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Effects of undernourishment, recurrent seizures and enriched environment during early life in hippocampal morphology

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ABSTRACT

It has been recently shown that enriched environment led to a significant benefit in learning and retention of visual–spatial memory, being able to reverse the cognitive impairment generated by undernourishment and recurrent seizures. We investigated the hippocampal morphological effects of recurrent seizures and undernourishment early in life in Wistar rats and the possible benefits produced by the enriched environment in these conditions. The morphological parameters stereologically evaluated were hippocampal volume, thickness of pyramidal stratum of the CA1 subfield and neuronal and glial densities in the same subfield. Male Wistar rats were divided into eight groups including nourished, nourished + enriched environment, nourished + recurrent seizures, nourished + recurrent seizures + enriched environment, undernourished, undernourished + enriched environment, undernourished + recurrent seizures and undernourished + recurrent seizures + enriched environment. Undernourishment model consisted in nutritional deprivation regimen from post-natal day 2 (P2) to P15. From P8 to P10, recurrent seizures group were induced by flurothyl three times per day. Enriched environment groups were exposed between P21 and P51. Our main findings were: (1) animals submitted to the enriched environment showed an increased hippocampal volume; (2) enriched environment promotes increases in the thickness of the pyramidal layer in hippocampal CA1 subfield in animals nourished and undernourished with recurrent seizures; (3) undernourishment during early development decreased neuronal density in CA1 and CA3 subfields. Our findings show that these three conditions induces important changes in hippocampal morphology, the most deleterious changes are induced by undernourishment and recurrent seizures, while more beneficial morphological changes are produced by enriched environment.

1. Introduction

An inadequate diet during early life has the potential to influence adversely in brain development in different regions, particularly in the neocortex and hippocampal formation (Hoffmann et al., 2004; Huang et al., 2003; Levitsky and Strupp, 1995). Undernourishment has been recognized to cause reductions in the numbers of neurons, synapses, dendritic arborization, and myelination, all of which result in decreased brain size (Andrade et al., 1996; Bedi, 1991; Hemb et al., 2010; Levitsky and Strupp, 1995; Morgane et al., 2002; Tonkiss et al., 1993). All these central nervous system (CNS) alterations are associated with delays in motor and cognitive functions (Fukuda et al., 2002; Gramsbergen and Westerga, 1992; Levitsky and Strupp, 1995).

Epilepsy is a common neurological disorder that occurs more frequently in children than in adults (Cowan, 2002). In addition, seizures during early development may be more detrimental than when occurring during adulthood (Bergman et al., 1983). The extent that prolonged seizure activity, i.e. status epilepticus (SE) and repeated seizures affect neuronal structure and function in the immature brain has been the subject of increasing clinical and experimental research.

Animal studies have demonstrated that the pathophysiological consequences of seizures in the developing brain can be associated with later cognitive and behavioral disturbances, which may be associated with hippocampal dysfunction (Lynch et al., 2000; Majak and Pitkanen, 2004; Sayin et al., 2004; Stafstrom, 2002). Recurrent or prolonged seizures during the neonatal period have been shown to reduce brain cell number (Wasterlain, 1976), synaptic reorganization with aberrant growth (Holmes et al., 1999;

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Liu et al., 1999), suppression of dendrite growth (Nishimura et al., 2008) and glutamatergic synapses (Swann et al., 2007).

The enriched environment is best conceptualized as a combination of social interaction, physical exercises and continued exposure to learning opportunities, which may alter brain structure and functions in rodents (Faverjon et al., 2002; Krech et al., 1960; Pinaud et al., 2001; Rampon et al., 2000; Rosenzweig and Bennett, 1972). Experimental studies suggested that enriched environment stimulates neuronal plasticity, facilitate learning and reduces cognitive impairment in animals with preliminary brain injury (Van Praag et al., 2000). We previously demonstrated that enriched environment was an effective treatment in the recovery of spatial memory in rats exposed to early undernourishment and seizures, being able to reverse the cognitive deficits generated by these disorders (Simão et al., 2012). Considering that environmental stimulation probably results in structural changes in neural cells to promote better cognitive outcome, it is reasonable to suggest that it could influence the hippocampal morphology.

The association of undernourishment and seizures, that is a common clinical situation, might augment the deleterious effects of seizures in the developing brain (Simão et al., 2012). Little attention has been given to the influence of environmental enrichment in immature rats with recurrent seizures and undernourishment in brain structures. Thus, the aim of this study was to investigated the hippocampal morphological effects of recurrent seizures and undernourishment early in life in Wistar rats and the possible benefits produced by the enriched environment in these conditions, evaluating the following parameters: hippocampal volume; thickness of pyramidal stratum of the CA1 subfield and neuronal and glial densities in the same subfield.

2. Methods

2.1. Experimental procedures

The experiments were conducted under conditions approved by the Scientific and Research Ethics Committees of the Pontifícia Universidade Católica do Rio Grande do Sul. All experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals adopted by the National Institute of Health (USA). All efforts were made to minimize animal suffering and reduce the number of animal needed. Pregnant female Wistar rats from our breeding colony were maintained on a 12-h dark–light cycle with food and water ad libitum. After delivery, each dam with the litter was housed individually. Each litter was culled to 10 pups. The day of birth was counted as P0. All animals were weaned at P21. The entire litters were randomly divided in 8 groups, conditions using only male pups in the following groups: N (nourished); N + EE (nourished + enriched environment); N + RS (nourished + recurrent seizures), N + EE + RS (nourished + enriched environment + recurrent seizures); U (undernourished); U + EE (undernourished + enriched environment); U + RS (undernourished + recurrent seizures); U + RS + EE (undernourished + recurrent seizures + enriched environment).

2.2. Undernourishment paradigm

The undernourishment paradigm consisted of limiting the offspring's access to nutrition by removing the dams from the cage starting at P2. The deprivation period was increased by 2 h for 6 consecutive days, from 2 h on P2 to 12 h on P7. The deprivation period remained at 12 h/day for the next 8 days (P8–P15). During deprivation, pups remained in a light heated cage, with room temperature maintained at 34 °C (measured with a thermometer placed in the room). After the deprivation period, the pups were housed with their respective dams. Age-matched control rats remained with their dams (Crnic, 1980; Florian and Nunes, 2011; Hemb et al., 2010; Ladd et al., 1996; Plotsky and Meaney, 1993).

2.3. Flurothyl-induced seizures

To provoke early recurrent seizures, we used flurothyl [bis(2,2,2-trifluoroethyl)ether] (99%), a volatile convulsive agent that rapidly stimulates the CNS, inducing generalized seizures (Nunes et al., 2000). Pups were exposed to liquid flurothyl (20 μL/min constant flow rate) delivered through a plastic syringe and dripped slowly onto filter paper in the center of the air-tight chamber (9.38 L), where the agent evaporates to provoke recurrent seizures. In rat pups recurrent flurothyl seizures in rats below the age of 10 days result in agitation, swimming movements, and tonic activity with clonic activity emerging at P8–P10. Experimental rats were exposed to flurothyl until tonic extension of both the forelimbs and hindlimbs were observed. Animals were submitted to recurrent seizures-3 exposures of flurothyl per day (1 h inter-exposure interval) from P8 to P10, until the appearance of the first tonic-clonic seizure. For all animals, the exposure to flurothyl took place immediately after separation from their dams. Between trials, the chamber was flushed with room air and cleaned.

2.4. Environmental enrichment

To test whether a period in an enriched environment has a beneficial effect following undernourishment and recurrent seizures, animals were exposed to this situation between P21 and P51. The enriched environment consisted of a large plastic cage measuring 100 cm (length) × 50 cm (width) × 40 cm (height) with various toys, wooden blocks, climbing platforms, plastic tubes, small shelters, and a running wheel. These objects were rearranged every week to facilitate exploratory behavior (Simão et al., 2012). Enriched rats were housed in groups of 7–10, which permitted extensive social interactions between cagemates. The nonenriched group remained in plastic cages but were handled the same amount of time as the enriched group.

2.5. Brain histology

On postnatal day 51, all rats were deeply anesthetized with ketamine (90 mg/kg i.p.) and xylazine (15 mg/kg i.p.). Using a peristaltic pump, the animals were transcardially perfused through the left cardiac ventricle with 200 mL of saline solution followed by 200 mL of fixative solution of 4% paraformaldehyde (Reagen, Brazil) diluted in 0.1 M phosphate buffer (PB), pH 7.4. Brains were removed from the skull, post-fixed for 24 h in the same fixative solution and cryoprotected in 30% sucrose solution in PB at 4 °C until they sank to the bottom and then frozen in isopentane cooled by means of liquid nitrogen. After these procedures, the brains were kept in a freezer (−70 °C) for further analyses. Coronal brain sections (20 μm) through the entire extent of the hippocampus were obtained using a cryostat (Leica, Germany) and one in every 10 sections was collected for analysis. Brain sections were mounted on gelatin-coated slides and were stained with cresyl violet method. Briefly, sections were air-dried, rehydrated, stained with 0.02% cresyl violet (Sigma) in acetate buffer, and then dehydrated through a series of alcohols, cleared in xylene, and, coverslipped.

2.6. Hippocampal volume estimation

The unbiased estimation of hippocampal volume was done using the Cavalieri principle associate with the counting point method (de Paula et al., 2009). The hippocampal coronal sections with a grid of equidistant points were observed using a stereo

microscope Zeiss, model Stemi V.6. The points located inside the hippocampus were counted and the hippocampal volume was estimated using the following equation: $V = T{\cdot}a/p{\cdot}\Sigma P$, were V, volume; T, distance between sections; a/p, area point (1 mm^2) and ΣP, sum of points. To estimate the hippocampal volume we considered sections located between the following coordinates of Paxinos and Watson (1998) (Bregma: −1.60 mm; Interaural: 7.40 mm) until (Bregma: −6.80 Interaural: 2.20 mm).

2.7. Neuronal and glial densities estimation

The neuronal and glial cells densities (number of neurons or glial cells/mm^3) were estimated in the pyramidal layer of CA1 and CA3 subfields using the histological sections obtained in the "anterior-dorsal" portion of the hippocampus located between the following coordinates (Bregma −1.60 mm/Interaural 7.40 mm; Bregma −3.8 mm/Interaural 5.2 mm). The stereological estimation of neuronal and glial cell densities was made using a microscope Olympus model BX51 bright field with a CCD camera coupled (Qimaging, model MicroPublisher 3.3 RTV). The selected images were scanned at fixed aperture (40×) by Peltier CCD system (Image Pro Plus Capture Media Cybernetics USA) and analyzed using the Image Pro Plus software 6.0.

Firstly, the morphological distinction of neurons and glia cells was performed. The neurons were identified by their large, pale nuclei surrounded by dark cytoplasm containing Nissl bodies and the glial cells were identified by their relative size to neurons and lack of stained cytoplasm. The nucleolus of the neurons and the nucleus of glial cells were used as the counting marker for neurons and glia, respectively. The neuronal and glial cells densities estimation were performed using an adaptation of the optical disector method (Stereo, 1984; Abreu-Villaça et al., 2002; Costa-Ferro et al., 2010). Briefly, ten equidistant coronal sections of the "anterior–dorsal" hippocampus were analyzed in the rostral–caudal direction per animal, in each section two disectors were analyzed, one in the CA1 subfield and another in CA3 subfield. In summary, the volume of disector is obtained multiplying the area of an area of interest (AOI) by the disector height.

In our study the slice height was used as the dissector height (Costa-Ferro et al., 2010). In an area of interest (AOI), the neurons and glia cells were counted at different focal planes obtained during the course of focusing through the tissue slice (in this case, Z axis = 20 μm). Neurons and glial cells found overlaying the left and upper borders of the AOI were counted together with the neurons and glial cells located within the analyzed square. Neurons and glial cells overlaying the right and lower border of AOI were not counted. Neuronal and glial densities were estimated using the following formula: $D(\text{est}) = (1/a\_h)\_(\text{PQ}/\text{PP})$, where $D(\text{est})$ = estimated neuronal or glial densities; a = AOI area (1888 μm^2); h = slice height or dissector height (20 μm); PQ = sum of neurons or glial cells counted; PP = sum of analyzed dissectors

2.8. Thickness of the pyramidal layer of CA1 subfield estimation

Using the same sections in which cell densities were evaluated, the thickness of the pyramidal layer of CA1 subfield was measured using the Image Pro Plus software 6.0.

2.9. Statistical analysis

Analysis of variance (ANOVA) followed by Tukey's test were used to compare the different study groups and values were expressed as mean ± SD. Statistical significance was defined as $p < 0.05$ for all tests.

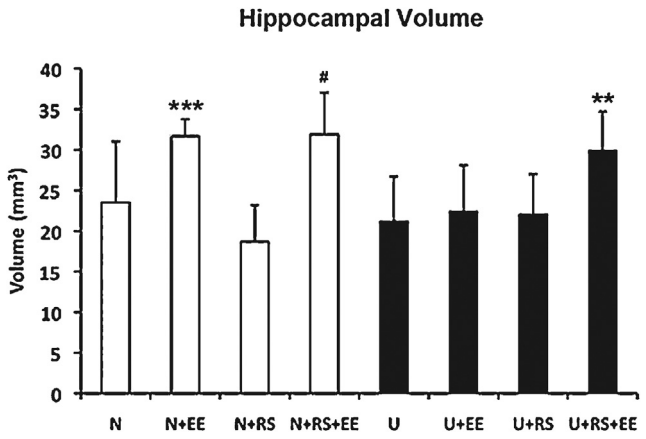

Fig. 1. Hippocampal volume estimation. Data are presented as the mean ± SD, $n = 6$ per group. N, nourished; N + EE, nourished + enriched environment; N + RS, nourished + recurrent seizures; N + EE + RS, nourished + enriched environment + recurrent seizures; U, undernourished; U + EE, undernourished + enriched environment; U + RS, undernourished + recurrent seizures; U + RS + EE, undernourished + recurrent seizures + enriched environment. $^{**}p < 0.01$ vs. U, $^{***}p < 0.001$ vs. N, $^{\#}p < 0.001$ vs. N + RS.

3. Results

3.1. Hippocampal volume

The Hippocampal volume was not influenced by undernourishment or seizures. However, expose to the enriched environment, resulted in an increase in the hippocampal volume of rats from N + EE ($p < 0.001$), N + RS + EE ($p < 0.001$) and U + RS + EE ($p < 0.01$) groups (Fig. 1).

3.2. Hippocampal CA1 subfield thickness

The thickness of the pyramidal layer in hippocampus CA1 subfield was not affected by undernourishment and recurrent seizures. However, as shown in Fig. 2, enriched environment increased the CA1 subfield thickness in the N + EE ($p < 0.001$) and in the U + RS + EE ($p < 0.01$) groups. Is possible to note the increased thickness after enriched stimulation in the CA1 subfield (Fig. 4).

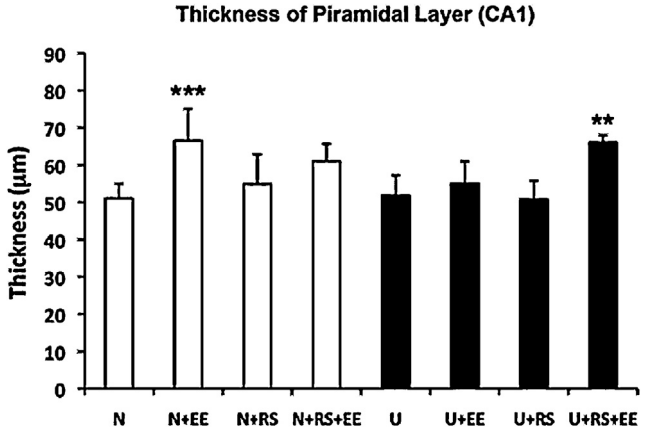

Fig. 2. Thickness of the pyramidal layer in the hippocampal CA1 subfield. Data are presented as the mean ± SD, n= 6 per group. N, nourished; N + EE, nourished + enriched environment; N + RS, nourished + recurrent seizures; N + EE + RS, nourished + enriched environment + recurrent seizures; U, undernourished; U + EE, undernourished + enriched environment; U + RS, undernourished + recurrent seizures; U + RS + EE, undernourished + recurrent seizures + enriched environment. $^{**}p < 0.01$ vs. U + RS, $^{***}p < 0.001$ vs. N.

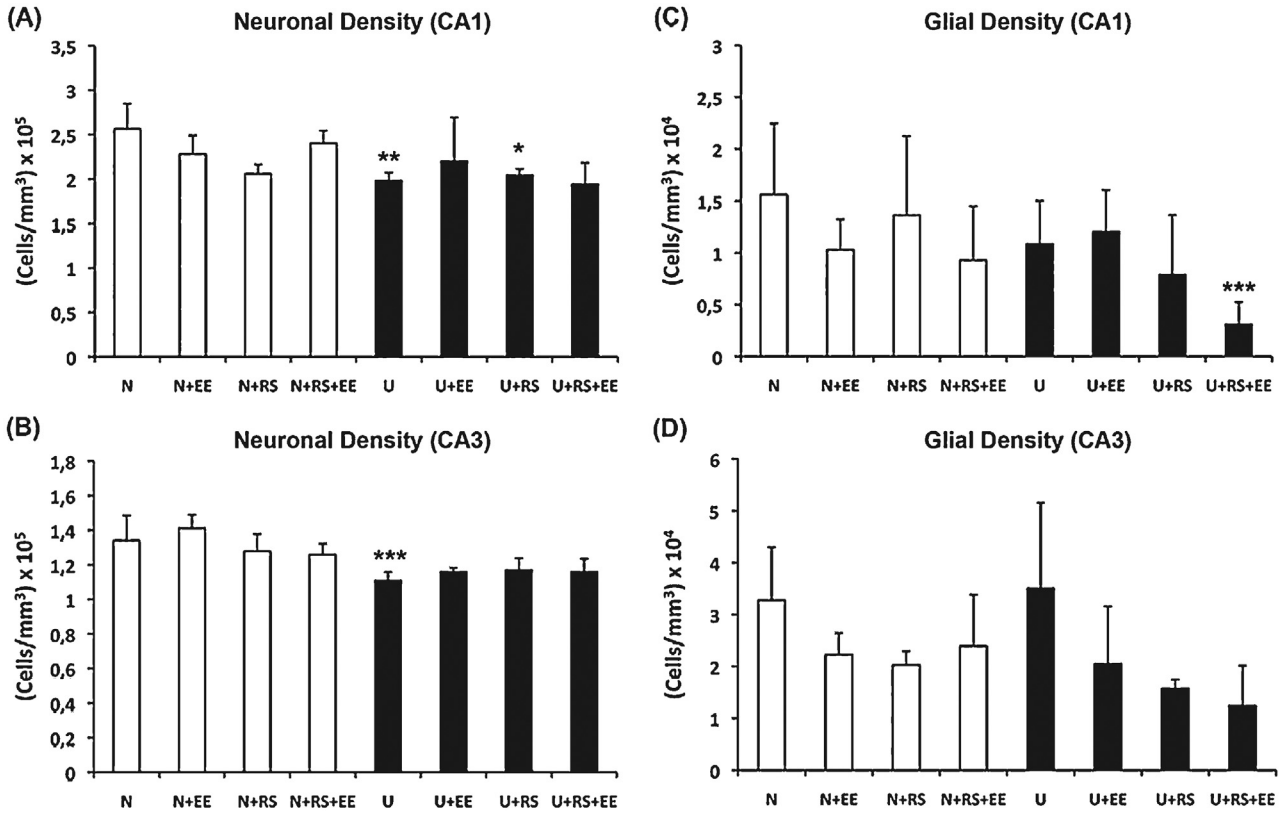

Fig. 3. Changes in neuronal and glial densities in CA1 and CA3 hippocampus subfields. Individual values of neuronal density measured in sublayers CA1 (A) and CA3 (B) are presented. Individual values of glial density measured in sublayers CA1 (C) and CA3 (D) are presented. Data are presented as the mean ± SD, $n = 6$ per group. N, nourished; N + EE, nourished + enriched environment; N + RS, nourished + recurrent seizures; N + EE + RS, nourished + enriched environment + recurrent seizures; U, undernourished; U + EE, undernourished + enriched environment; U + RS, undernourished + recurrent seizures; U + RS + EE, undernourished + recurrent seizures + enriched environment. (A) $^{*}p < 0.05$ vs. N, $^{**}p < 0.01$ vs. N; (B) $^{***}p < 0.001$ vs. N; (C) $^{***}p < 0.001$ vs. U + RS.

3.3. Neuronal density

We observed a significantly decreased neuronal density in the CA1 subfield of rats submitted to U ($p < 0.01$) when compared with N group (Fig. 3A). Animals submitted to U + RS ($p < 0.05$) showed a lower neuronal density when compared with N group. The average neuronal density in CA3 subfield of rats in the U group ($p < 0.001$) was lower than in the N group (Fig. 3B). Is possible to note the reduced hippocampal CA3 pyramidal cells in undernourished animals (Fig. 4).

3.4. Glial density

The glial density in the CA1 subfield of rats in the U + RS + EE group was significantly lower than in the U group (Fig. 3C).

4. Discussion

We report here that undernourishment during early development is associated with decreased neuronal density in the CA1 and CA3 subfield. Many investigators found biochemical alterations in the CNS in experimental malnutrition and seizures models in a developing brain in the hippocampal CA1 and CA3 subfield as well as the dentate gyrus (Ben-Ari and Holmes, 2006; Cintra et al., 1997; Florian and Nunes, 2011; Garcia-Ruiz et al., 1993; Liu et al., 1999; Lukoyanov and Andrade, 2000) related to malfunctions in neurotransmitter systems, such as the glutamatergic system (Marks et al., 1996; Millan et al., 1993), decreasing the number of synapses (Warren and Bedi, 1984) and dendritic spines (Leuba and Rabinowicz, 1979). All of these changes in the developing brain may promote a decrease in the neuronal density in CA1 and CA3 subfield in undernutrition and in recurrent seizures. The study of Andrade et al. (1995) have shown that all hippocampal population of neurons is affected by malnutrition. In a previous study of our group was demonstrated that seizures reduce the number of neurons in the CA3 region, however this reduction was not found in CA1 (Florian and Nunes, 2011).

Even after a period of nutritional rehabilitation, rats undernourished early in life may show structural changes in hippocampus relevant to adulthood and these changes may hamper the adaptation of animals to the environment in adult life. For example, Lukoyanov and Andrade (2000) demonstrated that the total number of the dentate granule cells and pyramidal neurons in the CA3 and CA1 hippocampal fields was significantly reduced in malnourished and rehabilitated animals. Furthermore, Andrade et al. (1995) showed that nutritional rehabilitation can neither reverses the deleterious effect of the morphological changes induced by undernourishment nor prevents neuronal degeneration. These data are in agreement with our results found in the neuronal density of undernourished animals and undernourished plus recurrent seizures during early periods of life. Even after nutritional rehabilitation, animals showed decreased neuronal density in CA1 and CA3 sublayers. Our findings may allow us to suggest that undernourishment and recurrent seizures during development has long-term adverse effects on some important cognitive functions (Hemb et al., 2010; Simão et al., 2012), allowing us to infer that these changes can be reflected directly by changes in neuronal and glial density in the hippocampus.

There is also no evidence of macroscopic brain atrophy in the brains of undernourished rats and it is conceivable that the

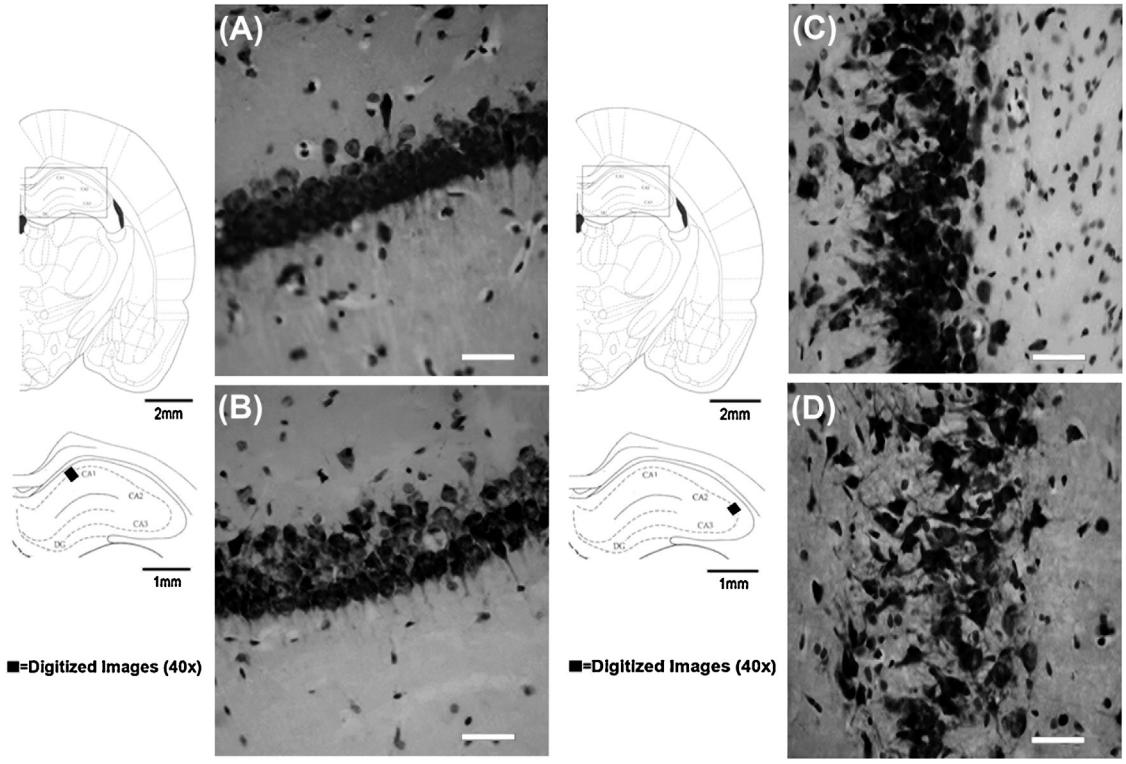

Fig. 4. Digitized images of the CA1 and CA3 subfields. It is possible to note the increased thickness after enriched stimulation in the CA1 subfield (B) when compared to nourished group (A). Animals submitted to undernourishment (D) showed reduced packing density in CA3 subfield when compared to nourished group (C). Scale bar = 50 μm. Adapted from Paxinos and Watson (1998).

hypothesis that decreased brain weight occurs due to neuronal loss, can be mitigated by regeneration mechanisms exhibited by surviving neurons (Coleman and Flood, 1986; Diaz-Cintra et al., 1994), as well as an increased number of glial cells and an enlargement of its processes mediated by neuronal degeneration (Yu et al., 1974). Our previous study showed no difference between nourished and undernourished brain weigh (Hemb et al., 2010). The reduction of neuronal density associated with comparatively normal volume of the hippocampus suggests that epilepsy and undernourishment results constitute an endorsement of the hypothesis that these factors affect brain development during neonatal period. In our study, CA1 and CA3 subfields in anterior-dorsal hippocampus were selected to perform the estimation of glial and neuronal densities because of their sensitivity to injury and cognitive deficits related to early undernourishment and recurrent seizures (Cintra et al., 1997; Garcia-Ruiz et al., 1993; Liu et al., 1999). The antero-dorsal hippocampus is deeply associated to learning and memory processes and it is more susceptible to morphological changes induced by enriched environment. Additionally, this region presents well defined anatomical limits of CA1 and CA3 regions (Viola et al., 2009; Yirmiya and Goshen, 2011; Rodrigues et al., 2013). Sustained impaired brain function could be the consequence of transient disturbances of critical developmental steps by several yet to be discovered factors including early undernourishment and epilepsy (Hemb et al., 2010). These abnormalities early in the hippocampus could represent the morphological basis of more durable and profound disturbances of neuronal circuitry and functioning, which would persist in adult animals.

We have also shown that the animals submitted to the enriched environment demonstrated an increased hippocampal volume. Likewise, nourished and undernourished plus recurrent seizures groups revealed an increased thickness of the pyramidal layer in hippocampus of CA1. Observed increase in dendritic spines (Globus et al., 1973; Ip et al., 2002), myelinated fibers (Qiu et al., 2002), enlargement of synaptic boutons and dentritic branching complexity (Greenough et al., 1973) and neuronal plasticity (Nakamura et al., 1999; Pinaud et al., 2001) may underlie many of the morphological changes observed in the brains of the animals exposed to an enriched environment. A remarkable improvement was previously observed in the rat's learning and memory ability (Simão et al., 2012), and this improvement is well correlated in this study with structural changes in hippocampus related to volume and thickness of the pyramidal layer of CA1 subfield. It is possible that enriched environment can affect the expression of genes that determine neuronal structure in the cerebral cortex and hippocampus (Rampon et al., 2000). These alterations could be both physiological or morphological, as well as changes in the neurotransmitter systems efficacy (Rosenzweig and Bennett, 1972), neurogenesis and gliogenesis (Kempermann and Gage, 1999; Van Praag et al., 2000) and increase in neurotrophic factors synthesis (Young et al., 1999). Our findings significantly advance the current understanding of the effects of enriched environment on undernourishment and recurrent seizures. These observations support the view that enriched environment may change the structural and functional modification of existing hippocampus formation.

An surprising outcome and intriguing question of our study was an increase in the total volume of hippocampus in animals submitted to enriched environment without changes in individual cell density in the same animals. It might be partially explained by the observed trend toward increased thickness of the pyramidal layer in hippocampus of CA1. We also cannot exclude the possibility that neuronal remodeling might have occurred in these animals. Thus, further studies may focus on possible on-going neuroplasticity processes (neurons and synapses). Limitations of this study may exist due to the number of seizures, since we could not show some alteration in the hippocampus promoted by seizures against

undernourishment and nourish animals. Comparative studies are needed to evaluate the number of seizures effects and to demonstrate the amount effective and recommended.

In summary, our study demonstrates that undernourishment and seizures early in life lead to morphological abnormality in pyramidal neurons in adulthood. Beneficial morphological changes showed by environmental enrichment indicates that rodent brain remain highly plastic and responsive to complex experiences in adulthood.

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