Effects of chronic food stress on morphometry and expression of nuclear organizing regions in the adult rats hippocampus

Chronic food stress on morphometry and expression of agnor in the rats hippocampus

Efeitos do estresse alimentar crônico na morfometria e expressão das regiões de organização nuclear nos ratos adultos hipocampo

Testemunho crônico de alimentação sobre morfometria e expressão do agnor no hippocampus das taxas

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ABSTRACT
The aim of this study was to verify the immediate and late effects of chronic food stress on the expression of Nuclear Organizing Regions (NORs) in hippocampal neurons. Twenty Wistar rats were separated into two groups: test (n = 10) and control (n = 10). Food stress started from the 60th day of life and lasted for a month. After this time the animals were anesthetized, euthanized and had their hippocampus dissected. The obtained hippocampus were histologically processed, stained by the HE technique for morphological description and by the AgNOR technique for NOR analysis. From each image the total number of neurons, the number of neurons with NOR in Dispersion (NND), the total number of NORs (NNOR), and then performed a ratio of NNOR by the total number of neurons to obtain the number Of NOR by neuron (NNN). Of the analyzed variants, the number of neurons was higher (p <0.001) in the stressed group (41.98 ± 17), when compared with the control group (33.57 ± 14). In addition, NND was also higher in the stressed group (4.523 ± 4.04) than the control group (1.4 ± 2.20) with p <0.001. Thus, we have that chronic food stress increases the number of granular neurons in the hippocampus in rats as well as increases the number of NOR in dispersion.

Keywords: Neuron, Hippocampus, Stress, Food restriction, Morphometry.

RESUMO
O objetivo deste estudo era verificar os efeitos imediatos e tardios do estresse alimentar crônico sobre a expressão das Regiões Organizadoras Nucleares (NORs) nos neurônios hipocampais. Vinte ratos Wistar foram separados em dois grupos: teste (n = 10) e controle (n = 10). O estresse alimentar começou a partir do 60º dia de vida e durou um mês. Após este tempo, os animais foram anestesiados, eutanizados e tiveram seus hipocampos dissecados. Os hipocampus obtidos foram processados histologicamente, corados pela técnica HE para descrição morfológica e pela técnica AgNOR para análise NOR. De cada imagem o número total de neurônios, o número de neurônios com NOR em dispersão (NND), o número total de NORs (NNOR), e então realizou uma proporção de NNOR pelo número total de neurônios para obter o número de NOR por neurônio (NNN). Das variantes analisadas, o número de neurônios foi maior (p <0,001) no grupo de estresse (41,98 ± 17), quando comparado com o grupo de controle (33,57 ± 14). Além disso, NND também foi maior no grupo de estresse (4,523 ± 4,04) do que no grupo de controle (1,4 ± 2,20) com p <0,001. Assim, temos que o estresse alimentar crônico aumenta o número de neurônios granulares no hipocampo em ratos, assim como aumenta o número de NOR em dispersão.

Palavras-chave: Neurônio, Hipocampo, Estresse, Restrição alimentar, Morfometria.
1 INTRODUCTION

The term stress can be defined as a state generated by the perception of stimuli that provoke emotional arousal and by disrupting a homeostasis, trigger an adaptation process characterized, among others, the increase of adrenaline secretion producing several systemic manifestations with physiological and psychological disorders (Herman and Cullinan, 1997; Schwabe et al, 2011). One of the physiological boots promoted by stress is the increase in the release of glucocorticoids (GCs) that are mainly responsible for the long-term responses promoted by stress (Schwabe et al, 2011).

Several experimental studies have been shown that dietary restriction and altered palatable dietary supply have been used as models to provoke eating disorders (Hagan et al., 2002; Corwin et al., 2011). These studies demonstrate that it is a presence of the food known as palatable to the animal, but without access to consumption and a stressful factor that raises corticosterone levels and alternately to periods of fasting and capable of provoking "binge eating" (Cifani Et al, 2009).

It does not refer to the central nervous system (CNS), several areas are vulnerable, an exhibitor of stressors, among which we highlight the hippocampus. The hippocampus contains high levels of GC receptors, which are more vulnerable to stressful events, both during the perinatal period and in adult life, and may lead to short- and long-term changes (Joel, 2008; Récamier-Carballo et al., 2017). Steroids related to stress affect the hippocampus in several different ways, where we evidence a reduction of neural proliferation in the gyrus-neurogenesis (Lathe, 2001; Herman and Muller, 2006; Schoenfeld et al., 2011). A NOR, which marks nucleolar organizing regions (NORs). These NORs contain genes that encode ribosomal RNA and have a peculiar affinity for silver, allowing their clear distinction and publication in cytological and histological preparations stained with silver nitrate, receiving a name of AgNORs (Derezine et al., 2000).

In the central nervous system, the use of AgNOR served to verify the negative effects of ethanol on the neurons, where the reduction of NORs was contacted (Gos et al., 2010). This reduction was also evident in Puree cells from neonates who suffered sudden perinatal death (Lavezzi et al., 2017). In addition, it was verified that the presence of NORs is related to a neural activity (Healy-Stoffel et al., 2012). Taking these data together, this study aimed to evaluate the immediate effects of chronic food stress on the density of granular hippocampal neurons and to analyze an expression of NORs neurons.
2 MATERIALS AND METHODS

2.1 ANIMALS AND EXPERIMENTAL GROUPS

Wistar rats were used from the colony of the vivarium of the Nutrition Department of the Federal University of Pernambuco. The animals were kept in an animal room under suitable conditions of temperature and humidity and water and feed ad libitum. At 60 days of age, the experimental groups were delineated according to the incidence of chronic stress in a stressed group (N = 10) and control group (N = 10).

2.2 CHRONIC FOOD STRESS

At 60 days after birth, submission to stress was started in the stressed group for one month. On the first day the animals had access to a palatable diet (30% labina, 55% nutela® and 15% water). On the second day the diet was offered, however in a container that allowed only visual and olfactory stimuli, but without access to consumption. On the third day, the animals were fasted for 24 hours. On the fourth day, they received the standard diet of the vivarium, being therefore considered a day of rest. The four steps were repeated until they reached the age of 90 days. After this period the animals were euthanized and had their hippocampus dissected and processed histologically.

2.3 EUTHANASIA AND BIOLOGICAL PROCESSING

To perform the euthanasia the animals were anesthetized with ketamine (60 mg / kg body weight) and xilasin (7.5 mg / kg body weight). The effect of the anesthetic was confirmed by the absence of the grip reflexes. This was performed with the animal lying down with the ventral region facing upwards and properly fixed by the anterior limbs on appropriate surface. From the xiphoid process, a V-shaped cut was performed on the musculature and ribs, opening the chest cavity so that it exposed the heart allowing access to the left ventricle, where the infusion cannula was inserted, which was attached to the Area by a Keller forceps.

By moving away from the lungs, the descending aorta was pinched, thus preventing all of its irrigation area from being perfused. 100 ml of saline solution (NaCl, 0.9%) were infused initially at room temperature for blood vessel removal, preventing clot formation and providing correct fixative penetration into tissues. 400 ml of fixative solution (4% paraformoldehdye, pH 7.4, at 4 ° C) was then infused. The attachment of the fixator to the region of the anterior limbs was evidenced by the contraction of these.

At the end of the infusion, the encephalons removed, the hippocampi were dissected and immersed in the same fixative solution for 24 hours. Then the hippocampi were submitted to
dehydration in alcohol solutions with increasing concentrations of 70 to 100%, diaphanized in xylol and paraffinized. For each fragment of hippocampus were made semi-serial cuts with thickness 5μm. The slides obtained were stained by haematoxylin and eosin as by the AgNOR staining technique.

2.4 AGNOR STAINING

For the AgNOR staining, the sections were pretreated in acetic acid / ethanol solution (1: 3) for five minutes, washed with absolute ethanol (three times) and left in standard deionized water for 15 minutes. They were then dried with filter paper and received the aqueous solution of silver nitrate (50%) mixed with 2% gelatin solution (2% in 1% formic acid). The silver solution was incubated for 30 minutes, at room temperature and in an oven at 45 °C.

From each photomicrograph obtained, the Number of Neurons with NOR in Dispersion (NND), the total number of point NORs per visual field (NNOR) and the number of NOR per neuron (NNN) were quantified. NND cells were considered whose NORs were scattered throughout cytoplasm, which characterizes high protein synthesis activity (Figure 1).

Figure 1: Photomicrography of the hippocampus stained by the AgNOR technique at 100x magnification. A- Control group; B- Stressed group. The arrows indicate some neurons that present NOR in dispersion.

2.6 HISTOMORPHOMETRIC ANALYSIS

The histological images of the slides were captured by digital camera coupled to the optical microscope, under fixed focus and field clarity, obtaining 10 fields per slide with final magnification of 400X.
2.7 ANALYSIS OF RESULTS

To perform the final analysis of the data, a comparison was made between the microscopic morphology of the granular neurons of the dentate gyrus of the hippocampus collected from the test and control groups. Student's t-test and Mann-Whitney test were used to compare the groups.

3 RESULTS

3.1 HISTOMORPHOMETRIC ANALYSIS

The results show that among the analyzed variables, the number of granular neurons was higher (p <0.001) in the stressed group (41.98 ± 17 - Student t test) when compared to the control group (33.57 ± 14).

3.2 ANALYZES OF NUCLEAR ORGANIZING REGIONS

The blades stained by the AgNOR technique showed morel and yellow coloration, with clear intensity in the cytoplasm and dark in the nucleus. The NORs were verified as dark spots inside the nucleus (Figure 1). Among the parameters measured, the number of neurons with NORs in NND dispersion showed significant differences (p <0.01) between the groups, being higher in the stressed group (4,523 ± 4,04) when compared to the group Control group (1.4 ± 2.20 - Mann-Whitney), as shown in table 1.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Number of Neurons</th>
<th>NND</th>
<th>NNOR</th>
<th>NNN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control Group</td>
<td>33.57 ± 14</td>
<td>1.4 ± 2.2</td>
<td>84,042 ± 24,035</td>
<td>1.2 ± 0.140</td>
</tr>
<tr>
<td>Stressed Group</td>
<td>41.98 ± 17*</td>
<td>4,523 ± 4,04**</td>
<td>87,928 ± 28,261</td>
<td>1.16 ±0.131</td>
</tr>
</tbody>
</table>

(*) p<0.001, student t test; (**) p<0.001, Mann-Whitney test.
4 DISCUSSION

In our work, we verified that chronic stress is capable of promoting morphological changes in the hippocampus of rats, increasing the number of neurons and NOR in dispersion. The increase in the number of neurons may be related to the increase of the neurogenesis or to the increase of the lifetime of these neurons. Most studies have suggested that stress is an event inducing reduction in hippocampal neurogenesis, which would decrease the number of neurons (Schoenfeld et al., 2011 and Mineur et al., 2007; Yang et al., 2011). However, there is evidence that some types of stress are ineffective in reducing neurogenesis in rats (Nicola et al., 2011).

Research suggests that dietary restriction, ie the imposition of periods without food, has neuroprotective effects against excitotoxic brain damage (Bruce-Keller et al., 1999; Sharma and Kaur, 2005; Manzanero et al., 2014). Neuroprotection promoted by dietary restriction arises as a consequence of increased expression of Derived Brain Neurotrophic Factor (BDNF). BDNF is known for its neurostimulating effect, whose action causes the newly generated cells to differentiate into neurons (Cameron et al., 1998). Further evidence of the neuroprotective effect of fasting was demonstrated by Kurmar et al. (2009), who found that this dietary restriction also protects neurons against the toxic effects of pilocarpine, a chemical compound used to induce epileptic seizures and consequently decreases the intensity of neurogenesis (Kurmar et al., 2009).

In addition to dietary restriction, one of the factors that may have been responsible for the findings in this research may have been the offer of a palatable diet. Palliative foods, so-called comfort foods, are able to attenuate the effects of induced stress by reducing neuroendocrine responses, such as reduced glucocorticoid release (Dallman et al., 2003; Zeeni et al., 2013; Ortolani et al. Et al., 2014). Added to this, the insertion of the capped pot with palatable diet may have functioned as a visual and olfactory food stimulant, causing the animal to explore the object in an attempt to access the palatable food and receive its food reward. Studies have shown that exploratory activity and enriched environment correlate positively with neurogenesis and plasticity of the hippocampus (Monteiro et al., 2013, Freund et al., 2015). In addition, olfactory and gustatory stimuli are currently known to activate the basolateral amygdala, which presents dense communications with the hippocampus, participating in the process of memory formation of new odors and flavors (Azuma et al., 1984; Nishijo et al. Cain and Bindra et al., 1972; Cain, 1975).

Thus, it is possible that the increase of the protein synthesis and the number of neurons in the hippocampus of the animals that undergo the food stress is an evolutionary adaptation to the food acquisition, involving neural circuits intrinsic and extrinsic to the hippocampus.
We concluded that the type of food stress used increases the neural density in the hippocampus of rats as well as the number of neurons with dispersed NOR immediately after the stressor event.

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