Basic nutritional investigation

Zinc and glutamine improve brain development in suckling mice subjected to early postnatal malnutrition

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ABSTRACT

Objective: The effect of zinc and glutamine on brain development was investigated during the lactation period in Swiss mice.
Methods: Malnutrition was induced by clustering the litter size from 6–7 pups/dam (nourished control) to 12–14 pups/dam (undernourished control) following birth. Undernourished groups received daily supplementation with glutamine by subcutaneous injections starting at day 2 and continuing until day 14. Glutamine (100 mM, 40–80 μL) was used for morphological and behavioral studies. Zinc acetate was added in the drinking water (500 mg/L) to the lactating dams. Synaptophysin and myelin basic protein brain expressions were evaluated by immunoblot. Zinc serum and brain levels and hippocampal neurotransmitters were also evaluated.
Results: Zinc with or without glutamine improved weight gain as compared to untreated, undernourished controls. In addition, zinc supplementation improved cliff avoidance and head position during swim behaviors especially on days 9 and 10. Using design-based stereological methods, we found a significant increase in the volume of CA1 neuronal cells in undernourished control mice, which was not seen in mice receiving zinc or glutamine alone or in combination. Undernourished mice given glutamine showed increased CA1 layer volume as compared with the other groups, consistent with the trend toward increased number of neurons. Brain zinc levels were increased in the nourished and undernourished-glutamine treated mice as compared to the undernourished controls on day 7. Undernourished glutamine-treated mice showed increased hippocampal gamma-aminobutyric acid and synaptophysin levels on day 14.
Conclusion: We conclude that glutamine or zinc protects against malnutrition-induced brain developmental impairments.

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Introduction

The human and rodent brain development relies in great part on postnatal plasticity, especially within highly dynamic brain regions such as cerebellum, hippocampus, and neocortex [1]. Nutritional insults during the first weeks of life in rodents (somewhat analogous to the first 2 y in humans) can delay the

acquisition of early motor reflexes [2], with associated deleterious effects in the cerebellum, mostly related to zinc deficiency [3]. Specific behavioral impairments due to nutritional deficits during the first weeks of life, such as iron-deficiency, might be retained even when these animals reach adulthood [4].

In our previous cohort studies of children living in impoverished Brazilian urban areas, we have shown a reciprocal "vicious cycle" between malnutrition and diarrheal illnesses with lasting physical and cognitive impairments, which are sustained into school age [5–7].

The pursuit of selective and time-effective nutritional interventions to ameliorate these deficits is critical to rescue, as much as possible, the normal course of child development. Zinc and glutamine are considered critical gut-trophic nutrients, which have protective roles, as demonstrated in various animal models of intestinal injury [8] and have been proven to be beneficial in clinical studies enrolling children afflicted with malnutrition and diarrhea in the developing world [9]. The recovery of the intestinal barrier function following nutritional therapy might improve the supply of brain-related nutrients (iron, fatty acids, and zinc) during critical time-windows of brain development.

Thus, the aim of this study was to evaluate the role of zinc and glutamine treatment, alone or in combination, in preserving the hippocampus morphology, with emphasis on the CA1 region, following early postnatal malnutrition, as assessed by unbiased stereological methods. The value of stereological analyses for understanding histological alterations in the hippocampus has been extensively highlighted [10]. In addition, we followed the ontogeny of some motor reflexes during the first 2 wk of life in mice to test the hypotheses that the effect of glutamine and zinc on brain morphological and biochemical changes would correlate with improved behavioral function.

Materials and methods

Malnutrition and treatment

Swiss mice were obtained from the Federal University of Ceara vivarium and were housed in breeding pairs with free access to standard chow diet and water in a temperature-controlled room. Pregnant mice were transferred to individual cages until delivery. All behavior tests were conducted in a silent room (14:00–16:00 h), under 12 h- light/dark cycle. Study protocols were in accordance with the Brazilian College for Animal Care guidelines and were approved by the Federal University of Ceara Animal Care and Use Committee. Malnutrition was induced by clustering the litter size (undernourished group, 12 to 14 pups per litter, and the nourished group with 7 to 8 pups per litter, since birth) during the first 2 wk. The undernourished groups received daily supplementation with glutamine (100 mM) by subcutaneous injections (40 µL during days 4–8 and 80 µL during days 9–14), starting at day 2 until day 14. Zinc acetate was added in the drinking water to the lactating dam in a concentration of 500 mg/L during the periods of lactation, which has been shown to be beneficial to the offspring immune system during the suckling time [11]. Therefore, we defined the following supplemented groups: glutamine + phosphate-buffered saline (PBS); zinc acetate + PBS; glutamine + zinc acetate (the latter given to the dams in their drinking water and presumably transferred to the pups by the breast-milk). The dose of glutamine (100 mM) was chosen based on a dose-response curve (50, 100, and 200 mM) during the first 2 wk (data not shown). Untreated nourished and undernourished groups received only PBS. Growth was monitored daily by recording tail length and body weight gains. Effort was made to keep the same degree of handling for all experimental groups. Although a gender effect is unlikely to constitute a strong factor in newborn mice, litters with high deviations from 1:1 ratio were not included in this study.

Behavioral studies

Behavioral studies, including cliff avoidance and swimming behaviors, were conducted in the first 2 wk of life and scored as described elsewhere [12,13]. The *cliff avoidance reflex* test [14,15] is used to assess the integration of exteroceptive input (vibrissae) and locomotor output, providing information concerning physical and motor development as well as sensory function and/or processing.

The offspring is placed on a platform elevated 10 cm above a table top. The forelimbs and snout of the animals are positioned so that the edge of the platform passes just behind an imaginary line drawn between the eye orbits. Avoidance is scored by reflex latency between being placed on the edge and turning until it is parallel to the edge of the table (0 = no response, latency > 60 s; 1 = response, < 15 s; 2 < 10 s; 3 < 5 s). The *swimming behavior* test is used to assess navigational and motor development. The offspring is placed into a tank with water temperature maintained at $27 \pm 1 \,^{\circ}C$ and swimming behavior is rated for direction (straight = 3, circling = 2, floating = 1) and head angle (ears out of water = 4, ears half out of water = 3, nose and top of head out of water = 2, and unable to hold head-up = 1). Limb movement is rated as either 1 = all four limbs used, or 2 = hind limbs only used.

Stereology

Tissue sampling and sectioning

A total of 19 male Swiss mice (5–10 g) were used in this protocol. Brain tissue was collected at day 14 (the end point), following a transcardiac perfusion-fixation with Palay's solution (containing 1% formaldehyde and 1% glutaraldehyde in 0.12 M phosphate buffer at pH 7.2) [16] and immediately immersed in the same solution for stereological analyses.

After euthanasia the hippocampus was dissected out from the brain and halved. Each hemi-hippocampus was systematically, uniformly, and randomly selected [17] between left and right and weighed. Subsequently, the selected hemi-hippocampus (left or right) was manually straightened along its septo-temporal axis to diminish the anatomical organ curvature.

Subsequently, the straightened hippocampus was embedded in a 10% agar solution and exhaustively cut into a thin section (100-µm-thick) followed by a thick section (1-mm-thick), alternately paired and used a vibratome (VT 1000 S; Leica, Wetzlar, Germany) [18]. Sections were orthogonal to hippocampus' long axis and a fraction (10^{-1}) of those paired sections (thin and thick section) was SUR selected. The average interval (K) between the section pairs was 400 µm. Thin sections were collected onto glass slides, stained with a 1% alcoholic toluidine blue solution, dehydrated in progressive ethanol concentrations, mounted under a coverslip with DPX (Fluka, Buchs, Switzerland), and used not only to record the exact position of CA1 layer in the hippocampus, i.e., mapping sections [18], but also for the estimate of postembedding hippocampus and CA1 layer volumes using Cavalieri's principle.

Thick sections were used to produce vertical, uniform, and random sections (VUR sections) [19]. First of all, thick sections were positioned onto a transparent plate of Silgard in the center of a circle with 36 (360°) equidistant divisions along the perimeter. Next step, a random number between 0 and 36 was generated using a random number table systematic uniform random sampling (SURS) [18].

Subsequently, a transparent cutting guide containing lines was placed onto the thick section at the same selected angle. Finally, a razor blade was used to produce bars from the sections guided by the lines in the cutting guide.

Each bar containing hippocampus was rotated 90° around the vertical axis to the sections and allowed for vertical, uniform, and random sections parallel to the vertical axis. The bars from each section were then re-embedded in a 10% agar solution and exhaustively sectioned 50-µm-thick using a vibratome, i.e., mean block advance (BA) was 50 µm.

Thick sections (from bars) were collected onto glass slides, stained with a 1% alcoholic toluidine blue solution, dehydrated in progressive ethanol concentrations, and mounted under a coverslip with DPX (Fluka) and were used to simultaneously estimate number and volume of the same CA1 neurons.

Section images were acquired using a Leica DMR Microscope coupled with a Digital Camera PLA622 (Pixellink, Ottawa, Ontario, Canada) and stereological software New Cast Visiopharm, Hoersholm, Denmark (version 2.16.1.0). The area of the unbiased counting frame used was 5000 µm² [20]. Before starting the counting procedure, a z-axis distribution (calibration) was performed to determine the neuron distribution throughout section thickness and establish the disector height, which was 20 µm. Section thickness was measured in every second field of view using the central point on the unbiased counting frame. The neuron nucleus was defined as the counting unit.

In this study the whole hippocampal structure including the granular cell layer of dentate gyrus and the pyramidal cell layer of CA1, CA2, CA3 was defined at all levels of sectioning according to the stereotaxic coordinates published elsewhere [21]. Hippocampus regions are represented in Figure 1. The CA1 area of the hippocampus was chosen for further stereological analyses because it constitutes one of the simplest and most examined cortical areas, because recent progress has been made in this area in explaining neuronal diversity, and because of the highly intricate gamma-aminobutyric acid (GABA)ergic interneuronal and glutamatergic principal circuitry supporting behavioral processing [22].

Hippocampus volume: V (HIP)

Hemi-hippocampus fresh weight was converted into volume to estimate tissue shrinkage. The formula used was: $V_{(volume)} = m_{(mass)}/d_{(density)}$. The specific
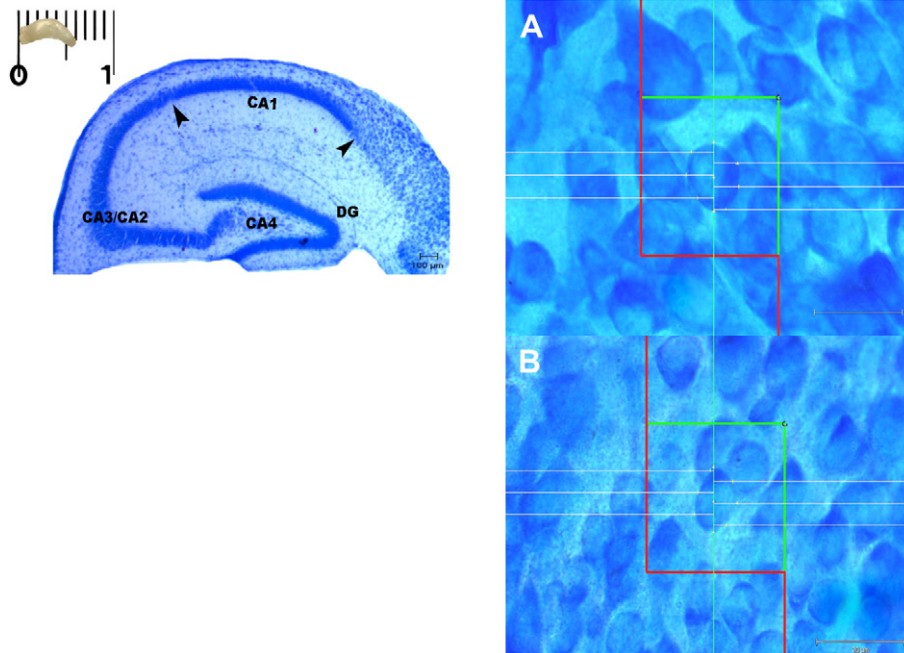
Fig. 1. Details of a hippocampus coronal section showing the whole layered organ structure. Arrowheads point to CA1-layer limits. Because of the small dimensions of CA2 layer, the latter was joined to CA3 pyramidal layer, named CA3/CA2. Toluidine blue. Scale bar: 100 μm. Cell volume differences between (A) undernourished and (B) nourished mice. CA1 cells undergo a malnutrition-related hypertrophy. White lines show the application of six-line vertical rotator. Toluidine blue. Scale bar: 20 μm for panels A and B.

density ($d_{(density)}$) was 1.04 g cm^{-3} [18]. In the Results we have reported the bilateral volume that was elicited multiplying V (HIP) by 2 because the left or right hemi-hippocampus was SUR sampled [17].

Volume of CA1 layer: V (CA1) and V(HIP$_{post-emb}$)

The volume of CA1 layer and the postembedding volume of the selected hemi-hippocampus were estimated using the Cavalieri principle on the thin sections (100-μm-thick). Then, V (CA1) or V (HIP$_{postemb}$): $= \Sigma\, P \cdot ap \cdot BA \cdot K$, where P is the number of test points hitting the tissue (CA1 or the whole hippocampus) (we have used on average 100 (CA1) and 200 (HIP) points per animal), ap is the area associated with each test point, BA is the mean block advance (or mean section thickness = 100 μm), and K is the distance between the sections sampled.

The error variance of Cavalieri's estimator (CE) was estimated according to Nyengaard [23]. Therefore, the error variance of Cavalieri's estimate was 0.04 for group 1, 0.04 for group 2, 0.03 for group 3, 0.03 for group 4, and 0.04 for group 5.

The volume shrinkage was then calculated as:

Volume shrinkage = 1 – volume after: volume before [18].

The hemi-hippocampus shrinkage volume (%) was estimated to be (mean ± SD): 4.75 ± 1.22 (group 1); 5.56 ± 1.52 (group 2); 4.16 ± 1.21 (group 3); 5.05 ± 1.17 (group 4), and 5.75 ± 1.12 (group 5). No correction for global shrinkage was performed because intergroup differences were not statistically significant ($P = 0.13$).

Numerical density of CA1 neurons: N$_V$ (CA1)

The optical disector was used to estimate the numerical density of CA1 neurons in a given hemi-hippocampus. The formula for NV estimation is: $N_v = \frac{\overline{tq}}{BA} \cdot \frac{\Sigma Q^-}{h.a(p) \cdot \Sigma P}$ where ΣQ^- is the total number of particles counted by disectors, a is the counting frame area, p is the number of reference points per counting frame and ΣP is the total number of reference points in each counting frame, which hit the reference volume, CA1 layer in this case, $\overline{tq^-}$, is the Q$^-$ weighted mean section thickness measured, and BA is the mean block advance in the vibratome. The mean number of disectors applied and particles counted (Q$^-$) per group was (24; 244) (group 1), (22; 241) (group 2), (23; 342) (group 3), (21; 274) (group 4), and (22; 282) (group 5), respectively.

Total number of CA1 neurons: N (CA1)

The total number of CA1 neurons was estimated by multiplying the numerical density of CA1 neurons by the volume of CA1 layer. Therefore:

$$N(CA1) = N_v(CA1) \cdot V(CA1)$$

The bilateral number was obtained by simply multiplying $N(CA1)$ 2 because the left or right hemi-hippocampus was SUR sampled [17].

The error variance of total neuron number (CE(N)) was estimated as shown in [23] and [24]. The error variance was 0.03 for group 1; 0.03 for group 2; 0.03 for group 3; 0.03 for group 4, and 0.02 for group 5.

Mean neuronal volume: $\overline{v_N}$ (CA1)

The mean perikaryal volume of CA1 neurons was estimate using a six-line vertical rotator [25]. On average, 200 neurons were sampled for the volume estimation, i.e., the same neurons which were sampled for number estimation.

Western blots

In brief, either the entire brain ($n = 7$/group) or the hippocampus ($n = 4$/group) from 14-d-old pups was carefully dissected and immediately dig-frozen in liquid nitrogen. Thawed specimens were pulverized with an electric homogenizer (ultra-Turrax homogenizer, Sigma, St. Louis, MO, USA), containing lysis buffer and then transferred to test tubes with protease inhibitor cocktail and centrifuged at 14 000 rpm. Supernatants were assayed using the bicinchoninic acid method, BCA Protein Assay Kit (Pierce, Rockford, IL, USA) to standardize 50 μg of protein product. Samples were loaded into 15% denaturing polyacryamide min gels (Bio-Rad, Hercules, CA, USA), and gels were transferred overnight and then blotted onto nitrocellulose membranes. Membranes were incubated with either rabbit synaptophisin or myelin basic protein (MBP) antibodies (at dilution of 1:500) for 1 h and then rinsed three times in rinsing buffer, then incubated in a secondary antibody and rinsed as described above. Each membrane was washed and exposed to Kodak X-Omat AR film (Kodak, Rochester, NY, USA). Stripped blots were later incubated with actin antibodies as an internal control.

Biochemical analyses

Analyses of brain and serum zinc levels were conducted using atomic absorption spectroscopy (SpectrAA 55 AAS, Varian, CA, USA). A standard calibration curve was obtained using a standard zinc solution (0.2 mg Zn/L; J.T. Baker). The whole brain from 7- and 14-d-old pups was removed quickly after decapitation, rinsed with deionized water, and stored frozen until analyzed. After thawing, the whole brain was weighed and mineralized using a heated HNO$_3$ ($\sim$110 °C) solution and thereafter analyzed by atomic absorption spectroscopy. Dissected hippocampi were obtained from another set of 14-d-old experimental pups and stored until analyzed. Analyses of amino acids (aspartate, glutamate, taurine, and GABA) were carried out from dissected hippocampus using a high-performance liquid chromatography apparatus (Shimadzu, Kyoto, Japan), and a fluorimetric detection method. Briefly, frozen tissue specimens were homogenized in 0.1 M perchloric acid and sonicated for 30 s at 25 °C. After sonication, samples were centrifuged at 15000 rpm, for 15 min at 4 °C. Supernatants were removed and filtered through a membrane (Millipore, Billerica, MA, USA,

0.22 μm), and the amino acids were derivatized with mercaptoethanol and *O*-phthaldialdehyde. *O*-phthaldialdehyde derivatives were then separated on a C18 column (150 mm × 4.6 mm; from Shimadzu, Japan) and after derivatization, amino acids were separated, using a mobile phase, consisting of sodium phosphate buffer (50 mM, pH 5.5) and 20% methanol. The area of each peak was determined with Shimadzu software and compared with the peak area of the corresponding external standard. Amino acid concentrations were expressed as μmol/mg of wet tissue.

Statistical analyses

Normal distribution was assured using Kolmogorov-Smirnov's test and equity of variances (homoscedasticity) was checked by means of Levene's test. Nutrition state-related effects among groups were assembled using one-way ANOVA through a statistical package, i.e., Minitab 15 (2007) and Graph Pad Prism 4.01 (2004). In the case of *P* < 0.05, Bonferroni's test was applied to draw multiple comparisons among groups. In some behavioral tests, independent Student's *t* test was used when appropriate.

Results

Growth and behavioral tests in Swiss mice

We have found a significant benefit of zinc treatment with or without glutamine on growth during the first 2 wk of life (Fig. 2). This zinc supplementation improved growth in the undernourished mice as early as the 5th day in weight gain and by the 7th day in tail length. Zinc treatment delayed the decrement in tail length as opposed to the other groups up to day 12 (Fig. 2B). Glutamine treatment alone was not sufficient to improve growth as compared to the untreated control. Zinc plus glutamine and zinc-alone-treated groups had better cliff avoidance scores at day 9 than the untreated undernourished controls, reaching similar performance to the nourished mice (Fig. 3A). The glutamine-treated undernourished mice did not show reductions in swim direction scores at day 9, as opposed to the untreated undernourished and zinc-treated groups (Fig. 3B). In addition, both undernourished zinc-treated groups had similar scores as the nourished controls regarding head position during pool swimming at day 10 (*P* < 0.05) and delayed the decrement of this reflex for 1 d (day 9) as compared with the untreated undernourished group (Fig. 3C). All treatments improve limb movement during swim at day 14, reaching similar score as the nourished control (Fig. 3D), although not different from the undernourished control.

Stereology

There was no significant difference in the hippocampus volume, *V* (HIP), total number of CA1 neurons, *N* (CA1), or in the numerical density of CA1 neurons, N_V (CA1), between the experimental groups (*P* > 0.05). The undernourished mice supplemented with glutamine showed significant increase in the volume of CA1 layer, *V* (CA1), as compared with all other groups (*P* = 0.018). Furthermore, the mean perikaryal volume of CA1 neurons, $\overline{v_N}$ (*CA1*), was found remarkably increased in the undernourished untreated group (1150.20 μm³) (0.13) as compared with all other groups (*P* = 0.001). All treatments prevented the increase in the neuronal volume to the level of the nourished controls (Table 1). Figure 1 illustrates the mouse
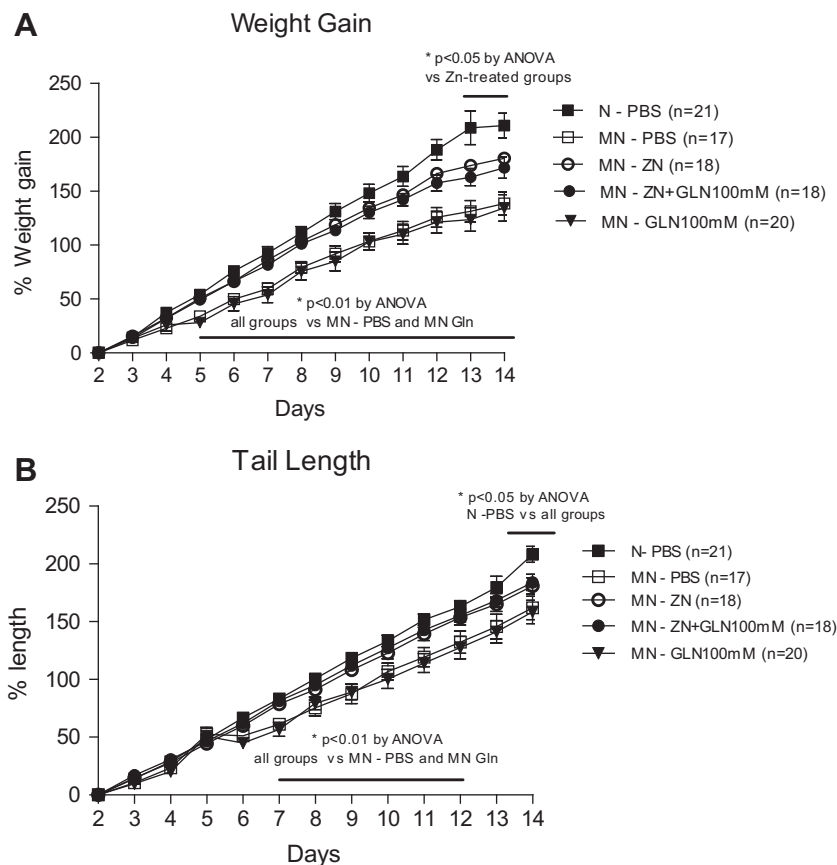

Fig. 2. Relative weight (A) and tail length (B) gain from the experimental mice during the malnutrition schedule. Statistical analyses were done from raw data using one-way analysis of variance corrected by Bonferroni's test. The significance level was set at *P* < 0.05. The results are shown as mean ± SEM. MN, malnourished mice; N, nourished mice.
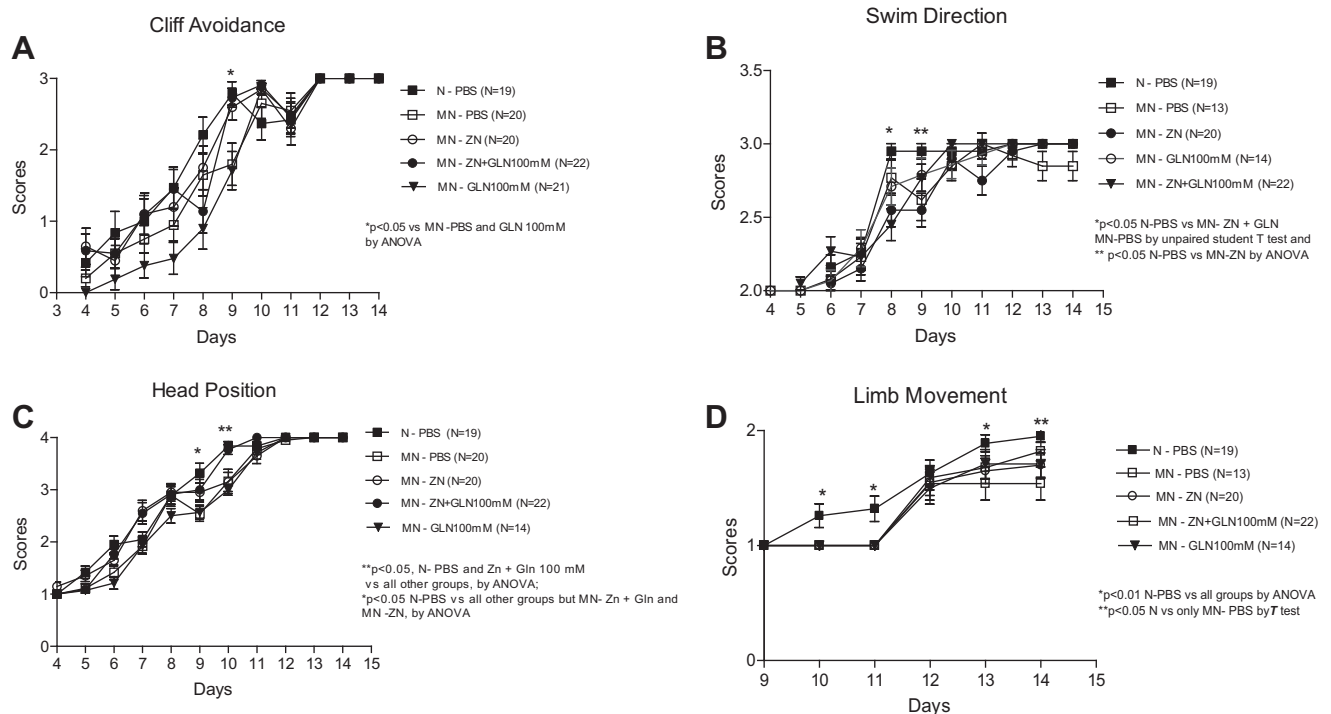
Fig. 3. Behavioral tests (cliff avoidance and swim reflexes: direction, head position, and limb movement) conducted in the first 2 wk of life in the nourished (N) and malnourished (MN) groups. Results are stratified in scores, accordingly. The significance level was set at $P < 0.05$.

hippocampus in coronal section with topographic regions and the application of a vertical rotator on nourished and undernourished subjects. Malnutrition-related neuronal hypertrophy is depicted in Figure 1A (right top panel).

Western blots

Although clear expressions of MBP were found in the whole brains and hippocampi of experimental mice, no differences were found between groups on day 14. However, we found higher expression of synaptophysin in the dissected hippocampus of the nourished group compared with the untreated undernourished group. In addition, glutamine treatment with or without zinc increased hippocampal synaptophysin expression, reaching similar expressions as the nourished control group (Fig. 4).

Biochemical analyses

To appreciate the importance of in the results mentioned above, we measured brain and serum zinc levels at days 7 and 14 of birth, during the malnutrition schedule. We found no differences in zinc serum levels between groups, although a trend of improvement was found. Furthermore, we found a significant increase in brain zinc levels in the glutamine (100 mM) -treated group at day 7 as compared to the untreated and zinc-treated malnourished mice ($P < 0.01$). A difference was also found between the nourished group and the undernourished control ($P = 0.02$) (Table 2). No difference was found between the groups at day 14, although a trend of increase zinc brain levels in the glutamine group was apparent ($P = 0.07$).

No changes were seen with the amino acids taurine and glutamate between groups. Aspartic acid brain levels were significantly reduced by litter size clustering. This effect was not improved by any treatment. The same effect occurs with glycine levels (data not shown). Glutamine and zinc treatments significantly increased GABA brain levels in undernourished mice as opposed to the PBS-untreated group ($P < 0.05$) (Table 2).

Table 1
The effects of mice postnatal diet supplementation on the volume of CA1 layer (mm^3), mean neuronal volume (μm^3), hippocampus volume (mm^3), numerical density of CA1 neurons (mm^{-3}), and total number of CA1 neurons

Variable		Group 1	Group 2	Group 3	Group 4	Group 5
Hippocampus volume		18.70 (0.24)	17.60 (0.25)	23.03 (0.36)	22.11 (0.25)	28.46 (0.43)
CA1	Layer volume	0.59 (0.10)	0.57 (0.19)	0.87[a] (0.34)	0.52 (0.04)	0.68 (0.25)
	Mean neuronal volume	1150.20[b] (0.13)	519.52 (0.06)	664.51 (0.40)	758.92 (0.23)	599.58 (0.33)
	Numerical density of neurons	2.20×10^5 (0.22)	2.15×10^5 (0.16)	2.20×10^5 (0.38)	2.60×10^5 (0.01)	2.78×10^5 (0.17)
	Total number of neurons	2.60×10^5 (0.29)	2.40×10^5 (0.22)	3.80×10^5 (0.36)	2.70×10^5 (0.04)	3.80×10^5 (0.36)

Group 1: undernourished mice; group 2, undernourished mice supplemented with zinc acetate; group 3, undernourished mice supplemented with glutamine (100 mM); group 4, undernourished mice supplemented with zinc acetate and glutamine (100 mM); group 5, nourished mice receiving PBS. Values are group means (CV) for $n = 4$ (groups 1, 2, 3, 5), and $n = 3$ (group 4). Nutrition state-related effects among groups were assembled using one-way ANOVA. In the case of $P < 0.05$, Tukey's test was applied to draw multiple comparisons among groups
[a] Group 3 is different from all other groups ($P = 0.018$).
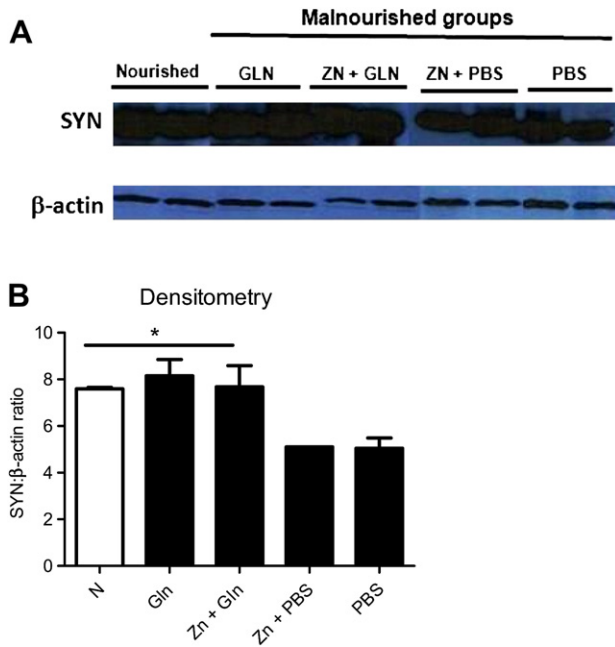[b] Group 1 is different from all other groups ($P = 0.001$).

Fig. 4. (A) Representative immunoblots of the hippocampus from the experimental mice for synaptophysin (SYN) on day 14. (B) Synaptophysin was normalized against β-actin and the densitometry data plotted. White bar represents the nourished group; black bars depict the malnourished groups. The significance level was set at $P < 0.05$, by Student's t test. The results are shown as mean ± SEM.

Discussion

Malnutrition during suckling stage in experimental rodents has been consistently recognized to cause lasting effects on behavior, growth, innate immunity, and endocrine functions, which may predispose to obesity and ultimately cardiovascular and neurodegenerative diseases [26–28].

In accordance with our findings of zinc benefit on growth, a recent meta-analysis of preventive zinc supplementation among infants and children has shown significant evidence of weight gain and linear growth improvements [29]. In support to our findings of lack of glutamine benefit on growth, most of the clinical data available have not shown growth advantages of glutamine parenteral nutrition to low-birth infants [30], although controversial findings exist. Recently, a double-blinded clinical trial has found a growth benefit of long-term (4-mo treatment) supplemented enteral nutrition on different nutritional parameters in very low birth-weight infants [31].

Our behavioral findings (delays in swim reflex) among undernourished mice in the first weeks of life are in agreement with classic studies of suckling mice reared in large litters [32]; however, our data are in disagreement with Tanaka [13]. Barros and colleagues using a low protein diet to the dams (with a formula similar to Brazilian regional diets accessible to children) also found delays of reflex ontogenesis in undernourished suckling pups [33].

Malnutrition can also disrupt the hippocampus, although a time-related vulnerability of the brain to malnutrition seems to occur; some alterations were observed only at birth (e.g., glial fibrillary acidic protein (GFAP)); others were observed on the 2nd and 15th postnatal days (e.g., ERK phosphorylation) [34].

Nutrients given by supplementation may or may not interact with each other in supporting brain development. For example, zinc supplementation given chronically to vitamin A-deficient mice improved nerve growth factor levels in the hippocampus,

Table 2
Hippocampal neurotransmitter levels and serum zinc and whole brain levels

Sample groups	Zinc				Glutamate	Aspartate	GABA	Taurine
	Serum (µg/dL)		Brain (µg of tissue/dL)		Hippocampus (µmol/g of tissue)			
	7th day	14th day	7th day	14th day	14th day			
Nourished	228 ± 12.6 (n = 7)	141 ± 4.91 (n = 16)	28.22 ± 0.56† (n = 7)	17.69 ± 0.56 (n = 8)	319.7 ± 68.08 (n = 6)	4937 ± 1135* (n = 3)	520.2 ± 738.3 (n = 5)	2480 ± 525.3 (n = 8)
PBS	202.7 ± 7.6 (n = 3)	150.7 ± 9.77 (n = 19)	23.66 ± 1.93 (n = 5)	18.08 ± 0.9 (n = 14)	538.8 ± 407.3 (n = 3)	788.5 ± 173.3 (n = 3)	117.6 ± 99.8 (n = 5)	1699 ± 820 (n = 5)
Gln	207 ± 10.7 (n = 6)	151.3 ± 10.29 (n = 19)	34.84 ± 1.29‡ (n = 6)	22.43 ± 2.28 (n = 12)	888.1 ± 250.7 (n = 6)	230 ± 102.7† (n = 6)	1194 ± 347.2† (n = 6)	2291 ± 570.3 (n = 6)
Zn	223.6 ± 9.47 (n = 7)	167.4 ± 13.3 (n = 14)	30.46 ± 2.87 (n = 7)	21.13 ± 1.63 (n = 9)	481.7 ± 74.54 (n = 5)	1525 ± 821.8 (n = 3)	702.1 ± 188.9† (n = 6)	2672 ± 299.3 (n = 7)
Zn + Gln	192 ± 6.45 (n = 6)	180.2 ± 12.4 (n = 11)	25.44 ± 1.37 (n = 7)	20.86 ± 1.07 (n = 8)	492.5 ± 112.2 (n = 6)	1047 ± 562.6 (n = 6)	134 ± 44.2 (n = 6)	2274 ± 475.2 (n = 6)

Data are presented as mean ± SEM

* $P < 0.05$ versus all other groups by one-way ANOVA and Bonferroni multiple comparison test.

† $P < 0.05$ versus malnourished PBS by unpaired Student t test.

‡ $P < 0.05$ versus malnourished PBS and Zn plus Gln-treated groups by one-way ANOVA and Bonferroni multiple comparison test.

cerebral cortex, and cerebellum extracts but did not enhance mouse memory outcomes when vitamin A was deprived [35]. Zinc deficiency during lactation in rats was found to have lasting growth deficits after dietary rehabilitation and delayed the first-week postnatal reflexes, including auditory startle, air righting, and rope descent as compared to nourished controls [36]. In our study, we have found a significant brain zinc deficiency due to litter clustering in 7-d-old mice but not later with 14-d-old. The reason might be that suckling mice have a faster growth pace at this age and are more dependent on breast milk-derived zinc to thrive. Additionally, intestinal absorption of zinc may come exclusively from the breast-milk-borne zinc-binding-protein transport, since the intestinal zinc-binding protein system is absent during the neonatal time [37]. We speculate that breast-milk restriction by litter clustering may have decreased zinc-binding proteins provided by the breast-milk and impaired zinc absorption in the pups. Zinc may especially be driven to the developing brain and this effect may compensate for the malnutrition challenge to the nervous tissue. This fact could explain the behavioral benefit of the zinc-treated groups.

Despite the lack of significant negative results regarding the MBP expression (an oligodendrocyte-derived protein) in the brains of undernourished mice compared to the nourished controls, we cannot rule out a regional effect, since our Western blot data come from the whole brain and the hippocampus from the suckling pups. Montanha-Rojas et al. found that rats submitted to malnutrition during the first half of the lactation period exhibited lower MBP expression in the temporal lobes, which was partially sustained in adulthood [38]. Gressens and colleagues giving a low protein diet (5% of casein) to early pregnant rats observed a moderate increased expression of synaptophysin in the basal ganglia of their undernourished offspring at birth and at postnatal day 7; however, they were not able to find changes in the neocortex, hippocampus, brain stem, and cerebellum in different time tables throughout the first and second week of postnatal development [39]. Our study likewise did not find changes in synaptophysin (a marker for synaptic density) in brain tissues of undernourished mice at day 14. However, we found a reduction in the hippocampal synaptophysin expression in suckling mice afflicted by malnutrition as compared with the nourished control at the same time. Furthermore, we were able to find increased synaptophysin expression among the glutamine-treated group as opposed to the undernourished PBS control. This finding deserves further clarification but may indicate an advantageous increase in the synaptic pool within the hippocampus rather than an abnormal burst of synaptogenesis, because it was found in similar levels in the nourished group. Interestingly, brain zinc levels were increased in the undernourished glutamine-treated group by a yet unknown mechanism. Interestingly, zinc is abundantly found in the hippocampal synaptic contacts, as previously stressed.

Our current data in Swiss mice provide new evidence of zinc supplementation early in life to prevent behavioral deficits due to malnutrition by restricting lactation. Zinc deficiency has been implicated to reduce the overall brain size with profound deficits in the cerebellum, including loss of cortical granular cells and immature Purkinje and basket cells [40,41]. In addition, one of the highest levels of zinc in the brain is found in the hippocampus in synaptic vesicles, boutons, and mossy fibers [42]. Improvements in motor-reflex development seen with zinc supplementation might be explained by the better plasticity in areas such as the hippocampus and cerebellum.

Breast-milk zinc levels seem to be affected by this malnutrition model, because oral supplementation to the dams improved most of the behavioral tests. Most of our findings suggest the more important role of zinc in improving early motor reflex acquisition than glutamine during suckling stage. In addition, we showed for the first time, to our knowledge, the benefit of both treatments in preventing malnutrition-associated changes in the neuronal volume.

The main morphoquantitative findings in this study were 1) CA1 neuron hypertrophy in undernourished mice; 2) increase in CA1 neuron layer volume in undernourished glutamine-supplemented mice; and 3) no malnutrition-induced changes in the total number of CA1 neurons.

CA1 neurons in undernourished mice are 92% bigger than in nourished subjects and also larger than in all supplemented mice. The size of those hypertrophied cells (1150 μm^3) is in line with that of a CA1 neuron of rat hippocampus, i.e., 1661 μm^3 [18]. That seems a compensatory mechanism to circumvent the deficiency in nutrients. Along similar lines, neuron hypertrophy may occur as a result of aging-related loss of human substantia nigra neurons, indicating cell degeneration or necrosis and characterized by perikaryon and dendrite swelling [43].

Another surprising outcome of this paper was a 28% increase in the volume of CA1 layer in undernourished glutamine supplemented mice when compared to untreated nourished and undernourished subjects, which might be partially explained by the observed trend toward increased cell number in this layer. To our knowledge, there is a lack of studies exploring the role of glutamine supplementation in brain plasticity during early development. Phosphate-activated glutaminase is present at high levels in the cerebellar mossy fiber terminals and glutamine availability may enable the synthesis of glutamate, an important excitatory neurotransmitter [44]. In addition, increased phosphate-activated glutaminase immunoreactivity was recorded in subpopulations of surviving neurons in hippocampal formation, particularly in CA1 and CA3, and in the polymorphic layer of the dentate gyrus in patients with temporal lope epilepsy [45].

In contrast to previous studies using either prenatal [46,47] or postnatal protein-deprivation models in rats [16], we have shown no reduction in the volume of CA1 layer in undernourished mice. Prenatal protein deprivation caused a 22% [46] and 17% [47] decrease in hippocampus CA1 layer volume, whereas postnatal protein deficit led to a 33% irreversible diminution in the same neuron layer.

A possible explanation for the non-reduction in CA1 layer volume may be the nature and length of our postnatal malnutrition model, which seems to be less severe than a 6-, 12-, or 18-m postnatal protein deprivation scheme [16,48].

It is widely known that protein deficiency yields an irreversible CA1 neuron loss in rats during either prenatal, e.g., 20% [46], 12% [47], or postnatal development even followed by refeeding, e.g., 120% [16] and 30% [49]. Paradoxically, this study depicted no changes in the total number of CA1 neurons, and it may be related to the malnutrition model used, which was performed using selective and time-effective nutritional interventions [50]. Similarly, a chronic food-restriction program (not protein deprivation) failed to show decrements in CA1 neuron number in rats [49].

An intriguing and remaining question is how to mechanistically explain the increase in CA1 region volume (undernourished and glutamine-supplemented mice) without changes in individual cell sizes (in the same animals). By the same token, the number of CA1 neurons in undernourished and glutamine-supplemented mice is exactly the same as that from nourished mice. Although CA1 neuron number did not change, we cannot exclude the possibility that neuron remodeling might have

occurred in these animals. Thus, further studies may focus on possible on-going neuroplasticity processes (neurons and synapses) using selective and less severe malnutrition programs as employed in this paper. In fact, thiamine re-administering reversed impaired hippocampal neurogenesis (CA1 and CA3 neurons) induced by thiamine deficiency [51].

Interesting data come from GABA levels changed by glutamine intervention in our litter size studies with Swiss mice. GABA, an inhibitory neurotransmitter in the adult brain, has been associated as an excitatory neurotransmitter in the neonatal brain, having a role with synaptic circuitry plasticity in the hippocampus [52], and may be protective in models of hippocampal injury in young mice [53]. Recently, Tamano et al. found GABA declines in the hippocampus following zinc deprivation and suggest that state of undernutrition could lead to glucose insufficiency for GABA synthesis [54]. Unexpectedly, glutamine intervention led to an early increase in zinc brain levels and may facilitate glucose bioavailability to the hippocampus. However, these findings were not accompanied by a significant behavior advantage by single glutamine treatment, suggesting that the components of our testing battery may not be revealing a consistent hippocampal-borne behavior. We are now planning Morris maze assessments in older cohort mice to evaluate this hypothesis.

Although we acknowledge that sociability issues due to different litter sizes may have a role in this model, we emphasize that crowded households and large numbers of children are often seen in shantytown communities in association with poor socioeconomic status and constitute as a risk factor for the enteric infection and malnutrition vicious cycle [55].

Conclusion

We concluded that glutamine or zinc protects against malnutrition-induced increased neuronal cell volume, increases that predispose to cell death. Glutamine also increased CA1 layer volume with a trend toward increased cell number, raised hippocampal synaptophysin expression, and transiently increased zinc brain levels. In addition, zinc improved several behavioral parameters as well as growth. These overall findings suggest the advantage of zinc and glutamine supplementation in undernourished children. More studies are needed to examine the mechanisms (e.g., apoptosis and neurogenesis in the hippocampus) related to these alterations, as well as their relevance to cognitive development in children, studies which are now being planned by our group.

Acknowledgments

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