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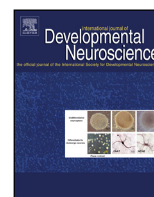

Effects of undernourishment, recurrent seizures and enriched environment during early life in hippocampal morphology

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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.

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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

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