

Unpredictable chronic prenatal stress and manifestation of generalized anxiety and panic in rat's offspring

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ABSTRACT

Often the manifestation of anxiety cannot be explained by known environmental or hereditary factors. With this perspective, it has been reported that prenatal stress may lead to emotional disturbances in the offspring. However, studies relating prenatal stress to anxiety are controversial and generally the stressors used do not mimick the reality experienced by mothers. Thus, this investigation evaluated the effects of an unpredictable chronic stress scheme applied in one of the three gestational weeks of rats on the manifestation of generalized anxiety and panic disorder in the progeny (males), analyzing, respectively, the avoidances and escapes in the elevated T-maze, at the 1st, 3rd or 6th month of progeny life. Control offspring showed increased generalized anxiety disorder and reduced panic at 6 months. The effects of prenatal stress depended on the gestational week where it occurred and on the progeny age: during the 1st gestational week the generalized anxiety decreased in 6 month old rats. Animals in the 3rd month, prenatally stressed during the last gestational week, showed anxiogenesis and panicogenesis, but effects reverted at the 6th month, when they presented anxiolysis and no changes related to panic. Together the results show that not only the gestational period in which the aversive experience occurred was important, but the age of the evaluated progeny, since the type and the intensity of behaviors related to anxiety may vary with the developmental stage. For the model of stress used in the present study, the effects of prenatal stress were more prominent when the exposure occurred during the 3rd gestational week in rats.

1. Introduction

Although the interaction between genes and the environment may increasingly explain the manifestation of mental disorders, in many cases it is impossible to determine precisely which factors are involved. In humans the history of postnatal life, from early stages after birth, has been widely discussed, but sometimes unsuccessfully to elucidate peculiarities still obscure, especially when one examines the manifestation of disorders such as anxiety.

It is already known that prenatal stress can cause pathologies in the embryo or fetus. Due to its rapid growth, the fetus is particularly vulnerable to insults and attendant changes in the hormonal milieu (Weinstock, 2005). Environmental adversity, whether emotional or physical, experienced by the mother during pregnancy can influence the growth of the fetus, affecting its physical and mental well-being throughout life (Van den Hove et al., 2013).

Although the mechanisms by which prenatal stress affects offspring

are not yet fully established, studies indicate its relationship to the exposure of the embryo or fetus to the action of catecholamines released by autonomic activation, decreasing oxygenation and the supply of basic nutrients to the fetus (Copper et al., 1996; Huizink et al., 2004), and mainly of glucocorticoids (Copper et al., 1996; Huizink et al., 2004; Van den Hove et al., 2013; Weinstock, 2005; Zagron and Weinstock, 2006) that culminate in alteration of the reactivity of the hypothalamic-pituitary-adrenal (HPA) axis (García-Cáceres et al., 2010; Green et al., 2011; Louvart et al., 2009; Wilson et al., 2013). Evidence has also shown that prenatal stress alters other neuroendocrine circuitry, such as serotonergic (Miyagawa et al., 2011; Van den Hove et al., 2014; Van den Hove et al., 2006; Wyrwoll and Holmes, 2012), noradrenergic (Green et al., 2011), GABAergic (Grigoryan and Segal, 2013; Laloux et al., 2012), glutamatergic (Laloux et al., 2012; Marrocco et al., 2012), as well as oxytocin and vasopressin (De Souza et al., 2013), indicating a modification of these systems, directly or indirectly, by changes in the progeny HPA axis due to prenatal stress.

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Table 1
Prenatal stress effects on offspring anxiety manifestation.

Species/Strain	Reference	Stressor	Gestational days in which stressor was applied	Age of assessed offspring	Was used a prior stressor on the offspring before the behavioral assessment?	Animal model (behavioral assessment)	Offspring anxiety (male)
Mouse / ddY	Nishio et al., 2006	Forced swim, 15 minutes, once a day	10 - 18	35 days	No	EPM	=
		Noise, 15 minutes, once a day	10 - 18	35 days	No	EPM	=
Mouse / ICR	Miyagawa et al., 2011	Restraint (1 hour, 3 times a day)	5,5 - 17,5	49 - 56 days	No	EPM	=
		Restraint (6 hours, once a day)			No	EPM	↑
Mouse / Swiss	Pallarés et al., 2007	Restraint (45 minutes, 3 times a day)	15 - parturition	90 days	No	EPM	=
Rat / CD	Richardson et al., 2006	1 random stressor per day: restraint under bright light (45 minutes, 3 times a day); foot shocks (one session of 30 minutes, once a day); 0.5 cc saline injection under bright light (once a day)	14 - 21	Adult (age not described)	No	EPM	=
		Restraint under bright light (45 minutes, 3 times a day)	14 - 21	Adult (age not described)	No	EPM	=
Rat / Sprague-Dawley	Polyrev et al., 2005	Restraint (45 minutes, 3 times a day)	15 - 20	77 - 84 days	No	EPM	=
Rat / Sprague-Dawley	Zuena et al., 2008	Restraint under bright light (45 minutes, 3 times a day)	11 - 21	90 days	No	EPM	↑
Rat / Sprague-Dawley	Laboux et al., 2012	Restraint under bright light (45 minutes, 3 times a day)	11 - parturition	22 days	No	EPM	↑
Rat / Sprague-Dawley	Van den Hove et al., 2013	Restraint under bright light (45 minutes, 3 times a day)	14 - 21	120 days	No	EZM	↑
Rat / Sprague-Dawley	Wilson et al., 2013	1 to 3 random stressors per day: restraint (1 hour); exposure to a cold environment (6 h); overnight food deprivation; forced swim (15 min); reversal of the light-dark cycle; social stress: induced by overcrowded housing during the dark phase of the cycle	14 - 22 or 23	63 days	No	EPM	=
Rat / Wistar	Rimondini et al., 2003	Restraint (60 minutes, 3 times a day)	14 - 21	127 days	No	EPM	=
				141 days	Yes (assessed 60 minutes after restraint for 1 hour).	EPM	↑
Rat / Wistar	Estanislau and Morato, 2005	80 foot shocks (session of 100 minutes)	Every other day for the entire gestation	60 days	No	EPM	↑
				60 days	Yes (assessed immediately after restraint for 1 hour).	EPM	↑
				80 days	No	ETM	↑
Rat / Wistar	Estanislau and Morato, 2006	80 foot shocks (session of 100 minutes)	Every other day for the entire gestation	30 days	No	EPM	=
				45 days	No	EPM	↓
				60 days	No	EPM	↑
Rat / Wistar	Zagron and Weinstock, 2006	Restraint (30 minutes per day)	14 - 21	35 days	No	EPM	=

EPM: Elevated plus-maze; EZM: Elevated zero-maze; ETM: Elevated T-maze; In relation to anxiety: ↑ increase; ↓ decrease; = unchanged.

Table 2

Unpredictable chronic stress scheme.

Adapted from Echandi et al. (1988); González et al. (1994).

Stressor Stimulus	Procedure	Length
Sleep deprivation	Constant darkness + platform	24 hours
Predator aversion	Cat odor on a flannel	4 hours
Restraint	Restraint box	2 hours
Exercise and novelty	Activity wheel	10 minutes
Sleep deprivation	Constant darkness + platform	24 hours
Predator aversion	Cat odor on a flannel	4 hours
Forced swim and exercise	Forced swim	5 minutes

Among the various effects observed due to prenatal stress, behavioral changes are notable, such as depressive-like behavior (Abe et al., 2007), attention deficit hyperactivity disorder (Van den Bergh and Marcoen, 2004), increased response to fear (Sadler et al., 2011; Tazumi et al., 2005), increased incidence of schizophrenia-like alterations (Holloway et al., 2013; Khashan et al., 2008) and increased anxiety-like behavior. The latter effect is rather controversial, since while some studies have found a positive relationship between prenatal stress and increased anxiety in male offspring, others did not find results in this same direction, as shown in Table 1.

Confirming the fluctuation of the findings with respect to anxiety, Weinstock (2008) demonstrated through a literature review that the effects of prenatal stress on the offspring depend on the gestational stage in which it is applied, the type, intensity and duration of stress, as well as the animal model used to evaluate anxiety and the species chosen (mice or rats). Even the strain can have an influence, since variations in anxiety in different strains of rats have been described (Rex et al., 2004). Another aspect not considered by the researchers was the evaluation of the effect of stressors during different stages of the gestational period, and also comparing the manifestation of anxiety in different phases of progeny life. Additionally, most stressors used do not simulate the reality experienced by mothers, exposed not to an aversive stimulus, but to a contingent of different and unpredictable stressors.

Moreover, most of the research that has evaluated prenatal stress effects on the manifestation of anxiety in the offspring used as an animal model the elevated plus-maze (EPM) (Pellow et al., 1985). As far as we are aware there is only one study assessing the effects of prenatal stress on offspring's anxiety using the elevated T-maze (ETM) (Estanislau and Morato, 2006), a test proposed by Graeff et al. (1993), Viana et al. (1994), which discriminates two types of anxiety based on avoidance and escape behaviors, respectively: generalized anxiety disorder (GAD) and panic disorder (PD) (Graeff et al., 1998; Graeff et al., 1993; Poltronieri et al., 2003; Viana et al., 1994; Zangrossi and Graeff, 1997; Zangrossi et al., 2001).

Thus, the objective of the present investigation was to evaluate the manifestation of GAD and PD in rat male offspring whose dams were subjected (or not) to an unpredictable chronic stress scheme in different stages of pregnancy, observing the defense repertoire of the animals in the ETM (avoidances and escapes). This study also aimed to determine which gestational period (1st, 2nd or 3rd gestational week) was more critical for the manifestation of these disorders in postnatal life and in which phase of offspring development (1st, 3rd or 6th month) the demonstration was evident.

2. Materials and methods

2.1. Dams

Virgin female *Wistar* rats with an average age of 75 days old, from UNESP Central vivarium (Botucatu/SP), were grouped (5 rats) in polypropylene boxes (32 × 38 × 18 cm) using sawdust as bedding material in the Female vivarium of the Physiology Laboratory, maintained under controlled conditions of temperature (21 °C ± 2 °C), lighting (50 lx at the center of the room and 12,12 h light-dark cycle, with lights on at 07:00 am), receiving chow and water ad libitum. The animals were handled only during box cleaning and in specific moments of the experiment. The study was approved by the local Ethics Committee on the Use of Animals (CEUA 011/2012). All procedures were conducted in accordance with international ethical standards concerning animal experimentation.

2.2. Mating

After a minimum period of 7 days in the vivarium of the Physiology Laboratory, the estrus cycle of rats was monitored by vaginal smear. When presenting proestrus or estrus, female rats were individually allocated with an experienced male (one couple per box). The criterion for statement of mating and determination of gestational day (GD) 1 was the presence of a vaginal plug. When not found, a vaginal smear was taken to observe the presence of sperm. After mating, all pregnant female rats (dams) were relocated in their boxes, remaining grouped (5 per box) or not, according to each experimental group.

2.3. Dams and unpredictable chronic stress

Dams were subjected to an unpredictable chronic stress scheme in one of the three gestational weeks: 1st week (from GD 1 to 7), 2nd week (from GD 8 to 14) or 3rd week (from GD 15 to 21). In this context, the rats, which were grouped into 5 animals per box, were separated into 1 animal per box during the designated gestational week and submitted, on each day of that week, to a different stressor, following the model adapted from Echandi et al. (1988) and González et al. (1994) (Table 2), being given 'forced swim' as the last stressor. Control groups (a total of three, one for each gestational week), consisting of dams grouped into 5 animals per box throughout the entire pregnancy, were not exposed to the unpredictable stress scheme. At the end of the designated gestational week, dams were assessed in the EPM in order to

verify whether the stress scheme caused alteration in their anxiety profile (Soliani et al., 2015). At the end of pregnancy, in the afternoon of GD 21, all dams were taken to the maternity of the Physiology Laboratory (1 animal per box), where parturition occurred. During lactation, there was no manipulation of the offspring, except for box cleaning (3 times a week), performed with a plastic shovel to avoid direct contact between the pups and the experimenter. At postnatal day 21, pups were weaned and in a separate room were sexed and identified by different marks on their tails. Subsequently, male progeny were placed in the Male vivarium of the Physiology Laboratory.

2.4. Offspring and experimental groups

In the Male vivarium (conditions of temperature and luminosity according to item 2.1), male pups were grouped into 5 animals per box (polypropylene, $41 \times 34 \times 17$ cm, lined with sawdust) according to the gestational week in which their dams were stressed (whenever possible, siblings were not allocated in the same box. To avoid a 'litter effect', at most 3 pups from the same dam were used in the same group). Considering that control dams consisted of non-manipulated animals (except for box cleaning and assessment in the EPM), we decided to evaluate only pups from control dams assessed in the intermediate gestational week, i.e., the 2nd week. In this way, 4 large groups were formed: *Control Group* (pups from dams assessed in the EPM at the end of the 2nd gestational week), *1st Gestational Week Group – 1st GW* (pups from dams stressed during the 1st gestational week), *2nd Gestational Week Group – 2nd GW* (pups from dams stressed during the 2nd gestational week) and *3rd Gestational Week Group – 3rd GW* (pups from dams stressed during the 3rd gestational week). Pups were still divided into 3 subgroups, according to the month of life in which they were submitted to behavioral assessment: *1st, 3rd and 6th month*. Once in the Male vivarium, animals received water and chow ad libitum and were not manipulated until the suitable age for assessment, except for box cleaning (3 times a week).

2.5. Behavioral assessment

When they were in the suitable month for evaluation, offspring were assessed in the ETM [1st month: age between 33 and 39 days old – n (control) = 9, n (1st GW) = 9, n (2nd GW) = 10, n (3rd GW) = 10]; [3rd month: age between 91 and 99 days old – n (control) = 10, n (1st GW) = 10, n (2nd GW) = 11, n (3rd GW) = 10]; [6th month: age between 181 and 189 days old – n (control) = 7, n (1st GW) = 9, n (2nd GW) = 10, n (3rd GW) = 10]. As mentioned, the ETM seeks to generate in the same animal two defensive responses: inhibitory avoidance and escape, which have been related, respectively, to generalized anxiety and panic (Graeff et al., 1993; Graeff et al., 1998; Poltronieri et al., 2003; Viana et al., 1994; Zangrossi and Graeff, 1997; Zangrossi et al., 2001).

We used an ETM made of wood, consisting of three arms of the same dimensions (50×12 cm), elevated 50 cm from the floor (Graeff et al., 1993). One of the arms was surrounded by lateral walls (40 cm tall) and was perpendicular to the two open arms. The open arms were delimited by a transparent acrylic protection of 1 cm height to keep the animals from falling. After 3 consecutive days of handling (5 min/day), 24 h before the behavioral assessment, animals were kept individually, for thirty minutes, in one of the open arms of the ETM (pre-exposure). For this, the open arms were separated by a wooden wall, disposed on the line between the central area of the maze and the proximal portion of the open arm. After this procedure, animals were returned to their boxes until the next day.

The test in the ETM was initiated by an inhibitory avoidance measurement. Each animal was placed at the distal end of the enclosed arm facing the intersection of the arms. The time taken by the rat to leave this arm with the four paws was recorded (Baseline latency). The same measurement was repeated in two subsequent trials (Avoidance 1 and

2) at 30 s intervals, during which time animals were placed in a polypropylene box with sawdust. Thirty seconds after the last avoidance, the rat was placed at the end of the same open arm used in the pre-exposure session and the time taken to leave this arm with the four paws was recorded in three consecutive trials (Escape 1 to 3), again with 30 s intertrial intervals. A cut off time of 300 s was established for avoidance and escape latencies. For each trial, the number of fecal boli was counted and when there was fecal and/or urinary excretion, the ETM was cleaned with a 20% ethanol solution before the next trial with the same animal and between different animals.

Thirty seconds after being tested in the ETM, animals were individually placed in the center of an open-field for the evaluation of locomotor activity, where the total number of squares crossed by the animal was counted for 5 min. The open-field was made of wood, measuring 60×60 cm, with marked squares of 20×20 cm.

The tests were carried out in an exclusive room for behavioral assessment in the Physiology Laboratory (temperature of $21^\circ\text{C} \pm 2^\circ\text{C}$), between 2 and 5 p.m. During the assessment, each rat was taken individually in a polypropylene box lined with sawdust to the experimental room. The apparatus was illuminated with an incandescent light bulb attached to the roof, providing a luminous intensity of 50 lx, avoiding shadow in the arms of the ETM. The apparatus was cleaned with a 20% ethanol solution after the end of the test of each animal. The experimenter stayed outside the room, tests were video recorded and then further analyzed with the program Etholog 2.25 (Otoni, 2000).

2.6. Statistical analysis

All data were analyzed with Statistica 6.0 software (Statsoft). The analyses were carried out by month (1st, 3rd or 6th month). For the ETM, to assess if there was learning during the avoidances or changes between the escape trials within the same group, we used ANOVA of repeated measures. For each trial of avoidance (Baseline, Avoidance 1 or Avoidance 2) or escape (Escape 1 to 3), experimental groups were compared to the control group using one-way ANOVA, according to the month of life analyzed. The number of crossed squares in the open-field, as well as the animals' body weight and total fecal boli eliminated on the ETM were analyzed by one-way ANOVA. In order to assess whether the profile of anxiety and other parameters naturally changed across age, control groups were also analyzed separately from experimental groups, using ANOVA of repeated measures and one-way ANOVA. *Post-hoc* comparisons were made with Duncan's test. In all analysis, a value of $p < 0.05$ was considered significant.

3. Results

3.1. Behavioral and physiological results presented by animals of control groups

In the analysis of avoidances (Fig. 1A, B and C), ANOVA of repeated measures showed that there were differences between the groups ($F_{2,23} = 6.46$; $p = 0.006$) and an effect of trials ($F_{2,46} = 7.16$; $p = 0.002$), but without an interaction between groups and trials ($F_{4,46} = 0.70$; $p = 0.599$). Duncan's test pointed out that animals in the 3rd month of life showed learning of avoidances, as evidenced by the increase of Avoidance 1 and 2 in relation to Baseline ($p = 0.042$ and $p = 0.007$, respectively). The same was observed with animals at 6 months (increased Avoidance 2 in relation to Baseline; $p = 0.049$), i.e., there was an increase of avoidance throughout the trials, featuring a defense behavioral repertoire, as expected for control groups. Although graphically (Fig. 1A) one can verify increased Avoidance 2 in relation to Baseline, the difference was not significant in the analysis of animals in the 1st month of life. Analyzing each trial, one-way ANOVA showed that there were differences between the groups for Baseline ($F_{2,23} = 5.20$; $p = 0.014$). Duncan's test pointed out that animals at 6 months of life showed increased latency in the enclosed arm in

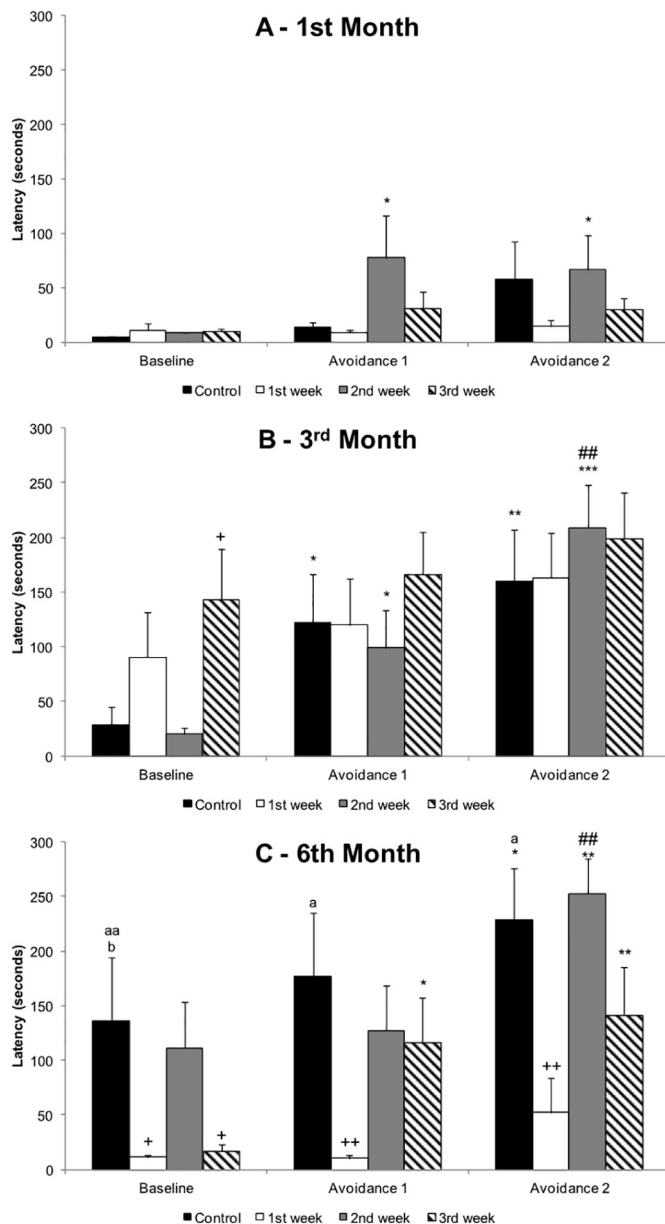


Fig. 1. A: 1st month of life; B: 3rd month of life; C: 6th month of life. Mean + SEM of latency in seconds for avoidances in the ETM. *Learning*: ANOVA of repeated measures followed by Duncan's test, where: * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ in relation to Baseline of the same group within the evaluated month; ## $p < 0.001$ in relation to Avoidance 1 of the same group within the evaluated month. *Differences between groups within the same month*: one-way ANOVA followed by Duncan's test, where: + $p < 0.05$ and ++ $p < 0.01$ in relation to the same trial of the control group. *Comparison among control groups (AxBxC)*: one-way ANOVA followed by Duncan's test, where: ^a $p < 0.05$ and ^{aa} $p < 0.01$ in relation to the same trial of the 1st month; ^b $p < 0.05$ in relation to the same trial of the 3rd month.

relation to animals at the 1st ($p = 0.006$) and 3rd month ($p = 0.016$). Regarding Avoidance 1 and 2 there were differences between the groups, respectively ($F_{2,23} = 4.10$; $p = 0.030$) and ($F_{2,23} = 3.65$; $p = 0.042$). Duncan's test pointed out that rats at 6 months showed an increased latency in the enclosed arm compared to animals in the 1st month for Avoidance 1 ($p = 0.012$), as well as for Avoidance 2 ($p = 0.015$).

Regarding the escapes (Fig. 2A, B, C), ANOVA of repeated measures showed that there were differences between the groups ($F_{2,23} = 8.01$; $p = 0.002$) and an effect of trials ($F_{2,46} = 3.95$; $p = 0.026$), without an

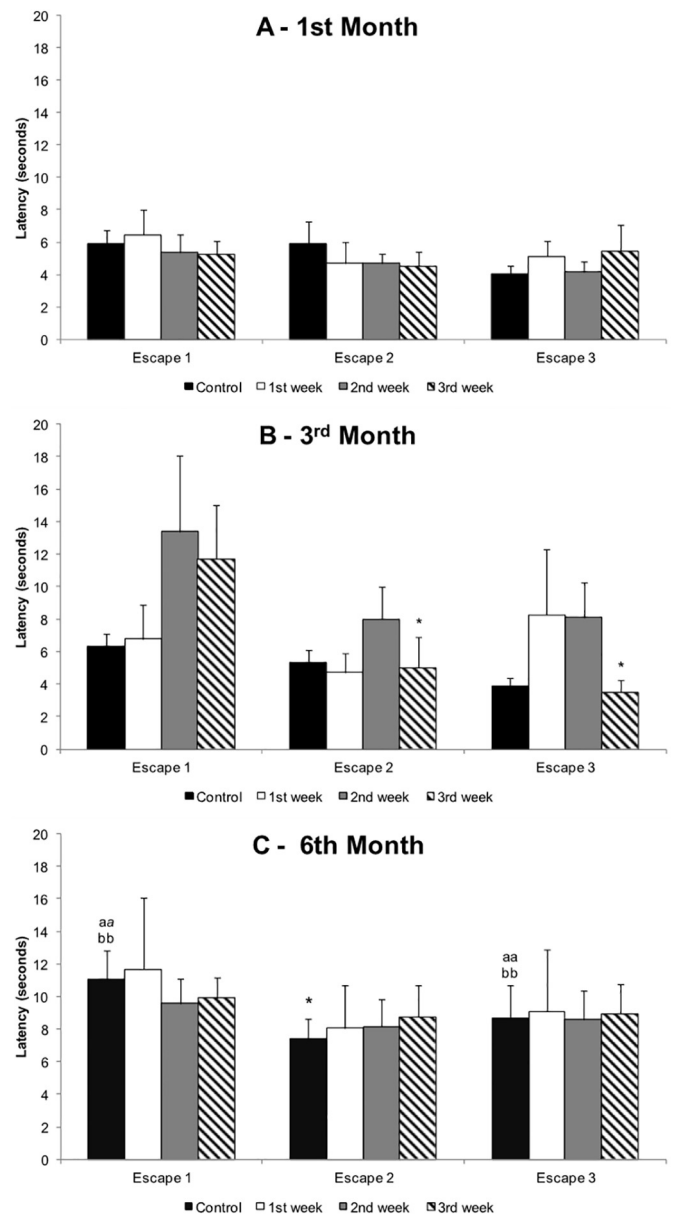


Fig. 2. A: 1st month of life; B: 3rd month of life; C: 6th month of life. Mean + SEM of latency in seconds for escapes in the ETM. *Changes among escapes*: ANOVA of repeated measures followed by Duncan's test, where: * $p < 0.05$ in relation to Escape 1 of the same group within the evaluated month. *Differences between groups within the same month*: one-way ANOVA followed by Duncan's test (there were no differences). *Comparison among control groups (AxBxC)*: one-way ANOVA followed by Duncan's test, where: ^{aa} $p < 0.01$ in relation to the same trial of the 1st month; ^{bb} $p < 0.01$ in relation to the same trial of the 3rd month.

interaction between groups and trials ($F_{4,46} = 1.04$; $p = 0.399$). Duncan's test did not point out differences between the 3 trials of animals in the 1st and 3rd month of life. However, for 6 month animals there was a reduction of latency in Escape 2 compared to Escape 1 ($p = 0.015$). Analyzing each trial separately, one-way ANOVA showed that there were differences between the groups for Escape 1 ($F_{2,23} = 6.06$; $p = 0.008$). Duncan's test pointed out an increase of latency for leaving the open arm for animals in the 6th month, in relation to animals in the 1st ($p = 0.004$) and 3rd month of life ($p = 0.006$). As for Escape 2 analysis, there were no differences between the groups ($F_{2,23} = 0.83$; $p = 0.450$). With Escape 3, there were differences between the groups ($F_{2,23} = 6.13$; $p = 0.007$), where Duncan's test showed an increased

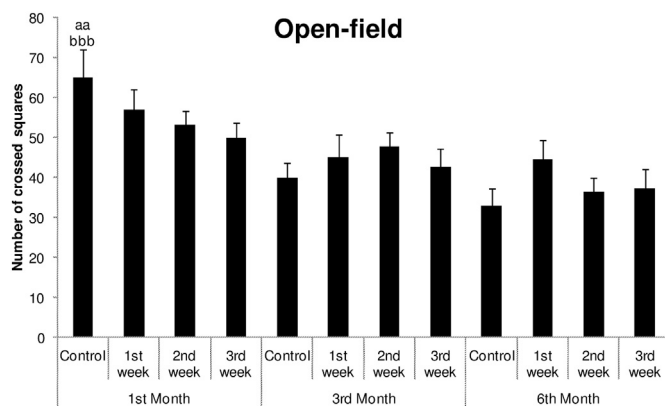


Fig. 3. Mean + SEM of number of crossed squares in open-field. One-way ANOVA followed by Duncan's test, where: ^{aa} $p < 0.01$; ^{bbb} $p < 0.001$, when compared to the control group of the 3rd and 6th month, respectively.

latency to leave the open arm for animals assessed in the 6th month, when compared to animals in the 1st and 3rd month of life ($p = 0.005$ for both).

Analyzing the number of crossed squares in the open-field (Fig. 3), one-way ANOVA showed differences between the groups ($F_{2,23} = 9.47$; $p = 0.001$). Duncan's test pointed out an increase in the number of crossed squares for control animals in the 1st month of life in relation to animals at 3 and 6 months old ($p = 0.004$ and $p < 0.001$, respectively).

As to body weight (Fig. 4), one-way ANOVA showed differences between the groups ($F_{2,23} = 118.64$; $p < 0.001$). Duncan's test revealed differences between all groups, as expected. Animals at 6 months were heavier than the animals at 1 and 3 months ($p < 0.001$ for both) and animals at 3 months, heavier than 1 month animals ($p < 0.001$).

For the number of fecal boli excreted on the ETM, one-way ANOVA showed no differences between the groups ($F_{2,23} = 1.97$; $p = 0.162$).

3.2. Effect of unpredictable chronic stress in different gestational weeks on the behavior and physiological variables of the offspring in three distinct stages of development (1st, 3rd and 6th month of life)

3.2.1. 1st month of life

For the avoidances presented by animals in the 1st month of life (Fig. 1A), ANOVA of repeated measures showed that there were no differences between the groups ($F_{3,34} = 1.49$; $p = 0.235$), but there was an effect of trials ($F_{2,68} = 4.78$; $p = 0.011$), without an interaction between groups and trials ($F_{6,68} = 1.47$; $p = 0.202$). Duncan's test pointed out that animals whose dams were stressed during the 1st or 3rd week

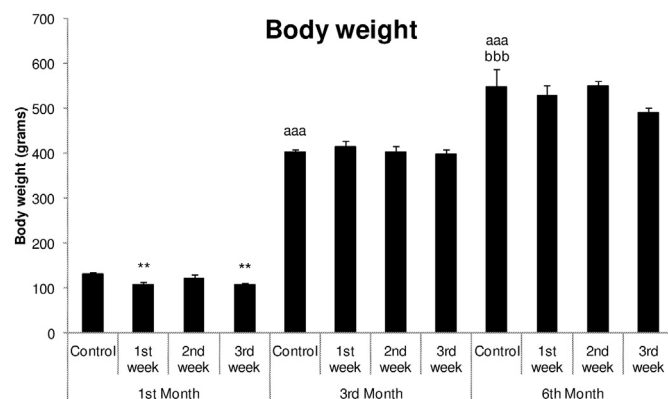


Fig. 4. Mean + SEM of body weight in grams. One-way ANOVA followed by Duncan's test, where: ^{**} $p < 0.01$ compared to the control group within the evaluated month; ^{aaa} $p < 0.001$ in relation to the control group of the 1st month and ^{bbb} $p < 0.001$ in relation to the control group of the 3rd month.

of gestation did not show learning with the avoidance trials. Only animals whose dams were stressed during the 2nd week showed this defensive behavior, as evidenced by the increase of Avoidances 1 and 2 in relation to Baseline ($p = 0.011$ and $p = 0.032$, respectively). Analyzing each trial separately, one-way ANOVA showed no differences between the groups for Baseline ($F_{3,34} = 0.83$; $p = 0.484$), neither for Avoidance 1 ($F_{3,34} = 2.06$; $p = 0.124$) nor for Avoidance 2 ($F_{3,34} = 1.00$; $p = 0.403$).

Regarding the escapes (Fig. 2A), ANOVA of repeated measures showed that there were no differences between the groups ($F_{3,34} = 0.14$; $p = 0.936$), neither an effect of trials ($F_{2,68} = 1.66$; $p = 0.197$), nor an interaction between groups and trials ($F_{6,68} = 0.66$; $p = 0.683$). In the analysis of each trial, one-way ANOVA showed no differences between groups: Escape 1 ($F_{3,34} = 0.25$; $p = 0.863$), Escape 2 ($F_{3,34} = 0.37$; $p = 0.777$) and Escape 3 ($F_{3,34} = 0.46$; $p = 0.711$).

As to the open-field (Fig. 3), one-way ANOVA showed no differences between the groups ($F_{3,34} = 1.66$; $p = 0.195$).

For body weight, one-way ANOVA showed differences between the groups ($F_{3,34} = 4.79$; $p = 0.007$). Duncan's test showed a decrease in body weight of animals whose dams were stressed during the 1st ($p = 0.005$) or 3rd gestational week ($p = 0.004$), compared to the control group, as can be seen in Fig. 4.

Regarding the elimination of fecal boli, one-way ANOVA showed no differences between the groups ($F_{3,34} = 1.50$; $p = 0.232$).

3.2.2. 3rd month of life

For the avoidances observed in 3 month old animals (Fig. 1B), ANOVA of repeated measures showed that there were no differences between the groups ($F_{3,37} = 0.84$; $p = 0.482$), but there was an effect of trials ($F_{2,74} = 21.94$; $p < 0.001$) without an interaction between groups and trials ($F_{6,74} = 1.87$; $p = 0.098$). Duncan's test revealed that unpredictable chronic stress during the 1st or 3rd gestational week impaired learning of avoidances because, in fact, Baseline was already high for these groups. However, animals whose dams were stressed during the 2nd week showed learning, as evidenced by the increase of Avoidances 1 and 2 compared to Baseline ($p = 0.035$ and $p < 0.001$, respectively) and also by the increase of Avoidance 2 in relation to Avoidance 1 ($p = 0.005$). Analyzing each trial separately, one-way ANOVA showed that for Baseline there were differences between the groups ($F_{3,37} = 3.53$; $p = 0.024$), but not for Avoidance 1 ($F_{3,37} = 0.51$; $p = 0.676$) and Avoidance 2 ($F_{3,37} = 0.35$; $p = 0.787$). Duncan's test pointed out an increase of latency for Baseline in animals whose dams were stressed during the 3rd gestational week, when compared to the control group ($p = 0.016$).

Regarding the escapes (Fig. 2B), ANOVA of repeated measures showed that there were no differences between the groups ($F_{3,37} = 1.25$; $p = 0.307$), but there was an effect of trials ($F_{2,74} = 4.36$; $p = 0.016$) without an interaction between groups and trials ($F_{6,74} = 1.18$; $p = 0.324$). Duncan's test revealed a reduction in Escapes 2 and 3 latencies in relation to Escape 1 in animals whose dams were stressed during the 3rd gestational week ($p < 0.05$ for both). In the analysis of each trial separately, one-way ANOVA showed no differences between the groups: Escape 1 ($F_{3,37} = 1.27$; $p = 0.300$), Escape 2 ($F_{3,37} = 0.97$; $p = 0.419$) and Escape 3 ($F_{3,37} = 1.24$; $p = 0.309$).

One-way ANOVA did not show differences between groups for the number of crossed squares in the open-field ($F_{3,37} = 0.58$; $p = 0.634$), neither for body weight ($F_{3,37} = 0.39$; $p = 0.763$) nor for elimination of fecal boli ($F_{3,37} = 1.86$; $p = 0.153$) (Fig. 3 and 4, respectively).

3.2.3. 6th month of life

In relation to the avoidances presented by animals at 6 months old (Fig. 1C), ANOVA of repeated measures showed differences between the groups ($F_{3,32} = 5.96$; $p = 0.002$) and an effect of trials ($F_{2,64} = 12.22$; $p < 0.001$), but without an interaction between groups and trials ($F_{6,64} = 1.28$; $p = 0.280$). Duncan's test pointed out that there was no learning of avoidances in animals whose dams were stressed

during the 1st gestational week, while those whose dams were stressed during the 2nd week showed learning, evidenced by the increase of Avoidance 2 in relation to Avoidance 1 and Baseline ($p = 0.007$ and $p = 0.003$, respectively). For animals whose dams were stressed in the 3rd week, learning was evidenced by the increase of Avoidances 1 and 2 compared to Baseline ($p = 0.008$ and $p = 0.028$, respectively). In the analysis of each trial, one-way ANOVA showed that there were differences between groups for Baseline ($F_{3,32} = 3.69$; $p = 0.022$), Avoidance 1 ($F_{3,32} = 2.92$; $p = 0.049$) and also for Avoidance 2 ($F_{3,32} = 5.61$; $p = 0.003$). Duncan's test revealed a reduction in Baseline latency for animals whose dams were stressed during the 1st or 3rd gestational week compared to the control group ($p = 0.020$ and $p = 0.018$, respectively), and a reduction in Avoidances 1 and 2 latencies for animals whose dams were stressed during the 1st gestational week in relation to the control group ($p < 0.01$ for both).

Regarding the escapes (Fig. 2C), ANOVA of repeated measures showed that there were no differences between the groups ($F_{3,32} = 0.04$; $p = 0.991$), neither an effect of trials ($F_{2,64} = 1.98$; $p = 0.146$) nor an interaction between groups and trials ($F_{6,64} = 0.15$; $p = 0.989$). Analyzing each trial, one-way ANOVA showed no differences between the groups: Escape 1 ($F_{3,32} = 0.15$; $p = 0.929$), Escape 2 ($F_{3,32} = 0.07$; $p = 0.976$) and Escape 3 ($F_{3,32} = 0.01$; $p = 0.999$).

As to the open-field, one-way ANOVA showed no differences between the groups ($F_{3,32} = 1.13$; $p = 0.351$) (Fig. 3). For body weight ($F_{3,32} = 1.80$; $p = 0.166$) and defecation ($F_{3,32} = 1.31$; $p = 0.289$) there were no differences either, as illustrated in Fig. 4.

4. Discussion

Knowing that puberty in male rats starts at about 46 days old (Engelbregt et al., 2000), animals evaluated in the 1st month were considered pre-pubescent, while animals evaluated in the 3rd and 6th month were considered young adults and mature adults, respectively. Observing only control groups, it was possible to verify the ontogeny of the anxiety manifestation of rats whose dams were not submitted to chronic stress during the gestational period. The expected behavior of an animal assessed in the ETM is that there is a learning of avoidances, i.e., that the latency to leave the enclosed arm increases with the three trials (Baseline, Avoidance 1 and Avoidance 2), and that there are no changes in escapes, i.e., that the latency to leave the open arm remains constant with the 3 trials (Escape 1 to 3). When the latency for avoidances increases, i.e., when the animal takes more time to leave the enclosed arm than a control, this effect is understood as anxiogenic. When the latency decreases, the effect is named anxiolytic. When the latency for escape increases, i.e., when the animal spends more time in the open arm than the control, the effect is known as panicolytic. If the animal leaves the open arm faster, the effect is named panicogenic (Graeff et al., 1993; Graeff et al., 1998; Poltronieri et al., 2003; Viana et al., 1994; Zangrossi and Graeff, 1997, 2014; Zangrossi et al., 2001). There was manifestation of a defense behavioral repertoire, as expected for control groups, for animals assessed in the 3rd and 6th months, evidenced by learning of avoidances, but not in those of 1 month old, showing that, under normal conditions, a certain degree of maturity is required for the learning of aversive and potentially dangerous situations (open arm experience).

The manifestation of GAD and PD in older animals was paradoxical, with an increase of the former and a reduction of the latter: 6 month old animals showed an increase of latency for leaving the enclosed arm in all trials analyzed compared to animals at 1 month and for Baseline compared to animals at 3 months, indicating that age was a trigger factor for generalized anxiety for control rats. In addition, animals at 6 months also showed an increase in escape latencies, i.e., there was a decrease of panic.

It is appropriate to emphasize that control animals in the 1st month presented an increase in motor activity and lower body weight in comparison to animals in the 3rd and 6th months, indicating that the

reduced latency for leaving the enclosed arm could be caused by these two factors. A study of Andrade et al. (2003), in which the evaluation of motor activity was made by counting the number of entries in the enclosed arms of the EPM, showed that, in fact, 1 month old male rats exhibit greater activity and less anxiety, illustrated by the greater exploration of open arms of the EPM, in comparison to animals of 3 and 6 months old, corroborating what was found in the present study. There were no differences for the elimination of fecal boli across the ages, indicating no alterations of autonomic activity due to stressful situations (Lavallo and Sollers, 2010).

In humans, the National Comorbidity Survey Reproduction (NCS-R) (National Comorbidity Survey Reproduction, 2007), using the criteria of the 4th edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV) (American Psychiatric Association, 1994), showed that the lifetime prevalence of GAD in men is 4.2%. The lifetime prevalence (without distinction of sex) increases with age (measurements taken starting from 18 years old), peaking between 45 and 59 years old (7.6%), and falling after that. As for panic disorder, NCS-R indicated a lifetime prevalence in men of 3.1%, with the highest prevalence (without distinction of sex) occurring between 30 and 59 years old (5.9% from 30 to 44 years old and from 45 to 59 years old) and lessening after 60 years old (Bandelow and Michaelis, 2015).

From a translational point of view, such data corroborate what was observed in the present study in relation to GAD (higher in mature adults), but we cannot make the same statement regarding the manifestation of PD. In adolescents, the National Comorbidity Survey: Adolescent Supplement (NCS-A) (National Comorbidity Survey: Adolescent Supplement, 2014), for boys between 13 and 17/18 years old, showed that the lifetime prevalence of GAD is 1.5%, while for PD, the prevalence is 2.0%. We did not find epidemiological data in the literature for individuals under 13 years old, making impossible the comparison of the manifestation of anxiety in pre-pubescent rats.

The high prevalence of anxiety in middle-aged men could be explained by the regression of the production of testosterone, the so-called 'andropause' (McHenry et al., 2014; Wespes and Schulman, 2002). In humans, levels of free or bioavailable testosterone may fall up to 40% between 40 and 70 years old (Seidman, 2007). The relationship between testosterone levels and depression and anxiety disorders in humans is evident in men with hypogonadism, a condition that reduces the functional activity of gonads and results in reduced levels of testosterone. These men exhibit a significantly higher prevalence of anxiety disorders and major depression compared with men who have normal physiological levels of androgens (Shores et al., 2004; Zarrouf et al., 2009). It has also been shown that the removal of testes in rats causes increased anxiety (Khakpai, 2014), suggesting a relationship between this disorder and low levels of testosterone.

The exposure of dams to unpredictable chronic stress in distinct stages of gestation differentially affected the offspring, according to the age at the behavioral assessment. For the learning of avoidances, it was possible to perceive that rats whose dams were subjected to the stressor scheme during the 1st or 3rd week of gestation showed deficiency in the acquisition of aversive information when evaluated in the 1st month of life. At the age of 3 months there was no learning either when stressors were applied during the 1st or 3rd gestational week, however, Baseline observed for these animals was already high, i.e., there was an increase in latency for leaving the enclosed arm when they were placed in that compartment of the ETM for the first time. Animals in the 6th month of life that suffered prenatal stress during the 1st week of gestation did not present this acquisition of aversive information and increase of avoidance with trials. Namely, the unpredictable chronic stress during the 1st gestational week impaired the progeny aversive conditioning, whereas there was no learning of avoidances in any of the evaluated ages.

As for the effect of unpredictable chronic stress on the manifestation of generalized GAD and PD, we found that the most critical prenatal period of exposure was the 3rd gestational week. Animals in the 3rd month of life, whose dams underwent the stress scheme during this

week, showed an increase of avoidance (Baseline) compared to control animals, and a reduction of latencies with escape trials, effects interpreted as anxiogenic and panicogenic.

In the 6th month, animals whose dams were subjected to unpredictable chronic stress during the 1st or 3rd gestational week left the enclosed arm faster than control animals, an effect that could be interpreted as a reduction in generalized anxiety. The same was observed in another study conducted in our laboratory (data to be published), which showed that when dams were subjected to a predictable chronic stressor (social separation) during the 1st or 2nd gestational week, there was impairment of avoidances in the offspring at 6 months of age, indicating that prenatal stress, in general, may lessen GAD in the progeny in more advanced age periods, where there may also be a morpho-functional involution process of the nervous system.

The interpretation of the results from the 6 month old animals (anxiolysis) exposed to prenatal stress during the 1st or 3rd week can lead to two reflections. In the first possibility, prenatal stress may have generated morphofunctional changes that allowed an increase of resistance in the progeny, i.e., when there was a demand during exposure to a test, preceded by prior confinement in one of the open arms of the apparatus (pre-exposure), animals did not interpret the situation as aversive, decreasing the latency for leaving the protected compartment. In fact this aspect has been discussed, justifying the hypothesis that prenatal stress would prepare the offspring to respond to the external world in a more adaptive way (Del Giudice, 2012). A second possibility would be exactly the reverse. The animals may have lost their protective mechanisms, showing not decreased anxiety, but increased impulsivity (Soubré, 1986). This would be very serious for their survival, resulting in high risk of exposure to predators or to other dangerous situations.

The current study using the ETM enabled an analysis of differential types of anxiety presented by male offspring. Similar investigations have already been conducted, however they were limited to assessing the effect of prenatal stress using uncontrollable electric foot shock sessions every other day of gestation, which led to an increase in generalized anxiety disorder in the offspring tested at 80 days old (Estanislau and Morato, 2005). Even with methodological differences, the results are in agreement with the present study, where as a consequence of prenatal stress we found increased generalized anxiety in animals with similar age (3 months), however, with unpredictable stressors applied only during the last gestational week. In that study (Estanislau and Morato, 2005), progeny were not assessed in the ETM in other stages of life, so it is not possible to make a comparison with our results for 1 and 6 month old animals.

At this point, one may ask whether the possible prejudice in maternal behavior of stressed dams had, by itself, changed the manifestation of anxiety in progeny throughout the different stages of development analyzed. Cross-fostering studies, where pups of stressed dams were raised by control dams showed reversal of some long-term behavioral and neurobiological effects caused by prenatal stress (Del Cerro et al., 2010; Maccari et al., 1995). However, these findings are complicated by the effects of cross-fostering itself, which can be characterized as a stressor, both for the offspring and mothers. The procedure of cross-fostering only provides reliable information if the quality and quantity of attention is the same for prenatally stressed and controls pups. This clearly has not been found in several studies (Weinstock, 2008). It was shown in rats, for example, that prenatally stressed male pups were less licked by their own mothers and by foster control mothers (Moore and Power, 1986; Power and Moore, 1986), while in mice, pups from control or stressed mothers, when fostered, received less attention than those kept with their biological mothers (Meek et al., 2001). The reasons for these results are not clear, but may depend, once again, on the strain used, the duration and severity of stress and the method used to assess maternal behavior (Weinstock, 2008).

One explanation for all these evidences is that the stressors trigger

the activity of the mother's HPA axis, culminating in the release of glucocorticoids. When these hormones reach the embryo/fetus, they have several actions on structures that regulate emotional tonus, such as the serotonergic system, modulating, for example, the expression of serotonergic receptors that have a key role in GAD, PD and stress resistance (for review, see Andrade et al., 2013; Chalmers et al., 1993; Deakin and Graeff, 1991; Graeff et al., 1996; Lanfumey et al., 2008). In addition, prenatal stress may alter the activity of the progeny's HPA axis (Abe et al., 2007; García-Cáceres et al., 2010; Green et al., 2011; Louvart et al., 2009; Wilson et al., 2013), which may by itself lead to anxiety disorders (Faravelli et al., 2012). Moreover, it is important to say that serotonin also plays a role in the modulation of the construction and plasticity of brain circuits during development, affecting neurogenesis, migration and initial axon targeting (Bonnin et al., 2007; Gaspar et al., 2003). These events can be negatively affected by the unbalance of serotonergic availability, altering adult brain function, and predisposing the individual to the manifestation of anxiety, for example (Bonnin et al., 2007). All these findings reinforce the relationship between the HPA axis and the serotonergic system (see more in Lanfumey et al., 2008), showing that they are closely related in the genesis of anxiety disorders and how prenatal stress, through glucocorticoid action, can affect the manifestation of these disorders during postnatal life.

5. Conclusions

Together, these data show that prenatal stress effects are not static and that not only the gestational period in which the aversive experience occurs must be taken into account, but also the age of the evaluated progeny, since the type and the intensity of behaviors related to anxiety may vary with the developmental stage.

Author contributions

TA suggested the idea; TA and FS designed the study; FS, RC, VK, MB, AA and TA conducted the experiment; TA and FS analyzed the results, wrote the whole manuscript and conducted the statistical analysis; TA, FS and MB edited the manuscript.

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