PROGRAMA DE POS GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS: BIOQUÍMICA

DEPARTAMENTO DE BIOQUÍMICA

INSTITUTO DE CIÊNCIAS BÁSICAS DA SAÚDE

UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

Tese de Doutorado

Avaliação do Potencial Ansiolítico e Antidepressivo da Guanosina

Roberto Farina de Almeida

Porto Alegre 2016

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Roberto Farina de Almeida

Orientador: Diogo Onofre Gomes de Souza

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Es ist nicht bequem, Gefühle
wissenschaftlich zu bearbeiten

It is not easy to deal scientifically
with feelings

Sigmund Freud, 1929

Dedico esta Tese

à minha família, e em especial

a minha amada mãe *Erica B. F. Almeida*

(in memorian)

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Apresentação

Esta tese está apresentada em três **partes**, cada uma constituída dos seguintes itens:

Parte I: Resumo, seguido de um Resumo em inglês (*abstract*), Lista de abreviações, Introdução e Objetivos;

Parte II: Metodologia e Resultados, dispostos em capítulos, onde cada capítulo contém um artigo científico;

Parte III: Discussão, Conclusão e Referências bibliográficas citadas na Introdução da Parte I e na Discussão da Parte III.

Os trabalhos elaborados nesta tese foram desenvolvidos no laboratório de Excitotoxicidade e Neuroproteção, no Departamento de Bioquímica da Universidade Federal do Rio Grande do Sul (UFRGS), sob a orientação do Prof. Dr. Diogo Onofre Gomes de Souza

Sumário

Parte I

Resumo

Almeida, Roberto Farina. Avaliação do Potencial Ansiolítico e Antidepressivo da Guanosina. Tese de Doutorado, Programa de Pós- graduação em Ciências Biológica: Bioquímica, Universidade Federal do Rio Grande do Sul. Porto Alegre, Brasil, 2016.

Os Transtornos psiquiátricos acompanham a história da humanidade. Classificados em categorias distintas podemos observar que dentre todas as patologias que constituem os transtornos mentais e de comportamento, as doenças mais prevalentes são as doenças de Ansiedade e de Transtorno Depressivo Maior (TDM). Mesmo com muitos avanços nas neurociências, assim como na terapêutica (psicofarmacologia), ainda hoje a fisiopatologia e o desenvolvimento farmacológico são áreas que necessitam intenso estudo. Avanços recentes tem sugerido que drogas capazes de modular os sistemas glutamatérgico e purinérgico possuem potencial efeito neuromodulador, sendo promissores candidatos para o desenvolvimento de novas drogas com ação ansiolítica e/ou antidepressiva. Dessa maneira, o objetivo desta tese foi investigar o potencial efeito ansiolítico da guanosina (GUO) em modelos animais preditivos para o estudo da potencial atividade ansiolítica de novos compostos, assim como seu potencial efeito antidepressivo no modelo animal de depressão da Bulbectomia Olfatória Bilateral (OBX). Inicialmente, nossos resultados demonstram que a administração de GUO foi capaz de produzir um consistente efeito ansiolítico modulando os níveis de adenosina (ADO) e glutamato cerebral. Ainda, pela primeira vez, foi observado que a GUO *per se* promoveu uma diminuição da liberação de glutamato em preparações de sinaptosomas de hipocampo, um efeito dependente da ativação dos receptores A_1 de ADO. Após a caracterização do potencial efeito ansiolítico da GUO, nosso objetivo foi avaliar o potencial efeito antidepressivo da GUO em um modelo animal com validade de face e de constructo, como o modelo da OBX. Contudo, após revisão da literatura em estudos que utilizaram o modelo da OBX, observou-se a necessidade de uma investigação a longo prazo, das principais alterações comportamentais e neuroquímicas induzidas pela OBX. Nossos resultados, mostram pela primeira vez, que camundongos submetidos a OBX apresentaram simultaneamente alterações comportamentais e neuroquímicas transitórias e de longa duração. Ademais, as evidências indicam que o hipocampo possui alta susceptibilidade aos danos induzidos pela OBX, visto que uma sinaptotoxicidade transitória, acompanhada de um duradouro desequilíbrio redox e aumento da resposta inflamatória foram observados. Por fim, o tratamento crônico com GUO foi capaz de reverter a maioria das alterações identificadas previamente como duradouras nos parâmetros comportamentais e neuroquímicos no modelo da OBX. Considerando os resultados neuroquímicos obtidos pelos diferentes protocolos de tratamento realizados neste estudo, novas hipóteses de mecanismos de ação exercidos pela GUO foram apresentadas, e mecanismos já estabelecidos foram reproduzidos. Por fim, de uma maneira geral os dados apresentados nesta tese reforçam a hipótese do envolvimento do sistema purinérgico nos transtornos psiquiátricos, e sugerem que a GUO apresenta uma potencial ação terapêutica para o tratamento destas doenças, abrindo assim novas perspectivas para elucidação dos mecanismos envolvidos na fisiopatologia da ansiedade e TDM.

Palavras-chave: Depressão Maior, Ansiedade, Guanosina, Bulbectomia Olfatória, Preparação Sinaptosomal

Abstract

Almeida, Roberto Farina. Investigation of the Potential Anxiolytic and Antidepressive of Guanosine. PhD Thesis, Post-graduation Program of Biological Sciences: Biochemistry, Universidade Federal do Rio Grande do Sul. Porto Alegre, Brazil, 2016.

Psychiatric disorder had accompanied the course of human history. Mental and behavioral disorders are classified in different categories and among all different psychiatric disorders; anxiety and major depressive disorder (MDD) are the most prevalents. Despite the substantial advances in our knowledge on the neurobiological bases of both anxiety and MDD, as well as in the therapeutic area (psychopharmacology), even today, the pathophysiology of these disorders as well as pharmacological development are still under investigation. Recent advances have suggested that drugs able to modulate glutamatergic and purinergic systems present a potential neuromodulatory effect, and are promising candidates for the development of new drugs with both anxiolytic and antidepressant effects. Thus, the aim of this work was to investigate the potential guanosine (GUO) anxiolytic-like effects in predictive animal models largely used to elucidate anxiolytic properties of new compounds, as well as investigate the potential GUO antidepressant effect in Olfactory Bulbectomy (OBX) model of depression. Initially, our results demonstrate that acute GUO administration was able to induce a consistent anxiolytic-like effect, by modulating the adenosine and glutamate cerebrospinal levels. Here, for the first time, it was observed that GUO *per se* was able to decrease the glutamate release in hippocampal synaptosome. After characterizing the potential anxiolytic-like effect promoted by GUO, our second goal was to evaluate the potential GUO antidepressant-like effect in an animal model with recognized face and construct validity as the OBX model of depression. However, given the lack of studies in the literature considering the time course of the behavioral and neurochemical changes after the depressive-like behavior onset induced by OBX we firstly characterize some important features regarding the OBX model. Collectively, mice submitted to the OBX model of depression and followed up to 8 weeks simultaneously presented transient and long-lasting deleterious effects in behavioral and neurochemical parameters. The evidences pointed that hippocampus was the most affected brain structure, since a transient hippocampal-related synaptotoxicity, accompanied by a long-lasting hippocampal imbalance in redox and inflammatory homeostasis were observed. Additionally, the neurochemical effects seem to strengthen our behavioral findings. Finally, chronic GUO treatment, similarly to the classical tricyclic antidepressant imipramine, was able to improve the long-term behavioral phenotype impairment induced by OBX, specifically improving behavioral performances that require cognitive functions, accompanied by reversion of hippocampal redox imbalance parameters, as well as in peripheral and central anti-inflammatory IL-10 release. Thus, in present study, the pre-clinical evaluation of GUO as a potential drug for treatment of the most prevalent psychiatric disorders (anxiety and MDD) presented promising results. Furthermore, additional GUO mechanisms of action were evidenced and new perspectives were established. Thus, the data presented in this thesis support the hypothesis of the involvement of the purinergic system in mood disorders, and suggest that GUO has a potential therapeutic activity for the treatment of psychiatric disorders.

Keywords: Major Depressive disorder, Anxiety, Guanosine, Olfactory Bulbectomy, Synaptosomal preparations.

Lista de Abreviaturas:

ΔΨ Potencial de membrana mitocondrial

ACTH Hormônio adrenocorticotrópico

ADO Adenosina

AMPA Ácido alfa-amino-3-hidroxi-metil-5-4-isoxazolpropiônico

BHE Barreira hemato-encefálica

BDNF Fator neurotrófico derivado do cérebro

CAF Cafeína

CCPA 2-Cloro-N^6-ciclopentiladenosina

CID-10 Classificação internacional de doenças e problemas relacionados à saúde

CGS21680 2-p-(2-carboxietil) fenetilamino-5′-N-etilcarboxamidoadenosina

CP101606 Inibidor seletivo da subunidade NR2B do receptor NMDA de glutamato

CRH hormônio liberador de corticotrofina

DCFH Dicloro-dihidro-fluoresceina diacetato

TDM Transtorno depressivo maior

DPCPX 8-ciclopentil-1,3- dipropilxantina

DSM V Manual estatístico e diagnóstico de doenças mentais 5° edição

EAAC1 Transportador neural de glutamato

EAAT1-5 Transportadores de aminoácidos excitatórios 1-5

EPM Labirinto em cruz elevado

GDP Guanosina difosfato

GFAP Proteína ácida fibrilar glial

GLAST Transportador glutamato-aspartato

GLT-1	Transportador de glutamato-1
GMP	Guanosina monofosfato
GPx	Glutationa peroxidase
GSH	Glutationa reduzida
GTP	Guanosina trifosfato
GUO	Guanosina
HPA	Hipotálamo-hipófise adrenal
HPLC	Cromatografia liquida de alta performance
iGLU	Receptores ionotrópicos de glutamato
IL-1	Interleucina-1
IL-6	Interleucina-6
IL-10	Interleucina-10
INF-γ	Interferon-gama
iNOS	óxido nítrico sintase induzível
ISRS	Inibidores seletivos da receptação de serotonina
KA	Cainato
MAO-A	Monoamina-oxidase A
MAPK	Proteína cinase ativada por mitógeno
MK-801	Antagonista do receptor NMDA de glutamato
mGLU	Receptores metabotrópicos de glutamato
NF-κB	Fator nuclear-kappaB
NK	Células natural killer
NMDA	N-metil-D-aspartato
NO	Óxido nítrico
NTPDase	Nucleosídeo trifosfato difosfoidrolases

OBX	Bulbectomia olfatória bilateral
OMS	Organização Mundial da Saúde
PC12	Células feocromocitoma
PDGs	Purinas derivadas da guanina
PI3K/AKT	Fosfatidilinositol-3-cinase / Homóloga celular ao oncogêne v-Akt
PNP	enzima nucleosídeo purina fosforilase
PSD-95	Proteína de Densidade Pós-sináptica 95
PPSE	Potencial pos-sináptico excitatório
ROS	Espécies reativas de oxigênio
RNS	Espécies reativas de nitrogênio
SANP-25	Proteína 25 associada a sinaptossoma
SOD	Superóxido dismutase
TNF-α	Fator de necrose tumoral
VAMP	Proteína de membrana associada a vesícula
ZM241385	Antagonista do receptor A_{2A} de adenosina

1. Introdução:

1.1. Transtornos Psiquiátricas

Os transtornos psiquiátricos, mentais e de comportamento acompanham a história da humanidade (Wong & Licinio, 2001). Normalmente, tais distúrbios geram grandes perturbações nos indivíduos capazes de afetar aspectos fisiológicos, comportamentais e sociais, gerando grandes prejuízos na qualidade de vida dos pacientes (Vos et al., 2012). Historicamente, os primeiros relatos de estudos que investigaram os fenômenos envolvidos nas perturbações psíquicas datam de 400 anos antes de Cristo, e buscaram a interpretação e entendimento dos mecanismos envolvidos nestas patologias objetivando claramente estratégias de prevenção (Wong & Licinio, 2001).

Com o passar do tempo, principalmente durante a segunda metade do século XX (meado dos anos 60), uma grande mudança no paradigma do cuidado em saúde mental tornava-se necessário, visto que um crescente número de pessoas acometidas por estes transtornos eram diagnosticadas (Wong & Licinio, 2001). Tais transformações foram influenciadas principalmente por 2 principais fatores: 1) o movimento de direitos humanos iniciado nos Estados Unidos, chamado de revolução psiquiátrica, na qual a organização dos serviços relacionados aos transtornos mentais tornou-se um tema consensual em todo o mundo; e 2) o desenvolvimento e progresso da psicofarmacologia, com a descoberta de novas classes de medicamentos, com destaque aos fármacos antidepressivos que ganharam grande atenção (Gmitrowicz & Kucharska, 1994; Wong & Licinio, 2001).

Atualmente, mesmo com muitos estudos buscando esclarecer a patogênese dos transtornos psiquiátricos, os diversos mecanismos envolvidos na sua etiologia e progressão ainda não são completamente conhecidas (Maes, Kubera, Obuchowiczwa, Goehler, & Brzeszcz, 2011; Moller et al., 2016). Mesmo com grandes avanços nas áreas

da saúde, pacientes acometidos por tais condições continuam sofrendo com a séria incapacidade causada pelos sintomas característicos desta condição, que estão entre as maiores causas de morbidade da sociedade moderna (Belmaker & Agam, 2008; Hofmeijer-Sevink et al., 2012; M. Y. Lee et al., 2016; Moller et al., 2016; Vos et al., 2012). Dentre as inúmeras consequências geradas por estas patologias, podem ser destacadas: a redução da qualidade de vida, da produtividade no trabalho e o alto prejuízo social dos pacientes. Por conseguinte, altos custos econômicos estão associados a alta prevalência destes transtornos, devido principalmente à alta demanda nos atendimentos de emergência, nas consultas médicas, na hospitalização e na prescrição de medicamentos (Vos et al., 2012).

Mesmo com a descoberta de inúmeros biomarcadores, assim como o enorme avanço na área da psiquiatria, ainda hoje são discutidos os critérios diagnóstico dos transtornos mentais e de comportamento (Doernberg & Hollander, 2016; M. Y. Lee et al., 2016; Smith, Summers, Dillon, Macatee, & Cougle, 2016). Para tal, a Organização Mundial da Saúde (OMS) publicou, em uma série de revisões com início em 1893, no ano de 1993 a décima Classificação Internacional de Doenças e Problemas Relacionados à Saúde (CID-10) o Capítulo V, que corresponde especificamente aos transtornos mentais e de comportamento (Doernberg & Hollander, 2016). Recentemente (2013), atualizando as definições de diagnóstico dos transtornos mentais, a Associação Americana de Psiquiatria publicou o Manual Estatístico e Diagnóstico de Doenças Mentais na sua 5° edição (DSM-V). No entanto, mesmo com muitos estudos nesta direção, os diagnósticos dos transtornos mentais e do comportamento permanecem sendo subjetivos e suscetíveis a erros (Doernberg & Hollander, 2016; Fried & Nesse, 2015; Moller et al., 2016; Smith et al., 2016).

Classificadas em categorias distintas podemos observar que dentre todas as patologias que constituem os transtornos mentais e de comportamento, os transtornos psiquiátricos mais prevalentes são os de ansiedade e Transtorno Depressivo Maior (TDM) (Griebel & Holmes, 2013; Hart et al., 2016; Machado-Vieira, Manji, & Zarate, 2009; Moller et al., 2016; Ortiz, Ulrich, Zarate, & Machado-Vieira, 2015; Stein & Sareen, 2015; Vos et al., 2012; Wieronska & Pilc, 2009). No entanto, mesmo que muitos estudos venham tentando esclarecer os mecanismos envolvidos na fisiopatologia destas desordens (MacNamara, Kotov, & Hajcak, 2016), devido a grande complexidade das alterações neuromorfológicas, neuroquímicas, neuroendócrinas e neuroimunes, ainda nos dias de hoje exige-se dos profissionais da saúde, através da investigação científica, a ampliação do conhecimento para responder com a máxima eficácia possível as exigências da área.

1.2. Ansiedade

Os transtornos de ansiedade, assim como a maioria das patologias neuropsiquiátricas, são de caráter multifatorial (Gross & Hen, 2004; Hoge, Ivkovic, & Fricchione, 2012; Stein & Sareen, 2015). Dados epidemiológicos indicam que a ansiedade é um transtorno psiquiátrico comum na prática médica, sendo na maioria dos casos acompanhado por outras comorbidades (Hofmeijer-Sevink et al., 2012; Hoge et al., 2012; Maes, Kubera et al., 2011). Atualmente, nos Estados Unidos, os transtornos de ansiedade estão entre as desordens psiquiátricas mais prevalentes, afetando cerca de 15,7 milhões de americanos a cada ano. Estimativas, indicam que cerca de 30 milhões de pessoas em algum momento de suas vidas serão acometidas por este transtorno (Riaza Bermudo-Soriano, Perez-Rodriguez, Vaquero-Lorenzo, & Baca-Garcia, 2012). Ainda, segundo demonstrado pelo *European Epidemiology of Mental Disorders*, em 2007, cerca de 20,6% das pessoas que sofriam com os transtornos de ansiedade procuraram os

serviços de saúde, e destes, 20,7% não receberam nenhum tratamento (Alonso & Lepine, 2007). No entanto, a real prevalência epidemiológica deste transtorno é de difícil obtenção, principalmente devido ao fato de que pequenas alterações nos critérios diagnósticos podem alterar os resultados (Lieb, 2005; Stein & Sareen, 2015).

Nos seres humanos, em condições fisiológicas, a ansiedade é produzida em resposta a uma variedade de eventos permitindo ao indivíduo avaliar seu ambiente e realizar ajustes em seu comportamento frente a situações de perigo potencial ou eminente (Hart et al., 2016; Riaza Bermudo-Soriano et al., 2012). Sugere-se que os transtornos de ansiedade são causados por uma hiper-estimulação dos comportamentos defensivos, que geram respostas fisiológicas (taquicardia, sudorese, midríase, etc) e psicológicas intensas (medo, por exemplo), causando grandes sofrimentos aos pacientes (Hoge et al., 2012; Olatunji, Cisler, & Tolin, 2007).

Avanços científicos sugerem que situações envolvendo estresse têm papel determinante para a causa deste transtorno (Campos, Fogaca, Aguiar, & Guimaraes, 2013; B. H. Harvey & Shahid, 2012). Considerando os aspectos neurobiológicos, as bases da ansiedade podem envolver diferentes vias neuroendócrinas e de neurotransmissão (Amiel & Mathew, 2007; B. H. Harvey & Shahid, 2012). Um componente neuroendócrino extremamente importante é o eixo Hipotálamo-hipófise-adrenal (HPA) que é regulado por diferentes áreas do cérebro, como a amígdala, substância cinzenta periaquidutal e o hipocampo (Borrow, Stranahan, Suchecki, & Yunes, 2016; Marques et al., 2016). Uma ruptura na homeostase deste sistema está intimamente relacionada ao medo e ansiedade (Borrow et al., 2016). Em situações de risco, os neurônios hipotalâmicos, regulados pela amígdala e hipocampo, secretam o hormônio liberador de corticotrofina (CRH) (Marques et al., 2016). Com isso, há um aumento nos níveis sanguíneos do hormônio adrenocorticotrófico (ACTH), que culmina

na liberação do hormônio cortisol (corticosterona em roedores), pela glândula adrenal (Ramos Ade, Homem, Suchecki, Tufik, & Troncone, 2014). Quando o núcleo central da amígdala é ativado, a resposta ao estresse é emitida levando à ativação inapropriada e contínua do eixo HPA (Marques et al., 2016). Ainda, períodos de estresse crônico, levam a hiper-estimulação dos receptores de glicocorticoides presentes no hipocampo, ativados pelo cortisol, o que pode levar a alteração da concentração de neurotransmissores presentes nesta região cerebral, acarretando grandes prejuízos na homeostase e plasticidade sináptica (Marques et al., 2016; Riaza Bermudo-Soriano et al., 2012).

Com relação ao envolvimento dos diferentes sistemas de neurotransmissores, reconhecidos alvos farmacológicos capazes de modular a neurotransmissão GABAérgica, assim como a serotoninérgica, possuem importantes efeitos ansiolíticos (Ball, Kuhn, Wall, Shekhar, & Goddard, 2005; Hoge et al., 2012; Riaza Bermudo-Soriano et al., 2012). Isto se deve ao fato dos benzodiazepínicos que atuam no sistema GABAérgico, assim como os inibidores seletivos da recaptação de serotonina (ISRS´s), serem os medicamentos de escolha e amplamente usados na terapêutica da ansiedade (Riaza Bermudo-Soriano et al., 2012). Porém, devido aos efeitos adversos que incluem os efeitos hipnótico-sedativos, propriedades músculo relaxante, prejuízo de memória, ganho de peso e síndrome de retirada, estes compostos vêm apresentando limitada aplicação terapêutica e a busca por novos alvos terapêuticos têm aumentado (Hoffman & Mathew, 2008; Roy-Byrne, 2005). Através de mecanismos já bem caracterizados, sabe-se que em condições de estresse o aumento dos níveis de glicocorticóides pode levar a um aumento de glutamato e/ou diminuição da depuração glutamatérgica na fenda sináptica (Riaza Bermudo-Soriano et al., 2012), acarretando uma hiper estimulação glutamatérgica, o que também seria um dos possíveis mecanismos envolvidos nos transtornos de ansiedade (B. H. Harvey & Shahid, 2012; Machado-Vieira et al., 2009;

Wieronska & Pilc, 2013). Dessa maneira, fármacos capazes de modular a neurotransmissão glutamatérgica são promissores alvos terapêuticos para o tratamento da ansiedade. Uma abordagem mais completa sobre o sistema glutamatérgico, assim como perspectivas do seu envolvimento nas doenças psiquiátricas abordadas nesta tese serão apresentadas na **seção 1.4**.

Em outra perspectiva, e ainda, como uma maneira indireta de modular os níveis de glutamato, o sistema purinérgico (compostos capazes de modular as vias das purinas derivadas da adenina, e da guanina) também tem sido associado com a etiologia das doenças psiquiátricas, assim como uma possível via de ação para promissoras drogas com ações ansiolíticas (Almeida et al., 2010; Jain, Kemp, Adeyemo, Buchanan, & Stone, 1995; Ortiz et al., 2015; Prediger, Batista, & Takahashi, 2004; Vinade et al., 2003). Com base nos efeitos neuromoduladores da adenosina (ADO) na liberação de glutamato, muitos efeitos modulando as respostas relacionadas com ansiedade já forma observados, seja por manipulação farmacológica, ou por manipulação genética dos receptores de ADO no cérebro (Burnstock, Krugel, Abbracchio, & Illes, 2011). Além disso, considerando que os receptores de ADO apresentam alta expressão em estruturas relacionadas com o sistema límbico, como o hipocampo, a neurotransmissão adenosinérgica vêm sendo um potencial alvo terapêutico para a ação de drogas ansiolíticas (Burnstock et al., 2011). Da mesma maneira, estudos investigando o potencial ansiolítico das bases púricas derivadas da guanina (PDGs) também são considerados potenciais alvos terapêuticos, contudo, por ser um dos principais focos desta tese, uma caracterização mais minuciosa será apresentada na **seção 1.5**.

1.3. Trantorno Depressivo Maior:

O Transtorno depressivo maior (TDM) é um transtorno psiquiátrico crônico que afeta cerca de 350 milhões de pessoas no mundo atual (Blazer, Kessler, McGonagle, &

Swartz, 1994; Bortolato et al., 2016; Karyotaki et al., 2016; Kessler, Chiu, Demler, Merikangas, & Walters, 2005; Kessler et al., 1994; Stahl, 2006; Thase, 2007). Segundo dados epidemiológicos da OMS estima-se que a prevalência da TDM tende a aumentar, sendo que projeções atuais apontam que em 2030 a TDM será a segunda maior doença incapacitante no mundo (Kaufman, DeLorenzo, Choudhury, & Parsey, 2016). Segundo os critérios diagnósticos definidos pelo DSM-V, para caracterização de TDM há a necessidade obrigatória da presença de humor depressivo, perda de interesse e/ou prazer durante pelo menos duas semanas (Karyotaki et al., 2016). Além destes, uma série de outras manifestações clínicas devem ser observadas em pacientes com TDM, como: apatia, tristeza, impotência, desesperança, alterações psicomotoras (retardo ou agitação), de sono (insônia ou hipersônia), redução no grau de concentração, de memória e de cognição, variação de peso corporal e perda de energia (Reus et al., 2016). A comunidade científica, em geral, acredita que a TDM não possui uma única causa, mas decorre de múltiplos fatores que somados desempenham um papel importante no desenvolvimento deste transtorno (Berton & Nestler, 2006; Czeh, Fuchs, Wiborg, & Simon, 2015; Nestler et al., 2002). Entre fatores já estabelecidos podemos citar experiências vividas pelos indivíduos (contato prévio com determinadas doenças e/ou uso de substâncias entorpecentes moduladoras do Sistema Nervoso Central – SNC), genética, idade, alterações hormonais e neuroquímicas (Czeh et al., 2015; Karalliedde, Wheeler, Maclehose, & Murray, 2000).

Por mais de três décadas, as bases biológicas dos transtornos depressivos têm sido explicadas por meio da hipótese monoaminérgica da depressão (Karyotaki et al., 2016). Essa teoria propõe que a TDM seja consequência de uma menor disponibilidade tanto de serotonina, noradrenalina e/ou dopamina (Schildkraut, 1965). Tal proposição é reforçada pelo conhecimento do mecanismo de ação dos antidepressivos atualmente

utilizados na clínica, que se baseiam principalmente, no aumento da disponibilidade desses neurotransmissores na fenda sináptica, seja pela inibição (seletiva ou não) de suas recaptações, ou pela inibição da enzima responsável por suas degradações (Nowak, Szewczyk, & Pilc, 2005; Stahl, Entsuah, & Rudolph, 2002).

Estudos atuais propõem que algumas estruturas cerebrais estejam envolvidas na fisiopatologia da TDM **(Figura 1)**. O hipocampo e o córtex pré-frontal são estruturas cerebrais implicadas diretamente nos mecanismos relacionados à memória, aprendizagem, atenção e impulsos, o que sugere que podem controlar muitos aspectos relacionados com a doença, como: défict cognitivo, sentimento de culpa, desesperança, assim como a tomada de decisão que pode culminar com o suicídio (Benedetti et al., 2009; Benedetti & Smeraldi, 2009; Brockmann et al., 2009; Halari et al., 2009; Klumpers et al., 2010; Peluso et al., 2009). Tais evidências são baseadas em achados que mostraram alterações morfológicas (atrofia de dendritos, e de células da glia), histológicas (perdas de neurônios) e neuroquímicas nestas regiões, como por exemplo: diminuição do volume hipocampal e do córtex pré-frontal principalmente em estudos *post-mortem* e em estudos pré-clínicos (Bremner et al., 2000; Colla et al., 2007; Czeh et al., 2015; Krishnan & Nestler, 2008; Nicholls, 2008; Ongur, Drevets, & Price, 1998; Pittenger & Duman, 2008; Rajkowska et al., 1999). Por outro lado, pesquisas indicam que a região da amigdala, outra região do cérebro bastante envolvida na fisiopatologia da TDM, teria sua atividade e morfologia aumentadas.

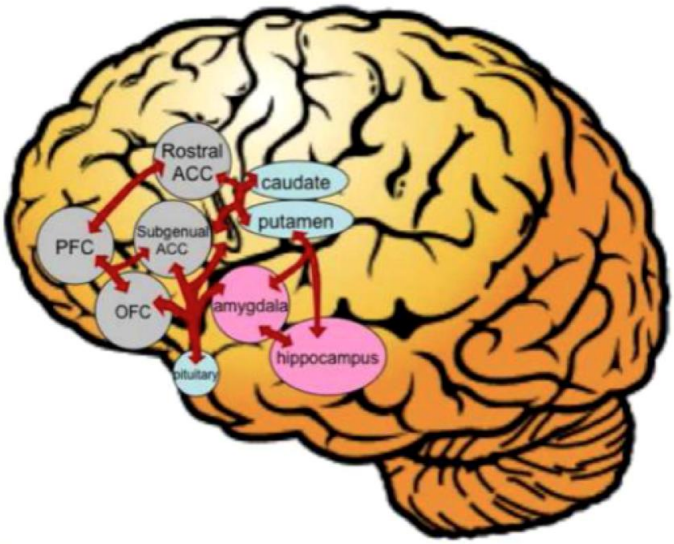

Figura 1. Regiões cerebrais implicadas na fisiopatologia do Transtorno Depressivo Maior.
Adaptado de Leslie Hulvershorn, Brain Imaging Behav. 2011

É de amplo conhecimento que assim como na teoria neuroendócrina dos transtornos de ansiedade, a etiologia da TDM pode estar relacionada com alterações hormonais dependentes do eixo neuroendócrino, HPA, o qual possui conexões com diversas estruturas do sistema límbico (Czeh et al., 2015; Song & Leonard, 2005) e pode ser regulado pelo estresse. Pesquisas indicam que alterações estruturais como atrofia e morte de neurônios são processos bastante evidentes dependendo da severidade e cronicidade do estresse. Além disso, o estresse diminui a neurogênese de neurônios granulares do giro denteado no hipocampo, principalmente por diminuir a expressão do fator neurotrófico derivado do cérebro (BDNF) no hipocampo (Kim, Nunes, Oliveira, Young, & Lafer, 2016), o que pode contribuir para as alterações estruturais já mencionadas. Dentre as vias relacionadas podemos destacar as vias de apoptose e/ou necrose celular, moduladas inicialmente por uma perturbação na homeostase do Ca^{+2}, com posterior ativação de diferentes vias enzimáticas relacionadas aos eventos de morte celular, como a ativação de proteases, fosfolipases, oxido nítrico sintase e/ou endonucleases (Li et al., 2011; Verkhratsky, 2007).

Além disso, estudos sugerem que a TDM acompanha a ativação de respostas imuno-inflamatórias. Pacientes com TDM apresentam aumento no número de leucócitos sanguíneos periféricos, aumento da razão $CD4^{+}/CD8^{+}$, redução do número de linfócitos e da atividade de células natural killer (NK), além de um aumento nos níveis sanguíneos de citocinas pró-inflamatórias e de seus receptores (Hannestad, DellaGioia, & Bloch, 2011; Hashioka, 2011). Juntamente com estas alterações, a desregulação na sinalização redox também poderia estar envolvida nas alterações progressivas envolvidas na TDM. Espécies reativas de oxigênio e de nitrogênio (ROS e RNS, respectivamente), são produzidos durante os processos fisiológicos normais (Black et al., 2016; Liu et al., 2015), e através da interação com proteínas, ácidos graxos e DNA, executam funções na regulação da função celular. No entanto, a superprodução de ROS e RNS pode acarretar alterações estruturais e funcionais com consequente comprometimento celular (Maes, Kubera et al., 2011; Moylan et al., 2014). Em situações normais, estes potenciais efeitos tóxicos são compensados por mecanismos antioxidantes intrínsecos, porém pesquisas conduzidas com pacientes acometidos pela TDM sugerem que estas populações apresentem aumento expressivo da produção de ROS e RNS (Maes, Galecki, Chang, & Berk, 2011), principalmente devido a redução da função dos sistemas antioxidantes (Black, Bot, Scheffer, Cuijpers, & Penninx, 2015; Moylan et al., 2014). Do mesmo modo, alterações em enzimas com função antioxidante têm sido relatados, reduzida atividade da superóxido dismutase (SOD) e da glutationa peroxidase (GPX) em pacientes acometidos pela TDM (Maes, Galecki et al., 2011; Maes, Kubera et al., 2011). Diante de todas as alterações mencionadas, um ilustração pode ser visualizada na **Figura 2**.

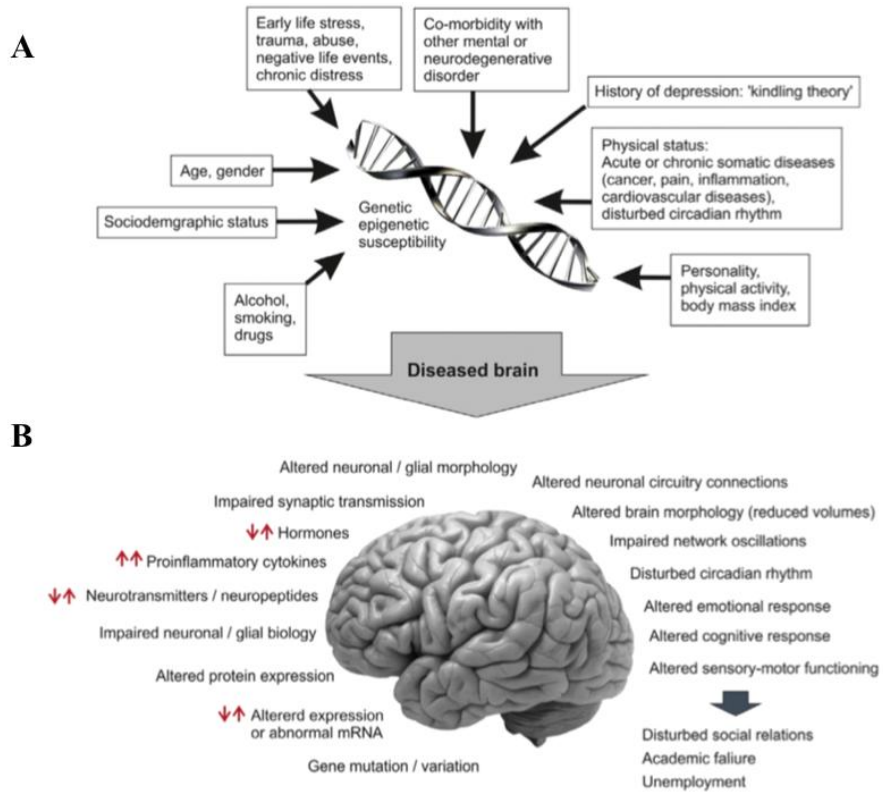

Figura 2. Fisiopatologia da Depressão Maior

Em (A) são apresentados os fenômenos que podem desencadear à DM, e em (B) são demonstradas possíveis alterações neuroquímicas encontradas em estudos que investigaram a fisiopatologia da DM. Adaptado de Boldizsár Czéh, Progress in Neuro-Psychopharmacology & Biological Psychiatry. 2015

A alteração da funcionalidade mitocondrial é uma hipótese bastante atrativa para explicar a fisiopatologia da TDM. Estudos investigando a bioenergética mitocondrial demonstraram uma significante diminuição na produção de ATP, assim como na atividade dos complexos mitocôndrias de I-IV em pacientes com TDM (Ben-Shachar & Karry, 2008), além de alterações no potencial de membrana ($\Delta\Psi$) mitocondrial (Avetisyan et al., 2016). Mesmo diante destes dados, o debate persiste, visto que estudos buscam esclarecer se a redução da bioenergética mitocondrial seria uma causa ou uma consequência da fisiopatologia da TDM.

A disfunção na bioenergética mitocondrial pode prejudicar o $\Delta\Psi$ e consequentemente a manutenção da homeostase tecidual e da neurotransmissão (principalmente a homeostase do glutamato), as quais consomem a maior parte da

energia gasta pelo cérebro (Sheng & Cai, 2015). Quando a produção de ATP é reduzida devido a alteração na atividade mitocondrial, a bomba de Na^+ /K^+ ATPase, que mantém o potencial de membrana, diminui a troca Na^+ /K^+. Isso irá aumentar a probabilidade dos canais de Na^+ e Ca^{+2} dependentes de voltagem se abrirem, elevando a concentração de Ca^{+2} (Sheng & Cai, 2015). O desequilíbrio na homeostase do Ca^{+2}, tem como consequência a liberação de aminoácidos excitatórios no espaço extracelular, como o glutamato (Streck et al., 2014). A disfunção no transporte desse neurotransmissor, que é dependente do gradiente eletrogênico, leva seu acúmulo na fenda sináptica, em um fenômeno denominado como excitotoxicidade glutamatérgica o qual está envolvido na etiologia e progressão das mais diversas doenças cerebrais, incluindo a TDM (Manji et al., 2012; Streck et al., 2014). Mesmo diante de tantas evidências, é importante enfatizar que a progressão destas alterações é complexa e bastante variável, sendo extremamente necessário novos estudos para melhorar a compressão dos mecanismos associados com a progressão temporal da doença. Uma ilustração abrangendo algumas vias de sinalização relacionadas a neuroprogressão da TDM é apresentada na **figura 3**.

Considerando que a sintomatologia da depressão é espécie específica, e que limitações nos modelos são previsíveis, torna-se possível verificar similaridade entre pacientes com TDM e roedores submetidos aos diferentes modelos para estudo da depressão como: alterações endócrinas, imunológicas, e de neurotransmissores o que torna tais modelos amplamente aceitos e utilizados (Chopra, Kumar, & Kuhad, 2011; Spijker & van Rossum, 2012; van Rossum & van den Akker, 2011). Atualmente, um modelo animal que preenche muitos dos critérios necessários para se estudar a TDM e que vêm ganhando atenção da comunidade científica é a ablação bilateral dos bulbos olfatórios no modelo da Bulbectomia Bilateral Olfatória (OBX) em roedores

(Hendriksen, Korte, Olivier, & Oosting, 2015; Mucignat-Caretta, Bondi, & Caretta, 2006; Song & Leonard, 2005).

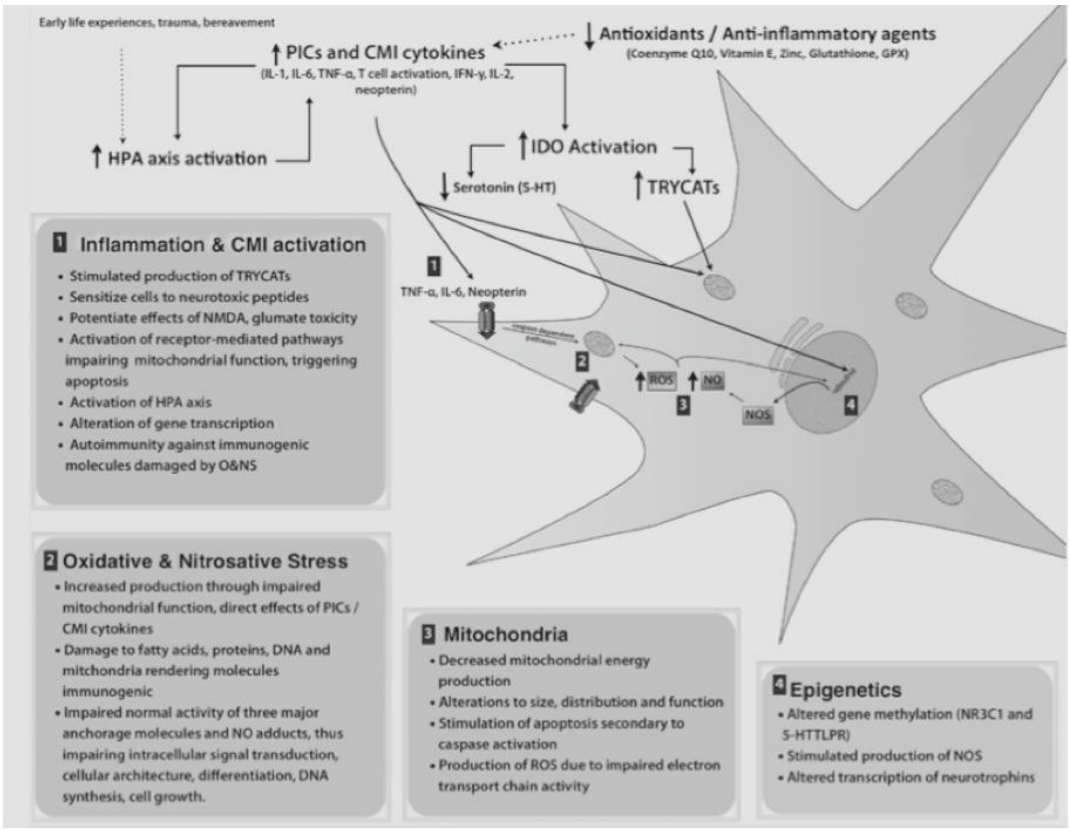

Figura 3. Exemplo de vias de sinalização que podem estar relacionadas com a neuroprogressão dos eventos associados à TDM. Adaptado de Moylan S. Molecular Pschiatry. 2013.

1.3.1 Bulbectomia olfatória bilateral (OBX) como modelo animal para o estudo do Transtorno Depressivo Maior

Os bulbos olfativos são projeções bilaterais do telencéfalo rostral e constituem cerca de 4% da massa total do cérebro de um roedor adulto (Song & Leonard, 2005). Devido as suas extensivas conexões eferentes em áreas cerebrais como a mesocorticolímbicas e subcorticais, postula-se que a remoção bilateral desta região tem um impacto maior no cérebro que apenas anosmia gerada pela ablação dos bulbos olfatórios **(Figura 4)** (Song & Leonard, 2005). Alterações na funcionalidade de diversas estruturas cerebrais são observadas após a OBX, principalmente devido a aspectos

relacionados com degeneração retrógrada, anterógrada e transneuronal principalmente em regiões centrais como a amígdala e o hipocampo (Hendriksen et al., 2015; Song & Leonard, 2005). Como consequência há prejuízo direto em componentes comportamentais, de memória e emoções. Dentre as alterações observadas destacam-se hiperatividade, anedonia, déficit cognitivo, alterações no comportamento exploratório, na ingesta alimentar e agressividade (Hendriksen et al., 2015; Song & Leonard, 2005). É importante ressaltar que a agitação psicomotora observada pela hiperlocomoção no teste do campo aberto, altamente explorada em estudos utilizando este modelo, parece ser uma das principais alterações fenotípicas desenvolvidas pelos animais submetidos à OBX, sendo este um dos motivos pelos quais este modelo também é considerado um modelo de depressão ansiosa por alguns autores (Song & Leonard, 2005). Entretanto, com relação as características comportamentais, principalmente nos parâmetros relacionados com anedonia, ainda há resultados contraditórios em estudos utilizando a OBX (Czeh et al., 2015; Song & Leonard, 2005). Além de alterações comportamentais, alterações endócrinas, imunes, neuroanatômicas e neuroquímicas que mimetizam as encontradas em pacientes acometidos pela depressão são observados nesse modelo (Czeh et al., 2015; Song & Leonard, 2005). Ainda, têm se demonstrado que a maior parte das alterações comportamentais e bioquímicas observadas nos roedores submetidos à supressão dos bulbos olfatórios são revertidas por tratamentos crônicos com antidepressivos com ação clínica comprovada (Hendriksen et al., 2015). Entretanto, mesmo com muitos estudos nesta e em outras direções, ainda há uma carência de estudos neuroquímicos no modelo da ablação do bulbo olfatório que podem auxiliar na compreensão da fisiopatologia da TDM, e por fim auxiliar no desenvolvimento de novos fármacos. Considerando que a progressão das alterações cerebrais e comportamentais em pacientes com TDM não está totalmente esclarecida, a utilização do modelo da OBX

pode ser uma excelente ferramenta para avançar neste conhecimento.

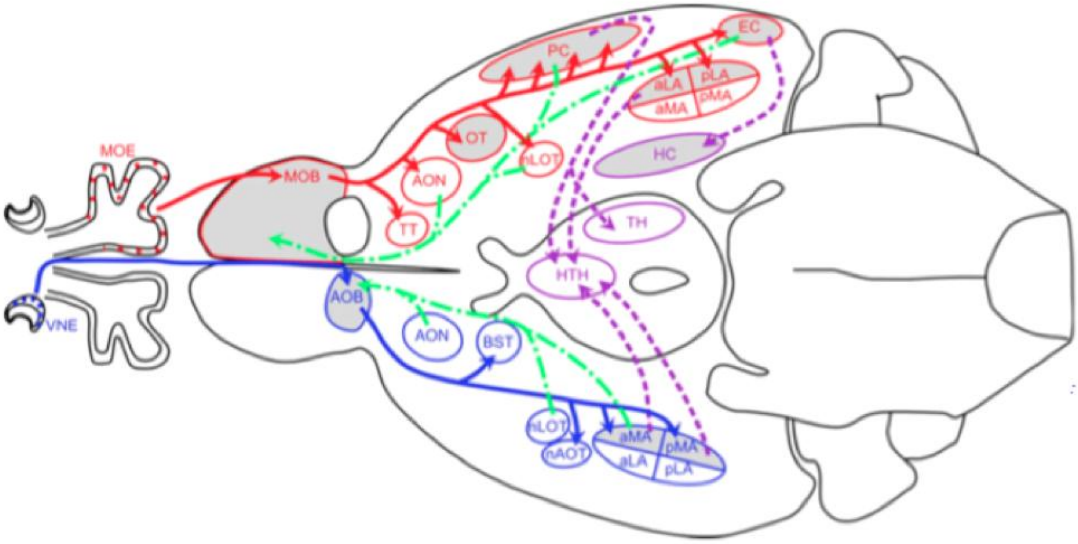

Figura 4. Conexões neuronais entre o bulbo olfatório e as diversas áreas do cérebro de camundongos

Demonstração das extensivas conexões eferentes entre as diferentes áreas cerebrais do cérebro de camundongos, onde após a OBX ocorrerá uma neurodegeneração retrógrada, anterógrada e transneuronal resultando em comprometimento funcional de diversos tecidos, principalmente em regiões centrais como a amígdala e o hipocampo. Adaptado de Canavan SV, Front Psychiatry. 2011

1.4. Sistema Glutamatérgico

Estima-se que 50% dos neurônios cerebrais sejam glutamatérgicos (Danbolt, Furness, & Zhou, 2016). O glutamato é o principal neurotransmissor excitatório do SNC. O glutamato está intimamente relacionado com os processos de aprendizagem e memória e também é fundamental para a diferenciação neural, migração, sobrevivência e desenvolvimento do cérebro, além de outros eventos que envolvem plasticidade neuronal (Izquierdo et al., 2006; Segovia, Porras, Del Arco, & Mora, 2001; Spitzer, Volbracht, Lundgaard, & Karadottir, 2016).

O glutamato tem sua ação pós-sináptica, realizada através da ativação de seus receptores. Estes podem ser classificados em dois grandes grupos: ionotrópicos (iGLU, - NMDA, AMPA and KA), que são permeáveis aos cátions Na^{+} e Ca^{+2}, e os

metabotrópicos (mGLU), que são acoplados a sistemas de segundos mensageiros através de proteínas G (Bruno et al., 2016; Maragakis & Rothstein, 2004, 2006; Sheldon & Robinson, 2007). Em condições fisiológicas, o glutamato vesicular é liberado na fenda sináptica por um mecanismo dependente de Ca^{+2}, ligando-se aos seus receptores. A concentração de glutamato nas vesículas é cerca de 100mmol/L e sua liberação produz um potencial pos-sináptico excitatório (PPSE) e sua ativação resulta na propagação do potencial de ação nos neurônios glutamatérgicos (Sattler & Rothstein, 2006; Sheldon & Robinson, 2007).

A homeostasia deste sistema de neurotransmissão, ainda, envolve a remoção do glutamato da fenda sináptica por transportadores específicos, os quais já foram caracterizados em cérebro de mamíferos: EAAT1-5 (Danbolt, 2001; Jensen, Fahlke, Bjorn-Yoshimoto, & Bunch, 2015). A atividade destes transportadores é dependente de Na^{+} (Chao, Fei, & Fei; Danbolt, 2001; Jensen et al., 2015; Tzingounis & Wadiche, 2007) e esta sujeita à regulação e plasticidade, sendo extremamente importantes na modulação do tônus fisiológico do sistema glutamatérgico (Chao, Fei, & Fei, 2010; Danbolt, 2001; Eulenburg & Gomeza, 2010; Tzingounis & Wadiche, 2007). Três destes transportadores são neuronais (EAAC1, EAAT4 e EAAT5), enquanto o GLT1 (EAAT2) e GLAST (EAAT1) estão predominantemente presentes em células gliais (Beart & O'Shea, 2007; Danbolt, 2001; Machado-Vieira et al., 2009; Swanson, Ying, & Kauppinen, 2004).

A excessiva ativação do sistema glutamatérgico, causada pelo excesso de glutamato extracelular, é altamente neurotóxica e está intimamente relacionada com doenças agudas (Acidente Vascular Cerebral, Convulsão e Traumas), assim como com doenças crônicas do cérebro (Doenças Neurodegenerativas, Demência e Transtornos Psiquiátricos) (Lipton & Rosenberg, 1994; Machado-Vieira et al., 2009; Maragakis & Rothstein, 2006; Meldrum, 1994; Segovia et al., 2001; Sheldon & Robinson, 2007). A

captação astrocitária de glutamato é o principal mecanismo endógeno para proteger neurônios da excitotoxicidade do glutamato (Robinson & Jackson, 2016). Vários estudos têm considerado que a disfunção de transportadores de glutamato pode ser o evento inicial ou parte de uma cascata implicada na patologia de doenças cerebrais (Machado-Vieira et al., 2009; Maragakis & Rothstein, 2004, 2006; Moussa et al., 2007; Robinson, 2006; Robinson & Jackson, 2016; Sheldon & Robinson, 2007; Tzingounis & Wadiche, 2007).

Um prejuízo na homeostase da neurotransmissão glutamatérgica parece ter um grande envolvimento nas doenças psiquiátricas. Com relação a ansiedade, diversos dados da literatura sugerem que a neurotransmissão glutamatérgica em estruturas relacionadas com o sistema límbico desempenham um papel crucial na patogênese dos transtornos de ansiedade (Amiel & Mathew, 2007; Bergink, van Megen, & Westenberg, 2004; Cortese & Phan, 2005; Marrocco et al., 2012; Riaza Bermudo-Soriano et al., 2012; Zimmer et al., 2015). Já foi observado que fármacos não competitivos, bem como antagonistas competitivos dos receptores glutamatérgico NMDA e AMPA produzem efeitos ansiolíticos (B. H. Harvey & Shahid, 2012; Marrocco et al., 2012; Riaza Bermudo-Soriano et al., 2012). Mais recentemente, vários compostos que atuam antagonizando os receptores metabotrópicos com alta seletividade e potência foram descobertos e seus efeitos estão sendo investigados em estudos comportamentais para avaliação de parâmetros de ansiedade (B. H. Harvey & Shahid, 2012).

Por outro lado, dados que corroboram com a hipótese glutamatérgica associada com a TDM já mostraram alterações nos níveis de glutamato em plasma/soro (Altamura et al., 1993; Mitani et al., 2006), liquor (Frye, Tsai, Huggins, Coyle, & Post, 2007; Frye, Watzl et al., 2007; Levine et al., 2000), e em córtex occipital/pré-frontal de pacientes que sofrem/sofriam de TDM (Dean, Scarr, Bradbury, & Copolov, 1999; Hashimoto,

2009; Hasler et al., 2007; Sanacora, Kendell, Fenton, Coric, & Krystal, 2004). Atualmente, é crescente o número de dados experimentais indicando que receptores glutamatérgicos NMDA estão envolvidos no mecanismo de ação de drogas antidepressivas. Resultados pré-clínicos, e clínicos (Machado-Vieira, Henter, & Zarate, 2015; Padovan & Guimaraes, 2004; Pittenger et al., 2008; Pittenger, Sanacora, & Krystal, 2007) sugerem que drogas capazes de antagonizar receptores NMDA – Ketamina, CP101606 e MK 801 – possuem propriedades antidepressivas (Machado-Vieira et al., 2009). Além disso, relatos mostram que o Riluzol, cujo mecanismo de ação se dá pela inibição da liberação de glutamato, estimulação de AMPA, aumento da recaptação de glutamato e estimulação da síntese de fatores tróficos (Frizzo, Dall'Onder, Dalcin, & Souza, 2004; Machado-Vieira et al., 2015; Machado-Vieira et al., 2009; Mizuta et al., 2001; Pittenger et al., 2008) apresentam efeitos antidepressivos em modelos animais para estudo da TDM, assim como em estudos clínicos com pacientes que acometidos pela TDM.

Diante de todas estas evidências, e considerando a grande complexidade envolvida na fisiopatologia da ansiedade e da TDM, ainda há uma grande necessidade de estudos visando o entendimento dos mecanismos envolvidos nestas doenças, assim como o desenvolvimento de novas alternativas terapêuticas.

1.5. Purinas Derivadas da Guanina

As Bases Púricas possuem importante função biológica, sendo amplamente distribuídas dentro e fora das células, dentres estas podem ser destacadas as Purinas Derivadas da Adenina (PDAs) e as Purinas Derivadas da Guanina (PDGs), com seus respectivos nucleotídeos e nucleosídeos **(Figura 5)**. As PDAs podem ser classificadas como ATP, ADP, AMP, Adenosina e Adenina, enquanto as PDGs podem ser classificadas como GTP, GDP, GMP, Guanosine e Guanina. Contemplando o restante

das purinas estão os metabólitos diretos da hidrólise dos nucleotídeos e nucleosídeos da adenina e guanina, como: Inosina, Xantina, Hipoxantina e Ácido Úrico. Dentre as diversas funções biológicas das purinas podemos destacar seu papel na construção do DNA e RNA (através da adenina e guanina), vias bioquímicas envolvidas no metabolismo energético celular (como os nucleotídeos ATP, e GTP), assim como nos mecanismos de transdução de sinal como segundos mensageiros (AMPc e GMPc) (Bourne, Sanders, & McCormick, 1990; Burnstock, 2007).

Nos últimos 25 anos, as PDGs vêm ganhando atenção dos pesquisadores devido ao seu potencial efeito neuroprotetor em modelos experimentais que envolvem a hiper-estimulação da neurotransmissão glutamatérgica (Frizzo et al., 2003; Schmidt, Avila, & Souza, 2005; Schmidt, Lara, de Faria Maraschin, da Silveira Perla, & Onofre Souza, 2000; Schmidt, Lara, & Souza, 2007). Estudos que já investigaram as possíveis ligações das PDGs evidenciaram que tais moléculas são capazes de inibir a ligação do glutamato e de seus análogos de preparações de membrana cerebral (Mendieta, Gago, & Ramirez, 2005; Paas, Devillers-Thiery, Changeux, Medevielle, & Teichberg, 1996). Dentre os resultados com grande destaque estão os trabalhos de Ramirez G. e Souza D.O., que demonstraram que os efeitos dos nucleotídeos da guanina não dependem de sua interação com a proteína-G (Souza & Ramirez, 1991).

Estudos posteriores, demonstraram que as PDGs podem atuar do lado externo da membrana plasmática celular, apresentando uma importante função fisiológica e neuromodulatória no sistema nervoso central; exercendo efeitos tróficos em células neurais (Bettio, Gil-Mohapel, & Rodrigues, 2016; Ciccarelli et al., 2001; Rathbone et al., 1999); e/ou como possível ação modulatória do sistema glutamatérgico (Baron et al., 1989; Bettio, Gil-Mohapel et al., 2016; Burgos, Barat, Souza, & Ramirez, 1998; Paz, Ramos, Ramirez, & Souza, 1994; Regner, Ramirez, Bello-Klein, & Souza, 1998), e

exercendo efeitos neuroprotetores em diferentes modelos de doenças cerebrais (Lara et al., 2001; Schmidt et al., 2007; Vinade et al., 2003).

A hidrólise dos nucleotídeos purinérgicos, como demontrado na **figura 5**, é realizada por uma variedade de enzimas, denominadas ecto-nucleotidases. Tais enzimas são cruciais para a modulação fisiológica de diversas funções do SNC (Gomes et al., 2009). Dentre essas enzimas podemos destacar a família das nucleosídeo trifosfato difosfoidrolases (NTPDases) e a ecto-5'-nucleotidase, as quais desempenham um importante papel regulador do sistema purinérgico em condições fisiológicas e patológicas (Vollmayer et al., 2001; Zimmermann, 2006a, 2006b). As NTPDases são enzimas que podem estar localizadas tanto na membrana plasmática das células hidrolisando nucleotídeos extracelulares, bem como ancoradas a organelas citoplasmáticas com o sítio ativo voltado para o lúmen das mesmas (Lavoie, Kukulski, Levesque, Lecka, & Sevigny, 2004). Já as ecto-5'-nucleotidases são responsáveis pela hidrólise de nucleotídeos 5' monofosfatados ao seu respectivo nucleosídeo (Strater, 2006).

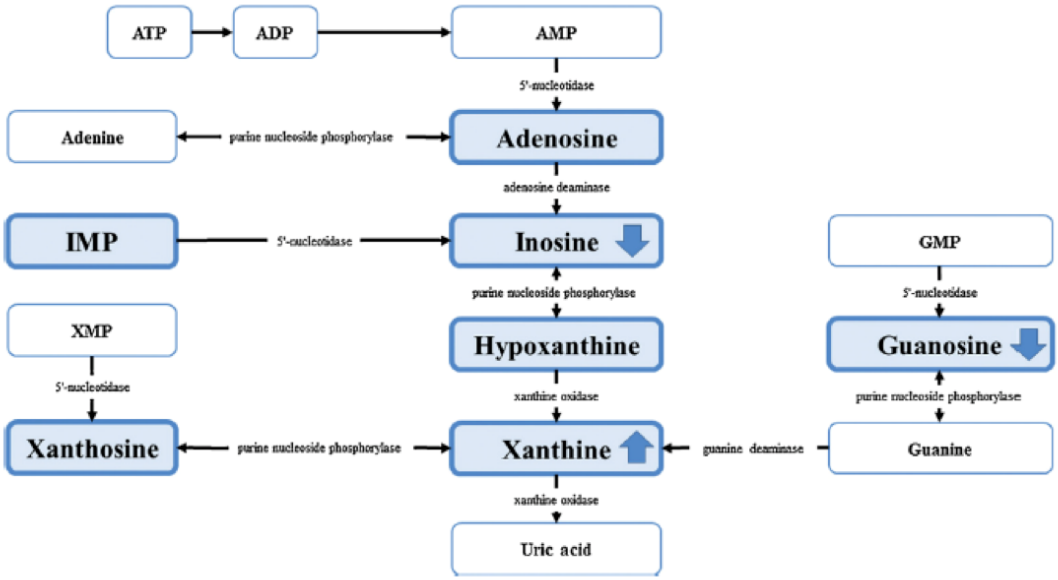

Figura 5. Demonstração das vias relacionadas com o catabolismo das Purinas Derivadas da Adenina e Guanina. Adaptado da Ali-Sisto, T., Neuropsychoendocrinology 2016

Uma vez formados, os nucleosídeos são potencialmente utilizados como substratos da enzima nucleosídeo purina fosforilase (PNP), uma outra família de enzimas do sistema purinérgico (Bzowska, Kulikowska, & Shugar, 2000; Bzowska et al., 1995). A PNP é uma enzima com ampla distribuição nos tecidos responsável pelo catabolismo de nucleosídeos, além de ser uma enzima chave na via da salvação, onde catalisa a fosforólise reversível de nucleosídeos púricos a uma ribose-1-fosfato (Bzowska et al., 2000; Bzowska et al., 1995; Yegutkin et al., 2008). Os principais substratos fisiológicos desta enzima são inosina (degradada a hipoxantina) e GUO (degradada a Guanina) - derivadas principalmente da hidrólise de ribonucleotídeos (Nakamura, Chu, Stoeckler, & Parks, 1986; Stoeckler, Cambor, & Parks, 1980; Stoeckler et al., 1986). Considerando especificamente a GUO, é importante enfatizar que estudos farmacocinéticos recentes demonstram que a GUO é rapidamente absorvida, atravessa a barreira hemato encefálica (BHE) sendo capaz de exercer efeitos neuromodulatores no cérebro (Bettio, Gil-Mohapel et al., 2016). Uma ilustração dos possíveis mecanismos de ação da GUO está apresentada na **figura 6**.

Além dos efeitos neuroprotetores frente aos insultos provocados pela hiperestimulação do sistema glutamatérgico, estudos recentes vêm demonstrando que as PDGs possuem efeitos neurotróficos/neuritogênicos em neurônios hipocampais, células da glia e células feocromocitoma (PC12), visto que mostraram aumento da proliferação, diferenciação, arborização e crescimento de neurônios. Também, estudos sugerem que o nucleosídeo GUO tenha ação antioxidante, impedindo o aumento de ROS, além de efeito anti-inflamatório, neutralizando o aumento de interleucina-6 (IL-6) e do fator de necrose tumoral (TNF- α). Além disso, a ativação induzida pela GUO das vias de sinalização da PI3K/Akt e MAPKs estaria associada com a inibição do fator nuclear-kappaB (NF-kB), com a inibição da óxido nítrico sintase induzível (iNOS) (Bellaver et al., 2015;

D'Alimonte et al., 2007; Dal-Cim et al., 2013), e com a indução da expressão da heme oxigenase-1 (Bau et al., 2005; Bellaver et al., 2015; D'Alimonte et al., 2007; Dal-Cim et al., 2012).

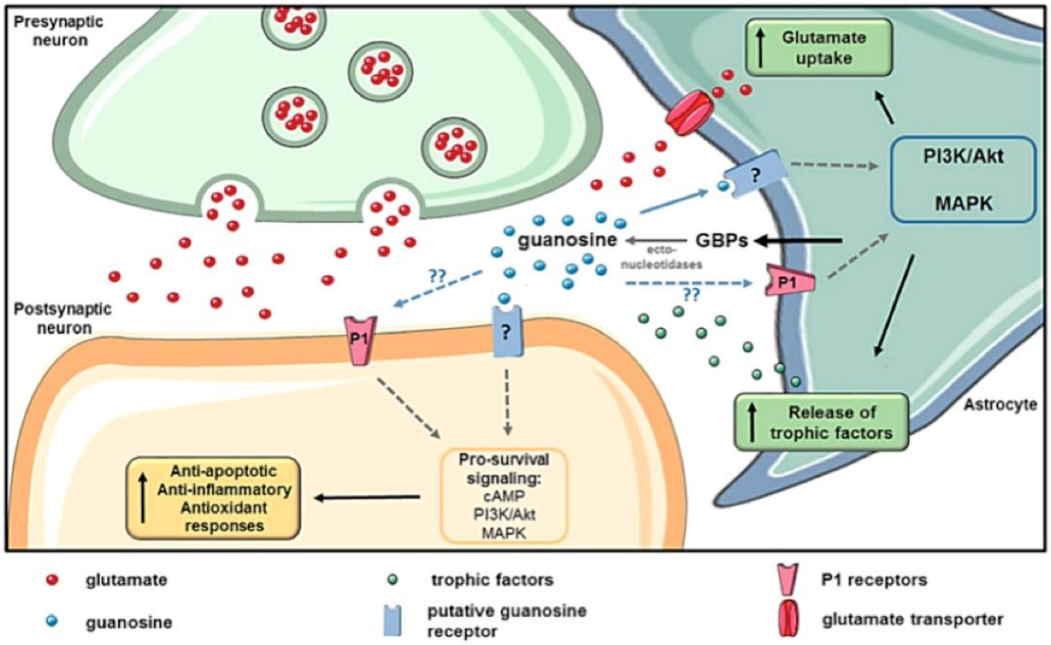

Figura 6. Ilustração do possível mecanismo de ação extracelular da GUO.
Os astrócitos são as principais fontes de Purinas Derivadas da Guanina (PDGs) no Sistema Nervoso Central (SNC). A GUO presente no espaço extracelular é gerada a partir do catabolismo de nucleotídeos da Guanina. Dentre os mecanismos já elucidados destacam-se os efeitos moduladores da neurotransmissão glutamatérgica e neurotrofinas, assim como o potencial efeito anti-inflamatório, antioxidante e antiapoptótico. Adaptado de Bettio, L.E. Purinergic Signalling, 2016.

Em relação aos possíveis efeitos das PDGs em modelos experimentais utilizados para avaliar o possível efeito ansiolítico de novas drogas, já foi demonstrado que a GUO administrada *ad libitum* na água de beber de camundongos por duas semanas foi capaz de apresentar um efeito ansiolítico no teste da placa perfurada (Vinade et al., 2003). Ainda, em um estudo que investigou especificamente a ação do GMP, nas doses de 10, 25, 50, 100 e 150mg/kg em relação ao comportamento relacionado com à ansiedade em ratos, foi demonstrado que a administração aguda e sistêmica de GMP na dose de 50mg/kg promoveu ação ansiolítica similar à administração de diazepam 2.0mg/kg em testes clássicos de ansiedade, como o claro/escuro e o labirinto em cruz elevado, sem alterar a locomoção dos animais no teste do campo aberto (Almeida et al., 2010).

Com relação a TDM, dados recentes evidenciaram um efeito típico antidepressivo da GUO em testes como nado forçado, suspensão pela cauda e de estresse agudo em roedores (Bettio et al., 2012; Bettio et al., 2014; Bettio, Neis et al., 2016). Nestes estudos, verificou-se que o a prévia administração de GUO foi capaz de prevenir o aumento induzido pelo estresse agudo no tempo de imobilidade no nado forçado. Além disso, também foi demonstrado que este efeito típico antidepressivo parece ser dependente, pelo menos em parte, das propriedades antioxidantes e relacionadas à neurogênese da GUO.

No entanto, mesmo sabendo que os testes preditivos para estudo das doenças psiquiátricas são extremamente importantes para investigar o mecanismo de ação de determinadas drogas, a utilização de modelos animais com validade de face e de constructo acaba sendo uma ferramenta mais completa, onde os animais acabam desenvolvendo os sintomas da depressão gradualmente ao longo do tempo em resposta a diferentes estímulos, possibilitando buscar um melhor entendimento da fisiopatologia dos transtornos mentais assim como novas alternativas de tratamento farmacológico. Assim, considerando que amplas evidências indicam que (1) dentre todas as patologias que constituem os transtornos mentais e de comportamento, as doenças psiquiátricas mais prevalentes são as doenças de Ansiedade e Depressão, (2) os mecanismos envolvidos na fisiopatologia das doenças psiquiátricas não estão totalmente elucidados, (3) que amplas evidências sustentam que o sistema purinérgico está fortemente relacionado com as bases neurobiológicas destes transtornos mentais, (4) que há uma grande necessidade do desenvolvimento de novas compostos com reconhecida ação terapêutica para o tratamento destas doenças e (5) que, embora sem ter o seu mecanismo de ação bem definido, estudos vem demonstrando um potencial efeito neuromodulador da GUO, estudos nesta direção se fazem-se necessário.

2. OBJETIVO

2.1. Objetivo Geral

Investigar o potencial ansiolítico e antidepressivo da GUO em modelos animais preditivos de atividade ansiolítica, assim como no modelo de depressão da Bulbectomia Olfatória Bilateral (OBX).

2.2. Objetivos específicos

- Investigar o efeito da GUO em modelos preditivos de atividade ansiolítica em ratos;
- Avaliar a participação dos sistemas glutamatérgico e adenosinérgico no potencial efeito ansiolítico da GUO;
- Investigar as bases neurobiológicas da TDM em um modelo animal com potencial aplicabilidade translacional, avaliando as alterações temporais relacionadas ao fenótipo comportamental e aos parâmetros neuroquímicos decorrentes da ablação bilateral dos bulbos olfatórios no modelo de depressão da OBX em camundongos. Esta fase preliminar visa a melhor caracterização do modelo de depressão da OBX para realização das etapas subsequentes.
- Avaliar e padronizar as diferentes alterações neuroquímicas, em parâmetros (1) mitocondriais, (2) relacionados à homeostase redox, e (3) às respostas inflamatória ao longo do tempo em camundongos submetidos a OBX
- Avaliar o potencial efeito antidepressivo da GUO no modelo da OBX em testes comportamentais para avaliação de parâmetros comportamentais relacionados especificamente com a função cognitiva.
- Investigar os a influência de diferentes parâmetros neuroquímicos envolvidos na resposta antidepressiva da GUO.

PARTE II

Nesta seção as metodologias e os resultados serão apresentados em capítulos, sendo estes compostos por artigos científicos. O Primeiro capítulo demonstra os resultados de um estudo experimental visando o entendimento dos mecanismos envolvidos na potencial ação ansiolítica da GUO. O Segundo capítulo, visa aumentar o entendimento sobre a fisiopatologia da TDM, avaliando a progressão das alterações cerebrais e comportamentais utilizando um modelo com elevado potencial translacional; no modelo da OBX. Por fim, o Terceiro capítulo desta tese visa a avaliação dos efeitos antidepressivos da GUO no modelo da OBX.

Capítulo I: *Guanosine Anxiolytic-Like Effect Involves Adenosinergic and Glutamatergic Neurotransmitter Systems*

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Guanosine Anxiolytic-Like Effect Involves Adenosinergic and Glutamatergic Neurotransmitter Systems

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Abstract Accumulating evidences indicate that endogenous modulators of excitatory synapses in the mammalian brain are potential targets for treating neuropsychiatric disorders. Indeed, glutamatergic and adenosinergic neurotransmissions were recently highlighted as potential targets for developing innovative anxiolytic drugs. Accordingly, it has been shown that guanine-based purines are able to modulate both adenosinergic and glutamatergic systems in mammalian central nervous system. Here, we aimed to investigate the potential anxiolytic-like effects of guanosine and its effects on the adenosinergic and glutamatergic systems. Acute/systemic guanosine administration (7.5 mg/kg) induced robust anxiolytic-like effects in three classical anxiety-related paradigms (elevated plus maze, light/dark box, and round open field tasks). These guanosine effects were correlated with an enhancement of adenosine and a decrement of glutamate levels in the cerebrospinal fluid. Additionally, pre-administration of caffeine (10 mg/kg), an unspecific adenosine receptors' antagonist, completely abolished the behavioral and partially prevented the neuromodulatory effects exerted by guanosine. Although the hippocampal glutamate uptake was not modulated by guanosine (both ex vivo and in vitro protocols), the synaptosomal K^+-stimulated glutamate release in vitro was decreased by guanosine (100 μM) and by the specific adenosine A_1 receptor agonist, 2-chloro-N^6-cyclopentyladenosine (CCPA, 100 nM). Moreover, the specific adenosine A_1 receptor antagonist 8-cyclopentyl-1,3-dipropylxanthine (DPCPX, 100 nM) fully reversed the inhibitory guanosine effect in the glutamate release. The pharmacological modulation of A_{2a} receptors has shown no effect in any of the evaluated parameters. In summary, the guanosine anxiolytic-like effects seem closely related to the modulation of adenosinergic (A_1 receptors) and glutamatergic systems.

Keywords Anxiety · Guanosine · Purines · Adenosine receptors · Glutamate · Glutamate release

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Introduction

Anxiety is a common mental disorder worldwide and is strongly associated with poor quality of life [1]. Despite advances in the elucidation of the pathophysiology of anxiety disorders, their causal factors, etiology, and mechanisms remain relatively poorly understood [2]. However, there is a consensus that the mechanisms involved in anxiety include multiple neurotransmitter systems, resulting in a wide range of pharmacological targets for treating anxiety disorders [3].

In addition to the classical pharmacological targets, the GABAergic [4] and serotoninergic [5] systems, particular emphasis has been placed on the development of novel drugs that focus on the glutamatergic and purinergic systems [6–8]. Glutamate (GLU) is the main excitatory neurotransmitter in the central nervous system (CNS) and acts through ionotropic and metabotropic receptors [9]. Changes in anxiety phenotypes

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have been observed after the administration of GLU receptor modulators [2, 3, 8, 10]. Moreover, glutamatergic neurotransmission is directly influenced by extracellular GLU levels, which are regulated by its release from neuronal pre-synaptic terminals and/or by its uptake mainly by astrocytic GLU transporters [8, 11]. Recently, the control of extracellular GLU levels has been proposed as a target for the development of novel anxiolytic drugs [2, 3, 8, 10].

Adenosine (ADO) neurotransmission has also been associated with anxiety-related responses [12] based on the modulatory effects of ADO on vesicular pre-synaptic GLU release [13]. ADO receptors have dual effects on GLU release: the activation of ADO A_1 receptors is associated with inhibition [13], whereas the activation of ADO A_{2A} receptors is more likely to facilitate GLU release [7, 14]. Therefore, anxiolytic/anxiety-related behaviors have been demonstrated by pharmacological and/or genetic manipulation of both receptors [7, 12, 15]. Moreover, although less abundant than A_1R, the ADO A_{2A}R as well as A_1R receptors play important roles in glutamatergic neurons of the hippocampus and other limbic structures, brain regions closely related to anxiety behavior, hence strengthening their potential as targets for treating anxiety [15].

Several findings support the hypothesis that guanine-based purines (GBPs) are able to modulate both adenosinergic and glutamatergic neurotransmission in vivo [16–18]. Indeed, the administration of GBPs, especially the nucleoside guanosine (GUO), counteracts the harmful effects of glutamatergic excitotoxicity in several experimental models [16–19]. Experimental evidence indicates that the neuroprotective effects exerted by GUO appear to be mediated by the stimulation of astrocytic GLU uptake, which clears excessive GLU from the synaptic cleft [20, 21] and keeps the extracellular GLU levels at physiological concentrations. Interestingly, at least some of the observed neuroprotective effects of GUO appear to be blocked by ADO receptor antagonists [17, 22]. However, the stimulation of astrocytic GLU uptake by GBPs is not influenced by caffeine (CAF), a nonspecific ADO receptor antagonist [20], suggesting additional mechanisms of action for GUO that involve both the adenosinergic and glutamatergic systems. Thus, to the best of our knowledge, the interplay between GUO and the adenosinergic and glutamatergic systems remains elusive.

Additionally, our group has already shown that systemic administration of guanosine-5′-monophosphate (GMP) induced anxiolytic-like behaviors in rats [6], and the induction of a similar effect by chronic GUO administration was also previously described in mice [23]. However, the mechanisms involved in these effects are still not clear. Thus, the present study aimed to investigate the anxiolytic potential of acute in vivo GUO administration in rats, with particular emphasis on mechanisms involving the adenosinergic and glutamatergic neurotransmitter systems. Here, the potential anxiolytic-like effect of GUO was evaluated in three anxiety-related paradigms: the elevated plus maze (EPM), and light/dark and round open field tasks. Additionally, ADO and GLU levels in cerebrospinal fluid (CSF), GLU uptake (ex vivo and in vitro protocols), and in vitro synaptosomal GLU release were also evaluated to investigate the putative molecular targets and signaling pathways recruited by GUO. The results presented here illustrate the involvement of the adenosinergic and glutamatergic systems on in vivo GUO anxiolytic-like behavior.

Materials and Methods

Animals

Male Wistar rats, 60–90 days old (250–300 g), were kept under a 12-h light/dark cycle (light on at 7:00 AM) at 22 ± 1 °C in plastic cages (5 per cage) with water and food available ad libitum. On the day of the behavioral tasks, they were acclimated to the behavioral room with appropriate lighting (25 lx) for 1 h before the behavioral procedures. The animals were maintained according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All protocols followed the Ethical Committee of the Federal University of Rio Grande do Sul (Project number 18236).

Chemicals

GMP, GUO, CAF, 2-chloro-N^6-cyclopentyladenosine (CCPA, an ADO A_1 receptor agonist), 2-*p*-(2-carboxyethyl) phenethylamino-5′-*N*-ethylcarboxamidoadenosine hydrochloride hydrate (CGS21680), 8-cyclopentyl-1,3-dipropylxanthine (DPCPX, an ADO A_1 receptor antagonist), and ZM241385 (an ADO A_{2A} receptor antagonist) were from Sigma Chemicals (St. Louis, MO, USA). L-[^{3}H] glutamate (specific activity of 50 Ci/mmol) was from Amersham International, UK. The anesthetic sodium thiopental was from Cristália (Itapira, SP, Brazil).

Drugs were dissolved in saline (0.9 % NaCl) and administered i.p. for in vivo evaluation. GUO and CAF were dissolved in Hank's buffered salt solution (HBSS) containing (in mM) the following: 137 NaCl, 0.63 Na_2HPO_4, 4.17 $NaHCO_3$, 5.36 KCl, 0.44 KH_2PO_4, 1.26 $CaCl_2$, 0.41 $MgSO_4$, 0.49 $MgCl_2$, and 5.5 glucose (pH = 7.4) for in vitro GLU uptake and release assays. CCPA, CGS21680, DPCPX, and ZM241385 were dissolved with DMSO (0.1 %) in HBSS.

For the Western blotting assay, bovine serum albumin, a protease and phosphatase inhibitor cocktail, and antibodies against glial fibrillary acid protein (GFAP) and synaptosomal-associated protein 25 (SNAP 25) were from Sigma Chemicals. The antibody against the *N*-methyl-D-aspartic receptor subunit (NR1) was from Chemicon; the antibody against postsynaptic density protein 95 (PSD 95) was

from Affinity BioReagents; the antibodies against excitatory amino acid carrier 1 (EAAC1) and glutamate transporter-1 (GLT-1) were from Alpha Diagnostic; the antibody against 2-amino-3-(5-methyl-3-oxo-1,2-oxazol-4-yl) propanoic acid (AMPA) receptor subunit 1 (GluA1; AMPA receptor subunit) was from UpState; and the antibodies against monoamine oxidase A (MAO A), β-tubulin III, and synaptobrevin (VAMP) were from Santa Cruz Biotechnology.

Drug Administration

The experimental design and drug administration schedules are shown in Fig. 1.

Protocol I

As depicted in Fig. 1—protocol IA, the rats were divided into five groups: saline, 50 mg/kg GMP (positive control), and 7.5, 30, or 60 mg/kg GUO. GMP 50 mg/kg was used as a positive control for anxiolytic-like effects [6]. The GUO doses were carefully chosen based on previously studies from our group, which demonstrated important neuromodulatory effects of GUO in different in vivo protocols [18, 19]. Sixty minutes after intraperitoneal (i.p.) administration of these compounds, the animals were subjected to an EPM task. After the behavioral procedure, CSF was immediately collected for purine content evaluation.

After assessing the potential anxiolytic GUO dose in the EPM, a well-established and widely used task to detect anxiolytic/anxiety-like phenotypes in rodents, the anxiolytic-like effect of 7.5 mg/kg GUO was also evaluated in two other anxiety-related paradigms, the light/dark and the round open field tasks (Fig. 1—protocol 1B). The rats were divided into two groups: saline or 7.5 mg/kg GUO. The same schedule of administration was maintained as previously mentioned.

Protocol II

As depicted in Fig. 1, the rats were divided into four groups (saline/saline, 10 mg/kg CAF/saline, saline/7.5 mg/kg GUO, and 10 mg/kg CAF/7.5 mg/kg GUO). The CAF dose was chosen based on the literature [24] and by preliminary pilot experiments (Material Supplementary 1). Pre-administration of saline or CAF i.p. was performed 15 min before i.p. administration of saline or GUO. Sixty minutes after the last administration, the EPM task was performed. It is important to highlight that 10 mg/kg CAF per se did not affect the anxiety-related behavior assessed by the EPM task. On the other hand, this CAF dose modulated rats' performance on the light/dark and round open field tasks (Material Supplementary 1), demonstrating that under different stimulus, CAF 10 mg/kg can act as a psychostimulant.

Immediately after the behavioral task, CSF was collected for the measurement of purines and GLU content and the brain was immediately processed for GLU uptake.

EPM Task

The EPM task was performed as previously described [25]. The apparatus consisted of two open (50 × 10 cm, length × width) and two enclosed (50 × 10 × 40 cm, length × width × height) arms that were separated by a central platform (5 × 5 cm); the height of the maze was 70 cm. The animal's behavior was recorded for 5 min using ANY-Maze software. The percentage of time spent in the open arms, the total distance travelled in open arms (cm), and the total

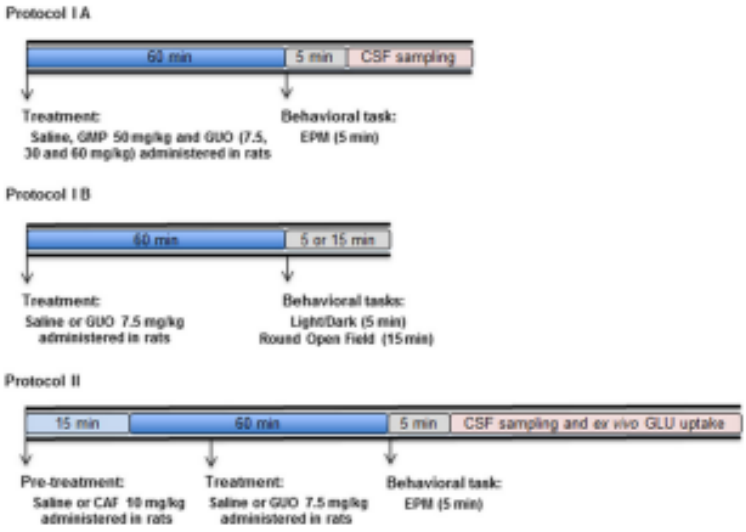

Fig. 1 Experimental design and drug administration. Schematic representation of the experimental protocols, including the drug administrations, behavioral tasks, and neurochemical analysis performed, for protocol I (A and B) and protocol II

distance travelled (cm) were determined. When administration of a drug significantly increased the first two behavioral parameters without changing the total number of transitions, the drug was considered anxiolytic.

Light/Dark Task

The light/dark task was performed as previously described [26]. The apparatus consisted of a rectangular acrylic box with two separated compartments. One compartment had black walls and a black floor (without illumination) with a size of 21 × 35 × 41 cm (height × length × width). The other had white walls and a white floor, with a size of 21 × 45 × 41 cm (height × length × width, illuminated by a 100-W lamp placed 45 cm above the center of the box). An 8 × 5 cm (height × length) opening joined both compartments. Each rat was placed in the light compartment facing away from the opening and allowed to explore the box for 5 min. The following behavioral parameters were analyzed by ANY-Maze software: the number of transitions between compartments and the time spent in the light compartment. When the administration of a drug increased these parameters, the drug was considered anxiolytic.

Round Open Field Task

The rats were placed in the center of a round open field apparatus (60 cm in diameter) and let to freely explore the arena for 15 min, as previously described with minor modifications [27]. Total locomotor activity, distance travelled in the center zone, and total time spent in the center zone were recorded for each animal using ANY-Maze software. The treatment was considered anxiolytic when it increases the time spent or the distance travelled in the center zone, in accordance with previous studies [27].

CSF Analysis

Preliminarily, two CSF sampling protocols were tested in order to avoid purine degradation (Supplementary Material 2). The following methodology (method II) showed to be more reliable and it was used in the experiments. The CSF was collected immediately after the EPM task to evaluate purines and GLU levels. The rats were anesthetized with sodium thiopental (40 mg/kg, i.p.) and placed in a stereotaxic apparatus. The CSF (100/150 μL) was collected by direct puncture of the cisterna magna with an insulin syringe (27 gauge × 0.5 in. in length). Samples with visible blood contamination were discarded. All samples were centrifuged at 10,000*g* at 4 °C for 10 min in an Eppendorf® centrifuge to obtain cell-free supernatants. After the centrifugation, the samples were immediately frozen in dry ice and stored at −80 °C until analysis.

High-Performance Liquid Chromatography

The high-performance liquid chromatography (HPLC) procedures were performed with cell-free CSF aliquots in a Shimadzu Class-VP chromatography system. The system consisted of a quaternary gradient pump with vacuum degassing and piston desalting modules, a Shimadzu SIL-10AF auto injector valve with 50 μL loop and UV and fluorescence detectors, which were used to detect purines and GLU, respectively (Shimadzu, Kyoto, Japan).

The HPLC for purine analysis was performed as previously described [16, 28]. Briefly, the levels of the following purines were determined: ADO, GMP, GUO, inosine (INO), hypoxanthine (HYPOX), xanthine (XANT), and uric acid (UA). The mobile phase flow rate was 1.2 mL/min, and the column temperature was 24 °C. The buffer compositions were unchanged (A: 150 mmol/L phosphate buffer, pH 6.0, containing 150 mmol/L KCl; B: the same buffer with 15 % acetonitrile). The gradient profile was modified according to the content of buffer B in the mobile phase: 0 % at 0.00 min, 2 % at 0.05 min, 7 % at 2.45 min, 50 % at 10.00 min, 100 % at 11.00 min, and 0 % at 12.40 min. Samples of 25 μL were injected. Absorbance was read at 254 nm in a UV detector.

The HPLC for GLU analysis was performed as previously described [29]. The samples were derivatized with *o*-phthalaldehyde, the mobile phase flow rate was 1.4 mL/min, and the column temperature was 24 °C. The buffer compositions were as follows: A: 0.04 mol/L sodium phosphate buffer, pH 5.5, containing 20 % methanol; and B: 0.01 mol/L sodium dihydrogen phosphate monohydrate buffer, pH 5.5, containing 80 % methanol. The gradient profile was modified according to the content of buffer B in the mobile phase: 0 % at 0.00 min, 25 % at 13.75 min, 100 % at 15.00–20.00 min, and 0 % at 20.01–25.00 min. Absorbance values were measured at 360 nm excitation and 455 nm emission in a fluorescence detector. Samples of 50 μL were injected.

The CSF levels of the purines and GLU were expressed in micromolar (as the mean ± S.E.M.).

Ex Vivo Na^+-Dependent Hippocampal GLU Uptake

Immediately after the EPM task (protocol II), the brain was processed for an ex vivo GLU uptake assay as previously described [30]. From each brain, hippocampal slices of 400 μm were obtained using a McIlwain chopper and individually placed into 24-well plates containing HBSS at 37 °C. The slices were washed once with 1 mL of 37 °C HBSS and then pre-incubated at 37 °C for 15 min. The incubation was started by the addition of 0.33 Ci/mL L-[^{3}H]GLU plus 100 μM (final concentration) GLU and stopped after 5 min with two ice-cold washes with 1 mL of HBSS. The washes were immediately followed by the addition of 0.5 N NaOH. Na^+-independent uptake was measured using the same

protocol, with modifications in the temperature (4 °C) and medium composition (choline chloride instead of sodium chloride). Na^+-dependent uptake was defined as the difference between both uptakes. The incorporated radioactivity was measured in a Hidex 300 SL scintillation counter.

Hippocampal Synaptosomal Preparation

Synaptosomes were prepared from the hippocampus of animals that were not subjected to any treatment or behavioral task according previously published procedures [31]. The hippocampus of each rat was manually homogenized (small capacity Teflon/glass homogenizer in 10 × mL/g) in 10 mM Tris buffer (pH 7.4) with 0.32 M sucrose, 1 mM EDTA, and 0.25 mM dithiothreitol (DTT). The homogenate (H) was centrifuged in microfuge tubes (1.5 mL per tube) at 1000×*g* for 10 min at 4 °C, using a fixed-angle rotor. The resulting supernatant (S1) was centrifuged at 11,000×*g* for 20 min at 4 °C to obtain a synaptosomal-enriched pellet (SP), which was washed twice with HBSS (pH 7.4) by centrifugation at 16,000×*g* for 10 min at 4 °C to remove excess sucrose. The final pellet was resuspended in HBSS buffer (900 μL).

To characterize the quality of the three different stages of the preparation of hippocampal synaptosomes (H, S1, and SP), we performed Western blot analysis to evaluate the immunocontent of neuronal and glial proteins, evaluating the enrichment of each protein in the SP. The following proteins were investigated: β Tub III for the neuronal cytoskeleton; SNAP 25 and VAMP for synaptic vesicles; PSD 95 for postsynaptic densities; EAAC1, NR1, and GLUA1 for neuronal plasma membranes; MAO A for mitochondrial membranes; and GFAP and GLT-1 for astrocytes (Supplementary Material 3).

In Vitro L-[^{3}H] GLU Uptake by Hippocampal Synaptosomal Preparations

Five minutes prior to the measurement of GLU uptake in the SP, 100 μM GUO was added to the incubation medium. Then, 1 μCi/mL L-[^{3}H]GLU plus 50 μM (final concentration) GLU was added, and the mixture was incubated for 15 min at 37 °C. The reaction was terminated by four washes of ice-cold HBSS through centrifugation at 14,000×*g* for 1 min at 4 °C. The final pellet was resuspended in HBSS, and an aliquot was separated. The radioactivity of this aliquot was determined using a Hidex 300 SL scintillation radioactivity counter.

In Vitro L-[^{3}H] GLU Release from Hippocampal Synaptosomal Preparations

L-[^{3}H] GLU release was measured according to [31], with minor modifications. First, the SP was loaded with L-[^{3}H] GLU using the in vitro L-[^{3}H] GLU uptake assay protocol (described above), without GUO in the incubation medium. Basal L-[^{3}H] GLU release was initiated by the addition of aliquots of loaded synaptosomes in HBSS buffer at 37 °C for 1 min and terminated by immediate centrifugation (14,000×*g* for 1 min at 4 °C). The percentage of previously loaded radioactivity present in the supernatant was considered the amount of GLU released. K^+-stimulated L-[^{3}H]GLU release was assayed as described for basal release, except that the incubation medium contained 40 mM KCl (NaCl decreased accordingly) to induce synaptosomal depolarization. The K^+-stimulated GLU release was calculated as the delta (Δ) between both GLU release activities. GLU release was increased by approximately 70 % by high K^+, indicating the viability of our preparations, in comparison between the nonstimulated L-[^{3}H]GLU release and the K^+-stimulated L-[^{3}H]GLU release (Fig. 6c, d).

To determine the effects of different drugs on GLU release, the final incubation medium (for basal or stimulated GLU release) contained GUO (100 μM), CCPA (100 nM), CGS21680 (30 nM), CAF (1 μM), DPCPX (100 nM), ZM241385 (50 nM), or one of the following combinations: CCPA/GUO, CGS21680/GUO, CAF/GUO, DPCPX/GUO, or ZM241385/GUO. The concentrations of CAF, CCPA, CGS21680, and ZM241385 were chosen based on literature [17, 32, 33], and the concentration of DPCPX was chosen based on our previous data (Supplementary Material 4). Radioactivity was separately determined for supernatants and pellets using a Hidex 300 SL scintillation counter.

Protein Determination

Protein content was measured using the BCA® protein assay kit with bovine serum albumin as a standard.

Statistical Analysis

One-way analysis of variance (ANOVA) followed by Tukey's post hoc test was used to analyze the effects of GUO on the EPM task. Student's *t* test was used to evaluate the effects of GUO on the light/dark and round open field tasks, on CSF purine concentrations, and on both ex vivo and in vitro GLU uptake and to compare basal versus K^+-stimulated L-[^{3}H]GLU release from the SP. Correlations between EPM behavioral parameters and ADO or GLU CSF levels were analyzed by Pearson's correlation. Two-way ANOVA followed by Bonferroni's post hoc test was used to analyze the influence of CAF pre-administration on the GUO effect, on the EPM task, and on ADO and GLU CSF levels [factors: (1) pre-administration with saline or CAF and (2) administration with saline or GUO] and to analyze

the effects of GUO, CCPA, CGS21680, CAF, DPCPX, and ZM241385 on the effect of GUO on synaptosomal GLU release [factors: (1) incubation with CCPA, CGS21680, CAF, DPCPX, or ZM241385 and (2) incubation with GUO]. Differences were considered statistically significant at $p < 0.05$.

Results

Systemic GUO Administration Induced Anxiolytic-Like Effects and Increased CSF ADO Levels

GMP (50 mg/kg) and GUO (7.5 mg/kg) presented anxiolytic-like effects in the EPM task by significantly enhancing the percentage of the time spent in the open arms (Fig. 2a, $^{**}p = 0.0029$ and $^{**}p = 0.0025$, respectively) and the open arms distance travelled (Fig. 2b, $^{*}p = 0.0130$ and $^{*}p = 0.0160$, respectively), without significantly affecting total distance travelled (Fig. 2c); higher doses of GUO had no anxiolytic-like effect. Based on these results, only 7.5 mg/kg GUO was used in subsequent experiments. In the light/dark task, 7.5 mg/kg GUO presented anxiolytic-like effects by significantly increasing the number of transitions between the compartments (Fig. 3a, $^{*}p = 0.0395$) and the time spent in the light compartment (Fig. 3b, $^{*}p = 0.0086$). In the round open field task, 7.5 mg/kg GUO presented an anxiolytic-like effect by significantly enhancing the time spent in the center zone (Fig. 3d, $^{*}p = 0.0278$), without affecting the total distance travelled or the distance travelled in the center zone (Figs. 3c and 4e, $p = 0.5342$ and $p = 0.0698$, respectively).

The administration of 7.5 mg/kg GUO simultaneously exerted an anxiolytic effect (Fig. 2) in the EPM task and increased the CSF levels of ADO (Fig. 5a, $^{**}p = 0.0041$) without affecting the levels of other purines (INO, GUO, HYPOX, XANT, and UA—Supplementary Material 5 A and B).

The Anxiolytic-Like Effects Promoted by Systemic GUO Administration Were Completely Prevented by Pre-administration of CAF, a Nonspecific ADO Receptor Antagonist

CAF (10 mg/kg) administration per se did not modulate any anxiety-like behavioral parameter in the EPM task (Fig. 4 and Supplementary Material 1). On the other hand, GUO 7.5 mg/kg, again, increases the percentage of time spent in the open arms (Fig. 4a, $^{**}p = 0.0010$) and in open arms distance travelled (Fig. 4b, $^{*}p = 0.0425$) in the EPM task. Additionally, the effect of GUO on the percentage of time spent in the open arms was completely abolished by i.p. pre-administration of 10 mg/kg CAF (Fig. 4a, $^{**}p = 0.0078$). The total distance travelled was not affected by any drug administration (Fig. 4c).

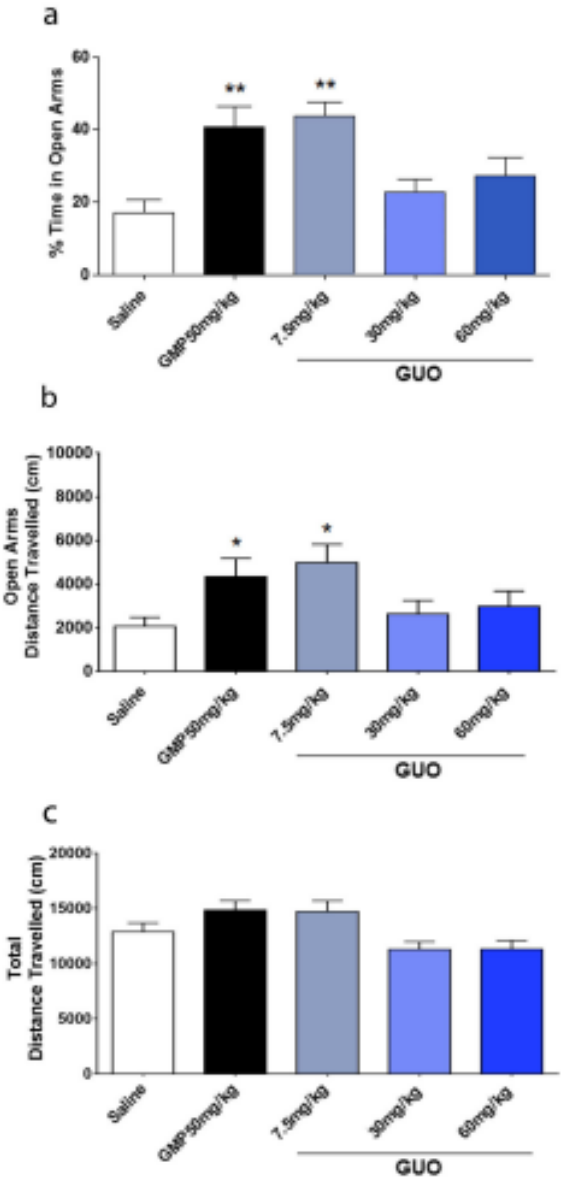

Fig. 2 Systemic administration of GUO-induced anxiolytic-like behavior in the EPM task. The percentage of time spent in the open arms (**a**), the total distance travelled in open arms (cm) (**b**), and the total distance travelled (cm) (**c**) were evaluated in the EPM task 60 min after i.p. administration of saline, 50 mg/kg GMP or 7.5, 30, or 60 mg/kg GUO. Data are reported as the mean ± S.E.M. and were analyzed by one-way ANOVA followed by Tukey's multiple comparisons test ($n = 10$ animals/group). $^{*}p < 0.05$ and $^{**}p < 0.01$ compared to the saline group

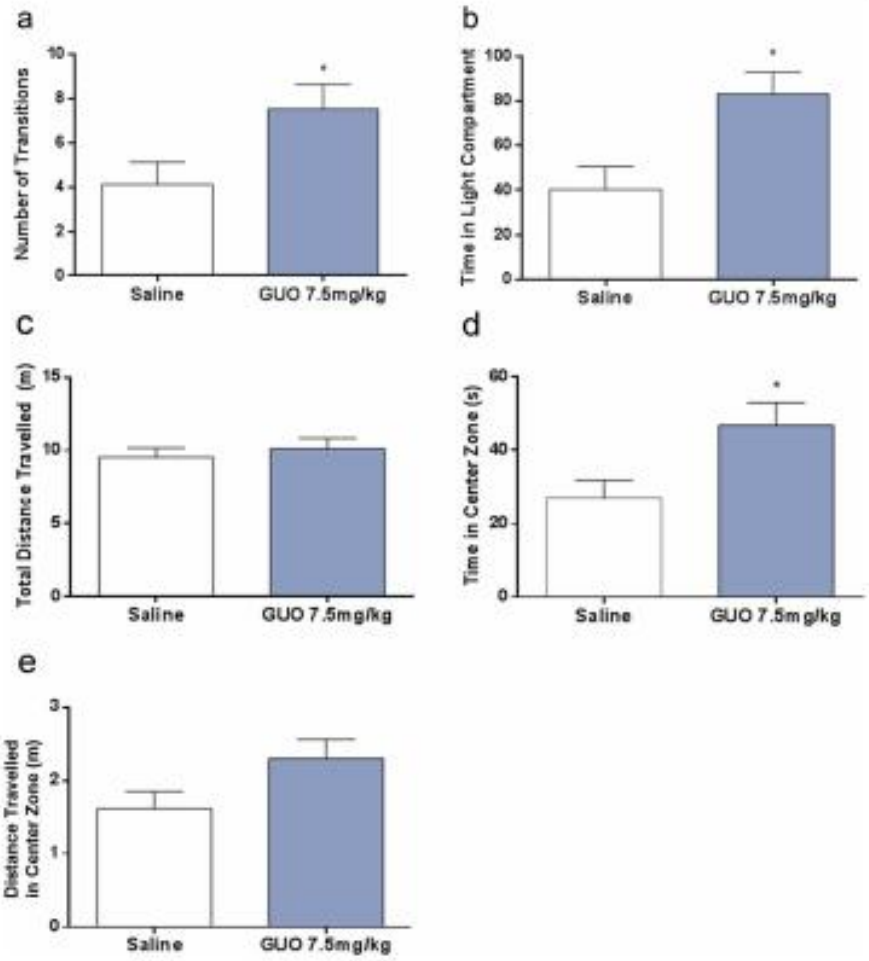

Fig. 3 Systemic administration of GUO-induced anxiolytic-like behavior in the light/dark task and in the round open field task. The number of transitions (**a**) and the time spent in the light compartment (**b**) were evaluated in the light/dark task 60 min after i.p. saline or 7.5 mg/kg GUO administration. The total distance travelled (**c**), the time spent in the center zone (**d**), and the distance travelled in center zone (**e**) were evaluated in the round open field task 60 min after i.p. saline or 7.5 mg/kg GUO administration. Data are reported as the mean ± S.E.M. and were analyzed by unpaired Student's *t* test. ($n = 12$ animals/group). $^{*}p < 0.05$ compared to the saline group

The Increase in CSF ADO Levels Promoted by GUO Administration Was Partially Prevented by CAF Pre-administration

CAF (10 mg/kg) did not present any effect per se on CSF ADO levels. However, GUO 7.5 mg/kg effect on increase CSF ADO levels (Fig. 5b, $^{***}p = 0.0001$) was partially prevented by pre-administration of CAF. A positive correlation between ADO levels and the percentage of time spent in the open arms in the EPM task can be observed regardless of CAF pre-treatment (Fig. 5c, $r^2 = 0.3228$, $^{**}p = 0.0016$), as well as between ADO levels and the open arm distance travelled (Fig. 5d, $r^2 = 0.2021$, $^{*}p < 0.0377$), without any correlation with total distance travelled in the EPM task ($r^2 = 0.1903$, $p = 0.5016$).

Interestingly, the animals that received CAF showed an increase in CSF levels of XAN and UA regardless of posterior administration of Sal or GUO (Supplementary Material 5 C and D). Accordingly, it is noteworthy that XAN and UA are products of CAF metabolism [34], which corroborates to the accuracy of our analysis.

Systemic GUO Administration Decreased CSF GLU Levels, an Effect that Was Partially Prevented by CAF Pre-administration

CSF GLU levels significantly decreased following systemic i.p. administration of 7.5 mg/kg Sal/GUO (Fig. 5e, $^{***}p = 0.0354$). CAF per se did not change significantly but partially blocked GUO effect on CSF GLU levels (Fig. 5e, $p = 0.9891$ and $p = 0.4345$, respectively). As opposed to CSF ADO levels, a negative correlation between CSF GLU levels and the percentage of time spent in the open arms in the EPM task (Fig. 5f, $r^2 = 0.2977$, $^{*}p = 0.0235$) could be observed. No significant correlations were observed with the open arm distance travelled ($r^2 = 0.1637$, $p = 0.2170$) or with the total distance travelled ($r^2 = 0.01949$, $p = 0.6652$) in the EPM task.

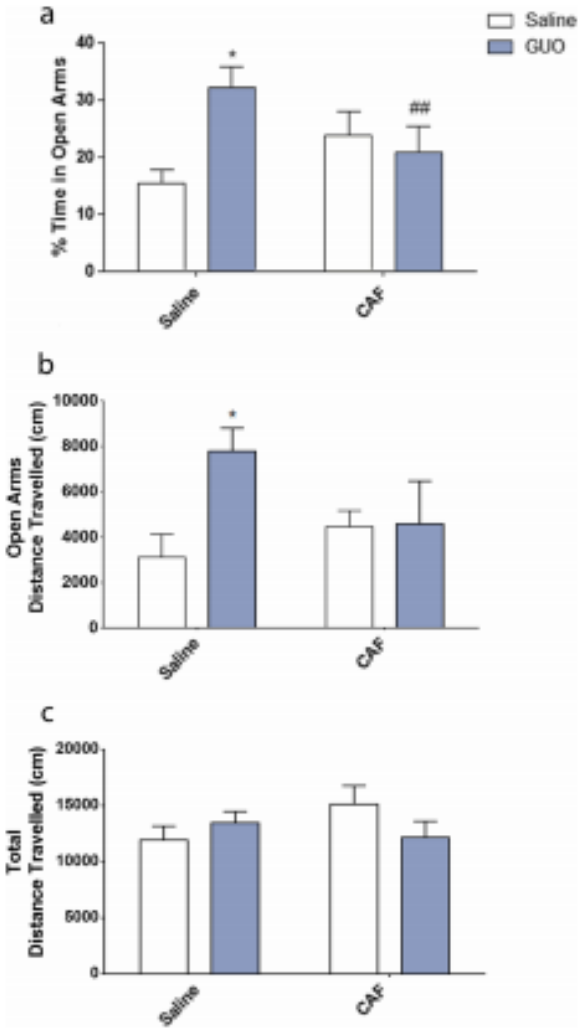

Fig. 4 Systemic CAF pre-administration inhibited the anxiolytic-like effects of GUO. The percentage of time spent in the open arms (**a**), the total distance travelled in open arms (cm) (**b**), and the total distance travelled (cm) (**c**) were evaluated in the EPM task 60 min after i.p. saline or 7.5 mg/kg GUO administration, which was preceded by 15 min of i.p. saline or 10 mg/kg CAF pre-administration. Data are reported as the mean ± S.E.M., and differences among groups were determined by two-way ANOVA followed by Bonferroni's post hoc test when applicable ($n = 12$ animals/group). $^{*}p < 0.05$ compared to the saline/saline group and $^{**}p < 0.01$ compared to the saline/GUO group

Ex Vivo and In Vitro Approaches to Investigate Putative GUO Mechanisms of Action

Considering that extracellular GLU levels are modulated by cellular uptake/release, we performed ex vivo GLU uptake assays in hippocampal slices, and no significant effect of in vivo GUO administration was observed (Fig. 6a, $p = 0.9933$). Thus, we further investigated the putative mechanisms of GUO action in vitro, using hippocampal SP preparations from naïve rats (not subjected to any behavioral task). The SP preparation was greatly enriched in synaptic components (Supplementary Material 3) and was sensitive to high K^+. GLU uptake by the hippocampal synaptosomal preparations (Fig. 6b, $p = 0.7212$) was not influenced by GUO. Interestingly, although basal GLU release was not affected by GUO (Fig. 6c, $p = 0.999$), K^+-stimulated GLU release was significantly decreased by 100 μM GUO in vitro (Fig. 6d, $^{***}p < 0.001$).

To examine the putative involvement of ADO receptors in the GUO effect on K+-stimulated GLU release, ADO receptor modulators were used, to identify putative adenosinergic involvement in the GUO effects. Basal GLU release was not affected by GUO, the ADO A_1 receptor agonist CCPA (100 nM), the ADO A_{2A} receptor agonist CGS21680 (30 nM), the nonspecific ADO receptor antagonist CAF (1 μM), the ADO A_1 receptor antagonist DPCPX (100 nM), the ADO A_{2A} receptor antagonist ZM241385 (50 nM), or their combinations with GUO (Fig. 6c). However, although none of the ADO receptor antagonists affected K^+-stimulated GLU release, the effect of GUO on reducing K^+-stimulated GLU release was not prevented by the A_{2A} antagonist ZM241385 (Fig. 6d, $^{**}p = 0.0076$), while CAF partially prevented (Fig. 6d, $p = 0.089$) and DPCPX totally prevented (Fig. 6d, $^{*}p = 0.013$) the GUO-mediated reduction in K^+-stimulated GLU release. Accordingly, the ADO A_1 receptor agonist CCPA also significantly decreased K^+-stimulated GLU release (Fig. 6d, $^{**}p = 0.008$) and the ADO A_{2A} receptor agonist CGS21680 did not affect the GUO effect (Fig. 6d, $p = 0.460$). Finally, the combination of GUO plus CCPA or CGS21680 continued to hold the decrease in glutamate release (Fig. 6d, $^{*}p = 0.0101$, $^{**}p = 0.0018$, respectively).

Discussion

Acute/systemic GUO administration in rats induced anxiolytic-like effects in three different anxiety paradigms; these effects were correlated with the increase of ADO nucleoside levels and with the decrease of GLU levels in CSF. Additionally, these GUO effects were prevented in vivo by CAF pre-administration. Interestingly, in hippocampal synaptosomal preparations, GUO and the ADO A_1 receptor agonist CCPA decreased K^+-stimulated GLU release, while the ADO A_1 receptor antagonist DPCPX and the nonspecific ADO receptor antagonist CAF reversed the GUO effect. Here, we provided experimental evidence in support to a new GUO mechanism of action involving the adenosinergic and glutamatergic systems, which seems to be closely related to the anxiolytic-like effects observed in vivo.

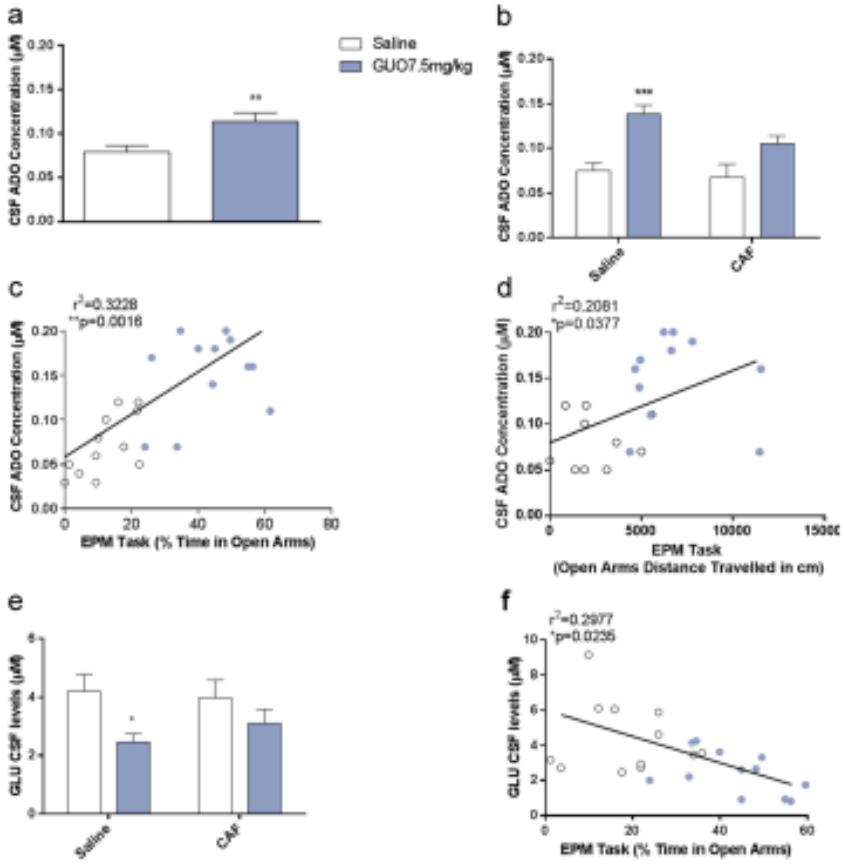

Fig. 5 CAF prevented the increase of ADO levels and the decrease of GLU levels on CSF of rats following systemic GUO administration induced anxiolytic-like effects. ADO levels (**a**) were measured on CSF collected immediately after EPM task performance from rats that received i.p. saline or 7.5 mg/kg GUO administration. Data are reported as the mean ± S.E.M. and were analyzed by unpaired Student's *t* test. ADO levels (**b**) and GLU levels (**e**) were measured on CSF collected immediately after EPM task performance from rats that received i.p. saline or 7.5 mg/kg GUO administration preceded by i.p. saline or 10 mg/kg CAF pre-administration. Data are reported as the mean ± S.E.M., and differences among groups were determined by two-way ANOVA followed by Bonferroni's post hoc test when applicable. Linear correlations between the percentage of time spent in the open arms or the total distance travelled in open arms (cm) in the EPM task and ADO CSF levels, and the percentage of time spent in the open arms in the EPM task and GLU CSF levels in the animals that received saline or GUO are presented in (**c**, **d**, and **f**, respectively). *Each point* represents one animal. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$, compared to the saline group ($n = 10$–12 animals/group)

Anxiolytic Potential of GUO Administration

The EPM is currently the first-choice task for screening anxiolytic/anxiety-like behavior. Here, in the EPM task, acute administration of only the lower dose of GUO produced an anxiolytic-like behavior. The U-shape dose curve response for GUO was similar to that previously reported for the anxiolytic-like behavior promoted by GMP [6]. However, comparing the effective anxiolytic dosages, GMP showed anxiolytic-like effects in a higher dose than GUO (GMP at 50 mg/kg and GUO at 7.5 mg/kg), which suggests that GUO is more potent than GMP. In addition, the anxiolytic potential of GUO was further demonstrated in the light/dark and round open field tasks. Thus, pharmacologically, all three paradigms presented reasonable sensitivity to 7.5 mg/kg GUO.

To the best of our knowledge, this is the first study to evaluate the potential anxiolytic effects of acute/systemic GUO administration. Importantly, it was previously demonstrated that chronic oral administration of GUO exerted anxiolytic-like effects in mice [23]. Regarding depression, for which the main comorbidity is anxiety, the administration of GMP or GUO also produces antidepressive-like effects in predictive tasks [35, 36]. It has been shown that some of the behavioral effects of GMP, such as its anticonvulsant and antinociceptive

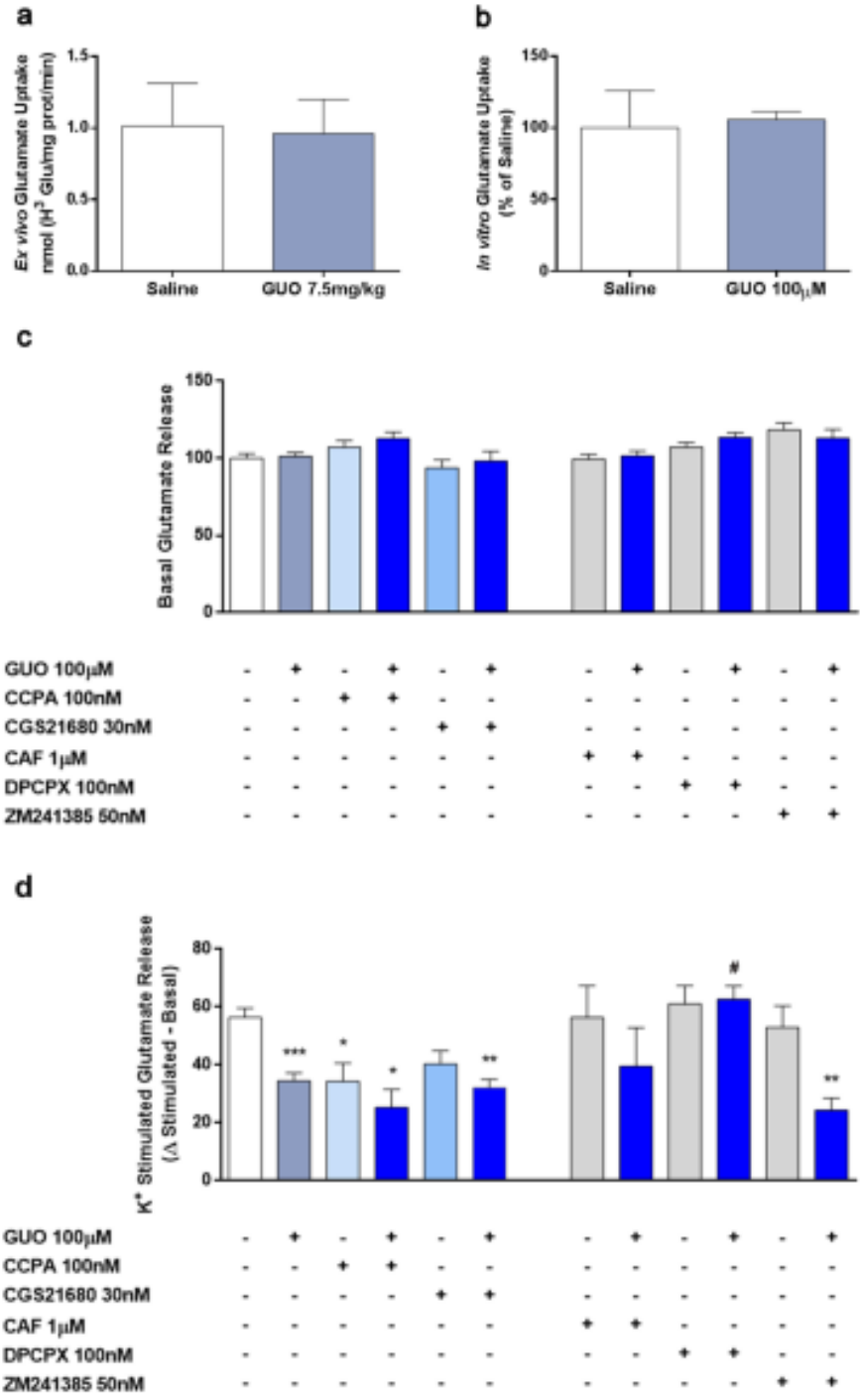

Fig. 6 In vitro GUO incubation decreased the release of GLU from hippocampal synaptosomal preparations, without any change in GLU uptake. Ex vivo hippocampal GLU uptake was evaluated 60 min after saline or GUO administration (a) (n = 12 animals/group), and in vitro hippocampal synaptosomal GLU uptake was evaluated 5 min after the synaptosomal incubation with 100 μM GUO (b) (n = 5 animals/group). Both methods were described in the "Materials and Methods" section. Data were compared by unpaired Student's t test. In vitro synaptosomal differences between the treatments on non-stimulated L-[3H]GLU release (basal) were measured in c), and in vitro K +-stimulated GLU release after 1 min of synaptosomal depolarization were evaluated in d, as described in the "Materials and Methods" section. The different treatments tested were GUO 100 μM, the ADO receptor agonists (CCPA 100 nM and CGS21680 30 nM), the ADO antagonists (CAF 1 μM, DPCPX 100 nM and ZM241385 50 nM) with or without GUO 100 μM. Data are reported as the mean ± S.E.M., and differences among groups were determined by two-way ANOVA followed by Bonferroni's post hoc test. (n = 25 animals). *p<0.05, **p<0.01 and ***p<0.001 compared to the saline group

effects, depend on the enzymatic conversion of GMP to GUO [37]. Although similarities exist, it remains to be investigated whether the mechanisms underlying the anxiolytic-like effects induced by GMP depend on its conversion to GUO. In fact, this topic is currently under investigation in our laboratory.

Interplay Between the Anxiolytic Effects of GUO and the ADO System

It has been previously demonstrated that CSF GUO levels increased 30 min after acute/systemic administration of 7.5 mg/kg GUO in rats [37]. By contrast, here, we did not observe any modulation in CSF GUO levels 60 min after systemic GUO administration. However, it has also been shown that GUO levels are increased in brain tissue homogenates 60 min after i.p. administration of GUO [38], suggesting that GUO uptake by neural cells could be at least partially involved in the decrease in GUO CSF levels, which could explain the unaltered CSF GUO levels observed in our study.

Additionally, we showed an increase in ADO CSF levels after GUO administration, and this increase was correlated with the anxiolytic parameters evaluated in the EPM task. The modulation of CSF ADO by GUO seems to occur later than 30 min after the GUO administration, as it cannot be observed earlier (30 min after systemic GUO 7.5 mg/kg administration) [37]. Intriguingly, our in vivo results are consistent with those of in vitro studies, in which GUO stimulates extracellular ADO enhancement in astrocytes and in other types of cells in culture [39, 40]. Furthermore, recently reported evidence indicates that the effect of GUO on regulating extracellular ADO levels may be a result of reducing its uptake by uncharacterized specific transporters [40, 41]. Together, all of these data suggest a close association between extracellular levels of GUO and ADO.

Interestingly, the increase in CSF ADO levels and the correlated anxiolytic-like effect promoted by GUO were sensitive to CAF. The literature supports the hypothesis that GUO presents effects that are either dependent on or independent of ADO receptors. In fact, using distinct CAF pre-administration doses, different results were obtained; in vivo, CAF pre-administration did not prevent the anticonvulsant (at a dose of 30 mg/kg) [42] and amnesic (at a dose of 5 mg/kg) effects of 7.5 mg/kg GUO [23] but prevented the antinociceptive effect (at a dose of 10 mg/kg) induced by 7.5 mg/kg GUO [16]. In vitro, some of the neural effects produced by exogenous GUO are prevented by ADO receptor antagonists, such as mitogenic activity, which could be partially inhibited by ADO A_1 and A_{2b} receptor antagonists [39], whereas other effects do not involve adenosinergic neurotransmission, such as the stimulatory effect on in vitro astrocytic GLU uptake activity. What seems clear is that GUO does not bind to ADO receptors, as shown in a preliminary study in which the binding of GUO to its putative G-protein receptor did not appear to be significantly sensitive to adenosinergic modulator compounds, including CAF [43].

Interactions Between the Anxiolytic Effects of GUO and Glutamatergic Neurotransmission

Acute systemic administration of GUO significantly decreased CSF GLU levels, an effect that was correlated with its anxiolytic-related behavior, as demonstrated by the increase in the percentage of time spent in the open arms of the EPM task. Previously, our group demonstrated that chronic systemic GUO administration prevented an increase in CSF GLU concentration in rats subjected to a chronic hepatic encephalopathy model [44]. Moreover, our group also showed that acute systemic administration of GUO prevented an increase in CSF levels of excitatory amino acids (including GLU) in the CSF of mice subjected to hyperalgesia induced by MK-801 [45]. Here, we provided the first report of a direct effect of systemic administration of GUO on CSF GLU levels in rats subjected to an anxiety-related paradigm.

No changes were observed in the GLU uptake activity induced by GUO in either the ex vivo (brain slices) or in vitro (synaptosomal preparations) protocols. These data indicated that there is at least one additional mechanism by which extracellular GLU levels are regulated by GUO in basal conditions. In this context, CAF pre-administration partially blocked the decrease in CSF GLU levels promoted by GUO, suggesting that the adenosinergic system is involved in the effects of GUO under physiological conditions.

In Vitro Effects of GUO on Hippocampal GLU Release

In vitro GUO significantly decreased K^+-stimulated GLU release in the hippocampal synaptosomal preparation, without affecting basal GLU release. Our data is in accordance with GUO effect on preventing extracellular GLU accumulation induced by excitotoxic conditions in a more complex system, i.e., hippocampal slices [46]. In fact, decreasing hippocampal GLU neurotransmission is a feasible mechanism of inducing anxiolytic-like effects [3] because the hippocampus is part of the limbic system, which is linked to emotional behaviors, and GLU is one of the neurotransmitters that is intimately responsible for exciting the limbic pathway during anxiety-related behaviors [47].

Notably, the GUO effect on the K^+-stimulated GLU release was similar to that of the ADO A_1 receptor agonist CCPA. Moreover, this GUO effect was completely blocked by an ADO A_1 receptor antagonist but not by an ADO A_{2a} antagonist. These data suggest that by inhibiting pre-synaptic GLU release through ADO A_1 receptor activation, GUO could decrease extracellular GLU levels. Interesting, ADO A_{2a} receptors seem to play a role in GUO stimulatory effect on glutamate uptake in excitotoxic conditions [17], corroborating with GUO effect on the interplay between adenosinergic and glutamatergic system.

Recently, it was also showed that GUO recovered the impairment caused by oxygen glucose deprivation in hippocampal slices through stimulation of glutamate uptake by a pathway that, among others, involves antagonism of ADO A_{2a} receptors [17]. Here we did not observe any counteractive effect of CGS21680, a specific ADO A_{2a} receptor agonist, on GUO effect. Collectively, these data suggest that the modulatory effects of GUO on different parameters of the glutamatergic neurotransmission through the adenosinergic system are regulated by different mechanisms, especially in comparison between physiologic and excitotoxic conditions.

In agreement with this hypothesis, the following should be highlighted: (i) an increase in endogenous ADO is capable of increasing the activation of ADO A_1 receptors [48]; (ii) ADO A_1 receptors are the most abundant of the four known ADO receptors in the brain [49], including in the hippocampus; and (iii) in some regions of the brain, one of the physiological roles of ADO is to inhibit GLU release via pre-synaptic ADO A_1 receptors, which consequently decreases neuronal excitability [50, 51]. Interestingly, there are other reports specifically related to the involvement of ADO A_1 receptors that are also consistent with the GUO effects described here: (i) the blockade of ADO A_1 receptors induces an anxiety phenotype in zebrafish [52], an effect opposite to the effect of GUO administration specified here; and (ii) the activation of ADO A_1 receptors in rats leads to memory impairment [53], an effect similar to that promoted by GUO [18, 23].

Considering these findings, our results indicate that GUO-induced enhancement in extracellular (CSF) ADO concentration might be able to increase the activation of ADO A_1 receptors, which could contribute to the inhibition of GLU excitability in the hippocampus, promoting attenuation of the anxiety-like phenotype.

Conclusions

In summary, the present work demonstrated the potential anxiolytic effects of acute/systemic GUO administration, which could be exerted by orchestration of the activities of the adenosinergic and glutamatergic systems. More specifically, a novel mechanism of action could be proposed for the anxiolytic effects of GUO, which include a modulatory effect on extracellular ADO and GLU levels. In addition, because high levels of extracellular GLU are involved in several neuropsychiatric conditions, these results reinforce the previously observed neuroprotective effects of GUO. Thus, we contributed to the goal of identifying the molecular targets and signaling pathways that are recruited by GUO and that promote anxiety-like behavior. Finally, GUO is an endogenous, orally active compound that is apparently well tolerated and that deserves attention as a potential novel therapeutic drug in anxiety disorders.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no competing interests.

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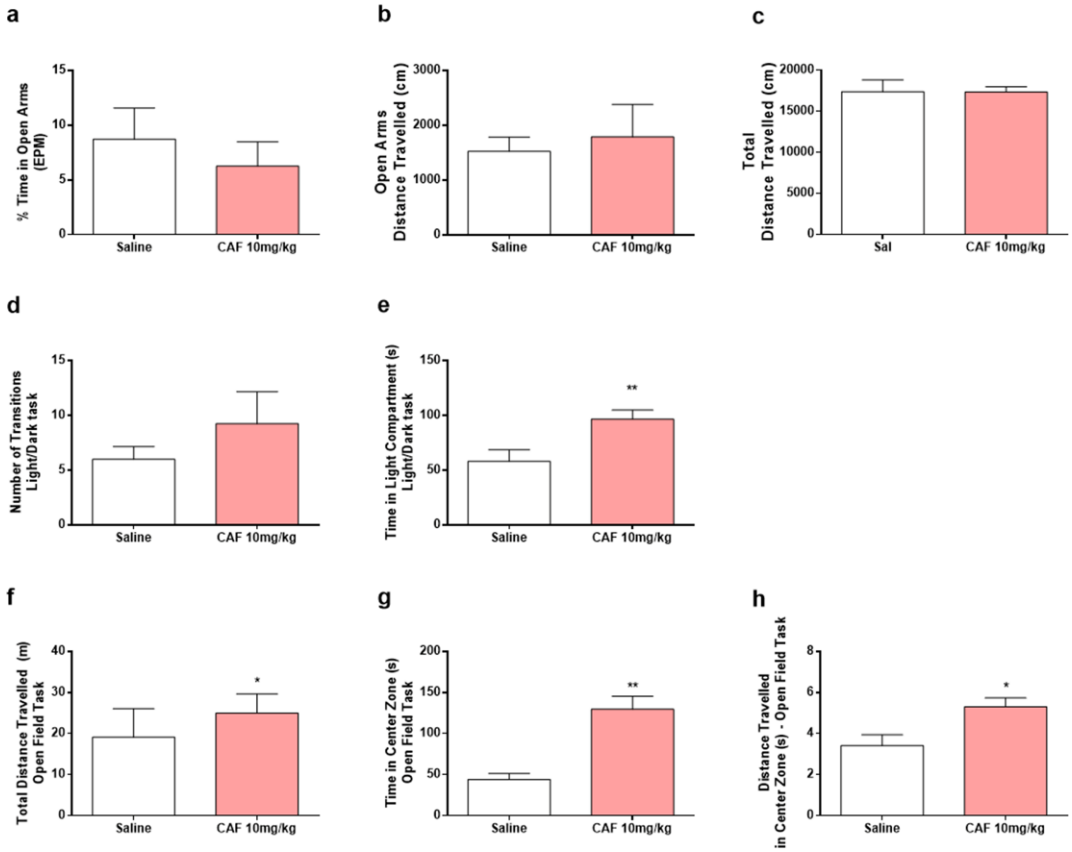
ESM 1 – CAF *per se* did not affect the anxiety-related behavior assessed by the EPM task.

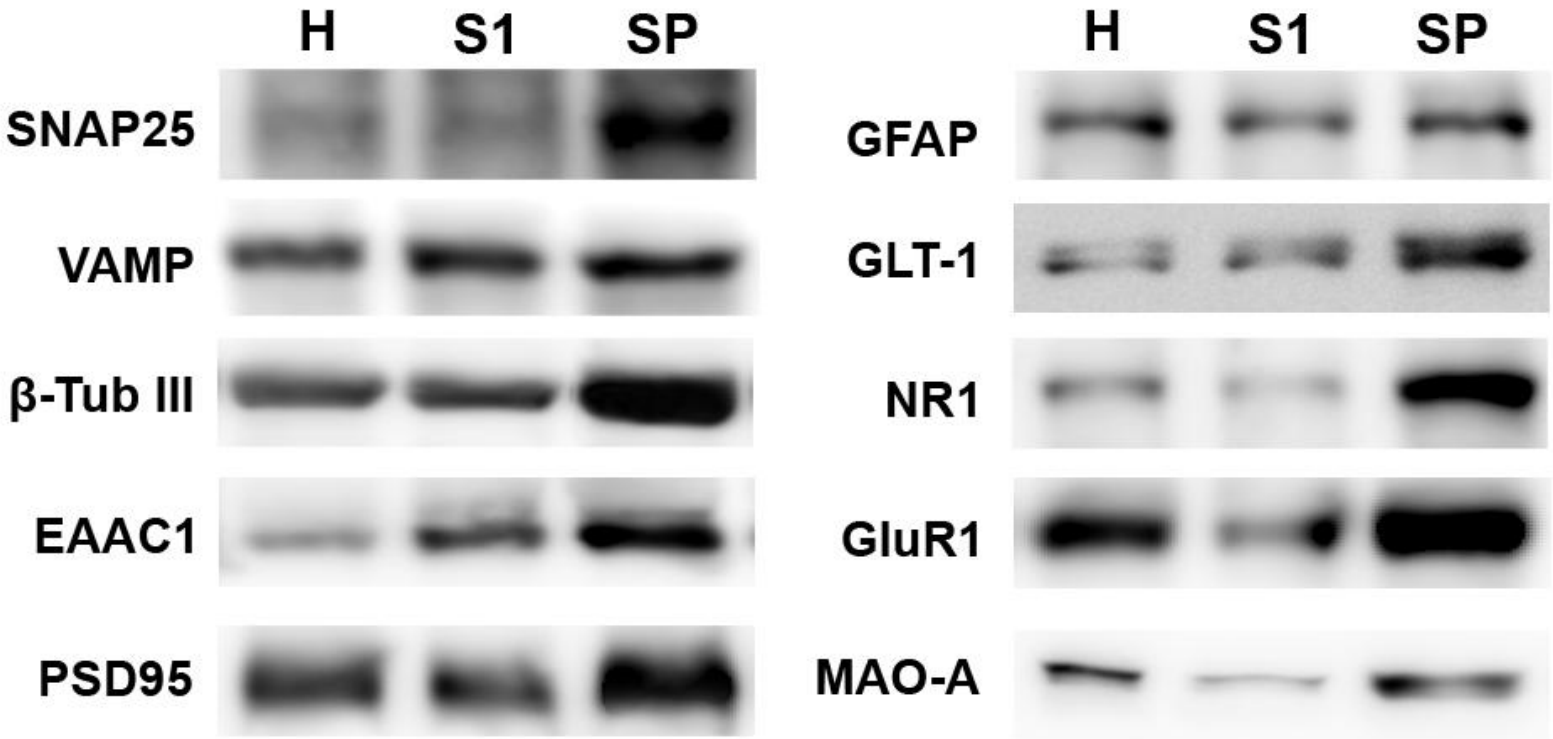


ESM 2 – CSF purine levels 60 minutes after i.p. saline administration: a comparison between two different protocols to preserve CSF purines.

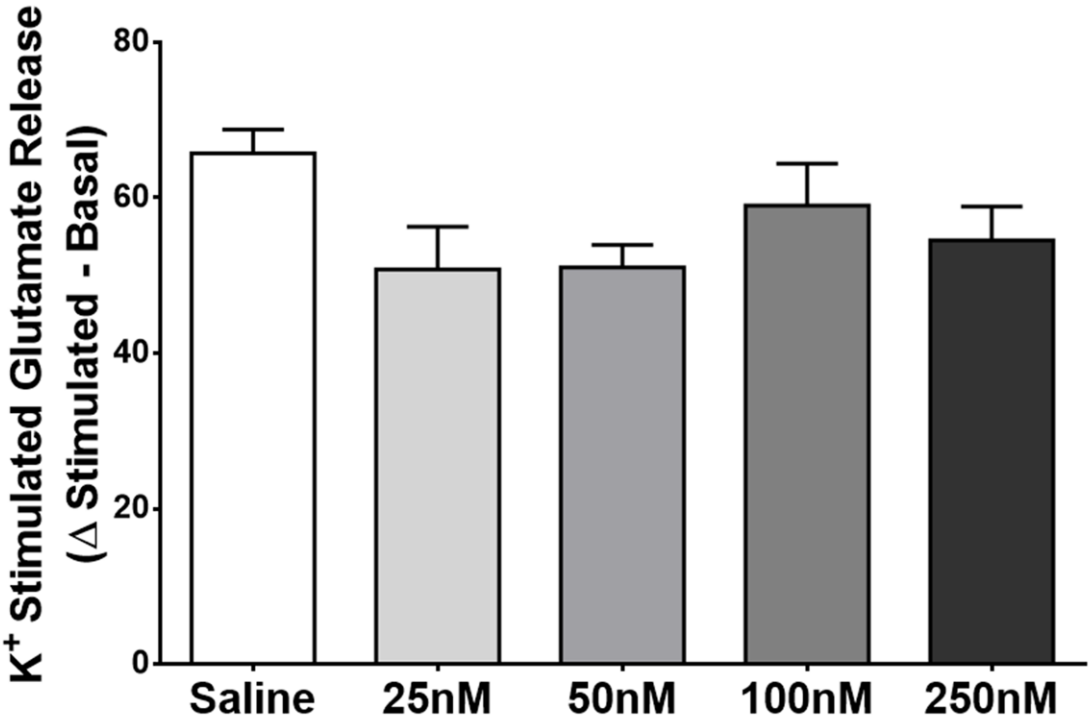
Table 1 – CSF purines levels 60 min after an i.p. injection of saline

(uM)	Method I	Method II
ADO	0.04 ± 0.02	0.10 ± 0.04*
INO	0.26 ± 0.07	0.55 ± 0.25*
HIPOX	2.80 ± 0.22	4.00 ± 0.63*
GUO	0.48 ± 0.05	0.52 ± 0.09
XANT	3.77 ± 0.25	2.60 ± 0.17*
UA	1.72 ± 0.17	1.36 ± 0.27*

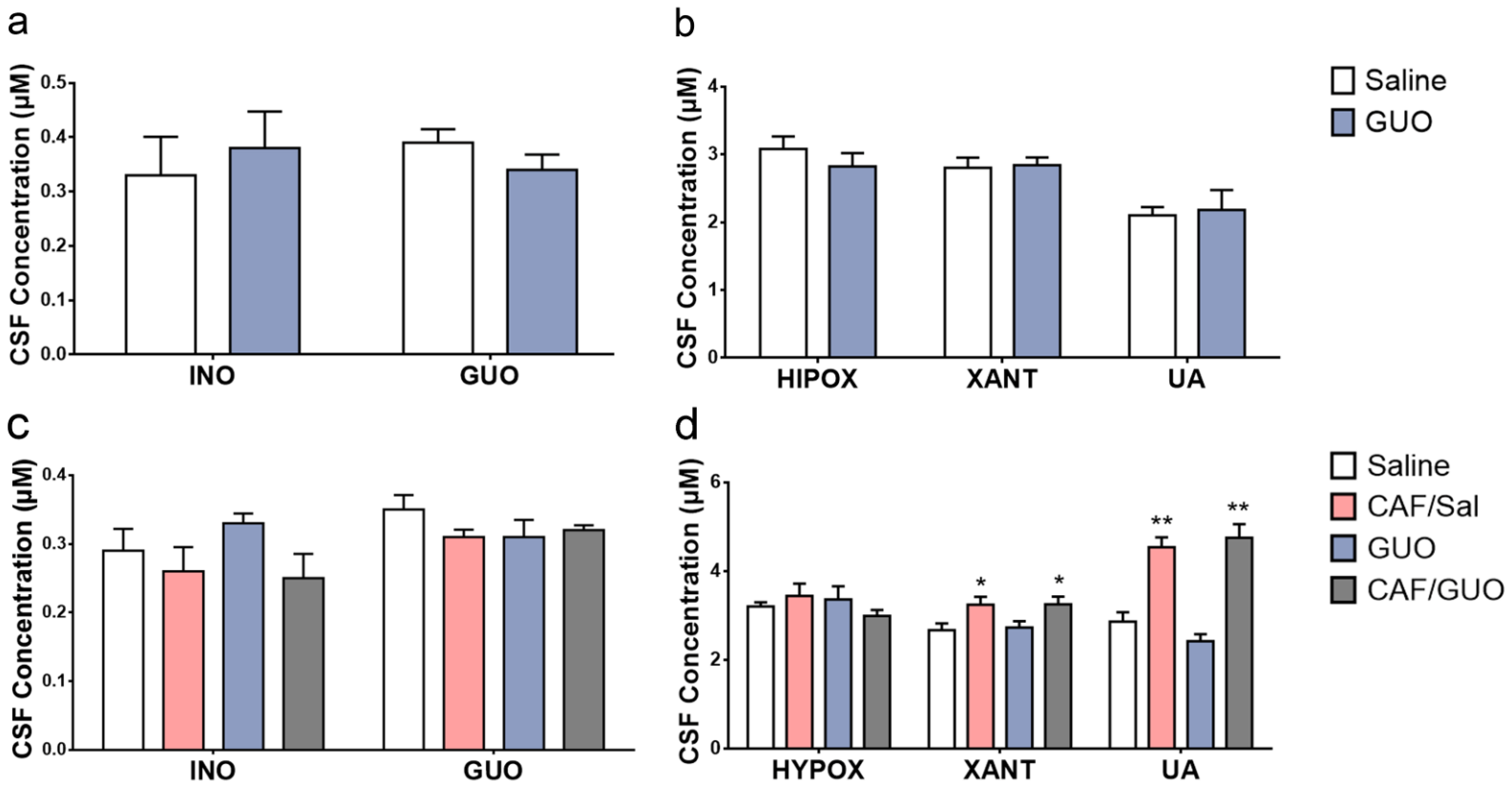
ESM 3 – Analysis of hippocampal synaptosomal preparations



ESM 4 – DPCPX dose response curve



ESM 5 – CAF modulates CSF levels of XAN and UA



Capítulo II: *Olfactory bulbectomy in mice triggers long-lasting and transient behavioral impairments and neurochemical hippocampal disturbances.*

No **capítulo II** apresentamos o artigo submetido ao periódico **Translational Psychiatry**

Detailed Status Information

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Title	Olfactory bulbectomy in mice triggers long-lasting and transient behavioral impairments and neurochemical hippocampal disturbances.
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Manuscript Type	Original Article
Corresponding Author	Dr. Diogo Souza (Universidade Federal do Rio Grande do Sul)
Contributing Authors	Ms. Roberto Almeida , Dr. Marcelo Ganzella , Dr. Daniele Machado , Dr. Samanta Loureiro , Mr. Douglas Leffa , Dr. André Quincozes-Santos , Dr. Letícia Pettenuzzo , Miss Marta Duarte , Mr. Tiago Duarte
Abstract	Major depression disorder (MDD) is a neuropsychiatric disease that is associated with profound disturbances in affected individuals. Elucidating the pathophysiology of MDD has been frustratingly slow, especially concerning the neurochemical events and brain regions associated with disease progression. Thus, we evaluated the time-course (up to 8 weeks) behavioral and neurochemical effects in mice that underwent an olfactory bulbectomy (OBX), which is used to model depressive-like behavior in rodents. Similar to the symptoms in patients with MDD, OBX induced long-lasting (e.g., impairment of habituation to novelty, hyperactivity and an anxiety-like phenotype) and transient (e.g., loss of self-care and motivational behavior) behavioral effects. OBX temporarily impaired synaptosomal mitochondria in the hippocampus but did not affect mitochondria from whole-cell preparations. Long-lasting pro-oxidative (i.e., increased levels of reactive oxygen species and nitric oxide and decreased glutathione levels) and pro-inflammatory (i.e., increased levels of pro-inflammatory cytokines IL-1, IL-6, TNF-α and decreased anti-inflammatory cytokine IL-10 levels) effects were induced in the hippocampus by OBX. Additionally, these parameters were transiently affected in the posterior and frontal cortices. This study suggests that the transient and long-lasting behavioral effects from OBX strongly correlate with mitochondrial, oxidative and inflammatory parameters in the hippocampus; furthermore, these effects show a weak correlation with these parameters in the cortex. Our findings highlight the underlying mechanisms involved in the neurochemical time course of events related to depressive behavior.
Techniques	Life sciences techniques, Cell/tissue technologies [Flow cytometry]; Life sciences techniques, Experimental organisms [Mouse]; Life sciences techniques, Protein techniques [Antibodies];
Subject Terms	Biological sciences/Neuroscience/Learning and memory/Hippocampus Health sciences/Diseases/Psychiatric disorders/Depression
Conflict of Interest Statement	The authors have declared there is **NO** conflict of interest to disclose
Clinical Trial	No
	Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Instituto Nacional de Ciência e Tecnologia (INCT) para Excitotoxicidade e Neuroproteção, Fundação de Amparo a Pesquisa do Estado do Rio Grande do Sul (FAPERGS), and Financiadora de Estudos e Projetos (FINEP) research grant BRede Instituto Brasileiro de Neurociências (IBN-Net)^, #01.06.0842-00. [Souza] Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Instituto Nacional de Ciência e Tecnologia (INCT) para Excitotoxicidade e Neuroproteção, Fundação de Amparo a Pesquisa do Estado do Rio Grande do Sul (FAPERGS), and Financiadora de Estudos e Projetos (FINEP) research grant BRede Instituto Brasileiro de Neurociências (IBN-Net)^, #01.06.0842-00. [Almeida] Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Instituto Nacional de Ciência e Tecnologia (INCT) para Excitotoxicidade e Neuroproteção, Fundação de Amparo a Pesquisa do Estado do Rio Grande do Sul (FAPERGS), and Financiadora de Estudos e Projetos (FINEP) research grant BRede Instituto Brasileiro de Neurociências (IBN-Net)^, #01.06.0842-00. [Ganzella] Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Conselho

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Olfactory bulbectomy in mice triggers long-lasting and transient behavioral impairments and neurochemical hippocampal disturbances.

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Abstract

Major depression disorder (MDD) is a neuropsychiatric disease that is associated with profound disturbances in affected individuals. Elucidating the pathophysiology of MDD has been frustratingly slow, especially concerning the neurochemical events and brain regions associated with disease progression. Thus, we evaluated the time-course (up to 8 weeks) behavioral and neurochemical effects in mice that underwent an olfactory bulbectomy (OBX), which is used to model depressive-like behavior in rodents. Similar to the symptoms in patients with MDD, OBX induced long-lasting (e.g., impairment of habituation to novelty, hyperactivity and an anxiety-like phenotype) and transient (e.g., loss of self-care and motivational behavior) behavioral effects. OBX temporarily impaired synaptosomal mitochondria in the hippocampus but did not affect mitochondria from whole-cell preparations. Long-lasting pro-oxidative (i.e., increased levels of reactive oxygen species and nitric oxide and decreased glutathione levels) and pro-inflammatory (i.e., increased levels of pro-inflammatory cytokines IL-1, IL-6, TNF-α and decreased anti-inflammatory cytokine IL-10 levels) effects were induced in the hippocampus by OBX. Additionally, these parameters were transiently affected in the posterior and frontal cortices. This study suggests that the transient and long-lasting behavioral effects from OBX strongly correlate with mitochondrial, oxidative and inflammatory parameters in the hippocampus; furthermore, these effects show a weak correlation with these parameters in the cortex. Our findings highlight the underlying mechanisms involved in the neurochemical time course of events related to depressive behavior.

1. Introduction

Major depression disorder (MDD) is a chronic and heterogeneous neuropsychiatric disease with a variable course and extremely high worldwide prevalence and incidence (Belmaker & Agam, 2008; Mann, 2005; Vos et al., 2012). MDD is characterized by profound disturbances in emotional regulation, motivation, social cognition and other systemic physiological aspects that result in a poor quality of life and disability (Belmaker & Agam, 2008; Black et al., 2016). The treatment for MDD patients commonly includes a combination of psychotherapy and pharmacotherapy (Karyotaki et al., 2016); however, despite the recent advances in antidepressive drug development, more than thirty percent of patients with MDD do not benefit from conventional antidepressant treatments and present with persistent symptomatology that leads to a chronic disease state (Balestri et al., 2016; Berton & Nestler, 2006). Progress in understanding the pathophysiology of MDD has been frustratingly slow (Berton & Nestler, 2006; Kim et al., 2016). Impairments in cognitive functioning (Black et al., 2016; Bora, Harrison, Yucel, & Pantelis, 2013) and evidence of neurodegenerative symptomatology in patients with MDD (Hurley & Tizabi, 2013; Kim et al., 2016) highlight the importance of identifying the molecular pathways that contribute to the progressive nature of this disorder. The pathogenesis and temporal course of MDD is complex and variable; thus, modeling human MDD in animals is extremely challenging but could significantly contribute to a better understanding of the mechanisms associated with the disease (Nestler & Hyman, 2010).

In this context, the bilateral olfactory bulbectomy (OBX) has garnered attention as an animal model of depression (Hendriksen et al., 2015; Kelly, Wrynn, & Leonard, 1997; Song & Leonard, 2005). This model is based on the hypothesis that

removal of the olfactory bulbs, which are part of the limbic system, affects their extensive efferent neuronal networks and disturbs the connection and function of the whole limbic system (Song & Leonard, 2005). The limbic circuit is essential for the maintenance of mood, emotional and memory components of behavior; thus, OBX induces depressive-like behaviors (Czeh et al., 2015; Hendriksen et al., 2015). Prominent behavioral changes that resemble the symptomatology observed in MDD patients (Hendriksen et al., 2015; Kelly et al., 1997; Song & Leonard, 2005) are apparent in the OBX animal model of depression, including anhedonia (Freitas et al., 2012) (e.g., an impairment in self-care and motivational behavior), increased sensitivity to stressful environments (Hendriksen et al., 2015; Song & Leonard, 2005; Zueger et al., 2005) (e.g., hyperactivity in the open field task), enhanced irritability (Song & Leonard, 2005) (e.g., increased murecidal behavior and territorial aggression), and memory and cognition impairments (Holubova et al., 2016) (e.g., deficits in the passive avoidance task and Morris water maze). Moreover, anatomical, cellular and biochemical changes similar to those observed in MDD patients were found in the central nervous system (CNS) of rodents that underwent an OBX, including a reduction in hippocampal volume (Wrynn et al., 2000), changes in synaptic strength (Czeh et al., 2015), impairments in mitochondrial metabolism (Rinwa, Kumar, & Garg, 2013), increased oxidative/nitrosative stress and inflammatory markers (Holzmann, da Silva, Correa da Silva, Steimbach, & de Souza, 2015; Yang et al., 2014), and enhanced cell death (Gomez-Climent et al., 2011; Jarosik, Legutko, Unsicker, & von Bohlen Und Halbach, 2007). Importantly, the chronic treatment of animals with antidepressants reverses the behavioral phenotypes and anatomical, cellular and biochemical changes induced by OBX (Freitas et al., 2012; Hendriksen et al., 2015; Song & Leonard, 2005). These data support the use of

OBX as an important animal model to investigate the pathophysiology of MDD.

Similar to many other psychiatric disorders, the neurochemical mechanisms involved in the progression of MDD remain elusive. The time course of changes in the brain that accompany long-lasting depressive behaviors in patients is unclear. OBX appears to be suitable animal model to explore the brain mechanisms associated with chronic depressive behaviors. Indeed, the OBX-induced disruption of neuronal connections between the olfactory bulbs and other brain regions resembles the neurodegenerative events in patients with MDD (Hendriksen et al., 2015). The majority of OBX studies focused on only one time point: 2 weeks after surgery. Thus, there is lack of information on the time course of the behavioral and neurochemical changes induced by OBX. To identify the putative pathways that contribute to the progression of MDD, we evaluated the time-course (up to 8 weeks) effects of OBX in mice by assessing behavioral patterns (i.e., hyperactivity, habituation to novelty and anhedonia) and neurochemical parameters (i.e., brain mitochondrial, oxidative, nitrosative and inflammatory markers) in MDD-related brain areas (i.e., hippocampus, posterior cortex and frontal cortex).

2. Material and Methods:

2.1. Animals

Male C57BL/6 mice (45-50 days old, 20-25 g) were obtained from Fundação Estadual de Produção e Pesquisa do Rio Grande do Sul, Porto Alegre, Brazil. Animals were housed 5 per cage and housed in a room under a 12-h/12-h light/dark cycle with a controlled temperature (22 ± 1 °C) and *ad libitum* access to food and water. The cages were placed in the experimental room 24 h prior to behavioral tasks, for acclimatization. All experiments were completed between 2:00 and 6:00 pm. All procedures were performed in accordance with the NIH Guide for the Care and Use

of Laboratory Animals and approved by the local Ethics Committee (project number 24577). All efforts were made to minimize animal suffering and the number of animals used in the experiments.

2.2. Experimental Schedule

To evaluate the long-term behavioral and neurochemical changes in an OBX model of depression, we designed 3 experimental schedules according to the time after surgery: 2 weeks (2W), 4 weeks (4W) and 8 weeks (8W). Naïve animals underwent an open field test (OFT) 1 day before the OBX (OFT1) to verify their baseline exploratory activity and discard any animals with behavioral abnormalities. Next, the animals were assigned to the Sham (mice that underwent the surgical procedure, but bulbs were left intact) or OBX (mice that underwent OBX) group.

The experimental scheme for the animals evaluated for 2 weeks after surgery (2W) is depicted in Fig. 1A. Accordingly, 2 weeks after surgery, the mice underwent a second OFT (OFT2). Two hours later, the mice underwent the Splash test. The mice were anesthetized and euthanized the following day, and brain samples were collected. Fig. 1B shows the experimental schedule for animals evaluated for 4 weeks after surgery (4W). Two weeks after surgery, the mice underwent OFT2. A third OFT was completed 4 weeks after surgery (OFT3). The mice were submitted to the splash test 2 hours after OFT3. On the following day, the mice were anesthetized and euthanized for sample collection. Fig. 1C shows the experimental schedule for animals evaluated for 8 weeks after surgery (8W). The schedule was similar to the 4W group, except OFT3 was performed 8 weeks (instead of 4 weeks) after surgery.

2.3. Bilateral olfactory bulbectomy (OBX)

2.3.1. Surgical procedure

The bilateral OBX was performed as previously described (Freitas et al., 2012) with minor modifications. Briefly, mice were anaesthetized via an intraperitoneal (i.p.) injection of xylazine (6 mg/kg) and ketamine (100 mg/kg) diluted in saline. The head was shaved and a burr hole (approximately 2 mm in diameter) was made in the skull above the olfactory bulbs 4 mm rostral to Bregma. Both olfactory bulbs were then dissected with surgical micro scissors and removed by suction with a glass Pasteur pipette. Animals were excluded from the study if the bulbs were not completely removed or the frontal cortex was injured (Freitas et al., 2012).

2.4. Behavioral tests

2.4.1. Open field test (OFT)

The OFT was used as previously described (Zueger et al., 2005) to investigate locomotor/exploratory activity, habituation and anxiety. Mice were placed near the sidewall in a gray wooden box (50×50×50 cm, length × width × height) with a 200 lux white light intensity and then recorded individually for 10 minutes with a video-camera (positioned above and at ca. 90° to the square arena) that was connected to a monitor. The behavioral performance of mice was analyzed using the AnyMaze® software (Stoelting Co., Wood Dale, IL). Multiple parameters were determined: i) the total distance traveled in the first 3 minutes was used to measure habituation to novelty; ii) the decrease in the distance traveled during the 1st to 3rd minute of the test was used to measure short-term habituation to novelty; iii) total time spent immobile during the first 3 minutes (the minimum duration of an immobile episode was set at 5 seconds); iv) the distance traveled during the last 7 minutes of the test was used to measure locomotor/exploratory activity; v) the total time spent immobile during the last 7 minutes; and vi) the time and the % of distance traveled in the center zone was

used to evaluate their anxiety-related phenotype. The apparatus was cleaned with 70% alcohol and dried after each test.

2.4.2. Splash test

The splash test was used to evaluate the loss of self-care and motivational behavior in mice (Freitas et al., 2012). A 10% sucrose solution was sprayed on the dorsal coat of mice. The sprayer delivered a fixed volume of 0.7 mL (each mouse received 3 sprays). Due its viscosity, the sucrose solution soils the fur of the mouse and induces the animal to initiate grooming behaviors. The grooming time (a grooming episode was defined as a mouse exhibiting behaviors such as licking, scratching or face-washing) during the first 5 minutes after application of the sucrose solution was recorded. This parameter was used as a measure of anhedonia.

2.5. Neurochemical assays

Mice were anesthetized, transcardially perfused with PBS and euthanized. Next, the brains were removed, and the hippocampus, posterior cortex and frontal cortex were dissected. The samples were immediately processed for flow cytometry or frozen at -80 ºC for other biochemical evaluations.

2.5.1. Flow cytometry

The mitochondrial mass and membrane potential ($\Delta\Psi$) were determined in whole neural cells or in synaptosomal-enriched preparations using flow cytometry (Becton Dickinson BD FACS Calibur cytometer). Mitochondrial mass (FL1-H) was detected with MitoTracker® Green FM (Life Technologies) labeling, and the mitochondrial membrane potential - $\Delta\Psi$ (FL-3H) was detected with Mitotracker ™ Red detection.

2.5.2. Dissociated whole neural cell preparations

For cell analyses, the tissue samples were mechanically dissociated using a fire polished Pasteur pipette in 0.1 M phosphate-buffered saline (PBS), pH 7.4 containing 0.1 mg/mL of collagenase IV. After dissociation, the samples were decanted for 15 minutes. An aliquot of supernatant was collected and incubated with MitoTracker™ Green FM and Red FM dyes (100 nM each) for 45 minutes at 37 °C. The mean fluorescence intensity of FL1-H and FL3-H was used to estimate mitochondrial mass and $\Delta\Psi$, respectively. The emission of fluorescence was measured using green (FL-1; 530 nm/30) and red (FL-3; 670 nm long pass) bandpass filters with a FACSCalibur platform and CellQuest Pro software (Becton Dickinson, Franklin Lakes, NJ, USA). Data from 10,000 events from dissociated cells of cerebral tissue were acquired for FL1-H and FL3-H using forward scatter (FSC) and side scatter (SSC) parameters with linear and log scales. All analyses were performed using Flow Jo software 7.6.3 (Treestar, Ashland, OR).

2.5.3. Synaptosomal preparations

Synaptosomal preparations were obtained as previously described (Almeida et al., 2016) with minor modifications. Briefly, tissue samples were homogenized (manual small capacity Teflon/glass homogenizer in 10 × volume/weight) in 10 mM Tris buffer (pH=7.4) containing 0.32 M sucrose, 1 mM EDTA and 0.25 mM DTT. The homogenates were centrifuged in microfuge tubes (1.5 mL per tube) at 1,000 *g for* 10 minutes at 4 °C using a fixed-angle rotor. The resulting supernatant was centrifuged at 11,000 *g* for 20 minutes at 4 °C using the same rotor. The synaptosomal-enriched pellet was then washed twice with HBSS (pH=7.4) by centrifugation at 16,000 *g for* 10 minutes at 4 °C to remove excess sucrose. The protocol for the whole-cell mitochondrial analysis was also used for synaptic

mitochondrial flow cytometry. Data from 40,000 events were acquired using a log scale for all parameters, including FSC and SSC.

2.5.4. Estimation of redox homeostasis

2.5.4.1. Intracellular levels of reactive oxygen species (ROS)

Tissue samples were homogenized in phosphate-KCl (20 mM/140 mM) buffer, pH=7.4, and centrifuged at 1,000 *g for* 5 minutes at 4 ºC. An aliquot of the supernatant was used to evaluate DCFH-DA oxidation. DCFH oxidation was used to measure intracellular ROS levels. 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) is hydrolyzed by intracellular esterases to produce dichlorofluorescin (DCFH). This non-fluorescent molecule is then oxidized to fluorescent dichlorofluorescin (DCF) by cellular oxidants. The fluorescence intensity was determined at an excitation wavelength of 488 nm and an emission wavelength of 520 nm using a plate reader (Spectra Max GEMINI XPS, Molecular Devices, USA) (Perez-Severiano et al., 2004).

2.5.4.2. Nitrite levels

NO levels were determined by measuring the nitrite levels (a stable oxidation product of NO) in tissue homogenates using the Griess reaction. The Griess reagent was a 1:1 mixture of 1% (w/v) sulphanilamide in 2.5% (w/v) phosphoric acid and 0.1% (w/v) N-(1-napththyl) ethylene diamine dihydrochloride in deionized water. Briefly, the tissue was homogenized in phosphate-KCl (20 mM/140 mM) buffer, pH=7.4 and centrifuged at 1,000 *g for* 10 minutes at 4 ºC. The supernatant was deproteinized with 20 µl TCA 25%, centrifuged at 2,000 *g* for 10 minutes at 4 ºC and immediately neutralized with 2 M potassium bicarbonate. After this procedure, the Griess reagent was added directly to the neutralized sample. The sample was then incubated in the dark for 15 minutes at 22 ºC (Hansel et al., 2014). Samples were

analyzed at 550 nm on a microplate spectrophotometer. Nitrite concentrations were calculated using a standard curve, and the results are expressed as percentages relative to the control conditions.

2.5.4.3. Glutathione (GSH) levels

GSH levels were assessed as previously described (Hansel et al., 2014). The tissues were homogenized in a phosphate-KCl (20 mM/140 mM) buffer, pH 7.4, containing 5 mM EDTA, and the protein was precipitated with 1.7% meta-phosphoric acid. The tissue homogenates were centrifuged at 1,000 *g for* 5 minutes at 4 ºC. The supernatant was mixed with o-phthaldialdehyde (1 mg/ mL methanol) and incubated at 22 °C for 15 minutes. The fluorescence intensity was measured using excitation and emission wavelengths of 350 nm and 420 nm, respectively. A calibration curve was created with standard GSH solutions. GSH concentrations were calculated as nmol/mg protein.

2.5.5. Inflammatory cytokine levels

The samples were homogenized in a PBS/Tris-HCl/SDS 5% solution, pH 7.4, and centrifuged at 5,000 *g* for 10 minutes at 4 °C. Commercial enzyme-linked immunosorbent assay (ELISA) kits for rat IL-1, IL-6, TNF-α and the anti-inflammatory cytokine (IL-10) were used according to the manufacturer's instructions (eBIOSCIENCE, San Diego, CA, USA). Briefly, 96-well microplates were incubated with the primary antibody at 4 °C overnight, washed and then blocked at room temperature for 1 h. The cytokine standards, calibrators, and samples were added to the plate in triplicate and incubated at room temperature for 2 h. After washing, the secondary antibody conjugated with peroxidase was added, and the plate was incubated at room temperature for 1 h. After this procedure, the samples were washed and a tetramethylbenzidine (TMB) chromogen was added. The reaction was allowed

to proceed for 15 minutes. The enzyme reaction was stopped by adding 1 M phosphoric acid (Stop solution). The absorbance was measured at 450 nm. The results for the tissue sample are expressed as picograms per milligram.

2.5.6. Protein determination

Protein content was measured using the Pierce BCA® protein kit (Thermo Scientific, Waltham, MA, USA) with bovine serum albumin as a standard.

2.6. Statistical analysis

A two-way ANOVA followed by Bonferroni post hoc test was used to analyze the effect of OBX surgery on behavioral and biochemical parameters and the time course changes induced by surgery in the Sham and OBX groups [factors: (1: surgery) Sham *versus* OBX within post-surgery time points and (2: time) comparison of different time points (naïve, 2W, 4W and 8W) within each group (Sham/OBX)]. Correlations among the grooming time in the Splash task and the first 3 minutes of distance travelled in the OFT, the last 7 minutes of distance travelled in the OFT and time in the center zone in the OFT and the correlation between the mitochondrial synaptosomal analysis with first 3 minutes of distance travelled in the OFT, the last 7 minutes of distance travelled in the OFT were analyzed by Pearson's correlation. The strength of the correlation was described using the guide suggested by Evans in 1996. Correlations were considered statistically significant at $r \geq 0.60$. All statistical procedures were carried out using Graph Pad Prism (Graph Pad Software, version 5, San Diego, CA, USA).

3. Results

Fig. 2 - OBX induced long-lasting impairments in habituation to novelty and hyperactivity in the OFT.

Fig. 2A and B shows the minute-by-minute distance traveled for mice from the naïve, Sham and OBX groups (see ESM_1 for a representative supplementary video).

During the first 3 minutes of the OFT, comparisons of naïve mice with the Sham groups (2W, 4W and 8W) revealed that repeated OFTs induced significant decreases in the distance traveled (Fig. 2C) and the difference in distance traveled between the 1st and 3rd minutes of testing (Fig. 2D) and a significant increase in the time spent immobile (Fig. 2E). Thus, the Sham groups presented a strong habituation to novelty. However, OBX groups presented a long-lasting impairment in the habituation to novelty compared with the naïve group, as evidenced by no decrease in the distance traveled during the first 3 minutes (Fig. 2B) and no change in the distance traveled between the 1st and 3rd minute of testing (Fig. 2C) or the time spent immobile (Fig. 2E).

During the last 7 minutes of testing, a long-lasting hyperactivity was observed in the OBX groups compared with their respective sham groups, as evidenced by a significant increase in the distance traveled (Fig. 2F). Additionally, OBX mice showed a significant increase in the total distance traveled compared with their respective naïve group (Fig. 2F). Sham animals from the 4W and 8W groups but not the 2W group showed a significantly longer immobility time compared with their naïve counterparts (Fig. 2G).

Fig. 3 - OBX induced a long-lasting anxiety-like phenotype (center avoidance in OFT)

OBX caused a significant long-lasting anxiogenic effect. OBX mice showed a decreased % of distanced traveled in the center zone when compared with the naïve group and the 2W, 4W and 8W Sham groups (Fig. 3A). OBX mice also showed a

decrease in the time spent in the center zone compared with the naïve group and the 2W and 4W Sham groups (Fig. 3B).

Fig. 4 - OBX induced a transient depressive-like behavior (anhedonia).

The splash test was used to measure anhedonic-like behavior (i.e., hedonic state, the ability to gain pleasure and motivational behavior). The transient anhedonic-like behavior induced by OBX was evidenced by a decrease in grooming time at 2 and 4 weeks; however, this effect was not present at 8 weeks (Fig. 4). There were transient correlations between grooming time and two OFT parameters, including the distance traveled during the first 3 minutes of testing (ESM_2A) and the distance traveled during the last 7 minutes of testing (ESM_2B). There were long-lasting correlations between grooming time and two OFT parameters, including the change in the distance traveled between the 1^{st} and 3^{rd} minute of testing (ESM_2C) and the time spent in the center zone of the arena (ESM_2D).

Fig. 5 and 6 – The effect of OBX on mitochondria from whole-cell and synaptosomal preparations from the hippocampus, posterior and frontal cortices. OBX produced transient changes in mitochondrial mass and ΔΨ in mitochondria from only hippocampal synaptosomal preparations.

No significant differences were observed in the mitochondrial mass or ΔΨ from whole-cell preparations of the hippocampus, posterior cortex and frontal cortex (Fig. 5). However, synaptosomal preparations from hippocampus showed a transient impairment in mitochondrial parameters, including a decrease in mitochondrial mass (Fig. 6A) and ΔΨ (Fig. 6B), in the 2W and 4W OBX groups but not the 8W group. There was transient correlation between the distance travelled in the first 3 minutes with synaptosomal mitochondrial ΔΨ (ESM_3C) and the distance travelled in the last 7 minutes with synaptosomal mitochondrial mass and ΔΨ (ESM_3B and D,

respectively).

Evaluation of the events from positively stained mitochondria (Mito+) in the hippocampal synaptosomal preparation revealed that OBX had no effect on mitochondrial mass (Mito+) (Fig. 6C), and a transient effect on mitochondrial ΔΨ (Mito+), as evidenced by a decrease in mitochondrial ΔΨ from the 2W and 4W groups but not the 8W group (Fig. 6D). There were transient negative correlations between the distance traveled during the first 3 minutes and the synaptosomal mitochondrial ΔΨ (ESM_3G) and the distance traveled during the last 7 minutes of testing *versus* the synaptosomal mitochondrial mass and ΔΨ (ESM_3F and_3H).

Fig. 7 - OBX induced a long-lasting imbalance in redox homeostasis in the hippocampus and a transient imbalance in the posterior and frontal cortices.

In the hippocampus, OBX induced a long-lasting effect on redox homeostasis. The OBX groups showed a significant increase in DCFH and NO levels (Figs. 7A-7B) and a significant decrease in GSH levels (Fig. 7C) compared with their respective Sham groups.

In the posterior cortex, OBX induced a transient imbalance in redox homeostasis. A significant increase in DCFH levels was observed in the 2W and 4W OBX groups but not the 8W OBX group (Fig. 7D). A significant increase in NO levels was observed in the 2W OBX group but not the 4W and 8W OBX groups (Fig. 7E). A significant decrease in GSH levels was observed in the 2W and 4W OBX groups but not the 8W OBX group (Fig. 7F). In the frontal cortex, a transient disruption in redox homeostasis was evidenced by the significant increase in DCFH levels (Fig. 7G) and significant decrease in GSH levels in the 2W OBX group but not the 4W and 8W OBX groups (Fig. 7I).

There were long-lasting correlations between the behavioral performance in

OFT *versus* the imbalance in hippocampal redox homeostasis (intracellular ROS and GSH levels) (ESM_4A, 4B and 4E), and a transient correlation between the behavioral performance in OFT *versus* NO intracellular levels (ESM_4C).

Fig. 8 - OBX induced a long-lasting inflammatory response in the hippocampus and a transient inflammatory response in the posterior and frontal cortices

OBX caused a long-lasting inflammatory response in hippocampus, as evidenced by a significant increase in the hippocampal pro-inflammatory cytokines IL-1 (Fig. 8A), IL-6 (Fig. 8B) and TNF-α (Fig. 8C) and a significant decrease in the anti-inflammatory cytokine IL-10 (Fig. 8D).

In the posterior cortex, OBX caused a mild but long-lasting inflammatory response. The OBX groups showed a significant increase in IL-1 (Fig. 8E) and a transient significant increase in TNF-α (Fig. 8G). In the frontal cortex, OBX caused a permanent significant increase in IL-1 (Fig. 8I) and IL-6 (Fig. 8J), a transient significant increase in TNF-α (Fig. 8K) and a transient significant decrease in IL-10 (Fig. 8L).

There was a long-lasting correlation between the distance traveled during the first 3 minutes of testing *versus* the hippocampal inflammatory response (i.e., IL-1, IL-6, TNF- α and IL-10 release) (ESM_5A, 5B, 5C and 5D). Additionally, there was a long-lasting correlation between the distance traveled during the last 7 minutes of testing and TNF-α release (ESM_5G).

4. Discussion

We are the first to describe the transient and long-lasting effects (up to 8 weeks) of the OBX mouse model of depression. We observed a long-lasting impairment in the habituation to novelty, hyperactivity and anxiety-like phenotype in

the OFT and a transient loss of self-care and motivational behavior in the splash test. The neurochemical analysis revealed that the hippocampus was the most affected brain structure compared with the posterior and frontal cortices. We observed multiple neurochemical changes in OBX mice: i) specific and transient impairment in synaptosomal (not in whole-cell) mitochondria mass and $\Delta\Psi$, which may be associated with hippocampal-related synaptotoxicity; and ii) long-lasting hippocampal imbalance in redox and inflammatory homeostasis. Our findings are strengthened by the presence of significant correlations between the behavioral and neurochemical parameters. The physiopathology of MDD and necessity for the novel therapeutics drugs remain under investigation; however, our data highlight promising future targets for the depression field.

OBX induced long-lasting behavioral changes: potential translational relevance.

The classical and the most widely accepted behavioral pattern in the OBX model of depression is the remarkable increase in locomotor/exploratory activity during the OFT (Czeh et al., 2015; Hendriksen et al., 2015; Kelly et al., 1997; Song & Leonard, 2005). Here, for the first time, the hyperactivity in OFT was evident for up to 8 weeks after OBX. OFT is a relevant tool for assessing behavioral disturbances in rodents (Gonzales, Barrett, Shumake, Gonzalez-Lima, & Lane, 2015; Padilla et al., 2010), and there is a wide diversity of symptoms present in mood disorders (Belmaker & Agam, 2008; Mann, 2005). However, there are lack of studies on the time-course of OBX-induced OFT behavioral changes (Mucignat-Caretta et al., 2006). To address this knowledge gap, we explored the long-term behavioral patterns of OBX.

Mice typically exhibit less exploratory behavior during the first few minutes of

testing in a familiar open field arena (Almeida et al., 2010; Padilla et al., 2010). This parameter is a measure of habituation to a novel environment. Here, we observed a normal habituation performance by Sham animals (i.e., a decrease in the total distance traveled, a lack of change in the distance traveled between the 1st and 3rd minute of testing, and an increase in immobility during the first 3 minutes of testing). In contrast, OBX mice showed long-lasting impairments and did not habituate to the open field up to 8 weeks post-surgery. OBX mice also showed chronic hyperactivity. Interestingly, all of these observations can be compared with clinical features that demonstrate a remarked cognitive decline in depressed patients (Cobb et al., 2016; Schmaal et al., 2015), which is predominantly diagnosed by strong declarative memory deficits (Bora et al., 2013; Papakostas, 2014; Vythilingam et al., 2004) rather than psychomotor agitation (Papakostas, 2014), a less frequent symptom of MDD. Considering that persistent cognitive decline is observed in MDD patients, the OBX model has face validity (symptomatic homology) and construct validity (theoretical rationale) as a depressive disorder model.

Another important aspect of mood disorders is the comorbidity between anxiety and MDD (Hofmeijer-Sevink et al., 2012; Stein & Sareen, 2015). Depression ranks among the top most frequent co-existing disorders with anxiety (Stein & Sareen, 2015). The co-existence of these disorders increases the tendency toward chronicity and severity (Balestri et al., 2016), suggesting that they may also share common pathophysiological mechanisms. The OFT is associated with increased stress and/or anxiety, which explains OBX-related hyperactivity (Song & Leonard, 2005). We demonstrated long-lasting anxiety-like behavior, including decreased exploration of the center zone of the OFT arena. Considering that hyperactivity is a putative sign of agitation-like behavior in anxious patients (Gupta, Radhakrishnan, Thangaraj, &

Kurhe, 2014) and that the comorbidity of anxiety disorder and MDD increases the chronicity of the disease (van Loo et al., 2014), we postulate that the long-lasting impairment in habituation to novelty and anxious phenotype induced by OBX is sustained by the interaction between anxious and depressive behaviors. Importantly, other behavioral tasks in rodents can be used to demonstrate depression-related phenotypes, such as object recognition, y-maze, passive avoidance, forced swim, tail suspension, light-dark and elevated plus maze tasks (Gupta et al., 2014; Han et al., 2008; Nakagawasai et al., 2016; Song & Leonard, 2005).

According to the DSM-5, a formal MDD diagnosis is characterized by a persistent depressive mood or anhedonia, which is one of the cardinal signs of depression in humans (Lally et al., 2014; Taylor, 2014). Furthermore, several studies indicate that the measurement of anhedonia in MDD patients is a complex process that encompasses aspects of personality, learning and biases (Rizvi, Pizzagalli, Sproule, & Kennedy, 2016). We are the first to demonstrate that OBX induced the transient loss of self-care and motivational behavior for up to 4 weeks after surgery. In contrast, previous studies suggested that anhedonic-like behaviors are not clearly observed in the OBX model of depression (Czeh et al., 2015). Significant correlations between anhedonic-like behavior, habituation to novelty and anxiety in the OFT strengthen the face and construct validity of the OBX model of depression (Czeh et al., 2015).

OBX triggered transient hippocampal mitochondrial impairments and long-lasting imbalances in redox homeostasis and the inflammatory response of the hippocampus.

Several studies demonstrated that mitochondrial cytopathies are a key feature in MDD (Aguiar et al., 2014; Scaglia, 2010). The mechanisms driving the observed

changes in the OBX model were shown to include alterations in mitochondrial metabolism (Rinwa et al., 2013). Here, we observed no OBX-related effects on the mitochondrial parameters of whole-cell preparations from any analyzed brain region. However, OBX profoundly affected hippocampal presynaptic mitochondria, indicating a remarkable specificity of OBX effects on presynaptic components.

Presynaptic mitochondria play important roles in synaptic transmission, plasticity and organization, including the movement of vesicles and calcium buffering (Mattson, Gleichmann, & Cheng, 2008; Nicholls, Brand, & Gerencser, 2015); thus, we conducted mitochondrial analyses of well-established preparations, including synaptosome-enriched preparations, to explore the putative specificity of mitochondrial parameters susceptible to OBX. Mitochondria from hippocampal synaptosome-enriched preparations uniquely showed a transient decrease in mitochondrial mass and $\Delta\Psi$. Positively-stained mitochondria (Mito+) also showed a transient decrease in the mitochondrial $\Delta\Psi$, highlighting the specific loss of hippocampal synaptic mitochondrial functionality in the OBX model of depression. The current results suggest that in addition to the decrease in synaptic mitochondria, there is also a decrease in mitochondrial functionality in the presynaptic terminals 2 weeks and 4 weeks after OBX. Interestingly, the OBX-induced effects on synaptic mitochondria were reversed by 8 weeks post-surgery. We observed significant negative correlations between mitochondria functionality ($\Delta\Psi$) *versus* the total distance traveled during the first 3 minutes of the OFT (habituation to novelty) and the distance traveled during the last 7 minutes of the OFT (hyperactivity) in the 2W and 4W groups, suggesting that the observed mitochondrial dysfunction in hippocampal synaptosomal-enriched preparation may contribute to synaptotoxicity-related effect. Mitochondria in the synaptosomal preparation (presynaptic terminals)

exhibited a significant lower content of electron transport components, which could lead to an increased susceptibility to neurodegenerative dysfunction and synaptotoxicity (Nicholls et al., 2015; Picard & McEwen, 2014). Mitochondria are strongly involved in neuroplasticity/synaptogenesis (Cheng, Hou, & Mattson, 2010; Picard & McEwen, 2014) and play a key role in regulating synaptic transmission, cognition and aging (Nicholls et al., 2015; Picard & McEwen, 2014); therefore, the transient impairment in synaptic mitochondria homeostasis may contribute to the behavioral disturbances observed in the OBX model of depression. Since, the impairment of the high metabolic requirement in presynaptic terminals was previously associated with changes in synaptic strength and/or loss of spine density in the limbic areas of OBX animals (Czeh et al., 2015), this data could suggest a close link between the transient mitochondrial changes and anhedonic-like behaviors.

The hippocampal selectivity of the OBX-induced mitochondrial alterations are in accordance with the structural modifications in specific brain regions that resulted in deficits in hippocampus-dependent learning and memory in OBX mice (Hendriksen et al., 2015). Some authors postulate that the cognitive phenotype induced by OBX (loss of spatial memory), accompanied by increased brain levels of tau-protein hyperphosphorylation, could be associated as a model for Alzheimer´s disease (Bobkova et al., 2014; Hu, Wang, Liu, Wang, & Zhu, 2012). Although both pathologies (Alzheimer's disease and MDD) have a chronic effect on cognitive performance, the neurotoxicity in animal models of Alzheimer's involves b-amyloid peptide deposition (Crimins, Pooler, Polydoro, Luebke, & Spires-Jones, 2013; Ferreira, Lourenco, Oliveira, & De Felice, 2015) and/or the abnormal phosphorylation of the microtubule-associated protein tau (Pooler, Noble, & Hanger, 2014). Although the precise synaptotoxic form of tau remains unclear, several studies suggest that

aggregated tau may from primary synaptotoxic insults (Kopeikina, Hyman, & Spires-Jones, 2012; Pooler et al., 2014; Spires-Jones, Kopeikina, Koffie, de Calignon, & Hyman, 2011). Notably, evidence suggests that tau regulates neuronal signal transduction by influencing the targeting and function of synaptic mitochondria; indeed, tau can bind to kinesin and compete with other cargo, which inhibits mitochondrial transport to the soma, axon, and pre-synaptic boutons (Pooler et al., 2014). At this time, although our results demonstrated the long-lasting effects on memory-related parameters (habituation to novelty), the transient neurochemical changes observed suggest that the OBX model of depression leads to a transient synaptotoxicity-related effect, which differs from the synaptotoxicity verified in Alzheimer's disease.

Several human and experimental studies, including meta-analyses (Black et al., 2015), suggested that an imbalance in several redox parameters contributed to the pathogenesis of MDD (Black et al., 2015; Hurley & Tizabi, 2013; Moylan et al., 2014; Yang et al., 2014). Here, our data show that OBX increased the production of ROS, NO and altered antioxidant defenses (e.g., GSH), particularly in the hippocampus, for up to 8 weeks. We postulate that these effects lead to dysfunction in intracellular signaling contributing to the hippocampal synaptotoxicity (Pooler et al., 2014). Indeed, the disruption of redox homeostasis is strongly associated with mitochondrial damage (Moylan et al., 2014) and may contribute to the transient mitochondrial $\Delta\Psi$ impairment demonstrated in our study. Moreover, OBX effects on intracellular ROS and NO levels in the posterior and frontal cortices were transient.

Several lives of evidence suggest that pro-inflammatory cytokines are produced in response to oxidative stress (Moylan et al., 2014) and play a critical role in the pathogenesis of MDD. Many studies have demonstrated that pro-inflammatory

cytokines, including IL-1, IL-6 and TNF-α, are elevated in the serum and CNS of patients with MDD (Hurley & Tizabi, 2013). These data are reinforced by recent work showing that inflammation illicits symptoms of anhedonia (Swardfager, Rosenblat, Benlamri, & McIntyre, 2016). Our temporal analysis revealed an increase in the levels of pro-inflammatory cytokines and a decrease in an anti-inflammatory cytokine in the hippocampus; furthermore, the majority of these changes were long-lasting. Previous studies showed that the inflammatory and redox state are intimately linked in the cell (Moylan et al., 2014); thus, our findings of GSH depletion, the main neuronal antioxidant defense, and increased pro-inflammatory cytokines are in accordance with the presence of a pro-oxidative state in the hippocampus. Additionally, *in vitro* and *in vivo* studies have shown that increases in pro-inflammatory cytokines can alter synaptic plasticity (Hurley & Tizabi, 2013). Thus, the pronounced and long-lasting redox imbalance and pro-inflammatory response displayed in hippocampus of OBX mice presented in our study could strongly influence the increased mitochondrial dysfunction observed at the presynaptic terminals.

Interestingly, the hippocampus redox status and cytokines levels significantly correlates with the distance traveled in the OFT, predominantly during the first 3 minutes of the task. These data suggest an association between long-lasting behavioral changes and the disruption of redox homeostasis and enhancement of the inflammatory response. Additionally, the hippocampus is intimately involved in emotional and spatial/topographical memory. Thus, our data reinforce the importance of separately evaluating OFT behavioral parameters and suggest that habituation to novelty in the OFT is an essential behavioral abnormality caused by OBX in mice.

Previous studies showed that the olfactory bulbs have defined regions that

communicate via neurotransmitters and projections to the amygdala, hippocampus, posterior pyriform cortex and entorhinal cortex (Song & Leonard, 2005). This description of anatomical connections among the main olfactory bulbs and other brain regions clarifies our data because the most pronounced neurochemical changes (mitochondrial mass and $\Delta\Psi$, redox imbalance and pro-inflammatory cytokines) that observed over the time occurred in the hippocampus and, to a lesser extent, in the posterior and frontal cortices. Thus, retrograde, anterograde and transneuronal degeneration may have occurred after the OBX surgery, leading to changes in the entire brain; however, some regions were more affected than others, such as hippocampus.

Therefore, we postulate that OBX surgery induces a number of maladaptive consequences in the hippocampus in a transient or long-lasting manner: (i) changes in hippocampal anatomical structure, (ii) transient hippocampal-related synaptotoxicity, (iii) long-lasting increases in ROS and pro-inflammatory cytokines, (iv) decreased hippocampal synaptic plasticity, (v) reduced hippocampal cellular resilience and (vi) impairment of hippocampal-dependent behavioral performance. Based on the aforementioned evidence, the structural changes in the hippocampus are consistent with the pronounced anxiety and depressive-like behavior in OBX mice. Our results provide additional characterization of the OBX model in mice and create new perspectives for the depression field, including future pharmacological studies, and potential targets for antidepressant drugs.

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Compliance with Ethical Standards

Conflict of Interest:

The authors declare that they have no competing interests.

Figure Legends:

Fig. 1 – Study protocols.

The experimental schedule for mice consisted of 3 time points: 2 (2W), 4 (4W) and 8 (8W) weeks after the Sham or OBX surgery. Mice were euthanized on day 16 (2W), 30 (4W) or 62 (8W). Brain structures were dissected for immediate analysis (flow cytometry) or stored at −80 °C for subsequent biochemical analyses of redox and inflammatory parameters. OFT1: 1st Open Field exposure – all groups were tested; OFT2: 2nd Open Field exposure – all groups were tested; OFT3: 3rd Open Field exposure – only the 4W and 8W groups were tested.

Fig. 2 – Effect of OBX on habituation to novelty and locomotor activity in the OFT

A minute-by-minute analysis of the locomotor activity of Sham (A) and OBX (B) groups during the OFT. Each point represents the mean of the group. The effect of time and surgery on the distance traveled during the first 3 minutes of testing (C), the change in the distance traveled between the 1st and 3rd minute of testing (D), the time spent immobile during the first 3 minutes of testing (E), the distance traveled during the last 7 minutes of testing (F) and the time spent immobile during the last 7 minutes of testing (G). Each column represents the mean±S.E.M. Data were analyzed using a

two-way ANOVA followed by Bonferroni post-hoc tests. *p<0.05, **p<0.01 and ***p<0.001 compared to the respective Sham group (surgery effect); #p<0.05, ##p<0.01 and ###p<0.001 compared to the respective naïve group (time effect) (n=25 animals/group).

Fig. 3 – The effect of OBX on anxiety-related behavior in the OFT

The effect of time and surgery on the time spent in the center zone (A) and the percentage of distance traveled in the center (B) by the Sham and OBX groups. Each column represents the mean ± S.E.M. Data were analyzed using a two-way ANOVA followed by Bonferroni post-hoc tests. *p<0.05 and ***p<0.001 compared to the respective Sham group (surgery effect); #p<0.05, ##p<0.01 and ###p<0.001 compared to the respective naïve group (time effect) (n=25 animals/group).

Fig. 4 – The effect of OBX on self-care and motivational behavior

The effect of time and surgery on grooming time in the splash test (A) for Sham and OBX mice. Data are reported as the mean ± S.E.M. and were analyzed using a two-way ANOVA followed by Bonferroni post-hoc tests. *p<0.05 and **p<0.01 compared to the respective Sham group (n=8-12 animals/group).

Fig. 5 – The effect of OBX on whole-cell mitochondrial mass and ΔΨ in multiple brain regions

The effect of OBX on mitochondrial mass and ΔΨ in the hippocampus (A and B), posterior cortex (C and D) and frontal cortex (E and F). Data are reported as the mean ± S.E.M. and were analyzed using a two-way ANOVA followed by Bonferroni post-hoc tests (n=6 animals/group).

Fig. 6 – The effect of OBX on the mass and ΔΨ of hippocampal synaptic mitochondria

The effects of OBX on hippocampal mitochondrial mass (A) and ΔΨ (B) and the mitochondrial mass (C) and ΔΨ (D) of only viable mitochondria (Mito+) from synaptosomal-enriched preparations. Data are reported as the mean ± S.E.M. and were analyzed using a two-way ANOVA followed by Bonferroni post-hoc tests. *p<0.05 and **p<0.01 compared to the respective sham group (n=6 animals/group).

Fig. 7 – The effect of OBX on redox homeostasis

Effects of OBX on the levels of DCFH (A), (D) and (G) in hippocampus, posterior cortex and frontal cortex, respectively; of NO (B), (E) and (H) in hippocampus, posterior cortex and frontal cortex, respectively; and of GSH (C), (F) and (I) in hippocampus, posterior cortex and frontal cortex, respectively. Data are reported as the mean ± S.E.M. and were analyzed using a two-way ANOVA followed by Bonferroni post-hoc tests. *p<0.05, **p<0.01 and ***p<0.001 compared to the respective Sham group (surgery effect); #p<0.05, ##p<0.01 and ###p<0.001 compared to the respective OBX 2W group (time effect) (n=5 animals/group).

Fig. 8 – The effect of OBX on inflammatory parameters

Effects of OBX on IL-1 (A), (E) and (I) in hippocampus, posterior cortex and frontal cortex, respectively; on IL-6 (B), (F) and (J) in hippocampus, posterior cortex and frontal cortex, respectively; on TNF-α (C), (G) and (K) in hippocampus, posterior cortex and frontal cortex, respectively; and on IL-10 (D), (H) and (L) in hippocampus, posterior cortex and frontal cortex, respectively. Data are reported as the mean ± S.E.M. and were analyzed using a two-way ANOVA followed by Bonferroni post-hoc tests. *p<0.05, **p<0.01 and ***p<0.001 compared to their respective Sham group (n=5 animals/group).

ESM_1 – Supplementary video

A video of the representative behavior of Sham and OBX mice in the OFT.

ESM_2 - Correlation between the grooming time in the splash test *versus* the behavioral performance in the OFT.

Linear correlations between grooming time in the splash test and 4 OFT parameters: the distance traveled during the first 3 minutes of testing (A), the distance traveled during the last 7 minutes of testing (B), the change in the distance traveled between the 1st and 3rd minute of testing (C) and the time spent in the center zone of the arena (D). Each point represents 1 animal. *$p<0.05$ and**$p<0.01$ (n=8-12 animals/group).

ESM_3 – Linear correlations between hippocampal synaptosomal mitochondrial parameters and the distance traveled during the first 3 minutes or last 7 minutes of the OFT.

The linear correlations between the hippocampal synaptosomal mitochondrial mass *versus* the distance traveled during the first 3 minutes (A) and last 7 minutes (B) of the OFT. The linear correlations between the hippocampal synaptosomal mitochondrial $\Delta\Psi$ and the distance traveled during the first 3 minutes (C) and the last 7 minutes (D) of the OFT. The linear correlations between the hippocampal synaptosomal (Mito+) mitochondrial mass and the distance traveled during the first 3 minutes (E) and the last 7 minutes (F) of the OFT. The linear correlations between the hippocampal synaptosomal mitochondrial $\Delta\Psi$ and the distance traveled during the first 3 minutes (G) and the last 7 minutes (H) of the OFT. Each point represents one animal. *$p<0.05$ and ***$p<0.001$ represent a significant correlation between the variables analyzed (n=6 animals/group).

ESM_4 – Linear correlations between hippocampal redox parameters and the distance traveled during the first 3 minutes and last 7 minutes of the OFT.

The linear correlations between DCFH levels *versus* the distance traveled in the first 3 minutes (A) and last 7 minutes (B) of the OFT. The linear correlations between NO levels and the distance traveled in the first 3 minutes (C) and last 7 minutes (D) of the OFT. The linear correlations between GSH levels and the distance traveled in the first 3 minutes (E) and last 7 minutes (F) of the OFT. Each point represents one animal. *$p<0.05$ and ***$p<0.001$ represent a significant correlation between the variables analyzed (n=6 animals/group).

ESM_5 – Linear correlations between hippocampal inflammatory parameters and the distance traveled during the first 3 minutes and last 7 minutes of the OFT.

(A), (B), (C) and (D), respectively, show the linear correlations between the IL-1, IL-6, TNF-α and IL-10 levels and the first 3 minutes distance travelled in the OFT. (E), (F), (G) and (H), respectively, show the linear correlations between the IL-1, IL-6, TNF-α and IL-10 levels and the distance traveled during the last 7 minutes of the OFT. Each point represents one animal. *$p<0.05$ and ***$p<0.001$ represent a significant correlation between the variables analyzed (n=6 animals/group).

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Fig. 1 – Study protocols.

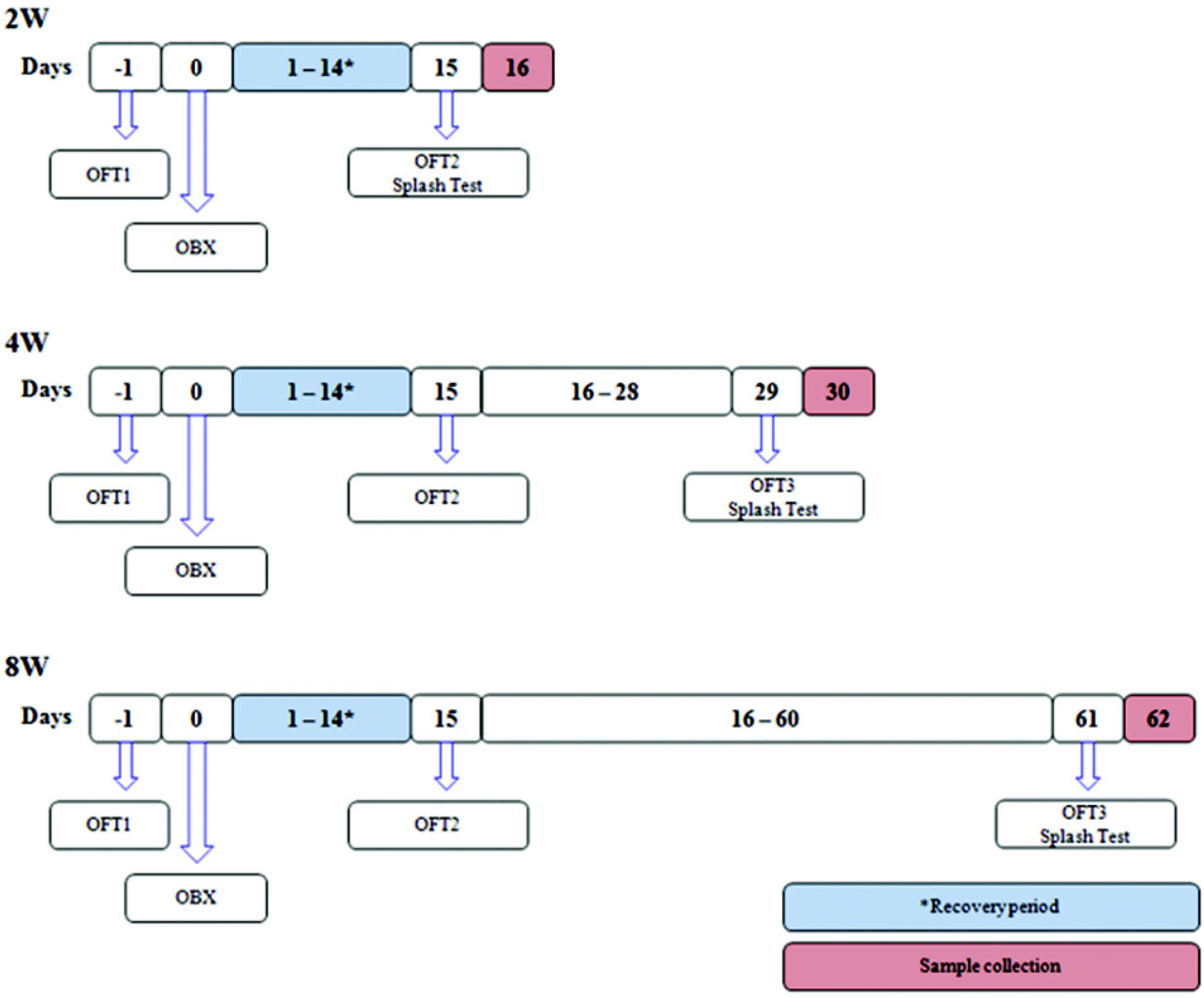


Fig. 2 – Effect of OBX on habituation to novelty and locomotor activity in the OFT

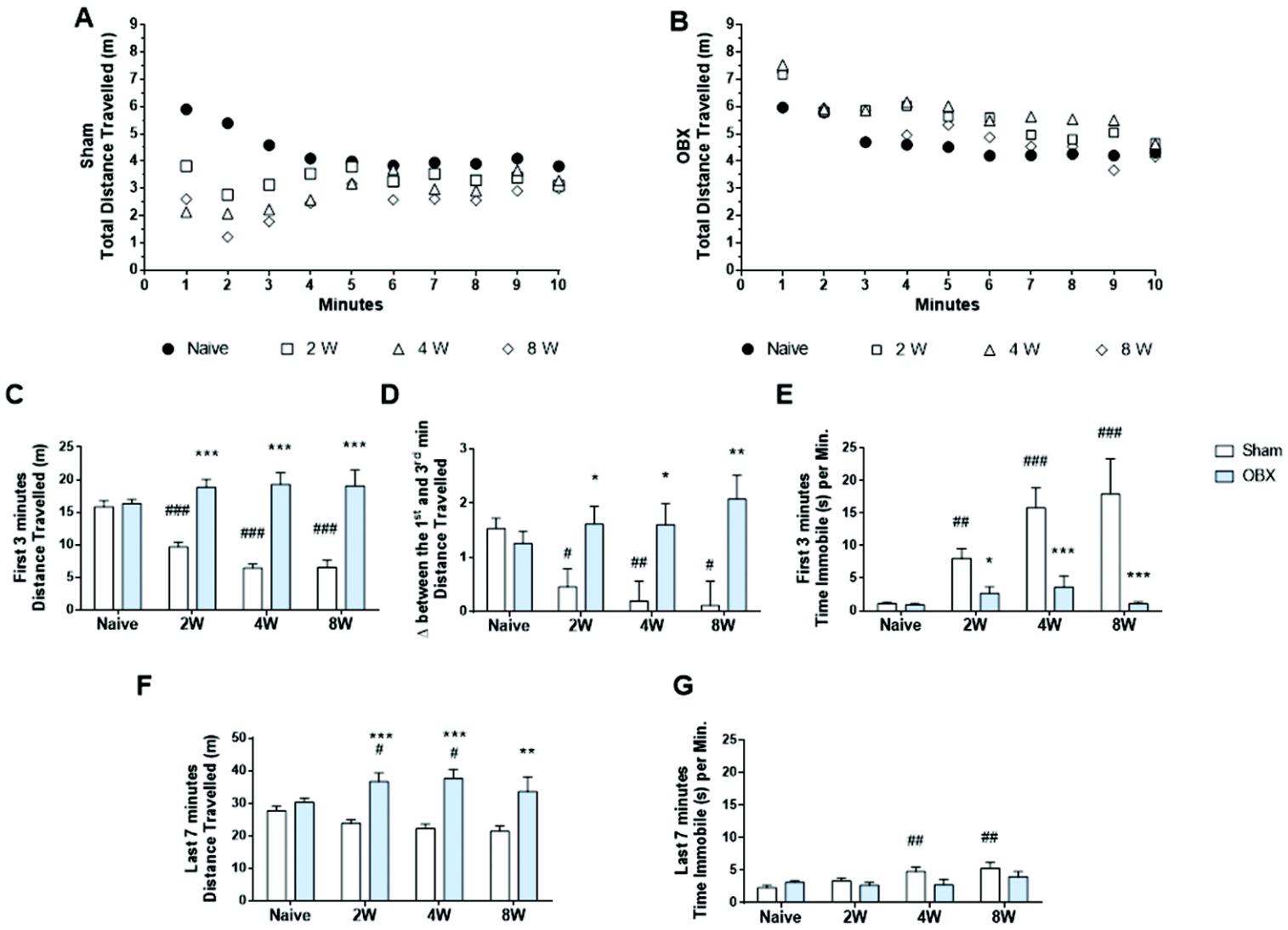


Fig. 3 – The effect of OBX on anxiety-related behavior in the OFT

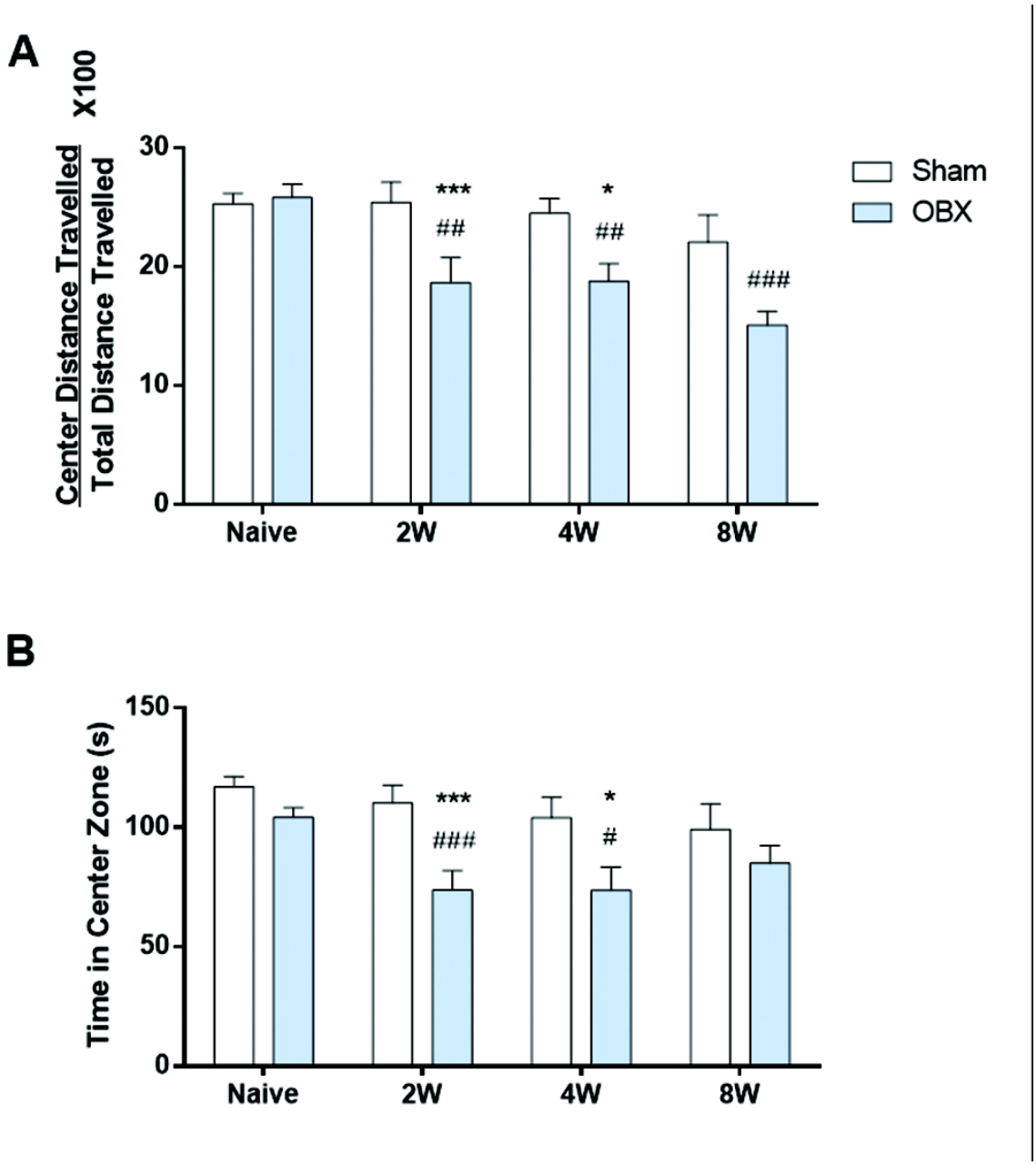


Fig. 4 – The effect of OBX on self-care and motivational behavior

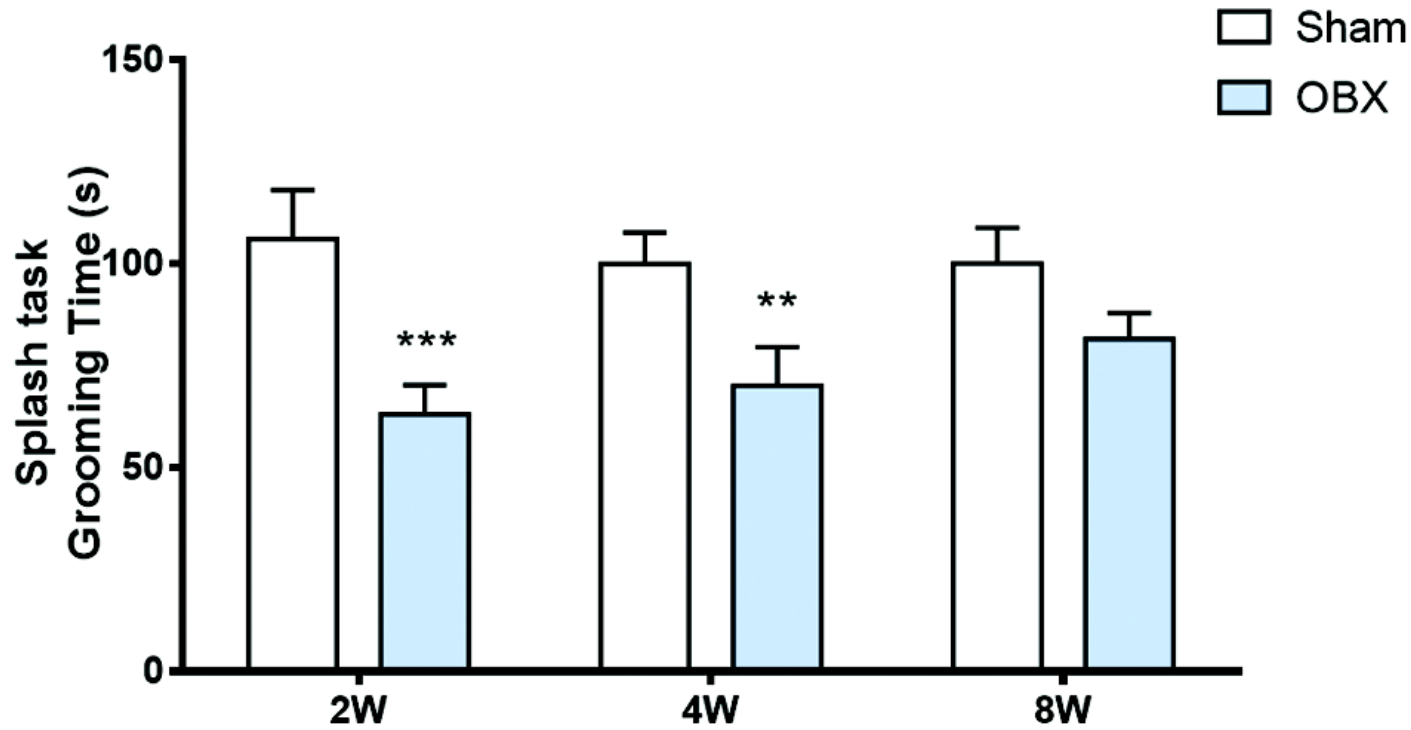


Fig. 5 – The effect of OBX on whole-cell mitochondrial mass and ΔΨ in multiple brain regions

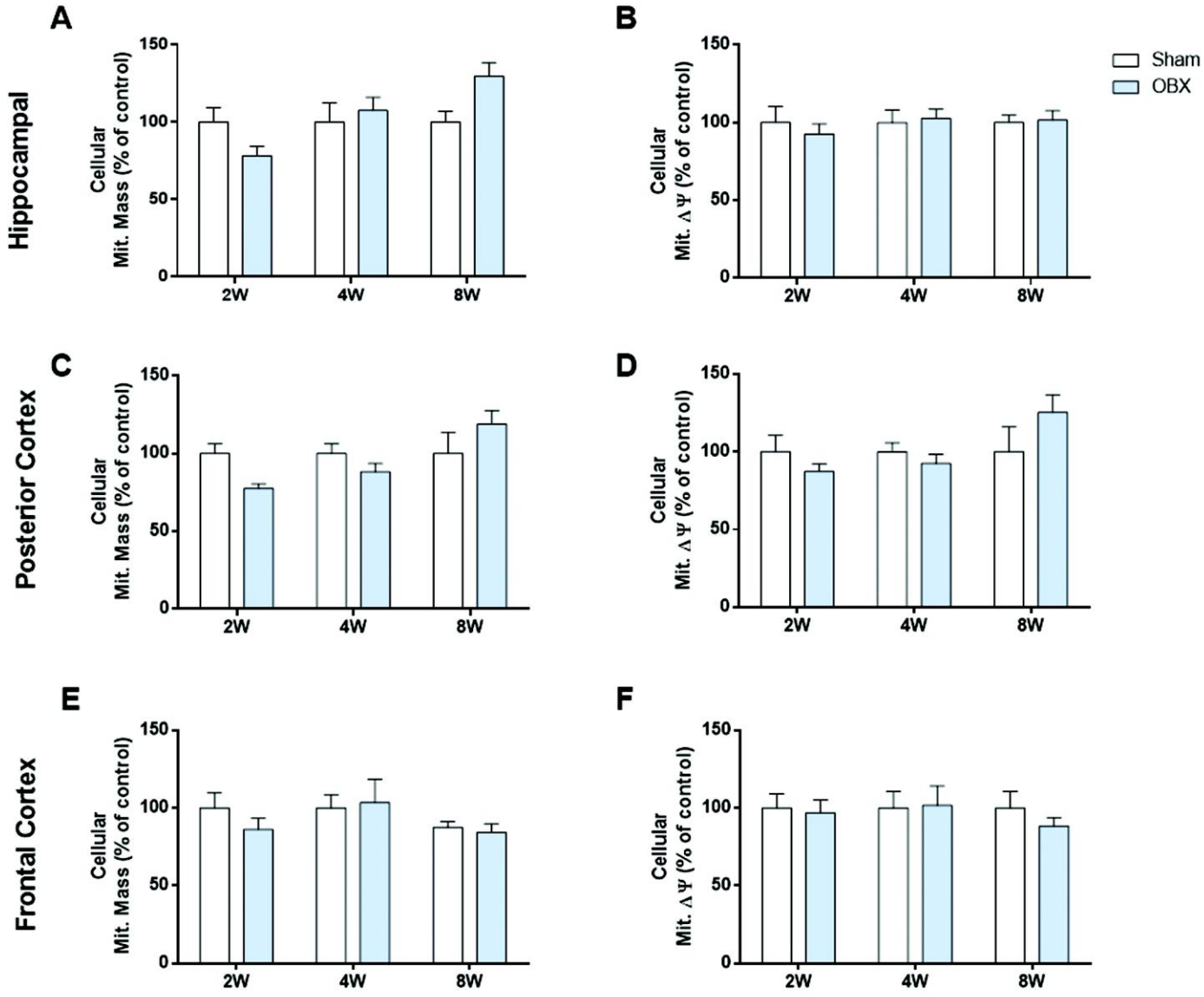


Fig. 6 – The effect of OBX on the mass and ΔΨ of hippocampal synaptic mitochondria

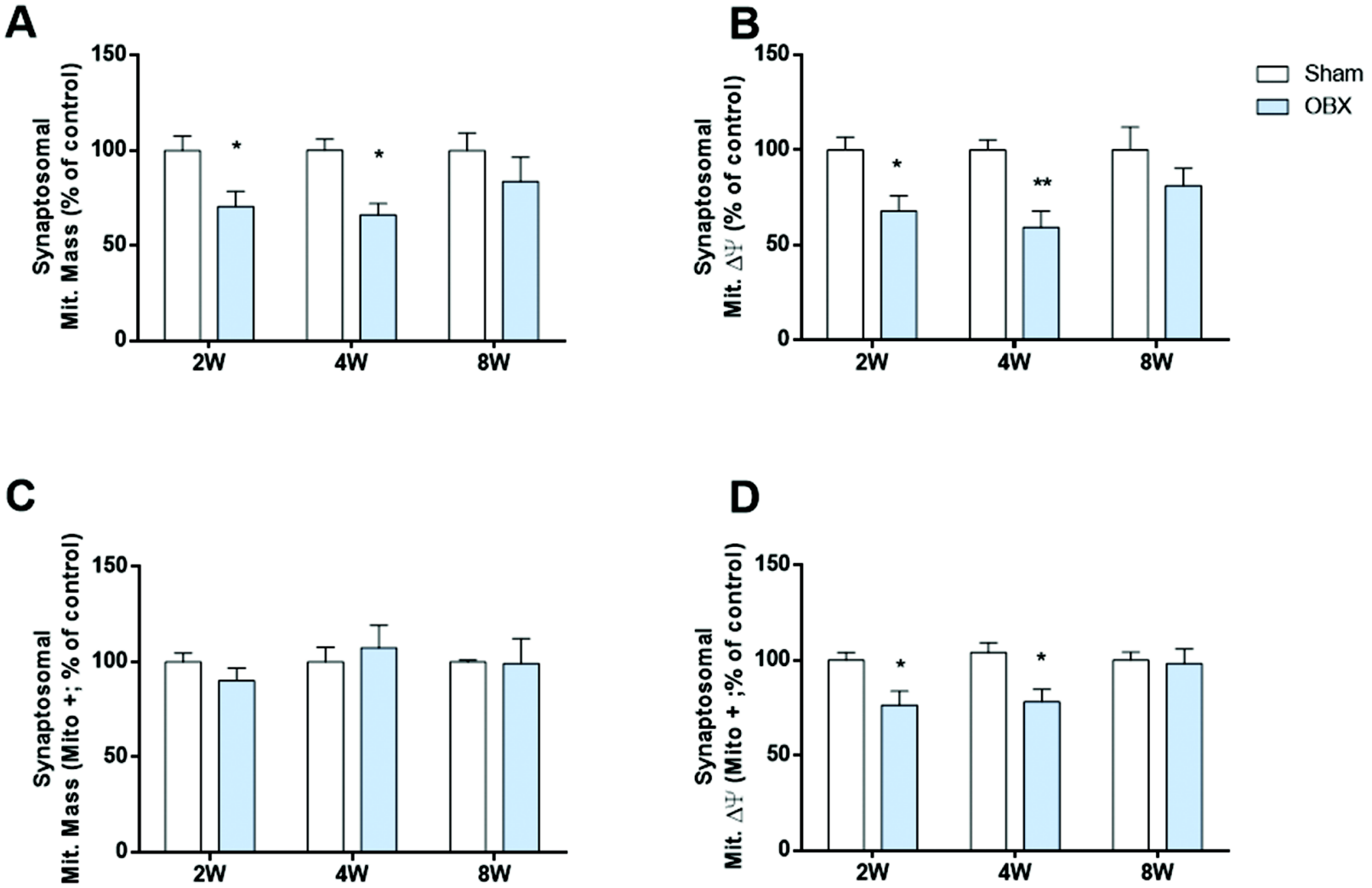


Fig. 7 – The effect of OBX on redox homeostasis

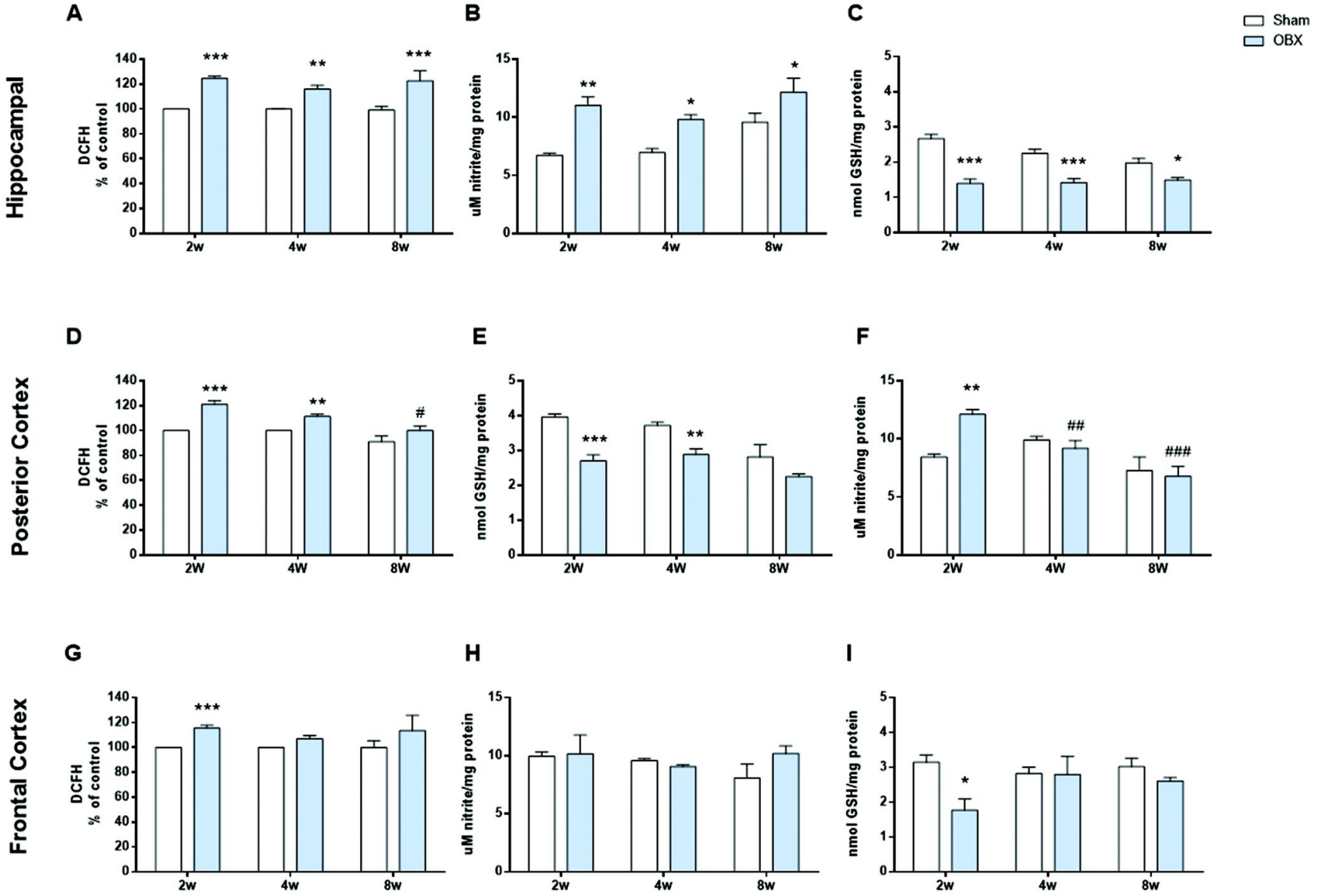
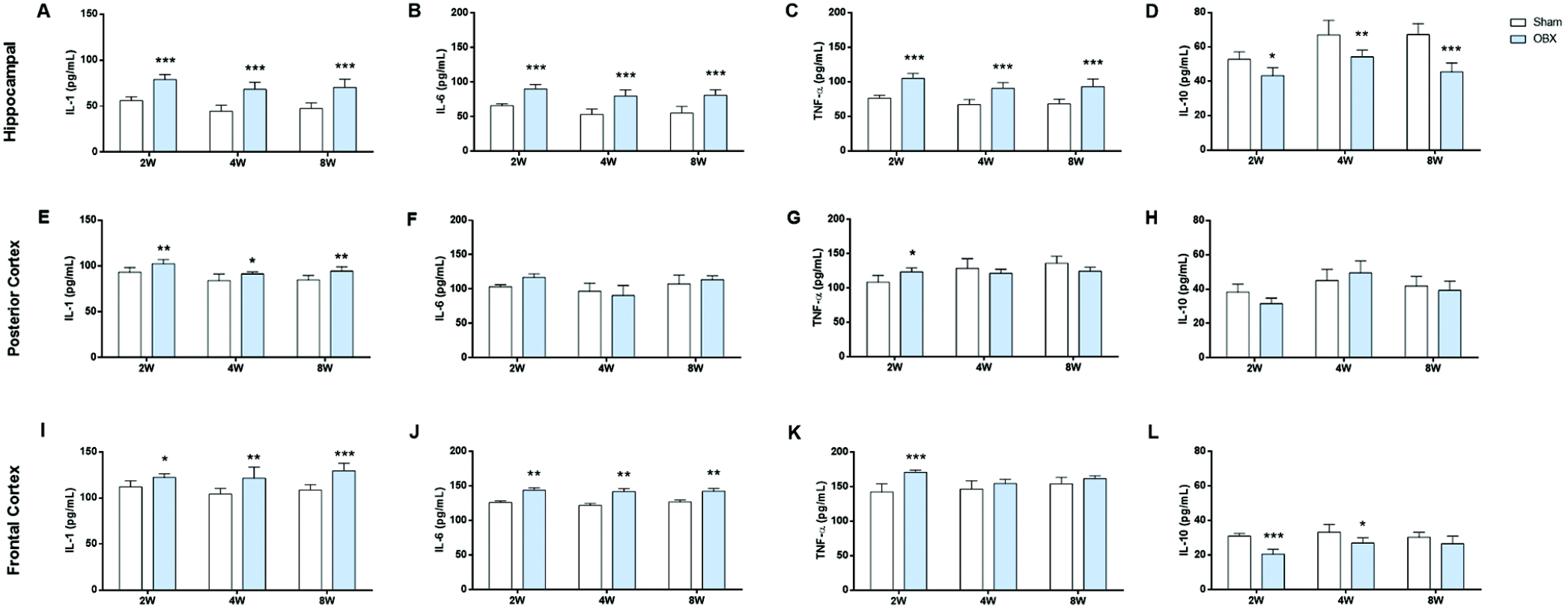
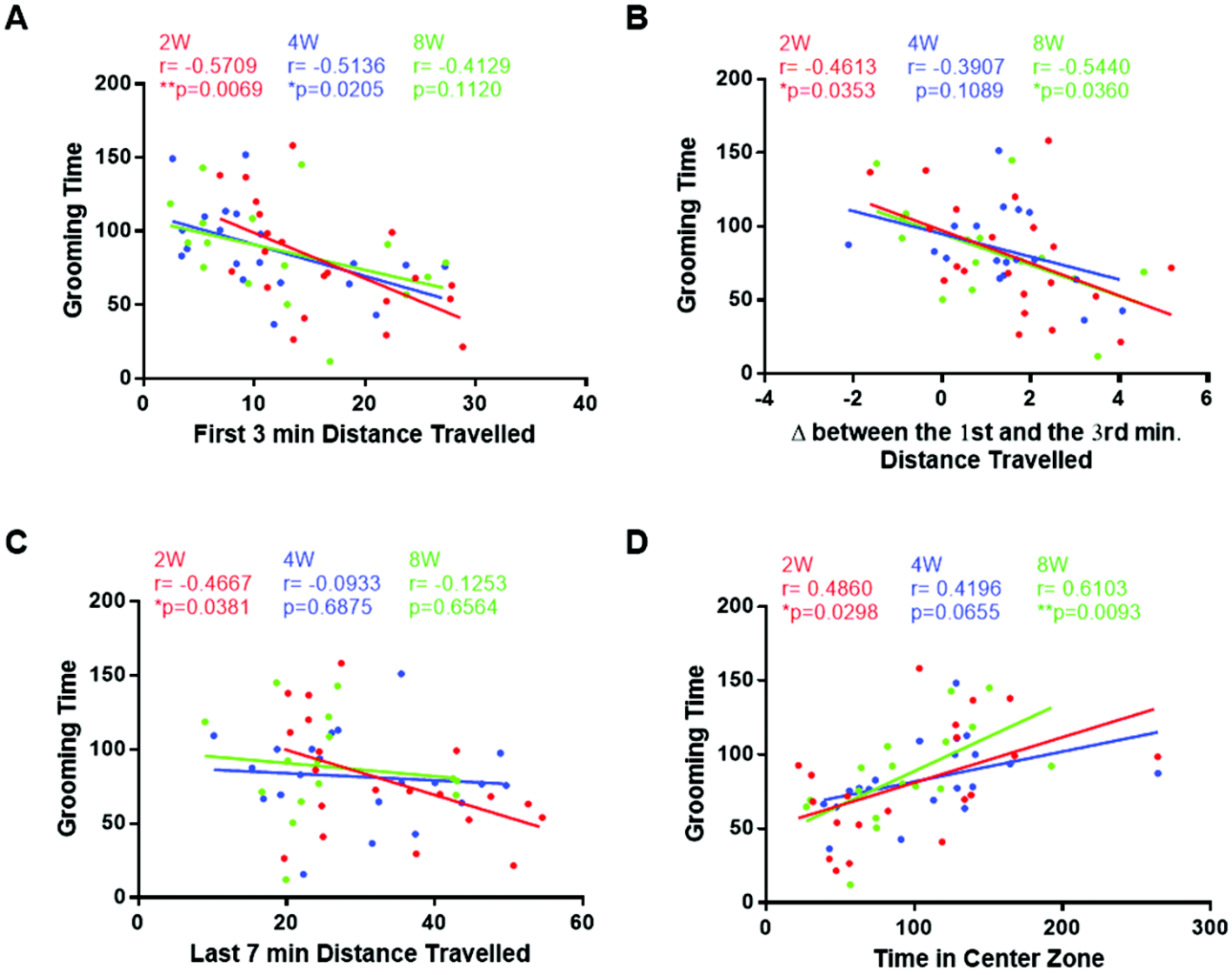


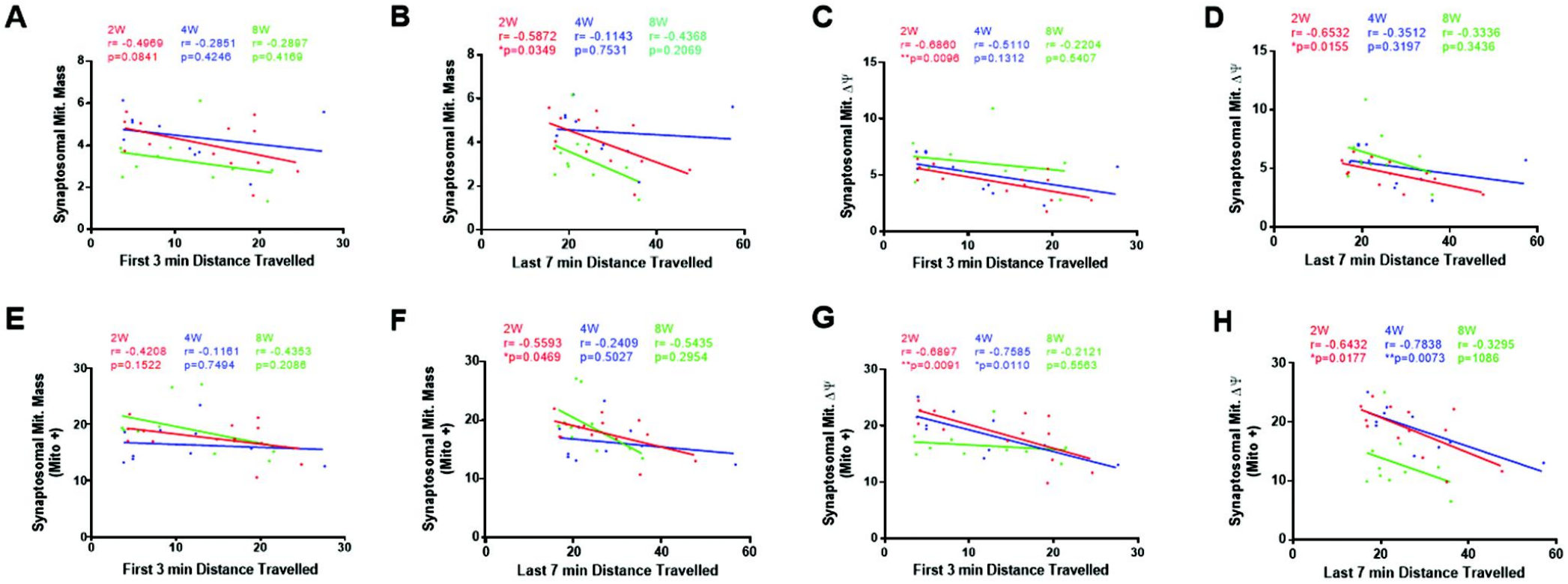
Fig. 8 – The effect of OBX on inflammatory parameters



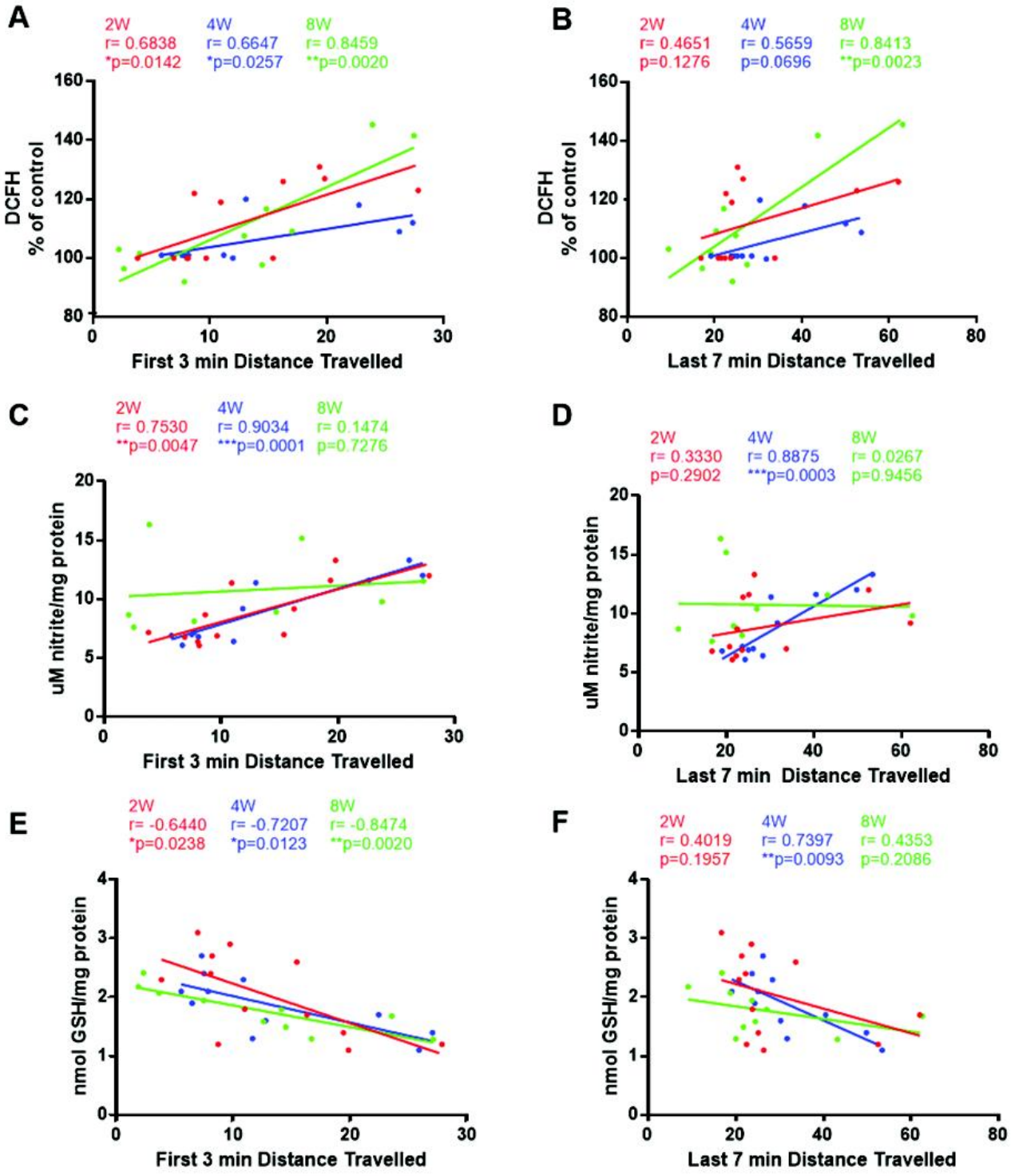
ESM_2 - Correlation between the grooming time in the splash test *versus* the behavioral performance in the OFT



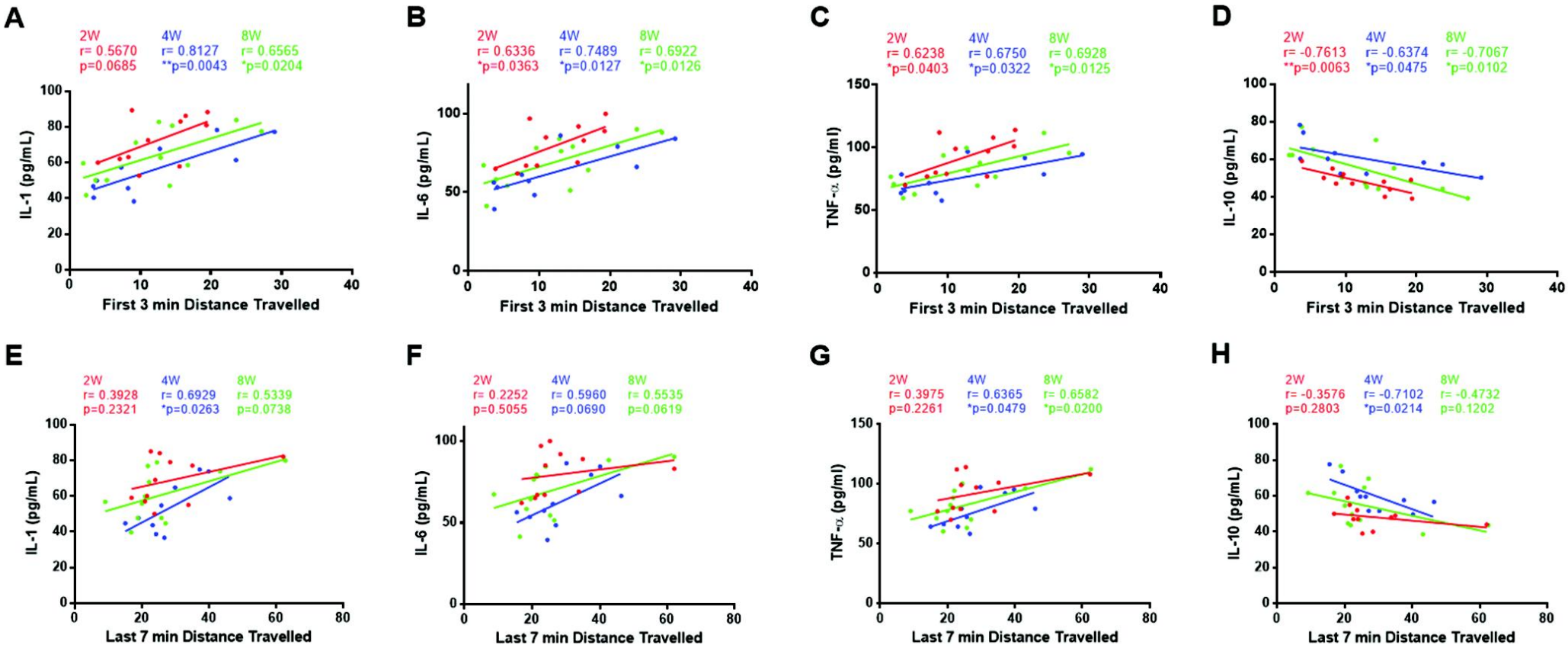
ESM_3 – Linear correlations between hippocampal synaptosomal mitochondrial parameters and the distance traveled during the first 3 minutes or last 7 minutes of the OFT.



ESM_4 – Linear correlations between hippocampal redox parameters and the distance traveled during the first 3 minutes and last 7 minutes of the OFT.



ESM_5 – Linear correlations between hippocampal inflammatory parameters and the distance traveled during the first 3 minutes and last 7 minutes of the OFT



Capítulo III: *Guanosine treatment improves the long-term behavioral changes and the impairment in hippocampal redox homeostasis induced by Bilateral Olfactory Bulbotectomy in mice.*

No **capítulo III** apresentamos o artigo que ainda está sob preparação.

Guanosine treatment improves the long-term behavioral changes and the impairment in hippocampal redox homeostasis induced by Bilateral Olfactory Bulbotectomy in mice.

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Abstract:

Major depressive disorder (MDD) is the most prevalent mental disorder and the leading cause of disability worldwide. Patients with recurrent episodes, present a notable intellectual (learning and memory abilities) compromising, which was at the same time an early and long-lasting prodromal sign, being an important focus to antidepressant drug development. Recently, we have proposed that Olfactory Bulbectomy (OBX) in mice is a suitable model to investigate long-lasting effects associated to depressive symptomatology, including some strictly parameters related to cognitive functions. Several lines of evidences have suggested that the purinergic signaling could be dysregulated in patients with MDD, and guanosine (GUO) signaling seems to be a promising target. Taken all these evidences, the present study aimed to investigate the potential antidepressant effect of chronic GUO treatment in mice submitted to OBX model of depression. The results presented here shows that chronic GUO treatment for 45 days, similarly to the classical tricyclic antidepressant IMI, was able to improve the long-term behavioral phenotype impairment induced by OBX, specifically improving behavioral performances that require cognitive functions, accompanied by reversion of hippocampal redox imbalance parameters (increase in intracellular ROS and NO levels, and decrease in the GSH levels), as well as in peripheral and central (hippocampal) anti-inflammatory IL-10 release. Thus, considering that our main findings, for the first time, pointed an improvement in memory components, accompanied by some hippocampal neuromodulatory effects promoted by GUO, which reinforce the GUO neuroprotective effect and establish news perspective in MDD therapeutic developments.

1. Introduction

Major depressive disorder (MDD) is the most prevalent mental disorder and the leading cause of disability worldwide (Schmaal et al., 2016; Vos et al., 2012). MDD is a multifactorial triggered disorder with complex symptomatology, with no validated biological markers that can be used to a definitive diagnostic, leading to a usually lately diagnosis (M. Y. Lee et al., 2016). Currently, MDD diagnosis is defined based on criteria published by the 5th edition of the Diagnostic and Statistical Manual of Mental Disorders (Bortolato et al., 2016; Czeh et al., 2015). As pointed, an individual need to have chronic (2 weeks or more) depressed mood (markedly diminished interest or pleasure in all, or almost all activities) accompanied by others behavioral changes (Czeh et al., 2015). Accordingly, in patients with recurrent episodes, the most frequent and at the same time an early and long-lasting prodromal sign that appears simultaneously with the depression mood is a notable intellectual (learning and memory abilities) compromising (R. S. Lee, Hermens, Porter, & Redoblado-Hodge, 2012; Williams et al., 2000). As described, short-term and long-term memories components are compromised in depressed patients and may contribute to the ineffectiveness of a chronic antidepressant therapy (Papakostas, 2014). Therefore, the cognitive deficit associated to depression is an important focus to antidepressant drug development (Bora et al., 2013; Bortolato et al., 2016; Maeshima et al., 2013).

In order to provide valuable information on the underlying pathophysiology of MDD and related symptoms, Bilateral Olfactory Bulbectomy (OBX) animal model of depression have been used (Czeh et al., 2015; Mucignat-Caretta et al., 2006; Song & Leonard, 2005; Yuan & Slotnick, 2014). After OBX surgery, a long-term disruption in compensatory pathways of the cortical–hippocampal–amygdala circuits occurs,

leading to dysfunctional signaling in the limbic brain circuitry, which induces prominent behavioral changes that resemble to the symptomatology observed in MDD patients (Czeh et al., 2015; Mucignat-Caretta et al., 2006; Song & Leonard, 2005; Yuan & Slotnick, 2014). Recently, we have proposed that OBX in mice is a suitable model to investigate long-lasting effects associated to depressive symptomatology, including some parameters related to behavioral performance (unpublished result). To note, OBX induced transitory anhedonic-like behavior, together with a long-lasting habituation to novelty impairment, hyperactivity and anxious-like phenotype (unpublished result). Moreover, OBX induced a long-lasting hippocampal imbalance in redox homeostasis and increased the inflammatory response, accompanied by a transitory impairment in the synaptosomal mitochondrial functionality (unpublished result).

Interestingly, several lines of evidences have suggested that the purine cycle, as well as the purinergic signaling could be dysregulated in patients affected with MDD (Ali-Sisto et al., 2016; Kesebir, Tatlidil Yaylaci, Suner, & Gultekin, 2014; Ortiz et al., 2015). In accordance with these data, it was demonstrated by a clinical and longitudinal study, a decreased in the serum guanosine (GUO) levels in MDD patients in comparison with non-depressed controls (Ali-Sisto et al., 2016). GUO is an endogenous nucleoside that presents a wide neuroprotective potential counteracting harmful effects observed in different animal models of brain disorders (Bettio, Gil-Mohapel et al., 2016; Schmidt et al., 2007). Regarding to MDD, GUO acutely administered in rodents has shown an antidepressive potential (Bettio et al., 2012; Bettio et al., 2014). In such conditions, GUO seems to play an important role as an extracellular signaling molecule that modulate some neurotrophins, display antioxidant activity, attenuate the increases in inflammatory response, and attenuate

the glutamatergic toxicity (Bettio, Gil-Mohapel et al., 2016). Therefore, considering that nowadays more than 30% percent of patients with MDD do not profit from conventional antidepressant treatments (Balestri et al., 2016; Berton & Nestler, 2006), new advances in drug development are extremely necessary and GUO signaling seems to be a promising target.

Taken all these evidences together, the present study aimed to investigate the potential antidepressant effect of chronic GUO treatment in mice submitted to OBX model, evaluating the long-lasting behavioral (exploring patterns closely related to cognitive function) and neurochemical (redox and inflammatory parameters) changes.

2. Materials and Methods:

2.1. Animals

Male C57BL/6 mice (45-50 days, 20-25g) were obtained from Fundação Estadual de Produção e Pesquisa do Rio Grande do Sul, Porto Alegre, Brazil. Animals were housed 5 per cage and allocated in a room with controlled temperature (22 ± 1 °C), under a 12h/12h light/dark cycle with *ad libitum* access to food and water. The cages were placed in the experimental room 24 h before behavioral tasks for acclimatization. All manipulations were carried out between 15:00 and 18:00h. All present procedures were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals and approved by the local Ethics Committee (project number 24577). All efforts were made to minimize animal suffering and the number of animals used in the experiments.

2.2. Drugs and Treatment Schedule

GUO and Imipramine (IMI) were purchased form Sigma Chemicals (St. Louis, MO, USA). All drug solutions were freshly prepared before administration

(dissolved in saline) and intraperitoneally injected (i.p.) at a dose of 40 mg/kg for IMI and 7.5 mg/kg for GUO (in a volume of 10ml/kg).

To evaluate the neuroprotective potential of GUO against long-term effects induced by OBX, the experimental protocol depicted in Fig 1 was used. Firstly, naive animals were submitted to Open Field Task (OFT) 1 day before the OBX surgery (OFT1) in order to verify the baseline activity and to discard any animal displaying behavioral abnormality. After 14 post-operative days (recovery period), the mice were re-evaluated in the OFT (OFT2) to evaluate the depressive-like condition. Then, the animals were randomly assigned to the following groups (10-12 mice per group): Sham Sal, OBX Sal, Sham IMI (40 mg/kg), OBX IMI (40 mg/kg), Sham GUO (7.5 mg/kg) and OBX GUO (7.5 mg/kg). In the same day after OFT2, the chronic treatments started with the drugs been injected once a day over the followed 45 days.

2.3. Bilateral Olfactory Bulbectomy

2.3.1. Surgical procedure

Bilateral olfactory bulb ablation was performed as previously described (Poretti et al., 2015). Briefly, mice were anaesthetized with a combination of xylazine (6 mg/kg) and ketamine (100 mg/kg) diluted in saline given by intraperitoneal (i.p.) route. The animals were fixed in a stereotactic frame (Stoelting Co., USA) and the skull was shaven and a burr hole (approximately 2 mm in diameter) was made above the olfactory bulbs, 4 mm rostral to the bregma. Both olfactory bulbs were then disconnected with a surgical micro scissors and removed by suction with a glass Pasteur pipette. Sham operations on mice serving as controls were done in the same way, but with the bulbs left intact. Animals were eliminated from the study if the bulbs were not completely removed or when the frontal cortex was injured (Roche, Harkin, & Kelly, 2007).

2.4. Behavioral tasks

2.4.1. Open Field Task (OFT)

OFT previously described by (Zueger et al., 2005) was used to investigate locomotor/exploratory activity, recognition activity and anxiety-like phenotypes. Mice were placed near to the sidewall in a gray wooden box (50×50×50 cm, length × width × height) with 200 lux light intensity, and then recorded individually during 10 minutes. All mice were recorded by a video-camera (positioned above and at ca. 90° to the square arena) connected to a monitor. The mice performance was analyzed using the AnyMaze® software, and the following parameters were determined: total distance traveled in the pre surgery, in the post-surgery and in the post treatment periods. Additionally, it was also evaluated the distance traveled during the first 3 minutes (considered as habituation to novelty) and the last 7 minutes (considered as locomotor /exploratory activity), and the total time of immobility (an immobile episode was only considered when the mouse stayed more than 5 seconds immobile). Finally, it was evaluated the percentage of the distance travelled in the center zone, to measure the anxiety-related phenotypes. The apparatus was cleaned with alcohol 70° and dried between animal trials.

2.4.2. Object Recognition Task (ORT)

The object recognition task (ORT), with minor modifications, was used to evaluate recognition memory (Figueiredo et al., 2013). ORT is based on the tendency of mice to discriminate a novel object (NO) from a familiar object (FO). ORT was performed at the same OFT apparatus. Mice were individually habituated to the OFT apparatus for 10 minutes in the OFT section performed in the day before. The task consists of an acquisition phase trial (training section – ORT1) and a test phase trial (test section – ORT2). The total distance exploring the arena was used to determine

the locomotor activity in the training and the test sections, which were analyzed by AnyMaze® software. During the acquisition phases, 2 identical objects were placed in a symmetric position in the center of the apparatus for 10 minutes. In the training section it was measured the total distance travelled and the time of exploratory activity in each object. Twenty-four hours after, a novel object replaced one of the familiar objects, and the exploratory activity was analyzed for 10 minutes. Again, in the test section the total distance travelled and the time of the exploratory activity in each object were evaluated for each animal. After each session, objects were thoroughly cleaned with 70% ethanol to prevent odor recognition. Exploration of an object was defined as rearing on the object or sniffing it at a distance of less than 1 cm, touching it with the nose, or both. Successful recognition of a previously explored object was reflected by preferential exploration of the NO. By definition, animals that recognize the FO explore the NO more than 50% of the total time.

2.4.3. Y-maze Test (YMT)

Y-maze test, with minor modifications, was conducted according to (Jaehne, Corrigan, Toben, Jawahar, & Baune, 2015). The apparatus consisted of grey wooden walls with 3 identical arms (30 × 8 × 15 cm each at an angle of 120° from the others). This experiment was a two-trial task with a training phase and a test phase trials, separated by an intertrial of 30 min. In the training phase trial, each mouse was individually placed in the maze with one of the 3 arms closed, and were allowed freely to explore the other 2 arms for 5 minutes, and then allocated back to the home cage. Thirty minutes after, the animal was again placed in the maze with all 3 arms opened, and was allowed freely to explore all the arms. The previously closed arm, opened in the test phase trial, was defined as the new arm. The animal performance

was video-recorded for later analysis. The time spent and the total distance travelled in the new arm was analyzed using AnyMaze® software.

2.5. Biochemical assays

After the behavioral protocols, mice were anesthetized, blood was collected by cardiac puncture, and mice were then transcardiacally perfused with PBS, and sacrificed by decapitation; brains were removed, and the hippocampi dissected at 4^0 C. The samples were frozen at -80 ºC for biochemical evaluations.

2.5.1. Estimation of redox homeostasis parameters

2.5.1.1. Reactive Oxygen Species (ROS) levels

To evaluate the levels of ROS, the hippocampi were homogenate in phosphate-KCl (20mM/140mM) buffer, pH=7.4, and centrifuged at 1,000×g×5 minutes at 4 ºC. An aliquot of supernatant was used to evaluate 20,70 - dichlorodihydrofluorescein diacetate (DCFH-DA) oxidation. DCFH-DA (7μM) oxidation was determined spectrofluorimetrically. Fluorescence was determined at 488 nm for excitation and 520 nm for emission. A standard curve was carried out using 20,70 - dichlorofluorescein (DCF) (Perez-Severiano et al., 2004). Results are shown as delta of DCFH-DA oxidation between 15 and 30 minutes of incubation.

2.5.1.2. Nitrite (NO) levels

NO levels were determined by measuring the amount of nitrite (a stable oxidation product of NO) in hippocampus homogenate, as indicated by the Griess reaction. The Griess reagent was a 1:1 mixture of 1% (w/v) sulphanilamide in 2.5% (w/v) phosphoric acid and 0.1% (w/v) N-(1-napththyl) ethylene diamine dihydrochloride in deionized water. Briefly, the tissue was homogenized in phosphate-KCl (20mM/140mM) buffer pH=7.4, and centrifuged at 1,000×g×10 minutes at 4 ºC. The supernatant was deproteinized with 20 μl TCA 25%, centrifuged

at 2,000×g×10 minutes at 4 ºC and immediately neutralized with 2 M potassium bicarbonate. After this procedure, the Griess reagent was added directly to the neutralized sample, and incubated in the dark for 15 minutes, at 22 ºC (Hansel et al., 2014). Samples were analyzed at 550 nm on a microplate spectrophotometer. Nitrite concentrations were calculated using a standard curve and the results are expressed as percentages relative to the control conditions.

2.5.1.3. Glutathione (GSH) levels

GSH levels were assessed as previously described (Hansel et al., 2014). Hippocampi were homogenate in a phosphate-KCl (20mM/140mM) buffer pH=7.4, containing 5 mM EDTA, and the protein was precipitated with 1.7% meta-phosphoric acid. The homogenate were centrifuged at 1,000×g×5 minutes at 4 ºC, the supernatant was mixed with o-phthaldialdehyde (at a concentration of 1 mg/ mL methanol) and incubated at 22 °C for 15 minutes. Fluorescence was measured using excitation and emission wavelengths of 350 nm and 420 nm, respectively. A calibration curve was performed with standard GSH solutions. GSH concentrations were calculated as nmol/mg protein.

2.5.2. Inflammatory Cytokine Levels

To obtain serum samples, the total blood was centrifuged at 5,000×g×10 minutes into silicone-coated tubes, and the serum was collected. Hippocampi were homogenized in PBS/Tris-HCl/SDS 5% pH 7.4 and further centrifuged at 5,000×g×10 minutes 4 °C, for collecting the supernatant.

Enzyme-linked immunosorbent assay (ELISA) kits for rat IL-1, IL-6, TNF-α, INF-γ and IL-10 were used accordingly to the manufacturer's instructions (eBIOSCIENCE, San Diego, CA, USA). Briefly, 96-well microplates were incubated with the primary antibody at 4 °C overnight, washed and blocked at room temperature

for 1 h. The cytokine standards, calibrators, and samples were added in triplicate to the plate and incubated at room temperature for 2 h. After washing, the secondary antibody conjugated with peroxidase was added and incubated at room temperature for 1 h. After this procedure, the samples were washed and tetramethylbenzidine chromogen was added. The enzyme reaction was stopped after 15 minutes, by adding phosphoric acid (H_3PO_4) 1M. The absorbance was measured at 450 nm. The results are expressed as pg/mg protein for tissue samples.

2.6. Protein determination

Protein content was measured using the Pierce BCA® protein kit (Thermo Scientific, Waltham, MA, USA) with bovine serum albumin as a standard.

2.7. Statistical analysis

Two-way analysis of variance (ANOVA) followed by Tukey´s post hoc test was performed to analyze the time course changes induced by the surgery and the treatment in Sham and OBX groups on OFT [factors: (1: time) comparison among naive and the different Sham/OBX treated groups]. One-sample Student´s *t* test was used to compare the mean exploration time for each object in the ORT test section with the fixed value of 50%. Two-way ANOVA followed by Tukey´s post hoc test was used to analyze the effect of OBX surgery on ORT total distance travelled and biochemical parameters [factors: (1: surgery) Sham *versus* OBX in the same group; and (2: treatment) comparison among different treatment groups]. One-way ANOVA followed by Tukey's post hoc test was used to compare the Δ between the both objects in the different Sham and OBX groups in ORT training and test sections, as well as to compare the different groups distance travelled and time spent in each of the 3 arms on the YMT test section. On the YMT training section two-way ANOVA, followed by multiple comparisons using Uncorrected Fischer´s LSD test were

performed to compare the different groups distance travelled and time spent in each of 2 arms.

3. Results:

3.1. OBX surgery induced long-lasting behavioral changes in the OFT.

In the ESM_1A and B it was showed the demonstrative minute-by-minute distance traveled for mice from the naïve and Sham; and from the naïve and OBX groups respectively.

3.2. Chronic GUO treatment, similarly to IMI, completely prevented the OBX induced habituation to novelty impairment and hyperactivity.

In a minute-by-minute analysis it was observed in Fig. 2A that all the Sham groups presented a significant decrease in the distance travelled in comparison with the naive group, while in comparison with the OBX groups (Fig. 2B) just the IMI and GUO groups presented a significant decrease in distance travelled on the 2^{nd} and 3^{rd} minutes.

In the first 3 minutes, comparing the naive mice with the treated Sham groups (Sal, IMI and GUO), it was observed that re-exposure of the mice to OFT induced a significant decrease in the distance travelled (Fig 2C), accompanied by a significant increase in time immobility (Fig. 2E). However, by comparing naive mice, with OBX Sal group a long-lasting impairment in the habituation to novelty was observed, since a significant increase in the distance travelled (Fig 2B), accompanied by no difference in an increase in the time immobile in the first 3 minutes was demonstrated (Fig. 2E). Additionally, chronic GUO treatment, similarly to IMI, on the Sham groups did not impair any behavioral feature compared with the Sham Sal group, however, on the OBX groups both treatments completely reverses only the distance travelled in the

first 3 minutes in the OFT, since both groups differ significantly from the OBX Sal group (Fig 2C).

In the last 7 minutes, Sham animals from Sal, IMI and GUO groups presented a significantly decrease in the distance travelled (Fig. 2F), while only the Sham IMI group presented a significant increase in the time immobile (Fig. 2G). On the other hand, a long-lasting hyperactivity was observed comparing OBX Sal mice with their naive group evidenced by a significant increase in the distance travelled in the last 7 minutes in the OFT (Fig. 2F). Moreover, chronic GUO treatment, similarly to IMI, completely reverses the OBX hyperactivity, while only the OBX IMI treatment significantly enhance the time immobile (Fig. 2G).

Finally, OBX caused a significant long-lasting anxiogenic effect by decreasing the % of distance travelled in the center zone in comparison to the naive group (Fig. 2H), and chronic IMI or GUO treatments were not able to revert this phenotype.

3.3. Chronic GUO and IMI treatments improved long-term recognition memory disrupted by OBX in the ORT.

In the ORT training section, as depicted in Fig. 3A and B (with the insert), respectively, no significant differences in the total distance travelled, and for a particular object preference were observed comparing the different experimental groups.

Specifically related to the ORT test section, none difference in the total distance travelled was observed comparing the different experimental groups (Fig. 3C). Moreover, it was evidenced that all groups could discriminate the novel object from the familiar object 24 hours after the training section (long-term recognition memory), considering the meaning exploration time higher than 50% (Sham Sal: mean=66.16, t=4.80, df=9; OBX Sal: mean=56.63, t=3.59, df=9; Sham IMI:

mean=69.13, t=10.16, df=9; OBX IMI: mean=69.54 t=7.49, df=9; Sham GUO: mean=66.73, t=7.92, df=11; OBX GUO: mean=69.28, t=9.91, df=11). However, analyzing the Δ between the NO and the FO a significantly lower exploratory behavior was observed only in the OBX Sal group (Fig. 3D - insert), suggesting that both treatments was able to completely reverses the OBX-induced recognition impairment in long-term recognition memory.

3.4. Chronic IMI treatment impairs the short-term memory in YMT only in OBX mice, while GUO did not influence the short-term memory.

Regarding to the YMT, no differences were verified on the total distance travelled (ESM_2A), as well as on the total time spent in each arm (ESM_2B) in the training section comparing all the experimental groups. In the test section, it was observed that all experimental groups, except the OBX IMI group, increase significantly both the total distance travelled (Fig. 4A) and the time spent in the new arm (arm 3), as depicted in Fig. 4B.

3.5. Chronic GUO treatment, similarly to IMI, completely prevented the redox homeostasis imbalance induced by OBX

As depicted in Fig. 5A, a significant increase in intracellular ROS production (DCFH) and NO levels (Fig. 5B), accompanied by a significant decrease in the antioxidant GSH levels (Fig. 5C) were observed in the hippocampus of OBX Sal group in comparison with Sham Sal group. On the other hand, chronic GUO, as well as IMI treatments, completely prevented the hippocampal intracellular ROS production and NO increases induced by OBX surgery (Fig. 5A and 5B, respectively). Furthermore, only the chronic GUO treatment on the OBX mice completely prevented the GSH decreases, while, chronic GUO treatment in the Sham group (*per se*) significantly decreases the GSH levels (Fig. 5C).

3.6. Chronic GUO and IMI treatments completely prevented the decreases in peripheral anti-inflammatory cytokine (IL-10) induced by OBX.

Analyzing the peripheral pro-inflammatory parameters (IL-1, IL-6, TNF-α and INF-γ) no statistical differences were pointed comparing the Sham and OBX groups (data not shown). However, regarding to the anti-inflammatory IL-10 cytokine level, the OBX surgery leads to a significantly decreased in its levels as observed in OBX Sal group, while the chronic GUO and IMI treatments completely prevented this reduction (Fig. 6).

3.7. Chronic GUO and IMI treatments completely prevented the decreases in hippocampal anti-inflammatory cytokine (IL-10) induced by OBX, without any change in the increases in the pro-inflammatory cytokines stimulated by OBX.

OBX surgery induces significant increases in the hippocampal pro-inflammatory cytokines IL-1 (Fig. 7A), IL-6 (Fig. 7B), TNF-α (Fig. 7C), and INF- γ (Fig. 7D) as observed in OBX Sal mice comparing with the Sham Sal group. At the same time, chronic GUO and IMI treatments were unable to modulate the OBX-induced increase in the pro-inflammatory cytokines. Finally, as in the serum analysis, OBX surgery also decreased significantly the hippocampal anti-inflammatory cytokine IL-10 (Fig 7E) levels and chronic IMI and GUO treatments completely reverse this effect (Fig. 7E).

Discussion:

Recently, we reported that OBX is a suitable animal model to investigate long-lasting behavior and neurochemical events related to MDD. Indeed, OBX surgery induces long-term cognitive deficit (habituation to novelty impairment) and psychomotor agitation (increases in locomotor activity), which are associated with an

early and transitory anhedonic-like behavior (loss of self-care and motivational behavior). Moreover, these behavioral disturbances were correlated to a pro-oxidative and a pro-inflammatory hippocampal state, also observed in MDD patients (unpublished results). Here, the present study shows that after 45 days, chronic GUO treatment similarly to the classical tricyclic antidepressant IMI, was able to reverse some of these long-term behavioral impairments and neurochemical disturbances induced by OBX, highlighting the improvement of the behavioral performance that require specifically cognitive skills in OBX mice, a well-characterized model of depression, which present good face and construct validity. Thus, our present data reinforce the recent findings that the purinergic system is intimately involved in human MDD physiopathology (Ali-Sisto et al., 2016), and even more, suggest GUO as an alternative treatment to depressive-related behaviors.

The intracellular role of the guanine-based nucleotides, including the nucleoside GUO, during signaling through G-protein-coupled receptors is well-known (Bettio, Gil-Mohapel et al., 2016; Schmidt et al., 2007). However, even today the extracellular roles promoted by GUO were not fully understood (Bettio, Gil-Mohapel et al., 2016; Schmidt et al., 2007). To note, several lines of evidences have highlighted the fact that GUO, has relevant extracellular signaling in both physiological and diseased conditions, acting mainly as a neuromodulator (Bettio, Gil-Mohapel et al., 2016; Schmidt et al., 2007). Indeed, ours and other groups reported neuroprotective effects of GUO both in *in vitro* and *in vivo* models of several neurological diseases, as cerebral vascular hypoperfusion (Ganzella et al., 2012), ischemia (Hansel et al., 2014; Hansel et al., 2015; Ramos et al., 2016), neuropathic pain (Schmidt, Paniz et al., 2010), epilepsy (Torres et al., 2010), Parkinson (Giuliani, Romano et al., 2012) and Alzheimer diseases (Pettifer et al., 2004; Tarozzi et al.,

2010). In line with the refereed effects, GUO also exerted neuroprotective effects in animal models of neuropsychiatry disorders. GUO injection prevented the hyperlocomotion induced by dizocilpine (a drug-based animal model of schizophrenia) (Tort et al., 2004) and presented anxiolytic-like effect in predictive behavioral tasks (Almeida et al., 2016). In addition, in predictive (Forced Swimming test and Tail Suspension test) or acute (acute restraint stress) rodent models of depression, it was already demonstrated that GUO administration produces a potential antidepressive-like effect mainly due to its capability of modulating the antioxidant defenses, as well as to mitigating oxidative damage induced by acute restraint stress (Bettio et al., 2014).

OBX in rodents is consistently used for the screening of potential novel therapeutic agents with antidepressant activity (Czeh et al., 2015; Mucignat-Caretta et al., 2006; Song & Leonard, 2005; Yuan & Slotnick, 2014). The abnormal behavioral phenotypes induced by OBX, usually ameliorated by chronic administration of antidepressants, providing valuable pre-clinical information (Czeh et al., 2015; Hendriksen et al., 2015). However, as for the first time pointed in our previous work, the OBX model has a limited time-window to explore the behavioral phenotypes, an early stages after the olfactory bulb removal (2 weeks post-surgery) anhedonic-like behavior, accompanied by cognitive deficit, hyperlomocotor activity and anxious phenotype, which were maintained up until 4 weeks after surgery (unpublished result), illustrating a complex symptomatology commonly observed in patients suffering from MDD (Czeh et al., 2015; Hendriksen et al., 2015). In fact, OBX model resembles some relevant findings from clinical studies that evidenced a long-lasting cognitive impairment in a significant proportion of MDD patients after the depressive-like behavior disappears (Bortolato et al., 2016; Rock, Roiser, Riedel, &

Blackwell, 2014). Indeed, this persistent cognitive deficit induced by MDD is one focus of treatment nowadays. Here, we demonstrated, for the first time, that chronic GUO treatment, similarly to IMI, improves some behavioral abnormalities induced by OBX in the OFT, including the habituation to novelty impairment (a component strictly associated to cognition) and the hyperactivity, suggesting, a potential antidepressant-like effect accompanied by a cognitive improvement promoted by GUO treatment.

Interestingly, although OBX Sal group presented impairment in habituation to novelty the results presented here demonstrate that at 8 weeks after surgery they displayed recognition memory in the ORT. However, they presented a lower discrimination rate, implying long-lasting learning and memory deficit, indicating an incomplete remission of the MDD symptomatology, an effect similar to observed with remised MDD patients. Interestingly, the related disturbance was prevented by GUO, as well as by IMI treatments. Taking to account that different mechanism are involved in memory components, and changes in short-term memory were also compromised in MDD (Culpepper, 2015; Williams et al., 2000), we investigate the putative short-term memory component in the YMT. Interestingly, OBX animals did not present any deficit in short-term memory. Additionally, GUO *per se* (in Sham group), as well as in the OBX-treated mice, did not interfere in the spatial memory in YMT. However, while the chronic IMI treatment *per se* did not interfere in any parameter of the YMT, in OBX mice IMI impaired the behavioral performance. Accordingly, rats chronic treated with IMI (10 mg/kg) for 28 days were unable to discriminate the familiar object from the novel object in the ORT (Naudon, Hotte, & Jay, 2007), disturbing the recognition memory. Additionally, and reinforcing our data,

clinical studies also shown that tricyclic antidepressant presented a negative influence in some memory components (Bortolato et al., 2016; Nagane et al., 2014).

Curiously, forty-five daily GUO injections did not modulate any effect *per se* on the behavioral tasks conducted here. To note, some behavioral modulatory effects were already reported by acute and or short-term treatments with GUO (Bettio, Gil-Mohapel et al., 2016; Schmidt et al., 2007). Acute GUO injection in rats, as well as 2 weeks orally treatment causes amnesic effects (Giuliani, Buccella et al., 2012; Vinade, Izquierdo, Lara, Schmidt, & Souza, 2004; Vinade et al., 2003). Conversely, and in agreement with our data, orally chronic GUO treatment (6 weeks) did not modulate the behavioral performance in rats submitted to Morris water maze task (Ganzella et al., 2012). Taken collectively all these above-mentioned data, it was suggested that as a neuromodulator compound, a short disturbance in GUO level can negatively influence learning and memory capability, but when exposed for a long period, the neural system homeostasis could be adapted and the amnesic effect could be dissolved. Remarkably, GUO modulates directly the glutamatergic system (Almeida et al., 2016; Bettio, Gil-Mohapel et al., 2016; Schmidt et al., 2007). Since glutamate is the main neurotransmitter in the brain, and their signaling is essential to diverse brain functions, including learning and memory (Robinson & Jackson, 2016), a long-term exposure to supra-physiological GUO level can restoration pivotal neural circuits of brain influencing positively the learning and memory impaired by OBX.

Besides the GUO effects on learning and memory, it was also evidenced that acute and short-term GUO treatment induces anxiolytic-like effect (Almeida et al., 2016; Vinade et al., 2003). Here, it was not verified any GUO effect on the % of center distance comparing the different experimental groups. To date, it is important to highlight that GUO accompanied the IMI effect. The majority of studies presented

in the literature using chronic IMI only to explore their potential antidepressant-like effect. Accordingly, the few studies found in the literature reported that the potential anxiolytic-like effect promoted by IMI remained controversial (Borsini F, 2000, (Hart et al., 2016). Collectively our behavior data shows that GUO, in general, modulates positively some long-lasting changes impaired in the OBX mice, especially parameters related to the short and long-term memory, while IMI chronic treatment could display a side effect, since it was demonstrated impairment in short-term memory.

It is notorious that to perform behavioral tasks aiming to investigate parameters involved in learning and memory, as the ORT, hippocampus plays a crucial role in memory encoded, consolidated and retrieved (Cohen & Stackman, 2015). Regarding to the normal functions sub served by hippocampus, as the emotionality and the spatial/topographical memory, impairment in hippocampal function might be expected to contribute to some of the cognitive abnormalities of depression (Bartsch & Wulff, 2015). Considering that GUO prevented the disturbance in recognition memory elicited by OBX (habituation to novelty in the OFT, and the lower discrimination rate in the ORT), we could hypotheses that GUO could be act in hippocampal signaling pathways which is essential to improve the OFT and ORT performance. In consonance with this proposition and sustained our hypothesis, Bettio *et al*., 2016 showed that the antidepressant-like effect of chronic GUO treatment in tail suspension task is associated with increased hippocampal neuronal differentiation (Bettio, Neis et al., 2016).

As a well-recognized mechanism in aging and in disease, the redox imbalance plays a pivotal role in the pathophysiology of psychiatric disorders including MDD, since increases the susceptibility to oxidative damage by reduced expression of

antioxidant enzymes and increased production of ROS (Kim et al., 2008) (Maes, Galecki et al., 2011; Maes, Kubera et al., 2011; Moylan et al., 2014). Here, chronic GUO treatment completely reversed redox imbalance induced by OBX in hippocampus, differently from IMI, which did not modulate the GSH levels. Particularly, it was demonstrated by our group that GUO has a potential antioxidant effect mainly by its modulation in the homeostasis of GSH, the major nonenzymatic antioxidant in the CNS (Quincozes-Santos et al., 2014). Additionally, *in vitro* GUO was able to prevent the LPS-induced decrease in GSH content in C6 astroglial cell and primary cultures of rat hippocampal astrocytes (Bellaver et al., 2015). Thus, it is possible that GUO may exert its effects by direct radical scavenging activity and may also modulate signaling pathways, avoiding the increases in both intracellular ROS production and NO levels (Gudkov, Shtarkman, Smirnova, Chernikov, & Bruskov, 2006), which was evidenced by us in the present work . It is worth pointing that, in their metabolic pathway, GUO is converted into uric acid, which could exert a potent antioxidant effect and clinical finding pointed a significantly decreased in plasma of depressed patients (Liu et al., 2015; Ortiz et al., 2015). Thus, the chronic treatment with GUO could increase activity of the purine degradation causing the consequent increase in uric acid levels, which leads to a compensatory mechanism to counteract the oxidative stress.

Finally, oxidative stress in MDD is also associated with some of the inflammatory changes in depression (Maes, Kubera et al., 2011). In addition, the depletion of GSH content is intimately associated with inflammatory response and cytotoxicity (Haddad, 2000). Several data has demonstrated that OBX induced robust increases in inflammatory response (Borre et al., 2014; Rinwa et al., 2013; Yang et al., 2014). Our results show that chronic GUO treatment was able to completely

reverse only the decrease of the anti-inflammatory cytokine IL-10 induced by OBX in serum and in hippocampus of mice. Conversely, previous reports indicate that GUO inhibited TNF-α release (D'Alimonte et al., 2007), and reduced inflammation response measured by macrophage invasion (Hansel et al., 2015; Jiang et al., 2007), but the exact mechanism underlying these effects are not established. However, an additional mechanism could be considered in future studies, since the nucleoside adenosine exert a well-established anti-inflammatory effect (Ali-Sisto et al., 2016), and different studies conducted by our group observed that GUO administration could lead to a significant increase in extracellular levels of adenosine (Almeida et al., 2016; Ciccarelli et al., 2000). So, this pathway could be involved in the mechanisms by which GUO prevents the reduction of the anti-inflammatory cytokines, and attenuates the glutamate release from synaptosomal preparation. Finally, considering that our main findings pointing for the improvement in the memory components, accompanied by some hippocampal neuromodulatory effects promoted by GUO, although we did not verified here, we could speculate that GUO effects were associated with some neurogenesis-related process stimulating by GUO, as the hippocampal neuronal differentiation already evidenced (Bettio, Neis et al., 2016; Di Iorio et al., 2004; Rathbone et al., 1998).

Conclusion:

In summary, the present work demonstrated for the first time that GUO was able to improve the long-lasting behavioral impairment (including some remised parameters) in animal paradigms that require specifically cognitive skills, acting as an antidepressant drug in a well-characterized model of depression, which presented both face and construct validity. Additionally, it was shown that GUO could exert their action by attenuating the hippocampal redox imbalance, accompanied by avoiding

peripheral and central (hippocampal) anti-inflammatory IL-10 decreases induced by OBX. Since the dysregulation of the purine cycle can be a relevant point in the course of MDD, our results together reinforce the potential neuroprotective effects promoted by GUO, establishing news perspective in therapeutic developments.

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Compliance with Ethical Standards

Conflict of Interest:

The authors declare that they have no competing interests.

Legends:

Fig. 1 – Diagrammatic representation of the study protocol

Experimental schedule of the protocol lasting 2 and 8 weeks after OBX surgery. At 61° days, mice were euthanized and the brain structures were dissected for biochemical analysis. The sample were stored at −80 °C. (OFT1: first Open Field exposure – all groups were tested; OFT2: second Open Field exposure – all groups were tested. In the behavioral tasks mice were submitted to OFT3: third Open Field exposure; Object Recognition task and y-Maze task.

Fig. 2 – Effect of chronic IMI and GUO treatments in the habituation to novelty, locomotor activity and anxiety-related behavior impairment induced by OBX surgery

Locomotor activity in a minute by minute analysis in the OFT of naïve and Sham groups (A), as well as naïve and OBX groups (B). Each point represents the mean of the group. Effect of the Sal, IMI and GUO chronic treatments on distance travelled in the first 3 minutes (C), on the Δ between the 1st and the 3rd minutes distance travelled (D), on the first 3 minutes of time immobility (E), on distance travelled in the last 7 minutes (F), in the last 7 minutes of time immobility (G) and on the percentage of center distance travelled (H) in mice submitted to the Sham or OBX surgery. Each column represents the mean±S.E.M. Data were analyzed by two-way ANOVA, followed by Tukey´s multiple range post-hoc test. #p<0.05, ##p<0.01 and ###p<0.001 comparing the respective naïve group (time effect) with their respective Sham or OBX groups. *p<0.05 comparing the Sham group with their respective OBX groups (treatment effect). (n=10-12 animals/group).

Fig. 3 – OBX effect on long-term memory in the ORT

Effect of the Sal, IMI and GUO chronic treatments in total distance travelled in the training (A) and in test (C) sections in mice submitted to the Sham or OBX surgery. Each column represents the mean±S.E.M. In (B) and (D) were demonstrated the average exploration time for each object in the training and test section respectively, in mice submitted to the Sham or OBX surgery. Each column represents the mean±S.E.M. In (B) and (D) on the inserts were demonstrated the Δ between the each objects. Each column represents the mean±S.E.M. Data were analyzed by one-way ANOVA, followed by Tukey´s multiple range post-hoc test. *p<0.05 and ***p<0.0001 comparing the NO with the FO in each group. On the inserts*p<0.05

comparing the OBX Sal group with the others Sham (Sal, IMI and GUO) and OBX (IMI and GUO) groups; (n=10-12 animals/group).

Fig. 4 – OBX effect on short-term memory in the YMT

Effect of the Sal, IMI and GUO chronic treatments in the distance travelled in each arm (A) and in the time spent in each arm (B) in the YMT in mice submitted to the Sham or OBX surgery. Each column represents the mean±S.E.M. Data were analyzed by one-way ANOVA, followed by Tuke´s multiple range post-hoc test. *p<0.05 and **p<0.01 comparing the differences among arm 1, 2 and 3 in each different experimental group. (n=10-12 animals/group).

Fig. 5 – OBX effect in redox homeostasis

Effect of Sal, IMI and GUO chronic treatments on hippocampal DCFH (A), NO (B) and GSH (C) levels in mice submitted to the Sham or OBX surgery. Data are reported as the mean ± S.E.M. and were analyzed by two-way ANOVA followed by Tukey´s multiple range post-hoc test. *p<0.05 and ***p<0.001 comparing the OBX mice with their respective Sham groups; #p<0.05, ##p<0.01 and ###p<0.001 comparing the different Sham or OBX groups with Sham or OBX Sal groups. (n=5-6 animals/group).

Fig. 6 – OBX effect on the serum anti-inflammatory IL-10 cytokine

Effect of Sal, IMI and GUO chronic treatments on serum IL-10 cytokine levels (A) in mice submitted to the Sham or OBX surgery. Data are reported as the mean ± S.E.M. and were analyzed by two-way ANOVA followed by Tukey´s multiple range post-hoc test. **p<0.01 comparing the OBX mice with their respective Sham groups; ##p<0.01 comparing the different OBX groups with the OBX Sal group. (n=5-6 animals/group).

Fig. 7 – OBX effect on the hippocampal inflammatory cytokines response

Effect of Sal, IMI and GUO chronic treatments on hippocampal IL-1 (A), IL-6 (B), TNF-α (C), INF-γ (D), and IL-10 (E) levels in mice submitted to the Sham or OBX surgery. Data are reported as the mean ± S.E.M. and were analyzed by two-way ANOVA followed by Tukey´s multiple range post-hoc test. **p<0.01 and ***p<0.001 compared to their respective Sham group, ##p<0.01 comparing the different OBX groups with the OBX Sal group. (n=5-6 animals/group)

ESM_1 – Effect of OBX on habituation to novelty, locomotor activity and anxiety-related behavior in OFT

A representative minute-by-minute analysis of the locomotor activity of Sham (A) and OBX (B) groups during the OFT. Each point represents the mean of the group. (n=32 animals/group).

ESM_2 – OBX effect in the training section of the YMT

Effect of the Sal, IMI and GUO chronic treatments in the distance travelled in each arm (A) and in the time spent in each arm (B) in the YMT in mice submitted to the Sham or OBX surgery. Each column represents the mean±S.E.M. Data were analyzed by Two-way ANOVA followed by multiple comparisons using Uncorrected Fischer´s LSD. *p<0.05, **p<0.01 and ***p<0.001 comparing the differences among arm 1, 2 and 3 in each different experimental group. (n=10-12 animals/group).

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Fig. 1 – Diagrammatic representation of the study protocol

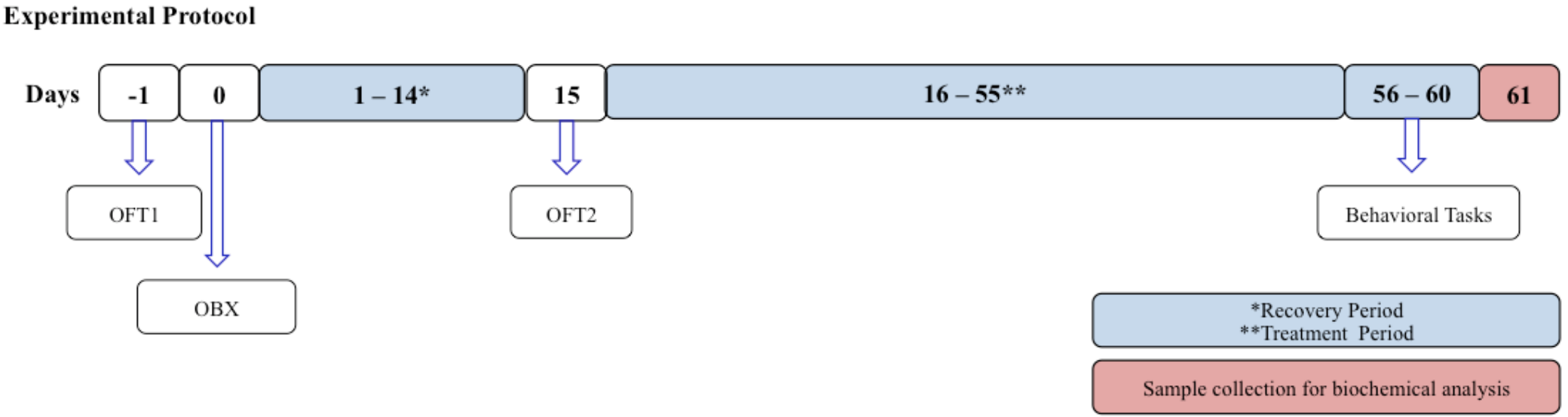


Fig. 2 – Effect of chronic IMI and GUO treatments in the habituation to novelty, locomotor activity and anxiety-related behavior impairment induced by OBX surgery

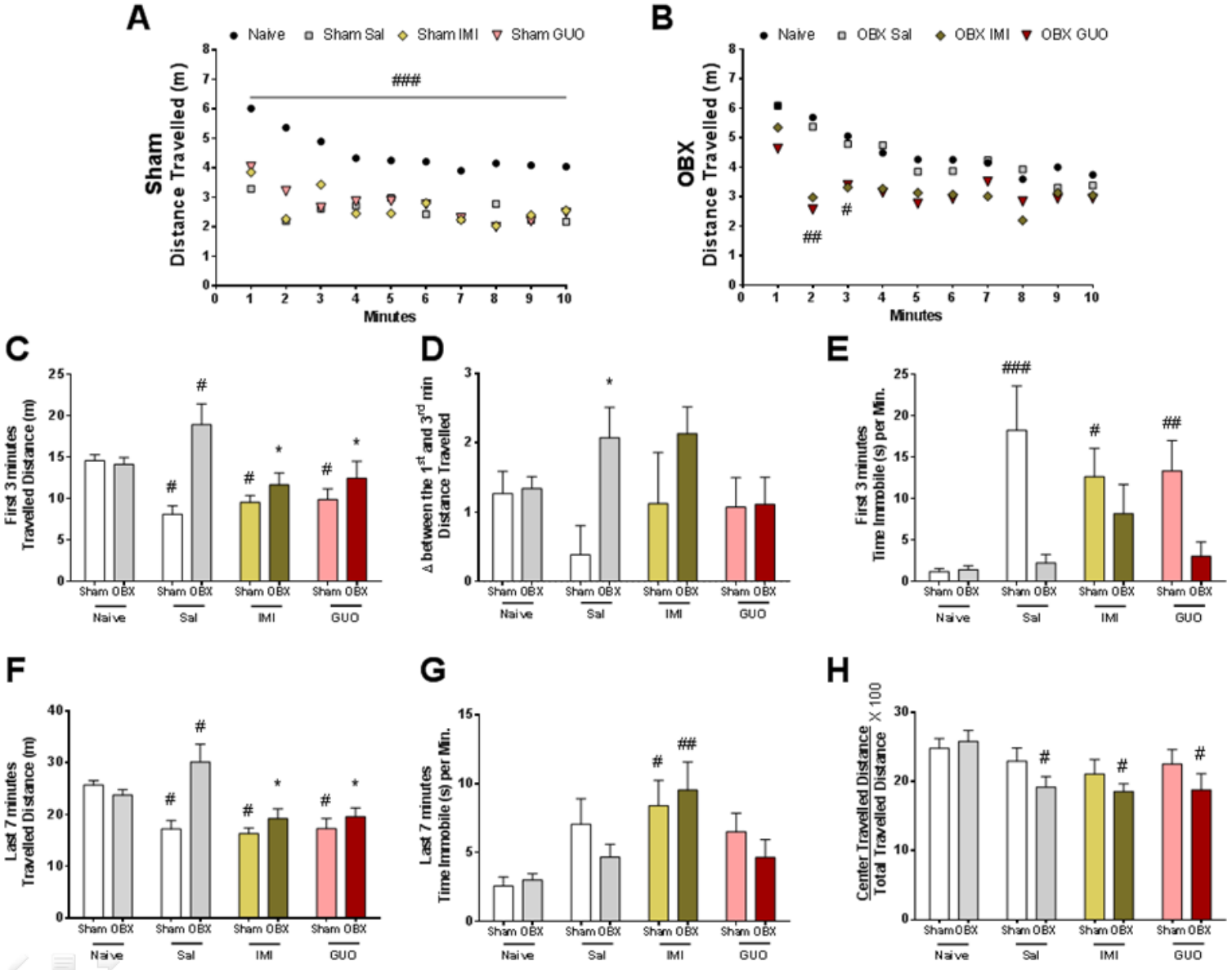


Fig. 3 – OBX effect on long-term memory in the ORT

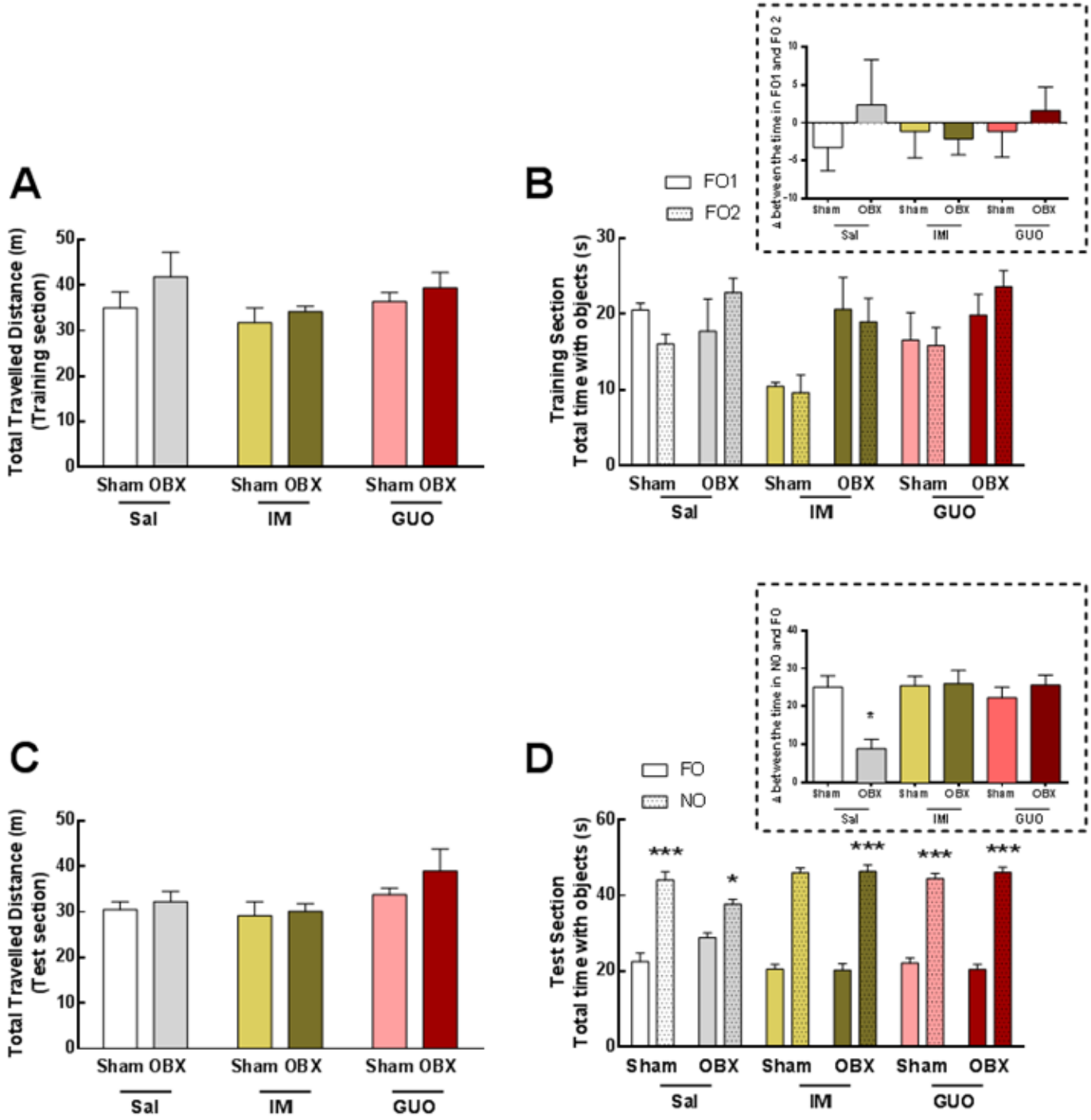


Fig. 4 – OBX effect on short-term memory in the YMT

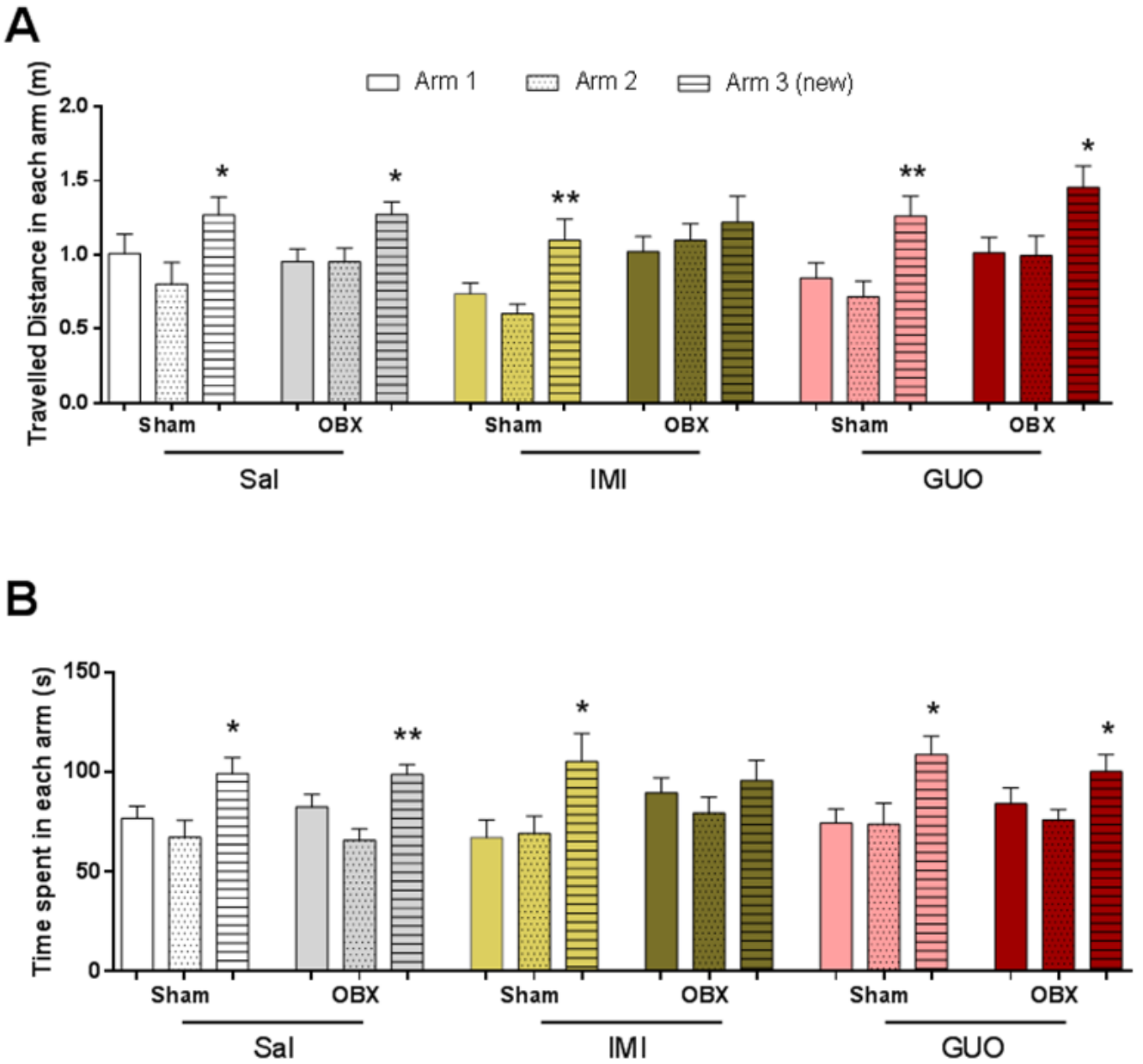


Fig. 5 – OBX effect in redox homeostasis

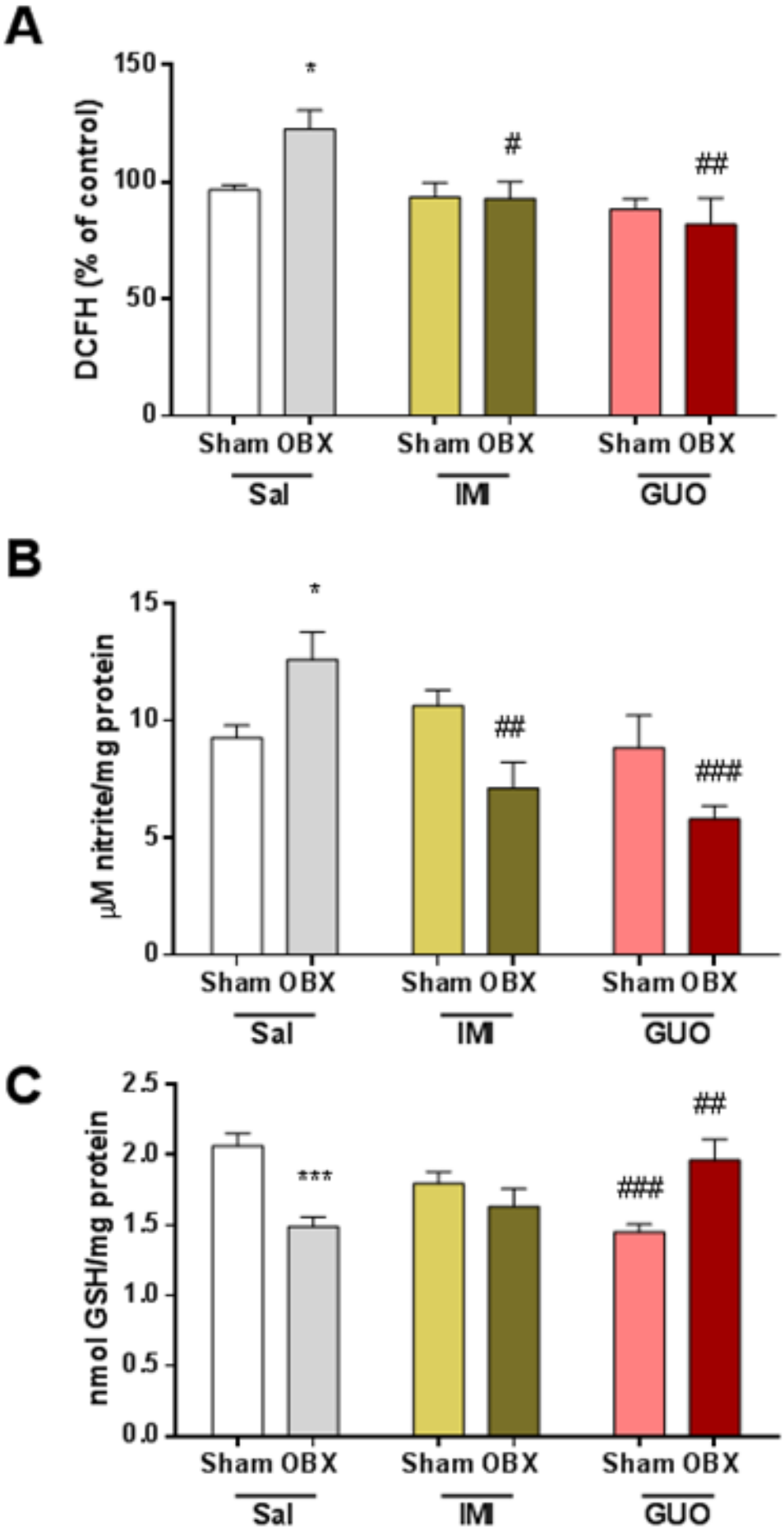


Fig. 6 – OBX effect on the serum anti-inflammatory IL-10 cytokine

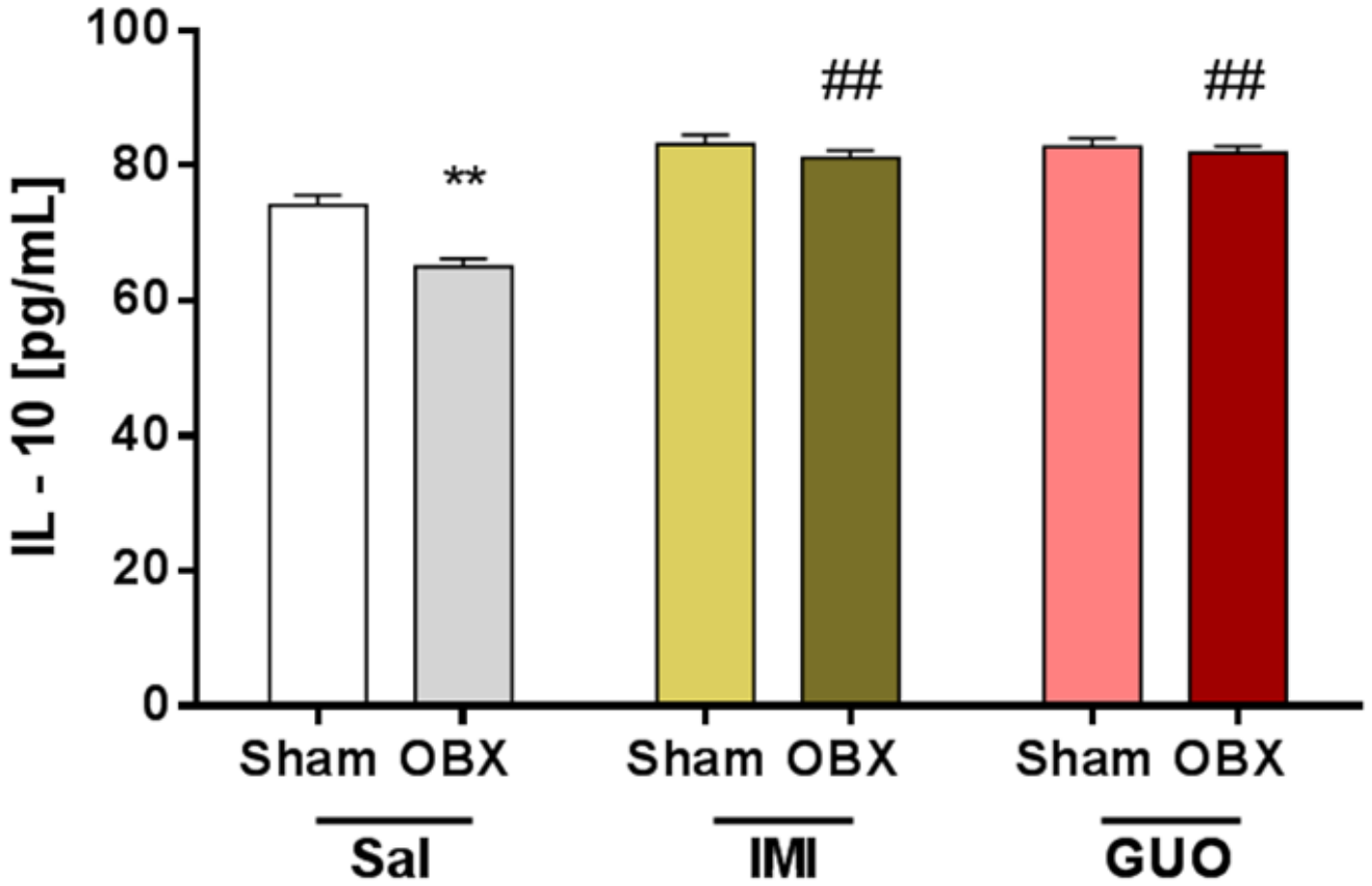
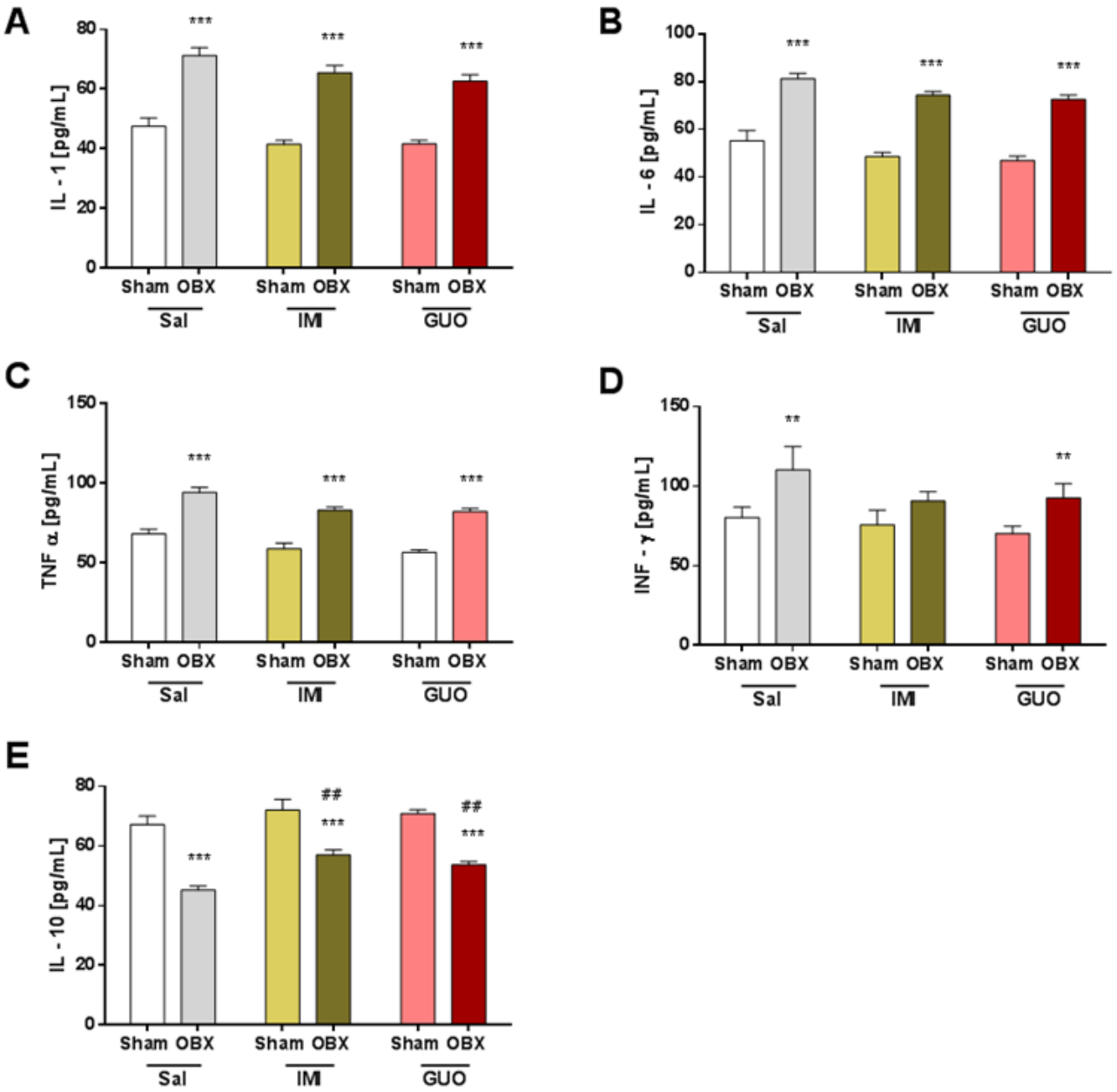
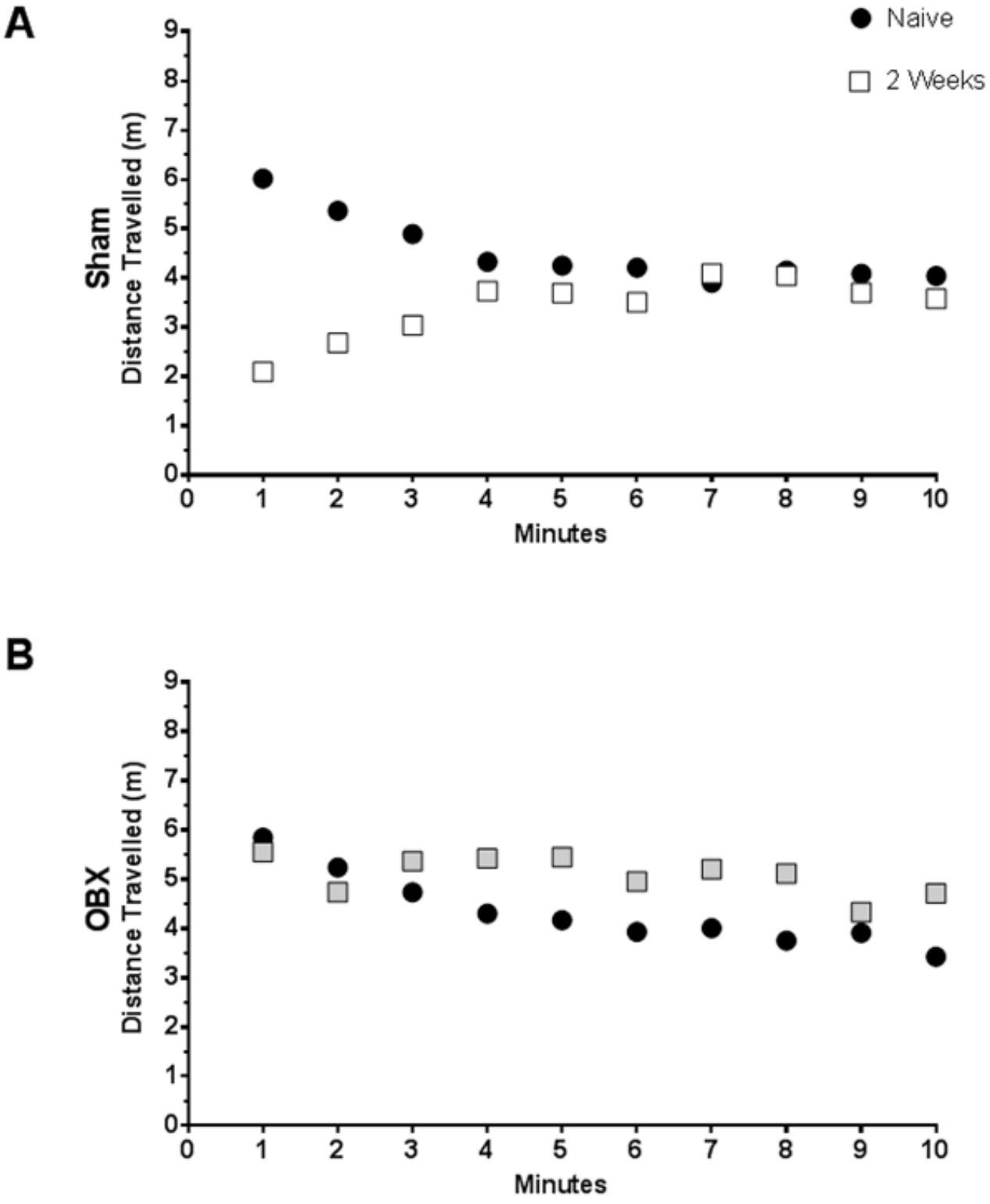


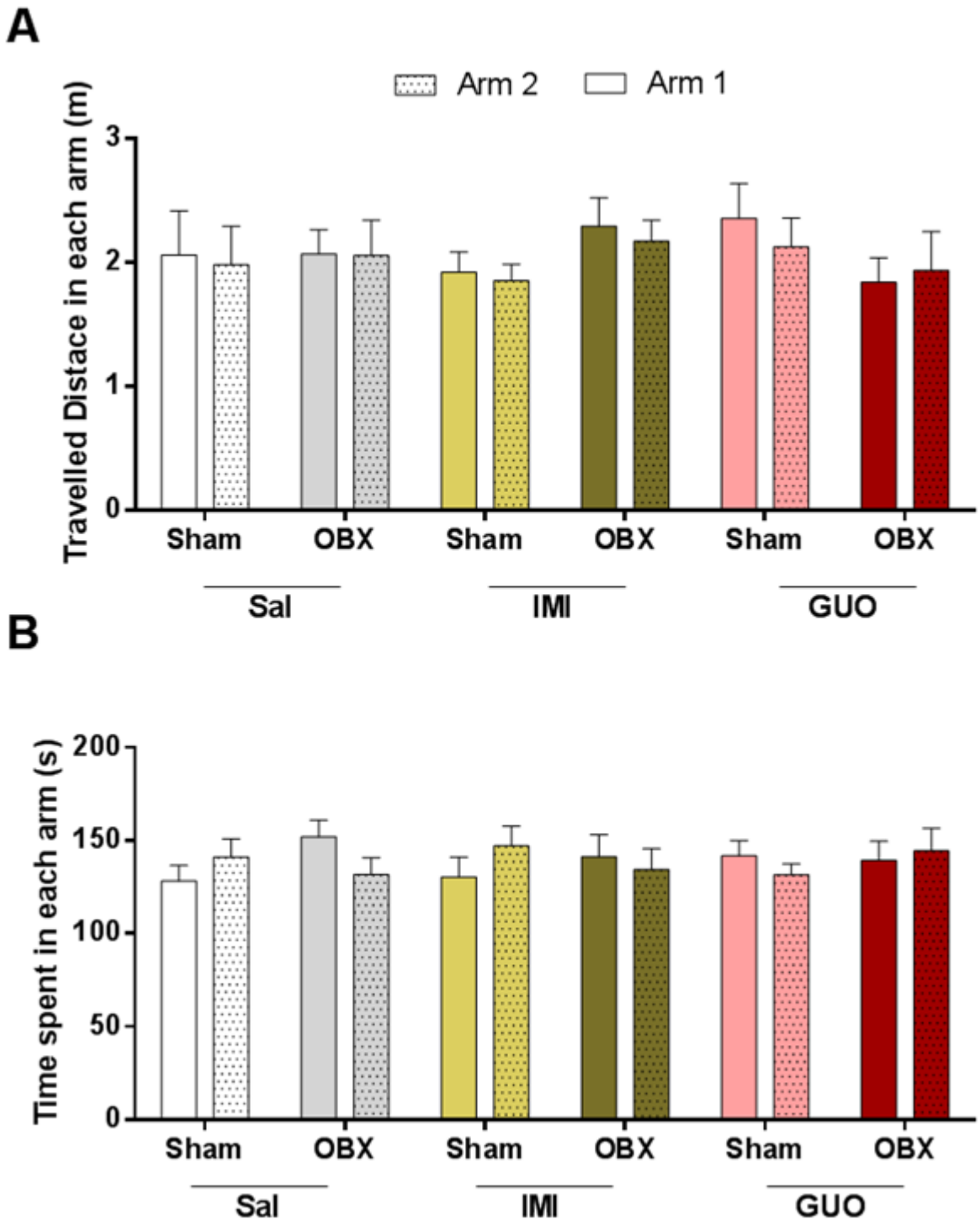
Fig. 7 – OBX effect on the hippocampal inflammatory cytokines response



ESM_1 – Effect of OBX on habituation to novelty, locomotor activity and anxiety-related behavior in OFT



ESM_2 – OBX effect in the training section of the YMT



PARTE III

3. DISCUSSÃO

O conhecimento crescente nas áreas da neurociências, especificamente nas vias de sinalização envolvidas na neurotransmissão glutamatérgica, renovaram a atenção para esse sistema de neurotransmissão como um possível alvo molecular para uma nova classe de drogas no tratamento de condições neuropsiquiátricas. Dessa maneira, estudos pré-clínicos têm sugerido que compostos capazes de modular a neurotransmissão glutamatérgica, através do antagonismo dos receptores ionotrópicos ou metabotrópicos de glutamato, ou ainda, diminuindo a liberação deste neurotransmissor nos terminais sinápticos, apresentam um potencial ansiolítico e/ou antidepressivo em modelos animais com validade preditiva para o estudo da ansiedade, assim como em modelos animais com validade preditiva, de face e de constructo para o estudo da depressão. (Cunha, Ferre, Vaugeois, & Chen, 2008; Lopes, Sebastiao, & Ribeiro, 2011; Machado-Vieira et al., 2009; Riaza Bermudo-Soriano et al., 2012).

Considerando a hipótese de que a GUO exerce suas ações neuroprotetoras através da modulação da neurotransmissão glutamatérgica, no primeiro estudo realizado nesta tese objetivou-se investigar o potencial ansiolítico da GUO através da sua administração sistêmica e aguda em ratos. Nossos resultados demonstram que a administração de GUO na dose de 7.5mg/kg foi capaz de induzir um consistente efeito ansiolítico em três diferentes paradigmas comportamentais com reconhecida validade preditiva (labirinto em cruz elevado, claro-escuro e campo aberto) para o estudo de fármacos com potencial efeito ansiolítico. Além disso, demonstramos que 60 minutos após a administração de GUO uma diminuição nos níveis de glutamato juntamente com um aumento nos níveis de adenosina (ADO) no líquor dos animais foram observados. Reforçando nossos resultados, o efeito comportamental foi

significativamente correlacionado com o aumento de ADO no líquor, assim como com a diminuição liquórica de glutamato. Tanto a GUO, quanto a ADO são nucleosídeos com importantes funções em diversos processos biológicos, e uma variação no tônus fisiológico da concentração destes nucleosídeos está intimamente associada com a modulação dos níveis de glutamato. Curiosamente, o pré-tratamento com cafeína foi capaz de prevenir os efeitos ansiolíticos *in vivo* observados após a administração de GUO, incluindo a parcial prevenção do aumento dos níveis de ADO e diminuição dos níveis de glutamato no líquor.

Considerando a neurotransmissão adenosinérgica, agonistas ou antagonistas deste sistema de neurotransmissão são efetivas vias para a modulação da sinalização glutamatérgica (Cunha et al., 2008; Dunwiddie & Masino, 2001; Eschke et al., 2001; Gomes et al., 2009; Lopes et al., 2011). Como já descrito, o aumento das concentrações de ADO no cérebro, pode influenciar a liberação vesicular de glutamato (Sperlagh & Vizi, 2011), efeito que tem sido associado com estratégias farmacológicas para combater os transtornos do humor (Lopes et al., 2011). No entanto, é importante ressaltar que a ação da ADO nos seus receptores tem um efeito dual, visto que a ativação dos receptores adenosinérgicos do tipo A_1 estão associados com a inibição da liberação de glutamato (Sperlagh & Vizi, 2011), enquanto a ativação dos receptores A_{2a} de adenosina estão relacionados com a facilitação da liberação do glutamato (Coelho et al., 2014; Costenla et al., 2011). Diante disto, estudos já demonstraram efeitos ansiolíticos ou ansiogênicos através da modulação farmacológica e/ou genética de ambos os tipos de receptores (Burnstock et al., 2011; Coelho et al., 2014; Lopes et al., 2011). Embora em diferentes níveis de expressão nas estruturas relacionadas com o sistema límbico, dentre elas com grande destaque o hipocampo, ações modulatórias da neurotransmissão adenosinérgica já elucidadas,

fortalecem o potencial alvo farmacológico de drogas para o tratamento da ansiedade (Burnstock et al., 2011).

No entanto, de acordo com alguns estudos do nosso grupo, o mecanismo de ação da GUO não parece estar envolvido com a modulação do sistema adenosinérgico (Lara et al., 2001; Vinade et al., 2004). Por outro lado, evidências suportam nossos presentes achados, onde, estudos demonstram que os efeitos tróficos promovidos pela GUO podem estar sim relacionados com o aumento de ADO em experimentos *in vitro* (Ciccarelli et al., 2000), assim como os efeitos antinociceptivos (*in vivo*) da GUO em camundongos parecem estar relacionados com a modulação dos receptores de ADO A_1 e A_{2a} (Schmidt, Bohmer et al., 2010).

Considerando que o aumento de ADO no liquor poderia estar influenciando as ações inibitórias da liberação pré-sináptica de glutamato e que preparações sinaptosomais são excelentes estratégias para investigar alterações nesta região, optamos por avaliar os efeitos da GUO em um protocolo de captação e liberação de glutamato. Mesmo que estudos indiquem que um dos mecanismos de ação da GUO seja pela sua capacidade de aumentar a captação de glutamato (Bettio, Gil-Mohapel et al., 2016; Schmidt et al., 2007), nossos resultados não demonstraram nenhuma alteração nestes parâmetros, tanto em modelos *in vitro*, quanto *ex vivo*. Por outro lado, pela primeira vez, observamos que a GUO *per se* foi capaz de promover uma diminuição da liberação de glutamato após estímulo com alto K^+ em homogeneizado de hipocampo, sem alterar a liberação basal.

Considerando que o hipocampo é uma região do sistema límbico e que está intimamente envolvido nos comportamentos que envolvem emoções (Femenia, Gomez-Galan, Lindskog, & Magara, 2012; Machado-Vieira et al., 2009), incluindo os comportamentos relacionados a ansiedade, e que o estímulo excitatório promovido

pelo glutamato pode ser uma via diretamente relacionada no transtorno de ansiedade (Femenia et al., 2012), uma diminuição da liberação de glutamato hipocampal promovida pela GUO pode ser uma possível via que explica o potencial ansiolítico deste composto. Por fim, os efeitos evidenciados pela GUO na liberação de glutamato frente ao estímulo com alto K^+ foram similares ao promovido pelo agonista A_1 adenosinérgico (CCPA), e completamente bloqueado pelo antagonista de receptores adenosinérgico A_1 (DPCPX). Com relação a modulação dos receptores A_{2a} de ADO nenhuma alteração dos efeitos promovidos pela GUO foi evidenciada.

Considerando estes dados, é possível sugerir num primeiro momento que através do aumento das concentrações de ADO no líquor, a GUO é capaz de diminuir a liberação de glutamato tendo como principal mecanismo de ação a ativação dos receptores A_1 de ADO. De acordo com esta hipótese, e levando em consideração que os receptores A_1 de ADO são os mais abundantes dos quatro tipos de receptores cerebrais de ADO (Rebola et al., 2005), e que sendo uma das funções fisiológicas da ADO inibir a liberação pré-sináptica de glutamato através da ativação dos receptores A_1 de ADO, o que têm como consequência a diminuição da excitabilidade neural (J. Harvey & Lacey, 1997; Lindberg et al., 2015; Oliet & Poulain, 1999; Rau, Ariwodola, & Weiner, 2014), este poderia de fato ser o mecanismo pelo qual a GUO exerce seu efeito ansiolítico.

Após caracterizar o potencial efeito ansiolítico da GUO, em testes preditivos para avaliação dos efeitos relacionados com ansiedade, e apontar alguns indícios sobre o seu mecanismo de ação, considerando ainda, que estudos utilizando testes animais com validade preditiva já demonstraram um potencial antidepressivo da GUO, nosso objetivo foi avaliar o seu potencial antidepressivo em um modelo animal de depressão que apresenta maior similaridade ao que realmente é observado em

pacientes que sofrem com a TDM (validade de face e de constructo). No entanto, é importante ressaltar que diante dos dados apresentados até o momento na literatura explorando as alterações decorrentes da OBX, sentiu-se a necessidade de investigar especificamente o modelo da OBX, principalmente no que diz respeito as alterações temporais relacionadas com: a agitação psicomotora, com a cognição e com a anhedonia, assim como em parâmetros neuroquímicos, principalmente na identificação de alguns mecanismos moleculares que poderiam contribuir para o entendimento da fisiopatologia deste prevalente transtorno. Dessa maneira, investigamos as possíveis alterações ao longo do tempo (até 8 semanas) promovidas pela ablação bilateral dos bulbos olfatórios em camundongos submetidos à OBX, avaliando características comportamentais (*e.g.* hiperatividade, habituação a novidade, e anedonia), e parâmetros neuroquímicos (*e.g.* marcadores mitocondriais, do *status* redox e da resposta inflamatória) em estruturas cerebrais intimamente envolvidas no desenvolvimento e progressão da TDM.

Coletivamente, os resultados apresentados no segundo estudo desta tese mostram, pela primeira vez, que camundongos submetidos ao modelo de depressão da OBX apresentaram simultaneamente alterações transitórias e de longa duração. Especificamente, pôde-se observar um comprometimento duradouro em aspectos relacionados a memória (habituação a novidade), a agitação psicomotora (hiperatividade) e a um fenótipo do tipo ansioso após subsequentes exposições ao teste do campo aberto. Acompanhando estas alterações, observamos que os animais submetidos a OBX apresentaram uma diminuição transitória da motivação e auto-cuidado em um teste comportamental para avaliação da anedonia.

Com relação aos estudos que abordam a OBX como modelo de depressão, faz-se necessário enfatizar que como característica comportamental marcante, a

hiperatividade observada no teste comportamental do campo aberto é uma alteração fenotípica bastante importante e reproduzível (Song & Leonard, 2005), contudo, há muito que se explorar neste teste como as alterações relacionadas com memória e parâmetros relacionados à ansiedade. Assim, sugerimos que futuros estudos abordando a OBX como modelo de TDM explorem estas avaliações, juntamente com um teste específico para avaliação do fenótipo anedonico (sintoma que deve obrigatoriamente estar presente no TDM). Por outro lado, analisando os parâmetros neuroquímicos, nossos resultados indicam claramente que dentre as estruturas cerebrais investigadas, o hipocampo foi a região cerebral onde as alterações foram mais expressivas. Através da análise de preparações sinaptosomais, observamos alterações transientes especificamente analisando massa e $\Delta\Psi$ mitocondrial na região pré-sináptica por citometria de fluxo, enquanto que em analisando parâmetros mitocondriais em células dissociadas nenhuma alteração foi observada nas diferentes estruturas avaliadas.

Com relação as alterações mitocondriais observadas é importante enfatizar que o prejuízo da função sináptica está intimamente relacionado às doenças cerebrais (Nicholls et al., 2015; Picard & McEwen, 2014). Considerando que a comunicação neural através da rápida e eficiente liberação de neurotransmissores em zonas ativas da pré-sinápse e justapostas à densidade pós-sináptica estão extremamente relacionadas com a disponibilidade de energia em terminais do axônio, o número e a funcionalidade mitocondrial nesta região é extremamente importante (Nicholls et al., 2015; Picard & McEwen, 2014; Sheng & Cai, 2015). Nos terminais pré-sinápticos, os neurotransmissores são empacotados em vesículas sinápticas e organizados em grupos ou em conjuntos funcionais que incluem a pronta liberação do *pool* vesicular de neurotransmissores, assim como a rápida reciclagem deste *pool* em um processo que

deve ser altamente controlado (Zhang et al., 2015). Nossos resultados indicam que animais submetidos ao modelo da OBX apresentam um prejuízo transiente nas mitocôndrias presentes no terminal pré-sináptico, sugerindo que a disponibilidade energética para todos os processos já mencionados está diminuída após a ablação bilateral dos bulbos olfatórios. Diante do fato que a disponibilidade de ATP tem um papel fundamental para a transmissão sináptica, organização e movimento de vesículas, assim como para o controle da homeostase do Ca^{+2} (Zhang et al., 2015) os resultados transientes presentes na análise dos parâmetros mitocondriais podem estar associados com uma sinaptotoxicidade transitória que provavelmente contribui para as alterações comportamentais observadas nos animais submetidos à OBX.

Além da modulação dos parâmetros mitocondriais, observamos que a OBX induz a um duradouro desequilíbrio na homeostase redox, assim como uma duradoura ativação das respostas inflamatórias. Em consonância com nossos resultados, muitos estudos na literatura indicam que pacientes acometidos pela TDM apresentam aumento nos biomarcadores relacionados com estresse oxidativo e inflamação, o que contribui para a patogênese deste transtorno (Hurley & Tizabi, 2013; Liu et al., 2015; Maes, Galecki et al., 2011; Moylan et al., 2014). Estudos sugerem que o aumento da atividade imuno-inflamatória, tais como, aumento dos níveis de IL-1 e TNF-α pode estar relacionado com distúrbios na cadeia transportadora de elétrons prejudicando a fosforilação oxidativa e consequentemente a diminuição dos níveis de ATP (Maes, Galecki et al., 2011; Maes, Kubera et al., 2011). Ainda, relacionando o aumento das ROS e RNS, assim como os marcadores de neuroinflamação, evidências indicam que a transcrição de IL-1, IL-6 e TNF-α são regulados pelo status redox da célula, e uma depleção da GSH estaria relacionada com o aumento da transcrição de citocinas pró-inflamatórias (Moylan et al., 2014). Recentemente, importantes informações

relacionando a liberação de citocinas pró-inflamatórias (tais como IL-1, IL-6 e TNF-α) pela micróglia vêm recebendo considerável atenção pelo seu papel nas doenças cerebrais (Duman, Aghajanian, Sanacora, & Krystal, 2016). Como as células mais comuns do sistema imune, a resposta exercida pela micróglia tem sido um ponto central na fisiopatologia das doenças neurodegenerativas, onde inclui-se a TDM (Duman et al., 2016). Em condições fisiológicas a atividade da micróglia está intimamente relacionada com a homeostase do sistema nervoso central, com mecanismos de neuroproteção, envolvendo principalmente a liberação de fatores neurotróficos, como o BDNF, assim como fator de transformação de crescimento (TGF- β) (Morris et al., 2016; Rojo et al., 2014). Por outro lado, quando a ativação da micróglia, bem como a ativação astrocitária é crônica, a liberação de citocinas, tem como consequência a estimulação de uma cascata inflamatória que está intimamente relacionada com os danos neuronais presentes nas doenças neurodegenerativas (Rojo et al., 2014).

Mesmo que a disfunção mitocondrial observada neste estudo tenha sido transiente e os prejuízos na homeostase redox, assim como na resposta inflamatória tenham sido duradouras, isto pode ser um indicativo da plasticidade cerebral. Embora os parâmetros relacionados com neuroplasticidade não tenham sido estudados neste momento, vias relacionadas com neurogênese/plasticidade sináptica devem ter seus mecanismos caracterizados em próximos estudos utilizando a OBX como uma ferramenta para estudo da progressão das alterações presentes na TDM. Ademais, sabe-se que a plasticidade sináptica representa uma das mais importantes e fundamentais funções do cérebro (Duman et al., 2016), a capacidade de perceber, avaliar e armazenar informações complexas, assim como realizar respostas apropriadas e adaptativas para estímulos externos é uma função extremamente nobre

(Duman et al., 2016). Esta função cerebral crítica exerce influência direta nas memórias de curta e longa duração, e alterações nos mecanismos relacionados com plasticidade sináptica têm sido associados com a TDM, principalmente pelo fato do considerável déficit cognitivo presente como sintomatologia (Bortolato et al., 2016; Culpepper, 2015; Femenia et al., 2012). Parâmetros relacionados com sinaptogênese são regulados por complexas vias de sinalização, e o rompimento dos principais mecanismos têm sido implicados como uma das vias susceptíveis na TDM, incluindo perda do suporte de fatores tróficos, e aumento duradouro dos níveis de citocinas inflamatórias (Duman et al., 2016).

Considerando estes achados, os dados apresentados neste estudo indicam que a modulação do estado redox e da resposta inflamatória têm uma relação bastante próxima com a progressão das alterações comportamentais apresentadas no modelo de depressão da OBX, assim como a disfunção mitocondrial observada em preparação sinaptosomal. Desta maneira, levando em consideração que a fisiopatologia da TDM, assim como o desenvolvimento de novas abordagens terapêuticas ainda estão sob investigação, os parâmetros apontados aqui contribuem para alvos promissores no campo das doenças psiquiátricas, e para futuros estudos, visto que novas perspectivas são necessárias.

Após uma investigação mais profunda no modelo da OBX, buscamos investigar o potencial efeito antidepressivo da GUO, explorando principalmente as vias de sinalização que apresentaram uma modulação duradoura no modelo da OBX. Como resultados deste terceiro estudo, mostramos que após 45 dias de tratamento com GUO, da mesma forma que o efeito do tratamento com o antidepressivo tricíclico imipramina (IMI), algumas alterações comportamentais duradouras foram completamente revertidas pela GUO, assim como os parâmetros neuroquímicos

relacionados com o equilíbrio redox no hipocampo, e a resposta anti-inflamatória em soro e hipocampo.

Visto que anteriormente havíamos identificado que as alterações cognitivas são duradouras no modelo da OBX (até 8 semanas), somado ao fato de que o prejuízo na função cognitiva é um dos principais sintomas presentes na TDM e, ainda, que estudos demonstram que o tratamento com os antidepressivos usualmente urilizados na clínica (ISRSS e os ATC) não apresentam remissão destes sintomas (Bortolato et al., 2016), optamos, neste momento, por realizar uma análise mais detalhada do efeito da GUO em parâmetros especificamente relacionados com cognição e memória. Dessa maneira os testes comportamentais utilizados levaram em consideração o envolvimento das vias relacionadas com a formação de memória de curta e de longa duração (Cohen & Stackman, 2015; Jaehne et al., 2015; Padilla et al., 2010). Nossos resultados demonstraram que 60 dias após à OBX, os camundongos do grupo OBX tratados com Salina apresentaram prejuízos nas memórias de habituação e de reconhecimento quando comparados com os seus respectivos controles (Sham Salina), sem nenhuma alteração nos fenômenos relacionados com memória de curta duração. Por outro lado, o tratamento crônico com GUO, foi capaz de melhorar os parâmetros relacionados com memória nos testes propostos, principalmente no que diz respeito às memórias de longa duração.

Embora estudos anteriores tenham observado que a GUO quando administrada de forma aguda apresenta efeito amnésico (Saute et al., 2006; Vinade et al., 2003), que evidentemente acabaria por influenciar parâmetros relacionados com aprendizado e memória, nossos resultados demonstram um efeito benéfico do tratamento crônico com GUO nos testes do campo aberto, reconhecimento de objeto e no labirinto em Y, onde diferentes vias relacionadas com memória foram mensuradas. Com comprovada

ação neuromoduladora frente às vias de sinalização cerebral, alterações agudas nos níveis de GUO podem influenciar negativamente as habilidades de aprendizagem e memória, principalmente pela sua modulação do tônus glutamatérgico (Ota, Zanetti, & Hallock, 2013), enquanto que por outro lado, quando um tratamento crônico é realizado, uma adaptação do sistema cerebral pode ocorrer e os prejuízos provocados pela modulação aguda se dissolverem.

Com relação aos efeitos relacionados com ansiedade que são exacerbados nos animais OBX, o tratamento crônico com GUO, não foi capaz de modular tal fenótipo. Embora estudos anteriores (Vinade et al., 2003), assim como o primeiro estudo desta tese, tenham mostrado um efeito do tipo ansiolítico após o tratamento com GUO, nesta abordagem experimental, seus efeitos não foram evidenciados. No entanto, da mesma maneira que o efeito amnésico promovido pela administração aguda de GUO se extingue após um tratamento crônico (45 dias), o efeito ansiolítico parece seguir o mesmo padrão. Diante da ação neuromodulatória do sistema glutamatérgico exercida pela GUO (Bettio, Gil-Mohapel et al., 2016; Schmidt et al., 2007), uma modulação aguda no tônus excitatório pode influenciar ambos parâmetros. Ainda, mesmo não sendo o foco deste estudo, onde a IMI foi utilizada apenas como um fármaco que apresenta propriedades reconhecidamente antidepressivas, o tratamento crônico com esta droga levou a um prejuízo no teste do labirinto em Y que avaliou memória de curta duração apenas nos animais que foram submetidos a OBX. Visto que este resultado encontra respaldo na literatura este pode ser considerado um efeito indesejado do tratamento com IMI (Nagane et al., 2014; Naudon et al., 2007), que não foi acompanhado pelo tratamento com GUO.

Por fim, com relação aos parâmetros neuroquímicos analisados, nossos resultados demonstram que a GUO e a IMI, foram capazes de reverter o aumento do

ROS, do óxido nítrico (NO), assim como a diminuição das respostas antioxidantes, avaliadas por GSH. Ademais, ambos tratamentos reverteram a diminuição dos níveis de IL-10, uma citocina anti-inflamatória, em soro e hipocampo dos animais submetidos à OBX. A opção por avaliar apenas a estrutura cerebral do hipocampo, se deu fundamentalmente pelo fato que nos experimentos realizados no estudo anterior, nossos resultados demonstraram que as alterações nestes parâmetros foram duradouros nesta região do cérebro, enquanto que em córtex posterior e frontal as alterações foram transientes. O potencial antioxidante da GUO já foi demonstrado, principalmente devido a modulação do antioxidante não enzimático GSH (Bellaver et al., 2015; Quincozes-Santos et al., 2014). Neste sentido, nossos dados corroboram com esses achados e reforçam a ação da GUO como um *scavenger*, que evita o aumento das ROS e do NO. Por outro lado, e considerando que a depleção de GSH estaria intimamente relacionada com a ativação das respostas inflamatórias e citotoxicidade, mesmo que o tratamento com GUO tenha revertido os efeitos deletérios na GSH, apenas a atividade anti-inflamatória da IL-10 foi revertida pelo tratamento com GUO. Assim, esses resultados podem sugerir uma atenuação das respostas inflamatórias induzida pela GUO que tem como consequência a melhora dos parâmetros comportamentais avaliados neste estudo.

4. CONCLUSÃO

Nesta tese, estudos pré-clínicos conduzidos com duas espécies de roedores foram realizados afim de investigar o potencial efeito da GUO como um promissor composto com atividade terapêutica para o tratamento da ansiedade e TDM, assim como, caracterizar os possíveis mecanismos de ação envolvidos nestes efeitos. Neste sentido, com o presente estudo foi possível caracterizar o potencial ansiolítico e antidepressivo da GUO, e avançar nos conhecimentos relacionados com o seu mecanismo de ação. Considerando os resultados neuroquímicos obtidos pelos diferentes protocolos experimentais utilizados, avançamos no entendimento das vias de sinalização envolvidas no mecanismo de ação da GUO, onde, pela primeira vez demonstra-se que a GUO é capaz de modular a liberação de glutamato, um efeito dependente da sinalização adenosinérgica, enquanto que por outro lado reforça-se o efeito da GUO como um composto com potente ação antioxidante. Coletivamente, de uma maneira geral, estes resultados reforçam as recentes evidências que sugerem um envolvimento do sistema purinérgico nos trantornos psiquiátricos. Por fim, considerando as necessidades de estudos que busquem avançar nos conhecimentos sobre os mecanismos fisiopatológicos da TDM, novas perspectivas relacionadas com sua fisiopatologia foram observadas em um modelo animal com elevado potencial translacional, onde a neuroprogressão das alterações neuroquímicas associadas com a TDM foram demonstradas. Neste sentido, uma avaliação temporal em camundongos submetidos ao modelo da OBX, demonstraram alterações transitórias em mitocôndrias localizadas especificamente na região pré-sináptica do hipocampo, acompanhadas de um duradouro desequilíbrio na homeostase redox e resposta inflamatória em hipocampo o que abre novas perspectivas para a elucidação dos mecanismos envolvidos na fisiopatologia da TDM.

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