UNIVERSIDADE FEDERAL DO PARANÁ
DEBORAH GALVÃO COELHO DA SILVA

EFEITOS DO PRAMIPEXOL NA VOCALIZAÇÃO ULTRASSÔNICA E COMPORTAMENTO TIPO-DEPRESSIVO EM MODELO ANIMAL DA DOENÇA DE PARKINSON INDUZIDO POR 6-OHDA EM RATOS

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Dissertação apresentada ao curso de Pós-Graduação em Farmacologia, Setor de Ciências biológicas Universidade Federal do Paraná, como requisito parcial à obtenção do título de Mestre em Farmacologia.

Orientador(a): Prof(a). Dr(a). Maria A.B.F Vital

Coorientador(a): Prof(a). Dr(a). Débora Dala Vecchia

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A outorga do título de mestre está sujeita à homologação pelo colegiado, ao atendimento de todas as indicações e correções solicitadas pela banca e ao pleno atendimento das demandas regimentais do Programa de Pós-Graduação.

CURITIBA, 31 de Agosto de 2019.

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RESUMO

A doença de Parkinson (DP) é a segunda mais frequente doença neurodegenerativa relacionada à idade, afetando geralmente indivíduos com mais de 60 anos. É caracterizada pelo comprometimento de diversas funções de controle sensório-motor, equilíbrio, marcha, função autonômica, cognição, comunicação e deglutição. Os principais sintomas motores, incluem tremor, rigidez, bradicinesia (lentidão na execução de movimentos) e alteração nos reflexos posturais, sendo estes sintomas causados pela perda neuronal dopaminérgica na substância negra pars compacta (SNc) e outras estruturas. Não obstante, outros fatores além da neurodegeneração dopaminérgica parecem estar relacionados à ocorrência dos sintomas da DP. O déficit de outros neurotransmissores parece estar envolvido na causa de vários sintomas não motores da doença, como dor, ansiedade, distúrbios do sono, depressão, entre outros que podem preceder os sintomas motores. A depressão é um dos sintomas não motores mais frequentes da DP, com uma prevalência média de 40% nestes pacientes. Alguns estudos têm sugerido que além dos tratamentos convencionais como os Inibidores Seletivos da recaptação de serotonina (ISRS), agonistas de receptores D2 da dopamina são efetivos no tratamento da depressão em pacientes com DP. A literatura mostra que o pramipexol parece modular a liberação espontânea de neurônios de dopamina, norepinefrina e serotonina em cérebros de ratos, sugerindo que a ação terapêutica deste fármaco pode ser atribuída ao aumento da neurotransmissão dopaminérgica e serotoninérgica. No entanto, apesar de demonstrar efeito antidepressivo ainda não se conhece o mecanismo farmacológico que leva a este efeito. No teste de preferência à sacarose, foi observado que após a lesão com 6-OHDA houve redução do consumo de sacarose no grupo tratado com veículo, apontando um caráter anedônico, de suma importância na caracterização do comportamento tipo deprimido, do mesmo modo, o grupo lesado tratado com PPX houve reversão parcial desse estado a partir do 21º dia de tratamento. No teste de natação forçada foi observado que os ratos tratados com PPX apresentaram um aumento no tempo de natação em relação aos grupos controles, indicando a reversão do comportamento tipo depressivo. No teste de vocalização ultrassônica foi observado que animais lesados tratados com PPX apresentaram aumento nas emissões de vocalizações de 50-kHz. Os dados

neuroquímicos indicam que o PPX foi capaz de promover o aumento de serotonina (5-HT) e dopamina (DA) no estriado e reduzir a expressão da enzima Indolamina-2,3-dioxygenase (IDO) no hipocampo.

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Palavras-chave: Depressão, Doenças neurodegenerativas, Agonista dopaminérgico, Neuroinflamação, 6-hidroxidopamina.

ABSTRACT

Parkinson's disease (PD) is the second-most common neurodegenerative disorder affecting individuals over 60 years of age. PD is characterized by the impairment of several functions of sensorimotor control, like balance, gait, autonomic functions, cognition, communication, and swallowing. The cardinal motor symptoms include resting tremor, rigidity, bradykinesia, and gait dysfunction. These symptoms are attributed mainly to a dramatic loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc). Regardless of the dopaminergic neurodegeneration, other factors may be related to the occurrence of PD symptoms. The deficit of other neurotransmitters is one of these factors, and it is linked to non-motor symptoms of the disease that can precede the motor ones, like pain, anxiety, sleep disturbances, and depression. Depression is one of the most frequent non-motor symptoms with a prevalence of 40% in PD patients. Studies have suggested that beyond the conventional treatments like the Selective Serotonin Reuptake Inhibitors (SSRI), agonists of the D2 dopamine receptors are effective in the treatment of depression in PD patients. Literature shows that pramipexole (PPX), a dopamine agonist, can modulate the release of neurotransmitters from dopamine (DA), noradrenaline (NA), and serotonin (5-HT) neurons in the rat brain, suggesting that the therapeutic action of this drug may be attributed to the increase of dopaminergic and serotonergic neurotransmission. However, despite demonstrating antidepressant effect, the pharmacological mechanism is not yet known. In the present study, the sucrose preference test showed that after the lesion with 6-hydroxidopamine (6-OHDA) the rat group treated with vehicle decreased the sucrose preference, pointing an anhedonic-like behavior; in the group lesioned with 6-OHDA and treated with PPX there was a partial recovery of this behavior after the day 21 of the treatment. In the forced swimming test, it was observed that the animals treated with PPX showed an increase in the swimming behavior in relation to control groups, indicating a significative improvement of the depressive-like behavior. In the ultrasonic vocalization test it was observed that the lesioned group treated with PPX had an increase in the 50-kHz vocalization emissions. The neurochemistry data indicate that PPX was capable of promoting the increase of 5-HT and DA in the striatum and reducing the expression of the enzyme indoleamine-2,3-dioxygenase (IDO) in the hippocampus.

Keywords: Depression, Neurodegenerative Diseases, Dopaminergic Agonist, Neuroinflammation, 6-Hydroxidopamine.

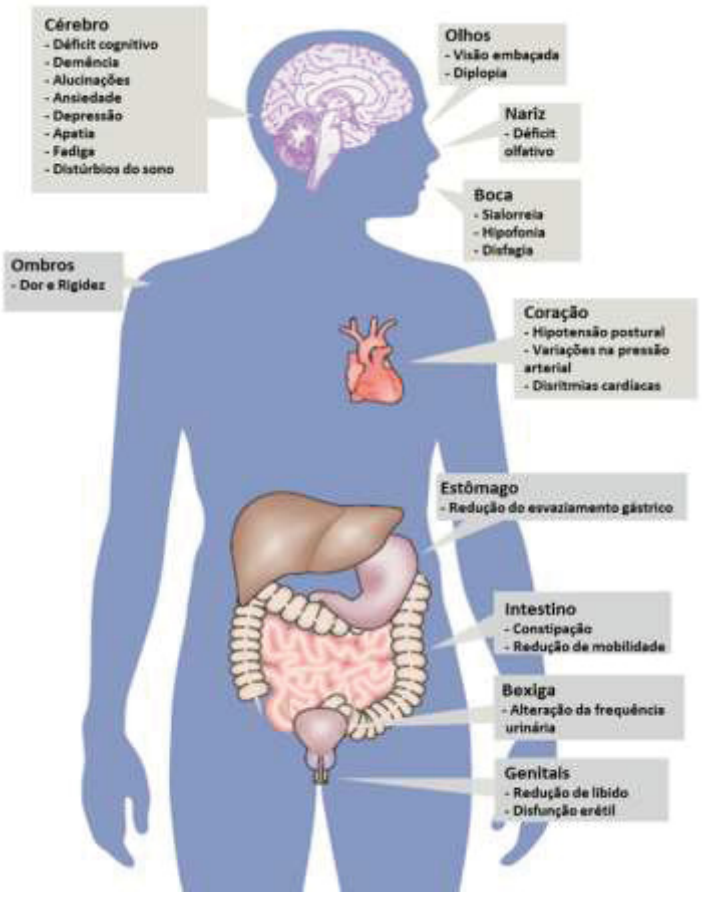
SUMÁRIO

1 INTRODUÇÃO

A doença de Parkinson (DP) é um dos distúrbios neurológicos mais frequentes, afetando aproximadamente 1% dos indivíduos com mais de 60 anos, causando incapacidade progressiva que pode ser retardada, mas não interrompida, por tratamento. Os sintomas motores são os principais utilizados no diagnóstico clínico da doença, sendo iniciados gradualmente de forma unilateral. Os tremores são comuns, mas a progressão da doença também causa rigidez e lentidão na execução dos movimentos (POEWE; SEPPI et al., 2017).

Os sintomas motores da DP são atribuídos à perda de neurônios dopaminérgicos do estriado, embora a presença de sintomas não motores também esteja relacionada a perda neuronal em áreas não-dopaminérgicas. Pesquisas sugerem que as alterações fisiopatológicas associadas à DP podem começar antes do início das características motoras e podem incluir uma série de apresentações não motoras, como distúrbios do sono, depressão e alterações cognitivas (DEL REY; QUIROGA-VARELA et al., 2018) (Figura 1).

FIGURA 1 – SINTOMAS DA DOENÇA DE PARKINSON



FONTE: Adaptado de Schapira et al. (2017).

De modo geral, os pacientes apresentam o início das características motoras da DP somente após cerca de 50% a 80% dos neurônios dopaminérgicos terem sido perdidos, sugerindo o envolvimento de um mecanismo compensatório nos estágios iniciais da doença (O'KEEFFE; SULLIVAN, 2018).

A progressão variável, mas pronunciada da DP, tem um impacto significativo nos pacientes, nas famílias e na sociedade, podendo levar a complicações sérias que requerem maiores cuidados. O tratamento atual é focado no tratamento sintomático sendo a levodopa o tratamento “ouro” no manejo dos sintomas motores, no entanto não há resposta efetiva para o tratamento dos sintomas não motores nesses pacientes (OBESO et al., 2017).

1.1 ETIOLOGIA

A etiologia da DP ainda é pouco compreendida apesar dos inúmeros avanços existentes. Os fatores que levam ao desenvolvimento da doença são complexos e envolvem desde alterações genéticas até fatores ambientais (Jellinger, 2015).

Dentre os fatores genéticos a alteração no gene da alfa-sinucleína (SNCA) é um dos principais conhecidos e foi descoberto há 22 anos atrás por Polymeropoulos e colaboradores (1997). Além desta alteração genética, outras mutações em genes também foram descobertas, como as dos genes LRRK2, VPS35, Parkin, PINK1 e DJ-1, os quais normalmente estão associados com a forma familiar da doença (Koros et al. 2017; Lill, 2016).

Alguns fatores ambientais também podem estar associados ao desenvolvimento da DP, dentre eles estão: exposição a pesticidas ou ambiente rural, consumo de água de poço, exposição a solventes orgânicos e poluentes, além disso exposições a infecções bacterianas e virais, traumas encefálicos, doenças como síndrome do intestino irritável, diabetes e até mesmo alterações na microbiota intestinal (Biosa et al. 2018; Jellinger, 2015; Liu, 2018).

Em 2003, Braak e colaboradores propuseram uma teoria que explicaria o desenvolvimento fisiopatológico da doença, baseando-se em autópsias de pacientes com DP. Nesta teoria foi proposto que o aparecimento de mutações na proteína alfa-sinucleína e a formação de corpos de Lewy se dariam primariamente em regiões do sistema nervoso entérico e bulbo olfatório e que esse aparecimento seria desencadeado por estímulos ambientais como patógenos, toxinas ambientais e proteínas infecciosas por exemplo (Stolzenberg et al. 2017). Após este primeiro estágio, a teoria de Braak propõem que haveria uma disseminação destes corpos de Lewy, através do nervo vago, para regiões da medula oblongata, tegmento pontino, regiões mesencefálicas (como a substância negra) além de outras regiões mesocorticais e neocorticais (Braak et al. 2003; Liu, 2018). Estes agregados proteicos desempenhariam um papel oxidativo e neuroinflamatório, levando a morte neuronal.

Diversos estudos mostram que a neuroinflamação da DP está associada ao aumento de citocinas pró-inflamatórias, como a IL-1β, IL-6 e TNF-α (Hirsch, 2009), e também com a hiperestimulação das células da glia, tais como a ativação microglial em regiões como a SNpc, estriado e hipocampo (Manocha et al. 2017).

Além da neuroinflamação, a doença está associada ao estresse oxidativo, que promove diversos efeitos prejudiciais sobre as células, levando a danos na membrana celular e até mesmo no DNA (Taylor et al. 2013). O estresse oxidativo na DP é resultante de diversos fatores, como a disfunção mitocondrial, formação de agregados proteicos, depleção de moléculas antioxidantes, dentre outros (Yan et al. 2013). Todos estes fatores contribuem para a degeneração neuronal e a depleção dopaminérgica.

1.2 EPIDEMIOLOGIA

O envelhecimento populacional é iminente em nossa sociedade atual. Dados da organização mundial da saúde mostram que a população atual tem vivido cerca de 20 anos a mais, comparado com gerações anteriores (WORLD HEALTH ORGANIZATION, 2015). Segundo dados do IBGE, a população com 65 anos ou mais no brasil representa cerca de 9,5%, e projeções para 2060 indicam que esta população representará 25% da população (IBGE, 2019). Em consequência desse envelhecimento populacional está o aumento de doenças associadas a idade, como a DP, que limitam a qualidade de vida do paciente (Franceschi et al. 2018).

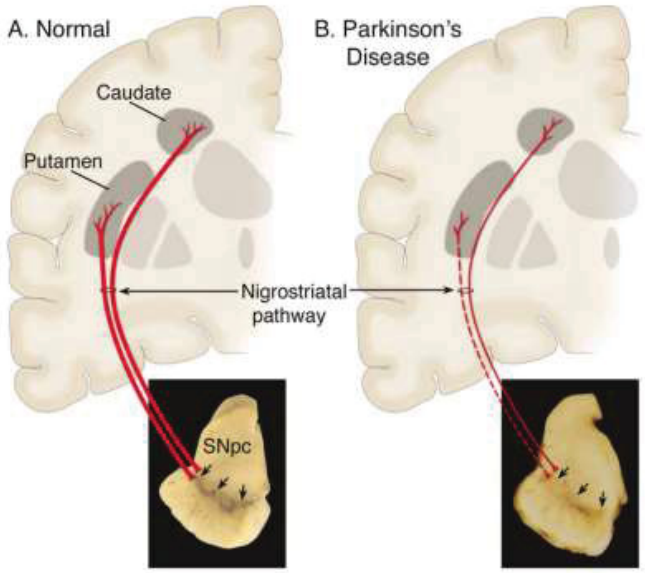
O número de pacientes com DP vem aumentado drasticamente nos últimos anos, correspondendo a mais de 6 milhões de pessoas em todo o mundo (Dorsey et al. 2018). Um estudo de meta-análise mostrou que a doença está intimamente relacionada com o avanço da idade (indivíduos com 80 anos ou mais apresentam maior prevalência da doença em comparação com indivíduos entre 65 e 79 anos) e, também, há uma maior prevalência da doença em indivíduos do sexo masculino, residentes de países Europeus e Estados Unidos (Pringsheim et al. 2014). Com base nestes dados e na severidade da doença torna-se importante a realização de novos estudos e a busca de tratamentos para a DP.

1.3 FISIOPATOLOGIA

As características histopatológicas da DP incluem a perda de neurônios dopaminérgicos pigmentados e a presença de corpos de Lewy. Corpos de Lewy são inclusões eosinofílicas intracitoplasmáticas, muitas vezes com halos, que são facilmente vistos em neurônios. Degeneração progressiva dos neurônios dopaminérgicos na substantia negra pars compacta (SNpc), que se projeta para o

estriado (a via nigrostriatal), resulta na perda da função dopaminérgica em indivíduos com DP (Figura 2). Tipicamente, os pacientes apresentam as características motoras da DP após cerca 50% a 80% dos neurônios dopaminérgicos terem sido perdidos, sugerindo o envolvimento de um mecanismo compensatório nos estágios iniciais da doença (WICHMANN, 2019).

FIGURA 2 – NEUROPATOLOGIA DA DOENÇA DE PARKINSON



FONTE: Dauer; Przedborski (2003).

Dois tipos de receptores de dopamina, D1 e D2 , influenciam a atividade motora no sistema extrapiramidal. Embora existam vários grupos de neurônios dopaminérgicos no sistema nervoso central (SNC), é a perda de células dopaminérgicas na SNpc que acredita ser responsável por algumas das manifestações motoras da DP (DEMAAGD; PHILIP, 2015).

1.4 DEPRESSÃO E DOENÇA DE PARKINSON

A depressão na DP é um dos distúrbios neuropsiquiátricos mais frequentes, e ocorre em cerca de 35% dos pacientes (AARSLAND et al., 2012). Entretanto, as estimativas de prevalência em relação à depressão na DP podem variar de 7 a 76%. Essa variação ocorre porque o distúrbio é subdiagnosticado nos pacientes com DP, ou ainda, a definição e o grau da depressão são avaliados de forma distinta entre os

estudos (AARSLAND et al., 2012; BOMASANG-LAYNO et al., 2015; DJAMSHIDIAN e FRIEDMAN, 2014). O correto diagnóstico da depressão em pacientes com DP é de grande importância, pois este distúrbio é o principal fator que piora a qualidade de vida, por causar a piora dos sintomas motores, contribuir para o declínio cognitivo e, assim, agravar a evolução da DP (BOMASANG-LAYNO et al., 2015; RIEDEL et al., 2010).

A fisiopatologia da depressão na DP ainda não está esclarecida. Estudos de imagem in vivo sugerem possíveis associações dos sistemas serotoninérgico, noradrenérgico e colinérgico, além do sistema límbico (MATSUI et al., 2007). Pacientes depressivos com DP podem apresentar perda neuronal maior e gliose em áreas catecolaminérgicas do cérebro (especificamente, locus coeruleus, nervo vago dorsal, e SNc) quando comparado com pacientes de DP sem depressão (CHAUDHURI; SCHAPIRA, 2009; OBESO et al., 2014). A depressão na DP se diferencia da depressão observada nos transtornos afetivos. A intensidade da depressão na DP varia de leve a moderada, e uma pequena parte dos pacientes apresenta transtorno depressivo maior. Pacientes com DP apresentam queixas de sensação de vazio e diminuída capacidade de demonstrar emoções. A anedonia – perda da capacidade de sentir prazer – é comum nos pacientes com DP. Pensamentos suicidas geralmente ocorrem, porém as tentativas de suicídio são raras (ZIEMSSEN; REICHMANN, 2007).

1.5 ENZIMA INDOLAMINA 2,3-DIOXIGENASE

Embora o déficit monoaminérgico seja a principal teoria sustentada a respeito da causa e agravamento da depressão, há estudos que apontam a participação da enzima Indoleamina 2,3-dioxigenase (IDO) no desenvolvimento da doença (MAZAREI; LEAVITT, 2015).

Ogawa et al (2014), apontam que pacientes deprimidos apresentam baixos níveis plasmáticos de triptofano (TRP), e a disponibilidade de TRP no plasma determina a taxa de síntese de 5-HT no cérebro. Este déficit está relacionado à redução da ingestão de alimentos, ou depleção serotoninérgica, promovendo intensificação da atividade enzimática da IDO, consequente à isso há redução dos níveis de 5-HT (ZINGER; BARCIA et al., 2011), desencadeando os sintomas depressivos.

Do mesmo modo, pacientes parkinsonianos apresentam alteração no metabolismo da via da quinurenina. Essa via é a principal envolvida na síntese e degradação do TRP, de modo que a ativação dessa via desvia o triptofano disponível da produção de serotonina para um catabolismo adicional. Isso ocorre quando a relação de quinurenina/TRP no plasma e no líquido cerebrospinal (CSF) é aumentada significativamente junto com 3-hidroxiquinurenina (3-HK), um composto neurotóxico que contribui para o dano oxidativo no putamên e na SNpc, podendo esse processo estar envolvido na neurodegeneração, além do quadro depressivo (SAVITZ, 2019).

Alguns estudos clínicos relataram que os metabólitos de KYN, TRP e 3-HK no soro e no LCR são mais altos em pacientes parkinsonianos do que controles, indicando um aumento na atividade da IDO. Em consequência, o aumento da produção de espécies reativas de oxigênio promove a atrofia do hipocampo, que pode ser associada à representação (LEWITT et al., 2013).

1.6 TRATAMENTO DA DEPRESSÃO ASSOCIADA À DP

Os tratamentos para a depressão na doença de Parkinson são geralmente insuficientes. Estima-se que cerca de 25% dos pacientes com DP são tratados com drogas antidepressivas (PICILLO et al., 2009). Os antidepressivos ISRS são os mais comumente usados em pacientes com a DP, entretanto, estudos clínicos, revisões sistemáticas e meta-análises sugerem que esses agentes não são mais eficazes que o placebo no tratamento dos sintomas principais da depressão nesses pacientes (AARSLAND et al., 2011). Além disso, alguns pesquisadores têm reportado um aumento dos sintomas parkinsonianos após o tratamento com antidepressivos em pacientes com DP deprimidos, enquanto outros reportam uma melhora de ambos os sintomas motores e depressivos (AVILA et al., 2015).

Ainda que as terapias para a reposição de dopamina para tratar a depressão na DP tenham se mostrado limitadas, alguns estudos sugerem que os agonistas dopaminérgicos como o pramipexol, o pergolide e o ropinirole podem ter propriedades antidepressivas significativas (BASSANI et al., 2014). Por exemplo, um estudo prospectivo, não controlado, de 6 meses mostrou que o ropinirole melhorou tanto sintomas de ansiedade quanto sintomas depressivos em pacientes com a doença de Parkinson com flutuações motoras, mas não nos pacientes sem essas flutuações (REKTOROVA et al., 2008). Em outro caso, uma meta-análise de estudos

controlados por placebo mostrou que o pramipexol melhorou o humor e sintomas motivacionais na DP em pacientes sem depressão (LEENTJENS et al., 2009), apesar de ser um estudo limitado pode ser mais uma indicação do papel dos agonistas dopaminérgicos na melhora da depressão na DP.

1.7 PRAMIPEXOL

O pramipexol (PPX) é um agonista dopaminérgico clinicamente utilizado no tratamento dos sintomas motores da DP. Alguns estudos têm sugerido que o PPX também é eficaz no tratamento da depressão em pacientes que não respondem aos tratamentos típicos (HORI; KUNUGI, 2013).

O PPX quando ligado pre-sinapticamente a neurônios dopaminérgicos, inibe a liberação de DA; no entanto, quando os neurônios dopaminérgicos são lesados, como acontece na DP, o PPX atua como um potente agonista pós-sináptico de receptor D2, demonstrando eficácia como tratamento (HORI; KUNUGI, 2012).

Estudos pré-clínicos e em humanos apontam que o PPX possui propriedades neuroprotetoras, provavelmente pela regeneração de neurônios dopaminérgicos, através da inibição da oligomerização da α-sinucleína e por suas propriedades antioxidantes (LUO et al., 2016).

Embora os estudos não sejam conclusivos a respeito dos efeitos do PPX no tratamento da depressão em modelos animais da DP, assim como o efeito neuroprotetor, sugere-se que o fármaco apresente características antidepressivas de acordo com estudos clínicos e pré-clínicos realizados.

1.8 MODELO DA 6-HIDROXIDOPAMINA (6-OHDA)

O uso de modelos animais induzidos por toxinas tem sido fundamental para a compreensão da fisiopatologia da DP. Com eles, é possível reproduzir os principais processos envolvidos na DP, como o estresse oxidativo, a neurodegeneração e a morte celular (HERNANDEZ-BALTAZAR et al., 2017). Estes modelos vêm sendo empregados para esclarecer os mecanismos patogênicos envolvidos na morte celular neural associada à DP, bem como as manifestações e alterações cerebrais, com a finalidade de que neuroprotetores possam ser desenvolvidos para controlar a progressão da doença (BLANDINI et al., 2008; BOVÉ; PERIER, 2012).

Dentre os modelos animais mais utilizados se encontram o 1-metil-4-fenil-1,2,3,6-tetrahidropiridina (MPTP), a 6-hidroxidopamina (6-OHDA), a rotenona e o

paraquat. De maneira geral, através de diversos mecanismos, todas essas toxinas simulam alterações fisiopatológicas, bioquímicas e histológicas da doença. Contudo, ainda não há um modelo que englobe todos os aspectos envolvidos na doença (VINGILL et al., 2018; ZENG et al., 2018).

A 6-OHDA é uma neurotoxina frequentemente utilizada como modelo de DP. Por não ser capaz de atravessar a barreira hemato-encefálica, é necessário que sua administração se dê diretamente na substância negra ou no estriado (JAKCSON-LEWIS et al., 2012). Após as injeções na substância negra, os neurônios dopaminérgicos começam a degenerar dentro de 24 horas e morrem progressivamente. Quando injetada no estriado, no entanto, a 6-OHDA produz uma degeneração dita retrógrada, mais prolongada, dos neurônios nigroestriatais, que dura de 1 a 3 semanas (DAUER; PRZEDBORSKI, 2003; BOVÉ et al., 2005; VINGILL et al., 2018).

A toxicidade induzida por 6-OHDA é relativamente seletiva para neurônios monoaminérgicos, resultante da captação preferencial por transportadores dopaminérgicos e noradrenérgicos (ZENG et al., 2018). Sendo semelhante à dopamina, a 6-OHDA apresenta alta afinidade pelo transportador deste neurotransmissor. Uma vez dentro do neurônio, a 6-OHDA se acumula no citosol e sofre auto-oxidação, promovendo uma alta taxa de formação de radicais livres. Além disso, como um mecanismo adicional, a toxina pode se acumular nas mitocôndrias, onde inibe a atividade da cadeia de transporte de elétrons, bloqueando o complexo I (BOVÉ et al., 2015; BLANDINI et al., 2008; WASIK et al., 2016). Embora a 6-OHDA não produza ou induza agregados proteicos ou inclusões do tipo Lewy como as descritas na DP, foi relatado que a 6-OHDA interage com a alfa-sinucleína. (JACKSON-LEWIS et al., 2012).

2 OBJETIVOS

Avaliar o efeito tipo-antidepressivo do pramipexol em um modelo de DP induzido pela infusão intranigral de 6-OHDA em ratos.

2.1 OBJETIVOS ESPECÍFICOS

- Induzir o comportamento tipo depressivo em ratos com a injeção de 6-OHDA
- Observar o efeito tipo-antidepressiva do PPX nos testes de natação forçada e preferência por sacarose;
- Avaliar o efeito do PPX no teste de vocalização ultrassônica
- Realizar imunohistoquímica para Tirosina Hidroxilase (TH) na SNpc.
- Avaliar expressão da enzima IDO no córtex e hipocampo de ratos lesados e tratados com PPX.

3 ARTIGO CIENTÍFICO

Os materiais e métodos, resultados e discussão do trabalho encontram-se no artigo científico anexo que foi submetido à revista Experimental Neurology.

EFFECTS OF PRAMIPEXOLE IN DEPRESSIVE-LIKE BEHAVIOR IN A 6-OHDA ANIMAL MODEL OF PARKINSON'S DISEASE

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Abstract

Parkinson's disease (PD) is the second most common age-related neurodegenerative disease, generally affecting individuals over 60 years of age. The major motor symptoms are linked to the dopaminergic neuronal loss in the substantia nigra pars compacta. Deficits of neurotransmitters other than dopamine (DA) seem to be involved in the etiology of non-motor symptoms of the disease with depression being one of the most common non-motor symptoms of PD. Pramipexole (PPX) a D2/D3 agonist approved both as a single therapy and as an adjunct to levodopa for the treatment of signs and symptoms of PD. Present study evaluated the depressive-like behavior in an animal model of PD and the effects of PPX as a potential therapy to reverse these signs. In the sucrose preference test, the 6-OHDA lesioned rats showed a reduction of sucrose consumption, indicating anhedonia. Besides a significant increase in the expression of indoleamine-2,3-dioxygenase (IDO) in the hippocampus and a reduction of tyrosine hydroxylase (TH) in the substantia nigra pars compacta (SNpc). In the ultrasonic vocalization (USV) test, 6-OHDA caused a significant reduction in the USV of 50-KHz. On the other hand, the lesioned rats that received PPX showed an increase in the 50-kHz USV calls and reduce the expression of indoleamine- 2,3-dioxygenase (IDO) in the hippocampus. Thus, PPX was able to increase the 5HT levels in the striatum.

Keywords: pramipexole, 6-OHDA, Parkinson’s disease, depressive-like behavior, ultrasonic vocalization, IDO.

Abbreviations: PD = Parkinson’s disease; 6-OHDA = 6-hydroxydopamine; aCSF = artificial cerebrospinal fluid; substantia nigra pars compacta (SNpc); tyrosine hydroxylase = TH

1. Introduction

Parkinson's disease (PD) is the second most common age-related neurodegenerative disease after Alzheimer's disease with about 1.5% of the world's elderly population over the age of 60 years old presenting PD (Lees et al., 2009). In addition to the occurrence of motor symptoms, non-motor symptoms such as mood disorders including depression and anxiety, hyposmia and REM sleep behavior disorder, can occur (Poewe et al., 2017; Mantri, Morley, 2018; O'Keee and Sullivan, 2018).

Depression is a frequent non-motor feature of PD affecting around 35% of patients (Aarsland et al., 2012). Several studies aimed to understand the characteristics of depression in PD. The current evidence indicates that depressive symptoms in PD patients may occur before the onset of motor symptoms, suggesting

that the neuropathological process of PD also increases the risk of depression (Hoek et al., 2011). There is a consensus for a critical involvement of monoamines, including the serotoninergic and noradrenergic pathways (Chernoloz et al, 2009; Frisina et al., 2009); however, in recent years the involvement of the dopaminergic system in this network of interactions has been suggested (Dunlop and Nemerof, 2007; Villas Boas et al., 2019).

Vocal changes are also observed in PD patients and they are grouped under the name of hypokinetic dysarthria (Sapir, 2014). Although hypokinetic dysarthria has been related to muscle rigidity, it can be also associated with impairment of non-motor factors of voice, such as internal cueing and vocal vigilance. In this line, lesion of nigral-striatal pathway causes an impairment of ultrasonic vocalizations of rats (Ciucci et al., Johnson et al., 2015; Vecchia et al., 2018).

Several clinical studies have shown that the use of dopaminergic agonists in the management of depression associated with PD has been effective due to the action on D2 and D3 receptors located in the nucleus accumbens (Nacc) (Sandoval-rincón et al., 2015). In this line, Pramipexole (PPX) is a full agonist at dopaminergic receptors of the D2 subfamily, namely the D3 receptors. Some studies have indicated that PPX is also effective in the beginning of depression treatment, and patients, who are refractory to other typical antidepressants, respond with improvements of depressive symptoms within four weeks after the initiation of treatment (Hori and Kunugi, 2012).

From another standpoint of view, some studies point at the indoleamine 2,3-dioxygenase (IDO) enzyme in the occurrence of depressive symptoms due to neuroinflammatory processes. Clinical trials reported that patients with depression showed an increase in the levels of metabolites of kynurenine pathways. However, few studies have specifically examined the relation between IDO expression and drugs (Myint and Kim, 2003; Souza et al., 2017).

Studies adopting the neurotoxin-induced animal models of PD, specifically intra-nigral injections of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) or 6-hydroxydopamine (6-OHDA), have demonstrated motor, cognitive, emotional, neurochemical, molecular, and sleep-related disruptions in animals (Ferro et al., 2005; Lima et al., 2007; Tadaiesky et al., 2008; Santiago et al., 2010). Furthermore, it was also found that the bilateral lesion of the substantia nigra pars compacta (SNpc)

with 6-OHDA induced prominent impairments in 50-kHz ultrasonic vocalization (USV) (Vecchia et al., 2018).

USV in rats and vocal communication in humans are controlled by similar physiological mechanisms. In rats, these vocalizations are communicative in character, associated with social and non-social conditions, which vary according to the age and context of the animal (Brenes and Schwarting, 2014). Adult and adolescent rats emit two different types of ultrasonic vocalizations. Low-frequency vocals called 22-kHz are emitted in situations considered aversive, such as predator exposure, foot-shocks, environmental noises, noxious stimuli, or punishment; And high-frequency vocalizations, called 50-kHz calls, which are emitted in positive emotional states such as sexual motivation, food intake, and exposure to psychostimulant drugs (Wohr and Schwarting, 2013; Simola, 2015).

Given the importance of study motor and non-motor symptoms of PD, the main goal of the present study was to evaluate the effect of PPX on ultrasonic vocalization and depressive-like behaviors in 6-OHDA-lesioned rats, an animal model of PD.

2. Material and methods

2.1. Animals

In this study were used male Wistar rats, three months old, weighing 280-320 g at the beginning of the experiment. All animals were obtained from the breeding colony of Federal University of Paraná. The animals had access to food and water *ad libitum*, were maintained in a temperature-controlled room (22°C ± 2°C) on a 12 h/12 h light/dark cycle (lights on at 7:00 AM), and were randomly housed in groups of 5 in polypropylene cages with wood shavings as bedding. All tests and procedures were conducted in accordance with national and international legislation (Brazilian Council of Animal Experimentation – CONCEA – and U.S. Public Health Service's Policy on Human Care and Use of Laboratory Animals – PHS Policy guidelines), and they were approved by the Ethics Committee for Animal Research of the Federal University of Paraná (CEUA- Protocol #1206). All efforts were made to minimize animal suffering and to reduce the number of animals used.

2.2. Drugs

The drugs used were: pramipexole (1.0 mg/kg i.p., Biossintética, MS-Brazil), Imipramine (20 mg/kg i.p., Novartis, Taboão da Serra-Brazil), 6-OHDA (6-hydroxydopamine hydrochloride, Sigma, St. Louis, MO, USA). Saline 0.9% was utilized as vehicle. The drugs were diluted in 0.9% saline.

2.3. Experimental design

The animals were first evaluated for their basal sucrose preference and ultrasonic vocalizations as described in detail in items 2.5 and 2.7. After this, the animals were divided into two groups (n = 7-11/group): (I) sham-operated group (bilaterally infused vehicle in the SNpc), and (II) 6-OHDA group (6 µg bilaterally infused into the SNpc). After the surgery the animals were subdivided into six groups: (a) sham+vehicle (n=9), (b) 6-OHDA+vehicle (n=10), (c) sham+imipramine (n=9), (d) 6-OHDA+imipramine (n=11), e) sham+PPX (n=7), f) 6-OHDA+PPX (n=8). The treatments with pramipexole (1 mg/kg, i.p.), imipramine (20 mg/kg, i.p.) or its vehicle (0.9% saline) was performed over 28 days, once a day in the afternoon, and started 1 hour after surgery. The animals were assessed for their sucrose preference test and ultrasonic vocalizations on days 14, 21 and 28 days after stereotaxic surgery). The same animals were also tested in the modified forced swim test 28 days (training session) and 29 days (test session) after surgery. At the end of these tests, a subset of rats (n=7-11/group) was euthanized by decapitation, followed by dissection of the striatum and hippocampus. These structures were stored at -70ºC until processed for neurochemical analysis. Another subset of rats (n=5-6/group) were deeply anesthetized with chloral hydrate (300 mg/kg, i.p.) and sodium thiopental (30 mg/kg, i.p.). The animals were intracardially perfused for posterior immunohistochemical evaluation tyrosine hydroxylase (TH; a marker of catecholaminergic neurons) in the substantia nigra pars compacta (SNpc) and IDO expression in the hippocampus.

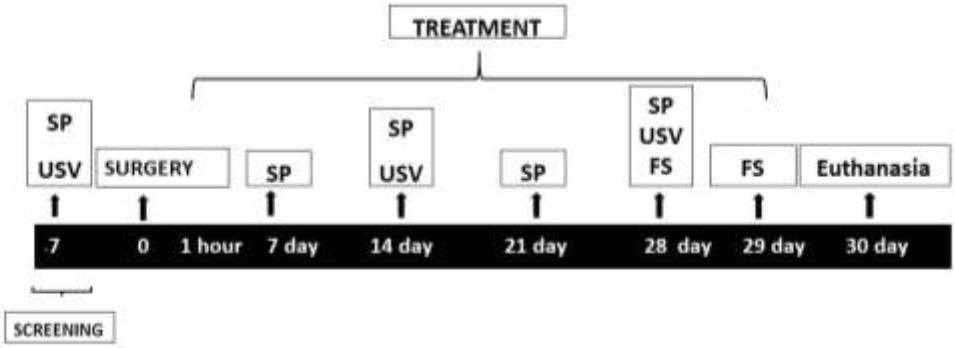


Figure 1. Experimental design. Sucrose preference test (SP); ultrasonic vocalizations (USV); Forced swimming test (FST).

2.4. Stereotaxic surgery

The neurotoxin 6-OHDA was prepared according to established doses that promote significant dopaminergic neuronal loss (Santiago et al., 2015, Ferro et al., 2005; Vecchia et al., 2018). The animals were anesthetized with Equithesin (3.0 ml/kg intraperitoneal, i.p.), and were bilaterally infused with 6 µg of 6-OHDA or saline on SNpc through a 27-gauge stainless steel needle according to the following coordinates: antero-posterior (AP): -5.0 mm from bregma; medio-lateral (ML): ± 2.1 mm from the midline; dorso-ventral (DV): -8.0 mm from skull (Paxinos and Watson, 2005). The flow of injection was controlled with an electronic pump (Harvard Apparatus, Holliston, USA) at a rate of 0.33 µL/min for 3 min and the needle was left in place for additional 2 min to avoid reflux. At the end of the injections, the needle was left in place for an additional 2 min to avoid reflux. After surgery, all the rats received Pentabiotic (0.1 ml, intramuscular) to prevent infection and were allowed to recover from anesthesia for 2-4 h in a heated and well-ventilated room. Food and water were placed inside the cage for 7-10 days so that the animals could easily access it without physical trauma caused by head surgery.

2.5. Sucrose preference test (SP)

The sucrose preference test is performed to evaluate anhedonia in rodents (Santiago et al., 2010). For this purpose, the animals were transferred to single housing cages with free access to food and water. Each rat was provided with two pre-weighed bottles of water, placed on the extreme sides of the cage during the 24h habituation phase. After this, one bottle position contained a 0.5% sucrose solution, as described previously, and 24h later, the bottle positions were reversed (Souza et

al., 2018). The tests were performed over 7 days before the stereotactic surgery to evaluate the basal sucrose preference, and after the surgery, they were evaluated weekly (on days 7, 14, 21 and 28) using the same concentration of sucrose.

2.6. Modified Forced Swimming Test (FST)

This procedure was a modification of the method proposed by Porsolt et al. (1978). The test was conducted in two sessions on day 28. In the first session, the rats were placed for 15 min into a tank (25-cm diameter, 65-cm height) that contained water at a temperature of 24±1ºC and a depth of 30 cm. Twenty-four hours later, the rats were submitted to the FST for 5 min which was videotaped for the later quantification of the following parameters: immobility (i.e., absence of motion of the entire body and only small movements necessary to keep the animal's head above the water), climbing (i.e., vigorous movements of the forepaws in and out of the water, usually directed against the wall of the tank), and swimming (i.e., large forepaw movements that displaced water and moved the body around the cylinder and which were necessary to keep the head above the water). The water was changed after each animal to avoid the influence of urinary or fecal material and temperature changes (Santiago et al., 2010; Bassani et al., 2014; Santiago et al., 2015).

2.7. Ultrasonic vocalization (USV)

USV were recorded in the so-called cage test (Schwarting et al., 2007). To perform the test, rats were habituated in a separate room for 30min. Then, they were transferred to the test room and placed individually into clean to cages (identical their homecage) containing fresh bedding under low illumination (4 lx). This test was first performed prior to surgery, when it served as a screening test (5min sessions on two consecutive days, performed at the same period of the day). This screening test was performed because rats showed a large inter-individual variability in the emission of 50-kHz USV. Those subjects that vocalized 1 standard deviation below the cohort mean were excluded from the following experiments. The test was repeated in the same conditions on days 14 and 28 after the drug treatment was started. Ultrasonic vocalizations were captured by a microphone (Ultrasound Gate Microphones, CM 16; Avisoft Bioacoustics) which was placed 45 cm above the center of the floor of the box where the animal was tested. The acoustic data were recorded using the Avisoft

Recorder Software program (version 2.7, Avisoft Bioacoustics; sampling rate: 214, 285Hz; format: 16 bit), and for the acoustic analysis, the recordings were transferred to SAS Lab Pro software (version 4.38, Avisoft Bioacustic; 512 FFT-length, 100% frame, Hamming window, 75% window overlap). Call numbers call types were determined. Call classification was made according to the shape of each vocalization (Schwarting et al., 2007; Burke et al., 2017; Vecchia et al., 2018). Fifty-kHz USV can be divided into simple and frequency-modulated calls. A flat (or simple) call was classified as such when the peak frequency changes within a single call element were equal to or, less than, 5 kHz. Nevertheless, the difference between the start and end peaks is greater than 5 kHz. In addition, calls with a flat shape in the up or down direction were also considered flat calls. Frequency-modulated (FM) calls consisted of trills, and step and mixed call. Trills: a unique call element with a peak frequency shift greater than 5 kHz or with two or more peak frequency changes in opposite directions with at least a 5 kHz distance. Step: when a basic flat call had at least one short flat element superimposed at the beginning or at the end of the call; it is necessary that one of these short "steps" is 5 kHz larger than the basic call. Mixed calls: calls that do not fall into the previous categories of step and trill calls, and consist of at least two types of components, e.g. trill and flat or step and flat. 50-kHz USV's were analyzed offline and classified by one experimenter who was blind to group conditions. The call number, subtype calls, and time durations of the calls were determined (Schwarting et al., 2007; Burke et al., 2017; Vecchia et al., 2018).

2.8. Tyrosine hydroxylase immunohistochemistry

At the end of the experiments (29 days), a subset of rats (n=5-6/group) were deeply anesthetized with chloral hydrate (300 mg/kg, i.p.) and sodium thiopental (30 mg/kg, i.p.) and intracardially perfused with saline, followed by the fixative solution (4% paraformaldehyde in 0.1M phosphate buffer, pH7.4). The brains were removed and immersed in the fixative solution for 24 h at 4°C. Next, the brains were placed in a 30% sucrose solution for at least 72h until they sank, and they were then stored at −70°C. Four series of 30μm frontal sections were cut in a cryostat and collected from the caudal diencephalon to caudal midbrain, according to Paxinos & Watson (Souza et al., 2018; Vecchia et al., 2018). Then sections were collected from the SNpc and placed into plates containing an anti-freeze solution. The tissue sections were incubated with primary anti-tyrosine hydroxylase (TH) monoclonal anti-bodies

(1:2000; Chemicon, Temecula, CA, USA) and diluted in phosphate-buffered saline (PBS). After overnight incubation at 4°C with primary antibodies, the sections were washed with PBS and incubated with the ready-to-use secondary antibody Envision plus (Dako Cytomation, Carpinteria, CA, USA) for 1 h at room temperature. The sections were then incubated with the anti-mouse IgG biotin-conjugated polyclonal secondary antibody (1:300; Sigma Aldrich, St Louis, MO) for 2h at room temperature. The antibody complex was detected using a modification of the ABC system (Vectastain ABCE lite kit, Vector Laboratories), followed by a reaction with 3,30-diamino-benzidine (Vector Laboratories DAB Substrate Kit for Peroxidase). The sections were placed on gelatin-coated slides, air dried, dehydrated in ascending ethanol concentrations, cleared in xylene and cover-slipped, and were then digitized under a 10× magnification with a DP71 Olympus Optical digital camera using a BX50 IX71 microscope (Olympus Optical, Tokyo, Japan). A digital area was created to delimitate the boundaries of the SNpc. For each analysis, the same area was adopted. All counts were performed in images obtained at a 40x magnification. A mean number of neurons in the SNpc was obtained for each group, and the results are expressed as a percentage of the sham+saline group. An unbiased quantification of the TH-labeled neuronal optical density in the SNpc was performed using ImageJ 1.51f (NIH, Bethesda, MD, EUA) software.

2.9. Evaluation of IDO expression

Animals were decapitated, their brains were rapidly removed and the prefrontal cortex (PFC) and hippocampus dissected. Until being processed for analysis, tissues were stored at -80 ºC. After that, a lysis buffer containing protease and phosphatase inhibitors (PBS-Triton X-100 1%, pH= 7.4, PMSF 1 mM, phenanthroline 1 mM, N-Ethylmaleimide 1 mM, Sodium Fluoride 5mM, sodium pyrophosphate 1mM, sodium orthovanadate 2 mM) was added and tissues were manually disrupted on ice using a micropestle (Eppendorf). After centrifugation, the supernatant was collected. Protein concentrations were determined by the Bradford protein assay (Biorad, Germany). From each sample, 40 ug was added to a well of a 15% bis-acrylamide gel and Sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed. Later, proteins were transferred to a nitrocellulose membrane (GE Healthcare). Membranes were blocked with 5% slime milk TBS-T for 30 minutes at room temperature and incubated overnight with the

desired antibody diluted in the same solution. Antibodies used were Santa Cruz Biotechnology anti-IDO (sc-53978) and Sigma anti-β actin (A5441). After incubation, membranes were extensively washed with TBS-T and incubated with the respective HRP-conjugated secondary antibody in blocking solution for 45 min at room temperature. Finally, membranes were washed again, and immune complexes were detected using the ECL chemiluminescent detection system (GE Healthcare Life Sciences, Brazil) and Westar ηC Ultra 2.0 (Cyanagen). Protein levels were quantified by densitometry using ImageJ v1.47 software (National Institutes of Health, USA). The antibodies used were Anti- IDO in proportion 1:400, Anti-rat: 1:10000, Anti-beta-actina: 1:10000, Anti-mouse: 1:6000.

2.10. Neurochemical analysis immunohistochemistry

After the end of experiments, a subset of rats (n=7-11/group) was euthanized by decapitation, followed by dissection of the striatum and hippocampus. The striatal tissue levels of the dopamine (DA); serotonin (5-HT); 3,4-dihydroxyphenylacetic acid (DOPAC); homovanillic acid (HVA) were quantified by using high performance liquid chromatography (HPLC-ED) with electrochemical detection. After weighing, the tissue samples were sonicated (Sonics) in 0.1 M perchloric acid containing 0.02% sodium metabisulfite (Sigma) and 50 ng/ml of the internal standard 3,4-dihydroxybenzylamine hydrobromide (Sigma). After centrifugation at 10,000 RPM for 30 minutes at 4°C, 20 µl of the supernatant were injected into a HPLC (Shimadzu) with a reverse phase C-18 column (Synergi Fusion-RP C-18; 150 x 4.6 mm ID) and an ESA Coulochem III detector equipped with a guard cell of 350 mV (ESA 5011A) and an LC-20AT HPLC pump (Shimadzu). The column was maintained at a controlled temperature (25°C). The cell contained two chambers in series: each chamber included a colorimetric graphite electrode, a double counting electrode and a dual reference electrode. Oxidation potentials were adjusted to 100 mV for the first electrode and 450 mV for the second electrode. The mobile phase was run at 1 mL / min and had the following composition: 20 g of citric acid monohydrate (Merck), 200 mg 1-octane sulfonic acid (Merck), 40 mg of ethylene diamine tetraacetic acid (EDTA, Sigma) in 900 mL of HPLC grade water. The pH of the buffer was adjusted to 4.0, then filtered through a 0.45 µm diameter pore filter. Then, methanol (Merck) was added until a final concentration of 10% (v/v) was reached. The concentrations of the neurotransmitters and their metabolites were calculated by the area under the curve

(current vs time) interpolated to a straight-line standard obtained from known concentrations of each chemical substance analyzed. The unit used to express these substances was ng/g tissue weight. (Bortolanza et al., 2010; Santiago et al., 2015).

2.11. Statistics

Data were expressed as means ± SEM. The FST, sucrose preference test and neurochemical data were analyzed using a two-way ANOVA for repeated measures (time and treatment as factors) followed by the Tukey post-hoc test when factor interaction was significant. The neurochemical and USV data were analyzed by unpaired Student's t-tests. The western blot results were analyzed using one-way ANOVA followed by Bonferroni post hoc tests. Values are expressed as mean ± standard error of mean (SEM). The level of significance was set at $p<0.05$.

3 Results

3.1. Sucrose preference test

In the sucrose preference test the results showed in the Figure 1 indicated that the 6-OHDA+vehicle group exhibited a significant reduction of the sucrose consumption on day 7 and 21 in comparison to the sham+vehicle group ($p<0.05$) $F(4, 188) = 5.470$. Thus, the 6-OHDA+PPX group showed a significant decrease in the consumption of sucrose on day 14 in comparison to sham+vehicle ($p<0.05$) $F(5, 47) = 5.053$.

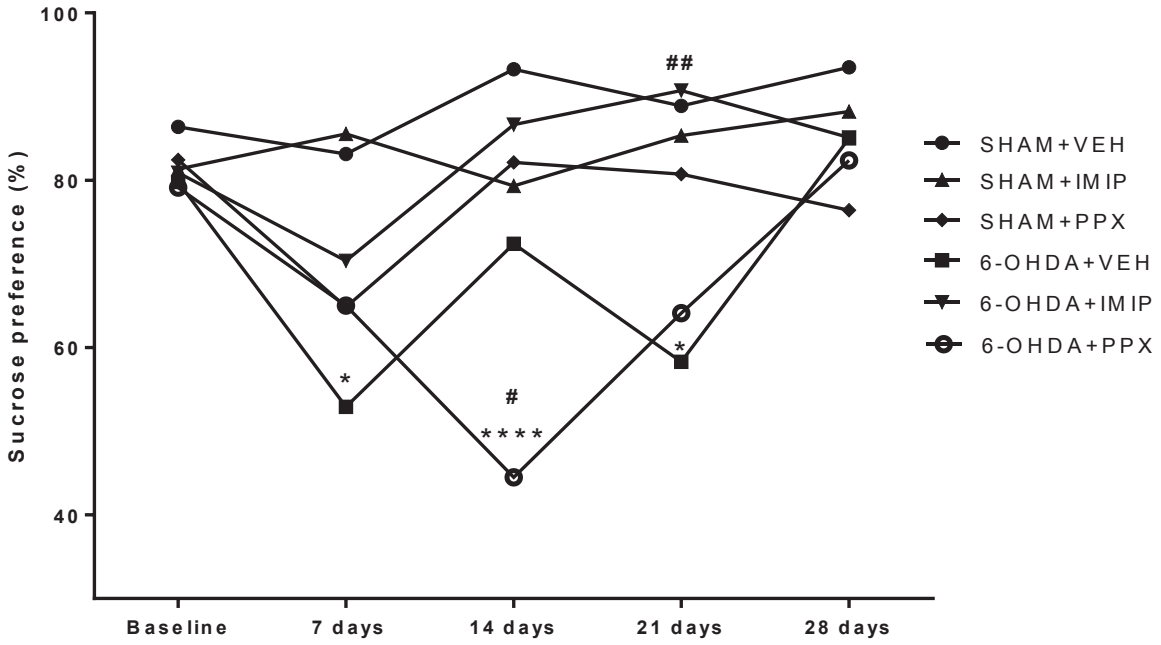


Fig. 1. Effect of PPX in the 6-OHDA lesioned rats in the sucrose preference test. The sham and 6-hydroxydopamine (6-OHDA) groups were treated with PPX (1.0 mg/kg i.p. daily), imipramine (20mg/kg i.p. daily) or vehicle (i.p., daily); Time points: 7, 14, 21 and 28 days after surgery. Data represent mean ± SEM; 6-OHDA+vehicle group: n=10 rats; sham+vehicle group: n=9 rats. 6-OHDA+ imipramine group: n=11; sham+imipramine group n=9; 6-OHDA+PPX group n= 8; sham+PPX group n= 7. *p< 0.05 compared with the sham+vehicle group,****p<0.05 compared with the 6-OHDA+vehicle group, and ## p<0.05 compared with 6-OHDA+vehicle group at same time point.

3.2. Forced swimming test

The immobility time in the Figure 2A shows the imipramine and PPX groups independently of the lesion showed a reduction in this parameter when these groups were compared to 6-OHDA+vehicle group (p<0.05, F (2, 54) = 13,48.) Regarding the swimming time (Figure 2B) it is possible to observe that PPX treated rats (both sham and 6-OHDA) had an increase in comparison to sham +vehicle rats (p<0.05, F (2, 54) = 34,12). Complementarily, the analysis of the climbing behavior demonstrated that imipramine was able to increase this parameter in both sham and lesioned group in

comparison to sham+vehicle and 6-OHDA+vehicle group ($p<0.05$, $F(2, 54) = 22,47$) (Figure 2C).

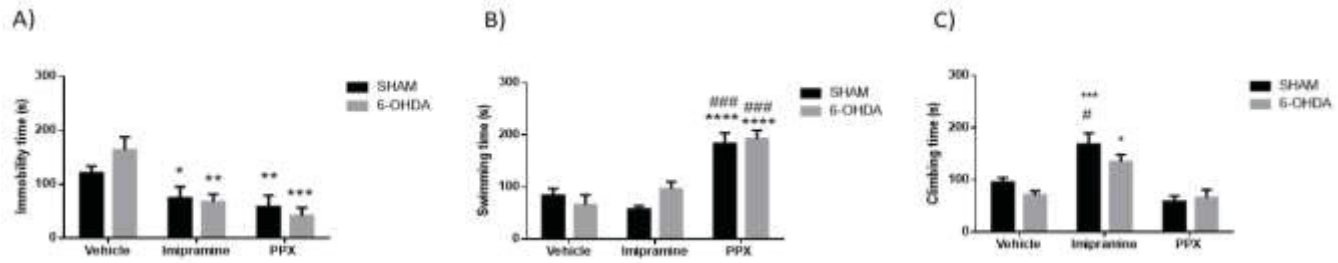


Fig. 2. Effect of administration of PPX (1.0 mg/kg/ i.p daily), imipramine (20mg/kg i.p. daily) or vehicle (i.p., daily), (A) Immobility time. (B) Swimming time. (C) Climbing time. Data represent mean ± SEM; 6-OHDA+vehicle group: n=10 rats; sham+vehicle group: n=9 rats. 6-OHDA+ imipramine group: n=11; sham+imipramine group n=9, sham+PPX group n= 7; 6-OHDA+PPX group n= 8; # $p<0.05$ compared with sham+vehicle group and *$p<0.05$ compared with 6-OHDA+vehicle group.

3.3. Ultrasonic vocalizations (USV)

Figure 3 illustrates the USV findings in the current study. The 6-OHDA+vehicle group showed a significant reduction in the numbers of 50-kHz calls when compared to the sham+vehicle on the 14th day ($F=87.42$, $p=0.001$; Fig. 3A). By contrast, the 6-OHDA+PPX group showed a significant increase in the numbers of 50-kHz calls when compared to the 6-OHDA+vehicle group in the 14th day ($F=56.72$, $p=0.0004$; Figure 3C) and 28th day ($F=8.316$, $p=0.0364$; Figure 3D). Moreover, on subtypes of USV, the 6-OHDA+PPX group showed an increase in the emission of flat subtype calls when compared to the 6-OHDA+vehicle group ($F=6.879$, $p=0.0255$; Fig 3G). There is no difference in USV between the sham+vehicle and sham+PPX groups (Figs 3E, 3F).

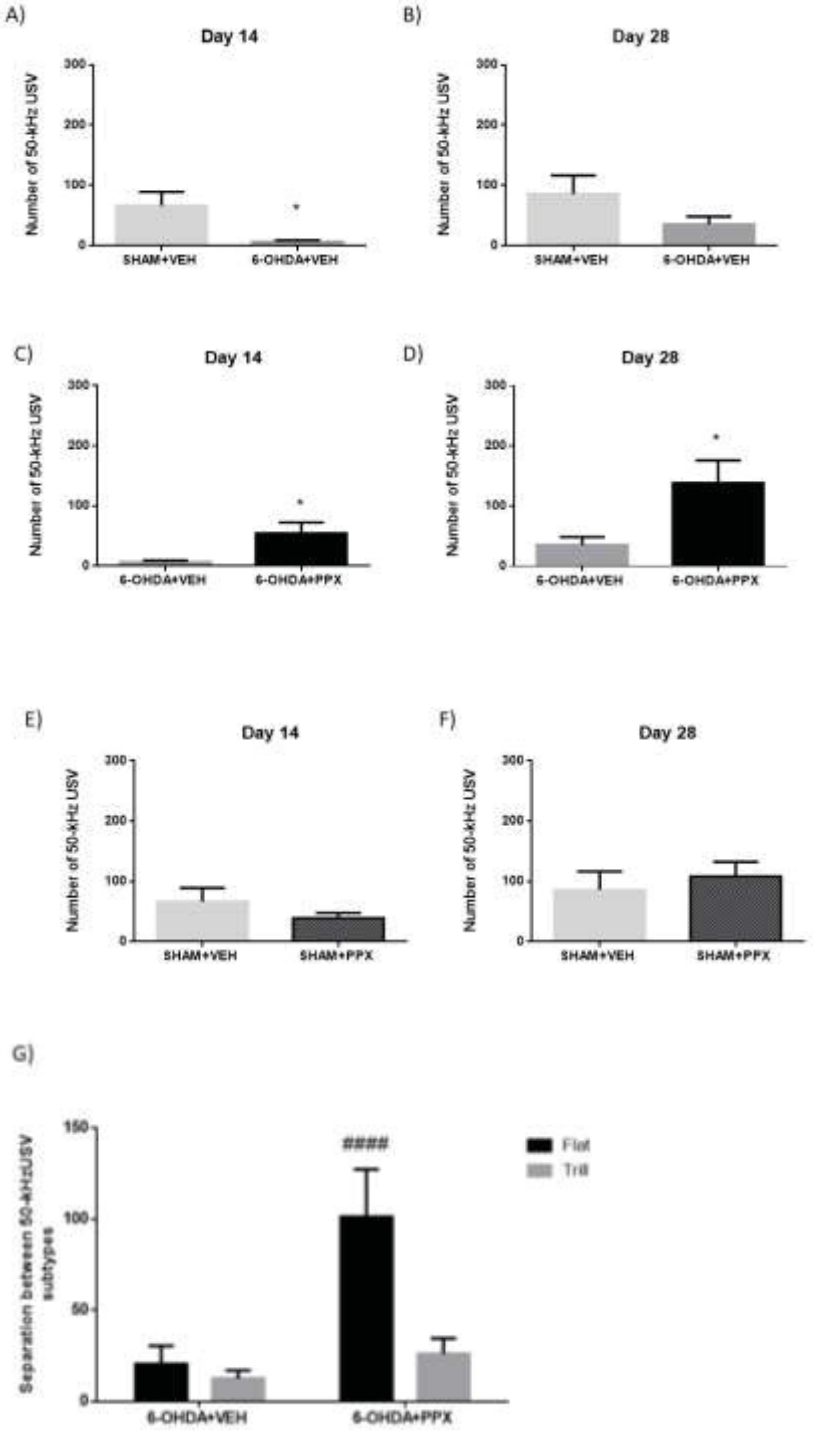
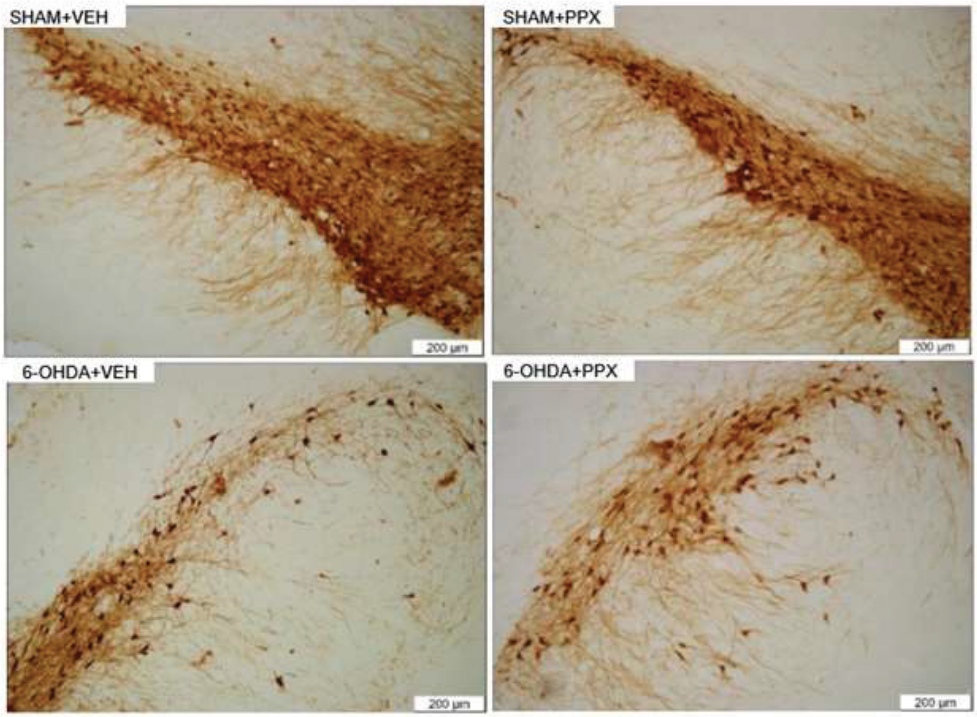


Fig. 3. Effect of bilateral lesion of SNpc with 6-OHDA on 50-kHz ultrasonic vocalizations. (A-F) Number of calls per 5 min; (G) Subtypes of USV. The 6-OHDA and sham groups were treated with PPX (1.0 mg/kg, i.p. daily) or vehicle (i.p. daily). Data represent mean ± SEM; sham+vehicle group: n=9, 6-OHDA+vehicle group: n=10 rats; 6-OHDA+PPX group: n=8 rats. Two-tailed unpaired Student's t-test. # $p<0.005$ when compared to the sham+vehicle group and * $p< 0.05$ in comparison with the 6-OHDA+vehicle group.

3.4 . Immunohistochemical analysis

Immunohistochemistry of tyrosine hydroxylase (TH) in the SNPc was performed at day 28 after surgery. As shown in Figure 4, there was a significant reduction in the number of TH-immunoreactive neurons both in the 6-OHDA+vehicle group (45%) and 6-OHDA+PPX (44%) when they were compared to the sham+vehicle group ($p<0.05$, $F(1, 11) = 38,89$).



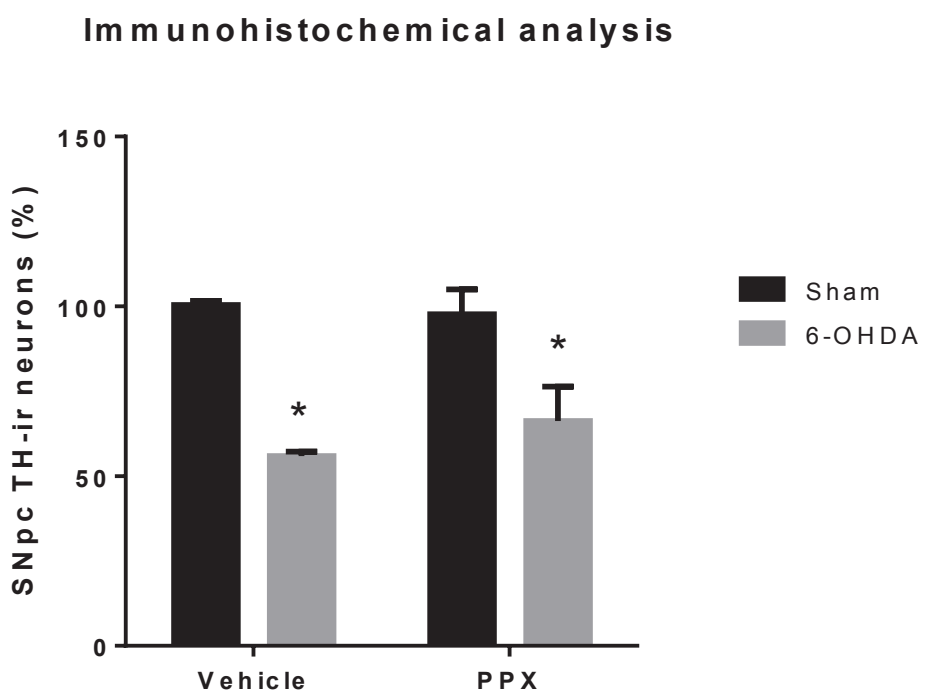


Figure 4. Effect of bilateral lesion of SNpc with 6-OHDA on immunohistochemical analysis of the SNpc 28 days after the stereotaxic surgery. Quantification of TH-ir neurons in the SNpc in each group (10x magnification). The data are expressed as a percentage of the control group. The sham and 6-OHDA groups were treated with PPX (1.0 mg/kg i.p. daily) or vehicle (i.p., daily) for 28 days. (A) colored sections of each group. (B) percentage of total TH in the SNpc. Sham+vehicle n=5, 6-OHDA+Vehicle n= 5, sham+PPX n=6, 6-OHDA+PPX n=6. Two-way Anova followed by the Bonferroni post-hoc test. * $p<0.05$ in comparison to sham+vehicle group.

3.5 Neurochemical analysis

According to Figure 5A the neurochemical data indicates that the 6-OHDA+PPX group showed an increase in levels of striatal DA when compared to the 6-OHDA+vehicle group ($F=10.35$, $p=0.0123$). Moreover, the sham+PPX group exhibited an increase in levels of striatal 5-HT when compared to the sham+vehicle and the 6-OHDA+vehicle groups ($F=18.62$, $p=0.0035$, Fig. 5D). There was no difference between the metabolites of both the amines on striatum of these animals.

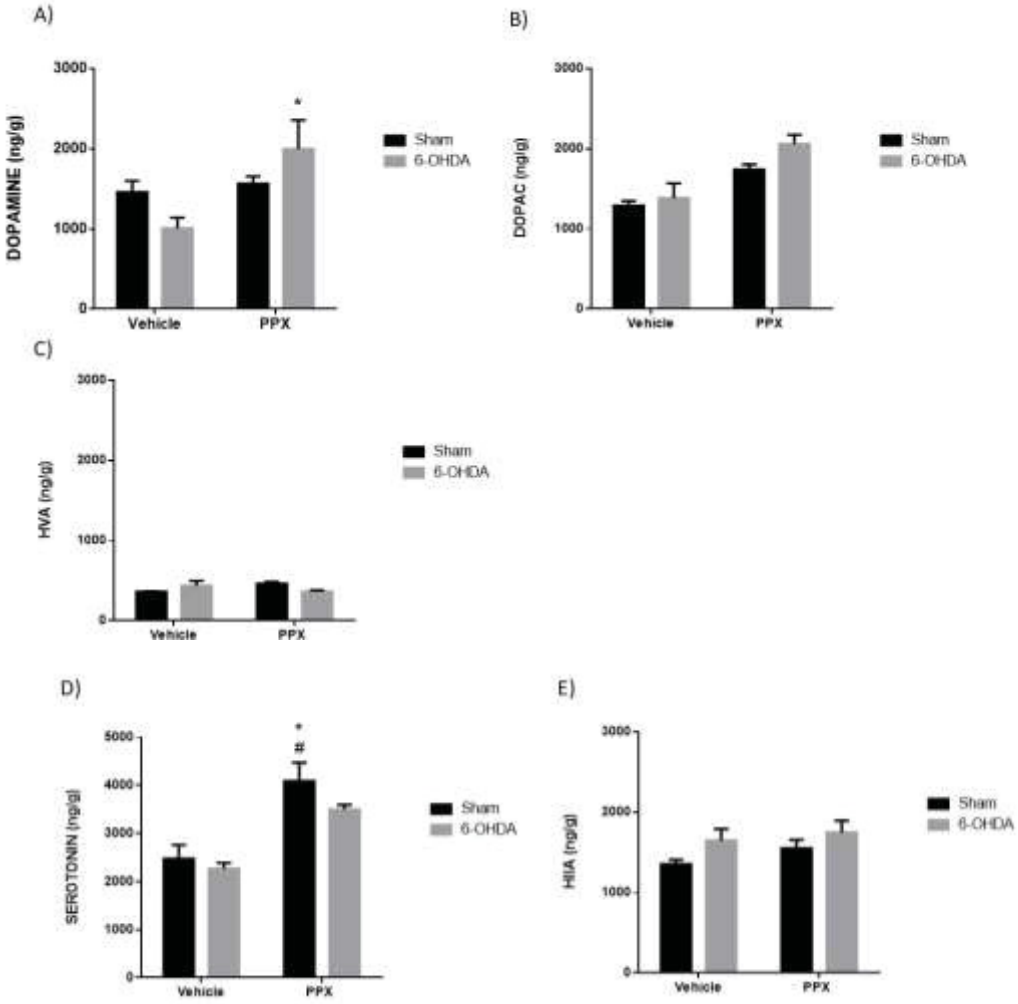


Figure 5. Striatal concentrations of (A) DA, (B) DOPAC, (C) HVA, (D) Serotonin and (E) HIAA at 28 days after the 6-OHDA infusion in the substantia nigra in animals treated with PPX (1.0 mg/kg, i.p). The data are expressed as mean ± SEM (n = 5/group). *p<0.05, compared with the 6-OHDA+vehicle group, # p<0.05 compared to the sham+vehicle group.

3.6. Western Blot

The data in the Figure 6A indicate that the 6-OHDA+vehicle group exhibited an increase in the expression of IDO in the hippocampus in comparison to sham+vehicle group. By contrast, 6-OHDA+PPX group showed a reduction in the expression of the IDO enzyme in the hippocampus when compared to the sham+vehicle and 6-OHDA groups ($p<0.05$, $F(1, 10) = 13.28$). Thus, in the Figure 6B the expression of IDO in the prefrontal cortex, the group treated with sham+PPX

showed a higher expression of the IDO in comparison to sham+vehicle and 6-OHDA+PPX groups ($p<0.05$, $F(1, 9) = 15.25$).

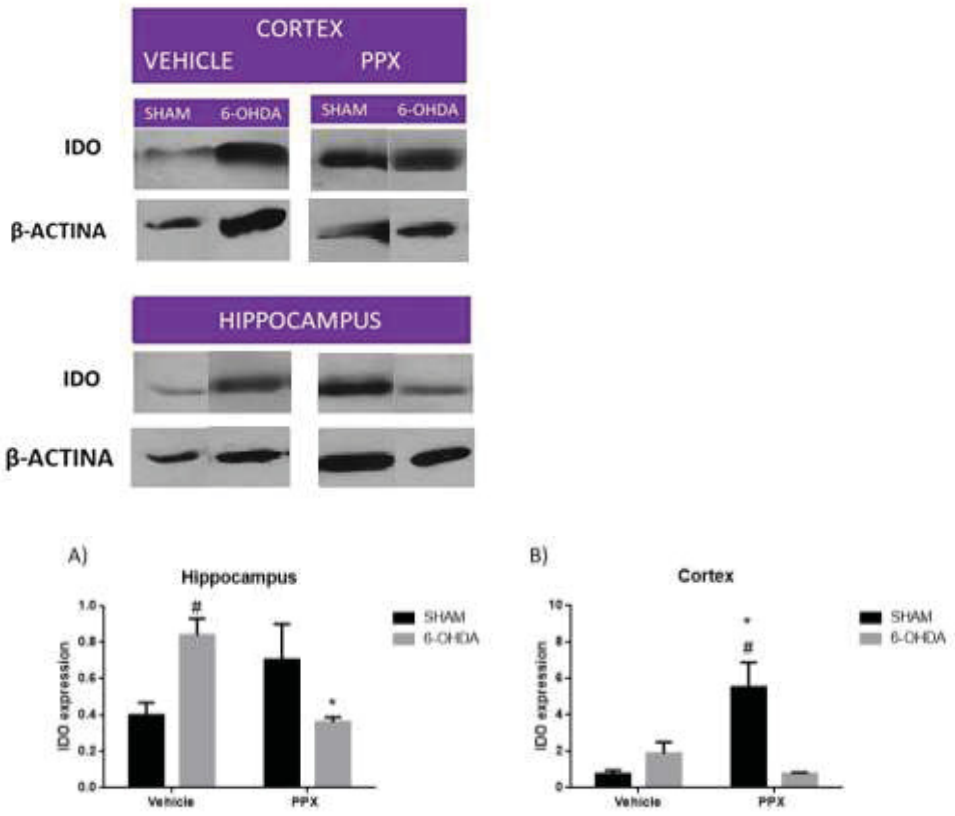


Figure 6. Effect of bilateral lesion of SNpc with 6-OHDA and the treatment with PPX on expression in the IDO enzyme in (A) hippocampus and (B) Cortex. The data are expressed as mean ± SEM (n:3-4/group). *p<0.05 (one-way ANOVA followed by the Tukey post hoc test). # p<0.05 compared to sham+vehicle group, *p<0.05 in comparison to 6-OHDA+vehicle group.

4. Discussion

The present study was conducted to better characterize the relationship among PPX, depressive-like behavior, neurochemical changes and IDO expression in the 6-OHDA-induced parkinsonism in rats. The results showed that depressive-like behavior was observed in sucrose preference test 21 days after the toxin infusion. Moreover, 6-OHDA+vehicle group present a reduction in the USV 50kHZ calls while 6-OHDA+PPX presented an increase in this parameter. Thus, despite the prolonged administration of PPX was not able to counteract the reduction of striatal TH

expression, this D2 agonist reduced the increased of hippocampal IDO expression observed in the 6-OHDA+vehicle.

In the sucrose preference test it was observed that 6-OHDA+vehicle group showed a significant decrease in the preference at 7 and 21 days after the neurotoxin infusion besides the 6-OHDA+PPX group exhibited a decrease of sucrose preference at 14 days. However, PPX reversed partially the anhedonia in the lesioned animals at 21 day. This result indicates a mild antidepressant effect in our experimental design. This last result corroborates clinical studies that indicate the antidepressant effect of PPX (Katz et al., 2004; Taylor et al., 2006; Tylee and Walters, 2007; Ramarker and Dulawa, 2017). Similarly, Barone et al. (2010), demonstrated in clinical studies that PPX has similar efficacy in the treatment of depression in comparison to SSRI. However, PPX was better tolerated and promoted improvement in the motor symptoms of PD patients.

In the present experimental schedule, the neurotoxin did not change the behavior of rats in the forced swimming test. One possible explanation to this unexpected result could be attributed to the small lesion inflicted by 6-OHDA in the SNpc. However, both imipramine and PPX were able to reduce the immobility time in this test and this result is suggestive of the antidepressant effect of PPX lesion and evidencing the influence of DA in the modulation of the depressive behavior. In fact, according to Yamada et al. (2004), dopaminergic drugs such as bupropion were able to reduce the time of immobility in the forced swimming test.

The antidepressant effect of PPX was corroborated by the analysis of the measurement of swimming time in the groups that received PPX. Considering the Pearson's correlation coefficients revealed a strong positive correlation ($r=+0.97$; $P=0.0015$) between the hippocampal 5-HT concentration and the swimming parameter (Santiago et al., 2010) it is possible to speculate that PPX interferes in the serotonergic pathways. Thus, it has already been demonstrated that the PPX also has a slight activity in the 5-HT1A receptor (Antonini; Powe, 2007; Newman-Tancredi et al., 2002a, 2002b). Moreover, these data suggest that there is a possible interaction of the dopaminergic system with serotonergic, both modulating the behavior. Tokunaga et al. (2009) showed that prolonged treatment of PPX in healthy rats promoted increased D2 and D3 receptor expression in the striatum, correlating these results with its antidepressant effects. Other studies have suggested that PPX seems to modulate the spontaneous release of DA, norepinephrine (NE) and 5-HT

neurons into rat brains, suggesting that the therapeutic action of this drug can be attributed to increased dopaminergic, serotonergic and noradrenergic neurotransmissions in the brain (Barone et al., 2010).

Regarding the climbing behavior, only the imipramine treated groups showed an increase in this parameter corroborating the correlation between NE and this behavior as described in a previous paper of our group (Santiago et al., 2010). In fact, imipramine that was used as a positive control in the current study is a tricyclic antidepressant that inhibits the serotonergic and noradrenergic transport. It has been previously suggested by (Renéric; Lucki, 1998) that an increase in both swimming and climbing behaviors in the FST occurs when the animal is treated by a drug which increases 5-HT, NE and DA levels in the nerve terminals.

In the ultrasonic vocalization test, the 6-OHDA+vehicle group showed a significant reduction in the number of 50-KHz calls emitted on the14th day during the test, in comparison to the sham+vehicle and 6-OHDA+PPX group. In relation to the lesion, these results corroborate with other studies that showed that the bilateral infusion of 6-OHDA in the SNpc promoted the reduction of emission of calls due to the reduced innervation of dopaminergic neurons (Ciucci et al., 2009; Vecchia et al., 2018).

On the other hand, on the 28th day of the test 6-OHDA+PPX group showed an increase in the number of 50-khz calls compared with the 6-OHDA+vehicle group indicating that this dopaminergic agonist was able to reverse the 6-OHDA-induced reduction in USV.

The effects of PPX are in line with other studies that showed the influence of monoaminergic systems on vocalization ultrasonic. Ringel (2013) showed that the emission of 50 kHz calls is related with the agonism of dopaminergic receptors D1 and D2, and that the antagonism of these receptors promotes the inhibition of these calls. In this is accordance with other studies that indicate that in addition to DA, NE and 5-HT influence the emission of ultrasonic vocalizations, with multiple influences on the emission and transmission of calls (Wright et al., 2012).

On the other hand, Battoszyk (1998) showed that D2 agonists such as pramipexole, roxindole, talipexole and 7-OH-DPAT dose-dependently inhibited USV of 22 kHz in rats. There are many differences between this study and the present in relation to the experimental schedule such as dose, treatment duration,

administration route of PPX and most importantly the type of USV that was performed that could contribute to these contraditory results.

According to Whor and Schwarting (2018), 50-kHz calls occur in situations that are highly rewarding and are associated with high positive emotional arousal, as rough-and-tumble play behavior, food, sexual experience, and exposition to psychostimulants. Previous studies have suggested that the trill subtype of frequency modulated (FM) 50-kHz may index positive emotional states, because rats emit these USVs at high numbers in response to rewarding stimulus, and the flat type calls, that are less complex in comparison to FM calls, are more related to the communicative state of rats (Brudzynski, 2018). Though PPX was not able to enhance subtypes of FM calls, the number of flat types was higher in comparison to group 6-OHDA treated with saline, indicating that this effect of PPX on vocalization is more of a communicative state than specifically emotional. At a neuroanatomical level, the emission of 50-kHz is regulated by the mesolimbic DA system originating from the ventral tegmental area (VTA), with the accumbens nucleus being a key brain target, thus, drugs that enhance the extracellular concentrations of DA, NA, and less so the indoleamine 5-HT, induce a robust increase in 50-kHz USV (Wöhr et al., 2015).

The data from the neurochemical analysis, in the Figure 5, show that PPX increased the striatal DA levels in the rats of 6-OHDA+PPX group but not in the vehicle+PPX. One possible explanation to this result may be related to the neuroplasticity mechanisms that occurred in the dopaminergic system after the lesion. It is important to highlight that in our experimental design the lesion inflicted by 6-OHDA caused a reduction of 45% in the dopaminergic neurons of SNpc; and 44% in the animals of 6-OHDA+PPX. This small lesion was corroborated by the discrete reduction of striatal DA levels in the 6-OHDA-induced lesion. Moreover, the striatal 5HT levels were increased in the sham+PPX group.

Preclinical studies previously performed in our laboratory demonstrated that the bilateral intranigral infusion of 6-OHDA in rats induced depressive-like behavior due to a reduction in striatal DA and hippocampal 5-HT levels (Santiago et al., 2010; Santiago et al., 2015). Similarly, Kaminska and co-workers (2017), showed that after the infusion of 6-OHDA in the forebrain of rats, there was a significant reduction in the levels of NE e, 5-HT and DA in regions of the CNS and striatum. The involvement of DA in depressive conditions has been implicated in a hypodopaminergic state, as well as in the dysregulation of the reward neural circuitry (Papakostas, 2006; Pecina

et al., 2017). However, in the present study this monoaminergic reduction was not so evident probably due to the small lesion observed in the present experimental schedule. It is important to point out that striatal 5HT levels were increased in the group sham+PPX. The serotoninergic system has strong anatomical and functional interactions with the dopaminergic system. Although PPX presents characteristics of the dopaminergic drugs, this effect on the serotoninergic system can be explained due to its agonist activity on the receptor 5-HT1$_A$.

Regarding the indoleamine-2,3-dioxygenase (IDO) expression, the data show that in the 6-OHDA+vehicle group there was a significant increase in the expression of this enzyme in hippocampus in comparison to sham+vehicle group. An increasing body of evidence indicates that IDO is responsible for degrading tryptophan leading a significant decrease of the serotonin synthesis and raises the production of tryptophan catabolites (TRYCATs) with important neurotoxic properties, such as kynurenine, xanthurenic acid, and quinolinic acid (Leonard and Maes, 2012). Moreover, the production of TRYCATs is more closely related to the onset of depression than the depletion is plasma tryptophan (Maes et al., 2011). More specifically, it has been related to patients with depression frequently show alterations on expression of proinflamatory cytokine levels in the plasma and cerebrospinal fluid (CSF) (Miller et al., 2009). In this way, Ogawa et al. (2014) showed that there are lower plasma tryptophan levels in patients with depression, and the availability of plasma tryptophan determines the rate of 5-HT synthesis in the brain. Moreover, the deficit of tryptophan is related to reducing food intake, or depletion by enhancing the activity of IDO enzymes, as it is the first enzyme in pathway of kynurenine, which degrades and converts tryptophan to kynurenine. The increase in the TRP metabolism causes increased oxygen-reactive species synthesis (ROS) that leads to neuronal death and hippocampal atrophy, in turn promoting depressive-like symptoms. For our knowledge, there are no studies investigating the possible interrelationship between the depressive-like behavior in parkinsonism models and IDO expression.

Interestingly, PPX was able to counteract the increased IDO expression in hippocampus. Considering that IDO is activated by some proinflammatory mediators (such as IFNγ, TNFα, IL-2, IL-1, prostaglandin PGE2, oxidative stress, and LPS) is it possible to speculate a PPX action in reducing the proinflammatory mediators or in the reduction of TRYCATs. In this sense, according to Lieberknecht et al. (2017) PPX

prevented the development of depression-like behavior in the forced swimming test, and the anhedonic behavior in the splash test in LPS induced a depressive- like behavior in mice and a reduction in the IL-1β. Moreover, the dopamine antagonists (haloperidol or sulpiride) were unable to abolish the antidepressant-like effect of PPX.

Although dopamine receptors have been associated to immune modulation, the obtained data indicate that the antidepressant-like action of PPX in LPS-treated mice may not be related to dopamine D2-like receptor activation.

There are no conclusive studies about the etiology of depression in PD and it is suggested that drugs able to suppress this inflammatory process are pharmacological potentials on the management of depression-associated to PD (Maddison and Giorgini, 2015). In the present study it was observed that PPX reduced one possible mechanism involved in the etiology of depressive-like behavior in the in the 6-OHDA lesioned rats.

5. Conclusion

Taken together, the results of the present study suggest that the depressive-like behavior induced by 6-OHDA may be partially related to the increased IDO expression in hippocampus. Moreover, PPX was able to reduce the IDO expression in the lesioned rats and showed a mild antidepressant effect in our model. However, we could not discard the effect of this D2/D3 agonist in the dopaminergic and serotoninergic systems.

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4 DISCUSSÃO

No presente estudo, o PPX promoveu reversão parcial do comportamento tipo depressivo induzido pela infusão bilateral de 6-OHDA na SNpc em ratos. No teste de preferência à sacarose houve aumento do consumo da sacarose no grupo 6-OHDA tratado com PPX partir do 21º dia de tratamento, aumento no tempo de natação no teste de natação forçada, indicando a reversão do comportamento tipo depressivo. Do mesmo modo, houve aumento no número de vocalizações ultrassônicas de 50-kHz em ambos os grupos que receberam PPX como tratamento. Nas análises imunohistoquímicas o PPX não foi capaz de inibir a morte de neurônios dopaminérgicos promovidos pela infusão de 6-OHDA na SNpc, no entanto reduziu a expressão da enzima IDO no hipocampo sugerindo a participação dessa via no manejo da depressão associada à DP.

A etiologia dos transtornos de humor na DP é um processo complexo, e apesar da causa da depressão em pacientes com DP ser desconhecida, estudos sugerem que o desequilíbrio neuroquímico de outros neurotransmissores além da dopamina, como a serotonina e noradrenalina especialmente na via mesocortical, contribuam para o desenvolvimento da depressão (SOUZA, et al., 2018).

Estudos pré-clínicos realizados anteriormente em nosso laboratório demonstraram que a infusão intranigral bilateral de 6-OHDA em ratos promove comportamento semelhante à depressão devido a uma redução dos níveis de DA e 5-HT no estriado (SANTIAGO, et al., 2010; SANTIAGO, et al., 2015). Da mesma forma, Kaminska e colaboradores (2017), mostraram que após a infusão de 6-OHDA no prosencéfalo de ratos, há uma redução significativa nos níveis de NE e, 5-HT e DA nas regiões do SNC e estriado. Sendo assim, um importante parâmetro para avaliar o comportamento tipo-depressivo em roedores.

No teste de preferência à sacarose, o PPX promoveu reversão parcial do consumo de sacarose a partir do 21º dia de tratamento, não havendo diferença entre o grupo controle 6-OHDA tratado com veículo. No entanto, no teste de natação forçada, houve aumento do tempo de natação em ambos os grupos que receberam o tratamento com PPX, sendo indicativo de reversão do comportamento tipo-depressivo em comparação aos grupos controles tratados com veículo. Esses resultados corroboram estudos pré-clinicos que indicam que fármacos agonistas dopaminérgicos promovem efeito “anti-imobilidade” por atuarem em receptores D1 e D2 (YAMADA, SUGIMOTO, YAMADA, 2004).

No teste de vocalização ultrassônica, o PPX promoveu o aumento da emissão de USVs de 50-kHz em comparação ao grupo 6-OHDA+veículo, corroborando com Whor e Scharting (2015) que indicam que agonistas dopaminérgicos promovem o aumento da emissão dessas USVs, podendo estar associado a estados afetivos positivos como, brincadeira, vencer uma luta, motivação sexual, ingestão de alimentos entre outros. Ciucci (2009) aponta que animais que receberam injeção 6-OHDA e foram posteriormente tratados com haloperidol promoveu a redução das chamadas de 50-kHz, provavelmente devido à redução da transmissão dopaminérgica. No entanto, Kelm-Nelson, et al, 2016, mostrou que a levodopa, agonista dopaminérgico não melhora esse prejuízo. Provavelmente, não existe recuperação do sistema dopaminérgico quando utilizado esse medicamento.

Os dados da análise neuroquímica mostram que o PPX promoveu aumento de DA e 5-HT estriatal em ambos os grupos tratados. Esses dados estão de acordo com outros estudos que apontam que o PPX parece modular a liberação espontânea de neurônios de dopamina, norepinefrina e serotonina em cérebros de ratos, sugerindo que a ação terapêutica do deste fármaco pode ser atribuída ao aumento da neurotransmissão dopaminérgica e serotoninérgica cerebral (Barone, et al., 2010), sugerindo ser efetivo na redução da depressão em pacientes com DP (BOMASANG-LAYNO, et al., 2015).

Corroborando estes dados, as análises do westerm blot mostram que o PPX foi capaz de reduzir a expressao da enzima IDO - enzima envolvida na degradação do triptofano e desencadeamento do processo inflamatório - no hipocampo em ambos os grupos tratados. Embora pouco seja conhecido a respeito da interação do sistema inflamatório e as influencias na DP e depressão, estudos sugerem que pacientes deprimidos apresentam aumeto da expressao de citocinas inflamatórias no hipocampo, e consequente a isso há aumento na formação de especies reativas ao oxigenio (ROS), desencadeando morte neuronal e atrofia hipocampal, sendo esse um dos fatores envolvidos no desenvolvimento dos sintomas depressivos (CHRISTMAS , et al., 2011)

Não há estudos conclusivos a respeito da interação entre o processo inflamatório, depressao e DP, no entanto, sugere-se que fármacos capazes de suprimir o processo inflamatórios são potenciais farmacológicos no tratamento da depressao associada à DP.

5 CONCLUSÃO

Os resultados do presente estudo sugerem o comportamento tipo-depressivo induzido pela 6-OHDA pode estar parcialmente relacionado com o aumento da expressão de IDO no hipocampo. Além disso, o PPX foi capaz de reduzir a expressão de IDO nos ratos lesados e mostrou um leve efeito tipo-antidepressivo em nosso modelo. Entretanto, não podemos descartar o efeito desse agonista D2/D3 nos sistemas dopaminérgico e serotoninérgico.

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