UNIVERSIDADE FEDERAL DO PARANÁ

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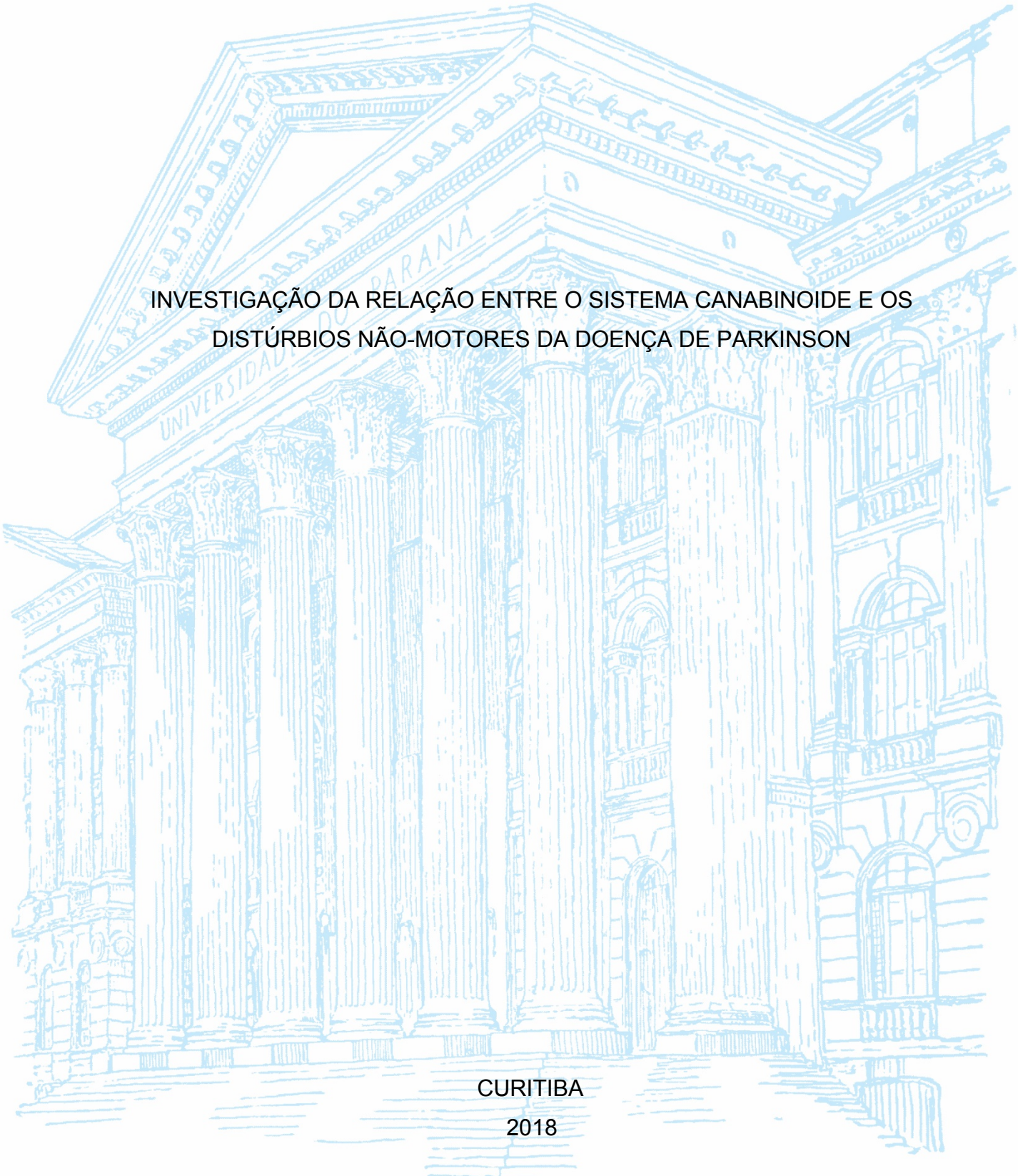

# INVESTIGAÇÃO DA RELAÇÃO ENTRE O SISTEMA CANABINOIDE E OS DISTÚRBIOS NÃO-MOTORES DA DOENÇA DE PARKINSON

CURITIBA

2018

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Tese apresentada ao curso de Pós-Graduação em Farmacologia, Setor de Ciências Biológicas, Universidade Federal do Paraná, como requisito parcial à obtenção do título de Doutor em Farmacologia.

Orientador: Prof. Dr. Marcelo de Meira Santos Lima.

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Os membros da Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação em FARMACOLOGIA da Universidade Federal do Paraná foram convocados para realizar a arguição da tese de Doutorado de **ADRIANO TARGA DIAS SANTOS** intitulada: **Investigação do papel dos receptores canabinóides da via nigroestriatal na regulação do sono REM no modelo de Parkinsonismo induzido por rotenona.**, após terem inquirido o aluno e realizado a avaliação do trabalho, são de parecer pela sua Aprovação no rito de defesa.

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Curitiba, 07 de Dezembro de 2018.

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Curitiba, 14 de janeiro de 2019.

Ao
Bibliotecário Responsável pelo Recebimento do Trabalho de Conclusão

Prezado (a),

O Programa de Pós-Graduação em Farmacologia, nível Mestrado e Doutorado, do Setor de Ciências Biológicas da Universidade Federal do Paraná, vem através deste, explicar que a tese de doutorado intitulada "INVESTIGAÇÃO DA RELAÇÃO ENTRE O SISTEMA CANABINOIDE E OS DISTÚRBIOS NÃO-MOTORES DA DOENÇA DE PARKINSON", entregue pelo aluno ADRIANO TARGA DIAS SANTOS teve o título alterado por sugestão da banca. Nos documentos internos, como na ata, está o título antigo "INVESTIGAÇÃO DO PAPEL DOS RECEPTORES CANABINÓIDES DA VIA NIGROESTRIATAL NA REGULAÇÃO DO SONO REM NO MODELO DE PARKINSONISMO INDUZIDO POR ROTENONA". Assim, solicito que a Biblioteca considere o novo título da tese, o qual está no trabalho todo, excetuando a ata de defesa e aprovação.

Atenciosamente,

Prof.ª Drª Maria Fernanda de Paula Werner
Coordenadora do Programa de
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Dedico esse trabalho à minha mãe, Jocasta Targa Dias, pela dedicação e apoio.

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Quando você chega ao limite de toda luz que você conhece,
e está a ponto de dar um passo na escuridão,
fé é saber que uma dessas coisas vai acontecer:
vai haver chão, ou você vai ser ensinado a voar.

Richard Bach

## RESUMO

A doença de Parkinson é uma doença neurodegenerativa caracterizada pela perda dos neurônios dopaminérgicos da substância negra pars compacta (SNpc), gerando os conhecidos sintomas motores, como tremor em repouso, bradicinesia, anormalidades de postura e de marcha, entre outros. Além disso, estão presentes os sintomas não-motores, como distúrbios do sono, déficits de memória, depressão, ansiedade e distúrbios olfatórios. Esses, apesar de menos conhecidos, podem aparecer anos ou décadas antes dos distúrbios motores, afetando drasticamente a qualidade de vida dos indivíduos com a doença de Parkinson. Dentre as causas dos distúrbios não-motores, está a própria degeneração inerente à doença. Nesse sentido, estudos investigam regiões e componentes importantes para, a longo prazo, desenvolver uma estratégia terapêutica eficaz. Recentemente, observou-se a presença de receptores canabinoides na via nigroestriatal, capazes de modular a neurotransmissão dopaminérgica. Além disso, diversos estudos demonstram efeitos de canabinoides na regulação do sono e na consolidação da memória em diferentes contextos. Considerando isso, o objetivo do presente estudo foi investigar o papel do sistema canabinoide na regulação do sono e na consolidação da memória no modelo animal de Parkinsonismo induzido por rotenona. Para isso, realizamos uma cirurgia estereotáxica em ratos Wistar machos, na qual administramos a neurotoxina rotenona diretamente na SNpc. Após sete dias de recuperação, os animais foram submetidos à fase de treino do teste de reconhecimento de objetos e, em seguida, houve a microinfusão estriatal de diferentes drogas moduladoras do sistema canabinoide. Foi realizado o registro de sono para, no dia seguinte, ser realizado o teste de reconhecimento de objetos e o teste do campo aberto. Ao final dos experimentos, os animais foram eutanasiados para coleta de amostras destinadas à imuno-histoquímica, western blot e PCR em tempo real. Observou-se uma diminuição no tempo despendido em sono NREM após a administração de rotenona. Interessantemente, o bloqueio dos receptores CB1 e CB2 reverteu o efeito induzido pela rotenona, parcialmente relacionado a um aumento compensatório nos níveis de mRNA do receptor CB1. Além disso, a administração de canabidiol (CBD) promoveu um aumento no tempo despendido em vigília, paralelamente a um aumento na expressão do receptor CB2, enquanto nenhum efeito foi observado após a administração do Δ9-tetraidrocanabinol (THC). A rotenona também prejudicou a memória de reconhecimento de objetos. Nesse caso, tanto o bloqueio dos receptores CB1, quanto a ativação dos receptores CB2, foram capazes de reverter esse déficit. Diferentemente, tanto a administração de CBD quanto de THC promoveram um prejuízo para a memória. Em conclusão, os achados do presente estudo demonstram um envolvimento do sistema canabinoide tanto na regulação do sono quanto na consolidação da memória de reconhecimento de objetos no modelo animal de Parkinsonismo induzido por rotenona.



Palavras-chave: Doença de Parkinson; canabinoides; sono; memória de reconhecimento; receptores canabinoides.

## ABSTRACT

Parkinson's disease is a neurodegenerative disorder characterized by the loss of the dopaminergic neurons within the substantia nigra pars compacta (SNpc), which leads to the well-known motor symptoms, such as resting tremor, bradykinesia, posture and gait abnormalities, among others. In addition, non-motor symptoms such as sleep disorders, memory deficits, depression, anxiety, and olfactory disorders are present. In fact, non-motor disturbances appear years or decades earlier than motor symptoms, drastically affecting the quality of life of individuals with Parkinson's Disease. The inherent degeneration of the disease is one of the leading factors for non-motors symptoms appearance. Regarding this, studies investigate important regions and components to develop an effective therapeutic strategy in the future. Recently, it was demonstrated the presence of cannabinoid receptors in the nigrostriatal pathway, modulating dopaminergic neurotransmission. In addition, several studies demonstrated the effects of cannabinoids on sleep regulation and memory consolidation in different contexts. Considering this, the objective of the present study was to investigate the role of the cannabinoid system in sleep regulation and memory consolidation in the animal model of Parkinsonism induced by rotenone. For this, we performed a stereotactic surgery in male Wistar rats, in which we administered the neurotoxin rotenone directly into the SNpc. After seven days, the animals were submitted to the training phase of the object recognition test and then we performed the striatal microinfusion of different drugs that modulate the cannabinoid system. The sleep was recorded and, in the next day, the animals performed the object recognition test and the open field test. At the end of the experiments, the animals were euthanized, and samples were collected for immunohistochemistry, western blot and real-time PCR analysis. A decrease in the time spent on NREM sleep was observed after administration of rotenone. Interestingly, CB1 and CB2 receptors blockade reversed the rotenone-induced effect, which was partially associated with a compensatory increase in CB1 receptor mRNA levels. In addition, cannabidiol administration increased the time spent awake, in parallel with an increase in CB2 receptor expression, while no effect was observed after Δ9-tetrahydrocannabinol (THC) administration. Rotenone also impaired object recognition memory. In this case, both CB1 receptors blockade and CB2 receptors activation reversed the deficit. Differently, both cannabidiol and THC administration impaired recognition memory. In conclusion, our findings demonstrate an involvement of the cannabinoid system both in sleep regulation and in the consolidation of object recognition memory in the animal model of Parkinson’s disease induced by rotenone.



Keywords: Parkinson’s disease; cannabinoids; sleep; recognition memory; cannabinoid receptors.

## LISTA DE ABREVIATURAS – LÍNGUA PORTUGUESA

6-OHDA – 6-hidroxidopamina

CBD – Canabidiol

GABA – Ácido gamma-aminobutírico

DMSO – Dimetilsulfóxido

L-DOPA - 3,4-dihidroxifenilalanina

MAO B – Monoaminaoxidase B

MPTP - 1-metil-4-fenil-1,2,3,6-tetrahidropiridina

OF – Teste do campo aberto

ORT – Teste de reconhecimento de objetos

PPT - Tegmento pedúnculo-pontino

SNpc – Substância negra pars compacta

TH – Tirosina hidroxilase

TH-ir – Tirosina hidroxilase-imunorreativo

THC - Δ9-tetraidrocanabinol

THCV - Δ9-tetraidrocanabivarina

**SUMÁRIO**

# 1. INTRODUÇÃO

## 1.3 DOENÇA DE PARKINSON

A doença de Parkinson é uma doença neurodegenerativa caracterizada pela perda dos neurônios dopaminérgicos da substância negra pars compacta (SNpc) (BRAAK et al., 2003). Essa doença afeta de 100-200 pessoas a cada 100.000 pessoas, com maior prevalência em indivíduos acima dos 65 anos de idade (MARRAS et al., 2018). De acordo com as projeções das Nações Unidas, o número de pessoas com 60 anos ou mais, em 2017, estimado em 962 milhões, irá crescer para aproximadamente 2,1 bilhões em 2050, aumentando drasticamente a probabilidade de indivíduos portadores da doença de Parkinson (UNITED NATIONS, DEPARTMENT OF ECONOMIC AND SOCIAL AFFAIRS, 2017).

As manifestações motoras da doença são as mais conhecidas e incapacitantes. Essas são representadas por bradicinesia, tremor em repouso, anormalidades de postura e marcha, entre outras (BRAAK et al., 2003). No entanto, existem as manifestações não-motoras, representadas pelos distúrbios do sono, prejuízos cognitivos, disfunções olfatórias, ansiedade e depressão. Apesar de serem menos conhecidas, essas são capazes de afetar drasticamente a qualidade de vida dos indivíduos portadores da doença e podem surgir muito antes das manifestações motoras (GRINBERG et al., 2010; LIMA, 2013).

Os distúrbios do sono são representados principalmente pela insônia, pela sonolência diurna excessiva e pelo distúrbio comportamental do sono REM (LIMA, 2013). Como exemplos de prejuízos cognitivos, estão os déficits na função executiva, distúrbios visuoespaciais e disfunções de memória. Em relação a isso, diferentes estudos sugerem prejuízo na memória de reconhecimento de objetos tanto em indivíduos com a doença de Parkinson como em modelos animais. No entanto, considerável controvérsia ainda existe, principalmente em relação aos demais tipos de memória e aos processos (aquisição, consolidação e evocação) afetados (TARGA et al., 2018).

Diversos fatores contribuem para o aparecimento dos sintomas não-motores, como idade, tratamento farmacológico ao qual o paciente está sendo submetido e a presença de outros sintomas, como depressão e ansiedade (DIEDERICH; MCINTYRE, 2012). Além disso, a neurodegeneração inerente à doença de

Parkinson tem papel fundamental nesse contexto. Braak e colaboradores (2003) propuseram um estagiamento para a doença de Parkinson, apontando que nas primeiras fases ocorre o acometimento da medula oblonga e do tegmento pedúnculo pontino (PPT). A partir disso, ocorre o comprometimento de alguns núcleos do mesencéfalo como a SNpc para, finalmente, regiões do córtex serem atingidas (BRAAK et al., 2003). Nesse sentido, os distúrbios do sono surgem quando ocorre o acometimento do PPT, o qual é muito importante na regulação do sono (MCCARLEY, 2004). Já os sintomas motores aparecem quando ocorre uma perda considerável dos neurônios dopaminérgicos da via nigroestriatal (BRAAK et al., 2003). No entanto, deve haver cautela ao simplificar ou generalizar esses contextos. Nas últimas décadas, por exemplo, alguns trabalhos têm demonstrado a associação da via nigroestriatal também aos distúrbios não-motores (LIMA et al., 2007; RODRIGUES et al., 2014). Estudos recentes do nosso laboratório demonstraram que a modulação dos receptores dopaminérgicos do tipo D2 presentes na via nigroestriatal alterou parâmetros de sono e da memória de reconhecimento de objetos no modelo animal de Parkinsonismo induzido pela administração de rotenona (TARGA et al., 2018, 2016).

A droga padrão ouro para o tratamento dos sintomas motores da doença de Parkinson é a levodopa ou 3,4-dihidroxifenilalanina (L-DOPA), um precursor da síntese de dopamina. Apesar de sua descoberta por volta de 1960, esse ainda é o fármaco de primeira escolha para o tratamento da doença (MUNCHAU; BHATIA, 2000). A utilização da levodopa geralmente está associada à administração de carbidopa ou benserazida, que inibem a conversão da L-DOPA à dopamina no tecido periférico, fazendo com que a primeira ultrapasse a barreira hematoencefálica e atinja o sistema nervoso central para, a partir disso, aumentar os níveis do neurotransmissor (BRICHTA; GREENGARD; FLAJOLET, 2013). Outros fármacos como anticolinesterásicos, inibidores da monoamina-oxidase (MAO) e agonistas dopaminérgicos também podem fazer parte do tratamento, de forma a aliviar os sintomas ou diminuir os efeitos adversos da L-DOPA (BRICHTA; GREENGARD; FLAJOLET, 2013; KALINDERI et al., 2011).

O tratamento dos sintomas não motores recebe menor atenção quando comparado aos sintomas motores, possivelmente em decorrência do menor entendimento a respeito das respectivas fisiopatologias. Em relação à memória, inibidores da colinesterase como rivastigmina e donepezila demonstraram um

aumento da função cognitiva (SADOWSKY et al., 2014). Além disso, alguns estudos apontam efeitos benéficos com o uso do inibidor seletivo da monoamina oxidase-B, rasagilina (STOCCHI; FOSSATI; TORTI, 2015). Em relação ao sono, agonistas dopaminérgicos como o pramipexol e a rotigotina, hipnóticos não-benzodiazepínicos como a eszopiclona e substâncias como a melatonina demonstraram melhora na qualidade do sono (AL-QASSABI; FERESHTEHNEJAD; POSTUMA, 2017). Além disso, canabinoides, como os nabiximols, diminuíram distúrbios relacionados ao sono e aumentaram a eficiência global do sono (BABAYEVA et al., 2016). De fato, o uso de canabinoides tem sido cada vez mais considerado dentro desses contextos (MORE; CHOI, 2015; PREDIGER, 2010).

## 1.4 SISTEMA ENDOCANABINOIDE

O sistema endocanabinoide é composto pelos endocanabinoides (ou canabinoides endógenos), pelas enzimas que promovem sua síntese e degradação e pelos seus alvos, os receptores canabinoides (GARCÍA et al., 2016). Os endocanabinoides são produzidos no organismo da maioria dos grupos de vertebrados, sendo a anandamida e o 2-araquidonoilglicerol os mais conhecidos (LU; MACKIE, 2016). Diferentemente, os canabinoides exógenos estão presentes nas plantas do gênero Cannabis, sendo representados principalmente pelo Δ9-tetraidrocanabinol (THC) e pelo canabidiol (CBD) (PANAHI et al., 2017). Esses compostos, tanto os exógenos quanto os endógenos, quando presentes no organismo, ligam-se predominantemente a dois tipos de receptores: receptores canabinoides do tipo 1 (CB1) e receptores canabinoides do tipo 2 (CB2). Ambos são acoplados à proteína Gi/o, inibindo a adenilato ciclase ou ativando a proteína quinase ativada por mitógeno (MAPK) (KENDALL; YUDOWSKI, 2017; PERTWEE et al., 2010). Os receptores CB1 estão presentes em abundância no Sistema Nervoso Central, por exemplo, nos gânglios da base, hipocampo, tálamo e cerebelo (PERTWEE et al., 2010). Dessa forma, atribui-se a eles a maioria dos efeitos psicoativos observados com o uso de canabinoides exógenos. Os receptores CB2 são classicamente descritos como receptores presentes em células do sistema imune, como macrófagos e monócitos (BERDYSHEV, 2000). No entanto, mais recentemente, tem se observado a presença desses no Sistema Nervoso Central,

como no estriado, SNpc e hipocampo (BRUSCO et al., 2008; GARCÍA et al., 2015; GONG et al., 2006; ONAIVI et al., 2006).

O sistema canabinoide apresenta um papel modulador em importantes processos do sistema nervoso central, como controle do movimento, processos cognitivos, nocicepção, entre outros (GARCÍA et al., 2016). Isso se dá por meio da sua participação em sinapses que controlam esses processos. Os receptores CB1 localizam-se tanto pós quanto pré-sinapticamente e, dessa forma, controlam a liberação de diferentes neurotransmissores, principalmente glutamato e GABA (BASAVARAJAPPA; SUBBANNA, 2014).

Estudos demonstram também a localização de receptores canabinoides CB1 e CB2 pós-sinapticamente nas sinapses entre os neurônios dopaminérgicos da SNpc e os neurônios GABAérgicos do estriado dorsal (GARCÍA et al., 2016; SIERRA et al., 2015). Além disso, García e colaboradores (2015) demonstraram a presença de receptores CB2 na SNpc em encéfalos *post-mortem*, sugerindo uma localização pré-sináptica, que controlaria diretamente a liberação de dopamina no estriado (GARCÍA et al., 2015). Ainda, receptores CB1 associam-se aos receptores dopaminérgicos D2 nas membranas estriatais pós-sinápticas, formando complexos denominados de heterômeros (MARCELLINO et al., 2008). Acredita-se que essa associação seja responsável pela habilidade de agonistas e antagonistas do receptor CB1 em reduzir ou potencializar, respectivamente, a transmissão dopaminérgica (ANDERSON et al., 1995a; MANEUF; CROSSMAN; BROTCHIE, 1997; MARCELLINO et al., 2008). Isso é de extrema relevância para a doença de Parkinson, considerando que a transmissão dopaminérgica está envolvida não apenas na função motora, mas também na regulação do sono e na consolidação da memória (TARGA et al., 2018, 2016).

## 1.5 DOENÇA DE PARKINSON E SISTEMA CANABINOIDE

Em termos de neuroproteção, o sistema canabinoide demonstrou papel importante, reduzindo o estresse oxidativo e a inflamação no modelo animal de Parkinsonismo induzido pela administração unilateral de 6-OHDA (6-hidroxidopamina) (GARCÍA-ARENCIBIA et al., 2007). Além disso, observou-se que tanto o THC quanto o CBD foram eficazes em diminuir a toxicidade promovida pela administração dessa mesma droga, tanto *in vivo* quanto *in vitro* (LASTRES-BECKER

et al., 2005). Em estudos posteriores, foi demonstrado que esse importante efeito é mediado principalmente pelos receptores CB2 (GÓMEZ-GÁLVEZ et al., 2016; PRICE et al., 2009) . De fato, Garcia et al. (2011) observaram a ocorrência de deterioração muito mais intensa em neurônios dopaminérgicos da SNpc em animais nocaute para o receptor CB2, quando comparados a animais selvagens (GARCÍA et al., 2011).

Em relação aos parâmetros motores, demonstrou-se que a administração sistêmica de CP 55,940 e WIN 55,212-2, ambos agonistas de receptores canabinoides, foi capaz de atenuar apenas o comportamento rotacional promovido pela ativação do receptor dopaminérgico D1 (SKF 38393), mas não pela ativação de D2 (quinpirol), em ratos unilateralmente lesionados com 6-OHDA, indicando uma interação entre os receptores D1 e o sistema canabinoide (ANDERSON et al., 1995b). Diferentemente, a administração sistêmica de WIN 55,212-2 foi capaz de reduzir o efeito anti-parkinsoniano promovido pela administração de quinpirol no modelo animal de Parkinsonismo induzido por reserpina, demonstrando uma interação entre o sistema canabinoide e os receptores D2 (MANEUF; CROSSMAN; BROTCHIE, 1997). Ainda, Garcia e colaboradores (2011) observaram que a administração do fitocanabinoide Δ9-tetraidrocanabivarina (Δ9-THCV), que promove ativação de receptores CB2 e inibição de receptores CB1, atenuou a inibição motora no modelo animal de Parkinsonismo induzido pela 6-OHDA (GARCÍA et al., 2011). Estudos realizados em humanos também apontam uma relação entre o sistema canabinoide e a doença de Parkinson no que tange os sintomas motores. Por meio de um questionário entregue aos pacientes do Centro de Distúrbios Motores de Praga, foi observado que 25% dos indivíduos fizeram uso de Cannabis e que desses, 45,9% relataram algum tipo de benefício, incluindo diminuição do tremor em repouso, da bradicinesia e da rigidez muscular (VENDEROVÁ et al., 2004). Os canabinoides parecem também atenuar os efeitos adversos decorrentes do uso de fármacos para o tratamento da doença de Parkinson, como as discinesias provenientes do uso da L-DOPA. Morgese e colaboradores (2007) demonstraram que a administração de WIN 55,212-2 atenuou as discinesias induzidas pela L-DOPA no modelo animal induzido por 6-OHDA (MORGESE et al., 2007). Outros autores observaram respostas semelhantes com o modelo animal de Parkinsonismo induzido por MPTP (1-metil-4-fenil-1,2,3,6-tetraidropiridina) (FOX et al., 2002).

Existem significativamente menos estudos investigando o papel do sistema canabinoide nos sintomas não-motores da doença de Parkinson. De fato, até o presente momento, não existe nenhum estudo que investigue a utilização de canabinoides para os déficits cognitivos associados à doença de Parkinson (WALTHER; HALPERN, 2010). No entanto, diversos estudos observam a relação entre canabinoides e memória em outros contextos. Em relação a memórias dependentes dos gânglios da base, observou-se que a infusão de WIN 55,212-2 sistemicamente ou diretamente no estriado dorsolateral foi capaz de prejudicar a consolidação de uma memória de procedimento (GOODMAN; PACKARD, 2014). Outros estudos demonstram um efeito deletério na aquisição e consolidação da memória a partir da administração do rimonabanto (um antagonista seletivo do receptor CB1) diretamente no estriado dorsolateral (GERDEMAN *et al*., 2006). Contrariamente, Bialuk e colaboradores (2016) observaram uma melhora na memória de reconhecimento de objetos após a administração de AM281 (um agonista inverso do receptor CB1) (BIALUK; WINNICKA, 2016). Essas divergências nos resultados são decorrentes de diferentes tipos de memórias analisados, administração de drogas com diferentes propriedades, em doses e vias variadas. No entanto, mesmo com resultados divergentes, é evidente o expressivo papel do sistema canabinoide na modulação de processos mnemônicos.

Assim como nos processos de memória, estudos demonstram um papel do sistema canabinoide na modulação do sono. De fato, com a presença dos receptores canabinoides em diversas estruturas encefálicas associadas à regulação do sono, é plausível discutir um possível envolvimento nesse sentido. Chagas e colaboradores (2013) demonstraram que a administração de diferentes doses de CBD por via intraperitoneal foi capaz de aumentar a porcentagem total de sono (CHAGAS et al., 2013). Contrariamente, foi observado que a administração intracerebroventricular de CBD aumentou o tempo despendido em vigília (MURILLO-RODRÍGUEZ et al., 2006).

Enquanto não se tem evidência do papel dos receptores CB2 no sono, estudos sugerem que os efeitos dos canabinoides sobre o sono sejam mediados pelo receptor CB1. Murillo-Rodriguez e colaboradores (2001) demonstraram que a ativação de receptores CB1 presentes no PPT aumentou o tempo despendido em sono não-REM (NREM) e sono REM (MURILLO-RODRÍGUEZ et al., 2001). Ainda, observou-se que a injeção intraperitoneal de anandamida foi capaz de aumentar os

níveis de adenosina no prosencéfalo basal e isso correlacionou-se temporalmente com um aumento de tempo despendido em sono NREM. No entanto, a administração de SR141716A (um antagonista do receptor CB1), precedendo a administração de anandamida, preveniu a ocorrência desse efeito (MURILLO-RODRIGUEZ et al., 2003). Em relação ao efeito dos canabinoides na modulação do sono em indivíduos Parkinsonianos, Lotan e colaboradores (2014) observaram que 20 de um total de 22 indivíduos descreveram uma melhora na qualidade do sono após fazer o uso de Cannabis (LOTAN et al., 2014). No entanto, além do estudo descrito carecer de medidas objetivas na análise, a literatura é escassa em relação ao tema, demonstrando a necessidade de novos estudos que investiguem essa questão.

Em resumo, sabe-se que os canabinoides apresentam efeitos tanto em processos relacionados à memória quanto na modulação do sono e que essas são funções alteradas na doença de Parkinson, afetando drasticamente a qualidade de vida dos pacientes. Além disso, sabe-se que os receptores canabinoides e dopaminérgicos interagem de forma indireta, estando co-localizados em neurônios dopaminérgicos da SNpc e GABAérgicos do estriado dorsal e de forma direta, por meio da formação de heterômeros. Assim, o objetivo do presente estudo foi investigar o efeito da modulação do sistema canabinoide, particularmente na via nigroestriatal, em parâmetros do sono e da memória em um modelo animal da doença de Parkinson. Esperava-se que, dependendo do receptor modulado e do tipo da modulação (ativação/inibição), houvesse um possível reestabelecimento do sono e da memória em animais com a doença de Parkinson. Esse estudo contribuirá para um melhor entendimento dos mecanismos envolvidos nos distúrbios não-motores da doença de Parkinson, estabelecendo a função do sistema canabinoide nesse contexto. Além disso, esses conhecimentos poderão contribuir para o delineamento de novas estratégias terapêuticas a partir desses alvos.

# 2 OBJETIVOS

## 2.1 OBJETIVO GERAL

Investigar o papel do sistema canabinoide na regulação do sono e na consolidação da memória de reconhecimento de objetos no modelo animal de Parkinsonismo induzido por rotenona.

## 2.2 OBJETIVOS ESPECÍFICOS

- Avaliar o efeito da modulação do sistema canabinoide[1] na regulação do sono em um modelo animal de Parkinsonismo induzido por rotenona por meio de registro encefalográfico.

- Avaliar o efeito da modulação do sistema canabinoide na consolidação da memória em um modelo animal de Parkinsonismo induzido por rotenona por meio do teste de reconhecimento de objetos.

- Avaliar o efeito da modulação do sistema canabinoide no comportamento motor em um modelo animal de Parkinsonismo induzido por rotenona por meio do teste de campo aberto.

- Avaliar o efeito da modulação do sistema canabinoide na expressão dos receptores CB1 e CB2 no estriado em um modelo animal de Parkinsonismo induzido por rotenona por meio da técnica de Western blot.

- Avaliar o efeito da modulação do sistema canabinoide nos níveis de mRNA dos receptores CB1 e CB2 no estriado em um modelo animal de Parkinsonismo induzido por rotenona por meio da técnica de PCR em tempo real.

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[1] Entenda-se “modulação do sistema canabinoide” por: 1) ativação/inibição dos receptores canabinoides CB1 no estriado, 2) ativação/inibição dos receptores canabinoides CB2 no estriado, 3) administração de CBD ou THC.

# 3 CAPÍTULOS

## 3.1 CAPÍTULO 1

# CB1 receptors blockade reverses sleep disturbances and memory impairments in an animal model of Parkinson's disease

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## Abstract

Sleep disturbances and cognitive dysfunctions are among the most prevalent non-motor symptoms of Parkinson's disease. Recent studies demonstrate a prominent role for dopaminergic system on these symptoms. In addition, a complex relationship between dopaminergic and cannabinoid systems has been observed, along with substantial reports on CB1 modulation effects in sleep and memory processes. Considering this, the objective of this study was to investigate the effects of striatal CB1 receptors modulation on sleep regulation and recognition memory consolidation in the rotenone model of Parkinson's disease. For this, rotenone (12 µg/µl) was administered within the Substantia nigra pars compacta of male Wistar rats. After seven days, the animals received CB1 receptor agonist (WIN 55,212-2, 400 ng/µl), CB1 antagonist (AM 251, 3.2 µg/µl) or vehicle (DMSO) directly in the striatum. In the next 3 hours, the animals were monitored for their sleep and, 24h later, they were submitted to the object recognition test and open field test. Finally, samples were collected for molecular analysis. The rotenone model of Parkinson's disease decreased the time spent in NREM sleep, affecting NREM sleep stability. In addition, rotenone administration impaired the object recognition memory. Interestingly, the blockade of CB1 receptors by AM251 administration reversed the rotenone-induced effects on sleep and recognition memory. A similar pattern was observed for CB1 receptors mRNA expression, but not at the protein level. These findings highlight the importance of cannabinoid system in the non-motor symptoms of Parkinson's disease, improving the understanding of the mechanisms that underlie these disturbances.


**Keywords:** Parkinson's disease, sleep regulation, recognition memory, cannabinoids, CB1 receptors.

## Introduction

Parkinson's disease is a neurodegenerative disorder characterized by the loss of dopaminergic neurons within the substantia nigra pars compacta (SNpc), which ultimately leads to the well-known motor symptoms. These are represented by bradykinesia, tremors, gait disturbances, among others[1,2]. In addition, non-motor symptoms like sleep disturbances and cognitive dysfunctions precede motor signs by years or decades, drastically affecting the quality of life of Parkinson's disease patients[3].

Different factors contribute to the appearance of the non-motor symptoms: depression and anxiety; age; pharmacological treatment; and the neurodegeneration that occurs as the disease progresses[4]. In fact, structures associated with sleep regulation like pedunculopontine tegmental nucleus (PPT), dorsal raphe nuclei and locus coeruleus are all affected before SNpc[1]. However, it was recently demonstrated that SNpc itself is involved in non-rapid eye movement (NREM) and rapid eye movement (REM) sleep regulation, contradicting previous reports that associated the nigrostriatal pathway exclusively with the motor symptoms[5,6]. Targa et al. (2016) demonstrated that ibotenic acid infusion within the PPT prevented the expected sleep rebound effect mediated by REM sleep deprivation (REMSD), which was reversed by striatal D2 receptors activation[6]. Also, striatal D2 receptors blockade by raclopride administration reversed the rotenone-induced impairment in object recognition memory[7]. This suggests that striatal D2 receptors play important roles in both sleep disturbances and recognition memory dysfunctions associated with Parkinson's disease.

Recent studies suggest that dopaminergic and cannabinoid receptors are part of a complex association[8]. CB1 are Gi/o-coupled receptors densely expressed within the

nigrostriatal pathway, particularly on the dendrites of GABAergic striatal neurons along with D2 and A2a receptors[9]. Also, the activation and blockade of CB1 receptors, respectively, decreased and increased dopaminergic transmission[8,10]. In fact, the use of Cannabis is associated with a decrease in resting tremors, bradykinesia and muscular rigidity[11]. Moreover, CB1 receptors activation decreased L-DOPA induced dyskinesias in different animal models of Parkinson's disease[12,13]. Thus, considering the recently demonstrated function of nigrostriatal pathway in the non-motor symptoms by Targa and collaborators (2016, 2018), the modulation of dopaminergic transmission by CB1 receptors may be important for cognition and sleep as well[7,14].

CB1 receptors modulation affects sleep and recognition memory in contexts that are not associated with Parkinson's disease[15,16]. In fact, it was demonstrated that CB1 receptors activation within the PPT increased the time spent in both NREM and REM sleep[17]. Anandamide administration also increased the time spent in NREM sleep, which was prevented by CB1 receptors blockade, suggesting a CB1 receptor-mediated effect[18]. Regarding recognition memory, CB1 receptors activation and blockade leads to, respectively, impairment and improvement of consolidation, in most of the reports. Indeed, Bialuk and collaborators (2011, 2016) demonstrated that blockade of CB1 receptors by AM251 or AM281 improved while activation by synthetic cannabinoids administration impaired memory parameters evaluated in the object recognition test (ORT)[15,19,20].

Considering that: 1) CB1 receptors modulation affects memory and sleep processes, which are important non-motor features in Parkinson's disease; 2) CB1 receptors modulate dopaminergic neurotransmission, which is associated with memory and sleep mechanisms; our objective was to investigate the effects of striatal CB1

receptors modulation on sleep regulation and recognition memory consolidation in the rotenone model of Parkinson's disease. To our knowledge, this is the first time that an association between Parkinson's disease non-motor symptoms and cannabinoid system is investigated. We hypothesised that the pharmacological modulation of striatal CB1 receptors could either potentiate or reverse the damage promoted by rotenone on sleep and memory. Such investigation will increase the understanding of the mechanisms that underlie sleep regulation and memory consolidation in Parkinson`s disease, possibly contributing to the development of novel therapeutic strategies.

## 2. MATERIALS AND METHODS

### *2.1 Subjects*

The experiments in this study were approved by the ethics committee of Federal University of Paraná (all experiments; approval ID #857) and Federal University of São Paulo (sleep experiments; approval ID #9022050417). They were carried out in accordance to the Guidelines of ethics and experimental care and use of laboratory animals (SBCAL). All efforts were made to minimize animal suffering and to reduce the number of animals used. Male Wistar rats, weighing approximately 280-320g were kept in a temperature-controlled room (22±2$^{o}$C), with a 12:12h light-dark cycle (lights on at 7:00 AM). They were maintained in groups of 5 animals in polypropylene cages containing bottles of water and pellets of food throughout the entire experiment.

### *2.2 Experimental design*

The experimental design is represented in Fig. 1. There were two sets of animals. The first set (n=70) underwent stereotaxic surgery for rotenone or dimethylsulfoxide (DMSO) administration within SNpc, and bilateral guide cannulas implantation within the striatum. The habituation phase of the object recognition test (ORT) took place on days 3, 5 and 7. After the last habituation (day 7), the animals were submitted to the training phase of the ORT and then, we administered the drugs that modulate cannabinoid receptors directly into the striatum. The effects of these drugs were evaluated in the next day (day 8), in which the ORT and the open field test (OF) were performed. Finally, the animals were euthanized through decapitation (samples collected for western blot and real-time PCR) or perfusion (samples collected for immunohistochemistry).

The second set of animals (n=30) were submitted to the same procedures on day 0, but with additional implantation of cortical electrodes for sleep-wake recording. After 7 days, we administered the drugs that modulate cannabinoid receptors directly into the striatum followed by a period of 3h of sleep-wake recording (9:00 AM-12:00 PM).

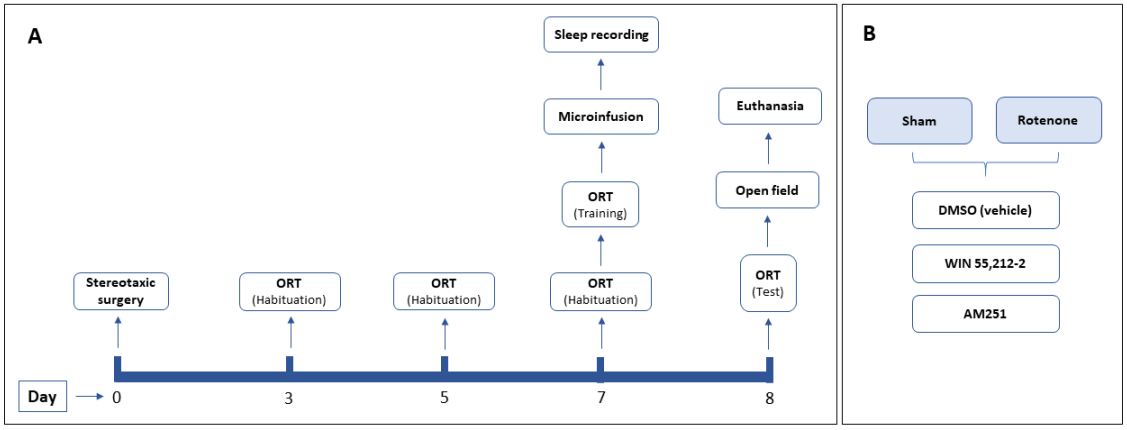


Figure 1. Experimental design (A), groups after stereotaxic surgery and microinfusion (B). ORT, object recognition test.

## *2.3 Stereotaxic surgery*

The animals were sedated with intraperitoneal xylazine (10 mg/kg; Syntec do Brasil Ltda, Brazil) and anesthetized with intraperitoneal ketamine (90 mg/kg; Syntec do Brasil Ltda, Brazil). For rotenone infusion within the SNpc, we used bregma as a reference for the following coordinates: (AP) = -5.0 mm, (ML)= ± 2.1 mm and (DV) = -8.0 mm[21]. Rotenone (12 µg/µl; Sigma-Aldrich®, United States) or DMSO 10% v/v (Sigma-Aldrich®, United States) infusions were made using an electronic infusion pump (Insight Instruments, Ribeirão Preto, Brazil) at a rate of 0.33 µl/min for 3 min[22]. Bilateral guide cannulas implantation within the striatum were made using bregma as reference for the following coordinates: (AP) = -1.0 mm, (ML)= ±3.0 mm and (DV) = -4.0 mm[21]. For electrodes positioning, the following coordinates were used, using bregma as a reference: (AP) = -1.8 mm, (ML) = -2.0 mm (first electrode) and (AP) = 3.0 mm, (ML) = 1.0 mm (second electrode); and lambda as a reference: (AP) = 1.0 mm, (ML) = -4.0 mm (third electrode) and (AP) = 4.0 mm, (ML) = 1.0 mm (fourth electrode)[21].

*2.4 Striatal microinfusion*

The awake animals were gently immobilized for infusions of the cannabinoid receptors agonist WIN 55,212-2 (400 ng/µl; Tocris, USA)[23], selective CB1 receptor antagonist AM 251 (3,2 µg/µl; Tocris, USA)[24] or vehicle (DMSO, 10% v/v; Sigma-Aldrich, USA) directly in the striatum. The infusions were made at the bilateral guide cannulas (implanted during stereotaxic surgery), at a rate of 0.33 µl/min for 3 min, with the assistance of an electronic infusion pump (Insight Instruments, Ribeirão Preto, Brazil)[22].

*2.5 Sleep recording procedure and quantification*

Electrophysiological signals were recorded on a digital polygraph (Neurofax QP 223A Nihon Kohden). After conventional amplification, the EEG signals were conditioned through analogical filters, using cut off frequencies of 1.0 Hz and 35.0 Hz, and were then sampled at 200 Hz using a 16 bits A/D converter. The recordings were divided in epochs of 10 seconds intervals and classified as wakefulness, NREM or REM sleep. In addition, the number of NREM and REM episodes and mean length of NREM and REM episodes were evaluated. Sleep efficiency was calculated as the time spent sleeping in relation to the total time of sleep recording.

Fast Fourier Transform (Hanning window) was computed on 256 points (corresponding to each vigilance state) with a resolution of 0.78 Hz. Fast Fourier Transform was applied in the frequency interval of 1.0 to 16.0 Hz and those above 16.0 Hz were discarded from analysis. Non-overlapping bands were set giving 0.5 Hz bins from 1.0 to 5.0 Hz, and 1.0 Hz bins from 5.1 Hz to 16.0 Hz. Delta power was calculated as mean power density on 1.0–4.0 Hz, theta power was calculated as mean power density on 5.0–8.0 Hz and alfa power was calculated as mean power density on 8.0–13.0 Hz[25].

*2.6 Object recognition test (ORT)*

The apparatus used to investigate object recognition memory consists of an open box (width ×length × height = 60 cm× 60 cm×50 cm) made of wood and covered with a black opaque plastic film. The objects to be discriminated were available in triplicate copies and were made of biologically neutral material such as glass, plastic or metal. Also, they are not known to have any ethological significance for the rats. This test is based on the tendency of the animals to explore new things instead of familiar things. Thus, when an animal remembers a familiar object and does not

know a new object, there is a tendency of this animal to explore the new object for a longer time when compared to the familiar object. The ORT in this study consisted of three phases: the habituation phase, the sample/training phase and the choice/test phase[26]. In the habituation phase, the animals had three minutes in day 3, 5 and 7 to explore the arena without the objects (Fig. 1). During the training phase (15 minutes after habituation in day 7), two identical objects were exposed in the back corners of the open box, 10 cm away from the sidewall. The rat was placed in the open box facing away from the objects and after 3 min of exploration, the rat was removed from the open box and returned to its cage. Twenty-four hours later (test phase), two objects were presented in the same locations that were occupied by the previous sample objects. One of the objects was identical to the object seen in the training phase and the other one was different. The tests were video recorded and analyzed by a blind experimenter. It was considered as exploration only when the rat touched the object with its nose or when the rat's nose was directed toward an object at a distance ≤2 cm. Delta value was obtained from the following formula: Delta value = Time spent exploring the new object – time spent exploring the familiar object.

*2.7 Open field test (OF)*

The apparatus consists of a circular arena (1 m of diameter) limited by a 40-cm-high wall and illuminated by four 60-W lamps situated 100 cm above the arena floor, providing illumination around 300 lx. The animals were gently placed in the center of the arena and could freely explore the area for 5 min. During the experiments, the OF was video recorded and the measure for ambulatory distance was computed online by an image analyzer (Smart Junior, PanLab, Harvard Apparatus, Spain).

*2.8 Western blot*

To determine CB1 and CB2 receptors expression within the striatum, the animals were decapitated, their brains were rapidly removed, and the striatum was dissected. Tissues were stored at -80ºC until processing. Samples were sonicated in RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% SDS, 50 mM Tris pH 8, 2 mM EDTA). After centrifugation (10 min, 12,000 rpm at 4°C), the supernatant was collected, and protein concentration was determined by the Bradford method (Bio-Rad, Germany). 40 µL of each sample were subjected to 10% SDS-PAGE gel (1.0 mm) and transferred to a PVDF membrane (GE Healthcare). The membranes were then blocked in 5% BSA diluted in TBS-T overnight at 4ºC, followed by incubation with anti-CB1 antibody (1:1000 diluted in TBS-T; Sigma-Aldrich®, USA) and/or anti-CB2 antibody (1:1000 diluted in TBS-T; Sigma-Aldrich®, USA) overnight at 4ºC. In the sequence, the membranes were incubated with anti-rabbit antibody (1:1000 diluted in TBS-T; GE Healthcare) for two hours. β-actin was used as the housekeeping protein (1:5000 diluted in TBS-T; Sigma-Aldrich®, USA). Signal was detected using the ECL chemiluminescent detection system (GE Healthcare Life Sciences, Brazil). The protein levels were quantified by densitometry using ImageJ v1.47 software (National Institutes of Health, USA).

*2.9 Real-time PCR*

To investigate the mRNA expression of CB1 and CB2 receptors within the striatum, the animals were decapitated, the striatum dissected and stored at -80ºC. Samples were processed for RNA extraction using phenol-based reagent Brazol (Labdel, SP, Brazil), followed by cDNA synthesis using qPCRBIO kit (PCRBiosystems, London, United Kingdom). The cDNA target sequences were amplified, in triplicate, using the

Fast Sybr Green dye (Applied Biosystems) in a ViiA7 Real Time PCR System (Applied Biosystems), according to the manufacturer's instructions. A dissociation cycle was performed after each run to check for non-specific amplification or contamination. The results were normalized by the normalization factor generated for each sample by the geNorm program, using HPRT and β-actin as housekeeping genes. The primers for CB1 receptor (forward AGGAGAACTTACTGTGAACAGGC, reverse ATGGGTGCTCCTTGCTTGAA), CB2 receptor (forward CCTACTCACTCTGGACAGGAA, reverse GCATAGATGTTTGCTGGGTGG), HPRT (forward CCCAGCGTCGTGATTAGTGA, reverse TGGCCTCCCATCTCCTTCAT) and β-actin (forward CGAGTACAACCTTCTTGCAGC, reverse ATACCCACCATCACACCCTGG) were designed using PrimerBLAST, and validated through BLAST.

*2.10 Immunohistochemistry*

For tyrosine hydroxylase (TH) immune-reactive neurons quantification, the animals were anesthetized with ketamine (90 mg/kg). After this, each animal was intracardially perfused with a saline solution, followed by a fixative solution of formaldehyde 4% in 0.1 M phosphate buffer (pH 7.4). Brains were removed from skulls and were immersed in fixative solution at 4$^{o}$C. Forty-eight hours later, the material was immersed in a 30% sucrose solution for three days and finally stored at 80$^{o}$C freezer before sectioning. At this stage, 40 µm sections corresponding to SNpc (4.92 mm and 5.28 mm) were collected[21]. SNpc slices were incubated with primary mouse anti-TH antibody (1:500; Chemicon, CA, USA). Biotin-conjugated secondary antibody (1:200 anti-mouse; Vector Laboratories, USA), was localized using the ABC system (Vectastain ABC Elite kit, Vector Laboratories, USA), followed by 3,30-

diaminobenzidine reaction with nickel enhancement. Neuronal density was determined by the software Image J (National Institutes of Health, USA). For the rotenone group, a mean value was calculated and converted to a percentage relative to the sham vehicle group. The images were obtained using a motorized Axio Imager Z2 microscope (Carl Zeiss, Jena, DE), equipped with an automated scanning VSlide (Metasystems, Altlussheim, DE).

*2.11 Statistical analysis*

The normal distribution of the data was assessed by the Kolmogorov-Smirnov test. Differences between groups were assessed by unpaired t-test (immunohistochemistry), two-way ANOVA (sleep recordings, ORT [delta value], open field test, western blot, Real-time PCR) and repeated measures two-way ANOVA (ORT). Fisher's post hoc test was carried when necessary. Values were expressed as mean ± standard error of mean (SEM). The level of significance was set at $P < 0.05$.

## 3. Results

*3.1 Cannabinoid receptors modulation on sleep regulation*

Regarding the macrostructure of sleep (Fig.2), we observed that WIN 55,212-2 administration decreased sleep efficiency compared to the sham vehicle and sham AM251 groups ($P<0.01$) (Fig.2A). Rotenone also decreased sleep efficiency ($P<0.05$), which was reversed by administration of AM251 ($P<0.05$). Thus, our results show effects of cannabinoid receptors modulation [$F(2,26) = 7.52$, $P<0.01$] and

interaction [F(2,26) = 5.24, *P*<0.05], but no effect of the lesion by itself [F(1,26) = 0.03, *P*=0.86].

In agreement, sham WIN 55,212-2 group spent more time awake compared to the sham AM251, sham vehicle and rotenone WIN 55,212-2 groups (*P*<0.01) (Fig.2B). This demonstrates an effect of the lesion [F(1,25) = 5.587, *P*<0.05], cannabinoid receptors modulation [F(2,25) = 6.039, *P*<0.01] and interaction [F(2,25) = 4.205, *P*<0.05].

Regarding the time spent in NREM sleep (Fig.2C), we observed effects of cannabinoid receptors modulation [F(2,25) = 9.386, *P*<0.001] and interaction [F(2,25) = 11.05, *P*<0.001]. No effect of the lesion was found [F(1,25) = 2.532, *P* = 0.1241]. In fact, there was a decrease in the sham WIN 55,212-2 group compared to sham vehicle (*P*<0.01), sham AM251 (*P*<0.001) and rotenone WIN 55,212-2 (*P*<0.001) groups. In addition, rotenone administration decreased NREM sleep (*P*<0.05), which was reversed by the administration of both WIN 55,212-2 (*P*<0.01) and AM251 (*P*<0.001). Such differences result from alterations in the mean length, but not in the number of NREM sleep episodes. In fact, a lesion effect was demonstrated for the number of NREM sleep episodes (Fig.2D) [F(1,27) = 10.33, *P*<0.01], but no effect of cannabinoid receptors modulation [F(2,27) = 2.181, P = 0.1324] or interaction [F(2,27) = 1.868, P = 0.1738] was found. Regarding the mean length of NREM sleep episodes (Fig.2E), we observed effect of the lesion [F(1,22) = 8.21, *P*<0.01], cannabinoid receptors modulation [F(2,22) = 3.56, *P*<0.05] and interaction [F(2,22) = 3.44, *P*<0.05].

We observed effects of cannabinoid receptors modulation [F(2,27) = 3.564, *P*<0.05], but no effects of lesion [F(1,27) = 0.03800, P = 0.8469] and interaction [F(2,27) = 2.305, P = 0.1190] in the time spent in REM sleep (Fig.2F). This was demonstrated

by a decrease in the sham WIN 55,212-2 group compared to the sham vehicle group ($P<0.01$), which results from a decrease in the number of REM sleep episodes ($P<0.01$) (Fig.2G), but not from a decrease in the mean length of REM sleep episodes (Fig.2H). In fact, we observed effect of cannabinoid receptors modulation [$F(2,27) = 4.836$, $P<0.05$], but no effect of lesion [$F(1,27) = 0.2247$, $P = 0.6393$] or interaction [$F(2,27) = 2.627$, $P = 0.0907$] in the number of REM sleep episodes. None of the treatments demonstrated an effect on the mean length of REM sleep episodes [$F(1,27) = 1.494$, $P = 0.2322$, for the lesion], [$F(2,27) = 0.1762$, $P = 0.8394$, for cannabinoid receptors modulation], [$F(2,27) = 1.612$, $P = 0.2182$, for the interaction]. Regarding the global spectral power, no differences among the experimental groups were observed (Fig.3A). However, we observed effects of the lesion [$F(1,24) = 4.572$, $P<0.05$] and cannabinoid receptors modulation [$F(2,24) = 5.382$, $P<0.05$], but no effects of the interaction [$F(2,24) = 1.723$, $P = 0.1998$] on alfa power (Fig.3B). This was demonstrated by a decrease in the alfa power of the rotenone WIN 55,212-2 group compared to its vehicle ($P<0.05$) and to rotenone AM251 group ($P<0.01$) and an increase in the rotenone AM251 compared to its sham group ($P<0.05$). In addition, no differences on the delta, theta and on the ratio theta/delta power were found (Fig.3C,D,E).

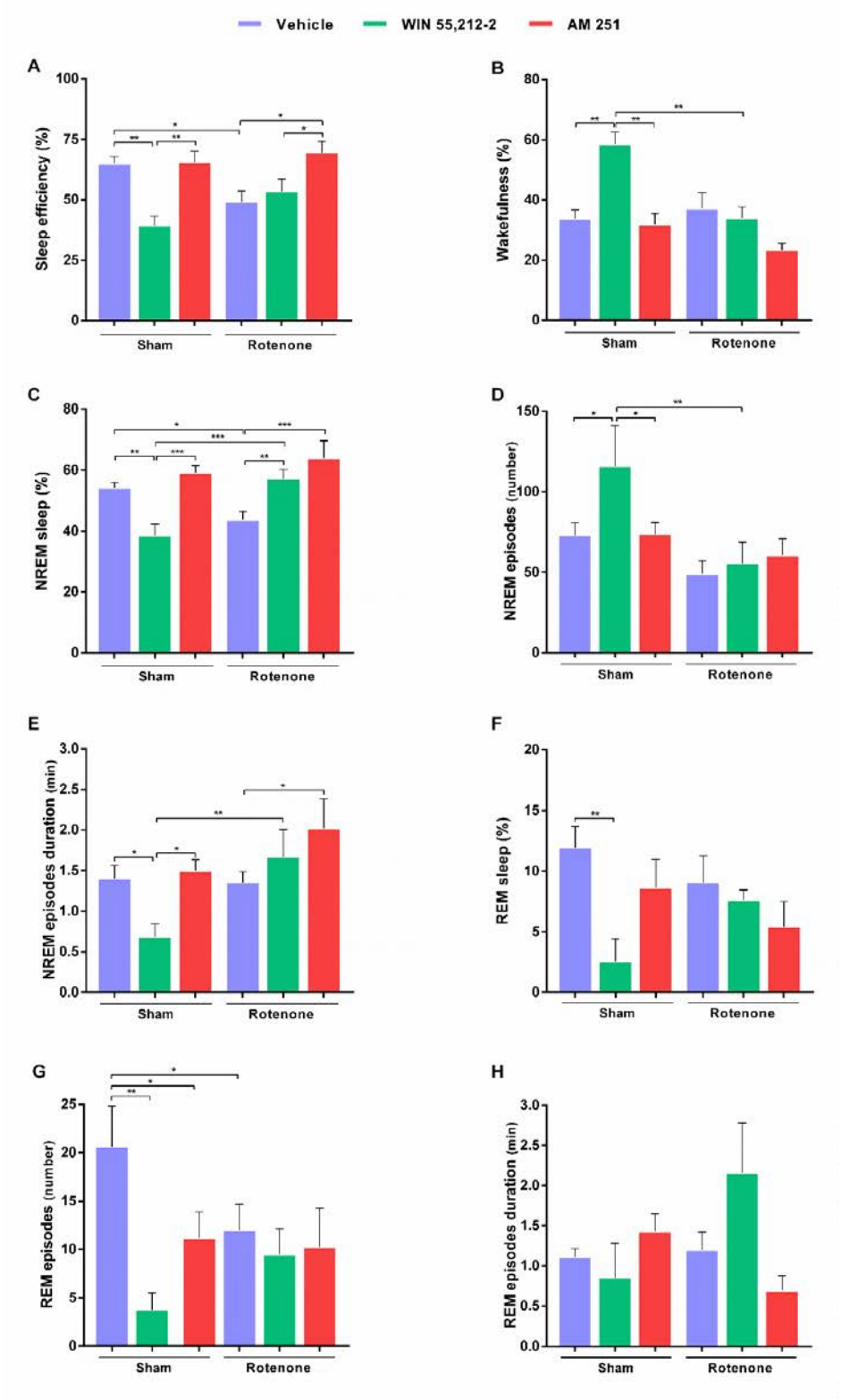


Figure 2. Cannabinoid receptors modulation on sleep regulation. Sleep efficiency (A), percentage of time spent in wakefulness (B), percentage of time spent in NREM sleep (C), number of NREM sleep episodes (D), mean length of NREM sleep episodes (E), percentage of time spent in REM sleep (F), number of REM sleep episodes (G), mean length of REM sleep episodes (H). Values are expressed as mean ± SEM. *P≤0.05, **P≤0.01, ***P≤0.001. Two-way ANOVA followed by Fisher's post hoc test. n = 4-6 animals/group.

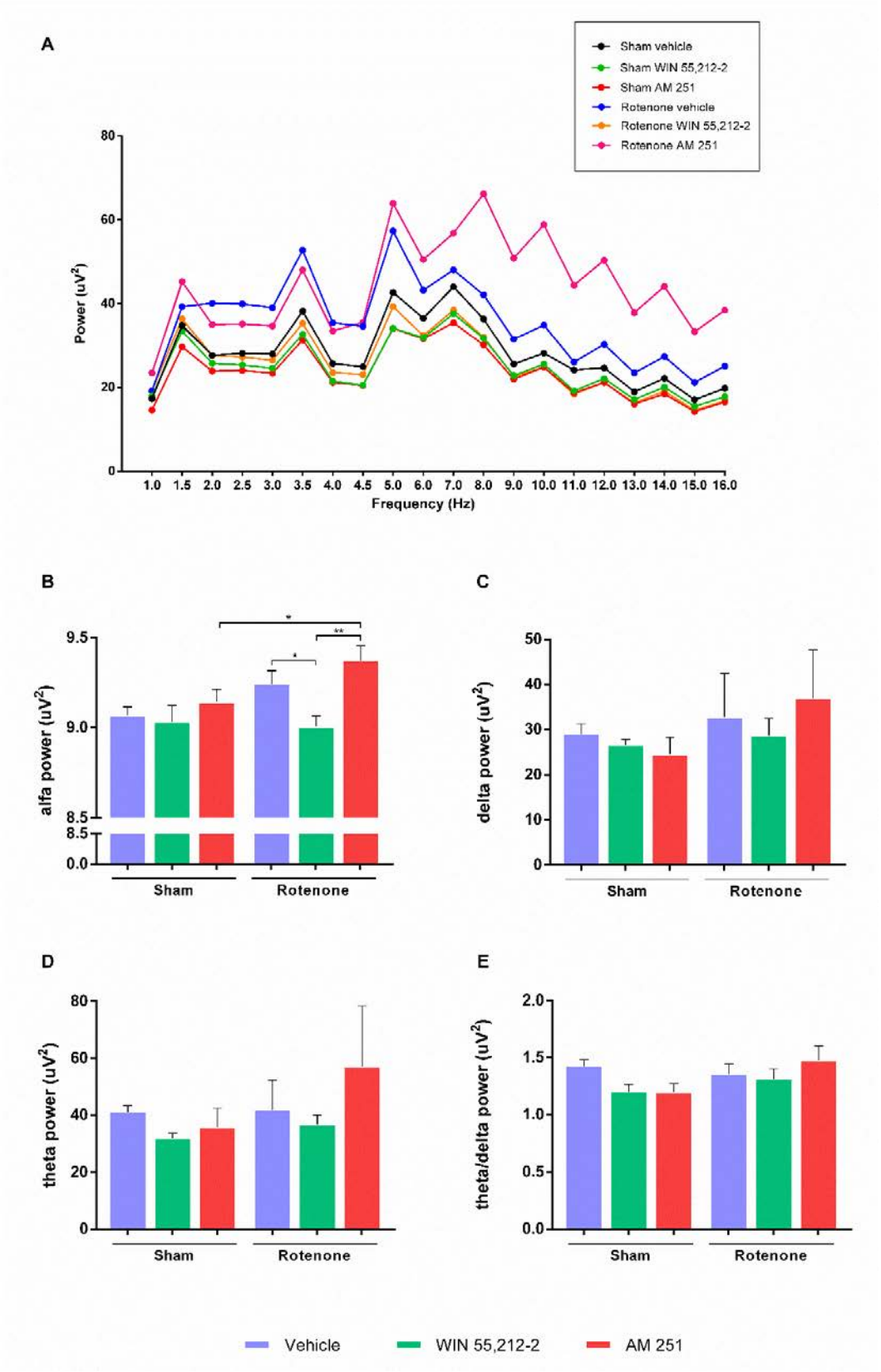


Figure 3. Cannabinoid receptors modulation on global spectral power. Spectral power in (A) different frequencies, (B) alfa range, (C) delta range, (D) theta range, (E) theta/delta ratio. Values are expressed as mean ± SEM. *P≤0.05, **P≤0.01, ***P≤0.001. Two-way ANOVA followed by Fisher's post hoc test. n = 4-6 animals/group.

*3.2 Cannabinoid receptors modulation on recognition memory*

The Fig. 4A shows that the sham groups spent more time exploring the new object compared to the familiar object ($P<0.05$, $P<0.01$ and $P<0.001$ for the sham vehicle, sham WIN 55,212-2 and sham AM251 groups, respectively), demonstrating that these animals differentiated the objects. Differently, rotenone administration impaired the object recognition memory, demonstrated by the similarity between the exploration of the new and familiar objects in the rotenone vehicle group. The blockade of CB1 receptor in the rotenone AM251 group reversed the rotenone-induced impairment ($P<0.05$). In fact, we observed an influence of the lesion [$F(1,71) = 6.11$, $P<0.05$], objects [$F(1,71) = 35.13$, $P<0.001$] and objects x lesion interaction [$F(1,71) = 6.54$, $P<0.05$], but no statistical influence of cannabinoid receptors modulation [$F(3,71) = 0.57$, $P=0.64$], lesion x cannabinoid receptors modulation interaction [$F(3,71) = 0.30$, $P=0.82$], objects x cannabinoid receptors modulation interaction [$F(3,71) = 1.78$, $P=0.16$] and lesion x cannabinoid receptors modulation x objects interaction [$F(3,71) = 0.43$, $P=0.73$] in our results.

Regarding the delta value (Fig.4B), no statistical differences among the groups were found. There was no influence of the lesion [$F(1,57) = 3.51$, $P=0.06$], cannabinoid receptors modulation [$F(2,57) = 1.78$, $P=0.17$] or the interaction between the two factors [$F(2,57) = 0.43$, $P=0.65$].

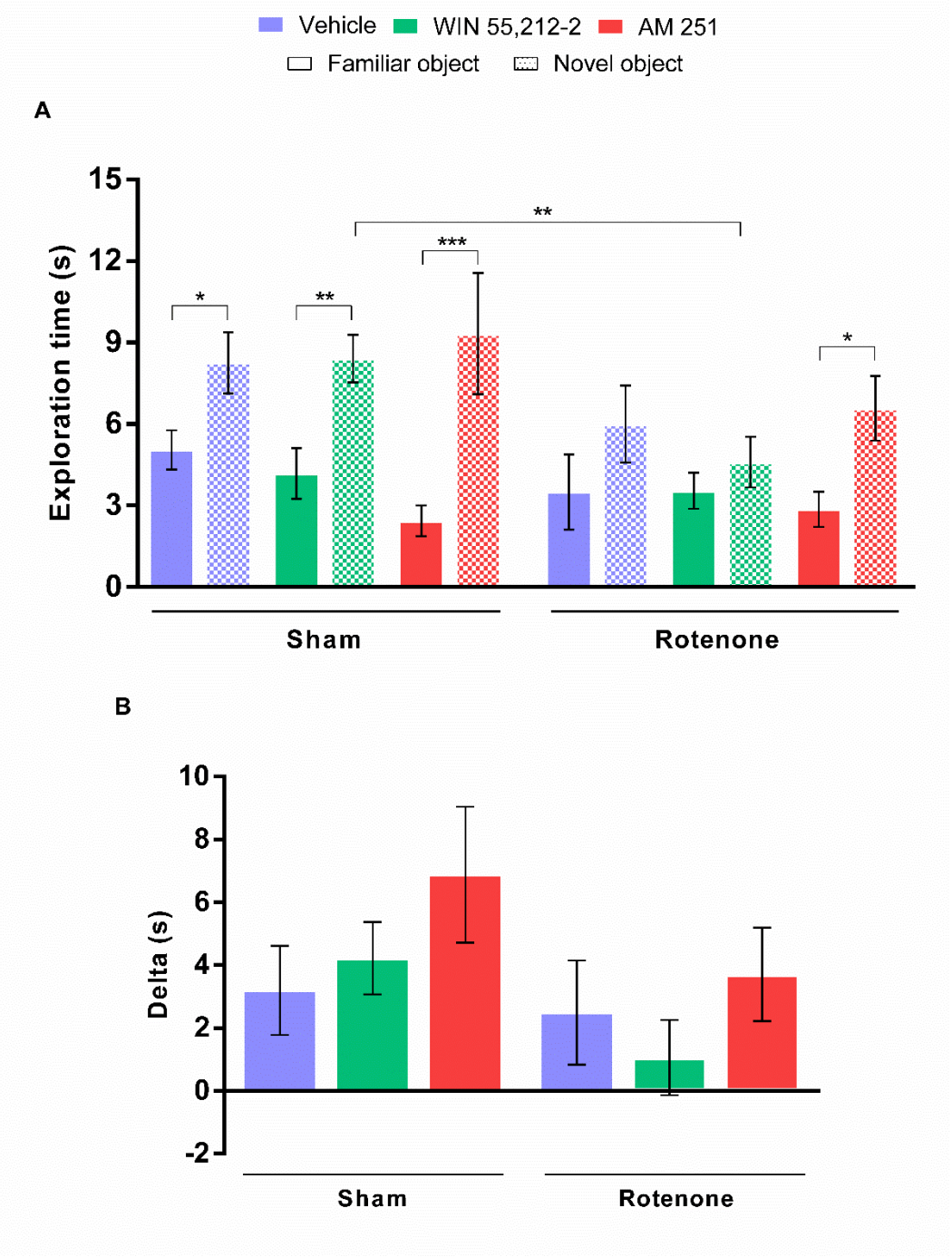


Figure 4. Object recognition test (ORT). Time spent exploring the objects (A) and delta value (B). Values are expressed as mean ± SEM. *P≤0.05, **P≤0.01, ***P≤0.001. Repeated measures two-way ANOVA (A) and Two-way ANOVA (B) followed by Fisher's post hoc test. n = 10-12 animals/group.

### *3.3 Open field*

Considering that CB1 receptors modulation reportedly affects motricity, we submitted the animals to the open field test (Fig.5). However, there was no effect of lesion

[F(1,45) = 0.2049, *P*=0.65], cannabinoid receptors modulation [F(2,45) = 0.1036, *P*=0.90] or interaction [F(2,45) = 2.190, *P*=0.12].

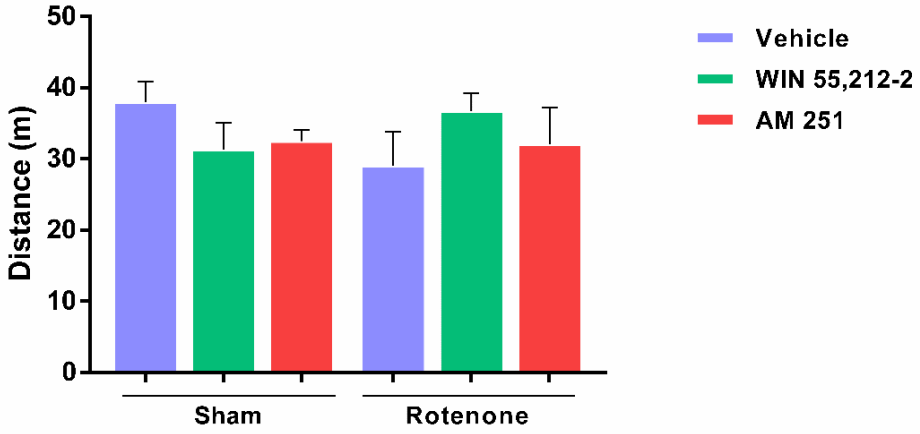


Figure 5. Open field test. Values are expressed as mean ± SEM. Two-way ANOVA. n = 10-12 animals/group.

*3.4 Cannabinoid receptors modulation on protein expression*

We observed effect of cannabinoid receptors modulation [F(2,18) = 4.888, *P*<0.05] and interaction [F(2,18) = 6.643, *P*<0.01], but no statistical effect of lesion itself [F(1,18) = 0.3501, P = 0.5614] in the CB1 receptor expression (Fig.6A). In fact, there was a decrease in the sham WIN 55,212-2 (*P*<0.05), sham AM251 (*P*<0.01) and rotenone vehicle (*P*<0.05) groups compared to sham vehicle group. Also, the rotenone AM251 group had an increased expression compared to the sham AM251 group (*P*<0.05). No differences were found regarding CB2 receptors expression (Fig.6B).

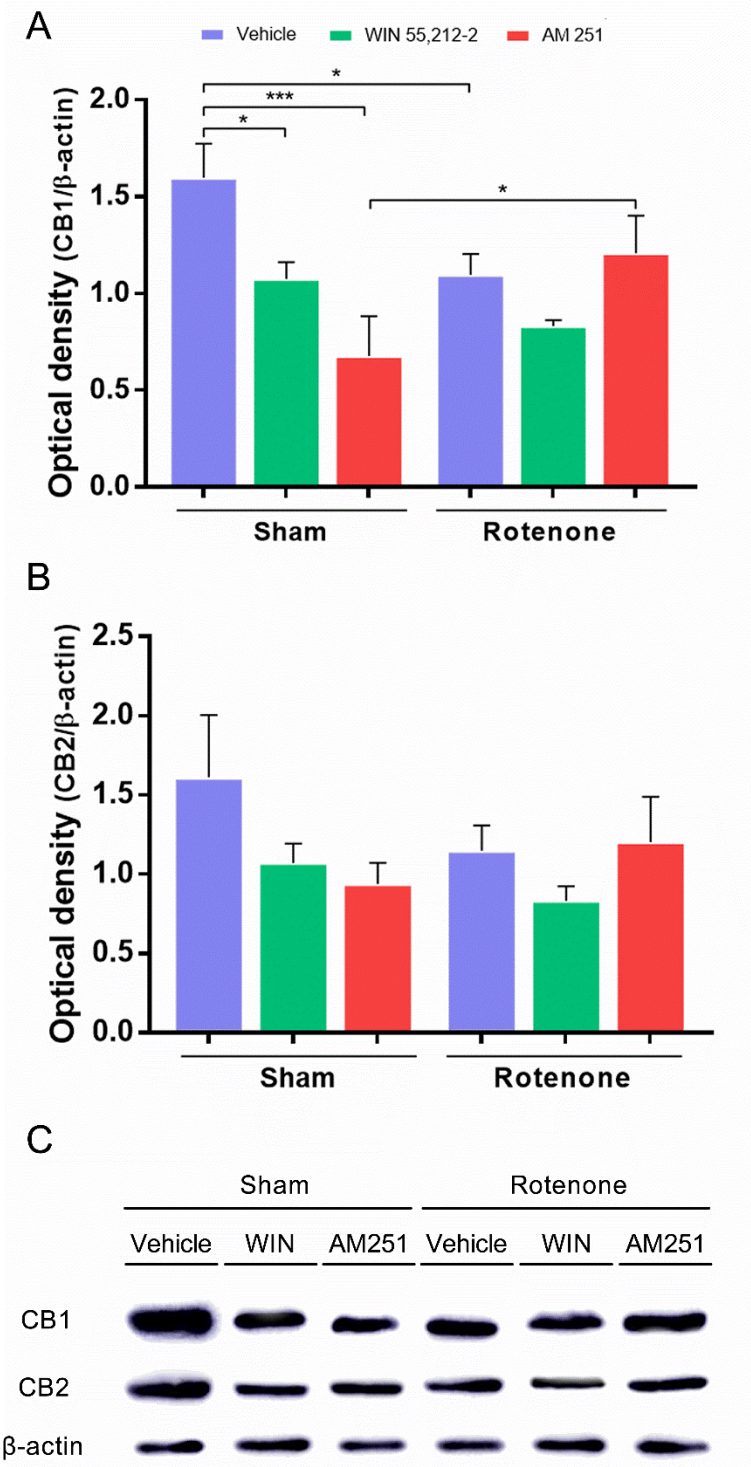


Figure 6. Cannabinoid receptors modulation on proteins expression. CB1 (A), CB2 (B) and representative bands (C). Values are expressed as mean ± SEM. *P≤0.05, ***P≤0.001. Two-way ANOVA followed by Fisher's post hoc test. n = 5 animals/group.

### *3.5 Cannabinoid receptors modulation on mRNA levels*

After Real-time PCR experiments, we observed an effect of cannabinoid receptors modulation [F(2,23) = 3.554, *P*<0.05], but no effect of the lesion [F(1,23) = 0.2885, P

= 0.5964] or interaction [F(2,23) = 1.110, P = 0.3464] on CB1 receptor mRNA levels (Fig.7A). This was demonstrated by an increase in both rotenone WIN 55,212-2 and rotenone AM251 groups compared to the rotenone vehicle group (*P*<0.05). We failed to observe any difference among groups regarding CB2 receptor mRNA levels (Fig.7B).

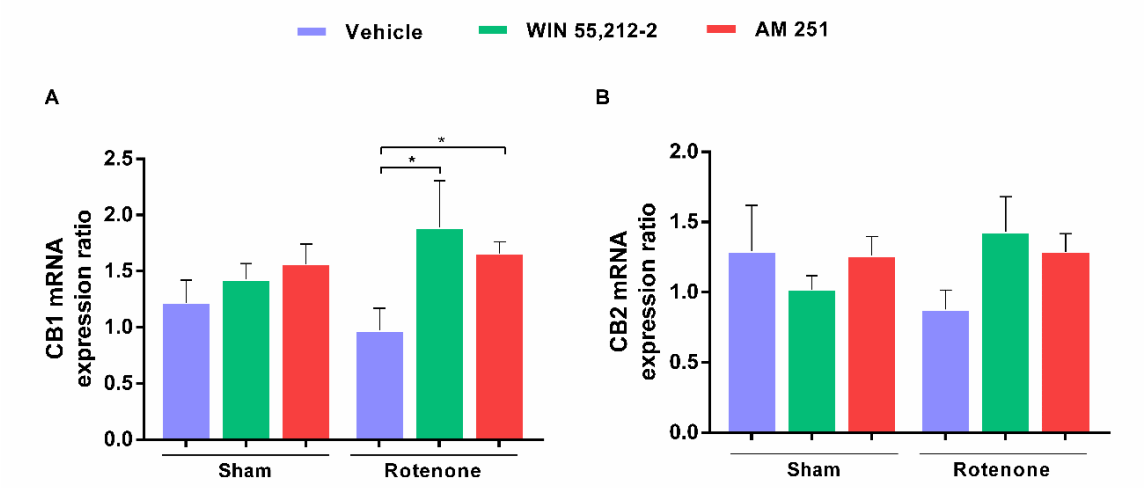


Figure 7. Cannabinoid receptors modulation on mRNA levels. CB1 (A) and CB2 (B) mRNA expression ratio. Values are expressed as mean ± SEM. *P≤0.05. Two-way ANOVA followed by Fisher's post hoc test. n = 5 animals/group.

### *3.6 Immunohistochemistry*

The Fig. 8 represents the density of tyrosine hydroxylase immune-reactive neurons within the SNpc. We observed a decrease of 55.1% in the rotenone vehicle group compared to the sham vehicle group (*P*<0.05).

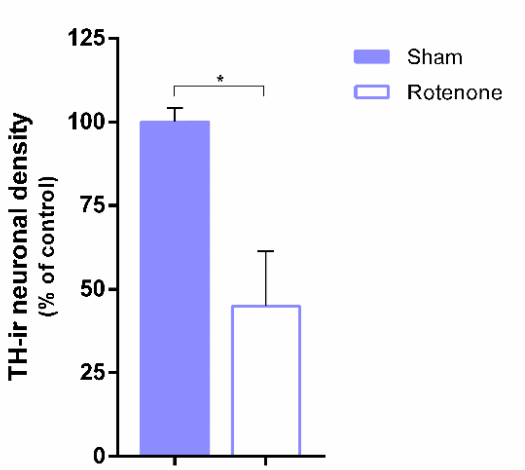


Figure 8. Percentage of TH immune-reactive neurons in SNpc. Values are expressed as mean ± SEM. *P≤0.05. Unpaired t-test. n = 4 animals/group.

## 4. Discussion

In the present study, we demonstrated that rotenone administration decreased the time spent in NREM sleep. Interestingly, the blockade of CB1 receptors by AM251 administration reversed the rotenone-induced effect. A similar outcome was observed regarding object recognition memory, in which the selective antagonism of CB1 receptors reversed the recognition memory impairment induced by rotenone.

WIN 55,212-2, a full agonist of CB1 receptors, increased the time spent awake, at the expense of a decrease in the time spent in both NREM and REM sleep, with no alteration in the global spectral power. Besides, there were no differences in the delta and theta power, suggesting a week association between macrostructure and spectral power in our study. Interestingly, while we found a decrease in the time spent in NREM sleep after WIN 55,212-2 administration, there was an increase in the number of NREM sleep episodes. However, there was a drastic decrease in the mean length of NREM episodes, which suggests substantial instability in NREM

sleep. Differently, the decrease in the time spent in REM sleep was consequence of a decrease in the number, but not in the mean length of REM sleep episodes.

Some reports contradicts our findings. Goonawardena and collaborators (2011) demonstrated that WIN 55,212-2 administration increased the time spent in NREM sleep[16]. Similarly, administration of anandamide, a partial agonist of CB1 receptors, also increased this parameter[27]. Some factors may explain the discrepancies between these outcomes and the present study. Firstly, the drugs used to modulate CB1 receptors have different properties. WIN 55,212-2, for example, is a full agonist that binds to both CB1 and CB2 receptors while anandamide, which is also used, is a partial agonist for these receptors. Also, drugs like anandamide binds to a variety of receptors, including the orphan GPR18 and GPR55, glycine, TRPV1 (Transient Receptor Potential Vanilloid 1) and TRPM8 (Transient Receptor Potential Menthol 8) receptors[28]. Furthermore, other factors including the chosen dose, route of administration, circadian variation and differences in the sleep analysis contribute for these differences, preventing a reliable comparison and interpretation of the data available in the literature.

As previously demonstrated, rotenone administration decreased the time spent in NREM sleep, highlighting the importance of the nigrostriatal pathway in NREM sleep regulation[6]. Moreover, our model did not affect the time spent in REM sleep but decreased the number of REM sleep episodes. In fact, REM sleep alterations induced by rotenone administration are not as robust as NREM sleep alterations[6]. Interestingly, AM251 administration reversed the rotenone-induced decrease in NREM sleep. This is apparently obtained by means of an increase in NREM sleep stability, but not by an increase in the number of NREM sleep episodes. Some studies report that AM251 administration in healthy conditions increases the time

spent awake, but others are in agreement to our findings, failing to observe such an effect[16,29]. In addition, this is the first time that AM251 administration is associated with an animal model of Parkinson's disease, especially for investigation of sleep parameters. Thus, we speculate that the beneficial effect of CB1 receptors blockade is dependent on a previous disruption in the nigrostriatal pathway, which was induced by rotenone administration in this case. Further studies are necessary to understand the mechanisms underlying this effect, which is very interesting considering the sleep disturbances in Parkinson's disease.

Rotenone also impaired the object recognition memory, demonstrated by a similar exploration between the familiar and non-familiar object. While a variety of studies show that the hippocampus has an important role in recognition memory, studies using Parkinson's disease animal models demonstrate that nigrostriatal pathway is of remarkable importance for this type of memory[7,30]. Using the rotenone model of Parkinson`s disease, Dos Santos and colleagues (2013) observed an impairment in object recognition memory 22 days after the lesion within SNpc, demonstrating that our model promotes a long-term cognitive decline[31]. Also, Targa and collaborators (2018) observed rotenone-induced impairment in recognition memory 7 days after the lesion, corroborating these findings[7].

AM251 administration also reversed the recognition memory impairment induced by rotenone. This demonstrates that the rotenone-induced impairment in object recognition memory is a reversible state, at least in this context. Similarly, previous findings demonstrated that raclopride-induced blockade of dopamine D2 receptors reversed the cognitive impairment after rotenone administration[7]. This led us to propose a speculative model for object recognition memory consolidation dependent of dopamine neurotransmission[7]. In this model, we proposed that a specific activity

level of dopaminergic neurotransmission is necessary to the expected performance on the ORT. Thus, modulation of this activity whether increasing or decreasing dopaminergic tone, leads to memory impairment. In fact, it was previously demonstrated that D1 receptors present an inverted U-shaped activity and memory consolidation may depend on the level of activation of D2 receptors[32–34]. Therefore, after rotenone administration within the SNpc, the dopaminergic tone decreased, reducing the binding of dopamine to postsynaptic D2 striatal receptors. This alteration in dopaminergic tone led to impairment in recognition memory. In this line, we suggest that the improvement in recognition memory after CB1 receptors blockade is associated with dopaminergic neurotransmission. CB1 cannabinoid receptors and D2 dopamine receptors are both G protein-couple receptors that are present in the striatal postsynaptic membranes[35]. In addition, these receptors form the heteromers, complexes of two or more functional receptor units exhibiting biochemical properties that are demonstrably different from those of its individual receptors. Heteromers formation promotes changes in dopamine receptor binding affinity, known as the “heteromer fingerprint”[9]. In fact, Marcellino and collaborators (2008) demonstrated that CB1 receptor activation blocked D2 activation-induced locomotor hyperactivity in rats[8]. Thus, we believe that antagonism of CB1 receptor may increase dopaminergic neurotransmission, reversing the rotenone-induced decrease in dopaminergic tone. Still, it is important to address that above-described hypothesis is speculative and further investigations are necessary to understand the mechanisms behind the observed outcomes. In addition, memory processes are affected by sleep[36]. Studies using sleep deprivation protocols demonstrate alterations in cognition, whether improving or impairing memory consolidation[7,31,37]. Thus, the

observed outcomes in recognition memory may reflect, at least in part, the alterations in sleep parameters.

The increase in CB1 receptors mRNA levels after AM251 administration can be associated with the increase in NREM sleep and to the improvement in recognition memory. However, we did not find the same outcomes at the protein level. The inverse pattern between protein and mRNA levels is expected, considering that one should only be synthesized when necessary, i.e., CB1 mRNA levels will increase once CB1 protein levels decrease[38]. Thus, we believe that CB1 receptors blockade led to an increase in mRNA CB1 levels to compensate the decreased tone in this neurotransmission. Further studies will be necessary to elucidate the relation between CB1 protein and mRNA levels and their possible role in memory and sleep parameters.

Our findings provide, for the first time, evidence on the influence of the cannabinoid system on sleep and recognition memory in an animal model of Parkinson's disease. We demonstrated that CB1 receptors modulation, specifically CB1 receptors blockade by AM251 administration, reverses the deleterious effects of rotenone administration in NREM sleep and in object recognition memory. In view of that, the modulation of the cannabinoid system becomes an interesting target to understand the mechanisms that underlie the non-motor symptoms of Parkinson's disease. This, in the last instance, will contribute to the development of possible therapeutic strategies.

**Conflict of interests**

The authors have declared that no conflict of interests exists.

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## 3.2 CAPÍTULO 2

### The CB-2-faced receptors: improvement of sleep or memory in Parkinson's disease

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## Abstract

Parkinson's disease non-motor symptoms drastically aggravate the quality of life of Parkinson's disease patients. The mechanisms that underlie these signs are still not fully elucidated and recent studies indicate that cannabinoid receptors play an important role. In this context, cannabinoid CB2 receptors are interesting targets, considering that their modulation would presumably lead to reduced psychoactive response, compared to CB1 receptors. In addition, these receptors were observed within the striatum and substantia nigra pars compacta (SNpc), with later evidence demonstrating a role in neuroprotection and motor function. Considering this, the objective of this study was to investigate the role of striatal CB2 receptors in two important non-motor symptoms of Parkinson's disease: sleep disturbances and cognitive deficits. For this, we administered rotenone in the SNpc of Wistar rats and, after 7 days, the animals received striatal infusions of a CB2 receptor agonist (GW 405833, 10 ug/ul), antagonist (AM630, 3 ug/ul) or vehicle (DMSO). We observed that rotenone administration decreased the time spent in NREM sleep. Interestingly, CB2 receptors blockade by AM630 administration reversed the rotenone-induced effect. On the other hand, the activation of CB2 receptors by GW405833 administration reversed the rotenone-induced impairment in object recognition memory. None of these events appeared to be related to CB1 and CB2 mRNA levels. Our findings demonstrate for the first time, a role for CB2 receptors in non-motor symptoms of Parkinson's disease. Such investigation will improve the understanding on the mechanisms that underlie these non-motor symptoms and consequently pave the way for future therapies.


**Keywords:** Parkinson's disease, sleep regulation, recognition memory, cannabinoids, CB2 receptors.

## Introduction

Parkinson's disease is a neurodegenerative disorder characterized by the loss of dopaminergic neurons within the substantia nigra pars compacta (SNpc), which leads to the well described motor symptoms, such as resting tremors, bradykinesia, gait disturbances, among others[1]. However, the non-motor symptoms, such as sleep disturbances and cognitive deficits, are becoming more frequently recognized as having a critical role particularly during the early onset[2,3].

The cannabinoid system is considered one of the major players in regulating the activity of several synapses, especially those involving GABAergic and glutamatergic neurotransmission[4]. Recent reports indicate a role in dopaminergic neurotransmission as well, which places the cannabinoid system as a promising target for Parkinson's disease[5,6]. Studies report interactions between cannabinoids and dopamine in the basal ganglia, demonstrating an inverse correlation between these two neurotransmission systems, in a way that increases of one neurotransmission decreases the other's activity[7–9]. Indeed, improvement in motor behavior after CB1 receptors blockade is observed in the animal model of Parkinson's disease induced by 6-OHDA (6-hydroxydopamine)[9]. In addition, we recently observed that blockade of CB1 receptors within the striatum decreased the rotenone-induced effects on sleep and memory (unpublished data).

Cannabinoids bind to the Gi/o-coupled CB2 receptors, which were thought to be restricted to cells of the immune system until reports demonstrating their presence on the brainstem[10]. Onaive and collaborators (2006) observed the transcripts of these receptors within the striatum, which was further supported by studies demonstrating their expression at the protein level in the same structure[11,12]. Recently, their

presence was described in the SNpc of *post-mortem* human brains[13]. Regarding the role of CB2 receptors within the central nervous system, it was demonstrated that the activation of these receptors decreased microglial activation and oxidative stress biomarkers, protecting against injuries within the central nervous system[14]. This suggests that CB2 receptors could act as neuroprotective agents. In fact, in the LPS (lipopolysaccharide) animal model of Parkinson's disease, there was an increase in CB2 mRNA levels in striatum and SNpc[15]. A similar outcome was observed after MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) administration, which is widely used as a model of Parkinson's disease[16]. Furthermore, CB2 receptors activation attenuated the motor inhibition and the loss of tyrosine hydroxylase–positive neurons induced by 6-OHDA administration within SNpc[9].

Currently, there is no evidence on the role of CB2 receptors in non-motor symptoms of Parkinson's disease. However, some studies report a function for these receptors in memory processes not associated with pathological conditions. Kruk-Slomka and collaborators (2016) demonstrated that both activation and blockade of CB2 receptors improved the performance in the passive avoidance test[14]. Also, the activation of these receptors protected against cognitive deficits following permanent ischemia[17]. Moreover, it was demonstrated that long-term fear contextual memory hippocampus-dependent is disrupted in knockout mice for CB2 receptors[18].

Considering the above-described, the objective of this study was to investigate the role of striatal CB2 receptors in two important non-motor features of Parkinson's disease: sleep disturbances and cognitive deficits. CB2 receptors scarcity in the central nervous system makes these receptors attractive as therapeutic targets as their modulation would presumably lead to reduced psychoactive response compared to CB1 receptors. Such investigation will improve the understanding of the

mechanisms that underlie these non-motor symptoms and possibly pave the way for future therapies.

## 2 Materials and Methods

### *2.1 Subjects*

The experiments in this study were approved by the ethics committee of Federal University of Paraná (all experiments; approval ID #857) and Federal University of São Paulo (sleep experiments; approval ID #9022050417). They were carried out in accordance to the Guidelines of ethics and experimental care and use of laboratory animals (SBCAL). All efforts were made to minimize animal suffering and to reduce the number of animals used. Male Wistar rats, weighing approximately 280-320g were kept in a temperature-controlled room (22±2$^{o}$C), with a 12:12h light-dark cycle (lights on at 7:00 AM). They were maintained in groups of 5 animals in polypropylene cages containing bottles of water and pellets of food throughout the entire experiment.

### *2.2 Experimental design*

The experimental design is represented in Fig. 1. There were two sets of animals. The first set (n=70) underwent stereotaxic surgery for rotenone or dimethylsulfoxide (DMSO) administration within SNpc, and bilateral guide cannulas implantation within the striatum. The habituation phase of the object recognition test (ORT) took place on days 3, 5 and 7. After the last habituation (day 7), the animals were submitted to the training phase of the ORT and then, we administered the drugs that modulate cannabinoid receptors directly into the striatum. The effects of these drugs were

evaluated in the next day (day 8), in which the ORT and open field test (OF) were performed. Finally, the animals were euthanized through decapitation and samples were collected for real-time PCR.

The second set of animals (n=30) were submitted to the same procedures on day 0, but with additional implantation of cortical electrodes for sleep-wake recording. After 7 days, we administered the drugs that modulate cannabinoid receptors directly into the striatum followed by a period of 3h of sleep-wake recording (9:00 AM-12:00 PM).

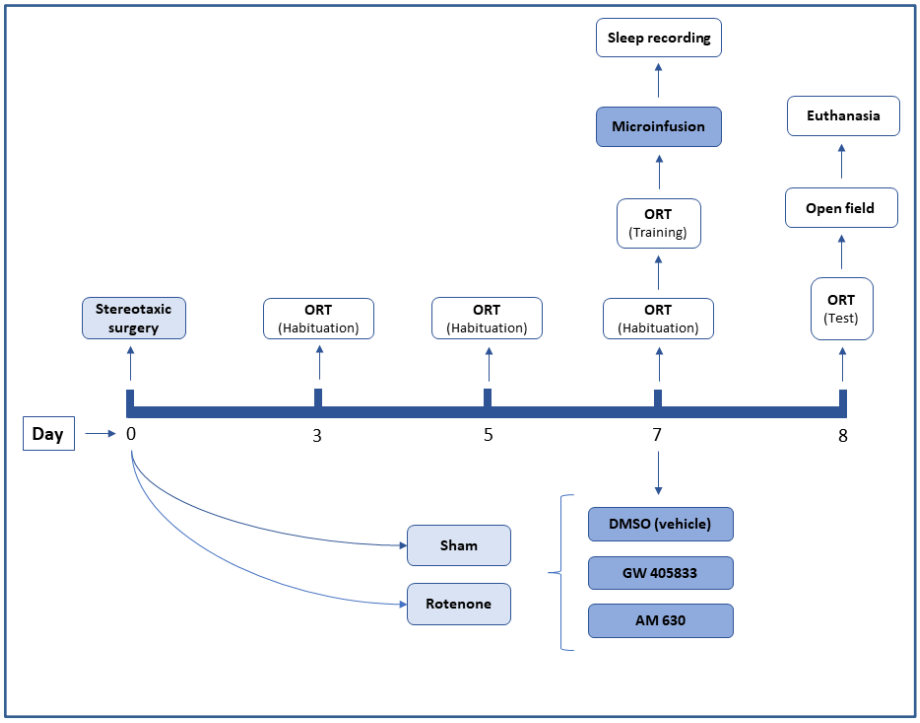


Figure 1. Experimental design. ORT, Object recognition memory.

*2.3 Stereotaxic surgery*

The animals were sedated with intraperitoneal xylazine (10 mg/kg; Syntec do Brasil Ltda, Brazil) and anesthetized with intraperitoneal ketamine (90 mg/kg; Syntec do Brasil Ltda, Brazil). For rotenone infusion within the SNpc, we used bregma as a reference for the following coordinates: (AP) = -5.0 mm, (ML)= ± 2.1 mm and (DV) = -8.0 mm[19]. Rotenone (12 µg/µl; Sigma-Aldrich®, United States) or DMSO 10% v/v

(Sigma-Aldrich®, United States) infusions were made using an electronic infusion pump (Insight Instruments, Ribeirão Preto, Brazil) at a rate of 0.33 µl/min for 3 min[20]. Bilateral guide cannulas implantation within the striatum were made using bregma as reference for the following coordinates: (AP) = -1.0 mm, (ML)= ±3.0 mm and (DV) = -4.0 mm[19]. For electrodes positioning, the following coordinates were used, using bregma as a reference: (AP) = -1.8 mm, (ML) = -2.0 mm (first electrode) and (AP) = 3.0 mm, (ML) = 1.0 mm (second electrode); and lambda as a reference: (AP) = 1.0 mm, (ML) = -4.0 mm (third electrode) and (AP) = 4.0 mm, (ML) = 1.0 mm (fourth electrode)[19].

*2.4 Striatal microinfusion*

The awake animals were gently immobilized for infusions of the CB2 receptor agonist GW 405833 (10 µg/µl; Sigma-Aldrich, USA), antagonist AM 630 (3 µg/µl; Sigma-Aldrich, USA) or vehicle (DMSO, 10% v/v; Sigma-Aldrich, USA) directly in the striatum. The infusions were made at the bilateral guide cannulas (implanted during stereotaxic surgery), at a rate of 0.33 µl/min for 3 min, with the assistance of an electronic infusion pump (Insight Instruments, Ribeirão Preto, Brazil).

*2.5 Sleep recording procedure*

Electrophysiological signals were recorded on a digital polygraph (Neurofax QP 223A Nihon Kohden). After conventional amplification, the EEG signals were conditioned through analogical filters, using cut off frequencies of 1.0 Hz and 35.0 Hz, and were then sampled at 200 Hz using a 16 bits A/D converter. The recordings were divided in epochs of 10 seconds intervals and classified as wakefulness, NREM or REM sleep. In addition, the number of NREM and REM episodes and mean length of

NREM and REM episodes were evaluated. Sleep efficiency was calculated as the time spent sleeping in relation to the total time of sleep recording.

Fast Fourier Transform (Hanning window) was computed on 256 points (corresponding to each vigilance state) with a resolution of 0.78 Hz. Fast Fourier Transform was applied in the frequency interval of 1.0 to 16.0 Hz and those above 16.0 Hz were discarded from analysis. Non-overlapping bands were set giving 0.5 Hz bins from 1.0 to 5.0 Hz, and 1.0 Hz bins from 5.1 Hz to 16.0 Hz. Delta power was calculated as mean power density on 1.0–4.0 Hz, theta power was calculated as mean power density on 5.0–8.0 Hz and alfa power was calculated as mean power density on 8.0–13.0 Hz[21].

*2.6 Object recognition test (ORT)*

The apparatus used to investigate object recognition memory consists of an open box (width ×length × height = 60 cm× 60 cm×50 cm) made of wood and covered with a black opaque plastic film. The objects to be discriminated were available in triplicate copies and were made of biologically neutral material such as glass, plastic or metal. Also, they are not known to have any ethological significance for the rats. This test is based on the tendency of the animals to explore new things instead of familiar things. Thus, when an animal remembers a familiar object and does not know a new object, there is a tendency of this animal to explore the new object for a longer time when compared to the familiar object. The ORT in this study consisted of three phases: the habituation phase, the sample/training phase and the choice/test phase[22]. In the habituation phase, the animals had three minutes in day 3, 5 and 7 to explore the arena without the objects (Fig. 1). During the training phase (15 minutes after habituation in day 7), two identical objects were exposed in the back corners of

the open box, 10 cm away from the sidewall. The rat was placed in the open box facing away from the objects and after 3 min of exploration, the rat was removed from the open box and returned to its cage. Twenty-four hours later (test phase), two objects were presented in the same locations that were occupied by the previous sample objects. One of the objects was identical to the object seen in the training phase and the other one was different. The tests were video recorded and analyzed by a blind experimenter. It was considered as exploration only when the rat touched the object with its nose or when the rat's nose was directed toward an object at a distance ≤2 cm. Delta value was obtained from the following formula: Delta value = Time spent exploring the new object – time spent exploring the familiar object.

*2.7 Open field test (OF)*

The apparatus consists of a circular arena (1 m of diameter) limited by a 40-cm-high wall and illuminated by four 60-W lamps situated 100 cm above the arena floor, providing illumination around 300 lx. The animals were gently placed in the center of the arena and could freely explore the area for 5 min. During the experiments, the OF was video recorded and the measure for ambulatory distance was computed online by an image analyzer (Smart Junior, PanLab, Harvard Apparatus, Spain).

*2.8 Real-time PCR*

To investigate the mRNA expression of CB1 and CB2 receptors within the striatum, the animals were decapitated, the striatum dissected and stored at -80ºC. Samples were processed for RNA extraction using phenol-based reagent Brazol (Labdel, SP, Brazil), followed by cDNA synthesis using qPCRBIO kit (PCRBiosystems, London, United Kingdom). The cDNA target sequences were amplified, in triplicate, using the

Fast Sybr Green dye (Applied Biosystems) in a ViiA7 Real Time PCR System (Applied Biosystems), according to the manufacturer's instructions. A dissociation cycle was performed after each run to check for non-specific amplification or contamination. The results were normalized by the normalization factor generated for each sample by the geNorm program, using HPRT and β-actin as housekeeping genes. The primers for CB1 receptor (forward AGGAGAACTTACTGTGAACAGGC, reverse ATGGGTGCTCCTTGCTTGAA), CB2 receptor (forward CCTACTCACTCTGGACAGGAA, reverse GCATAGATGTTTGCTGGGTGG), HPRT (forward CCCAGCGTCGTGATTAGTGA, reverse TGGCCTCCCATCTCCTTCAT) and β-actin (forward CGAGTACAACCTTCTTGCAGC, reverse ATACCCACCATCACACCCTGG) were designed using PrimerBLAST, and validated through BLAST.

*2.9 Statistical analysis*

The normal distribution of the data was assessed by the Kolmogorov-Smirnov test. Differences between groups were assessed by two-way ANOVA (sleep recordings, ORT [delta value], open field test, Real-time PCR) and repeated measures two-way ANOVA (ORT). Fisher's post hoc test was carried when necessary. Values were expressed as mean ± standard error of mean (SEM). The level of significance was set at $P < 0.05$.

## 3 Results

*3.1 CB2 receptors in sleep regulation*

The Fig. 2 represents the macrostructure of sleep after CB2 receptors modulation. We observed that rotenone administration decreased sleep efficiency ($P$<0.05) while activation of CB2 receptors increased this parameter ($P$<0.05) (Fig.2A). Thus, there was an effect of the lesion [F(1,24) = 9.146, $P$<0.01] and interaction between lesion and CB2 receptors modulation [F(2,24) = 3.334, $P$<0.05], but no effect of CB2 modulation by itself [F(2,24) = 1.693, P = 0.2051].

Regarding the time spent awake (Fig.2B), we observed a lesion effect [F(1,24) = 4.299, P $P$< 0.05], but no effect of CB2 modulation [F(2,24) = 0.5326, P = 0.5939] or interaction [F(2,24) = 2.516, P = 0.1019]. This was demonstrated by a decreased time spent awake in the sham GW405833 compared to the rotenone GW405833 group ($P$<0.01). In addition, activation of CB2 receptors in the sham group decreased the time spent awake compared to its vehicle group and to the sham AM630 group ($P$<0.05).

The decreased time spent awake after CB2 receptors activation in the sham group led to an increase in NREM sleep ($P$<0.05) (Fig.2C), demonstrating an effect of CB2 receptors modulation [F(2,24) = 4.122, $P$<0.05]. A lesion effect was observed [F(1,24) = 5.561, $P$<0.05] with the decreased time spent in NREM sleep in the rotenone GW405833 and rotenone vehicle groups compared to its respective sham groups ($P$<0.05, for both). Interestingly, the blockade of CB2 receptors by AM630 administration reversed the rotenone-induced decrease in NREM sleep ($P$<0.05). In addition to lesion and CB2 receptors modulation effects we observed an effect of the interaction between these factors [F(2,24) = 3.659, $P$<0.05]. We did not find any differences among the groups regarding the number [F(1,22) = 0.1792, P = 0.6761, lesion] [F(2,22) = 1.507, P = 0.2436, CB2 modulation] [F(2,22) = 1.495, P = 0.2462, interaction] and duration [F(1,22) = 0.2171, P = 0.6458, lesion] [F(2,22) = 0.1194, P =

0.8881, CB2 modulation] [F(2,22) = 1.941, P = 0.1674, interaction] of NREM sleep episodes (Fig.2D and E, respectively).

There was a lesion effect in the time spent in REM sleep [F(1,24) = 6.394, *P*<0.05) (Fig.2F), represented by a decrease in the rotenone GW405833 group compared to its sham group (*P*<0.05). No effect of CB2 receptors modulation [F(2,24) = 0.833, P=0.4466] or interaction between the factors [F(2,24) = 1.366, P=0.2743] was demonstrated statistically. A similar outcome was observed regarding the number of REM sleep episodes (*P*<0.01) (Fig.2G), demonstrating a lesion effect [F(1,24) = 13.05, *P*<0.01], but no effects of CB2 modulation [F(2,24) = 0.3658, P = 0.6975] or interaction between the factors [F(2,24) = 1.188, P = 0.3222]. None of the treatments had any effect on the duration of REM sleep episodes (Fig.2H) [F(2,22) = 1.806, P = 0.1878, lesion] [F(2,22) = 0.8704, P = 0.4327, CB2 modulation] [F(1,22) = 0.6015, P = 0.4463, interaction].

No differences among the experimental groups were observed in the global spectral power (Fig.3A). Conversely, we observed effects of the lesion [F(1,22) = 5.021, *P*<0.05] on alfa power (Fig.3B), but no statistical differences were observed among the groups. There was no effect of CB2 receptors modulation [F(2,22) = 0.3000, P = 0.7438] or interaction [F(2,22) = 0.2887, P = 0.7520]. In addition, no differences on delta power (Fig.3C), theta power (Fig.3D) or on the ratio theta/delta (Fig.3E) were found.

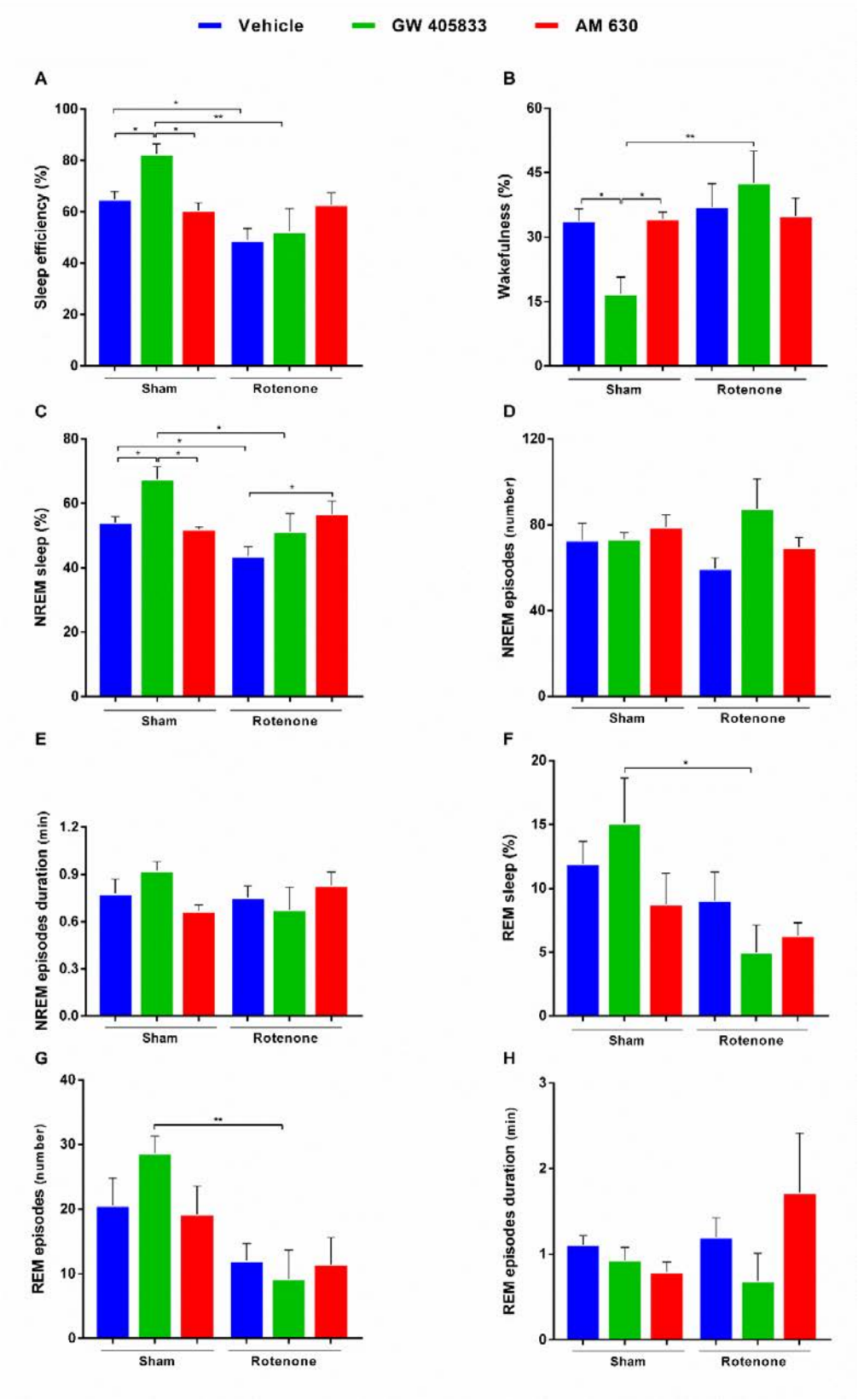


Figure 2. CB2 receptors modulation on sleep regulation. Sleep efficiency (A), percentage of time spent in wakefulness (B), percentage of time spent in NREM sleep (C), number of NREM sleep episodes (D), mean length of NREM sleep episodes (E), percentage of time spent in REM sleep (F), number of REM sleep episodes (G), mean length of REM sleep episodes (H). Values are expressed as mean ± SEM. *P≤0.05, **P≤0.01. Two-way ANOVA followed by Fisher's post hoc test. n = 4-6 animals/group.

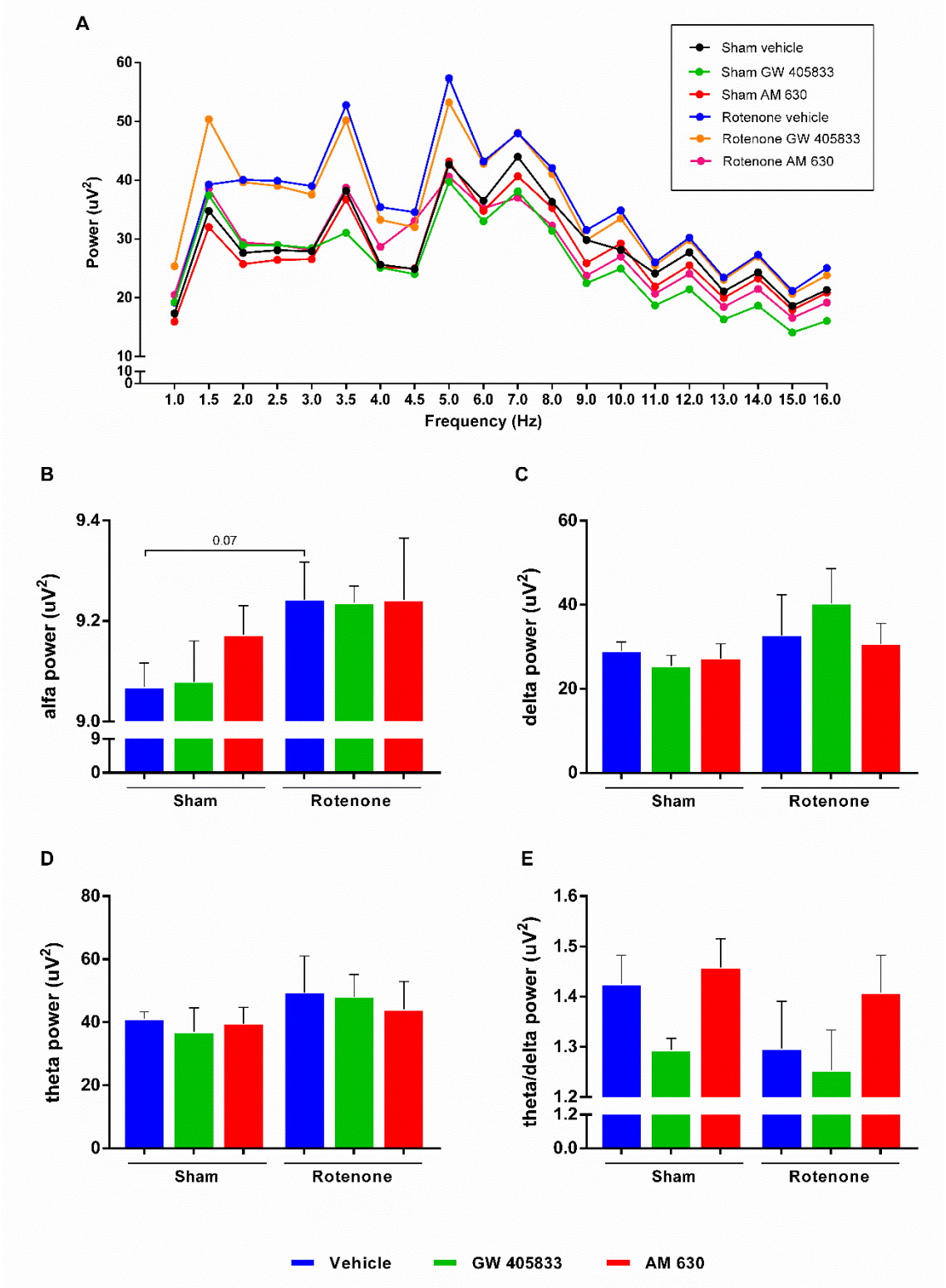


Figure 3. CB2 receptors modulation on global spectral power. Spectral power in (A) different frequencies, (B) alfa range, (C) delta range, (D) theta range, (E) theta/delta ratio. Values are expressed as mean ± SEM. Two-way ANOVA. n = 4-6 animals/group.

## *3.2 CB2 receptors in recognition memory*

In the Fig. 4A, we observed that the sham groups spent more time exploring the new object compared to the familiar object ($P$<0.05, $P$<0.001 and $P$<0.001 for the sham vehicle, sham GW405833 and sham AM630 groups, respectively). Also, rotenone impaired the object recognition memory, which was reversed by activation of CB2 receptors in the rotenone GW405833 group, demonstrated by an increase in the time exploring the new object compared to the familiar object ($P$<0.001). In this context, we observed an influence of the objects [F(1,71) = 29.51, $P$<0.001], objects x CB2 modulation [F(2,71) = 3.93, $P$<0.05] and objects x lesion [F(1,71) = 5.35, $P$<0.05], but no statistical influence of lesion [F(1,71) = 2.26, $P$=0.14], CB2 receptors modulation [F(2,71) = 1.18, $P$=0.31], lesion x CB2 modulation [F(2,71) = 2.55, $P$=0.08], and lesion x CB2 modulation x objects [F(2,71) = 1.50, $P$=0.23].

CB2 receptors modulation also led to statistical differences among groups in the delta value (Fig.4B). This was represented by a decrease in the rotenone vehicle and rotenone AM630 groups compared to their respective sham groups ($P$<0.05, for both), which corroborates the findings presented in the Fig. 4A. Also, rotenone GW405833 group presented an increase in the delta value compared to the rotenone vehicle group ($P$<0.01). In fact, we observed an influence of both lesion [F(1,71) = 5.353, $P$<0.05] and CB2 modulation [F(2,71) = 3.930, $P$<0.05], but no influence of the interaction between these factors [F(2,71) = 1.501, $P$=0.23].

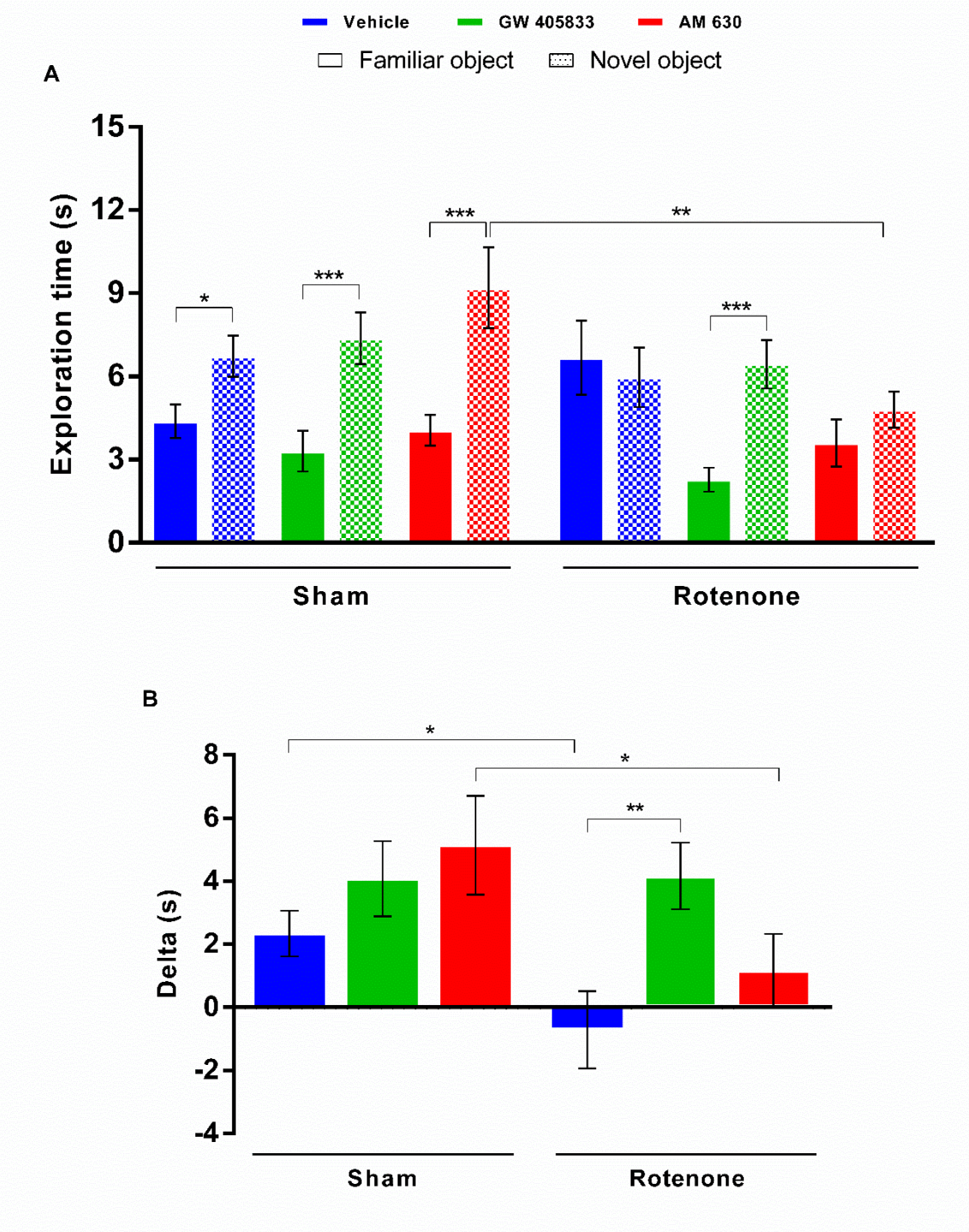


Figure 4. Object recognition test (ORT). Time spent exploring the objects (A) and delta value (B). Values are expressed as mean ± SEM. *P≤0.05, **P≤0.01, ***P≤0.001. Repeated measures two-way ANOVA (A) and Two-way ANOVA (B) followed by Fisher's post hoc test. n = 10-12 animals/group.

### *3.3 CB2 receptors modulation on striatal mRNA levels*

We did not observe effect of any intervention in the CB1 and CB2 mRNA expression [F(1,24) = 0.8613, P = 0.3626, lesion; F(2,24) = 0.1927, P = 0.8260, CB2 modulation; F(2,24) = 0.4291, P = 0.6560, interaction] (Fig.5).

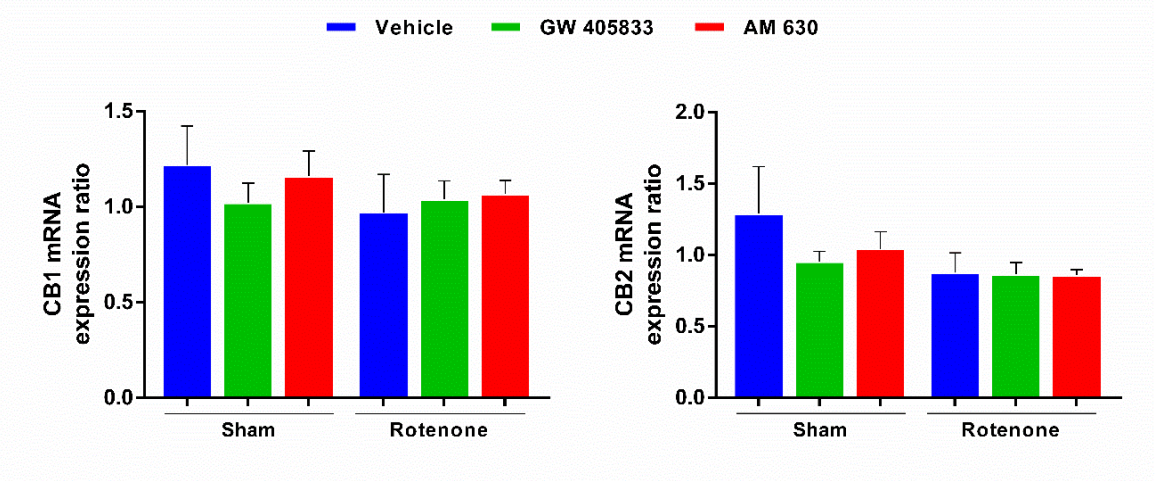


Figure 5. CB2 receptors modulation on mRNA levels. CB1 (A) and CB2 (B) mRNA expression ratio. Values are expressed as mean ± SEM. Two-way ANOVA. n = 5 animals/group.

*3.4 CB2 receptors modulation on the open field test*

There was no effect of the lesion [F(1,35) = 0.3315, P = 0.5684], CB2 receptors modulation [F(2,35) = 1.228, P = 0.3053] or interaction between the factors [F(2,35) = 1.737, P = 0.1909] in the open field test (Fig.6).

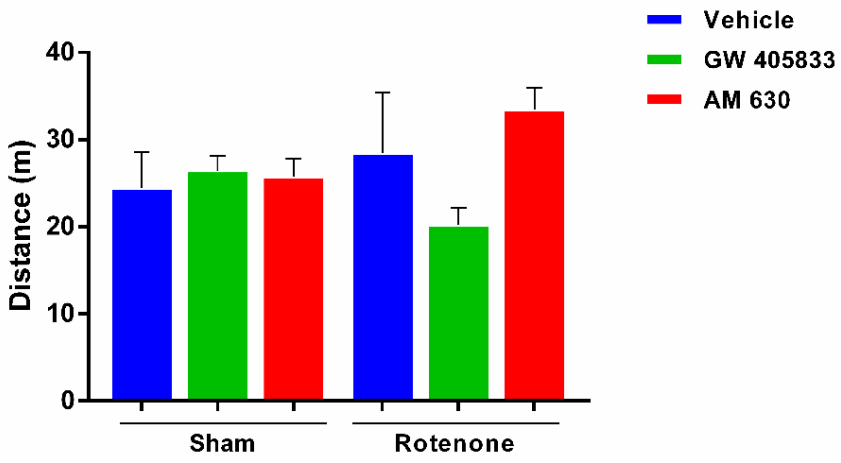


Figure 6. Open field test. Values are expressed as mean ± SEM. Two-way ANOVA. n = 10-12 animals/group.

## Discussion

We observed that rotenone administration decreased the time spent in NREM sleep. Interestingly, CB2 receptors blockade by AM630 administration reversed the rotenone-induced effect. On the other hand, the activation of CB2 receptors by GW405833 administration reversed the rotenone-induced impairment in object recognition memory. None of these events appeared to be related to CB1 and CB2 mRNA levels in the striatum.

CB2 receptors activation by GW405833 (a partial agonist at CB2 receptors) administration decreased the time spent awake in the sham group, at the expense of an increase in both NREM and REM sleep. Conversely, we recently observed that WIN 55,212-2 (a full agonist of CB1/CB2 receptors) administration increased the time spent awake (unpublished data). This suggests that a specific level of CB2 activation may be necessary for a given response. In addition, GW405833 is a selective CB2 agonist while WIN 55,212-2 exhibits affinity for other receptors, including CB1 receptors (full agonist), which may account to the observed results[23,24].

The localization of CB2 receptors was thought to be restricted to cells of the immune system until reports demonstrating their presence on the brainstem and striatum[10,12]. In addition, García and collaborators (2015) recently demonstrated CB2 receptors expression within the SNpc in *post-mortem* human brains[13]. These authors observed a reduced expression of these receptors in *post-mortem* brains of Parkinson's disease patients compared to control subjects as a consequence of a decrease in the dopaminergic cells, but also due to a decreased expression in the reminiscent cells. Thus, we speculate that the decrease in dopaminergic cells after rotenone administration reduced the target for GW405833, which prevented its effects on the

rotenone group. Further investigations on the protein expression will be necessary to confirm this hypothesis.

Our data on the mRNA levels do not corroborate previous findings showing an increase of CB2 expression in Parkinson's disease[15]. However, most of the studies evaluated the protein levels while we observed the transcripts. Also, the time frame between neurotoxin administration and evaluation of mRNA levels might account for such differences. Moreover, studies report that LPS and MPTP models of Parkinson's disease increase CB2 receptors expression and, consequently, are prone to respond to CB2 receptors agonists[15,16]. On the other hand, 6-OHDA, which induces a much lower inflammatory response, fails to do the same[5,9]. Considering this, we believe that our rotenone model did not increase CB2 mRNA expression due to the comparably lower inflammatory response induced by it.

As previously demonstrated, rotenone decreased the time spent in NREM sleep[25]. In addition, the administration of AM630, blocking CB2 receptors, reversed the rotenone-induced decrease in NREM sleep. In parallel, despite not reaching statistical significance, we observed an increase in the global spectral power (1.5-4.0 Hz range) in the rotenone vehicle and rotenone GW405833 groups, which was reversed to control levels after AM630 administration. We believe that this increase indicates the pressure for NREM sleep, as it is decreased in those groups.

We observed that CB2 receptors activation by GW405833 administration reversed the rotenone-induced impairment in object recognition memory. Despite differences in protocols and types of memory investigated, CB2 receptors modulation is commonly associated with improvement of memory[14,17,18]. The mechanisms underlying these effects are far from being understood, but recent reports assign the beneficial effects of CB2 modulation to its anti-inflammatory properties, especially in

contexts of neurodegenerative diseases[14]. Kruk-Slomka and collaborators (2016) demonstrated that modulation of CB2 receptors improved cognition in parallel with an increase in antioxidant capacity[14]. Also, CB2 receptors activation reduced MPTP-induced microglial activation[16]. Considering this, CB2 receptors activation by GW405833 administration could have reversed the rotenone-induced impairment in recognition memory by a similar mechanism, possibly decreasing oxidative stress. Another possible mechanism for the observed effect, is that GW 405833, as a partial agonist, acted like a competitive antagonist for endogenous ligands due to its supposedly decreased expression after dopaminergic lesion. Further studies will be necessary to address these questions.

A previous study from our group demonstrated that CB1 receptors blockade by AM251 administration also improved recognition memory (unpublished data). This suggests that both CB1 blockade and CB2 activation are interesting approaches to reverse rotenone-induced impairments in recognition memory. In addition, CB1 blockade, but not CB2 activation, reversed the rotenone-induced decrease in NREM sleep. $\Delta^9$-THCV ($\Delta^9$-tetrahydrocannabivarin) is a cannabinoid with the ability to activate CB2 receptors and block CB1 receptors. Garcia and collaborators (2011) demonstrated that $\Delta^9$-THCV administration attenuated the characteristic motor inhibition observed in Parkinson's disease, counteracting the loss of tyrosine hydroxylase–positive neurons within SNpc after 6-OHDA lesion[9]. This result supports our data suggesting that CB2 receptor activation associated to CB1 blockade could be a potential therapeutic target against sleep and cognitive disfunctions in Parkinson´s disease.

Altogether, our findings showed for the first time, a role for CB2 receptors in non-motor symptoms of Parkinson's disease. The CB2 receptors blockade, within the

striatum, demonstrated to be effective in counteract the rotenone-induced decrease in NREM sleep while the activation was effective in reversing rotenone-induced impairment in recognition memory. Further studies will be necessary to understand the mechanisms underlying these effects. In addition, it will be interesting to optimize the modulation of CB2 receptors aiming to improve both symptoms concomitantly.

**Conflict of interests**

The authors declare that no conflict of interests exists.

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## 3.3 CAPÍTULO 3

# A gift that comes with a price: cannabidiol increases wakefulness and impairs recognition memory in an animal model of Parkinson's disease

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## Abstract

Sleep disturbances and cognitive dysfunctions significantly affect the quality of life of Parkinson's disease patients. Although effective treatments are available for motor symptoms, proper treatments for non-motor symptoms are necessary. The main components of Cannabis, cannabidiol (CBD) and $\Delta^9$-tetrahydrocannabinol (THC), are promising agents in this context, considering recent reports demonstrating their effects on sleep and memory. To investigate this, we administered rotenone within the substantia nigra pars compacta (SNpc) of male Wistar rats and, after 7 days, we performed infusions of CBD (10 µg/µl), THC (30 µg/µl) or dimethylsulfoxide (10% v/v) directly in the striatum. Then, sleep was recorded and, after 24 hours, the animals performed the object recognition test. We observed that CBD infusion within the striatum increased the time spent awake in our rotenone model of Parkinson's disease. This was concomitant with an increase in striatal CB2 receptors expression. In addition, we demonstrated that rotenone, CBD and THC impaired object recognition memory. Future studies are necessary to unveil the mechanisms that underlie the effects of CBD on these two non-motor symptoms and to define whether the increased wakefulness is beneficial or harmful.


**Keywords:** Parkinson's disease, sleep regulation, recognition memory, cannabidiol, $\Delta^9$-tetrahydrocannabinol.

## Introduction

Parkinson's disease is the second most prevalent neurodegenerative disorder in the world, and current estimates are that more than ten million people suffer from this disease[1]. It is characterized by a loss of dopaminergic neurons within the substantia nigra pars compacta (SNpc), which leads to the well-known motor symptoms such as tremors, bradykinesia, disturbances in balance, among others. In addition, non-motor symptoms such as sleep disturbances and cognitive dysfunctions appear years or decades before the onset of motor symptoms, drastically affecting the quality of life of Parkinson's disease patients[2,3].

Although effective treatments are available for motor symptoms, proper treatments for sleep disturbances and cognitive dysfunctions are still lacking[4]. Some factors contribute to this, including insufficient knowledge regarding the mechanisms underlying each disturb and the multifactorial identity of these disturbances. However, growing number of evidence shows that cannabinoids are promising agents in this context[5]. $\Delta^9$-tetrahydrocannabinol (THC), one of the main components of Cannabis, promotes an improvement in sleep parameters while it leads to an impairment in different types of memory, including object recognition memory[6,7]. Another important cannabinoid, the cannabidiol (CBD), also affects sleep, improving the time spent sleeping or increasing the wakefulness[8,9]. Regarding cognition, studies show a beneficial effect of CBD in different types of memory, including object recognition memory[10,11]. Peres and collaborators (2016) demonstrated that CBD reversed the cognitive deficits induced by the reserpine model of Parkinson's disease[12]. In addition, this cannabinoid reversed the iron-induced impairment in recognition memory[13].

The plethora of effects after cannabinoids administration is a consequence of the variety of targets. THC, for example, acts as a partial agonist for CB1 and CB2 receptors[14], also targeting the orphan GPR55 and GPR18 receptors[15–17]. In addition, THC binds the transient receptor potential vanilloid (TRPV) type 1 and 2[18], glycinergic receptors and transient receptor potential menthol (TRPM) 1[14]. On the other hand, CBD is a low-affinity antagonist for CB1 and CB2 receptors[14,19], with substantial affinity for the orphan GPR55, also binding GPR18, TRPV2, TRPV3, TRPM8, and 5HT1A receptors[14,16,20,21]. Moreover, CBD inhibits the reuptake and enzymatic degradation of the endogenous cannabinoid anandamide[22]. Most of these targets are present within the nigrostriatal pathway, which is extremely important for non-motor symptoms in Parkinson's disease[23,24]. In fact, recent studies show that modulation of CB1 and CB2 receptors within the nigrostriatal pathway leads to interesting outcomes related to sleep and memory (unpublished data).

Considering this, the objective of this study was to investigate the effects of the two main constituents of Cannabis, CBD and THC, on the non-motor symptoms of Parkinson's disease, particularly on sleep and memory. For this, we administered rotenone within the SNpc to model Parkinson's disease and, after 7 days, we administered CBD or THC directly in the striatum. We choose such protocol to evaluate the specific role of the nigrostriatal pathway in these parameters after THC/CBD administration. We hypothesised that THC would be more effective on sleep, reversing the rotenone-induced decrease in non-rapid eye movement (NREM) sleep and CBD would be more effective on memory, reversing the rotenone-induced impairments in recognition memory.

## 2 Materials and Methods

### *2.1 Subjects*

The experiments in this study were approved by the ethics committee of Federal University of Paraná (all experiments; approval ID #857) and Federal University of São Paulo (sleep experiments; approval ID #9022050417). They were carried out in accordance to the Guidelines of ethics and experimental care and use of laboratory animals (SBCAL). All efforts were made to minimize animal suffering and to reduce the number of animals used. Male Wistar rats, weighing approximately 280-320g were kept in a temperature-controlled room (22±2$^{o}$C), with a 12:12h light-dark cycle (lights on at 7:00 AM). They were maintained in groups of 5 animals in polypropylene cages containing bottles of water and pellets of food throughout the entire experiment.

### *2.2 Experimental design*

The experimental design is represented in Fig. 1. There were two sets of animals. The first set (n=70) underwent stereotaxic surgery for rotenone or dimethylsulfoxide (DMSO) administration within SNpc, and bilateral guide cannulas implantation within the striatum. The habituation phase of the object recognition test (ORT) took place on days 3, 5 and 7. After the last habituation (day 7), the animals were submitted to the training phase of the ORT and then, we administered the drugs that modulate cannabinoid receptors directly into the striatum. The effects of these drugs were evaluated in the next day (day 8), in which the ORT was performed. Finally, the animals were euthanized through decapitation and samples collected for western blot and real-time PCR.

The second set of animals (n=30) were submitted to the same procedures on day 0, but with additional implantation of cortical electrodes for sleep-wake recording. After 7 days, we administered the drugs that modulate cannabinoid receptors directly into the striatum followed by a period of 3h of sleep-wake recording (9:00 AM-12:00 PM).

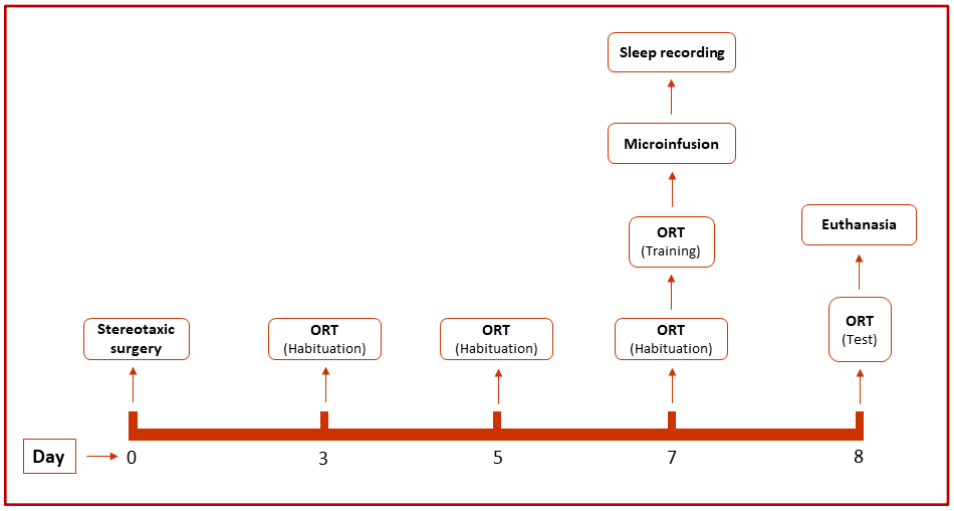


Figure 1. Experimental design. ORT, Object recognition memory.

*2.3 Stereotaxic surgery*

The animals were sedated with intraperitoneal xylazine (10 mg/kg; Syntec do Brasil Ltda, Brazil) and anesthetized with intraperitoneal ketamine (90 mg/kg; Syntec do Brasil Ltda, Brazil). For rotenone infusion within the SNpc, we used bregma as a reference for the following coordinates: (AP) = -5.0 mm, (ML)= ± 2.1 mm and (DV) = -8.0 mm[25]. Rotenone (12 µg/µl; Sigma-Aldrich®, United States) or DMSO 10% v/v (Sigma-Aldrich®, United States) infusions were made using an electronic infusion pump (Insight Instruments, Ribeirão Preto, Brazil) at a rate of 0.33 µl/min for 3 min[26]. Bilateral guide cannulas implantation within the striatum were made using bregma as reference for the following coordinates: (AP) = -1.0 mm, (ML)= ±3.0 mm and (DV) = -4.0 mm[25]. For electrodes positioning, the following coordinates were used, using bregma as a reference: (AP) = -1.8 mm, (ML) = -2.0 mm (first electrode) and (AP) = 3.0 mm, (ML) = 1.0 mm (second electrode); and lambda as a reference: (AP) = 1.0

mm, (ML) = -4.0 mm (third electrode) and (AP) = 4.0 mm, (ML) = 1.0 mm (fourth electrode)[25].

*2.4 Striatal microinfusion*

The awake animals were gently immobilized for infusions of CBD (10 µg/µl; Phytoplant, Spain), THC (30 µg/µl; isolated from the plant) or vehicle (DMSO, 10% v/v; Sigma-Aldrich, USA) directly in the striatum. The infusions were made at the bilateral guide cannulas (implanted during stereotaxic surgery), at a rate of 0.33 µl/min for 3 min, with the assistance of an electronic infusion pump (Insight Instruments, Ribeirão Preto, Brazil).

*2.5 Sleep recording procedure*

Electrophysiological signals were recorded on a digital polygraph (Neurofax QP 223A Nihon Kohden). After conventional amplification, the EEG signals were conditioned through analogical filters, using cut off frequencies of 1.0 Hz and 35.0 Hz, and were then sampled at 200 Hz using a 16 bits A/D converter. The recordings were divided in epochs of 10 seconds intervals and classified as wakefulness, NREM or REM sleep. In addition, the number of NREM and REM episodes and mean length of NREM and REM episodes were evaluated. Sleep efficiency was calculated as the time spent sleeping in relation to the total time of sleep recording.

Fast Fourier Transform (Hanning window) was computed on 256 points (corresponding to each vigilance state) with a resolution of 0.78 Hz. Fast Fourier Transform was applied in the frequency interval of 1.0 to 16.0 Hz and those above 16.0 Hz were discarded from analysis. Non-overlapping bands were set giving 0.5 Hz bins from 1.0 to 5.0 Hz, and 1.0 Hz bins from 5.1 Hz to 16.0 Hz. Delta power was

calculated as mean power density on 1.0–4.0 Hz, theta power was calculated as mean power density on 5.0–8.0 Hz and alfa power was calculated as mean power density on 8.0–13.0 Hz[27].

*2.6 Object recognition test (ORT)*

The apparatus used to investigate object recognition memory consists of an open box (width ×length × height = 60 cm× 60 cm×50 cm) made of wood and covered with a black opaque plastic film. The objects to be discriminated were available in triplicate copies and were made of biologically neutral material such as glass, plastic or metal. Also, they are not known to have any ethological significance for the rats. This test is based on the tendency of the animals to explore new things instead of familiar things. Thus, when an animal remembers a familiar object and does not know a new object, there is a tendency of this animal to explore the new object for a longer time when compared to the familiar object. The ORT in this study consisted of three phases: the habituation phase, the sample/training phase and the choice/test phase[28]. In the habituation phase, the animals had three minutes in day 3, 5 and 7 to explore the arena without the objects (Fig. 1). During the training phase (15 minutes after habituation in day 7), two identical objects were exposed in the back corners of the open box, 10 cm away from the sidewall. The rat was placed in the open box facing away from the objects and after 3 min of exploration, the rat was removed from the open box and returned to its cage. Twenty-four hours later (test phase), two objects were presented in the same locations that were occupied by the previous sample objects. One of the objects was identical to the object seen in the training phase and the other one was different. The tests were video recorded and analyzed by a blind experimenter. It was considered as exploration only when the rat touched

the object with its nose or when the rat's nose was directed toward an object at a distance ≤2 cm. Delta value was obtained from the following formula: Delta value = Time spent exploring the new object – time spent exploring the familiar object.

*2.7 Western blot*

To determine CB1 and CB2 receptors expression within the striatum, the animals were decapitated, their brains were rapidly removed, and the striatum was dissected. Tissues were stored at -80ºC until processing. Samples were sonicated in RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% SDS, 50 mM Tris pH 8, 2 mM EDTA). After centrifugation (10 min, 12,000 rpm at 4°C), the supernatant was collected, and protein concentration was determined by the Bradford method (Bio-Rad, Germany). 40 µL of each sample were subjected to 10% SDS-PAGE gel (1.0 mm) and transferred to a PVDF membrane (GE Healthcare). The membranes were then blocked in 5% BSA diluted in TBS-T overnight at 4ºC, followed by incubation with anti-CB1 antibody (1:1000 diluted in TBS-T; Sigma-Aldrich®, USA) and/or anti-CB2 antibody (1:1000 diluted in TBS-T; Sigma-Aldrich®, USA) overnight at 4ºC. In the sequence, the membranes were incubated with anti-rabbit antibody (1:1000 diluted in TBS-T; GE Healthcare) for two hours. β-actin was used as the housekeeping protein (1:5000 diluted in TBS-T; Sigma-Aldrich®, USA). Signal was detected using the ECL chemiluminescent detection system (GE Healthcare Life Sciences, Brazil). The protein levels were quantified by densitometry using ImageJ v1.47 software (National Institutes of Health, USA).

*2.8 Real-time PCR*

To investigate the mRNA expression of CB1 and CB2 receptors within the striatum, the animals were decapitated, the striatum dissected and stored at -80ºC. Samples were processed for RNA extraction using phenol-based reagent Brazol (Labdel, SP, Brazil), followed by cDNA synthesis using qPCRBIO kit (PCRBiosystems, London, United Kingdom). The cDNA target sequences were amplified, in triplicate, using the Fast Sybr Green dye (Applied Biosystems) in a ViiA7 Real Time PCR System (Applied Biosystems), according to the manufacturer's instructions. A dissociation cycle was performed after each run to check for non-specific amplification or contamination. The results were normalized by the normalization factor generated for each sample by the geNorm program, using HPRT and β-actin as housekeeping genes. The primers for CB1 receptor (forward AGGAGAACTTACTGTGAACAGGC, reverse ATGGGTGCTCCTTGCTTGAA), CB2 receptor (forward CCTACTCACTCTGGACAGGAA, reverse GCATAGATGTTTGCTGGGTGG), HPRT (forward CCCAGCGTCGTGATTAGTGA, reverse TGGCCTCCCATCTCCTTCAT) and β-actin (forward CGAGTACAACCTTCTTGCAGC, reverse ATACCCACCATCACACCCTGG) were designed using PrimerBLAST, and validated through BLAST.

*2.9 Statistical analysis*

The normal distribution of the data was assessed by the Kolmogorov-Smirnov test. Differences between groups were assessed by two-way ANOVA (sleep recordings, ORT [delta value], open field test, western blot, Real-time PCR) and repeated measures two-way ANOVA (ORT). Fisher's post hoc test was carried when necessary. Values were expressed as mean ± standard error of mean (SEM). The level of significance was set at $P < 0.05$.

## 3 Results

### *3.1 Phytocannabinoids and sleep regulation*

We observed a decrease in the sleep efficiency of sham CBD and rotenone vehicle groups compared to the sham vehicle group ($P<0.05$) (Fig.2A). In addition, there was an increase in the rotenone THC group compared to the rotenone CBD group ($P<0.05$). In fact, we observed effect of the lesion [$F(1,28) = 5.652$, $P<0.05$], cannabinoids administration [$F(2,28) = 3.904$, $P<0.05$], but no effect of the interaction [$F(2,28) = 0.3130$, $P = 0.7338$].

Regarding the time spent awake (Fig.2B), there was only an effect of cannabinoids administration [$F(2,28) = 4.585$, $P<0.05$], demonstrated by an increase in rotenone CBD group compared to rotenone vehicle and rotenone THC groups ($P<0.05$). No effect of the lesion [$F(1,28) = 1.097$, $P = 0.3040$] or interaction between the factors [$F(2,28) = 0.03934$, $P = 0.9615$] was found.

Conversely, there was an effect of the lesion [$F(1,28) = 4.659$, $P<0.05$], but no effect of cannabinoids administration [$F(2,28) = 2.824$, $P = 0.0764$] or interaction [$F(2,28) = 0.3292$, $P = 0.7222$] on the time spent in NREM sleep (Fig.2C). In fact, we observed that the rotenone vehicle group spent significantly less time in NREM sleep compared to the sham vehicle group ($P<0.05$). No differences among groups were found regarding the number and mean length of NREM sleep episodes (Fig.2D,E). In addition, no differences were observed regarding REM sleep-related parameters (Fig.2F,G,H).

The global spectral power presented no differences among the experimental groups (Fig.3A). In addition, no differences were observed in alfa, delta, theta and in the ratio theta/delta power (Fig.3B-E).

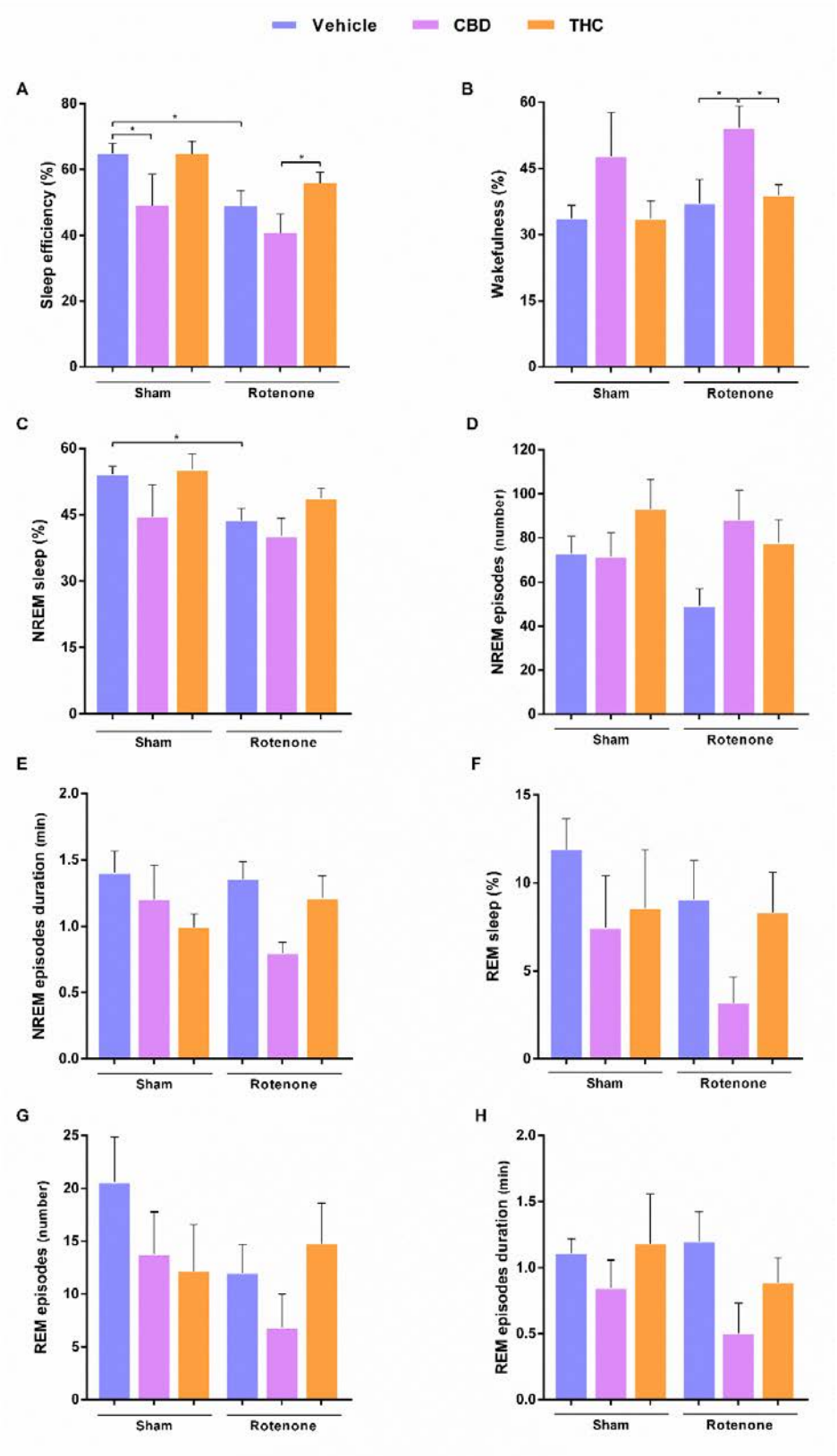


Figure 2. Phytocannabinoids and sleep regulation. Sleep efficiency (A), percentage of time spent in wakefulness (B), percentage of time spent in NREM sleep (C), number of NREM sleep episodes (D), mean length of NREM sleep episodes (E), percentage of time spent in REM sleep (F), number of REM sleep episodes (G), mean length of REM sleep episodes (H). Values are expressed as mean ± SEM. *P≤0.05. Two-way ANOVA followed by Fisher's post hoc test. n = 4-6 animals/group.

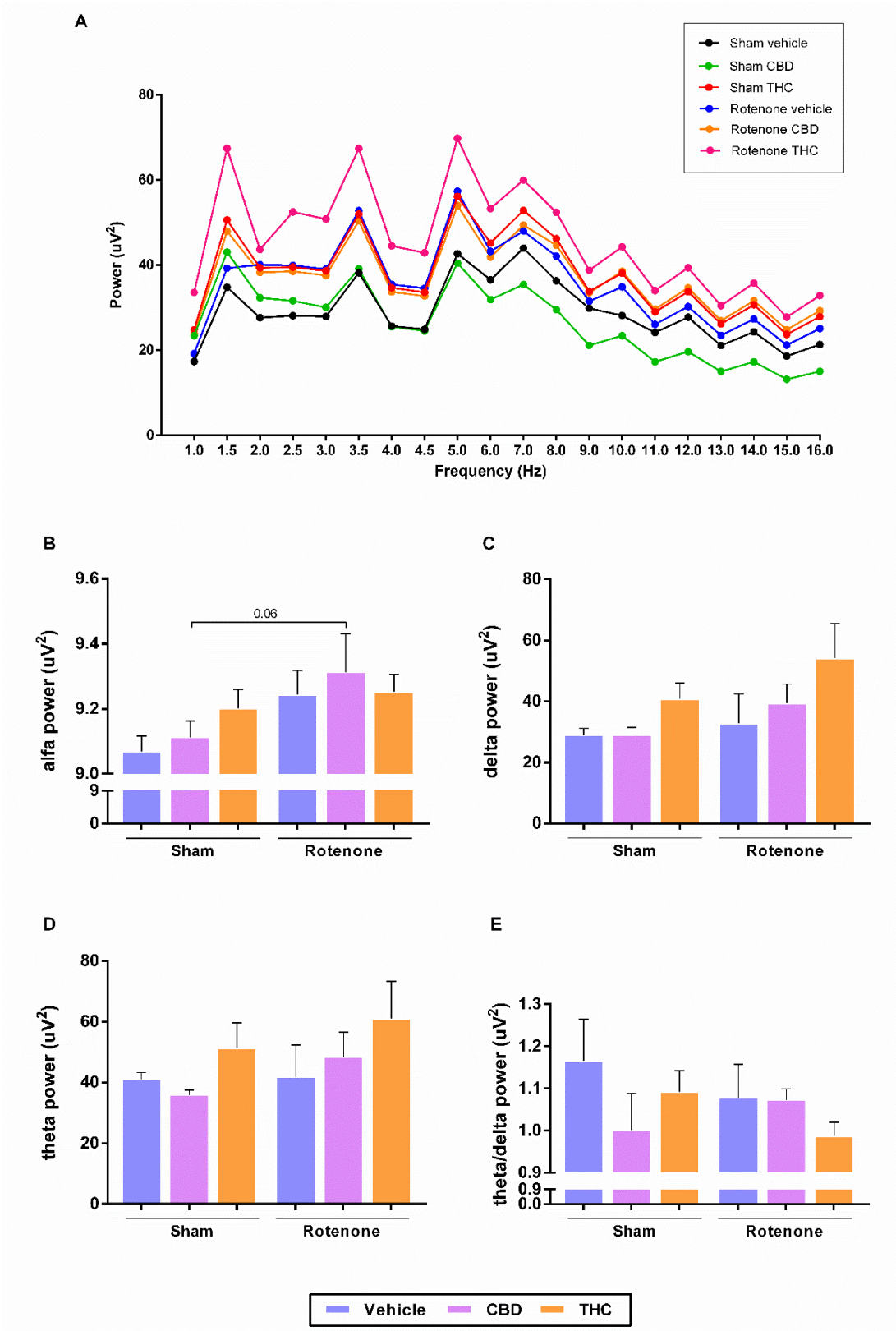


Figure 3. Phytocannabinoids on global spectral power. Spectral power in (A) different frequencies, (B) alfa range, (C) delta range, (D) theta range, (E) theta/delta ratio. Values are expressed as mean ± SEM. Two-way ANOVA. n = 4-6 animals/group.

*3.2. Phytocannabinoids and recognition memory*

We observed that the sham vehicle group spent more time exploring the new object compared to the familiar one, demonstrating that these animals differentiated the objects ($P$<0.01) (Fig.4A). As expected, rotenone impaired object recognition memory, demonstrated by a similar time exploring familiar and new objects. Interestingly, both THC and CBD administration also impaired recognition memory. In fact, we observed effects of the lesion [F(1,50) = 5.01, $P$<0.05], the object [F(1,50) = 6.49, $P$<0.05] and lesion x cannabinoids interaction [F(2,50) = 3.90, $P$<0.05]. No effects of cannabinoids [F(2,50) = 0.83, P=0.438], object x lesion [F(1,50) = 0.77, P=0.381], object x cannabinoids [F(2,50) = 2.34, P=0.106], lesion x cannabinoids x objects [F(2,50) = 0.82, P=0.444] were found.

There was an effect of cannabinoids administration [F(2,68) = 4.225, $P$<0.05], but no effects of the lesion [F(1,68) = 1.869, P = 0.1761] or interaction between the factors [F(2,68) = 2.471, P = 0.0921] on the delta value (Fig.4B). This was demonstrated by a decrease in the sham CBD and rotenone vehicle groups compared to the sham vehicle group ($P$<0.001 and $P$<0.05, respectively).

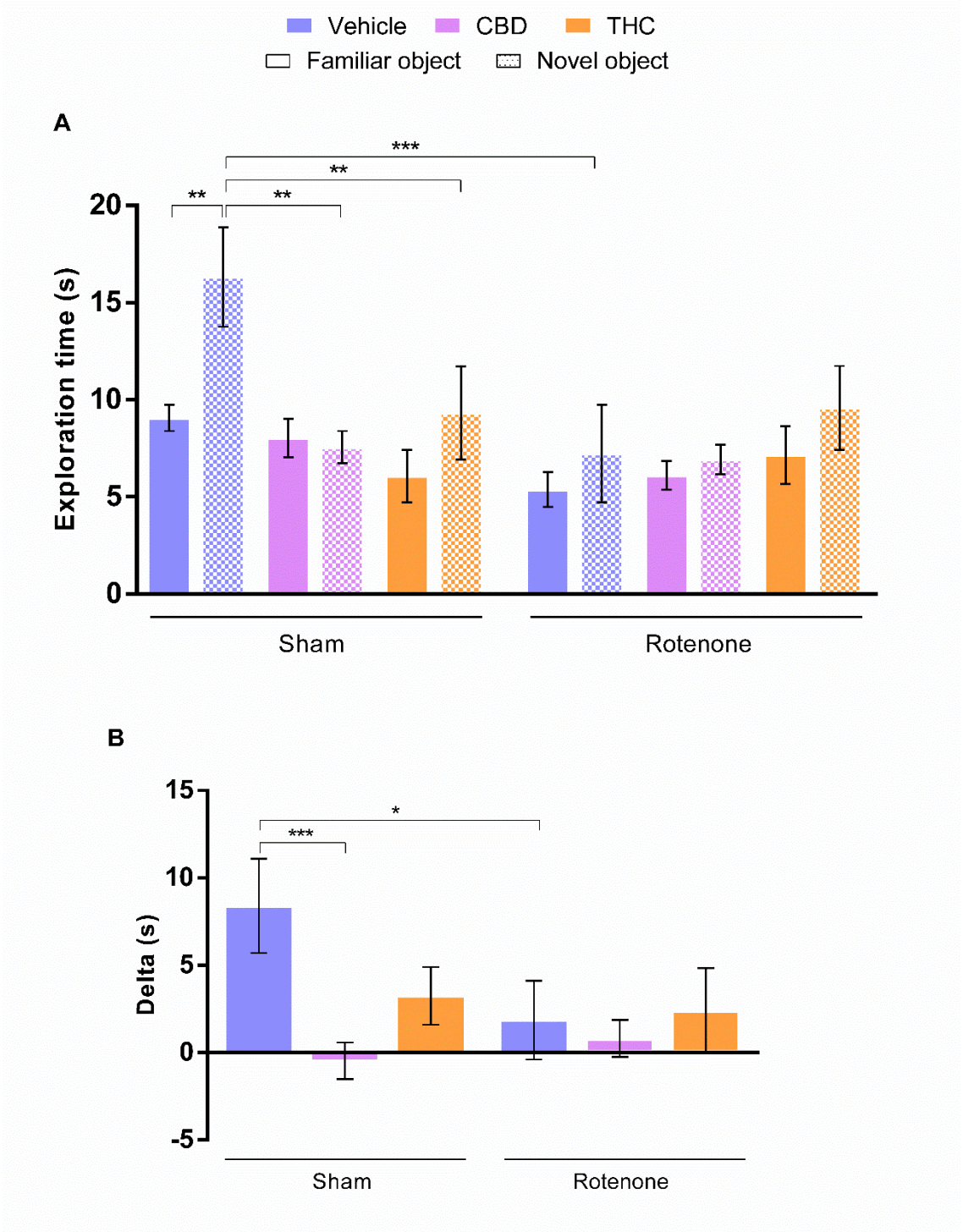


Figure 4. Object recognition test (ORT). Time spent exploring the objects (A) and delta value (B). Values are expressed as mean ± SEM. *P≤0.05, **P≤0.01, ***P≤0.001. Repeated measures two-way ANOVA (A) and Two-way ANOVA (B) followed by Fisher's post hoc test. n = 10-12 animals/group.

### *3.3 Phytocannabinoids administration and western blot*

We did not find statistical differences among the groups in relation to CB1 receptor expression after cannabinoids administration (Fig.5A). On the other hand, we

observed increased CB2 receptor expression in the rotenone CBD group compared to rotenone vehicle ($P<0.05$) and rotenone THC groups ($P<0.001$) (Fig.5B). In fact, there was an effect of cannabinoids administration [$F(2,18) = 4.623$, $P<0.05$], but no effects of the lesion [$F(1,18) = 0.1998$, $P = 0.6602$] and interaction between the two factors [$F(2,18) = 1.929$, $P = 0.1742$].

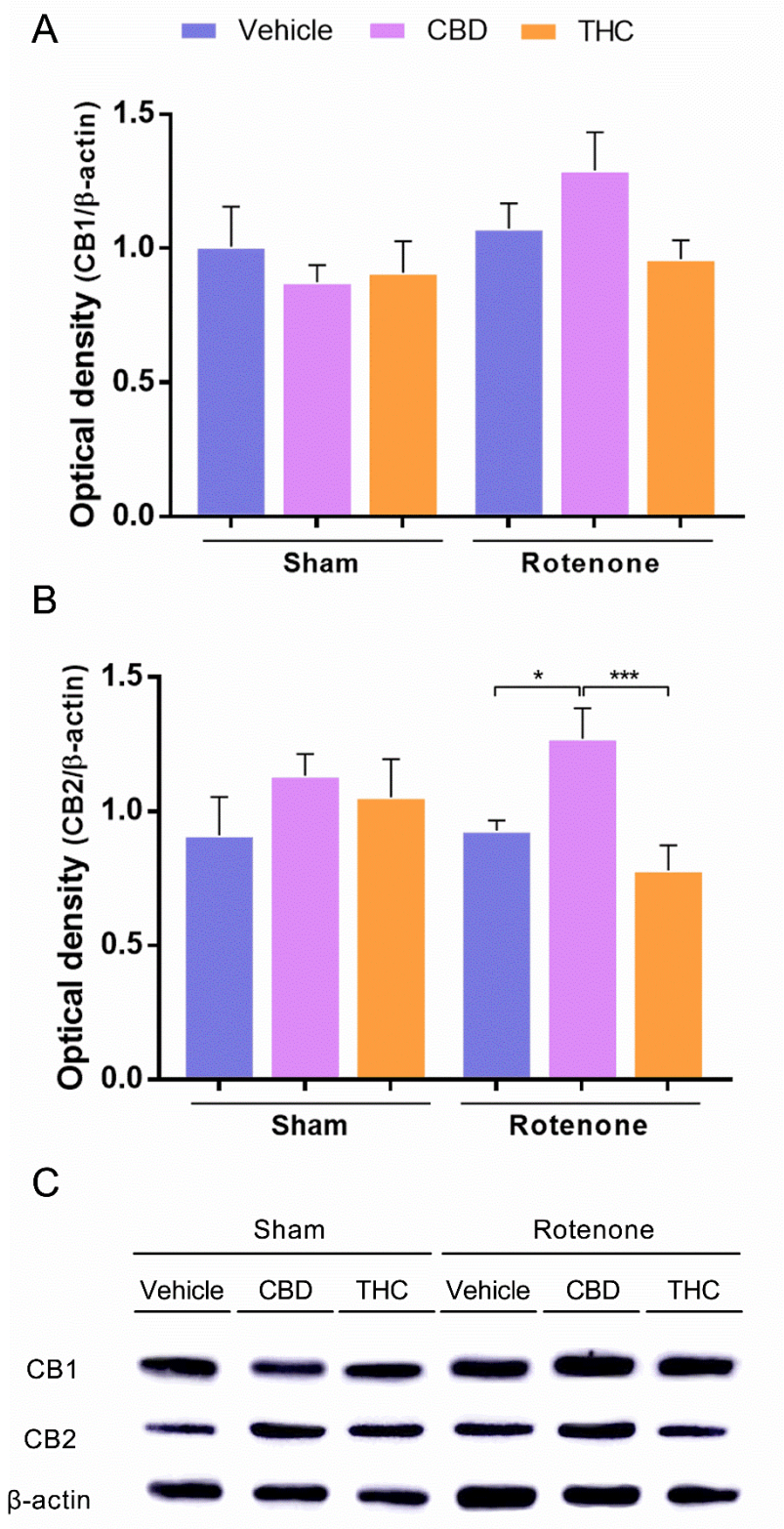


Figure 5. Phytocannabinoids administration on proteins expression. CB1 (A), CB2 (B) and representative bands (C). Values are expressed as mean ± SEM. *P≤0.05, ***P≤0.001. Two-way ANOVA followed by Fisher's post hoc test. n = 5 animals/group.

*3.4 Phytocannabinoids administration and real-time PCR*

No statistical differences among the groups were found for CB1 and CB2 mRNA levels after cannabinoids administration (Fig.6).

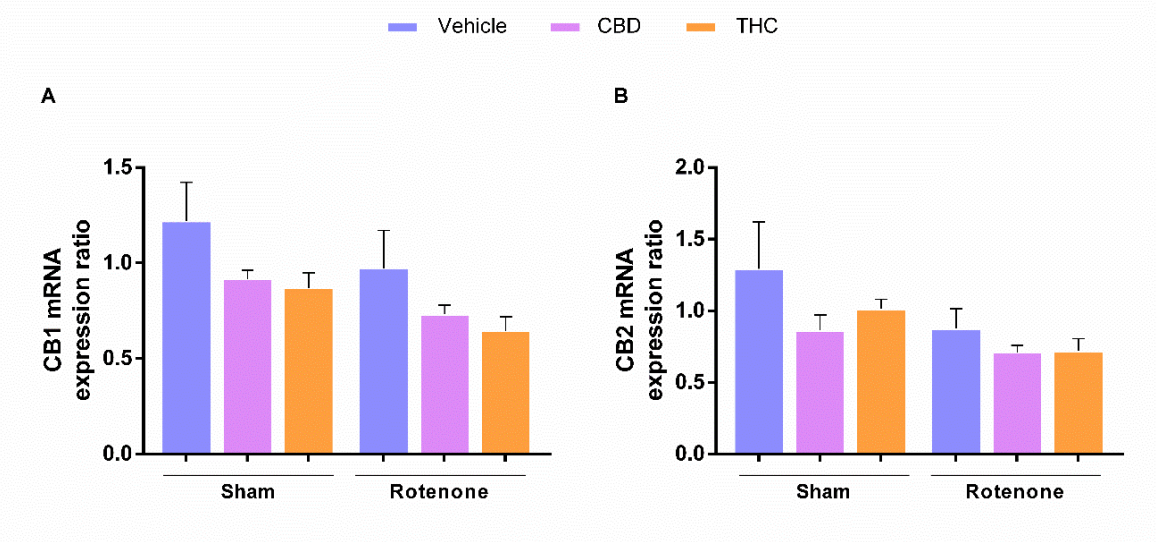


Figure 6. Phytocannabinoids administration and mRNA levels. CB1 (A) and CB2 (B) mRNA expression ratio. Values are expressed as mean ± SEM. Two-way ANOVA. n = 5 animals/group.

## Discussion

We demonstrated that CBD infusion within the striatum increased the time spent awake in an animal model of Parkinson's disease. Such result was concomitant with an increase in striatal CB2 protein expression. In addition, we observed that both CBD and THC infusion within the striatum impaired object recognition memory in healthy animals, without any effect on rotenone-lesioned animals.

CBD increases wakefulness in different experimental conditions. Murillo-Rodriguez (2006) observed that intracerebroventricular administration of CBD increased wakefulness and decreased the time spent in REM sleep, concomitantly with an increase in extracellular dopamine within the nucleus accumbens. In addition, there was an increase in the neuronal activity marker, c-fos, in wake-related nuclei, including the hypothalamus and dorsal raphe nuclei[29]. Later studies demonstrated

similar effects after CBD administration within the lateral hypothalamus and dorsal raphe nuclei[9,30]. In fact, we also observed an increase in wakefulness after CBD administration, which was concomitant to a trend of increase in alfa power. However, differently from the described studies, we demonstrated the effects of CBD administration directly in the dorsal striatum, which is not a wake-related structure as the lateral hypothalamus and dorsal raphe nuclei. The possible mechanisms by which CBD is exerting its effects may be associated to other than cannabinoid receptors, including the orphan GPR18 (partial agonist)[16], GPR55 (antagonist)[17], TRPV (Transient Receptor Potential Vanilloid) type 2 and 3[20], and TRPM8 (Transient Receptor Potential Menthol 8)[14] receptors. In addition, despite the low affinity for cannabinoid receptors, it behaves as an antagonist of CB1 receptors and an inverse agonist of CB2 receptors[14,31]. In this regard, we observed an increase in CB2 protein expression after CBD administration. However, further studies are necessary to confirm if this is the mechanism by which CBD exerted its effects.

To our knowledge, there is only one study investigating the effects of CBD on non-motor symptoms of Parkinson's disease. Chagas and collaborators (2014) demonstrated that four patients treated with CBD had a substantial reduction in the frequency of REM behavior disorder-related events without side effects[32]. Thus, based on our findings, CBD could be an interesting agent for the excessive daytime sleepiness that affects Parkinson's disease patients. However, it is important to address that the sleep recording took place around 9:00-12:00 p.m., when the animals are predominantly sleeping. Thus, the increase in wakefulness after CBD administration could be in fact increasing insomnia.

We did not observe effects of THC administration on sleep parameters of healthy and rotenone-lesioned animals. Conversely, THC is normally associated with increased

sleep in most of the studies[7]. We believe that the discrepancy between our findings and the literature relies on differences on the doses of THC used, route of administration and methodological differences. In fact, to our knowledge, this is the only study that administered THC within the striatum to investigate its effects on sleep and memory.

As previously reported, rotenone administration impaired object recognition memory[24,33]. Surprisingly, CBD and THC administration had a similar effect, preventing the animals to differentiate the objects. THC affects different types of memory, at different phases in a variety of doses, routes of administration and protocols[34,35]. On the other hand, CBD is often associated with an improvement in cognition. Osborne and collaborators (2017) demonstrated that CBD improved object recognition memory in a schizophrenia animal model[11]. In addition, a similar outcome was observed in an animal model of Alzheimer's disease[10]. Moreover, despite assessment of a different type of memory, Peres and collaborators (2016) observed that four injections of CBD (0.5 or 5 mg/kg) improved the performance in the discriminative avoidance task in the reserpine model of Parkinson`s disease[12]. We believe that the contradictions among the described studies and our results are a consequence of different factors. First, the studies administered the drug systemically, while we performed a specific infusion within the striatum. This is closely related to the dose and to the concentration of drug that effectively reaches its target. In this regard, as already mentioned, CBD has a plethora of targets[14]. Moreover, differences in the type of memory being investigated and in the ORT protocol account for differences among studies.

Considering that we did not perform a behavioral test to investigate motor activity, one could say that the observed impairment in object recognition memory is a

consequence of a decrease in motor activity induced by CBD and, especially, THC. We believe that this is not the case due to two reasons. First, the time exploring the familiar object is similar among the groups that received the drugs and the groups that received the vehicle, indicating that the animals were not static, but explored the objects, however indiscriminately. In addition, the animals received the drugs after the training phase of ORT and were only evaluated for their recognition memory 24h later. Thus, we believe that the possible deleterious effect on motor behavior after the drugs administration disappeared by the time the animals were tested.

Memory consolidation is demonstrably affected by sleep[36]. We previously demonstrated the influence of REM sleep deprivation on recognition memory using a similar test protocol as the one used in this study[24,33,37]. However, based on the present findings, there is not a direct relation between sleep parameters and recognition memory consolidation. This suggests that a discreet modulation of striatal receptors is not as effective as REM sleep deprivation to affect memory. Considering that our treatments affected both sleep and memory, but these effects appear to be dissociated, further studies to investigate the mechanisms underlying each of the effects will be very important. By doing so, it would be possible to target the mechanism that improves wakefulness, for example, without affecting recognition memory consolidation.

For the first time, we demonstrated that striatal infusion of CBD and THC, the two main components of Cannabis, affect sleep and memory. This confirms previous reports demonstrating the importance of the nigrostriatal pathway in the non-motor symptoms of Parkinson's disease, besides the established role in motor function[23,24]. THC, at the chosen dose, did not demonstrate an effect in sleep modulation and impaired memory consolidation. Conversely, CBD demonstrated an effect on sleep

modulation but also a deleterious effect on recognition memory. This is interesting, considering that CBD does not present the psychoactive effects induced by THC. In this regard, future studies are necessary to unveil the mechanisms that underlie the effects of CBD on these two non-motor symptoms and to define whether the increased wakefulness is beneficial or harmful.

**Conflict of interests**

The authors declare that no conflict of interests exists.

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## CONSIDERAÇÕES FINAIS

Esse estudo buscou investigar o envolvimento do sistema canabinoide na regulação do sono e consolidação da memória em um modelo animal de Parkinsonismo. Nossos achados confirmam uma influência desse sistema nesses importantes sintomas não-motores que afetam drasticamente a qualidade de vida de indivíduos com a doença de Parkinson.

Os dados apresentados sugerem que a inibição de receptores canabinoides CB1 e CB2 é capaz de reverter a diminuição do tempo despendido em sono NREM promovida pela administração de rotenona. Esses resultados contradizem achados da literatura, que demonstram um aumento do tempo despendido em vigília após o bloqueio dos receptores CB1 (GOONAWARDENA et al., 2011). No entanto, a modulação dos receptores canabinoides no presente estudo foi realizada diretamente no estriado dorsal, afim de observar o papel dos receptors CB1 presentes nessa via, especificamente. Além disso, o efeito promovido pelo bloqueio dos receptores CB1 foi observado somente após a lesão da via nigroestriatal, sugerindo que esse efeito seja dependente de um insulto prévio. Diferentemente, não se tem registro do efeito da modulação de receptores CB2 no sono. Nesse sentido, nossos achados são inéditos, porém existe uma carência de estudos para corroborá-los ou contrariá-los. De qualquer forma, são achados muito interessantes, especialmente considerando que a modulação dos receptores CB2 presumivelmente leva a menos efeitos psicoativos, se comparada à modulação de receptores CB1.

Estudos demonstram que os receptores canabinoides e dopaminérgicos interagem de forma indireta, uma vez que estão localizados nas mesmas regiões, como nos neurônios GABAérgicos estriatais pós-sinápticos (GARCÍA et al., 2016). Além disso, interagem de forma direta, formando os heterômeros. Essa interação direta é responsável pela influência da modulação dos receptores canabinoides na transmissão dopaminérgica (MARCELLINO et al., 2008). Dessa forma, agonistas e antagonistas do receptor CB1 reduzem e potencializam, respectivamente, a transmissão dopaminérgica (ANDERSON et al., 1995b; MANEUF; CROSSMAN; BROTCHIE, 1997; MARCELLINO et al., 2008). Em um contexto similar ao apresentado nesse estudo, nós observamos que a ativação de receptores dopaminérgicos do tipo D2 estriatais reverteu déficits na capacidade de recuperar o sono após a privação de sono REM (TARGA et al., 2016). Assim, observa-se que a

transmissão dopaminérgica é, de fato, importante para a manutenção da função normal do sono. Nesse sentido, nós hipotetizamos que o bloqueio de receptores canabinoides realizado no presente estudo levou a um aumento na transmissão dopaminérgica, normalizando o sono.

Não foi possível observar efeito da administração de THC no sono dos animais, diferentemente do demonstrado pela maioria dos estudos na literatura (CALIK; CARLEY, 2017; GORELICK et al., 2013). Isso pode ser atribuído a uma série de fatores como as doses utilizadas, vias de administração, protocolos experimentais, entre outros. Nesse caso, acreditamos que o principal determinante tenha sido a diferença entre a administração sistêmica realizada na maioria dos estudos e a administração diretamente no estriado, realizada no nosso estudo. Por outro lado, a administração de CBD promoveu um aumento no tempo despendido em vigília, corroborando os dados da literatura (MURILLO-RODRÍGUEZ et al., 2008, 2014). Isso foi observado após a lesão com rotenona, sugerindo um possível papel do CBD em distúrbios como a sonolência diurna excessiva. Paralelamente, houve um aumento na expressão de receptores CB2 no estriado. O CBD possui diversos alvos no sistema nervoso central, possuindo pouca afinidade pelos receptores CB1 e CB2, nos quais atua como um inibidor alostérico (LAPRAIRIE et al., 2015). De qualquer forma, assim como observado após o bloqueio dos receptores CB1, o aumento na expressão dos receptores CB2 poderia indicar uma resposta compensatória após o bloqueio desses, promovido pelo CBD. No entanto, o mecanismo pelo qual o CBD exerceu esses efeitos possivelmente envolve seus outros alvos, uma vez que o bloqueio de receptores CB2 levou a um aumento do tempo em sono NREM, o que não foi observado com a administração do CBD. Estudos futuros serão necessários para investigar essas questões.

O bloqueio dos receptores CB1 demonstrou um efeito benéfico na memória de reconhecimento de objetos, assim como havia demonstrado no sono. Da mesma forma, houve uma reversão do efeito deletério induzido pela administração de rotenona, paralelamente a um aumento nos níveis de mRNA do receptor CB1. Além de corroborar dados da literatura, que indicam que um bloqueio do receptor CB1 pode ser benéfico para diferentes tipos de memória, esses dados sugerem uma possível influência do sono na consolidação da memória. Diferentemente, os achados a partir da modulação dos receptores CB2 e da administração de CBD/THC sugerem relativa independência entre o sono e a memória, pelo menos no contexto

estudado. A administração de CBD e THC prejudicou a memória em animais saudáveis e não apresentou efeito em animais que receberam rotenona. Ao contrário do THC, os resultados encontrados a partir da administração do CBD não eram esperados (FAGHERAZZI et al., 2012; OSBORNE et al., 2017). Atribuímos essas diferenças entre os nossos dados e os achados em outros estudos aos fatores anteriormente mencionados, como via de administração, dose e protocolo experimental utilizado.

A ativação de receptores CB2 reverteu o prejuízo na consolidação da memória de reconhecimento de objetos promovido pela rotenona. Alguns fatores podem explicar esses achados. Primeiramente, a ativação dos receptores CB2 está associada a uma diminuição da neuroinflamação e do estresse oxidativo (JAVED et al., 2016; RONCA et al., 2015). Dessa forma, ao diminuir a neuroinflamação e estresse oxidativo sabidamente promovido pela infusão de rotenona, a ativação desses receptores também reverteu os déficits causados por essa neurotoxina. Kruk-Slomka e colaboradores (2016) demonstraram que a modulação de receptores CB2 promoveu uma melhora cognitiva em paralelo a um aumento na capacidade oxidativa do encéfalo (KRUK-SLOMKA et al., 2016). Além disso, a droga utilizada para ativar os receptores CB2, GW405833, é um agonista parcial (PERTWEE et al., 2010). Dessa forma, existe a possibilidade de que, por ser um agonista parcial e estar em uma concentração aumentada nessa sinapse, essa droga possa ter atuado como um antagonista competitivo em relação aos agonistas endógenos desse receptor, levando a esse efeito inesperado.

Os resultados acima descritos sugerem que tanto o bloqueio dos receptors CB1 quanto a ativação dos receptores CB2 são estratégias interessantes para reverter os déficits causados pela administração de rotenona na memória de reconhecimento de objetos. Além disso, o bloqueio do receptor CB1 reverte a diminuição do tempo em sono NREM promovida pela rotenona. Nesse sentido, a administração de $\Delta^9$-THCV, um canabinoide com a habilidade de ativar receptores CB2 e bloquear receptores CB1, parece ser uma abordagem interessante a ser investigada em estudos futuros (GARCÍA et al., 2011).

Em conclusão, os achados do presente estudo demonstram um envolvimento do sistema canabinoide tanto na regulação do sono quanto na consolidação da memória de reconhecimento de objetos no modelo animal de Parkinsonismo induzido por rotenona.

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