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Retinal development impairment and degenerative alterations in adult rats subjected to post-natal malnutrition

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ABSTRACT

Background: The early stages of central nervous system (CNS) development are extremely important. Key events such as neurogenesis, gliogenesis, synaptogenesis, and ontogenesis occur. Malnutrition promotes alterations in CNS development, including the retinal development. During retinal development, malnutrition can induce a delay in some important events, such as neurotransmitter expression and neurogenesis.
Methodology/Principal findings: Postpartum Wistar rats were fed either a commercial diet or a multideficient diet. Pups were breastfed by these rats, and from PND21 were kept with the same diet until PND45. We investigated the effects of malnutrition on adult retinal tissue with regard to (1) endogenous gamma-amino butyric acid (GABA) release induced by excitatory amino acids (EAAs) and (2) the expression of cellular markers related to degenerative events, such as reactive gliosis, microglial activation, cell proliferation and cell death. Endogenous GABA release induced by EAAs was higher in the retina of malnourished rats. The Müller cell population was reduced and displayed alterations in their phenotype profile compatible with reactive gliosis. The expression of glutamine synthetase and markers of cellular proliferation were higher in the retina of malnourished rats. Additionally, retinal dysplasia-like structures were present, indicating disturbance in the cell cycle machinery.
Conclusion/Significance: The current study provides evidence that the adult retina shows degenerative processes induced by long-term malnutrition during the postnatal development. These findings have high clinical significance with regard to the identification of possible targets for interventions in malnourished patients.

1. Introduction

Malnutrition is a worldwide problem that affects millions of children during the most vulnerable stages of development. Inadequate food intake is a major non-genetic factor that can affect the central nervous system (CNS) (Wainwright, 2001) because CNS development depends on multiple metabolic nutrition-related reactions (Morgane et al., 1993; Rao and Jacobson, 2004). Malnutrition reduces the total number of neuronal and glial cells and alters synaptic connections and myelination (Almeida et al., 2005; Guedes et al., 1996). Neurotransmitters and neuromodulators are synthesized from food-derived precursors, and malnutrition can therefore affect the quantities of these basic functional elements.

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L-glutamate and gamma-amino butyric acid (GABA) are, respectively, the major excitatory and inhibitory neurotransmitters in the CNS, including the retina (Caruso et al., 1989; Massey and Redburn, 1987; Mosinger et al., 1986; Yazulla et al., 1986). The vertebrate retina is formed by six neuronal cell types—rod, cone, horizontal, bipolar, amacrine and ganglion cells— and a major glial cell type—Müller cells (Young, 1985). The diversity of studies on the retinal cell types and molecules involved in synaptic transmission, including neurotransmitters, provides a robust platform for the use of this tissue as an appropriate model for the study of both CNS neurotransmission and pharmacology as well as for understanding its own circuitry and its role in visual processing.

Neurotransmitter expression plays an important role during retinal development (Martins et al., 2006; Martins and Pearson, 2008) with important implications for the appropriate functioning of the mature retina. In adult vertebrate retinal tissue, glutamate mediates neurotransmission along the vertical pathway, which consists of photoreceptors and bipolar and ganglion cells (Das et al., 2006; Kalloniatis and Fletcher, 1993; Massey and Redburn, 1987). This pathway is responsible for the first processing of visual information and for projections to the central visual regions in the brain through the ganglion cells axons. There are three classes of glutamate ionotropic receptors: those activated by *N*-methyl-D-Aspartate (NMDA), those activated by alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and those activated by kainate (KA). In the GABAergic system, GABA in the rat retina is expressed by displaced and topic amacrine cells and by approximately 6% of ganglion cells. These cell populations have an important role in modulating glutamatergic signaling (Caruso et al., 1989; Vaughn et al., 1981).

In situ stimulation of the retina by glutamate or its agonists causes the release of endogenous GABA in monkey, chicken and opossum retinas (Andrade da Costa et al., 2000; Calaza et al., 2001, 2006; Calaza Kda et al., 2003). This phenomenon is believed to be induced by the stimulation of NMDA and non-NMDA ionotropic receptors (Andrade da Costa et al., 2000; do Nascimento and de Mello, 1985; Moran and Pasantes-Morales, 1983; Schwartz, 1987; Tapia and Arias, 1982; Yazulla, 1983; Yazulla et al., 1986). This pharmacological approach can be very useful for identifying the $GABA^{+}$ cell population sensitive to ionotropic glutamate receptor stimulation as well as for assessing possible alterations in tissue excitability.

Appropriate neurotransmission and maintenance of excitability homeostasis largely depend on glial cells. Müller cells are a specialized type of glial cell only found in the retina. This cell population is implicated in several physiological processes, including neuronal signaling, neuropigment recycling, and neuroactive and vasoactive factor release (Bringmann et al., 2006). Similar to other glial cells, Müller cells exhibit reactive gliosis, displaying changes in their morphology, and function in non-physiological situations. In some cases, Müller cells exhibit a proliferative type of reactive gliosis (Ahmad et al., 2011, 2000; Das et al., 2006; Dyer and Cepko, 2000, 2001; Lewis et al., 2010) in which newly generated cells can differentiate into resident retinal cell types (Fischer and Reh, 2003). This proliferation can be mediated by the disinhibition of cyclin D3; after injury, the expression of $p27^{kip1}$, a Cip/Kip cyclin-dependent kinase inhibitor protein, decreases, and a subset of Müller cells re-enters the cell cycle and proliferates (Dyer and Cepko, 2000).

Reactive gliosis is primarily characterized by changes in gene expression (Ridet et al., 1997; Roesch et al., 2012). The upregulation of intermediate filament glial fibrillary acidic protein (GFAP) expression is one of the first steps observed in the retina after some injury events. This upregulation is a hallmark of reactive gliosis (Dyer and Cepko, 2000; Nork et al., 1986). Additional changes can also be observed, such as the upregulation of nestin, glutamine synthetase and microglia activation (Bringmann et al., 2006). Microglia is present throughout the CNS, including the retina. Microglia have phagocytic capacity and act as an immune-competent cell type with strong involvement in responses to lesions (Wohl et al., 2011).

The retinal tissue may suffer various deleterious effects of malnutrition, such as the delayed expression of acetylcholine, GABA and calcium-binding proteins (Almeida et al., 2001) and the neurogenesis of retinal GABAergic cells (Almeida et al., 2001; Silveira et al., 2007). These alterations have been observed during development, but there are no data on the deleterious effects on adult retinal tissue after malnutrition during the postnatal development. The purpose of this study was to identify the morphological and functional alterations that occur in the adult rat retina with a multideficient diet. Specifically, we aimed to investigate the effects of stimulating ionotropic glutamate receptors on the retinal GABAergic cell population and to characterize morphological alterations related to degenerative events induced by malnutrition.

2. Materials and methods

2.1. Animals

Fifty-six Wistar rats (UFRJ animal facility) were used. All procedures were performed in accordance with the guidelines for the use of animal experimental research published by the Brazilian Society of Neuroscience and Behavior (SBNeC) and the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications). All procedures were reviewed and approved by the Committee for Animal Care and Use of the Centro de Ciências da Saúde—Universidade Federal do Rio de Janeiro (CCS/UFRJ)—CEUA protocol # IBCCF133). Animals were housed under a 12-h light/dark cycle at $23 \pm 2\,^{\circ}C$. An intraperitoneal injection of sodium pentobarbital (50 mg/kg) was used for euthanasia. Eyes were enucleated, and the ocular globes were hemisected at the cornea level. The posterior eyecup containing the retina was removed.

2.2. Experimental groups and diet composition

In this study, two groups of pregnant Wistar rats were submitted to different diets: (1) control group in which rats were submitted to standard diet during the whole period. (2) malnourished group in which rats were submitted to RBD diet from the birth day on. After birth, pups from these different groups were breastfeeding up to PND21 and then kept in the same diet condition—control or malnourished—until PND45. For the retinal dysplasia analysis a set of animals were kept until PND60.

The components of these diets are described in Almeida, 2001 (Almeida et al., 2001). Briefly, the control diet contained 23% protein from vegetable and animal origin (Labina, Purina Alimentos Ltda, Brasil), and the multideficient diet (regional basic diet—RBD Table 1) contained low levels of protein (7.87%), fat (0.95% compared to 8.52% in the control diet) and essential and non-essential amino acids and high levels of carbohydrates and fiber. The two diets were isocaloric (Teodosio et al., 1990).

2.3. Treatment of the retinal tissue with excitatory amino acids (EAAs)

Retinas from control and malnourished animals were separated into two different groups: “stimulated retinas” and “non-stimulated retinas”. After enucleation and dissection, retinas were kept in a saline solution containing 125.0 mM/L NaCl, 2.5 mM/L KCl, 2.0 mM/L $CaCl_2$, 25.0 mM/L $NaHCO_3$, 1.25 mM/L NaH_2PO_4, 25.0 mM/L glucose, and 10.0 mM/L $MgCl_2$ and were continuously aerated with 95% O_2 and 5% CO_2. For experiments in which NMDA was used, the solution was magnesium-free and con-