UNIVERSIDADE FEREAL DO PAMPA

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A INFUSÃO DE DOPAMINA NA REGIÃO CA1 DO HIPOCAMPO REVERTE O DÉFICIT DE MEMÓRIA INDUZIDO PELA DEPRIVAÇÃO MATERNAL

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DOPAMINE INFUSION IN HYPOCAMPUS CA1 REGION REVERTS MEMORY DEFICT INDUCED BY MATERNAL DEPRIVATION

Trabalho de conclusão de curso apresentado à Universidade Federal do Pampa, como parte das exigências para a obtenção do título de Bacharel em Fisioterapia.

Orientadora: Prof. Dr. Pâmela Billig Mello Carpes

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BANCA EXAMINADORA

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APRESENTAÇÃO

O presente trabalho de conclusão de curso é apresentado na forma de um artigo cientifico, conforme normas de TCC do Curso de Fisioterapia da UNIPAMPA. Trata-se de um trabalho experimental, que foi desenvolvido após aprovação da Comissão de Ética para o Uso de Animais da Unipampa (ANEXO I). O artigo está de acordo com as normas da Revista Physiology and Behavior (ANEXO II).

DOPAMINE INFUSION IN CA1 REGION OF HYPOCAMPUS REVERTS MEMORY DEFICTS INDUCED BY MATERNAL DEPRIVATION

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Highlights

1. Maternal deprivation causes object recognition memory deficits.
2. Intra-hipocampal dopamine infusion reverts memory deficits related to maternal deprivation.
3. Maternal deprived rats do not present alterations on hippocampal dopamine levels.

DOPAMINE INFUSION IN CA1 REGION OF HYPOCAMPUS REVERTS MEMORY DEFICTS INDUCED BY MATERNAL DEPRIVATION

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Abstract

Previous research showed that maternal deprivation (MD) lead to memory deficits during adulthood. The hippocampus, an important brain structure that participates in the memory processes, receives dopaminergic afferents from other brain areas. Here we demonstrated that, although dopamine levels on hippocampus are not altered by MD, the memory deficits resultant from MD in object recognition memory task are reversed by intra-hippocampal (CA1) infusion of dopamine.

Keywords: Early-life stress; Object recognition; Dopaminergic system; Memory deficits.

1. Introduction

Maternal care during the neonatal period is fundamental for the development of the Central Nervous System (CNS). Human [1, 2] and animals studies [3-6] suggest that stress during this sensitive period may exert modifications in the organization of the neural network [7] , which can lead to psychiatric disorders such as anxiety, depression and schizophrenia [8-12]. In addition, neurochemical changes such as imbalance in oxidative parameters [3, 4], hormonal [13] and immunological changes [14] can be caused by prolonged stress, especially in the neonatal period. Changes in different neurotransmitter systems, such as the histaminergic [15], cholinergic [16], serotoninergic [17, 18] e glutamatergic [19] systems can also be observed in animal models submitted to different neonatal stress protocols.

Maternal deprivation (MD) is a potent natural stressor that can lead to behavioral changes and cognitive deficits that persist during adulthood [3, 4]. Studies have shown that stress models during the neonatal period, such as MD, can cause damage to both short and long-term memory in different cognitive tasks dependent on brain structures such as prefrontal cortex (PFC) and hippocampus (HP) [4, 15, 20, 21]. These structures, important for the organization and retention of memory [22-24], receive dopaminergic afferents from the ventral tegmental area (VTA), substance nigra (SN) [25, 26] and the locus coeruleus (LC) [11, 27].

The dopaminergic system plays an important role in emotional responses to stress [28, 29]. Studies have shown that exposure to glucocorticoids in pre and postnatal periods affects the development of the dopaminergic system and have suggested that increased sensitivity to drugs of abuse is related to interactions between prenatal stress,

glucocorticoids and dopaminergic neurons [17, 30]. Thus, changes in molecular mechanisms may reflect the variation in the risk of psychopathologies, particularly as a result of maternal neglect or child abuse.

Considering that the hippocampus is an important structure for acquiring and consolidating different types of memories [31], and which may be affected by neonatal stress protocols [15, 16, 19], we demonstrated in previous studies that MD can induce mnemonic deficits by different mechanisms, as the increase of oxidative stress [3, 4]. In addition, we verified the neuroprotective effects of different interventions to attenuate the oxidative imbalance in the PFC and HP [3, 4]. However, despite the involvement of various neurotransmitter systems in memory damage induced by MD were studied, little is known about the dopaminergic system role. We hypothesized that MD causes changes in hippocampal dopaminergic neurochemistry, which are related to the memory deficits observed in rats submitted to MD.

2. Materials and methods

2.1. Animals

Pregnant female Wistar rats were obtained from the Central Vivarium of the Federal University of Santa Maria (RS/Brazil). All animals were kept in a 12h light/12h dark cycle (light phase started at 7h), with controlled temperature (23 ± 2ºC) and air humidity (60% ± 5%). Pregnant female rats were individually housed with sawdust bedding with food and water available *ad libitum*. The day of delivery was considered the day 0. At postnatal day 1 (PND-1) the MD protocol was initiated with half of the pups and lasted until PND-10. Animals were weaned at 21 days of age (PND-21) and were housed 5 per cage in regular cages. Only the males were used in the following experiments; the females were donated to other studies in progress. All experiments were conducted in accordance with the principles of laboratory animal care [32] and were approved by the Institutional Animal Care and Use Committee of the Local Institution (Protocol #005/2017).

Male rats were divided into six groups:

(i) control, in which the rats were submitted to behavioral tests, and immediately after the training session in object recognition task (OR) received an intra-hippocampal infusion of saline (n = 14);

(ii) deprived, in which the rats were submitted to the MD protocol and to the behavioral tests, and immediately after the training in OR received an intra-hippocampal infusion of saline (n = 14);

(iii) dopamine, in which the rats were submitted to behavioral tests, and immediately after the training session in OR received an intra-hippocampal infusion of dopamine (n = 10);

(iv) deprived + dopamine, in which the rats were submitted to the MD protocol and the behavioral tests, and immediately after the training session in OR received an intra-hippocampal infusion of dopamine (n = 10);

(v) *naive* control, the rats hippocampus were isolated to measurement of dopamine levels without any behavioral or drug intervention (n = 4); e

(vi) *naive* deprived, the rats hippocampus were isolated to measurement of dopamine levels in rats submitted to the MD protocol without any behavioral or drug intervention (n = 4).

Four rats from groups *(i)* and *(ii)* were euthanized after training in OR for hippocampal dissection and subsequent biochemical analysis of dopamine levels.

2.2. Maternal deprivation protocol (MD)

Pregnant rats were kept in their individual boxes until the delivery day (considered day zero). Rats from groups (ii), (iv) and (iv) were submitted to MD, what consisted in removing the mother from the residence box to other room for the period of 3h per day from PND-1 to PND-10. The pups were maintained in their home cage, without suffering any manipulation, while the mothers were absent the room temperature was increased to 32ºC to compensate the absence of the mother's body heat [33]. At the conclusion of each daily deprivation session, the mothers were returned to their home boxes. The rats in groups (i), (iii) and (iii) remained in their resident boxes together with their mothers during the first ten days of life. On PND-21, the animals were weaned, and the males were maintained in groups of 5 in plastic boxes with food and water available ad libitum. Female rats were donated to other ongoing studies

2.3. Surgical procedure and drug infusion

For the implantation of cannulas in the CA1 region of the hippocampus, necessary for infusion of the drugs, the rats of groups (i) to (iv) were anesthetized with ketamine and xylazine (i.p., 75 mg / kg and 10 mg / kg respectively) and 27 gauge guide

cannulae were implanted by means of stereotactic surgery to the CA1 region of the dorsal hippocampus (AP = - 4,2; LL = ± 3.0, DV = – 2.0 mm) according to the coordinates of the anatomy atlas Paxinos e Watson [34]. The cannulae were fixed with dental cement. Post-surgical recovery time was 4 days.

For drug infusion, 30 gauge cannulae were fitted into the guide cannulae. Infusions (1 μl / side) in the CA1 region of the dorsal hippocampus were performed for a period of 60 seconds using an infusion pump, and the cannulas were left in place for an additional 60 seconds to minimize backflow. Groups (iii) and (iv) received 1 μl / side of dopamine at the concentration of 1 μg / μl diluted in saline solution; groups (i) and (ii) received only infusion of saline solution; the groups (i) and (ii) received only saline infusion. Doses and volumes were determined by previous studies showing the effect of the compound on learning and behavioral performance [27, 35, 36].

2.4. Drugs and reagents

Dopamine (DA) was purchased from Sigma-Aldrich (St. Louis, MO) and diluted in 0.9% saline (pH 7.2) and stored at -20 ° C, protected from light until use. Other reagents used in this experiment were of analytical grades and obtained from standard commercial suppliers.

2.5. Object recognition (OR) task

The training and test of the task of object recognition were carried out in a wooden box with the front in transparent glass (50 x 50 x 50 cm) [37]. Object recognition (OR) is based on the animal's natural tendency to explore more of the new object than the familiar in a known environmental context. Firstly, the animals were habituated to the OR apparatus, for this, during 4 consecutive days, the animals were allowed to explore the environment (box) for 20 minutes per day. On the 5th day (training session), the animals were again placed in the apparatus with two different objects (named objects A and B) for free operation for 5 min. The long-term memory test session was evaluated 24 hours after the training session. For test session, one of the objects was replaced by a new object (named object C), and again the animals were placed in the apparatus for free operation for 5 min. The time spent exploring each object was recorded and expressed as a percentage of total exploration time [38]. The objects were made of metal or plastic. Exploration was defined as when the object was touched or sniffed with the nose and / or front paws of the animals. To avoid olfactory

preferences, objects and apparatus were cleaned with 70% alcohol after testing each animal. The effect of DA and / or saline intra-hippocampal infusions was studied by administration immediately after training session in OR [39, 40].

2.6. Behavioral control tests

2.6.1. Open field (OF)

In order to evaluate the locomotor and exploratory activity of the animals and to ensure that the infusion of DA or other procedure did not impair these behaviors, altering the results of the RO memory test, the animals were submitted to the Open Field (OF). The apparatus consists of a wooden box (50 x 50 x 50 cm) with a front wall made of transparent glass. Black lines divided the floor of the box into 12 equal quadrants. The animals were placed in the apparatus for free operation for 5 minutes. The number of crossings (locomotor activity) and the number of rearing (exploratory activity) were recorded [16].

2.6.2. Elevated plus maze (EPM)

To evaluate the state of anxiety, which could affect the results of the memory tests, the animals were submitted to the EPM. The apparatus consists of a cross-shaped table raised 60 cm above the ground, with two open arms and two closed arms, each measuring 50 x 10 cm. The closed arms had also side walls 20 cm high. The animals were placed in the center of the apparatus and during the 5 minute session were recorded: (i) the entries number in the open and closed arms; and (ii) time spent on open and closed arms [41].

2.7. Determination of dopamine levels

The determination of hippocampal levels of DA was measured by HPLC (*High Performance Liquid Chromatography*) in homogenates prepared from the hippocampus using the reverse-phase HPLC system. Rats' brains were removed and bilateral hippocampus were quickly dissected out in an iced surface and homogenized in 50 mM TrisHCl, pH 7.4 (1/10, w/v). Afterwards, samples were centrifuged at 2400g for 20 min; supernatants were filtered and then stored at −80 °C until use [42]. The HPLC system consisted of a Vacuum Degasser (YL9101) and quaternary pump (YL9110) connected to a reversed phase column (SYNERGI 4l FUSION-RP 80 Å 250 ×4.60 mm; Phenomenex) on a Column Compartment (YL9131) coupled to a Diode Array Detector

(YL9160). The mobile phase consisted of methanol and water (12/88, v/v) adjusted to pH 3 with phosphoric acid. To separate DA, we used the programming isocratic with a flow rate of 0.8 ml/min. The sample was filtered through 0.22 μm syringe filters. We injected 20 μl samples into the HPLC system by an auto sampler device (YL9150). The detection was at 198 nm by DAD. Chromatograms were recorded and integrated by PC integration software (YLClarity). All analyses were run in triplicate. The analytical parameters were as follows: linear range, 0.1–10.0 μg/ml; determination coefficient, 0.999; and calibration equation, y =628.12 x− 34.342. Norepinephrine for HPLC was supplied by Sigma-Aldrich Brazil. Other reagents used in this experiment were of analytical grades and obtained from standard commercial suppliers.

2.8. Statistical analysis

The time of exploration of the objects in the OR task was converted to a percentage of the total exploration time and the t-test of a sample was used to compare the percentage of the total exploration time spent on each object with a theoretical mean (50%). The results of OF, EPM and HPLC were analyzed using one-way ANOVA followed by post-hoc, if necessary. All data were expressed as mean ± standard deviation (SD). The differences were considered statistically significant when $P < 0.05$.

3. Results

3.1. Hippocampal dopamine infusion reverses the memory deficit of object recognition caused by MD

As expected, rats from all groups explored each of the objects (A and B) by about 50% each during the training session ($P > 0.05$ for all groups and objects; Fig. 1). Rats from control group explored significantly more than 50% of the total exploration time of the new object (C) on test day ($t_{(6)} = 0.32$; $P = 0.018$; Fig. 1, Control), which demonstrates a preserved memory. The rats from the deprived group spent a percentage similar to 50% of the total exploration time by exploring the family object (A) and the new object (C) on test day ($t_{(9)} = 1.11$; $P = 0.293$; Fig. 1, MD), which suggests OR memory deficit. Hippocampal infusion of dopamine maintained the memory of non-deprived rats ($t_{(6)} = 2.78$; $P = 0.032$; Fig. 1, DA) and was able to reverse memory deficit of OR induced by MD, since that rats from the dopamine group that were submitted to MD explored significantly more than 50% of the total time of exploration the new object (C) on test day ($t_{(9)} = 5.59$; $P = 0.0003$; Fig. 1, MD + DA).

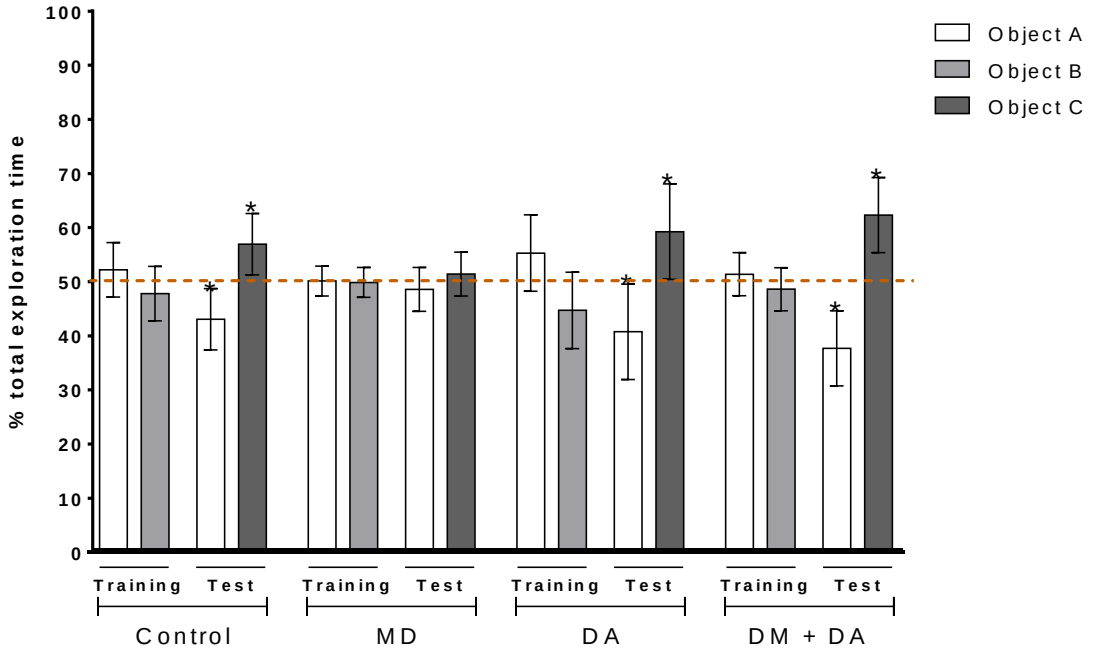

Fig. 1. Maternal deprivation causes object recognition (OR) memory deficits. Dopamine infusion into the CA1 region of the hippocampus reverses this deficit. The animals were trained in the OR task and tested 24 hours after. In the training session, the animals of the different groups were exposed to object A and B and explored about 50% of the total exploration time of each object. In the test session, the animals were exposed to the family object (A) and to a new object (C). The MD group was not able to differentiate in the new object from the familiar object, but the MD rats that received dopamine infusion after the OR training session were. Data are expressed as mean ± SD of the percentage of the total exploration time; * $P < 0.05$ in the one simple t-test, considering a theoretical average of 50%; n = 10-14 per group.

3.2. The MD and dopamine infusion do not cause changes in locomotor and exploratory behavior and anxiety of animals.

The open fields tests, to evaluate locomotor and exploratory activity, and elevated plus maze, to evaluate anxiety, were performed the next day of DA or saline infusion. Intra-hippocampal DA infusion did not affect the number of crossings and rearings over 5 minutes of the free-arm session in the OF task (Tab. 1 – Open Field; $F_{(3,33)} = 0.79$; $P > 0.50$ for crossings; $F_{(3,33)} = 0,32$; $P > 0,81$ for rearings). Similarly,

intra-hippocampal DA infusion did not affect the number of entries or time spent in the open arms over 5 minutes of the EPM session (Tab. 1 – Elevated Plus Maze; $F_{(3,33)}$ = 0.45; P > 0.71 for entries; $F_{(3,33)}$ = 0.57; P > 0.63 for time).

Table 1.

Dopamine or saline infusion, as well as MD, did not alter the locomotor and exploratory activity in the Open Field task (P > 0.05; one-way ANOVA), and neither the behavior of anxiety in the Elevated Plus Maze (P > 0.05; one-way ANOVA). The data are expressed as mean ± SD (n = 10 / group).

	Control	MD	DA	MD + DA
Open Field				
Crossings (n)	84.38 ± 23.31	81.50 ± 54.59	63.22 ± 23.06	64.70 ± 35.08
Rearings (n)	35.50 ± 12.22	36.70 ± 27.82	29.56 ± 10.64	30.80 ± 17.63
Elevated Plus Maze				
Entrances in open arms (n)	6.12 ± 1.35	5.70 ± 2.35	5.33 ± 1.65	5.10 ± 2.18
Entrances in closed arms (n)	5.87 ± 1.126	5.50 ± 2.014	5.55 ± 2.007	5.10 ± 2.378
Time in open arms (s)	121.0 ± 37.84	129.1 ± 51.32	99.78 ± 51.45	121.8 ± 57.14
Time in closed arms (s)	179.0 ± 37.84	170.9 ± 51.32	200.2 ± 51.45	178.2 ± 57.14

3.3. The MD and training in the OR task do not cause detectable changes in hippocampal dopamine levels

The OR task training, as well as MD, did not promote detectable changes in dopamine (DA) levels in the hippocampus (Fig. 2; $F_{(3,12)}$ = 2.47; P = 0.11 ANOVA).

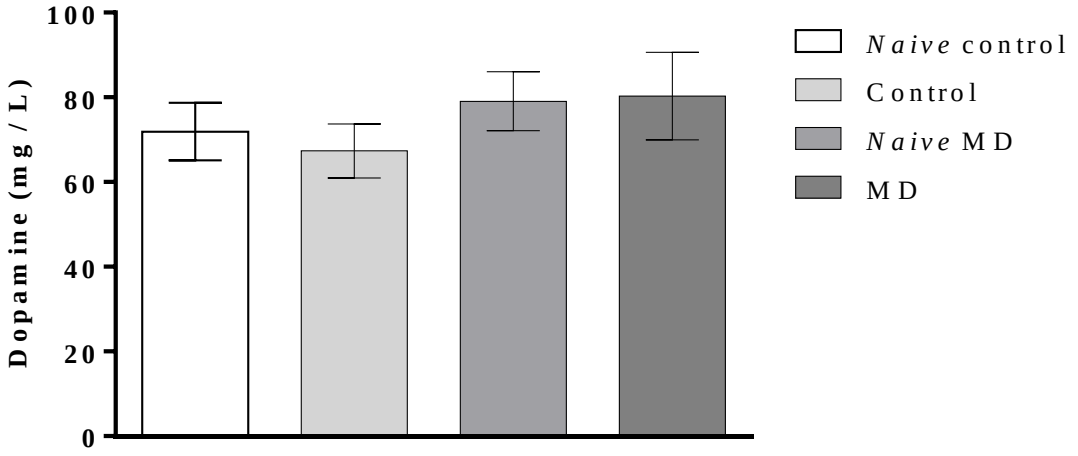

Fig. 2. The maternal deprivation and training the recognition task did not alter the levels of dopamine (DA) measured in hippocampal homogenate HPLC (P = 0.111, one-way ANOVA; n= 4/group).

4. Discussion

Our results demonstrate that infusion of dopamine into the CA1 region of the hippocampus immediately after learning the OR task is able to reverse the memory deficit induced by MD. In last decades, it has been consistently shown that stress during the neonatal period leads to changes in brain development, primarily through the effects of glucocorticoids [43-45]. In particular, the hippocampus, an important structure that participates in memory formation and consolidation, contains high concentrations of glucocorticoid and mineralocorticoid receptors [46]. Thus, the increase in circulating glucocorticoids, especially during the CNS development period, may affect hippocampal function [47, 48].

Among the effects induced by MD in different CNS structures that may be related to memory deficits observed in this model are also: the imbalance between antioxidant and pro-oxidant mechanisms [3, 4]; altered levels of Brain Derived Neurotrophic Factor (BDNF) [19, 49, 50]; and the modulation that MD exerts on different neurotransmitter systems, such as the histaminergic [15], cholinergic [33], glutamatergic [19], serotoninergic [18, 51] e dopaminergic system [17, 52].

In addition to the molecular effects, the functional effects of MD affect the behavior of animals during adulthood. In previous studies, we demonstrated that adult rats submitted to MD present both short-term and long-term memory deficits in

different cognitive tasks, and that this cognitive deficit is caused, at least in part, by changes in oxidative parameters in the hippocampus, both that antioxidant strategies, such as exercise and green tea, were able to protect these deficits [3, 4].

The results of the present study demonstrate that dopamine infusion immediately after learning the recognition task reverses the mnemonic damage induced by MD, evidencing the involvement of the dopaminergic system in MD-related mnemonic dysfunction. Considering that the hippocampus receives dopaminergic afferents from the ventral tegmentar area (VTA), substance nigra (SN) [25, 26] and locus coeruleus (LC) [11, 27], it is likely that these structures are affected by stress during the neonatal period. In addition, previous studies have shown that stress in the neonatal period may affect the expression of dopaminergic receptors D1, D2, D3 and D5 [53, 54], impairing spatial [55, 56] and aversive memory [56, 57].

Despite the evident effect of dopamine administration on the memory of deprived animals, no differences were found in the hippocampal levels of this neurotransmitter between control and deprived animals. These results may be related to the methodology used in the present study, which involved the *post mortem* analysis, when the ideal would be the *in vivo* analysis, by microdiálise, although other studies have already used this type of technique [27, 58, 59]. Another possible explanation for the results is that dopaminergic changes are not related to the availability of the neurotransmitter, but to changes in the expression of dopaminergic receptors [53, 54] . Thus, additional studies will be conducted in an attempt to better elucidate the dopaminergic mechanisms involved in memory deficit related to MD.

Thus, our results reinforce the findings of previous studies about memory deficit induced by maternal deprivation [3, 4], and provides new evidence that the dopaminergic system is involved, since that intra-hippocampal administration of dopamine immediately after learning reverses the recognition deficits of object recognition. However, since there has been no increase in dopamine levels in the learning-induced hippocampus, it is likely that this deficit is caused by other changes, such as changes in expression of dopaminergic receptors. All of these mechanisms should be the subjects of future research.

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Pró-Reitoria de Pesquisa, Pós-Graduação e Inovação (PROPII)

COMISSÃO DE ÉTICA NO USO DE ANIMAIS - CEUA

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CERTIFICADO DE APROVAÇÃO DE PROTOCOLO PARA USO DE ANIMAIS EM PESQUISA

Número de protocolo da CEUA: 005/2017

Título: Envolvimento do sistema dopaminérgico no dano de memória induzido pela deprivação maternal.

Data da aprovação: 08/05/2017

Período de vigência do projeto: 08/05/2019

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CEUA

Comissão de Ética em Uso de Animais

Finalidade	**( ) Ensino (X) Pesquisa**
Espécie/Linhagem/Raça	Ratos wistar
Nº de animais	20 e 52
Peso/Idade	250g-300g /120dias (fêmeas) e do nascimento até 120dias (machos)
Sexo	Fêmeas e Machos
Origem	Biotério da Universidade Federal de Santa Maria e prole das fêmeas

Profª. Drª. Vanusa Manfredini
Coordenadora CEUA/UNIPAMPA