

Quantitative changes in the V2 lateral visual cortex in developing rats with induced protein malnutrition

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SUMMARY

In this paper we have analyzed the quantitative changes in the lateral visual cortex V2 (V2L) from developing rats with induced isocaloric protein malnutrition that looks like Kwashiorkor condition, as well as the changes induced after refeeding. Using stereological techniques the cortical volume, numerical density and the absolute number of neurons and the average neuronal volume were estimated.

The result obtained led us to the following conclusions: i) A significant enlargement in the ratio between brain weight and body weight was detected, disappearing after refeeding. ii) The volume of the cerebral cortex, and V2L was reduced. This was partially reversed by the refeeding at the level of the cerebral cortex, but there was not significant reversal in V2L. iii) The neuronal density was enlarged in V2L. Globally considered, this parameter was unchanged after refeeding. Nevertheless, in those animals refed from post-gestational age P0 the numerical density of neurons was significantly lower than in rats refed from post-gestational age P21. iv) The absolute number of neurons and their average volume was unchanged in V2L. Nevertheless, the average neuronal volume was significantly lower in rats with more than thirty days of post-gestational age (>P30) in comparison with the rats with less than thirty days of post-gestational age (≤

P30). v). No differences regarding sex were observed at the level of the studied parameters.

Key words: Lateral visual cortex V2 – Protein malnutrition – Stereology – Neurons

INTRODUCTION

It is considered important to study the effects of malnutrition during brain development, because various circumstances, including the fact that brain development ranges widely in the postnatal period in humans, or that histogenesis of the cerebral cortex is extremely complex and slow, with asynchrony in maturation of different cortical areas (Sarnat, 1992; Rakic et al., 1994). Briefly, cell differentiation in the developing central nervous system is the cumulative result in every cell of the concrete history of activation / repression of genes and their products occurred over time (Sur et al., 2005).

Much of this sequence is not predetermined, but results from the dynamic interaction of intracellular signalling cascades and intercellular signals located in time and space. Such signals may have place either on neuronal membranes, or in the extracellular matrix or the synaptic cleft, and may interact with various neurotransmitters and other substances present in the blood, such as hormones, where nutrient levels play a key role for this interaction (Fame et al., 2011).

This study presents a model of isocaloric low-protein malnutrition that recalls Kwashiorkor condition in men (Levitsky et al., 1995). From the point

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Submitted: 18 December, 2015. Accepted: 30 March, 2016.

of view of cytoarchitecture, the lateral visual cortex V2 (V2L) is quite homogenous (Cenquizca et al., 2007) and an optimal region to perform morphometric studies. With respect to neuronal distribution, layers from I-IV show the same laminar pattern, with a thickness similar to that in other brain areas, such as the primary motor or somatosensory (Skoglund et al., 1996).

The rat's nervous system in general, and the cerebral cortex in particular, is very immature at birth, and the process of anatomical and functional maturation is completed during the postnatal period (Robertson et al., 2000), making it a good model to study the effects of postnatal malnutrition.

The V2L area is also known as Brodmann area 18, 18a in rodents. On the whole, it can be recognized as an elongated region, smaller and more myelinated than area 17 (primary visual area). It is located medial, anterior and lateral to the same visual area 17 and to the limbic area between V1 and hippocampal formation (Rosa et al., 2005) above the secondary associative temporal, and behind the associative auditory cortex rostral part (Krieg, 1947; Zilles et al., 1995). The ends of the area are surrounded by thalamocortical afferent fibres that circumscribe the boundaries of the area (Antonini et al., 1999). Functionally, the visual cortex V2L is defined as an association cortex of sensory perception related to the processes of learning and memory (Bai et al., 2004; Lopez-Aranda et al., 2009).

In relation to the first quantitative studies on the effect of malnutrition in the brain, we should mention those by Angulo-Colmenares et al. (1979), who observed that the offspring of rats subjected to a diet low in proteins during pregnancy showed changes in dimensional aspects, such as the anteroposterior length, width and height of the cerebral hemispheres and the thickness of the area 3 (somatosensory cortex), as well as reduction of neuropil volume in layers II and IV.

Later, some authors (Bhide et al., 1984; Bedi et al., 1989; Bedi, 1994; Peeling et al., 1994; Bedi, 2003) carried out stereological estimates of the number of neurons in order to determine the effects of periods of malnutrition during early life on the size of neuronal population in several regions of the rat brain, as well as the changes in numerical density of neurons and synapses in the visual cortex. Desai et al. (1996) states that the return to the optimal diet after P21 (weaning) did not correct brain disorders. However, these changes were partially reversed when the refeeding begins at P0. It has also been noted that early and prolonged malnutrition adversely affects nutritional rehabilitation. In fact, the deleterious effect of malnutrition on growth and orientation of the apical dendrites of some pyramidal neurons persisted in the adult and could not be reversed by the early refeeding (Cordero et al., 2003).

In this paper and in order to accurately identify

the neurons analyzed, immunohistochemical techniques were used to demonstrate a specific nuclear marker of neuronal population (NeuN) that is almost exclusively expressed in the nervous system. It appears during the development stage and remains in the adult. It manifests itself in mice, rats, chicken and humans. The NeuN appears to be a marker of neuronal maturation and differentiation both in vivo and in vitro (Mullen et al., 1992; Arias et al., 2002).

Stereological methods were performed to estimate: cortical volumes, counting (relative and absolute) of neurons and the average volume of perikaria. These studies were applied on the V2L visual cortex from the control group, undernourished rats and animals with refeeding after malnutrition.

This study pursues the following aims: i) to check whether quantitative changes in undernourished rats are given at different stages after birth (<P30, ≥P30) compared to controls in the V2L area affecting the next variables: volume of the cerebral cortex, V2L volume, average volume of neuron perikaria, numerical density and absolute number of neurons; and, ii) to assess the extent to which the reversibility of quantitative changes occurs in rats re-fed through diet of 25% protein content, from P0 and from P21.

MATERIALS AND METHODS

Animals

The rats were selected from litters obtained as follows: Forty mature female Sprague-Dawley rats with an average weight of 200 gr were taken, and subjected to isocaloric diets with a protein content of 25% (20 animals) and 6% (the other 20) respectively. The diet was maintained for 5 weeks prior to mating with male adult, fed on a standard diet. A total of 2 litters for each group was obtained.

During pregnancy the feed is kept on demand by placing fodder in a container of 150 gr. An average of 10 pups were left for each mother. The lactation was on demand, and at 21 days weaning was performed. From the undernourished group, a number of pups were randomly selected to be fed with 25% protein diet (group re-fed). A set of animals in this group were refeed from P21 (21 days post-pregnancy). To get refeeding from P0 (at birth) of the remaining animals of the refeed group, mothers were fed after delivery with diet of 25% protein.

The pups were grouped as follows:

- i) Group N (controls): Fourteen rats (6 females, 8 males) from mothers nurtured with the 25% protein diet for 5 weeks prior to mating with normal males.
- ii) Group M: Fourteen rats (6 females, 8 males) from undernourished mothers fed with 6% protein diet for 5 weeks prior to mating with normal males.

- iii) Group R1: Fifteen rats (6 females, 9 males) from undernourished mothers that were re-fed from P0 to P20 with 25% protein diet supplied to mothers during lactation.
- iv) Group R2: Sixteen rats (7 females, 9 males) from undernourished mothers that were re-fed after weaning to P21.
- v) Besides, in order to compare the re-fed animals with groups N and M, a pool of R1+R2 (group R) was also employed.

In summary, a total of 59 brains of pups (25 females and 34 males) were studied, of which 10 animals per nutritional group were sacrificed with a post-gestation age between P25 and P30 (age \leq 30 days old), while the remaining rats (an average of 5 rats per nutritional group) were sacrificed in a range from P30 to P42 post-gestation age (age $>$ 30 days old).

The entire procedure was performed in the animal facility of the Faculty of Medicine at the Autonomous University of Madrid. They were kept in a controlled environment (20-22°C temperature and 45-55% relative humidity) under 12-hour cycles of light and darkness.

The animals were bred and manipulated in accordance with the bioethical standards of international organizations (WMA Statement on Animal Use in Biomedical Research), European Union guidelines, and Spanish State and Local regulations for the use of animals in research, and approved by the Ethical and animal Studies Committee of the Autonomous University of Madrid.

Diets

The development and preparation of diets was carried out by Harlan Interfauna (Barcelona, Spain), according to the composition indicated in Table 1, as formulation developed by Dr. Irma Herrera, Research Department of the National Institute of Nutrition, Caracas, Venezuela. The diet with a 25% of proteins is normo-proteinic, and was used to normally fed and re-fed animals. The 6% proteinic diet was carbohydrate-enriched, compensating therefore the protein deficiency with glucids, as happens in social groups with malnutrition

Table 1. Percentage composition of the diet of malnutrition (Diet M) and for the controls and animals referred to (Diet N / R)

INGREDIENTS	DIET M 6% Proteins	DIET N/R 25% Proteins
lactalbumin	7.69	32.05
corn oil	6.00	6.00
vitamins	1.00	1.00
minerals	3.50	3.50
starch	81.81	57.45
total	100.00	100.00

(Schurch, 1995). Both were prepared in batches, in order to prevent contamination, and stored at 4°C. They were supplied on demand. Both diets are isocaloric, i.e. provide 4 Kcal/gr of food, at the expense of the proportion of carbohydrates (corn starch), and lipids (corn oil). Supplementation with trace elements are made according to the composition of mineral salts of Williams-Briggs, modified to 3.5%.

Obtaining samples

The pups were weighed and after being sacrificed, brain weight was obtained in all groups of animals. The animals were killed by decapitation after anaesthesia with an overdose of sodium pentobarbital (Vetoquinol, Lure, France). The brain was removed by careful dissection.

Tissue processing

The brains were fixed by immersion in 10% paraformaldehyde in 0.1M PBS (phosphate buffered saline) at pH 7.4 for 72 hours. Then they were cut from the middle coronal plane towards the rear, in coronal slices of 2 mm thickness. Then were dehydrated in ethanol and afterwards embedded in paraffin. From each coronal slice, 4 μ m thick sections were made. Some sections were routine-stained with hematoxylin-eosin and others processed to immunohistochemistry.

Immunohistochemistry

To detect NeuN immunoreactivity, sections were incubated with a monoclonal anti-NeuN antibody (Chemicon International, USA) diluted at 1:100, pretreatment of sections by heat in citrate buffer pH 6.0 (using a pressure cooker) (Martin et al., 2001) was performed to enhance immunostaining. The primary antiserum was diluted in PBS pH 7.4 containing 1% bovine serum albumin (BSA) plus 0.1% sodium azide. The incubation with primary antiserum was overnight at 4°C. The second antibody employed was a biotin-caproyl-anti-mouse immunoglobulin (Biomed, Foster City, CA, USA). The second antibody was diluted at 1/400 in PBS containing 1% BSA without sodium azide, and incubated for 30 min at room temperature. Thereafter, sections were incubated with a streptavidin-biotin-peroxidase complex (Biomed). The immunostaining reaction product (was developed using 0.1 g diaminobenzidine (DAB) (3,3',4,4' - Tetraminobiphenyl, Sigma, St Louis, USA) in 200 mL of PBS, plus 40 mL of hydrogen peroxide 30% (w/v). All the specimens were processed with the same batch of primary and secondary antibodies and revealed with the same reagents.

All slides were dehydrated in ethanol, and mounted in a synthetic resin (Depex, Serva, Heidelberg, Germany).

Setting of V2L anatomical limits

The coordinates used to select the area where

the stereological estimations were performed have been obtained from the Atlas of Paxinos and Watson, 1998: from the interaural of 4.20 mm to interaural of 1.70 mm (Paxinos et al., 1998). We took into account the topographic relationship of V2L with: the pole of the dorsal geniculate nucleus, the posterior end of the nucleus of the thalamus and the hippocampal CA1 (Fig. 1).

Quantitative methodology

i) Once calculated the body and brain weights of animals, the ratio between them was estimated: brain weight / body weight (WR).
 ii) Before fixing, the brain volume was estimated by liquid displacement method (by immersion of fresh brain in a graduated cylinder filled with 0.1M PBS) and expressed in cm^3 . Changes in volume during tissue processing were calculated in order to transform the volumes that were measured in paraffin-embedded material into their actual measurements in fresh visual cortex. The amount of shrinkage (17% volume) was calculated previously (Gonchar et al., 1997). To determine whether the shrinkage in the thickness of the section is homogeneous in all areas from V2L, a pilot study was done in five additional normal rats by plotting the number of neurons counted against the depths at which the neurons were counted (West, 2013). The distribution of counts was uniform along the z axis, that is, the same at all depths of the section, as expected if homogeneous shrinkage has taken place. Consequently, this shrinkage factor was applied in the calculations of the number of neurons, to adjust V2L volume for shrinkage.

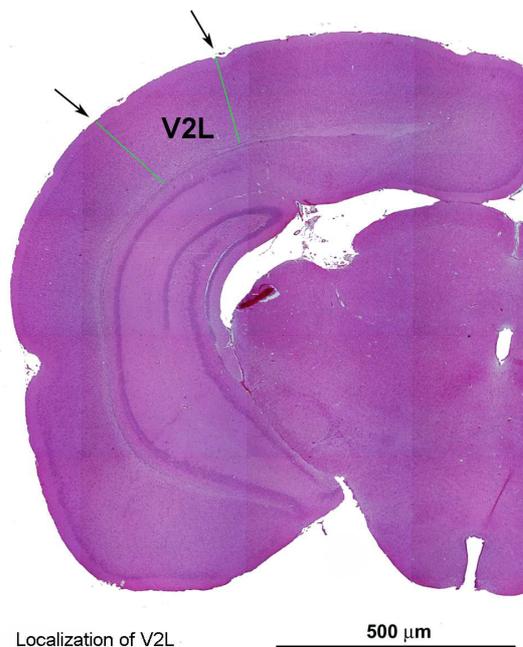


Fig. 1. Localization of the region of interest. Image of a coronal section of rat brain, where the limits of the visual cortex V2L (black arrows and green lines) are indicated. Stained with HE.

iii) Estimation of the volume of visual area V2L (V2L volume): On each coronal section of the brain, stained with hematoxylin-eosin, a grid of points was overlapped, to measure the volume fraction occupied by V2L (V_V V2L), using the following formula:

$$V_V \text{ V2L} = \frac{\sum \text{ points on V2L}}{\sum \text{ points on coronal section}}$$

For this estimate, 10 randomly selected sections per specimen were employed, at a final magnification of x2 (Santamaría et al., 2009).

V2L volume was then calculated by multiplying V_V V2L by the brain volume. The result was expressed in mm^3 .

iv) The estimate of relative and absolute numbers of neurons was performed by stereological methods, namely the optical disector (Bjugn, 1993; Bjugn et al., 1993; Howard et al., 1998; Howard et al., 2005). The optical disector is a three-dimensional probe, delimited by two optical tissue planes and edges of exclusion and inclusion intended to avoid over- or under-counts. The application of optical disector is based on the unbiased brick-counting rule proposed by (Howard et al., 1998). A particle is counted if it is fully contained in the probe or cut on any of the planes of inclusion and does not touch any of the prohibited areas (Sterio, 1984). We mean by particle any discrete tissue element that can be distinguished unambiguously. In the present study the neuron nuclei were employed as counting unit, assuming the mononuclear character of the neurons we are counting.

Five 15-mm-thick sections per specimen were chosen by systematic random sampling (Howard et al., 1998) from the pool of sections obtained by complete sectioning of each specimen. Measurements were carried out using an Olympus microscope equipped with a x100 oil immersion lens (numerical aperture of 1.4) at a final magnification of x1200, (Rodríguez et al., 2003). This program allows the selection of fields to be studied by random systematic sampling after the input of an appropriate sampling fraction. An average of 20 fields / section was selected and used to count the number of cells. This intensity of sampling seems adequate because the distribution of NeuN immunoreactive neurons is quite homogeneous in V2L (Herculano-Houzel et al., 2013).

The numerical density (number of neurons per unit of tissue volume) of immunoreactive neurons to NeuN (N_V neu) in V2L was calculated and expressed as number of neurons $\times 10^3 / \text{mm}^3$. Then, the absolute number of NeuN immunoreactive neurons (N neu), was obtained multiplying N_V neu by the V2L volume, and expressed as number of neurons $\times 10^6$.

v) Determining the average neuronal volume: A grid of points was superimposed on sections of V2L for estimation of the volume fraction occupied

Table 2. Two-Way ANOVA to study the effects of factor post-gestational age (≤ 30 days and > 30 days) on the variables studied in the different study groups

Two ways ANOVA (1)	Age	
	≤ 30 days old	> 30 days old
N	+	+
M	+	+
R	+	+

nourished animals (N); malnourished rats (M) and re-fed rats (R)

by immunoreactive neurons NeuN (V_V neu) using the following formula:

$$V_V \text{ neu} = \frac{\sum \text{points on perikaria NeuN positive}}{\sum \text{points on V2L}}$$

For this estimate, 10 randomly selected sections per specimen were employed, at a final magnification of $\times 40$.

Afterwards, the average neuronal volume (U_N) was obtained by the formula:

$$U_N = V_V \text{ neu} * V2L \text{ volume} / N \text{ neu}$$

The result was expressed in μm^3 .

All stereological estimates were made using the stereological software CAST-GRID (Interactivision, Silkeborg, Denmark).

Statistical analyses

We obtained the mean values \pm SD of all the variables studied for each group (N, M and R), in each age group (≤ 30 days, > 30 days), and subdividing the animals into males and females.

Furthermore, in the group R the mean values of the variables in animals re-fed to P0-P20, and P21 (R1 and R2 respectively) were assessed separately. With all these data the following analyses were performed:

i) Two-way ANOVA to evaluate: a) The influence of age groups on nutritional status, and b) the influence of sex on nutritional status. The distribution of the two factors in each of the indicated ANOVAS was summarized in Tables 2 and 3. Therefore, the following sources of variation were analyzed:

Two-way ANOVA (1): age, nutritional status, interaction between the two factors.

Two-way ANOVA (2): Sex, nutritional status, interaction between the two factors. To compare the means of each row Bonferroni test was used.

ii) One-way ANOVA to evaluate the influence of nutritional status without regard neither age nor sex. For the comparison of means test Student-Newman-Keuls was used.

iii) In the group R, the means of the variables were compared between rats re-fed to P0-P20 (Group R1) and P21 (Group R2), using the Stu-

Table 3. Two-Way ANOVA to study the effects of factor sex (males and females) on the variables studied in the different study groups

Two ways ANOVA (2)	Sex	
	males	females
N	+	+
M	+	+
R	+	+

nourished animals (N); malnourished rats (M) and re-fed rats (R)

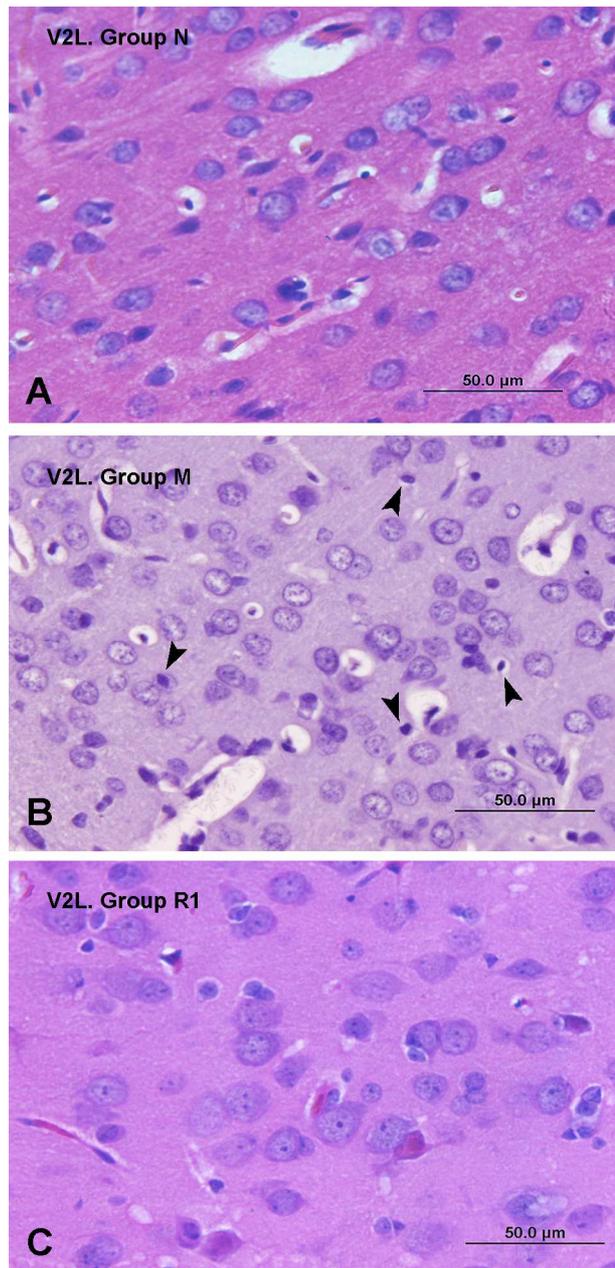


Fig. 2. Visual cortex from V2L. **(A)** Image of an animal from the group N (nourished rat) at P42 post-gestational age. **(B)** Image of a rat from group M (undernourished rat) at the same age as in A. No histological changes were observed, except for a slight rise of glial nuclei (arrowheads). **(C)** Image of an animal of the group R1 (rat re-fed from P0 to P20), showing a similar appearance with the group N. Stained with HE.

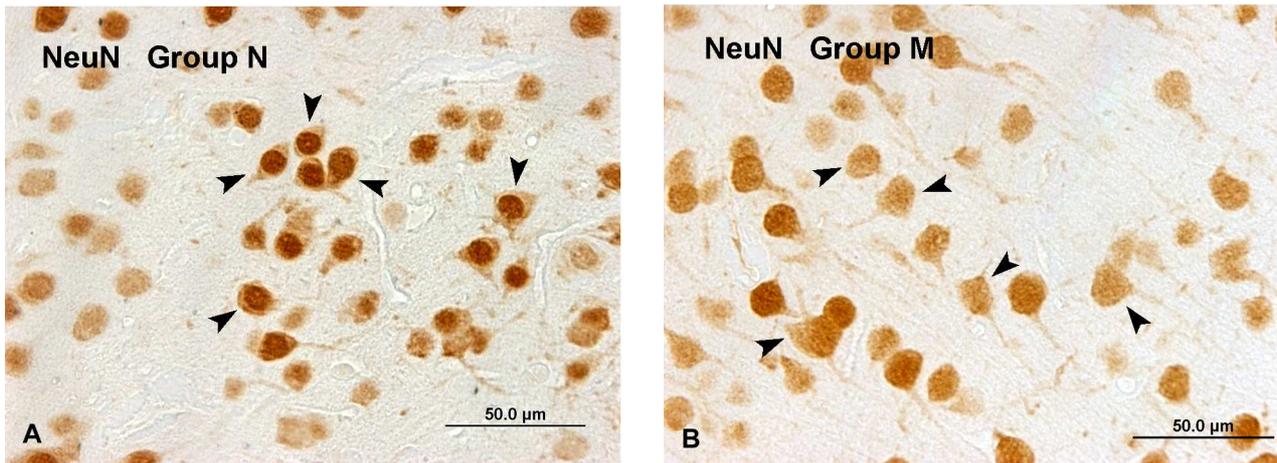


Fig. 3. Immunohistological study of the NeuN expression in the visual cortex V2L at P42 post-gestational age. **(A)** Image from an animal in the group N (nourished rat). Predominant nuclear immunopositive staining (arrowheads) is detected. **(B)** Image of an animal from group M (undernourished rat). No histologic abnormalities were observed, except a slight reduction in the intensity of the nuclear immunostaining and augmented immunoreactivity in the cytoplasm (arrowheads).

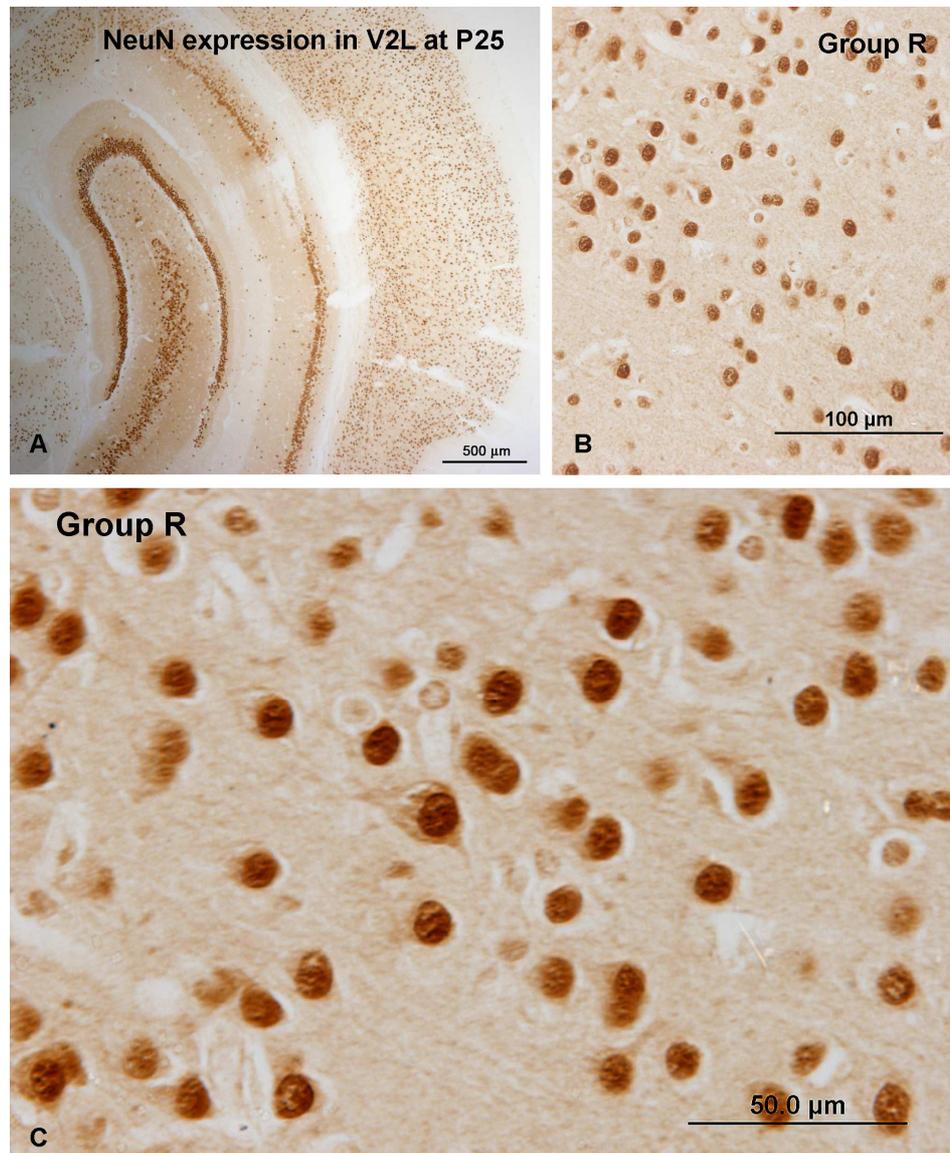


Fig. 4. Immunohistological study of the NeuN expression in the visual cortex V2L at P25 post-gestational age. **(A)** Image of an animal of the group R (rats refed from P0). **(B)** Detail of (A): Note the strong neuronal immunostaining detected. **(C)** Higher magnification to appreciate nuclear immunoreactivity similar to that observed in group N (data not showed).

Table 4. Summary P values for each variable studied for the two factors considered (nutrition and age) and the possible interaction between them.

VARIABLE	NUTRITION	AGE	INTERACTION
WR	< 0.0001***	< 0.0001***	0.021*
Brain volume V2L volume	< 0.0001*** 0.011*	0.002** 0.045*	0.803 0.025*
N _V neu	0.014*	0.330	0.043*
N neu	0.808	0.916	0.175
U _N	0.612	0.018*	0.459

(*) significant differences; (**) Very significant differences; (***) Highly significant differences.

Table 5. Summary P values for each variable studied for the two factors considered (nutrition and sex) and the possible interaction between them

VARIABLE	NUTRITION	SEX	INTERACTION
WR	<0.0001***	0.253	0.185
Brain volume V2L volume	<0.0001*** 0.054	0.094 0.711	0.029* 0.058
N _V neu	0.029*	0.196	0.975
N neu	0.866	0.509	0.321
U _N	0.438	0.074	0.980

(*) significant differences; (**) Very significant differences; (***) Highly significant differences.

dent t-test.

In all the analyses the level of signification was $p < 0.05$. The statistics was performed using SPSS 9.0 (SPSS Inc., Chicago ILL USA).

RESULTS

Descriptive findings

Regarding conventional histology, no qualitative differences between groups (Fig. 2 A-C) were observed. However, in the rats of group M, from a qualitative point of view, an apparent rising of glial cellularity can be observed (Fig. 2B).

With respect to NeuN immunostaining no relevant differences between the three groups (N, M, R) have been detected, except for a discreet but consistent shift of the immunoreactivity from the nuclei to the cytoplasm in the group M (Fig. 3 A,B). In the group of re-fed animals (R), NeuN immunostaining was similar to that described in nourished rats (N group), with predominant immunoreactivity detected in the nuclei (Fig. 4 A-C).

Quantitative observations

A significant rise of WR was observed in the M group in comparison to N. In re-fed animals (R), the WR drops significantly in relation to group M, but remains significantly higher than in the group N (Fig. 5A). The brain volume declines significantly in group M with respect to N and R.

The brain volume from group R was partially recovered with respect to M, but still significantly

lower than in N (Fig. 5B). V2L volume drops significantly in M in comparison to N. The group R has a V2L volume intermediate between N and M, but shows no significant difference with both. (Fig. 5C).

The numerical density of neurons (N_V neu) is significantly lower in the N group in relation to M and R. There are no significant differences between M and R (Fig. 6A). However, the absolute number of neurons (N neu) and the average neuronal volume does not change significantly in relation to nutritional status (Fig. 6 B,C).

Regarding to the start of the re-feeding: WR, brain volume and V2L volume, remain unchanged, because no significant differences were detected between P0 and P21 (Fig. 7 A-C).

However, N_V neu is significantly lower in R1 (rats re-fed to P0-P20) in comparison to R2 (animals re-fed to P21) (Fig. 8A). On the other hand, the absolute number of neurons and the average neuronal volume were not affected by the start of re-feeding (Fig. 8 B,C).

The summary of two-way ANOVA for nutritional state and age was shown in Table 4 indicating that both nutritional condition and age have significant influence on: WR, brain volume, and V2L volume. The numerical density of neurons was significantly affected by nutrition and average neuronal volume by age respectively. The interaction of the two factors is significant in WR, N_V neu, and V2L volume.

It can be observed (Fig. 9) significant differences to WR, in relation to age and nutritional condition, exclusively to N and M. Also, it can be observed (Fig. 10) significant differences in brain volume in relation to the age and nutrition exclusively for N and for the group under 30 days post-gestation.

In the group N, there is a significant rise in volume V2L volume in relation to age and nutritional condition, and above 30 days post-gestation (Fig. 11).

Neither N_V or N neu have significant differences for nutrition when the two age groups are compared (Figs. 12 and 13) were also observed.

In Fig. 14, it appears that there are no significant differences for average neuronal volume,

jointly considering the age and nutritional status.

The summary of two-way ANOVA for nutrition and sex is indicated in Table 5, showing that nutritional condition significantly influences the WR, brain volume, and N_V neu. Sex factor has not significantly influence on the studied variables. The interaction of the two factors is only significant for brain volume.

DISCUSSION

The diet provided does not appear to cause significant histological changes. However, we have observed in V2L neurons from the group of undernourished rats a displacement of NeuN immunoreactivity from the nucleus to the cyto-

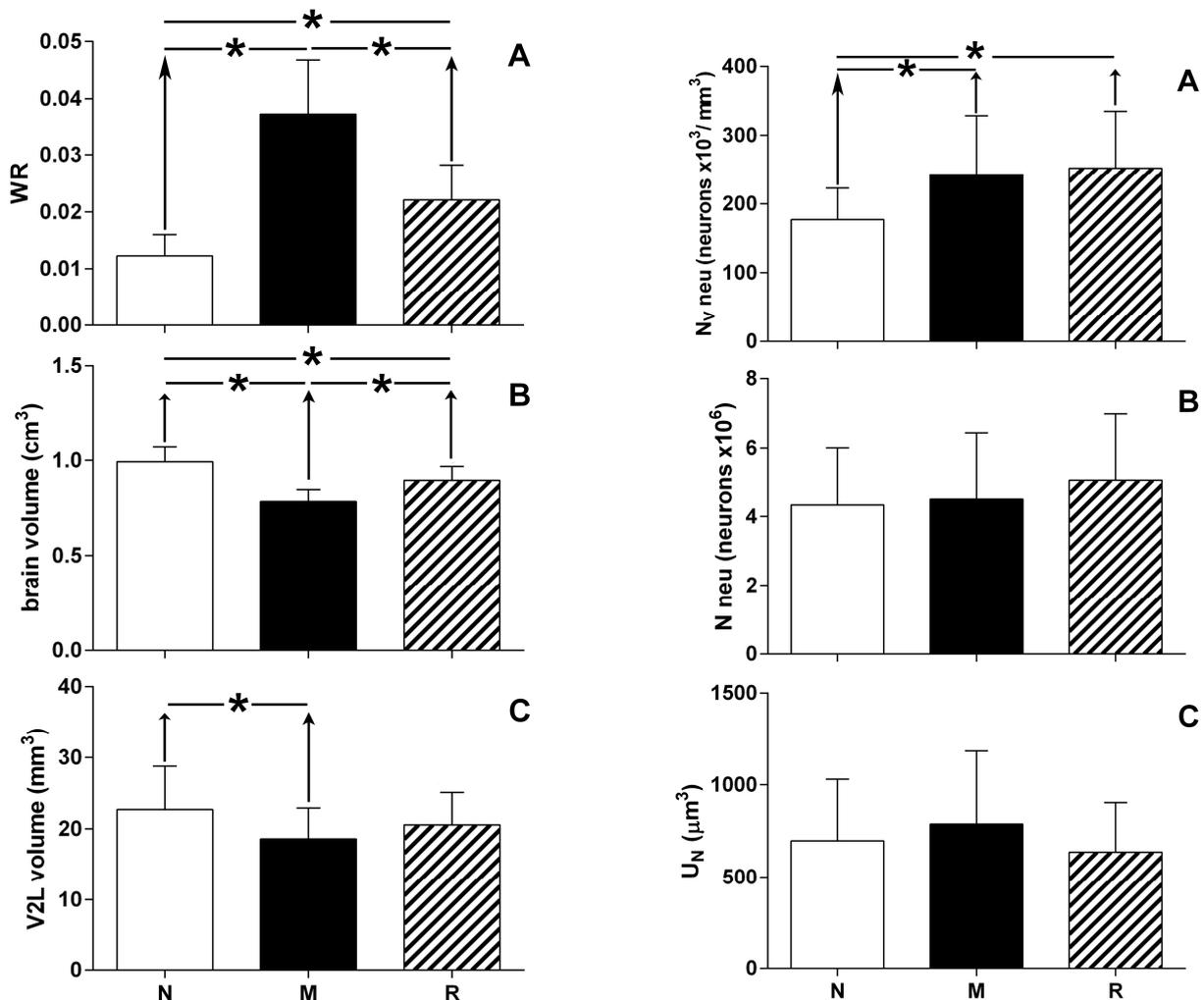


Fig. 5 (left). Bar diagrams representing mean \pm SD values in V2L for the different groups: N (nourished rats, n=14), M (undernourished rats, n=14), R (refed rats, n=31): **(A)** Ratio between brain and body weights (WR); **(B)** Brain volume; **(C)** V2L volume. The values with significant differences ($p < 0.05$) between them are indicated by asterisks and lines connecting the error bars.

Fig. 6 (right). Bar diagrams representing mean \pm SD values in V2L for the different groups: N (nourished rats, n=14), M (undernourished rats, n=14), R (re-fed rats, n=31): **(A)** Numerical density of neurons (N_V neu); **(B)** Absolute number of neurons (N neu); **(C)** Average neuronal volume (U_N). The values with significant differences ($p < 0.05$) between them are indicated by asterisks and lines connecting the error bars.

plasm. Cytoplasmic immunoreactivity has been observed in normal neurons of the nervous plexus of the guinea-pig's gastrointestinal tract (Van Nassauw et al., 2005). The metabolic changes caused by malnutrition could explain this shift in the NeuN immunoreactivity, because some authors suggest changes in the location and intensity of the immunoreaction for NeuN related to metabolic disturbances (Lind et al., 2005; Dredge et al., 2011) or pathological changes such as cerebral ischemia (Unal-Cevik et al., 2004).

On the other hand, the diet supplied produced differences in both cerebral and body weight detected in the group of undernourished rats (Kanarek et al., 1986); and the effect that refeeding had on these parameters, as noted by other authors in similar experimental models (West et al., 1976; Morgane et al., 1978; Warren et al.,

1985; Cordero et al., 1986).

In principle, we have to emphasize the fact that the changes appreciated from the standpoint of statistical analysis, were far more significant in relation to the ponderal and volumetric parameters in regard to the parameters of relative and absolute number of neurons.

We should draw attention to the presence of a lower WR and a greater brain volume in the group N than in group M. Both parameters show a tendency to be inversely affected with age, that is, in the group > P30, WR tends to be lower whereas brain volume tends to be higher in comparison to the group \leq P30 where the inverse occurs. This may suggest that the effect of the nutritional factor in young age affects more importantly the development of brain structures than in older ages as was suggested by other

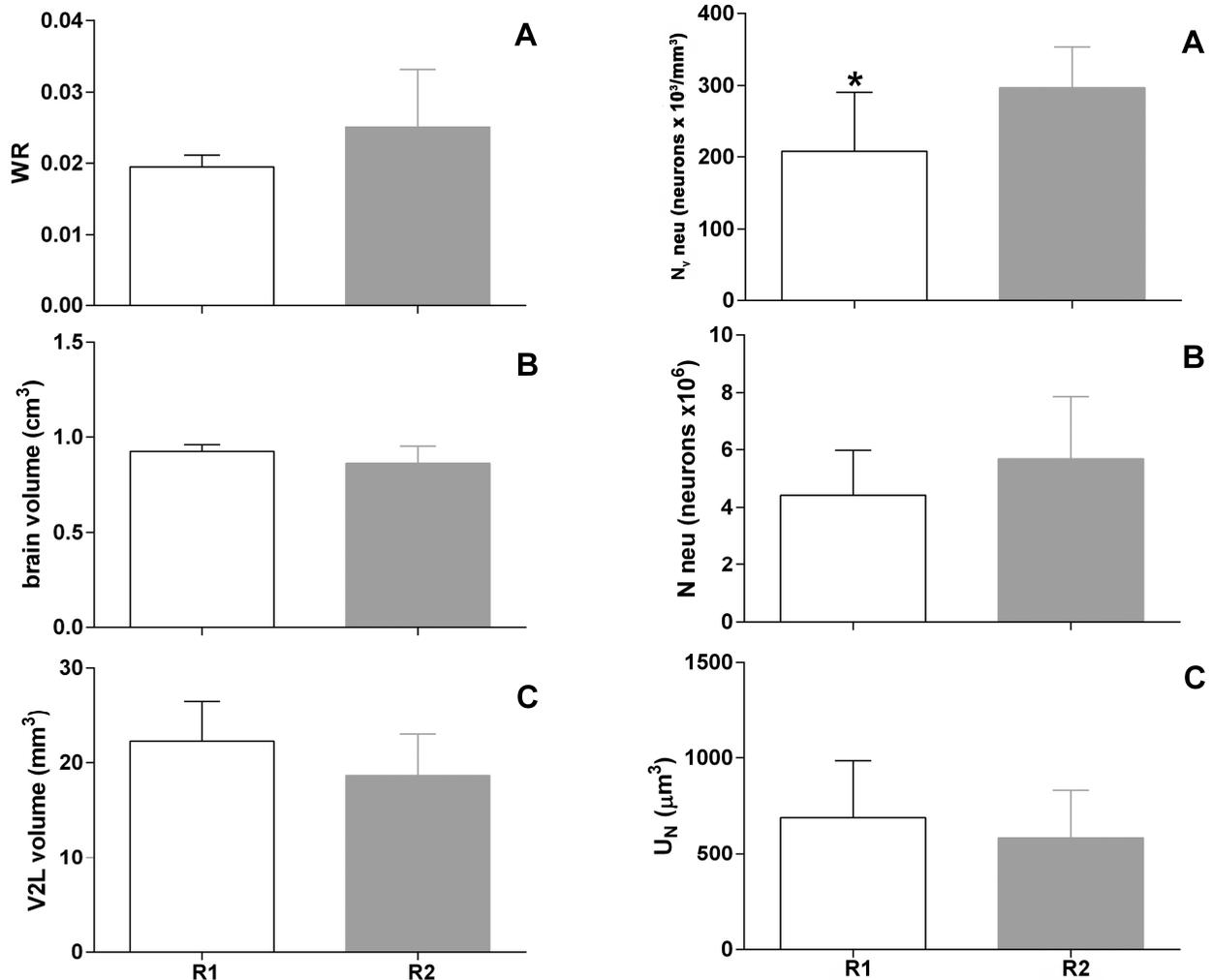


Fig. 7 (left). Bar diagrams representing mean \pm SD values in V2L for re-fed rats at P0-P20 (Group R1, n=15) and P21 (Group R2, n=16) post-gestational age: **(A)** Ratio between brain and body weights (WR); **(B)** brain volume and **(C)** V2L volume.

Fig. 8 (right). Bar diagrams representing mean \pm SD values in V2L for re-fed rats at P0-P20 (Group R1, n=15) and P21 (Group R2, n=16) post-gestational age. **(A)** Numerical density of neurons (NV neu); **(B)** Absolute number of neurons (N neu); and **(C)** average neuronal volume (UN). The asterisk on the top of error bars indicates significant differences between adjacent bars.

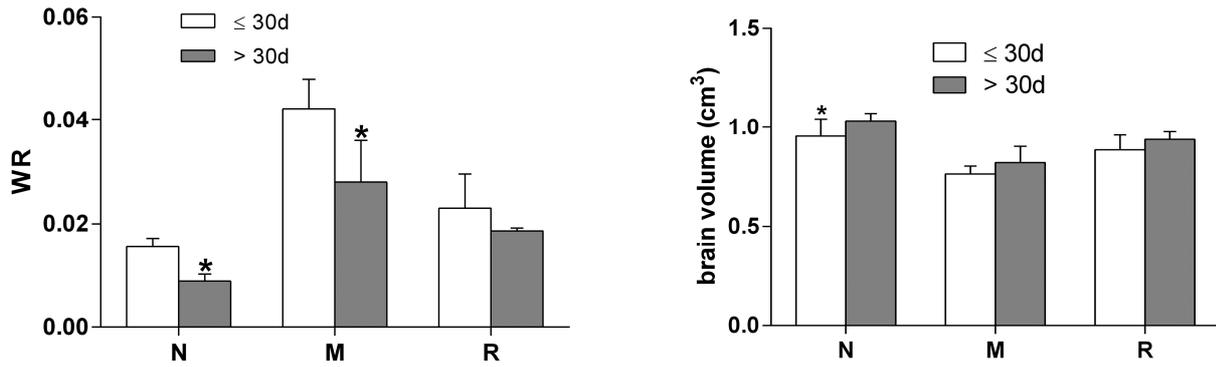


Fig. 9 (left). Bar diagrams representing mean \pm SD values of the ratio between brain and body weights (WR) for the different groups: N (nourished rats, n=14), M (undernourished rats, n=14), R (refed rats, n=31), considering for each of them, the age groups studied (≤ 30 days, > 30 days). Asterisks placed above error bars indicate the presence of significant differences between adjacent bars.

Fig. 10 (right). Bar diagrams representing mean \pm SD values of the brain volume for the different groups: N (nourished rats, n=14), M (undernourished rats, n=14), R (refed rats, n=31), considering for each of them, the age groups studied (≤ 30 days, > 30 days). Asterisks placed above error bars indicate the presence of significant differences between adjacent bars.

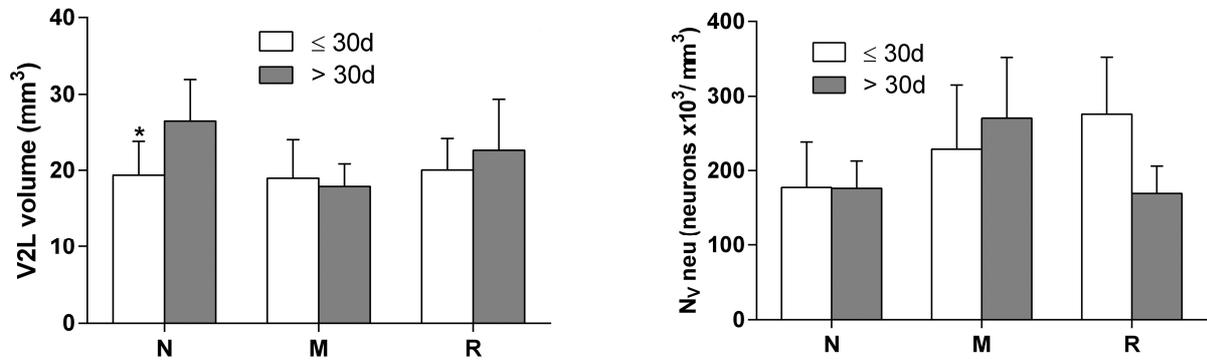


Fig. 11 (left). Bar diagrams representing mean \pm SD values of the V2L volume for the different groups: N (nourished rats, n=14), M (undernourished rats, n=14), R (refed rats, n=31), considering for each of them, the age groups studied (≤ 30 days, > 30 days). Asterisks placed above error bars indicate the presence of significant differences between adjacent bars.

Fig. 12 (right). Bar diagrams representing mean \pm SD values of the numerical density of neurons (NV neu) in V2L for the different groups: N (nourished rats, n=14), M (undernourished rats, n=14), R (refed rats, n=31), considering for each of them, the age groups studied (≤ 30 days, > 30 days).

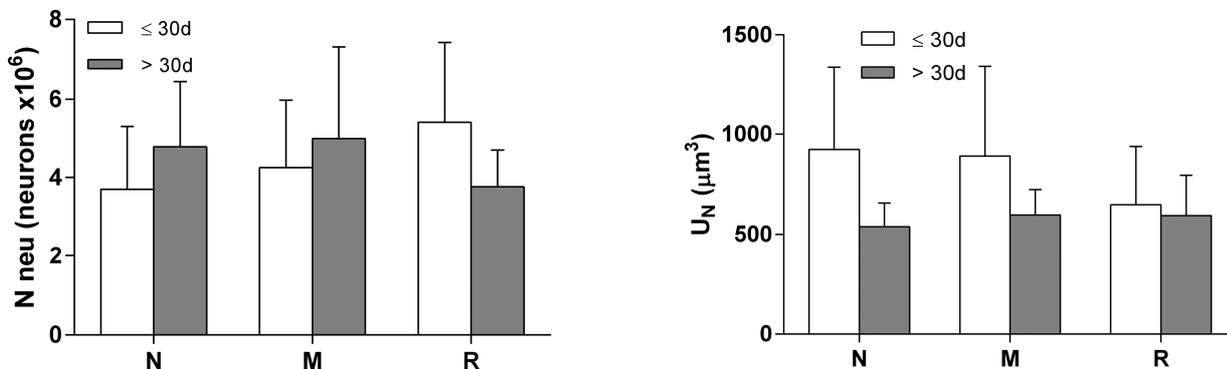


Fig. 13 (left). Bar diagrams representing mean \pm SD values of the absolute number of neurons (N neu) in V2L for the different groups: N (nourished rats, n=14), M (undernourished rats, n=14), R (re-fed rats, n=31), considering for each of them, the age groups studied (≤ 30 days, > 30 days).

Fig. 14 (right). Bar diagrams representing mean \pm SD values of the average neuronal volume (UN) in V2L for the different groups: N (nourished rats, n=14), M (undernourished rats, n=14), R (re-fed rats, n=31), considering for each of them, the age groups studied (≤ 30 days, > 30 days).

authors (Cragg, 1972; Smart et al., 1973; Noback et al., 1981; Bhide et al., 1982; Davies et al., 1983; Valadares et al., 2010).

V2L volume was also reduced in group M, where greater neuronal density was detected compared to group N. This was not followed by a rise in the absolute number of neurons in V2L. The rise of numerical neuronal density in M was probably due to decreasing of V2L volume.

This agrees with the findings of other authors (Dobbing et al., 1971; Cragg, 1972; West et al., 1976; Morgane et al., 1978; Bhide et al., 1984) that determined the cerebral cortex displays a reduction in volume and extent after the neonatal malnutrition.

The decreasing of brain and V2L volumes, in both M group and, in a minor degree, in the group of re-fed rats (R) may be interpreted as due to the decreasing of neuropil, caused by the reduction the axonal tree and / or dendrites and spines in the ages studied, being able to compromise connections of V2L cortex. If the thickness of the cortex is affected, it is likely that the condition of hypoproteinemia and other consequences of malnutrition also affect to the myelination processes (Thomson et al., 2003), with functional consequences such as those identified by Salas et al. (1974), in the sense that the reduced complexity of neocortical structures may be partially responsible for the reduced learning ability and reducing adaptive behavior patterns, generally seen in adult mammals food deprived during early life; and more recently by Stepanyants et al. (2002) with regard to changes in neuronal circuitry or plasticity.

Neither age nor sex influenced the neuronal numerical density. The average neuronal volume is lower in the older group ($P > 30$), which might suggest a decreasing of the size of neurons with the development, consistent with the fact pointed out by Shankle et al. (1999). In this regard, Lister et al. (2005) note that, despite the paradigm of the loss of neurons that is to produce the prenatal protein malnutrition, they did not observe it, and yet they appreciated reduction in the size of the soma of granular cells of the dentate gyrus. The sex of the animals does not seem to influence in this regard.

It was seen that the effect of re-feeding (R group) had a clear tendency towards normalization from both WR and brain volume, while the V2L volume recovered less than the cerebral total volume. These parameters in the R group showed an intermediate position between N and M. Although the dimensional parameters (WR, brain volume, V2L volume) show a tendency to be similar to the normal rats (group N), differences are still relevant, and this suggest that the recovery in the span of time considered was not complete, perhaps would be necessary to do ex-

periments to longer times to confirm such tendency.

It seems that the time of onset of refeeding does not influence the change of some parameters such as V2L volume and the absolute number of neurons. For example, a significant difference was not obtained in these parameters between the re-fed from P0 with respect to the re-fed from P21.

The N_V neu is greater in the rats re-fed to P21. Therefore, the atrophy of the V2L cortex was not improved when the start of the refeeding happened later. Several authors (Bass et al., 1970; Bedi et al., 1980; Bhide et al., 1982; Angulo-Colmenares, 1987; Bedi et al., 1988; Gundappa et al., 1988; Diaz-Cintra et al., 1990) note that with refeeding recovery of cortical width occurs, as well as other parameters altered by early undernutrition.

Several authors (Brown et al., 1968; Warren et al., 1985; Desai et al., 1996; Soto-Moyano et al., 1999) also note that the return to the optimal diet after P21 (weaning) did not correct the body deficit; instead, it is corrected if started at P0. The brain weight was the least affected. These results agree with those of the present study.

In our results the average neuronal volume does not vary with the onset of re-feeding. The rest of the parameters studied showed no significant differences between re-fed and undernourished, although a significant difference was seen in terms of neuronal density between the re-fed from P0-P20, and P21, suggesting that the effect of re-feeding is most effective while start earlier, as mentioned already by Desai et al. (1996).

To summarize, the next conclusions can be drawn. The protein malnutrition model applied:

i) Induces a significant rise in the ratio between the brain and body weights tending to correction after re-feeding. ii) Decreases the volume of the cerebral cortex, and V2L volume; refeeding partially reverses the change in the cerebral cortex, but not significantly in V2L. iii) Increases neuronal density in V2L. Refeeding produced no reversion in the ages studied. However, a significantly lower value in this parameter was observed in rats re-fed from P0 with respect to the re-fed from P21. iv) Neither the absolute neuron number nor average neuronal volume was modified in V2L, although it could be seen a significantly lower value of this last parameter in the $> P30$, regarding $\leq P30$. v) Neither interaction nor differences were observed in the studied parameters with respect to sex of the rats.

ACKNOWLEDGEMENTS

This study was partially funded by Consejo de Desarrollo Científico y Humanístico de la Universidad de Carabobo, Venezuela.

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