Behavioral Profile and Dorsal Hippocampal Cells in Carioca High-Conditioned Freezing Rats

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Running title: Behavioral profile and dorsal hippocampus in CHF rats

Abstract

Contextual fear conditioning selection is an important behavioral paradigm for studying the role of genetic variables and their interaction with the surrounding environment in the etiology and development of anxiety disorders. Recently, a new line of animals selectively bred for high levels of freezing in response to contextual cues previously associated with footshock was developed from a Wistar population. The purpose of the present study was to evaluate the emotional and cognitive aspects of this new line of animals, which has been named Carioca High-Freezing (CHF). For the characterization of anxious behavior, CHF and control animals were tested in the elevated plus-maze (EPM) and the social interaction test. CHF animals were significantly more anxious than control rats in terms of both the number of entries into EPM open arms and the percentage of time spent in these arms. The time spent in social interaction behavior was also significantly decreased. No statistical differences were found in locomotor activity, as measured by both the number of entries into the closed arms of the EPM and the number of crossings into the social interaction test arena. No differences between CHF and control groups were found in the depression forced swimming test, suggesting that the anxiety trait selected in the CHF line interacted with other emotional systems such as depression. Cognitive aspects of the CHF rats were evaluated in the object recognition task. Results from this test indicated no difference between the two groups. The present study also performed histological analysis of the dorsal hippocampus from CHF and control animals. Results revealed an absence of qualitative and quantitative differences between these two groups of animals in cells located in the dentate gyrus, CA1, and CA3 areas. Therefore, future studies are required to further investigate the possible neural mechanisms involved in the origin and development of the anxious phenotype observed in this model.

1- Introduction

Fear and anxiety traits are believed to have been selected in human evolutionary history for their crucial role in protecting our hunter-gatherer ancestors when facing adverse environments [1]. Indeed, appropriate anxious reaction has an adaptive role in dealing with threatening situations. However, chronic anxious responses, especially in the absence of the feared stimuli, can characterize dysfunctional or pathological processes.

Freezing response to contextual cues previously associated with footshock seems to be one of the most reliable animal models of anxiety disorders [2;3]. Specific and complex circuits in the brain are known to underlie this conditioned response. The hippocampus is considered to be one of the major brain structures involved in the mediation of learned fear responses, likely via descending projections to the amygdala [4], as electrolytic lesions in hippocampal regions that connect to the amygdala prevent contextual fear learning [5;6]. The hippocampus is believed to be responsible for gathering contextual stimuli into representational units and then sending this information to the amygdala. Efferent projections from the central nucleus of the amygdala to the brain stem seem to be responsible for the motor output of the conditioned freezing response [7]. In particular, the dorsal portion of the hippocampus has been found to be involved in the modulation of this response [8], although the ventral hippocampus is also implicated, with anxiogenic effects when stimulated by serotonergic agonists [9].

Important molecular and pharmacological aspects of mental disorders are difficult to investigate in humans. Moreover, the study of fear and anxiety in animals from normalized populations might not always mimic the pathophysiology of clinical conditions, but natural and adaptive behavioral and physiological reactions to drugs and

aversive events [10]. For this reason, animals selectively bred for high emotionality have been considered to be important tools for understanding the neurobiology of anxiety disorders. Different behavioral paradigms have been employed for this purpose. Among these paradigms are the ambulation and defecation in the open field, such as in the Maudsley reactive rats [11;12], open arm entrance in the elevated plus-maze, as in the high-anxiety related behavior rats [10], and active avoidance behavior, as in the high-avoidance rats referred to as Roman [13], Syrakuse [14], Koltushi [15], and Hatano [16].

Recently, Gomes and Landeira-Fernandez [17] developed two new lines of Wistar rats, termed Carioca High- and Low-Freezing (CHF, CLF), that were selectively bred for high and low levels of freezing in response to contextual cues previously associated with footshock. After three generations of breeding, CHF rats are considered to naturally have a greater propensity for exhibiting higher freezing responses when compared to the low-freezing line. Since the characterization of this animal model may be an important tool for investigating the role of genetic variables and their interaction with the surrounding environment in the etiology and development of anxiety disorders, the major objective of this study was to validate behaviorally the CHF line (Carioca High-Freezing) in an innate animal model of anxiety (i.e., the elevated plus-maze and the social interaction test). The forced swimming test and the object recognition task were also employed in order to evaluate whether traits from other emotional or cognitive systems, such as depression or memory, were co-selected during the CHF breeding procedure. Finally, the present study also investigated whether CHF and control animals presented qualitative and/or quantitative differences in cells located in the dorsal hippocampus.

2- Materials and Methods

2.1 Animals

Experimental procedures reported herein were performed under the guidelines for the use of animal experimental research established by the Brazilian Society of Neuroscience and Behavior (SBNeC), in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications). Animal handling and sacrifice methods were reviewed and approved by the Committee for Animal Care and Use of the CCS/UFRJ (protocol # IBCCF002). Experimental animals (Carioca High-Freezing [CHF], a line selectively bred for high contextual fear conditioning) were obtained according to procedures described in previous work [17]. In the present work, CHF rats from the S4 (elevated plus-maze, social interaction, and forced swimming tests, as well as histological experiments) and the S6 (object recognition test) were used. Males from both groups were housed in acrylic cages (31 cm x 38 cm) in groups of 3-6 in an animal room under a 12-h light-dark cycle (lights on at 8:00 h), and food and water were provided *ad libitum*. For both groups, 2-3-month-old animals were used. Body weight varied from 250-348 g (control) and 323-363 g (CHF). Both the control (CTRL) and the experimental groups were reared under the same environmental conditions. Experimental animals used in this work did not undergo a line selecting test (contextual fear conditioning) and did not go through any other stressful events.

2.2 Behavioral tests

Animals were tested in the elevated plus-maze (EPM) and social interaction test for anxious behavior screening, in the forced swimming test for depressive behavior

identification, and in the object recognition test for cognitive performance assessment. Animals that were tested in the EPM were later perfused for histological analysis; those placed in the social interaction test were also tested in the forced swimming apparatus, with a minimum of two weeks latency between tests; animals used for object recognition measures did not go through any other tests.

2.2.1 Elevated plus-maze

Twelve control rats and 16 CHF rats were tested in the elevated plus-maze test. The test was first developed by Handley and Mithani [18] and was validated as a model for anxious-related behavior by Pellow *et al.* [19]. The test is based on the natural conflict faced by rodents when exploring a new environment and the innate aversion of being exposed to open areas [20]. The apparatus consists of two wooden opposed closed arms, two opposed open arms surrounded by an acryclic protection in order to prevent animals from falling down, and an open square in the center. The maze was elevated 50 cm above the floor. All animals were handled for two minutes on five consecutive days prior to the experimental session. The same experimenter was responsible for both handling and placing the animals into the maze in order to reduce human contact bias. The apparatus was cleaned with ethanol 98% before each rat was placed within in. The animals were placed into the center of the plus-maze facing one of the closed arms. The experimental session (5 minutes) was recorded by a camera located 70 cm above the maze, and the following measures were later analyzed: the number of entries into closed arms, and both the number of entries and the percentage of time spent in open arms.

2.2.2 Social interaction

The social interaction protocol was a modified version of that presented by Henniger *et al.* [20]. The test arena was made of black PVC (54 x 36x 27 cm) and the floor was divided into six squares (18 x 18 cm). The test was conducted under cold light. All animals (n = 12; 6 pairs for each group) were individually familiarized with the apparatus on the two days prior to testing for 10 minutes each day. Animals were divided in weight-matched pairs. In the experimental session, rats were placed in the center of the arena facing each other. Both members of a pair belonged to the same line of rats, but were unknown to each other. The testing session was recorded by a Sony Video Hi8 TRV238 camera placed vertically over the apparatus. The following parameters were recorded: (1) time spent in active social interaction (sniffing, following, grooming, kicking, mounting, jumping on, wrestling and boxing with, crawling under or over the partner); (2) the number of line crossings of both rats. The arena was cleaned before each trial with ethanol 98%.

2.2.3 Forced swimming test

The forced swimming test was first designed by Porsolt, LePichon, and Jalfre [21], but the protocol used here was an adapted version from Zangen et al. [22]. The arena consisted of a glass cylindrical tank (42 cm high and 17.5 cm in diameter) that contained enough water (25^{o}C) so that the rat could not touch the bottom of the tank with its hind paws. Rats (n = 9 for each group) were placed in the tank for a 10-minute habituation session on each of the two days prior to testing; rats' performance was recorded with a Sony Video Hi8 TRV238 camera, located 90 cm from the apparatus. Before each trial, water from the apparatus was changed. The following swimming behaviors were used as measures of coping: diving, vigorous

paddling with all four legs, circling the tank, and clambering at the tank walls. "Immobility" was scored as floating and treading water just enough to keep the nose above the water's surface [23;24]. After both habituation and testing sessions, rats were gently dried and returned to their respective home cages.

2.2.4 Object recognition

The object recognition test was first established by Ennaceur and Delacour [25]. A 40cm x 40cm wooden arena was used for this test and the experimental procedures performed were similar to those previously described by De Lima *et al*. [26]. Animals (n = 12/group) were individually habituated to the apparatus in the four consecutive days prior to testing, for 20 minutes each day. In the habituation session, there were no objects in the arena. Twenty-four hours after the last habituation session, animals were individually placed in the center of the arena for 5 minutes, where two similar objects (A1 and A2) were available for free exploration. Typically, objects are made of plastic, glass, or metal. In this study, soft drink cans and colorful glass cookie jars were used. Several samples of each object were used in order to avoid olfactory cues. Exploration was defined as sniffing or touching objects with either the nose or the forepaws. Twenty-four hours after this first testing session, long-term memory was evaluated. At this stage, rats were re-exposed to the arena for 5 minutes in the presence of a familiar object (A) and a novel one (B). For half of the animals from each group, A was familiar and B was novel. For the other half, the opposite was presented. The aim of this procedure was to avoid spatial or object preference. This test provides three different measures: index of recognition (TB / (TA + TB) [TA = time spent exploring the familiar object; TB = time exploring the novel object]; index of exploration 1 (time spent in

both familiar objects exploration), and index of exploration 2 (time spent in both types of object exploration). Exploration indexes validate the index of recognition as they show that the ability to explore, which is a basic condition for recognizing the object the next day, is unaltered. Both testing session were recorded by a Sony Video Hi8 TRV238 camera, located 130 cm vertically above the apparatus.

2.3 Hippocampal histology

Animals were anesthetized (n = 3 for each group) with chloroform perfused through the left ventricle of the heart with 4% paraformaldehyde and 10% saccharose in 0.1 phosphate buffer (pH = 7.4), followed by 20% and 30% saccharose in 0.1 phosphate buffer (pH = 7.4). Brains were removed and kept immersed in 30% saccharose in 0.1 phosphate buffer (pH = 7.4) solution for cryoprotection for one week. Serial 40 µm brain sections were cut in Leica CM 3050 S cryostat apparatus and thaw-mounted in poly-L-lysine-treated slides. After two days under room temperature, slides were kept at -20ºC. Brain sections from interaural 6.2 mm/ bregma -2.8mm to interaural 4.7 mm / bregma -4.3mm from each animal were then washed with phosphate buffer saline (PBS) for 5 minutes and stained with 4',6-diamidino-2-phenylindole (DAPI), a fluorescent stain that binds to DNA, for 1.5 minutes. Slides were washed with PBS once more for 5 minutes and mounted in N-propyl galate. Next, staining was observed using a fluorescent microscope (Zeiss Standard 20) and photos from the three hippocampal areas (dentate gyrus, CA1, and CA3) were taken (10x magnification; 3.6 focus) using a digital camera attached to the microscope. Using Image ProPlus 4.0 software, cells were counted in eight 100-μm^2 fields drawn in each picture (total of 27 pictures of

each hippocampal region/group). Manipulation of the digital images was restricted to threshold and brightness adjustments to the entire image.

2.4 Statistics

Data were analyzed in the Graph Prism 4.0 program, using t test for unpaired samples and results are expressed as mean ± S.E.M. Histological analyses are expressed in mm^3, according to estimative calculus based on the thickness of each brain slice (40 μm).

3. Results

3.1 *Elevated plus-maze*

Figure 1 illustrates performance on the EPM. CHF animals displayed a significantly lower number of entries into open arms ($t(26)=2.31$; $p<0.05$) and percentage of time spent in these arms ($t(26)=2.16$; $p<0.05$). There were no significant differences in general locomotor activity as the number of entries into the closed arms did not differ significantly between the two groups ($t(26)=1.96$; $p>0.05$). Therefore, the CHF group exhibited a significantly more anxious phenotype, according to two parameters measured in this test, and these differences cannot be accounted for by variations in locomotor activity.

INSERT FIGURE 1 HERE

3.2 *Social interaction*

Congruent with the results observed in the EPM, CHF rats showed higher anxiety scores in the social interaction test. The experimental group spent significantly less time exhibiting active social interactive behavior with an unknown partner from the same line ($t(10)=4.91$; $p<0.05$), as can be seen in Figure 2. Additionally, there were no significant differences in locomotor activity, as measured by the number of line crossings in the arena ($t(10)=1.60$; $p>0.05$).

INSERT FIGURE 2 HERE

3.3 *Forced swimming*

Behavior of CHF and control animals in the forced swimming test is illustrated in Figure 3. As can be seen, the experimental group did not significantly differ from control rats in this depressive behavior paradigm ($t(16)=0.99$; $p>0.05$), as measured by the time spent in escaping behaviors.

INSERT FIGURE 3 HERE

3.4 *Object recognition*

Cognitive aspects associated with memory in CHF and controls animals were assessed by the object recognition test, as illustrated in Figure 4. No significant differences were observed in any of the three measures taken from this paradigm. Therefore, CHF did not differ from control group in the recognition index

(t(22)=0.81; p>0.05), the index of exploration 1 (t(22)=1.75; p>0.05), or the index of exploration 2 (t(22)=0.34; p>0.05).

INSERT FIGURE 4 HERE

3.5 *Histological analysis*

3.5.1 Qualitative analysis

Qualitative analysis of the three cell layers of the dorsal hippocampus (dentate gyrus, CA1, and CA3) revealed no variations between groups concerning the hippocampal tissue conformation, as seen in Figure 5.

INSERT FIGURE 5 HERE

3.5.2 Quantitative analysis

Quantitative analysis of total dorsal hippocampal cells stained with DAPI was performed for the same three hippocampal layers: dentate gyrus, CA1, and CA3. No significant differences were observed for these measures (Dentate gyrus: t(69)=1.58; p>0.05; CA1: t(68)=0.14; p>0.05; CA3: t(68)=0.38; p>0.05), as illustrated in Figure 6.

INSERT FIGURE 6 HERE

Discussion

The ability of an organism to evaluate stimuli and adaptively respond to them is one of the most important processes involved in survival in continuously changing environmental conditions [27]. Anxiety is a complex trait that has been conserved during evolution so that animals may assess potentially dangerous situations in order to enhance the probability of survival [28]. Anxious states in humans are characterized by avoidance behavior and by a tendency to perceive threatening stimuli [29]. Attentional and mnemonic processes are enhanced and ambiguous situations are interpreted as potentially dangerous [30].

The analysis of the neural substrates underlying anxiety and fear is, in great part, based on the investigation of behavioral inhibition induced by natural or learned aversive stimuli in animal models [5;31]. In this work, we used an animal line of Wistar rats selectively bred for contextual fear conditioning [17], an animal model for anxiety disorder [2]. The evaluation of anxiety-related behavioral and physiological aspects is crucial for the line to be considered an appropriate model for study of these psychopathologies. According to Landgraf and Wigger [32], an anxious phenotype should present features related to behaviors and coping strategies characteristic of this condition. In this sense, the present work behaviorally validates the CHF line as a model for studying anxiety and conditioned fear.

Two animals models of anxiety used in this work revealed that the CHF line can be considered behaviorally validated as a model for anxiety disorder. In the elevated plus-maze test, both the total number of entries and the time spent in open arms were significantly decreased in comparison to the control group. The anxious behavioral pattern was also observed in the social interaction test, where CHF rats spent significantly less time exhibiting active social interaction behaviors with their pairs.

This observation indicates higher anxiety, which is in accordance with the performance of rats selected for anxiety in the elevated plus-maze [27].

A commonly stated issue underlined by researchers that work with behavioral tests of anxiety based on exploratory behaviors is that factors unrelated to anxiety conditions might alter locomotor activity [33] and compromise data interpretation. The results obtained by both anxiety paradigms used here suggest that this issue did not introduce bias in this work. Interestingly, the observed difference in the number of entries and the time spent in open arms, as well as the decreased rate of social interactive behavior in CHF rats, cannot be attributed to differences in locomotor activity as both the number of entries in closed arms of the elevated plus-maze and the number of line crossings in the arena of the social interaction test did not differ between groups. Therefore, performance on the elevated plus-maze and social interaction test revealed that CHF rats exhibit a significantly more anxious phenotype when compared to the control group. The behavioral difference between CHF and control rats can be considered a stable and robust trait in conflict situations that elicit fear and anxiety, and not only a behavioral profile observed in the test used for selection [20].

The forced swimming test, used for depressive behavior screening, did not show differences between groups. This result corroborates the consistency of the CHF line as an anxious model as traits related to other phenotypes, such as depression, were not selected concomitantly. Landgraf and Wigger [32] underline the importance of this feature and postulated that an animal model must be able to capture the specific symptom or mechanism of the studied psychopathology, without modeling other processes or functions. According to Gorman [34], there is a high level of co-morbidity between anxiety disorders and depression, with an over 60% rate of co-occurrence. This rate of occurrence suggests that co-morbidity is the rule rather than the exception.

Therefore, even with this high probability of co-occurrence of anxious and depressive characteristics, the present model was most highly related to the anxious/fearful phenotype.

Additionally, results from the forced swimming test demonstrated that, although there might be elevated rates of co-morbidity between anxiety and depression, the two systems are biologically distinct, which is in accordance with the hypothesis that these states have different etiologies [35]. The results of the CHF line in the forced swimming test differ from those obtained with some animal lines that were selected for innate fear as the latter show depressive behaviors in this paradigm [10;36;27]. However, data from this study are in accordance with those presented by Ho et al. [37], in which rats selected for anxiety in the elevated plus-maze did not differ from the less anxious line in terms of depression, as measured by the forced swimming test. In this sense, it can be stated that the CHF line can be considered a good predictor of behavioral phenotypes in anxiety models, but cannot be considered a model for studying depression.

Since the emotional assessment of a new situation and the utilized coping strategies can depend on cognitive functions [28], the hypothesis that non-emotional memory differs between CHF and control rats was also investigated. For this purpose, the object recognition test was used. The task of recognizing objects has been widely used as a model for investigating the neurobiological mechanisms of learning and memory [26]. Interestingly, differences in the indices obtained from this test were not observed between groups, indicating that the memory systems selected during breeding of the CHF line were restricted to the emotional memory system.

The hippocampus was chosen as the neural structure responsible for the anxiety differences observed among CHF animals. This choice was made considering the fact that this region seems to be associated with the etiology of certain types of anxiety

disorder. In fact, several pieces of evidence indicate that patients with symptoms of post-traumatic stress disorder present smaller hippocampal volume in comparison to control subjects [38]. Moreover, experimental research indicates that the dorsal hippocampus modulates several anxiety-like responses. For example, Gonzalez *et al.* [39] found anxiolytic effects in the social interaction test after microinjection of benzodiazepinic sites in $GABA_A$ into the dorsal hippocampus. Additionally, Rezayat et al. [40] showed an interaction between GABA and cholecystokinin during modulation anxiety in the elevated plus-maze after injections of agonists and antagonists of both neurotransmitters into the dorsal hippocampus. Nazar *et al.* [41] also pointed out that GABA and serotonergic systems within the dorsal hippocampus are intimately involved in emotional behaviors. When they microinjected picrotoxin (a non-competitive antagonist of the $GABA_A$ receptor) into this hippocampal portion, the anxiolytic effect caused by serotonin depletion was attenuated. File *et al.* [36] also showed that the High DPAT Sensitive line (HDS) (an animal model selected for high sensitivity to the hypotermic response induced by the serotonergic agonist 8-OH-DPAT) presents reduced scores in the social interaction test, accounting for, at least in part, the abnormal functioning of 5-HT_{1A} receptors in the dorsal hippocampus. Kjelstrup *et al.* [42], in turn, demonstrated that this hippocampal portion is involved in fear conditioning since lesions in this area prevent contextual fear conditioning.

Qualitative analysis of the dorsal hippocampal tissue, specifically the dentate gyrus and CA1 and CA3 areas, revealed that development of the hippocampal formation of CHF rats did not differ from that of control animals in relation to the morphological organization of the tissue in these three main cell layers. Therefore, no qualitative damage of the tissue was detected, indicating that the tissue was preserved as a whole and that behavioral differences between groups cannot be explained in terms of

hippocampal injury. Cell quantification in the three mentioned areas of the dorsal hippocampus was not different between groups, which suggests that differences in the hippocampal circuitry in CHF animals, as hypothesized, might occur at the molecular level of this structure.

It is important to mention that the ventral portion of the hippocampus was not addressed in the present study. This issue is important because there are some reports indicating that this region might be involved in anxiety regulation. For example, Kjelstrup *et al.* [42] reported that lesions within the ventral hippocampus alter unconditioned fear responses in the elevated plus-maze test. Moreover, activation of 5-HT2C receptors within the ventral hippocampus induced anxiety responses in the elevated plus-maze. Therefore, future studies of the ventral hippocampus of CHF rats might produce additional data in the investigation of the mechanisms involved in the anxious trait exhibited by this animal model.

In conclusion, these data show that the CHF line represents a robust animal model of anxiety disorder, as differences in the experimental group were observed in two different anxiety tests. Motor activity did not account for the differences between CHF and control animals. The absence of reliable differences between CHF and control animals in the forced swimming test and object recognition task indicated that the breeding procedure that increased the occurrence of conditioned freezing to contextual cues did not interfere with other emotional or memory systems. Possible neurophysiological differences between CHF and control animals might be more specific than the total amount of cells within the dorsal hippocampus. Thus, future studies are required to examine the possible mechanisms involved in the origin and development of the anxious phenotype observed in this model.

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Figure captions

Figure 1. Baseline scores (MEAN ± S.E.M.) of CTRL and CHF animals in the elevated plus-maze test (EPM). Top: number of entries into the open arms; middle: percentage of time spent in the open arms; bottom: number of entries into the closed arms. CTRL, control (n = 12); CHF, carioca high-freezing (n = 16). *P<0.05.

Figure 2. Baseline scores (MEAN ± S.E.M.) of CTRL and CHF animals in the social interaction (top) and locomotor activity (bottom) tests. CTRL, control (n = 6 pairs); CHF, carioca high-freezing (n = 6 pairs). *P<0.05.

Figure 3. Baseline scores (mean ± S.E.M.) of CTRL and CHF animals in the forced swimming test. CTRL, control (n = 9); CHF, carioca high-freezing (n = 9).

Figure 4. Baseline scores (MEAN ± S.E.M.) of CTRL and CHF animals in the object recognition test. Top: object recognition. Middle: index of exploration 1 (time spent in exploration of both familiar objects). Bottom: index of exploration 2 (time spent in exploration of both types of objects). CTRL, control (n = 12); CHF, carioca high-freezing (n = 12).

Figure 5. Qualitative analysis of coronal sections of the dorsal hippocampus (dentate gyrus, CA1, and CA3 – 40 µm) of CTRL and CHF animals. CTRL, control (n = 3); CHF, carioca high-freezing (n = 3). A = Dentate gyrus CTRL; B = CA1 CTRL; C = CA3 CTRL; D = Dentate gyrus CHF; E = CA1 CHF; F = CA3 CHF.

Figure 6. Quantitative analysis of coronal sections of the dorsal hippocampus (dentate gyrus, CA1, and CA3 – 40 µm) of CTRL and CHF animals. Top: dentate gyrus. Middle: CA1 layer. Bottom: CA3 layer. CTRL, control (n=3); CHF, carioca high-freezing (n=3).

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