Mutation Research 772 (2015) 30–37

Contents lists available at ScienceDirect

Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis

journal homepage: www.elsevier.com/locate/molmut
Community address: www.elsevier.com/locate/mutres

Anxious phenotypes plus environmental stressors are related to brain DNA damage and changes in NMDA receptor subunits and glutamate uptake

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ARTICLE INFO

Article history:
Received 14 October 2014
Received in revised form
17 December 2014
Accepted 18 December 2014
Available online 27 December 2014

Keywords:
DNA damage
Glutamatergic system
Anxiety
Stress
Depression

ABSTRACT

This study aimed at investigating the effects of chronic mild stress on DNA damage, NMDA receptor subunits and glutamate transport levels in the brains of rats with an anxious phenotype, which were selected to represent both the high-freezing (CHF) and low-freezing (CLF) lines. The anxious phenotype induced DNA damage in the hippocampus, amygdala and nucleus accumbens (NAc). CHF rats subjected to chronic stress presented a more pronounced DNA damage in the hippocampus and NAc. NMDAR1 were increased in the prefrontal cortex (PC), hippocampus and amygdala of CHF, and decreased in the hippocampus, amygdala and NAc of CHF stressed. NMDAR2A were decreased in the amygdala of the CHF and stressed; and increased in CHF stressed. NMDRA2A in the NAc was increased after stress, and decreased in the CLF. NMDAR2B were increased in the hippocampus of CLF and CHF. In the amygdala, there was a decrease in the NMDAR2B for stress in the CLF and CHF. NMDAR2B in the NAc were decreased for stress and increased in the CHF; in the PC NMDAR2B increased in the CHF. EAAT1 increased in the PC of CLF + stress. In the hippocampus, EAAT1 decreased in all groups. In the amygdala, EAAT1 decreased in the CLF + stress and CHF. EAAT2 were decreased in the PC for stress, and increased in CHF + control. In the hippocampus, the EAAT2 were increased for the CLF and decreased in the CLF + stress. In the amygdala, there was a decrease in the EATT2 in the CLF + stress and CHF. These findings suggest that an anxious phenotype plus stress may induce a more pronounced DNA damage, and promote more alterations in the glutamatergic system. These findings may help to explain, at least in part, the common point of the mechanisms involved with the pathophysiology of depression and anxiety.

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1. Introduction

Depression is seen globally as a major public health concern, with a lifetime prevalence of approximately one in five [33]. In addition, many patients with depression present comorbidities, among which, the most common is anxiety [12]. It is well known that associated comorbidities can lead to mood disorders following a more severe course, and also to less positive treatment responses, when

Abbreviations: CHF, Carioca high-freezing; CLF, Carioca low-freezing; Ac, nucleus accumbens; NMDA, N-methyl-D-aspartate; PC, prefrontal cortex.

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http://dx.doi.org/10.1016/j.mrfmmm.2014.12.005
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compared with individuals who have experienced just depression or anxiety alone [61,68]. Although some theories have been proposed, including models which have pointed toward stress or cognitive vulnerability as a cause for depression and anxiety symptoms, or indeed to cognitive vulnerability and anxiety symptoms being a cause of depressive symptoms [11], it is difficult to establish a common point for the mechanisms involved with the pathophysiology of both depression and anxiety. Thus, animal models of psychiatric dysfunctions have been described as important tools to investigate the neurobiology involved with mood disorders, as well as aiding with the development of effective therapeutic targets for its treatment [1]. For studies related to stress or depression, animal models such as chronic mild stress (CMS) have been used. CMS is an animal model based on environmental manipulations, which induce anhedonia and depressive-like behavior in rodents, as well as physiological alterations, similar to those found in individuals suffering from depression [5,54]. In relation to anxiety, some animals models have been developed to generate both conditioned and unconditioned fears in the same rat, and normally anxious rodents are selectively bred for both high and low levels of freezing in response to contextual cues previously associated with footshock [19,21].

Recently, basic and clinical studies have shown an important role for the glutamatergic system in both depression and anxiety [22,34,49]. Glutamate is a neurotransmitter which is important to neural plasticity, cell proliferation and migration beyond memory and learned processes, including the acquisition and extinction of fear-conditioning [45,58]. Glutamate acts in specific receptors located on the surface of the cell membrane, classified according to their pharmacological and functional properties [60]. Its action occurs in three different cellular compartments: presynaptic neurons, postsynaptic neurons and glia, and it acts through two types of receptors: (1) ionotropic (iGluR), including NMDA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPA) and kainate; and (2) metabotropic (mGluR) [43]. Interestingly, patients with depression presented significantly increased levels of serum glutamate, compared with healthy controls [34,47]. Glutamate decarboxylase 1 is an enzyme that catalyzes the decarboxylation of glutamate to GABA, and is associated with determining any predisposition to anxiety disorders in adulthood after childhood stress exposure [22]. In addition, glutamate receptor knockout mice manifested anxiety and depressive-like behavior [65]. It is well known that an increase in glutamate levels, dysfunction in their receptors, or dysfunction in uptake can cause excitotoxicity and neuronal death, which involves mitochondrial permeability and elevated levels of free radicals, as well as DNA damage [35,44]. DNA damage may activate several intracellular signaling pathways such as the phosphorylation of p53 at the serine and threonine residues, leading to cell apoptosis [18,24]. In this way, studies have shown that DNA damage may play a role in the pathophysiology of mood disorders [3,7,32]. It has also been shown that the frequency of DNA damage is correlated with the severity of depressive symptoms [4]. Moreover, genes involved in the repair of oxidative DNA damage, telomere dysfunction and regulation were directly correlated with anxiety scores in depressive patients [63].

As there is consistent data to indicate that the susceptibility to stress increases with a vulnerability to anxiety and vice versa, and that the importance of both the glutamatergic system and DNA in pathophysiology of mood disorders are also evident. Thus, this study was aimed at investigating the impact of stress-induced damage to DNA and NMDA receptor subunits, and also at glutamate transporters levels in the prefrontal cortex, hippocampus, nucleus accumbens and amygdala of rats with anxious phenotype. The prefrontal cortex, hippocampus, amygdala and nucleus accumbens were chosen due to their significance in both stress and anxiety [21,23,50].

2. Materials and methods

2.1. Animals

All 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) and also with approval by the local Ethics Committee (Universidade do Extremo Sul Catarinense) under protocol number 020/2013. Experimental animals (Carioca High-Freezing [CHF], a line selectively bred for high contextual fear conditioning, and Carioca Low-Freezing [CLF], a line selectively bred for low contextual fear conditioning) were obtained from the Pontifical Catholic University of Rio de Janeiro (PUC-Rio) according to procedures described in previous work [19]. Briefly, albino Wistar rats were selectively bred for differences in defensive freezing behaviors in a contextual fear conditioning paradigm. Significant differences in their freezing response were acquired after three generations of selective breeding (S3). In the present work, CHF rats from the fourth generation (S4) were used in the chronic mild stress protocol and biochemical experiments. Control animals corresponded to albino Wistar rats from populations that had not undergone any pretreatment or any other experimental conditions. Males from both groups were born in the same animal room and housed in acrylic cages (31 cm × 38 cm) in groups of 3–6, under a 12 h light/dark cycle (lights on at 8:00 h), with food and water provided ad libitum. 2–3-month-old animals were used in both groups. The animals' body weights varied from 250 to 394 g (control), and 254–379 g (CHF). Both the control 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. Chronic mild stress protocol

The chronic mild stress (CMS) protocol was adapted from the procedure as described previously [26]. The animals were divided into 6 groups: (1) no anxiety + control; (2) no anxiety + stress; (3) CLF + control; (4) CLF + stress; (5) CHF + control; (6) CHF + stress. The control groups (no stress) were kept undisturbed in their home cages during the 20 days of treatment, receiving only ordinary daily care with supports of food and water. The 20-days of chronic mild stress paradigm were applied to the animals in the stressed group [48] (Fig. 1). Individual stressors and the length of time they were applied each day were as follows. The following stressors were used: (i) food deprivation; (ii) water deprivation; (iii) restraint; (iv) restraint at 4 °C; (v) flashing light for duration of 120–210 min; and (vi) isolation. Stressor stimuli were applied at different times every day, in order to minimize its predictability. The restraint test was carried out by placing the animal in a 25 cm × 7 cm plastic tube and adjusting it with plaster tape on the outside, so that the animal was unable to move. There was a 1 cm hole at the far end for breathing. The exposure to a flashing light test was undertaken by placing the animal in a 60 cm × 60 cm × 25 cm plywood box divided into 16 cells of 15 cm × 15 cm × 25 cm with a frontal glass wall. A 40 W lamp, flashing at frequency of 60 flashes/min, was used.

2.3. Comet assay

Comet assay as described previously [64] were carried out under alkaline conditions. Brain tissue from the prefrontal cortex, hippocampus, nucleus accumbens and amygdala were placed in cold phosphate-buffered saline, before being minced with a syringe

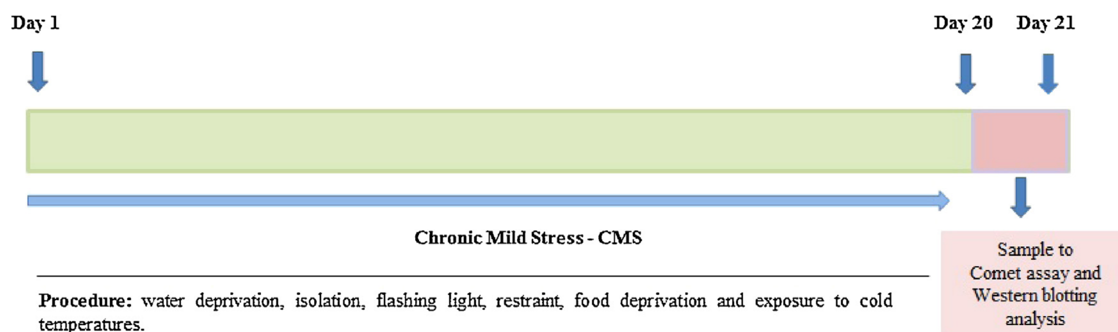

Fig. 1. Schematic drawing of the stress protocol undertaken on rats with an anxious phenotype. CMS procedures were performed for 20 days. On 21st day, one day after last stressor, brain samples were removed for biochemical analysis.

plunger in order to obtain a fine cell suspension. Tissue sample cell aliquots (20 μL) were embedded in low melting agarose (0.75%, w/v; 95 μL or 80 μL, respectively). This mixture was then deposited onto a microscope slide which had been pre-coated with normal melting point agarose (1.5%, w/v), and furnished with a cover slip (two slides per sample). The slides were briefly placed on ice for the agarose to solidify, so that the cover slip could be carefully removed. Next, the base slide was immersed in freshly prepared lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, pH 10.0–10.5). The slides were then immersed in freshly prepared alkaline buffer for 20 min (300 mM NaOH, 1 mM EDTA, pH > 13), before an electrophoresis experiment (15 min/300 mA; 25 V; 0.7 V/cm) was performed in the same buffer. All these steps were carried out under minimal indirect light. Following electrophoresis, slides were neutralized in 400 mM Tris (pH 7.5) and stained with ethidium bromide solution (10 mg/mL). To calculate the damage index (DI), cells were visually separated into five classes according to tail size (0 = no tails to 4 = maximum-length tails). Accordingly, a single DNA damage score was obtained for each sample and consequently for each group studied. The DI for the group could range between 0 (completely undamaged = 100 cells × 0) and 400 (maximum damage = 100 cells × 4). The damage frequency (DF in %) was calculated for each sample, based on the number of cells with tails, compared to those without. Visual scoring of comet assays is considered a reliable evaluation method and usually results in high correlation values with computer-based image analysis methods [14]. All slides were coded for blind analysis.

2.4. Western blotting analysis

To perform immunoblotting, tissue samples taken from the rats prefrontal cortex, hippocampus, amygdala and nucleus accumbens were homogenized in Laemmli buffer (62.5 mM Tris–HCl, pH 6.8, 1% (w/v) sodium dodecyl sulfate (SDS),10% (v/v) glycerol), and equal amounts of protein (100 μg/well) were fractionated by polyacrylamide gel electrophoresis-sodium dodecyl sulfate (SDS-PAGE) and electro transferred to nitrocellulose membranes. The efficiency of the electro transfer was then verified by Ponceau S staining, and the membrane was then blocked in Tris-Tween buffer saline (TTBS: 100 mM Tris–HCl, pH 7.5, containing 0.9% NaCl and 0.1% Tween 20) with 5% albumin. The membranes were then incubated overnight at 4 °C with rabbit polyclonal anti-NMDAR1, anti-NMDAR2A, anti-NMDAR2B, anti-EAAT1 and anti-EAAT2 (1:1000). Secondary anti-rabbit IgG was incubated with the membrane for 2 h (1:1000). The membrane was then washed again with TTBS, and immunoreactivity was detected by chemiluminescence using ECL. Densitometry analysis of the films was performed using the Image J® v.1.34 image analysis software. All results were expressed as a relative ratio between NMDAR1, NMDAR2A, NMDAR2B, EAAT1 and EAAT2 and immunocontent protein β-actin.

2.5. Statistical analysis

All data are presented as mean ± S.E.M to Western blotting analysis, and as mean ± SD to comet assay. The normality of variables was evaluated using the Kolmogorov–Smirnov test. Differences among experimental groups in the comet assay and Western blotting analysis tests were determined by one-way ANOVA, followed by Tukey post-hoc test when ANOVA was significant; P values < 0.05 were considered to be statistical significant.

3. Results

Fig. 2 shows the effects of chronic stress on frequency (A) and index (B) DNA damage in the prefrontal cortex, hippocampus, amygdala and nucleus accumbens of rats with the anxious phenotype. In the hippocampus, we observed an increase in the DNA damage index for all groups, compared to the no anxiety + control group, a decrease in the no anxiety + stress group, and an increase in the CHF groups when compared to CLF + control group ($F = 74.618$; $p < 0.05$; Fig. 2A). In the amygdala, there was an increase in the DNA damage index for the CLF and CHF groups, compared to the no anxiety + control group ($F = 55.284$; $p < 0.05$; Fig. 2A). The DNA damage index was increased in the nucleus accumbens of CHF groups, compared to the no anxiety + control and CLF + control groups ($F = 10.074$; $p < 0.05$; Fig. 2A). We did not observe any DNA damage index ($F = 0.881$; $p = 0.511$; Fig. 2A), or damage frequency ($F = 0.618$; $p = 0.689$; Fig. 2B) in the prefrontal cortex for all groups tested. The DNA damage frequency in the hippocampus was increased in the CLF and CHF groups compared to the no anxiety + control groups, and also in the CHF groups, compared to the CLF + control ($F = 66.441$; $p < 0.05$; Fig. 2B); in the amygdala, we observed an increase in DNA damage frequency in the CLF and CHF groups when compared to the no anxiety + control group ($F = 64.472$; $p < 0.05$; Fig. 2B); in the nucleus accumbens, there was an increase in the CHF groups, compared to the no anxiety + control and CLF + control groups ($F = 10.995$; $p < 0.05$; Fig. 2B).

Fig. 3 illustrates the effects of the stress and anxiety phenotypes on NMDAR1, NMDAR2A, NMDAR2B, EAAT1 and EAAT2 levels in the prefrontal cortex, hippocampus, amygdala and nucleus accumbens. The NMDAR1 subunit was increased in the prefrontal cortex in the CHF + control group, when compared to the no anxiety + control group ($F = 7.78$; $p < 0.001$; Fig. 3A). In the hippocampus, there was an increase in NMDAR1 within the CLF + control and CHF groups, compared to the no anxiety + control group, and also in the CHF groups, compared to CLF + control group; there was a decrease in NMDAR1 within the CHF + stress group, when compared to the CHF + control group ($F = 86.56$; $p < 0.001$; Fig. 3A). In the amygdala, we observed

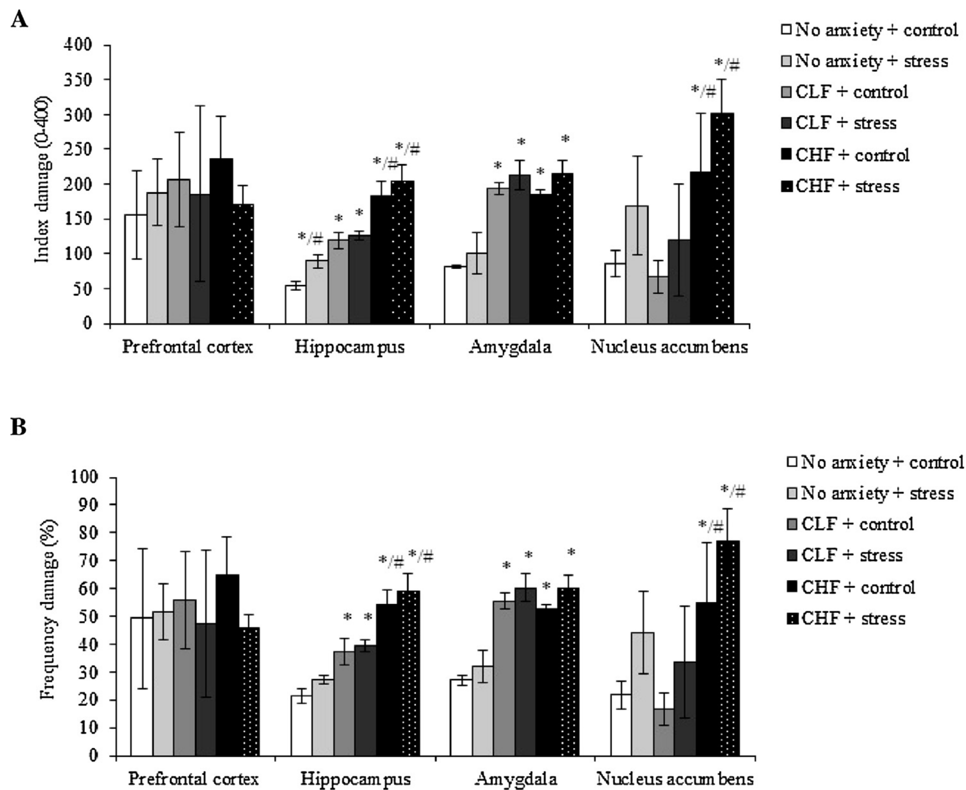

Fig. 2. The effects of the chronic mild stress protocol on frequency (A) and index (B) DNA damage in prefrontal cortex, hippocampus, amygdala and nucleus accumbens of rats with anxious phenotype. Bars represent mean ±S.D. *$p < 0.05$ vs. no anxiety + control; #$p < 0.05$ vs. CLF + control; and $p < 0.05$ vs. CHF + control according to ANOVA, followed by the Tukey post-hoc test.

an increase in NMDAR1 within the CHF + control group, when compared to the no anxiety and CLF control groups, and a decrease in the CHF + stress group, compared to the CHF + control group ($F = 26.52$; $p < 0.001$; Fig. 3A). In the nucleus accumbens, there was a decrease in NMDAR1 within the no anxiety + stress and CHF + stress groups, compared to the no anxiety + control group, and a decrease in the CHF + stress group, compared to CHF + control group ($F = 15.93$; $p < 0.001$; Fig. 3A).

We did not observe any significant differences in the prefrontal cortex relating to NMDAR2A ($F = 2.904$; $p = 0.035$; Fig. 3B), or in the hippocampus ($F = 0.0256$; $p = 0.933$; Fig. 3B), when compared to the control groups. However, in the amygdala, there was a decrease in NMDAR2A within the no anxiety + stress and CHF + control groups, compared to the no anxiety + control group, and also in the CHF + stress group, compared to the CHF + control group ($F = 11.862$; $p < 0.001$; Fig. 3B). NMDAR2A was found to have increased in the nucleus accumbens of the no anxiety + stress group when compared to the control group; and it had decreased in the CLF + stress group, compared to the CLF + control group ($F = 114.197$; $p < 0.001$; Fig. 3B).

In relation to NMDAR2B, an increase was shown in the hippocampus of the CLF and CHF groups, when compared to the no anxiety + control group, and in the CLF + stress group, when compared to the CLF + control group ($F = 31.30$; $p < 0.001$; Fig. 3C). In the amygdala, there was a decrease in NMDAR2B in the no anxiety + stress and CLF + control groups, when compared to the no anxiety + control group, and also in the CHF + stress group, when compared to the CHF + control group; on the other hand, an increase was observed in the CLF + stress and CHF + control groups, when compared to the no anxiety + control group, and also in the CLF + stress group, when compared to the CLF + control group ($F = 34.97$; $p < 0.001$; Fig. 3C). The levels of NMDAR2B in the nucleus accumbens were decreased in the no anxiety + stress group and increased in the CHF groups, when compared to the no anxiety + control group ($F = 26.94$; $p < 0.001$; Fig. 3C). In the prefrontal cortex, alterations were found in NMDAR2B levels only in the CHF + control group, when compared to the CLF + control group ($F = 6.58$; $p = 0.001$; Fig. 3C).

The EAAT1 amino acid transporter levels were increased in the prefrontal cortex of the CLF + stress group, when compared to the no anxiety + control group ($F = 7.687$; $p < 0.001$; Fig. 3D). In the hippocampus a decrease was found in EAAT1 levels in all groups, when compared to the no anxiety + control group ($F = 13.95$; $p < 0.001$; Fig. 3D). In the amygdala, an increase was observed in EAAT1 levels in the CLF + stress and CHF groups, when compared to the no anxiety + control group, and also in the CLF + stress group, when compared to the CLF + control group; and a decrease in CHF + stress group, when compared to the CHF + control group ($F = 188.91$; $p < 0.001$; Fig. 3D). No significant alterations in EATT1 levels were found in the nucleus accumbens, for any of the groups tested ($F = 2.25$; $p = 0.081$; Fig. 3D).

The EAAT2 amino acid transporter levels were found to have decreased in the prefrontal cortex of the no anxiety + stress group, and increased in the CHF + control group, when compared to the no anxiety + control group ($F = 16.54$; $p < 0.001$; Fig. 3E). In the hippocampus, the levels of EAAT2 were increased in the CLF + control group, when compared to the no anxiety + control group, and decreased in the CLF + stress group when compared to the CLF + control group ($F = 7.97$; $p < 0.001$; Fig. 3E). In the amygdala, a decrease was observed in EATT2 levels within the CLF + stress and CHF groups when compared to the no anxiety + control group; and also in the CLF + stress group, when compared to the CLF + control group ($F = 46.02$; $p < 0.001$; Fig. 3E). No alterations were observed in the nucleus accumbens for any of the groups

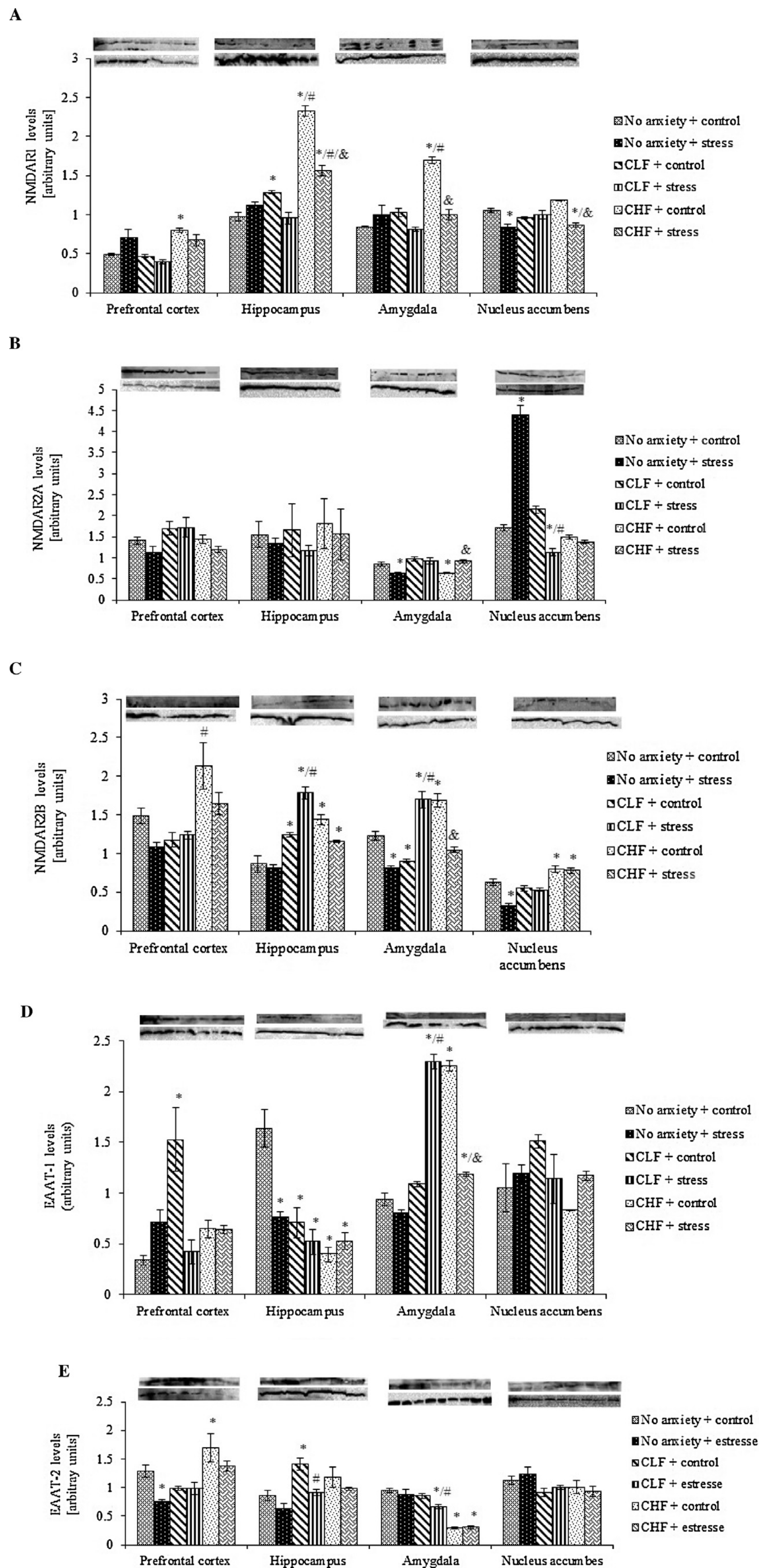

Fig. 3. The effects of the chronic mild stress protocol on NMDAR1 (A), NMDAR2A (B), NMDAR2B (C), EAAT1 (D) and EAAT2 (E) levels in the prefrontal cortex, hippocampus, amygdala and nucleus accumbens of rats with an anxious phenotype. Representative images of each protein and NMDAR1 (A), NMDAR2A (B), NMDAR2B (C), EAAT1 (D) and EAAT2 (E), and β-actin respectively are shown in the upper panels. Bars represent mean ±S.E.M. $^{*}p < 0.05$ vs. no anxiety + control; $^{\#}p < 0.05$ vs. CLF + control; and $p < 0.05$ vs. CHF + control according to ANOVA, followed by the Tukey post-hoc test.

tested ($F = 3.40$; $p = 0.018$; Fig. 3E), when compared to control groups.

4. Discussion

In the present study, we reported for the first time that both the stress and anxiety phenotypes could induce DNA damage index and frequency in the hippocampus, amygdala and nucleus accumbens, but not in the prefrontal cortex. Still, we observed that rats which were selectively bred for high contextual fear conditioning were subjected to chronic stress; they presented a more pronounced level of DNA damage in the hippocampus and nucleus accumbens. DNA damage caused by both anxiety and depression has been related to previous researches. In fact, it has been reported that there was DNA damage in the amygdala and hippocampus of Wistar rats subjected to acute stressors such as the restraint and forced swimming test (FST), and in the prefrontal cortex of rats subjected to the FST [15], suggesting that the DNA damage to different brain structures is dependent on the stressor that is applied. In the present study, we did not show any DNA damage in the prefrontal cortex, and FST was not applied as a stressor. DNA damage can lead to high and deleterious mutation rates, and to DNA toxicity in the nuclear and the mitochondrial genome [40]. Moreover, a study has shown that the expression of two genes, specifically STMN1 and $P16^{ink4a}$, which are markers of telomere dysfunction, cellular senescence and DNA damage were upregulated in the blood leucocytes of women with major depressive disorder. Also, the STMN1 levels were correlated with anxiety scores in the depression group, and $p16^{INK4a}$ with the depression and anxiety score [63]. Thus, it is possible that DNA gene dysfunction in specific genes can be related to anxiety and depression, which are frequently found in the same individual. It is well known that DNA alterations may originate from glutamate-induced Ca^{2+} influx, and consequently, a mitochondrial reactive oxygen species-mediated mechanism [67]. Therefore, the results found in the present study detailing the higher levels of DNA damage found in the CHF rats may be related, at least in part, to oxidative stress. The current CHF generation, when compared to CLF, is shown to be higher in lipid peroxidation and lower in the antioxidant enzymes catalase and glutathione peroxidase within the brain [30].

Glutamate excitotoxicity has been highlighted as having an important role in the pathology of mood disorders, including anxiety and depression [2,42,59]. In addition, the importance of the NMDA receptor and its subunits NR1, NR2A-D and NR3, which are coded by different genes [58], have been reported in many basic and clinical studies. The NR1 subunit is important for the function of the NMDA receptor channels, and the NR2 subunits determine the features of NMDA by forming different heteromeric arrangements with the NR1 subunit [17,31]. The NMDA receptor is implicated in neural development, plasticity and excitotoxicity, beyond memory and behavior [8,13,45]. Acute stress increases glutamate release in the amygdala [57]. Moreover, knockout mice specific to ionotropic glutamate receptors present hyperactivity, lower anxiety and depressive-like behaviors [65,66], suggesting that dysfunction within glutamate receptors may be involved with the development of mood disorders. NMDA receptors in the amygdala and hippocampus are intricate in the acquisition, as well as extension and expression of contextual and fear conditioning [16]. In fact, an NMDA receptor antagonist injected directly into the amygdala was able to block retention of extinction fear [25]. Also, the amygdala has been seen to be crucial for the expression of contextual fear conditioning in both CLF and CHF rats [19]. Previously, a series of studies demonstrated that NMDA receptor antagonists, such as ketamine and memantine, can present antidepressant effects in animal models of depression, as well as in humans suffering from major depression [6,53–56]. In the present work, we related that the subunit of the NMDA receptor R1, which is a critical component of the functional ionophore complex, was increased in the CHF groups in the prefrontal cortex, hippocampus and amygdala. However, there was a decrease in the CHF groups which had been subjected to stress within the hippocampus, amygdala and NAc, suggesting that the low levels of NMDAR1 may be related to a commonality between anxiety and depression. Interestingly, it was also shown that there was a reduction in NMDAR1 mRNA levels within the dentate gyrus and CA3 regions of post-mortem samples taken from schizophrenics, depressives and bipolar patients [38].

In the present study, we did not find any large alterations in NMDAR2A levels, only a decrease in the amygdala of the CHF and stress groups, an increase in CHF plus stress group, and an increase in stressed and a decrease in CLF groups in the NAc. Cicek et al. [10] demonstrated that isoniazid, which is known to be neurotoxic, induced a decrease in NR2A levels in the hippocampus that was correlated to an increase in lipid peroxidation, and that endosteine, which is an antioxidant, was able to reverse these effects. In addition, mice carrying a mutation at the Tyr 1325 phosphorylation site, which is largely found in NR2A, showed reduced depression-related behavior [62], suggesting that NR2A may be an important target in studying the pathogenesis of mood disorders and in developing therapeutic drugs.

Previously, it has been shown that regulation of NMDAR2B phosphorylation was associated with anxiety-like behavior and corticotropin-releasing factor (CRF) expression in the amygdala [20]. In this study we showed alterations in NMDAR2B induced by both stress and anxious phenotypes; however, the effects were dependent on the brain area. The activation of NMDAR2B is pointed to being neurotoxic [37,41]. In fact, an increase in N2B expression was found in the prefrontal cortex, hippocampus and amygdala of CHF rats, when compared to CLF rats, suggesting that animals which are more anxious have altered patterns of N2B subunit expression [39], these findings are correlated by our study, which demonstrated larger alterations in rats with higher levels of anxiety, mainly when they were submitted to chronic stress. Differences between NMDA subunits may be attributed to their unlike properties. In fact, NMDA subunits present electrophysiological and pharmacological differences, beyond the differences in their distribution and expression profiles [70]. Recently it was demonstrated that glutamate deactivation triheteromers are distinct from NR1/N2B and NR1/NR2B by the subunit-selective antagonist's ifenprodil, CP-101, 606, TCN-201, and extracellular Zn^{2+} [27]. Furthermore, intracellular signaling effects after NMDA activation are dependent on its localization, for example, the activation of the extrasynaptic NMDA receptor is neurotoxic and the activation of synaptic NMDARs is neuroprotective [28,29]. Thus, the different effects found in NMDA subunits in the present study within anxious and/or stressed rats may be attributed to the site from which the NMDA is studied. This then, in course, could become a limitation of this work, and future studies are necessary to evaluate the effects of mood disorders specific to NMDA and its subunit localizations. However, it is known that pro-death pathways or events, including oxidative stress and DNA damage are preferentially activated by extrasynaptic NMDAR [28]. Besides the changes in NMDA receptors, many studies have suggested that impairment in the reuptake of glutamate may also be involved in glutamate-induced toxicity to neurons, and that alterations in the uptake system may be a marker for depression [51]. Glutamate is removed from the synaptic cleft present in the glial astrocytes by specialized transporters such as EAAT1, EAAT2 and EAAT3 [51]. Reductions in EAAT2 and EAAT3 in the hippocampus, striatum and frontal cortex are associated with depressive-like behavior in rats subjected to prenatal restraint stress [69]. Suppressed expression of EAAT2 was found in the hippocampus and cerebral cortex of rodents that were submitted to

learned helplessness, an animal model of depression [71]. Moreover, postmortem brain samples from patients suffering with major depression also demonstrated reductions in EAAT1 and EAAT2 [9,46]. Accordingly, we also related a decrease in EAAT1 within the hippocampus of stressed or anxious rats, and in the amygdala of CHF rats subjected to stress. However, EAAT1 levels were increased in the prefrontal cortex of CLF rats subjected to stress, and in the amygdala of stressed CLF and CHF rats. These results showed in the amygdala may be related to a compensatory down regulation of EAAT2 in the amygdala. Furthermore, the hippocampus and amygdala present different effects; in depression, the hippocampus was reduced in volume and activity, while the amygdala presented both increased morphology and activity [23]. Still, increased levels of EAAT2 mRNA and protein expression in the hippocampus were found in rats subjected to chronic restraint stress, and EAAT2 levels were decreased via treatment with the antidepressant tianeptine [52]. Thus it appears that the effects on EAAT activity are dependent on the brain area and stressor applied. Altered or reduced glutamate reuptake may lead to impaired NMDA-receptor function and neural alteration [9,36], as pointed out by our findings.

In conclusion, our data shows, for the first time to our knowledge, that an anxious phenotype plus stress may induce a more pronounced level of DNA damage and cause alterations within the glutamatergic system in the brain areas involved with mood disorders. These findings may explain, at least in part, the common point of the mechanisms involved with the pathophysiology of both depression and anxiety. Future studies are necessary to investigate the relationship between impairment of glutamatergic neurotrasmission and intracellular signaling cascades that lead to DNA damage. Moreover, these approaches might help to develop new therapeutic targets in treating anxiety and depression.

Conflict of interest statement

The authors declare any conflict of interest.

Acknowledgements

Laboratory of Neurosciences (Brazil) is a center within the National Institute for Translational Medicine (INCT-TM) and is also a member of the Center of Excellence in Applied Neurosciences of Santa Catarina (NENASC). This research was supported by grants from CNPq (JQ, FDP and GZR), FAPESC (JQ), Instituto Cérebro e Mente, UNESC (JQ, FDP and VMA), and LíOréal/UNESCO/ABC Brazil Fellowship for Women in Science 2011 (GZR). JQ and FDP are CNPq Research Fellows. HMA has CAPES studentships. The Center for Experimental Models in Psychiatry (USA) is funded by the Department of Psychiatry and Behavioral Sciences, The University of Texas Medical School in Houston.

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