Worldwide, millions of people are exposed to dietary imbalance that impacts in health and quality of life. In developing countries, like in Brazil, in poor settings, dietary habits, traditionally hypoproteic, are changing rapidly to western-type high-fat foods. These rapidly changing dietary habits are imposing new challenges to human health and there are many questions in the field that remain to be answered. Accordingly, we currently do not know if chronic consumption of hypoproteic (regional basic diet, RBD) or high-fat diets (HFD) may impact the brain physiology, contributing to blood-brain barrier (BBB) dysfunction and neuroinflammatory events. To address this issue, mice were challenged by breastfeeding from dams receiving standard, RBD or HFD from suckling day 10 until weaning. Immediately after weaning, mice continued under the same diets until post-natal day 52. Herein, we show that both RBD and HFD cause not only a peripheral but also a consistent central neuroinflammatory response, characterized by an increased production of Reactive Oxygen Species (ROS) and pro-inflammatory cytokines. Additionally, BBB hyperpermeability, accounted by an increase in hippocampal albumin content, a decrease in claudin-5 protein levels and collagen IV immunostaining, was also observed together with an upregulation of vascular cell adhesion molecule 1 (VCAM-1). Interestingly, we also identified a significant astrogliosis, manifested by upregulation of GFAP and S100β levels and an intensification of arbor complexity of these glial cells. In sum, our data show that dietary imbalance, related with hypoproteic or high-fat content, impairs BBB properties potentially favoring the transmigration of peripheral immune cells and induces both a peripheral and central neuroinflammatory status. Noteworthy, neuroinflammatory events in the hippocampus may cause neuronal malfunction leading to cognitive deficits and long-term persistence of this phenomenon may contribute to age-related neurodegenerative diseases.

Keywords: high-fat diet, regional basic diet, blood-brain barrier, neuroinflammation, oxidative stress, malnutrition
INTRODUCTION

According to the World Health Organization (WHO), about 45% of deaths, among children under 5 years old, are directly related to various degrees of malnutrition in poor settings of the middle- and low-income countries (1). Interestingly, in the same settings, overweight and obesity rates are escalating during early childhood, a scenario particularly evident in Brazilian favelas [shantytowns; (2)]. Characteristics of this dual burden are also seen largely elsewhere in low resource countries (3). Worldwide, surveillance data from WHO shows that 155 million pre-school children suffer from impairment in physical and cognitive development, while 41 million are overweight or obese (4). Obesity is particularly worrisome, since the impact in human health is expected to greatly increase in the coming years. This scenario raises public health concerns related to malnutrition, short and long-term illness and their increasing costs.

The effects of malnutrition may be endemic in low-income families. Many people cope with poor education, hygiene and precarious access to adequate health care, which could create a poor environment for optimal brain development (5). During the last decades obesity has reached epidemic proportions in developing countries, causing deaths and permanent sequelae (6). The magnitude of this burden, especially on children's cognitive development, is still mostly unknown.

Blood-brain barrier (BBB) is a dynamic and specialized structure composed of endothelial cells, connected with each other by tight (TJs) and adherens (AJs) junctions, and associated with pericytes, basement membrane and astrocyte endfeet, that together with microglia and neurons comprise the neurovascular units. The brain endothelium, with the lack of fenestrations and low fluid-phase endocytosis (pinocytosis), is the first “physical barrier” where intercellular complexes confer low paracellular permeability and high electrical resistance to the BBB (7). The core importance of the BBB is demonstrated by its role in the maintenance of brain homeostasis and protection against toxic compounds and blood composition fluctuations, but simultaneously providing essential nutrients for the normal brain function (7, 8). In fact, the presence of selective transporters allows the passage of specific molecules into the brain parenchyma that are essential for its function.

Malnutrition (hypoproteic diets) and obesity (high-fat diets) may be both associated with systemic inflammation (9, 10). Moreover, these effects may mount an environmental enteropathy vicious cycle (with microbiota and epigenetics changes) (11) that may lead to different levels of central nervous system (CNS) dysfunction, including BBB disruption (12) that is the gatekeeper of the brain. In addition, oxidized-LDL, which may be increased by malnutrition states (13), can induce apoptosis to brain endothelial cells (14). Despite these interesting observations, there is still scarcity of studies evaluating the impact of high-fat and low-protein diets (especially the latter one) on the BBB integrity and neuroinflammatory responses. Therefore, in this study, we bring novel data on mice BBB challenged by two models of malnutrition, based upon chronic feeding with a Brazilian northeast regional basic diet [RBD; (15)], and a high-fat content-diet (HFD) (16).

MATERIALS AND METHODS

Animals

In this study, C57BL/6j pregnant female mice from Charles River, Inc. were acclimated and fed with a standard chow diet for 6 days at the Faculty of Medicine, University of Coimbra’s vivarium, with free access to water and food. Thereafter, pregnant females were housed individually in nursery cages to give birth. Newborn suckling mice (standardized to 6–8 litter/dam) were randomized to three experimental groups, with challenged dams receiving either regional basic diet (RBD) or high fat diet (HFD), starting 10 days after birth delivery. The controls received the chow diet. The RBD is moderately deficient in proteins and fats (82% carbohydrates, 6% protein, 2% fat), while the HFD contains approximately 60% fat (21% carbohydrates, 18% proteins, 60% fat). On day 21, pups were weaned from their mothers and continued with the initial diet until 52th day of age. After anesthesia, mice were transcardially perfused with phosphate buffered saline (PBS) or with 4% paraformaldehyde (PFA) in PBS, and the hippocampi harvested for western blotting or total protein lysates were added to 140 µL of the mixed DEPPD solution and ferrous sulfate (Biotek, Synergy HT), at 505 nm. ROS levels were calculated from an optical density graph.

Degradation of Oxidative Stress Markers

Reactive oxygen species (ROS) levels in the hippocampus were measured by N, N-diethyl-pera-phenylenediamine (DEPPD) assay as previously described (17). In brief, 5 µL of hippocampi lysates were added to 140 µL of 0.1 M sodium acetate buffer (pH 4.8) at 37°C in a 96-well plate. Samples were taken in triplicate and 100 µL of the mixed DEPPD solution and ferrous sulfate at a ratio of 1:25 was added to each well to initiate reaction. The microtiter plate was then incubated at 37°C, for 5 min, and absorbance was measured by a spectrophotometer plate reader (Biotek, Synergy HT), at 505 nm. ROS levels were calculated from a calibration curve and expressed as hydrogen peroxide (H2O2) equivalent (1 unit = 1.0 mg H2O2/L). The calibration curve for standard solution was obtained by calculating slopes from an optical density graph.

Abbreviations: WHO, World Health Organization; BBB, Blood-brain barrier; RBD, Regional Basic Diet; HFD, High Fat Diet; PFA, Paraformaldehyde; PBS, Phosphate buffered saline; PVDF, Polyvinylidene difluoride membrane; DEEPPD, Diethyl-pera-phenylenediamine; H2O2, Hydrogen Peroxide; TBA, Thiobarbituric Acid Reactive; MDA, Malondialdehyde; PND, Pos-natal day; GFAP, Glial Fibrillary Acidic Protein; MPO, Myeloperoxidase.
The thiobarbituric acid reactive-species (TBARs) assay was used to assess products of lipid peroxidation, via malondialdehyde (MDA) (17). Briefly, 100 µL of tissue supernatant were incubated at Room Temperature (RT) in the dark for 1 h in a TBA solution together with butylhydroxytoluene (BHT; Sigma-Aldrich) and a catalyst (Iron III chloride; Sigma-Aldrich). Afterwards, samples were incubated at 95–100°C for 60 min and followed by butanol extraction. The supernatants were read spectrophotometrically at 532 nm (Biotek, Synergy HT) and the concentration of MDA was calculated with respect to a calibration curve using 1,1,3,3-tetramethoxypropane (Sigma-Aldrich) (range: 0.1–83.5 µM). Results were expressed as µM/mg of hippocampal tissue.

**Enzyme-Linked Immunosorbent Assay**

Blood samples were withdrawn by cardiac puncture into BD Vacutainer SST Tubes (BD Bioscience, Franklin Lakes, NJ, USA). Serum was separated by centrifugation at 1,100 x g for 15 min and stored at −80°C until analysis. The released levels of IL-1β, TNF-α, and IL-10 from the three animal groups were quantified by using an ELISA Ready-SET-Go kit (eBioscience, San Diego, CA, United States), as specified in the datasheet. The results were expressed as pg/mL.

**Western Blotting**

Hippocampal tissue was processed for western blotting according to standard procedures (17, 18). Briefly, the tissue was homogenized in lysis buffer (0.15 M sodium chloride, 0.05 M Tris-base, 0.005 M ethyleneglycoltetraacetic acid, 0.5% sodium deoxycholate, 0.1% SDS and 1% X-Triton, pH 7.5) supplemented with protease inhibitor cocktail tablets (Roche Applied Sciences). Total protein content was quantified using the bicinchoninic acid method (BCA; Pierce) and stored at −20°C until further use. Afterwards, the processed samples were boiled (at 95°C) in sample buffer (Tris-HCl 0.5 M, 10.4% SDS pH 6.8) for 5 min. Different concentrations of sample, containing specific proteins, have been analyzed. Each sample was submitted to electrophoresis in polyacrylamide gel (10% and 12%), proteins, have been analyzed. Each sample was submitted to electrophoresis in a polyacrylamide gel (10% and 12%), followed by incubation with alkaline phosphatase-conjugated secondary antibody anti-goat IgG (1:10,000, GE Healthcare), anti-rabbit (1:20,000, GE Healthcare), or anti-mouse (1:10,000, GE Healthcare), 1h at RT. Internal control for protein content in each sample was evaluated by mouse anti-GAPDH (1:10,000, Sigma-Aldrich). Targeted protein bands were visualized following membrane incubation with enhanced chemifluorescence (ECF, Amersham) using a Typhoon FLA 9000 (GE-Healthcare Biosciences) and quantified using Image Studio software (version 5.2, Licon).
(mean body weight = 4.4 ± 0.3 g), until day 52 of age. Results showed that until post-natal day (PND) 25, all groups present a similar development. After that, we observed that control group developed, as expected, with a slow and sustained weight gain, reaching a mean value of 14.9 ± 0.2 g (332.7 ± 4.9% of control). Regarding RBD group, the body weight slightly increased over time reaching 10.6 ± 0.05 g (248.5 ± 1.2% of control) at 52 days of age, whereas there was a marked increase in body weight gain for the HF diet group reaching 26.2 ± 0.2 g (605 ± 6.1% of control; Figure 1).

Afterwards, we evaluated several inflammatory mediators in blood serum of these animals. First, we observed a significant increase in lipid peroxidation products (measured by MDA reaction products) in HFD animals (Figure 2A; 15.3 ± 0.2 μM/mg; P < 0.001), with no effect on RBD diet animals (Figure 2A; 2.18 ± 0.1 μM/mg). Regarding pro-inflammatory cytokines, there was an increase in both TNF-α and IL-1β serum levels not only in RBD (Figures 2B,C; 16 ± 2.5 pg/mL TNF-α, P < 0.001; 8.9 ± 1.5 pg/mL IL-1β, P < 0.05; respectively), but also in HFD animals (Figures 2B,C; 12.5 ± 1.6 pg/mL TNF-α P < 0.01; 11 ± 1.6 pg/mL IL-1β, P < 0.01, respectively). Moreover, the serum levels of the anti-inflammatory cytokine IL-10 decreased with both diets (Figure 2D; RBD 7.8 ± 0.2 pg/mL IL-10, P < 0.05; HFD 6.5 ± 0.4 pg/mL IL-10, P < 0.01). Such results clearly show that both diets induce a peripheral pro-inflammatory profile.

It has already been demonstrated that blood composition fluctuations can have an impact on brain function. Therefore, we also evaluated the inflammatory status of hippocampi of the animals fed with both diets. In fact, we observed a significant increase in ROS production (Figure 3A; RBD 648.5 ± 4.7, P < 0.001; HFD 964.3 ± 23.3, P < 0.001) and TNF-α protein levels (Figure 3B; RBD 137 ± 10% of control, P < 0.05, HFD 153 ± 7.6% of control, P < 0.01) by both diets. Regarding IL-1β of its protein levels with HFD and only a tendency, yet not significant, by RBD diet (Figure 3C, 119 ± 3.5% of control, P < 0.01). Overall, we concluded that both diets triggered a peripheral inflammatory state that was accompanied by a concomitant hippocampal neuroinflammation.

The (neuro)inflammatory processes have been described as key players in BBB disruption and transmigration of peripheral immune cells into brain parenchyma (7, 20). To evaluate BBB alterations induced by RBD and HFD, we measured the protein levels of albumin in brain tissue, since this blood-borne protein does not cross the BBB under normal conditions. Interestingly, albumin content in the hippocampus was significantly increased in mice chronically exposed to RBD (Figure 4A; 139.3 ± 10.5%; P < 0.05) and HFD (Figure 4A; 179.4 ± 25.3%; P < 0.01), indicating a hyperpermeability of BBB triggered by both diets. To further unravel the transport across the BBB that is being altered, we analyzed the protein levels of endothelial claudin-5 and caveolin-1 (Cav-1), the latter has been associated with brain injury-related BBB breakdown (21). The transport at the BBB can occur through paracellular and transcellular pathways, with the first being controlled by intercellular complexes present between

![Figure 1](image1.png)  
**Figure 1** | Effect of regional basic (RBD) and high-fat (HFD) diets on mice body weight from postnatal day 10 to postnatal day 52. Data were analyzed by using two-way ANOVA followed by Dunnett’s multiple comparisons test. *P < 0.0001 and **P < 0.0001 vs. nourished control group (CTR). The results are shown as mean ± SEM.

![Figure 2](image2.png)  
**Figure 2** | Effect of regional basic (RBD) and high-fat (HFD) diets on inflammatory mediators. (A) Malondialdehyde (MDA) formation, a marker of oxidative stress in hippocampal tissue, is only increased in HFD animals. ELISA quantification on blood serum protein levels of pro-inflammatory cytokines (B) TNF-α and (C) IL-1β shows an upregulation after chronic exposure to both diets. Additionally, ELISA quantification of the anti-inflammatory cytokine (D) IL-10 shows a significant decrease of its protein levels in both diet feeding animals. Data were analyzed by using Kruskal-Wallis test, followed by Dunn’s post-test for multiple comparisons, n = 5–8. *P < 0.05, **P < 0.01, and ***P < 0.001 vs. nourished control group (CTR). The results are shown as mean ± SEM.
adjacent endothelial cells, such claudin-5 a tight junction (TJ) protein, and the second pathway occurs through the endothelial cell and involves the formation of vesicles, including caveolin-1-coated vesicles. Herein, we observed a significant downregulation of claudin-5 protein levels (Figure 4B) in both RBD (63.5 ± 6.7%, P < 0.01) and HFD animals (56.8 ± 6.5%, P < 0.01), suggesting an increase in paracellular permeability of the BBB. After that, we investigated the effect of both diets on Cav-1 and concluded that only HFD was able to induce a significant upregulation of its protein levels (Figure 4C, 131 ± 6.5%, P < 0.05). Further, to corroborate the weakening of BBB we also evaluated alterations in collagen IV (coll IV) immunoreactivity (Figures 4D,E; 47,997 ± 5,101, P < 0.001, when compared with the control 23,784 ± 2,412) in the hippocampus. RBD had also an effect on both parameters analyzed, but not so significant when compared to HFD (Figure 5B, 135 ± 8.2% of control, P < 0.05; Figure 5C, 36,195 ± 3,758, P < 0.05, when compared with the control 23,784 ± 2,412). Moreover, when the protein levels of S100β, a specific marker of astrocytes reactivity, were measured, an upregulation was observed after exposure to both diets (Figure 5D; RBD 131 ± 4.79% of control, P < 0.05, HFD 149 ± 11.9% of control, P < 0.01). Taking into consideration the importance of a simultaneously analysis of cellular morphological alterations, we further concluded that both diets induced an increase in the total length of astrocytic processes (Figure 5F; CTR 70.2 ± 3.6 µm; RBD 88.8 ± 5.5 µm, P < 0.05; HFD 88.6 ± 5; P < 0.05), and promoted an arbor complexity of these cells (Figure 5G; P < 0.05). Curiously, only in HFD animals was possible to observe an increase of the total number of astrocytic processes (Figure 5E; CTR 4.1 ± 0.1; RBD 4.3 ± 0.1; HFD 5.1 ± 0.3; P < 0.01).

**DISCUSSION**

In recent years, a westernization of diets, with an increase in fat content, on developing countries has been observed, and has a huge socio-economic impact (24). Despite several preclinical studies exploring this issue, there is a scarcity of information regarding the impact of protein malnutrition on BBB properties. The BBB is known as the gatekeeper of the brain, being extremely selective and so protecting the brain from variations in blood composition and toxins. Nevertheless, it has also a role in providing essential nutrients for proper brain function. Thus, BBB dysfunction may lead
FIGURE 4 | Effect of regional basic (RBD) and high-fat (HFD) diets on blood-brain barrier properties. An increase of albumin (A) and a decrease of claudin-5 (B) protein levels were observed with both RBD and HFD diets. (C) Regarding caveolin-1 (Cav1) protein levels, there was also a significant increase of its total levels in HFD group. (D) Representative immunohistochemistry images for Collagen IV (green, brain vessels), and Hoechst 33342 (blue, nuclei). Scale bar = 40 µm. (E) DRB and HFD induced a significant decrease in collagen IV immunoreactivity. Moreover, both diets induced a significant upregulation in (F) VCAM-1 (vascular cell adhesion molecule-1). Above the bars, representative western blot images of Albumin (66 kDa), claudin-5 (25 kDa), caveolin-1 (20 kDa), VCAM-1 (110 kDa), and GAPDH (37 kDa) are shown. Data were analyzed by using Kruskal-Wallis test, followed by Dunn’s post-test for multiple comparisons, n = 5–7. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 vs. nourished control group (CTR). The results are shown as mean ± SEM.

to brain damage and indeed is known to be a common feature in all neurodegenerative diseases. Moreover, under aggression related with trauma, ischemia, metabolic disorders or malnutrition, neuroinflammatory cascades are initiated involving glial cells and molecular pathways that may interfere with BBB function (12, 17, 18, 25). In fact, a chronic long-term inflammatory environment in the brain usually leads to chronic impairment of the BBB, which will contribute to long-term neurodegeneration and neuropathology, including neurodegenerative conditions like Alzheimer’s disease (26).
FIGURE 5 | Effect of regional basic (RBD) and high-fat (HFD) diets on astrocytes properties. Representative immunohistochemistry images for (A) GFAP (red, astrocytes), and Hoechst 33342 (blue, nuclei). Scale bar = 40 µm. Both diets induced an increase in (B) GFAP protein levels, (C) immunoreactivity, and (D) S100β protein levels. Yet, HFD had a more pronounced effect than RBD. Regarding morphological alterations, only HFD increased the (E) total number of processes, with no effect on RBD animals. However, both diets induced a significant increase in (F) the total length of such processes and (G) arbor complexity. Results are expressed as mean ± S.E.M., n = 5–7 [for (B,D)], n = 10 [for (C,G)], n = 30 [for (E,F)]. Data were analyzed by using Kruskal-Wallis test, followed by Dunn’s post-test for multiple comparisons. *P < 0.05, **P < 0.01, and ***P < 0.001 vs. nourished control group (CTR). A two-way ANOVA followed by Bonferroni’s Multiple comparison test was used for Sholl analysis, where *P < 0.05 and **P < 0.01 vs. CTR.

In addition, long-term undernutrition (27), similar to our RBD and HFD feeding protocol (28, 29), induces a systemic inflammation with brain deleterious effects, being the hippocampus one of the most susceptible brain regions (29). Curiously, calorie intake, meal frequency, texture and content seem to be associated with modification in hallmarks of hippocampal physiology and cognitive performance associated with neurogenesis (30). It was recently shown that a preexisting protein malnutrition worsens the motor deficits observed after stroke (31). Moreover, post-ischemia protein malnutrition can aggravate neuroinflammatory processes and inhibits neuroplasticity in the hippocampus (32). Accordingly, in our
chronic model of malnutrition or high-fat content diet we also observed both peripheral and central inflammatory markers, with an increase in both ROS production and pro-inflammatory cytokines.

Long-term feeding with RBD (27, 33) and HFD (34) may lead to intestinal barrier leakage with increased gut-to-blood bacterial translocation, and continued LPS systemic circulation. Alterations in the gut microbiota toward more LPS-releasing Gram-negative bacteria by both diets may contribute to low-grade systemic inflammation with brain deleterious effects (27, 28), including BBB disruption and oxidative stress (35). In addition, systemic inflammation could lead to BBB dysfunction by increased myeloperoxidase (MPO)-releasing neutrophil activity (36). This potential MPO release from circulating blood neutrophils may be due to increased LPS binding to the endothelial surface of the brain capillaries (37). Interestingly, rats challenged with cecal ligation and puncture (as a model of sepsis) showed increased BBB permeability to Evans Blue brain staining and reduced endothelial claudin-3 and claudin-5 levels, effects that were ameliorated by the cholesterol-lowering drug simvastatin (38).

It is also known that systemic inflammation can lead to BBB dysfunction by increasing the activity of proinflammatory cytokine-releasing neutrophils (36), white blood cells that are an essential part of the innate immune system. In fact, it has been reported that HFD contributes to BBB disruption either in young and aged mice, as shown by increased permeability of brain microvessels to IgG and to sodium fluorescein and changes in tight junction proteins including occludin and claudin-5 (39, 40). Further, the same authors concluded that HFD triggers neuroinflammation, as evaluated by reactive microglia and release of pro-inflammatory cytokines (40). Moreover, in an animal model of HFD for 9 weeks, an increase in leukocyte transmigration into the brain parenchyma was observed (41). Accordingly, in the present study we showed a downregulation of collagen IV, the most important protein of the BBB basement membrane, and the tight junction protein, claudin-5, which is a strong indicator of BBB dysfunction. Moreover, there was an increase in VCAM-1 protein levels, an adhesion molecule specifically expressed in endothelial cells and necessary to immune cells transmigration into the brain parenchyma. Therefore, hippocampal Cav1 immunoreactivity was increased, suggesting active endothelial transcytosis, further supporting the BBB hyperpermeability induced by the diets. Thus, we showed that both RBD and HFD feeding protocols induced a very robust (neuro) inflammatory process as well as a BBB hyperpermeability.

Glial cells, such as astrocytes and microglia, are involved in neuroinflammatory responses playing also an important role in BBB function. Tucsek et al. (40) concluded that HFD triggers neuroinflammation, demonstrated by activated microglia and release of pro-inflammatory cytokines (40). In addition, mice chronically feeding with a HFD has been associated with increased oxidative stress and GFAP in the cerebral cortex and in the hippocampal dentate gyrus with impaired recognition memory (42). These data support our observation that particularly HFD chronic consumption causes a significant astrocyte reactivity in the hippocampus, as shown by increased expression of GFAP and S100β. Nevertheless, RBD had also an effect but not so strong as with HFD. Curiously, Feoli et al. (43) did not observe alterations in hippocampal GFAP and S100β immune contents on the 60th postnatal day in undernourished rats. Such contradiction can be explained by differences related with diet feeding protocols and animal strains. Still, the authors observed an increase of S100β levels in the cerebrospinal fluid (43).

The evaluation of astrocytic morphological alterations is of crucial importance to understand the role of these glial cells on the brain function. Thus, in order to stratify the possible different types of astrocytes, a classification of A1 and A2 astrocytes has been arisen by Ben Barres lab based on different gene expression profiles (44–46). Both A1 and A2 astrocytes are reactive glial cells, in which A1 type seems to be induced by exposure to IL-1α, TNF-α and complement proteins (45). Therefore, we can hypothesize that our malnutrition models are shifting astrocytes to an A1 profile. Accordingly, we observed a significant astrogliosis proved by an increase of GFAP and S100β expression, and by significant morphological alterations of astrocytes. Still, a genomic analysis is necessary to correctly classified the type of reactive astrocytes.

In summary, the present report shows that RBD and HFD chronic feeding impacts BBB permeability, and leads to brain oxidative stress and neuroinflammation with a prominent role of astrocytes. Systemic inflammation was also observed and may underly central alterations. However, more studies are warranted to dissect the mechanisms associated with these effects.

**AUTHOR CONTRIBUTIONS**

CdA, RL, LO, and VC-S: performed the experiments and wrote the paper; RG and CR: provided scientific advice and revised the paper; JM, AS, and RO: designed the scientific concept, provided financial support, wrote and revised the paper.

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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