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Effects of early life interventions and palatable diet on anxiety and on oxidative stress in young rats

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ABSTRACT

Early life events can change biochemical, endocrine and behavioral aspects throughout the life of an animal. Since there is a strong relationship between stress, neonatal handling and feeding behavior, the aim of this study was to investigate the effects of these three factors on behavioral parameters (anxiety and locomotion), oxidative stress in brain structures (prefrontal cortex and hippocampus) and on plasma glucose. Nests of Wistar rats were handled (10 min/day), or not (control groups), on days 1–10 after birth. Males from these groups were divided into 4 subgroups: 1) stressed by isolation in childhood (pre-puberty) and with access to a highly palatable diet 2) stressed by isolation and receiving standard lab chow 3) not isolated and receiving a highly palatable diet and 4) not isolated and receiving standard chow. The animals were kept under these conditions for 7 days. Rats receiving the highly palatable diet consumed more food, more calories, gained more weight and had a higher plasma glucose level, but had a lower caloric efficiency than the standard chow groups. Both handling and palatable diet were able to increase food consumption on the first day of isolation. Isolation stress had an anxiogenic effect in the plus maze, which was counteracted by handling. Palatable diet increased time spent in the central area of the open field apparatus and in the open arms of the elevated plus maze, showing an anxiolytic effect. The use of both these conditions, however, does not appear to bring additional protection against the effects of stress during this particular period of life, i.e. pre-puberty. In the prefrontal cortex, handling reduced thiol content and appears to imbalance the antioxidant enzymes system, which is counteracted by a palatable diet. Hippocampus seems to be more sensitive than the prefrontal cortex to early interventions, especially to the highly palatable diet, and both handling and diet appear to imbalance the antioxidant enzyme system. Thus, measurements of antioxidant enzymes activities indicate that handling may endanger some brain structures and that the palatable diet was able to prevent some handling effects on antioxidant enzymes, depending on the brain structure.

1. Introduction

There are critical periods in the development of offspring in which several body systems are not yet mature [1]. Interventions in the neonatal period may influence the mother's relationship with the pups, affecting the development of the offspring nervous system and modifying biochemical, endocrine and behavioral aspects throughout the animal's life [2–5]. Experimental models have been developed to study the effects of interventions in early life and its consequences. One such model is the neonatal handling model; a brief, repeated and seemingly harmless separation of pups from their mothers. As adults, handled pups have less fear in new environments, an increased intake of palatable foods [6] and a smaller increase in the secretion of glucocorticoids by the adrenal gland in response to stressors, when compared to non-handled animals [7].

The social environment is a source of stress, both for humans and for rodents, especially during puberty. Nevertheless, many studies in rodents have shown that isolation for a period of time that includes puberty has an impact on behavior, emotionality and stress reactivity in adults [8–10]. In the natural environment, rodents live in groups and exhibit high levels of social behavior, both with younger and older animals [11]. Social interactions are rewarding to them [12], while social isolation is an aversive event and increases the activity of the HPA axis [8,10].

After exposure to stressful events, there are increased levels of circulating glucocorticoids. If this increase persists for a long time (e.g., chronic stressors), deleterious effects begin to appear, which are especially damaging to the nervous system. These deleterious effects have been reported to increase the generation of reactive oxygen

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species (ROS) [13]. Although ROS participate in normal physiological processes, when in excess they can cause oxidative damage to biomolecules and cellular structures. If, in addition to the increased production of ROS, there is also a decrease in antioxidant defenses, a state of oxidative stress develops, which is involved in the pathogenesis of many diseases, including those caused by everyday stress [14]. The brain is especially vulnerable to free radicals and many studies have demonstrated their effects on the hippocampus and prefrontal cortex [15–17]. Furthermore, the hippocampus and the prefrontal cortex are deeply involved in the stress response [18,19], being particularly susceptible to interventions during puberty [20].

Feeding behavior involves complex mechanisms that include caloric demand of the body as well as hedonic and cognitive aspects [21–24]. Moreover, it can be changed by different factors such as nutrient availability and stress [25]. The hormones released in response to stress may affect appetite in different ways. Norepinephrine and corticotropin-releasing hormone (CRH) have been reported as appetite suppressants facing stress [26], whereas cortisol stimulates the appetite during recovery from stress [27]. The increased influence of stress on daily life has been associated with a higher motivation for foods rich in lipids and carbohydrates [28,29]. However, excessive sugar intake, leading to an elevated level of plasmatic glucose, has been associated with free radical production [30].

Since there is a strong relationship between stress, neonatal handling and feeding behavior, the aim of this study was to investigate the effects of these three factors on behavioral parameters (anxiety and locomotion) and oxidative stress in the hippocampus and prefrontal cortex. In addition, we measured a metabolic parameter (plasma glucose) in young Wistar rats, which were neonatally handled and stressed by isolation in childhood (pre-puberty), and had access to a highly palatable diet concomitant to stress. Our hypothesis was that neonatal handling and the access to a palatable diet could protect these animals against consequences of stress exposure.

2. Materials and methods

2.1. Subjects

All animal procedures were approved by the Institutional Ethical Committee and followed the recommendations of the International Council for Laboratory Animal Science (ICLAS) and of the Federation of Brazilian Societies for Experimental Biology. All efforts were done to minimize animal suffering, as well as to reduce the number of animals needed for this study.

Fourteen pregnant Wistar rats bred at our own animal facility were randomly selected on gestational day 18, and housed alone in home cages made of Plexiglas (65×25×15 cm) with the floor covered with sawdust and were maintained in a controlled environment: lights on between 07:00 h and 19:00 h, temperature of 22±2 °C, cage cleaning twice a week, food and water provided *ad libitum*. The day of birth was considered day 0 and the litters were culled in 8 pups within 24 h.

The litters were divided into two groups: handled and non-handled. The neonatal handling occurred between days 1–10 after birth, between 11:00 h and 14:00 h, for 10 min/day [6]. Once during this period, dirty sawdust was carefully removed from one side of the cage, without disturbing the mother and the nest, and replaced by clean sawdust at that side by the main researcher.

Litters were weaned on postnatal day 21. Only the male offspring were used in this study. Male pups were weighed and distributed into four groups, in such a way that only one animal per litter was used in each group, with 5–8 animals/group. The following groups were used: (1) receiving standard lab chow, and stressed by isolation (one animal in a smaller home cage, 27×17×12 cm) [8]; (2) receiving a highly palatable diet [30], and stressed by isolation; (3) receiving standard lab chow, and not isolated; and (4) receiving a highly palatable diet, and not isolated. These interventions occurred between postnatal days 21–28 and the daily food consumption was measured [31].

At postnatal day 28, the food was removed from the cages at the beginning of light cycle. Two hours later, two behavioral tests were performed consecutively: Open Field [32] and Plus Maze [33]. Since the open field exposure has no impact on the animal's subsequent behavior, at least for some tasks [34–36], this test was performed before the plus maze experiment. Both tests were recorded and analyzed by computer programs.

After 8 h of fasting, the animals were weighed again and killed by decapitation. Trunk blood was collected and the brain was removed, dissected (prefrontal cortex and hippocampus) and frozen at −70 °C for further analysis. Adrenal glands were also dissected and weighed.

2.2. Neonatal handling

Pups of the handled group were gently removed from the nest and placed in an incubator at 32 °C. The cages with the mothers remained in the same room and, after 10 min, pups were returned to their home cages. The researcher changed gloves for the manipulation of each litter to avoid the spread of any kind of odor from nest to nest. Pups of non-handled groups were kept with their mothers without interventions until weaning.

2.3. Highly palatable diet

The highly palatable diet used in this study is enriched with simple carbohydrates, and made from condensed milk, sucrose, vitamins and salts, powdered standard lab chow, purified soy protein, soy oil, water, methionine and lysine. The nutritional content of this diet is similar to that of a standard lab chow (including 22% protein and 4–6% fat), however most carbohydrates in the palatable diet were sucrose (from condensed milk and from sucrose). In contrast, the standard lab chow was composed of carbohydrates that were mainly from starch. The palatable diet was made on postnatal day 20 and the pellets were daily switched.

2.4. Food consumption and caloric efficiency

Previously weighed amounts of standard lab chow and highly palatable diet were offered and the remaining amounts of pellets were measured each day to evaluate consumption. The food consumption was measured per cage and, in the control cages, the amount of food consumed was divided by the number of animals per cage to determine mean consumption per animal.

To verify how much weight gain was due to food consumption, we calculated the caloric efficiency, dividing the weight gain in milligrams by the total amount of kilocalories consumed in the period. To verify the amount of kilocalories consumed per animal, we multiplied the amount of food ingested by the caloric content per gram of chow or diet. The standard lab chow has a caloric content of 3.24 kcal/g, whereas the highly palatable diet has a caloric content of 4.5 kcal/g (being 38% more caloric than the standard chow).

2.5. Behavioral tests

2.5.1. Open Field Test

The open field consisted of an open wooden arena (60×40 cm) with 12 equally divided squares measuring 15×13.3 cm. Fifty-centimeter high walls bordered the field. The frontal wall was made of glass, which allowed the observation of the animal by the researcher. The behavioral test was conducted in an observational room using red light illumination. The animals were observed for 5 min and the locomotion activity (number of line crossings), rearing (standing upright on the hind legs) and time of grooming were analyzed. After the

observational time, the animals were placed in the Plus Maze apparatus.

2.5.2. Plus Maze

The elevated plus maze apparatus was made of wood and consisted of two opposing open arms (48.5 × 10 cm), two opposing enclosed arms with no roof (48.5 × 9.5 × 49 cm), and an open square (13 × 10 cm) in the center. The maze was elevated 50 cm above the floor. The behavioral test was conducted in the same observational room using red light illumination. The animal was placed in the center of the plus maze, facing one of the open arms, and remained in the apparatus for 5 min. We analyzed the number of entries and the time spent in the open or enclosed arms, the frequency of head dipping, the frequency of rearings and time of grooming.

2.6. Biochemical analysis

For determination of antioxidant enzyme activities in the prefrontal cortex and hippocampus, each brain structure was homogenized in 10 volume (w:v) ice-cold 50 mM potassium phosphate buffer (pH 7.4), containing 1 mM EDTA. The homogenate was centrifuged at 960 ×*g* for 10 min at 4 °C and the supernatant was analyzed. For determination of plasma glucose, the trunk blood was collected into tubes with EDTA and centrifuged at 960 ×*g* at 4 °C for 10 min. The plasma was separated and frozen at −70 °C for further analysis.

2.6.1. Superoxide dismutase activity

SOD activity was determined using a RANSOD kit (Randox Labs., USA). This method employs xanthine and xanthine oxidase to generate superoxide radicals that react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride to form a formazan dye that is assayed spectrophotometrically at 492 nm at 37 °C. The inhibition of the production of the chromogen is proportional to the activity of SOD present in the sample [37]. SOD activity was expressed as U per mg of protein. One unit of SOD was defined as the amount that caused 50% inhibition of the rate of reduction of INT, under the conditions of the assay.

2.6.2. Glutathione peroxidase activity

GPx activity was determined according to Wendel [38], with modifications [39]. The reaction was carried out at 37 °C in a solution containing 20 mM potassium phosphate buffer (pH 7.7), 1.1 mM EDTA, 0.44 mM sodium azide, 0.5 mM NADPH, 2 mM glutathione and 0.4 U glutathione reductase. The activity of GPx was measured taking tert-butylhydroperoxide as the substrate at 340 nm. The contribution of spontaneous NADPH oxidation was always subtracted from the overall reaction ratio. GPx activity was expressed as nmol NADPH oxidized per minute per mg protein.

2.6.3. Catalase activity

CAT is an enzyme that degrades hydrogen peroxide (H_2O_2), and its activity assessment is based upon establishing the rate of H_2O_2 degradation spectrophotometrically at 240 nm at 25 °C [40]. CAT activity was calculated in terms of μmol of H_2O_2 consumed per minute per mg of protein, using a molar extinction coefficient of 43.6 M^{-1} cm^{-1}.

2.6.4. Total thiol content

Total thiol content was determined spectrophotometrically based on the reduction of 5,5 '-dithiobis-2-nitrobenzoic acid (DTNB) by thiol groups, which become oxidized (disulfide), yielding a yellow compound (TNB), whose absorption is measured at 412 nm [41].

2.6.5. Protein assay

The total protein concentrations were determined by the Lowry method with bovine serum albumin as the standard [42], which was used to normalize previous analyses.

2.6.6. Plasma levels of glucose

Plasma glucose was measured using a commercial kit from Wiener, Rosario, Argentina [31].

2.7. Statistical analysis

Statistical analyses were performed by repeated measures ANOVA (the within subjects factor was time; between subjects factors were handling, stress and diet) for food consumption. A three-way ANOVA was also performed, with handling, diet and stress as factors. Data are expressed as mean ± standard error of mean and significance was given by $p<0.05$.

3. Results

3.1. Body and adrenal gland weight

The body weight of all animals increased with time [$F(1,46) = 1609.23$, $p<0.005$] (data not shown). On day 21, no difference was found between groups, but on day 28, groups receiving the palatable diet had higher body weights, compared to groups receiving chow [$F(1,46) = 10.05$, $p<0.005$]. Weight gain was also increased by palatable diet [$F(1,46) = 26.19$, $p<0.005$] and there was an interaction between handling and diet [$F(1,46) = 4.47$, $p<0.05$], since handling increased weight gain and the diet's effect was less evident (Fig. 1). There was no difference regarding adrenal gland weight (data not shown).

3.2. Food consumption and caloric efficiency

Over the seven days, the consumption of all groups increased with time [$F(1,20) = 25.93$, $p<0.001$] (data not shown), and groups receiving the highly palatable diet had a higher consumption than the groups receiving the standard lab chow [$F(1,26) = 17.79$, $p<0.0005$] (Fig. 2). Since the consumption data from isolated animals is more precise and has more power (n) than controls, and since the first day of isolation has a special impact on the animals, we analyzed the consumption during the first day of isolation, considering only isolated animals. We observed a positive effect of neonatal handling [two-way ANOVA, $F(1,22) = 5.03$, $p<0.05$] in addition to the diet's positive effect [$F(1,22) = 6.7$, $p<0.05$], since handling increased consumption when compared to non-handled animals (Fig. 3). Despite the increase in weight gain and in caloric intake (data not shown), groups receiving the highly palatable diet had a lower caloric efficiency [$F(1,46) = 32.9$, $p<0.001$] (Fig. 4).

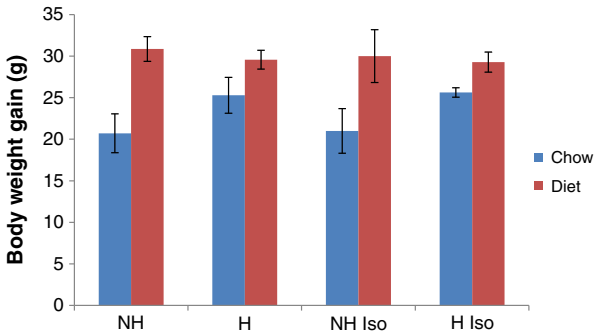

Fig. 1. Body weight gain of non-handled (NH), handled (H) and stressed (Iso) animals receiving standard chow or palatable diet. Data expressed as means ± S.E.M. Three-way ANOVA showed a significant effect of diet ($p<0.005$) and an interaction between handling and diet ($p<0.05$).

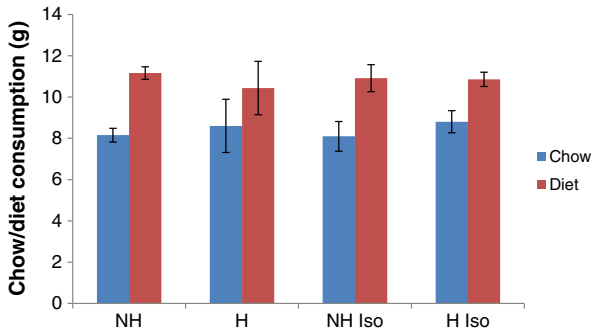

Fig. 2. Average consumption during seven days of non-handled (NH), handled (H) and stressed (Iso) animals receiving standard chow or palatable diet. Data expressed as means ± S.E.M. Three-way ANOVA showed a significant effect of diet ($p<0.0005$).

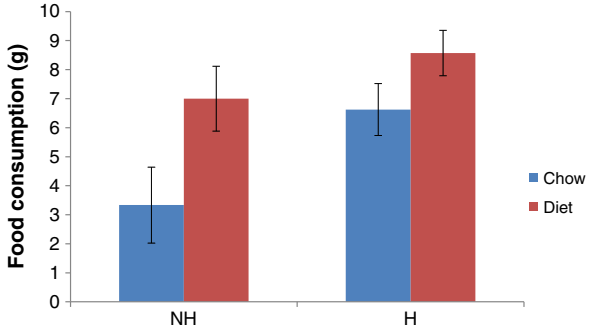

Fig. 3. First day consumption of standard chow or palatable diet of non-handled (NH) and handled (H) isolated animals. Data expressed as means ± S.E.M. Two-way ANOVA showed a significant effect of handling ($p<0.05$) in addition to the diet effect ($p<0.05$).

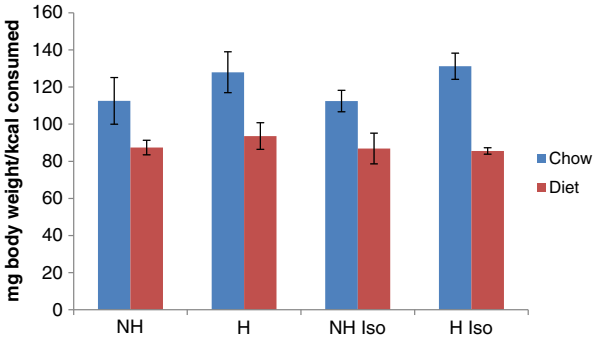

Fig. 4. Caloric efficiency of non-handled (NH), handled (H) and stressed (Iso) animals receiving standard chow or palatable diet. Data expressed as means ± S.E.M. Three-way ANOVA showed a significant effect of diet ($p<0.001$).

3.3. Behavioral tests

3.3.1. Open Field

Time in the central squares of the Open Field was increased by exposure to the palatable diet [$F(1,46)=6.568$, $p<0.05$], but no difference was found in total crossings, crossings in the peripheral area, time for the first crossing, frequency of rearings and time of grooming (Table 1).

3.3.2. Plus Maze

Time in the open arms of the Plus Maze was decreased by stress [$F(1,38)=5.224$, $p<0.05$], increased by palatable diet [$F(1,38)=6.589$, $p<0.05$] and there was an interaction between handling and stress [$F(1,38)=5.816$, $p<0.05$], with handling reducing the effect of stress. Stress also decreased the frequency of head dipping [$F(1,46)=12.362$, $p<0.001$]. Time of grooming was increased by stress [$F(1,46)=9.089$, $p<0.005$] and decreased by handling [$F(1,46)=5.001$, $p<0.05$]. No difference was found in the frequency of rearings (Table 2).

3.4. Biochemical measurements

3.4.1. Prefrontal cortex

An interaction was found between handling and diet on SOD activity [$F(1,36)=8.382$, $p<0.01$] and on SOD/GPx ratio [$F(1,34)=11.637$, $p<0.005$], since handling only increased enzyme activity in chow groups, and not in groups receiving the palatable diet (Fig. 5a and Table 3). CAT activity was increased by diet [$F(1,43)=4.481$, $p<0.05$] (Fig. 5b). For GPx activity, we found an interaction between stress and diet, since stress only increased the activity in the chow groups [$F(1,37)=4.627$, $p<0.05$] (Fig. 5c). Total thiol content was decreased by handling [$F(1,31)=5.004$, $p<0.05$] and increased by diet [$F(1,31)=8.995$, $p<0.001$], and no difference was found in the SOD/CAT ratio (Table 3).

3.4.2. Hippocampus

SOD activity was increased by handling [$F(1,39)=8.366$, $p<0.01$] and decreased by diet [$F(1,39)=8.673$, $p<0.01$] (Fig. 6a). For CAT activity, we found an interaction between handling and diet [$F(1,36)=9.143$, $p<0.005$] and another between the three variables [$F(1,36)=7.496$, $p<0.01$] (Fig. 6b). Diet decreased GPx activity [$F(1,37)=9.251$, $p<0.005$] and SOD/CAT ratio [$F(1,34)=4.93$, $p<0.05$], and in both there were interactions between handling and diet [$F(1,37)=6.432$, $p<0.05$ and $F(1,34)=4.27$, $p<0.05$, respectively], with handling accentuating the diet's decreased effect (Fig. 6c and Table 4). SOD/GPx ratio was increased by handling [$F(1,34)=9.33$, $p<0.05$] and no difference was found in total thiol content (Table 4).

3.4.3. Plasma glucose

Glucose level was increased by diet [$F(1,37)=12.81$, $p<0.001$] (data not shown).

Table 1
Effect of neonatal handling, isolation and palatable diet on behavior in the Open Field.

Groups			Time in central area	Total crossings	Crossings in peripheral area	Time for first crossing	Frequency of rearings	Time of grooming
Non-handling	Standard chow	Non-stress	5.35 ± 0.62	112.71 ± 11.43	103.57 ± 10.80	3.08 ± 0.68	42.00 ± 6.85	10.15 ± 1.56
		Stress	3.82 ± 0.78	97.67 ± 6.87	89.00 ± 6.22	6.35 ± 2.40	44.17 ± 4.20	6.66 ± 0.96
	Palatable diet	Non-stress	7.43 ± 1.39	113.14 ± 6.32	101.71 ± 5.52	5.70 ± 1.57	45.14 ± 4.37	7.12 ± 1.91
		Stress	7.73 ± 1.14	117.60 ± 6.38	106.40 ± 6.99	6.02 ± 0.88	48.80 ± 7.27	6.45 ± 2.52
Handling	Standard chow	Non-stress	5.88 ± 1.33	96.71 ± 9.93	87.14 ± 9.23	8.80 ± 1.49	53.43 ± 4.85	7.01 ± 2.71
		Stress	4.8 ± 0.95	119.50 ± 5.52	109.00 ± 5.68	5.56 ± 0.86	54.88 ± 2.97	6.68 ± 2.07
	Palatable diet	Non-stress	7.02 ± 1.19	112.71 ± 7.69	100.57 ± 6.35	6.91 ± 1.77	50.57 ± 4.43	4.80 ± 1.51
		Stress	5.51 ± 0.85	114.71 ± 5.39	102.29 ± 4.96	8.37 ± 2.63	49.57 ± 5.34	6.35 ± 1.64

Data are expressed as mean ± S.E.M. Three-way ANOVA showed a significant effect of diet ($p<0.05$) on time in central area. No difference was found in total crossings, crossings in the peripheral squares, time for the first crossing, frequency of rearings and time of grooming ($p>0.05$).

Table 2
Effect of neonatal handling, isolation and palatable diet on behavior in the Plus Maze.

Groups			Time in open arms	Frequency of head dipping	Frequency of rearings	Time of grooming
Non-handling	Standard chow	Non-stress	73.83 ± 20.03	9.71 ± 3.50	15.57 ± 2.96	19.61 ± 5.62
		Stress	38.82 ± 13.91	5.67 ± 2.59	15.50 ± 3.63	19.25 ± 6.62
	Palatable diet	Non-stress	117.93 ± 19.9	18.00 ± 4.33	14.14 ± 1.40	8.59 ± 3.49
		Stress	54.94 ± 21.61	4.00 ± 1.64	18.00 ± 3.29	33.12 ± 11.05
Handling	Standard chow	Non-stress	59.36 ± 17.34	14.29 ± 2.66	17.14 ± 2.58	5.53 ± 2.75
		Stress	68.11 ± 12.01	8.63 ± 2.00	22.88 ± 2.46	10.61 ± 4.63
	Palatable diet	Non-stress	116.24 ± 18.16	18.14 ± 4.13	18.57 ± 3.20	9.63 ± 2.26
		Stress	96.93 ± 10.47	10.43 ± 2.43	16.86 ± 1.67	12.07 ± 2.83

Data are expressed as means ± S.E.M. Three-way ANOVA showed a significant effect of stress ($p<0.05$), diet ($p<0.05$) and an interaction between handling and stress ($p<0.05$) on time in open arms. An effect of stress was found on the frequency of head dipping ($p<0.001$), and time spent on grooming ($p<0.005$). An effect of handling on time of grooming was also observed ($p<0.05$). No difference was found in frequency of rearings ($p>0.05$).

4. Discussion

Juvenile animals receiving a highly palatable diet showed increased caloric intake and gained more weight at the end of the week. However, their caloric efficiency was lower than that of groups receiving standard lab chow. These results suggest that, even though animals were consuming more calories, groups receiving this diet did not gain as much weight as expected. Therefore, this excess of calories was possibly used in other processes, such as increased basal metabolism. Another possibility may be a different intestinal absorption in these animals, an observation that may be worthy of future studies. Another study by our group [31], using adult animals receiving chocolate (rich in fat and sugar), did not observe any reduced caloric efficiency, and this difference could be related to the age of the animals (in the present study we used animals in the pre-puberty phase). The type of palatable food used may also be important, as well as the fact that the stress in the previous study was chronic (50 days), and it is known that chronically stressed animals do not show the same behavior and do not experience the consequences that animals exposed to acute or sub acute stress do [25,43–45]. Furthermore, we observed that, when animals are isolated, they show a reduced consumption during the first day, which increases over time. On the first day of isolation, in addition to the effect of the palatable diet (with higher consumption), a positive handling effect appears, since handled animals showed a different pattern of consumption. It is possible that these animals are less susceptible to isolation stress consequences; however, it is important to remember that stress may, in some cases, increase food consumption [25,46]. Thus, it is also possible that handled animals could increase food consumption in response to stress, when compared to non-handled animals. Reports from the literature show reduced stress responses in handled adults [7,47], but it is important to consider that the present results were observed before puberty.

Both the elevated plus-maze and open field tasks have been used to assess neurobehavioral profiles of animals under the influence of anxiogenic/anxiolytic agents [36,48]. In the present study, the open-field task was used to assess locomotion capacity and anxiety-like behavior. Results suggest that the access to a highly palatable diet during the pre-puberty period had an anxiolytic effect (since it increased time spent in central squares of the open-field apparatus), which was also observed in the plus maze apparatus (increasing time spent in open arms), while isolation was anxiogenic in the plus maze. Therefore, this diet appears to have the properties of "comfort food", as postulated in the literature [49,50]. These results are in agreement with the hypothesis that reward-based eating may decrease the response to stress when animals have access to a palatable diet [21,49]. In addition, the highly palatable diet had no influence on crossings and rearings in the open field, which is consistent with Souza [30].

It is well established that neonatally handled rats exhibit decreased stress responses in adulthood [3,5,51] when facing both acute [52] and chronic stress situations [51,53]. When adults, handled rats show less fear in novel environments, a greater exploratory

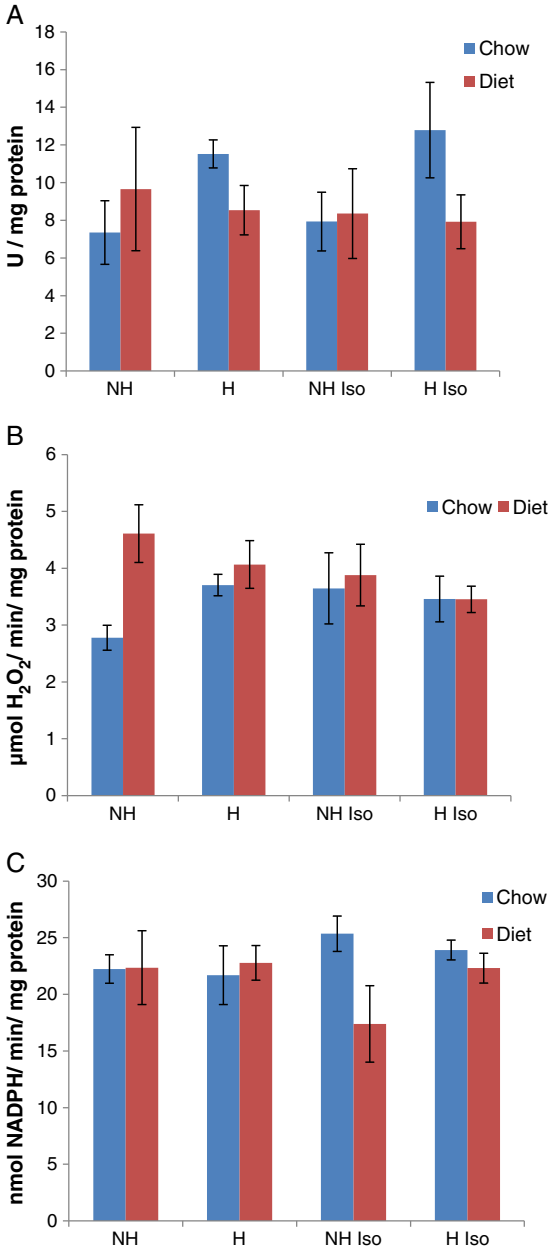

Fig. 5. Antioxidant enzyme activities in the prefrontal cortex of non-handled (NH), handled (H) and stressed (Iso) animals receiving standard chow or palatable diet. (a) SOD (U/ mg protein), (b) CAT (µmol H_2O_2 consumed/ min/ mg protein) and (c) GPx (nmol NADPH oxidized/ min/ mg protein). Data expressed as means ± S.E.M. Three-way ANOVA showed an interaction between handling and diet on SOD activity ($p<0.01$), a diet effect on CAT activity ($p<0.05$) and an interaction between stress and diet on GPx activity ($p<0.05$).

Table 3
Effect of neonatal handling, isolation and palatable diet on biochemical measures in prefrontal cortex.

Groups			SOD/CAT	SOD/GPx	Thiol
Non-handling	Standard chow	Non-stress	2.54 ± 0.46	0.32 ± 0.06	60.05 ± 2.38
		Stress	2.57 ± 0.66	0.31 ± 0.05	64.33 ± 3.53
	Palatable diet	Non-stress	2.04 ± 0.56	0.36 ± 0.09	68.87 ± 8.45
		Stress	2.82 ± 1.26	0.46 ± 0.08	66.27 ± 10.12
Handling	Standard chow	Non-stress	3.14 ± 0.22	0.59 ± 0.09	58.56 ± 3.50
		Stress	3.76 ± 0.68	0.53 ± 0.09	60.12 ± 3.25
	Palatable diet	Non-stress	2.33 ± 0.56	0.35 ± 0.05	65.58 ± 2.87
		Stress	2.26 ± 0.34	0.34 ± 0.05	58.01 ± 3.31

Data are expressed as means ± S.E.M of SOD/CAT, SOD/GPx and total thiol content (nmol TNB/mg de protein). Three-way ANOVA showed an interaction between handling and diet on SOD/GPx ratio ($p<0.005$), no difference in SOD/CAT ratio ($p>0.05$) and a handling and a diet effect on total thiol content ($p<0.05$ and $p<0.001$, respectively).

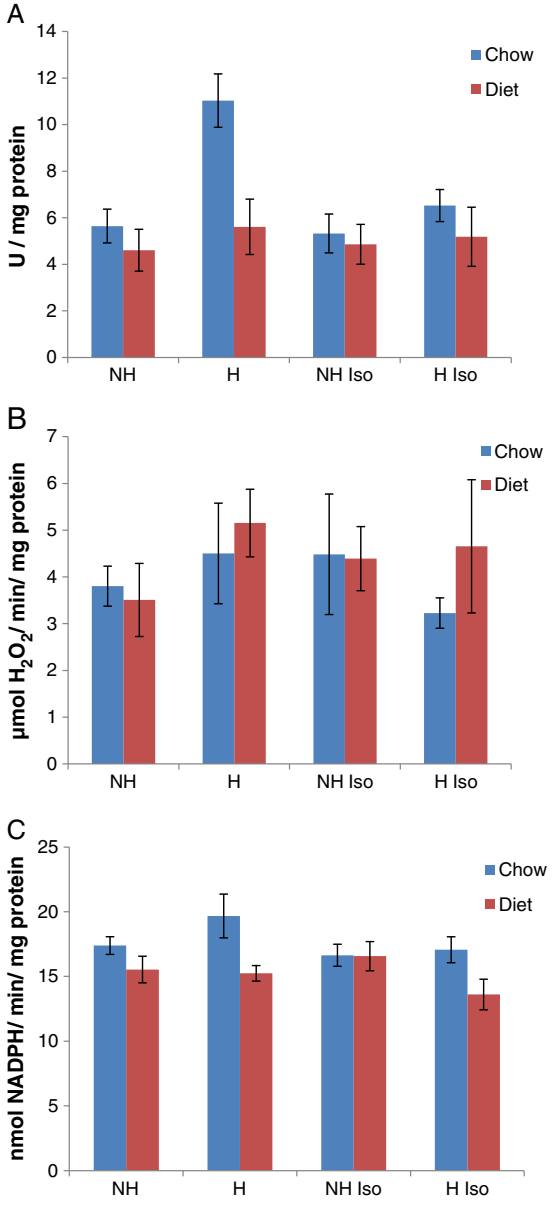

Fig. 6. Antioxidant enzyme activities in the hippocampus of non-handled (NH), handled (H) and stressed (Iso) animals receiving standard chow or palatable diet. (a) SOD (U/ mg protein), (b) CAT (µmol H_2O_2 consumed/ min/ mg protein) and (c) GPx (nmol NADPH oxidized/ min/ mg protein). Data expressed as means ± S.E.M. Three-way ANOVA showed a significant effect of handling and diet on SOD activity (both $p<0.01$), an interaction between handling and diet and another interaction between the three variables on CAT activity ($p<0.005$ and $p<0.01$, respectively), a diet effect and an interaction between handling and diet on GPx activity ($p<0.005$ and $p<0.05$ respectively).

behavior and lower anxiety [42,54–56]. In the present study, handling partially prevented stress-induced anxiogenic effects in the plus-maze apparatus, thus adding further support to the suggested protective effect of handling on some adverse environmental effects.

Evidence points to a relationship between anxiety-like behavior and oxidative stress [57–60], and some studies have suggested that psychosocial stress may lead to alterations in some cellular processes, which may cause oxidative stress [61–63]. The brain is especially vulnerable to free radical production and to oxidative damage because of its high oxygen consumption, abundant lipid content and a relative paucity of antioxidant enzymes [64–66]. On the other hand, there is also evidence, in rodents, showing that administration of high fat or high caloric diets increases free radical generation in the brain [53]. In this study, we analyzed antioxidant enzyme activities (SOD, CAT and GPx) in the hippocampus and prefrontal cortex of juvenile rats. The ROS scavenging activity of SOD is effective only when it is followed by the actions of CAT and GPx, because the dismutase activity of SOD generates hydrogen peroxide from the superoxide ion, which requires further scavenging by CAT and GPx [67]. An excess of H_2O_2 facilitates the production of hydroxyl radical ($OH^{\bullet}$), the most powerful oxidant molecule, through a reaction with iron or copper (Fenton chemistry) [68]. Therefore, it has been hypothesized that an imbalance in the SOD/CAT and SOD/GPx ratios might be responsible for oxidative alterations in the brain [69].

In the prefrontal cortex, there was an interaction between handling and diet, with handling increasing SOD activity and SOD/GPx ratio only in the chow groups, and not in the palatable diet groups. This interaction did not occur for CAT and GPx activities, possibly unbalancing the antioxidant enzyme system, which could lead to an increase in H_2O_2 production. In addition, the palatable diet increased CAT activity, suggesting efficient degradation of H_2O_2. Thus, it is possible that the palatable diet prevents the imbalance of antioxidant enzymes that is caused by handling. Handling itself decreased thiol content (measured both in protein and non-protein thiol, mainly represented by the reduced form of glutathione). Therefore, a reduction in total thiol content may indicate possible protein damage or reduction in reduced glutathione [70–72]. On the other hand, the palatable diet increased the thiol content, further suggesting a beneficial role for this diet in this structure.

In the hippocampus, handled groups also presented an increase in SOD activity and SOD/GPx ratio, while no influence on CAT and GPx activities was observed, possibly leading to an increase in H_2O_2 production. The palatable diet was capable of decreasing the SOD and SOD/CAT ratio, besides reducing GPx activity. A reduction in antioxidant enzymatic defense could endanger the cell; however, as we observed no effect on thiol content, other studies are warranted to evaluate other parameters of oxidant damage. Thus, the hippocampus seems to be more sensitive to early interventions, especially to this highly palatable diet. When palatable diet groups were also handled, there was an even greater decrease in GPx activity and SOD/CAT ratio. Although these interventions were able to increase CAT activity,

Table 4
Effect of neonatal handling, isolation and palatable diet on biochemical measures in hippocampus.

Groups			SOD/CAT	SOD/GPx	Thiol
Non-handling	Standard chow	Non-stress	2.05 ± 0.22	0.42 ± 0.03	52.69 ± 2.98
		Stress	2.17 ± 0.42	0.44 ± 0.04	52.57 ± 4.81
	Palatable diet	Non-stress	2.07 ± 0.48	0.36 ± 0.02	52.83 ± 4.17
		Stress	1.73 ± 0.52	0.38 ± 0.03	52.32 ± 3.61
Handling	Standard chow	Non-stress	2.81 ± 0.35	0.57 ± 0.05	52.63 ± 4.56
		Stress	2.78 ± 0.38	0.49 ± 0.05	53.25 ± 4.96
	Palatable diet	Non-stress	1.49 ± 0.18	0.46 ± 0.06	52.18 ± 2.48
		Stress	1.78 ± 0.32	0.47 ± 0.08	50.83 ± 3.53

Data expressed as means ± S.E.M of SOD/CAT, SOD/GPx and total thiol content (nmol TNB/mg de protein). Three-way ANOVA showed a significant effect of diet and an interaction between handling and diet on SOD/CAT ratio (both $p<0.05$), a handling effect on SOD/GPx ratio ($p<0.05$) and no difference in thiol content ($p>0.05$).

which may counteract the decrease in GPx activity, preventing an increase of H_2O_2 concentration, CAT and GPx have a distinct cellular localization [73], and this could represent an imbalance in the antioxidant defenses. Thus, it is not possible to say that the palatable diet prevents the imbalance of antioxidant enzymes caused by handling in this structure, as appears to happen in the prefrontal cortex. A previous study from our group found no difference between handled and non-handled animals in antioxidant enzyme activities [38], but these analyses were made in adult animals, instead of in young animals, as is the case of this study. However, since no measurements of damage to other macromolecules and production of free radicals were made, it is not possible to conclude whether there was a state of oxidative stress.

Glucose is the brain's main substrate of energy, and changes in its extracellular concentration may affect metabolic and behavioral aspects, such as feeding and memory [74–77]. Since food rich in sugar may cause deleterious effects, including changes in metabolic parameters, we also evaluated plasma glucose. Palatable diet increased plasma glucose, and this increase is considered a risk factor for some conditions, such as metabolic syndrome [78]. Another study from our group showed that the metabolic profile, exhibited by handled adult animals, suggests a particular metabolic response concerning energy storage and expenditure when exposed long term to a highly palatable diet [79], and handling has been suggested to protect animals from the potential risks of a hypercaloric diet, which does not appear to occur in young animals. On the other hand, it is possible that handled animals may consume more calories because of a high metabolic demand, compared to controls. These results need further investigation.

In conclusion, both handling during the neonatal period and access to a highly palatable diet were able to reduce the effects of stress on anxiety-like behavior due to isolation. The use of both these conditions, however, does not appear to bring additional protection against the effects of stress in this particular period of life, pre-puberty. Additionally, despite these effects on behavior, measurements of antioxidant enzymes activities indicate that handling may endanger some brain structures, leading to a state of imbalance in the antioxidant defense system, and that the palatable diet is able to prevent some handling effects on antioxidant enzymes, depending on the brain structure. This study also points to the importance of previous life events when studying behavioral and physiological disturbances. Understanding how interventions and the type of diet eaten during development affect brain and behavior may help to elucidate the pathophysiological mechanisms related to eating disorders.

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