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ORIGINAL PAPER

Long-Lasting Effects of Maternal Separation on an Animal Model of Post-Traumatic Stress Disorder: Effects on Memory and Hippocampal Oxidative Stress

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Abstract Adverse early life events, such as periodic maternal separation, may alter the normal pattern of brain development and subsequently the vulnerability to a variety of mental disorders in adulthood. Patients with a history of early adversities show higher frequency of post-traumatic stress disorder (PTSD). This study was undertaken to verify if repeated long-term separation of pups from dams would affect memory and oxidative stress parameters after exposure to an animal model of PTSD. Nests of Wistar rats were divided into intact and subjected to maternal separation (incubator at 32°C, 3 h/day) during post-natal days 1–10. When adults, the animals were subdivided into exposed or not to a PTSD model consisting of exposure to inescapable footshock, followed by situational reminders. One month after exposure to the shock, the animals were exposed to a memory task (Morris water maze) and another month later animals were sacrificed and DNA breaks and antioxidant enzymes activities were measured in the hippocampus. Rats exposed to shock or maternal separation plus shock showed long-lasting effects on spatial memory, spending more time in the opposite quadrant of the water maze. This effect was higher in animals subjected to both maternal separation and shock. Both shock and maternal separation induced a higher score of DNA breaks in the hippocampus. No differences were observed on antioxidant enzymes activities. In conclusion, periodic maternal separation may increase the susceptibility to the effects of a stressor applied in adulthood on performance in the water maze. Increased DNA breaks in hippocampus was induced by both, maternal separation and exposure to shock.

Keywords Maternal separation · Post-traumatic stress disorder · Comet assay · Memory · Oxidative stress

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Introduction

Early life stressors have a permanent effect on the organism. This organizational effect of environment on physiological systems is known as perinatal "programming" [1]. In the rat, the first two weeks of life are a critical period for the maturation of the hypothalamic–pituitary–adrenocortical (HPA) axis, which is one of the major neuroendocrine systems activated in response to environmental challenges, and early environmental manipulations have long-lasting effects on behavioral parameters related to coping with stress [2, 3]. One of these manipulations is long-term separation of the pups from the dam, which is considered one of the most potent naturally occurring stressors to which rat pups can be exposed during the neonatal period [4]. In this procedure, neonatal rats are removed from the mother for several hours daily during the first 2 weeks of

life [3, 5, 6]. When tested as adults, maternally separated (MS) offspring exhibited behavioral and neuroendocrine signs similar to those observed in patients with depression and anxiety disorders [7, 8].

Early stressful situations may increase the vulnerability to cognitive deficits and psychiatric disorders in adult life [9], including post-traumatic stress disorder (PTSD). PTSD is a serious and debilitating anxiety disorder in which a person exposed to a traumatic event (or events) develops symptoms in three domains: avoiding stimuli associated with the trauma, experiencing symptoms of increased autonomic arousal and reexperiencing the trauma, with the pathological replay of the emotional memory formed in response to painful, life-threatening, or horrifying events [10]. In order to understand the neurobiology of PTSD, animal models of this disorder have been used, in which different aspects of this condition may be studied. The exposure to uncontrollable stressors, such as inescapable footshock, produces many behavioral changes, and this paradigm has been proposed as model of depression and of anxiety-related disorders such as PTSD [11]. Some authors use a re-exposure to a traumatic stressor [12, 13] or repeated exposures to situational reminders [14, 15], which are believed to induce re-experiencing of the aversive event.

Imaging studies in PTSD patients have demonstrated volume reductions in the hippocampus that seems to be correlated with illness severity and the degree of cognitive deficit [16, 17]. The hippocampus is involved in the response to stress and in memory performance [18]. Altered activities of the antioxidant enzymes and levels of free radical scavengers, as well as other parameters of oxidative stress in hippocampus have been found to be related to stress exposure [19, 20], suggesting that the stress response leads to increased production of free radicals in hippocampus [21, 22].

The aim of the present study is to verify if maternal separation in rats alters the susceptibility to the effects of an intense stressor applied in adult age, verifying its long-lasting effects on cognitive aspects (spatial memory, evaluated by the performance in Morris water maze task) and also verifying oxidative stress parameters as antioxidant enzymes activities and DNA breaks in the hippocampus.

Materials and Methods

Subjects

Pregnant Wistar rats bred at our own animal facility were randomly selected. They were housed alone in home cages made of Plexiglas (65 × 25 × 15 cm) with the floor covered with sawdust and were maintained in a controlled environment: lights on between 07:00 and 19:00 hours, temperature of 22 ± 2°C, cage cleaning twice a week, food and water provided ad libitum. All litters were culled within 24 h from birth to eight pups and were maintained intact unless for maternal separation procedures, which were carried out between 10:00 and 14:00 hours.

Weaning was on postnatal day 21. In this study only males rats were used respecting a maximum of two pups per litter per experiment. The animals were housed four to five per cage. Rats had free access to food (standard lab rat chow) and water, except during the period when the behavioral task was applied. Tasks were performed between 13:00 and 16:00 hours, after animals had reached adult life.

All animal treatments were approved by the Ethical Committee of our University and followed the recommendations of the International Council for Laboratory Animal Science (ICLAS).

Maternal Separation

Non-separated group—Pups were left undisturbed with the dam until weaning. It was stated on the cage that these animals should not be touched, not even for cage cleaning. Dirty sawdust was carefully removed from one side of the cage, without disturbing the mother and the nest, and replaced by clean sawdust at that side by the principal researcher.

Maternal separation group—Pups were removed from their home cage and were placed into a clean cage lined with clean paper towel, inside an incubator at 32°C next to the dam's cage. After 3 h, pups were returned to their dams. This procedure was carried out during the first ten days of life, after which pups were left undisturbed until weaning.

Exposure to a Stressor During Adulthood

After reaching 60 days of age, the animals were exposed to a PTSD model (adapted from [11]), which consisted of a single exposure to footshock, followed by three weekly exposures to a situational reminder (SR). The animals (maternal separated and non-separated) were subdivided into two other groups: no shock and shock.

The apparatus consisted of a 50 × 25 × 25 cm box, which was divided in two equal compartments, both compartments with a frontal glass wall. The first compartment presented a smooth floor, and the second compartment presented a grid floor consisting of 1 mm bronze bars spaced 10 mm apart. The animals were gently held by their body and lowered in the first compartment, with their nose pointing to the rear left corner. After 2 min, a guillotine door was opened until the animal crossed to the

second compartment. The door was then closed and a 1 mA 60 Hz footshock was delivered during 20 s. The no shock group was subjected to the same treatment, but no shock was delivered.

For the exposure to situational reminders (SR), 1 week after exposure to the apparatus described above, the animals were placed in the box for 2 min, but just in the first compartment. This procedure was repeated during 3 weeks, with a seven-days interval between each SR.

Morris Water Maze

About 10 days after the last SR, rats were submitted to the Morris water maze, to evaluate spatial memory. The maze consisted of a black circular pool with 180 cm in diameter filled with water (temperature 22°C, depth 40 cm) situated in a room with visual cues on the walls. A transparent platform with 10 cm in diameter was submerged in the water (2 cm below the water surface). The pool was conceptually divided in four quadrants and had four points designed as starting positions (N, S, W or E). Rats received five training sessions (one per day) and a probe trial in the 6th day. Each session consisted of four trials with a 10 min intertrial interval. A trial began when the rat was placed in the water at one of the four starting positions, chosen at random, facing the wall. The order of starting position varied and any given sequence was not repeated on acquisition phase days. The rat was given 60 s to locate the platform; if the animal did not succeed, it was gently guided to it and left on it for 20 s.

Rats were dried and returned to their home cages after each trial. The probe trial consisted of a single trial, with the platform removed. The time spent in the target quadrant (where the platform used to be), as well as in the opposite quadrant, were measured [23].

Biochemical Measurements

Preparation of the Samples

One month after the behavioral task, animals were sacrificed between 10:00 and 14:00 hours and the hippocampus was dissected and used to assess DNA breaks through the comet assay, or frozen at −70°C, until evaluation of antioxidant enzymes activities. All animals were sacrificed within this interval of time in a random order considering groups.

Single Cell Gel Electrophoresis: Comet Assay

A standard protocol for comet assay preparation and analysis was adopted [24], as described in [25]. Cells were scored from 0 (undamaged) to 4 (maximal damage), according to the tail intensity (size and shape), resulting in a DNA breaks score [26].

Antioxidant Enzymes Activities

For evaluating antioxidant enzymes activities, the hippocampus was stored at −70°C until analysis, when it was homogenized in 10 vol (w:v) ice-cold 50 mM potassium phosphate buffer (pH 7.4), containing 1 mM EDTA for determination of Superoxide Dismutase (SOD) and Glutathione Peroxidase (GPx) activities. To determinate catalase (CAT) activity, samples were homogenized in 10vol (w:v) ice-cold potassium phosphate buffer 10 mM (pH 7.0). The homogenate was centrifuged at $960\times g$ for 10 min at 4°C and the supernatant was used.

Superoxide Dismutase Activity SOD activity was determined using a RANSOD kit (Randox Labs., USA) which is based on the procedure described by [27].

Catalase Activity Catalase is an enzyme able to degrade hydrogen peroxide (H_2O_2), and its activity assessment is based upon establishing the rate of H_2O_2 degradation spectrophotometrically at 240 nm at 25°C [28]. CAT activity was calculated in terms of micromoles of H_2O_2 consumed per minute per milligram of protein, using a molar extinction coefficient of 43.6 M^{-1} cm^{-1}.

Glutathione Peroxidase Activity GPx activity was determined according to [29], with modifications. The reaction was carried out at 37°C in 200 µL of solution containing 20 mM potassium phosphate buffer (pH 7.7), 1.1 mM EDTA, 0.44 mM sodium azide, 0.5 mM NADPH, 2 mM glutathione and 0.4 U glutathione reductase. The activity of GPx was measured taking tert-butylhydroperoxide as the substrate at 340 nm. The contribution of spontaneous NADPH oxidation was always subtracted from the overall reaction ratio. GPx activity was expressed as nmol NADPH oxidized per minute per mg protein.

Protein Assay

The total protein concentrations were determined using the method described by [30] using bovine serum albumin as the standard.

Statistical Analysis

Data were expressed as mean + SE of the mean, and were analyzed by a two way ANOVA, using maternal separation and exposure to shock as factors. The significance level was accepted as different when the P value was equal or

less than 0.05. Sample size varies in each experiment and is showed individually in the Results section.

Results

One month after exposure to footshock, rats were submitted to a spatial memory task, using the Morris water maze. The mean time to find the platform in the training days is showed in Fig. 1. Two-way ANOVA showed differences in the time spent in the target and opposite quadrants (Fig. 2). Animals that were subjected to shock spent less time in the target quadrant [F (1, 28) = 8.702; $P < 0.01$]. Significant effects of shock [F (1, 28) = 33.01; $P < 0.001$] and a significant interaction shock × maternal separation [F (1, 28) = 5.407; $P < 0.05$] were observed in the time spent in the opposite quadrant, since animals exposed to shock showed an increase in this parameter and this effect was further increased by maternal separation.

A higher score of DNA breaks was observed in the hippocampus of animals subjected to shock or to maternal separation. A two-way ANOVA showed significant effects of shock [F (1, 23) = 5.503; $P < 0.05$] and maternal separation [F (1, 23) = 35.473; $P < 0.001$], as displayed in Fig. 3. On the other hand, no differences were found among the groups on the antioxidant enzymes activities (SOD, CAT, GPx) (two-way ANOVA; $P > 0.05$; Table 1).

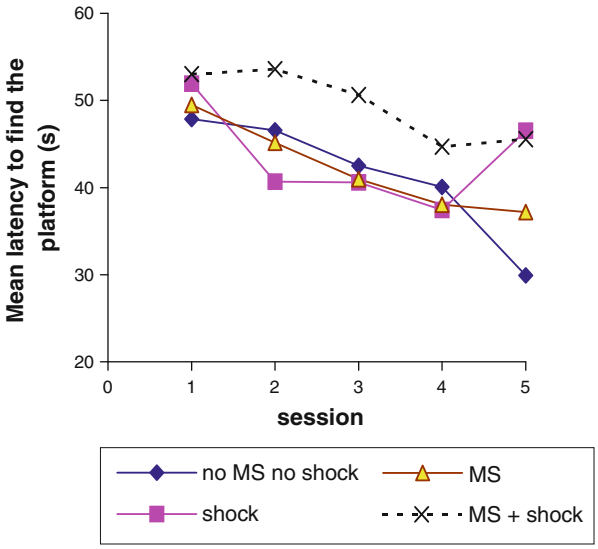

Fig. 1 Latency to find the platform (s) during the training sessions in the Morris Water Maze of the no-shock, shock, non-maternal separation and maternal separation groups. Data are shown as the mean latency (in s) to find the platform in the training days. Two-way ANOVA, N = 7–10 animals per group. There was no significant difference between groups (maternal separation and shock) on this parameter (Two-way ANOVA, $P > 0.05$ for both factors, maternal separation and for exposure to shock)

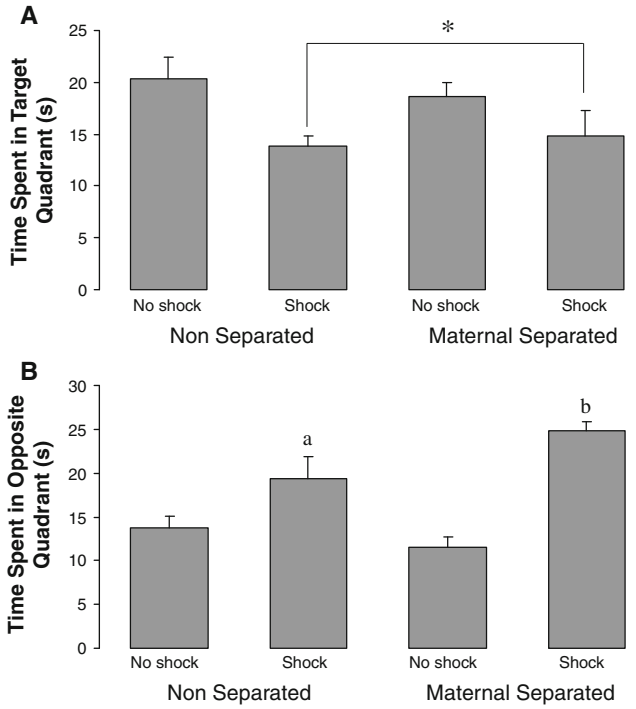

Fig. 2 Performance in the Morris Water Maze of the no-shock, shock, non-maternal separation and maternal separation groups. Data are shown as mean + SEM of the time (in s) spent in the target (**a**) and opposite (**b**) quadrants during the 60 s exposure to the maze in the test session. N = 7–10 animals per group. *A two-way ANOVA showed a shock effect for the target quadrant (**a**). In (**b**), there was a shock effect (**a**) and an interaction between shock × maternal separation for the opposite quadrant (**b**)

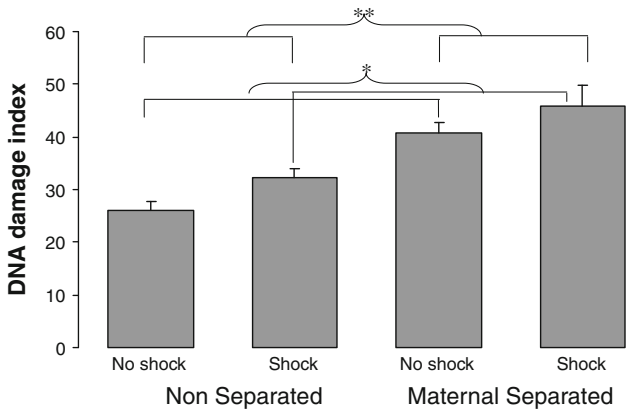

Fig. 3 Effect of shock and maternal separation on DNA breaks index in hippocampus 2 months after subjecting the animals to an inescapable shock as a PTSD model. Data are expressed as mean+SEM N = 6–8 animals/group. A two-way ANOVA showed significant effects of both exposure to shock (*) and maternal separation (**)

Discussion

Environmental conditions during the neonatal period may affect adult behavioral and neuroendocrine responsiveness,

Table 1 Antioxidant enzymes activities superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT)

Groups	SOD	GPx	CAT
No MS no shock	12.52 ± 2.85	56.78 ± 8.56	1.03 ± 0.55
MS	13.90 ± 4.07	58.81 ± 4.71	1.94 ± 0.77
Shock	15.31 ± 1.65	49.90 ± 6.52	1.70 ± 0.69
MS + shock	13.01 ± 1.62	66.76 ± 9.43	0.97 ± 0.52

Groups are maternal separated or not and exposed or not to shock as a PTSD model in the adulthood. Data are expressed as mean ± SEM of SOD (U/mg protein), GPx (nmol NADPH oxidized/min/mg protein), and CAT (μmol H2O2 transformed/min/mg protein) activities. N = 6/group. A two-way ANOVA showed no differences between the groups ($P > 0.05$)

and may influence the pathogenesis of psychiatric disorders. In this sense, risk factors for Post-Traumatic Stress Disorder (PTSD) may include past experiences as well as individual neurobiology, and early adverse experiences are considered a major risk factor for the development of anxiety disorders. Therefore, this study investigated the effects of maternal separation (MS), in male rats, on the outcome of exposing them, as adults, to a stressful experience. Behavioral observations were made at least 1 month after the stressful experience, in an attempt to model long-term effects of the exposure to a severe stressor, as observed in humans with PTSD. We observed that both treatments, maternal separation during the first 10 days of life and exposure to a stressful event in adult life, had important effects on memory and DNA breaks index in the hippocampus, while no differences were found in antioxidant enzymes activities.

Neonatal maternal separation of rat pups leads to an altered stress responsive phenotype [31]. It is known that the majority of hippocampal granule neurons develops and extends their axons between days 1 and 21 of life [32–34]. This peak period of neurogenesis overlaps the stress hyporesponsive period (days 4–14), and exposure to elevated levels of corticosterone during the neonatal period may affect hippocampal development [31]. Glucocorticoids may be capable of affecting hippocampal development by directly or indirectly influencing the balance between neurogenesis and apoptosis of granule neurons throughout life in many species [35, 36].

Studies of structural brain abnormalities in PTSD have focused in particular on the hippocampus, a structure critically involved in memory [37–39], indicating that PTSD is associated with atrophy of the hippocampus [37, 40]. The hippocampus has also a neuroendocrine role in the hypothalamic–pituitary adrenal axis, in the feedback control of the stress response [41]. Because of its critical role in learning and memory as well as in stress regulation, alterations in the hippocampus have been proposed as contributing to the etiology of PTSD [37]. Although glucocorticoids, the adrenal hormones secreted during situations of stress, can damage the hippocampus [42], the mechanisms that explain trauma-related hippocampal atrophy are not clear.

Stress hormones released during emotionally arousing experiences regulate memory storage especially in the hippocampus, but also in other brain regions [43–45]. Thus, emotion can significantly modify the accuracy and retention of new memories. Evidence from another animal model of PTSD leading to memory impairment in the water maze (as measured 7-days post-stress) suggests a correlation between memory effects and neurochemical effects in hippocampus [46]. In the present study, we observed long-lasting effects of exposure to a shock and to situational reminders, both on behavior and on hippocampal DNA breaks index.

In PTSD, the patients may develop memory impairments [47, 48]. In this study, rats exposed to a PTSD model showed long-lasting effects (observed at least 1 month after exposure to the shock), suggesting impairments on spatial memory, since they spent more time in the opposite quadrant in the water maze task, while spending less time in the target quadrant. Besides, rats subjected to maternal separation and to this model of PTSD (shock) spent even more time in the opposite quadrant, suggesting that maternal separation worsened the impairment observed after exposure to shock. In the present study, although MS worsened the effects of another stressor, it did not have appreciable effect per se on memory. Chronic neonatal maternal separation has been shown to produce impairments in learning and memory [31]. However, adult memory performance is dependent on the nature and intensity of the early intervention, which may lead to distinct effects on memory [49], or these differences could still be attributed to different rat lineages or different MS schedules.

Deficits in learning and memory have been associated with damage to the hippocampus, which may be caused by stress [50–55]. One mechanism suggested as a factor inducing hippocampal neuroendangerment after stress is an increased oxidative stress [22]. Increased production of reactive oxygen species (ROS) could induce an altered antioxidant enzymes activities profile, since ROS have been reported to directly increase SOD expression [56, 57]. Increased ROS production could also lead to an increased DNA breaks index. In the present study, however, no differences were observed in antioxidant enzymes activities. It is possible that the unchanged antioxidant enzymes activities observed in the present study could be preceded by an earlier increase, at the time of stress exposure, and subsequent return to control values. Besides, changes in oxidative balance have been reported with unchanged levels of antioxidant enzymes [58]. Thus, these results do not mean

absence of oxidative stress, since other parameters may have been altered, such as damage to lipids or proteins. In addition, increased DNA breaks have been induced in the hippocampus of SM rats and rats subjected to shock. DNA breaks measured by comet assay under alkaline conditions (pH > 13), (as it was performed here), can detect single and double-stranded breaks, incomplete repair sites, alkali labile sites, and also possibly both DNA–protein and DNA–DNA cross-links in eukaryotic cells [59]. Increased DNA breaks have been considered a suggestion of increased risk of lesion in a particular tissue [60, 61], and in this context it could be related to the effects observed in spatial memory in the present study. However, single-strand breaks can arise either directly (e.g. from attack of deoxyribose by reactive oxygen species) or indirectly via enzymatic cleavage of the phosphodiester backbone during DNA base excision repair [62]. In order to understand the causes of the increased DNA breaks showed here, more studies are needed.

There are many evidences that exposure to adverse early life events may increase vulnerability to psychopathology in adult life. Individuals who experience early trauma, such as parental loss, sexual abuse or physical assault in childhood show higher tendency in adulthood to develop (PTSD), major depression or generalized anxiety. Maternally separated rats exhibit a dysfunction of the HPA axis reactivity to stress and, therefore, the MS model in rat is considered actually as a model of enhanced stress responsiveness and depressive-like behaviour [8].

Maternal separation is considered a useful model to study childhood neglect and abuse. Experiments have shown that maternal separation may lead to behavioural and neuroendocrine abnormalities reminiscent of behavioural disorders such as depression and anxiety disorders [63, 64]. These studies therefore clearly showed that stress early in life may have profound long-lasting effects on the central nervous system, and these effects may lead to various behavioural abnormalities and long-term biochemical changes in the brain [59, 62]. There are important memory disturbances in MS-related psychiatric disorders, and memory deficits induced by maternal separation could be related to comorbidity between depression and PTSD [65].

Concluding, our findings showed that early adverse life events may enhance the susceptibility to the effects of a stressor applied in adulthood regarding spatial memory. Long-lasting effects of exposure to a shock and to situational reminders were observed both on behavior and on hippocampal DNA breaks index. In addition, long lasting effects of maternal separation procedure were also observed on DNA breaks.

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