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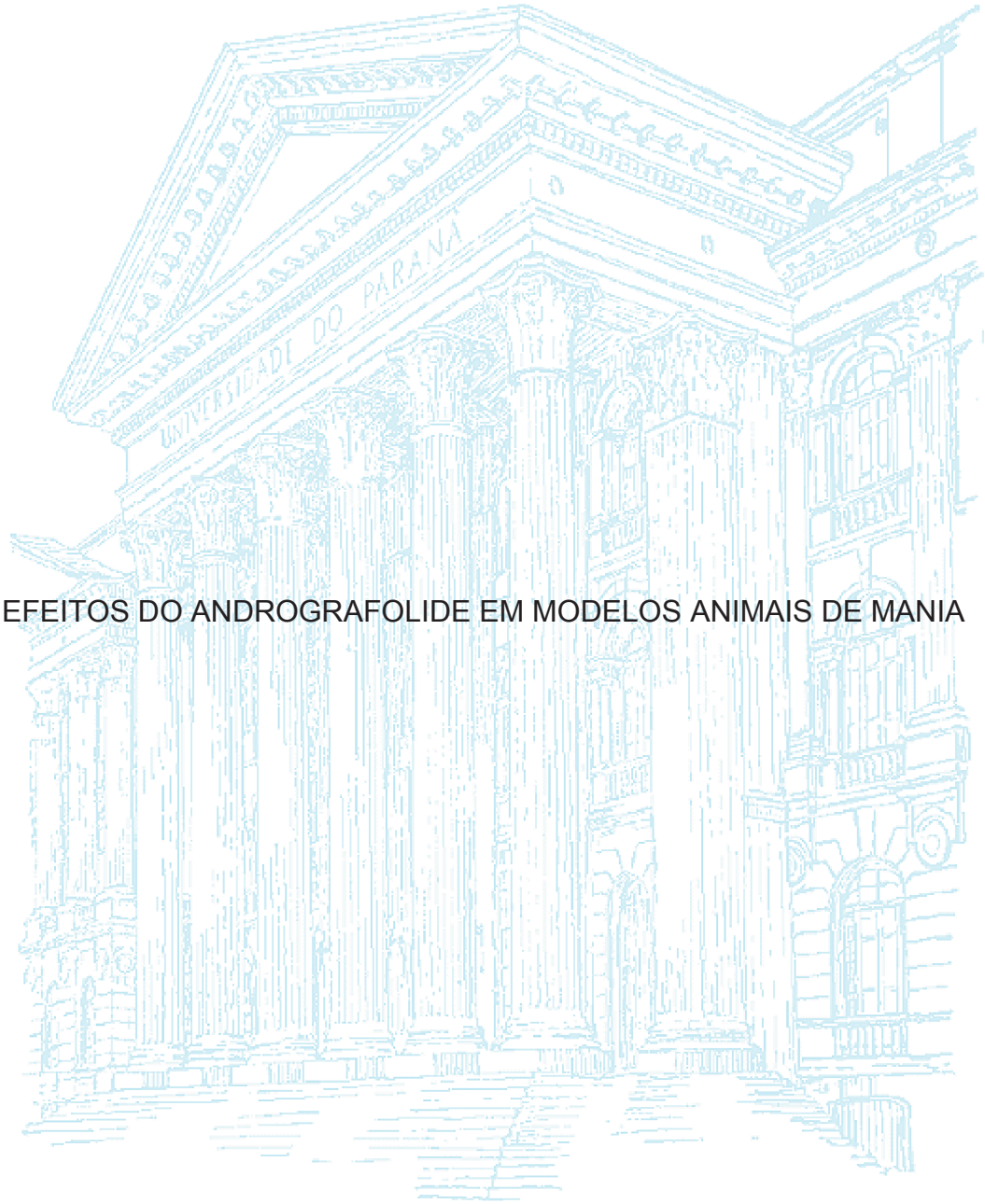
LUIZ KAE SALES KANAZAWA



EFEITOS DO ANDROGRAFOLIDE EM MODELOS ANIMAIS DE MANIA

CURITIBA

2021

LUIZ KAE SALES KANAZAWA

EFEITOS DO ANDROGRAFOLIDE EM MODELOS ANIMAIS DE MANIA

Tese de Doutorado em Farmacologia, pelo Programa de Pós-Graduação em Farmacologia, Setor de Ciências Biológicas, Universidade Federal do Paraná

Orientador: Prof. Dr. Roberto Andreatini

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TERMO DE APROVAÇÃO

Os membros da Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação em FARMACOLOGIA da Universidade Federal do Paraná foram convocados para realizar a arguição da tese de Doutorado de **LUIZ KAE SALES KANAZAWA** intitulada: **Efeitos do andrografolide em modelos animais de mania**, sob orientação do Prof. Dr. ROBERTO ANDREATINI, que após terem inquirido o aluno e realizada a avaliação do trabalho, são de parecer pela sua APROVAÇÃO no rito de defesa.

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

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08/04/2021 14:23:52.0
MARIA APARECIDA BARBATO FRAZÃO VITAL
Avaliador Interno (UNIVERSIDADE FEDERAL DO PARANÁ)

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RUBIA MARIA MONTEIRO WEFFORT
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Assinatura Eletrônica
09/04/2021 11:13:11.0
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NOTA EXPLICATIVA

Esta tese é apresentada em formato alternativo – artigo para publicação – de acordo com as normas do Programa de Pós-Graduação em Farmacologia da Universidade Federal do Paraná.

RESUMO

A atividade aumentada da enzima glicogênio sintase quinase 3β (GSK3β) tem um papel importante na fisiopatologia do transtorno bipolar (TB). Nesta linha, a inibição da GSK3β tem sido associada à ação antimaníaca do lítio, fármaco antimaníaco padrão. Inibidores da GSK3β inibem comportamentos tipo maníacos, mimetizando os efeitos do lítio. Atualmente, o manejo terapêutico do TB consiste na administração de estabilizadores de humor e antipsicóticos e frequentemente acarreta diversos efeitos colaterais e não-responsividade, que afetam a adesão ao tratamento e a qualidade de vida do paciente. A pesquisa de novos agentes terapêuticos para o TB se faz necessária. Considerando que a inibição da atividade da GSK3β possui efeito tipo-antimaníaco, os efeitos do andrografolide (ANDRO), um inibidor da GSK3β isolado da *Andrographis paniculata*, foram estudados em modelos de mania: (a) hiperlocomoção induzida por metilfenidato; (b) hiperlocomoção induzida por privação de sono; (c) hiperlocomoção e aumento de vocalizações ultrassônicas (USVs) de 50-kHz induzidos por lisdexanfetamina (LDX); (d) aumento de atividade exploratória induzida por metilfenidato. Também foram avaliados os efeitos do ANDRO nos níveis de p-Ser^{9}-GSK3β, a forma fosforilada e inativa da GSK3β, no córtex pré-frontal (CPF) e estriado dos animais após privação de sono ou metilfenidato e as alterações nos índices de estresse oxidativo induzidas por LDX. A privação de sono levou à hiperlocomoção e o tratamento com lítio e ANDRO (0.5 e 2.0 mg/kg) preveniram a hiperlocomoção induzida por privação de sono. A privação de sono diminuiu a razão p-Ser^{9}-GSK3β/GSK3β no CPF. Tanto o lítio quanto ANDRO (2.0 mg/kg) aumentaram a razão p-Ser^{9}-GSK3β/GSK3β no CPF. A administração de metilfenidato aumentou a atividade locomotora, comparado aogrupo controle e o tratamento com lítio e ANDRO (0.5 e 2.0 mg/kg) preveniram a hiperlocomoção induzida por metilfenidato. Metilfenidato diminui a razão p- Ser^{9}-GSK3β/GSK3β no estriado e tanto o lítio quanto ANDRO (2.0 mg/kg) aumentaram a razão p-Ser^{9}-GSK3β/GSK3β no estriado. LDX aumentou a atividade locomotora e as USVs de 50-kHz em ratos, o que foi prevenido pelo tratamento crônico com lítio e ANDRO (2.0 mg/kg). Lítio e ANDRO (2.0 mg/kg) também preveniram o aumento na peroxidação lipídica (um parâmetro de estresse oxidativo) induzido por LDX no estriado dos ratos. Houve uma correlação positiva entre a hiperlocomoção induzida por LDX e os aumentos nasUSVs de 50-kHz com a peroxidação lipídica induzidos por LDX. A administraçãode metilfenidato também levou à hiperlocomoção e ao aumento na atividade exploratória (*nosepokes*) no monitor de padrão comportamental (BPM), o que foiprevenido pelo tratamento crônico com lítio e ANDRO (2.0 mg/kg). No geral, os resultados mostram que o tratamento crônico com ANDRO preveniu os comportamentos tipo maníacos, aumentou a razão p-Ser^{9}-GSK3β/GSK3β e reduziu o estresse oxidativo. Portanto, ANDRO aparenta possuir efeitos tipo- antimaníacos e é um agente promissor a ser investigado de forma mais aprofundada para o manejo da mania no TB.

Palavras-chave: Andrografolide. GSK3β. Mania. Metilfenidato. Privação de sono. Transtorno bipolar. Vocalizações ultrassônicas.

ABSTRACT

Increased activity of the enzyme glycogen synthase kinase 3β (GSK3β) is shown to play a pivotal role in the pathophysiology of bipolar disorder (BD). In this line, the inhibition of GSK3β has been associated to the antimanic effect of lithium, the standard antimanic drug. GSK3β inhibitors block manic-like behaviors, mimicking the effects of lithium. Nowadays, the pharmacological management of BD consists of the administration of mood stabilizers or antipsychotics and it often involves a myriad of adverse effects or non-responsiveness, which affect medication adherence and the patient's quality of life. The search for new therapeutic agents for BD is necessary. Considering that the inhibition of GSK3β activity may display antimanic-like effects, we tested the effects of andrographolide (ANDRO), a GSK3β inhibitor isolated from *Andrographis paniculata,* in animal models of mania: (a) methylphenidate-induced hyperlocomotion; (b) sleep deprivation-induced hyperlocomotion; (c) lisdexamfetamine (LDX)-induced hyperlocomotion and increases in 50-kHz ultrasonic vocalizations (USVs); (d) increased exploratory activity induced by methylphenidate. We also evaluated the effects of ANDRO on the levels of p-Ser^{9}-GSK3β, the phosphorylated and inactive form of GSK3β, in the pre-frontal cortex (PFC) and striatum of mice after sleep deprivation or methylphenidate administration and alterations in levels of oxidative stress induced by LDX. Sleep deprivation resulted in hyperlocomotion and treatment with lithium, 0.5 mg/kg and 2.0 mg/kg ANDRO blocked sleep deprivation-induced hyperlocomotion. Sleep deprivation decreased the p-Ser^{9}-GSK3β/GSK3β ratio in the PFC. Both lithium and 2.0 mg/kg ANDRO increased the p-Ser^{9}-GSK3β/GSK3β ratio in the PFC. Methylphenidate administration increased locomotor activity compared to the control group and treatment with lithium, 0.5 mg/kg and 2.0 mg/kg ANDRO blocked methylphenidate-induced hyperlocomotion. Methylphenidate reduced the p-Ser^{9}-GSK3β/GSK3β ratio in the striatum and both lithium and 2.0 mg/kg ANDRO increased the p-Ser^{9}-GSK3β/GSK3β ratio in the striatum. LDX increased locomotor activity and 50-kHz USVs in rats, which was prevented by chronic treatment with lithium or 2.0 mg/kg ANDRO. Lithium and 2.0 mg/kg ANDRO also prevented LDX-induced increases in lipid peroxidation (an oxidative stress parameter) in the rat striatum. There was a positive correlation between LDX-induced hyperlocomotion and LDX-induced increases in 50-kHz USVs and with LDX-induced lipid peroxidation. Methylphenidate administration also led to hyperlocomotion and increased exploratory activity (nosepokes) in the behavioral pattern monitor (BPM), which was prevented by chronic treatment with lithium and 2.0 mg/kg ANDRO. Overall, the results show that chronic treatment with ANDRO prevented manic-like behaviors, increased the p-Ser^{9}-GSK3β/GSK3β ratio and reduced oxidative stress levels. Thus, ANDRO appears to possess antimanic-like effects and it is a promising agent to be more thoroughly investigated for the management of mania in BD.

Key-words: Andrographolide. Bipolar disorder. GSK3β. Mania. Methylphenidate. Sleep deprivation. Ultrasonic vocalizations.

SUMÁRIO

1. INTRODUÇÃO

1.1 Transtorno Bipolar

O Transtorno Bipolar (TB) é uma doença mental crônica que afeta entre 1 e 3% da população mundial e é uma das maiores causas de incapacitação no mundo (Goodwin e Jamison, 2007). O TB é caracterizado por episódios recorrentes de depressão e de mania ou hipomania (Abrial et al., 2015). O TB pode ser subdividido em TB-I e TB-II, sendo que o TB-I é caracterizado por episódios de mania e depressão, enquanto que o TB-II é caracterizado por episódios de hipomania e depressão. Os episódios maníacos no TB-I são períodos de humor persistentemente e anormalmente elevado, expansivo ou irritável, com aumento de atividades direcionadas a um objetivo e energia. O mesmo se aplica à hipomania no TB-II. No entanto, o episódio é caracterizado como maníaco se ele durar, pelo menos, uma semana (ou qualquer período, caso haja necessidade de hospitalização), e estiver presente na maior parte do dia, quase todos os dias, enquanto que o episódio hipomaníaco deve durar, pelo menos, quatro dias consecutivos e estar presente na maior parte do dia, quase todos os dias. Três ou mais dos seguintes sintomas devem estar presentes em um grau significativo e serem uma mudança notável do comportamento usual do indivíduo nos episódios maníacos ou hipomaníacos: auto-estima inflada ou sentimento de grandiosidade; menor necessidade de dormir; verborragia; fuga de ideias; distratibilidade; aumento de atividades direcionadas a um objetivo ou agitação psicomotora; envolvimento em atividades com alto potencial de consequências negativas. Porém, na hipomania, o episódio não é severo o suficiente para causar incapacitação ou problemas sociais ou ocupacionais, ou que o paciente necessite de hospitalização (American Psychiatry Association, 2013).

Os episódios de depressão maior presentes nos TB-I e TB-II são caracterizados por cinco ou mais dos seguintes sintomas, presentes por um período de, pelo menos, duas semanas, com mudanças consideráveis da funcionalidade normal do indivíduo (sendo que, pelo menos, um sintoma seja humor deprimido ou anedonia): humor deprimido na maior parte do dia, diminuição significativa do interesse ou prazer nas atividades; significativa perda ou ganho de peso; insônia ou hipersonia; agitação ou retardo psicomotor; fadiga

ou perda de energia; sentimentos de inutilidade ou culpa; capacidade diminuída de pensar ou se concentrar, ou indecisão; pensamentos recorrentes de morte ou ideação suicida (American Psychiatry Association, 2013).

Outras formas de TB incluem: transtorno ciclotímico, caracterizado por flutuações crônicas de humor com períodos de sintomas hipomaníacos e depressivos, por, pelo menos, dois anos; episódios mistos, no qual episódios maníacos ou hipomaníacos ocorrem com sintomas depressivos; TB induzido por medicamentos ou substâncias, no qual substâncias como álcool ou psicoestimulantes, como a anfetamina, induzem episódios de mania ou hipomania; TB não especificado, quando não há todos os critérios para nenhum dos subtipos de TB; TB de ciclagem rápida, em que ocorre mais de quatro episódios de mudanças de humor dentro de doze meses; e TB com sintomas psicóticos, em que há alucinações ou delírios nos episódios de mania ou hipomania (American Psychiatry Association, 2013).

De acordo com Nivoli (2011) e Judd et al. (2008), pacientes bipolares passam aproximadamente 2/3 de suas vidas com humor deprimido e 1/3 em outros tipos de humor (maníaco, hipomaníaco ou eutímico). Portanto, existe maior atenção para as fases depressivas do transtorno e não tanto para as fases maníacas ou hipomaníacas. No entanto, assim como nas fases depressivas, os sintomas de mania afetam significativamente o bem-estar dos pacientes, estando associados a diversos prejuízos sociais e pessoais, grandes perturbações e problemas na funcionalidade social e ocupacional do paciente (American Psychiatry Association, 2013; Müller-Oerlinghausen et al., 2002). Comportamentos suicidas são frequentes em pacientes com TB, sendo que entre 20 e 60% deles tentam suicídio pelo menos uma vez na vida e, entre 4 e 19% acabam por cometer suicídio (Dome et al., 2019). No TB, o risco de morte por suicídio pode ser 30% maior quando comparado à população geral (Bauer et al., 2018). Além disso, apesar de haver diversos medicamentos para o manejo dos sintomas depressivos, existem menos opções farmacológicas para o manejo dos sintomas maníacos e que apresentam diversas limitações quanto ao seu uso clínico, como intolerância aos efeitos colaterais e refratariedade ao tratamento (Cipriani et al., 2011; Keck, 2003). Ademais, mesmo quando o tratamento é adequado, o curso do TB envolve altas taxas de recorrência de episódios maníacos ou depressivos, recaídas e hospitalizações (Souza, 2011) e, mesmo

com a remissão dos sintomas, ainda pode haver sintomas subsindrômicos que persistem em muitos pacientes (Knapp e Isolan, 2005). O grande número de efeitos adversos resulta em menor aderência ao tratamento (Castro-Costa e Silva, 2011; Miklowitz e Johnson, 2006; Sajatovic et al., 2004), o que contribui para menor responsividade ao tratamento (Osterberg e Blaschke, 2005), aumento de recaídas (Gutiérrez-Rojas et al., 2010), tentativas de suicídio e suicídio (Pompili et al., 2009); hospitalizações (Hong et al., 2011) e envolvimento com delitos (Robertson et al., 2014).

Dentre os principais tratamentos farmacológicos para o TB, os estabilizadores de humor lítio e valproato de sódio são os principais (Chiu et al., 2013; Miklowitz e Johnson, 2006). Antipsicóticos como risperidona, olanzapina, quetiapina, clozapina e aripiprazol, além de anticonvulsivantes como a lamotrigina e oxcarbazepina, também foram aprovados para o manejo terapêutico do TB, em combinação com antidepressivos (Bai et al., 2019; Bauer et al., 2019). Os antipsicóticos atuam em receptores de dopamina e de serotonina. Antipsicóticos de segunda geração (atípicos) têm menos afinidade por receptores D_2 de dopamina do que antipsicóticos de primeira geração, e maior afinidade por receptores 5-HT_{2A} de serotonina (Jauhar e Young, 2019). Uma das vantagens dos antipsicóticos de segunda geração é a menor tendência de causar problemas motores, em comparação aos antipsicóticos de primeira geração (Carbon et al., 2017). Os principais efeitos adversos dos antipsicóticos incluem ganho de peso, alterações no metabolismo de glicose, colesterol e prolactina, sedação, disfunções sexuais, e sintomas extrapiramidais (Young et al., 2015). No geral, os medicamentos para o tratamento do TB causam uma série de efeitos adversos, como ganho de peso, acatisia, náuseas, vômitos, sonolência, tremores, tontura, astenia, alterações hematológicas, dentre outros, o que afeta a qualidade de vida do paciente e a adesão ao tratamento (Bai et al., 2019; López-Muñoz et al., 2018). Monoterapia com antidepressivos não é recomendada, devido ao risco de virada maníaca ou indução de ciclagem rápida (Bauer et al., 2012; Malhi et al., 2012).

As alterações bioquímicas envolvidas na fisiopatologia do TB ainda não estão totalmente elucidadas (Szabo et al., 2009). Contudo, estudos demonstraram o envolvimento de estresse oxidativo, além de aumento na

atividade da enzima glicogênio sintase quinase 3 beta (GSK3β) na fisiopatologia do TB.

1.2 Estresse Oxidativo

Estudos indicam que, dentre diversos fatores, um aumento de estresse oxidativo está envolvido na fisiopatologia do TB, havendo um aumento na produção de radicais livres e depleção de enzimas e moléculas antioxidantes (Berk et al., 2011). Um dos principais processos envolvidos na geração de radicais livres, espécies reativas de oxigênio (ERO) e de nitrogênio (ERN), é a fosforilação oxidativa. Dentre as EROs, as mais relevantes são o oxigênio singleto ($^1O_2$), peróxido de hidrogênio (H_2O_2), ozônio (O_3), ácido hipocloroso (HOCl) e os radicais livres ânion superóxido (O^{2-}) e grupo hidroxila (OH^-). Óxido nítrico (NO) e o radical livre peroxinitrito ($ONOO^-$) são exemplos de ERNs (Kohen e Nyska, 2002). EROs e ERNs são capazes de reagir com praticamente qualquer biomolécula, incluindo DNA, RNA, proteínas, carboidratos e lipídios, causando danos a estas moléculas (Diplock et al., 1998). O sistema de defesa antioxidante endógeno protege as células do dano causado pelos radicais livres, EROs e ERNs. Uma das moléculas antioxidantes mais importantes na prevenção do dano oxidativo é a glutationa reduzida (GSH), que é capaz de reduzir proteínas com grupamentos sulfidril oxidados e também reduzir os níveis de ânions superóxido e H_2O_2 com a co-atividade de outras enzimas, como a glutationa peroxidase (GPx), glutationa redutase (GR), glutationa-S-transferase (GST) e a catalase (CAT) (Rosa et al., 2014).

Estudos mostram que há alterações nos níveis de enzimas antioxidantes na mania no TB. A atividade da GR e GST encontra-se aumentada em pacientes bipolares em estágios avançados, quando comparado a pacientes bipolares em estágios menos avançados (Andreazza et al., 2009). Estudos demonstram o consistente aumento na peroxidação lipídica (LPO) por radicais livres e EROs e alteração nos níveis de enzimas antioxidantes em pacientes bipolares independente do estágio (Berk et al., 2011; Andreazza et al., 2007; Machado-Vieira et al., 2007; Ozcan et al., 2004). Brüning et al. (2012), em um modelo animal de mania, demonstraram correlação positiva entre hiperlocomoção e níveis aumentados de LPO em ratos. Valvassori et al. (2020) mostraram que a

injeção intracerebroventricular de lítio, tamoxifeno e outros inibidores de PKC preveniram comportamentos tipo-maníacos, como aumento de cruzamentos, *rearings* e *groomings* no teste do campo aberto, induzidos pela administração intraperitoneal de metanfetamina, um modelo de mania, em ratos. Além disso, estas drogas também preveniram estresse oxidativo, regulando parâmetros como níveis de GPx, GR, 4-hidroxi-2-nonenal (4-HNE), 8-isoprostano (8-ISO), grupos carboxil e 3-nitrotirosina (3-NT) no córtex pré-frontal (CPF), hipocampo e estriado de ratos. Machado-Vieira et al. (2007) avaliaram parâmetros de estresse oxidativo como os níveis de substâncias reativas do ácido tiobarbitúrico (TBARS) e proteínas carbonil e malondialdeídos (MDA) no soro de pacientes bipolares e mostraram que os pacientes bipolares tratados com lítio apresentaram níveis significativamente mais baixos de TBARS e MDA do que pacientes não tratados.

1.3 GSK3β

Além de estresse oxidativo, vias nas quais a enzima GSK3β atua como reguladora também foram relacionadas ao TB e o aumento na atividade da GSK3β está relacionada à ocorrência de comportamentos tipo-maníacos em animais (Prickaerts et al., 2006; Gould et al., 2004).

A enzima GSK3 é uma quinase serina/treonina que regula diversas vias de sinalização (Prickaerts et al., 2006), incluindo vias que envolvem proteínas metabólicas, estruturais e de sinalização relaciondas à modulação de receptores acoplados a proteínas G, receptores de hormônios ou ionotrópicos e tráfego de receptores (Beurel et al., 2016; Enman e Unterwald, 2012). A GSK3 possui duas isoformas: GSK3α e GSK3β, que são quinases serina/treonina associadas coma regulação da síntese de glicogênio em resposta à insulina (Dandekar et al., 2018; Frame e Cohen, 2001). Estudos mostram que a GSK3β geralmente tem ação pró-apoptótica e pró-inflamatória, estimulando a produção de diversas citocinas pró-inflamatórias e fator de necrose tumoral. A inibição da GSK3β mostrou ter efeitos benéficos em condições inflamatórias (Jope et al., 2007; Rowe et al., 2007). A GSK3β fosforila mais de cem substratos e estando envolvida em diversas vias de sinalização e expressão gênica, afetando neurogênese, sobrevivência neuronal e ritmo circadiano (Jope et al., 2006). Inibição de neurogênese e de plasticidade neuronal são fatores cruciais em

transtornos de humor e a GSK3β interfere em ambos processos no cérebro (Li e Jope, 2010).

A GSK3β é um componente da via de sinalização da Wnt, que é essencial no desenvolvimento embrionário, e também em síntese proteica, proliferação celular, diferenciação celular, dinâmica de microtúbulos e adesão celular, por exemplo (Frame e Cohen, 2001). A GSK3β forma um complexo de destruição da β-catenina, uma proteína relacionada com a transcrição de proteínas anti-apoptóticas, fosforilando-a e facilitando sua degradação (Serrano et al., 2014). Na ausência de ligantes da Wnt, a GSK3β e a proteína APC se ligam diretamenteà proteína Axina, formando um complexo que facilita a fosforilação da β-catenina pela GSK3β, direcionando-a à degradação via proteassomo (Valvezan e Klein,2012). A via da Wnt é ativada pela ligação da Wnt a um receptor da família Frizzled, ocorrendo a ativação da proteína Dishevelled (Zhang et al., 2010), induzindo à fosforilação das proteínas LRP5 e LRP6, resultando na inibição da GSK3β e na estabilização da β-catenina, que não sofre mais degradação via proteassomo e acumula no citoplasma. A β-catenina, então, migra ao núcleo dacélula e interage com famílias de fatores de transcrição como os LEF/TCF ativando a transcrição de genes envolvidos com neuroproteção e proliferação celular (Valvezan e Klein, 2012; MacDonald et al., 2009; Clevers, 2006).

Estudos de intervenções farmacológicas e modelos genéticos demonstram o envolvimento da atividade da GSK3β no TB. Camundongos *knockout* para GSK3β, ou tratados com inibidores de GSK3β exibem reversãode comportamentos tipo maníacos e tipo depressivos, o que sugere que a inibição da GSK3β tenha efeito benéfico em transtornos de humor (Gould et al.,2004; O'Brien et al., 2004). Prickaerts et al. (2006) sugerem que o uso de camundongos transgênicos com superexpressão de GSK3β possa ser um modelo animal de mania, pois tais animais apresentam comportamentos tipo maníacos, como hiperlocomoção.

Lítio, o estabilizador de humor padrão ouro no manejo do TB, é um inibidor direto e indireto da GSK3β (Cole, 2013). Em ratos, inibidores de GSK3β induziram efeitos similares aos efeitos antimaníacos do lítio. Superexpressão de GSK3β anula os efeitos benéficos do tratamento com lítio em camundongos. Polimorfismos na região promotora do GSK3β, em humanos, está relacionada

com desenvolvimento precoce do TB e com a responsividade ao tratamento com lítio (Polter et al., 2010).

A fosforilação da GSK3 em resíduo de serina-9 na porção N-terminal (Ser^{21} na isoforma α e Ser^{9} na isoforma β), é o maior mecanismo de regulaçãoda enzima (Polter et al., 2010). Isto gera um pseudosubstrato que inibe a GSK3β, possibilitando a ativação de proteínas como a glicogênio sintase e a mTOR (Proud, 2006). Algumas proteínas capazes de fosforilar a GSK3β em Ser^{9} incluem a proteína quinase A (PKA) e a proteína quinase B (Akt) (Rowe et al., 2007). O impedimento da fosforilação inibitória em Ser^{9} da GSK3β pode promover comportamentos tipo maníacos e estudos mostram que a fosforilação em Ser^{9} da GSK3β encontra-se, de fato, diminuída em animais e pacientes bipolares em episódios maníacos (Polter et al., 2010).

Uma das ações do lítio é inibir a atividade da GSK3β diretamente, ao competir com íons Mg^{2+} pelo sítio de ligação da enzima (Ryves e Harwood, 2001) ou indiretamente, por estimular a via da PI3K/Akt, induzindo à fosforilação em Ser^{9} da GSK3β (Chiu et al., 2013; Kitagishi, 2012), que está associada à inibição da GSK3β. Isto também ocorre como mecanismo de ação de drogas como o valproato e antipsicóticos (Kozlovski et al., 2006; DeSarno et al., 2002; Chalecka-Franaszek e Chuang, 1999).

Pacientes bipolares apresentam níveis aumentados de GSK3β, quando comparados a indivíduos saudáveis (Li e Jope, 2010). A inibição da GSK3β por fosforilação em Ser^{9} encontra-se diminuída em células mononucleares do sangue periférico de pacientes bipolares sintomáticos, quando comparado com indivíduos controle saudáveis (Polter et al., 2010). A razão de GSK3β fosforilada/ não fosforilada (razão p-Ser^{9}-GSK3β / GSK3β) é um índice de atividade da GSK3β e pode ser correlacionada com aspectos comportamentais (Grabinski eKanaan, 2016; De Sousa et al., 2015).

1.4 Lítio

Lítio tem sido o medicamento mais importante no tratamento do TB desde os anos 60 (Jauhar e Young, 2019). Lítio regula o transporte de membrana celular, distribuição de íons e regulação de neurotransmissores, por inibir neurotransmissão excitatória, diminuindo a neurotransmissão dopaminérgica e

por modular a neurotransmissão glutamatérgica, causando *downregulation* de receptores NMDA. O lítio regula a sinalização intracelular e enzimas, como a GSK3β, pela inibição da fosforilação da Akt, ativando vias de neuroproteção (Alda et al., 2015), além de inibir a GSK3β por estimular sua fosforilação inibitória em resíduos de Ser^9 (Malhi et al., 2013). A enzima GSK3β é ativada em condições de estresse crônico, como a excessiva neurotransmissão dopaminérgica na mania e leva à hiperatividade em animais (Prickaerts et al., 2006; Beaulieu et al., 2004). O tratamento com lítio antagoniza o desenvolvimento de comportamentos tipo maníacos dependentes de dopaminaem roedores por interferir na regulação da via de sinalização de Akt-GSK3β porreceptores D_2 (Beaulieu et al., 2009). Tais resultados indicam que a inativação da GSK3β está envolvida no mecanismo de ação antimaníaco do lítio em pacientes bipolares (Dandekar et al., 2018).

Outra enzima alvo de ação inibitória do lítio e que está envolvida na fisiopatologia do TB é a enzima proteína quinase C (PKC) (Einat et al., 2007). O lítio inibe a PKC por interferir no ciclo do fosfoinositol (Malhi et al., 2013). Após a ativação de acoplados à proteína G_q, a fosfolipase C é estimulada, catalisando a conversão do fosfatidilinositol 4,5-bifosfato (PIP_2) em dois segundos mensageiros: o inositol trifosfato (IP_3) e o diacilglicerol (DAG). O IP_3 estimula a mobilização de cálcio intracelular, enquanto o DAG ativa a PKC (Manji et al., 2001). Uma vez ativada, a PKC migra do citosol à membrana plasmática, sendo que a forma ligada à membrana representa a enzima ativada, capaz de fosforilar seus subtratos de forma eficiente (Parker e Murray-Rust, 2004). A enzima inositol monofosfatase (IMPase) e inositol polifosfato fosfatase (IPPase) facilitam a reciclagem de IP_3 de volta à forma de mioinositol, permitindo que o ciclo do fosfoinositol continue. O lítio inibe o ciclo do fosfoinositol por inibir a recaptação de inositol (induzindo à sua depleção) e inibindo as enzimas IMPase e IPPase (Malhi et al., 2013), culminando na inibição da atividade da PKC. Estudos mostram que pacientes maníacos apresentam maior razão membrana:citosol da PKC em plaquetas, que é normalizada após tratamento com lítio (Friedman et al., 1993). A administração aguda e crônica de anfetamina induz o aumento da atividade da PKC e da razão membrana:citosol da PKC, e aumento da fosforilação de GAP-43, um substrato da PKC, acarretando em alterações de

neurotransmissão, relacionadas à ocorrência de comportamentos tipo maníacos (Einat et al., 2007).

No entanto, o tratamento com lítio envolve a ocorrência de diversos efeitos adversos importantes, como náuseas, vômitos, ganho de peso, xerostomia, polidipsia, poliúria, falência renal, disfunções tireoidianas, sonolência, resistência à insulina, dentre outros (Bai et al., 2019; Kemp et al., 2014; Chiu et al., 2013; Price e Marzani-Nissen, 2012; Souza, 2011; Müller-Oerlinghausen et al., 2002). Öhlund et al. (2018) mostraram que 44% dos pacientes bipolares em tratamento com lítio descontinuaram o tratamento devido aos efeitos colaterais ou à falta de responsividade.

A ação inibitória do lítio sobre a enzima GSK3β é uma das responsáveis por sua ação antimaníaca (Chiu et al., 2013). Vários estudos indicam que a GSK3β é, de fato, um alvo interessante no contexto do TB e que inibidores de GSK3β possuem propriedades antimaníacas. Portanto, a pesquisa envolvendoa eficácia terapêutica de inibidores de GSK3β como possíveis agentes antimaníacos é de grande relevância (Kozikowski et al., 2007).

1.5 Inibidores de GSK3β

Existem vários estudos que demonstram os efeitos benéficos de moléculas inibidoras de GSK3β. Kalinichev e Dawson (2011) avaliaram as propriedades antimaníacas dos inibidores de GSK3β, indirubina, alsterpaulona e SB-627772, TDZD-8, AR-A014418, dentre outros. Tais moléculas foram capazesde reverter a hiperlocomoção induzida por anfetamina, um modelo animal de mania, assim como lítio ou valproato, que são drogas utilizadas no manejo terapêutico do TB. Além disso, outros inibidores de GSK3β, como CHIR99021, 6-BIO, SB216763 e alsterpaulona também diminuíram a hiperlocomoção induzida por anfetamina (Muneer, 2017). Enman e Unterwald (2012) mostraramque o ácido valpróico e o inibidor de GSK3β SB216763 diminuíram a hiperlocomoção e estereotipia induzidos por anfetamina. Valvassori et al. (2017)mostraram que o lítio e o valproato inibem a GSK3β, revertendo comportamentos tipo maníacos induzidos pela administração de ouabaína, um modelo animal demania. Estes dados, portanto, reforçam a importância da pesquisa de agentes

inibidores de GSK3β e/ou PKC, com ação antioxidante na pesquisa de novos agentes terapêuticos para o tratamento do episódio maníaco do TB.

1.6 Andrografolide

Neste contexto, o diterpeno andrografolide (ANDRO) possui atividade inibitória sobre a GSK3β (Serrano et al., 2014). Este composto é o principal constituinte bioativo da planta *Andrographis paniculata*, amplamente utilizada há séculos na Medicina Tradicional Chinesa, além da Medicina Tradicional Tailandesa, no Japão, Escandinávia e Indonésia (Lu et al., 2019); Jayakumar et al., 2013). A *A. paniculata* também é conhecida como “kalmegh” na Índia, ou como “rei dos amargos” devido ao seu gosto amargo (Mussard et al., 2019). A planta mede entre 30-110 cm de altura, é encontrada em locais escuros e úmidos e possui folhas glabras e flores brancas com pontos púrpuras nas pétalas. A planta inteira possui valor medicinal, porém as folhas contêm a maior concentração do principal composto bioativo, ANDRO (Sareer et al., 2014).

ANDRO é uma lactona labdano diterpenóide, que foi mencionado pela primeira vez na Indian Gazette em 1951, como um ativo da *A. paniculata.* Porém, somente em 1984 o potencial terapêutico do ANDRO foi descrito, em um estudo de dano hepático (Choudhury e Poddar, 1984). Muitos efeitos terapêuticos do ANDRO já foram reportados: efeitos anti-diarreicos, anti-virais, anti-maláricos, antioxidantes, antiinflamatórios, hepatoprotetores, efeitos benéficos para disfunções sexuais, para problemas cardiovasculares, no tratamento de carbúnculos, úlceras, colite, herpes e picadas de cobras venenosas (Lim et al., 2012; Bharati et al., 2011). A planta é usada em mais de vinte e cinco formulações Ayurvédicas, sendo bastante relevante na farmacopéia indiana (Pandey et al., 2019). A *A. paniculata,* inclusive, está presente na farmacopéia Americana e é comercializada e utilizada nos Estados Unidos como suplemento nutricional (Aromdee et al., 2014).

As propriedades farmacológicas do ANDRO estão relacionadas principalmente aos seus efeitos anti-inflamatórios e antioxidantes. Mittal et al. (2016) mostraram que, por seus efeitos antioxidantes, ANDRO apresentou efeitos protetores contra morte celular induzida por H_2O_2, espécies reativas de oxigênio e LPO em células HepG2. Os autores mostraram que a administração

de ANDRO levou à inibição da enzima GSK3β, levando à retenção de Nrf2 no núcleo e expressão sustentada de HO-1, por se ligar a elementos de resposta antioxidante (Are). Vários estudos, *in vivo* e *in vitro*, mostram o efeito antioxidante do ANDRO. Eles envolvem as propriedades de sequestrador de ROS, efeitos protetores de mitocôndrias, inibição de enzimas produtoras de radicais livres, como a NADPH oxidase ou xantina oxidase, além da ativação de sistemas antioxidantes envolvendo SOD, CAT e GPx (Mussard et al., 2019).

Pan et al. (2017), por exemplo, demonstraram que os efeitos hepatoprotetores do ANDRO estão relacionados às suas propriedades anti-inflamatórias e antioxidantes, pois ele foi capaz de melhorar a histologia hepática, diminuindo níveis de AST, ALT, MPO, MDA, IL-1β, TNF-α e ROS. Li et al. (2017) mostraram que ANDRO apresentou efeito benéfico em modelos de artrite reumatoide, diminuindo a severidade da artrite e a destruição das juntas em modelo de artrite induzida por colágeno em camundongos, diminuindo os níveis de IL-6, IL-1β, TNF-α no soro e reduzindo a fosforilação da p38 MAPK eexpressão de ERK1/2. Tan et al. (2016) mostraram que o ANDRO reverteu infiltração leucocitária pulmonar e produção de citocinas pró-inflamatórias (TNF-α, IL-1β e CXCL1, por exemplo) em um modelo de inflamação pulmonar em camundongos.

Além disso, o tratamento com ANDRO mostrou resultados promissores em estudos de doenças neurodegenerativas, como doença de Parkinson e doença de Alzheimer (Rivera et al., 2016; Zhang et al., 2014). Rivera et al. (2016) mostraram que o tratamento com ANDRO resultou na recuperação da transmissão sináptica basal e parcial ou completa proteção de certas proteínas sinápticas em modelos animais de disfunções cognitivas. Serrano et al. (2014) demonstraram que o ANDRO foi capaz de induzir proteção de potenciação de longa duração (*long-term potentiation* LTP) e proteínas sinápticas contra oligômeros Aβ em modelo animal de Alzheimer e que o ANDRO foi capaz de inibir a depressão de longa duração (*long-term depression*, LTD) de maneira concentração-dependente, levando ao acúmulo de β-catenina e à redução da forma ativa da GSK3β, uma enzima chave associada à LTD.

ANDRO inibe a GSK3β por interagir com o sítio de ligação da enzima. ANDRO ativa a via de sinalização da Wnt/β-catenina, que é importante para uma variedade de processos biológicos, incluindo adesão celular, regulação sináptica

e funcionamento sináptico. ANDRO induz à transcrição de genes relacionados à Wnt e induz à fosforilação de GSK3β no resíduo de serina-9, levando à sua inativação (Tapia-Rojas et al., 2015). Serrano et al. (2014) mostraram que ANDRO foi capaz de aumentar os níveis da forma inativa de GSK3β e também aumentar os níveis de β-catenina, uma proteína capaz de estimular a transcrição de genes de proteínas anti-apoptóticas.

Atualmente, ANDRO e extratos de *A. paniculata* são administrados via oral pela população geral (Tan et al., 2017), sendo avaliados clinicamente para diversas doenças. Por exemplo, *A. paniculata* foi testada em um estudo duplo-cego, placebo-controlado para o tratamento de infecções do trato respiratório superior (Gabrielian et al., 2002). Além disso, o Paractin(R), uma composição de *A. paniculata*, mostrou efeitos benéficos promissores em um estudo clínico de fase II placebo-controlado para artrite reumatoide, reduzindo os níveis de fator reumatoide, IgA e C4 (Identificador: NCT00749645).

Produtos naturais sempre foram fontes de uma diversidade de moléculas biologicamente ativas, impulsionando descobertas farmacêuticas por séculos (Kandanur et al., 2019). Levando em consideração que o ANDRO possui atividade inibitória sobre a enzima GSK3β, como a droga antimaníaca lítio, é relevante avaliar se o ANDRO possui atividade tipo antimaníaca testando seus efeitos em modelos animais de mania no TB.

1.7 Modelos animais de mania

Modelos animais de doenças humanas devem satisfazer três critérios: validade de constructo, validade de face e validade preditiva. A validade de constructo refere-se aos fatores comuns entre os mecanismos envolvidos no modelo e na doença humana. A validade de face refere-se aos fatores comuns entre as características do modelo e aos sintomas da doença humana. Avalidade preditiva refere-se à eficácia do tratamento com drogas utilizadas na doença humana no fenótipo do modelo animal (Kato et al., 2007).

Vários modelos animais de mania ou depressão já foram desenvolvidos e padronizados. Eles podem ser classificados em diferentes categorias: modelos farmacológicos, nutricionais, ambientais ou genéticos (Kato et al., 2007). Algumas razões para a falta de desenvolvimento de medicamentos eficazes no

tratamento do TB são a falta de modelos animais estabelecidos para o TB e a dificuldade em avaliar o efeito profilático de estabilizadores de humor. Para o estudo de antidepressivos, diversos testes comportamentais já foram estabelecidos (Kato et al., 2007).

A administração de ouabaína, um inibidor da Na+/K+ ATPase, é um modelo de mania, pois ratos apresentam hiperatividade após a administração de ouabaína (Decker et al., 2000). Modelos nutricionais de mania também podem ser empregados, como a administração de homocisteína ou a privação de ácidos graxos poliinsaturados n-3, que leva a agressividade (DeMar et al., 2006; Levine et al., 2005). Modelos ambientais também podem ser utilizados, como a privação de sono (Gessa et al., 1995), pois perturbações no ciclo sono-vigília e no ritmo circadiano fazem parte dos sintomas de pacientes bipolares e utilizados como critérios diagnósticos (Gonzalez et al., 2014). Modelos genéticos, como o uso de linhagens específicas de animais, podem ser empregados (Einat, 2007). Camundongos mutantes para CLOCK ou animais transgênicos com superexpressão de GSK3β mostram hiperatividade (Roybal et al., 2007; Prickaerts et al., 2006).

No entanto, o modelo de mania mais utilizado é a administração aguda de psicoestimulantes, como a anfetamina (Frey et al., 2006), metanfetamina (Gould et al., 2001) ou dimesilato de lisdexanfetamina (LDX) (Macêdo et al., 2013). Eles causam hiperatividade e este modelo é usado para testar a eficácia de tratamentos antimaníacos, como lítio ou valproato.

1.7.1 Hiperlocomoção induzida por metilfenidato

A administração de psicoestimulantes afeta diversos sistemas de neurotransmissão, o que corresponde ao fato de que os níveis de vários neurotransmissores são afetados em pacientes com TB (Beyer e Freund, 2017). O sistema catecolaminérgico é o principal envolvido nos sintomas tipo maníacos. Níveis elevados de dopamina ou noradrenalina foram observados em pacientes com TB (Berk et al., 2007). Psicoestimulantes aumentam os níveis sinápticos de dopamina ou noradrenalina por inibir ou reverter mecanismos de recaptação (Berk et al., 2007). A anfetamina não só induz comportamentos tipo-maníacos em animais, como também causa sintomas de mania em indvíduos saudáveis

ou pacientes bipolares, como menor necessidade de dormir, humor elevado, aumento de energia, hipersexualidade, afetando funções sensoriais e motoras, aprendizado e memória, por exemplo (Corp et al., 2014; Cousins et al., 2009; Berk et al., 2007; Asghar et al., 2003; Jacobs e Silverstone, 1986). Psicoestimulantes podem, inclusive, antecipar episódios maníacos em pacientes bipolares (Young et al., 2011). Lítio e valproato podem atenuar comportamentos maníacos induzidos por anfetamina (Frey et al., 2006; Flemenbaum et al., 1974).

A hiperlocomoção induzida por psicoestimulantes é o modelo animais mais frequentemente empregado (Young et al., 2011). A indução farmacológica de comportamento tipo maníaco apresenta validade de face, de constructo e validade preditiva (Einat, 2006; Machado-Vieira, 2004). Para mensurar a hiperlocomoção, o número de cruzamentos no campo aberto pode ser avaliado como índice de atividade locomotora. A inibição ou atenuação da hiperlocomoção após a administração de metilfenidato, por exemplo, é indicativo de efeito tipo antimaníaco, em doses que não afetem a atividade locomotora *per se* (Sabioni et al., 2008; Gould et al., 2001).

1.7.2 Hiperlocomoção induzida por privação de sono

Outro modelo de mania é a hiperlocomoção induzida por privação de sono, sendo que a privação de sono geralmente antecipa episódios maníacos (Kato et al., 2007). Após a privação de sono, animais demonstram insônia, hiperatividade, irritabilidade, agressividade, hipersexualidade e estereotipias (Gessa et al., 1995). Tais alterações comportamentais são revertidas por haloperidol ou prevenidas por lítio (Gessa et al., 1995). O objetivo do protocolo é que, os animais estando em plataformas rodeadas por água por 24h, 72h ou 96h, ao atingirem o sono REM, eles apresentam relaxamento muscular, caindo na água, tendo seu sono interrompido (Machado-Vieira et al., 2004). Após o período de privação de sono, os animais mostram comportamentos tipo maníacos, como hiperlocomoção e aumento da emissão de vocalizações ultrassônicas (USVs) apetitivas (Wendler et al., 2019; Gessa et al., 1995).

O modelo de privação de sono induz várias alterações neuroquímicas, como o *downregulation* da expressão da enzima tirosina hidroxilase na substantia nigra pars compacta, assim como a diminuição da neurotransmissão

dopaminérgica na substantia nigra pars compacta e estriado (Lima et al., 2012), aumento da expressão de receptores D_2 no estriado (Lima et al., 2007), assim como supersensibilidade de receptores dopaminérgicos (Tufik et al., 1978). Este protocolo de indução não-farmacológica de comportamentos tipo maníacos possui validade de face, de constructo e preditiva (Einat, 2006; Gessa et al., 1995).

1.7.3 Aumento de vocalizações ultrassônicas de 50-kHz induzido por lisdexanfetamina

O dimesilato de lisdexanfetamina (LDX) é uma pró-droga de *d*-anfetamina de longa ação, utilizada no manejo terapêutico do transtorno do déficit de atenção e hiperatividade (TDAH) (Ermer et al., 2016). Foi desenvolvido para melhorar os efeitos terapêuticos de estimulantes como a *d*-anfetamina ou metilfenidato, que são tratamentos já estabelecidos para o TDAH, porém cujos efeitos não são prolongados (Swanson et al., 2011). Na molécula da LDX, um peptídeo liga o grupo amino da *d*-anfetamina a um grupo carboxila da L-lisina. Após a absorção no intestino, hidrólise enzimática deste peptídeo por uma peptidase eritrocitária desconhecida libera a *d*-anfetamina bioativa e o subproduto L-lisina (Ermer et al., 2016). A *d*-anfetamina age primariamente aumentando os níveis sinápticos de dopamina e noradrenalina (Ward e Citrome, 2018). Em adultos com TDAH, a meia-vida da LDX é de 0.5 horas e a meia-vida da *d*-anfetamina liberada é 17h, o que permite a administração única diária ao paciente (Adler et al., 2017).

Como a administração de psicoestimulantes é um modelo de mania, a administração de LDX também tem sido utilizada para mimetizar comportamentos tipo maníacos em animais. Bristot et al. (2016) mostraram que a administração diária por 14 dias de LDX (10 mg/kg) causa hiperlocomoção, que foi prevenida por pré-tratamento com 47.5 mg/kg de lítio por sete dias. Eger et al. (2016) utilizaram a administração de LDX como modelo de mania para induzir hiperlocomoção e desbalanço oxidativo em ratos, o que foi prevenido por pré-tratamento crônico com sinvastatina. Outro parâmetro que pode ser analisado para avaliar mudanças comportamentais neste contexto são alterações na emissão de USVs pelo animal. Wendler et al. (2016) utilizaram a

administração de LDX como modelo de mania para induzir aumentos de USVs de 50-kHz em ratos.

Os roedores emitem espontaneamente USVs acima do alcance auditivo humano (> 20 kHz) em contextos positivos ou negativos para expressar estados emocionais ou para comunicação com outros roedores (Brudzynski, 2015; Burgdorf et al., 2011; Knutson et al., 2002). Ratos adultos emitem USVs de alta frequência (50-kHz) em situações apetitivas, como ao brincar com outros ratos, acasalamento ou por administração de psicoestimulantes (Burgdorf et al., 2011). Em situações aversivas, como em estímulos dolorosos ou estressantes, os ratos emitem USVs de baixa frequência (22-kHz) (Wöhr e Schwarting, 2013). Assim, tanto as USVs apetitivas de 50-kHz e as USVs aversivas de 22-kHz representam diferentes aspectos afetivos e comportamentais dos ratos (Wöhr e Schwarting, 2013). Diferentes tipos de USVs ocorrem em diferentes idades, sendo que durante a infância predomina-se as USVs de 40-kHz e durante a adolescência e fase adulta predominam as USVs de 22 ou 50-kHz, que diminuem em frequência na senescência (Knutson et al., 2002).

Ratos adolescentes e adultos podem emitir USVs de 50-kHz, mais curtas e de maior frequência, em contextos de brincadeiras, como “rough-and-tumble", e exploração entre machos e fêmeas (Brudzynski e Pniak, 2002), atividade locomotora, *rearing* e exploração (Fu e Brudzynski, 1994). É menor em duração, variando de 20 a 80 ms, e maior em frequência, variando de 35 a 70-kHz, com largura de banda de 1 a 6-kHz (Blanchard et al., 1993).

Baseado no perfil de frequência e duração de USVs, três subtipos de vocalizações apetitivas (de 50-kHz) foram descritas até agora: “flat”, frequência modulada (que podem ser subdivididas em “step” ou “mixed”), e “trills” (Brudzynski, 2015), que podem ser vistas nas Figuras 1 e 2. Tanto os tipos *flat* como os frequência modulada estão no mesmo pico de frequência, porém variam em perfil sonográfico e duração (Brudzynski, 2015). USVs *flat* de 50-kHz parecem ser chamadas de contato, ocorrendo em maior frequência em interações afetivas sociais não-positivas (Burgdorf et al., 2008). USVs frequência modulada de 50-kHz parecem ser seletivas para interações afetivas sociais positivas (Burgdorf et al., 2011). USVs *step* são duas chamadas adjacentes com um “jump” entre elas (Grant et al., 2018). *Trills* representam oscilações do tipo “onda” na frequência de chamada e representam estados de excitação e

motivação (Burgdorf et al., 2008). Administração repetida de anfetamina causa sensibilização com aumento da porcentagem de emissões de USVs *trill*, mas não de *flat* (Ahrens et al., 2009).

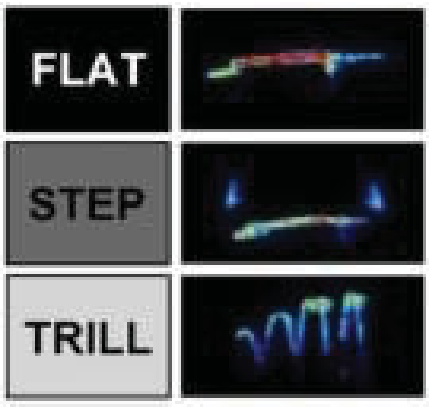

Fig. 1 Exemplos de USVs dos subtipos flat, step e trill. Adaptado de: Kisko et al., 2018.

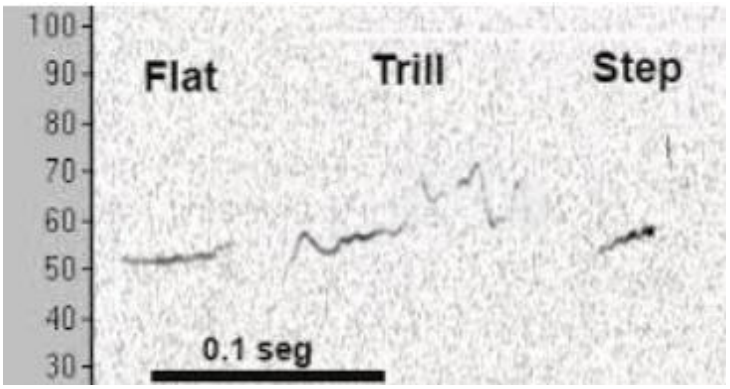

Fig. 2 Espectrograma de USVs dos subtipos *flat, step* e *trill* de 50 kHz. Adaptado de: Trein, 2017.

Estímulos altamente aversivos como odor de predadores, choque nas patas e iluminação excessiva, diminuem as taxas de emissão de USVs de 50-kHz, enquanto que estímulos de recompensa aumentam as taxas de USVs de 50-kHz (Burgdorf et al., 2011; Knutson et al., 2002). A descoberta de que as USVs de 50 kHz refletem um estado positivo em ratos permite que tais medidas possam monitorar estados hedônicos em modelos animais de diversos transtornos psiquiátricos (Burgdorf et al., 2011). O aumento da emissão de USVs de 50-kHz induzido pela administração de anfetamina é geralmente relacionada em paralelo com a hiperlocomoção (Ahrens et al., 2013). Outros psicoestimulantes que afetam a liberação de catecolaminas, como a cocaína,

também aumentam as USVs de 50-kHz (Wright et al., 2012). A administração periférica ou central de anfetamina aumenta incondicionalmente a emissão de USVs de 50-kHz de maneira dose-dependente (Burgdorf et al., 2001; Wintink e Brudzynski, 2001). Este efeito pode ser revertido com administração periférica do antagonista dopaminérgico haloperidol, por exemplo (Wintink e Brudzynski, 2001).

Rippberger et al. (2015) mostraram que a administração de *d*-anfetamina levou ao aumento de todos os subtipos de USVs de 50-kHz. Drogas utilizadas no tratamento do TB, como antipsicóticos (como risperidona e haloperidol), lítio e tamoxifeno inibem o aumento nas USVs de 50-kHz induzido por *d*-anfetamina ou LDX (Wendler et al., 2016; Barker et al., 2015; Rippberger et al., 2015; Pereira et al., 2014). Pereira et al. (2014) avaliaram os efeitos da droga antimaníaca lítio e dos inibidores de PKC tamoxifeno e miricitrina no aumento de USVs de 50-kHz e hiperlocomoção induzidos por anfetamina em ratos Wistar machos adultos. Lítio, tamoxifeno e miricitrina aboliram o aumento de USVs de 50-kHz e hiperlocomoção induzidos por anfetamina.

Neste contexto, levando em consideração que a GSK3β é uma enzima envolvida na fisiopatologia do TB e que a inibição da GSK3β, como a induzida por lítio ou valproato, melhora sintomas maníacos, é relevante testar o efeito do inibidor de GSK3β ANDRO em diferentes modelos animais de mania, como a hiperlocomoção e aumento de atividade exploratória induzidos por privação de sono e/ou metilfenidato, além do aumento de USVs de 50-kHz por LDX. Além disso, avaliar os efeitos do ANDRO nos níveis de GSK3β e p-Ser^{9}-GSK3β e em parâmetros de estresse oxidativo no CPF e estriado dos animais.

2. OBJETIVOS

2.1 Objetivo geral

Avaliar os possíveis efeitos tipo antimaníacos do ANDRO em diferentes modelos animais de mania, como a hiperlocomoção induzida por privação de sono ou metilfenidato, além do aumento de USVs de 50-kHz por LDX e no aumento da atividade exploratória induzida por metilfenidato no monitor de padrão comportamental (BPM), assim como avaliar os efeitos do ANDRO nos níveis de GSK3β e p-Ser^{9}-GSK3β e em parâmetros de estresse oxidativo no CPF e estriado dos animais.

2.2 Objetivos específicos

- Avaliar os efeitos do tratamento crônico com ANDRO (0.5 mg/kg ou 2.0 mg/kg) ou lítio (100 mg/kg) em camundongos submetidos ao modelo de hiperlocomoção induzida por privação de sono ou metilfenidato
- Avaliar os efeitos do tratamento crônico com ANDRO (0.5 mg/kg ou 2.0 mg/kg) ou lítio (100 mg/kg) nos níveis de GSK3β e p-Ser^{9}-GSK3β no CPF e estriado dos animais submetidos aos modelos de hiperlocomoção induzida por privação de sono ou por metilfenidato.
- Avaliar os efeitos do tratamento crônico com ANDRO (2.0 mg/kg) ou lítio (100 mg/kg) em ratos submetidos ao modelo de aumento de USVs de 50-kHz e hiperlocomoção induzidos por LDX.
- Avaliar os efeitos do tratamento crônico com ANDRO (2.0 mg/kg) ou lítio (100 mg/kg) em parâmetros de estresse oxidativo (níveis de GSH e LPO) no CPF e estriado de ratos submetidos ao modelo de aumento de USVs de 50-kHz e hiperlocomoção induzidos por LDX.
- Avaliar os efeitos do tratamento crônico com ANDRO (2.0 mg/kg) ou lítio (100 mg/kg) na atividade exploratória de camundongos submetidos ao modelo de hiperlocomoção induzida por metilfenidato no monitor de padrão comportamental (BPM).
- Correlacionar todos os resultados obtidos para analisar os possíveis efeitos tipo antimaníacos do ANDRO.

3. ARTIGO 1

Andrographolide prevents sleep deprivation- and methylphenidate-induced manic-like behavior mediated via GSK3β inhibition

Kanazawa, Luiz Kae Sales[a]; Nelissen, Ellis[b]; Aguiar, Rafael Pazinatto[c]; Prickaerts, Jos[b]; Andreatini, Roberto[a].

[a] *Laboratory of Physiology and Pharmacology of the Central Nervous System, Department of Pharmacology, Federal University of Paraná, Centro Politécnico, 81540-990 Curitiba, PR, Brazil*

[b] *Department of Psychiatry and Neuropsychiatry, School for Mental Health and Neuroscience (MHeNS), Maastricht University, Universiteitssingel 50, 6229 ER Maastricht, The Netherlands*

c Department of Pharmacology and Therapeutics, State University of Maringá, 87020-900, Maringá, PR, Brazil

ABSTRACT

The pathophysiological aspects of bipolar disorder (BD) are not yet completely elucidated. However, studies indicate that it involves increased activity of the enzyme glycogen synthase kinase 3 beta (GSK3β) in the brain. Inhibition of GSK3β activity has been shown to reduce manic symptoms, with the antimanic effects of lithium, a GSK3β inhibitor, as a clear example. Andrographolide (ANDRO), the major bioactive compound of the plant *Andrographis paniculata*, is also an inhibitor of GSK3β and, therefore, might possess antimanic-like properties. Thus, we aimed to investigate the effect of a 21 days chronic treatment with 0.5 mg/kg or 2 mg/kg ANDRO or 100 mg/kg lithium on mice submitted to different models of mania: 24-h paradoxical sleep deprivation (SD) and acute administration of 5 mg/kg methylphenidate (s.c.). Both models are known to induce hyperlocomotion and increased activity of GSK3β. The results showed that SD induced hyperlocomotion in mice, which was reversed by chronic treatment with lithium and both doses of ANDRO. We also found that SD decreased Ser^9 phosphorylation of GSK3β in the prefrontal cortex (PFC) of these mice, indicative of increased GSK3β activity. Chronic treatment with lithium or 2.0 mg/kg ANDRO was able to reverse this SD-induced decrease in p-Ser^9-GSK3β. In addition, chronic administration of lithium as well as 0.5 mg/kg and 2.0 mg/kg ANDRO reduced methylphenidate-induced hyperlocomotion. Methylphenidate induced decreased Ser^9 phosphorylation of GSK3β in the striatum, which was prevented by lithium and 2.0 mg/kg ANDRO. These results indicate that ANDRO has antimanic-like properties that may be mediated by inhibiting GSK3β activity in the frontostriatal system.

Key words: andrographolide, bipolar disorder, glycogen synthase kinase 3 beta, mania, methylphenidate, sleep deprivation.

Abbreviations: ANDRO, andrographolide; BD, bipolar disorder; GSK3β, glycogen synthase kinase-3 beta; OF, open-field; PFC, pre-frontal cortex; SD, sleep deprivation.

1. INTRODUCTION

Bipolar disorder (BD) is a disease defined by episodes of mania and depression with euthymic states in between (Logan and McClung, 2016). There is a high prevalence of psychiatric and medical comorbidities among BD patients, as well as high rates of suicide attempts, disability and mortality (Grande et al., 2016). The pathophysiological factors underlying BD are not completely elucidated. However, several studies show the involvement of molecular targets in intracellular signaling pathways, such as glycogen synthase kinase 3 beta (GSK3β), protein kinase C (PKC) and inositol monophosphates (Grande et al., 2016). The enzyme GSK3β is a constitutively active serine/threonine kinase, inactivated by phosphorylation of serine residues at serine 9 of the regulatory amino-terminal domains (Frame and Cohen, 2001). The enzyme acts as a downstream regulatory switch that determines the output of several signaling pathways (Prickaerts et al., 2006). This enzyme is involved in many complex biological alterations of BD, such as neuroinflammation, oxidative stress and alterations in membrane ion channels and in the circadian system (Luca et al., 2016). An increase in GSK3β expression is linked to the occurrence of manic-like behaviors in animals (Prickaerts et al., 2006; Gould et al., 2004). Manic bipolar patients showed higher levels of GSK3β compared to healthy controls (Li et al., 2010), and mood stabilizers such as lithium and valproate have been associated with inhibition of GSK3β in preclinical studies (Cechinel-Recco et al., 2012; Jope, 2011). In fact, the inhibitory activity of the mood stabilizer lithium over GSK3β is believed to be essential for its antimanic action (Chiu et al., 2013). Furthermore, it is known that GSK3β inhibitors reduced locomotor hyperactivity both in DAT knockout and amphetamine-treated wild-type mice (Beaulieu et al., 2004; Gould et al., 2004), which are models for manic-like behavior. Additionally, the inhibition of GSK3β seems to have a neuroprotective effect (Serrano et al., 2014).

The pharmacological management of BD usually consists of a mood stabilizer alone or in combination with antipsychotics or antidepressants (Vieta et al., 2013). However, many patients take years to achieve stabilization and the life-long treatment may lead to the occurrence of several adverse effects (Alda and Manchia, 2018). Therefore, research on alternative therapeutic options is

needed and molecular targets in intracellular signaling pathways might act as a starting point to develop future treatments (Geddes and Miklowitz, 2013).

Andrographolide (ANDRO), which is the major bioactive compound isolated from *Andrographis paniculata*, a medicinal plant with anti-inflammatory and antioxidant properties (Yang et al., 2017; Jayakumar et al., 2013; Das et al., 2009), is known to induce GSK3β inhibition (Tapia-Rojas et al., 2015). This diterpenoid possesses anti-inflammatory, antiapoptotic and antioxidantproperties which can be beneficial in many disorders (Graverini et al., 2018; Daset al., 2009). Serrano et al. (2014) showed that, similarly to the antimanic drug lithium, ANDRO was capable of increasing the levels of the inactive, serine 9 phosphorylated form of GSK3β in an Alzheimer's disease mouse model. Therefore, the possible antimanic-like effect of ANDRO, a GSK3β inhibitory drug, was investigated in sleep deprivation (SD)- and methylphenidate-induced animalmodels for mania. The administration of psychostimulants is considered to be a valid model of mania, as it induces behavioral and molecular changes seen in mania (Logan and McClung, 2016). SD also produces neurochemical alterationsseen in bipolar patients (Arent et al., 2015). Both models also induce increased expression of GSK3β in different brain regions (Andrabi et al., 2020; Mines andJope, 2012). Thus, the evaluation of the effects of ANDRO on SD- and methylphenidate-induced manic-like behavior can provide a better understandingof the neurobiology of mania and potentially offer perspectives on ANDRO as a treatment for the management of BD.

2. MATERIALS AND METHODS

2.1 Animals

Male Swiss mice (30 – 40 g) were housed socially (6-8/cage) with controlled temperature (22±2 ºC) in a 12h:12h light/dark cycle (with lights on between 7 a.m. and 7 p.m.) with free access to water and food. The animals were allowed to acclimate for one week before testing commenced. All experiments were performed in accordance with the Brazilian Law for Animal Experimental Ethics and Care (11.794/8 October 2008) and the Local Committee on the Care and Use of Laboratory Animals. The experimental procedures were approved by

the Institutional Ethics Board (CEUA/BIO – protocol #1109). All efforts were made to minimize animal suffering and the number of animals used.

2.2 Drugs

Andrographolide (Sigma, São Paulo, Brazil) was administered at doses of 0.5 and 2 mg/kg, intraperitoneally (i.p.) (Chan et al., 2010; Niranjan et al., 2010). ANDRO was dissolved in saline with dimethyl sulfoxide (DMSO). The repeated treatment was performed throughout 21 days, 3 times a week.

Lithium carbonate (Eurofarma, Itapevi, Brazil) was used as positive control at a dose of 100 mg/kg. Lithium was dissolved in saline and the pH was adjusted to 7.4 by adding 2N HCl. Repeated treatment was performed throughout 21 days, once a day, i.p.

Methylphenidate (Novartis, São Paulo, Brazil) was used for the induction of manic-like behavior at a dose of 5 mg/kg, subcutaneously (s.c.), 30 minutes before the experiments, in a single administration.

All drugs were administered in a constant volume of 10 ml/kg body weight.

2.3 Sleep deprivation protocol

For the non-pharmacological induction of manic-like behavior, mice were submitted to the 24h SD protocol (Silva et al., 2004). Groups of six animals were placed in polypropylene cages (41 x 34 x 16 cm), each cage containing 12 platforms (3 cm in diameter x 5 cm in height), surrounded by water up to 1 cm below the surface of the platforms. The animals could move freely, jumping from one platform to the other. Food and water were available the whole time.

The objective of the protocol is that, when animals reach REM sleep, they display muscle relaxation, falling into the water and, thus, having their sleep interrupted (Machado-Vieira et al., 2004). After this period of SD, animals show some behaviors such as hyperlocomotion and an increase in appetitive ultrassonic vocalizations (USVs), which can be correlated with manic-like behaviors (Wendler et al., 2019; Armani et al., 2012; Gessa et al., 1995). SD also reproduces aspects of the manic episode such as hyperactivity, hypersexuality and aggressiveness of manic patients (Valvassori et al., 2017).

The animals were chronically treated for 21 days with either vehicle (saline + DMSO), 100 mg/kg lithium or 0.5 or 2.0 mg/kg ANDRO. On the last day of

treatment, the animals were submitted to the 24h SD protocol as previously described. After this period, the animals were placed in the open-field, where the number of crossings was measured for 5 minutes. The open-field apparatus is a round arena (42 cm in diameter x 28 cm in height) divided in three circles subdivided into 25 equal regions (Barbosa et al., 2011). Following the test, the animals were euthanized for the removal of their pre-frontal cortex (PFC) and striatum for further analysis.

2.4 Methylphenidate-induced hyperlocomotion

Psychostimulant-induced hyperlocomotion is the most frequently used animal model of mania (Einat, 2006). This pharmacological induction of manic-like behavior is reliable and shows face, construct and predictive validity (Einat, 2006; Machado-Vieira et al., 2004). Psychostimulants that are capable of increasing the levels of dopamine cause behavioral effects that resemble mania, such as hyperlocomotion (Hasler et al., 2006).

To measure hyperlocomotion, the number of crossings in the open field was analyzed as an index of locomotor activity. The blocking or attenuation of hyperlocomotion after methylphenidate administration is indicative of an antimanic-like effect, at doses that do not impair locomotor activity *per se* (Sabioni et al., 2008; Gould et al., 2001).

The animals were chronically treated for 21 days with either vehicle (saline + DMSO), 100 mg/kg lithium or 0.5 or 2.0 mg/kg ANDRO. On the test day, either vehicle or 5 mg/kg methylphenidate (s.c.) was administered to the animals. After 30 minutes, they were placed in the center of the open-field apparatus for the evaluation of the locomotor activity within 5 minutes (Barbosa et al., 2011). Following the test, the animals were euthanized for the removal of their PFC and striatum for further analysis.

2.5 Western Blot

Mice were euthanized by decapitation and the PFC and striatum were removed, immediately frozen in liquid nitrogen and kept at -80ºC. The samples were homogenized in 200 µl ice-cold lysis buffer (1 mM EDTA, 1 mM EGTA, 1% glycerol, 0.1% triton, and 1% IGEPAL CA-630 in phosphate-buffered saline (PBS)) containing protease and phosphatase inhibitors (Roche, Mannheim,

Germany) by the use of a SpeedMill PLUS tissue homogenizer (Analytik Jena AG, Jena, Germany). The homogenates were subsequently centrifuged at 14000 rpm for 20 minutes at 4ºC. The supernatant was used for protein determination (DC™ Protein Assay, Bio-Rad laboratories, Veenendaal, the Netherlands). 30µg of each sample was incubated at 100ºC for 7 min and separated on a 10% SDS-PAGE gel. After electrophoresis, proteins were transferred to nitrocellulose membranes (Bio-Rad Laboratories) and subsequently blocked with Odyssey blocking buffer in PBS (Li-Cor, Lincoln, NE, USA) for 1h at room temperature. Afterwards, the membranes were incubated overnight at 4ºC with the following primary antibodies in blocking buffer and PBS: rabbit anti-GSK3β (#9315S, Cell Signaling Technologies, Beverly, MA, USA, 1:1000); rabbit anti-p-GSK3β (p-Ser^{9}) (#9336S, Cell Signaling Technologies, 1:1000) or mouse anti-β-actin (#A5441, Sigma-Aldrich, Darmstadt, Germany, 1:20000), for normalization. Membranes were washed with PBS or PBS-0.1% Tween 20 (PBS-T), incubated with goat anti-rabbit IRDye 800 and donkey anti-mouse IRDye 680 secondary antibodies (Li-Cor, 1:10000) for 1h at room temperature, and washed again. The membrane was dried and bands were visualized using an Odyssey CLx Infrared Imaging System (Li-Cor) and quantification was performed using the software Image Studio Lite Ver 5.2.

2.6 Statistical analysis

Data were analyzed by one-way ANOVA. The statistical analysis for the groups treated with lithium was performed separately from the groups treated with ANDRO. The differences between the groups were analyzed by the least significant difference (LSD) *post hoc* test. Differences were considered to be statistically significant when $p < 0.05$. Data was expressed as mean ± SEM. SPSS 16.0 (SPSS Inc., Chicago, IL) was used for the statistical analysis.

3. RESULTS

3.1 The effects of lithium and ANDRO on sleep deprivation-induced hyperlocomotion

Hyperlocomotion was measured in mice by recording the number of crossings in an open-field test. A one-way ANOVA ($F_{3,36} = 4.98$, $p < 0.01$)

revealed that chronic treatment with lithium successfully reversed SD-induced hyperlocomotion (*post-hoc* LSD, $p < 0.01$; Figure 1A). Similar results were found for chronic ANDRO treatment ($F_{5,53} = 2.52$, $p < 0.05$) at both 0.5 mg/kg (*post-hoc* LSD, $p < 0.05$) and 2.0 mg/kg ($p < 0.01$; Figure 1B). This confirms that ANDRO exerts effects similar to lithium on locomotor behavior of mice in a SD-induced model for mania.

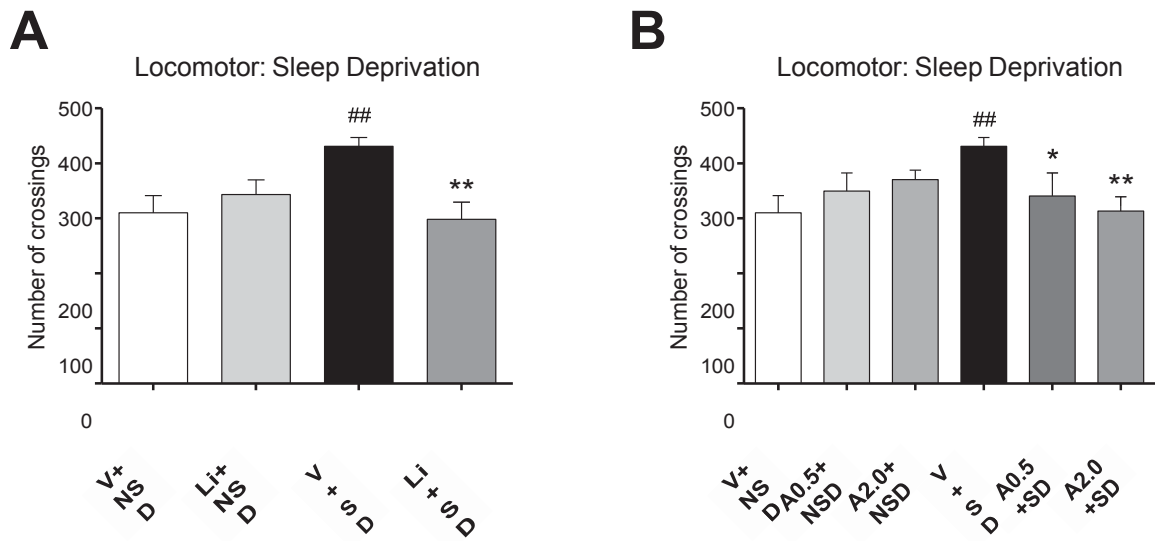

Fig 1. Effects of lithium and ANDRO on SD-induced hyperlocomotion. SD induced hyperlocomotion in mice, as was measured by an increased number of crossings in the open-field test (one-way ANOVA with *post-hoc* LSD, $p < 0.01$). Both A) lithium (i.p.) and B) 0.5mg/kg and 2.0 mg/kg ANDRO (i.p.), successfully reversed this SD-induced hyperlocomotion ($p < 0.01$). Data are represented as mean ± SEM; n=8-10. Hashes represent a significant difference from the V+NSD group, asterisks represent a significant difference from the V+SD group. $^{\#\#}$ $p < 0.01$; * $p < 0.05$, ** $p < 0.01$. V+NSD: vehicle + non-sleep deprivation; Li+NSD: lithium + non-sleep deprivation; V+SD: vehicle + sleep deprivation; Li+SD: lithium + sleep deprivation; A0.5+NSD: 0.5 mg/kg ANDRO + non-sleep deprivation; A2.0+NSD: 2.0 mg/kg ANDRO + non-sleep deprivation; A0.5+SD: 0.5 mg/kg ANDRO + sleep deprivation; A2.0+SD: 2.0 mg/kg ANDRO + sleep deprivation.

3.2 The effects of lithium and ANDRO on GSK3β phosphorylation in a SD model

Ser^9 phosphorylation levels of GSK3β as an indicator of its inactivity were measured in the PFC and striatum of mice chronically treated with lithium or ANDRO, alone or in combination with SD. A one-way ANOVA ($F_{3,35} = 10.08$, $p < 0.001$) revealed that SD reduced Ser^9 phosphorylation of GSK3β (*post-hoc* LSD,$p < 0.001$) and that GSK3β inhibitor lithium prevented this decrease ($p < 0.001$, Figure 2A) in the PFC. Similar results ($F_{5,53} = 6.22$, $p < 0.001$) were found for 2.0 mg/kg ANDRO, which successfully prevented a decrease in GSK3β Ser^9-phosphorylation ($p < 0.001$, Figure 2B) in the PFC. This effect was dose-

dependent, since 0.5 mg/kg ANDRO did not affect phosphorylation of Ser^{9}. This shows that ANDRO is able to dose-dependently inhibit GSK3β similar to lithium in a SD model for mania in the PFC of mice.

In the striatum, a one-way ANOVA revealed both treatment effects for the lithium groups ($F_{3,36}$ = 5.30, p < 0.01), as well as for the ANDRO groups ($F_{5,52}$ = 2.93, p < 0.05). However, contrary to the findings in the PFC, SD did not affect Ser^{9} phosphorylation of GSK3β in the striatum. Interestingly, both lithium (Figure 2D) and 2.0 mg/kg ANDRO (Figure 2E) enhanced GSK3β Ser^{9}-phosphorylation with and without SD. This shows that lithium and ANDRO both exert unspecific drug effects in the absence of SD model effect, or a disease model altogether. Additionally, the absence of SD induced effects on GSK3β Ser^{9}-phosphorylationin the striatum suggests that such effects are more specific to the PFC in comparison to the striatum.

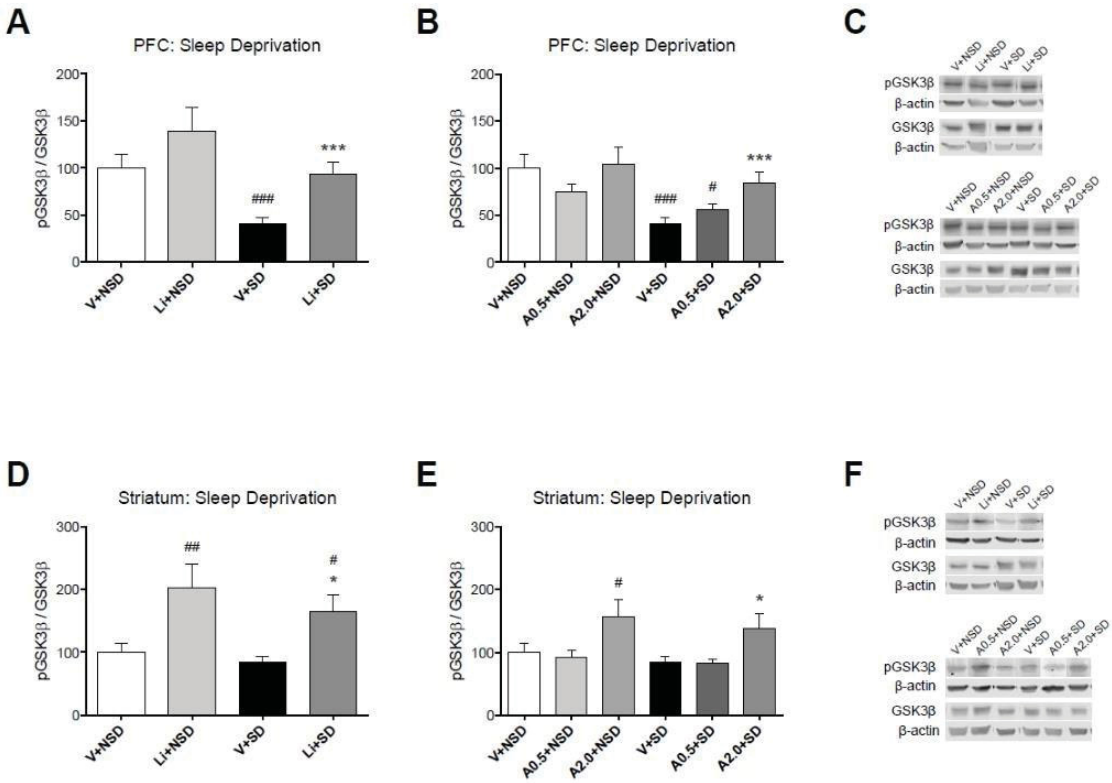

Fig 2. Effects of lithium and ANDRO on Ser^{9} phosphorylation of GSK3β in a SD model for mania. A) SD induced a reduction in Ser^{9} phosphorylation of GSK3β (one-way ANOVA with *post-hoc* LSD, p < 0.001) in the PFC, suggesting increased GSK3β activity. Chronic treatment with lithium (i.p.) or B) 2.0 mg/kg ANDRO (i.p.) was able to prevent this decrease in phosphorylation (p < 0.001). 0.5 mg/kg ANDRO did not affect GSK3β Ser^{9}-phosphorylation levels, suggesting a dose-dependent effect of ANDRO. C) Representative western blot bands for the PFC. D) Contrary to the PFC, SD did not affect serine-9 phosphorylation of GSK3β in the striatum. Yet, chronic treatment with either lithium (i.p.) or E) 2.0 mg/kg ANDRO (i.p.) enhanced GSK3β Ser^{9}-phosphorylation independent of SD model effects, or a disease model altogether, suggesting disease-unspecific effects of both drugs. F) Representative western blot bands for the striatum.

Data are represented as mean ± SEM; n=8-10. Hashes represent a significant difference from the V+NSD group, asterisks represent a significant difference from the V+SD group. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$; * $p < 0.05$, *** $p < 0.001$. V+NSD: vehicle + non-sleep deprivation; Li+NSD: lithium + non-sleep deprivation; V+SD: vehicle + sleep deprivation; Li+SD: lithium + sleep deprivation; A0.5+NSD: 0.5 mg/kg ANDRO + non-sleep deprivation; A2.0+NSD: 2.0 mg/kg ANDRO + non-sleep deprivation; A0.5+SD: 0.5 mg/kg ANDRO + sleep deprivation; A2.0+SD: 2.0 mg/kg ANDRO + sleep deprivation.

3.3 The effects of lithium and ANDRO on methylphenidate-induced hyperlocomotion

Similar to the SD model, hyperlocomotion was measured in mice by recording the number of crossings in an open-field test. A one-way ANOVA ($F_{3,36} = 20.38$, $p < 0.001$) revealed that chronic lithium treatment successfully reversed methylphenidate-induced hyperlocomotion (*post-hoc* LSD, $p < 0.001$; Figure 3A). Similar results were found for chronic treatment with ANDRO ($F_{5,54} = 13.71$, $p < 0.001$) at both 0.5 mg/kg and 2.0 mg/kg (*post-hoc* LSD, $p < 0.001$; Figure 1B). Similar to the SD findings, this confirms that ANDRO exerts effects on locomotor behavior of mice in a methylphenidate-induced model for mania that are similar to conventional treatment with lithium.

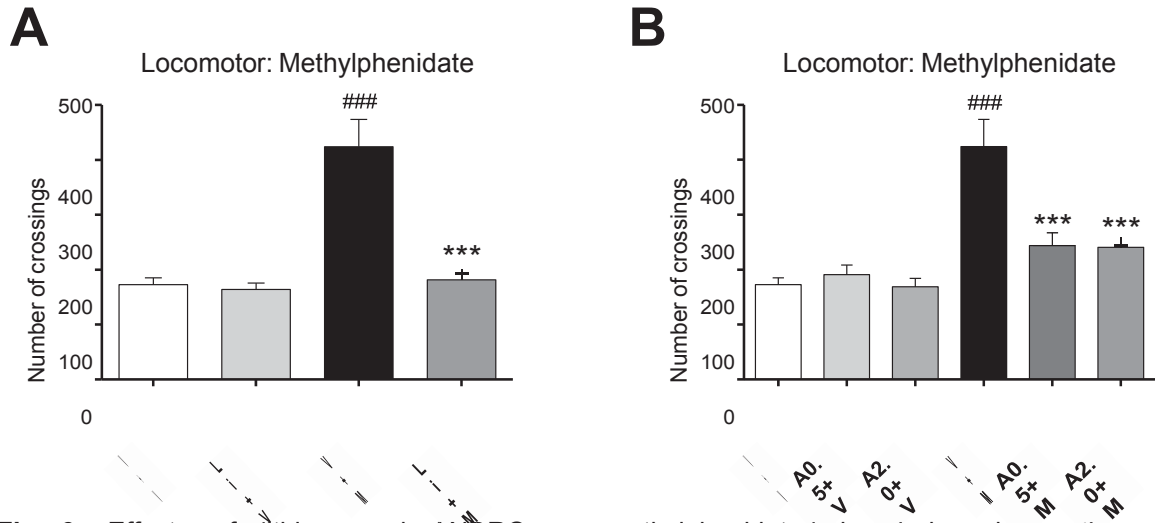

Fig 3. Effects of lithium and ANDRO on methylphenidate-induced hyperlocomotion. Methylphenidate treatment (s.c.) induced hyperlocomotion in mice, as was measured by an increased number of crossings in the open-field test (one-way ANOVA with *post-hoc* LSD, $p < 0.001$). Both A) lithium (i.p.) and B) 0.5mg/kg and 2.0 mg/kg ANDRO (i.p.), successfully reversed this methylphenidate-induced hyperlocomotion ($p < 0.001$). Data are represented as mean ± SEM; n=8-10. Hashes represent a significant difference from the V+V group, asterisks represent a significant difference from the V+M group. $^{\#\#\#}p < 0.001$; *** $p < 0.001$. V+V: vehicle + vehicle; Li+V: lithium + vehicle; V+M: vehicle + methylphenidate; Li+M: lithium + methylphenidate; A0.5+V: 0.5 mg/kg ANDRO + vehicle; A2.0+V: 2.0 mg/kg ANDRO + vehicle; A0.5+M: 0.5 mg/kg ANDRO + methylphenidate; A2.0+M: 2.0 mg/kg ANDRO + methylphenidate.

3.4 The effects of lithium and ANDRO on GSK3β phosphorylation in a methylphenidate model

Again, phosphorylation levels of GSK3β on Ser^9 were measured in the PFC and striatum of mice as an indication of GSK3β protein inactivity levels, but now in the methylphenidate model for mania. A one-way ANOVA revealed chronic treatment effects for both the lithium groups ($F_{3,30} = 16.78$, $p < 0.001$) and the ANDRO groups ($F_{5,49} = 2.49$, $p < 0.05$) in the PFC of mice. However, methylphenidate treatment did not affect Ser^9 phosphorylation of GSK3β in the PFC. Still, both GSK3β inhibitor lithium ($p < 0.01$, Figure 4A), as well as 2.0 mg/kg ANDRO ($p < 0.05$, Figure 4B) showed unspecific treatment effects by enhancing GSK3β Ser^9 phosphorylation in the absence of a model effect of methylphenidate. Moreover, lithium even enhanced Ser^9 phosphorylation in the absence of methylphenidate treatment altogether, suggesting a drug-effect completely unspecific to the presence of GSK3β activity deficits. Interestingly, 0.5 mg/kg ANDRO in combination with methylphenidate reduced Ser^9 phosphorylation ($p < 0.05$), suggesting a differential dose-effect of ANDRO.

A one-way ANOVA ($F_{3,36} = 7.24$, $p < 0.001$) revealed that methylphenidate treatment did reduce Ser^9 phosphorylation (*post-hoc* LSD, $p < 0.05$) in the striatum, contrary to the PFC. Chronic treatment with GSK3β inhibitor lithium successfully prevented this decrease ($p < 0.001$; Figure 4D) and similar results ($F_{5,54} = 5.88$, $p < 0.001$) were found for 2.0 mg/kg ANDRO ($p < 0.001$), but not 0.5 mg/kg (Figure 4E). This suggests that, similar to lithium, 2.0 mg/kg ANDRO inhibited GSK3β activity through enhanced Ser^9 phosphorylation. Additionally, methylphenidate-induced enhancement of GSK3β activity through reduced Ser^9 phosphorylation seems to be specific for the striatum in comparison to the PFC. This is in contrast to sleep deprivation, which seems to specifically affect the PFC rather than the striatum.

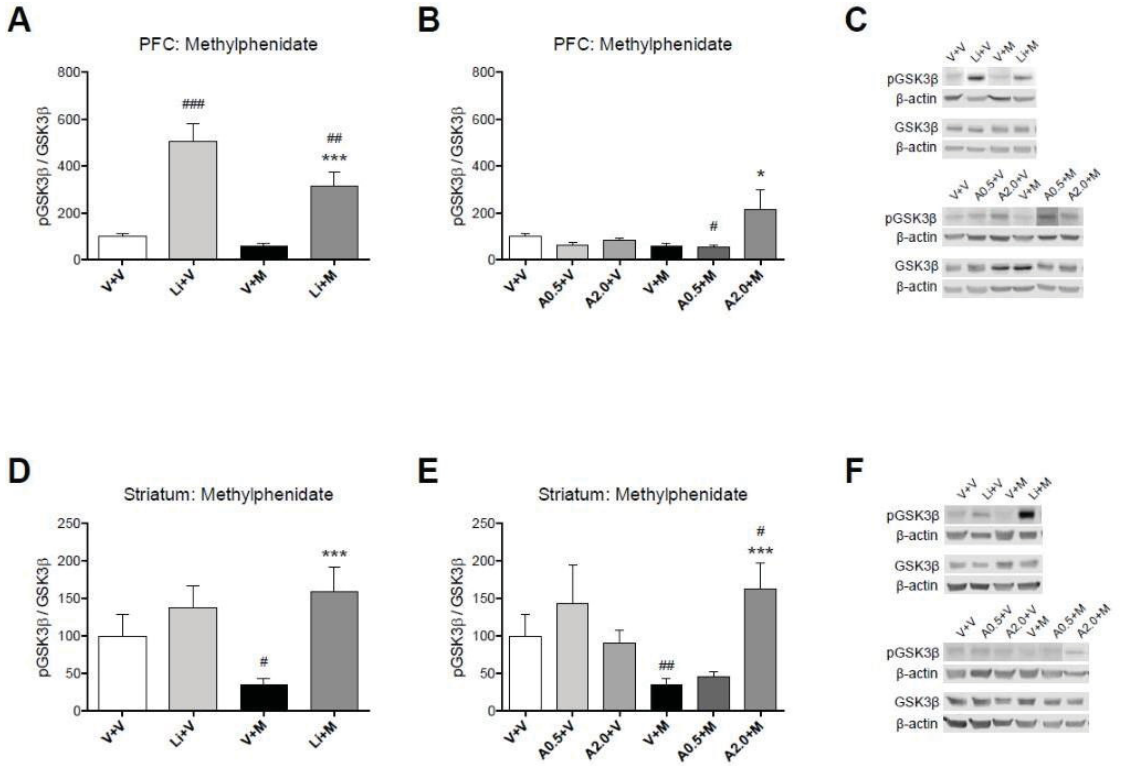

Fig 4. Effects of lithium and ANDRO on Ser^{9} phosphorylation of GSK3β in a methylphenidate model for mania. A) Methylphenidate treatment (s.c.) did not affect Ser^{9} phosphorylation of GSK3β (one-way ANOVA with *post-hoc* LSD, $p < 0.05$) in the PFC of mice. Still, both chronic treatment with lithium (i.p.) and B) 2.0 mg/kg ANDRO (i.p.) increased Ser^{9} phosphorylation of GSK3β ($p < 0.001$), suggesting an unspecific drug effect regardless of a methylphenidate model effect. Surprisingly, 0.5 mg/kg ANDRO reduced GSK3β Ser^{9} phosphorylation levels in the presence of the methylphenidate model, suggesting differential dose-effects of ANDRO. C) Representative western blot bands. D) Contrary to the PFC, methylphenidate treatment (s.c.) reduced GSK3β Ser^{9} phosphorylation in the striatum of mice. Both lithium (i.p.) and E) 2.0 mg/kgANDRO (i.p.) chronic treatment prevented this decrease in Ser^{9} phosphorylation. 0.5 mg/kg ANDRO did not affect GSK3β Ser^{9} phosphorylation, suggesting dose-dependent effects of ANDRO. F) Representative western blot bands. Data are represented as mean ± SEM; n=8-10. Hashes represent a significant difference from the V+V group, asterisks represent a significant difference from the V+M group. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$; $^{*}p < 0.05$, $^{***}p < 0.001$. V+V: vehicle + vehicle; Li+V: lithium + vehicle; V+M: vehicle + methylphenidate; Li+M: lithium + methylphenidate; A0.5+V: 0.5 mg/kg ANDRO + vehicle; A2.0+V: 2.0 mg/kg ANDRO + vehicle; A0.5+M: 0.5 mg/kg ANDRO + methylphenidate; A2.0+M: 2.0 mg/kg ANDRO + methylphenidate.

4. DISCUSSION

4.1 Chronic ANDRO treatment reverses SD-induced changes in locomotor behavior and p-Ser^{9}-GSK3β phosphorylation in the PFC of mice

Our results showed that 24-h SD induced hyperlocomotion in mice. Chronic treatment with lithium was able to reverse this SD-induced hyperlocomotion. Importantly, this effect was seen at doses that did not affect spontaneous locomotor activity. These results are similar to reports in literature showing that SD induced hyperactivity in mice, which was reversed by lithium administration (Valvassori et al., 2017; Armani et al., 2012). Additionally, Wendler

et al. (2019) showed that rats submitted to the SD model, displayed increased locomotor activity, increased emission of 50-kHz USVs, as well as a change in the call profile characterized by an increase in the percentage of frequency modulated 50-kHz USVs, which is related to the SD-induced manic-like behaviors. All these behaviors were reversed by treatment with lithium. Abrial et al. (2015) showed also that SD triggers manic-like behaviors such as hyperlocomotion and increased sleep latency, which were reversed by lithium and aripiprazole. Interestingly, we found that chronic treatment with 0.5 mg/kg as well as 2.0 mg/kg ANDRO, which can inhibit GSK3β activity, displayed an antimanic-like effect by reversing SD-induced hyperlocomotion similar to lithium.

The enzyme GSK3β has numerous cellular and molecular functions, including cell development, gene transcription, metabolic homeostasis, neurogenesis, and apoptosis (Doble and Woodgett, 2003). However, animals that overexpress GSK3β show behaviors which can be correlated with mania in humans (Chen et al., 2009; Prickaerts et al., 2006). It was shown that phosphorylation of GSK3β at Ser^9 inhibits GSK3β activity, and previous studies have used the increase or decrease in p-Ser^9 levels as changes of cellular GSK3β activity (Jope and Johnson, 2004). Lithium, the prototype mood stabilizer, is both a direct and indirect inhibitor of GSK3β, which is proposed to be involved in the anti-manic action of the drug (Phiel and Klein, 2001). At therapeutic concentrations, lithium treatment can lead to inhibition of GSK3β by increasing phosphorylation at Ser^9 (Costemale-Lacoste et al., 2016; Sani et al., 2012). Li et al. (2007) showed an 8-fold increase in p-Ser^9-GSK3β levels in peripheral blood mononuclear cells of BD patients treated with lithium, compared to healthy controls. Therefore, we investigated the effects of SD on the Ser^9 phosphorylation status of GSK3β as an indicator of reduced GSK3β activity.

Our results showed that SD decreased Ser^9 phosphorylation of GSK3β in the PFC of mice, indicative of increased GSK3β activity. Chronic treatment with lithium or 2.0 mg/kg ANDRO were able to reverse this SD-induced decrease in p-Ser^9-GSK3β. Li et al. (2010) showed that the levels of total GSK3β were higher whereas Ser^9 phosphorylation of GSK3β was reduced in blood cells from manic patients than in those cells of healthy controls, and that chronic antimanic treatment increased the Ser^9 phosphorylation of GSK3β. Likewise, Andrabi et al. (2020) showed that SD induced an upregulation in GSK3β levels in the rat

hippocampus, which was prevented by lithium treatment. Therefore, the increase in the levels of inactive Ser^9-phosphorylated GSK3β by lithium and 2.0 mg/kg ANDRO can have a beneficial effect on manic-like behaviors. One of the antimanic actions of lithium involves indirect inhibitory effects on GSK3β activity, by activating Akt directly, which increases the levels of inactive p-Ser^9-GSK3β (Freland and Beaulieu, 2012).

Interestingly, in the striatum, SD did not affect the Ser^9 phosphorylation status of GSK3β. However, both lithium and 2.0 mg/kg ANDRO increased phosphorylation of Ser^9-GSK3β, indicative of GSK3β inhibition, despite this absence of SD-induced effects on GSK3β in the striatum. This is indicative of a non-specific drug effect independent of the presence of a disease model, both for lithium and ANDRO. Our lack of an effect on Ser^9-GSK3β phosphorylation in the striatum is in contrast to SD-induced alterations in the PFC, so it might be argued that the rodent SD-induced model for mania is mainly affecting GSK3β activity in the PFC.

4.2 Chronic ANDRO treatment reverses methylphenidate-induced changes in locomotor behavior and Ser^9-GSK3β phosphorylation in the striatum of mice

The mechanism of action of methylphenidate is mediated by its ability to block the DAT and thus increase levels of extracellular dopamine in brain regions such as the striatum and the PFC (Bymaster et al., 2002; Volkow et al., 2002. Volkow et al., 2001). The administration of methylphenidate also induces hyperactivity which is reversed by lithium, sodium valproate and carbamazepine treatment (Logan and McClung, 2016; Tonelli et al., 2013; Barbosa et al., 2011). Methylphenidate monotherapy in bipolar patients is associated with increases in manic episodes, which are not observed when bipolar patients are using mood stabilizers (Viktorin et al., 2017). Moreover, in healthy volunteers, methylphenidate induced euphoric mood (Smith and Davies, 1977). These results indicated the validity of the methylphenidate administration model of mania. In our study, chronic administration of lithium as well as 0.5 mg/kg and 2.0 mg/kg ANDRO reduced methylphenidate-induced hyperlocomotion, which is an

indicative of antimanic-like effect of ANDRO at doses that did not affect spontaneous locomotor activity.

Studies have shown that psychostimulant administration leads to an increased activity of GSK3β (Mines and Jope, 2012). For instance, GSK3α/β knock-in mice with serine-to-alanine mutations to block serine phosphorylation, show increased sensibility to manic-like amphetamine-induced locomotor hyperactivity (Polter et al., 2010). Additionally, mice lacking one allele of the GSK3β gene show a significant reduction in locomotor responses to amphetamine (Beaulieu et al., 2004). In humans, the activity of GSK3β in the blood of BD patients is increased, when compared to healthy control subjects (Jacoby et al., 2016). Li et al. (2010) observed that antimanic treatment increases p-Ser^{9}-GSK3β in peripheral blood mononuclear cells of bipolar patients with a manic episode. Our study indicates that methylphenidate administration led to a reduction in the phosphorylation of Ser^{9}-GSK3β in the striatum of mice, as seen by a reduction in the p-GSK3β/GSK3β ratio. Additionally, chronic treatment with 2.0 mg/kg ANDRO and lithium reversed the methylphenidate-induced reduction of phosphorylation of Ser^{9}-GSK3β in the striatum of mice. In several rodent models of mania, synthetic inhibitors of GSK3β mimic the therapeutic effects of lithium (Kozikowski et al., 2011; Kozikowski et al., 2007). Mines and Jope (2012) showed that 8-day i.p. administration of 2 mg/kg amphetamine or 20 mg/kg methylphenidate led to decreased levels of p-Ser^{9}-GSK3β in the striatum of mice. Therefore, the attenuation of a methylphenidate-induced decrease in Ser^{9} phosphorylation of GSK3β in the striatum after treatment with ANDRO may be linked to ANDRO's behavioral antimanic-like effect.

Interestingly, methylphenidate did not affect Ser^{9} phosphorylation of GSK3β in the PFC of mice, while both lithium and 2.0 mg/kg ANDRO showed a non-specific drug effect independent by increasing phosphorylation of Ser^{9}-GSK3β in this absence of a methylphenidate-induced effects in the PFC. Additionally, 0.5 mg/kg ANDRO actually reduced phosphorylation of Ser^{9}-GSK3βin the presence of methylphenidate treatment, suggesting a dose-dependent differential effect of ANDRO, yet independent of any methylphenidate treatment. Due to the absence of methylphenidate-induced effects on p-Ser^{9}-GSK3β in the PFC, contrary to a reduction in phosphorylation of Ser^{9}-GSK3β in the striatum, it

could be concluded that the rodent methylphenidate model for mania might mainly exerts its effects through striatal mechanisms over the PFC.

4.3 Differential roles of the striatum and PFC in methylphenidate and SD models for mania in mice

As already briefly pointed out above, we observed that SD-induced enhancement of GSK3β activity through reduced Ser^9-phosphorylation seems to be specific for the PFC in comparison to the striatum. This is in contrast to the methylphenidate effect, which seems to affect Ser^9-phosphorylation specifically in the striatum rather than the PFC.

Most human studies involving the effects of SD measure parameters such as verbal fluency, logical reasoning, working memory, planning, inhibitory capabilities and decision-making, which are functions related to the PFC (Muzur et al., 2002; Harrison and Horne, 2000; Harrison et al., 2000). Indeed, defective frontal functioning was detected in these parameters after SD (Muzur et al., 2002). Neuroimaging studies showed detrimental effects of 24h SD on the blood flow in frontal brain areas, which were related to poor prefrontal task performance afterwards (Thomas et al., 2000; Drummond et al., 1999). Finally, electroencephalogram studies show that the PFC is particularly sensitive to SD, as the PFC shows the greatest changes in brain wave pattern from sleep to waking, as it is relatively inactive all through sleep (Maski and Kothare, 2013; Muzur et al., 2002). Therefore, it is not surprising that the PFC is mainly affected by SD in our study, contrary to the striatum which appears more resilient to the SD model.

In our study, methylphenidate administration did not significantly affect the levels of p-Ser^9-GSK3β in the PFC, as it did in the striatum, suggesting the striatum is more sensitive to the methylphenidate model for mania compared to the PFC. Indeed, Quansah and Zetterström (2019) showed that chronic methylphenidate administration in young rats increased the levels of dopamine-related genes and D_1 receptors mainly in the ventral striatum, in comparison to other brain areas such as the PFC. This indicates that methylphenidate affects primarily the striatum as opposed to the PFC, thereby supporting the findings in our current study. Additionally, a study in monkeys by Kodama et al. (2017), demonstrated that in the striatum, both high and low doses of methylphenidate

induced consistent increases in DA release approximately 30 minutes after the administration. In the PFC on the other hand, a consistent increase in DA release was observed 1 hour after the administration of a high dose of methylphenidate, but not low doses. Finally, Gray et al. (2007) showed that methylphenidate decreased tyrosine hydroxylase-immunoreactivity in the striatum but increased in the mPFC. These results demonstrate that methylphenidate administration in different doses affects the striatum and/or the PFC in different manners, supporting our findings that the striatum is more sensitive to methylphenidate opposed to the PFC.

5. CONCLUSION

Overall, our results show that SD and methylphenidate administration induced hyperlocomotion in mice, which was reversed by chronic treatment with lithium and 0.5 and 2.0 mg/kg ANDRO. Additionally, SD as well as methylphenidate administration led to a reduction in the p-Ser^{9}-GSK3β/GSK3β ratio in the PFC and striatum, respectively, indicating an increased activity of the enzyme. Chronic treatment with 2.0 mg/kg ANDRO and lithium increased the p-Ser^{9}-GSK3β/GSK3β ratio in the PFC and striatum. Overall, these results indicate that ANDRO has antimanic-like properties that may be mediated by the GSK3β pathway, though via divergent effects in the PFC and striatum dependent on the animal model of mania.

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3. ARTIGO 2

Andrographolide prevents increases in 50-kHz ultrassonic vocalizations, hyperlocomotion and oxidative stress induced by lisdexamfetamine in rats, an animal model of mania

Kanazawa, L. K. S.[a]; Radulski, D. R.[b]; Pereira, G. S.[b]; Prickaerts, J.[c]; Schwarting R.K.W.[d]; Acco, A.[b]; Andreatini, R[a].

[a] *Laboratory of Physiology and Pharmacology of the Central Nervous System, Department of Pharmacology, Federal University of Paraná, Centro Politécnico, 81540-990 Curitiba, PR, Brazil*

[b] *Laboratory of Pharmacology and Metabolism, Department of Pharmacology, Federal University of Paraná, Centro Politécnico, 81540-990, Curitiba, PR, Brazil*

[c] *Department of Psychiatry and Neuropsychiatry, School for Mental Health and Neuroscience (MHeNS), Maastricht University, Universiteitssingel 50, 6229 ER Maastricht, The Netherlands*

[d] *Behavioural Neuroscience, Experimental and Biological Psychology, and Center for Mind, Brain and Behavior (CMBB), Philipps-Universität Marburg, 35032 Marburg, Germany*

ABSTRACT

In rats, lisdexamfetamine (LDX) induces manic-like behaviors such as hyperlocomotion and increase in appetitive 50-kHz ultrasonic vocalizations (USVs), which are prevented by antimanic drugs, such as lithium. Inhibition of glycogen synthase kinase 3 beta (GSK3β) has been associated with antimanic effect. Thus, the aim of the present study was to evaluate the possible antimanic-like effect of andrographolide (ANDRO), a GSK3β inhibitor, on LDX-induced hyperlocomotion and 50-kHz USVs increase. In addition, the effect of ANDRO was studied on LDX-induced oxidative stress. Lithium was used as positive control. Adult Wistar rats were treated with vehicle, lithium (100 mg/kg i.p.) or ANDRO (2.0 mg/kg i.p.) 3 times a week for 21 days. On the test day, either 10 mg/kg LDX or saline was administered i.p. and USV calls and locomotor activity were recorded. LDX administration increased the number of 50-kHz calls as well as locomotor activity. Repeated treatment with lithium or ANDRO prevented these effects of LDX on 50-kHz USVs and locomotor activity. LDX increased lipid peroxidation (LPO) levels in rat striatum and both lithium and ANDRO prevented this effect. LPO levels in rat striatum were positively correlated with increases in 50-kHz USV emission as well as hyperlocomotion. In conclusion, the present results indicate that ANDRO has antimanic-like and antioxidant effects in an animal model of mania.

Key words: andrographolide, bipolar disorder, glutathione, GSK3β, lisdexamfetamine, lipid peroxidation, mania, oxidative stress, ultrassonic vocalizations

Abbreviations: ANDRO: andrographolide; GSH: reduced glutathione;GSK3β : glycogen synthase kinase-3β; LDX: lisdexamfetamine; LPO: lipid peroxidation; PFC: pre-frontal cortex; USVs: ultrassonic vocalizations.

1. INTRODUCTION

Manic episodes of bipolar disorder (BD) consist of elevated or irritable mood with enhanced energy, psychomotor agitation, risk behavior, pressured speech and tachylalia, for example (American Psychiatry Association, 2013). The pharmacological treatments for the management of manic phases of BD include mood stabilizers, such as lithium or sodium valproate, as well as antipsychotics and tamoxifen (Geddes and Miklowitz, 2013). However, these treatments are associated with the occurrence of several adverse effects, which negatively affect treatment adhesion (Baldessarini et al., 2018).

Oxidative stress has been associated with mania and antimanic drugs (Saxena et al., 2017; Malhi et al., 2013). Machado-Vieira et al. (2007a) found an increase in thiobarbituric acid reactive substances (TBARS) in manic non-medicated patients, which was reduced after lithium treatment. Lv et al. (2020) observed that malondialdehyde levels are higher in manic patients, decreasing after 6 weeks of effective electroconvulsive therapy. Moreover, increased oxidative stress was found in different animal models of mania, which could be reduced by treatment with lithium, valproate or antipsychotics (Menegas et al., 2020; Dal-Pont et al., 2019; Valvassori et al., 2019; Hodes et al., 2018; Valvassori et al., 2017; Souza et al., 2015; Arunagiri et al., 2014; Gazal et al., 2014; Brocardo et al., 2010; Frey et al., 2006).

Oxidative stress has been linked to the enzyme glycogen synthase kinase 3β (GSK3β), which is proposed as the target of the antimanic effects of lithium (Dal-Pont et al., 2019; Dandekar et al., 2018; Valvassori et al., 2017; Malhi et al., 2013). Furthermore, GSK3β activity is enhanced in peripheral blood mononuclear cells of bipolar patients in manic states (Li et al., 2010) and mice that overexpressed GSK3β showed manic-like behaviors (Prickaerts et al., 2006). In this line, it had been suggested that lithium and valproic acid prevented ouabain-induced manic-like behavior in rats through GSK3β inhibition (Valvassori et al., 2017). Moreover, the antimanic-like effect of GSK3β inhibition was associated to an antioxidant effect (Dal-Pont et al., 2019; Machado-Vieira et al., 2007a). Andrographolide (ANDRO), the main bioactive constitutive of the plant *Andrographis paniculata*, possesses anti-inflammatory, antioxidant and neuroprotective effects (Mittal et al., 2016; Tan et al., 2016; Serrano et al., 2014;

Lim et al., 2012). Importantly, ANDRO also inhibits GSK3β (Varela-Nallar et al., 2015; Serrano et al., 2014). Thus, it can be hypothesized that ANDRO might display antimanic-like effects.

Psychostimulant administration is the pharmacologically-induced animal model of mania most frequently used (Young et al., 2011). This model is based on the drug-induced increase in locomotor activity mainly (Hernandez-Miranda et al., 2017; Young et al., 2011). More recently, we proposed that 50-kHz ultrasonic vocalizations (USVs), which are related to positive affect, can serve as additional readouts for manic-like states (Wendler et al., 2019; Engelhardt et al., 2017; Hernandez-Miranda et al., 2017; Wendler et al., 2016; Pereira et al., 2014). In this line, lisdexamfetamine (LDX), a pro-drug of amphetamine, induces an increase of locomotor activity and 50-kHz USVs that are prevented by lithium or valproate administration (Bristot et al., 2019; Wendler, et al., 2016; Souza et al., 2015; Macêdo et al., 2013).

The aim of the present study was to evaluate the possible antimanic-like effect of repeated treatment with ANDRO on LDX-induced increases in 50-kHz USVs and locomotor activity of rats. In addition, the effect of ANDRO was studied on LDX-induced decreases in glutathione (GSH) levels and increases in lipid peroxidation (LPO) levels in rat prefrontal cortex (PFC) and striatum. Lithium was used as a positive control.

2. METHODS

2.1 Animals

Adults Wistar male rats (280-300 g) were socially housed (3-4 rats per cage) in polycarbonate cages (41 x 34 x 16 cm) and maintained in a room with controlled temperature (22±2 ºC) and constant 12h:12h light/dark cycle (with lights on between 7 a.m. and 7 p.m.), with water and standard laboratory chow access *ad libitum*. The experiments started one week after the rats arrived in our facility. All experiments were performed in accordance with the Brazilian Law for Animal Experimental Ethics and Care (11.794/8 October 2008) and the Local Committee on the Care and Use of Laboratory Animals. The experimental procedures were approved by the Institutional Ethics Board (CEUA/BIO –

protocol # 1109). All efforts were made in order to minimize the number of animals used and their suffering.

2.2 Drugs and treatment protocol

ANDRO (Sigma, São Paulo, Brazil) was administered at a dose of 2 mg/kg, intraperitoneally (i.p.) (Chan et al., 2010; Niranjan et al., 2010). ANDRO was dissolved in saline with dimethyl sulfoxide (DMSO 2% v/v). The repeated treatment was performed throughout 21 days, 3 times/week (Monday, Wednesday and Friday). Lithium carbonate (Eurofarma, Itapevi, Brazil) was used as positive control at a dose of 100 mg/kg. Lithium was dissolved in saline and the pH was adjusted to 7.4 by adding 2N HCl. Repeated treatment was performed throughout 21 days, once a day. LDX (Venvanse®, Shire, São Paulo, Brazil) was dissolved in saline at a dose of 10 mg/kg. All drugs were administered i.p in a constant volume of 1 ml/kg body weight

The animals were treated with vehicle (saline + DMSO), 100 mg/kg lithium or 2.0 mg/kg ANDRO for 21 days. On the test day, 10 mg/kg LDX or saline was administered (i.p.) 1 h before the test. One hour after LDX administration, the rats were placed individually in an acrylic box (40 x 40 x 40 cm) for the recording of USV calls and locomotor activity, as shown in Figure 1.

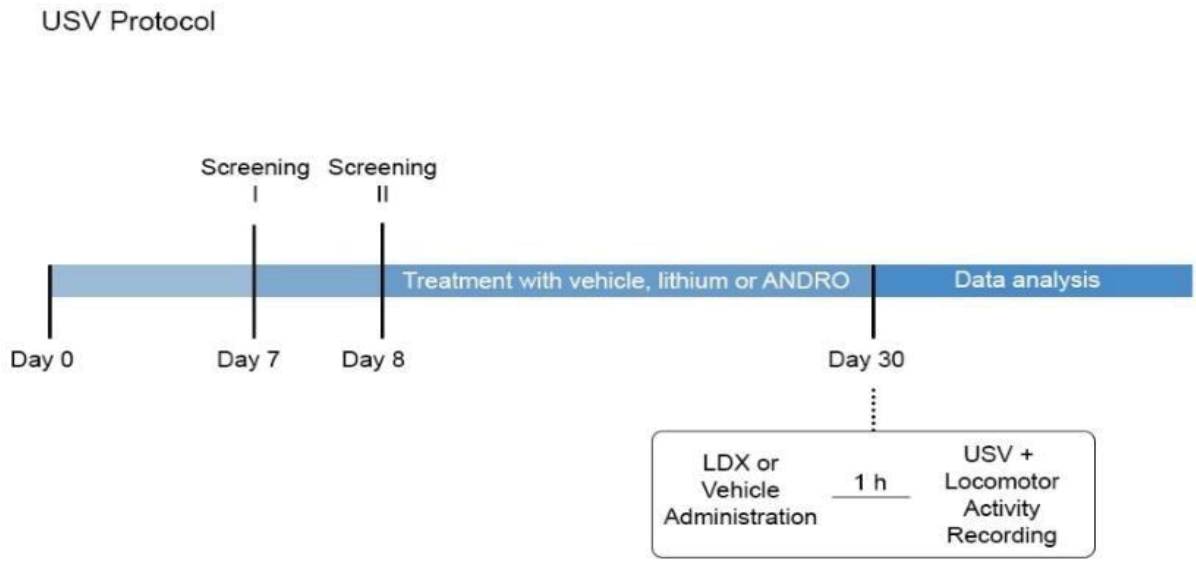

Fig 1. Experimental protocol. ANDRO: andrographolide; LDX: lisdexamfetamine; USV: ultrasonic vocalization recording. Screening test: individual USV recording in a clean homecage.

2.3 Screening test

In order to control for inter-individual variability that could affect USV, the rats were tested for their levels of spontaneous USV in a polycarbonate cage with clean bedding as a screening test. This test was performed on two consecutive days (5 minutes each) and the number of spontaneous 50-kHz USV calls were recorded. The rats were divided into the experimental groups (vehicle + saline, lithium + saline, ANDRO + saline, vehicle + LDX, lithium + LDX, ANDRO + LDX) according to the average number of 50-kHz USVs emitted on the two test days. Lights were dimmed to 4 lux for all tests (Natusch and Schwarting, 2010).

2.4 Locomotor activity test

USV calls and locomotor activity were recorded simultaneously. On the test day (day 30), 1h after LDX (or saline) injection, the rats were placed individually in an acrylic box (40 x 40 x 40 cm), with fresh bedding, and observed for 20 minutes. Recordings of the locomotor activity were made by a camera placed on top of the acrylic box. On the monitor screen the box image was divided virtually into 9 equally sized squares and a blind observer counted the number of squares crossed by the rats.

2.5 Ultrasonic vocalizations and analysis

USV emission were recorded by an UltraSound Gate Condenser Microphone (CM16; Avisoft Bioacustics, Berlin, Germany), sensible to frequencies between 15 and 180-kHz, which was placed 45 cm above the acrylic box and connected to a computer with the Avisoft Recorder 2.7 software (Wendler et al, 2019; Wendler et al., 2016; Pereira et al., 2014). Spectrograms from the USV recordings were generated at a frequency resolution of 488-Hz and a time resolution of 0.512 ms and were manually quantified, according to previous studies (Wendler et al., 2019; Pereira et al., 2014; Natusch and Schwarting, 2010). All USV emitted over 33-kHz were considered as 50-kHz USV (Wendler et al., 2016; Wöhr et al., 2015; Pereira et al., 2014). The USV emission from the first 20 seconds of each minute (out of the 20 minutes in total) were analyzed (Wendler et al., 2019; Wendler et al., 2016).

2.6 Evaluation of oxidative stress parameters in the mouse brain

2.6.1 Brain samples

The animals were euthanized by decapitation immediately after the recording of USV emission and locomotor activity. The PFC and striatum were dissected, frozen in liquid nitrogen, and stored at -80ºC until further analysis. The brain samples were homogenized in potassium phosphate buffer (0.1 M, pH 6.5) in a 1:10 dilution. One part of the homogenate was used to determine the GSH levels, and the other was centrifuged at 9000 × *g* in a micro-high-speed refrigerated centrifuge (VS-15000 CFNII, Vision Scientific, Daejeon, South Korea) for 20 min. The supernatant was used to evaluate LPO.

2.6.2 Evaluation of GSH levels

To measure GSH levels, 100 μl of the homogenate was mixed with 80 μl of 12.5% trichloroacetic acid and centrifuged at 7600 x *g* for 15 min at 4ºC. Next, 20 μl of the supernatant was mixed with 280 μl of Tris buffer (0.4 M, pH 8.9) and 5 μl of DTNB (5,5'-dithiobis-[2-nitrobenzoic acid] in methanol, following the protocol originally described by Sedlak and Lindsay (1968), with minor modifications. Absorbance was read at 415 nm using a multi-mode microplate reader (BioTek Synergy HT, BioTek Instruments, Highland Park, VT, USA). The individual values were interpolated in a standard curve of GSH (0.375-3.0 μg) to corroborate the linearity of the reaction (r^2 must be > 0.99), and the values were divided by a correction factor. The results are expressed as μg/g of tissue.

2.6.3 Evaluation of LPO levels

Lipid peroxidation was determined according to the protocol described by Jiang et al. (1992), with minor modifications. First, 100 μl of the supernatant was suspended in 100 μl of methanol, vortexed, and centrifuged at 5400 x *g* for 5 min at 4ºC. Next, 100 μl of the supernatant was added to 900 μl of FOX2 reagent (Wolff's reagent; 4 mM BHT, 250 μM $FeSO_4$, 250 mM H_2SO_4, and 100 mM xylenol orange). The samples were then vortexed and incubated for 30 min in the dark at room temperature. Absorbance was read at 560 nm using a multi-mode

microplate reader (BioTek Synergy HT, BioTek Instruments, Highland Park, VT, USA). The results are expressed as nmol.mg.protein^{-1}.

2.6.4 Quantification of proteins

The quantification of proteins (mg.ml^{-1}) in the PFC and striatum samples was performed according to the method designed by Bradford (1976) and used to express the LPO data. The reaction was examined at 595 nm in a microplate reader (BioTek Synergy HT, BioTek Instruments, Highland Park, VT, USA) using bovine serum albumin (BSA) as protein standard.

2.7 Statistical analysis

Data were analyzed by two-way ANOVA (factor LDX treatment: saline or LDX; factor repeated treatment: vehicle, lithium or ANDRO) followed by the Newman-Keuls. Since some variables (total 50-kHz USVs, total time, and number of trill and flat subtypes) did not show homoscedasticity, the raw data were transformed in square root before statistical analysis. Differences were considered statistically significant when $p < 0.05$. Pearson's correlation index was used to evaluate the degree of association between variables. Data was expressed as mean ± SEM of raw data. Statistica 7.0, StatSoft (Tulsa, USA) was used for the statistical analysis.

3. RESULTS

3.1 Repeated lithium and ANDRO treatment reversed LDX-induced increases in 50-kHz USV

Two-way ANOVA of the number of USVs showed effects of LDX administration ($F_{1,37} = 8.570$, $p < 0.001$), of repeated treatment ($F_{2,37} = 13.87$, $p < 0.001$) and LDX administration x repeated treatment interaction ($F_{2,37} = 4.42$, $p < 0.05$; Figure 3A). The *post hoc* test indicated that LDX administration significantly increased the number of calls ($p < 0.001$). Treatment with lithium and ANDRO prevented the increases in the number of 50-kHz calls induced by LDX ($p < 0.001$ and $p < 0.01$, respectively). Lithium or ANDRO alone did not reduce the number of calls ($p > 0.05$).

Call subtypes: regarding *flat calls*, there was an effect of LDX administration ($F_{1,37}$ = 14.23, p < 0.001), of repeated treatment ($F_{2,37}$ = 9.17, p < 0.001) and a significant LDX administration x repeated treatment interaction ($F_{2,37}$ = 3.92, p < 0.05; Table 1). LDX increased flat calls (p < 0.001) and repeated lithium and ANDRO prevented this effect (both p < 0.01). On *trill calls*, there was an effect of LDX administration ($F_{1,37}$ = 6.36, p < 0.001), of repeated treatment ($F_{2,37}$ = 11.01, p < 0.01) and an LDX administration x repeated treatment interaction ($F_{2,37}$ = 5.06, p < 0.05). LDX increased trill calls (p < 0.001) and repeated lithium and ANDRO prevented this effect (p < 0.001 and < 0.05, respectively). On *step calls* there was an effect of LDX treatment ($F_{1,37}$ = 9.28, p < 0.01), of repeated treatment ($F_{2,37}$ = 3.27, p < 0.05) but not for LDX administration x repeated treatment interaction ($F_{2,37}$ = 2.89, NS). LDX increased step calls (p < 0.01) independently from repeated treatment (Table 1).

On temporal parameters of 50-kHz USV (Table 1), there were no effects of LDX administration or repeated treatment with lithium or ANDRO on the duration of calls (LDX administration: $F_{1,37}$ = 1.09, NS; repeated treatment: $F_{2,37}$ = 1.00, NS; factors interaction: $F_{2,37}$ = 1.28, NS) and on the latency for the first call (LDX administration: $F_{1,37}$ = 0.04, NS; repeated treatment: $F_{2,37}$ = 0.40, NS; factors interaction: $F_{2,37}$ = 0.33, NS). On the other hand, on total calling time there was an effect of LDX administration ($F_{1,37}$ = 10.75, p < 0.01), of the repeated treatment ($F_{2,37}$ = 6.16, p < 0.01) and a significant LDX administration x repeated treatment interaction ($F_{2,37}$ = 4.74, p < 0.05). LDX increased total calling time (p < 0.001), an effect that was prevented by lithium and ANDRO treatment (both p < 0.01; Table 1).

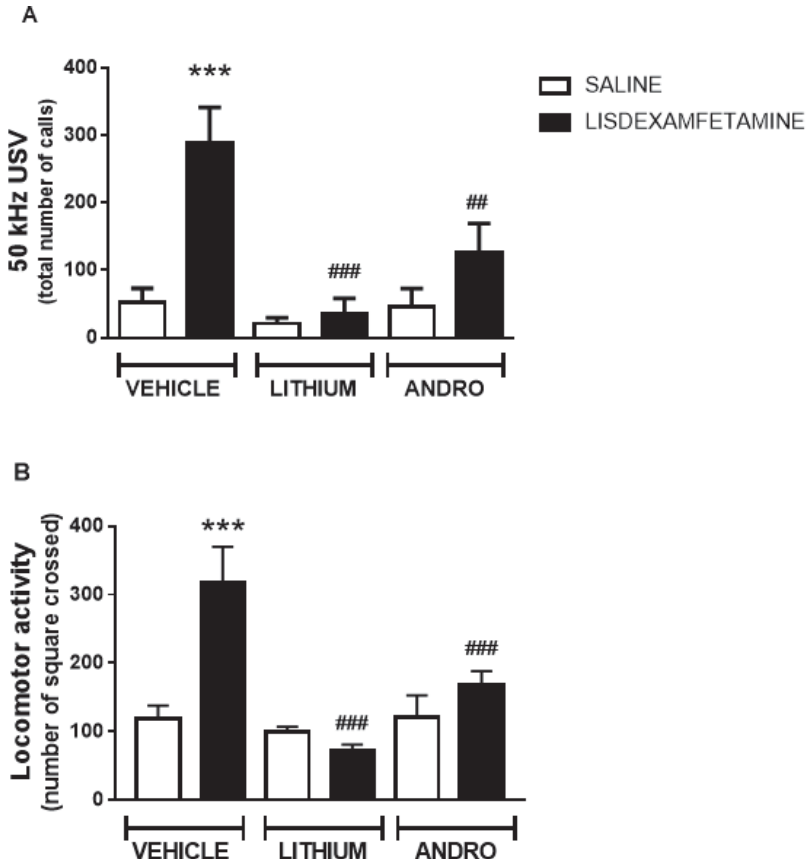

Fig 2. Effects of 21 days treatment with lithium (100 mg/kg i.p.), ANDRO (2.0 mg/kg i.p.) or vehicle on LDX (10 mg/kg i.p.)-induced increase in the number 50-kHz USV calls (A) and locomotor activity (B). Vehicle: saline + DMSO. Data are expressed by mean ± SEM. *n* = 5-8 rats/group. ***p < 0.001, compared with rats treated with vehicle + saline; ##p < 0.01 and ###p < 0.001, compared to the vehicle + LDX (two-way ANOVA followed by the Newman-Keuls *post hoc* test).

3.2 Repeated lithium and ANDRO treatment prevented LDX-induced hyperlocomotion

There were effects of LDX administration ($F_{2,37}$= 19.80, p < 0.001), of repeated treatment ($F_{1,37}$= 9.35, p < 0.001) and LDX administration x repeated treatment interaction ($F_{2,37}$= 4.07, p < 0.05). LDX administration increased locomotor activity (p < 0.001; Figure 3B), and treatment with lithium and ANDRO prevented such hyperlocomotion (p < 0.001 and p < 0.01, respectively). Lithium or ANDRO alone did not reduce locomotor activity (both p > 0.05).

Table 1 – Effects of repeated lithium and ANDRO on acute effects of LDX on call subtypes and temporal parameters of 50-kHz USVs.

	Saline			Lisdexamfetamine		
	Vehicle	Lithium	ANDRO	Vehicle	Lithium	ANDRO
Call subtypes						
Flat	40 ± 16	13 ± 5	28 ± 14	204 ± 39*	26 ±17	84 ± 32
Trill	6 ± 4	4 ± 1	10 ± 5	49 ± 11*	3 ± 2	22 ± 8
Step	4 ± 1	3 ± 2	4 ± 3	20 ± 31$	5 ± 3$	10 ± 4$
Temporal Parameters						
Call duration	5.2± 2.8	11.8±8.2	3.7±2.2	8.2±4.6	7.8±6.1	7.1±3.5
Latency 1st call	0.03±0.01	0.08±0.05	0.03±0.02	0.04±0.01	0.03±0.01	0.03±0.01
Total Time	1.69±0.67	1.14±0.40	1.73±1.19	11.46±2.56*	1.26±0.71	4.54±1.75

Data represent mean ± SEM.
Call duration, latency to first call, and total time in seconds; call subtypes: number of calls.
*$p < 0.05$ compared to all other groups
$^{\$}p < 0.05$ compared to all saline treated rats (LDX administration factor)

3.3 Repeated ANDRO administration prevented LDX-induced lipid peroxidation in rat striatum

LPO levels

In the striatum, two-way ANOVA showed effects of repeated treatment ($F_{2,37}$ = 6.33, $p < 0.01$), LDX administration ($F_{1,37}$ = 71.52, $p < 0.001$) and LDX administration x repeated treatment interaction ($F_{2,37}$ = 13.94, $p < 0.001$) in LPO levels. The Newman-Keuls test indicated that LDX administration increased LPO levels ($p < 0.001$) and lithium and ANDRO repeated treatment prevented LDX-induced increases in LPO levels ($p < 0.001$ and $p < 0.05$, respectively; Figure 3A).

In PFC, two-way ANOVA indicated that there were no effects of repeated treatment ($F_{2,37}$ = 2.38, NS), LDX administration ($F_{1,37}$ = 0.22, NS) or LDX administration x repeated treatment interaction ($F_{2,37}$ = 1.08, NS) in the levels of LPO (Figure 3B).

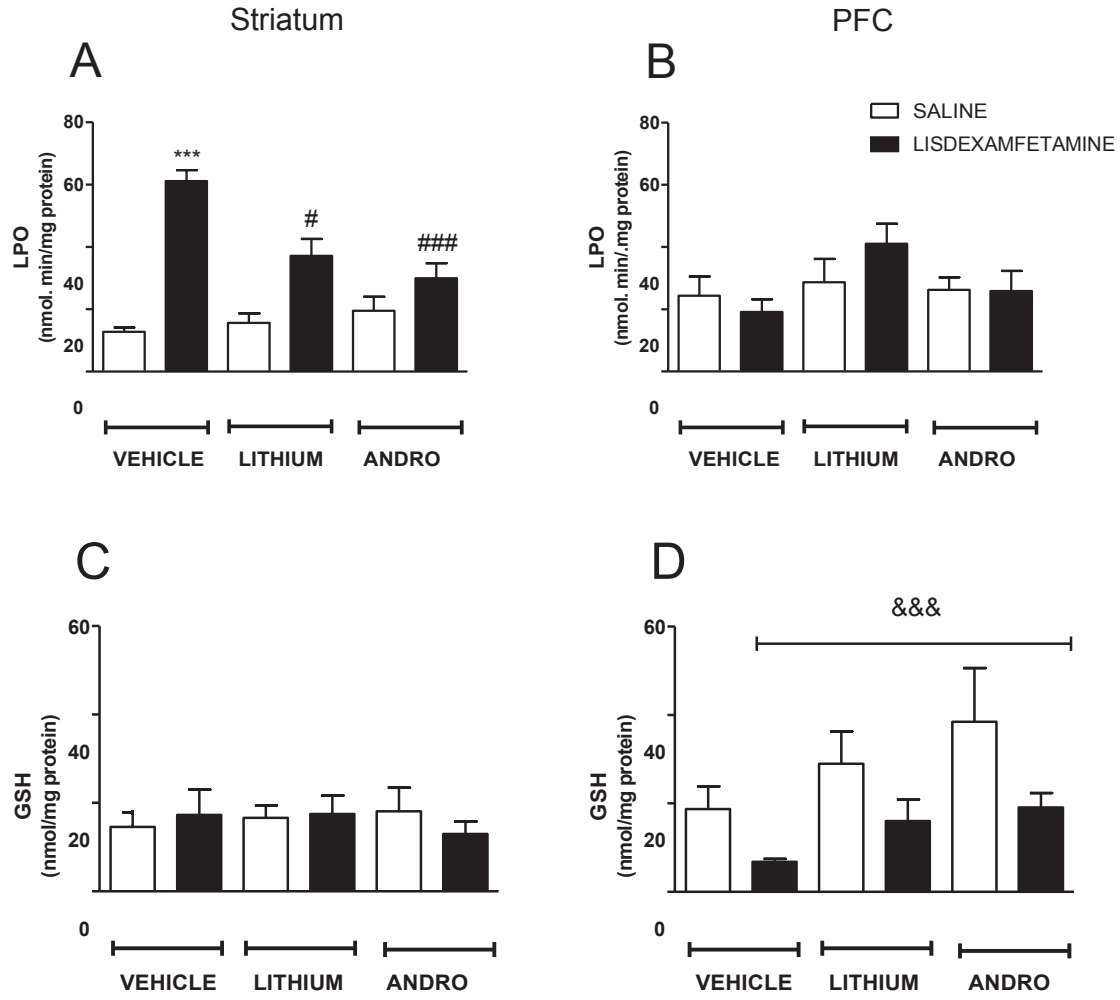

Fig 3. Effects of 21 days treatment with lithium (100 mg/kg i.p.), ANDRO (2.0 mg/kg i.p.) or vehicle on LDX (10 mg/kg i.p.)-decreases in GSH and increases in LPO levels in the PFC and striatum. A) LPO levels in the striatum; (B) LPO levels in the PFC; (C) GSH levels in the striatum; (D) GSH levels in the PFC. Vehicle: saline + DMSO. Data are expressed by mean ± SEM. *n* = 5-8 rats/group. ***$p < 0.001$, compared with rats treated with vehicle + vehicle; $^{\#}p < 0.05$ and $^{\#\#\#}p < 0.001$, compared with rats treated with vehicle + LDX; $^{\&\&\&}p < 0.05$ compared to vehicle treated rats (LDX administration factor). Two-way ANOVA followed by the Newman-Keuls test).

GSH levels

In striatum, two-way ANOVA did not indicate effects of repeated treatment ($F_{1,37}$ = 0.055, NS), LDX administration ($F_{1,37}$ = 0.02, NS) or LDX administration x repeated treatment interaction ($F_{1,37}$ = 0.02, NS) in the levels of GSH (Figure 3C).

In PFC, two-way ANOVA showed effects of repeated treatment ($F_{2,37}$ = 3.35, $p < 0.05$) and LDX administration ($F_{1,37}$ = 7.59, $p < 0.01$) but not repeated treatment-LDX administration interaction $F_{1,37}$ = 0.20, NS). The Newman-Keuls test indicated that LDX administration decreased GSH levels in rat PFC ($p < 0.01$; Figure 3D).

3.4 Correlations

The Pearson's correlation test showed positive correlations between LPO levels in rat striatum with 50-kHz USVs calls (r= 0.62, p < 0.001) and with locomotor activity (r= 0.61, p < 0.001), as well as between locomotor activity with 50-kHz USV calls (r= 0.62, p < 0.001), as shown in Figure 4.

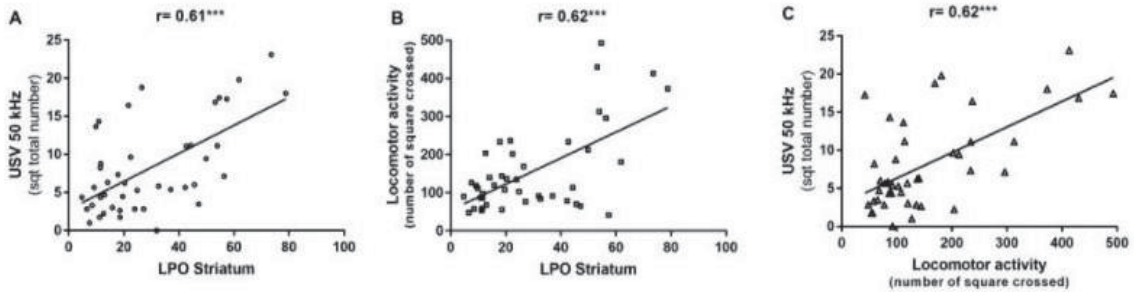

Fig 4. Pearson's correlation between the LPO levels in the striatum with 50-kHz USV calls (A), LPO levels with locomotor activity (B) and between locomotor activity with 50-kHz USV calls (C).

4. DISCUSSION

The present study showed that repeated treatment with ANDRO prevented LDX-induced manic-like behaviors (hyperlocomotion and increases in 50-kHz USVs) and striatal LPO levels. Similar results were obtained with repeated treatment with lithium, a clinically effective antimanic drug, which was used as a positive control. These results suggest that ANDRO possesses an antimanic-like behavioral effect and exerts antioxidant activity in the LDX model of mania.

The administration of psychostimulants (e.g., *d*-amphetamine) is a common method for inducing manic-like behavior in animal models, such as hyperlocomotion. LDX is a long-acting *d*-amphetamine pro-drug employed in the therapeutic management of attention deficit/hyperactivity disorder (Ermer et al., 2016). LDX administration has also been used to mimic manic-like behavior in animals (Bristot et al., 2019; Ascoli et al., 2017; Eger et al., 2016; Wendler et al., 2016; Souza et al., 2015; Macêdo et al., 2013). These manic-like behaviors were reversed or prevented by treatment with lithium or valproate (Lv et al., 2020; Bristot et al., 2019; Ascoli et al., 2017; Souza et al., 2015; Macêdo et al., 2013; Malhi et al., 2013; Machado-Vieira et al., 2007a; Cunha et al., 2006). LDX administration also increases oxidative stress and reduced BDNF levels, as observed in plasma as well as serum of bipolar patients in manic state (Caldiroli

et al., 2020; Machado-Vieira et al., 2007b). Thus, LDX is a valid model for the study of mania and antimanic-like drugs. In the present study, repeated ANDRO and lithium administration prevented LDX-induced hyperlocomotion at a dose that did not affect spontaneous locomotor activity, a profile suggestive of an antimanic-like effect (Young et al., 2011).

In addition to hyperlocomotion, LDX administration also showed an increase in 50-kHz USVs. Adult rats emit high-frequency 50-kHz USVs in appetitive situations, such as playing with other rats, mating or after psychostimulant administration (Rippberger et al., 2015; Burgdorf et al., 2011) Therefore, it is proposed that 50-kHz can represent a positive affective state in rats (Brudzynski et al., 2018; Wöhr and Schwarting, 2013). Considering that 50-kHz USVs may reflect a positive state in rats, this behavior can be used to monitor hedonic states in animal models of various psychiatric disorders (Burgdorf et al., 2011). In this line, 50-kHz USVs were found to be increased in different animal models of mania such as amphetamine or LDX administration and sleep deprivation (Wendler et al., 2019; Engelhardt et al., 2017; Wendler et al., 2016; Pereira et al., 2014). This increase in 50-kHz USVs was blocked by the antimanic drugs lithium, tamoxifen and antipsychotics (Wendler et al., 2019; Wendler et al., 2016; Barker et al., 2015; Pereira et al., 2014; Wintink and Brudzynski, 2001). Thus, 50-kHz USVs have been proposed as a new marker in animal models of mania, representing the increase in the positive affect (Wendler et al., 2019; Engelhardt et al., 2017; Hernandez-Miranda et al., 2017; Wendler et al., 2016). In the present study, repeated treatment with ANDRO and lithium prevented LDX effects in the total number of 50-kHz calls, in the number of trill and flat calls subtypes and in total calling time. ANDRO and lithium alone did not affect 50-kHz USVs. These results indicated an antimanic-like effect of ANDRO on 50-kHz USVs.

The present study also shows that LDX administration can lead to increased LPO in striatum and decreased GSH levels in PFC. Repeated lithium and ANDRO treatment prevented LDX-induced increases in LPO in rat striatum. Increased generation of reactive oxygen species and free radicals are involved in the pathophysiology of BD, as LPO markers are present in different phases of BD in the serum/plasma of BD patients and are associated to illness severity and/or the number of manic episodes (Akarsu et al., 2018; Sowa-Kucma et al.,

2017; Brown et al., 2014; Andreazza et al., 2007; Machado-Vieira et al., 2007a). In this line, high concentrations of phospholipids in brain tissues make the brain more vulnerable to oxidative stress induced by LPO (Banerjee et al., 2012). Lipid hydroperoxide chain reactions eventually cause the formation of reactive aldehydes and this can damage lipid membranes (Maes et al., 2018). Increased oxidative stress leads to derangement of signal transduction, structural plasticity and cellular resilience in brain tissue (Schäfer et al., 2004). Lv et al. (2020) showed that LPO levels were higher in the plasma of treatment-resistant BD patients and that decreased after 6 weeks of electroconvulsive therapy.

Oxidative stress in the brain was observed in different models of mania such as psychostimulants (Chaves Filho et al., 2020; Valvassori et al., 2019; Hodes et al., 2018; Sharma et al., 2016; Frey et al., 2006), ouabain (Dal-Pont et al., 2019; Valvassori et al., 2017), sleep deprivation (Kanazawa et al., 2016) and ketamine (Gazal et al., 2014). The observation that mood stabilizing agents such as lithium and sodium valproate also exert antioxidant effects, reinforces the idea that oxidative stress is involved in the pathophysiology and treatment of mania (de Queiroz et al., 2018; Valvassori et al., 2017; Kanazawa et al., 2016; Brown et al., 2014; Banerjee et al., 2012; Andreazza et al., 2007). However, the antioxidant effects of lithium may be specific to certain brain regions and they can vary depending on experimental variables including type of drug and age of animals. For example, in mice, repeated administration of amphetamine increased LPO in PFC, hippocampus and amygdala and doxycycline, but not lithium, reversed LPO increasing in hippocampus (Chaves Filho et al., 2020). On the other hand, Hodes et al. (2018) found that acute administration of amphetamine increased LPO in the hippocampus of mice but not in the PFC. Repeated methylphenidate administration led to LPO and protein damage in the PFC, but not in the striatum, cerebellum or hippocampus of juvenile rats (Schmitz et al., 2012). Particularly to LDX, it increased LPO in rat PFC, hippocampus and striatum, and lithium and valproate were able to prevent and reverse the effects of repeated LDX administration on LPO in these brain areas (Macêdo et al., 2013; de Souza et al., 2015). In the present study, acute LDX administration increased LPO levels in the striatum, but not in the PFC. Treatment with ANDRO and lithium reduced LDX-induced increase in LPO levels in rat striatum. In addition, there was a positive correlation between LPO levels in rat striatum and increases in 50-kHz

USVs, as well as LDX-induced hyperlocomotion. Menegas et al. (2020), using the m-amphetamine administration model of mania, also observed correlations between hyperlocomotion and lipid damage parameters in the striatum of rats. Clinically, a positive correlation between mania severity (Young Mania Rating Scale) and oxidative stress index was also (Akarsu et al., 2018) observed which supports the relevance of our pre-clinical approach.

GSH is a non-enzymatic antioxidant molecule and its depletion can lead to neuronal dysfunctions and various disorders (Gaucher et al., 2018). In the present study, LDX administration reduced GSH levels in rat PFC. ANDRO and lithium treatment, however, had no effects on LDX-induced decrease on GSH level. Studies also showed divergent results regarding GSH levels in the brain. Macêdo et al. (2013), for example, showed that LDX administration induced hyperlocomotion and decreased GSH content in rat PFC and striatum, which was prevented by lithium in both brain areas. However, only lithium reversed the reduction of GSH in the PFC. These heterogeneous results can be dependent of methodological differences including subjects, experimental protocol or acute/repeated drug administration.

The antimanic effect of lithium has been partly related to its inhibitory activity on the enzyme GSK3β, which is upregulated in the brain of BD patients (Li et al., 2010). Moreover, GSK3β has been linked to oxidative stress in mania models. AR-A014418, a GSK3β inhibitor, reverted ouabain-induced hyperlocomotion and oxidative stress in mice brain (Dal-Pont et al., 2019). In this line, ANDRO also inhibits GSK3β (Varela-Nallar et al., 2015; Serrano et al., 2014) and, thus, inhibition of GSK3β may also contribute to the antimanic-like effect of ANDRO observed in the present study.

5. CONCLUSION

In conclusion, the present results suggest that ANDRO possesses antimanic-like effects in the LDX-administration model of mania, preventing LDX-induced increases in 50-kHz USVs and hyperlocomotion, and its antioxidant effects may mediate these effects.

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5. ARTIGO 3

Chronic andrographolide administration prevents methylphenidate-induced manic-like behavior in the behavioral pattern monitor

Kanazawa, L. K. S.[a]; Andreatini, R.[a]

[a] *Laboratory of Physiology and Pharmacology of the Central Nervous System, Department of Pharmacology, Federal University of Paraná, Centro Politécnico, 81540-990 Curitiba, PR, Brazil*

ABSTRACT

Because many symptoms of bipolar disorder (BD) are subjective, it is very difficult to mimic BD in animal models. The most commonly used model of mania is the single administration of psychostimulants, which induce manic-like behavior, such as hyperlocomotion. Another parameter that can be evaluated in manic-like behavior is exploratory behavior. The behavioral pattern monitor (BPM) collects information about the locomotor activity and exploratory behavior (rearings and nosepokes) of rodents. This evaluation is important in pre-clinical tests for the research of new drugs. As pharmacological options for the management of the manic phases show many limitations regarding their clinical use, it is relevant to search for other alternatives. Andrographolide (ANDRO) is an inhibitor of the enzyme glycogen synthase kinase 3 beta (GSK3β), a similar mechanism of action as the mood stabilizer lithium. Thus, the present study aimed to evaluate a possible antimanic-like effect of chronic treatment with ANDRO by evaluating its effect on methylphenidate-induced increased locomotor and exploratory activity in mice in the BPM. Methylphenidate administration led to hyperlocomotion (increased number of crossings) and increased exploratory activity (increased number of nosepokes) in 1 h in the mouse BPM. These parameters were prevented by treatment with ANDRO and lithium. There were no significant effects of ANDRO or lithiumon the latency for the first crossing. Methylphenidate administration increased the number of rearings and climbings,although there were no effects of ANDRO or lithium on these parameters. Theseresults suggest a possible antimanic-like effect of ANDRO and reinforce the notion that the BPM is a useful tool in the evaluation of the antimanic-like effect of drugs.

Key-words: andrographolide, behavioral pattern monitor, bipolar disorder, exploratory behavior, mania, nosepokes

Abbreviations: ANDRO – andrographolide, BD – bipolar disorder, BPM – behavioral pattern monitor

1. INTRODUCTION

Animal models can be useful tools in elucidating neuronal mechanisms related to certain behaviors (Mack et al., 2019). However, because many symptoms of bipolar disorder (BD) are subjective, such as euphoria, grandiosity, guilt or helplessness, or are human-related, such as racing thoughts and pressured speech, it is very difficult to mimic BD in animal models (Mack et al., 2019). A variety of models for BD have been proposed, such as pharmacological, environmental, nutritional or genetic models (Kato et al., 2007). The most commonly used model of mania is the single administration of psychostimulants such as amphetamine (Frey et al., 2006), metamphetamine (Gould et al., 2001) or lisdexamphetamine dimesylate (Macêdo et al., 2013), for example. Psychostimulants increase synaptic dopamine and noradrenaline through inhibition or reversing the corresponding reuptake mechanisms (Berk et al., 2007). They not only induce mania-like behavior in animals but also cause manic symptoms in healthy humans and BD patients, such as decreased need for sleep, elevated mood, increased sexual behavior and hyperactivity (Corp et al., 2014; Cousins et al., 2009; Berk et al., 2007; Asghar et al., 2003). Hyperactivity is present in nearly all manic states (Young et al., 2011a).

To measure psychostimulant-induced hyperlocomotion, the number of crossings in the open field can be analyzed as an index of locomotor activity. The blocking or attenuation of hyperlocomotion after methylphenidate administration is indicative of an antimanic-like effect, at doses that do not impair locomotor activity *per se* (Gould et al., 2001). Besides hyperlocomotion, another parameter that can be evaluated in manic-like behavior is exploratory behavior. The assessment of unconditioned locomotor and exploratory behavior has become one of the most widely used paradigms in rodents to determine the effects of various experimental manipulations (Young et al., 2007). Several investigators have recognized the necessity for analyses of multivariate profiles and/or spatio-temporal patterns of motor activity and proposed different approaches to quantify the various components of the open field behavior (Young et al., 2007; Drai and Golani, 2001).

The behavioral pattern monitor (BPM) collects information about the locomotor activity, locomotor patterns and exploratory behavior (rearings and nosepokes) of rodents (Tanaka et al., 2012). Initial uses of the multivariate profiles of locomotor and investigatory behaviors provided by the BPM helps to elucidate the behavioral characteristics and neuropharmacological mechanisms of psychoactive drugs, for example (Adams and Geyer, 1982). DAT knockout mice exhibit increased locomotor activity and increased exploratory behavior, as seen by increases in the number of nosepokes (Young et al., 2007).

One approach to bridge the gap between animal research and clinical research in BD is to develop translational models that extend human studies to animal paradigms that examine analogous constructs. The BPM allows sensitive quantification of the characteristics of human hyperactive and exploratory behavior (Young et al., 2007). Measurements of the locomotor activity and exploratory behavior are also important in pre-clinical tests for the research of new drugs (Perry et al., 2009).

As pharmacological options for the management of the manic phases of BD show many limitations regarding their clinical use, such as intolerance to several side effects or refractoriness to treatment (Cipirani et al., 2011; Keck, 2003), it is relevant to search for other alternatives. The wide variety of biological effects of andrographolide (ANDRO) have been shown in several papers describing both pre-clinical and clinical tests. These biological effects of ANDRO include its anti-inflammatory, antioxidant, antimicrobial, hepato- and neuroprotective properties, for example (Mussard et al., 2019; Tan et al., 2016; Lim et al., 2012; Chern et al., 2011; Gabrielian et al., 2002). ANDRO is an inhibitor of the enzyme glycogen synthase kinase 3 beta (GSK3β), as the antimanic drug lithium is (Varela-Nallar et al., 2015).

Considering that ANDRO possesses antioxidant and inhibitory activity over GSK3β, a similar mechanism of action as the mood stabilizer lithium, the present study aimed to evaluate a possible antimanic-like effect of chronic treatment with ANDRO by evaluating its effect on methylphenidate-inducedincreased locomotor and exploratory activity in mice in the BPM.

2. MATERIALS AND METHODS

2.1 Animals

Male Swiss mice (30 – 40 g) were housed in polycarbonate cages (41 x 34 x 16 cm) and kept in a room with controlled temperature (22±2 ºC) in a 12h:12h light/dark cycle (with lights on between 7 a.m. and 7 p.m.) with free access to water and food. The cleaning of the cages was performed 3 times per week. The animals were allowed to acclimate for one week before testing commenced. All experiments were performed in accordance with the Brazilian Law for Animal Experimental Ethics and Care (11.794/8 October 2008) and the Local Committee on the Care and Use of Laboratory Animals. The experimental procedures were approved by the Institutional Ethics Board (CEUA/BIO – protocol #1109). All efforts were made to minimize animal suffering and the number of animals used.

2.2 Drugs

Andrographolide (Sigma, São Paulo, Brazil) was administered at a dose of 2 mg/kg, intraperitoneally (i.p.) (Chan et al., 2010; Niranjan et al., 2010). ANDRO was dissolved in saline with dimethyl sulfoxide (DMSO). The chronic treatment was performed throughout 21 days, 3x/week. Lithium carbonate (Eurofarma, Itapevi, Brazil) was used as positive control in a dose of 100 mg/kg.Lithium was dissolved in saline and the pH was adjusted to 7.4 by adding 2N HCl.Chronic treatment was performed throughout 21 days, once a day. Methylphenidate (Novartis, São Paulo, Brazil) was used for the induction of manic-like behavior in a dose of 5 mg/kg, subcutaneously (s.c.), 30 minutes before the experiments, in a single administration. All drugs were administered ina constant volume of 10 ml/kg body weight.

2.3 Behavioral pattern monitor (BPM)

The BPM consists of an acrylic apparatus, a rectangular and transparent box (30 x 60 x 48 cm) with eight holes in the side walls and three holes on the floor (Fig. 1). Each hole is 1.2 cm in diameter. The floor is divided by nine squares. The central square is 114 x 268 mm long. The four squares in the corners are 96 mm x 172 mm long. The two superior and inferior squares are 96 x 268 mm long,

while the two lateral squares are 114 x 172 mm long (Fig.1). These measurements are in accordance to Tanaka et al. (2012).

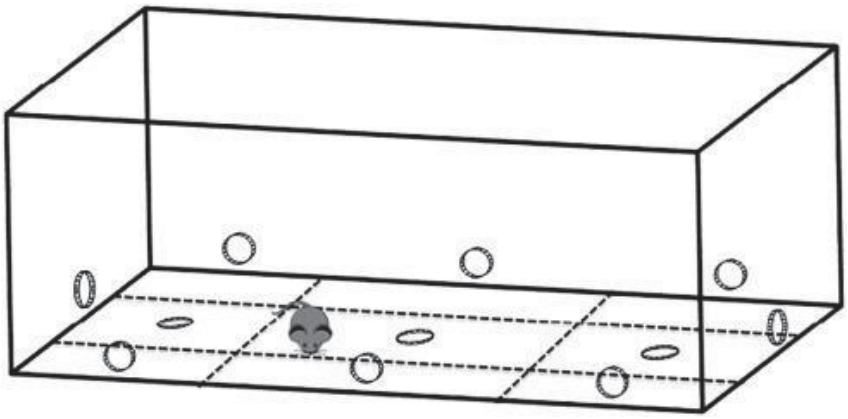

Fig 1: The behavioral pattern monitor (BPM) (Kwiatkowski et al., 2019).

2.4 Experimental protocol

The animals were treated for 21 days with either vehicle (saline + DMSO), 100 mg/kg lithium or 2.0 mg/kg ANDRO. On the test day, the animals were treated with either vehicle or 5 mg/kg methylphenidate. Thus, six groups were formed: vehicle + vehicle, vehicle + methylphenidate, lithium + vehicle, lithium + methylphenidate, ANDRO + vehicle and ANDRO + methylphenidate. Twenty minutes after vehicle or methylphenidate administration, the animals were individually put in the BPM apparatus, where, for one hour, locomotor and exploratory activity were recorded. Locomotor activity was measured by the number of crossings performed by the animals, and the exploratory activity was measured by the number of nosepokes (when the animal inserts its nose inside the holes), rearings (erect position without support on the lateral walls) and climbings (erect position with support on the lateral walls) during the 60-minute session. The latency for the first crossing (in seconds) was also measured. The measurements were made by observation of the recorded videos of the experiments.

2.5 Statistical analysis

Data were analyzed by two-way ANOVA. The differences between the groups were analyzed by the Bonferroni *post hoc* test. Differences were considered to be statistically significant when $p < 0.05$. Data was expressed as mean ± SEM. Statistica 7.0, StatSoft (Tulsa, USA) was used for the statistical analysis.

3. RESULTS

3.1 Chronic treatment with ANDRO and lithium prevented methylphenidate-induced increased number of crossings and nosepokes in the mouse BPM

Two-way ANOVA showed that there was a significant effect of methylphenidate administration ($F_{1,30} = 32.947$, $p < 0.001$), repeated treatment ($F_{2,30} = 5.215$, $p < 0.05$) and a significant methylphenidate administration x repeated treatment interaction ($F_{2,30} = 9.553$, $p < 0.001$), on the locomotor activity (Fig. 1A). Methylphenidate increased locomotor activity ($p < 0.001$), and this effect was prevented by lithium ($p < 0.001$) and ANDRO treatment ($p < 0.01$) (Fig. 1A). In the evaluation of exploratory behavior, results indicate a significant effect of methylphenidate administration ($F_{1,30} = 9.708$, $p < 0.01$) and a significant methylphenidate administration x repeated treatment interaction ($F_{2,30} = 12.372$, $p < 0.001$) on the number of nosepokes. Methylphenidate administration increased the number of nosepokes ($p < 0.001$), which was prevented by lithium ($p < 0.001$) and ANDRO repeated treatment ($p < 0.05$) (Fig. 1B).

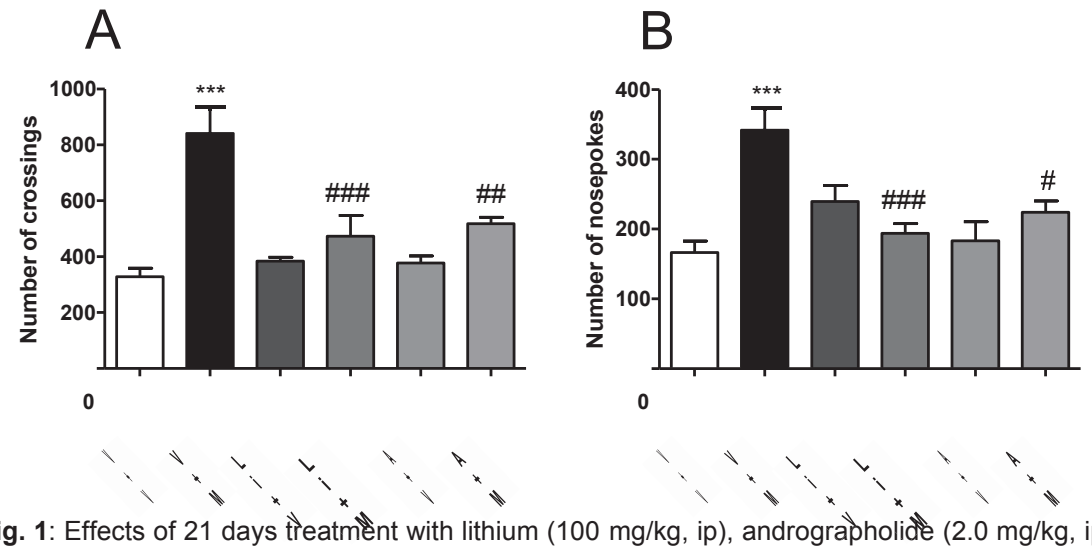

Fig. 1: Effects of 21 days treatment with lithium (100 mg/kg, ip), andrographolide (2.0 mg/kg, ip, ANDRO) or vehicle on methylphenidate (5 mg/kg, sc)-induced increase in locomotor and exploratory activity in the BPM, as represented by the number of crossings (A) and number of nosepokes (B), respectively. Vehicle: saline + DMSO. Data are expressed by mean ± SEM. *n* = 6 mice/group. ***$p < 0.001$, compared with rats treated with vehicle + saline; #$p < 0.05$, ##$p < 0.01$ and ###$p < 0.001$, compared to the vehicle + methylphenidate (two-way ANOVA followed by the Bonferroni *post hoc* test). V+V: vehicle + vehicle; Li+V: lithium + vehicle; V+M: vehicle + methylphenidate; Li+M: lithium + methylphenidate; A+V: 2.0 mg/kg ANDRO + vehicle; A+M: 2.0 mg/kg ANDRO + methylphenidate.

3.2 Methylphenidate administration increased the number of rearings and climbings in the mouse BPM, which was not prevented by chronic treatment with ANDRO or lithium

As shown in Fig. 2A, there were no significant effects of methylphenidate administration or repeated treatment with lithium or ANDRO on the latency for the first crossing in the BPM (methylphenidate administration: $F_{1,30} = 0.002$, NS; repeated treatment: $F_{2,30} = 1.178$, NS; factors interaction: $F_{2,30} = 0.403$, NS). Two-way ANOVA showed that there was a significant effect of methylphenidate administration on the number of rearings ($F_{1,30} = 14.038$, $p < 0.001$). However, treatment with lithium or ANDRO did not affect this parameter (Fig. 2B). There was also a significant effect of methylphenidate administration on the number of climbings ($F_{1,30} = 19.831$, $p < 0.001$). Repeated treatment with lithium or ANDRO did not affect the number of climbings (Fig. 2C).

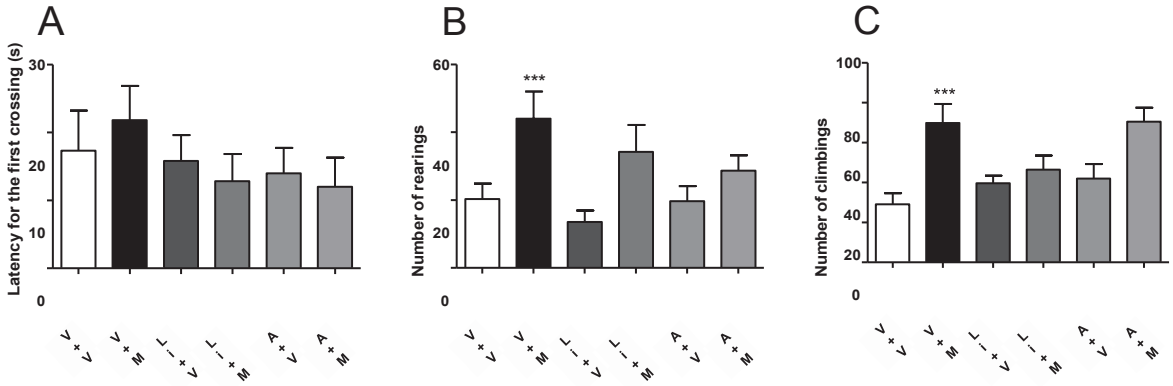

Fig. 2: Effects of 21 days treatment with lithium (100 mg/kg, ip), andrographolide (2.0 mg/kg, ip, ANDRO) or vehicle on the latency for the first crossing (A), number of rearings (B) and number of climbings (C) in the methylphenidate (5 mg/kg, sc)-induced increase in locomotor and exploratory activity model in the BPM. Vehicle: saline + DMSO. Data are expressed by mean ± SEM. *n* = 6 mice/group. ***$p < 0.001$, compared with rats treated with vehicle + saline (two-way ANOVA followed by the Bonferroni *post hoc* test). V+V: vehicle + vehicle; Li+V: lithium + vehicle; V+M: vehicle + methylphenidate; Li+M: lithium + methylphenidate; A+V: 2.0 mg/kg ANDRO + vehicle; A+M: 2.0 mg/kg ANDRO + methylphenidate.

4. DISCUSSION

In the present study, methylphenidate administration led to hyperlocomotion, as it increased in the number of crossings and enhanced exploratory behavior, by increasing the number of nosepokes. Chronic treatment with lithium or ANDRO prevented methylphenidate-induced increases in locomotor activity and exploratory behavior. Methylphenidate administration also increased the number of rearings or climbings, which was not prevented by chronic treatment with neither lithium nor ANDRO.

Hyperactivity is a cardinal symptom of mania. Psychostimulant induced hyperlocomotion is the most frequently used animal model of mania (Young et al., 2011a). This pharmacological induction of manic-like behavior is reliable and shows face, construct and predictive validity (Einat, 2006; Machado-Vieira et al., 2004). Psychostimulants that are capable of increasing the levels of dopamine cause behavioral effects that resemble mania, such as hyperlocomotion (Hasler et al., 2006). As amphetamine can produce mania-like symptoms in healthy human subjects and can precipitate manic episodes in patients with BD, amphetamine-induced alterations in the behavioral repertoire of rats, most notably amphetamine-induced hyperlocomotion, are commonly used in pre-clinical research (Young et al., 2011a).

Evaluation of the locomotor activity with the BPM has shown that hyperactivity includes complex multifaceted behaviors. The BPM has been used to demonstrate differential effects of drugs on locomotor activity and exploratory behavior in rodents (Young et al., 2007). The BPM allows the evaluation of various features of behavioral exploration in rodents, such as nosepoking, rearing, locomotor activity and locomotor patterns (Tanaka et al., 2012). In rodents, the multivariate behavioral parameters evaluated in the BPM have allowed to distinguish and quantify the effects of pharmacological, environmental and genetic manipulations (Kwiatkowski et al., 2019; Risbrough et al., 2006; Ralph-Williams et al., 2003; Geyer and Paulus, 1992).

In our study, methylphenidate induced increases in the number of crossings, which was prevented by lithium and ANDRO, and also on the number of rearings and climbings, which was not prevented by lithium or ANDRO. These heterogeneous results have been shown in the literature. Van Enkhuizen et al. (2013), for example, showed that the administration of 1.5% valproic acid in mice chow for 28 days prevented hyperlocomotion in DAT knockout mice or mice submitted to the administration of GBR12909, but did not affect the number of nosepokes or rearings in neither group. In rats, MDMA, which induces serotonin release, increased the locomotor activity and the spatial coefficient of variation but decreased rearing, nosepoking, center entries and the spatial *d* (Geyer and Paulus, 1992). While both amphetamine and apomorphine increased locomotor activity in rats, amphetamine treatment increased the frequency of nosepoking, whereas apomorphine decreased it (Geyer et al., 1979). Thus, different stimuli may affect locomotor activity and exploratory activity differently and the BPM allows a more thorough analysis of these effects.

Apart from locomotor activity, the evaluation of exploratory behavior is also important in the context of manic-like behaviors. Rearing depends not only on exploration *per se* but also on the amount of locomotor activity. Rearings may represent both inspective and diversive exploration of an environment, while nosepoking is more likely to reflect inspective, investigatory exploration. Studies show that the effect of psychostimulants on locomotor activity and exploratory behavior can vary. DAT knockout mice exhibited increased locomotor activity and increased exploratory behavior, as seen by increases in the number of nosepokes (Young et al., 2007). GBR12909 and modafinil differentially affect

rearing and nosepoking in C57BL/6J and 129 strain mice, respectively (Young et al., 2011b; Young et al., 2010). In mice, amphetamine administration also increased motor activity but reduced exploration (rearings and nosepokes), in both male and female mice (Minassian et al., 2016). Perry et al. (2009) showed that DAT knockdown mice on a 129Sv/J background exhibited increased nosepoking, while GBR12909 administration to C57BL/6J mice resulted in more prominent effects on locomotor activity and rearing behavior. Thus, the effects of different psychostimulants on mice can affect locomotor and exploratory behaviors (nosepokes and rearings or climbings) differently, depending on the stimuli or dose, for example. Nevertheless, in our study, methylphenidate induced increases in locomotor activity and exploratory activity, some of which were prevented by repeated treatment with ANDRO and lithium.

Besides showing an antimanic-like effect of ANDRO, this study also shows the utility of the BPM as a tool for the evaluation of locomotor activity and exploratory behavior in the research for new antimanic drugs. The development of the BPM from rat to mice is important because of cross-species generalization, with supporting evidence of similar locomotor pattern effects of psychostimulants in both rats and mice, and also, because BPM for mice allows the use of genetically modified animals (Young et al., 2007).

Perry et al. (2009) developed a human BPM, by which they showed that manic patients had altered exploratory behavior, with high motor activity and increased object exploration. These results were different from schizophrenic patients, who did not show the expected habituation of motor activity. Thus, this model identifies characteristics of bipolar mania that differ from schizophrenia, which is very helpful in the development of more accurate studies (Perry et al., 2009). The current version of the human BPM, has been established as a room on a locked inpatient psychiatric unit, containing a file cabinet, bookcase, desk and no chairs, while interesting and manipulable objects in the room. Thepatient's behavior is monitored and the results show that manic patients display dramatic increases in motor activity (Minassian et al., 2007), and interactions with the objects in the room (Kincaid et al., 2007). The approach demonstrates the value of bi-directional development of objective translational tests that assess similar responses in both rodents and humans to improve the process of drug development and validation (Kwiatkowski et al., 2019).

In this context of drug validation, the methylphenidate administrationmodel was used to test the possible antimanic-like effects of ANDRO, with lithiumas positive control, in the mouse BPM. Our results show that ANDRO displayed antimanic-like effect as it prevented methylphenidate-induced hyperlocomotion and increased exploratory behavior (nosepokes). Also, we showed that the BPM is a useful resource in the evaluation of antimanic-like effects of antimanic drug candidates.

5. CONCLUSION

The present study demonstrated that the BPM is a useful tool in the evaluation of the antimanic-like effect of drugs, as it allows the analysis of locomotor activity and exploratory behavior. This study also showed that the chronic ANDRO treatment prevented methylphenidate-induced hyperlocomotion and increases in nosepoking, displaying an antimanic-like effect, as did the antimanic drug lithium. Our findings show promising antimanic-like properties of ANDRO, which, however need further investigation.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

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6. DISCUSSÃO GERAL

O número reduzido de opções farmacológicas eficientes e seguras para o tratamento do TB torna necessária a pesquisa para novas drogas. Considerando que o lítio, a droga antimaníaca padrão ouro para o TB, inibe a enzima GSK3β, e tem efeito antioxidante, seria possível que o ANDRO, um componente da planta *A. paniculata*, que também inibe a enzima GSK3β e possui ação antioxidante (Mussard et al., 2019; Serrano et al., 2014), possuísse propriedades tipo-antimaníacas. Tal hipótese surgiu levando em conta que a expressão aumentada da enzima GSK3β e o estresse oxidativo cerebral têm papéis importantes na fisiopatologia do TB (Valvassori et al., 2020; Polter et al., 2010).

Os resultados do presente estudo mostraram que o tratamento crônico com ANDRO (0.5 mg/kg e 2.0 mg/kg) e lítio (100 mg/kg) foram capazes de prevenir a hiperlocomoção induzida por administração de metilfenidato e privação de sono de 24h. Estes são modelos farmacológicos e não-farmacológicos, respectivamente, de indução de comportamento tipo maníaco em animais. Ambos são considerados modelos animais de mania validados, pois apresentam validade de face, de constructo e preditiva (Einat, 2006; Machado-Vieira et al., 2004). A administração de metilfenidato em animais mimetiza os efeitos da administração de psicoestimulantes em humanos, que induzem comportamentos maníacos em indivíduos sadios ou com TB, como necessidade diminuída de dormir, humor elevado, hipersexualidade, alterações sensorimotoras e alterações no aprendizado e memória, por exemplo (Corp et al., 2014; Cousins et al., 2009; Berk et al., 2007; Asghar et al., 2003; Jacobs e Silverstone et al., 1986). Após privação de sono de 24 h, animais demonstram insônia, hiperatividade, irritabilidade, agressividade, hipersexualidade, estereotipia e aumento na emissão de USVs de 50-kHz, sendo que alguns destes parâmetros são vistos em humanos após privação de sono (Pereira et al.,2014; Gessa et al., 1995). Isto demonstra a validade de face de ambos modelosde mania. A administração de metilfenidato afeta a transmissão de vários neurotransmissores, por aumentarem a concentração sináptica de dopamina e noradrenalina pela inibição ou reversão de mecanismos de recaptação, que reflete o que ocorre com os níveis destes neurotransmissores no paciente com TB. O modelo de privação de sono de 24 também envolve diversas alterações

de neurotransmissores, como pelo aumento da expressão de receptores D_2 no estriado e hipersensibilidade de receptores dopaminérgicos, levando a um aumento da neurotransmissão dopaminérgica em animais, que reflete o que é visto em pacientes bipolares (Lima et al., 2007; Tufik et al., 1978). Isto mostra a validade de constructo de ambos modelos de mania (Beyer e Freund et al., 2017; Berk et al., 2007). Lítio e valproato, que são drogas utilizadas na terapia do TB, podem atenuar comportamentos induzidos pela administração de metilfenidato ou por privação de sono de 24 h, o que mostra a validade preditiva de ambos modelos (Einat, 2006; Machado-Vieira et al., 2004; Gessa et al., 1995). Portanto, o efeito preventivo do ANDRO na hiperlocomoção induzida por administração de metilfenidato e por privação de sono de 24 h denota um possível efeito tipo antimaníaco.

Em paralelo, foi avaliado o efeito do ANDRO nos níveis de GSK3β e p-Ser^9-GSK3β no CPF e estriado de camundongos submetidos aos modelos de hiperlocomoção induzida por privação de sono de 24h ou administração de metilfenidato. Os resultados indicam que a privação de sono de 24h induziu hiperlocomoção nos camundongos, que foi prevenida pelo tratamento crônico com ANDRO (0.5 mg/kg e 2.0 mg/kg) e lítio (100 mg/kg). Os resultados mostram que a privação de sono de 24h reduziu a fosforilação em Ser^9 da GSK3β no CPF de camundongos, o que indica atividade aumentada da GSK3β. O tratamento crônico com ANDRO (2.0 mg/kg) e lítio (100 mg/kg) preveniu a diminuição de p-Ser^9-GSK3β induzida por privação de sono. Além disso, o tratamento crônico com ANDRO (0.5 mg/kg) e ANDRO (2.0 mg/kg) e lítio preveniram a hiperlocomoção induzida por metilfenidato. A administração de metilfenidato reduziu a fosforilação em Ser^9 da GSK3β no estriado, o que foi prevenido por ANDRO (2.0 mg/kg) e lítio.

No geral, a GSK3β é considerada uma molécula pró-inflamatória, estimulando a produção de diversas citocinas pró-inflamatórias e fator de necrose tumoral. A inibição da GSK3β mostrou ter efeitos benéficos em condições inflamatórias (Jope et al., 2007). O impacto da GSK3β na neurotransmissão ainda foi totalmente elucidado, mas estudos mostram que a GSK3β interfere na neurotransmissão por afetar canais de cálcio e potássio. Também está envolvida na fosforilação de proteínas relacionadas ao gene

CLOCK e ao ritmo circadiano (Luca et al., 2016). Níveis aumentados de foram mostrados em pacientes em episódios maníacos, quando comparados a indivíduos saudáveis (Luca et al., 2016). Após o tratamento, foi mostrado que os níveis de GSK3β não foram alterados, porém a concentração de p-Ser^{9}-GSK3β aumentou (Li et al., 2010). Estudos mostram uma relação entre a atividade aumentada de GSK3β e hiperatividade decorrente da diminuição da fosforilação inibitória em Ser^{9} em pacientes bipolares (Li e Jope, 2010). Dal-Pont et al. (2019) demonstraram que a privação de sono de 24h induz comportamentos tipo maníacos em camundongos e diminui os níveis de fatores neurotróficos no CPF e hipocampo, o que foi revertido com tratamento com lítio e valproato. Xue et al. (2019) mostraram que a privação de sono inibe a via da PI3K/Akt/GSK3β, o que sugere uma ativação da GSK3β, que induziu neuroinflamação e estresse oxidativo. A administração aguda de psicoestimulantes ativa a GSK3β por reduzir a fosforilação inibitória em Ser^{9} no estriado e CPF de camundongos, o que está envolvido com comportamentos como hiperlocomoção (Enman e Unterwald, 2012). Removendo a GSK3β no estriado de camundongos utilizando a técnica CRISPR-Cas9, Kim et al. (2019) mostraram que houve supressão da hiperlocomoção induzida por anfetamina. Mines e Jope (2012) mostraram que o efeito de psicoestimulantes pode ser região-seletivo e que processos de sinalização ocorrem de diferentes formas no CPF e no estriado. Eles demonstraram que a administração intraperitoneal de anfetamina (2 mg/kg) ou metilfenidato (20 mg/kg) por 8 dias levou à diminuição dos níveis de p-Ser^{9}-GSK3-β no estriado de camundongos C57BL/6J.

No presente estudo, também foram avaliados os efeitos do ANDRO na hiperlocomoção induzida pela administração de LDX, que também induz aumentos na emissão de USVs de 50-kHz. LDX é uma pró-droga da *d*-anfetamina de longa duração (Ermer et al., 2016) e sua administração pode ser utilizada como modelo de mania (Eger et al., 2016; Wendler et al., 2016). A emissão de USVs pode ser considerada um reflexo de estados hedônicos dos ratos (Burgdorf et al., 2011). Estudos mostram que a privação de sono induz hiperlocomoção e aumento no número de *rearings*, e também na emissão de USVs de 50-kHz em ratos, o que denota comportamentos tipo-maníacos, mostrando a validade de face do modelo (Wendler et al., 2019).

Psicoestimulantes induzem ao aumento da emissão de USVs de 50-kHz pela ativação de receptores D_1 e D_2 (Rippberger et al., 2015) e isso está envolvido com o aumento da neurotransmissão dopaminérgica presente na mania (Ashok et al., 2017). A aplicação intra-accumbens de quinpirol, um agonista de receptores D_2 e D_3, induz à emissão aumentada de USVs de 50-kHz (Brudzynski et al., 2012). A administração de LDX induz comportamentos tipo-maníacos por aumentar a neurotransmissão dopaminérgica e por induzir estresse oxidativo, sendo que ambos parâmetros estão envolvidos na fisiopatologia do TB, portanto mostrando a validade de constructo do modelo de mania da administração de LDX (Macêdo et al., 2013). A administração de LDX induz à emissão aumentada de USVs de 50-kHz, o que é prevenida por tratamento com lítio (Wendler et al., 2016). Pereira et al. (2014) mostraram que o lítio foi capaz de reverter o aumento das USVs de 50-kHz induzido por anfetamina. Estes estudos mostram a validade preditiva do modelo de mania de administração de LDX, um psicoestimulante. No presente estudo, o tratamento crônico com ANDRO (2.0 mg/kg) e lítio preveniram o aumento no número de USVs de 50-kHz induzida por LDX e no tempo total de vocalização. Em paralelo, o tratamento com as drogas preveniu a hiperlocomoção induzida por LDX em ratos.

Além disso, foi avaliado o efeito antioxidante do ANDRO. Sabe-se que tanto ANDRO quanto lítio possuem efeito antioxidante. O tratamento com lítio reduz a razão de SOD e CAT, reduzindo estresse oxidativo. Lítio também possui efeitos benéficos em disfunções mitocondriais e do retículo endoplasmático (Machado-Vieira et al., 2009; Maurer et al., 2009). A inibição da GSK3β pela administração de lítio aumenta a resistência de células neuronais hipocampais de murinos ao estresse oxidativo (Schäfer et al. 2004). Zhang et al. (2019) demonstraram que a bupivacína aumenta os níveis de EROs e diminui os níveis de GSH em células SH-SY5Y de neuroblastoma, o que foi prevenido pela pré-incubação das células com ANDRO. Thakur et al. (2016) mostrou que ratos diabéticos, induzida por estreptozotocina, apresentaram níveis aumentados de LPO no CPF, o que foi reduzido pelo tratamento com ANDRO. No presente estudo, a administração de LDX induziu ao aumento de LPO no estriado dos ratos, o que foi prevenido pelo tratamento crônico com ANDRO (2.0 mg/kg) e lítio. Houve também uma correlação positiva entre os níveis de LPO induzidos

por LDX no estriado dos ratos com a hiperlocomoção e o aumento das USVs de 50-kHz induzidos por LDX.

Na avaliação dos efeitos do ANDRO no aumento da atividade exploratória induzida por metilfenidato em camundongos, o tratamento com lítio e ANDRO (2.0 mg/kg) preveniram a hiperlocomoção e o aumento do número de *nosepokes* induzido por metilfenidato no BPM.

Apesar das diversas propriedades terapêuticas do ANDRO, a baixa solubilidade e relativa baixa potência são empecilhos para que ANDRO atinja seus efeitos terapêuticos (Sharma et al., 2017). Modificações na estrutura química e otimização de sistemas de distribuição podem melhorar e facilitar sua atividade farmacológica do ANDRO e seus derivados (Dai et al., 2018). ANDRO, inclusive, já é comercalizado a nível mundial como suplemento dietético, como pílulas de *A. paniculata* (planta pulverizada), principalmente por suas propriedades antiinflamatórias e antioxidantes (Kataky e Handique, 2010). Além disso, a pesquisa dos efeitos terapêuticos do ANDRO não se baseiam somente em estudos pré-clínicos, pois existem vários estudos clínicos sendo realizados. Como exemplo, pode-se citar um estudo randomizado, duplo-cego, controlado com placebo, o efeito terapêutico de tabletes de extrato de *A. paniculata* (170 mg de *A. paniculata* contendo 85 mg de ANDRO) foi avaliado em indivíduos com esclerose múltipla remitente recorrente recebendo terapia com interferon e o tratamento reduzindo em 44% os sintomas de fadiga associados à esclerose múltipla, comparado com o grupo placebo (Bertoglio et al., 2016). Outro estudo randomizado, duplo-cego, controlado por placebo de fase II avaliou os efeitos de tabletes orais de *A. paniculata* em pacientes com eslcerose múltipla. Os resultados ainda não foram publicados (NCT02280876).

Vários estudos também demonstraram a segurança do ANDRO. Em um estudo de toxicidade, uma dose oral de 5 g/kg de ANDRO foi administrada via oral por 14 dias em ratos, e não houve efeitos adversos (Bothiraja et al., 2012). A LD_{50} de ANDRO via intraperitoneal em camundongos é de 11.46 g/kg. Prakash e Manavalan (2011) mostraram que a administração aguda de 2000 mg/kg de ANDRO v.o. em camundongos não alterou o peso corporal, níveis de creatinina, colesterol total, glicemia nem parâmetros hematológicos, comparado ao grupo controle. Tais estudos demonstram que ANDRO parece ser relativamente

seguro, o que aumenta o interesse no seu uso terapêutico (Lu et al., 2019; Tan et al., 2017).

Vale lembrar que o tratamento convencional para o TB, ainda possui respostas em níveis inadequados para tratar mania aguda ou episódios depressivos ou como tratamento preventivo de manutenção a longo prazo (Gitlin, 2006). Os medicamentos incluem lítio, valproato, antipsicóticos e anticonvulsivantes, além de antidepressivos (López-Muñoz et al., 2018; Gitlin, 2006). Tais medicamentos causam uma série de efeitos adversos, como ganho de peso, acatisia, náuseas, vômitos, sonolência, tremores, tontura, astenia, alterações hematológicas, dentre outros (Bai et al., 2019; López-Muñoz et al., 2018). Tais aspectos, assim como problemas na posologia, medo de dependência medicamentosa e medo de efeitos colaterais afetam a aderência do paciente ao tratamento (Levin et al., 2020; Levin et al., 2016; Chang et al., 2015.; Devulapalli et al., 2010). Neste contexto, é importante que haja mais opções de drogas antimaníacas, mais eficazes e mais seguras.

Pacientes com TB apresentam maior morbidade, maior risco de incapacitação e diminuição de longevidade (Baldessarini et al., 2020). Pacientes com TB possuem maior risco de mortalidade, podendo chegar a um risco quinze vezes maior, quando comparado à população geral (Staudt-Hansen et al., 2019; Ösby et al., 2018). Portanto, é de grande relevância a pesquisa contínua por novas drogas antimaníacas que possam ser incluídas no arsenal farmacológico para o manejo do TB e o presente estudo mostrou que o ANDRO possui atividade tipo-antimaníaca (efeitos mostrados na Tabela 1), sendo um candidato promissor neste contexto.

Tabela 1: Resumo dos efeitos do tratamento crônico com ANDRO nos diferentes modelos animais de mania

Modelo de mania	**Experimento/análise**	**Resultados**
Hiperlocomoção induzida por metilfenidato em camundongos	Teste do campo aberto	MPH: ↑ cruzamentos Li: ↓ cruzamentos 0.5 ANDRO: ↓ cruzamentos 2.0 ANDRO: ↓ cruzamentos

Hiperlocomoção induzida por metilfenidato em camundongos	Razão de p-Ser^{9}-GSK3β/GSK3β no estriado	MPH: ↓ Li: ↑ 0.5 ANDRO: ↓ 2.0 ANDRO: ↑
Hiperlocomoção induzida por privação de sono de 24 h em camundongos	Teste do campo aberto	PS: ↑ cruzamentos Li: ↓ cruzamentos 0.5 ANDRO: ↓ cruzamentos 2.0 ANDRO: ↓ cruzamentos
Hiperlocomoção induzida por privação de sono de 24 h em camundongos	Razão de p-Ser^{9}-GSK3β/GSK3β no CPF	PS: ↓ Li: ↑ 0.5 ANDRO: ↓ 2.0 ANDRO: ↑
Hiperlocomoção induzida por LDX em ratos	Avaliação da atividade locomotora	LDX: ↑ cruzamentos Li: ↓ cruzamentos 2.0 ANDRO: ↓ cruzamentos
Aumento da emissão de USVs de 50-kHz induzida por LDX em ratos	Avaliação da emissão de USVs de 50-kHz em ratos	LDX: ↑ número de USVs de 50-kHz Li: ↓ número de USVs de 50-kHz 2.0 ANDRO: ↓ número de USVs de 50-kHz
Hiperlocomoção induzida por LDX em ratos	Avaliação de parâmetros de estresse oxidativos	LDX: ↑ LPO no estriado Li: ↓ LPO no estriado 2.0 ANDRO: ↓ LPO no estriado
Hiperlocomoção e aumento da atividade exploratória induzidas por metilfenidato	Avaliação da atividade locomotora e atividade exploratória no behavioral pattern monitor (BPM)	MPH: ↑ cruzamentos, ↑ *nosepokes,* ↑ *rearings*, ↑ escaladas Li: ↓cruzamentos, ↓ *nosepokes* ANDRO: ↓ cruzamentos, ↓ *nosepokes*

ANDRO: andrografolide; BPM: behavioral pattern monitor; CPF: córtex pré-frontal; LDX: lisdexanfetamina; Li: lítio; LPO: peroxidação lipídica; MPH: metilfenidato; PS: privação de sono; USV: vocalizações ultrassônicas.

7. CONCLUSÃO

No geral, os resultados mostraram que o tratamento com ANDRO foi capaz de prevenir comportamentos tipo-maníacos, como hiperlocomoção, aumento de USVs de 50-kHz e aumento de atividade exploratória, induzidos por administração de metilfenidato, por privação de sono de 24h ou por administração de LDX em camundongos e ratos. Além disso, o tratamento com ANDRO, assim como com lítio, preveniu o aumento da expressão da enzima GSK3β, por aumentar a razão de p-Ser^{9}-GSK3β/GSK3β e também por prevenira indução de LPO (parâmetro de estresse oxidativo). Portanto, ANDRO parece apresentar atividade tipo-antimaníaca e pode ser um agente promissor a ser investigado para uma nova alternativa de agente terapêutico para a mania no TB.

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