INFLUENCE OF THE NORADRENERGIC SYSTEM ON BEHAVIORAL PARAMETERS OF ANXIETY AND EXPLORATION

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ABSTRACT
Introduction: Parkinson's disease (PD) is a progressive and chronic neurodegenerative motor disorder, which results in the selective loss of dopaminergic neurons. Its motor symptoms include stiffness, bradykinesia and tremor at rest. Degeneration of the noradrenergic system has great relevance in the development of the disease and the appearance of non-motor symptoms that are observed in the preliminary stages of PD. Objective: In this way, the objective is to create an animal model to degenerate the noradrenergic system of rats and to evaluate the influence of this system in behavioral tests. Methodology: An animal model wasperformed using the neurotoxin DSP-4 in order to degenerate the noradrenergic system of wistar rats. After a period of 12 days, open field tests were performed to assess locomotion andexploration, and the elevated plus maze test was used to assess anxiety and fear. Results: In anopen field test, the DSP-4 model showed reduced locomotor and exploratory activity in relation to the control group, confirming the expected animal model. In the elevated plus-maze test, there was an increase in anxiety and fear in relation to the control group. Conclusion: The depletion of noradrenergic neurons contributes to the appearance of a behavioral pattern that could be related to the non-motor symptoms of PD.

Keywords: DSP-4; Parkinson's diseases; Non-motor symptoms; Behavioral tests.

INFLUÊNCIA DO SISTEMA NORADRENÉRGICO EM PARÂMETROS COMPORTAMENTAIS DE ANSIEDADE E EXPLORAÇÃO

RESUMO
Introdução: A Doença de Parkinson (DP), é um distúrbio motor neurodegenerativo de modo progressivo e crônico, na qual resulta na perda seletiva de neurônios dopaminérgico. Seus sintomas motores incluem rigidez, bradicinesia e tremor em repouso. A degeneração do sistema noradrenérgico tem grande relevância no desenvolvimento da doença e aparecimento dos sintomas não motores que são observados em fases preliminares da DP. Objetivo: Desta forma, tem-se como objetivo realizar um modelo animal para degenerar o sistema noradrenérgico de cobaias e avaliar a influência deste sistema em testes comportamentais. Metodologia: Foi realizado um modelo animal a partir do uso da neurotoxina DSP-4 com a finalidade de degenerar o sistema noradrenérgico das cobaias, e também fez-se o uso de agonista ß adrenérgico Clembuterol para fins de recuperação da via. Após um período de 12 dias foram realizados os testes de campo aberto, para avaliação da locomoção e exploração, e o teste de labirinto em cruz elevado, para avaliação de ansiedade e medo. Resultados: Em testede campo aberto o modelo com DSP-4 mostrou-se com atividade locomotora e exploratória reduzida em relação ao grupo controle, confirmando o modelo animal esperado. Já no teste do labirinto em cruz elevado mostrou-se aumento de ansiedade e medo em relação ao grupo controle. Conclusão: A depleção de neurônios noradrenérgicos contribui para o aparecimentode um padrão comportamental que poderia ser relacionado com os sintomas não motores da DP.

Palavras-chave: DSP-4; Parkinson; Sintomas não motores; Testes comportamentais.

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INTRODUCTION

Parkinson's disease (PD) is a neurodegenerative disorder that occurs in the central nervous system (CNS) in a chronic and progressive way. According to data from the World Health Organization (WHO), the estimated prevalence is 100 to 200 cases per 100 thousand inhabitants, and about 3% of the world population over 65 years is affected by PD[1]. PD is characterized by a degenerative CNS disorder that impairs long-term motor skills and cognitive processes[2]. The main symptoms are tremor at rest, stiffness and bradykinesia[3]. In addition to motor deficits, the disease also has characteristic non-motor symptoms, whichmay arise before motor problems[3,4]. The onset of PD it is characterized by the depletion of dopaminergic neurons in the substance nigra *pars compacta*. And, alone degeneration of dopamine (DA) in animal models have failed to elicit both simultaneously and not motor deficits - PD engines, possibly because the condition is a disorder multi-systemic featuring a profound loss in other systems[2.5].

Another region involved in PD that also suffers degeneration is the noradrenergic nucleus called the locus coeruleus (LC). This structure projects axons to different regions of the brain where it releases neurotransmitters, among them the most studied, noradrenaline (NA) that acts on alpha and beta receptors coupled to the G protein6. Their roles and functions are well characterized in attention activity, and in the long term in motor control7. A series of studies have raised the possibility of a time lag between the degeneration of LC and SN in PD, where previous noradrenergic degeneration appears to predispose SN dopaminergic neurons to also degenerate, consequently leading to PD. The loss of noradrenergic neurons could contribute to the appearance of symptoms such as sleep disorders, anxiety and depression, all identified in the early stages of PD. This loss of neurons raises intriguing questions about the NA system, which can be a marker and / or a potential target for new therapies, in which it targets pre- motor symptoms 10-20 years before the onset of PD[10].

Studies show that in rodents, LC degeneration can be induced using the noradrenergic neurotoxin, N-ethyl-N-(2-chloroethyl)-2-bromobenzylamine hydrochloride (DSP-4). The DSP-4 contained well established in the literature, specific for NO and can easily cross the blood-brain barrier[11].

Currently, the progress of research on non-motor symptoms and neuronal loss of PD is hampered by the lack of adequate animal models. In view of this, the present research project aims to evaluate the influence of the noradrenergic system in tests behavioral disorders where there is degeneration induced by the administration of the neurotoxin DSP-4.

METHODOLOGY

Animals and experimental design

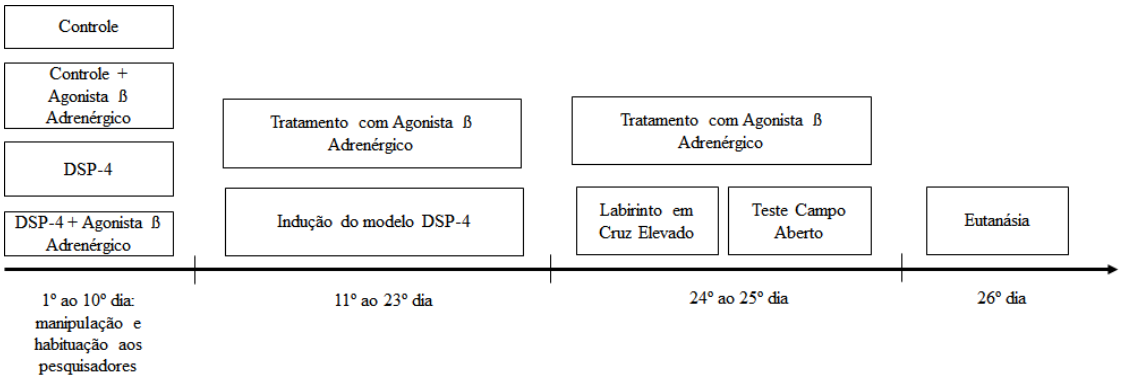
For the experiment, 40 male Wistar - Kyoto rats (150g-200g) were obtained from the Center for Experimental Biological Models, of the Pontifical Catholic University of Rio Grande do Sul (RS / Brazil) and housed four animals per cage, where the temperature 22 ± 1 ° C was maintained and the 12h light-dark cycle was respected with water and feed *ad libitum* , all animal handling is in accordance with the Animal Use Ethics Committee of the University of Vale do Taquari (Ceua Approved) / Univates: 034/2020). Adrenergic ß agonist or saline were administered via gavage for 13 days in rats allocated according to the following groups (n = 10 / group):

Control group + saline;

Control group + ß adrenergic agonist; Group DSP-4 + saline; Group DSP-4 + beta adrenergic agonist;

The animals remained 10 days after arrival in a period of manipulation and habituation to researchers and the vivarium (illustrated in image 1). On day 11, the DSP-4 model (50mg / kg) was induced and treatment with Clenbuterol (ß Adrenergic Agonist) (10 µg/ kg / day) or saline was initiated until euthanasia. After 12 days for induction of the DSP-4 model, the animals started the behavioral tests first with the Elevated Cross Maze and the Open Field Test. 24h after the last test the animals were euthanized, where samples of brain tissues were collected.

Image 1. Experimental design



Model DSP-4

The use of the neurotoxin DSP-4 (N- (2-chloroethyl) -N-ethyl2-bromobenzylamine) served to induce noradrenergic system degeneration. Thus, the neurotoxin has a specific action on noradrenergic axon terminals that are originated in the LC, since their use is well established in the literature [10,11] . An intraperitoneal injection was made with the neurotoxin DSP-4 (50mg / kg) dissolved in a saline solution, and after the treatment started for 12 days with the administration of the ß adrenergic agonist and or saline always at the same time (7:30 - 8:00) including during tests for behavioral assessment of animals. Finally, the animals were euthanized on the 14th day.

Adrenergic ß agonist (dose 10ug / kg / day)

In studies it has been suggested that the significant loss of noradrenergic neurons in the LC it contributes to the progression of PD, and that the use of pharmacotherapies aimed at restoring NA is likely to have therapeutic potential[3]. Clenbuterol was used as an ß adrenergic agonist for administration in two groups of the experiment as a form of pharmacotherapy to restore this loss of NA.

Behavioral tests

The animals were evaluated from the 12th day of treatment in the Labyrinth in Cruz Elevado and in the Open Field Test, the equipment was always cleaned with 70% alcohol after each animal tested. All behavioral tests were conducted by a trained observer, in a room with attenuated sound and under low light intensity.

Open Field Task

To assess the effects of DSP-4 on locomotor activity and exploratory behavior of animals, we used the analysis of locomotion / exploration behavior in the scheme known as "open field" [12]. For this, a black wooden box was used whose floor is divided into 12 quadrants with equal surface area. The animal was gently placed in the open field arena (ie, the box), which he can explore freely for 5 min. During this time, the following data were collected: the number of crossed lines and the number of elevations on the hind legs, behaviors that in rodents denote locomotion and exploration [13].

Elevated cross maze

The Elevated Cross Labyrinth (LCE) test was used to check anxiety and fear in animals. The experimental apparatus consists of a central platform (5 cm to 5 cm) with two open orthogonal arms (50 x 10 x 0.5 cm) and two closed orthogonal arms (50 x 10 x 10 cm), raised 50 cm from the floor . The rats were placed on the central platform facing the open arm and left free for exploration for 5 min. The following data were collected: the total number of entries in the open and closed arms, the time spent in the open and closed arms, in addition to other parameters related to anxiety behavior [14].

Statistical analysis

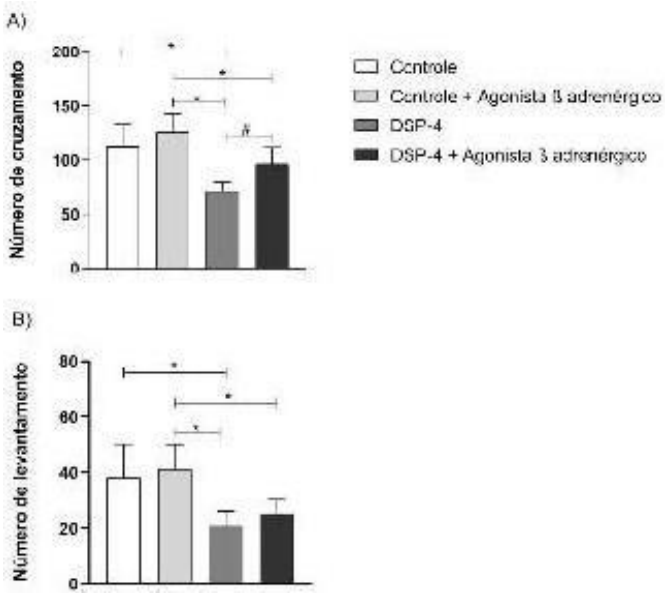
The data were checked for normal distribution using the Shapiro- Wilk test. Behavioral data are presented as mean ± standard error of the mean (SEM). Task data fromthe Open Field Test and Elevated Cross Maze groups were compared using one-way ANOVA with Bonferroni post hoc or T test, according to the data distribution. The level of significance was set at 0.01 for all analyzes.

RESULTS

Open field

Locomotor and exploratory activity was altered between groups (Figure 1). For locomotor activity (crossings) (F (3.40) = 22.09, P <0.0001) and for exploratory activity (lifting of front legs) (F (3, 39) = 14.47, P <0, 0001), (Figure 1). The number of crossings were higher in the Control group compared to the DSP-4 group (P <0.0001, Figure 1- A.). The Control + Adrenergic Agonist group showed an increase in the number of crossings compared with the DSP-4 and DSP-4 + Adrenergic Agonist groups (P <0.0001; P = 0.0008, respectively, Figure 1-A). Finally, we observed an increase in locomotor activity in the DSP-4 + ß-adrenergic agonist group compared to the DSP-4 group (P = 0.0058, Figure 1-A). For exploratory activity, the number of forelegs was higher in the Control group compared to the DSP-4 group (P = 0.0002, Figure 1- B). The Control + adrenergic agonist group showed an increase in the number of surveys compared to the DSP-4 and DSP-4 + adrenergic agonist groups (P <0.0001; P = 0.0004, respectively, Figure 1-B). For the lifting of paws, we found no differences between the DSP-4 group versus DSP-4 + ß adrenergic agonist.

Figure 1 - Open field

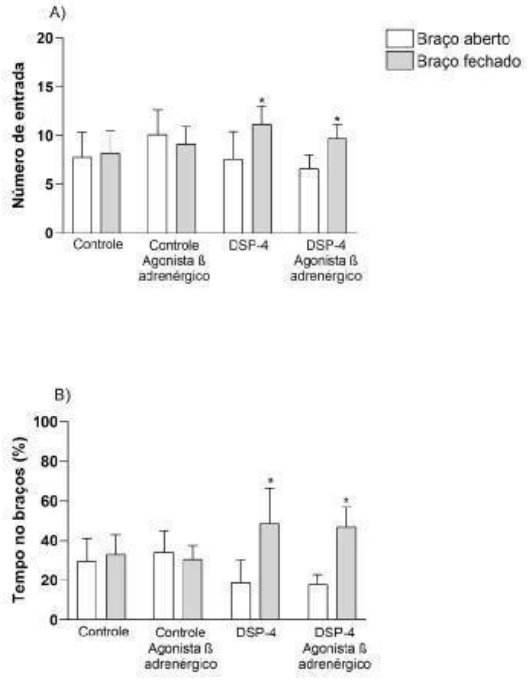


A) Number of crossings. B) Front leg lift number. The DSP-4 model impaired locomotor and exploratory activity as well as treatment with ß-adrenergic Agonist only improved locomotor activity and not for exploratory activity. * P <0.01; Control or Control + ß adrenergic agonist versus DSP-4 or DSP-4 + ß adrenergic agonist; # P <0.01; DSP-4 verses DSP-4 + ß adrenergic agonist. ANOVA followed by Bonferroni's multiple comparison; (n = 10).

Elevated cross maze

In the assessment of anxiety and fear behavior (Figure 2), we found differences both in the number of entries and in the percentage of time in the arms for the DSP-4 and DSP-4 +ß adrenergic agonist groups. We found no differences for the control groups. For the numberof entries in the arms, we found an increase in entries in the closed arms for DSP-4 and DSP-4 + ß-adrenergic agonist (P = 0.0043 and P = 0.0001, respectively, Figure 2 - A). For the percentage spent on the arms, we found a higher expenditure on the closed arms for DSP-4 and DSP-4 + ß adrenergic agonist (P = 0.0002 and P <0.0001, respectively, Figure 2 - B).

Figure 2 - Elevated Cruz Maze



A) Number of entries. B) Percentage of time spent in the arms. The DSP-4 model impaired anxiety and fear behavior as well as treatment with the ß adrenergic Agonist was not able to change the anxiety and fear behavior in animals. * P<0.01. T test comparing entries and / or time between the open arm versus the closed arm; (n= 10)

DISCUSSION

After performing the tests, it was possible to determine the differences between the groups of the experiment on the applied tests. In the open field test, differences were found in the control groups in relation to the DSP-4 groups, thus confirming the animal model, where its noradrenergic system is blocked, causing

the effect of decreased locomotion and exploration. However, when comparing the DSP-4 group with the DSP-4 + agonist group, there was a significant increase in locomotion, which was expected, due to the action of the agonist and its role in reestablishing noradrenergic pathways [4]. In the elevated plus- maze test, the DSP-4 group showed greater anxiety behavior compared to the control groups. Thus confirming that the significant loss of the noradrenergic system ends up generating symptoms such as fear and anxiety [9,12]. However, the DSP-4 + agonist group was not able to reverse anxiety and fear behavior.

Since the noradrenergic system is directly related to patterns of behavior that involve attention and focus, that is, the more noradrenergic activation the more the subject is able tofocus on an activity and the better his cognitive performance [2,4,11]. And also considering thatthe noradrenergic system is closely related to the control of the circadian cycle [9]. And perhaps mainly, the noradrenergic system is also involved in behavioral patterns of affectivedisorders such as depression and anxiety, where less activity of this system is proven [8]. The results presented in the present study can establish a direct parallel between the non-motor symptoms observed at the beginning of PD with a decrease in the activity of the noradrenergic system. Where cognitive decline, lack of attention, sleep disorders, and especially anxiety and depression, which are relevant non-motor symptoms that affect patients' lives, could be directly related to the loss of neurons in the locus coeruleus and decreased activity of the noradrenergic system as a whole [4.7].

Our study opens a new path for the use of the noradrenergic system as a diagnostic tool for PD, where clinical symptoms related to noradrenergic functions could be evaluated in periodic medical consultations in the population under study age of development of PD. Another fact is that we correlate the non-motor symptoms already described for PD directly with the loss of neurons in the noradrenergic system [8]. Thisfinding also opens up possibilities for exams such as CT scans to be used as tools for early diagnosis of PD.

CONCLUSION

Our results demonstrate that the loss of the noradrenergic system can be directly related to the decrease in locomotor and exploratory activity, as well as, increased anxiety and fear in the studied animal model. Co-treatment with the ß2

adrenergic agonist clenbuterol was able to partially reverse some of the parameters evaluated. Based on our results, we conclude that the non-motor symptoms seen in Parkinson's disease can be directly related to the loss of the noradrenergic system previously reported for this pathology. Also, this knowledge can be used for the development of new tools for previous diagnosis of PD.

REFERENCES

1. Wirdefeldt K, Adami HO, Cole P, Trichopoulo D, Mandel J. Epidemiology and etiology of Parkinson's disease: a review of the evidence. European Journal of Epidemiology.2011; 26 (S1): 1-58.

2. Goedert M. Parkinson's Disease: Alpha-Synuclein and Neurodegeneration. Lancet, The. 2009; 461–7

3. COLLINS, LM et al. Contributions of central and systemic inflammation to the pathophysiology of Parkinson's disease. Neuropharmacology, 2012;62(7):2154-2168.

4. WILLIAMS-GRAY, CH et al. The CamPaIGN study of Parkinson's disease: 10-year outlook in an incident population-based cohort. Journal of Neurology, Neurosurgery & Psychiatry, 2013;84(11):1258–1264.

5. PETRUCELLI, L.; DICKSON, DW Neuropathology of Parkinson's Disease. Parkinson's Disease, p. 35–48, 2008.

6. VARDJAN, N .; ZOREC, R. Locus Coeruleus Noradrenergic Neurons and Astroglia in Health and Disease. In: .Noradrenergic signaling and astroglia. Academic Press, 2017; 01-24. cap. 01. E-book

7. FEINSTEIN, DL; KALININ, S .; BRAUN, D. Causes, consequences, and cures for neuroinflammation mediated via the locus coeruleus: noradrenergic signaling system. Journal of Neurochemistry, 2016;139:54–178.

8. BENARROCH, EE The locus ceruleus norepinephrine system: Functional organization and potential clinical significance. Neurology, 2009; 73(20):1699–1704.

9. WEINSHENKER, D. Long Road to Ruin: Noradrenergic Dysfunction in Neurodegenerative Disease. Trends in Neurosciences, 2018;41(4):211–223.

10. ITOI, Keiichi et al. Targeting of Locus Ceruleus Noradrenergic Neurons Expressing Human Interleukin-2 Receptor-Subunit in Transgenic Mice by a Recombinant Immunotoxin anti- Tac (Fv) -PE38: A Study for Exploring

Noradrenergic Influence upon Anxiety-Like and Depression-Like Beha. Journal of Neuroscience, 2011;31(16):6132 - 6139.

11. Kostrzewa RM. Evolution of neurotoxins: from research modalities to clinical realities. Curr Protoc Neurosc. 2009.

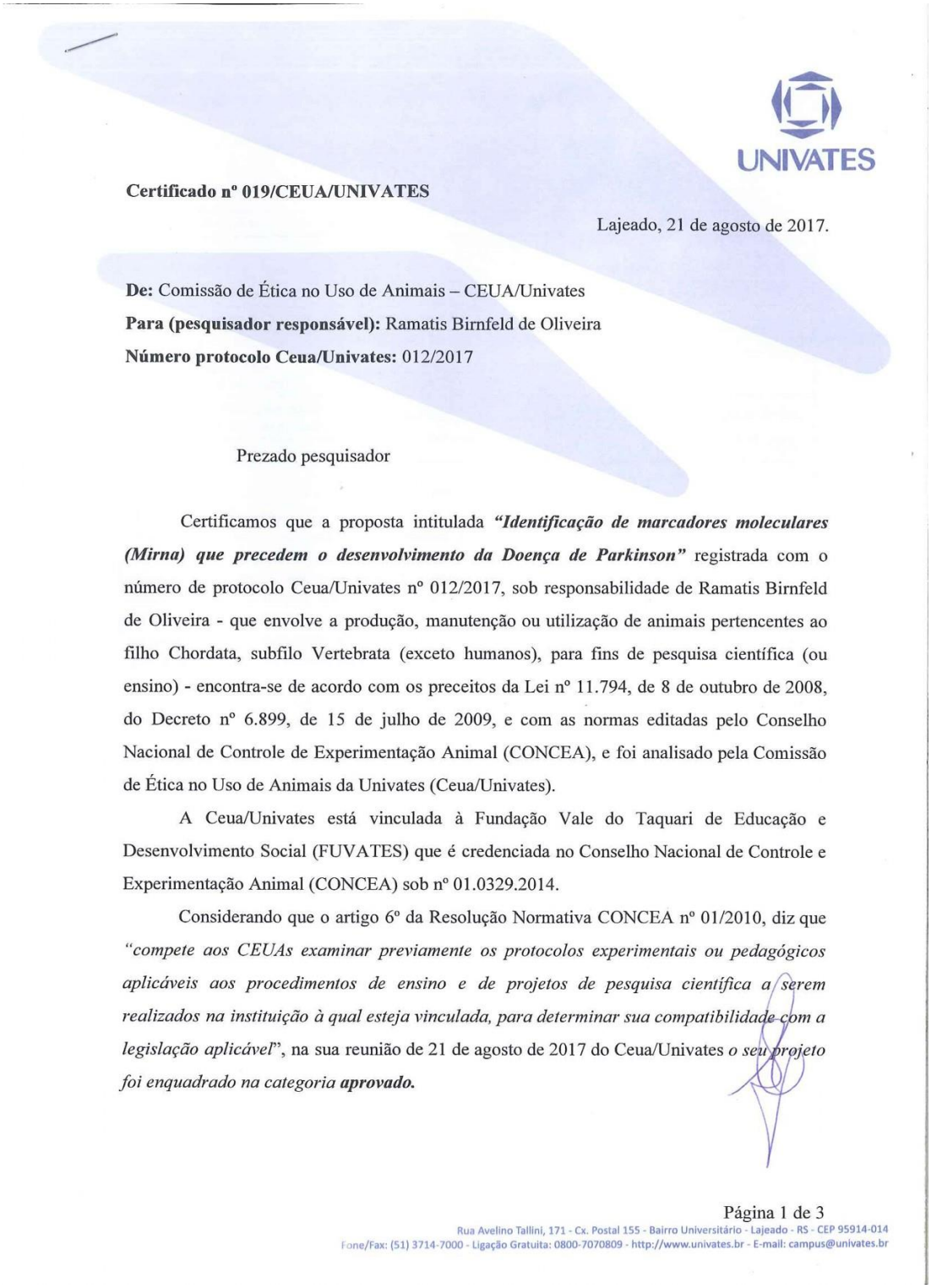
12. Verma P, Hellemans KGC, Choi FY, Yu W, Weinberg J. Circadian phase and sex effects on depressive / anxiety-like behaviors and HPA axis responses to acute stress. Physiol Behav. 2009; 99: 276–285.

13. Martins, A. et al. Supplementation with different teas from *Camellia sinensis* prevents memory deficits and hippocampus oxidative stress in ischemia-reperfusion. Neurochemistry International. 108, September 2017, Pages 287-295.

14. Pellow, S; Chopim, SE File, M Briley. Validation of open: closed arm entries in an elevated plus-maze as a measure of anxiety in the rat. J Neurosci Methods. 1985 Aug; 14 (3): 149-6.

11 ANEXOS

Anexo 1 – Carta de aceite do COEP – UNIVATES.



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Certificado nº 019/CEUA/UNIVATES

Lajeado, 21 de agosto de 2017.

De: Comissão de Ética no Uso de Animais – CEUA/Univates
Para (pesquisador responsável): Ramatis Birnfeld de Oliveira
Número protocolo Ceua/Univates: 012/2017

Prezado pesquisador

Certificamos que a proposta intitulada ***"Identificação de marcadores moleculares (Mirna) que precedem o desenvolvimento da Doença de Parkinson"*** registrada com o número de protocolo Ceua/Univates nº 012/2017, sob responsabilidade de Ramatis Birnfeld de Oliveira - que envolve a produção, manutenção ou utilização de animais pertencentes ao filho Chordata, subfilo Vertebrata (exceto humanos), para fins de pesquisa científica (ou ensino) - encontra-se de acordo com os preceitos da Lei nº 11.794, de 8 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA), e foi analisado pela Comissão de Ética no Uso de Animais da Univates (Ceua/Univates).

A Ceua/Univates está vinculada à Fundação Vale do Taquari de Educação e Desenvolvimento Social (FUVATES) que é credenciada no Conselho Nacional de Controle e Experimentação Animal (CONCEA) sob nº 01.0329.2014.

Considerando que o artigo 6º da Resolução Normativa CONCEA nº 01/2010, diz que *"compete aos CEUAs examinar previamente os protocolos experimentais ou pedagógicos aplicáveis aos procedimentos de ensino e de projetos de pesquisa científica a serem realizados na instituição à qual esteja vinculada, para determinar sua compatibilidade com a legislação aplicável"*, na sua reunião de 21 de agosto de 2017 do Ceua/Univates *o seu projeto foi enquadrado na categoria* ***aprovado.***

Página 1 de 3

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miRNAs em estágio prévio da DP poderá contribuir para o desenvolvimento de novos protocolos e metodologias que busquem detectar uma DP iminente. Orçamento: O projeto contempla plenamente o atendimento das necessidades para o desenvolvimento das atividades de pesquisa e todas as exigências com relação à metodologia. Metodologia: O modelo animal que será utilizado baseia-se na degeneração do sistema noradrenérgico com a neurotoxina DSP-4, que é capaz de degenerar o sistema noradrenérgico especificamente sem alterar outras estruturas cerebrais. A caracterização do perfil de miRNAs será feita pela técnica de PCR array com confirmação por RT-PCR.

De acordo legislação, cabe aos membros do Ceua/Univates, determinar a validade dos certificados por eles emitidos e portanto, já adiantamos que, o novo certificado a ser emitido após regularização da pendência acima descrita, terá validade máxima de 1 ano. Esclarecemos que é possível atualização de informações e prorrogação da validade do Certificado a qualquer tempo.

Após o recebimento do presente Certificado pelo pesquisador responsável, a secretaria da Ceua/Univates inserirá as informações sobre o experimento no Cadastro das Instituições de Uso Científico de Animais (CIUCA) do Conselho Nacional de Controle de Experimentação Animal (CONCEA).

No projeto apresentado consta Termo de Responsabilidade devidamente assinado onde o pesquisador responsável certifica que: a) leu o disposto na Lei nº 11.794, de 8 de outubro de 2008, e nas demais normas aplicáveis à utilização de animais em ensino e/ou pesquisa, especialmente as Resoluções Normativas do Conselho Nacional de Controle de Experimentação Animal – CONCEA; b) o experimento apresentado não é desnecessariamente duplicativo, possuindo mérito científico e a equipe participante foi treinada e é competente para executar os procedimentos descritos neste protocolo; e c) que não existe método substitutivo que possa ser utilizado como uma alternativa ao experimento.

Cátia Viviane Gonçalves
Coordenadora do Ceua/Univates
Portaria nº 393/Reitoria/Univates de 22/set/2014

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