artificial light-dark cycle or temperature control, and with water and food *ad libitum*.

Diets

Three types of diets of varying protein (casein) content were used throughout the experiments: control (20% casein) and two protein-deficient (5% or 8% casein) diets. In order to maintain diets isocaloric, the protein-deficient ones were prepared with proportional increase of sucrose (45.8% to the 5.0% diet and 42.8% to the 8.0% casein diet). The constituents of control diet were (g/1000g): casein 20.0; corn starch 30.8; sucrose 35.0; soybean oil 8.0; choline 0.2; saline mixture 5.0 (Association of Official and Agricultural chemist, Washington, DC); vitamin mixture 1.0 (Vitamin Diet Fortification Mixture, Nutritional Biochemicals Co., Cleveland, Ohio).

Experimental Design

The experimental design is shown in detail in Table I. Briefly, control (20.0 %) and low-protein

(5.0% casein) diets were offered to two different groups of rats for 6 weeks. At the end of this period, nine rats were taken at random from each group and killed. The remaining animals were used for a further experimental period of 6 weeks. The controls continued to receive the same diet, while the rats receiving the low-protein diet were divided into two groups: 1) group continuing on the low-protein diet (modified from 5.0 % to 8.0 % casein) 2) group recovered from the low-protein diet, receiving the control diet. Rats which died spontaneously during the experimental periods were not taken into account when the data were calculated.

Biochemical Studies

The evolution of animal body weight was recorded weekly throughout the experimental periods. The rats were killed by decapitation under light ether anaesthesia. The weights of heart, lever, total brain and different brain regions (telencephalon, cerebellum, brain stem and hypothalamus) were recorded. The different brain regions were further subdivided as precisely as possible to provide left and right portions of each region, except for the hypothalamus. The right portions were studied for nucleic acids contents[25] and the left portions and hypothalamus were studied for norepinephrine[6] and 5-hydroxytriptamine contents[33].

Behavioral Studies

On the day prior to sacrifice, all rats were submitted to two daytime behavioral studies:

1) Locomotor activity: the test consisted of a single session in an open field cage (45.0 x 32.0 x 20.0cm, provided with 5 infrared photocells. The cells were installed at 15.0cm interval along the longest side of the cage. To record locomotion two of the cells were placed 2.0cm above the floor and to record the righting behavior the 3 remaining cells were installed 12.0cm above the floor. The interruptions of the infrared flashing light between cells were individually recorded with an automatic programming circuit. The 20 minutes session of the open field test were divided in 8 blocks of 2.5 minutes for recording purpose.

2) Platform test: an adapted Mower cage of 30.0 x 15.0 x 20.0cm (Funbec model, São Paulo) with a grid floor of stainless steel bars of 0,3 cm in diameter, and spaced 1.3cm apart, connected to an shock generator (model 700, Grasson-Stadler, Inc, USA) was used for this test. A wooden platform of 5.0 x 15.0 x 5.0cm was installed in one side of the cage to avoid the grid shock. The animal was placed over the wood platform at the beginning of the test. The time until the step-down to the grid floor (pre-shock time) and the shock duration, (time until the animal returned to the wooden platform) – active avoidance - were recorded. The time for a second step-down onto the grid floor after the shock (passive avoidance latency) was also recorded. When the animal remained on the wooden platform after the shock without stepping down to the grid floor for 1.000 seconds the session was interrupted.

Statistical Analysis

The results were analyzed taking into consideration the duration of the experimental session, comparing the results of the 2 groups of diets studied over a period of 6 weeks and the results of the 3 groups maintained in the special diets for 12 weeks. The differences between results of the controls and animals malnourished for 6 weeks were analyzed using the *t* test. The differences between controls and malnourished animals for 12 weeks and animals recovered with control diet were compared using one way analysis of variance, and the Newman-Keuls test was used for *post hoc* analysis of differences between groups[63]. The level of significance of 0.05 was used

Table I - Experimental Design

Six week period		Six week period
Control diet		
	10 ——————————————→	Control diet, behavioral studies + sacrifice*
	1 died	
20 rats	9 behavioral studies + sacrifice* (N6)	
Low-protein diet (5% casein)		
	10 ——————————————→	Low-protein diet (8% casein) behavioral studies + sacrifice* (H12); 2 rats died before sacrifice (8 rats left)
	11 died	
40 rats	9 behavioral studies + sacrifice* (H6)	
	10 ——————————————→	Control diet, behavioral studies + sacrifice* (R12)

*Sacrifice at the end of the experimental period; N6, N12, H12 H6 and R12 are used to identify each experimental group.

Results

Body weight

Mean body weight data for the various groups assessed during the experimental period are shown in the figure 1. Malnourished rats (groups H6 and H 12) showed less increases in body weight gain than the control group. The recovered group (R 12) exhibited an increase of body weight when the diet was changed from a low protein level (5.0%) to a higher protein level (20.0%). However, at the end of experimental period this group (R 12) showed a lower mean body weight than the control group (N 12).

Brain Weight

Data of brain weight and mean weight of different brain regions obtained for the biochemical studies are presented in table II. The brain weight was consistently lower in the rats fed with low-protein diets.

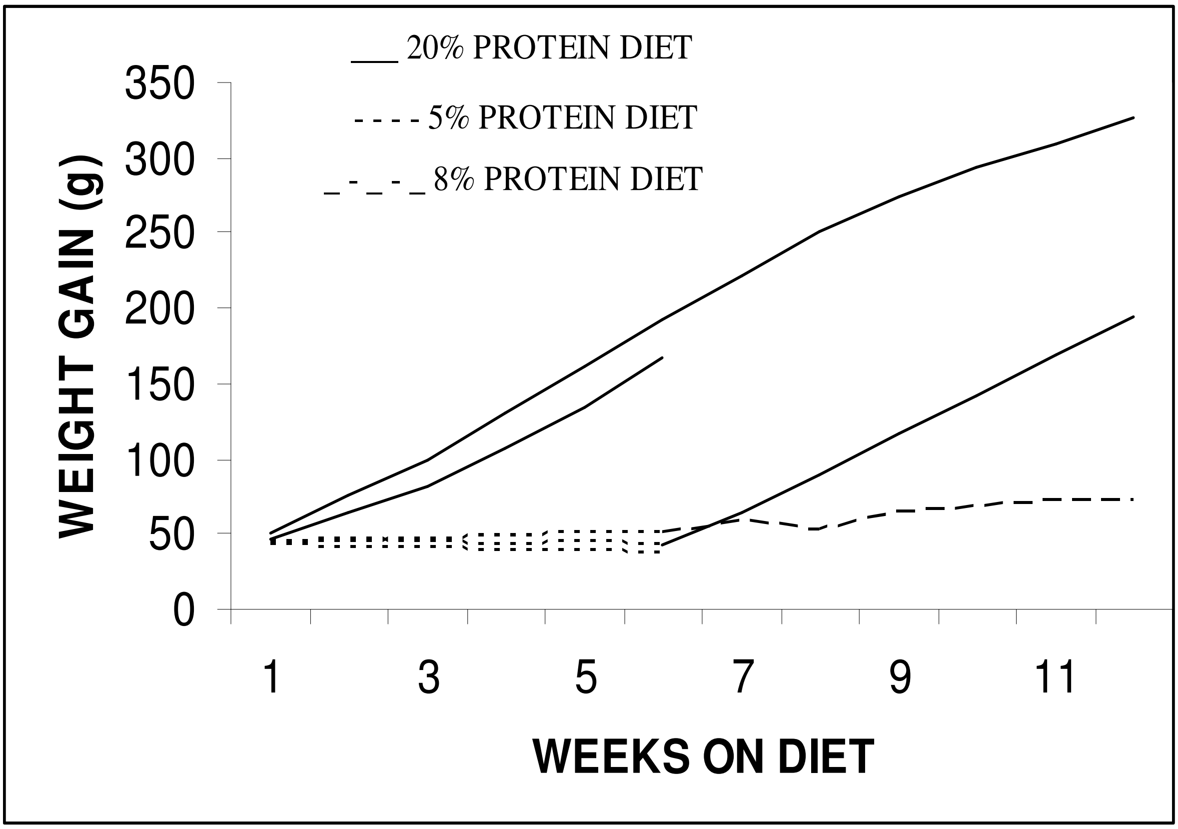

Figure 1. Mean body weight gain curves for the various rat groups.

Table II - Weight of brain and of different brain regions (mean±SD) of the groups studied

	Groups studied for 6 weeks		Groups studied for 12 weeks		
Weight	**Control (N6)**	**Low- protein (H6)**	**Control (N12)**	**Low- protein (H12)**	**Recovered (R12)**
Brain (g)	$1,6 \pm 0,10$	$1,33 \pm 0,07^{*}$	$1,76 \pm 0,08$	$1,45 \pm 0,05^{\Delta}$	$1,61 \pm 0,07^{\circ\dagger}$
Telencephalon (mg)	$902,2 \pm 78,8$	$773,3 \pm 73,4^{*}$	$980,0 \pm 72,1$	$791,2 \pm 49,6^{\Delta}$	$893,0 \pm 43,1^{\circ\dagger}$
Brain stem (mg)	$417,7 \pm 54,5$	$326,6 \pm 52,9^{*}$	$474,0 \pm 57,3$	$393,7 \pm 27,8^{\Delta}$	$409,0 \pm 27,7^{\circ\dagger}$
Cerebellum (mg)	$222,2 \pm 15,4$	$187,7 \pm 20,9^{*}$	$251,0 \pm 25,8$	$212,5 \pm 22,2^{\Delta}$	$240,0 \pm 30,33^{\circ\dagger}$
Hypothalamus (mg)	$28,3 \pm 3,6$	$16,4 \pm 3,9^{*}$	$38,3 \pm 3,6$	$23,3 \pm 3,9^{\Delta}$	$31,6 \pm 3,1^{\circ\dagger}$

N6 versus H6 $^{*}p < 0.05$ (Student test). N12 versus_H12 $^{\Delta}$ $p < 0.05$;H12 versus R12 $^{\circ}p < 0.05$; N12 versus R12 $^{\dagger}p < 0.05$
(Newman-Keuls test)

Biochemical Studies (measures)

The biochemical results are shown in table III. Higher norepinephrine concentration was detected in the brain stem of rats fed with low-protein diet for 6 weeks and in the hypothalamus of rats fed with low protein for 12 weeks. The telencephalon and hypothalamus of rats fed the low protein diet for 6 weeks presented a higher 5-hydoxytriptamine concentration. A consistently lower nucleic a lower nucleic acids concentration (DNA and RNA) was found in the 3 brain regions of rats fed with low-protein diet for 6 weeks. In the group fed with low protein for 12 weeks and the recovered group for 12 weeks, DNA levels were decreased in all 3 brain regions, when compared to the control group, but RNA concentration did not showed significant differences.

Table III - Concentrations (mean +SD) of biogenic amines and nucleic acids in different brain regions of the groups studied

Substance	Region	Groups studied for 6 weeks		Groups studied for 6 weeks		
		Control (N6)	Low-protein (H6)	Control (N12)	Low-protein (H12)	Recovered (R12)
Nor (μg/g)	Telencephalon	0,21± 0,07	0,27 ± 0,09	0,19 ± 0,06	0,24 ± 0,11	0,23 ± 0,06
	Brain stem	0,36 ± 0,13	0,57 ± 0,18*	0,27 ± 0,11	0,37 ± 0,14	0,32 ± 0,09
	Cerebellum	0,15 ± 0,05	0,15 ± 0,06	0,10 ± 0,03	0,15 ± 0,07	0,13 ± 0,05
	Hypothalamus	0,82 ± 0,43	1,18 ± 0,47	0,48 ± 0,16	0,91 ± 0,33$^{\Delta}$	0,55 ± 0,10°
5HT (μg/g)	Telencephalon	0,28 ± 0,06	0,36 ± 0,07*	0,22 ± 0,05	0,29 ± 0,08	0,25 ± 0,08
	Brain stem	0,57 ± 0,16	0,61 ± 0,15	0,32 ± 0,08	0,37 ± 0,10	0,36 ± 0,13
	Cerebellum	0,46 ± 0,19	0,47 ± 0,21	0,20 ± 0,11	0,23 ± 0,13	0,20 ± 0,05
	Hypothalamus	2,02 ± 0,34	3,04 ± 0,63*	0,56 ± 0,12	0,73 ± 0,39	0,65 ± 0,37
DNA (μg/g)	Telencephalon	1,68 ± 0,04	1,24 ± 0,11*	1,87 ± 0,05	1,49 ± 0,07$^{\Delta}$	1,53 ± 0,11†
	Brain stem	2,41 ± 0,29	2,02 ± 0,08*	2,94 ± 0,17	2,24 ± 0,20$^{\Delta}$	2,66 ± 0,26$^{\circ\dagger}$
	Cerebellum	3,64 ± 0,33	3,24 ± 0,40*	3,55 ± 0,28	2,83 ± 0,29$^{\Delta}$	2,86 ± 0,18†
RNA (μg/g)	Telencephalon	2,04 ± 0,21	1,77 ± 0,20*	2,26 ± 0,17	1,94 ± 0,41	1,95 ± 0,33
	Brain stem	2,22 ± 0,21	1,78 ± 0,30*	2,60 ± 0,41	2,31 ± 0,43	2,32 ± 0,38
	Cerebellum	2,70 ± 0,37	2,13 ± 0,31*	2,76 ± 0,29	2,32 ± 0,58	2,48 ± 0,44

N6 versus H6 $^{*}p < 0.05$ (Student test). N12 versus_H12 $^{\Delta}$ $p < 0.05$;H12 versus R12 $^{\circ}p < 0.05$; N12 versus R12 $^{\dagger}p < 0.05$ (Newman-Keuls test)

Behavioral Measures

The data of locomotor activity of the different groups of rats are showed in figure 2 as a mean number of impulses recorded the infrared photocells during the experimental session. All animals submitted to the low-protein diet (including the recovered group) showed higher locomotor activity when compared to the control groups.

The information obtained by platform test is summarized in the figure 3. Malnourished rats took longer time to step down from the platform to the grid floor (longer pre-shock latency). Rats fed with control diet escaped from the electric shock faster than the malnourished rats. The passive avoidance latency (period on the platform) was extremely short in both the control groups, in contrast with the longer duration observed in the malnourished and recovered animals. In both situations the recovered rats behaved like the control group.

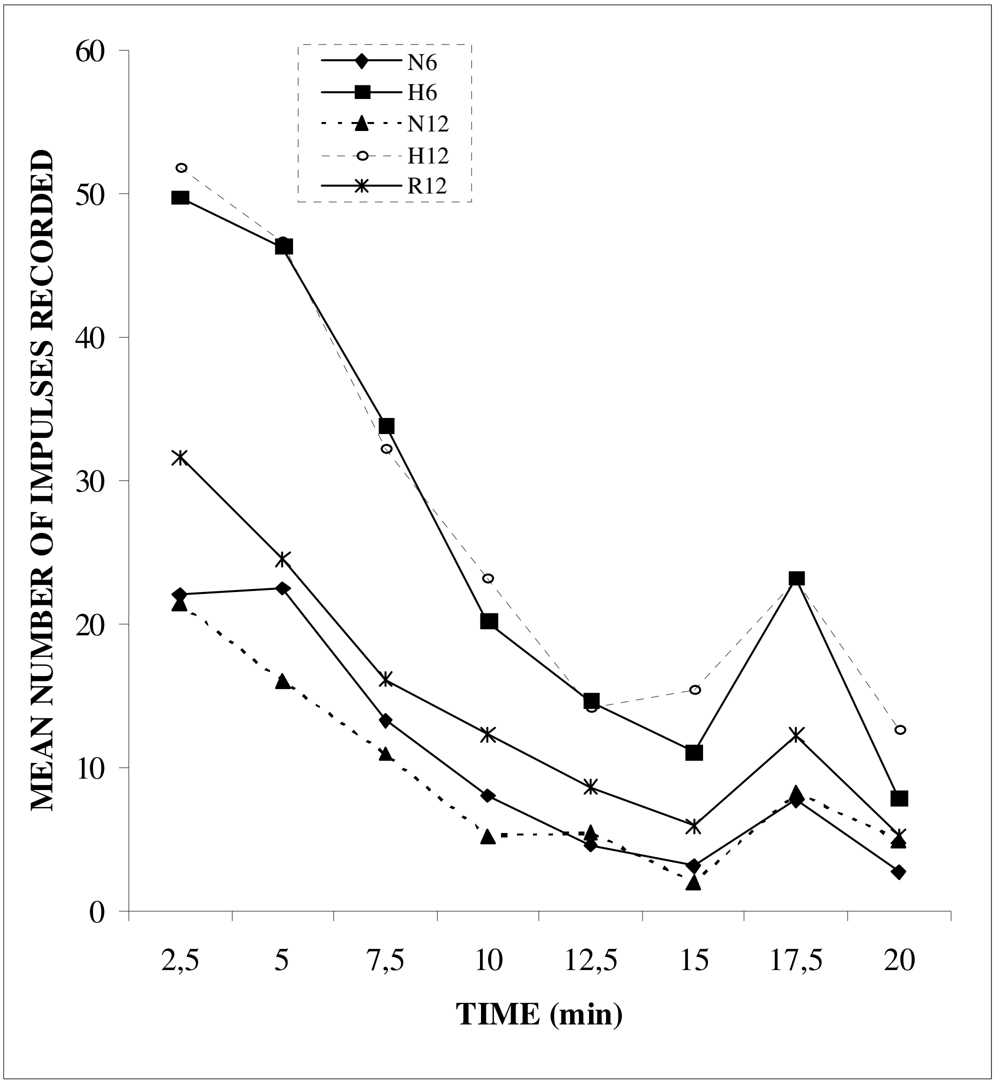

Figure 2. Locomotor Activity of the various rat groups expressed as the total number of impulses detected by the five infrared photocells.

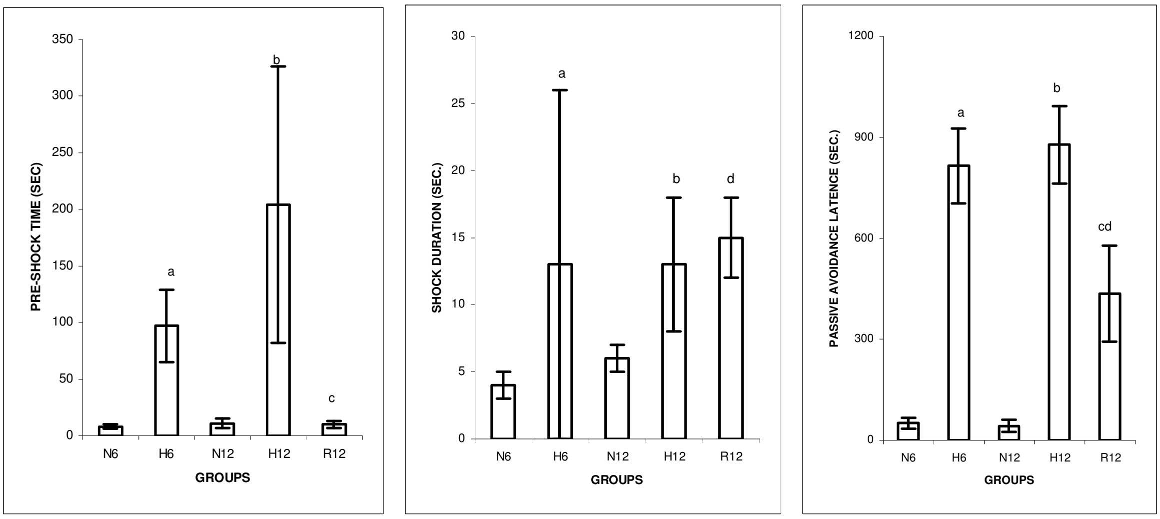

Figure 3. Platform test: mean (± SD) duration of pre-shock time and passive avoidance latency time for the rat groups studied. N6 *versus* H6 $^{a}p < 0.05$ (Student test). N12 *versus* H12 ^{b} $p < 0.05$; H12 *versus* R12 $^{c}p < 0.05$; N12 *versus* R12 $^{d}p < 0.05$ (Newman-Keuls test).

DISCUSSION

The deleterious effects of protein-calorie malnutrition on the rat central nervous system are more evident during the prenatal period[39] or during lactation[72] and almost no effect on brain growth has been shown after weaning[52,40,41,42]. Thus, most investigators prefer to work on the lactating dam during lactation and gestation. The stress caused to the lactating dam by impairing the mother-pup interaction affects the study of behavior regardless of the direct effects of malnutrition on the brain[12,61,51]. These factors can be eliminated by working with after-weaning malnutrition by feeding the animals a low-protein diet "ad libitum" under the same environmental conditions.

The diet used[43] had already been show to be effective in previous studies from this laboratory[53,47]. The high mortality rate at the end of the first phase due to the low initial weight of the rats forced us to change the low-protein diet from 5% to 8% casein during the 2nd phase. Weight gain was markedly reduced in the malnourished groups (H6 and H12), and body weight was partially restored in the group allowed to recover (R12).

The literature has shown that the brain is relatively protected in relation to body weight[18], with permanent somatic effects occurring in the central nervous system when malnutrition takes place during the most vulnerable period[70,42]. Our experiment showed a significant weight deficit in the brain of malnourished rats even when malnutrition had occurred outside the critical period, with partial recovery only in the "recovered group", and therefore with a permanent deficit. Widdowson and Dobbing[68] using less drastic post-weaning protein malnutrition, found a similar deficit in brain weight, but obtained full recovery after repletion. With respect to the various brain regions, the deficit is permanent mainly at the level of the cerebellum[21,64,39]. In our study, the deficit occurred in all regions, with no predominance for the cerebellum, probably because the rats were submitted to malnutrition when the phase of greatest cerebellar growth had already passed at the end of lactation.

Our results showed significantly higher concentrations of both noradrenaline and serotonin in the hypothalamic of malnourished rats (groups H12 and H6, respectively). Contradictory results have been reported in the literature, such as decreased concentration of noradrenaline[66,54,9,35] or 5HT[22,19,24,35], and increased noradrenaline[65,66,34] and 5HT[65,66,67,64,45,23,44], as well as no significant differences[45,2]. In the present experiment, concentration returned to normal values after recovery of nutritional status (group R12). We believe that the discrepant data in the literature in terms of biogenic amines are due to the different methodologies used to the variability of animal response even in groups submitted to the same treatment.

The literature reports reduction of brain nucleic acid contents when malnutrition occurs during lactation[69,71,46,3], but no change when malnutrition occurs after weaning[20]. However, by modifying a low-protein diet[17] induced a permanent reduction in brain nucleic acids. In the present study, we detected significantly lower DNA levels in the forebrain, brain stem and cerebellum of malnourished rats (groups H6 and H12). The recovered groups (R12) showed irreversible brain damage at the forebrain and stem level. No significant differences were observed in RNA content. Since the amount of DNA is an index of cell number[37], we could interpret these results for malnourished rats as a smaller number of cells, with no significant recovery after repletion. However, we do not know whether there is a smaller number of neurons or only of glial cells, or whether this deficiency has a significant effect on normal brain functional activity. Indeed, we do not know how many neurons or other cells are needed for normal brain functioning[10].

The behavioral study showed greater locomotion in the activity cage of malnourished rats, as compared to control group. The recovered group showed intermediate results. Activation of photoelectric cells was more intense during the first five minutes of each session, and rapidly decreased throughout the session for all animals. The control animals, however, always showed lower activation of the cells, which may indicate more rapid adaptation to the new environment. According to Klein[27], malnourished animals are more excitable and react more intensely to stimuli. In contrast, Sobotka[64] found greater activity in the control rats and considered it to be exploratory activity. We believe that well-nourished rats are more adaptable, showing good initial exploratory activity, but soon quieting down and adapting more easily to the new environment. Other reports in the literature agree with the present results[31,49,59,61,3,4].

With respect to behavior in response to electric shock, malnourished rats took longer to step down from the platform (pre-shock time), probably due to the delay in adapting to the new environment, thus confirming data obtained for the same group in the activity test. Well-nourished rats quickly located the platform after receiving the electric shock, thus escaping the aversive stimulus, whereas the malnourished rats jumped, cried, climbed the cage walls, bit the bars, urinated and defecated more during the shock, taking longer to escape the aversive stimulus. The recovered groups took even longer than the malnourished animals to escape shock, but did not show such intense aggressive responses. In terms of returning latency, most malnourished rats did not step down again onto the grid floor within a period of 1000 seconds after escaping the shock, whereas the well-nourished rats stepped down again within a shorter time, and the recovered group behaved in an intermediate way between malnourished animals. This latency for return to the platform is considered as a passive escape from the aversive stimulus. Smart et al.[58] found inhibited motion by malnourished rats in terms of avoiding electric shock, and Sobotka[64] found considerably greater returning latency in malnourished rats. Our results may be considered to agree with those reported in the literature.

The integration between the effects of malnutrition on the structure of the central nervous system and the functional alterations due to these modifications has not yet been fully elucidated. In the present study we made an attempt to integrate the different ramifications on this subject. We believe that the behavioral changes found here are directly related to structural deficiency of the central nervous system caused by post- weaning malnutrition since the environment was held constant for each groups. However, this interpretation deserves a more precise study for a better understanding of the correlations between brain and behavioral changes.

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