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Effects of Preweaning Polysensorial Enrichment upon Development of the Parietal Cortical Plate of Undernourished Rats: A Stereological Study

Efectos del Enriquecimiento Ambiental Predestete sobre el Desarrollo de la Placa Cortical Parietal de Ratas Desnutridas: Estudio Estereológico

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SUMMARY: This investigation was undertaken in order to quantify the effects of early polysensorial enrichment on the development of cortical pyramids, located in the parietal cortex of rats simultaneously submitted to protein-energy undernutrition. A short period of stimulation during suckling significantly decreases the cellular density in the cortical plate (phylogenetic-ontogenetic evolutionary index). Results suggest that the cerebral cortex develops according to a sophisticated neuronal network, which exhibits a notable degree of structural specificity, regulated by genetic and environmental clues. The most obvious prediction is that environmental influences are probably transduced as a structural expression in the developing parietal cortical plate.

KEY WORDS: Environmental enrichment; Cortical gradients; Undernutrition; Parietal cortex; Stereology.

INTRODUCTION

A number of studies, particularly in rodents, provide evidence that early postnatal enrichment determines a significant increase on cortical dendritic expansions during the preweaning period (Fernandez *et al*., 1997, 1998, 2003; Inzunza *et al*., 2003; Venable *et al*., 1989). Although there exists already a remarkable body of literature on the influence of enriched environments on brain structure, most of these studies have been focused on postweaning influences (Angenstein *et al*., 1998; Faherty *et al*., 2003; Greenough *et al*., 1985; Irwin *et al*., 2005; Nithianantharajah *et al*., 2004; Restivo *et al*., 2005; Weiler *et al*., 1997), that is, when the rat's brain is essentially mature. In contrast, only a few works have employed preweaning stimulation in spite of previous evidence showing that the greatest environmental effects occur when enrichment is induced soon after birth (Fernandez *et al*., 1998; Inzunza *et al*.; Venable *et al*., 1988). The mechanisms through which early environmental enrichment produces improvement in neuronal cytodifferentiation and plasticity are not at all clear, but a reasonable assumption would be that such morphological changes are at least partially due to stimulation of endogenous growth factors (Das *et al*., 2001; McAllister, 1997; Tropea *et al*., 2001; Wirth *et al*., 2003).

The purpose of the present investigation was to analyze the effects and possible interactions between undernutrition and environmental enrichment during the development of parietal cortical pyramids, in a period ranging from the 2nd to the 21st postnatal day and evaluated later at the 30th and 120th postnatal days. Neuronal density was chosen as a reliable index for assessing neocortical cytodifferentiation. As it has been pointed out in the literature (Altman, 1967; Wolff, 1978), preliminary lamination beginning in the rat during prenatal life is subsequently modified during an early postnatal developmental stage characterized by a progressive decrease of the packing density of cortical neurons, as a consequence of growth in both the dendritic processes of neurons and the number and caliber of axons reaching the cortex. Neuron packing density decreases rapidly from birth until the 16th postnatal day, then there is a decelerated decrease until weaning, and in 45-day-old rats, neuron packing density is similar to that

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found in adults (Brizzee *et al*., 1964). Thus, changes in density scores are thought to reflect deficits in dendritic growth during the early postnatal life, such as reduced amount of terminal dendritic branching, reduction of the surface occupied by their appendages, decrease in the number and length of segments as well as delayed development of their dendritic fields. The anatomical substratum observed in this study was the parietal cortical plate, an area of the neocortex that shows in the rat a significant developmental acceleration of neuronal differentiation after enriched rearing (Fernández *et al*., 1998, 2003; Inzunza *et al*.). The effects of early environmental enrichment on the structure of visual and motor cortices of eutrophic rats have already been addressed in other studies (Beaulieu & Colonnier, 1989; Venable *et al*., 1989). In particular, the effect of specific preweaning experience-induced structural changes in the parietal cortex of normal eutrophic rats has previously been documented elsewhere (Fernández *et al*., 2003). In the light of the above data, the aim of the present study was to elucidate whether the arrangement of parietal neuronal density, present during the early postnatal periods, may be modified under the influence of environmental enrichment. Further, our finding could give experimental support to the idea that the brain of the rats reared in complex habitat persistence after a period without environmental stimulation.

MATERIAL AND METHOD

In the present investigation, 64 male pups Sprague-Dawley albino rats were used. On postnatal day 1, male pups arising from 16 different litters were cross-fostered and culled to 16 pups per mother, thus forming four groups of animals that were randomly assigned to environmental enrichment (two groups, n=32) or to a social non-enriched condition (two groups, n=32). All pups from these litters were exposed to suffer protein-energy undernutrition due to the competition for mother's milk (Wurtman & Miller, 1976). At the postnatal day 21st, all experimental groups were reduced from 16 to 8 subjects, with food and water ad libitum, to be later sacrificed for histological studies at the 30th and 120th postnatal days. All the litters were kept under controlled light-dark conditions, at constant temperature (21±1.2ºC) and relative humidity (60±5%). The experimental protocol and animal management were in accordance with the NIH Guide for the care and use of laboratory animals (Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council, 2006).

From day 2nd to 21st, pups in the enriched environment were exposed, without the mother, to three 20-min sessions per day of exploration in a 70x70x40 cm wire mesh cage provided with a large variety of objects for play and exploration, which were changed or rearranged daily (Fernández *et al*., 1997). These included running wheels, tunnels, ladders, sand boxes, balls, rattle, leafy plants, long grass, tree branches, sawdust, furry and rough surfaces, toys of different sizes and textures (wood, metals or plastic) and two elevated platforms along opposite sides of the cage, 16 cm above the level of the floor. Recorded classic music was also provided. Each enriched pup underwent gentle handling for 2 min before each testing/training session, while 24 small lights flashed intermittently. Afterwards, the pups were placed in a water bath at 37ºC for at least 10 seconds, time in which they swam. The non-enriched rats were left undisturbed except for removal of the mother from the litter during the stimulation periods.

To evaluate structural changes in the parietal cortex, half of pups of each group were sacrificed at the 30th and the other half at the 120th postnatal day. For this purpose the animals were deeply anesthetized with sodium pentobarbital (6 mg/100 g body weight) and perfused through the left ventricle with a 0.9% saline solution followed by a 4% formaldehyde/saline solution. The brains were removed immediately from the cranium, post-fixed in the above fixative and cryoprotected for 48 h in a 30% sucrose solution. Frozen serial coronal sections (45 mm thick) were obtained using a sliding microtome. The sections were stained with cresyl violet, dehydrated and mounted with Entellan (Merck).

In order to get data as comparable, sections obtained at different postnatal development periods were processed under identical conditions. Eight sections from each rat were selected. These sections correspond to a region situated between the anterior commissure and anterior aspect of the hippocampus. This region is located from A=6.5 to A=3.2 for 30-day-old rats (Sherwood & Timiras, 1970), and from 0.30 to -2.12 in subjects 120-days-old according to the Stereotaxic Atlas of Paxinos, as related to the Bregma.

Neuronal density quantification was based in previously described optical disector methods (Sterio, 1984; West & Gundersen, 1990). We sample one out of three sections, counting the neuronal nuclei with an ocular micrometer grid under 100X oil immersion objective. Criteria for recognition of neurons were based on previously research (Ling *et al*., 1973; Braendgaard *et al*., 1990). Optical disector of a known area (0.1x0.1 mm^2) was applied focusing down the microscope through 8mm in depth, as read from the fine focusing knob. We discarded all the nuclei that intersected the left and the bottom sides of the frame, as well as the first nuclei that came into the focus in the upper surface of the brain's sections. For each section, four unbiased counting frames were sampled in a systematically

random fashion (in both medial and lateral parietal cortical sectors). Counting fields were oriented perpendicularly to the pial surface and involved cortical layers II-III and V. The numerical density of neurons (Nv) was estimated by using the equation: Nv= Q- /h x a(fra), where Q- is the total average of neurons counted, h is the height of the optical dissector (8 mm) and a(fra) corresponds to the disector area (10000 μm²).

Initially, the data obtained from the different cortical layers was described uni-dimensionally by using appropriate statistical, average neuronal density and standard deviation. In a second phase, the multivariate analysis of variance (MANOVA) was applied to study the effects of the factors: treatment, time, and section, based on findings obtained in four consecutive observations (layers x area). The described analysis relied on the SAS statistical program (version 6.06, SAS Institute, Inc., Cary, NC).

RESULTS

An interaction statistically significant was detected between time (30 versus 120 postnatal days) and stimulation; therefore, partial analysis for 30 and 120 days were separately conducted. Figures 1 and 2, show the effects of early polysensorial stimulation upon neuronal density, measured on postnatal days 30 and 120, in the medial and lateral parietal cortices of undernourished rats. Figure 1A shows that on postnatal day 30 early stimulation (U.E 30 versus U.NE 30) induces a significant reduction of neuronal density in the supragranular layers of the medial parietal cortex ($p<0.0001$). Ninety days after discontinuing environmental enrichment, a significant decrease in neuronal density still remained (U.E 120 versus U.NE 120) in the supragranular layers of the parietal cortex ($p<0.001$), indicating that the effects of early enrichment

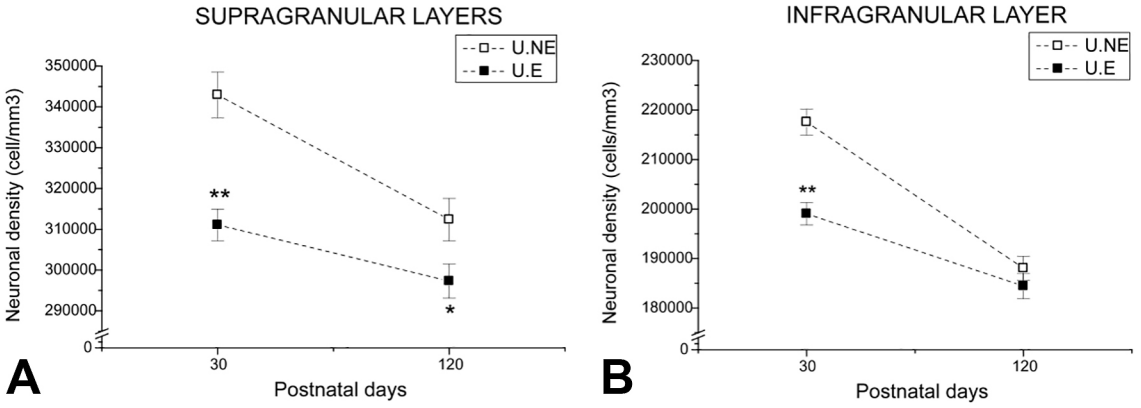

Fig. 1. Average neuronal density (neurons/mm3 ± SEM) found in the medial parietal cortex on postnatal day 30 and 120, in pups grown under social (open square) or enriched condition (solid square). U.NE: Undernourished/non-enriched condition; U.E: Undernourished-enriched condition. A. Supragranular II–III layers; B. Infragranular V layer. Significant effects of the environment are indicated as follow: *p<0.001, **p<0.0001.

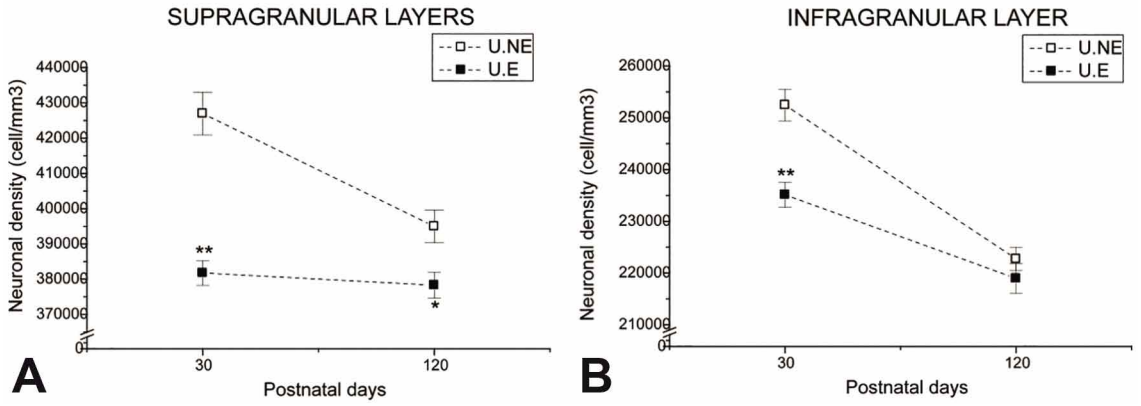

Fig. 2. Average neuronal density (neurons/mm3 ± SEM) found in the lateral parietal cortex on postnatal days 30 and 120, in pups grown under social condition (open square) o enriched condition (solid square) U.NE:Undernourished/ non-enriched condition; U.E: Undernourished-enriched condition. A. Supragranular layers II–III; B. Ingrafranular layer V. Significant effects of the environment are indicated as follow: *p<0.001, **p<0.0001.

involved a persistent morphological expression. In the infragranular layer V (Fig. 1B), the changes induced by early polysensorial stimulation were statistically significant only on the postnatal day 30 ($p<0.0001$).

The lateral parietal cortex (Fig. 2A) of subjects under the influence of an enriched environment (U.E 30 versus U.NE 30) presented a significant reduction of neuronal density in the supragranular layers II–III on the postnatal day 30 ($p<0.0001$). This difference was still significant by postnatal day 120 ($p<0.001$). In contrast (Fig. 2B), the changes induced by early polysensorial stimulation in the infragranular layer V were statistically significant only on the postnatal day 30 ($p<0.0001$).

DISCUSSION

The foregoing results indicate that exposure to environmental enrichment markedly ameliorates the morphological development of parietal cortical pyramids in rats simultaneously submitted to an undernutrition regimen, thus suggesting a possible beneficial effect of early polysensorial enrichment on morphological parameters that are thought to be affected by adverse early postnatal nutritional paradigms. In fact, previous studies have shown that animals submitted to undernutrition during the critical period of brain growth experienced a reduction of neuronal cytodifferentiation (Leuba & Rabinowicz, 1979; Cordero *et al.*, 1993; Noback & Eisenman, 1981), while early polysensorial stimulation generated opposite effects to that induced by undernutrition: growth of the basal dendritic tree and increases in neuronal geometric complexity (Fernández *et al*., 1997, 2003), thereby reducing the neuronal density (Beaulieu & Colonnier, 1989). In particular, environmental enrichment accelerated the neuronal cytodifferentiation in sectors of high sensibility such as the lateral parietal cortex (Fernández *et al*., 2003; Inzunza *et al*.), a feature that may be related to the early arrival of afferent signals coming from the enriched environment to the lateral sector that contains the representation of the face and vibrissae. This notion could be supported by previous studies linking the process of cytodifferentiation and maturation with the arrival of specific inputs to the cerebral cortex during the early postnatal period (Fernández *et al*., 2003). Such effect on neuronal development seems, however, to be relatively independent from corporal dimensions, as can be established by comparing data obtained in both parameters (independently) in Table I. Thus, pups that were reared in enriched environmental conditions displayed decreased corporal averages with increased geometrical arrangements and noteworthy territorial expansions of dendrites (Fernández *et al*., 1997, 2003).

The present results also indicate that the decreases in neuronal density induced by a short time period of environmental enrichment (90 min daily for 20 days) during the preweaning period are still present at adult age, at least in the supragranular layers of the medial parietal cortex. This is indicative of, on the one hand, that the effects of early enrichment involved a persistent morphological expression and, on the other hand, that enrichment early in life affects differentially supra- and infragranular layers of the neocortex. Interestingly, postnatal undernutrition seems also to affect differentially pyramidal cells of deep and upper layers in the cerebral cortex of rodents, the effect being more pronounced (higher neuronal density, lower dendritic branching and spine development) in layers II and III than in layers V and VI (Leuba & Rabinowicz). The greater sensitivity of upper cortical layers (II/III) to nutritional and environmental postnatal influences might be explained because these layers appear later in the ontogeny and are phylogenetically more recent. Therefore, as layers II/III are less differentiated at birth and have a considerable postnatal differentiation, both postnatal protein-restriction and enrichment might have a stronger developmental effect.

Table I. Distribution of experimental groups submitted to environmental and nutritional influences during the early postnatal period.

Group	Corporal development		Neuronal density (neurons x 10^4 / mm^3 mean)			
			Medial		Lateral	
	Weight (g)	Size (cm)	II – III	V	II – III	V
U.NE	100.1±4.21	16.2±0.18	42.6±0.60	25.2±0.29	34.2±0.55	21.7±0.25
U.E 30	71.8±2.59	15.7±0.42	38.1±0.34	23.5±0.24	31.1±0.38	19.9±0.22
U.NE	372.7±13.04	26.1±0.40	39.5±0.45	22.2±0.22	31.2±0.51	18.8±0.23
U.E 120	324.9±10.09	25.4±0.31	37.8±0.35	21.8±0.28	29.7±0.41	18.4±0.25

Values are means ± SEM. n=7 in all groups. U.NE 30: undernourished/non enriched (from the 2nd to the 21st postnatal day), sacrificed at the 30th postnatal day. U.E 30: undernourished/enriched (from the 2nd to the 21st postnatal day), sacrificed at the 30th postnatal day. U.NE 120: undernourished/non enriched (from the 2nd to the 21st postnatal day), sacrificed at the 120th postnatal day. U.E 120: undernourished/enriched (from the 2nd to the 21st postnatal day), sacrificed at the 120th postnatal day.

How polysensorial experience during the brain growth spurt can influence long-term neuronal organization in the cerebral cortex is not clear at present, but a reasonable assumption would be that such morphological changes are at least partially due to stimulation of endogenous growth factors. As it is largely known, BDNF plays a key role in activity-dependent gene induction in the brain and activation of TrkB receptors by BDNF have long-term effects on transcription of a variety of genes related to brain plasticity (Cheng *et al*., 2003; Das *et al*.; Sale *et al*., 2004; Tropea *et al*.). In this respect it is known that environmental enrichment increases the expression of BDNF (Rocamora *et al*., 1996; Sale *et al*.) and NGF (Pham *et al*., 1999) in the cerebral cortex, while the physical activity present in the exploratory behavior of rats, in our enriched experimental paradigm, could induce a significant effect on the expression of BDNF-mRNA in the somatosensory and visual cortices (Rocamora *et al*.). The distribution of neurotrophins in the cerebral cortex conditions complex responses to the environmental contingencies. For instance, the neurotropic factors BDNF and NT-3 have highly specific effects and topographical dependence. For example, in layer IV NT-3 inhibits the dentritic stimulation caused by BDNF, while in the layer VI, BDNF inhibits the dendritric growth stimulated by NT-3 (McAllister *et al*.). These topographical findings are related with the differences shown in this study between the supragranular and infragranular layers. It is then evident that during the early postnatal period, the cerebral cortex develops a highly sophisticated neural network that exhibits a notable degree of structural specificity, regulated by molecular and environmental clues.

In conclusion, the present study showed that a relatively short period of stimulation significantly decreases the cellular density in the cortical plate (phylogenetic-ontogenetic evolutionary index), structural modification that is still present in adult life (Altman). The most obvious prediction is that environmental influences are probably transduced as a structural expression in the developing parietal cortical plate.

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RESUMEN: La presente investigación se realizó con la finalidad de cuantificar los efectos del enriquecimiento polisensorial temprano sobre el desarrollo de células piramidales de la corteza parietal, en ratas que simultáneamente fueron sometidas a desnutrición calórico-proteica. Un breve periodo de estimulación durante el periodo de lactancia disminuyó significativamente la densidad celular en la placa cortical (índice evolutivo filogenético-ontogenético). Los resultados sugieren que la corteza cerebral se desarrolla de acuerdo con una red neuronal altamente sofisticada, la cual exhibe un notable grado de especificidad estructural regulada por claves genéticas y ambientales. La predicción más obvia es que las influencias ambientales son probablemente traducidas como expresión estructural en la placa cortical parietal.

PALABRAS CLAVE: Enriquecimiento ambiental; Gradientes corticales; Desnutrición; Corteza parietal; Estereología.

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