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Juvenile stress of short frequency and intensity does not affect rats´ brain white matter

Estresse juvenil de curta frequência e intensidade não afeta a substância branca cerebral de ratos

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ABSTRACT
The current study evaluates the lasting effects of two types of stress on the corpus callosum (CC). Forty-two male Wistar rats were randomly divided into three groups: Control Group (CG), Physical Stress (FS, immobilization) and Psychological Stress (PS, exposure to predators). Stress procedures occurred for three consecutive days at the juvenile stage (P25-P27) and analyzed at the adult age (P74); brains were retrieved and processed by Klüver-Barrera technique and sections were analyzed by morphometry. Results showed that there were no changes in the general aspects such as animal weight, and in the histological aspects such as CC thickness and quantity of the region´s glia nuclei. Current research suggests that the lasting effects of both models of juvenile stress of short frequency (3 days) and intensity (90 minutes/FS and 20 minutes/PS) were neither detrimental nor protective, featuring a positive adaptation.

Keywords: Corpus callosum. Immobilization. Myelin. Oligodendrocytes. Psychological stress.

RESUMO
O objetivo deste estudo foi avaliar os efeitos duradouros de dois tipos de estresse sobre o corpo caloso (CC). Foram estudados 42 ratos Wistar machos divididos aleatoriamente em três grupos: Grupo Controle (GC), Estresse Físico (EF, imobilização) e Estresse Psicológico (EP, exposição ao predador). Os procedimentos de estresse ocorreram durante três dias consecutivos na idade juvenil (P25-P27) e foram analisados na idade adulta (P74). Os cérebros foram coletados, processados com a técnica de Klüver-Barrera, e secções foram analisadas por meio de morfometria. Os resultados demonstraram que não houve alterações em aspectos gerais como peso dos animais, e histológicos como espessura do CC e quantidade dos núcleos gliais nesta região. O estudo sugere que os efeitos duradouros de ambos os modelos de estresse juvenil de curta frequência (3 dias) e intensidade (90 minutos/EF e 20 minutos/EP) não foram nem prejudiciais e nem protetores, o que pode ser considerado uma adaptação positiva.

Palavras-chave: Corpo caloso. Estresse psicológico. Imobilização. Mielina. Oligodendrócitos.

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INTRODUCTION

The corpus callosum (CC) is the principal structure of the brain´s white matter, mainly responsible for the brain´s inter-hemispherical connection[1]. The band is made up of approximately two hundred million myelinated axions[2] and its cell

composition changes according to neurodevelopment. Glioblasts abound immediately after rats´ birth and later glia cells predominate, mainly comprising oligodendrocytes, astrocytes and microglia[3].

Oligodendrocytes, the most numerous cell type in CC, are essential for the production of myelin in the Central Nervous System, a process called myelination[4] and remyelination after axonal lesion[5]. The structure which involves the neurons´ axions and which consequently forms the myelin´s sheath is a crucial evolutionary trait since it increases the conduction speed of nerve impulses and helps the control of development and neuroplasticity of neural circuits in adults[4].

Neurodevelopment is a long process. The juvenile or pre-pubescent phase in laboratory mice corresponds to age between P22 and P34[6], which is a rather sensitive period due to its intense neuroplasticity[7]. During this phase stress has been associated to changes in different regions of the brain including the corpus callosum, hippocampus and cortex areas, such as anterior, pre-frontal, dorsolateral and orbitofrontal cingulate[1].

It has been suggested that morphological and molecular abnormalities in the brain´s white matter are involved in neuropsychiatric disorders[1]. Further, stress is associated with the intensification of mental disorders such as anxiety[8] and the white matter´s microstructural development is susceptible to environmental influence[9].

Therefore, several studies have reported structural changes and volume of CC in children and in adults[1]. Decrease in CC has also been described in non-human primates exposed to stress in the early stages of development, whilst volume decrease has been related to cognitive deficits[10]. Further, early stress in non-human primates has been linked to changes in the microstructure of the brain´s white matter[11]. A possible connection of this morphological effect may be the glucocorticoid receptors in oligodendrocytes and parent cells that mediate the differentiation and myelination processes while transforming the cells into targets of glucocorticoid hormones and their co-factors[12]. The above may cause disorders in myelin production and, consequently, in the function of the nervous system.

The lasting effects of two stress models (physical and psychological), featuring short frequency and intensity, on the number of glia cells were verified in different regions of the corpus callosum and in their thickness in the anterior region.

METHODOLOGY

Current study was undertaken to investigate the lasting effects of two

different types of juvenile stress on rats´ corpus callosum by histological analysis.

ANIMALS

Male Wistar rats (n = 42), aged 21 days, were retrieved from the Central Vivarium of the State University of Maringá, Maringá, Brazil, and placed in groups of five in standard boxes. After adaptation in the Sectorial Vivarium of the Department of Morphological Sciences, they were randomly distributed in experimental groups: Physical Stress (FS; n = 14), Psychological Stress (OS; n = 14) and Control (CG; n = 14), and maintained under standard conditions of constant temperature (22°C ± 1°C) and light/dark cycle (12/12h), with lights on at 7h. Feed (Nutrilab-CR1, Nuvital Nutrients, Curitiba PR Brazil) and water were given *ad libitum*. At their respective age, 25 days after birth (P25 - P27), animals of groups FS and PS were retrieved from the sectorial vivarium and placed in an annexed room where stress procedures were applied. The animals were them replaced in their original boxes with their respective mates in the sectorial vivarium. CG animals were kept within the sectorial vivarium and received routine cleaning care. At 45 days, all animals were regrouped (with mates). Four or three animals were kept up to 75 days of birth and then euthanized. Assays were undertaken according to experimental procedures approved by the Committee for Ethics in Animal Experimentation (CEUA) (Protocol n. 499.305.061.7) of the State University of Maringá.

EXPERIMENTAL DESIGN

Immobilization stress

Immobilization stress is a physical stress factor[13] applied between days 25 and 27 after birth (P25-P27). Animals was placed in a plastic tube (10 cm long by 4 cm diameter) for 90 minutes (three 30-min periods, with 15-min intervals) during three days. During these sessions the rats were kept in a room next to their colony abode and after the end of the stress session they were returned to their original boxes.

Stress by predator

The model, considered to be a psychological stress agent[14] which occurred between P25-P27, consisted of two separate adjacent boxes: the rat´s box (a transparent propylene box, 15 cm long x 27 cm wide x 21 cm high, with holes in its walls); the cat´s box (a box made of wire mesh, 80 cm long x 80 cm wide x 60 cm high). Juvenile animals were placed one by one in the rat´s box for 20 minutes (two 10-minute periods, separated by 5-minute intervals) during three days. Three adult cats were used as predatory stimuli, one each day, coupled to a bottle of their urine

which was kept in the cat´s box during experimental days.

All stress procedures were undertaken under white light, between 7h and 17h. After each stress session, the devices (tubes and rat´s box) were sterilized by a solution of ethanol 70%. The cleaning of the cage of the Control Group was the only intervention made. After stress procedures, the animals were kept alone till euthanasia.

ANATOMICAL ANALYSES

Euthanasia and coloration

As adults, at 74 days of birth, the animals were anaesthetized (thiopental 100 mg + lidocaine 10 mg/kg, i.p.; 0.1 mL/100 pc), weighed and perforated in the intercardium with a buffered saline solution (phosphate buffer pH 7.4; 0.1M) and a paraformaldehyde solution 4% (phosphate buffer pH 7.4; 0.1M). The encephalon of each animal was removed and sectioned in the regions of olfactory bulb, optic nerves and spinal medulla and fixed in paraformaldehyde 4%, dehydrated in ethanol (70 - 100%), clarified in xylene and placed in paraffin. Sections were cut at the coronal plane, thickness 16 µm, and stained by Klüver-Barrera–Luxol Fast Blue, contra-colored by cresylic violet.

Quantitative analysis of glia cells

Two regions, the anterior region (Bregma 2.28 to 1.56 mm) and the posterior region of CC (Bregma 1.92 to –4.92 mm) were employed for the quantification of the glia [15], comprising five semi-serial sections and two fields in each section, totaling ten fields for each animal. Nuclei of glia cells were counted by the grade test system inserted into the eyepiece of an optical microscope Nova Optical Systems, objective 40X. These were disposed in the first visual plane (focused) and also comprised those in the field´s external lines. The nuclei of the second plane (not focused) within the test field were discarded. Further, according to the calibration of the microscope with the test field, each ocular unit of the test field was equivalent to 12.5 µm, with total area analyzed per field measuring 15,625 µm². Results were given as means ± standard deviation (n/10,000 µm²) (Figure 1a).

Analysis of the corpus callosum´s thickness

Five sections of the CC´s anterior region of each animal were employed to measure the thickness of the corpus callosum, in a position adjacent to the brain´s longitudinal fissure (Figure 1b), by a millimetric ruler inserted in the eyepiece of the optic microscope Olympus BX40. Objective 10X was used for visualize the

region where each ocular unit of the ruler measured 10.26 µm. Results were given as means and standard deviation (µm).

STATISTICAL ANALYSIS

The type of distribution of data was verified by Shapiro-Wilk test with BioEstat 5.0, and found normal, followed by one-way variance analysis (ANOVA One-way), with Tukey´s post hoc test to compare groups with GraphPad Prism 5.0. Significance level was 5% ($p<0.05$) and data were given as means ± standard deviation.

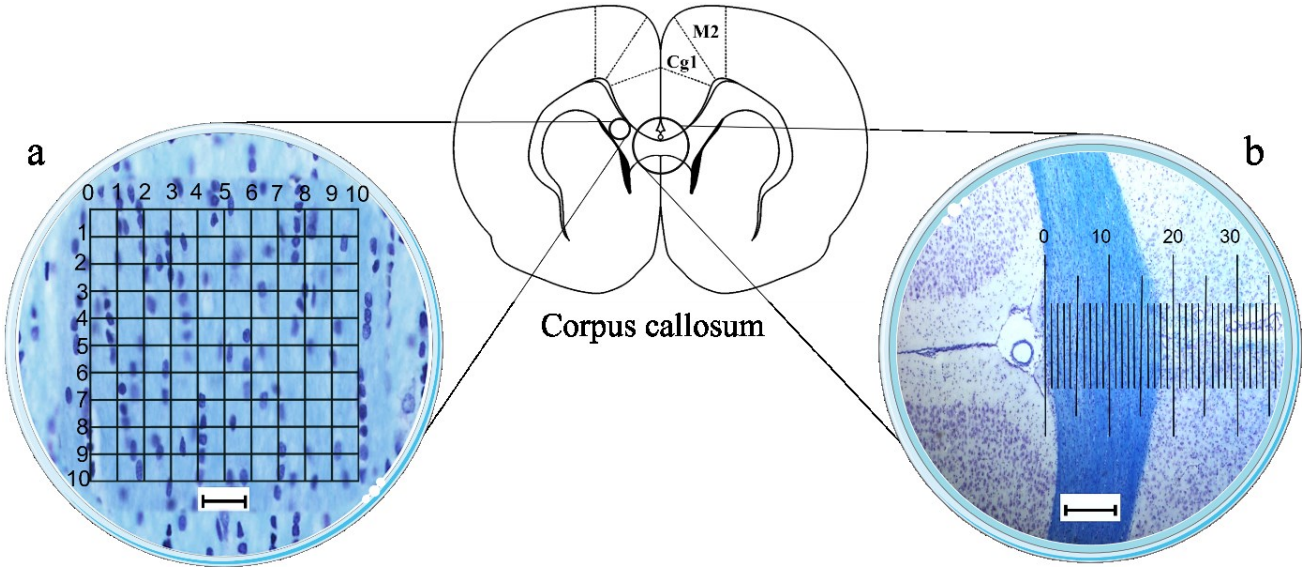

Figure 1. Representation of methodology for the qualitative analysis of glia cells and for the thickness of the corpus callosum. Test system 10x10 used (total area 15,625 µm²), measured randomly between the two peaks of CC, divisions **M2** (Cortex secondary motor) and **Cg1** (cingulate cortex, area 1) were reference for the delimitation of the analyzed region (**a**)**.** Illustration of CC will millimetric ruler showing the method for thickness analysis of CC (n = 6-7) (**b**). Histological technique: Klüver-Barrera. Scale: 20 µm (**a**); 200 µm (**b**).

RESULTS

BODY AND BRAIN WEIGHT

Statistical analysis did not show any significant difference between experimental groups for body weight (CG: 297.20 ± 13.48 g; FS: 308.70 ± 20.48 g; PS: 318.00 ± 31.90 g; $F_{2.39} = 2.813$, $p = 0.723$) and brain weight (CG: 1.88 ± 0.10 g; FS: 1.87 ± 0.08 g; PS: 1.84 ± 0.16 g; $F_{2.39} = 0.2998$, $p = 0.7427$) (Figure 2).

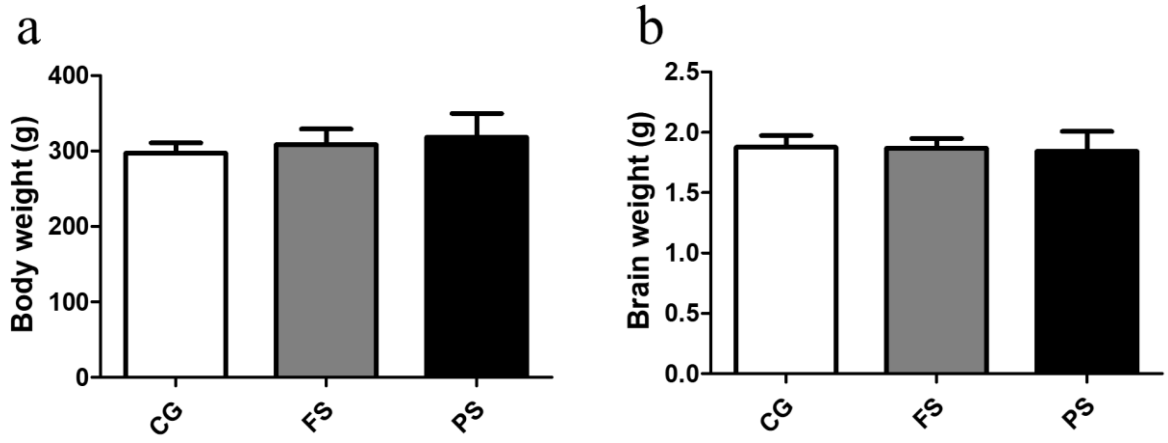

Figure 2. Graphs show thickness data as means ± standard deviation of body weight (**a**) and brain weight (**b**) of each group (n = 14), at significance level p<0.05. It has been verified that there was no significant difference between groups Control (**CG**), Physical stress (**FS**) and Psychological stress (**PS**).

QUANTITATIVE ANALYSIS OF GLIA CELLS

There were no statistical differences in the amount of glia cells in the anterior region of the CC among the experimental groups, FS (47.09 ± 5.25 /10,000 µm²), PS (50.07 ± 1.32 /10,000 µm²) and CG (47.64 ± 4.83 / 10,000 µm²) ($F_{2.12}$ = 0.7178; p = 0.5076) (Figure 3a). Likewise, no changes were reported in the posterior region of CC when compared to groups FS (34.24 ± 10.22 / 10,000 µm²) and PS (39.18 ± 6.48 / 10,000 µm²) and CG (35.49 ± 6.06 / 10,000 µm²) ($F_{2.12}$ = 0.5402; p = 0.5962) (Figure 3b).

ANALYSIS OF CC THICKNESS

There was no significant difference in the thickness of the CC anterior region between groups FS (320.1 ± 60.01 µm) and PS (327.6 ± 82.46 µm) and CG (283.2 ± 28.56 µm) ($F_{2.16}$ = 1,054; p = 0.3717) (Figure 3c).

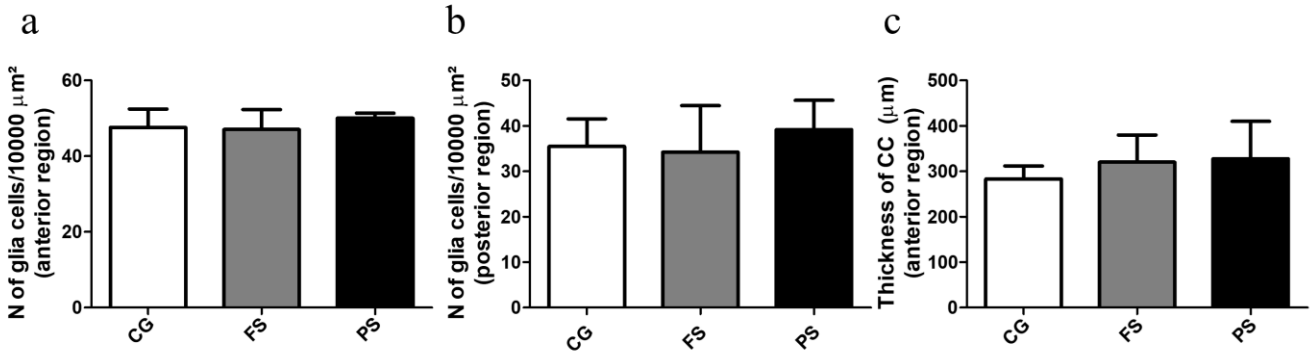

Figure 3. Graphs show data as means ± standard deviation of the number of glia cells in 10,000 µm² in the anterior region (**a**) and in the posterior region (**b**) of CC (n = 5), and of CC thickness in the anterior region, in µm (**c**), featuring no alterations among groups under analysis. Significance level at $p<0.05$. Control Group (**CG**); Physical Stress group (**FS**) and Psychological Stress group (**PS**).

DISCUSSION

Current study investigated the lasting effects of two stress models on the morphology of the brain´s white matter, CC and measurements such as brain and body weight. Stress models during the juvenile phase, immobilization and exposure to predators, characterized as physical and psychological stress, did not have any lasting alteration in the measurement analyzed. The above suggests that their effects were neither deleterious nor protective.

BODY AND BRAIN WEIGHT

The suprarenal gland of the animals described in current paper had been analyzed in a recent study with regard to weight, cortex thickness and adrenal medulla. Coherent to CC morphological results, the study reported that the suprarenal gland did not reveal any alterations in weight and in its morphology with regard to cortex and adrenal medulla[16]. Data suggest that models were not harmful to the point of changing measurements such as weight. Our results may be compared to those by Saber *et al.*[17] who reported that body weight of rats submitted to acute immobilization stress (1 day/6 h) failed to have any significant changes. However, the chronic stress model (10 days/2 h a day) caused a decrease in animal weight. According to our results, there is evidence that weight change of the suprarenal gland occurs when stress if highly toxic and probably is related to a greater production of corticosteroid hormones. Further, the authors have detected that after a 5-day recovery period, the parameter was restored. However, current study did not identify changes in body and brain weight of rats. The above may be associated to the

recovery period of 47 days and stress intensity (90 minutes of physical stress and 20 minutes of psychological stress during three consecutive days).

CORPUS CALLOSUM

The maintenance of thickness in the CC´s median region shows that the two types of stress experienced during the juvenile stage were not sufficiently harmful to cause lasting morphological consequences. On the contrary, studies in humans have reported a decrease in CC volume under different types of abuse[1], suggesting that the juvenile phase is highly sensitive and that the CC is modified by stress. Current analysis, however, showed that the morphological response to stress depends on its type and suggests relationship with recovery period. For the first time current research has compared stress models according to intensity and lasting responses, limiting scientific comparisons. However, current study underpins that stress failed to cause morphological changes in CC. The chronic model by Miyata *et al.*[18] showed that immobilized mice or mice immersed in water (21 days/2 h a day) did not show any changes in CC thickness, although a decrease in the diameter of axions in the brain region has been detected. Current study has shown that intensity of chronic stress did not affect CC thickness but caused microstructural changes in the brain region and, therefore, triggered a debate on the necessary mechanisms for structural changes.

Specialized literature suggests that neuroplasticity of the white matter depends on stress frequency and intensity, underpinned by several studies. Rats submitted to immobilization stress of long frequency and intensity (28 days/4 h a day) presented structural changes in myelin, comprising distortion, disintegration and reduction of the rats´ anterior brain (telencephalon + diencephalon) and decrease in the levels of myelin´s basic protein (MBP)[19]. Protein decrease has also been reported after stress by social defeat (exposure to aggression by a former reproducer) in the medial pre-frontal cortex of juvenile rats[20].

Results of current study and by other authors show that stress effect on the brain´s White matter is stress-dependent where stress type, frequency and intensity determine the type of morphological response.

CC GLIA CELLS

The two stress models did not damage the number of nuclei of glia cells. Results showing that the lasting effects of immobilization and exposure of the

predator during the juvenile phase on the general count of glia cells in CC are being published for the first time. Oligodendrocytes during the adult phase are prevalent in CC, with more than 70% of cell types in the region. Since current study analyzes this phase, one may infer that the number of nuclei analyzed is coherent to the analysis of CC thickness, since both did not have any morphological changes. The conservation of the number of glia cells, specifically oligodendrocytes, has also been reported in a chronic model of immobilization stress associated with immersion in water of adult rats[18]. The above evidenced that the intensity of stress by immobilization did not interfere in the parameter under analysis.

Several studies demonstrate that recovery is related to the nervous system regeneration capacity. Por instance, rats exposed to acute stress model (immobilization + exposure to the predator´s odor) showed a decrease in the number of oligodendrocytes in the hippocampus and in the pre-frontal cortex of females alone after a short recovery period (12 days). However, in the long term (67 days), a decrease in the amygdaloid corpus alone has been detected in males and females[21]. Another study on rats revealed that, after a 21-day recovery, the increase detected after stress of the inter-fiber space, mainly occupied by oligodendrocytes, reversed to the level of control animals[18].

Current study greatly contributes towards responses to different stress types on the brain´s white matter since the literature is still at a fledging stage on whether there is a standard in oligodendrocytes´ responses. Evidences exist that chronic immobilization stress (7 days/3 h a day) increased oligodendrogenesis in rats[22]. However, with greater frequency and intensity (14 days/4 h a day) the same model decreased oligodendrocytes in rats´ hippocampus[23]. The above and current result suggest that responses in the white matter do not follow a simple standard and require further analyses. One study shows that different stress frequencies and intensities have different impacts on behavior and oligodendrocytes. Por instance, chronic stress (14 days/1 h a day) caused adaptation to stress (hole-board behavior test) without influencing the oligodendrocytes. However, acute (1 day/1 h and 4 h) and chronic (14 days/4 h) stress failed to cause adaptation with a decrease in the number of these cells[23].

Literature and comparison with results of current study underscore that there are several factors associated to stress responses such as 1) intensity and stress duration; 2) recovery period; 3) sex-dependent responses; and 4) responses

dependent on the brain region. Present study has been restricted to evaluate CC region, to employ low-cost techniques and revealed that results of short-term investigation, when compared to long term ones, may cause interpretation mistakes. This is true when different types of stress and sex are compared. Current investigation used models and exposure periods standard in the literature[13,14], and according to the hypothesis that models would have lasting harmful effects in general (morphology of white matter and weight). These were discarded from the morphological point of view but other issues arose. What about short-term effects (soon after the stress event)? What are the physiological and molecular effects? Would females have the same responses?

CONCLUSION

Current study suggests that the lasting effect of low frequency stress (3 days) and intensity (90 minutes of physical stress and 20 minutes of psychological stress) experienced during the juvenile phase was neither harmful nor protective. From the morphological stance, it did not cause quantitative alterations in the brain´s white matter (CC region) of male rats, neither in the amount of glia cells nor in CC thickness. The above is actually a positive adaptation. Consequently, current study is a contribution towards the investigation of stress effects during the juvenile phase and benefits neurobiology of mental disorders.

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