Available online at www.sciencedirect.com
SciVerse ScienceDirect
www.elsevier.com/locate/brainres

Research Report

Association between oxidative stress and contextual fear conditioning in Carioca high- and low-conditioned freezing rats

Waseem Hassan[a,b], *Vitor de Castro Gomes*[a], *Simone Pinton*[c], *Joao Batista Teixeira da Rocha*[c], *J. Landeira-Fernandez*[a,d,*]

[a]*Pontifícia Universidade Católica do Rio de Janeiro, Rio de Janeiro, RJ, Brazil*
[b]*Institute of Chemical Sciences, University of Peshawar, Peshawar 25120, Khyber Pakhtunkhwa, Pakistan*
[c]*Departamento de Química, Centro de Ciências Naturais e Exatas, Universidade Federal de Santa Maria, Santa Maria, CEP 97105-900, RS, Brazil*
[d]*Universidade Estácio de Sá, Rio de Janeiro, RJ, Brazil*

ARTICLE INFO

Article history:
Accepted 20 March 2013
Available online 6 April 2013

Keywords:
Breeding lines
CHF and CLF rats
Freezing
Elevated plus maze
Oxidative stress

ABSTRACT

We recently reported two novel breeding lines of rats known as Carioca high-and low-conditioned freezing (CHF and CLF), based on defensive freezing responses to contextual cues previously associated with electric footshock. The anxiety-like profile of these animals from the 7th generation was tested in the elevated plus maze. The results indicated that CHF animals presented a significantly more "anxious" phenotype compared with CLF animals. Animals from the 12th generation were used to evaluate the oxidative stress status of the cortex, hippocampus, and cerebellum. Reactive oxidative species (ROS) were evaluated using 2,7-dichlorofluorescin diacetate (DCFH-DA; a sensor of reactive oxygen species [ROS]), and the levels of malondialdehyde (MDA), an early marker of lipid peroxidation, were assessed. The results indicated that free radical concentrations and MDA levels were significantly higher in all three brain structures in CHF rats compared with CLF rats. Our data also showed that the hippocampus had the highest reactive species and MDA concentrations compared with the cortex and cerebellum in CHF rats. Animals from the 16th generation were used to evaluate the antioxidant enzyme activity of catalase (CAT) and glutathione peroxidase (GPx) within these three brain structures. The results indicated that CAT activity was lower in the cortex and hippocampus in CHF rats compared with CLF rats. No significant difference was observed in the cerebellum. The enzymatic activity of GPx was significantly decreased in all three structures in CHF rats compared with CLF rats. The hippocampus exhibited the highest GPx activity compared with the other two brain structures. These findings suggest the involvement of a redox system in these two bidirectional lines, and the hippocampus might be one of the prime brain structures involved in this state of oxidative stress imbalance.

*Corresponding author at: Pontifícia Universidade Católica do Rio de Janeiro, PUC-Rio, Laboratório de Neurociência Comportamental, Departamento de Psicologia, Rua Marquês de São Vicente, 225, 22453-900 Rio de Janeiro, RJ, Brazil. Fax: +55 21 35271187.
E-mail address: landeira@puc-rio.br (J. Landeira-Fernandez).

0006-8993/$ - see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.brainres.2013.03.039

1. Introduction

The nervous system has tremendous reservoirs of polyunsaturated and saturated fatty acids and is extremely prone to the damaging effects of oxidative stress, resulting in a loss of membrane integrity, protein damage, and neuronal dysfunction. Cells have their own defense mechanisms in the form of reducing molecules and different antioxidant enzymes that prevent the escalating effects of reactive oxygen species (ROS; i.e., excessive levels of molecular oxygen or its chemical derivatives). However, when ROS concentrations exceed the antioxidative capacity of an organism, the cells enter a state of oxidative stress, in which excessive ROS induce oxidative damage incellular components.

Oxidative stress has been linked to the pathological manifestations of many neurological disorders. Strong evidence indicates that social phobia, depression, anxiety, and other psychiatric disorders are partially related to oxidative stress, such as increased reactive species production (Dean et al., 2009; Liu et al., 2008). A linear relationship between oxidative stress markers in peripheral blood and anxiety-like behavior have been established in mice (Bouayed et al., 2007). These findings support previous studies that showed a close relationship between brain oxidative stress markers and anxiety-related phenotypes in six inbred mouse strains (Hovatta et al., 2005). Several brain areas, such as the hippocampus, appear to be strongly affected by the deleterious effects of oxidative insult (Bonatto et al., 2005). Similarly, data from the literature have indicated that exposure to stressful stimuli produces widespread physiological and behavioral effects in animals, and oxidative stress has been recently shown to be associated with anxiety in different behavioral models (Gingrich, 2005).

The literature has demonstrated significant differences between “anxious” and “non-anxious” mice with regard to their oxidative status in both neuronal and glial cells in various brain tissues, particularly the cerebellum, cortex, and hippocampus, and peripheral blood cells. Glial cells have been reported to play a key role in the protection of the nervous system by detoxifying ROS released by neurons and acting as debris scavengers and killers of microbial pathogens (Mosley et al., 2006; Morale et al., 2006). The presence of oxidative stress in glial cells in the cerebellum and hippocampus in “anxious” mice may therefore alter their protective function.

Anxiety is a complex and multifunctional trait, and individuals present a wide range of trait anxiety, varying from extremely low to extremely high levels. Relevant animal models are needed to study the neuroendocrine, neurochemical, and neurogenetic mechanisms of intricate behavioral phenotypes, such as anxiety. Various strategies, including genetic manipulations, selective breeding for extremes in a particular behavioral phenotype, environmental modifications, and combinations of these, are normally employed to gain better insights into the complex cascade of anxiety and related issues. One of the most promising of these approaches is the generation and development of two breeding lines derived from the same rat strain with extreme differences in anxiety-related behavior to explore the genetic basis of extreme emotionality. The process of selective breeding began in the middle of the 20th century. Since then, a wide range and large number of different lines have been reported in the literature (for review, see Ramos and Mormède, 2006).

Our laboratory (Gomes and Landeira-Fernandez, 2008) produced two rat lines, named Carioca high- and low-conditioned freezing (CHF and CLF), that were selectively bred for high and low levels of defensive freezing response to contextual cues previously associated with footshock. Contextual fear conditioning represents one of the most efficient and simplest forms of producing aversive learning (Landeira-Fernandez, 1996) historically associated with one of the main causes of pathological anxiety (Pavlov, 1927). In a typical experiment, a rat is exposed to a novel chamber. After a few minutes of habituation, a brief and unsignaled footshock is delivered. When returned to the same chamber in the absence of the aversive stimulus, the animal exhibits a permanent fear reaction to contextual cues previously associated with the footshock. Defensive freezing behavior has been considered one of the most reliable measures of contextual fear conditioning. This defensive response depends on the association between the cues of the experimental chamber and footshock (Landeira-Fernandez et al., 2006) and is directly related to shock intensity (Sigmundi et al., 1980). The results of our ongoing breeding program have already shown a clear divergence of the conditioned freezing phenotype after only three generations (Gomes and Landeira-Fernandez, 2008). Behavioral and biochemical characterization of this animal model may be an important tool for investigating the involvement of the underlying neural mechanisms involved in anxiety-related disorders.

The purpose of the present study was to first verify the behavioral divergence between CHF and CLF animals in the elevated plus maze test for anxiety. Considering the sparse data about oxidative stress in bidirectional rat lines bred for extreme emotionality, the second purpose of the present study was to biochemically characterize these two new rat lines by examining different oxidative stress parameters. In contrast to previous studies that used different inbred strains (Hovatta et al., 2005) and lines bred on the basis of the elevated plus maze paradigm (Krömer et al., 2005; Ditzen et al., 2006), the present study was performed in the same rat lines. CHF and CLF animals from the 7th, 12th, and 16th generation were used for the different experiments in the present study.

2. Results

Fig. 1 presents the mean and standard error of the mean (SEM) percentage of time spent freezing in CHF and CLF animals from the 7th (left), 12th (center), and 16th (right) generations during the contextual fear conditioning test session. As shown in the figure, CHF animals froze more that CLF animals across all three generations used in the present study. This impression was confirmed statistically. Student's *t*-test revealed a significant difference between CHF and CLF animals in the 7th ($t_{28}=15.71$, $p<0.001$), 12th ($t_{12}=13.83$, $p<0.001$), and 16th ($t_{22}=65.70$, $p<0.001$) generations.

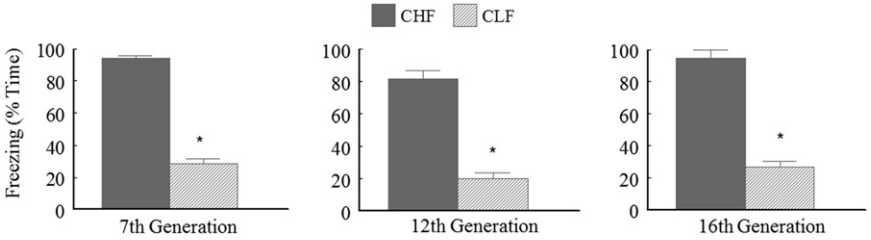

Fig. 1 – Mean (±SEM) percentage of freezing in CHF and CLF animals during the 8 min contextual fear conditioning test in animals from 7th (left), 12th (center), and 16th (right) breeding generations. *$p<0.001$, significant different between CHF and CLF animals from each generation.

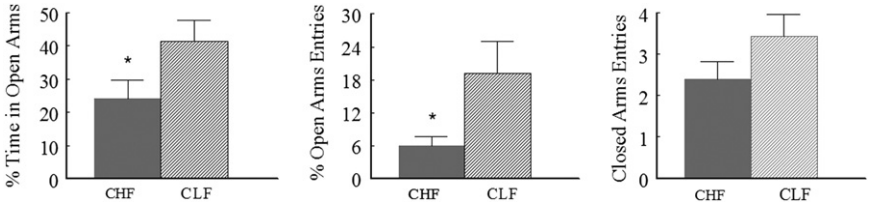

Fig. 2 – Mean (+SEM) percentage of open-arm entries (left), percent time spent in the open arms (center), and closed-arm entries (right) in the elevated plus maze in CHF and CLF animals. *$p<0.05$, significant difference between CHF and CLF animals.

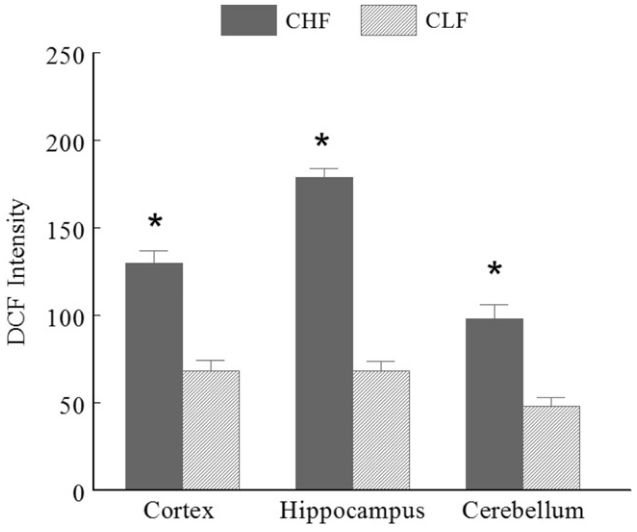

Fig. 3 – Mean (+SEM) reactive species (RS) levels expressed as fluorescence emission intensity of dichlorofluorescein (DCF-RS) in the cortex, hippocampus, and cerebellum in CHF and CLF rats. *$p<0.001$, significant difference between CHF and CLF animals across different brain structures.

Fig. 2 presents the elevated plus maze results. One CLF animal fell from the maze during the experiment and was excluded from the analysis. Student's *t*-test indicated that CHF rats had a reduced percentage of open-arm entries ($t_{27}=2.01$, $p<0.05$) and reduced time spent in the open arms ($t_{27}=2.27$, $p<0.05$) compared with CLF animals. No difference in closed-arm entries was observed between CHL and CLF animals ($t_{27}=1.52$, $p>0.05$).

Fig. 3 depicts the ROS results as fluorescence emission intensity of DCF-RS in the cortex, hippocampus, and cerebellum in CHF and CLF animals. A two-way 3×2 analysis of variance (ANOVA) was used to analyze these results. The first factor, with three levels, was related to brain structure. The second factor, with two levels, was related to rat line. This analysis indicated a significant interaction between the two factors ($F_{2,52}=20.76$, $p<0.0001$) and significant main effects of brain structure ($F_{2,52}=50.32$, $p<0.0001$) and rat line ($F_{1,26}=124.61$, $p<0.0001$). Pairwise *post hoc* comparisons indicated that CHF rats consistently exhibited more DCF fluorescence in all three brains structures compared with CLF rats (all $p<0.001$). The results also indicated that the hippocampus in CHF rats had the highest DCF-RS emission intensity, followed by the cortex and cerebellum (all $p<0.05$).

Fig. 4 presents the extent of lipid peroxidation in the three brain structures in CHF and CLF animals, reflected by TBARS formation. A two-way ANOVA was used to analyze these results. Similar to the DCF-RS results, a significant interaction between the two factors was found ($F_{2,52}=17.31$, $p<0.0001$), with significant main effects of brain structure ($F_{2,52}=20.76$, $p<0.0001$) and rat line ($F_{1,26}=124.61$, $p<0.0001$). Pairwise *post hoc* comparisons indicated that CHF rats consistently exhibited higher TBARS levels in all three brain structures compared with CLF rats (all $p<0.001$). The hippocampus in CHF rats had the highest TBARS level, followed by the cortex and cerebellum (all $p<0.05$).

The CAT activity results in the three brain structures in CHF and CLF animals are depicted on the left in Fig. 5. The two-way ANOVA showed a significant interaction between the two factors ($F_{2,44}=5.52$, $p<0.01$), with significant main effects of brain structure ($F_{2,44}=3.30$, $p<0.05$) and rat line ($F_{1,12}=43.88$, $p<0.01$). Pairwise *post hoc* comparisons indicated that CHF rats consistently presented a significant reduction of CAT activity in the cortex and hippocampus compared with CLF animals (all $p<0.001$). No significant difference was observed in the cerebellum between these two rat lines ($p>0.05$).

The GPx activity results in the three brain structures in CHF and CLF animals are shown on the right in Fig. 5.The two-way ANOVA revealed a significant interaction between the two factors ($F_{2,44}=32.69$, $p<0.0001$) and a main effect of rat line ($F_{1,22}=187.06$, $p<0.0001$) but no main effect of brain structure ($F_{2,44}=3.13$, $p>0.05$). Pairwise *post hoc* comparisons indicated that CHF rats reliably presented lower GPx activity in all three brains structures compared with CLF rats (all $p<0.001$). The results also indicated that the hippocampus in CHF animals presented lower GPx activity compared with the cortex and cerebellum (all $p<0.01$).The hippocampus in CLF animals presented higher GPx activity compared with the cortex and cerebellum (all $p<0.01$).

3. Discussion

Consistent with our previous report (Gomes and Landeira-Fernandez, 2008), CHF rats exhibited higher freezing behavior in response to contextual cues associated with electric footshock compared with CLF rats in all three generations used in the present study. The results obtained from animals of the 7th generation indicated that CHF animals presented a significantly more anxious phenotype, reflected by open-arm parameters in the elevated plus maze, compared with CLF animals. No differences were found between CHF and CLF rats in the number of closed-arm entries, suggesting that the anxiety-like profile of CHF animals was not attributable to locomotor impairment but rather to increases in aversion to the open arms. These behavioral differences between these two lines of animals in the elevated plus maze are consistent with a previous study that investigated randomly selected CHF and control animals (Dias et al., 2009) and indicate that the conditioned freezing parameters used for breeding selection remained stable in different threatening situations.

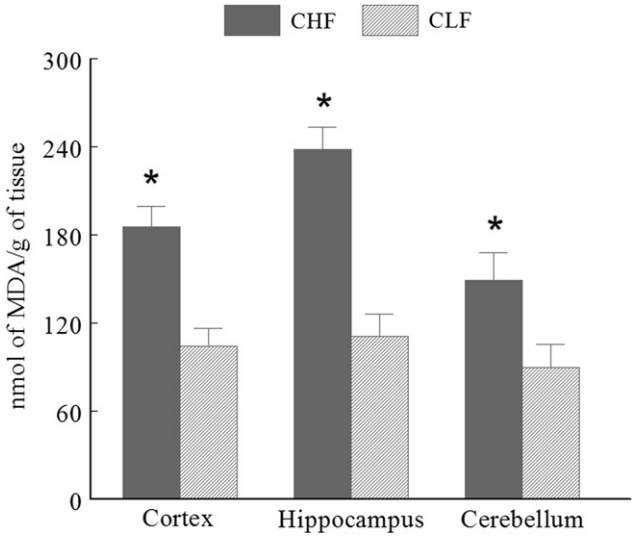

Fig. 4 – Mean (+SEM) extent of lipid peroxidation in the cortex, hippocampus, and cerebellum in CHF and CLF rats, reflected by TBARS formation. TBARS are expressed as nanomoles of MDA per gram of tissue. *$p<0.001$, significant difference between CHF and CLF animals across different brain structures.

Reports in the literature suggest an important association between anxiety and oxidative stress. For example, recent studies have shown the direct involvement of oxidative stress in anxiety-like behavior in rodents (de Oliveira et al., 2007; Salim et al., 2010a,b; Souza et al., 2007). Berry et al. (2007) showed that pain sensitivity and emotional behavior in wild type mice increased with age, likely attributable to the accumulation of oxidative damage. These authors showed that deletion of the *p66Shc* gene resulted in lower levels of oxidative stress, reduced pain sensitivity, and reduced anxiety-like behavior. Notably, the *p66Shc* gene is responsible for the regulation of reactive species metabolism. Desrumaux et al. (2005) showed that vitamin E deficiency resulted in increased levels of central oxidative stress markers that in turn resulted in anxiogenic-like behavior in mice, with no abnormalities in locomotor performance.

The limbic region, comprising the frontal cortex, hippocampus, amygdala, and hypothalamus, is an important system involved in behavioral regulation, forming parts of several well-defined anxiety- and fear-related circuits in the forebrain (Singewald et al., 2003; Hovatta et al., 2005). The present study suggests that anxiety and fear might be associated with increased free radical production in the cortex, hippocampus, and cerebellum, in which CHF animals displayed high levels of ROS and lipid peroxidation in all three of these brain structures. The hippocampus appears to be the most important target, reflected by the highest free radical concentrations compared with the cortex and

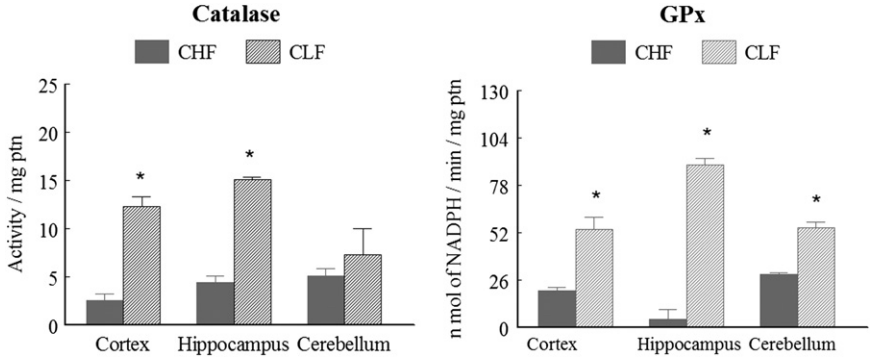

Fig. 5 – Mean (+SEM) GPx and CAT activity in the cortex, hippocampus, and cerebellum in CHF and CLF rats.*$p<0.001$, significant difference between CHF and CLF animals across different brain structures.

cerebellum. Considerable data indicate that free radicals are involved in the biochemical mechanisms that underlie neuropsychiatric disorders in humans. Antioxidant therapy has profound recuperative effects on oxidative damage (Ozcan et al., 2004). Free radicals usually affect biomolecules, such as proteins, lipids, and DNA, and mitochondria (Valko et al., 2004). The present results are consistent with Bonatto et al. (2005), Gabbita et al. (1998), and Serrano and Klann (2004), who also showed that the hippocampus is strongly affected by the harmful effects of oxidative bursts. Lipid peroxidation was also significantly higher in the hippocampus and inferior parietal lobule in elderly individuals who exhibited mild cognitive impairment (Butterfield et al., 2006).

The present study also investigated the status of two antioxidant enzymes in CHF and CLF animals. The enzyme CAT decomposes hydrogen peroxide to water and oxygen. Our data indicated that CHF rats had significantly reduced CAT activity in the cortex and hippocampus. No significant difference was found in the cerebellum. Further evidence of the involvement of oxidative stress came from the GPx enzyme analysis. The biochemical function of GPx is to reduce various hydroperoxides to alcohols and hydrogen peroxide to water, thus offering protection against oxidative bursts. Consistent with free radical concentrations in various brain structures and TBARS formation, GPx activity was significantly decreased in CHF animals compared with CLF animals. The drastic reduction was observed in the hippocampus in CHF rats compared with CLF rats, further suggesting that the hippocampus may be a sensitive target of toxic oxidative bursts. Similarly, recent studies have shown the direct involvement of oxidative stress in anxiety-like behavior in rodents (de Oliveira et al., 2007; Salim et al., 2010a,b; Souza et al., 2007). More direct evidence of the involvement of oxidative stress in anxiety was provided by Bouayed et al. (2007). These authors showed that naive Swiss albino male mice had large heterogeneity in anxiety levels, and they provided baseline data for the involvement of oxidative stress in anxiety-like behavior.

The formation of reactive species, both nitrogen and oxygen, can alter protein conformation through reactions with amino acid residues apart from damaging the lipid profile and DNA sequence. For example, oxidatively modified proteins and lipids may contribute to the formation of neurofibrillary tangles, together with extracellular deposits of amyloid β peptide, a molecular hallmark of Alzheimer's disease, a neurodegenerative disorder in which the involvement of ROS in hippocampal and cortical degeneration appears to be undeniable (Castegna et al., 2002; Smith et al., 1997; Volkel et al., 2006).

Important for the present study is the fact that the hippocampus is an important brain structure involved in contextual fear conditioning. For example, several studies have shown that hippocampal lesions disrupted conditioned freezing in response to contextual cues previously associated with footshock (Maren and Fanselow, 1997; Richmond et al., 1999; Rogers et al., 2006; Yoon and Otto, 2007). Therefore, the highest free radical concentrations in the hippocampus in conjunction with the lowest GPx activity in the hippocampus in CHF animals would be expected to enhance the susceptibility of hippocampal neurons and thus disrupt the neural circuitry involved in this type of emotional learning. However, our group recently showed that the morphological organization of the dentate gyrus and CA1 and CA3 subfields of the hippocampus in CHF rats was not different from control animals (Dias et al., 2009). These results indicate the absence of qualitative damage to the tissue and show that behavioral differences between groups cannot be explained by hippocampal injury. Additionally, the cell quantification experiments found no significant differences between CHF and control animals. Therefore, the involvement of hippocampal oxidative stress in CHF animals in the present study might occur at the molecular level.

Studies that have investigated the association between antioxidant enzymes and phenotypic alterations in bidirectional lines are scarce and contradictory. Hovatta et al. (2005) investigated the relationship between antioxidative defense mechanisms and anxiety-related phenotypes in six inbred mouse strains. They found that the activity of the antioxidative enzymes glutathione reductase 1 and glyoxalase 1 were higher in the more anxious lines. However, other studies that used two Swiss CD1 mouse lines that were selectively bred for anxiety-like behavior found that glyoxalase 1 was less expressed in the line with a high anxiety-related phenotype (Krömer et al., 2005; Ditzen et al., 2006). Although genetic variability may be at least partially responsible for interspecies and inter individual differences, the precise mechanisms that underlie the relationship between emotional stress and oxidative stress in the genesis of anxiety require further investigation.

In summary, the present study indicated that the bidirectional Carioca line appears to represents a robust animal model of anxiety. CHF rats exhibited higher oxidative stress in the cortex, hippocampus, and cerebellum compared with CLF rats. The antioxidative enzyme activity results further indicated a bidirectional imbalance of redox status. The fact that CHF animals presented lower CAT and GPx activity compared with CLF animals also suggests a disruption of antioxidative defense mechanisms in this bidirectional line. The hippocampus appears to be the main structure involved in the imbalance of redox status. Further studies are needed to precisely identify the biochemical mechanisms that lead to differences in and the prevalence of this extreme behavior.

4. Experimental procedures

4.1. *Animals*

The present study used animals selectively bred for high and low contextual fear conditioning according to procedures described in our previous work (Gomes and Landeira-Fernandez, 2008). Briefly, albino Wistar rats were selectively bred for differences in defensive freezing behavior using contextual fear conditioning previously associated with footshock. The elevated plus maze experiment reported in the present study used 15 CHF and 15 CLF male animals from the 7th generation. The biochemical experiment that evaluated ROS parameters used seven CHF and seven CLF male rats from parallel breeding of the 12th generation. The activity of

two antioxidant enzymes was examined in 12 CHF and 12 CLF male rats from the 16th generation.

The animals were 15–20 weeks old and weighed 350–480 g. They were bred and maintained in the colony room in the PUC-Rio Psychology Department with controlled room temperature (24±1 °C) and a 12 h/12 h light/dark cycle (lights on 7:00 AM–7:00 PM). The animals were housed in groups of three to five, according to their respective lines, in polycarbonate cages (18 × 31 × 38 cm) with food and water available ad libitum. All of the behavioral experiments were conducted during the light phase of the light/dark cycle. The animals were handled once daily for a period of 2 min for 5 days before the fear conditioning experiment. The experimental procedures reported herein were performed in accordance with the guidelines for experimental animal research established by the Brazilian Society of Neuroscience and Behavior (SBNeC) and the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Animal handling and the methods of sacrifice were reviewed and approved by the Committee for Animal Care and Use of PUC-Rio (protocol no.20/2009).

4.2. *Apparatus*

Fear conditioning occurred in four observation chambers (25 × 20 × 20 cm), each placed inside a sound-attenuating box. A red light bulb (25 W) was placed inside the box, and a video camera was mounted to the back of the observation chamber so the animal's behavior could be observed on a monitor outside the experimental chamber. The floor of each chamber consisted of 15 stainless steel rods (4 mm diameter) spaced 1.5 cm apart (center-to-center) that were wired to a shock generator and scrambler (Insight, São Paulo, Brazil). An interface with eight channels (Insight) that connected the shock generator to a computer allowed the experimenter to apply an electric footshock. Ammonium hydroxide solution (5%) was used to clean the chamber before and after each subject.

The elevated plus maze consisted of two wooden, opposing open arms (50 × 10 cm) arranged perpendicular to two other closed arms of the same size enclosed by 40 cm high walls. These four arms delimited a central area of 10 × 10 cm. The open arms were surrounded by an acrylic protection (1 cm high) to prevent the animals from falling from the apparatus. The maze was elevated 50 cm above the floor. Illumination was provided by a dim light bulb (60 W) on the ceiling of the experimental room, and the light intensity in the center of the maze was adjusted to 55 lux. A video camera linked to a monitor and computer in an adjacent room videotaped the experimental session. The apparatus was cleaned with 98% ethanol before each rat was placed in the maze.

4.3. *Contextual fear conditioning procedure*

The contextual fear conditioning protocol consisted of one training session and one test session. During training, each animal was placed in the observation chamber for 8 min. At the end of this period, three unsignaled 0.6 mA (1 min) electric footshocks were delivered, with an inter shock interval of 20 s. Two minutes after the last foot shock, the animal was returned to its home cage. The test session occurred approximately 24 h after training and consisted of placing the animal for 8 min in the same chamber in which the three footshocks were delivered on the previous day. No footshock or other stimulation occurred during this period. A time-sampling procedure was used to evaluate fear conditioning in response to contextual cues. The animal was observed every 2 s, and a well-trained observer recorded episodes of freezing, defined as the total absence of non-respiratory movements. The animals used in the present study were selected from each breeding line based on their freezing response during the test session.

4.4. *Elevated plus maze procedure*

Each animal was placed in the center of the elevated plus maze facing one of the closed arms. The experimental session lasted 5 min. A highly trained observer who remained blind to the treatment conditions recorded the number of entries into and time spent on the open and closed arms with the help of computer software. From these measures, the percentage of open arm entries (100 × open arm entries/total arm entries) and percentage of time spent on the open arms (100 × time open/[time open+time closed]) were calculated for each animal as indices of anxiety-like behavior. The absolute number of closed arm entries was interpreted as a reliable index of locomotor activity (File, 1992; Cruz et al., 1994).

4.5. *Sample preparation for oxidative stress parameters*

All of the rats were sacrificed by decapitation, and the brains were quickly removed, dissected to separate various structures (i.e., cortex, hippocampus, and cerebellum) on an ice pad, and further homogenized in a 10 vol of 50 mM Tris–HCl, pH 7.4. The homogenate was centrifuged at 4000 × *g* at 4 °C for 10 min, and a low supernatant fraction (S1) was used for *ex vivo* assays.

4.5.1. *Free radical concentration measurements*

To estimate the level of total ROS production, 50 μl samples were diluted 1:10 in Tris-buffer (pH 7.4). The oxidation of 2,7-dichlorofluorescein diacetate (DCHF-DA) to fluorescent dichlorofluorescein (DCF) was measured for the detection of DFC reactive species (DCF-RS) using the method described by Colpo et al. (2008). The DCF fluorescence emission intensity was recorded at 520 nm (with 480 nm excitation) using a fluorescence spectrophotometer (Hitachi F-2000; Hitachi, Tokyo, Japan) 60 min after the addition of 12 μl DCHF-DA to the medium.

4.5.2. *Rate of lipid peroxidation*

The rate of lipid peroxidation was measured as described by Ohkawa et al. (1979). The low supernatant fraction (S1) from various structures was mixed with incubation medium that contained 0.01 mM Tris–HCl (pH 7.4) and incubated at 37 °C for 60 min. Thiobarbituric acid reactive substances (TBARS) production was stopped by adding 100 μl of acetic acid buffer (pH 3.5), and lipid peroxidation products were measured by adding 100 μl of 0.6% TBA. The tubes were then incubated in

boiling water for 60 min, and their contents were subjected to spectrophotometric analysis. The amount of TBARS produced was measured at 532 nm using malondialdehyde (MDA) as an external standard. TBARS levels are expressed as nanomoles of MDA per gram of tissue.

4.5.3. *Glutathione peroxidase assay*

GPx activity in S1 was assayed spectrophotometrically according to the method of Wendel (1981) through the Glutathione (GSH)/Nicotinamide adenine dinucleotide phosphate (NADPH)/glutathione reductase (GR) system. Hydrogen peroxide (H_2O_2) was used as the substrate. S1 was added to the GSH/NADPH/glutathione reductase system, and the enzymatic reaction was initiated by adding H_2O_2. In this assay, enzyme activity is indirectly measured by determining NADPH decay. H_2O_2 is decomposed, generating Glutathione disulfide (GSSG) from GSH. GSSG is regenerated back to GSH by the glutathione reductase that is present in the assay medium at the expense of NADPH. Enzymatic activity is expressed as nanomoles of NADPH per minute per milligram of protein.

4.5.4. *Catalase activity*

For CAT activity, the S1 supernatant was assayed spectrophotometrically according to the method of Aebi (1984), which involves monitoring of the disappearance of H_2O_2 in the presence of brain structures homogenate at 240 nm. An aliquot of S1 was added to 50 mM potassium phosphate buffer, pH 7.0, and the enzymatic reaction was initiated by adding H_2O_2. One unit of enzyme was defined as the amount of enzyme required to monitor the disappearance of H_2O_2. Enzymatic activity is expressed as units (U) per milligram of protein, in which1Udecomposes 1molH_2O_2 per minute atpH7 and 25°C.

Acknowledgments

JLF and JBTR are supported by grants from the Brazilian National Research Council (CNPq). WH is a recipient of a FAPERJ-CAPES postdoctoral fellowship. VCG had a graduate student fellowship from CNPq.

REFERENCES

Aebi, H., et al., 1984. Catalase in vitro. Methods Enzymol. 105, 121–126.

Berry, A., Capone, F., Giorgio, M., Pelicci, P.G., de Kloet, E.R., Alleva, E., Minghetti, L., Cirulli, F., et al., 2007. Deletion of the life span determinant p66^{Shc} prevents age-dependent increases in emotionality and pain sensitivity in mice. Exp. Gerontol. 42, 37–45.

Bonatto, F., Polydoro, M., Andrades, M.E., Frota Jr., M.L.C., Dal-Pizzol, F., Rotta, L.N., Souza, D.O., Perry, M.L., Moreira, J.C., et al., 2005. Effect of protein malnutrition on redox state of the hippocampus of rat. Brain Res. 1042, 17–22.

Bouayed, J., Rammal, H., Younos, C., Soulimani, R., et al., 2007. Positive correlation between peripheral blood granulocyte oxidative status and level of anxiety in mice. Eur. J. Pharmacol. 564, 146–149.

Butterfield, D.A., Reed, T., Perluigi, M., De Marco, C., Coccia, R., Cini, C., Sultana, R., et al., 2006. Elevated protein-bound levels of the lipid peroxidation product 4-hydroxy-2-nonenal, in brain from persons with mild cognitive impairment. Neurosci. Lett. 397, 170–173.

Castegna, A., Aksenov, M., Thongboonkerd, V., Klein, J.B., Pierce, W.M., Booze, R., Markesbery, W.R., Butterfield, D.A., et al., 2002. Proteomic identification of oxidatively modified proteins in Alzheimer's disease brain: Part II. Dihydropyrimidinase-related protein 2, α-enolase and heat shock cognate 71. J. Neurochem. 82, 1524–1532.

Colpo, E., De Bem, A.F., Pieniz, S., Schettert, S.D., dos Santos, R.M., Farias, I.L., Bertoncello, I., Moreira, C.M., Barbosa, N.V., Moretto, M.B., Rocha, J.B.T., et al., 2008. A single high dose of ascorbic acid and iron is not correlated with oxidative stress in healthy volunteers. Ann. Nutr. Metab. 53, 79–85.

Cruz, A.P., Frei, F., Graeff, F.G., et al., 1994. Ethopharmacological analysis of rat behavior on the elevated plus-maze. Pharmacol. Biochem. Behav. 49, 171–176.

de Oliveira, M.R., Silvestrin, R.B., Mello, E.S.T., Moreira, J.C., et al., 2007. Oxidative stress in the hippocampus, anxiety-like behavior and decreased locomotory and exploratory activity of adult rats: effects of sub acute vitamin A supplementation at therapeutic doses. Neurotoxicology 6, 1191–1199.

Dean, O.M., van den Buuse, M., Bush, A.I., Copolov, D.L., Ng, F., Dodd, S., Berk, M., et al., 2009. A role for glutathione in the pathophysiology of bipolar disorder and schizophrenia? Animal models and relevance to clinical practice. Curr. Med. Chem. 16, 2965–2976.

Desrumaux, C., Risold, P.Y., Schroeder, H., Deckert, V., Masson, D., Athias, A., Laplanche, H., Le Guern, N., Blache, D., Jiang, X.C., Tall, A.R., Desor, D., Lagrost, L., et al., 2005. Phospholipid transfer protein (PLTP) deficiency reduces brain vitamin E content and increases anxiety in mice. FASEB J. 19, 296–297.

Dias, G.P., Bevilaqua, M.C., Silveira, A.C., Landeira-Fernandez, J., Gardino, P.F., et al., 2009. Behavioral profile and dorsal hippocampal cells in Carioca high-conditioned freezing rats. Behav. Brain Res. 205, 342–348.

Ditzen, C., Jastorff, A.M., Keler, MS., Bunck, M., Teplytska, L., Erhardt, A., Krömer, S.A., Varadarajulu, J., Targosz, B.S., Sayan-Ayata, E.F., Holsboer, F., Landgraf, R., Turck, C.W., et al., 2006. Protein biomarkers in a mouse model of extremes in trait anxiety. Mol. Cell. Proteom. 5, 1914–1920.

File, S.E., et al., 1992. Behavioural detection of anxiolytic action. In: Elliot, JM, Heal, DJ, Marsden, CA (Eds.), Experimental Approaches to Anxiety and Depression. John Wiley, Chichester, pp. 25–44.

Gabbita, S.P., Lovell, M.A., Markesbery, W.R., et al., 1998. Increased nuclear DNA oxidation in the brain in Alzheimer's disease. J. Neurochem. 71, 2034–2040.

Gingrich, J.A., et al., 2005. Oxidative stress is the new stress. Nat. Med. 11, 1281–1282.

Gomes, V.C., Landeira-Fernandez, J., et al., 2008. Amygdaloid lesions produced similar contextual fear conditioning disruption in the Carioca high- and low-conditioned freezing rats. Brain Res. 1233, 137–145.

Hovatta, I., Tennant, R.S., Helton, R., Marr, R.A., Singer, O., Redwine, J.M., Ellison, J.A., Schadt, E.E., Verma, I.M., Lockhart, D.J., Barlow, C., et al., 2005. Glyoxalase 1 and glutathione reductase 1 regulate anxiety in mice. Nature 438, 662–666.

Krömer, S.A., Keβler, M.S., Milfay, D., Birg, I.N., Bunck, M., Czibere, L., Panhuysen, M., Pütz, B., Deussing, J.M., Holsboer, F., Landgraf, R., Turck, C.W., et al., 2005. Identification of glyoxalase-I as a protein marker in a mouse model of extremes in trait anxiety. J. Neurosci. 25, 4375–4384.

Landeira-Fernandez, J., et al., 1996. Context and Pavlovian conditioning. Braz. J. Med. Biol. Res. 29, 149–173.
Landeira-Fernandez, J., DeCola, J.P., Kim, J.J., Fanselow, M.S., et al., 2006. Immediate shock deficit in fear conditioning: effects of shock manipulations. Behav. Neurosci. 120, 873–879.
Liu, C.F., Yu, L.F., Lin, C.H., Lin, S.C., et al., 2008. Effect of auricular pellet acupressure on antioxidative systems in high-risk diabetes mellitus. J. Altern. Complement. Med. 14, 303–307.
Maren, S., Fanselow, M.S, et al., 1997. Electrolytic lesions of the fimbria/fornix, dorsal hippocampus, or entorhinal cortex produce anterograde deficits in contextual fear conditioning in rats. Neurobiol. Learn. Mem. 67, 142–149.
Morale, M.C., Serra, P.A., L'episcopo, F., Tirolo, C., Caniglia, S., Testa, N., Gennuso, F., Giaquinta, G., Rocchitta, G., Desole, M. S., Miele, E., Marchetti, B., et al., 2006. Estrogen, neuroinflammation and neuroprotection in Parkinson's disease: glia dictates resistance versus vulnerability to neurodegeneration. Neuroscience 138, 869–878.
Mosley, R.L., Benner, E.J., Kadiu, I., Thomas, M., Boska, M.D., Hasan, K., Laurie, C., Gendelman, H.E., et al., 2006. Neuroinflammation, oxidative stress and the pathogenesis of Parkinson's disease. Clin. Neurosci. Res. 6, 261–281.
Ohkawa, H., Ohishi, N., Yagi, K., et al., 1979. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal. Biochem. 95, 351–358.
Ozcan, M.E., Gulec, M., Ozerol, E., Polat, R., Akyol, O., et al., 2004. Antioxidant enzyme activities and oxidative stress in affective disorders. Int. Clin. Psychopharmacol. 19, 89–95.
Pavlov, I., et al., 1927. Conditioned Reflexes: An Investigation of the Physiological Activity of the Cerebral Cortex. Oxford University Press, London.
Ramos, A., Mormède, P., et al., 2006. Genetic analysis of emotional behaviors using animal models. In: Jones, B.C., Mormède, P. (Eds.), Neurobehavioral Genetics: Methods and Applications second ed. CRC Press, Boca Raton, pp. 291–306.
Richmond, M.A., Yee, B.K., Pouzet, B., Veenman, L., Rawlins, J.N., Feldon, J., Bannerman, D.M., et al., 1999. Dissociating context and space within the hippocampus: effects of complete, dorsal, and ventral excitotoxic hippocampal lesions on conditioned freezing and spatial learning. Behav. Neurosci. 113, 1189–1203.
Rogers, J.L., Hunsaker, M.R., Kesner, R.P., et al., 2006. Effects of ventral and dorsal CA1 subregional lesions on trace fear conditioning. Neurobiol. Learn. Mem. 86, 72–81.
Salim, S., Asghar, M., Chugh, G., Taneja, M., Xia, Z., Saha, K., et al., 2010a. Oxidative stress: a potential recipe for anxiety, hypertension and insulin resistance. Brain Res. 1359, 178–185.
Salim, S., Sarraj, N., Taneja, M., Saha, K., Tejada-Simon, M.V., Chugh, G., et al., 2010b. Moderate treadmill exercise prevents oxidative stress-induced anxiety-like behavior in rats. Behav. Brain Res. 208, 545–552.
Serrano, F., Klann, E., et al., 2004. Reactive oxygen species and synaptic plasticity in the aging hippocampus. Ageing Res. Rev. 3, 431–443.
Sigmundi, R.A., Bouton, M.E., Bolles, R.C., et al., 1980. Conditioned freezing in the rat as a function of shock intensity and CS modality. Bull. Psychonom. Soc. 15, 254–256.
Singewald, N., Salchner, P., Sharp, T., et al., 2003. Induction of c-Fos expression in specific areas of the fear circuitry in rat forebrain by anxiogenic drugs. Biol. Psychiatry 53, 275–283.
Smith, M.A., Harris, P.L.R., Sayre, L.M., Perry, G., et al., 1997. Iron accumulation in Alzheimer's disease is a source of redox-generated free radicals. Proc. Nat. Acad. Sci. U.S.A. 94, 9866–9868.
Souza, C.G., Moreira, J.D., Siqueira, I.R., Pereira, A.G., Rieger, D.K., Souza, D.O., Souza, T.M., Portela, L.V., Perry, M.L., et al., 2007. Highly palatable diet consumption increases protein oxidation in rat frontal cortex and anxiety-like behavior. Life Sci. 81, 198–203.
Valko, M., Izakovic, M., Mazur, M., Rhodes, C.J., Telser, J., et al., 2004. Role of oxygen radicals in DNA damage and cancer incidence. Mol. Cell. Biochem. 266, 37–56.
Volkel, W., Sicilia, T., Pahler, A., Gsell, W., Tatschner, T., Jellinger, K., Leblhuber, F., Riederer, P., Lutz, W.K., Gotz, M.E., et al., 2006. Increased brain levels of 4-hydroxy-2-nonenal glutathione conjugates in severe Alzheimer's disease. Neurochem. Int. 48, 679–686.
Wendel, A., et al., 1981. Glutathione peroxidase. Methods Enzymol. 77, 325–333.
Yoon, T., Otto, T, et al., 2007. Differential contributions of dorsal vs. ventral hippocampus to auditory trace fear conditioning. Neurobiol. Learn. Mem. 87, 464–475.