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Large litters rearing changes brain expression of GLUT3 and acetylcholinesterase activity in adult rats

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HIGHLIGHTS

- Undernutrition during the brain period of growth spurt reduces brain weight and ability to transport glucose.
- Early postnatal malnutrition in rats changes GLUT3 neuronal expression in adult life.
- Malnutrition increases acetylcholinesterase activity in cerebral cortex.

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ABSTRACT

Effects of malnutrition in the brain are more pronounced during the period of growth spurt, corresponding to the suckling in rodents. Neuronal glucose transporter GLUT3 expression and acetylcholinesterase activity were studied in the brain of adult young rats (84 days old) suckled in litters formed by 6 (control group) or 12 pups (malnourished group). In the adult rats, brain weight, blood glucose levels and GLUT3 expression were decreased in malnourished group (5%, 18%, 58%, respectively, $P < 0.001$, Student's *t* test) compared to the control. Increased activity of acetylcholinesterase was found in cerebral cortex homogenates and a significant interaction ($P = 0.019$, ANOVA two-way, Tukey's test) was found between nutritional state and homogenate fraction. In summary, malnutrition during suckling period decreased GLUT3 expression and increased acetylcholinesterase activity in the rat brain that could contribute to possible cognitive deficits and changes of brain metabolic activity.

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1. Introduction

Several studies report the effects of malnutrition on the central nervous system (CNS) reducing brain weight [3,23], modifying neurotransmitter systems [31], inducing morphological changes of neurons [17]. These effects are more severe if the malnutrition is long-termed or if it occurs during the "brain growth spurt" which corresponds to the highest rate of neurogenesis during development. In rats this period corresponds to 21 days of postnatal life that is during suckling [17].

Among the experimental models to induce malnutrition, the increase of litter size during suckling was successful used by Rocha-de-Melo et al. [20]. These authors observed that malnutrition caused by the reduction in food intake due to competition among pups of large litters led to reduce body and brain weight besides changes of cortical spreading depression velocity of propagation in the cerebral cortex of these animals. Effects of short- and long-term malnutrition on the CNS development, even after nutritional recovery with balanced diet, evidenced changes of energy metabolism in the adulthood with reduced expression of glucose transporters in cardiac and skeletal muscle [1,9] and increased activity of acetylcholinesterase (AChE) in regions like the cerebellum, striatum and hypothalamus [19].

Acetylcholine is a major neurotransmitter of the nervous system, associated with the maintenance of processes of learning and memory [32,34] and the control of cerebral blood flow [21]. The decrease in cholinergic activity and increased activity of fractions of AChE has been associated with states of dementia in elderly patients and the loss of cognitive function in patients with Alzheimer's disease [4]. Malnutrition can also lead to a reduction in density of muscarinic receptors in brain regions, such as motor and somatosensory cortex in rats submitted to protein-energy restriction [30].

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In the CNS, the astrocytes have an important role in the metabolism of glucose [18], particularly during brain activation. Glutamate released in synaptic cleft is recaptured by astrocytes leading to an increased need of ATP to restore ionic gradient in these cells. Then glucose will be primary uptake by astrocytes and metabolized through glycolitic pathway to supply neurons with lactate which is converted to piruvate to furnish intermediates to tricarboxilic acid cycle to produce energy. In malnourished rats it was demonstrated an increased glutamate concentration on synaptic clefts that will stimulate oxidative stress and excitotoxicity in brain cells of these animals [6].

In the first 2 weeks of postnatal life the levels of glucose transporters, GLUT1 and GLUT3 in the rat brain are low but increase significantly with brain maturation and synaptogenesis [29]. Therefore, malnutrition occurring in this period could change brain glucose transporters expression and in consequence glucose metabolism. Neuronal glucose transporter and acetylcholinesterase activity in brain of malnourished rats from large litters have not been studied yet. Thus, this study aims to investigate if malnutrition during the critical development of the CNS changes the expression of the main glucose transporter in neurons, GLUT3 and, if the effects of malnutrition in early life could also affect acetylcholinesterase activity in brain of adult rats.

2. Methods

The experiments were conducted in accordance with protocols established in the "Principles of Laboratory Animal Care" (NIH) and with the norms of the Ethics Committee for Animal Research, of the Universidade Federal de Alagoas. Wistar rats were kept in an environmentally controlled room at 25 ± 2 °C with a 12:12 h light/dark cycle (lights on at 06:00 h). Male Wistar rats from dams feed with a commercial rat chow diet containing 22% of protein (Labina®) were randomly distributed, at birth, to form litters of two different sizes: medium, composed by 6 and large by 12 pups. After weaning (at postnatal day 21), pups were housed 4–5 per cage and fed with the same maternal lab chow diet until 12 weeks-old when experiments were performed. Animals from medium litters constituted control ($n = 6$) and that from large ($n = 7$) undernourished group. The body weights were determined weekly until the day of experimental procedures.

2.1. Tissue preparations and assays

In the adulthood animals were fasted for 12 h and blood glucose levels were measured collecting a drop of blood from the tail using a glucometer (Prestige IQ®). After, animals were anesthetized with sodium pentobarbital (50 mg/kg body weight) and sacrificed by decapitation. The brain and cerebellum were quickly removed, weighed, frozen in isopentane −70 °C and after stored at −20 °C to AChE activity determination and GLUT3 expression studies, except when measures were immediately proceeded. The right hemisphere of 6 control and 7 undernourished animals were used for analysis of activity of acetylcholinesterase and determination of protein by a colorimetric method [5,14].

The acetylcholinesterase activity was evaluated in tissue homogenate divided into three samples: homogenate of cells (HC) which was homogenized in 0.9%NaCl (pH 7.4) solution and mechanically disrupted using a Polytron®; homogenate centrifuged of cells (HCC, centrifuged at 12,000 rpm, 4 °C for 10 min) and homogenate free of cells with triton (HFCT) where samples were homogenized by mechanical disruption in a buffer containing 0.5% Triton X-100. The samples were immediately subjected to the assays described below.

Specific dilutions of homogenates were used for AChE activity: the HC was diluted in a proportion of 1:100; the extract centrifuged HCC 1:50 and cell-free extract with 0.5% triton – HFCT 1:100 dilutions. AChE activity was determined spectrophotometrically according to the method of Ellman et al. [5] at 412 nm. One unit (U) of enzyme activity was defined as the amount of AChE catalyzing the hydrolysis of 1 μmole of substrate per minute under assay conditions. AChE activity was expressed in U/mL and the specific activity of AChE in U/mg protein. Protein was measured according to Lowry et al. [14].

2.2. Glucose transporter GLUT3 expression

The glucose transporter GLUT3 expression was assayed in the left cerebral cortex of 4 animals for each group by Western blotting. Briefly, cerebral cortex of each animal was dissected and homogenized by mechanical processing in a buffer containing 40 mM Tris–HCl (pH 7.4), 250 mM sucrose, 5 mM dithiothreitol, 2 mM EGTA, 2 μg/mL protease inhibitors cocktail (Sigma). The homogenate was centrifuged at 1000 × *g* for 10 min, supernatant was recovered, diluted in the same buffer and then centrifuged at 16,000 × *g* for 10 min at 4 °C. Pellets were diluted in Tris–HCl buffer and protein dosage was proceeded using BCA kit essay.

Samples of protein (25 μg) in triplicate were diluted in electrophoresis gel to 10% SDS-PAGE according to the method of Laemmli [12]. The proteins were transferred by electrophoresis in Tris–glycine, pH 7.4 buffer (30 V at 4 °C, overnight) to nitrocellulose membranes (BioRad) and the sites of non-specific binding was blocked in a solution containing 1% BSA (bovine serum albumin) + 10% skim milk (Sigma) at room temperature for 1 h. Then, the membranes were incubated with primary antibodies anti-GLUT3 (1:1500, Millipore) and anti-beta actin (1:5000) diluted in 0.1% PBST + 1% skim milk and incubated at 4 °C overnight. After rinsing in PBST the membranes were incubated with fluorescent secondary antibodies anti-goat IRDye (1:5000) and anti-mouse Alexa Fluo (1:5000) (LI-COR) diluted in PBST + 1% milk powder for 2 h at room temperature. The detection and quantification of the signal was performed by the Odyssey® infrared detection system (LI-COR).

2.3. Statistics

Data of body weight, blood glucose levels, protein quantification and activity of acetylcholinesterase were evaluated using the Student's *t* test. Analysis of variance of one or two ways followed by Tukey's test was also used for activity assays.

3. Results

The larger number of pups per litter to induce protein-energy malnutrition caused significant reduction compared to the control group in body weight of malnourished animals at weaning. Body weight in this period was: weaning, 35.50 ± 5.16 g control, 28.00 ± 2.00 g, malnourished $P < 0.001$. After weaning until adult life there were no differences in body weight of malnourished rats (296.71 ± 17.39 g) compared to control group (298.67 ± 15.11 g). The weight of the brain showed a significant (1.186 ± 0.016 g) difference when compared with the control animals (1.245 ± 0.025 g) $P < 0.001$. However, the weight of the cerebellum showed no significant difference (0.285 ± 0.02 g control and 0.2719 ± 0.01 g malnourished groups).

Blood glucose levels were significantly lower in the malnourished group 52.79 ± 6.30 mg/dL when compared to the control group 64.4 ± 6.73 mg/dL mg (Student's *t* test).

The effects of malnutrition on the expression of GLUT3 protein in the cerebral cortex of adult animals show that malnutrition caused a significant reduction in the expression of GLUT3 compared to control animals (Fig. 1). Analysis of protein content in the three extracts of brain tissue preparations used for activity measures showed no

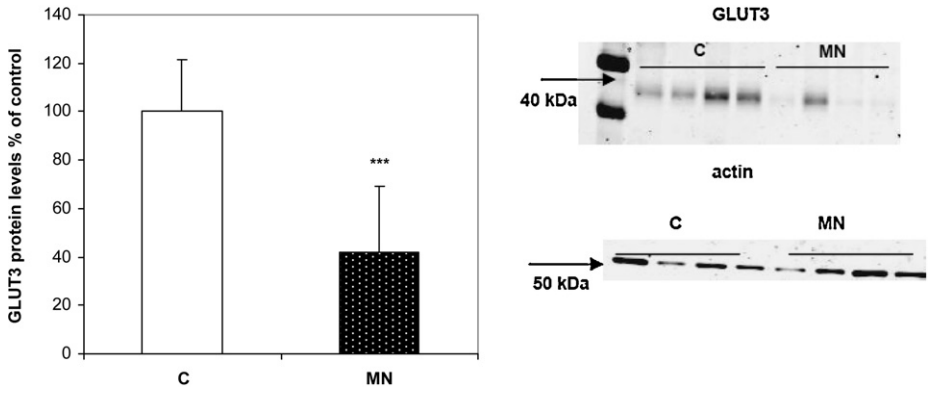


Fig. 1. Expression of the GLUT3 transporter in cerebral cortex of control (C, $n = 4$) and malnourished (MN, $n = 4$) animals obtained of at least three Western blots. The bars represent the average percentage variation of GLUT3 protein after the values had been normalized using β-actin signal as a reference. In the left, infrared Western blot analysis showing on the top GLUT3 (band at 39 kDa), and in the bottom β-actin (β-actin, band at 50 kDa) in the cerebral cortex of control and malnourished rats. ***$P < 0.001$, Student's t test.

significant difference in protein content between the experimental groups when compared with the same types of treatment between them. However, we observed a significant difference in protein content between the HCC homogenate and the other two treatments (Table 1).

The evaluation of the activity of acetylcholinesterase in malnourished and control animals was performed in order to express the enzyme activity in U/mL (Table 1) and its specific activity in U/mg protein (Fig. 2). The differential AChE activity found in HC homogenate between control and malnourished rats reflects diluted fractions whilst HFCT characterize membrane-bound and diluted fraction of AChE enzyme. Analysis showed a significant difference among the 3 different types of homogenates in the control ($P < 0.0001$) and malnourished ($P < 0.001$, Student's t test) groups (Table 1). The activity of the HCC homogenate was significantly lower than in other treatments, while in HFCT there was a significant increase when compared to the others. Increased activity of HFCT was possible due to the fact that the membranes were solubilized in triton 0.5%, increasing availability of AChE fraction bound to the membrane.

Overall data in Fig. 2 show a tendency to higher AChE activity in the 3 different homogenates although only HC homogenate from malnourished rats have presented higher AChE specific activity (U/mg protein) in compared to the control group ($P < 0.05$, Student's t test). Comparing the effects of the type of extract and nutritional status on the activity of acetylcholinesterase, was found a significant interaction ($P = 0.019$, two-way ANOVA, Tukey's test) between the two factors influencing the activity of the enzyme.

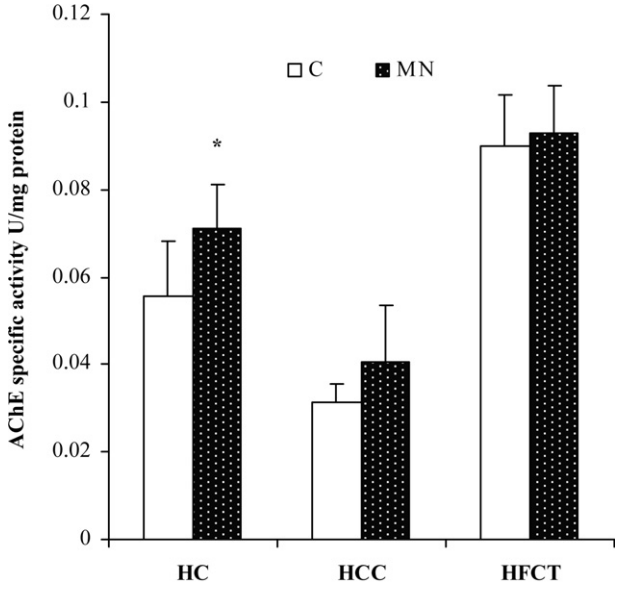


Fig. 2. Specific activity of acetylcholinesterase (AChE) in brain tissue extracts from control (C, $n = 5$) and malnourished (MN, $n = 6$) animals. The bars represent the average enzyme activity in milligrams of protein for each type of extract ± the standard deviation ($P < 0.05$, Student's t test). HC, homogenate of cells; HCC, homogenate centrifuged of cells; HFCT, homogenate free of cells with triton.

4. Discussion

Nutrients restriction during the suckling period in our study induced reduction of the brain weight in the adulthood. Suckling animals of dams submitted to different protein restriction diets or whose food supply was reduced by increasing the size of the litter, also showed reduction in brain weight when compared to control animals [15]. Comparing these results with ours, whose energy restriction was during the whole period of suckling, we emphasize that reduction of nutrients availability during brain growth sprout has important effects on brain development.

Table 1
Effects of litter size and brain homogenate treatment on brain protein content and acetylcholinesterase (AChE) activity.

Groups	Protein content (mg/mL)			AChE (U/mL)		
	Brain homogenate					
	HC	HCC	HFCT	HC	HCC	HFCT
Control	12.83 ± 1.34[a]	3.65 ± 0.21[a]	12.77 ± 1.44[a]	0.70 ± 0.11[b]	0.11 ± 0.03[b]	1.19 ± 0.13[b]
Malnourished	12.68 ± 1.74[a]	3.55 ± 0.75[a]	12.42 ± 0.90[a]	0.91 ± 0.04[b]	0.13 ± 0.02[b]	1.19 ± 0.16[b]

HC, homogenate of cells; HCC, homogenate centrifuged of cells; HFCT, homogenate free of cells with triton.
[a] $P < 0.0001$ significant differences among homogenates compared within control and malnourished groups.
[b] $P < 0.001$ significant differences among homogenates compared within control and malnourished groups.
$n = 6$ for control and $n = 7$ for malnourished groups.

Food restriction in the animals of our study induced changes in the availability of primary energy substrate used by the brain: the glucose. Studies show that malnutrition in the intra-uterine and post-natal life cause resistance to insulin action [13,16] resulting in changes of blood glucose levels and resistance to insulin resulting in elevated glucose levels that persist even in adult life and characterize diabetes type II [8]. In contrast, in a study in rats where malnutrition was caused by the consumption by dams of free-protein diet during the lactation showed that the malnourished rats had higher insulin secretion induced by glucose than that of the control group [13]. In another study, the effects of malnutrition on the expression of mRNA for insulin showed that maternal malnutrition caused increased levels of insulin mRNA of the offspring and when malnutrition was prolonged until adulthood a reduction in these levels was observed [16].

Studies on the expression of glucose transporters in peripheral tissues show that malnutrition affects the expression of glucose transporters. Gavete et al. [9] reported that in skeletal muscle there was no change in GLUT4 protein but an increase in insulin stimulated translocation of the transporter. Lopes da Costa et al. [13] reported in malnourished animals during the weaning, increased expression of GLUT2 in hepatocyte and pancreatic beta cells. Stuart et al. [26] have reported that 50% reduction of GLUT3 glucose transporters have no effect on glucose transport to the brain, however, previous reports of one our studies [35] have demonstrated that a 30% reduction in GLUT3 expression in α-linolenic deficient diet Wistar rats have pronounced effect in reducing glucose uptake and utilization as essayed by 2-[C^{14}]-deoxyglucose and cytochrome oxidase activity in rat brains respectively. The present study has reported a 58% reduction of GLUT3 expression on cerebral cortex of larger litter animals which is a significant effect of reduced brain ability to transport glucose into neurons.

The important reduction of GLUT3 in cerebral cortex of animals malnourished in our study could be explained in part by the decrease in blood glucose levels and lower brain weight of these animals compared to the control group. This could induce decreased glucose availability to neurons and thus a lower expression of GLUT3. On the other hand we cannot exclude the contribution of other glucose transporters such as GLUT1 in the blood–brain barrier and astrocytes as major carriers of glucose to the brain parenchyma and the cerebral metabolism in general. In fact, according to Pellerin and Magistretti's hypothesis [18] during brain activation, the glucose is primary uptake by astrocytes and then metabolized to lactate that is transported to neurons, converted to pyruvate to produce energy via oxidative phosphorylation.

Rats from large litter presented in our study smaller brain weight which was most likely a result of reduced nutrient supply due to higher competition among pups during weaning. In fact, studies [10,17] have demonstrated that minor availability of nutrients during brain sprout development can change membrane composition and impacting the lipid association of some membrane-bound enzymes such acetylcholinesterase [28,33].

In our study, the activity of HFCT (on the membrane fraction) showed no significant difference between control and malnourished groups, similar to that occurs in ischemic states. However, the relationship between HFCT/HC was lower in malnourished animals caused by the increase in cholinesterase activity of the HC animals.

Effects of changed acetylcholinesterase activity in cerebral cortex of large litter size rats showed in our results could be attributed to nutritional status during weaning more than a possible stress effect changing brain weight and cholinergic system.

Environmental enrichment and social stimuli during pre- and postnatal life provides interaction between individuals of the same species and stimulate the development of the nervous system and cognitive functions like learning and memory [24,27]. Moreover, morphological changes such as increased neurogenesis, dendritic and synaptic interactions were observed in rodents raised in enriched environment with sensory stimuli [24]. Agreeing with this, animals raised in sensory enriched environments exhibited increased brain glucose metabolism [11]. Conversely, stressors such as maternal deprivation during weaning are known to accentuate stress response characterized by morphological and functional changes such as the reduction of cortical thickness and memory deficits [7]. Also, changes in neurotransmitter systems such as the increased availability of acetylcholine in the synaptic cleft and morphological changes that reverberate throughout adulthood [24,36].

Recent findings have demonstrated that large litter rearing could ameliorate anxiety and stress responses. Bulfin et al. [2] studying sex differential response to stress demonstrated that male Wistar rats raised in a mixed very large litter (20 animals) had attenuated response from hypothalamic–pituitary–adrenal axis (HPA). Lean animals from very large litters had reduced body weight through adult life and exhibited reduced anxiety reaction to stressor stimuli as tested in elevated plus maze. Spencer and Tilbrook [25] showed that female rats raised in litters of only four animals had long-term effect in enhanced exploratory test demonstrated in elevated plus maze as well as enhanced activity of hypothalamic paraventricular nucleus to stress.

Acetylcholine role on stress response is mediated by HPA stimulation through corticotrophin releasing hormone (CRH). Indeed, rats submitted to restriction stress showed increased acetylcholine release in hippocampus and hypothalamus mediating hypothalamic adrenocorticotrophic release in response to stress. Though, enhanced activity of cholinergic system is involved in stress response [36].

Segovia et al. [22] demonstrated enhanced acetylcholine release in the rat prefrontal cortex induced by handling stress during aging and environmental stimuli reduced this effect, which corroborate with our data showing that animals from larger litters had increased acetylcholinesterase activity, consequently a probable reduced availability of acetylcholine in synaptic cleft of malnourished animals, in opposing to stress effects that stimulates acetylcholine release.

Malnutrition when occurs during the critical period of brain development leads to structural and metabolic changes observed in adulthood by reduction of expression of GLUT3 and the increased activity of acetylcholinesterase in rats raised in large litters, indicating that nutritional status may be a determining factor to induce long-term changes in cerebral metabolic activity.

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