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Effects of long-term malnutrition and rehabilitation on the hippocampal formation of the adult rat. A morphometric study

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ABSTRACT

We have previously shown that the numerical density of dentate granule and CA3 pyramidal cells of adult rats is reduced after lengthy periods of low-protein diet. In this study, the total number of these neurons was estimated, together with those for the hilar and CA1 pyramidal cells in order to obtain a complete and unbiased insight into the effects of malnutrition and rehabilitation from malnutrition on the structure of the hippocampal formation. Groups of 2-month-old rats were fed a low protein diet (8% casein) for 6, 12 and 18 months and compared with age-matched control and recovery rats. The recovery group was fed a low protein diet for 6 months and then switched to normal diet during the same period. Total numbers of neurons of each hippocampal region were calculated from their numerical density, estimated with the physical disector, and from the volume of the respective cell layers, after correction for the tissue shrinkage factor. The total number of granule, hilar, CA1 and CA3 pyramidal cells was reduced in all groups of malnourished rats including the recovery group. No differences were found between malnourished and recovery groups. These findings indicate that a prolonged low protein diet, started in adult life, leads to a deficit in neuronal numbers in the hippocampal formation, and that it may also disrupt the normal process of cell acquisition in the dentate gyrus. Moreover, our data support the view that the morphological alterations induced by a low protein intake are irreversible.

Key words: Hippocampus; protein malnutrition; neuronal degeneration; nutritional rehabilitation; stereology.

INTRODUCTION

There is ample evidence that a balanced diet is of primary importance for the normal development and function of the brain (Morgane et al. 1978, 1992, 1993; McConnell, 1980). Although an inadequate consumption of carbohydrates, lipids, vitamins and trace elements might alter the structure of the central nervous system (Morgane et al. 1978, 1992, 1993), apart from vitamin deficiency, a reduced protein intake is, among all nutritional deficiencies, the major condition leading to such alterations (Morgane et al. 1978, 1992, 1993). It has been reported, furthermore, that the effects of malnutrition are not uniform throughout the brain: the visual cortex (Warren & Bedi, 1982, 1984; Díaz-Cintra et al. 1990), nucleus locus coeruleus (Díaz-Cintra et al. 1984), nucleus raphe dorsalis (Díaz-Cintra et al. 1981) and other brain areas (West & Kemper, 1976; Angulo-Colmenares et al. 1979; Bedi et al. 1980; Katz et al. 1982; Escobar & Salas, 1993; Bedi, 1994; Tolley & Bedi, 1994) are not markedly sensitive, whereas the hippocampal formation is particularly vulnerable (Lewis et al. 1979; Jordan et al. 1982; Katz et al. 1982; Ahmed et al. 1987; Cintra et al. 1990; Bedi, 1991; Díaz-Cintra et al. 1991, 1994; García-Ruiz et al. 1993).

Following protein deprivation, especially when instituted in the perinatal period, the hippocampal formation has been recognised as one of the most affected regions, and the granule cells of the dentate gyrus as the most sensitive of its constituent neurons

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(Cintra et al. 1990; Bedi, 1991; Díaz-Cintra et al. 1991). Indeed, the total number of granule cells (Bedi, 1991) and the molecular layer synapse-to-granule cell ratio (Ahmed et al. 1987) are reduced in this condition, as are the soma size (Cintra et al. 1990; Díaz-Cintra et al. 1991), the dendritic branching density (Cintra et al. 1990; Díaz-Cintra et al. 1991) and the number of dendritic spines (Cintra et al. 1990; Díaz-Cintra et al. 1991) of these neurons. In addition, alterations induced by perinatal malnutrition have also been observed in the hippocampal CA3 region; these include reductions in the areal density of pyramidal cells (Jordan et al. 1982) and in their soma size (García-Ruiz et al. 1993) and dendritic trees (García-Ruiz et al. 1993).

In contrast to the developing brain, the mature brain has long been thought to be resistant to malnutrition (Dobbing, 1968; Morgane et al. 1978, 1992, 1993). Recently, using a long-term low protein diet model started in adult life, we have provided evidence that this leads to a decrease in the numerical density of the dentate granule and CA3 pyramidal cells (Paula-Barbosa et al. 1988, 1989). We were aware that it could be argued that numerical densities of neurons are ratio estimates and, consequently, that they lack the biological meaning attributable to absolute values, i.e. to total number of neurons (West & Gundersen, 1990; West et al. 1991; Mayhew, 1992). Besides, no information is available as to the effects of protein deprivation on the remaining neuronal populations of the hippocampal formation. Since the hilar and the CA1 pyramidal cells are also important links in the hippocampal circuitry (Zimmer et al. 1983; Amaral & Witter, 1989), we thought it of interest to extend our observations to these cells, inasmuch as there is ample evidence that hippocampal neurons often display a selective vulnerability to the same aggressive agent (Madeira et al. 1991, 1992; Schmidt-Kastner & Freund, 1991).

In this way, we expected to obtain a better insight into the effects of malnutrition on the rat hippocampal formation by answering the following questions: (1) does prolonged low-protein intake interfere with the total number of neurons of the hippocampal formation of the adult rat? (2) is there a selective vulnerability of these neurons to malnutrition? and (3) does malnutrition interfere with neurogenesis in adulthood?

Because the effects of rehabilitation from malnutrition are still a matter of controversy, we incorporated in this study a group of low-protein fed animals later switched to standard conditions. In so deciding, we attempted to evaluate if the reestablishment of a normal diet permits the restoration of affected neuronal populations, especially in regions, such as the dentate gyrus, in which there is protracted postnatal neurogenesis (Bayer, 1980, 1982; Altman & Bayer, 1990).

MATERIALS AND METHODS

Animals and treatment regimes

Two-month-old Wistar rats from the colony of the Gulbenkian Institute of Science (Oeiras, Portugal), weighing 320 ± 20 g at the beginning of the experiment, were used. At 2 months of age, rats were individually caged and 3 experimental groups were formed by pooling animals from 7 separate litters. All these litters had been reduced, by culling on the day of birth, to a standard composition of 6 male and 2 female pups. After weaning, all rats were fed with standard laboratory diet. At 8, 14 and 20 months of age, i.e. 6, 12 and 18 months after starting treatment, each experimental group was reduced to a standard composition of 5 animals each by selecting animals at random. Animals were treated as follows. (1) *Malnourished rats.* Animals were fed with an 8% low protein diet (ICN, Cleveland, USA) for 6, 12 and 18 months. The main features in the composition of the diet were the following: 8% casein, which is the only source of protein in the diet, 78% carbohydrate and 10% fat. The diet was supplemented with 0.3% methionine, since casein has a low content of sulphur aminoacids. A supplement of Salt Mixture U.S.P. XIV (4%) (ICN) was provided. (2) *Recovery rats.* Animals were fed as for the previous group for 6 months and then switched to standard laboratory diet for further 6 months. (3) *Control rats.* Animals were fed for 6, 12 and 18 months with standard laboratory chow (Letica, Spain) containing 17% protein supplemented with 0.7% lysine, 0.5% methionine and cystine, 57% carbohydrates, 4% fat and 7% salts. Both diets were supplemented with ICN Diet Vitamin Fortification Mixture.

All rats had free access to food and water throughout the experimental periods. Daily food intake, measured by weighing food at 09.00 h each day, was 26.8 ± 3.2 g in controls and 25.3 ± 2.9 g in the experimental groups.

The body weights for the malnourished and control groups were recorded each week during the first month of the experiment; for the recovery group, an identical procedure was followed during the first month after switching to the standard diet. Thereafter, body weights were recorded every 15 d and the last

determination was performed on the day the animals were killed.

General procedures

At the end of the experimental periods, animals were anaesthetised with ether, and killed by transcardiac perfusion of a solution containing 1% paraformaldehyde and 1% glutaraldehyde in 0.12 M phosphate buffer at pH 7.2 (Palay & Chan-Palay, 1974). The brains were removed from the skulls, weighed, blocked by hand into left and right halves, immersed for 2 h in the perfusion solution and codified. The left hemispheres were used for the estimation of the volumes of the hippocampal formation; the right hemispheres were dissected in order to free the hippocampal formations, which were used for neuronal quantifications and for the estimation of the tissue shrinkage factor (SFv).

For the volumetric estimations, the blocks containing the hippocampal formation were dehydrated in an ascending series of ethanols and embedded in celloidin. These blocks were sectioned on a sliding microtome, at a nominal thickness of 60 μm, in the horizontal plane (Fig. 1). The sections were collected, mounted serially and stained with cresyl violet (Fig. 1 *b–h*). The mean thickness of the sections was estimated in each animal from measurements of the thickness of every 10th section, using a Microcator (Olympus) attached to the stage of a microscope and a × 100 oil immersion objective lens (West et al. 1991). The mean section thickness determined was 58.4 ± 4.1 μm.

The determination of the SFv of the celloidin-embedded sections was calculated separately for each group. For this purpose, the septal and temporal poles of the dissected right hippocampal formation were separated by a horizontal cut from the mid-septotemporal part of the hippocampal formation. A section from each pole was immediately obtained on a Vibratome from the surface facing the site of transection, and photographed. The remaining portions of the septal and temporal poles were embedded in celloidin, and the first section obtained from each block, immediately adjacent to the Vibratome section, was also photographed. The areas of the photographed sections were measured, and the SFv calculated from the ratio of the areas of the sections obtained before and after embedding (Uylings et al. 1986; Madeira et al. 1991, 1992).

For neuronal quantifications, the right hippocampal formation was sectioned in the horizontal plane on a tissue chopper. The blocks obtained were processed, at room temperature, as though being prepared for electron microscopic observation. After processing, 4 Epon-embedded blocks were selected from each hippocampal formation, using a systematic random sampling procedure (Gundersen & Jensen, 1987). From each block, 4 groups of 3 consecutive 2 μm semithin sections were cut, mounted serially and stained with toluidine blue. In order to determine the actual thickness of the sections 10 semithin sections were randomly collected during sectioning of the material. They were reembedded and 3 cross-sections (2 μm) of each semithin section were cut; the mean thickness estimated was 2.1 ± 0.2 μm. The SFv of Epon-embedded material was not calculated because it is almost nonexistent (Eins & Wilhelms, 1976; Schüz & Palm, 1989).

Volume estimations

The volumes of the layers were estimated based on the principle of Cavalieri (1966) and following the sampling procedures described by Gundersen & Jensen (1987). Sections used for measurements were sampled systematically at regular intervals of 300 μm from the entire sets of serially mounted celloidin-embedded sections. To ensure that the estimator would be unbiased, the first section was randomly sampled from the first 5 sections, containing the hippocampal formation, which provided a total of 13–14 sections for each hippocampal formation. The cross-sectional areas of the layers were estimated by point counting using a × 4 objective lens and a camera lucida, at a final magnification of × 62. The test systems used were previously chosen in order to ensure that approximately 100 points would hit the entire set of sectional profiles of the hippocampal formation layers. The volume of the layers was then calculated from the total number of points that fell on the layers and the distance between the systematically sampled sections (Gundersen & Jensen, 1987; Gundersen et al. 1988). Data obtained were then corrected for SFv.

The boundaries of the neuron-containing layers were defined consistently at all levels of the hippocampal formation on the basis of their cytological organisation and following the criteria described in detail by West et al. (1978). Accordingly, the discrete granular layer of the dentate gyrus, which is typically horseshoe-shaped in the midseptotemporal part of the hippocampal formation, is clearly defined at all levels by the tight packing and small size of its constituent neurons (Fig. 1 *b–h*). The hilus, a crescent-shaped area between the two limbs of the granule cell layer which

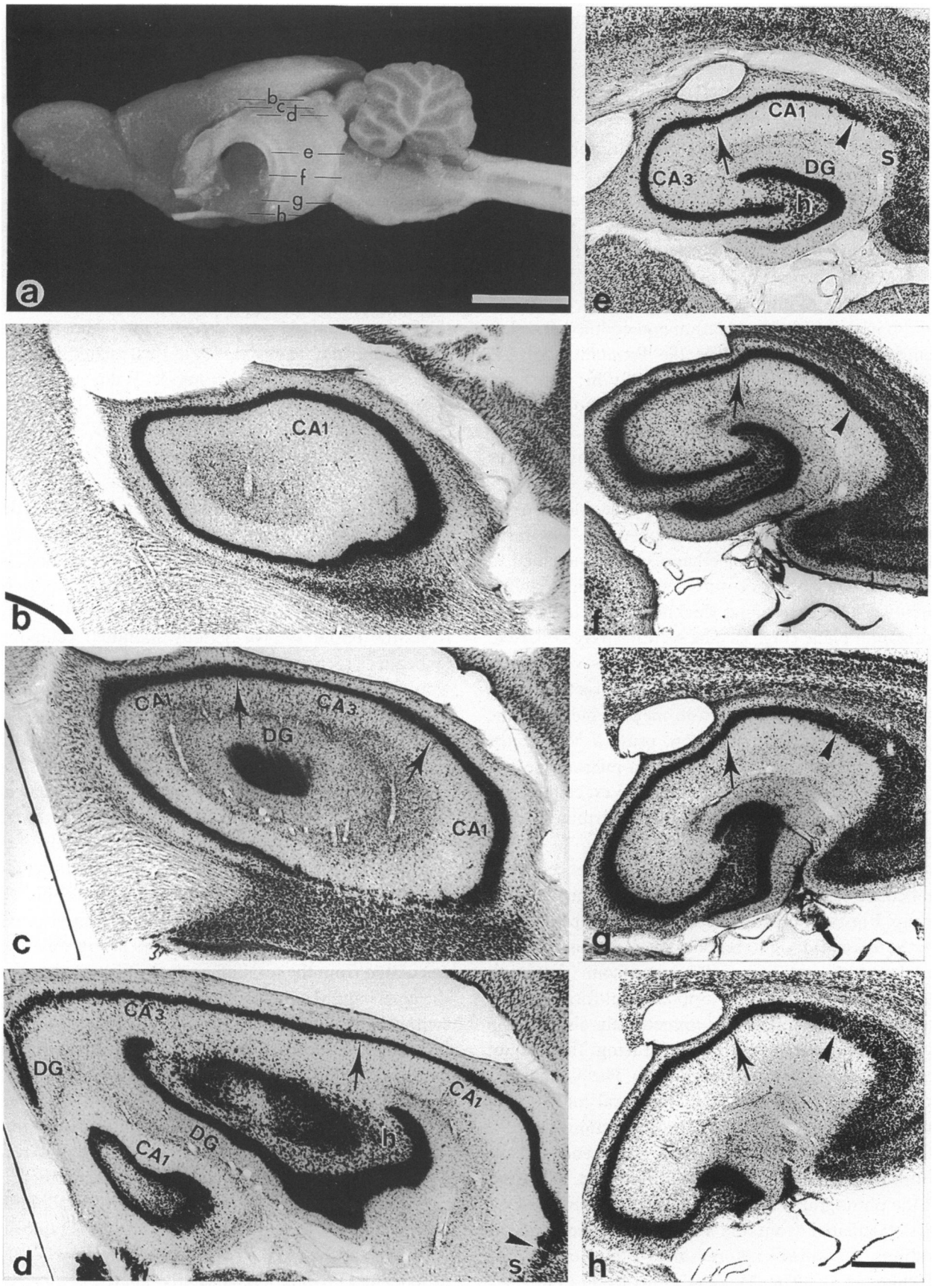

Fig. 1. (*a*) Lateral view of the left hippocampal formation of an 8-month-old control rat, exposed by removal of the neocortex. The lines (*b–h*) indicate the location from where the sections present in *b–h* were obtained. Bar, 5 mm. (*b–h*) Photographs of horizontal celloidin sections of the hippocampal formation representative of those used to estimate the volume of the neuron-containing layers. The main structural features of the hippocampal formation can be seen: CA1–CA3, fields of Ammon's horn; DG, dentate gyrus; h, hilus; S, subiculum.

has a concave border with the regio inferior, was delineated based on the abrupt transition between the scattered polymorphic hilar neurons and, proximally, the inner border of the granular layer and, distally, the tightly packed neurons of the CA3 pyramidal cell layer (Fig. 1*b–h*). The hilus, as defined here, corresponds to the CA4 field of Lorente de Nó (1934). Ammon's horn is divided into a regio superior and a regio inferior that correspond to fields CA1 and CA2+CA3 of Lorente de Nó (1934), respectively. For volumetric estimation purposes, the CA2 region was considered as belonging to the CA3 region because the boundaries between these two fields of the hippocampus are not discrete in conventionally stained sections. Thus, discrimination between CA1 and CA3 was made taking as reference the distal part of the CA2 region, which is easy to differentiate from CA1 by the sudden transition of the large pyramidal neurons, similar to but more loosely organised than those seen in CA3, to the tightly packed pyramidal cell layer CA1 (Fig. 1*b–h*). Distally, this layer is continuous with the subiculum and the border between these two regions of the hippocampal formation was drawn at the point where the single, narrow and heavily packed row of small pyramidal neurons of CA1 gives way to the wider and less cell dense layer of the subiculum (Fig. 1*b–h*).

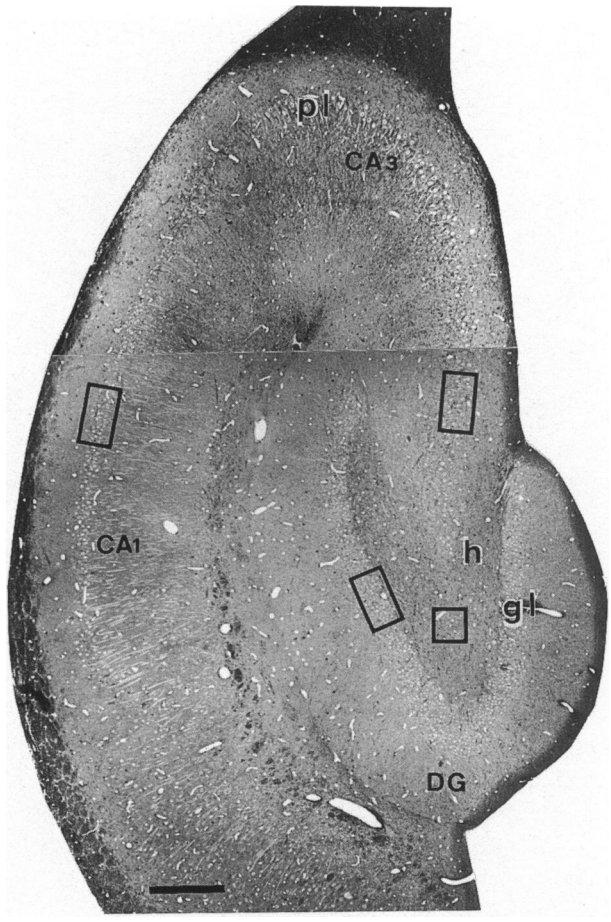

Fig. 2. Light micrograph of horizontal semithin section of the hippocampal formation of a 14-month-old control rat. The boxes represent the places were disectors were performed: CA1–CA3, fields of Ammon's horn; DG, dentate gyrus; gl, granular layer; h, hilus; pl, pyramidal layer. Toluidine blue. Bar, 250 μm.

Neuronal quantification

Numerical densities. The numerical density (N_V) was estimated by applying the physical disector method (Sterio, 1984; West et al. 1988; Mayhew, 1992). From the sets of semithin sections, obtained as described above, photographs of the same area of every region studied were taken from 2 consecutive sections. The photographs of the granule cell layer were analysed at a final magnification of ×640, whereas those from the remaining hippocampal regions were analysed at a final magnification of ×320. In all animals, photographs were taken approximately from the same region of each layer analysed, i.e. the middle part of the lateral (suprapyramidal) limb of the granule cell layer, proximal part of the CA3 pyramidal cell layer (CA3c), proximal part of the CA1 pyramidal cell layer, and central part of the hilus, i.e. midway between the tip of CA3 and the apex of the dentate gyrus granular layer (Fig. 2). There are reasons to assume that the estimations performed are representative of each neuronal population, because it is well established that no significant variations in the numerical density of the different neuronal populations occur along either the septotemporal or the transverse axis of the hippocampal formation of control (West et al. 1988, 1991; Paula-Barbosa et al. 1989; Brandão et al. 1992) and low protein fed rats (Paula-Barbosa et al. 1989).

For each neuronal population studied, a total of 32 disectors was made per animal.

Total number of cells. The total number of each neuronal type was calculated by multiplying their numerical density by the volume of the respective layer, after correction for the SFv.

Statistical analysis

A single two-way analysis of variance (ANOVA) was carried out in data from controls and rats mal-

The borders between CA1 and CA2–3 are indicated by arrows, and those between CA1 and the subiculum by arrowheads. Cresyl violet. Bar, 500 μm.

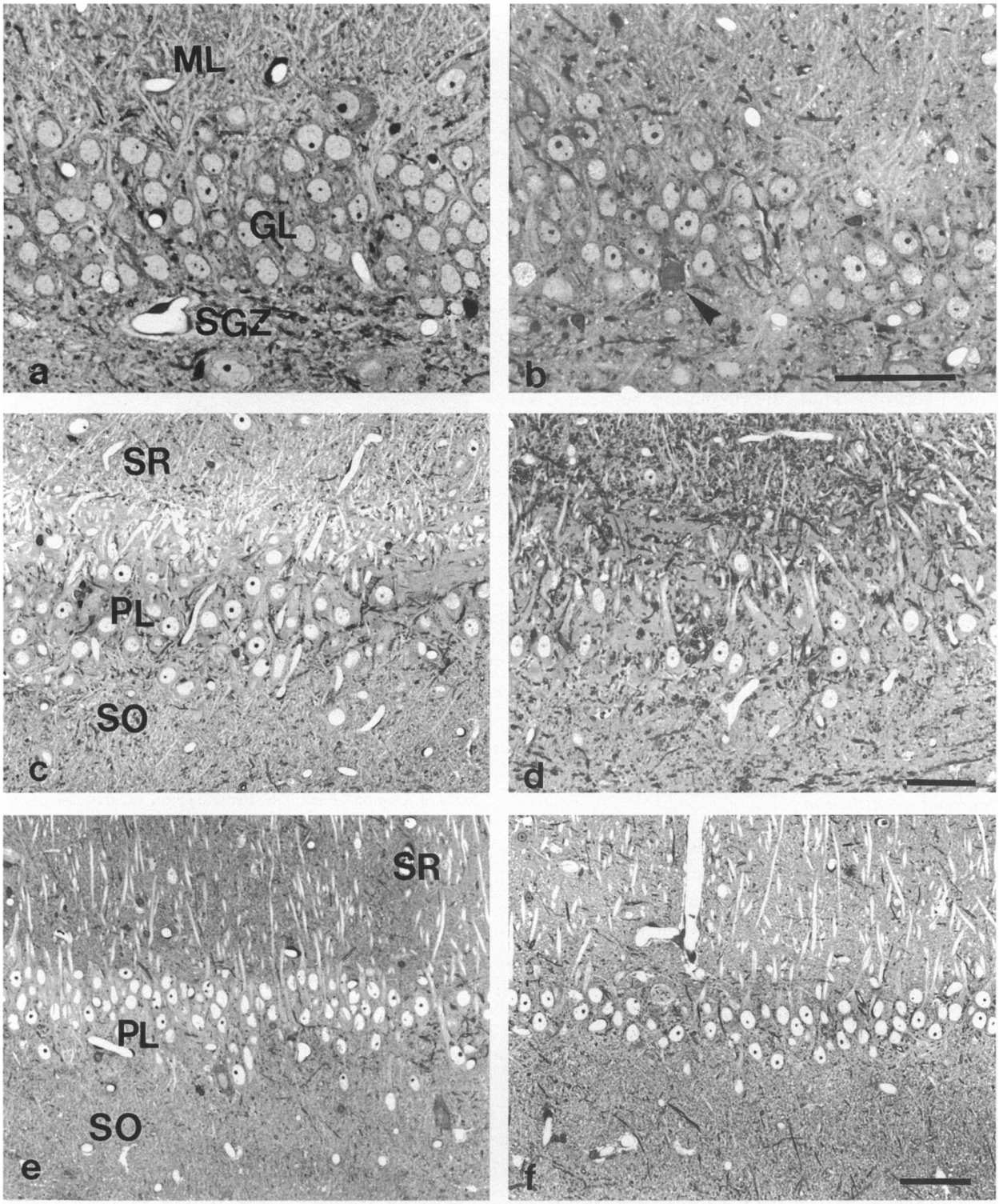

Fig. 3. (*a*, *b*) Light micrographs of semithin sections from the dentate gyrus granular layer of a 14-month-old control rat (*a*) and a 14-month-old malnourished rat (*b*). Note the reduced packing density after protein restriction. The arrowhead in (*b*) points to a dark neuronal profile which may correspond to a degenerated granule cell. ML, molecular layer; GL, granular layer; SGZ, subgranular zone. Toluidine blue. Bar, 25 μm. (*c*, *d*) Light micrographs of semithin sections from the CA3 pyramidal layer of a 14-month-old control rat (*c*) and a 14-month-old malnourished rat (*d*). The number of cells is reduced in the protein-restricted animal. PL, pyramidal cell layer; SO, stratum oriens; SR, stratum radiatum. Toluidine blue. Bar, 25 μm. (*e*, *f*) Light micrographs of semithin sections from the CA1 pyramidal layer of a 14-month-old control rat (*e*) and a 14-month-old malnourished rat (*f*). In the latter neuronal packing is smaller. PL, pyramidal cell layer; SO, stratum oriens; SR, stratum radiatum. Toluidine blue. Bar, 25 μm.

nourished for 6, 12 and 18 months to discern the main effects. Treatment and duration of treatment were used as independent variables. To test for the effect of treatment in animals malnourished for 12 months and in age-matched recovery and control rats a one-way ANOVA was performed. Animals were used as replicates and the remainder mean square as the error term. To test whether pairs of group means differed significantly from each other, post hoc linear polynomial contrasts were applied (Moore & McCabe, 1989).

Throughout this study, values are expressed as mean $\pm$ S.D. A level of $P < 0.05$ was considered statistically significant.

RESULTS

Qualitative observations

In celloidin-embedded material, the basic morphology of the hippocampal formation was similar in all groups. In semithin sections, however, the dentate granule and the hippocampal CA3 and CA1 pyramidal cells appeared to be less closely packed in malnourished and recovery animals than in controls (Fig. 3). Conversely, the density of hilar cells appeared to be identical in all groups analysed.

Dark neurons were more numerous and more frequently observed in animals of the experimental groups than in controls. These profiles might correspond to degenerated neurons, a hypothesis that must be regarded cautiously because of the possibility of artefact (Cammermeyer, 1978).

Quantitative results

Body and brain weights. During the first month of the experiment, the mean body weight increase of controls and malnourished rats was 67.1 ± 13.2 g and 65.6 ± 14.5 g, respectively. Thereafter, the mean body weight increase of the control rats was 30.4 ± 8.5 g/month, whereas that of the malnourished animals was 28.3 ± 7.3 g/month. In the recovery group, the mean body weight increase during the first month of normal diet was 38.8 ± 10.3 g and thereafter 20.0 ± 8.2 g/month. No significant differences were found in the mean body weights among age-matched malnourished, recovery and control rats (Table 1; Fig. 4*a*). A progressive and statistically significant

Table 1. *Results of two-way and one-way ANOVA on the body and brain weights of experimental and control groups of rats*

	Two-way ANOVA						One-way ANOVA	
	Treatment (D.F. 1,24)		Duration of treatment (D.F. 2,24)		Interaction (D.F. 2,24)		Treatment (D.F. 2,12)	
	F	*P*	F	*P*	F	*P*	F	*P*
Body weight	0.63	0.435, N.S.	40.46	$< 5 \times 10^{-5}$	0.40	0.673, N.S.	3.4	0.068, N.S.
Brain weight	0.81	0.377, N.S.	2.76	0.08, N.S.	2.84	0.078, N.S.	1.83	0.202, N.S.

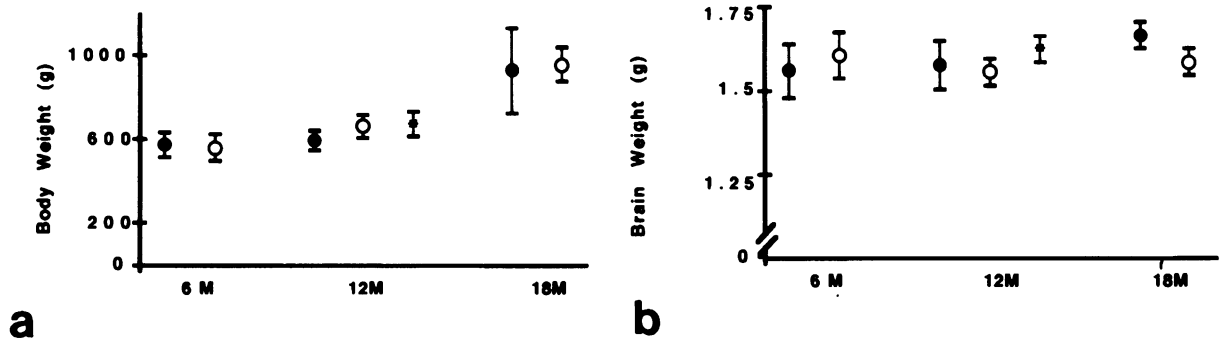

Fig. 4. Body and brain weights for control (C, ●), malnourished (UN, ○) and recovery (R, *) groups of rats at 6 (6M), 12 (12M) and 18 months (18M) stages of the experiment. Vertical bars represent 1 standard deviation (S.D.). (*a*) Mean body weights; 6MC vs 18MC, 12MC vs 18MC, 6MUN vs 18MUN, 12MUN vs 18MUN, $P < 0.0005$. (*b*) Mean brain weights.

Table 2. *Results of two-way and one-way ANOVA on the morphometric data obtained from the hippocampal formation of experimental and control groups of rats*

	Two-way ANOVA						One-way ANOVA	
	Treatment (D.F. 1,24)		Duration of treatment (D.F. 2,24)		Interaction (D.F. 2,24)		Treatment (D.F. 2,12)	
	F	*P*	F	*P*	F	*P*	F	*P*
Volume of the layers								
Granular layer	5.20	0.030	0.09	0.896, N.S.	0.36	0.703, N.S.	3.01	0.085, N.S.
Hilar region	4.28	0.063, N.S.	2.79	0.080, N.S.	0.606	0.575, N.S.	2.45	0.124, N.S.
CA3 pyramidal	46.38	$< 5 \times 10^{-5}$	2.36	0.112, N.S.	4.54	0.030	16.82	3×10^{-4}
CA1 pyramidal	33.42	$< 5 \times 10^{-5}$	0.17	0.852, N.S.	0.45	0.628, N.S.	8.62	0.005
Numerical densities								
Granule cells	88.14	$< 5 \times 10^{-5}$	1.44	0.257, N.S.	0.459	0.637, N.S.	30.54	$< 5 \times 10^{-5}$
Hilar cells	33.93	$< 5 \times 10^{-5}$	1.50	0.243, N.S.	0.140	0.870, N.S.	7.46	0.008
CA3 pyramidal cells	59.66	$< 5 \times 10^{-5}$	1.05	0.366, N.S.	0.935	0.454, N.S.	12.06	0.001
CA1 pyramidal cells	186.26	$< 5 \times 10^{-5}$	0.06	0.942, N.S.	1.50	0.242, N.S.	24.32	1×10^{-4}
Total numbers								
Granule cells	94.81	$< 5 \times 10^{-5}$	1.94	0.178, N.S.	0.137	0.825, N.S.	59.25	$< 5 \times 10^{-5}$
Hilar cells	32.03	$< 5 \times 10^{-5}$	1.08	0.315, N.S.	0.795	0.463, N.S.	17.08	3×10^{-4}
CA3 pyramidal cells	338.25	$< 5 \times 10^{-5}$	2.10	0.080, N.S.	0.924	0.394, N.S.	45.12	$< 5 \times 10^{-5}$
CA1 pyramidal cells	186.50	$< 5 \times 10^{-5}$	0.526	0.624, N.S.	0.725	0.497, N.S.	51.33	$< 5 \times 10^{-5}$

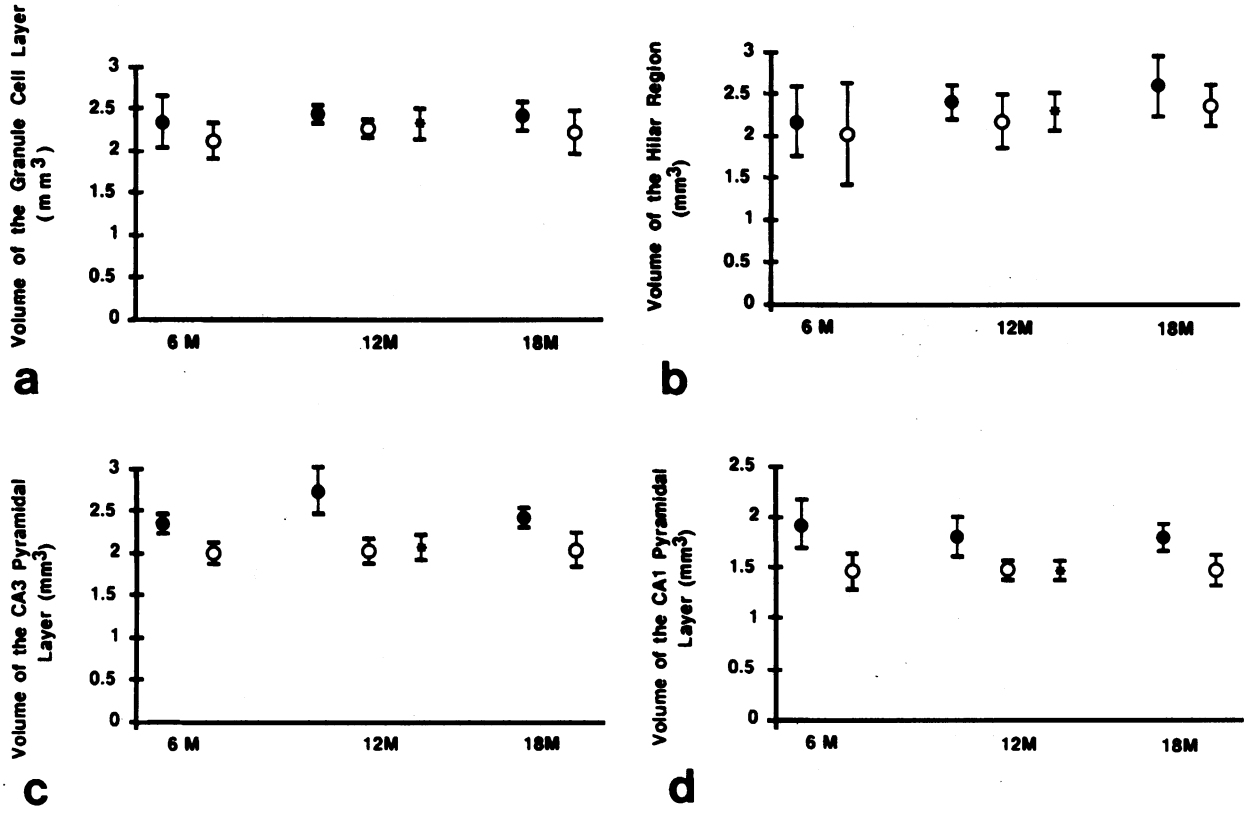

Fig. 5. Volume data obtained from the hippocampal formation of control (C, ●), malnourished (UN, ○) and recovery (R, ✱) groups of rats at 6 (6M), 12 (12M) and 18 months (18M) stages of the experiment. Vertical bars represent 1 S.D. (*a*) Mean volumes of the dentate gyrus granular layer; 12MC vs 12MUN $P < 0.02$. (*b*) Mean volumes of the hilar region. (*c*) Mean volumes of the CA3 pyramidal cell layer; 12MC vs 12MUN, 12MC vs 12MR, $P < 0.0005$; 6MC vs 6MUN, $P < 0.001$; 18MC vs 18MUN, $P < 0.02$. (*d*) Mean volumes of the CA1 pyramidal cell layer; 6MC vs 6MUN, $P < 0.0005$; 12MC vs 12MUN, 12MC vs 12MR, $P < 0.0025$; 18MC vs 18MUN, $P < 0.01$.

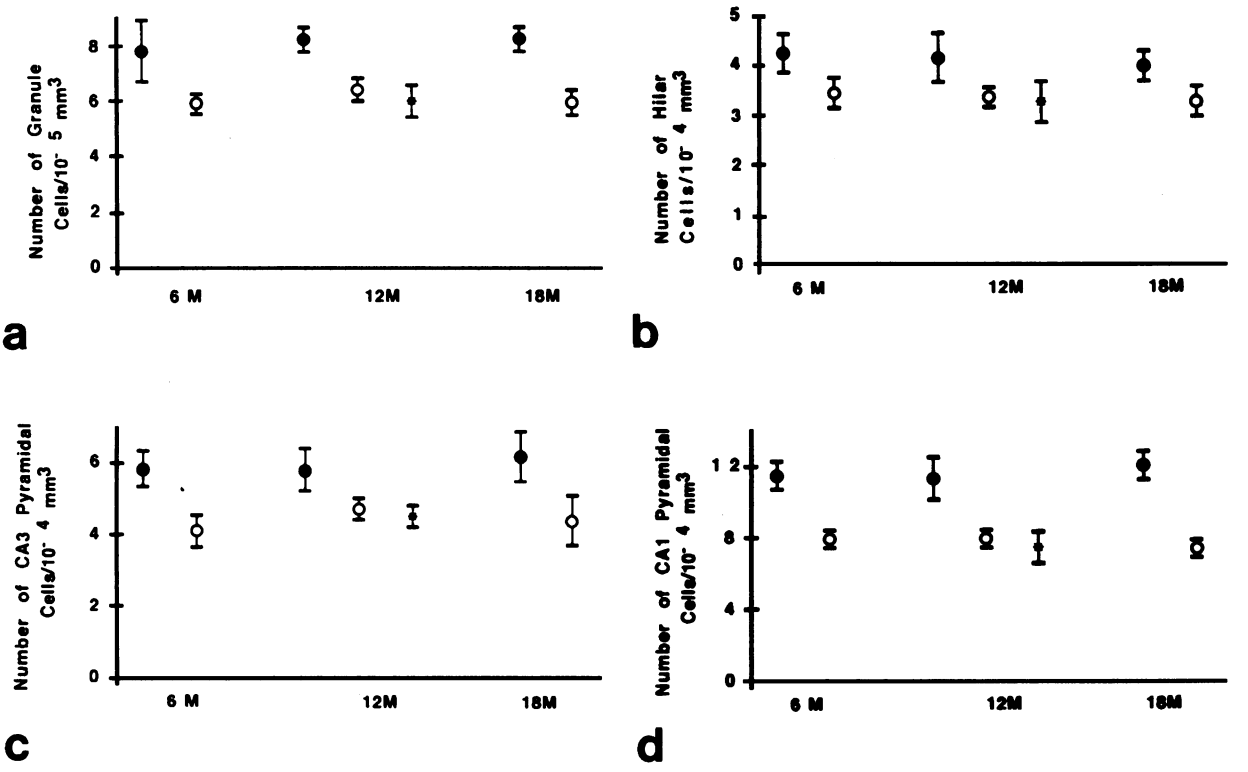

Fig. 6. Numerical density data obtained from the hippocampal formation of control (C, ●), malnourished (UN, ○) and recovery (R, *) groups of rats at 6 (6M), 12 (12M) and 18 months (18M) stages of the experiment. Vertical bars represent 1 S.D. (*a*) Numerical density of granule cells; 6MC vs 6MUN, 12MC vs 12MUN, 12MC vs 12MR, 18MC vs 18MUN, $P < 0.0005$. (*b*) Numerical density of hilar cells; 6MC vs 6MUN, $P < 0.001$; 12MC vs 12MUN, 12MC vs 12MR, $P < 0.0025$; 18MC vs 18MUN, $P < 0.005$. (*c*) Numerical density of CA3 pyramidal cells; 6MC vs 6MUN, 12MC vs 12MR, 18MC vs 18MUN, $P < 0.0005$; 12MC vs 12MUN, $P < 0.001$. (*d*) Numerical density of CA1 pyramidal cells; 6MC vs 6MUN, 12MC vs 12MUN, 12MC vs 12MR, 18MC vs 18MUN, $P < 0.0005$.

increase in body weights occurred with increasing age in control and experimental groups.

No significant differences were found between the mean brain weights of age-matched experimental and control rats nor between the brain weights of progressively older animals (Table 1; Fig. 4*b*).

Volume of the cell layers. ANOVA revealed that the variations in the volume of the granular layer were dependent on the effect of treatment (Table 2). Only in rats malnourished for 12 months was the volume of the layer smaller than in the respective age-matched controls (Fig. 5*a*). Nutritional rehabilitation had no effect on the volume of the granular layer (Table 2; Fig. 5*a*). No effect of treatment or duration of treatment on the volume of the hilar region was found (Table 2; Fig. 5*b*).

ANOVA showed that the variations in the volumes of the CA3 and CA1 pyramidal cell layers were dependent on the effect of treatment (Table 2). The volumes of the CA3 (Fig. 5*c*) and CA1 (Fig. 5*d*) pyramidal cell layers were smaller in malnourished and recovery rats than in the respective age-matched controls. No differences were found in the volumes of the CA3 and CA1 pyramidal cell layers when the recovery animals were compared with the age-matched malnourished rats.

Numerical density of cells. ANOVA showed a significant effect of treatment on the numerical density of granule and hilar cells (Table 2). The numerical density of the granule (Fig. 6*a*) and hilar (Fig. 6*b*) cells was smaller in malnourished and recovery groups than in the respective age-matched controls. No differences were found in the numerical density of granule and hilar cells between recovery and age-matched malnourished groups.

ANOVA revealed a significant effect of treatment on the numerical density of CA3 and CA1 pyramidal cells (Table 2). The numerical density of both cell populations was smaller in malnourished and recovery groups than in the respective age-matched controls (Fig. 6*c*, *d*). No significant differences were found in the numerical density of CA3 and CA1 pyramidal cells when the recovery and the age-matched malnourished groups were compared.

Total number of cells. ANOVA showed that the variations in the total number of granule and hilar cells were dependent on the effect of treatment (Table 2). The number of these neurons was smaller in

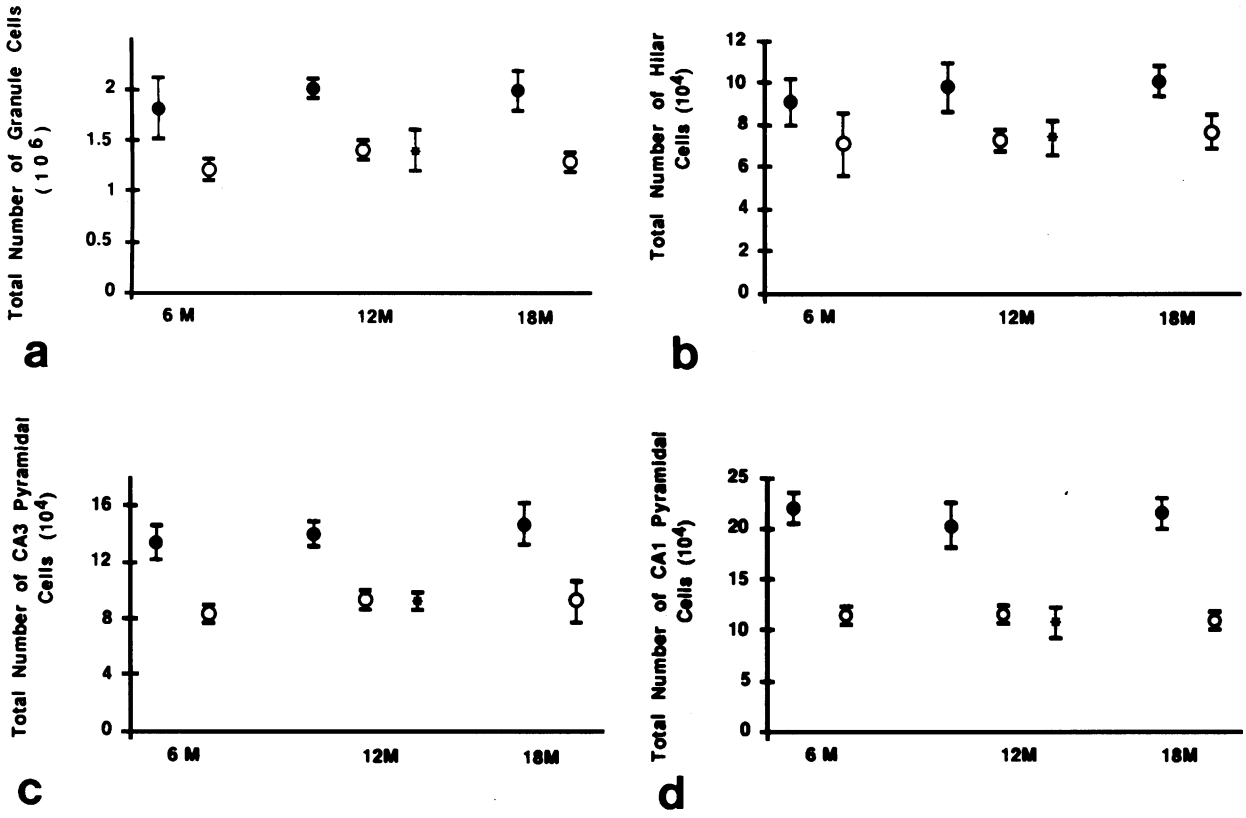

Fig. 7. Total numbers obtained from the hippocampal formation of control (C, ●), malnourished (UN, ○) and recovery (R, *) groups of rats at 6 (6M), 12 (12M) and 18 months (18M) stages of the experiment. Vertical bars represent 1 S.D. (*a*) Total number of granule cells; 6MC vs 6MUN, 12MC vs 12MUN, 12MC vs 12MR, 18MC vs 18MUN, $P < 0.0005$. (*b*) Total number of hilar cells; 12MC vs 12MUN, 12MC vs 12MR, $P < 0.0005$; 18MC vs 18MUN, $P < 0.0025$; 6MC vs 6MUN, $P < 0.05$. (*c*) Total number of CA3 pyramidal cells; 6MC vs 6MUN, 12MC vs 12MUN, 12MC vs 12MR, 18MC vs 18MUN, $P < 0.0005$. (*d*) Total number of CA1 pyramidal cells; 6MC vs 6MUN, 12MC vs 12MUN, 12MC vs 12MR, 18MC vs 18MUN, $P < 0.0005$.

malnourished and recovery animals than in the respective age-matched controls (Fig. 7*a*, *b*). No differences were found in the total number of granule and hilar cells when the recovery and age-matched malnourished groups were compared.

ANOVA showed a significant effect of treatment on the total number of pyramidal cells (Table 2). The total number of CA3 (Fig. 7*c*) and CA1 (Fig. 7*d*) pyramidal cells was smaller in malnourished and recovery animals than in the respective age-matched controls. No differences were found when the recovery and the age-matched malnourished groups were compared.

DISCUSSION

The timing of onset of protein deprivation is a determining factor of its effects on the structure of the central nervous system (Dobbing, 1968; Morgane et al. 1978, 1992, 1993). The vulnerability is maximal when the brain is developing at the highest rate, i.e. during the so called 'brain growth spurt period' (Dobbing, 1968), which takes place in early postnatal life (Dobbing, 1968). The effects of malnutrition on the developing brain are particularly well characterised in rodents; reductions in neuronal numbers (West & Kemper, 1976; Leuba & Rabinowicz, 1979*a*; Jordan et al. 1982; Bedi, 1991), impoverishment of dendritic trees and decreases in the number of dendritic spines (Leuba & Rabinowicz, 1979*b*; McConnell, 1980; Díaz-Cintra et al. 1981, 1984, 1990, 1991) are the most striking alterations described. Conversely, malnutrition initiated in adult life has not been considered to be a probable cause of major alterations of the brain structure (Dobbing, 1968; Morgane et al. 1978, 1992, 1993). It is not surprising, therefore, that the investigative imagination of researchers has not been captured by a domain of the utmost importance as postweaning malnutrition, and that most of the existing reports are confined to short periods of experimentation (Cordero et al. 1985; Peters et al. 1987; Warren et al. 1989; Brock & Prasad, 1992; Yucel et al. 1994).

Quantitative studies performed in adult rats have shown that following 1 month of undernutrition, the

number of synapses per neuron was increased in the visual cortex, in spite of the absence of alterations in the numerical density of its neurons (Warren et al. 1989). In the same vein, low-protein feeding of adult rats, during the same experimental period, has been reported to increase the spine density in the medium sized spiny neurons of the striatum and in the neurons located ventral to the perirhinal fissure of the entorhinal cortex (Brock & Prasad, 1992). Conversely, under the same experimental conditions, no alterations in spine density were found in pyramidal neurons of the frontal and parietal cortices nor in the spiny neurons of the medial septum (Brock & Prasad, 1992). Moreover, in a dietary restriction model in which the body weights reached at 2 months of age were kept constant during life span, no neuronal loss was demonstrated in the visual cortex of 26-month-old rats (Peters et al. 1987); however, in rats that succeeded in surviving until 46–48 months there was evidence of neuronal loss, a finding which was correlated with the ageing process (Peters et al. 1987).

In previous studies, using long-term protein deprivation begun in adult life as the experimental paradigm, we have demonstrated that in layer III of the prelimbic area there was no cell loss after 18 months of low protein intake (Paula-Barbosa et al. 1988). However, in the cerebellar cortex the numerical density of granule cells was reduced after 12 months of the experiment (Paula-Barbosa et al. 1989), as was the numerical density of dentate granule and CA3 pyramidal cells after 6 months of low protein diet, a reduction not aggravated by extension of the experimental period (Paula-Barbosa et al. 1989). In a subsequent study we provided evidence that the numerical density of the synapses established by mossy fibres and the thorny excrescences of CA3 pyramidal cells was also decreased (Andrade et al. 1991).

In the present work we demonstrate that besides granule and CA3 pyramidal cells the remaining neuronal populations of the hippocampal formation of the adult rat are unequivocally affected by long-term protein deprivation, a finding which, to the best of our knowledge, is described for the first time. In the dentate gyrus, the total number of granule cells was decreased in low-protein fed rats, an alteration found to be mainly dependent on the decrease of the numerical density of its neurons, which accords with our previous data (Paula-Barbosa et al. 1989). Because these alterations are identical to those reported in studies in which undernutrition was started in the perinatal period (Jordan et al. 1982; Ahmed et al. 1987; Bedi, 1991) we can conclude that granule cells are highly vulnerable to a low protein diet, irrespective of the time when it is introduced. Furthermore, there are reasons to assume that the severity of the resulting alterations is highest during the early months of deprivation and that they are not progressive because cell loss does not significantly increase after 6 months of treatment.

In rodents, granule cell neurogenesis continues during adult life (Bayer, 1980, 1982; Altman & Bayer, 1990). Therefore, the possibility cannot be safely excluded that the decrease in the total number of granule cells in malnourished rats might derive from a deficient cell accretionary process during the experiment. In other words, malnutrition might affect the proliferation of the precursor cells, and the migration of the newly-generated neurons from their site of origin to the places they were destined to settle in, thus leading to a reduction in the number of granule cells. This assumption is supported by data from experiments undertaken in rats submitted to perinatal malnutrition, which indicate that in this condition the division rate of the neuronal precursor cells is reduced (Deo et al. 1978; Lewis et al. 1979; Morgane et al. 1992, 1993) and the migration of the granule cells slowed (Deo et al. 1978; Lewis et al. 1979; Debassio & Kemper, 1985) in regions which display a predominant postnatal neurogenesis, namely the cerebellar cortex, the olfactory bulb and the dentate gyrus. In spite of this, perinatal undernutrition did not seem to alter the number of granule cells in the olfactory bulb (Tolley & Bedi, 1994). There is evidence, however, that experimentally induced inhibition of protein synthesis leads to a reduction of the number of granule cells in the cerebellar cortex and dentate gyrus of both young and adult rats (Pasquini et al. 1967; Rami et al. 1986; Madeira et al. 1988*a*, *b*, 1991), which provides support for the hypothesis of a disturbed process of neurogenesis in these brain areas.

A deficient process of cell acquisition, however, cannot be regarded as the sole possible underlying factor for the numerical reduction of granule cells in the dentate gyrus. Should this be so, then the largest expected decrease in the number of granule cells following 6 months of undernourishment could never exceed 22%, i.e. the percentage of granule cells generated during the same period (Bayer, 1982). As the reduction found reached 35%, neuronal degeneration must also be taken into account as a major factor underpinning the decrease in the number of granule cells.

The total number of hilar neurons was also found to be reduced in malnourished rats at all ages

analysed. This alteration is exclusively dependent on the decrease in the numerical density of hilar cells as the volume of the hilar region does not differ between malnourished and control groups. In this hippocampal region, cell death emerges as the only explanation for the reduction of the total number of cells because, although some hilar neurons may proliferate during adulthood, they are essentially formed prenatally (Bayer, 1980; Altman & Bayer, 1990).

The effects of a long-term low protein diet on pyramidal cells mirror those reported for the dentate gyrus, as the number of neurons in both CA1 and CA3 hippocampal fields is smaller in malnourished than in control rats. However, in addition to a reduction in the numerical density, a parallel decrease in the volume of the cells layers was also detected. As the latter alteration was not observed in the dentate gyrus and cell loss is of identical magnitude in the entire hippocampal formation, it is conceivable that the alteration in the volume of the pyramidal cell layers might result from a higher vulnerability of the components of the neuropil of these regions to malnutrition. Moreover, in the hippocampus proper, cell loss must be regarded just as a consequence of neuronal degeneration since the process of cell acquisition of both CA1 and CA3 pyramidal cells is accomplished before birth (Bayer, 1980).

In the present work, and utilising as the main estimator the total number of cells, we have demonstrated without ambiguity that all main neuronal populations of the hippocampal formation of the mature rat are affected by long-term low protein intake. It is possible, therefore, that in protein deprived rats there is an abnormal synthesis and degradation of specific proteins, including enzymes and receptors, that once altered might ultimately lead to neuronal death (Brock & Prasad, 1992; García-Ruiz et al. 1993; Morgane et al. 1993). Other factors cannot be disregarded as possible underlying causes of the neuronal degeneration observed in the present experimental conditions. For example, there is evidence that the switch from standard conditions to a low protein diet is by itself a considerable stress (Morgane et al. 1978; García-Belenguer et al. 1993) and that malnutrition activates the hypothalamo–pituitary–adrenal axis (García-Belenguer et al. 1993). As a result the plasma levels of glucocorticoids increase (García-Belenguer et al. 1993), an alteration which induces marked hippocampal neuronal degeneration (Sapolsky, 1986, 1987; Gould et al. 1992) and exacerbates the hippocampal damage inflicted by several aggressive agents (Sapolsky, 1986, 1987).

Investigations in which models of malnutrition instituted in the perinatal period were used have shown that rehabilitation from malnutrition reverses the alterations in cell packing (Leuba & Rabinowicz, 1979*a*; Bedi et al. 1980; Thomas et al. 1980; Warren & Bedi, 1984), cortical width (Angulo-Colmenares et al. 1979) and synapse-to-neuron ratio (Bedi et al. 1980; Thomas et al. 1980; Warren & Bedi, 1984; Ahmed et al. 1987) induced by undernutrition in several regions of the brain. Nevertheless, other studies failed to detect such improvement in parameters such as neuronal numbers or densities (Ahmed et al. 1987; Bedi, 1991) and dendritic branching and spine density (Leuba & Rabinowicz, 1979*b*; McConnell, 1980). In the present study, we have provided evidence that rehabilitation from malnutrition does not lead to an improvement of the morphological alterations induced by malnutrition as data obtained are similar in recovery and malnourished rats. Furthermore, the reestablishment of an adequate protein intake does not prevent the degenerative processes initiated by the deprivational status because in all regions analysed, namely in the dentate gyrus in which there is postnatal neurogenesis, neuronal numbers do not differ between recovery and malnourished groups.

It is somehow surprising that the substantial neuronal loss herein described is not reflected in changes in brain weight. No significant differences were observed between the brain weights of low protein fed and control rats, a finding which fits observations from other authors (Morgane et al. 1978; Resnick et al. 1982; Cordero et al. 1985; Yucel et al. 1994). As no evidence of macroscopic brain atrophy was detected in malnourished rats, it is conceivable that a brain weight decrease, which would have been anticipated on the basis of the neuronal loss, might have been blunted by regrowth mechanisms displayed by the processes of the surviving neurons (Coleman & Flood, 1986; García-Ruiz et al. 1993; Díaz-Cintra et al. 1994) as well as by an increase in the number of glial cells and enlargement of their processes triggered by the neuronal degeneration (Yu et al. 1974). In addition the selective vulnerability of the mature brain to malnutrition must also be considered as a probable additional cause for such a finding. Indeed, information gleaned from the existing literature suggests that degeneration throughout the brain (Paula-Barbosa et al. 1988; Warren et al. 1989; Yucel et al. 1994) might not reach the magnitude of that found in the hippocampal formation.

It can be concluded, therefore, that in rodents long-term protein deprivation, even when established in

adult life, markedly disrupts the structural organisation of the hippocampal formation and, furthermore, that the affected neuronal populations are not restored after the reestablishment of a normal diet. Consequently, all links of the trisynaptic circuit of the hippocampal formation (Zimmer et al. 1983; Amaral & Witter, 1989) may be irreversibly damaged. The functional and behavioural implications of these alterations are at the moment not clear, but if a homology with the effects observed in perinatally malnourished animals is justified it is possible that a number of cognitive functions, including learning and memory, might be irreversibly damaged (Morgane et al. 1978, 1992, 1993; Jordan et al. 1982; Katz et al. 1982).

The ultimate goal of these experiments is the extrapolation of results to the human brain; caution should be taken, as human malnutrition is usually a complex combination of several types of nutritional deficiency (Morgane et al. 1993), whereas in the present experimental situation a specific deprivation was evaluated. However, it is conceivable that the damage inflicted by malnutrition in the adult human brain might be severe and irreversible. To assess this possibility, the study of brains of malnourished humans is of paramount importance, inasmuch as with the stereological methodologies recently described (West & Gundersen, 1990; West et al. 1991) it is possible to overcome the problems in the analysis of postmortem material.

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