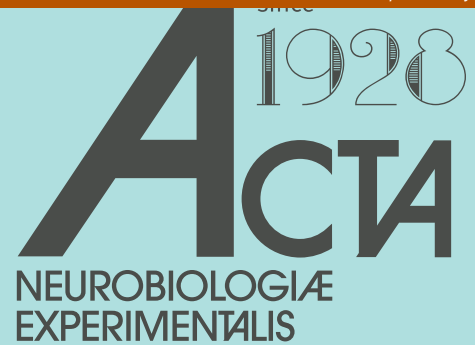


RESEARCH PAPER

Acta Neurobiol Exp 2020, 80: 331–343
DOI: 10.21307/ane-2020-029



Decreased serotonin content and release in the ventral hippocampus of prenatally stressed male rats in response to forced swim test

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Prenatal stress modifies the serotonergic system by altering the synthesis, metabolism, receptors and serotonin content in the hippocampus. However, it is currently unknown whether serotonin release in the ventral hippocampus of prenatally stressed rats is altered. In this study, serotonin (5-HT) and its metabolite, 5-hydroxyindoleacetic acid (5-HIAA) levels were analysed in dialysates (*in vivo*) and in homogenates (*in vitro*) of the ventral hippocampus. This was made after the sucrose preference test and after forced swim test (FST) in male adult progeny from mothers that were stressed by immersion in cold water during the last week of gestation. Serum concentration of corticosterone was also evaluated in control and in prenatally stressed males. Sucrose preference was differently affected in prenatally stressed males: 69% showed decreased sucrose consumption, and were considered anhedonic; 31% exhibited sucrose consumption similar to control and were considered non-anhedonic. During the FST, increased immobility and decreased swimming were observed in prenatally stressed males. After sucrose test, content and release of 5-HT in prenatally stressed rats were similar to those in the control group, with higher metabolite. After the FST, 5-HT content increased, but its release increased slightly in anhedonic rats and did not change in non-anhedonic rats, with lower metabolite. The response of the adrenal axis to the FST was larger in anhedonic prenatally stressed males, than in control and non-anhedonic males. These data show that behavioural disruption caused by prenatal stress is related to low release and lower metabolism of serotonin in the ventral hippocampus in adult male offspring, as well as to hyperactivity and hyperreactivity of the adrenal axis.

Key words: prenatal stress, serotonin, depression, ventral hippocampus, corticosterone

INTRODUCTION

Prenatal brain development is critical for shaping adult behavior (Pallarés and Antonelli, 2017), with growth and differentiation of major brain structures occurring during fetal development (Entringer et al., 2015). Many environmental factors, such as prenatal maternal stress can alter brain structure development, which has been associated to cognitive, behavioral

and psychosocial disturbances, in both animals and humans (St-Pierre et al., 2016). One of the alterations caused in prenatally stressed offspring in animal models is the low capacity to coping with stress due to the de-regulation of the HPA axis, thus increasing susceptibility to adult disorders, such as hyperactivity and hyperreactivity of HPA axis (Fan et al., 2009), metabolic diseases (Tamashiro et al., 2009), cognitive deficits (Guerrero et al., 2016), anxiety-like behavior

(Brunton and Russel, 2010) or depressive-like behavior (Brunton, 2013). The ventral hippocampus is related to the stress response (Herman et al., 2005) and emotional behavior (Fanselow and Dong, 2010; Lee et al., 2017). CA1 ventral hippocampal projections to the bed nuclei of the stria terminalis (BST) are important for neuroendocrine dysfunctions associated with psychiatric disorders, such as depression, anxiety, and post-traumatic stress disorder (PTSD) (Fanselow and Dong, 2010; Sheline et al., 2019). The ventral hippocampus in the rat receives projections from the median raphe nucleus, predominantly innervating CA1 and CA3 regions and the dentate gyrus (Mamounas et al., 1991; Vertes, 1991; Berumen et al., 2012), with the highest density of serotonergic axons in CA3 (Hensler, 2006). Prenatal stress disrupts the hippocampal serotonergic system by decreasing 5-HT content at weaning (Ishiwata et al., 2005) or at day 35 of age (Hayashi et al., 1998) and increasing the concentration of its metabolite, 5-hydroxyindoleacetic acid (5-HIAA), thus increasing its metabolic rate (Ishiwata et al., 2005), leading to a depressive-like phenotype (Mueller and Bale, 2008). Other studies however, did not find differences in 5-HT content at weaning (Gemmel et al., 2016) or in adulthood (Ishiwata et al., 2005; Van den Hove et al., 2014). Prenatal stress also increases serotonin transporter (SERT) (Bielas et al., 2014), decreases 5-HT_{1A} receptor binding (Van den Hove et al., 2006) and its expression (Huang et al., 2012) in the ventral hippocampus of male offspring. In contrast, the immunoreactivity of tryptophan hydroxylase in the raphe nucleus increases (Miyagawa et al., 2011; Van del Hove et al., 2014). In association with decreased 5-HT content in the hippocampus, prenatal stress causes anxious- and depressive-like behavior in male and female rats (Soares-Cunha et al., 2018). Similarly, prenatally stressed rats show decreased sucrose consumption, which is known as anhedonic behavior (Basta-Kaim et al., 2014; Głombik et al., 2015; Ślusarczyk et al., 2015), together with what has been defined as learned helplessness behavior, with a longer time of immobility in the forced swim test (FST) (Alonso et al., 1991; Morley-Fletcher et al., 2003; Morley-Fletcher et al., 2004; Soares-Cunha et al., 2018). However, it is not clear whether these behaviors are related to changes in 5-HT release, rather than to its content in the ventral hippocampus of prenatally stressed animals. Therefore, the aim of this study was to investigate the effects of prenatal stress on 5-HT and 5HIAA levels in dialysates (*in vivo*), and in homogenates (*in vitro*) of ventral hippocampus, and its possible correlation with sucrose preference and the FST in adulthood. Serum corticosterone concentration was also evaluated in order to assess adrenal response to the FST.

METHODS

Subjects

Three-month-old female Wistar rats weighing 250 grams were provided by the vivarium of the Autonomous Metropolitan University, Iztapalapa. Rats were kept under standard laboratory conditions, under an inverted light/dark cycle (lights off at 09:00), temperature 23°C ±1, food and water *ad libitum*. Animal handling and the experimental protocols were in accordance with Mexican official standards (NOM-062-ZOO-1999), as well as the guidelines for ethical research, teaching and information dissemination at the Biology and Health Sciences Division of Autonomous Metropolitan University (2010). This study was approved by the Ethics Commission of the Biology and Health Sciences Division of Autonomous Metropolitan University.

Experimental design

Experimental design is shown in Fig. 1. Female rats bred with sexually experienced males. Pregnancy was confirmed by the presence of sperm in females' vagina. That day was considered as day zero of gestation. Pregnant females were placed in individual acrylic boxes and weighed daily throughout gestation. Control pregnant females (n=12) were kept in their boxes with routine care by the vivarium staff and were weighed. Pregnant females in the maternal stress group (n=12) were exposed to stress by immersion in cold water.

Gestational stress procedure

During the last week of gestation (day 15 to 21), pregnant females from the stress group were taken to another room and placed individually in a tank filled with water to a 15 cm height. Water temperature was 15°C. The rats remained there for 15-min; this process was performed twice a day (09:00 AM and 15:00 PM) (García-Vargas et al., 2019). At birth, litters from each experimental group were sexed, weighed, and standardized in number by cross-fostering within each experimental group. Pups were weaned at postnatal day 22, and males were separated from the females. Male descendants only were used for this study. One male from each of ten litters per group was tested in each experiment, as is appropriate for studies using mammals that have litters (Holson and Pearce, 1992; Williams et al., 2017). Hence, 10 (non-siblings) prenatally stressed or control males were used separately in the behavioral tests at 3 months of age.

Behavioral tests

Sucrose preference

Sucrose intake was assessed in males from the control and prenatal stress groups (n=15, each group). Sucrose concentration used are variable in several studies, 1% (Muscat and Willner, 1992); 2% (Amchova et al., 2014); 2.5% (Tang et al., 2013); 3% (Kalueff et al., 2006); 4% (Barr and Phillips, 1999); 7% (Vollmayr et al., 2004). In this study, an average concentration of 3% sucrose was used. First, rats had access to a 50 mL drinking bottle containing a 3% sucrose solution for 2 days continuously, instead of water. For the next 3 days, one-hour daily tests were done: rats had access to two drinking bottles, one containing water, and the other containing a 3% sucrose solution. The bottles were exchanged after 30 min to prevent place preference. Before the last day of sucrose intake test, rats were food and water deprived for 21 h. On the 6th day, at the beginning of the dark phase, rats had access to bottles containing 50 mL of the sucrose solution. The volume consumed in millilitres was evaluated and expressed as a percentage (Tang et al., 2013). According to the endpoint sucrose intake, rats from the prenatal stress group were classified into anhedonic (those which consumed less than 32 mL, $\leq 65\%$), and non-anhedonic (those which consumed sucrose in a similar way to the control rats, more than 33mL, $\geq 65\%$), as has been previously defined by other authors (Bergström et al., 2007; Strekalova et

al., 2011). After the sucrose test, males were euthanized by decapitation, the ventral hippocampus was dissected, weighed and stored at -80°C until 5-HT and 5-HIAA content evaluation by HPLC-ED. Trunk blood was also collected for corticosterone assessment.

Forced swim test (FST)

In other control (n=15) and prenatally stressed males (n=15), the FST was performed (Porsolt et al., 1977) 24 h after the sucrose test. Tests were done during the dark phase of the light-dark cycle. Rats were individually placed in an acrylic cylinder (60 cm height, 30 cm diameter) containing water (40 cm height, 25°C) (Sunal et al., 1994). Before the test, each rat received a first session, during which they were placed in the cylinder for 15 min. 24 h after the pre-test, a 5 min experimental session was done. Rats were individually placed in the cylinder, immobility (rat remained floating making only movements necessary to keep its head above water) was scored, as well as the time it spent swimming and climbing. Rat behavior was video-recorded for further analysis. All tests were carried out by an experimenter blinded to treatment.

Tissue preparation for serotonin and metabolite concentrations in homogenates

At the end of the 5-min FST session, rats were euthanized by decapitation. The right ventral hippocampus

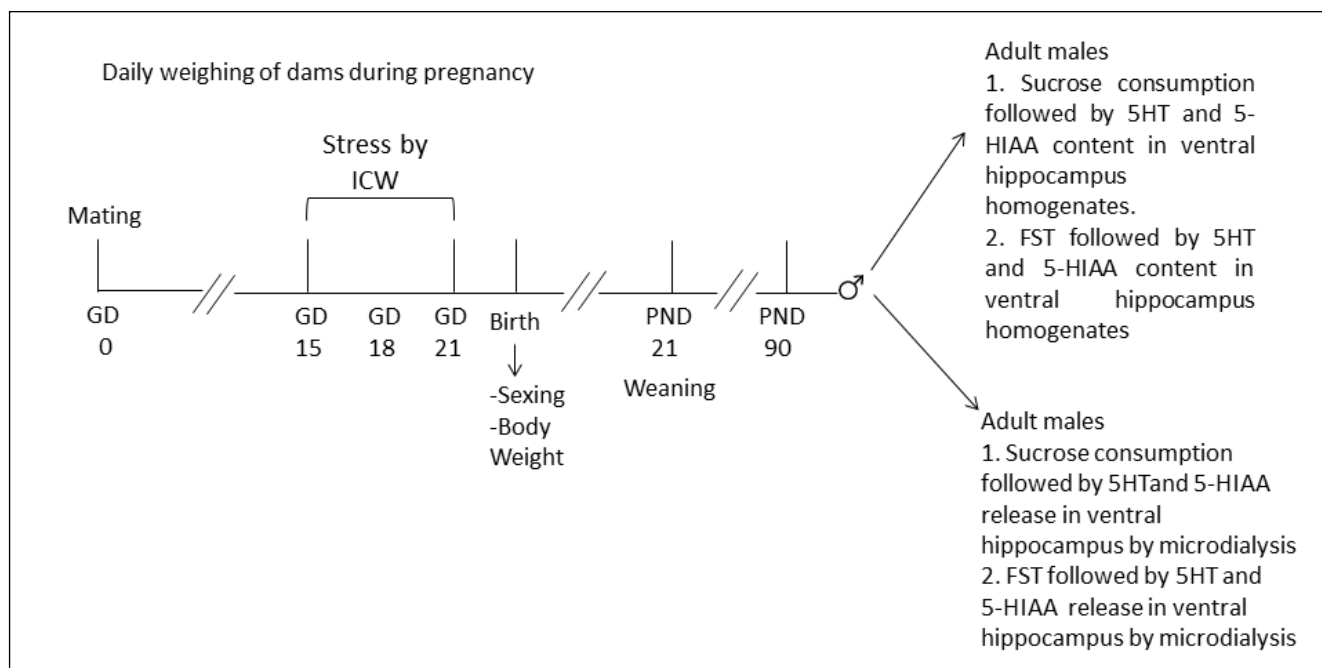


Fig. 1. Timeline of experimental design. GD: Gestational day; ICW: Immersion in cold water; PND: Postnatal day; 5-HT: Serotonin; 5-HIAA: 5-Hidroxiindoleacetic acid; FST: Forced swim test.

was dissected, weighed and stored at a temperature of -80°C until the samples were evaluated. Weights from ventral hippocampus were 35 mg on average. Subsequently, the samples were thawed and 300 μL of perchloric acid were added, the tissue was homogenized in ice and centrifuged for 15 min at 8000 rpm at 4°C . The supernatant was filtered with a 0.45 μm membrane (Sigma), and 10 μL of the filtered sample was injected into the chromatographic system (HPLC-ED) for quantification of tissue serotonin and its metabolite. Also, trunk blood samples were obtained for corticosterone evaluation.

Microdialysis procedure

In other rats the sucrose test was performed. After identifying anhedonic and non-anhedonic rats, cannulas directed toward the right ventral hippocampus were implanted to perform microdialysis in order to quantify 5-HT release and its metabolite, 5-HIAA. Control ($n=10$), anhedonic ($n=10$), and non-anhedonic ($n=5$) males were anesthetized with a single dose of ketamine (PISA) (80 mg/kg, i.p) and xylazine (PISA) (20 mg/kg, i.m). A stereotaxic apparatus (Stoelting, 51600, Wood Dale, IL, USA) was used to secure the head of the rat. The guide cannula was placed toward the ventral hippocampus with the following coordinates: antero/posterior (A/P) -5.3 mm from bregma, medial/lateral to midline (M/L) -5.2 mm, and dorsal/ventral from dura (D/V) -7.5 mm (Paxinos et al., 1980). It was secured with screws and dental acrylic. A scheme presenting the section of the brain with the probe positioning, as well as a photomicrograph are shown

in Fig. 2. After a 10-day recovery period, the microdialysis probe (polyacrylonitrile membrane, pore size 40,000 D) was introduced, protruding 3 mm below the guide cannula to the ventral hippocampus, then the cannula was secured to the skull with dental acrylic. A period of two hours allowed for stabilization of the cellular environment in the ventral hippocampus. Then, two samples were taken every h (10 μL each) and were injected to HPLC-ED system. In other control ($n=10$) and prenatally stressed males ($n=15$), the sucrose test was performed and prenatally stressed males were identified as anhedonic or non-anhedonic. Subsequently, cannulas directed toward the ventral hippocampus were implanted to perform microdialysis. After a 10-day recovery period, a 15 min session of the FST was carried out in all animals. The following day, two hours before a 5 min session, a microdialysis cannula was introduced, protruding 3 mm below the guide cannula to the ventral hippocampus, and secured to the skull with dental acrylic; two samples were obtained every h. Immediately after the 5 min session of FST, two samples were collected every h, and 10 μL from each sample were injected into the chromatographic system.

Biochemical procedures

Corticosterone quantification

The steroids extraction was performed according to the method reported by Woodward and Emery (1987). Serum (1 mL) was mixed with 100 μL of a 19-nortestos-

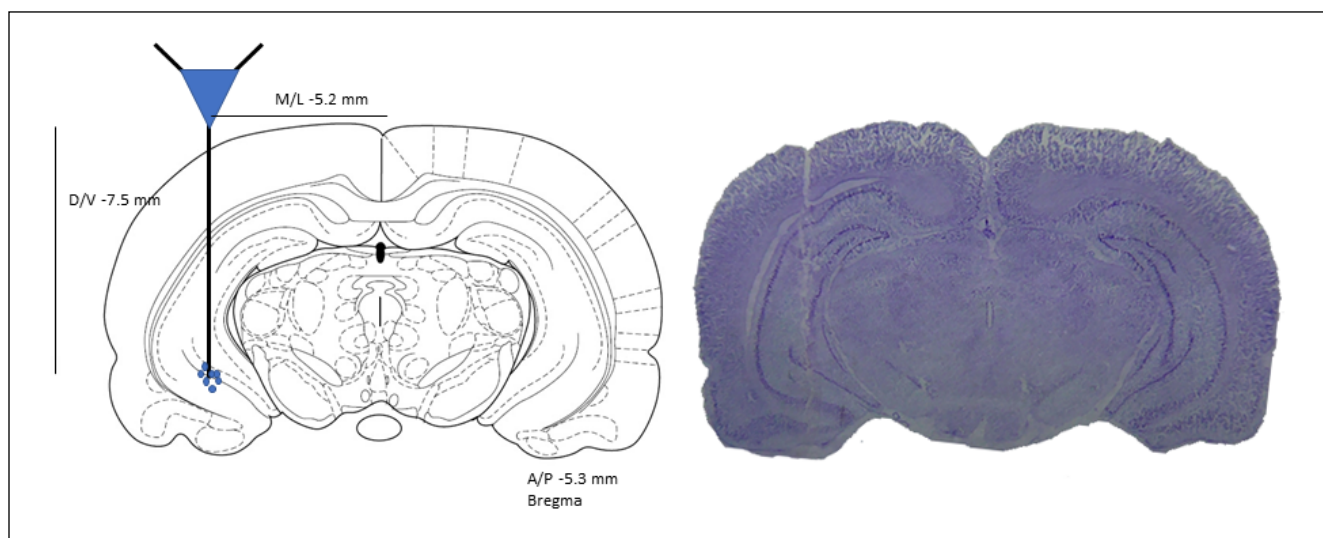


Fig. 2. Scheme and photomicrograph presenting the section of the brain with probe positioning. Coordinates: Antero/Posterior (A/P) -5.3 mm from bregma, Medial/Lateral to midline (M/L) -5.2 mm, and Dorsal/Ventral from dura (D/V) -7.5 mm. Magnification of coronal cut of brain: 4x.

terone (5 µg/mL in methanol) solution as an internal control. Corticosterone was extracted in 5 mL of diethyl ether-dichloromethane (60:40 v/v) and centrifuged for 5 min at 8,000 rpm. The organic phase was obtained and mixed with HPLC-grade water and centrifuged for five min. The organic phase (3 mL) was obtained and evaporated at room temperature. The residue was dissolved in 100 µl of methanol-water (60:40 v/v). Corticosterone was evaluated in a HPLC system with a precolumn (Symmetry C18, particle size 3.5 µm, 2.1 × 10 mm, Waters Corp., Milford, MA, USA). Separation of the analytes was done at 40°C, in a Waters Symmetry C18 column (particle size 5 µm, column size 2.0 × 150 mm, Waters Corp., Milford, MA, USA), with water-acetonitrile HPLC grade (65:35 v/v) at a flow of 0.4 mL/min. A 600-MS controller system was used for the mobile phase and the steroids were detected with a Waters 486 UV detector (250 nm). The results were analysed using the Millennium 32 program (Waters Corp., Milford, MA, USA).

Serotonin assessment

A Waters pump model 515 (Waters Corp., Milford, MA, USA) was used for delivery of the mobile phase of phosphates, pH 3.1, at a 0.3 mL/min flow rate. The samples were injected with a Rheodyne manual injector (Waters Corp., Milford, MA, USA). Separation of the analytes was done on a Symmetry C18 column, particle size 2.5 mm × 25 mm (Waters Corp., Milford, MA, USA). The analytes were detected by an electrochemical detector, model 2476 (Waters Corp., Milford, MA, USA) at + 800 mV and a sensitivity of 2 nA. The results were analysed using the Millennium 32 program (Waters Corp., Milford, MA, USA).

Statistical analysis

Body weight gain in pregnant dams was analysed by linear regression and the slope of the curves was compared. Length of pregnancy, number in litters, male pups, and body weight at birth were analysed with Student's t-test. The percentage of sucrose preference was analysed by Chi square test. Forced swimming test was analysed using a one-way analysis of variance (ANOVA), followed by a *post hoc* multiple comparison Tukey test. Corticosterone concentrations, serotonin and its metabolite content and release after sucrose preference test and after FST, as well as the metabolic rates, were analysed by repeated measures two-way ANOVA, followed by a *post hoc* multiple comparison Tukey-Kramer test. Pearson correlation test was used to analyze the correlation between corticosterone vs. serotonin

content and release, as well as vs. sucrose intake. The correlation between content and release of serotonin vs. immobility, swimming and sucrose intake was also evaluated. The differences were considered significant when $P < 0.05$. The statistical analysis was performed by a GraphPad PRISM version 6.01 statistical software (GraphPad Software Inc., USA).

RESULTS

Body weight gain in pregnant females and offspring birth weight

Stressed pregnant mothers showed lower body weight gain compared to control pregnant females. The slopes were different for control (10.66 ± 0.8477) and maternal stress (7.784 ± 0.6725) groups ($P = 0.018$). Body weight gain was lower in stressed pregnant mothers during the last week of pregnancy compared to non-stressed mothers. The correlation coefficient (r^2) for the control group was 0.9816 and for the prenatal stress group it was 0.9749. (Fig. 3). No differences were found in the length of pregnancy in control (22.62 ± 0.17 d) and stressed dams (23.37 ± 0.17 d).

Regarding offspring, there were no differences in the litter size between control (10.62 ± 0.34) and prenatally stressed rats (9.87 ± 1.56), nor in the number of males in the control (5.5 ± 0.99) and prenatally stressed offspring (4.62 ± 1.08). Birth weight of prenatally stressed males was lower (6.03 ± 0.07 g) than that of control males (6.84 ± 0.13 g), [$t_{98} = 5.407$, $P = 0.0001$].

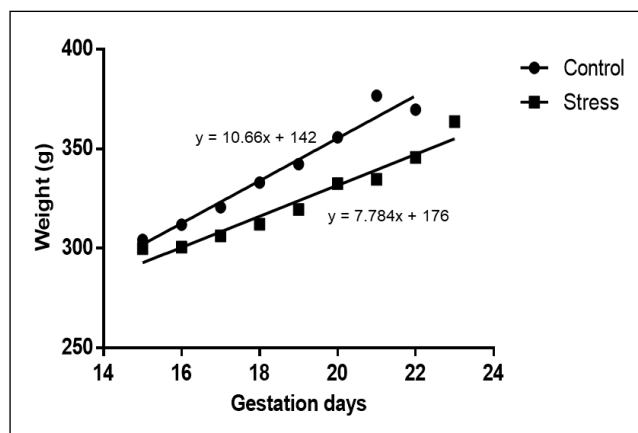


Fig. 3. Body weight gain in pregnant rats from gestational days 15 to 23. Stressed mothers had less body weight gain during pregnancy. Correlation coefficient for control line: 0.9898; Correlation coefficient for stress line: 0.9749. The slopes indicate the average increase in body weight per day, which were significantly lower in stressed dams compared to control dams ($P = 0.01$). $n = 12$ per group.

Sucrose preference test

The sucrose consumption test showed that 69% of the prenatally stressed rats were anhedonic, as shown by lower sucrose consumption; 31% were considered non-anhedonic, as their sucrose consumption and preference were similar to that of control rats. Anhedonic rats showed lower sucrose preference (30%) than control and non-anhedonic rats (70%) [$X^2_2=38.84$, $P=0.003$] (Fig. 4).

Forced swim test

The duration of immobility observed in prenatally stressed rats (both anhedonic and non-anhedonic) during FST was significantly higher than that of con-

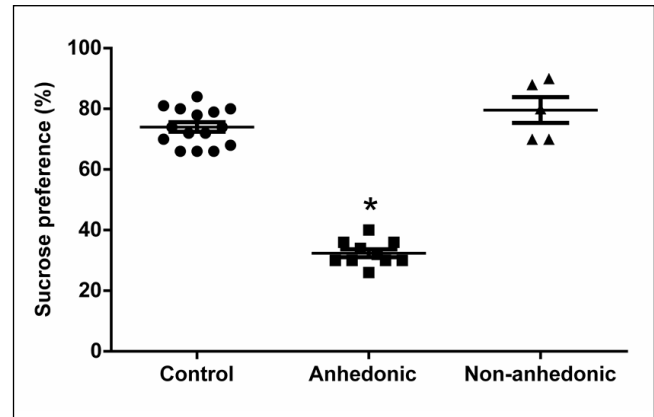


Fig. 4. Sucrose preference in control, anhedonic, and non-anhedonic rats. Percentage of preference was similar in control and non-anhedonic rats. * $P=0.01$, compared to control and non-anhedonic males. Control group: $n=15$; Anhedonic: $n=10$; Non-anhedonic: $n=5$.

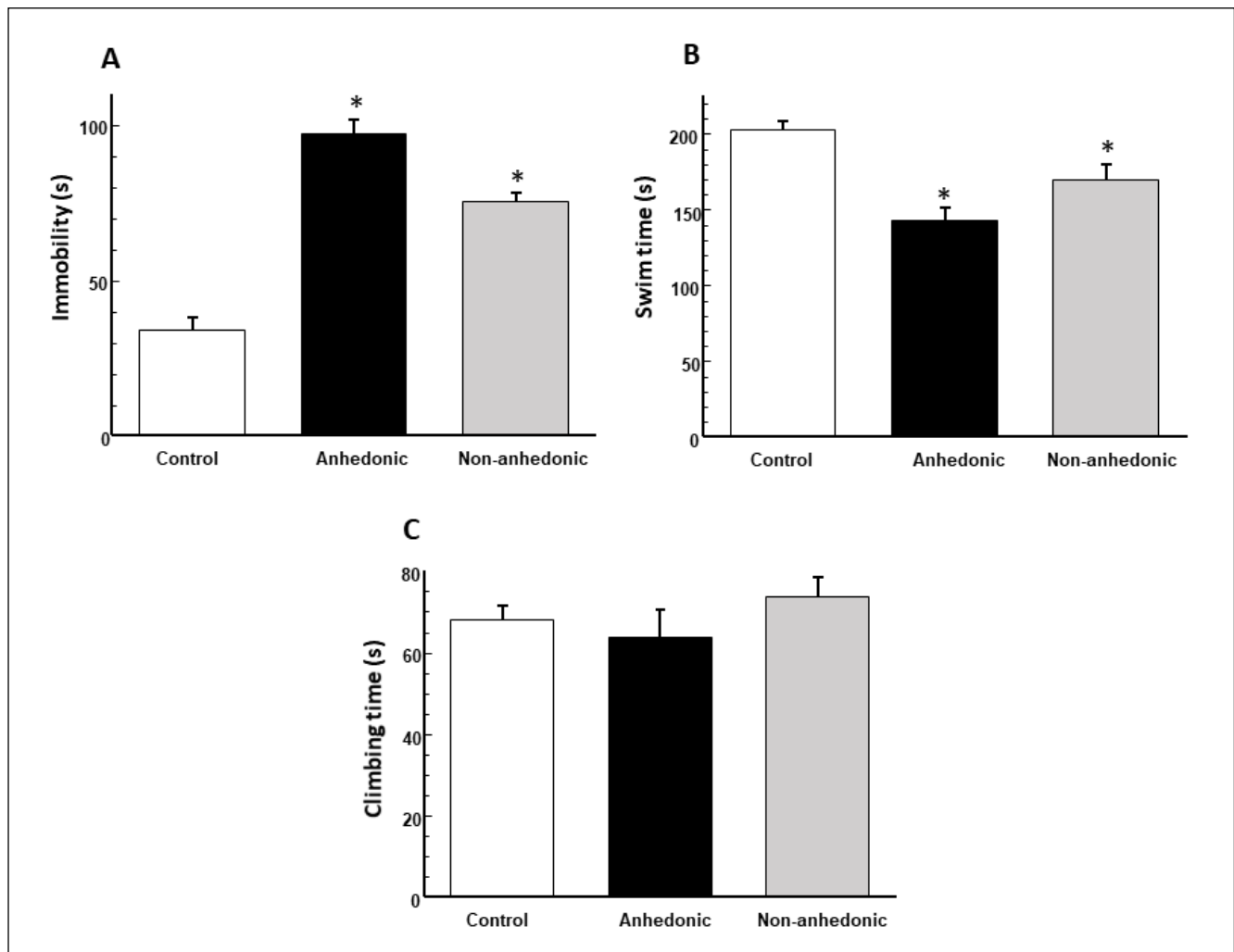


Fig. 5. Forced swim test (FST) parameters. (A) Immobility increased in prenatally stressed anhedonic and non-anhedonic rats. (B) Swim time was shorter in prenatally stressed rats. (C) Climbing time was similar in all groups. Data shown as Mean \pm S.E.M. * $P=0.01$, compared to the control group. Control group: $n=15$; Anhedonic: $n=10$; Non-anhedonic: $n=5$.

control males [$F_{2,27}=70.09$, $P=0.0001$] (Fig. 5A). In contrast, time spent swimming decreased in the anhedonic and non-anhedonic rats [$F_{2,27}=18.02$, $P=0.0001$] (Fig. 5B). No differences were observed in the time for climbing behavior during the 5-min FST [$F_{2,27}=0.760$, $P=0.476$] (Fig. 5C).

Serotonin release and 5-HIAA

After sucrose preference test, 5-HT release in the ventral hippocampus was similar among control, anhedonic and non-anhedonic males. After FST, 5-HT release increased in the ventral hippocampus of control rats ($F_{2,45}=713.33$, $P=0.0001$). In anhedonic rats, a slight but significant increase was observed, whereas no change in 5-HT release was observed in non-anhedonic rats (Fig. 6A). After sucrose preference test, extracellular 5-HIAA in the ventral hippocampus of prenatally stressed rats was higher, compared to control rats. After FST, extracellular 5-HIAA in the control group increased with respect to extracellular concentration after sucrose preference test. In comparison, the metabolite decreased in the ventral hippocampus of anhedonic and non-anhedonic rats, being lower than in control rats after the FST [$F_{2,45}=31.17$, $P=0.001$] (Fig. 6B). In the control group, 5-HT release correlated negatively with

immobility duration in the FST ($r=-0.8651$, $P=0.0119$) and positively with swim duration ($r=0.9702$, $P=0.0003$). In contrast to controls, low 5-HT release correlated negatively with immobility duration ($r=-0.8066$, $P=0.0284$) and positively with swim duration during the FST in anhedonic rats ($r=0.8226$, $P=0.0231$). In a similar way to anhedonic males, a negative correlation between 5-HT release and immobility duration ($r=-0.9295$, $P=0.0024$) and positive correlation with swim duration ($r=0.7729$, $P=0.0416$) were observed in non-anhedonic rats. 5-HT metabolic rate, obtained of dialysates from microdialysis, showed higher 5-HT metabolism in the ventral hippocampus of anhedonic rats, compared to control and non-anhedonic rats, after sucrose preference test ($F_{2,45}=9.77$; $P=0.001$). After FST, however, metabolic rate decreased in all groups, but was higher in prenatally stressed males compared to controls (Table I).

Content of 5-HT and 5HIAA in the ventral hippocampus

5-HT content in ventral hippocampus was similar in the rats of all three groups: control, anhedonic and non-anhedonic after sucrose preference test. After FST, 5-HT content decreased in the ventral hippocampus of control rats, while in anhedonic rats it increased

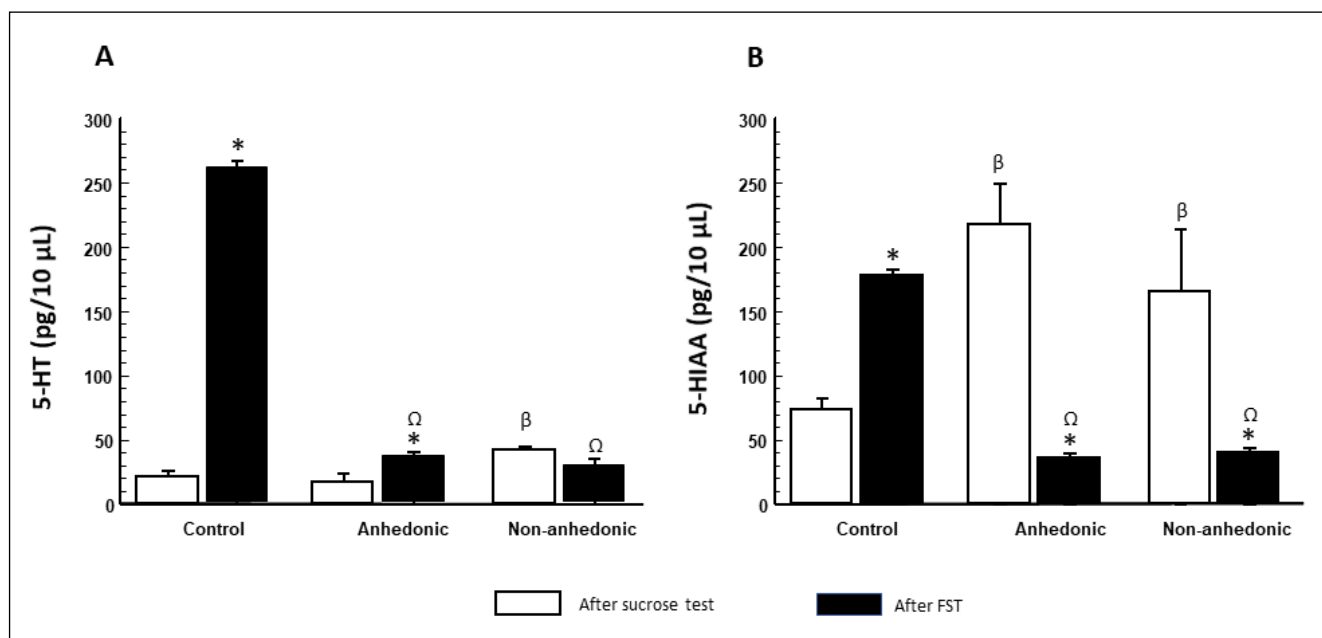


Fig. 6. (A) Serotonin (5-HT) levels in dialysates of ventral hippocampus after sucrose preference test and after the forced swim test (FST). 5-HT release increased in control rats after the FST, but in anhedonic rats a slight increase was observed. 5-HT levels did not modify in non-anhedonic rats. (B) Extracellular 5-HIAA in dialysates of ventral hippocampus. After sucrose preference test, the metabolite was higher in prenatally stressed rats. After the FST, metabolite concentration in anhedonic and non-anhedonic males decreased and were lower than in control males. Data shown as mean \pm S.E.M. * $P=0.01$ compared to levels after sucrose preference test; $\beta P=0.01$ compared to control group after sucrose preference test; $\Omega P=0.01$, compared to control group after FST. Control group: $n=10$; Anhedonic: $n=10$; Non-anhedonic: $n=5$.

Table I. Serotonin (5-HT) metabolic rates in the ventral hippocampus after sucrose preference test (SPT) and after forced swimming test (FST).

	5HIAA/5HT			
	Homogenates		Dialysates	
	After SPT	After FST	After SPT	After FST
Control	0.44±0.07	2.18±0.35*	3.91±0.41 [⊙]	0.68±0.01*
Anhedonic	0.85±0.21*	0.35±0.04 ^{⊙β}	9.11±1.75*	1.13±0.12 ^{⊙β}
Non-anhedonic	0.60±0.07	0.39±0.03 ^β	4.74±1.42 [⊙]	1.56±0.15 ^{⊙β}

Data shown as mean ± S.E.M. Repeated measures ANOVA. * $P=0.01$, compared to control after SPT; [⊙] $P=0.01$ compared to anhedonic after SPT; ^{⊙β} $P=0.01$ compared to anhedonic or non-anhedonic after SPT; ^β $P=0.01$ compared to control after FST.

significantly; in non-anhedonic rats 5-HT did not modified after FST [$F_{2,55}=44.10$; $P=0.0001$], (Fig. 7A). 5-HIAA content in the ventral hippocampus of anhedonic rats was higher than in control rats after sucrose preference test, whereas metabolite content in non-anhedonic rats was similar to controls. After FST, metabolite content increased significantly in control rats ($F_{2,55}=10.59$, $P=0.001$); in anhedonic and non-anhedonic rats, however, there were no changes after FST with respect to their values after sucrose preference test (Fig. 7B). Hippocampal serotonin content in anhedonic rats after sucrose preference test correlated positively with longer immobility times in these rats ($r=0.8398$, $P=0.0364$).

Metabolic rate (5HIAA / 5HT)

5-HT metabolic rate in homogenates from ventral hippocampus of control animals increased after the FST. In prenatally stressed, anhedonic rats, the metabolic rate was higher than in control rats after sucrose preference test, but decreased significantly after FST [$F_{2,55}=51.36$, $P=0.0001$] (Table I).

Serum corticosterone

Corticosterone concentrations in prenatally stressed, anhedonic male rats were significantly

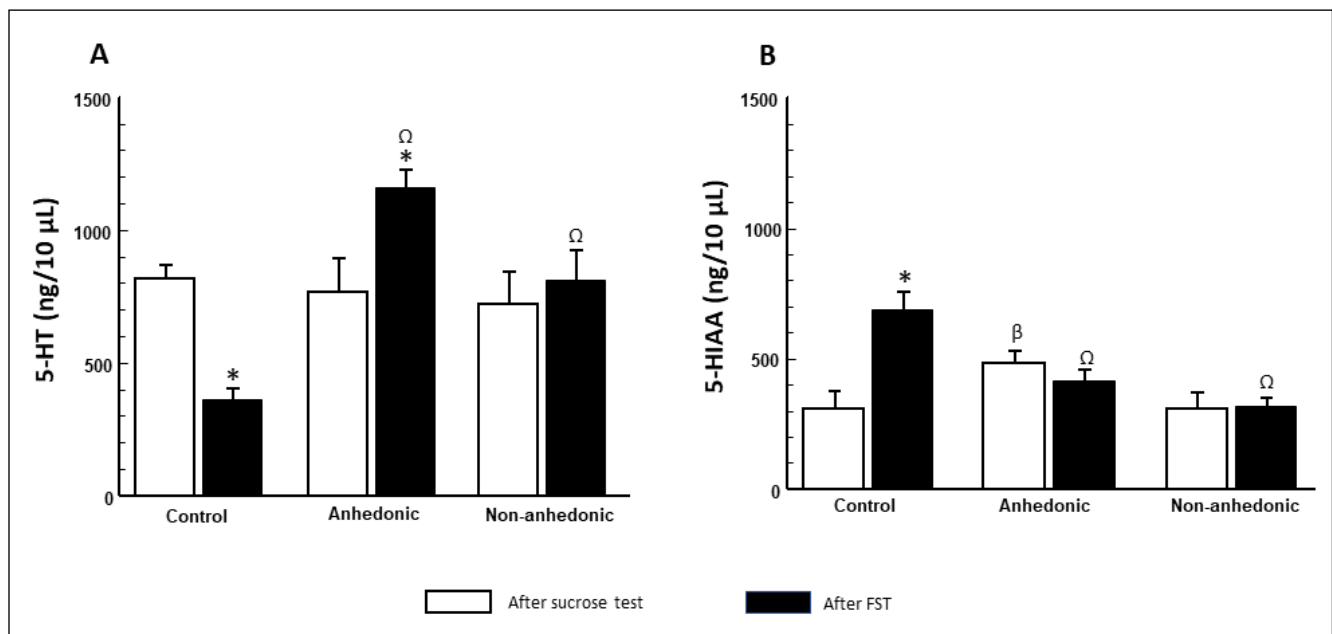


Fig. 7. (A) Serotonin (5-HT) content in homogenates from ventral hippocampus after sucrose preference test and after FST conditions. 5-HT content after sucrose preference test was similar in control and prenatally stressed rat. After the FST, 5-HT levels decreased in control males, but in anhedonic males 5-HT content increased. No changes were observed in non-anhedonic rats. (B) 5-Hydroxiindoleacetic acid (5-HIAA) content in homogenates from ventral hippocampus. Metabolite concentration increased after the forced swim test (FST) in the control group, but it did not change in prenatally stressed rats. Data shown as Mean ± S.E.M. * $P=0.01$, compared with levels after the sucrose preference test; ^β $P=0.01$ compared to control group after sucrose preference test; [⊙] $P=0.01$ compared to control group after the FST. Control group: $n=5$; Anhedonic: $n=0$; Non-anhedonic: $n=5$.

higher than those of control male rats after sucrose preference test. Corticosterone levels increased in all groups after FST, with the highest adrenal axis response in anhedonic rats compared to control rats. [$F_{2,55}=102.90, P=0.0001$]. Anhedonic and non-anhedonic males showed higher corticosterone levels than control males, after sucrose preference test and after FST (Fig. 8). Corticosterone levels in anhedonic rats after the FST correlated positively with immobility duration ($r=0.9578, P=0.0001$), and negatively with swim duration ($r=-0.9645, P=0.0001$). In non-anhedonic animals, no correlation was observed between the above-mentioned parameters. Corticosterone concentration in anhedonic rats also correlated negatively with sucrose consumption ($r=-0.9463, P=0.0001$).

DISCUSSION

The results of the present study show that prenatal stress does not modify 5HT and 5-HIAA levels after sucrose preference test, but it increases serotonin metabolism in anhedonic rats. Behavioral alterations in sucrose preference test and FST in prenatally stressed rats are related to alterations in the content, release, and metabolism of serotonin in the ventral hippocampus. These effects correlate to the high levels of corticosterone in anhedonic offspring, but not in non-anhedonic offspring.

The lower body weight gain observed in stressed mothers during the last week of gestation (Fig. 3) is similar to that reported in other studies (Amugongo and Hlusko 2014; Van den Hove et al., 2014; Franko et al., 2017). This result could be explained by the decrease in food intake, as has been previously reported in pregnant females submitted to stress (Ward and Wainwright, 1988). Activation of the HPA axis during stress can interfere with body weight gain since, in addition to stimulating the release of ACTH in the pituitary gland, corticotropin-releasing hormone (CRH), also acts as an anorexigenic peptide in the hypothalamus, suppressing appetite and food intake in stressed subjects (Charmandari et al., 2005).

The lower birth weight observed in prenatally stressed males compared to control males (6.03 ± 0.07 g vs. 6.84 ± 0.13 g), is consistent with other stress protocols that have been previously reported (Van den Hove et al., 2014). Other studies, however, did not find changes in offspring birth weight (Abe et al., 2007; Zohar and Weinstock, 2011; Amugongo and Hlusko 2014). The differences in this result could be explained by the type of stressor used. During fetal development, the placenta provides oxygen and nutrients to the fetus through diffusion and other types of

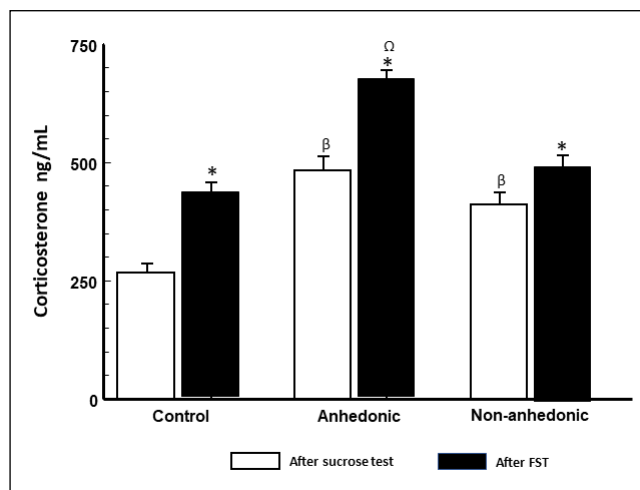


Fig. 8. Serum corticosterone levels after sucrose preference test and after forced swim test (FST). Hormone levels after sucrose preference test were higher in anhedonic and non-anhedonic rats compared to controls. After FST, corticosterone increased to a greater extent in anhedonic rats than in control and non-anhedonic rats. Non-anhedonic rats showed a slight increase in corticosterone after FST. Data shown as Mean \pm S.E.M. * $P=0.01$ compared to corticosterone levels after sucrose preference test; ^β $P=0.01$ compared to control after the sucrose preference test; ^Ω $P=0.01$, compared to levels in control and anhedonic rats after FST. Control group: $n=15$; Anhedonic: $n=10$; Non-anhedonic: $n=5$.

regulated transport, and these processes can be disturbed by glucocorticoids (Brunton et al., 2010). Cold water immersion stress significantly increases the level of glucocorticoids during pregnancy by 200%, compared with non-stressed dams (Guerrero et al., 2016; García-Vargas et al., 2019), modifying placental functionality. High maternal glucocorticoids during gestational stress might lead to decreased nutrient transport to the fetus, decreasing glucose transporter GLUT1, and consequently, glucose transport to the fetus (Viltart and Vanbesien-Mailliot, 2007).

The lower sucrose preference observed in prenatally stressed rats (Fig. 4) is in agreement with that reported in other studies (Alonso et al., 1991; Zhang et al., 2017). In those studies, however, no differentiation was made between anhedonic and non-anhedonic rats. In this study, 69% of prenatally stressed rats were considered anhedonic due to their low sucrose preference. The remaining 31% of prenatally stressed males showed sucrose consumption and preference similar to control males, thus being considered as non-anhedonic (Bergström et al., 2007; Strekalova et al., 2011). Several studies indicate that in animal models, there is a significant variation, among individuals, in their response to stress (Schmidt et al., 2010; Scharf and Schmidt, 2012). Rats that have experienced prenatal stress can be separated in those that are

vulnerable (anhedonic) and those that are resilient (non-anhedonic) (Krishnan and Nestler, 2011), allowing a more precise analysis of depression-like behavior generated by prenatal stress. Probably, this difference can be explained by differential gene expression in the hippocampus of anhedonic and non-anhedonic rats (Stekalova et al., 2011). The fact that not all the individuals exposed to stress develop stress-related problems indicates resilience, showing better outcomes despite severe stress exposure (Russo et al., 2012). DNA methylation is an epigenetic mechanism for reprogramming the genome after early life stress. Recently, it has been reported in children that prenatal stress may epigenetically shape resilience by methylating genes such as those encoding the glucocorticoid receptor (*NR3C1*) and its repressor (*FKBP41*). The methylation of these genes indicates an enhanced ability to terminate hormonal stress responses in prenatally stressed children, and more DNA methylation have been observed in heterochromatin regions associated with stress/disease resilience (Serpeloni et al., 2019). This may explain the lower hyperactivity and hyperreactivity of the adrenal axis observed in non-anhedonic rats after the FST, compared to anhedonic rats. Anhedonic behavior is associated with a decrease in the activity of the reward system (pleasure), and brain areas involved in anhedonic behavior are found in the mesolimbic system. The main projections are dopaminergic, which go from the ventral tegmental area to the nucleus accumbens (NAc), in the ventral striatum (Wise, 2008). Anhedonic behavior, shown by decreased response to reward stimuli, is associated with a lower release of dopamine in the nucleus accumbens (Di Chiara et al., 1999) and in the bed nucleus of the stria terminalis (Soares-Cunha et al., 2018). Serotonin modulates dopamine release (Yan, 2000), and could have a regulatory role in the reward process and in anhedonic behavior (Der-Avakian and Markou, 2012). Chronic treatment with Selective Serotonin Reuptake Inhibitor (SSRI) antidepressants increases sucrose preference in mice (El Yacoubi et al., 2011), and 5-HT synthesis blockage results in partial impairment in reward experiments (Liu et al., 2014). The light 5-HT release in the ventral hippocampus of non-anhedonic rats after sucrose preference test, compared to control group, is consistent with that evidence, since they had a sucrose preference similar to control rats. In anhedonic rats, the low sucrose preference could be explained by other mechanisms. For example, it has been reported that a decrease in 5-HT transporter (5-HTT) expression is associated with a stress-induced anhedonic state, whereas an increase in the transporter is associated with stress-induced non-anhedonic state (Tang et al., 2013). Thus,

studies on 5-HTT expression should be done in prenatally stressed animals, anhedonic and non-anhedonic.

The longer duration of immobility observed in adult prenatally stressed, anhedonic and non-anhedonic rats, as well as shorter swimming durations in the FST, are consistent with other studies despite differences in the protocol of prenatal stress: immobilization and exposure to intense light (Morley-Fletcher et al., 2003; 2004). Other studies report effects of prenatal stress in females, but not in males (Alonso et al., 2000; Frye and Wawrzycki, 2003). The discrepancies could be explained by the stress protocol used. In those reports, stress was caused by exposure to bright light while immobilization was used only on day 18 of gestation. In contrast, in this study, the stressor was applied from day 15 to day 21 of gestation and consisted in cold-water immersion, which is a more intense stressor than others, as it causes a drastic elevation of corticosterone in pregnancy (Guerrero et al., 2016; García-Vargas et al., 2019), and it does not allow habituation to the stressor (Retana-Márquez et al., 2003). In many studies, immobility is the variable that is quantified in the FST to confirm depression-like behavior (learned helplessness) (Porsolt et al., 1977; 1979), since the animal cannot escape, it stops trying and remains immobile, which has been anthropomorphically interpreted as “despair”, a depression-like phenotype. In spite of the FST has been considered a depression-like behavior, it is rather a behavioral adaptation for survival (Porsolt et al., 1978; Cryan and Mombereau, 2004; Molendijk and Kloet, 2015; Campus et al., 2015; De Kloet and Molendijk, 2016), and it is a useful behavioral test for the assessment of adrenal and serotonergic response to acute stress, since this test assess coping strategies to an acute inescapable stressor (Commons et al., 2017). The results of the present study indicate that prenatal stress alters the functionality of the serotonergic system, which is consistent with evidence indicating that stress during pregnancy causes neurochemical changes that affect the hippocampal serotonergic system (Charil et al., 2016), modifying the content of serotonin (Hayashi et al., 1998). The high content and low release of serotonin in anhedonic rats correlated negatively with immobility durations and shorter swim durations observed in the FST.

In contrast to other studies reporting low 5-HT and high 5-HIAA content in ventral hippocampus of prenatally stressed rats (Hayashi et al., 1998; Soares-Cunha et al., 2018), no changes were observed in 5-HT, but higher 5-HIAA content was found after the sucrose preference test in our study. After the FST, 5-HT content increased, without changes in 5-HIAA. These differences can be due to differences in the type of stressor used: crowding and saline injection (Hayashi

et al., 1998) or chronic unpredictable mild stress (Soares-Cunha et al., 2018) in those studies vs. immersion in cold water in the present study, which might activate the HPA axis differently, causing different maternal corticosteroid release. Another difference is the age of rats at which 5-HT was evaluated: 3 weeks (Hayashi et al., 1998) or 2 months (Soares-Cunha et al., 2018), and 3 months in the present study. Possibly, 5-HT turnover rate differs with age. In our study, we observed that the metabolic rate of serotonin was higher (0.85 ± 0.21) in prenatally stressed anhedonic rats than in control male rats (0.44 ± 0.07). Although the content of 5-HT after the sucrose preference test was similar in anhedonic and control rats, higher 5-HIAA content in anhedonic males, compared to controls (491.55 ± 39.82 ng/10 μ L, and 319.96 ± 55.02 ng/10 μ L, respectively) were observed. In microdialysis study, higher metabolic rate was observed in anhedonic males (9.11 ± 1.75), compared to controls (3.91 ± 0.41) and non-anhedonic rats (4.74 ± 1.42) after sucrose preference test. These results are consistent with previous reports studying histological 5-HT and its metabolite (Hayashi et al., 1998), in which a higher baseline metabolic rate was reported in prenatally stressed rats compared to their controls. In the current work, the metabolic rate in control males increased, contrasting with prenatally stressed animals, in which metabolic rate decreased, suggesting that serotonin levels were not modified by the FST. This was confirmed by the microdialysis study.

Microdialysis results show that 5-HT release and extracellular concentration of 5-HIAA increased in response to the FST in control males. These data indicate that, under normal conditions, 5-HT is released in response to the FST. Immobility and swimming behaviors have been related to serotonin release (Porsolt et al., 1979). The increase in 5-HT levels in control rats after FST explains the shorter immobility and longer swim durations. In comparison, only a slight increase in 5-HT levels was observed in anhedonic rats, after FST. In non-anhedonic rats, no change was observed in 5-HT, with decreased levels of 5-HIAA after the FST. These results indicate that prenatal stress disrupts the serotonergic system, as shown by negative correlation between immobility and serotonin release in anhedonic and non-anhedonic rats. In addition to low 5-HT release, it is possible that SERT increases 5-HT recapture in prenatally stressed rats, since an increase in SERT expression has been reported in other studies (Bielas et al., 2014). As far as we know, this is the first study that reports the *in vivo* basal serotonin metabolism in the ventral hippocampus and how it changes after the FST in prenatally stressed rats.

The stressful nature of the FST, due to its water component, allows us to evaluate the adrenal response. High corticosterone levels observed in prenatally stressed, anhedonic males with no FST and after the FST, compared with control animals, confirm that prenatal stress causes adrenal axis hyperactivity (high baseline corticosterone levels) and hyperreactivity (increased response to stressors), which is consistent with what was previously reported with other stressors (Koehl et al., 1999). The high concentration of the steroid interferes with serotonergic transmission, causing longer immobility durations and less time swimming (Mitchel and Meaney, 1991; Morley-Fletcher et al., 2003). The higher activity and reactivity of adrenal axis have been attributed to a lower number of GR in the ventral hippocampus, leading to higher corticosterone concentration in prenatally stressed animals (Joëls et al., 2008). As mentioned before, non-anhedonic or resilient rats showing corticosterone response to the FST, similar to that of controls, might be related with epigenetic changes in the expression of the MR gene (Serpeloni et al., 2019). This confirms the differences in the stress response among individuals (Huizink et al., 2004; Bergström et al., 2007; Schmidt et al., 2008). It is likely that GR and MR are not diminished in non-anhedonic animals, allowing an adrenal axis response similar to control animals. This remains to be proven in later studies.

CONCLUSION

The results of this work show that prenatal stress causes anhedonic behavior, shown as low sucrose preference in 69% of the male offspring, and 31% were non-anhedonic rats. Also, longer immobility durations and less swimming behavior in the FST are observed in prenatally stressed males. These behavioral alterations correlate with low 5-HT content and release in the ventral hippocampus, suggesting that disruption of the serotonergic system contributes to anhedonic behavior and lower swimming durations. Hyperactivity (higher basal levels of corticosterone) and hyperreactivity (increased levels of corticosterone in response to FST) of the adrenal axis in anhedonic rats seem to be involved in the disturbances in 5-HT metabolism.

ACKNOWLEDGEMENTS

Authors want to express their gratitude to Edith Monroy for her advice in the language of the text.

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