Effect of Environmental Enrichment during Nutritional Rehabilitation on Body Growth, Blood Parameters and Cerebral Cortical Development of Rats[1]

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ABSTRACT Environmental enrichment has been reported to aid recovery from behavioral deficits associated with malnutrition in infants and young rats. This study investigated whether corresponding neuroanatomical changes could be detected. Rats were suckled either by well-fed dams or dams malnourished during lactation. At weaning, well-fed males were either housed in pairs (standard condition, SC) or 12 per large cage with toys (enriched condition, EC) and fed a 17% protein diet (SC control and EC control, respectively). Malnourished pups were fed either a 17% (rehabilitation; "rehab") or a 6% (low protein) protein diet and housed in the SC or EC environment (SC rehab, EC rehab, SC low protein, and EC low protein). After 30 d there were no differences in hematocrit, serum total protein and albumin levels between SC and EC animals. Rehab rats had significantly lower serum total protein and albumin levels than did controls. Cortical thickness and dendritic branching of occipital cortex pyramidal cells were evaluated. Early malnutrition did not permanently affect cortical thickness. EC rehab rats had thicker cortices than did SC rehab rats at almost all locations measured. SC rehab rats had fewer high order dendrites than did SC controls. The difference in dendritic branching between EC and SC rats was 44% among rehab rats, 21% among controls and 11% (not significant) among low protein–fed rats. Environmental enrichment during nutritional rehabilitation enhances dendritic branching and thickness of the occipital cortex. *J. Nutr.* 119: 2005–2016, 1989.

INDEXING KEY WORDS:

- *malnutrition* • *protein restriction*
- *environmental enrichment* • *cortical thickness*
- *dendritic branching* • *male rats*

Numerous reports have shown that children who suffered from severe protein-energy malnutrition in early childhood have persistent deficits in their mental development (1, 2). Controlled laboratory studies have repeatedly related malnutrition during the early postnatal period with long-term behavioral abnormalities in experimental animals (3, 4). These seemingly irreversible changes may be the most serious consequences of severe malnutrition.

Longitudinal studies where environmental modification was added to child rehabilitation/supplementation programs showed that when a program of environmental stimulation was incorporated into the treatment, significant progress in tests for mental development could be achieved (5–9). Similar evidence of the relationship among malnutrition, the environment and behavior has been obtained from controlled laboratory studies where functional deficiencies caused by malnutrition were reduced by an early experience in an enriched environment (10–14).

Environmental stimulation or enrichment appears to help in the recovery from some of the behavioral deficits due to malnutrition. The purpose of the present study was to investigate whether the combined effects of good nutrition and enriched environments are reflected in measurable structural changes within the rat cerebral cortex. Cortical thickness and dendritic branching of pyramidal neurons in the occipital cortex were evaluated.

Both of these parameters of cortical development have been shown to be affected by malnutrition early in life and to respond to environmental enrichment. In the rat (15–18) and in the mouse (19) cortical thickness was significantly reduced by preweaning malnutrition. While most of the studies that assessed the long-term effect of malnutrition indicate that nutritional rehabilitation is effective in restoring deficits in cortical thickness (20, 21), this finding has not been demonstrated conclusively in the entire dorsal cortex. On the

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other hand, many studies have demonstrated the long-lasting effect of malnutrition early in life on dendritic branching in the neocortex (22, 23), reticular formation (24–26) and cerebellum (27, 28).

The plasticity of the morphology of the cerebral cortex in response to environmental conditions has been clearly demonstrated (29, 30). Measuring cortical thickness from magnified, projected brain sections, Diamond and co-workers (31, 32) found that rats housed in an enriched environment for different lengths of time had significantly thicker cortices than those housed in either isolated or standard environments. Similarly, environmental enrichment has been shown to enhance dendritic branching of stellate neurons in layer IV (33, 34) and of pyramidal neurons in layers II, III, IV and V (35, 36).

Hypoalbuminia and low serum total protein are invariably found in protein-energy malnutrition (37). These results have been demonstrated experimentally in the rhesus monkey (38), rat (39), dog (40) and pig (41). A positive correlation between the clinical severity and the serum albumin levels has been found in children with kwashiorkor (42) and in experimental rats (43). Many investigators have studied the effect of nutritional rehabilitation on the recovery of low serum albumin and total protein caused by various types of protein restriction (44, 45). In these studies, however, the restriction occurred during adulthood, not during early life. Given the critical nature of malnutrition at a young age and its effect on biochemical and metabolic parameters (46, 47) later in life, it is surprising that studies during early ages have been ignored. Thus, we were interested in studying whether the deleterious effects of pre-weaning malnutrition on serum albumin and serum total protein levels were still evident after 30 d of nutritional rehabilitation, and if environmental enrichment had an effect on recovery of these parameters.

MATERIALS AND METHODS

Animals and diets. Twenty-one female Sprague-Dawley rats (outbred, Bantin and Kingman, Fremont, CA) were shipped to the campus when 12 d pregnant. They were housed in wire-covered polypropylene cages with wood shavings as bedding material and were fed a 17% protein, purified powdered diet. The composition of all the diets is described in **Table 1**. On d 17 of gestation, the dams were matched by weight and randomly assigned to one of two groups: control (6 dams, fed the 17% protein diet) and protein restricted (15 dams, fed the 8% protein diet). Both diets were designed to be well balanced for nutrients other than protein. Protein restriction was initiated on d 17 of gestation, so that dams would be depleted by the onset of lactation. This procedure did not affect dam or litter weights at parturition (20 d). The animals were fed their respective diets throughout the remaining days of pregnancy and during lactation.

TABLE 1

Composition of diets

	Protein level		
	17%	8%	6%
		%	
Casein[1]	20.0	9.4	7.0
Corn starch[2]	15.0	15.0	15.0
Sucrose	49.5	60.1	62.5
Cellulose[1]	5.0	5.0	5.0
Corn oil	5.0	5.0	5.0
Mineral mix UCB-1Rb[3]	3.5	3.5	3.5
Vitamin mix[4]	1.5	1.5	1.5
DL-Methionine[5]	0.3	0.3	0.3
Choline bitartrate[5]	0.2	0.2	0.2

[1]91.2% crude protein; Tekland Test Diets, Hazleton Laboratories of America, Madison, WI.

[2]Monarch Institute Foods, Brisbane, CA.

[3]Provided the following (g/100 g diet): $CaCO_3$, 0.725; $CaHPO_4$, 1.130; Na_2HPO_4, 0.651; KCl, 0.730; $MgSO_4$, 0.230; $MnSO_4 \cdot H_2O$, 0.0510; $CuSO_4$, 0.0013; ferric citrate (16.7% FeO), 0.0151; $ZnCO_3$, 0.0021; and KIO_3, 0.0001.

[4]AIN-76 vitamin mix (48).

[5]Sigma Chemical, St. Louis, MO.

On the day after parturition all litters were culled to eight pups. At weaning (21 d postnatally), 24 male pups were taken at random from the control litters, matched by weight and assigned to one of two environmental conditions: *1*) standard (SC control 12 pups) or *2*) enriched (EC control, 12 pups). Both groups were fed the 17% protein diet. Male pups from the 15 protein-restricted litters were allowed to continue the experiment only if their weight was between 26.5 and 33.5 g (89% of the male pups). All of the litters had no less than six surviving pups each. The pups were stratified by weight and each stratum randomly assigned to one of four groups: *1*) a group fed the 17% protein diet and kept in a standard environment (SC rehab), *2*) a group fed the 17% protein diet and kept in an enriched environment (EC rehab), *3*) a group fed the 6% protein diet and kept in a standard environment (SC low protein), or *4* a group fed 6% protein diet and kept in an enriched environment (EC low protein). Each group contained 12 pups. **Figure 1** illustrates the experimental design. The animal room was kept at 22.2 ± 1.1°C with a relative humidity of 50 ± 5% and a 12-h light period (0600 to 1800 h). The pups were kept in their respective conditions for 30 d and then anesthetized with sodium phenobarbital (Sigma Chemicals, St. Louis, MO) and decapitated.

Environment. For the standard condition (SC), rats were housed in pairs in standard wire-mesh cages (32 × 20 × 20 cm). For the enriched condition (EC), rats were housed 12 per group in large wire-mesh cages (70 × 70 × 45 cm) and were provided with various

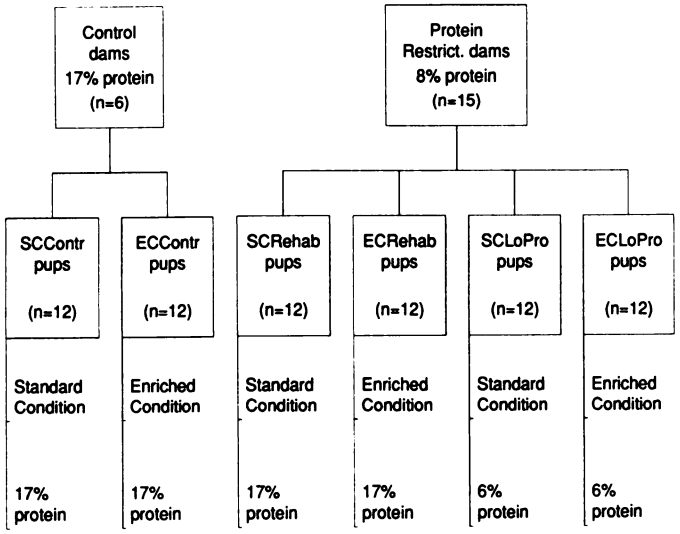

FIGURE 1 The experimental design.

objects (toys), which were changed daily. These consisted of small mazes, ladders, swings, plastic and metal cubes, spheres, cylinders and trays of small objects, which could be carried about by the rats.

Blood analyses. Blood was collected from the neck of the decapitated rats for hematocrit, albumin and total protein analyses. For the latter, blood was centrifuged at 4,000 × *g* and serum albumin and total protein were determined by measuring absorbance at 625–635 nm and 540–545 nm, respectively (Sigma procedures no. 630 and 540, Sigma Diagnostics, St. Louis, MO).

Histological procedures. After decapitation, the brains were removed and processed following the Ramon-Moliner (49) modification of the Golgi-Cox technique. Cedukol (E. Merck, Darmstadt, West Germany) was used as the embedding material. Alternate coronal sections, 50-μm and 150-μm thick, were obtained with representative cortical samples (**Fig. 2**): *1*) immediately anterior to the crossing of the corpus callosum (frontal cortex), *2*) immediately anterior to the crossing of the anterior commissure (parietal cortex), and *3*) at the crossing of the posterior commissure (occipital cortex). The 50-μm sections were counterstained with cresylechtviolet (Fluka, Ronkonkoma, NY) as described by Ramon-Moliner (49) and used for cortical thickness measures. The 150-μm sections were used for dendritic branching studies.

Diencephalon measures. The outline of the diencephalon was traced from projected images of the sections representing the occipital cortex (Fig. 2). A vertical line was drawn from the top margin of the posterior commissure to the ventral edge of the diencephalon following ventricle III (dorsoventral axis). Another line was made perpendicular to ventricle III, where the diencephalon was widest (laterolateral axis). The cross-sectional area was estimated by multiplying the two axes.

Measurements of hippocampal thickness. The outline of the hippocampus was traced from the sections

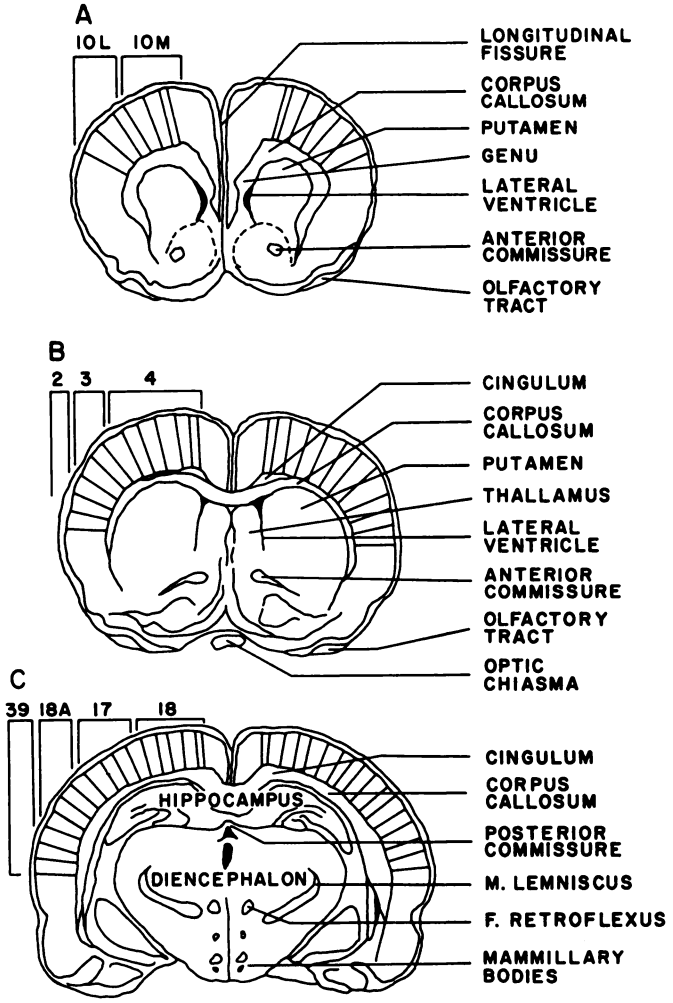

FIGURE 2 Schematic representation of coronal sections of the (*A*) frontal, (*B*) parietal, and (*C*) occipital rat cortex showing where measurements were made.

representing the occipital cortex (Fig. 2). Measurements were made in both hemispheres perpendicular to the tangent of the pial surface at three points on the medial, intermediate and lateral edge of the stratum granularis.

Measurements of cortical thickness. Outlines of the brain and cortex were traced from microslide projected images (22.4×) (Fig. 2). Three sections (i.e., one most nearly corresponding to each of the three subcortical landmarks) were selected for analysis. On the projected drawings, cortical thickness was measured on both hemispheres from the dorsal aspect of layer II to the ventral aspect of layer VI. The experimental treatment received by any particular rat was unknown to the person collecting the data. On each hemisphere, the first reading was taken 4 mm lateral to the medial elevation of the corpus callosum; the second, 6 mm lateral to the first. Additional readings were taken 10 mm apart, the number of which depended on the region being measured: in the frontal region seven readings were made, including Krieg's (50) area 10; in the parietal region 12 readings were made, including Krieg's (50) areas 4, 3 and 2; and in the occipital region 17 readings were

made, including Krieg's (50) areas 18, 17, 18a and 39.

The measurements were analyzed as described by Diamond et al. (32). For the frontal cortex, the first four measurements on each hemisphere were averaged and termed area 10M. The remaining three were averaged on each hemisphere and termed 10L. For the parietal cortex, the mean of the first six measurements was termed area 4; that of the next three, area 3; and that of the remaining three, area 2. For the occipital cortex, the mean of the first six measurements was termed area 18; that of the next four, area 17; that of the next four, area 18a; and that of the remaining three measurements, area 39.

Analysis of dendritic branching. Camera lucida drawings were made of the basal dendrites of pyramidal cells from layers II and III of the occipital cortex (areas 18, 17 and 18a) of each rat. Neurons were selected on the basis of completeness of impregnation and clarity of the dendritic tree. A maximum of 21 and a minimum of 17 pyramidal cells were analyzed from both hemispheres for each animal. Annotations were made on the appearance of neurons and staining quality of each section. The centrifugal method was used to classify branching segments by order. Thus, dendrites leaving the cell body were termed first-order until the point at which they bifurcate into second-order branches, the second-order branches bifurcate into third-order branches, and so on. To further analyze dendritic arborization, the number of dendritic segments that intersected concentric rings at 20-μm intervals, from 20 μm to 140 μm, were counted (50).

Statistical analysis. For the blood parameters, two-way analyses of variance were performed with two factors, diet and environment. A Tukey Test (52) with an associated procedure-wise error rate of 5% was done when significant differences were found. Pearson correlation coefficients were computed to test the strength of the relationships between final weight and hematocrit, serum albumin and serum total protein.

Cortical data from rats fed the low protein diet were analyzed separately from those of rehab and control animals, because the variability within the former group was greater than that in the other two groups. Also, when scatter-plots were made with cortical thickness and body weight in the axes, it was found that cortical thickness was linearly related to body weight in the low protein–fed animals only. Analysis of variance between cortical thickness and body weight revealed that in these animals a great amount of the within-group variability could be explained by differences in body weight.

Cortical values from control and rehab rats were analyzed using a three-factor, repeated-measure analysis of variance with two grouping factors, diet and environment, and one "within factor," side. When significant differences were found, this was followed by a Tukey Test (52). For low protein–fed animals, an analysis of covariance was performed, the covariates being cortical thickness and final body weight. Once the effect of body weight was removed, the effect of the environment was tested.

To analyze dendritic branching, nested two-way analysis of variance with two factors, diet and environment, was performed. Dendrites within each order were analyzed in groups as low order (orders 1, 2 and 3) and high order (4, 5 and 6). The number of dendritic segments intersecting concentric rings was tested, using a nested two-way analysis of variance and a Tukey Test (52) when significant differences were found.

RESULTS

Body weight. **Figure 3** shows the mean body weight of pups during lactation [litter weight (males and females)/number of pups per litter]. The difference in weight between those from control dams and those from protein-restricted dams became significant after the 10th d of lactation, when they were 25.1 ± 2.3 (SEM) and 17.6 ± 2.4 g, respectively. At weaning, pups from protein-restricted litters (i.e., males and females) were much lighter than those from the control litters (29.9 ± 3.7 and 56.8 ± 3.3 g, respectively). By the end of the experiment, rehab rats (SC + EC) were 24% lighter than control rats (181.0 ± 20.5 and 238.0 ± 15.3 g, respectively, **Table 2**). Among the rehab groups, five rats (three from the EC group and two from the SC group) lost weight during this period and three died a few days before the experiment's completion. The weights of the pups that died were not included in the means shown in Table 2. All rats fed the low protein diet survived. They were, on an average, 26 and 35% as heavy as those in the control and rehab groups, respectively.

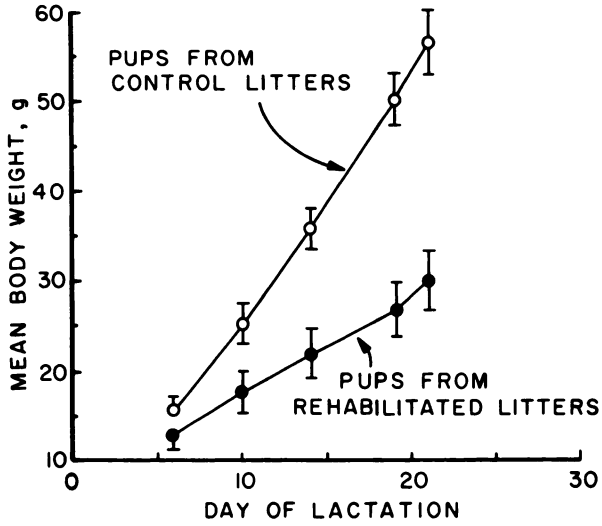

FIGURE 3 Mean body weight ± SEM of pups during lactation. Mean body weight was calculated as litter weight/number of pups per litter (six control and 15 rehab litters).

TABLE 2

Mean weight and blood parameters of control, rehab and low protein–fed rats housed in standard (SC) or enriched (EC) environments[1]

Treatment	*n*	Initial weight	Final weight	Hct[2]	Serum albumin	Serum protein
		g		*ml %*	*g/dl*	
SC control	12	61.6 ± 4.1[a]	243.8 ± 13.5[a]	42.3 ± 1.9[a]	3.77 ± 0.17[a]	6.13 ± 0.30[a]
EC control	12	60.0 ± 4.1[a]	232.2 ± 17.1[a]	41.3 ± 1.8[a]	3.71 ± 0.15[a]	6.00 ± 0.34[a]
SC rehab	12	29.6 ± 2.5[b]	181.8 ± 18.8[b]	39.2 ± 3.0[a]	3.34 ± 0.17[b]	5.43 ± 0.23[b]
EC rehab	9	30.0 ± 3.0[b]	180.1 ± 22.2[b]	39.9 ± 2.3[a]	3.39 ± 0.14[b]	5.48 ± 0.12[b]
SC low protein	12	29.8 ± 2.1[b]	66.1 ± 30.3[c]	32.7 ± 3.5[b]	2.25 ± 0.38[c]	3.95 ± 0.64[c]
EC low protein	12	30.0 ± 2.5[b]	60.3 ± 25.0[c]	35.8 ± 2.9[b]	2.54 ± 0.39[c]	3.56 ± 0.54[c]

[1]Values are means ± SEM. Means in each column not sharing a common superscript letter are significantly different from each other ($P < 0.05$).

[2]Hct, hematocrit. There was a significant interaction of diet × environment, $P < 0.05$.

For each dietary treatment there were no significant weight differences between SC- and EC-reared rats.

Blood parameters. Control rats had significantly higher serum albumin levels than did rehab rats (3.74 and 3.39 g/dl, respectively, environments combined), and these in turn had significantly higher values than did rats fed the low protein diet (2.39 g/dl for low protein–fed SC and EC rats combined, Table 2). Similarly, control rats had significantly higher total serum protein levels than did rehab rats (6.07 and 5.16 g/dl, respectively), and these in turn had significantly higher values than did rats fed the low protein diet (3.76 g/dl).

Hematocrit values from control and rehab groups were significantly higher than those from the low protein–fed groups. A significant interaction between diet and environment was found when analyzing this parameter.

For each dietary treatment there were no significant differences in blood parameters between SC- and EC-reared rats. Only serum albumin levels were significantly correlated with final weight in rehab and low protein–fed groups (**Table 3**).

Brain parameters. *Diencephalon.* Control and rehab rats had significantly larger estimated diencephalon cross-sectional areas than did the low protein–fed rats (**Table 4**). The effect was due mostly to the difference in laterolateral width: when analyzing the axes independently, we found that the dorsoventral axis was not significantly affected by dietary treatment (data not shown).

SC control and SC rehab rats tended to have larger diencephalon cross-sectional areas than did EC control and EC rehab rats, respectively. The difference, however, was not significant.

Hippocampus. There were no differences in hippocampal thickness between control and rehab rats (Table 4). Both control groups and the EC rehab group had significantly thicker hippocampi than did both of the groups fed the low protein diet. There was a 7% difference between values for the control and low protein–fed groups (environments combined). For each dietary treatment, there were no significant differences between environmental rearing conditions or between right and left hemispheres (data not shown), and there were no significant interactions between side and diet, side and environment, or diet and environment.

Cortical thickness. In both control and rehab rats, the right hemisphere was significantly thicker than the left at all locations measured, except areas 2 and 39 (data not shown). There were no interactions between side and environment nor between side and diet at any location. For low protein–fed rats there was no signif-

TABLE 3

Pearson's correlation coefficients of final weight and blood parameters within dietary treatments[1]

Blood parameter	Control (EC + SC)	Rehab (EC + SC)	Low protein (EC + SC)
Serum total protein	−0.1106 (24)	0.3749 (19)	0.1108 (24)
Serum albumin	−0.1029 (24)	0.6171[2] (19)	0.5766[3] (24)
Hematocrit	0.2914 (23)	0.2311 (19)	0.3158 (24)

[1]Animals in both environments (EC and SC) were combined. The number of animals contributing to each mean is shown in parentheses.

[2]$P < 0.005$.

[3]$P < 0.03$.

TABLE 4

Dimensions of diencephalon and hippocampus from control, rehab and low protein–fed rats raised in standard (SC) or enriched (EC) environments[1]

	Diencephalon: estimated cross-sectional area	Thickness of hippocampi (mean of right and left)
	mm^2	*mm*
SC control	177.8 ± 9.42^a (9)	2.73 ± 0.09^a (10)
EC control	174.0 ± 9.4^a (11)	2.83 ± 0.13^a (8)
SC rehab	171.0 ± 9.6^a (9)	2.70 ± 0.08^{ab} (10)
EC rehab	168.7 ± 8.2^a (7)	2.81 ± 0.12^a (8)
SC low protein	150.4 ± 12.9^b (8)	2.58 ± 0.12^c (8)
EC low protein	150.2 ± 14.0^b (8)	2.61 ± 0.18^{bc} (8)

[1]Values are means ± SEM. Within a column means not sharing a superscript letter are significantly different from each other ($P < 0.05$). The number of animals contributing to each mean is shown in parentheses.

icant difference between the thickness of right and left hemispheres.

For all three dietary treatments, the frontal cortex at areas 10M and 10L was significantly thicker in EC-reared rats than in SC-reared rats (**Table 5, 6**). The differences at 10M were 4% between SC control and EC control and 6% between SC rehab and EC rehab. At area 10L these differences were 4 and 6%, respectively. For low protein–fed rats, the differences between SC and EC groups were 5 and 7% at 10M and 10L, respectively.

No significant differences were found between SC control and EC control rats at any of the locations in the parietal and visual cortices measured. EC rehab rats, however, had significantly thicker cortices than did SC rehab rats at areas 4, 2, 18, 17 18a and 39 (Table 5). The differences at these locations were 6, 6, 9, 9, 8 and 9%, respectively. Among the low protein–fed groups, cortices of EC-reared rats were significantly thicker than those of SC-reared rats at areas 4 and 3 in the parietal cortex and only at area 18 in the visual cortex (Table 6). The differences at these locations were 4, 5 and 5%, respectively.

There were no significant differences in cortical thickness at any location measured, except area 18 between control and rehab rats reared in the corresponding environments (i.e., SC control vs. SC rehab and EC control vs. EC rehab). At area 18, SC control rats had significantly thicker cortices than did SC rehab rats.

Significant interactions between diet and environment were found in the visual cortex at areas 18, 17 and 39. Here, cortices of EC rehab rats were larger than those of EC control rats, whereas the cortices of SC rehab rats were smaller than those of the SC control rats. Thus, amongst rehab rats the environmental effect on the occipital cortex was greater than between the control rats.

TABLE 5

Cortical thickness of control and rehab rats housed in standard (SC) or enriched (EC) environments[1]

Area[2]	Overall mean	Pooled estimate of SEM	Proportion of the overall mean			
			SC control	EC control	SC rehab	EC rehab
	mm					
Frontal						
10M	3.54	0.13	0.98 (10)	1.02* (12)	0.97 (9)	1.03* (7)
10L	3.63	0.16	0.98 (10)	1.02* (12)	0.97 (9)	1.03* (7)
Parietal						
4	3.55	0.12	0.99 (12)	1.01 (9)	0.98 (9)	1.04* (7)
3	3.60	0.15	0.99 (11)	1.03 (9)	0.98 (9)	1.02 (7)
2	3.20	0.18	1.01 (10)	1.03 (10)	0.95 (9)	1.02* (5)
Occipital						
18	2.43	0.09	0.99 (11)	1.03 (11)	0.95 (7)	1.03* (8)
17	2.70	0.10	0.99 (11)	1.01 (12)	0.96 (10)	1.04* (8)
18a	2.91	0.13	0.97 (10)	1.01 (9)	0.97 (10)	1.06* (8)
39	2.97	0.12	1.00 (9)	1.01 (9)	0.96 (9)	1.04* (7)

[1]Mean thickness of the left and right hemispheres combined. An asterisk indicates that the mean EC value was significantly (*$P < 0.05$) greater than the corresponding SC value for the same dietary treatment. The number of animals contributing to each mean is shown in parentheses.

[2]Area of the cortex from which measurements were taken.

TABLE 6

Cortical thickness of SC and EC low protein–fed rats[1]

Area[2]	Overall mean	Pooled estimate of SEM	Proportion of the mean	
			SC low protein	EC low protein
	mm			
Frontal				
10M	3.30	0.20	0.98 (9)	1.02* (12)
10L	3.50	0.21	0.96 (9)	1.04* (12)
Parietal				
4	3.24	0.15	0.98 (9)	1.02* (12)
3	3.24	0.16	0.97 (9)	1.03* (12)
2	3.00	0.22	0.98 (6)	1.02 (12)
Occipital				
18	2.30	0.14	0.98 (11)	1.02* (12)
17	2.49	0.17	0.98 (11)	1.02 (12)
18a	2.66	0.15	0.98 (7)	1.02 (12)
39	2.76	0.22	0.98 (7)	1.02 (12)

[1]Mean thickness of the left and right hemispheres combined. An asterisk indicates that the mean EC value was significantly ($^*P < 0.05$) greater than the corresponding SC value for the same dietary treatment. The number of animals contributing to each mean is shown in parentheses.

[2]Area of the cortex from which measurements were taken.

Dendritic branching. The following observations were made during microscopic analysis:

1. Impregnation of the cortical tissue immediately adjacent to the longitudinal fissure was poor. Consequently, few neurons were taken from this area.
2. In general, there were more stained neurons in the right hemisphere than in the left.
3. For drawings, there was a temptation for the observer to select neurons with simpler dendritic trees in those brains where there was a dense dendritic mass. Conversely, in those brains that had fewer, not richly branched neurons, there was the temptation to select those with a more complex dendritic tree. This would have the effect of making the observed differences less than what they actually were.
4. As mentioned briefly in Materials and Methods, impregnation of brains from low protein–fed rats was generally substandard. The same has been noted by Escobar (53) in the "association cortex" of undernourished, 12-mo-old rats. A large proportion of the neurons had beaded or incompletely stained dendrites. On the other hand, glial cells and capillaries were well impregnated.
5. The cortical architecture of the brains of rats fed the low protein diet was not normal. Stratification was poor and the cortical layers thinner and difficult to identify. Cell bodies were round and small, and dendrites were usually extremely thin and, in many cases, lacking the characteristic spines. Sometimes the upper 20% of the cortex was completely devoid of stained cells. Because of this, even if the slides were coded, the investigator believed she could sometimes recognize the sections from low protein–fed rats. For this reason no statistical comparisons of these sections were made between low protein–fed rats and those from control and rehab groups.

It was striking that no differences in number of low order dendrites were found among the control and rehab groups. However, EC rats had significantly more high order dendrites than did SC rats in both the control and rehab groups (**Table 7**). SC and EC low protein–fed rats had the lowest numbers of high order dendrites.

There was a significant interaction between diet and environment for high order dendrites. EC control and EC rehab animals had 21 and 44% more high order dendrites than did SC control and SC rehab animals, respectively. Even though EC low protein–fed rats had 11% more high order dendrites on average than SC low protein–fed rats, this difference was not significant.

Another way of stating the differences between the low order dendrites and the high order dendrites was to do measurements at successive distances from the neuron soma. No significant differences in the number of dendrites intersecting concentric rings at 20 and 40 μm from the neuron soma were found among the control and rehab groups. However, at 80, 100 and 120 μm, values for EC control and EC rehab rats were significantly higher than those for SC rehab rats (Table 7). The difference between EC control and SC control rats was significant only at a distance of 120 and 140 μm from the cell soma. Neurons from SC control animals tended to have more dendrites intersecting concentric rings at all distances than did those from SC rehab animals; however, the difference was never significant.

TABLE 7

Mean number of dendritic segments in each order and mean number of dendritic segments intersecting concentric rings at different distances from neuron soma for control and rehab rats[1]

Parameter	SC control (n = 11)	EC control (n = 11)	SC rehab (n = 10)	EC rehab (n = 9)
Order[2]				
Low	27.9 ± 1.8[a]	27.8 ± 1.2[a]	27.9 ± 1.3[a]	27.7 ± 1.3[a]
High	18.6 ± 1.8[b]	22.6 ± 1.0[a]	15.3 ± 2.0[c]	22.1 ± 1.8[a]
Distance[3]				
20 μm	15.0 ± 1.1[a]	14.3 ± 1.1[a]	14.4 ± 0.9[a]	15.1 ± 1.9[a]
40 μm	20.9 ± 1.4[a]	21.0 ± 1.5[a]	20.2 ± 1.1[a]	21.9 ± 2.0[a]
60 μm	21.9 ± 1.5[ab]	22.4 ± 1.3[ab]	20.8 ± 1.4[b]	23.6 ± 1.9[a]
80 μm	19.5 ± 1.5[ab]	20.7 ± 1.8[a]	17.8 ± 1.5[b]	21.5 ± 1.7[a]
100 μm	15.2 ± 1.5[ab]	17.1 ± 1.9[a]	12.9 ± 1.7[b]	16.8 ± 2.1[a]
120 μm	9.7 ± 1.4[bc]	12.1 ± 2.0[a]	7.7 ± 1.6[c]	11.3 ± 2.1[ab]
140 μm	5.1 ± 1.4[b]	7.3 ± 1.9[a]	3.7 ± 1.2[b]	6.4 ± 1.4[ab]

[1]Values are means ± SEM. Within a row, for each order and each distance, means not sharing a common superscript letter are significantly different from each other, $P < 0.05$.
[2]Dendrites within each order have been grouped as low order dendrites (1 + 2 + 3) and high order dendrites (4 + 5 + 6).
[3]Distance from neuron soma.

There were no differences between EC and SC low protein–fed rats in the number of dendrites intersecting concentric rings (**Table 8**).

At the ages studied, malnutrition during lactation did not affect the dimensions of the diencephalon, the thickness of the hippocampus, or the cerebral cortex (**Table 9**). For all cortical and hippocampal measures, except occipital thickness at areas 18 and 17, SC rehab rats had values slightly, but not significantly, lower than did SC control animals.

TABLE 8

Mean number of dendritic segments in each order and mean number of dendritic segments intersecting concentric rings at different distances from neuron soma for rats fed the low protein diet.[1]

	SC low protein (n = 12)	EC low protein (n = 10)
Order[2]		
Low	27.8 ± 1.8	27.9 ± 2.3
High	13.2 ± 2.3	14.7 ± 1.5
Distance[3]		
20 μm	14.9 ± 1.3	15.3 ± 1.4
40 μm	19.7 ± 1.2	20.0 ± 1.3
60 μm	18.7 ± 1.7	18.5 ± 1.8
80 μm	14.5 ± 2.4	13.9 ± 2.8
100 μm	9.3 ± 2.2	8.8 ± 2.8
120 μm	5.0 ± 1.8	4.7 ± 2.3
140 μm	2.3 ± 1.2	1.9 ± 1.0

[1]Values are means ± SEM. There were no significant effects of environment for these measures.
[2]Dendrites within each order have been grouped as low order dendrites (1 + 2 + 3) and high order dendrites (4 + 5 + 6).
[3]Distance from neuron soma.

DISCUSSION

Weights and Blood Parameters

Lowering the level of protein in the dams' diet in late pregnancy and for the period of lactation reduced mean pup body weight. This observation agrees with reports by several investigators where malnutrition/undernutrition was attained by using a variety of methods to interfere with normal milk availability (15, 21). In the literature, common weights for pups malnourished by reducing the protein content of the dam's diet range from 40 to 60% (54, 55) of the weight of well-fed pups.

Even after 1 mo of free access to a good quality diet, the serum total protein and albumin levels of rehab animals were significantly lower than those of control animals. There are no reports of the effects of malnutrition during lactation on these serum parameters later in life. Weimer (44) and Weimer, Nishihara and Ling (45) found that recovery of normal values for serum total protein and albumin levels after drastic protein restrictions in adult rats was quite rapid—faster, in fact, than weight repletion. He also found that the longer the depletion period, the slower the rehabilitation. However, even after periods of as long as 2 mo of consuming a 2% protein diet, adults recovered their blood protein levels completely after consuming a high protein diet for 20 d. We found that the effect of malnutrition during lactation on these serum parameters was still evident after 30 d of nutritional rehabilitation. It is possible that malnutrition at an early age in the rat affects serum albumin and total protein levels later in life, much as it affects growth and other metabolic and biochemical characteristics. It is also possible that complete recovery requires a longer period of rehabil-

TABLE 9

Summary of results from control and rehab rats: Percentage difference between treatment groups

Parameter	Rehab vs. control[1]	EC rehab vs. SC rehab[2]	EC control vs. SC control[2]
		%	
Final body weight	−24	−1	−5
Hematocrit	−5.4	+1.8	−2.5
Albumin	−10.0	+1.0	−1.6
Total protein	−10.0	0.6	−2.1
Diencephalon (area)	−3.5	−1.3	−2.1
Hippocampal thickness	−0.4	+4.1	+3.7
Cortical thickness			
Frontal (areas 10M + 10L)	−0.2	+6.1*	+4.5*
Parietal (areas 4 + 3 + 2)	−0.3	+5.5*	+1.7
Occipital (18 + 17 + 18a + 39)	+0.2	+12.9*	+2.5
Mean cortical thickness	(−0.1)	(+8.2)	(+2.9)
Dendritic branching			
Low order	−0.3	−0.7	−0.3
High order	−9.2	+44.4**	+21.5*
Ring analysis			
20 and 40 μm	+0.6	+4.0	−1.7
60 and 80 μm	−1.0	+16.8*	+4.1*
100, 120 and 140 μm	−11.6	+42.0**	+21.7*

[1]No probability values from the analyses of variance are given to the differences between marginal means in this column because they have been given to the differences between cell means.

[2]Significant differences between EC and SC values are indicated by asterisks: $^{*}P < 0.05$, $^{**}P < 0.01$.

itation when malnutrition takes place early in life because of the high protein requirement for growth. This hypothesis requires further testing.

It must be noted that rats were shipped to our laboratory on the 12th d of pregnancy. This might have resulted in some stress to the dams. All groups, however, were exposed equally.

Brain Parameters

Effects of dietary treatment. Consistent with our findings (Table 9), no differences between animals malnourished during lactation and well-fed animals have been reported with respect to the thickness of the frontal cortex after 25 (16) and 45 d (15) of rehabilitation; the thickness of the frontal, parietal and occipital cortices after 30 d of rehabilitation (20); and of the medial occipital cortex after 1.5 (56), 2 (57) and 5 (56) mo of rehabilitation. Katz and Davies (21) found a small but significant difference in the thickness of the hippocampus after 1 mo of nutritional rehabilitation between previously malnourished and control rats. After 3 mo of rehabilitation there was no difference between the groups.

Unlike cortical thickness, nutritional deficits in dendritic branching persisted after 30 d of nutritional rehabilitation. SC rehab animals had 21% fewer high order dendrites than did SC control animals. Davies and Katz (57) also found that at 2 mo of age rats malnourished during lactation had 29% fewer high order dendrites.

The devastating effect of persistent protein malnutrition on cortical development was seen in animals fed the low protein diet. The striking decrease in the mass of fibers and dendrites impregnated by the Golgi-Cox stain may be due to the fact that the affinity of dendrites to the stain can depend on factors concerned with the process of maturation, such as an increase in thickness of fibers or a change in their chemical constitution (58). Brains of neonatal (59) and of hypothyroid (60) rats have been found to have a reduced affinity for the Golgi-Cox stain.

Effects of environmental treatment. In our study, environmental enrichment had a significant effect on thickness of most cortical areas measured in rehab animals (Table 9). For control rats, though differences between EC and SC groups were within the range reported in the literature (31, 32), they were only significant in the frontal cortex (areas 10M and 10L). Here the environmental effect was greater than in the occipital cortex. It has been generally found that the occipital cortex is more responsive to environmental enrichment than is the frontal cortex (differences between EC and SC groups being around 4 and 2%, respectively). The section of the frontal cortex that was analyzed is concerned with motor function. As part of the enriched environment, we included in each cage a tray containing small objects (screws and bolts, small cubes, marbles, etc.). The animals were seen to pick these up with both their paws and hop with them to other parts of the cage. Since this study was completed, another investigator has suggested that this could enhance the

environmental effect on frontal cortical thickness (D. A. Levitski, personal communication).

Surprisingly, the cortical response to environmental enrichment was greater among rats fed the low protein diet than among controls. The EC low protein–fed rats were quite active. It has been reported that male rats malnourished during lactation and then weaned to a 5% protein diet show a dramatic increase in exploratory and spontaneous activity by 50 d of age (61). Guthrie (62) also reported that rats malnourished during lactation and then weaned to a 3% casein diet were significantly more active than well-fed pups when tested at 3, 5, 7 and 9 wk of age. Increased activity among EC low protein–fed rats might account for their greater cortical response. It must be kept in mind that at all cortical locations measured the cortex of low protein–fed animals was thinner than that of controls.

Environmental enrichment had a significant effect on dendritic branching. Among control animals, EC-reared rats had 22% more high order dendrites than did SC-reared rats. Dendrites from EC control animals also made 22% more intersections with concentric rings at distances of 100 μm or greater from the neuron soma (Table 9). These findings are consistent with those of Greenough, Volkmar and Juraske (35) and Volkmar and Greenough (34), who found that pyramidal neurons in the occipital cortex had more high order basal dendrites in EC-reared rats than in SC-reared rats. Low order branching was not affected. They also found that dendrites from EC animals made more intersections with concentric rings at distances greater than 80 μm from the neuron soma. In 1966 Holloway (33), and in 1978 Uylings et al. (36), had also reported dendritic effects similar to those found in the present study in rats due to differential housing.

Interactive effects between environment and diet. Cortical response to environmental enrichment was greater among rehab animals than among controls. Our study showed a significant interaction between diet and environment in the thickness of the occipital cortex in three of four locations, and in dendritic branching in the number of high order dendrite segments and in the number of concentric ring intersections made by dendrites at distances greater than 60 μm from the neuron soma. Most of the studies with a design similar to ours have not found an interaction between nutrition and the environment on brain weight, total protein and DNA (63, 64). These studies, however, showed that environmental enrichment enhanced performance in tests assessing certain aspects of learning behavior. Katz and Davies found significant environmental effects, but no interactions with diet, when measuring brain weight (65), cerebral dimensions (21), thickness of the hippocampus (21), medial occipital cortex (56) and dendritic branching (57). Bhide and Bedi (20) also found no interaction between environment and diet on forebrain weight and cortical thickness at 11 locations. They failed, however, to find any significant or consistent environmental effect among the well-fed pups reared in isolated conditions (IC) or enriched conditions (EC), with IC-EC cortical thickness differences ranging from 11.2% to −12.2%. Among the previously malnourished rats, they found that cortices of EC-reared rats were significantly thicker than those of IC-reared rats in four of 10 locations. Unfortunately, in a second experiment they were unable to replicate these findings.

In each area of the cortex, the response to environmental enrichment tended to be greater with rehab rats than with controls (Table 9). The difference in response was large enough for the interaction to be significant in the occipital cortex with respect to thickness at areas 18, 17 and 39 and with both measures of dendritic branching.

The discrepancy between our results and those mentioned above could possibly be due to different methods of measuring the thickness of the occipital cortex. They assessed the medial portion of the cortex, which would correspond to area 18 and part of area 17 in our experiment. Also, in our study rehabilitation and environmental treatment were both initiated at 21 d of age so that animals went directly from their litters to either EC or SC environments. In their study (57), animals were rehabilitated at 21 d of age, but they were kept with their mothers and siblings until 28 d of age and then housed in cages of four pups each for 3 d before being assigned to different environments. Thus, for the first 9 d of rehabilitation, animals that would be assigned to SC environments were, in fact, in quite social environments. Malkasian and Diamond (66) have shown that the greatest environmental effect occurs when enrichment begins during the first 28 d of life, the time when the rats' cortices are growing the fastest. It could be that this first week of rehabilitation is crucial for cortical "catch-up" growth. Finally, the nutritional restriction imposed on the dams differed. We fed dams an 8% protein diet, while they fed dams 50% of the amount of diet consumed by control dams.

In our study, animals rehabilitated in an enriched environment showed significantly greater mean values for cortical thickness and dendritic branching than did animals rehabilitated in standard environments. Moreover, the environmental cortical effect experienced by rehabilitated animals was significantly greater than that experienced by control animals with respect to the thickness of the occipital cortex and dendritic branching. Our present studies provide neuroanatomical support to previous behavioral findings.

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