

EFEITOS DE DESNUTRIÇÃO PROTÊICA E ESTIMULAÇÃO AMBIENTAL PRECOSES E CONCORRENTES  
SOBRE O SISTEMA NERVOSO CENTRAL E COMPORTAMENTO\*

EFFECTS OF EARLY CONCURRENT PROTEIN MALNUTRITION AND ENVIRONMENTAL STIMULATION  
ON THE CENTRAL NERVOUS SYSTEM AND BEHAVIOR

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**ABSTRACT**

A complex program of environmental and sensory stimulation was developed to study its potential effects in reversing some of the alterations produced by early protein malnutrition in the brain and behavior of rats. Litters (dam plus 6 male pups) were fed diets containing 8% (malnourished) or 25% (well-nourished) casein. After weaning, the animals were maintained on the same diets as their respective dams until 50 days of age. Environmental stimulation consisted of 3-min daily handling from birth to 50 days of age by rearing the rats in an enriched living cage and exposing the animal to visual, auditory, and olfactory stimuli. At 50 days of inhibitory avoidance tests. Animal of the same age were sacrificed, the brain removed and divided in telencephalon, brain stem and cerebellum. DNA and RNA were assessed in telencephalon and cerebellum. Protein malnutrition produced brain weight deficits that were partially reversed by environmental stimulation. The behavioral measure showed lower locomotor activity and higher latencies in inhibitory avoidance for malnourished animals as compared to well-nourished animals. Environmental stimulation reduced the aversiveness in the inhibitory avoidance test as showed by lower latencies in the stimulated group of animals. These results suggest that early protein malnutrition impairs brain and behavior of rats and a complex program of environmental stimulation is beneficial to reverse some of those impairments.

*Keywords:* Protein malnutrition, Environmental enrichment, Brain weight, Locomotor activity, Inhibitory avoidance.

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It has been consistently demonstrated that protein or protein-calorie malnutrition early in life causes body and brain changes both in humans and experimental animals (Dobbing, 1987; Levitsky, & Strupp, 1995; Lima et al., 1993; Morgane et al., 1992; 1993; Patel, 1983). Regarding the brain changes, long-term effects of malnutrition on brain morphology (Bedi, 1987; Cintra et al., 1989; Cordero et al., 1985; Morgane et al., 1992; 1993) and neurochemistry (Almeida et al., 1996; Chen et al., 1992; Blatt et al., 1994; Wiggins et al., 1984) have been reported. Malnutrition early in life also causes a decrease in the total number of cells in the central nervous system of malnourished animals when compared to well-nourished controls (Leuba & Robinowicz, 1979; Ishimura, 1985). The content of DNA (a classical index of cell number) is frequently reported to be reduced by protein malnutrition in rats (Bedi, 1986; Sobotka et al., 1974; Srivastava, 1985; Winick & Noble, 1966; Zamenhof et al., 1971).

Protein malnutrition imposed early in life also changes the behavior of the animals. Malnourished rats consistently present a lower threshold in response to painful aversive stimuli (Lynch, 1976; Smart et al., 1975) and higher passive avoidance latencies (Almeida et al., 1988; 1992; Lynch, 1976). In addition, locomotor activity has been shown to be changed by both concurrent (Almeida et al., 1992; Frankova, 1975; Villescascas et al., 1979) and previously imposed protein malnutrition (Cowley & Griesel, 1964; Frankova, 1975; Frankova & Barnes, 1968; Levitsky & Barnes, 1970; Smart, 1974; Tonkiss & Smart, 1983; Villescascas et al., 1979).

It has also been suggested that early environmental stimulation (enriched environment, social stimulation and/or handling) can be efficient in correcting some of the alterations produced by protein malnutrition in the brain (Adaro et al., 1986; Bedi et al., 1988; Bhide & Bedi, 1982; 1984a, b; Carughi et al., 1989) and in behavior (De Oliveira & Almeida, 1985; Frankova, 1968; Levitsky & Barnes, 1972; Santucci et al., 1994). Environmental stimulation increases brain weight and protein synthesis (Renner & Rosenzweig, 1987), as well as the levels of growth hormones and ornithine decarboxylase in both animals and humans (Schanberg & Field, 1987). However, the issue is controversial and some studies did not report any improvements in the brain and behavior of malnourished animals produced by some kind of environmental stimulation (Rocha & Mello, 1994).

Thus, the main aim of the present study was to develop a complex program of environmental and sensory stimulation and to study its potential effects in reversing some of the alterations produced by protein malnutrition in the brain and behavior of rats. This program consists of tactile manipulation (handling), social interaction (housing the animals in groups) in large cages with objects to explore, as well as visual, auditory and olfactory stimulations.

## METHODS

### Subjects

Forty-eight litters of Wistar rats from the animal house of the Campus of Ribeirão Preto, University of São Paulo, were used. Within 12h of birth, male pups were weighed and randomly assigned to a litter of six per dam. The dams and pups were placed in transparent plastic cages (40 x 31 x 17 cm) and randomly assigned to receive either diets were isocaloric and prepared as previously described (Almeida et al., 1991). The protein deficient diet contained 8% casein, 5% salt mixture, 1% vitamin mixture, 8% corn oil, 0.2 choline, and 77.8% cornstarch. The normoprotein diet contained 25% casein, 60.8% cornstarch, and the same percentage of the other constituents as the in protein-deficient diet. The two diets were supplemented with L-methionine (2.0g/Kg of protein) since casein is deficient in this amino acid. The litters were maintained on these diets until the end of lactation (21 days). After weaning, the animals were maintained on the same diets as their respective dams until 50 days of age. The rats were maintained under a 12hL : 12hD cycle (lights on at 0700h) and room temperature was kept at 23-25°C. These conditions meet the standard for the care of laboratory animals outlined in the Guide for the Care and Use of Laboratory Animals (NRC, 1996).

### Environmental Enrichment and Stimulation

During the lactation period (0-21 days of age) half of the pups assigned to each diet condition were submitted to daily stimulation by handling while the other half was maintained without manipulation. Handling consisted of placing the animal in one hand and stroking its dorsal region cranio-caudally for 3 min with the thumb of the other hand. After weaning (22-49 days of age) the stimulated and non-stimulated animals assigned to each diet condition were housed individually in 25 x 19 x 15 cm metal cages or in groups in 50 x 42 x 29 cm metal cages. Additionally, the stimulated animals were maintained in cages enriched with a variety of objects such as wooden blocks, plastic platforms, stairs, mirrors and marbles. The non-stimulated animals were maintained in similar metal cages without the objects. Thus, eight groups were formed: well-nourished individually housed and stimulated, well-nourished individually housed and non-stimulated, well-nourished housed in group and stimulated, well-nourished housed in groups and non-stimulated.

The same group composition was applied to malnourished animals. After eye opening, the handling was conducted outside the animal room to provide additional visual and auditory stimulation. After weaning, the animals were also exposed to olfactory stimulation by coating the hands of the experimenter with a deodorant (Miss France, Gressy Lever, Ltd.) prior handling.

### Locomotor Activity

A Plexiglas cage (45 x 32 x 20 cm) provided with a system of five infrared photocells was used. The photocells were connected to programming equipment

(Grason Stadler, USA), so that experimental sessions could be run automatically. Three photocells were located at a height of 6 and 12 cm for malnourished and control animals respectively, in order to record the rearing behavior, and two photocells were located at a height of 4 and 6 cm to record ambulatory behavior. At 47 days of age the animals were placed in the center of the cage and the rearing and ambulatory behaviors were recorded in one session of 20 min divided into blocks of 2.5 min.

### **Inhibitory Avoidance**

For the inhibitory avoidance test, a wooden platform (15 x 12 x 3 cm) was placed on the right side of a modified Mowrer cage (FUNBEC, Brazil, 91 x 15 x 31 cm). A sliding door activated by the experimenter separated the platform from the electrified grid floor. The animals were placed on top of the wooden platform and after 10s the sliding door was opened. The latency to step down from the platform to the grid floor was measured (pre-shock latency). This procedure was repeated three times at 30-min intervals. On the third trial, immediately after the animals stepped down only one shock of 0.6-mA intensity (Grasson-Stader shock generator, model 700, USA) and 2-s duration was applied to the paws of the animals. After the end of the shock, the animals were immediately replaced on the wooden platform and 10s later the door was opened again and the latency step down to the grids was measured (post-shocking latency). The maximum time allowed for one trial was 900 s.

### **Brain Weight and Biochemical Measurements**

At 50 days of age, three animals from each group were weighed and sacrificed. The brain was removed and divided into three regions: telencephalon, brainstem and cerebellum. DNA and RNA were assessed in telencephalon and cerebellum using procedure described by Karsten and Wollenberger (1972).

### **Statistical Analysis**

The body weight of the pups and food intake during the lactation period were analyzed by a three-factor (diet, environmental stimulation and days of the lactation) analysis of variance (ANOVA), with repeated measures for the last factor (days 0,7,14 and 21 of lactation). The brain weight and biochemical measures (DNA and RNA) of pups at 50 days of age were analyzed by a three-factor (diet, housing and environmental stimulation) ANOVA. The locomotor activity was analyzed by a four-factor (diet, housing, environmental stimulation and blocks of experimental session) ANOVA, with repeated measures for the last factor (blocks 1-8 of the experimental session). Passive avoidance was analyzed by a four-factor (diet, housing, environmental stimulation and phase of testing) ANOVA, with repeated measures for the last factor (pre- and post-shocking). When appropriate, post-hoc

analysis was conducted using the Newton-Keuls test with the alpha level set at 0.05.

## **RESULTS**

### **Food Intake**

The food intake (data not shown) recorded on days 0,7,14 and 21 of lactation showed that both malnourished and well-nourished animals increased their food intake from day 0 to day 21 as shown by a significant main effect on days  $F(3,191) = 2031.14$ ,  $p < 0.001$ . However, the well-nourished animals showed both higher intake and a higher increase in food intake from day 0 to day 21 as shown by a significant effect of diet  $F(3,191) = 167.13$ ,  $p < 0.001$  and a significant diet by day interaction  $F(9,191) = 170.97$ ,  $p < 0.001$ . A post-hoc analysis showed that well-nourished dams had higher food intake ( $p < 0.05$ ) than malnourished dams on days 7, 14 and 21.

### **Pup Body Weight**

Pup body weight determined during the lactation period showed that both malnourished and well-nourished animals increased their body weight from day 0 to day 21 as represented by a significant main effect on days  $F(3,191) = 2031.14$ ,  $p < 0.001$ . However, the well-nourished animals had both a higher weight and a higher increase in the body weight from day 0 to day 21 as shown by a significant effect of diet  $F(3,191) = 167.13$ ,  $p < 0.001$  and a significant diet by day interaction  $F(9,191) = 170.97$ ,  $p < 0.001$ . A post-hoc analysis showed that well-nourished pups had a higher body weight ( $p < 0.05$ ) than malnourished pups on days 7,14 and 21. At the end of the lactation period (day 21) the malnourished animals had a 57% deficit in body weight compared to the well-nourished group (Figure 1).

Body weight determined during the post-lactation period (22-49 days of age) showed that both malnourished and well-nourished animals increased their body weight from day 28 to day 49 as shown by a significant effect of diet  $F(3,239) = 1199.15$ ,  $p < 0.001$ . However, the well-nourished animals had both a higher weight and a higher increase in body weight from day 28 to day 49 as shown by a significant effect of diet  $F(3,239) = 171.34$ ,  $p < 0.001$  and a significant diet day by day interaction  $F(28,239) = 94.46$ ,  $p < 0.05$ ) than malnourished animals had in all days analyzed. At the end of post-lactation period (day 49) the malnourished animals had an 78% deficit in body weight a compared to the well-nourished group (Figure 1).

The handling and environmental enrichment procedures during the lactation and post-lactation period did not affect the body weight of animals.

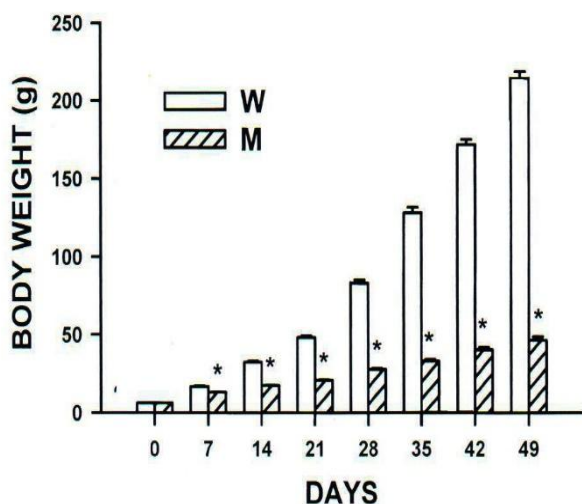


Figure 1. Weekly mean body weight of well nourished (W) and malnourished (M) from birth to 49 days of age. Values represent the mean  $\pm$ SEM. \* $p < 0.05$  compared to well-nourished animals of the same age.

### Pups Brain Weight

Well-nourished animals showed a higher total brain weight than malnourished animals as evidenced by a significant effect of diet factor  $F(1,23) = 108$ ,  $p < 0.001$ . In addition, stimulated animals showed a higher total brain weight compared with non-stimulated animals as evidenced by significant effect of stimulation factor  $F(1,23) = 27.77$ ,  $p < 0.001$ . The total brain weight of individually housed animals was higher than the total brain weight of group-housed animals in the situation of environmental stimulation. However, in non-stimulated animals the result was the opposite, i.e., grouped animals presented higher total brain weights. These differential effects showed a significant housing  $\times$  stimulation interaction  $F(1,23) = 5.66$ ,  $p < 0.05$  (Figure 2).

ANOVA also showed higher weights of both brainstem,  $F(1,23) = 36.16$ ,  $p < 0.001$ , and cerebellum  $F(1,23) = 38.65$ ,  $p < 0.001$ , in well-nourished animals than in malnourished animals. The environmental stimulation also produced higher weights of brainstem,  $F(1,23) = 10.53$ ,  $p < 0.01$ , and cerebellum,  $F(1,23) = 10.53$ ,  $p < 0.01$ , and cerebellum,  $F(1,23) = 28.39$ ,  $p < 0.001$  in well-nourished animals than in malnourished animals (Figure 2). No interaction effects were observed.

### Biochemical Measurements

The total amount of DNA (Table I) was higher in well-nourished animals than in malnourished animals as evidenced by a significant effect of diet on both telencephalon  $F(1,16) = 55.93$ ,  $p < 0.001$  and cerebellum  $F(1,16) = 55.93$ ,  $p < 0.001$  and cerebellum  $F(1,16) = 26.33$ ,  $p < 0.001$ . The stimulated animals showed increases in total DNA compared with non-stimulated animals as evidenced by a significant effect of environmental stimulation on

both telencephalon  $F(1,23) = 17.11$ ,  $p < 0.001$  and cerebellum  $F(1,23) = 17.13$ ,  $p < 0.001$ . Regarding RNA content, ANOVA showed higher amounts in well-nourished animals in the telencephalon as evidenced by a significant effect of diet  $F(1,23) = 5.84$ ,  $p < 0.05$ , as well as higher amounts of RNA in stimulated animals than in non-stimulated animals  $F(1,23) = 8.16$ ,  $p < 0.01$ . No significant differences in RNA levels were found in the cerebellum.

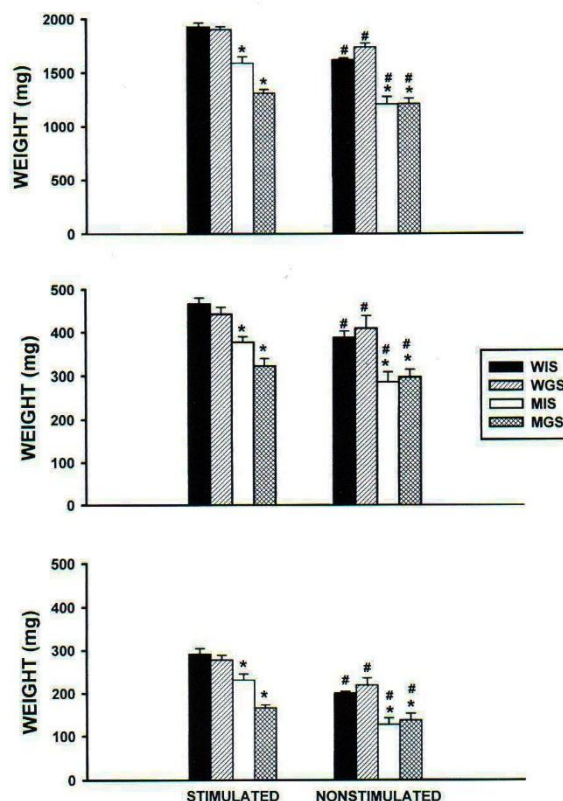


Figure 2. Mean total brain weight (upper) and brain stem (middle) and cerebellum (bottom) weight of well-nourished and malnourished animals. WIS = well-nourished individually housed and stimulated group; WGS = well-nourished group-housed and stimulated group; MIS = malnourished individually housed and stimulated group. Left columns represent weights from stimulated animals and right columns represent weights from nonstimulated animals. Values represent the mean  $\pm$ SEM. \* $p < 0.05$  compared to the well-nourished groups and # $p < 0.05$  compared to the stimulated groups.

### Locomotor Activity

The locomotor data are presented in Figure 3. The well-nourished animals showed a higher locomotor activity than malnourished animals as evidence by a significant effect of diet  $F(1,136) = 31.62$ ,  $p < 0.001$ . Grouped animals demonstrated a lower locomotor activity than individually housed animals as evidence by a significant effect of housing  $F(1,136) = 5.84$ ,  $p < 0.01$ . In addition, the locomotor activity decreased across the 8 blocks of 2.5 min of the experimental session as evidenced by a significant effect of blocks  $F(1,136) = 55.68$ ,  $p < 0.001$ . However, the decrease in locomotor activity within the experimental session was higher in well-nourished animals compared with the decrease seen in

malnourished animals. This differential effect due to diet led to a significant diet x block interaction  $F(7,952) = 5.52$ ,  $p < 0.001$ . Finally, a significant effect of housing x stimulation interaction  $F(1,136) = 28.70$ ,  $p < 0.001$ , emphasizes the differential effects of the stimulation in grouped and individually housed animals. The post-hoc analysis showed that animals housed in groups and

submitted to stimulation had a higher locomotor activity than non-stimulated grouped animals ( $p < 0.05$ ). The opposite was observed with animals housed individually, i.e., non-stimulated animals showed higher locomotor activity than stimulated animals ( $p < 0.05$ ).

Table 1. Total amount of DNA and RNA in the telencephalon and cerebellum of experiment and control groups.

	DNA		RNA	
	Telencephalon	Cerebellum	Telencephalon	Cerebellum
WIS	0.601 ± 0.07	0.379 ± 0.03	1.126 ± 0.14	0.301 ± 0.07
WGS	0.680 ± 0.03	0.402 ± 0.03	1.583 ± 0.31	0.482 ± 0.11
MIS	0.439 ± 0.03	0.304 ± 0.04	0.789 ± 0.07	0.465 ± 0.06
MGS	0.438 ± 0.03	0.279 ± 0.07	0.773 ± 0.10	0.187 ± 0.03
WIN	0.503 ± 0.03	0.343 ± 0.03	0.827 ± 0.11	0.401 ± 0.04
WGN	0.507 ± 0.03	0.288 ± 0.02	0.606 ± 0.04	0.458 ± 0.07
MIN	0.308 ± 0.03	0.147 ± 0.09	0.535 ± 0.07	0.302 ± 0.11
MGN	0.393 ± 0.01	0.195 ± 0.04	0.637 ± 0.08	0.561 ± 0.08

WIS = well-nourished individually housed and stimulated group; WGS = well-nourished group-housed and stimulated group; MIS = malnourished individually housed and stimulated group; MGS = malnourished group-housed and stimulated group; WIN = well-nourished individually housed and non-stimulated group; WGN = well-nourished group-housed and non-stimulated group; MIN = malnourished individually housed and non-stimulated group; MGN = malnourished group-housed and non-stimulated group. Values represent the mean ± SEM.

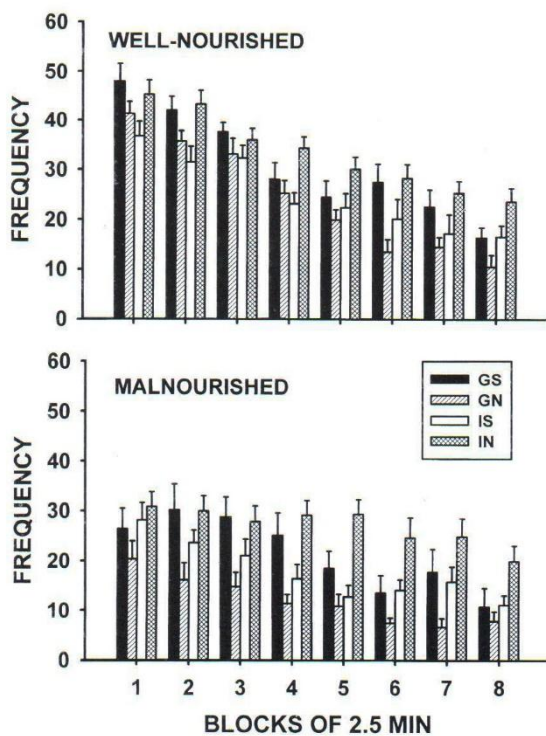


Figure 3. Mean frequency of locomotor activity in blocks of 2.5min for well-nourished (upper) and malnourished (bottom) groups. GS = group-housed and stimulated group; GN = group-housed and non-stimulated group; IS = individually housed and stimulated group and IN = individually housed and non-stimulated group. Values represent the mean ± SEM.

### Inhibitory Avoidance

The inhibitory avoidance data are represented in Figure 4. The malnourished animals showed a higher latency than malnourished animals during both the pre- and post-shock phases as evidenced by a significant effect of diet  $F(1,76) = 34.49$ ,  $p < 0.001$ . Animals housed in groups showed higher latencies than individually housed animals as demonstrated by a significant effect of housing  $F(1,76) = 8.86$ ,  $p < 0.01$ . Stimulated animals presented a lower latency than non-stimulated animals as evidenced by a significant effect of environmental stimulation  $F(1,76) = 7.24$ ,  $p < 0.01$ . Finally, animals of all groups increased latency in the post-shock phase as indicated by a significant effect of phase  $F(1,76) = 92.54$ ,  $p < 0.001$ . However, a greater increase in the latency of the post-shock phase for malnourished animals than for well-nourished animals led to a significant diet x phase interaction  $F(1,76) = 23.27$ ,  $p < 0.001$ . In addition, animals housed in groups showed a greater increase in the latency of post-shock phase as compared with individually housed

animals, as evidenced by a significant housing x phase effect  $F(1,76) = 5.54, p < 0.05$ .

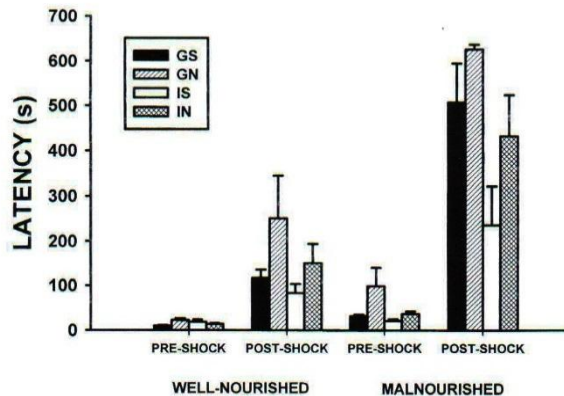


Figure 4. Mean latency of inhibitory avoidance during the pre- and post-shock phases for the well-nourished and malnourished groups. Group denominations as in Figure 3. Values represent the mean  $\pm$  SEM.

## DISCUSSION

Exposure of the litters to the balanced diets during the lactation period produced a higher food intake in controls when compared to malnourished dams. Thus, the lower food intake in the malnourished groups also produced a lower intake of the other nutritional elements of the diet rather than simply the protein source. In this way, the malnutrition procedure caused protein-calories malnutrition in the animals, since malnourished rats consumed significantly lower amounts of the low-protein diet. The lower intake of the low-protein diet produced lower body and brain weights in the malnourished animals indicating the effectiveness of the procedure in producing malnutrition, as previously demonstrated by our group (Almeida et al., 1991; 1992; Moreira et al., 1997) and by other (Dobbing, 1987). In addition, the amount of DNA was significantly lower in malnourished animals than in controls in both telencephalon and cerebellum. These results may indicate a lower number of nervous cells in the malnourished animals, as previously reported in the literature (Bedi, 1986; Sobotka et al., 1974; Srivastava, 1985; Winick and Noble, 1966; Zamenhof et al., 1972).

The environmental stimulation produced an increase in total brain weight, suggesting that environmental enrichment can increase the number and/or size of the nervous cells, as supported by increased amounts of DNA and RNA observed in the present study after the procedure of environmental and sensory stimulation. However, the environmental stimulation procedure interacts with housing situation, producing higher brain weights in individually housed animals than in group-housed stimulated animals. The opposite was observed in non-stimulated animals. The reason for these interactional effects cannot be easily understood based only on the present data. Finally, the absence of interactions between nutritional and environmental factors on body and brain measurements, as well as on the biochemical measurements indicates that both malnourished and control animals benefit from a complex

program of environmental and sensory stimulation. Independent of the similar effects of our program environmental enrichment on malnourished and control animals, the improvement caused by this program clearly reduced the deficits caused by early protein malnutrition. These results stress the need for more experimental studies in humans to determine what kind of environmental enrichment is more efficient to help children to recover from protein or protein-calorie deficits imposed early in life.

The lower locomotor activity of the malnourish animals agrees with previously reported studies (Almeida et al., 1992) indicating that concurrent protein malnutrition impairs locomotion in a new stressful environment. However, the lower decay in the levels of exploration across session by the malnourished animals suggests that these animals have difficulties to habituate to a new stressful environment. This lower decrease in locomotion of the malnourished animals across sessions could be the result of an altered emotional response to new environments, suggesting that protein malnutrition could lead to injuries in neural substrates underlying emotional responsiveness. This interpretation finds support in a series of studies showing that protein malnutrition alters the morphology and neuro-chemistry of cerebral believed to participate in emotional responses such as hippocampus (Díaz-Cintra et al., 1991; Garcia-Ruiz et al., 1993) and amygdala (Escobar and Salas, 1993). In addition, in the present study group-housed animals showed lower locomotor activity than single-housed animals, indicating a possible beneficial effect of social contact on the emotional response to a novel environment, as reported previously (De Oliveira and Almeida, 1985; Kelly et al., 1996; Smith et al., 1997). The same argument could be used to explain why single-housed non-stimulated animals had higher locomotor activity than single-housed stimulated animals, showing the beneficial effect of environmental stimulation on the emotional response to a novel environment.

The suggestion of altered emotional responses following protein malnutrition early in life is reinforced by the results of the inhibitory avoidance test. In this test malnourished animals showed higher latencies to step-down both pre- and post-shock. In addition, malnourished animals show higher increments in the latency to step-down from the pre to post-shock phase as compared with control animals. This higher post-shock latency indicates a higher emotional response following shock delivery. Another possibility is higher sensitivity to the electric shocks since it has been previously demonstrated that early protein malnutrition leads to a lower threshold in a response to painful aversive stimuli (Almeida et al., 1992; Lynch, 1976; Smart et al., 1975).

The effects environmental enrichment observed in the present study show that complex environment (social, environmental and sensorial stimulation) is beneficial to both malnourished and control animals reinforcing previously reported studies on the importance of a rich environment for the development of the central nervous system and behavior (De Oliveira and Almeida,

1985; Levitsky and Barnes, 1972; Renner and Rosenzweig, 1987; Schanberg and Fields, 1987). Thus, the present data reinforce the need for careful environmental arrangements in order to supply both social and environmental stimulation in programs designed to permit recovery from deficits produced by early malnutrition in brain and behavior of both animals and humans.

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