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Title Page

Hippocampal biomarkers of fear memory in an animal model of generalized anxiety disorder

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Running title: Biomarkers of fear memory in an animal model of generalized anxiety

Abstract

Generalized anxiety disorder (GAD) is highly prevalent and incapacitating. Here we used the Carioca High-Conditioned Freezing (CHF) rats, a previously validated behaviorally selected animal model for GAD, to identify biomarkers and structural changes in the hippocampus that could be part of the underlying mechanisms of their high-anxiety profile. Spatial and fear memory was assessed in the Morris water maze and passive avoidance test. Serum corticosterone levels, immunofluorescence for glucocorticoid receptors (GR) in the dentate gyrus (DG), and western blotting for hippocampal brain derived neurotrophic factor (BDNF) were performed. Immunohistochemistry for markers of cell proliferation (bromodeoxiuridine/Ki-67), neuroblasts (doublecortin), and cell survival were undertaken in the DG, along with spine staining (Golgi) and dendritic arborization tracing. Hippocampal GABA release was assessed by neurochemical assay.

Fear memory was higher among CHF rats whilst spatial learning was preserved. Serum corticosterone levels were increased, with decreased GR expression. No differences were observed in hippocampal cell proliferation/survival, but the number of newborn neurons was decreased, along with their number and length of tertiary dendrites. Increased expression of proBDNF and dendritic spines was observed; lower ratio of GABA release in the hippocampus was also verified. These findings suggest that generalized anxiety/fear could be associated with different hippocampal biomarkers, such as increased spine density, possibly as a compensatory mechanism for the decreased hippocampal number of neuroblasts and dendritic arborization triggered by high corticosterone. Disruption of GABAergic signaling and BDNF impairment are also

proposed as part of the hippocampal mechanisms possibly underlying the anxious phenotype of this model.

Keywords: fear memory; anxiety; adult hippocampal neurogenesis; BDNF; dendritic arborization; dendritic spines.

1. Introduction

Anxiety disorders, such as generalized anxiety disorder (GAD), are among the most prevalent psychiatric conditions [1]. GAD is characterized by generalized, chronic and excessive worry and anxiety, accompanied by disruptive somatic symptoms and cardiovascular changes [2]. It has been associated with significantly reduced quality of life [3], and it is estimated to be as incapacitating as depressive disorders [4]. Effective interventions require the investigation of the underlying structural and functional psychobiology of this condition, with appropriate animal models being a key tool for this.

As proposed for psychiatric disorders in general, GAD is a multifactorial condition, with disruptive functioning of different brain areas – such as the prefrontal and cingulate cortex, as well as the amygdala [5] - possibly participating together in its etiology and development. Furthermore, hippocampal abnormalities in GAD patients have also been proposed [6]. In rodents, some of the neurobiological aspects demonstrated to be involved in anxiety also include those related to hippocampal function and plasticity, such as components of the GABAergic and glutamatergic systems [7], the expression of brain-derived neurotrophic factor (BDNF) [8], spinogenesis [9], as well as adult hippocampal neurogenesis (AHN) [10]. In addition, anxiety has been linked with elevated glucocorticoid levels [11], which have also been associated with reduced dendritic arborization in the hippocampus [12].

The hippocampus is a crucial structure for contextual fear learning, which is in turn considered an appropriate paradigm for establishing animal models of GAD [2, 13,

14]. The present work investigated biomarkers of hippocampal plasticity in the Carioca High-Conditioned Freezing rats (CHF), an established and validated animal model of generalized anxiety. The CHF rodent population is an experimental group behaviorally selected for high freezing response in contextual fear conditioning [15]. A previous work established the CHF as a behaviorally validated model for the study of spontaneous high-anxiety [16], as differences were found only in anxiety-related paradigms and not in the forced swimming test of depression or the object recognition test, used as a paradigm for measuring cognitive skills. However, spatial and fear memory had yet to be investigated in this model. For these purposes, in the present work animals were exposed to the Morris water maze (MWM) and passive avoidance test (PAT). Importantly, the neural aspects underlying their anxious phenotype remained unclear. To investigate some of the possible hippocampal-related aspects underlying this model, 5-bromodeoxiuridine (BrdU) and Ki-67 were utilized as markers of proliferating cells in the dentate gyrus (DG), and immunohistochemical analysis of doublecortin (DCX) was performed to label neuroblasts [17] and to characterize dendritic arborization of these newly born neurons. BrdU positive cells in hippocampal sections of animals sacrificed 5 weeks after the last injection of this marker were taken as a measure of newborn cell survival.

As corticosterone levels are believed to modulate hippocampal function, serum corticosterone and expression of glucocorticoid receptors (GR) in the DG were determined. In addition, levels of hippocampal precursor proBDNF were identified and expression of dendritic spines was evaluated, as well as GABA release.

We propose that generalized anxiety can result in part from multiple structural and biochemical plastic mechanisms in the hippocampus possibly orchestrated to

protect the individual from chronic exposure to high corticosterone levels, but leading to facilitation of fear memory retention.

2. Materials and Methods

2.1. Animals

Experimental procedures followed the Brazilian Society of Neuroscience and Behavior (SBNeC) guidelines, in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications). Handling and methods of sacrifice were approved by the Committee for Animal Care and Use of the CCS/UFRJ (protocol # IBCCF002). Experimental animals (Carioca High-Freezing [CHF], a line of Wistar rats selectively bred for their high conditioned freezing response in contextual fear learning) were obtained as described previously [15]**.** Briefly, albino Wistar rats were exposed to the contextual fear conditioning paradigm, and selectively bred for differences in defensive freezing behaviour in response to the conditioned context. Significant differences in freezing response were acquired after three generations of selective breeding (S3). In order to preserve the spontaneous differences between the experimental and the control groups, the animals used herein were not exposed to contextual fear conditioning or any aversive stimuli prior to experimentation. However, other individuals from all breeding generations were tested in the contextual fear conditioning paradigm for confirmation of the fear trait across generations. Breeding generations S9, S13 and S14 were used in the present study (Fig. 1).

INSERT FIGURE 1 HERE

The control group (CT) was composed of the offspring of randomized cross-breeding populations, thus encompassing animals with high, low and average conditioned freezing responses. Two- to three-month-old males were used, kept in acrylic cages (31 cm x 38 cm) in groups of 6 under a 12 h light/dark cycle, with food and water *ad libitum*. The animals' dark/light cycle was inverted one week prior to behavioral testing, and procedures occurred during their dark cycle. Animals were used once in each experiment, except for those exposed to the MWM, which underwent the PAT 3 days after the MWM probe trial. Since both groups were exposed simultaneously to the same environmental conditions and experimental design, it is expected that baseline differences between the two groups are preserved.

2.2. Behavioral Analysis

2.2.1. Morris water maze

A polyethylene tank (1200 mm diameter x 500 mm height) and crystal acrylic platform (130 mm diameter x 300 mm height) were used. Experimental procedures followed those described elsewhere [18]. To verify locomotor activity, animals (n = 12 CHF/ 12 CT) were individually placed in the tank with transparent water and allowed to swim for 2 minutes or until they reached the platform. Five trials were performed, each with the platform placed in different locations; latency was registered. On days 2 - 6, animals performed 5 trials, each with different starting points. During these, water in the tank received non-toxic white paint treatment, and the platform was placed 2 cm below

water level. On day 7, the platform was removed and the probe trial was performed. Animals' performance was recorded and later analyzed by AnyMaze software (version 4.84).

2.2.2. Passive avoidance test

The apparatus consisted of an aluminum arena containing a safe platform (200 mm x 75 mm) and a grid area (200 mm x 225 mm) where electrical stimulation could be administered. On day 1, each animal (n = 19 CHF/ 18 CT) was placed in the platform and a 0.5 mA footshock was administered for 1s every time it stepped down. The amount of footshocks received until the animal remained in the platform for 2 uninterrupted minutes was registered (acquisition index). On day 2, each animal was placed again on the platform and the latency to step down was registered (retention index). Maximum time established = 120 s.

2.3. Neurobiological Analysis

2.3.1. BrdU administration

Animals received three intraperitoneal injections of BrdU (100 mg/kg) administered at 10 mg/ml, approximately every 16 h. Animals were perfused 24 h (n = 08 CHF/ 05 CT) or 5 weeks (n = 03 CHF/ 03 CT) after the last injection.

2.3.2. Euthanasia and brain sectioning

Animals used for immunohistochemistry were anesthetized and perfused through the left ventricle of the heart with saline and 4 % paraformaldehyde in 0.1 M phosphate buffer. Brains were removed and cryoprotected in 30 % sucrose for 1 week. Serial 40 μm coronal brain sections were obtained on a cryostat and stored in TBS-AF

(tris buffered saline + anti-freezing solution of 0.05 % sodium azide, 30 % glycerol and 15 % sucrose) at 4 °C.

2.3.3. BrdU immunohistochemistry

Sections were washed (n = 08 CHF/ 05 CT for analysis of cell proliferation; n = 03 CHF/ 03 CT for analysis of cell survival), exposed to 1 % hydrogen peroxide for 30 minutes, washed again and kept for 30 minutes at 37 °C in 2 N HCl, following 2 x 10-minute immersions in 0.1 M borate buffer at room temperature. Sections were washed and exposed to 3 % normal horse serum (NHS) (Vector Laboratories, S-2000) for 2 h. Sections were incubated overnight with anti-BrdU primary antibody (Serotec MCA2060B; 1:500 in 3 % NHS), washed, and incubated with goat anti-rat secondary antibody (Vector Laboratories, BA-9400; 1:250 in 3 % NHS) for 2 h. Sections were washed, and incubated in ABC complex (Vector Laboratories, PK-6100, Vectastain® Elite ABC-Peroxidase Kits; 1:1,000) for 2 h. Following washes, the reaction was revealed by diaminobenzidine (DAB) (Sigma, D5637-5G). Slides were mounted in Entellan (Merck, HX075822).

2.3.4. Ki-67/ DCX immunohistochemistry

Sections were washed, exposed to 1 % hydrogen peroxide for 30 minutes, washed and kept for 2 h in 15 % NHS. Sections were incubated overnight with anti-Ki-67 primary antibody (ABCAM, AB16667; 1:500 in 10 % NHS) (n = 08 CHF/ 08 CT) or anti-DCX primary antibody Santa Cruz, SC-8066; 1:200 in 10 % NHS) (08 CHF/ 08 CT). Sections were washed, and incubated with horse anti-rabbit biotinylated secondary antibody (Vector Laboratories, BA-1100; 1:200 in 10 % NHS), in the case of Ki-67, or

with peroxidase horse anti-goat antibody (Vector Laboratories, PI-9500; 1:200), for DCX staining. Finally, Ki-67 sections were washed, and incubated in ABC 1:1000 for 2 h. Revelation and mounting followed procedures described for BrdU immunostaining.

2.3.5. Stereological analysis

Cells were counted by stereology [19], in a 1-in-6 series of sections (240µl apart), starting rostrally and progressing caudally, through a 40x objective. The DG was traced using a semiautomatic stereology system (StereoInvestigator, MicroBrightfield) and a 2.5x objective. For each section, the system was set to randomly place a 50 µm x 50 µm counting frame throughout the traced DG. Only those stained cells that were either within the counting frame or touching the green border of the counting frame were counted. The total number of positive cells was estimated using the optical fractionator method, by relating the number of cells counted to the DG sectional volume.

2.3.6. Corticosterone radioimmunoassay

Animals (n = 10 CHF/ 10 CT) were euthanized by decapitation, and blood was collected for hormone concentration analysis. Serum was obtained after centrifugation of the blood at 1,200 g for 20 minutes and stored at -20 °C. Serum corticosterone was determined using a specific coated tube RIA kit; intra- and inter-assay coefficients of variation were 4.0 - 12.2 % and 4.8 - 14.9 %, respectively, and sensitivity was 5.7 ng/mL (Coat-a-Count Rat Corticosterone, Siemens Medical Solutions Diagnostics). Procedures followed the manufacturer's recommendations.

2.3.7. GR immunofluorescence

Sections were washed and exposed to 5 % bovine serum albumin (BSA) for 1 h, following overnight incubation with anti-GR primary antibody (Santa Cruz GR (H-300): sc-8992; 1:200) (n = 09 CHF/ 12 CT). Sections were then washed and incubated with anti-rabbit secondary antibody (Alexa 555; 1:500) in a dark chamber. Slides were mounted in N-propyl galate and imaged through a 40x objective with a fluorescent microscope (Zeiss Axioskop, Axiovision software). GR expression in the DG was analyzed with ImageJ software (http://rsbweb.nih.gov/ij/) and expressed as Corrected Total Cell Fluorescence (CTCF).

2.3.8. Dendrite visualization and quantitative morphometric analysis

Granule neurons expressing DCX were imaged using a 40x objective, and micrographs were acquired using a Zeiss AxioCam MR Rev3 camera, following procedures previously described [20]. Dendrites were traced, categorized and analyzed using the NeuronJ plugin for ImageJ (http://rsbweb.nih.gov/ij/) by a blinded experimenter (8 neurons traced per animal; n = 08 CHF/ 08 CT).

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2.3.9. Dendritic spine density

Animals (04 CHF/ 04 CT) were euthanized by decapitation, and brain hemispheres underwent Golgi impregnation method (Kit FD Rapid GolgiStainTM - PK401), following the manufacturer’s instructions. Sections were obtained in a cryostat (100 µm; -22 ºC). After staining completion, slides were mounted in Entellan, and kept protected from light. Tertiary dendritic shafts (8 per animal) were visualized using a 63x objective (Axiovert 35 microscope) and imaged by a Zeiss Axiocam MRm camera.

Individual spines were distinguished by contrast adjustment and quantified in ImageJ software.

2.3.10. BDNF expression

Hippocampi of both groups (n = 04 CHF/ 04 CT) were dissected and transferred to lysis buffer. The tissue was sonicated (3 cycles of 10 s at 50 Hz) and protein concentration was determined [21]. Samples were diluted in lysis and sample buffer to a final concentration of 3µg protein/µl, and heated for 5 minutes at 100 °C. Samples were loaded on a 10 % polyacrylamide gel and later transferred to a PVDF membrane at 10 V for 40 minutes, using a semi-dry system (Bio-Rad 170-3940). Next, the membrane was washed, blocked for 2 h with 2 % BSA, and incubated overnight with anti-BDNF primary antibody (Santa Cruz (N-20): sc-546, 1:1,000). After washing, it was incubated for 2 h with anti-rabbit HRP-conjugated secondary antibody (Sigma, A0545, 1:10,000). For the loading control, the membrane was stripped with glycine buffer for 30 minutes at room temperature, blocked with 5 % milk for 30 minutes, washed and incubated with anti-α-tubulin primary antibody for 30 minutes (Sigma, T5168, 1:50,000). Next, it was washed and incubated for 30 minutes with anti-mouse HRP-conjugated secondary antibody (Sigma, A5278, 1:5,000). All reactions were detected by chemiluminescence (Millipore, Luminata® system).

2.3.11. GABA release

Hippocampi of both groups (n = 05 CHF/ 06 CT) were dissected and sectioned (400 µm). Loading was made with reaction solution (GABA 100 µM + ^{3}H-GABA 0.25 µCi). Slices were washed 3 x with Hank`s at 37 °C. The supernatant was collected in 6 series, with 5 - minute intervals. Two Hank's washing series were collected, as well as

two with high potassium Hank's, and the last two with Hank's. Total GABA is represented by lysate GABA + released GABA. Radioactivity was quantified by liquid scintillation counting.

2.4. Preparation of figures

Digital images were created by using GIMP 2.8.6 (GNU Image Manipulation Program; www.gimp.org) and assembled into final figures. General adjustments of contrast and brightness were made where appropriate.

2.5. Statistics

Data were analyzed in GraphPad Prism 5 using Student's t-test for unpaired samples (expressed as mean ± S.E.M), one- and two-way analysis of variance (ANOVA) with post-hoc Bonferroni test. Differences were considered significant when $p \leq 0.05$.

3. Results

3.1. Spatial learning is preserved in CHF rats

A reliable model of anxiety should only feature aspects directly related to the phenotype it intends to reproduce. Therefore, to strengthen the characterization of the CHF as a strict model for anxiety without signs of cognitive degeneration/impairment, animals were tested in the MWM. With regard to the latency to reach the invisible platform, there was a significant effect of day ($F_{(4,352)} = 11.3739$; $p < 0.0001$), but no effect of groups ($F_{(1,13)} = 0.1039$; $p = 0.752$) or of the interaction ($F_{(4,352)} = 0.8974$; $p = 0.466$) (Fig. 2A), showing that both groups similarly improved their performance

along the test days and trials. Accordingly, there was a significant effect of total distance travelled with regard to day ($F (4,568) = 26.6464$; $p < 0.0001$), but not to groups ($F (1,22) = 3.1891$; $p = 0.088$) or to the interaction ($F (4,568) = 0.7712$; $p = 0.544$) (Fig. 2B), suggesting that both CT and CHF rats present decreased distance travelled along trials (Fig. 2C), and thus are not impaired in this spatial learning task.

INSERT FIGURE 2 HERE

No differences were observed between CT and CHF rats with regard to the number of entries in the target zone in the probe trial (7.833 ± 0.4578 CT, n = 12; 10.17 ± 1.296 CHF, n = 12; $p = 0.1037$) (Fig. 3A), demonstrating that spatial memory is preserved in the anxious group, despite their chronic stress phenotype. Interestingly, the latency to enter the target zone was significantly decreased among CHF animals (3.858 ± 0.7718 s CT, n = 12; 1.900 ± 0.2923 CHF, n = 12; $p = 0.0268$) (Fig. 3B). They also spent less time in this quadrant (23.45 ± 1.307 CT, n = 12; 17.84 ± 1.373 CHF, n = 12; $p = 0.0073$) (Fig. 3C), showing more exploration of other locations of the tank when facing the absence of the safe platform. No differences were observed in the visible platform trials (Supplementary data S1).

INSERT FIGURE 3 HERE

3.2. CHF rats display enhanced fear memory

The enhanced ability to recall aversive information in emotional contexts is a phenomenon observed among anxious patients [22, 23]. To test the hypothesis of enhanced ability to form and/or store aversive memories among CHF animals, the PAT was used. No difference was observed in the number of shocks administered on day 1 (2.611 ± 0.2003 CT, n = 18; 2.211 ± 0.1636 CHF, n = 19; p = 0.1287) (Fig. 4A), showing similar capacity for aversive learning in both groups. However, CHF animals presented a significantly increased latency to step down from the platform (64.94 ± 12.57 s CT, n = 18; 107.7 ± 7.096 s CHF, n = 19; p = 0.0049) (Fig. 4B), demonstrating enhanced rate of fear memory retention.

INSERT FIGURE 4 HERE

3.3. Basal serum corticosterone levels are increased in CHF animals

Although corticosteroids do not directly regulate emotional behavior, they are believed to weaken or strengthen certain neural pathways, leading to altered behavioral outcomes [24] [25]. As shown in Fig. 5A, basal corticosterone levels were significantly higher in CHF animals (118.9 ± 27.97 CT; 339.0 ± 49.38 ng / mL CHF; n = 10; p = 0.0011), consistent with the anxious behavioral profile displayed by the experimental group.

INSERT FIGURE 5 HERE

3.4. CHF rats have decreased expression of GR in the DG

Considering that, at high circulating levels, corticosteroids enhance the acquisition, conditioning and consolidation of stressful experiences via GR [26], analysis of this receptor expression was performed. A significant decrease was verified in the DG of CHF animals (CTCF, 3,604 ± 298.3 CT, n = 12; 2,385 ± 266.5 CHF, n = 9; p = 0.0085) (Fig. 5B), probably resulting from a negative feedback loop from the increased corticosterone levels.

3.5. Hippocampal cell proliferation and survival are not altered in CHF rats

A reduction in AHN has also been proposed to underlie dysfunctionality in anxiety disorders [27]. As a first step to investigate this aspect in CHF rats, quantification of cell proliferation markers in the DG was performed. As shown in Fig. 6A, no differences were found in the total estimated number of BrdU+ (cells / mm^3: 3,061 ± 483.0 CT, n = 5; 3,545 ± 396.3 CHF, n = 8; p = 0.4592) or Ki-67+ cells (cells / mm^3: 2,097 ± 214.7 CT, n = 8; 2,563 ± 291.1 CHF, n = 8; p = 0.2184). In addition, quantification of BrdU+ cells in animals sacrificed 5 weeks after the last injection revealed no differences in cell survival between groups (cells / mm^3: 2,721 ± 542.6 CT, n = 3; 2,519 ± 341.2 CHF, n = 3; p = 0.7693) (Fig. 6B). This shows that the behavioural profile observed in the CHF group may be due to other mechanisms rather than cell proliferation and survival in the DG.

INSERT FIGURE 6 HERE

3.6. Hippocampal number of neuroblasts is decreased in CHF rats

Analysis of DCX, a microtubule-associated protein expressed exclusively in immature neurons, was performed to verify differences in newborn neurons. Indeed, DCX quantification revealed a decrease in the number of neuroblasts in the DG of CHF rats (cells / mm^3: 16,310 ± 1630 CT, n = 8; 11,870 ± 1115 CHF, n = 8; p = 0.0411) (Fig. 7), suggesting a possible decrease in AHN in the CHF group.

INSERT FIGURE 7 HERE

3.7. Neuroblasts in CHF rats display altered dendritic morphology

Given that the dendritic field largely defines the synaptic input of the cell [20], and thus, its functionality, the dendritic architecture of these young neurons was analyzed. As shown in figure 8, the analysis of dendrite number and length revealed alterations in the dendritic morphometry of newly born neurons of CHF animals, especially in total dendrite number (6.938 ± 0.3164 CT, n = 64; 5.719 ± 0.2611 CHF, n = 64; p = 0.0036, fig. 8A) and length of dendrites (µm: 336.9 ± 17.21 CT, n = 64; 289.2 ± 13.75 CHF, n = 64; p = 0.0324, fig. 8B). Further investigation revealed that decreased dendritic arborization was due to reduced tertiary dendrite number (3.906 ± 0.3147 CT, n = 64; 2.703 ± 0.2604 CHF, n = 64; p = 0.0038, fig. 8A) and length (µm: 183.1 ± 16.90 CT, n = 64; 138.3 ± 14.77 CHF, n = 64; p = 0.0482, fig. 8B). This parallels with studies showing an association between stress and modifications in dendritic architecture,

especially dendritic retraction in the hippocampus [12, 28-31], and may suggest a protective response to reduce the contact surface of the neuron with the neurochemical environment altered by the chronic stress condition these animals present.

No differences were seen in the length of primary dendrites (µm: 40.96 ± 3.598 CT, n = 64; 38.14 ± 3.393 CHF, n = 64; p = 0.5691, fig. 8a) or in the number (2.031 ± 0.02192 CT, n = 64; 2.016 ± 0.01563 CHF, n = 64; p = 0.5627, fig. 8a) or length (µm: 112.5 ± 7.136 CT, n = 64; 112.1 ± 6.952 CHF, n = 64; p = 0.9706, fig. 8b) of secondary dendrites.

INSERT FIGURE 8 HERE

3.8. CHF animals have more dendritic spines in the DG

Dendritic arborization, however, is not the only important morphological indicator of the ability of a neuron to receive synaptic inputs. Another important aspect is the expression of dendritic spines, micro-specializations of the dendritic shafts to establish functional contacts with other cells. Alterations in spine density are associated with changes in synaptic strength [32], especially in the context of excitatory synapses [33], which are crucial for memory formation [34-36]. In contrast to what was observed for dendritic architecture of neuroblasts, quantitative analysis of dendritic spines in tertiary branches of DG neurons revealed that CHF rats displayed higher dendritic spine linear density compared to the CT group (spines / 10 µm: 14.64 ± 0.4105 CT, n = 32; 17.10 ± 0.5231 CHF, n = 32; p = 0.0005) (Fig. 9), supporting the hypothesis that the

anxious group displays enhanced hippocampal machinery for certain plasticity-related functions.

INSERT FIGURE 9 HERE

3.9. Expression of hippocampal proBDNF in the CHF group is increased

Other parameters of hippocampal plasticity were also investigated, in the search for neural aspects that could, at least partly, explain the enhanced fear memory and anxiety traits displayed by the CHF model. One of these parameters was hippocampal BDNF expression, known to underlie newborn cell survival, synaptic formation and plasticity [37]. A significant increase in hippocampal expression of proBDNF (32kDa) was observed in CHF animals (0.1731 ± 0.05977 CT, n = 4; 1.107 ± 0.1659 CHF, n = 4; p = 0.0018) (Fig. 10), a feature that could contribute to the preserved cell survival rates found and for the enhanced retention of fear memory if it also reflects increased cleavage into mature BDNF.

INSERT FIGURE 10 HERE

3.10. The hippocampus of CHF animals release less GABA under stimulation with K^+

Since the GABAergic system exerts a classic inhibitory effect, with its receptors being the target of widely used anxiolytic drugs [38], it was considered important to investigate some functional aspect of this system in the CHF model. For this, a neurochemical assay of GABA release was performed. Results showed that, under stimulation with K^+, CHF hippocampal samples released less GABA than those from CT (2.953 ± 0.3489 CT, n = 6; 1.668 ± 0.2151 CHF, n = 5; p=0.0155) (Fig. 11). This is strong evidence for a decreased inhibitory activity in the hippocampus of the CHF group, which may be part of the mechanism underlying the highly anxious behavioural profile previously reported [16].

INSERT FIGURE 11 HERE

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4. Discussion

Although GAD is highly prevalent and incapacitating, the neural correlates underlying its symptoms remain unclear. Previously, the behavioral aspects of CHF rats, an experimental model for GAD, have been investigated [16]. This group spontaneously displays an anxious phenotype in the elevated plus-maze and social interaction tests, with no differences in the forced swimming test of depression, or in the declarative memory assessed by the object recognition test. Additionally, no differences in the total

number of cells in the dorsal hippocampus were found. However, the involvement of the hippocampus in risk assessment, the cognitive component of fear memory, and in contextual fear conditioning [39] indicated the need for additional investigations of this brain structure in the CHF. Moreover, further assessment of cognitive and fear memory was considered crucial for strengthening the characterization of the model.

Confirming previous data on locomotor activity, visual perception and cognitive learning [16], the MWM showed similar performance of CHF and CT rats in all days of visible platform testing (Supplementary data S1). Interestingly, in the probe trial, CHF animals presented decreased latency to enter the target zone, which could indicate enhanced ability to retain information on the environment, especially in aversive conditions lacking escape alternatives. Also, CHF animals spent less time in the target quadrant, a parameter that could indicate enhanced perception of the environment as threatening or even some difficulty to adapt to it, since after all trials, some environmental habituation would be expected. This hypothesis of increased memory in emotional contexts was explored in the PAT, where CHF rats presented enhanced retention index. Taken together, both the MWM and PAT confirmed that the learning ability of CHF animals is preserved, but memory retention in emotional contexts is enhanced. Interestingly, the tendency to associate stimuli in stressful conditions is a feature consistently observed in anxious patients [22, 23].

Components of the hypothalamic-pituitary-adrenal axis (HPA) are among the most important biomarkers of stress in rodents [40], and particularly in anxious rat lines [41]. For instance, high-anxious rats from a line selected in the elevated plus-maze exhibited increased secretion of adrenocorticotropic hormone and corticosterone [42, 43]. The higher HPA response in conditioned, fear-selected rats [42] suggests that

hyperanxious animals may over-interpret certain stimuli, a behavior that also occurs in psychiatric patients. Corticosterone, likely acting via GR in the hippocampus, also plays a facilitatory role in acute freezing behavior [26], which is one of the behavioral characteristics of the CHF line [15]. Here, we report that CHF rats exhibit significantly higher levels of corticosterone, a feature that correlates with the decreased expression of GR in their DG.

Besides alterations in the HPA axis, reduced hippocampal volume has been observed in anxiety and mood disorders [44]. However, this aspect was found to be unaltered in CHF animals (Supplementary data S2), despite the reduced hippocampal dendritic arborization of neuroblasts observed. It is possible that the maintenance of cell proliferation and survival could have contributed to the preservation of hippocampal volume. This hypothesis is supported by previous data showing that over-expression of BDNF in the amygdala and hippocampus correlates with high anxiety and unaltered volume [8]. Additionally, we previously reported that the total number of hippocampal cells was similar between CHF and CT [16], pointing to more molecular, rather than macro-structural, alterations in this model. Accordingly, we investigated cellular markers in the context of AHN, and showed that cell proliferation and survival are not altered among CHF animals. This could, at least in part, explain the non-depressive profile of these animals [16], since differences in BrdU labelling have been consistently correlated with depression models [45, 46].

On the other hand, the number of neuroblasts was found to be decreased in CHF rats. Interestingly, a reduced number of newly born hippocampal neurons in aged rats was shown to be sufficient for fear learning [47], which might also be applicable in our model. In fact, it can be hypothesized that a reduced number of neurons is not only

sufficient for fear conditioning in the CHF rats but is one of the neural features that could be responsible for their strong fear memory retention and behaviorally anxious phenotype. It has been shown that early life stress can lead to altered dendritic morphology and enhanced contextual fear learning in maternally deprived animals [48]. Additionally, under high levels of corticosterone, even long-term potentiation (LTP) in the DG of these stressed animals was facilitated. Considering these, alterations in neurogenesis, synaptogenesis, neurotransmission and/or the neuroendocrine system could modulate and enhance fear memory, leading animals to exhibit increased fear responses. Strong evidence for this comes from the findings of dendritic spine expression and decreased GABAergic inhibition in the hippocampus. Opposite roles for pro- and mature BDNF have been proposed [49], with the former being associated with long-term depression and apoptosis [32], and the latter with enhanced cell survival and plasticity [50]. If the increased expression of proBDNF observed in the CHF hippocampus reflects increased availability for cleavage into mature BDNF, it could also contribute for the hypothesis that the over-expression of certain biomarkers of hippocampal plasticity could result in enhanced fear memory retention and anxiety. Although further studies are needed to confirm the involvement of each of these aspects with fear and anxiety in the CHF rats, other features, including LTP and the involvement of brain regions like the amygdala and medial pre-frontal cortex, might be present and could interact, resulting in the spontaneously anxious phenotype observed in this rat line. Increased gliogenesis and/ or faster maturation of granule neurons could also take place in the DG of the CHF group, given the findings of decreased number of neuroblasts with unaltered rates of cell proliferation and survival showed herein. In this sense, further assessment of neurogenesis/ gliogenesis by double-labeling of BrdU+NeuN (NeuN – neuronal nuclei, marker of mature neurons) and BrdU+GFAP

(GFAP - glial fibrillary acidic protein, marker of astrocytes) is still needed to confirm this hypothesis and contribute for the understanding of the anxious phenotype observed in the CHF model.

An association between anxiety and decreased cell proliferation/ survival, and spinogenesis has been consistently proposed [51]. However, this overall reduction of structural plasticity appears not to fully link with the increased fear learning observed in clinical and experimental anxiety. Here, we show that increased anxiety does not always correlate with reduced cell proliferation/survival and spine expression. Furthermore, we propose that given that natural compensatory mechanisms must take place in the brain to prevent the deleterious effects of glucocorticoids [52], dendritic arborization could be seen as a buffering process by the hippocampal system to avoid cell loss [44]. Consistent with previous data on stress and fear [53], decreased dendritic arborization in the CHF rats was associated with an increase in spine density . Along with other mechanisms such as decreased GABAergic inhibition, this increase in spinogenesis could, in turn, facilitate fear memory retention and anxiety. Thus, the findings presented here challenge our current view of decreased cell proliferation/survival and spinogenesis as biomarkers underlying all kinds of fear/anxiety-related conditions. In turn, it is proposed that the variability and complexity of anxious/fear traits must be addressed by the view that a differential expression of biomarkers must accompany the particular features of each set of behaviors modeled by a given paradigm.

Although much remains to be unraveled, the findings reported here represent consistent indices for some novel neurobiological understanding of generalized anxiety in the context of hippocampal plasticity. Moreover, the particular features found may contribute to the important concept that a more complete view of symptoms observed in

clinical practice must come from different paradigms, each modeling specific anxiety-related behaviors through specific neural substrates.

5. Conclusion

The data presented herein reinforces the CHF group as a consistent model for the study of anxiety and fear, since these are the only behavioral aspects found to be differentially expressed by the experimental group. In addition, some candidates to compose part of the neurobiological basis underlying the vulnerability of this group to higher levels of anxiety were identified. Among these, it can be highlighted the increased levels of corticosterone, accompanied by decreased expression of GR in the DG, reduced number and arborization of hippocampal neuroblasts, as well as disrupted inhibitory signaling in the hippocampus. Unexpectedly, cell proliferation and survival in the DG were unaltered and spine density in the DG was found to be increased. Additional assays aiming to identify the levels of mature BDNF and the role of proBDNF within the context of anxiety are needed. Together, these data suggest that some biomarkers – such as decreased spines - usually found in stress models cannot always apply to anxiety. Future studies are needed to further explore and confirm the specific neurobiological features - in the hippocampus and in other brain structures - that confer the fear trait of this model, so that it can be used as a tool for the screening of novel translational interventions for generalized anxiety.

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Conflict of interest

The authors declare that they have no conflicts of interest.

Figure Captions

Fig. 1. Experimental design. In a previous work, albino Wistar rats have been selected for their high-conditioned freezing response in the contextual fear paradigm; after three breeding generations (S3), significant differences were observed between high- and low- freezing animals, and the experimental group used herein was named the Carioca High-Conditioned Freezing rats [15]. In the present work, breeding generations S9, S13 and S14 were used. Animals that were exposed to the contextual fear conditioning paradigm for confirmation of the fear-trait phenotype in each given generation were not used in the experiments described here but their offspring. In this way, spontaneous differences between groups could be preserved and baseline behavioral and neurobiological differences could be identified, as opposed to differences due to the stress of undergoing the fear conditioning paradigm.

Fig. 2. Spatial learning was assessed by the invisible platform test in the MWM. Latency to reach the platform in this spatial learning task of the MWM along the test days is shown in (A) and the total distance travelled in (B). There was no significant difference between groups in any trial, suggesting that spatial learning here is preserved in the anxious group (n = 12CT; 12

CHF). The pathway travelled by a CT and CHF animal in the beginning and end (after 72 trials) of hidden platform testing in the MWM is plotted in (C). Note that the pathway travelled by both animals, representing their respective groups, decreases after trials. *MWM* Morris Water Maze, *s* seconds, *m* meters, *CT* control, *CHF* Carioca high-conditioned freezing

Fig. 3. Spatial memory parameters were assessed in the probe trial of the MWM. Bar graphs show mean ± S.E.M number of entries in the target zone for CT (black) and CHF (grey) in (A). There was no difference between groups with regard to the number of entries in the target zone. Mean ± S.E.M latency to first entry in the target zone for CT (black) and CHF (grey) is shown in (B). CHF rats spent significantly less time to first entry into this quadrant, possibly indicating enhanced spatial memory in aversive conditions and/or less tolerance to being in an adverse environment. Time spent in the target zone for CT (black) and CHF (grey) is shown in (C), also as mean ± S.E.M. CHF rats spent significantly less time in the target zone, raising the hypothesis that these anxious animals present enhanced perception of the tank as an aversive context and/or display some difficulty to adapt to this kind of context (n = 12 CT; 12 CHF). Track plot of CT and CHF animal in the probe trial of the MWM is illustrated in (C). In the figure, the target zone corresponds to the left superior quadrant (zone SW). Although it is not possible to analyze in the figure all the parameters assessed in this trial, note the intensity of the plot of the CHF animal in quadrants that are not the target zone, indicating more time spent in other locations of the tank. *SW* southwestern quadrant, *MWM* Morris Water Maze, *CT* control, *CHF* Carioca high-conditioned freezing. $^{*}p \leq 0.05$, $^{**}p \leq 0.01$

Fig. 4. Acquisition and retention of fear memory were analysed in the PAT. Bar graphs in (A) show the mean ± S.E.M number of shocks applied until the animal remained for 2 uninterrupted minutes in the safe platform (measure of acquisition) for CT (black) and CHF (grey). CHF rats do not differ from CT in the fear learning parameter assessed in this test, as

can be observed by the similar amount of footshocks delivered in the first day of test. The latency to first step down from the safe platform (measure of retention) for CT (black) and CHF (grey) is shown in (B), as mean ± S.E.M. CHF animals display enhanced fear memory retention, as shown by the time spent in the safe platform before first step down in the second day of test (n = 18 CT; 19 CHF). *PAT* passive avoidance test, *CT* control, *CHF* Carioca high-conditioned freezing. $^{**}p \leq 0.01$

Fig. 5. Bar graphs in (A) show serum corticosterone levels (ng / ml) at basal condition for CT (black) and CHF (grey). There was a significant difference between CT and CHF groups, with the anxious animals presenting higher levels of the stress hormone. Values represent mean ± S.E.M (n= 10 CT; 10 CHF). Expression of GR measured by immunofluorescence for CT (black) and CHF (grey) was found to be significantly reduced in the dentate gyrus of the CHF group, as shown in (B) (n = 12 CT; 09 CHF). *GR* glucocorticoid receptor, *CTCF* Corrected Total Cell Fluorescence, *CT* control, *CHF* Carioca high-conditioned freezing. $^{**}p \leq 0.01$

Fig. 6. Cell proliferation and survival in the dentate gyrus of adult CT and CHF animals were assessed by immunohistochemistry. Quantification of BrdU-positive cells in the dentate gyrus (DG)1 day after the last BrdU injection, and of Ki-67, are shown in (A) for CT (black) and CHF (grey). Stereological analysis revealed no differences between groups with regard to quantification of $BrdU^+$ and $Ki\text{-}67^+$ cells. (BrdU, n = 05 CT; 08 CHF; Ki-67, n = 08 CT; 08 CHF). Stereological quantitative analysis of $BrdU^+$ cells in the DG of animals sacrificed 5 weeks after the last BrdU injection is shown in (B) for CT (black) and CHF (grey). This analysis revealed no differences between CT and CHF rats with regard to cell survival (n = 03 CT; 03 CHF, values represent mean ± S.E.M). *BrdU* bromodeoxiuridine, *CT* control, *CHF* Carioca high-conditioned freezing, *DG* dentate gyrus

Fig. 7. Bar graphs show the mean ± S.E.M number of neuroblasts in the adult dentate gyrus (DG) of CT and CHF rats. Quantification of DCX+ cells in the DG of CT (black) and CHF (grey) is shown in (A). Stereological analysis showed a significant reduction of DCX+ cells in the DG of CHF animals (n = 08 CT; 08 CHF), suggesting decreased levels of neuronal differentiation in this group. Photomicrographs of the DG of both groups, stained for DCX are shown in (B). Note the reduction in DCX expression in the DG of CHF animal. *Scale bars* indicate 250 μm in (B) upper panels (4x objective) and 25 μm in (B) lower panels (40x objective). *DCX* doublecortin, *CT* control, *CHF* Carioca high-conditioned freezing, *DG* dentate gyrus. $^{*}p \leq 0.05$

Fig. 8. Morphometric analysis of neuroblast dendrites was performed in order to evaluate dendritic arborization of immature neurons in the dentate gyrus (DG) of CT and CHF animals. Mean ± S.E.M number of total, secondary and tertiary dendrites in the newly born neurons in the DG of CT (black) and CHF (grey) is shown in (A). A significant difference was found in the total number of dendrites, reflecting a specific reduction in the number of tertiary dendrites in the CHF group (n = 64cells / group). Total, primary, secondary and tertiary length (μm) of dendrites of newly born neurons in the DG of CT (black) and CHF (grey) is shown in (B). CHF neuroblasts present decreased total dendritic length, as a reflection of the reduction observed in the length of tertiary dendrites (n = 64cells / group). In (**C**), the tracing of a neuroblast of CT and CHF animal illustrate the reduced number and length of dendrites in the experimental group. *Scale bar* indicates 40 μm. *CT* control, *CHF* Carioca high-conditioned freezing. *$p \leq 0.05$, **$p \leq 0.01$

Fig. 9. Bar graphs in (A) show dendritic spine linear density in tertiary dendrites of the dentate gyrus of CT and CHF animals, as mean ± S.E.M. Data show a significant increase in the expression of dendritic spines in the experimental group (n = 32 dendritic shafts / group).

Photomicrograph in (B), along with its high contrast representation used for spines quantification, shows the dendritic shafts from CT and CHF neurons; higher density of spines can be noted in the anxious group. *Scale bar* indicates 10 µm. *CT* control, *CHF* Carioca high-conditioned freezing. ***$p \leq 0.001$

Fig. 10. Expression of BDNF in the hippocampus of CT and CHF animals was analysed by western blotting. A significant increase in the expression of this trophic factor in the experimental group was observed, as quantitatively shown in (A) (n = 04 CT (black bars); 04 CHF (grey bars)). Quantification was made using ImageJ software and normalized to α-tubulin expression, as shown in (B). *BDNF* brain derived neurotrophic factor, *CT* control, *CHF* Carioca high-conditioned freezing. **$p \leq 0.01$

Fig. 11. Statistical analysis of [^{3}H]-GABA release with depolarizing stimulus (KCl, 80 mM) by basal GABA release in 400 µm hippocampal slices of CT (black) and CHF animals (grey). The ratio of [^{3}H]-GABA released is inferior in CHF slices (n = 06CT; 05 CHF), suggesting altered inhibitory response in the hippocampus of the anxious model. *CT* control, *CHF* Carioca high-conditioned freezing. *$p \leq 0.05$

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