UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

Faculdade de Farmácia

Disciplina de Trabalho de Conclusão de Curso de Farmácia

Intervenções precoces e avaliação de parâmetros oxidativos em diferentes estruturas do sistema nervoso de ratas adultas

Danusa Mar Arcego

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Porto Alegre, dezembro de 2010

Quem nada conhece, nada ama. Quem nada pode fazer, nada compreende.

Quem nada compreende, nada vale. Mas quem compreende também ama, observa, vê...

Quanto mais conhecimento houver inerente numa coisa, tanto maior o amor...

Aquele que imagina que todos os frutos amadurecem ao mesmo tempo,

como as cerejas, nada sabe a respeito das uvas.

Paracelso

Agradecimentos

À minha orientadora, Carla, pelo carinho, cuidado, paciência e por contribuir com a minha formação.

À minha co-orientadora, Cristie, pela ajuda, orientação e ensinamentos passados nesses anos de iniciação científica.

À todos os colegas do laboratório 37, pela ajuda, força, carinho e amizade, em especial a Rachel, pela colaboração no desenvolvimento deste trabalho.

Aos meus pais, os maiores incentivadores, por todo o amor e apoio compartilhado, sempre foram e serão o alicerce da minha vida.

Aos amigos, colegas de graduação, e a todos que contribuíram de alguma forma para a realização desse trabalho.

À Universidade Federal do Rio Grande do Sul, Faculdade de Farmácia e ao Departamento de Bioquímica por tornarem possível a minha formação.

Para esses e todos os outros a quem sou grata e não mencionei aqui, deixo meu sincero obrigado pelo seu tempo, conselhos e talento.

EARLY LIFE INTERVENTIONS: EVALUATION OF OXIDATIVE PARAMETERS IN DISTINCT CNS STRUCTURES IN ADULT FEMALE RATS

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Este artigo foi elaborado segundo as normas da revista International Journal of Developmental Neuroscience, apresentadas em anexo.

Abstract

Early life events lead to a large number of behavioral and biochemical alterations in adulthood. The aim of this study is to verify wheter the release of gonadal hormones during puberty affects parameters of oxidative stress observed in adulthood in cerebral cortex, striatum and hypothalamus of female rats subjected to neonatal handling. Rats were exposed or not to neonatal handling (10min/day, first 10 days of life). Between 21-28 post-natal days, females from each litter were divided into the following groups: ovariectomy, sham, and intact (no surgery). When adults, parameters of oxidative stress were analyzed. The groups subjected to surgery (ovx and sham), showed increased production of free radicals by the method of oxidation of dichlorodihydrofluorescein (DCFH) in cerebral cortex and striatum. Decreased catalase activity was observed in the cerebral cortex, in the same groups. No effects of neonatal manipulation were observed in these structures. We conclude that the period after weaning constitute a critical window for stressful interventions during development, leading to alterations in parameters of oxidative stress in adulthood, and these effects are not influenced by estradiol and neonatal handling.

Key-words: neonatal handling, estradiol, oxidative stress, ovariectomy, surgical stress, pre-puberty period.

1. Introduction

Early life events may affect the relationship between mother and pups (Levine, 1993; Levine, 1994), leading to changes in behavioral, neurochemical and neuroendocrine aspects in adult life. Neonatal handling is an experimental model that can be used to evaluate the effects of a short separation of pups from their mother. Rats submitted to this procedure, show many alterations in adult life (Meaney and Aitken, 1985; Meaney et al., 1991; Viau et al., 1993; Meaney et al., 1994; Silveira et al., 2004; Branchi and Alleva, 2006; Kosten et al., 2007; Stamatakis et al., 2008; Daskalakis et al., 2009), and it has been suggested that neonatal handling is a protective factor against aged related impairments (Meaney et al., 1988). However, there are no studies evaluating the influence of gonadal hormones on parameters of oxidative stress in adult neonatally handled rats.

During development and, to some degree throughout life, gonadal hormones, like estradiol, stimulate changes in brain. Estradiol effects include increased size, nuclear volume, dendritic length and branching, density of dendritic spines and, therefore, degree of synaptic connection, as well as synaptic plasticity (Foy, 2010). According to some studies, estrogens may reduce anxiety (Mora et al., 1996; Fernandez and Picazo,1999; Bowman et al., 2002; Walf and Frye, 2010) and depressive behavior (Sherwin, 1994; Walf and Frye, 2010), and also enhance performace in some cognitive tasks, including increased frontal activation during a working memory (Joffe et al., 2006; Smith et al., 2006; Dumas et al., 2010). Moreover, estradiol protects from oxidative stress (Ejima et al., 1999; Borrás et al., 2010; Giddabasappa et al., 2010).

Estrogens have antioxidant properties, what might contribute directly to their neuroprotective effects (Niki and Nakano, 1990; Huh et al., 1994; Lacort et al., 1995; Sawada et al., 1998). These hormones have been shown to modulate tissue redox status

in brain (Kume-Kick and Rice, 1998), and changes in ovarian steroids levels have regionally distinct effects on antioxidants levels (Kume-Kick and Rice, 1998). In this context, gender differences on antioxidants content and antioxidant enzymes activities have been reported (Carrillo et al., 1992; Taskiran et al., 1997).

Free radicals and reactive oxygen species (ROS) are constantly formed in the organism as part of normal and essential biological processes (Halliwell and Cross, 1994). The brain is especially vulnerable to free radicals-induced damage because of its high oxygen consumption, abundant lipid content and relative paucity of antioxidant enzymes (Olanow, 1992; Halliwell and Gutteridge, 2007). Oxidative stress occurs when there is an imbalance between antioxidant defenses and oxidative species, in such a way that the enzymatic antioxidant defenses, such as the enzymes superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx), and the non-enzymatic defenses, are not able to neutralize the reactive species efficiently (Halliwell and Gutteridge, 2007). As a consequence, cellular proteins, lipids and DNA may be damaged (Cochrane, 1991).

In the present study, the effect of deprivation of gonadal hormones before puberty on oxidative stress parameters in the brain of neonatally handled and non-handled adult females is investigated.

2. Material and Methods

2. 1. Experimental subjects

All animal proceedings 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.

Thirty-two pregnant Wistar rats bred at our own animal facility were randomly selected. They were housed alone in home cages made of Plexiglas (65 x 25 x 15 cm) with the floor covered with sawdust and were maintained in a controlled environment: lights on between 07:00 and 19:00 h, temperature of 22 ± 2º C, cage cleaning twice a week, food and water provided *ad libitum*. The day of birth was considered as day 0. All litters were culled within 24 h of birth to eight pups and were maintained undisturbed, except for handling procedures, which were carried out between 10:00 and 15:00 h. Several litters were submitted to the handling procedures in the same day. The researcher changed gloves between the handling procedures of each litter to avoid any kind of odor to be spread from nest to nest. Litters were weaned and separated by sex on postnatal day 21. Rats had free access to food (standard lab rat chow) and water. Between 24-28 post-natal days (PND), females from each litter were divided into the following groups: OVX (subjected to ovariectomy), sham, and intact (no surgery). Only one animal per litter was used per group. When adults (around 4 months-old), biochemical measurements were performed.

2.2. Neonatal Handling

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

Handled group: The dam was gently pulled to one side of the cage and the pups were removed from their home cage and were placed into a clean cage lined with clean paper

towel. This cage was placed into an incubator set to maintain an ambient temperature of 30–32°C. After 10 min, pups were returned to their dams that were in the same room. This procedure was performed from day 1–10 following birth, and then pups were left undisturbed until the 21st day of life (weaning). It was also stated on the cage that these animals should not be touched, not even for cage cleaning. The same procedure of non-handled group was done to change dirty sawdust (Silveira et al., 2006).

2.3. Surgery

Ovariectomy (OVX) was performed between 24 and 28 PND. Rats were anesthetized with 120 mg/kg ketamine HCl (Dopalen: Agribrands, Campinas, SP, Brazil) and 16 mg/kg xylazine (Anasedan: Agribrands, Campinas, SP, Brazil) and bilateral ovariectomy was performed through a single abdominal incision. The abdominal skin was then cut, the peritoneum was opened, both ovarian arteries were linked, and both ovaries were removed. The muscle and the skin were sutured. Sham animals were subjected to surgery, but the ovaries were not removed.

2.4. Preparation of the Samples for Biochemical Measurements

When they were around 4 months old, animals were killed by decapitation and the prefrontal cortex, hypothalamus and striatum were quickly dissected out. The brain structures were stored at -70°C until analysis, when they were homogenized in 10 vol (w:v) ice-cold 50 mM potassium phosphate buffer (pH 7.4), containing 1 mM EDTA. The homogenate was centrifuged (at 3.000 rpm) for 10 min at 4°C and the supernatant was used for the evaluation of free radicals production by the chemical oxidation of dichlorodihydrofluorescein (DCFH), the determination of total thiol content and the evaluation of antioxidant enzymes activity.

2.5. Superoxide Dismutase (SOD) Activity

SOD activity was determined using a RANSOD kit (Randox Labs., USA) which is based on the procedure described by Delmas-Beauvieux et al. (1995). This method employs xanthine and xanthine oxidase to generate superoxide radicals that react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (INT) to form a formazan dye that is assayed spectrophotometrically at 492 nm at 37^{o} C. The inhibition in production of the chromogen is proportional to the activity of SOD present in the sample.

2.6. Catalase (CAT) 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^{o} C (Aebi, 1984). CAT activity was calculated in terms of micromoles of H_2O_2 consumed per minute per mg of protein, using a molar extinction coefficient of 43.6 M^{-1} cm^{-1}.

2.7. Glutathione Peroxidase(GPx) Activity

GPx activity was determined according to Wendel (1981), with modifications. The reaction was carried out at 37^{o} 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.

2.8. Evaluation of free radicals production by the chemical oxidation of DCFH (Lebel et al., 1992)

Samples were incubated with 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA 100μM) at 37 ° C for 30 minutes. DCFH-DA is cleaved by cellular esterases and the

DCFH formed is eventually oxidized by reactive oxygen species (ROS) or reactive nitrogen species (RNS) presented in samples. The formation of the oxidized fluorescent derivative dichlorofluorescein (DCF) was monitored using excitation and emission wavelength of 488 and 525nm, respectively, using a spectrophotometer. The amount of reactive oxygen/nitrogen species was quantified using a DCF standard curve and results were expressed as nmoles of DCF formed per mg of protein.

2.9. Determination of total thiol content

This essay is based in 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 spectrophotometrically at 412 nm (Aksenov and Markesbery, 2001).

2.10. Protein Assay

The total protein concentrations were determined using the method described by Lowry et al. (1951), with bovine serum albumin as standard.

2.11. Statistical Analysis

Data were expressed as means ± SE of the mean, and were analyzed using two-way ANOVA, with neonatal handling and surgery as factors (ANOVA using blocks was used when indicated, to account for variations between distinct groups of rats). Significance level was accepted as different when the P value was equal or less than 0.05.

3. Results

There were no statistical differences between groups on the determination of total thiol in the striatum, hypothalamus and cerebral cortex (Two-way ANOVA, P>0.05) (Fig. 1). A significant effect of surgery during infancy (sham and ovx) was observed on the production of free radicals, evaluated by the method of oxidation of DCFH, when compared with the intact group, both in the cerebral cortex [Two-way ANOVA, $F(2,28)=6.34$, $P=0.005$] (Figure 2A), and striatum [$F(2,27)=6.20$, $P=0.006$] (Figure 2B), showing that surgical intervention at this period of life increased DCF later in adulthood. No differences were found on this measure in the hypothalamus ($P>0.05$, Figure 2C).

Tables 1, 2 and 3 show the antioxidant enzymes activities (SOD, CAT and GPx) in the cerebral cortex, striatum and hypothalamus, respectively. Two-way ANOVA showed no statistical differences between non-handled and handled animals, neither between intact and surgery groups, on these enzymes activities in striatum and hypothalamus (Two-way ANOVA, P>0.05). However, there was a significant effect of surgery (sham and OVX) in the cerebral cortex, decreasing CAT activity when compared to the intact group [ANOVA using blocks, $F(2,21)=3.74$, $P=0.04$].

4. Discussion

The major findings of this study show that early life interventions, such as surgery during the infancy (before 28th PND), affected parameters of oxidative stress in the cerebral cortex and striatum in adult rats. Neonatal handling did not affect the parameters evaluated, neither the absence of estradiol.

In this study, surgery was shown to increase the production of free radicals in the cerebral cortex and striatum. Furthermore, there was a decrease on catalase activity in

the cerebral cortex of rats that underwent surgical procedure in early life, suggesting that pre-puberty events may affect the oxidative balance of these animals in a long term basis. It is important to observe that it was the surgical procedure (possibly the stress of the surgery), and not the ovariectomy, that showed these effects, since sham animals had the same effects as OVX animals.

The brain is very susceptible to genetic influences and environmental experiences and conditions that occur in early life (Singer, 1995; Katz and Shatz, 1996; Grossman et al., 2003; Hensch, 2005; Friederici, 2006). Specific experiences potentiate or inhibit neural connectivity at key developmental stages and these time points are referred to as critical periods (Knudsen, 2004). During the pre-puberty and adolescence periods, neuronal rearrangements occur and the neural circuitry are structurally remodeled resulting in fined connectivity and functionality of brain regions in adulthood (Andersen, 2003; Buwalda et al., 2010). In addition, some studies support the hypothesis that the adolescent brain is particularly vulnerable to stress and the prefrontal cortex would be more susceptible to development during this period (Buwalda et al., 2010).

It is known that interventions in the neonatal period, such as handling modify the relationship of mother and pups and thus may lead to changes in adult life. According to Meaney et al. (1988), nervous system degeneration would be less pronounced in handling animals with age. Also, previous study evaluated the antioxidant enzymes activities in the hippocampus of female and male adult neonatal handled rats compared to non-handled and did not find any differences between these animals (Noschang et al., 2010). This is in agreement with our results that found no differences in the activity of antioxidant enzymes comparing handled and non-handled animals. It is also important to notice that we have not found differences between non-handled and handled groups

on free radicals production and neither on total thiol, suggesting that this early intervention (neonatal handling) does not affect the oxidative balance in adult life. However, more studies related to oxidative damage on lipids, proteins and DNA are necessary to confirm that. On the other hand, exposure to a stressor later on (but still before puberty), was able to induce long-term effects on oxidative stress parameters of some structures of the nervous system, and neonatal handling was not protective in this case.

Stress exposure and high glucocorticoid levels lead to a series of biochemical and behavioral changes that modify normal body homeostasis (Tsigos and Chrousos, 2002). It has been shown that in conditions related to stress exposure, the antioxidant enzymes activities, the levels of free radical scavengers, and other parameters of oxidative stress (Das and Banerjee, 1993; Fontella et al., 2005; Derin et al., 2006; de Vasconcellos et al., 2006; Noschang et al., 2009) are modified, suggesting that the stress response leads to increased production of free radicals (McIntosh et al., 1998; Liu et al., 1996; Fontella et al., 2005). In addition, a significant increase in catalase activity and a decrease in GPx activity in brain have been reported in response to immobilization stress (180 min/day for 15 days) (Sahin and Gumuslu, 2007). Atif et al., (2008) showed that restraint stress (for 1 h and 4 h) decreased the activity of GPx and CAT, and increased the lipid peroxidation in cerebral cortex, striatum and hippocampus. Another study in hippocampus showed that repeated restraint stress (1h/day during 40 days) increased the lipid peroxidation and GPx activity, and decreased total antioxidant reactivity in male adult rats, and did not find differences in the activities of CAT and SOD, neither on DCF levels (Fontella et al., 2005). In our case, we suggest surgery might be acting as a stressor, leading to the decreased CAT activity in the cerebral

cortex in sham and OVX rats, and the increased DCF levels in cerebral cortex and striatum, in rats that underwent a surgical procedure.

Effects of sex hormones on oxidative stress have been reported, particularly estradiol, which has been shown to have antioxidant and neuroprotective properties (Niki and Nakano, 1990; Huh et al., 1994; Lacort et al., 1995; Sawada et al., 1998; Ozgonul et al., 2003). In a study where adult ovariectomized rats were exposed to a chronic stressor the authors observed an increased lipoperoxidation (TBARS) in hypothalamus. Estradiol replacement was able to counteract this effect by a significant interaction between these two treatments (estradiol and chronic stress). In agreement with this possible neuroprotective effect of estradiol replacement, total antioxidant reactivity is increased in hippocampus, both in control and stressed groups receiving estradiol (Prediger, et al., 2004). On the other hand, estradiol replacement decreased GPx activity in spinal cord homogenates from adults ovariectomized female rats (Crema et al., 2010). In the present study, ovariectomized rats (absence of estradiol) showed no alteration on the oxidative stress parameters evaluated. It should be pointed out, however, that OVX was performed before the onset of puberty, and organizational effects of gonadal hormones during this period of development did not occur in these rats, what may account for some differences when comparing studies performing OVX during adulthood.

In conclusion, this study evidences that interventions in early stages of life, as pre-puberty period, induce changes in parameters of oxidative stress that are observed during adult life; besides, these effects are independent from the action of ovarian hormones and neonatal handling. This study contributes to the understanding of how early life events can influence neurochemistry in adult life.

Acknowledgements

Financial support: National Research Council of Brazil (CNPq) and FAPERGS-PRONEX.

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Table 1. Effects of neonatal handling and surgery on the antioxidant enzymes activities in cerebral cortex of rats.

Antioxidant enzyme	Group	Non-handled	Handled
Catalase	Intact	2.12 ± 0.43	1.94 ± 0.53
	Sham	1.56 ± 0.38	1.66 ± 0.40
	Ovx	1.30 ± 0.33	1.33 ± 0.29
SOD	Intact	8.37 ± 1.43	10.53 ± 1.49
	Sham	14.63 ± 2.48	9.17 ± 0.81
	Ovx	11.48 ± 2.40	10.24 ± 1.24
GPx	Intact	26.25 ± 2.03	29.27 ± 4.17
	Sham	28.58 ± 2.80	24.28 ± 2.81
	Ovx	26.25 ± 1.63	25.48 ± 2.59

Data are expressed as mean ± SEM of SOD (U/mg protein), GPx (nmol NADPH oxidized/min/mg protein), and CAT (μmol H_2O_2 transformed/min/mg protein) activities. N= 6-8/group. There was a significant effect of surgery on CAT activity (P = 0.04).

Table 2. Effects of neonatal handling and surgery on the antioxidant enzymes activities in striatum of rats.

Antioxidant enzyme	Group	Non-handled	Handled
Catalase	Intact	1.25 ± 0.12	2.19 ± 0.25
	Sham	2.05 ± 0.53	1.93 ± 0.42
	Ovx	1.95 ± 0.78	1.57 ± 0.42
SOD	Intact	9.76 ± 0.82	7.96 ± 0.85
	Sham	8.24 ± 1.53	9.67 ± 0.71
	Ovx	10.30 ± 0.38	9.77 ± 1.44
GPx	Intact	36.38 ± 4.73	29.27 ± 1.89
	Sham	31.63 ± 4.52	35.30 ± 4.05
	Ovx	35.47 ± 4.77	34.29 ± 2.47

Data are expressed as mean ± SEM of SOD (U/mg protein), GPx (nmol NADPH oxidized/min/mg protein), and CAT (μmol H_2O_2 transformed/min/mg protein) activities. N= 5-7/group. There were no differences between the groups ($P > 0.05$).

Table 3. Effects of neonatal handling and surgery on the antioxidant enzymes activities in hypothalamus of rats.

Antioxidant enzyme	Group	Non-handled	Handled
Catalase	Intact	2.27 ± 0.31	2.52 ± 0.64
	Sham	2.53 ± 0.34	2.69 ± 0.41
	Ovx	2.34 ± 0.35	2.13 ± 0.28
SOD	Intact	12.89 ± 1.15	15.56 ± 1.20
	Sham	14.25 ± 2.09	13.45 ± 0.88
	Ovx	13.56 ± 1.67	14.10 ± 1.06
GPx	Intact	36.12 ± 4.33	31.51 ± 2.54
	Sham	42.52 ± 5.22	37.46 ± 3.49
	Ovx	43.16 ± 4.55	37.29 ± 5.02

Data are expressed as mean ± SEM of SOD (U/mg protein), GPx (nmol NADPH oxidized/min/mg protein), and CAT (μmol H_2O_2 transformed/min/mg protein) activities. N= 5-8/group. There were no differences between the groups ($P > 0.05$).

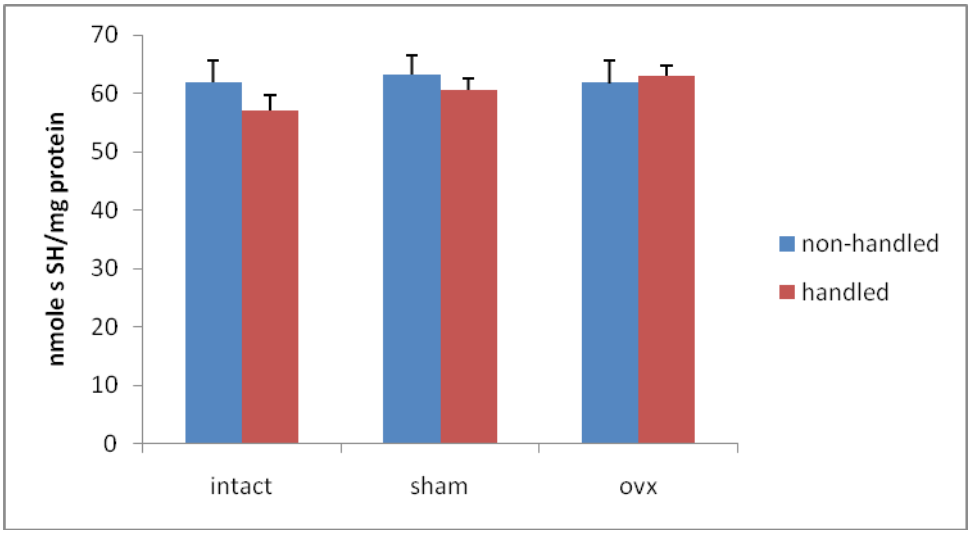
Legends to Figures

Figure 1 Determination of total thiol. Data are expressed as mean ± SEM. N=4-7/group. **A** Striatum, **B** Hypothalamus and **C** Cerebral Cortex. Two-way ANOVA showed no differences between the groups (P>0.05).

Figure 2 Production of free radicals in cerebral cortex (A), striatum (B), and hypothalamus (C), evaluated by the method of oxidation of DCFH. Data are expressed as mean percentage of control ± SEM. N= 5-6 /group. There was a significant effect from surgery (sham and ovx), when compared to the intact group, both in cerebral cortex (Two-way ANOVA, P=0.005) and in the striatum (Two-way ANOVA, P=0.006), while no difference was observed in the hypothalamus (Two-way ANOVA, P>0.05).

Figure 1

A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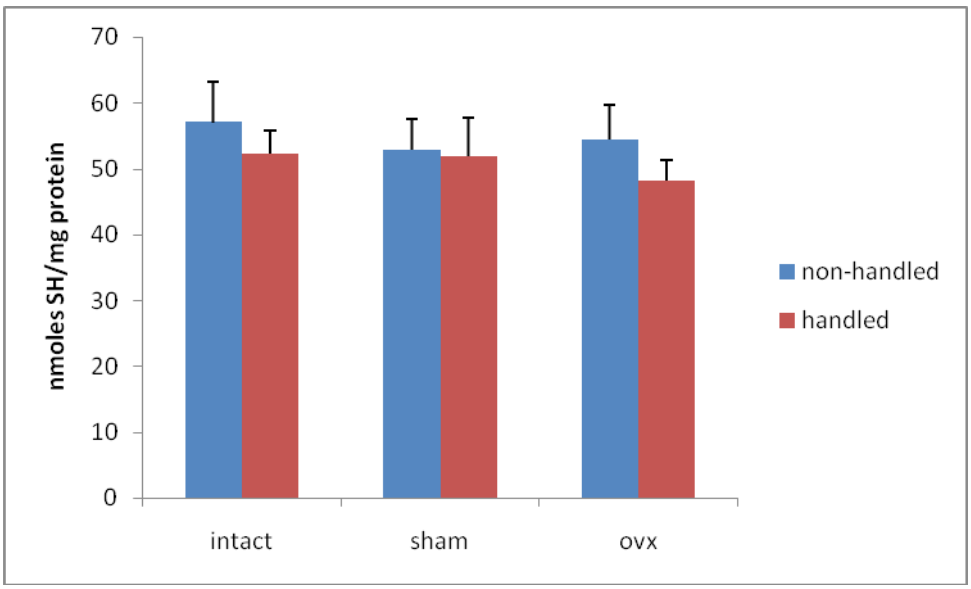
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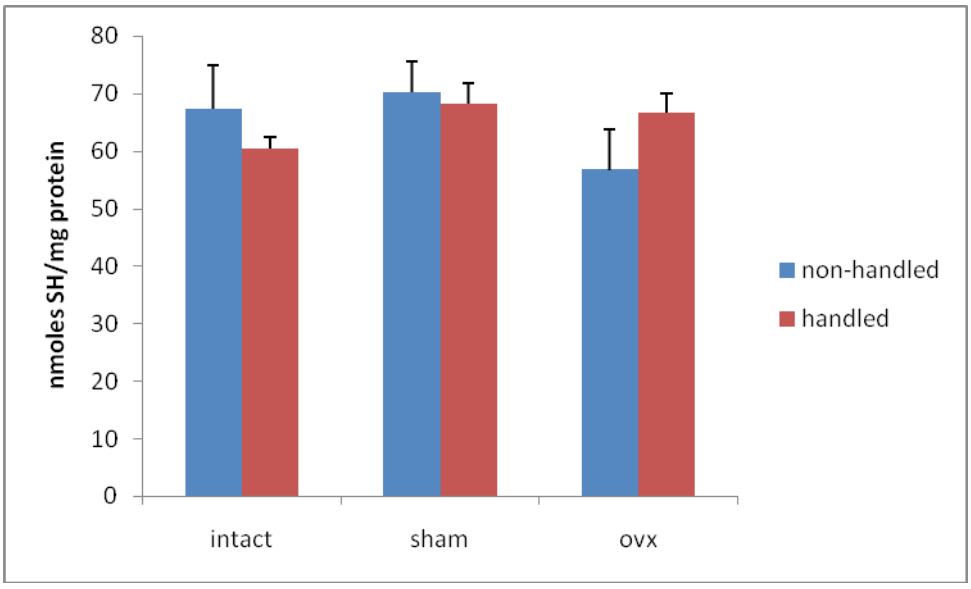
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Figure 2

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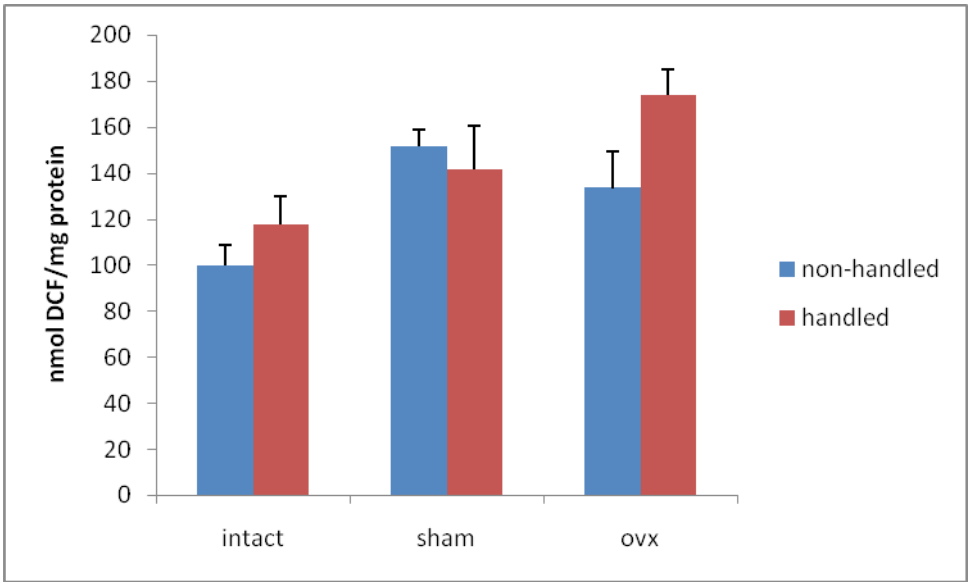
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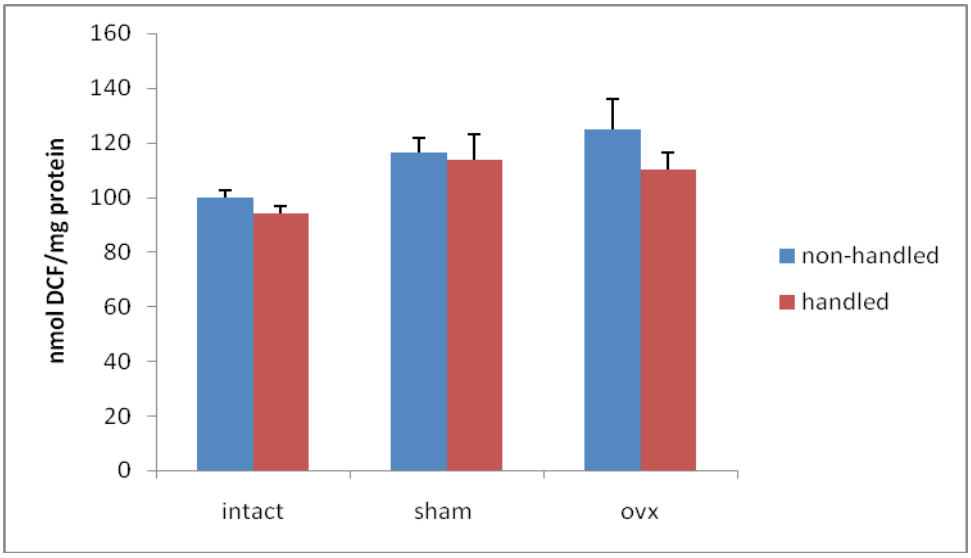
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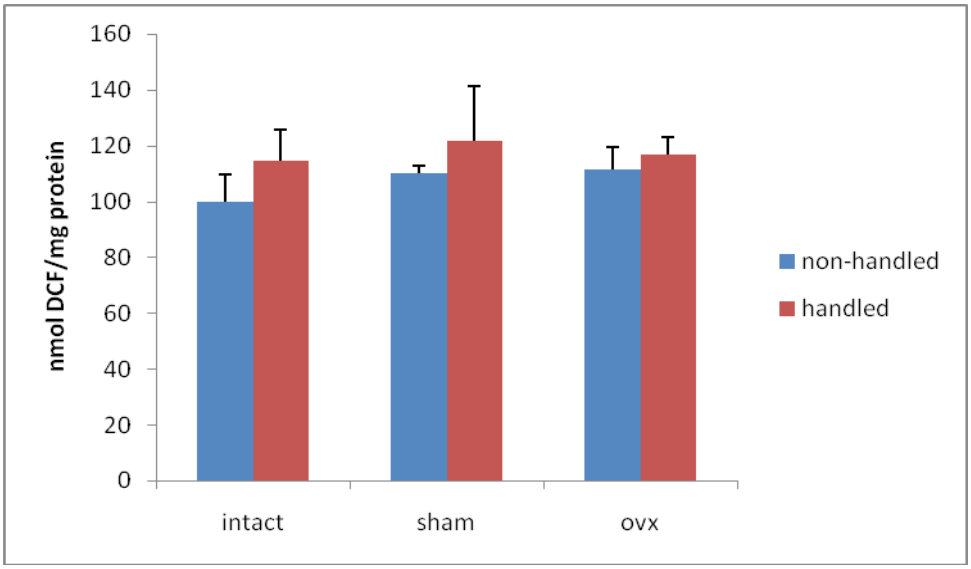
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Anexo

International Journal of Developmental Neuroscience – Guide for Authors

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