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Efeitos do Nitroprussiato de Sódio e do Canabidiol no modelo animal de psicose
por Cetamina S+ e Cetamina R em ratos machos e fêmeas

RIBEIRÃO PRETO

2024

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Tese apresentada ao Programa de Pós-graduação em Medicina (Neurologia) da Faculdade de Medicina de Ribeirão Preto da Universidade de São Paulo como parte das exigências para obtenção do título de Doutora em Ciências.

Área de concentração: Neurociências

Orientador: Prof. Dr. Jaime Eduardo Cecílio Hallak

RIBEIRÃO PRETO

2024

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da Silva Dias, Isabella Caroline

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"Identifico a humildade, e não a arrogância, como o princípio fundamental da maturidade científica. O ideal não é a verdade ou a certeza, mas a busca contínua e pluralista do conhecimento"

Hasok Chang

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RESUMO

da Silva Dias, Isabella Caroline. **Efeitos do Nitroprussiato de Sódio e do Canabidiol no modelo animal de psicose por Cetamina S+ e Cetamina R em ratos machos e fêmeas.** 2024. Tese (doutado em Medicina (Neurologia) – Faculdade de Medicina de Ribeirão Preto – Universidade de São Paulo – Ribeirão Preto – SP.

A esquizofrenia (SZ) é um dos transtornos mentais mais debilitantes que existem. Os sintomas são geralmente classificados em três grandes categorias; sintomas positivos, negativos e déficits cognitivos. Os tratamentos atuais do transtorno permanecem insatisfatórios em abranger todos os tipos de sintomas. Além disso, os antipsicóticos disponíveis produzem efeitos adversos significativos relacionados ao controle sensorio-motor e endócrino, dentre outros. Nesse sentido, o canabidiol (CBD) e o nitroprussiato de sódio (NPS) têm demonstrado efeitos interessantes na pesquisa pré e clínica, por apresentarem efeitos mais abrangentes e relativamente mais seguros. A causa da SZ é desconhecida, mas a principal hipótese aponta para anomalias ocorridas durante o neurodesenvolvimento que podem culminar no aparecimento de sintomas durante a adolescência. O conhecimento da fisiopatologia do distúrbio é o principal aliado na busca por tratamentos mais eficazes, portanto, a pesquisa em animais torna-se uma ferramenta valiosa. Nesse sentido, o modelo de cetamina (KET) é um dos mais utilizados, pois consegue mimetizar os três tipos de sintomas em roedores. A KET possui dois isômeros ópticos, R(-) e S(+) (KET S). O isômero S tem mais afinidade pelos receptores NMDA, por isso acredita-se que pode produzir mais sintomas semelhantes aos da SZ do que os outros tipos de KET. Quando misturados produz-se a forma mais popular de KET, a racêmica (KET R). Uma das principais características do transtorno é a diferença entre os sexos. Pesquisas indicam que a SZ se manifesta de forma diferente em homens e mulheres em termos de fisiopatologia, epidemiologia, resposta ao tratamento e prognóstico. No entanto, poucos trabalhos foram realizados para investigar sistematicamente essa questão. Considerando o exposto, nesta tese nos propomos investigar novas abordagens terapêuticas durante a fase do neurodesenvolvimento, com o pre tratamento de CBD e NPS, isolados e em associação (artigo 1); investigar se há diferença entre as cetaminas (mistura racêmica e isômero S+) em produzir sintomas do tipo SZ em ratos adolescentes (artigo 2); e por fim, investigar a eficácia terapêutica do tratamento com CBD e NPS (isolados e em associação) no tratamento agudo em ratos adolescentes (short communication, artigo 3). Importante ressaltar que os 3 trabalhos foram realizados em ratas fêmeas e ratos machos para investigar a diferença da variável sexo nos parâmetros investigados.

Palavras-chave: Esquizofrenia, cetamina, neurodesenvolvimento, sexo, canabidiol, nitroprussiato de sódio

ABSTRACT

da Silva Dias, Isabella Caroline. **Sodium Nitroprusside and Cannabidiol Effects in the animal model of psychosis caused by Ketamine S+ and Ketamine R in male and female rats.** 2024. Thesis (doctorate in Medicine (Neurology)) – Faculty of Medicine of Ribeirão Preto – University of São Paulo – Ribeirão Preto – SP.

Schizophrenia (SZ) is one of the most debilitating mental disorders that exists. Symptoms are generally classified into three broad categories; positive and negative symptoms and cognitive deficits. Current treatments for the disorder remain unsatisfactory in covering all types of symptoms. Furthermore, available antipsychotics produce significant adverse effects related to sensorimotor and endocrine control, among others. In this sense, cannabidiol (CBD) and sodium nitroprusside (SNP) have demonstrated interesting effects in pre- and clinical research, as they have more abrange and relatively safer effects. The cause of SZ is unknown, but the main hypothesis points to anomalies occurring during neurodevelopment that may culminate in the appearance of the first symptoms during adolescence. The knowledge of the pathophysiology of the disorder is the main ally in the search for more effective treatments, therefore, research on animals becomes a valuable tool. In this sense, the ketamine (KET) model is one of the most used, as it can mimic the three types of symptoms in rodents. KET has two optical isomers, R(-) and S(+) (KET S). The S isomer has more affinity for NMDA receptors, so it is believed that it may produce more SZ-like symptoms than the other types of KET. When mixed, the most popular form of KET is produced, racemic (KET R). One of the main characteristics of the disorder is the difference between the sexes. Research indicates that SZ manifests differently in men and women in terms of pathophysiology, epidemiology, response to treatment, and prognosis. However, little work has been carried out to systematically investigate this issue. Considering the above, in this thesis we propose to investigate new therapeutic approaches during the neurodevelopment phase, with the pre-treatment of CBD and SNP, isolated and in combination (manuscript 1); investigate whether there is a difference between ketamines (racemic mixture and S+ isomer) in producing SZ-like symptoms in adolescent rats (manuscript 2); and finally, investigate the therapeutic efficacy of treatment with CBD and SNP (alone and in combination) in an acute treatment in adolescent rats (short communication, manuscript 3). It is important to highlight that the 3 studies were carried out on female and male rats.

Keywords: Schizophrenia, ketamine, neurodevelopment, sex, cannabidiol, sodium nitroprusside

LISTA DE ABREVIATURA E SIGLAS

AMPA - α -amino-3-hidroxi-5-metil-4-isoxazolpropiónico

ANOVA - *Analysis of variance*

BDNF - *Brain-derived neurotrophic factor*

CA - Campo aberto

CBD – Canabidiol

cGMP - Monofosfato de guanosina cíclico

DSM – *Diagnostic and Statistical Manual of Mental Disorders*

F – *Females*

GABA - Ácido gama-aminobutírico

i.p. - Intraperitoneal

KET – Cetamina (do inglês, *Ketamine*)

KET R – *Ketamine R, S*

KET S – *Ketamine S⁺*

L-DOPA – Levodopa

LDA - *Linear discriminant analysis*

LO - Localização de objetos

LOF - *Local Outlier Factor*

LTP - *Long Term Potentiation*

M - *Males*

NMDA - Ácido N-metil-D-aspartato

NK – *Newman-keuls post hoc*

NOR - *Novel object recognition*

NPS – Nitroprussiato de Sódio

OFT - *Open field test*

PET - *Positron Emission Tomography*

PKG - Proteínas quinase dependente de cGMP

PS - Teste de preferência de sacarose

PPI - Inibição pré-pulso

RO - Reconhecimento de novos objetos

SAL - Saline 0.9%

SNP – *Sodium nitroprusside*

SPT - *Sucrose preference test*

SZ – Esquizofrenia (do inglês, *Schizophrenia*)

TEPT - Transtorno de estresse pós-traumático

THC – Tetrahydrocannabinol

UHR - *Ultra High Risk*

VEH - *Vehicle*

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1. INTRODUÇÃO

1.1. ESQUIZOFRENIA: A EVOLUÇÃO DO DIAGNÓSTICO

A esquizofrenia é um dos transtornos mentais mais debilitantes que existe, acarretando sérias consequências na qualidade de vida não só dos pacientes acometidos, mas também em seu círculo sócio-familiar (Maibing et al., 2015; Tandon et al., 2009). Além disso, devido à incapacidade da sociedade em incorporar esses indivíduos em tarefas que geram renda, a esquizofrenia tem impacto direto na economia global (Hoseinipalangi et al., 2021). Seu diagnóstico certo é parte fundamental para essas pesquisas epidemiológicas, mas nem sempre foi assim.

O conceito atual de esquizofrenia tem raiz nas definições de Kraepelin (1899), Bleuler (1911) e Schneider (1959). Ao longo da história essas definições suscitaram as discussões necessárias para se chegar nos critérios diagnósticos atuais (Hoenig, 1983; Tandon et al., 2009). No final do século 19, Kraepelin observava certo padrão de semelhanças entre pacientes com catatonia, hebefrenia e demência paranoide, que se iniciava na adolescência ou início da idade adulta, com tendência a deteriorização dos sintomas, embotamento afetivo e consequente demência ao passar dos anos. Então primeiramente ele dividiu dois grupos: os que apresentavam sintomas episódicos, ausência de deteriorização e um prognóstico mais favorável foram classificados como maníacos-depressivos (posteriormente transtorno bipolar); e o outro grupo como demência precoce. Portanto, o princípio do diagnóstico da esquizofrenia se baseou em início dos sintomas na adolescência, curso crônico com deteriorização significativa e prejuízo permanente da função mental (Berrios and Hauser, 1988).

Já Eugen Bleuler (1911) considerava que o principal dos sintomas da esquizofrenia era a desintegração de diferentes funções psíquicas, levando aos seus sintomas de dissociação, embotamento afetivo, e ambivalência de sentimentos. Classificava delírios e alucinações como

sintomas acessórios. Com isso aumentou o espectro de sintomas e cunhou o termo esquizofrenia (Tandon et al., 2009).

Na década de 1950, Kurt Schneider definiu 11 sintomas de primeira ordem para o diagnóstico do transtorno, o que mais tarde ficou classificado como sintomas positivos. Levava em conta elementos de “incompreensibilidade” da experiência individual e foi criticado por levar mais em consideração a forma e não o conteúdo para fechar critério de psicose (Mellor, 1970).

Existem diferenças claras entre essas três perspectivas de definição de esquizofrenia. Kraepelin não forneceu critérios específicos para seu diagnóstico, mas enfatizou a importância do curso longitudinal e seu resultado desfavorável. Em contraste, tanto Bleuler como Schneider forneceram critérios transversais específicos, e se concentraram em diferentes aspectos da doença. Então as definições atuais de esquizofrenia incorporam a cronicidade Kraepeliniana, os sintomas negativos Bleulerianos e sintomas positivos Schneiderianos (Andreasen, 2007; Tandon et al., 2009).

A oitava e a nona edição da Classificação Internacional de Doenças (CID-8 e CID-9, Organização Mundial da Saúde, 1967, 1978) enfatizaram os sintomas positivos, a cronicidade e o prognóstico desfavorável como características definidoras da esquizofrenia, enquanto a segunda edição do Diagnóstico e o Manual Estatístico de Transtornos Mentais (DSM-II, *American Psychiatric Association*, 1968) definiram-a com base na “perda dos limites do ego”. Estas diferenças levaram a grandes discrepâncias nas taxas de diagnóstico de esquizofrenia entre os Estados Unidos e o resto do mundo, algo que ficou bem claro em estudo realizado nos Estados Unidos e Reino Unido na década de 1970 (Cooper et al., 1972). Este estudo apontou que embora pacientes internados nos hospitais públicos de Nova Iorque e Londres apresentassem sintomas semelhantes, a casuística de esquizofrenia era o dobro no hospital da América do Norte. Este foi um dos fatores que deixou a terceira edição do DSM mais enxuta e rigorosa em relação ao diagnóstico de esquizofrenia e o

transtorno maníaco-depressivo (DSM-III, Associação Americana de Psiquiatria, 1980; Cooper et al, 1972). Fazendo um retrospecto nos dias atuais, historicamente os critérios diagnósticos mais abrangentes pertencem ao DSM-II enquanto os mais restritos pertencem ao DSM-III (Tandon et al., 2009).

Atualmente, em seu quinto e último volume (DSM-5), a esquizofrenia é diagnosticada na presença de dois ou mais sintomas como delírios, alucinações, discurso desorganizado, comportamento desorganizado ou catatônico; sintomas negativos como diminuição da expressão emocional, avolição e anedonia (lista A); comprometimento funcional acentuado, incluindo interpessoal, acadêmico e ocupacional (B); perturbação contínua durante pelo menos 6 meses, incluindo 1 mês dos sintomas da lista A e pode incluir período de sintomas prodrômicos ou residuais (C); transtorno de humor excluído como diagnóstico primário (D); sintomas não causados por efeitos de alguma substância ou outra condição médica (E); no contexto do espectro do autismo ou distúrbio da comunicação, problemas proeminentes, delírios ou alucinações devem estar presentes para fazer um diagnóstico (F) (DSM-5, *American Psychiatric Association*, 2014).

Como citado anteriormente é comum o início dos sintomas aparecerem na adolescência/início da vida adulta, o que levou pesquisadores a hipotetizarem que a causa do transtorno se dava durante a fase do neurodesenvolvimento (Eyles, 2021; Fatemi and Folsom, 2009).

1.2. NEURODESENVOLVIMENTO E ADOLESCÊNCIA

A causa exata do transtorno ainda é desconhecida, mas a principal hipótese aponta para anomalias ocorridas durante o neurodesenvolvimento que geralmente culminam com o aparecimento dos sintomas na fase da adolescência (2) (Eyles, 2021; Fatemi and Folsom, 2009).

Inclusive, indivíduos adolescentes com alta probabilidade de desenvolverem esquizofrenia formam um grupo clínico específico cada vez mais protagonista de pesquisas de prevenção do transtorno, os *ultra high risk* (UHR) (2) (Fusar-Poli et al., 2017; Sommer et al., 2016). Os sintomas são geralmente classificados em três grandes categorias: sintomas positivos, negativos e déficits cognitivos. No primeiro tipo de sintoma encontra-se alucinações, delírios e percepções alteradas de maneira geral. Em sintomas negativos encontra-se o embotamento social, avolição e pobreza de discurso. Já na última categoria, apresenta-se sintomas cognitivos com dificuldade de concentração, desorientação no tempo e espaço, dificuldades em funções executivas e déficits mnemônicos (Green et al., 2004).

Na natureza, encéfalos em desenvolvimento são caracterizados por um alto grau de plasticidade sináptica, o que é evolutivamente vantajoso pois permite o refinamento das células do sistema nervoso central em consonância com as demandas do ambiente circundante. Entretanto, essa mesma labilidade torna o indivíduo extremamente vulnerável pois alterações nessa fase implicam resultados a longo prazo, principalmente para o funcionamento cognitivo e emocional (Meyer and Lee, 2019).

Recentemente aumentou-se o interesse em investigar o impacto de fatores sociais, comportamentais e neurobiológicos que caracterizam os estágios do desenvolvimento. Muito devido ao fato de até três quartos de todos os transtornos psiquiátricos geralmente surgirem antes dos 24 anos, como a esquizofrenia (Giedd et al., 2008; Kessler et al., 2005; Meyer and Lee, 2019). Além disso, cada vez mais as pesquisas associam pior prognóstico, comorbidades e taxas de recorrência mais altas em indivíduos que apresentam sintomas ainda na juventude. Pois na maioria das vezes as respostas cognitivas, emocionais e comportamentais baseiam-se em experiências anteriores que se sucedem nas primeiras fases da vida (Andersen and Teicher, 2008; Welsh et al., 2017).

1.2.1. Fase pré-mórbida

Dados apontam que indivíduos que desenvolvem esquizofrenia apresentavam uma série de limitações cognitivas, comportamentais e emocionais durante o desenvolvimento. Dentre elas destaca-se o atraso no desenvolvimento motor, baixo desempenho acadêmico, isolamento social, e déficits na atenção e na linguagem (Schenkel and Silverstein, 2004). Isto foi demonstrado em estudos de coorte (Cannon et al., 2002; Done et al., 1994; Jones et al., 1994), estudos prospectivos (Cornblatt et al., 1999; Fish, 1992; Keshavan et al., 2005), e relatos de caso (Schmael et al., 2007; Walker et al., 1993).

Entretanto, embora as características deste período pré-mórbido evidenciem a vulnerabilidade precoce e latente para a esquizofrenia, elas não estão presentes em 100% dos casos e nem sempre quando presentes culminam no aparecimento do transtorno na juventude (Tandon et al., 2009).

1.2.2. Fase prodrômica

A fase prodrômica corresponde ao período de tempo que precede o primeiro episódio de psicose, sendo marcada por sintomas psicóticos subliminares, déficits cognitivos e alterações de humor (Cornblatt et al., 1999). Esta fase foi inicialmente caracterizada através de estudos retrospectivos de pacientes no primeiro episódio (Chapman, 1966). Mais tarde as informações foram obtidas através de estudo longitudinais com indivíduos UHR para desenvolverem a esquizofrenia, seja devido ao histórico familiar ou por apresentarem sinais suspeitos (Addington et al., 2007; Olsen and Rosenbaum, 2006; Thomas and Woods, 2006). Os sintomas cognitivos, negativos e depressivos aparecem em média cerca de cinco anos antes do primeiro contato clínico e a incapacidade social surge aproximadamente de um a três anos após (An Der Heiden and Häfner, 2000; Klosterkötter et al., 2008). Dentre os indivíduos que procuram o sistema de saúde e que

atendem aos critérios de alto risco de desenvolver o transtorno, cerca de um sexto até a metade desenvolvem esquizofrenia. Neste sentido, quando os sintomas positivos são mais graves e ocorre maior grau de comprometimento social aumentam o grau da “conversão” (Cannon et al., 2008; Yung et al., 2008, 2003).

Recentemente houve um aumento no interesse em tentar prevenir a evolução desses sintomas prodrômicos para os sintomas clássicos da esquizofrenia, utilizando uma série de abordagens psicológicas e psicofarmacológicas entre os indivíduos que atendem aos critérios de UHR, mas os resultados vêm sendo inconclusivos até o momento (Häfner and Maurer, 2006; Lee et al., 2005).

1.2.3. Primeiro episódio psicótico

Definir o início dos sintomas da esquizofrenia é um desafio devido a vários fatores como dificuldade em detectar o primeiro sinal de distúrbio mental, disfunção social, primeira psicose e até mesmo o primeiro contato com o serviço de saúde. Sendo assim, para fins práticos, o desenvolvimento de sintomas psicóticos marca o início formal do transtorno (Fleming et al., 1993; Häfner et al., 1998).

Assim definida, o início da esquizofrenia ocorre tipicamente entre 15 a 45 anos de idade, sendo raros casos antes da puberdade ou após os 50 anos de idade (Häfner et al., 1998). É um dado importante a se levar em consideração para o prognóstico, visto que indivíduos que apresentaram início precoce (antes dos 13 anos de idade) manifestam sintomas mais graves e resposta empobrecida aos tratamentos atuais (Luoma et al., 2008; Masi et al., 2006).

O uso de substâncias químicas e os estressores da vida podem precipitar o primeiro episódio (Corcoran et al., 2003; Luoma et al., 2008), embora nenhum gatilho específico possa ser identificado na maioria dos casos. Assim como os episódios subsequentes de psicose, o primeiro

episódio geralmente começa com um aumento do humor e dos sintomas negativos (fase pré-psicótica), acompanhado de um aumento dos sintomas positivos (fase psicótica em si), seguido pela substituição desses sintomas em depressivos e negativos (depressão pós-psicótica) caminhando para a resolução da crise que pode ou não reincidir (Drake et al., 2003; Owens et al., 2005).

Interessante ressaltar que o início do episódio psicótico tende a ser mais precoce em homens, de 5 a 7 anos mais cedo, o que evidencia outro fator de extrema importância no estudo da esquizofrenia, o sexo (Tandon et al., 2009).

1.3. SEXO: O FATOR SIGNIFICATIVO E POUCO INVESTIGADO

A incidência da esquizofrenia é ligeiramente maior em homens do que em mulheres, com estimativas de razão aproximadamente de 1,5 (Abel et al., 2010; Wu et al., 2013). Inclusive, desde o início do século XX, Kraepelin já descrevia que a hospitalização por esquizofrenia ocorria mais em homens jovens (Hill, 2016). Entretanto, esta discrepância aumenta ou diminui de acordo com a idade (Mendrek and Mancini-Marie, 2016). Uma das possíveis explicações seria os níveis fisiológicos elevados de estradiol em mulheres na idade reprodutiva, pois a idade precoce da menarca foi associada com a idade posterior de início da esquizofrenia no sexo feminino (Cohen et al., 1999; Hill, 2016). Em relação aos sintomas, mulheres tendem a apresentar mais sintomas positivos enquanto homens expressam consistentemente mais sintomas negativos e déficits cognitivos (Abel et al., 2010; Leung, 2003). Dados de uma série de estudos de pacientes com esquizofrenia descreveram desempenho inferior do sexo masculino na atenção, linguagem e função executiva em comparação com o sexo feminino (Goldstein et al., 1998; Seidman et al., 1997). Também foi demonstrado que as mulheres se sobressaem em aprendizados verbais e tarefas de memória (Bozikas et al., 2010; Hill, 2016). Ademais, os homens parecem apresentar um curso do transtorno

mais grave, o que se deve, possivelmente, ao início mais precoce (Abel et al., 2010; Franceschini and Fattore, 2021). Já as mulheres, tendem a responder melhor aos tratamentos antipsicóticos disponíveis e requerem doses mais baixas para a eficácia terapêutica. No entanto, isso depende do tipo de antipsicótico, do estágio da fase reprodutiva e da progressão da esquizofrenia (Abel et al., 2010).

Os primeiros estudos tentaram vincular as diferenças encontradas entre os sexos segundo anormalidades estruturais e funcionais do encéfalo, porém chegaram à conclusão que as disparidades ocorrem em áreas que normalmente apresentam dimorfismo sexual fisiologicamente. Ou seja, naturalmente o cérebro masculino apresenta anomalias morfológicas relacionadas a esquizofrenia, com volumes frontais e temporais reduzidos (Abel et al., 2010; Choleris et al., 2018; Leung, 2003; Mendrek and Mancini-Marie, 2016).

Outros trabalhos se propuseram a investigar o papel dos hormônios sexuais na fisiopatologia do transtorno (Markham, 2012; Mendrek and Stip, 2011; Seeman, 2012). Assim, foi demonstrado que pacientes se beneficiaram de tratamentos hormonais como estrogênio, progesterona e testosterona (McGregor et al., 2017; Sun et al., 2016). Também foi estabelecida a correlação positiva entre hormônios reprodutores femininos com o início tardio e sintomas mais moderados (Begemann et al., 2012; Sun et al., 2016). Mulheres em fases de baixo estrogênio apresentam maior probabilidade de desenvolver psicose quando comparadas as mulheres em idade reprodutiva, então a administração de hormônios pós menopausa reduziu o risco de desenvolver a esquizofrenia, como observado também em outras patologias (Gogos et al., 2015; Markham, 2012). Além disso, foi detectado que níveis elevados de estrogênio durante a gravidez podem prevenir recaídas de crises psicóticas. Curiosamente, também conseguiu se estabelecer correlação entre os níveis plasmáticos de estrogênio e os sintomas da esquizofrenia em pacientes

do sexo masculino (Kaneda and Ohmori, 2005). Desde então a ação do estrogênio vem recebendo significativa atenção nas pesquisas de prováveis agentes terapêuticos (Ziemka-Nalecz et al., 2023).

Com isso foi comprovado o efeito neuroprotetor do estrogênio, influenciando os principais sistemas de neurotransmissores envolvidos na fisiopatologia do transtorno como a dopamina. A ação no sistema dopaminérgico se dá tanto no nível da transcrição genética quanto em efeitos indiretos não genômicos por ativação de vias de sinalização específicas (Brand et al., 2021). Com isso surgiu a hipótese de que o efeito do estrogênio na via dopaminérgica está relacionado ao comportamento de menor impulsividade e ao risco diminuído de abuso de substâncias em mulheres quando comparadas aos homens (Riecher-Rössler et al., 2018). Além disso, o estrogênio modulou vias serotoninérgicas (aumentando a densidade de receptores 5HT2a) (Busatto et al., 1997) e glutamatérgicas (Vollenweider et al., 1998) em modelos animais.

Em contraste com o número de publicações relacionados a estrogênio e esquizofrenia, poucos trabalhos se propuseram a investigar o papel da progesterona. Halari e colaboradores relataram uma correlação entre maior concentração de progesterona circulante com pior desempenho em tarefas de memória espacial (Halari et al., 2004). Já outros estudos relataram que estes hormônios não exerceram efeito significativo em pacientes com o transtorno (Rubin et al., 2010).

À semelhança dos receptores de estrogênio, os receptores de testosterona também já foram implicados na modulação dos estados de humor e do desempenho cognitivo (Mendrek et al., 2007). Resultados mais recentes demonstraram que os níveis plasmáticos de testosterona estavam inversamente correlacionados com a gravidade dos sintomas negativos em pacientes do sexo masculino (Mendrek et al., 2011).

Outro hormônio importante em termos de função reprodutiva, a ocitocina, também foi especulada como potencial alvo terapêutico para a esquizofrenia. De acordo com esta premissa, os pacientes que apresentavam níveis elevados de ocitocina plasmática desenvolviam menos sintomas psicóticos (Cochran et al., 2013) e melhoravam a performance em testes cognitivos (Frost et al., 2014). Este efeito antipsicótico pode estar relacionada à sua capacidade de atuar como regulador da dopamina, neurotransmissor importante na fisiopatologia do transtorno (Feifel, 2011).

Um número pequeno de estudos foi realizado na tentativa de identificar as variantes genéticas que poderiam explicar as diferenças entre os sexos na esquizofrenia (Borsboom et al., 2019). Bristow e colaboradores analisaram diversos genes relacionados com a transmissão GABAérgica no córtex cingulado anterior em indivíduos post-mortem. No grupo dos homens com esquizofrenia a expressão desses genes foi menor quando comparados ao grupo controle. Especificamente os genes GABA-A α 5, GABA-A β 1, e GABA-A ϵ . Ao contrário, nas mulheres com esquizofrenia houve um aumento na expressão dos genes GABA-A β 1 e GAD67 (Bristow et al., 2015).

Está claro o dimorfismo sexual no estudo da esquizofrenia. Entretanto, a discussão está muito limitada em hormônios reprodutivos e causas genéticas, fatores importantes como epigenética e contexto psicossocial não estão sendo levados em consideração. O que ressalta a urgência de mais trabalhos neste sentido.

1.4. DOPAMINA E A HIPÓTESE CLÁSSICA

A dopamina é um neurotransmissor da classe das monoaminas que medeia várias funções fisiológicas como sensação de prazer, controle motor, regulação do humor e funções endócrinas. No ano de 1963, o farmacologista sueco Arvid Carlsson detectou em animais que as drogas com

ação antipsicótica na clínica interferiam na sinalização deste neurotransmissor, e então propôs que esse efeito se devia ao bloqueio de receptores dopaminérgicos (Carlsson et al., 1963; Coyle et al. 2020). Anos mais tarde, Solomon Snyder constatou haver uma correlação positiva entre a eficácia clínica dos antipsicóticos com a sua afinidade específica pelo receptor do tipo D2 (Burt et al., 1976). Essas observações foram a base para a “hipótese dopaminérgica da esquizofrenia”, a qual propôs que a causa do transtorno seria devido a uma disfunção na neurotransmissão de dopamina (Snyder et al., 1976). Com o passar do tempo houve pequenas vitórias nesse sentido, enfim encontraram um tratamento farmacológico eficaz para tratar psicose (Milev et al., 2005). Mas também veio a frustração de não performar a cura esperada surtindo pouco efeito nos sintomas negativos e déficits cognitivos, além de efeitos adversos significativos. Com isso os pacientes não tinham autonomia para receber alta, continuavam separados de seus familiares e sem possibilidade de inserção no mercado de trabalho (Buchanan et al., 2007).

Além do tratamento farmacológico para a psicose, a hipótese dopaminérgica impulsionou as pesquisas do mecanismo de ação de outra droga promissora na época, a clozapina (Kane et al., 1988). Era um fármaco sem patente que havia demonstrado efeitos terapêuticos em pacientes que não respondiam ao tratamento disponível, reduzindo o risco de suicídio e a taxa de abuso de substâncias (Green et al., 2008). Devido ao efeito mais amplo da clozapina de interagir com outros receptores além da dopamina, jogou luz na necessidade de investigação além do sistema dopaminérgico (Coward et al., 1992). Neste sentido a clozapina inaugurou os antipsicóticos classificados de segunda geração que além de antagonizar receptores D2 também interagem com receptores serotoninérgicos e atenuavam efeitos adversos extrapiramidais (Rummel-Kluge et al., 2012; Coyle et al., 2020).

1.4.1. Os achados dos estudos de imagem

Considerando ser a esquizofrenia um distúrbio cerebral, suas assinaturas fisiopatológicas deveriam ser encontradas no encéfalo. Seguindo este racional vários estudos foram realizados com esse intuito. A dopamina é sintetizada a partir de seu precursor levodopa (L-DOPA) por descarboxilase do ácido aromático, com isso a L-DOPA radiomarcada por emissão de pósitron na tomografia (em inglês – PET, *positron emission tomography*) pôde servir como medida da síntese de dopamina (Daubner et al., 2011). Através dessa técnica foi possível identificar altos níveis de glutamato e dopamina nos gânglios da base, e baixos níveis não só de glutamato e dopamina, mas também de GABA nas regiões corticais, especialmente no córtex frontal em comparação ao grupo controle de indivíduos saudáveis (Howes et al., 2023).

Além disso, verificou-se que a maioria dos corpos celulares dos neurônios dopaminérgicos se localizam no mesencéfalo, particularmente na substância negra e na área tegmental ventral, se projetando para regiões como o corpo estriado e o córtex frontal (Haber et al., 2014). Inicialmente, estudos post-mortem e de neuroimagem indicavam que os níveis de marcadores de dopamina estavam alterados no mesencéfalo de pessoas com esquizofrenia (Howes et al., 2013). Contudo, o foco principal dos estudos tem sido o estriado, onde evidências de alterações foram demonstradas em mais de 50 estudos de imagem (Howes et al., 2012). Até a última década, as alterações moleculares e de circuito específicas no sistema dopaminérgico de pessoas com esquizofrenia não eram claras. A aplicação de técnicas de imagem ajudou a preencher esta lacuna do conhecimento. Pessoas com esquizofrenia também apresentam níveis elevados de dopamina nas terminações sinápticas do estriado, corroborando a hipótese de sua liberação excessiva (Kageles et al., 2010).

Numerosas regiões cerebrais projetam-se para o corpo estriado em um padrão topográfico definido. Uma teoria de longa data propõe que a hiperatividade da dopamina na esquizofrenia ocorre nas regiões límbicas. No entanto, esta hipótese não é apoiada pelos dados *in vivo*, que mostram que as maiores diferenças na síntese e liberação de dopamina em pessoas com

esquizofrenia estão no estriado dorsal, e não no estriado límbico (ventral) (McCutcheon et al., 2018). Especificamente sobre o estriado, as maiores diferenças são observadas na região associativa que recebe projeções do córtex frontal, embora uma elevação estatisticamente significativa também seja observada no estriado sensório-motor (McCutcheon et al., 2018).

Apesar dos esforços na procura de assinaturas fisiopatológicas mais claras pouco se obteve, principalmente quando comparados a outras áreas da medicina (Kageles et al., 2010). Tanto que no final do século 19 o neurologista Fred Plum chegou a afirmar que a “esquizofrenia era o cemitério para os neuropatologistas”, iniciando provavelmente aí a premissa de que a psiquiatria já nasceu 30 anos atrasada em relação a outras especialidades (Coyle et al., 2020; Howes et al., 2023).

1.5. GLUTAMATO E NOVAS EVIDÊNCIAS

A hipótese glutamatérgica para explicar a fisiopatologia da esquizofrenia teve início em um estudo publicado por Kim e colaboradores em 1980, no qual afirmaram que pacientes com o transtorno apresentavam diminuição da concentração de glutamato no líquido cefalorraquidiano (Kim et al., 1980). Como toda novidade na ciência alguns grupos reproduziram este achado (Bjerkstedt et al., 1985), outros não (Gattaz et al., 1982). Mas apesar da falta de consenso foi o suficiente para hipotetizarem o déficit glutamatérgico como precursor dos sintomas (Bressan and Pilowski, 2003).

O glutamato desempenha um papel fundamental na mediação do equilíbrio homeostático entre excitação e inibição no sistema nervoso central, desenvolvimento neuronal, neurodegeneração, plasticidade sináptica, aprendizagem e memória (Vinnakota et al., 2023). É conhecido por ser o principal neurotransmissor excitatório do cérebro e atuar tanto em receptores ionotrópicos e quanto metabotrópicos (Howes et al., 2023). Os receptores inotrópicos incluem o

ácido α -amino-3-hidroxi-5-metil-4-isoxazolpropionico (AMPA), o ácido N-metil-D-aspartato (NMDA), e o cainato. Estes receptores contêm canais iônicos que quando ativados permitem a entrada de Na^+ e K^+ favorecendo a despolarização rápida do neurônio (Willard et al., 2013). Os receptores metabotrópicos são subdivididos em três grupos: receptores do grupo I, metabotrópicos de glutamato 1 (mGluR1) e mGluR5; receptores do grupo II, mGluR2 e mGluR3; e receptores do grupo III, mGluR4, mGluR6, mGluR7 e mGluR8. Os grupos II e III são principalmente pré-sinápticos e regulam a liberação de neurotransmissores, enquanto o grupo I é principalmente pós-sináptico (38). Em condições fisiológicas, a ativação de receptores metabotrópicos produz correntes pós-sinápticas lentas. Com isso esses receptores estão presentes em todas as regiões cerebrais e são considerados os maiores moduladores de segundos mensageiros em mamíferos (Bressan and Pilowski, 2003).

Atualmente, técnicas mais modernas nos permitem aprofundar a investigação do papel do glutamato na esquizofrenia. Especificamente o desenvolvimento da espectroscopia de ressonância magnética de prótons (^1H -MRS) nos permitiu a medição in vivo do glutamato e de seu metabólito glutamina. Como os espectros de glutamato e glutamina se sobrepõem, pode ser difícil separar suas medições, de modo que o sinal combinado, denominado Glx, é frequentemente relatado. Até o momento, mais de 120 estudos foram realizados utilizando ^1H -MRS para medir esses índices glutamatérgicos em pacientes com esquizofrenia e foram resumidos em uma meta-análise de 2023 feita por Merritt e colaboradores (Merritt et al., 2023). A análise estatística encontrou níveis mais baixos de glutamato no córtex frontal medial, níveis mais elevados de glutamina no tálamo e níveis mais elevados de Glx nos gânglios da base naqueles com esquizofrenia em relação ao grupo de voluntários saudáveis. Além disso, encontraram associação positiva entre gravidade total dos sintomas e níveis de glutamato nos gânglios da base e lobo temporal (Merritt et al., 2023). Estes resultados indicam que a disfunção glutamatérgica pode estar subjacente aos sintomas e que

a relação é oposta no córtex frontal em relação aos gânglios da base e ao córtex temporal. No entanto, ainda não foi determinado se as alterações do glutamato necessariamente levam aos sintomas (Vinakkota et al., 2023).

Em contraste com o grande número de estudos de 1H-MRS sobre glutamato, poucos foram realizados levando em conta outros aspectos do sistema glutamatérgico. No entanto, foram desenvolvidos traçadores de imagem molecular para alguns receptores e outros estão em desenvolvimento (McCluskey et al., 2020). Neste sentido, o receptor NMDA tem sido uma área chave de interesse devido a capacidade de seus antagonistas induzirem sintomas de psicose (Beck et al., 2020).

Nas últimas três décadas, acumulou-se uma grande quantidade de dados pré-clínicos, indicando que alterações nos receptores NMDA levam a anormalidades comportamentais relevantes para a esquizofrenia (Javitt et al., 2022). No entanto, apenas dois estudos in vivo foram publicados investigando os receptores NMDA em pacientes com o transtorno (Pilowsky et al., 2006). Ambos os estudos encontraram evidências de menor disponibilidade de receptores NMDA no hipocampo em relação a outras regiões do cérebro em pessoas com esquizofrenia (Pilowsky et al., 2006).

1.5.1 Receptores do tipo NMDA

Os receptores NMDA são canais formados por diferentes proteínas heteroméricas chamadas subunidades NMDAR1 (GluN1), NMDAR2 (GluN2), e NMDAR3 (GluN3). Majoritariamente a estrutura do receptor é composta por duas subunidades GluN1 e duas GluN2 ou GluN3 (Paoletti et al., 2013). Eles controlam a condutância de Na⁺, K⁺ e principalmente Ca⁺ através da membrana neuronal. Quando em repouso, íons de Mg⁺ ficam ligados a um sítio dentro do canal iônico impedindo o influxo dos outros íons. Tanto é que, o canal só é ativado se 3 eventos ocorrerem

simultaneamente: 1) ligação do agonista, o neurotransmissor glutamato + 2) ligação do co-agonista (glicina ou d-serina) + 3) despolarização da membrana pós-sináptica. Somente após a ocorrência desses eventos o íon de Mg^{+} é deslocado de seu sítio permitindo o fluxo iônico. Quando ativados, os receptores NMDA agem no sentido de aumentar ainda mais a despolarização iniciada pelos receptores AMPA; fenômeno conhecido como *long term potentiation* (LTP) que é extremamente importante em processos de aprendizagem e armazenamento de memória (Bressan and Pilowski, 2003; Paoletti et al., 2013).

Existem diversos agonistas e antagonistas para os diferentes sítios de ligação dos receptores NMDA. Especificamente por possuírem sítio de ligação comum, a fenciclidina, o MK801 e a cetamina ganham destaque no estudo da esquizofrenia por sua relação com os sintomas psicóticos. Interessante ressaltar que este sítio só pode ser acessado quando o Mg^{+} foi descolado, ou seja, quanto o canal está aberto. Por isso esses fármacos são conhecidos por antagonistas não competitivos, por não competir com o glutamato em seu sítio de ligação (Bressan and Pilowski, 2003; Paoletti et al., 2013).

1.5.2. Psicose induzida por antagonistas do receptor NMDA

A fenciclidina foi desenvolvida para ser um anestésico dissociativo, mas foi amplamente utilizada como droga de abuso no coquetel conhecido como *angel dust* por suscitar sintomas psicóticos, e assim mimetizar os sintomas da esquizofrenia (Luby et al., 1959). Quando esses achados foram reproduzidos em voluntários saudáveis se formulou o modelo explicativo da esquizofrenia chamado "psicose induzida por fenciclidina". Somente após 30 anos mais tarde, Lodge e Anis foram capazes de estabelecer que o bloqueio de receptores NMDA era o mecanismo pelo qual a fenciclidina fazia seu efeito (Lodge and Anis, 1972). Posteriormente, os trabalhos apontaram que inúmeros antagonistas NMDA agindo no mesmo sítio da fenciclidina (como o

MK801 e a cetamina) ou não (CPP e CPP-ene), induziam sintomas do tipo da esquizofrenia (Olney et al., 1995).

Uma hipótese sugere que o antagonismo de receptores NMDA promove um aumento da liberação de glutamato que age em outros receptores (Moghaddam et al., 1997). Ela é corroborada pelo fato de que substâncias que inibem a liberação de glutamato, como os agonistas de receptores metabotrópicos, reverterem o efeito da fenciclidina em roedores (Moghaddam et al., 1998). Estudos clínicos também caminharam nessa direção. A lamotrigina, droga anticonvulsivante que possui este mecanismo de ação, também atenuou os efeitos psicomiméticos da cetamina (Anand et al., 2000).

1.5.3. Interação entre os sistemas dopaminérgico e glutamatérgico

Evidências atuais nos direcionam no sentido de que os dois sistemas de neurotransmissores apontados até aqui interagem entre si para desencadear os sintomas da esquizofrenia (Maksymetz et al., 2017; Vinnakota et al., 2023).

Estudo com roedores mostrou que a atividade dopaminérgica desencadeada pelo estresse é regulada por projeções glutamatérgicas provenientes do córtex pré-frontal (Talpalar et al., 2010). Outro avaliou a integridade celular através de ressonância magnética e achou uma correlação entre lesão no córtex frontal com hiporresponsividade do sistema dopaminérgico a anfetamina (Li et al., 2010). Trabalhos mais recentes corroboram esses achados ao avaliar os fluxos sanguíneos dessas regiões (Hou et al., 2017). Em conjunto, estes achados apontam que as alterações do sistema dopaminérgico observadas na esquizofrenia estão associadas primariamente as disfunções glutamatérgicas, sugerindo que antes mesmo do proposto pela hipótese clássica dopaminérgica para explicar o transtorno, ocorra o desarranjo das vias do glutamato (Li et al., 2010; Vinnakota et al., 2023).

1.5.4. Alterações GABA

Além da dopamina e glutamato outro neurotransmissor merece destaque no estudo da fisiopatologia da esquizofrenia, o GABA (Vinakkota et al., 2023). O ácido γ -aminobutírico (GABA) é o neurotransmissor inibitório mais comum no cérebro humano (56,57). Estudos post-mortem revelaram níveis mais baixos de mRNA e/ou proteína de numerosos marcadores GABA, como a descarboxilase do ácido glutâmico que sintetiza GABA e o transportador de membrana GABA 1, no córtex frontal de pacientes com esquizofrenia comparados a voluntários saudáveis (Krystal et al., 1994). Além disso, evidências de menor atividade de interneurônios GABAérgicos positivos para parvalbumina, que têm um papel fundamental na coordenação do disparo rápido de neurônios glutamatérgicos, também são relatadas em indivíduos com o transtorno (Vicini et al., 1998).

Uma meta-análise realizada em 2022 com 30 estudos de imagem 1H-MRS *in vivo* de GABA65, encontrou níveis mais baixos no córtex médio cingulado em pessoas com primeiro episódio psicótico e no córtex occipital em pessoas com esquizofrenia crônica, em comparação com indivíduos saudáveis. Entretanto, não foram observadas alterações nas demais regiões corticais frontais examinadas ou nos gânglios da base (Nakahara et al., 2022). Esses achados, juntamente com a evidência de níveis mais baixos de proteínas envolvidas na produção e recaptação de GABA, sugerem níveis mais baixos de GABA cortical na fisiopatologia da esquizofrenia (Curley et al., 2011; Howes et al., 2023). Com isso, cada vez mais esforços estão sendo realizados no sentido de performar modelos do transtorno de maneira mais fidedigna com a realidade.

1.6. MODELO DE ESQUIZOFRENIA INDUZIDA POR CETAMINA

Considerando o exposto acima, um dos modelos farmacológicos que mais se assemelham ao que acontece na esquizofrenia é o da hipofunção de receptores NMDA ocasionada por seus

antagonistas (Bressan and Pilowski, 2003). Além dos sintomas positivos (que também são induzidos por agonistas serotoninérgicos como anfetaminas e o ácido lisérgico), os antagonistas NMDA foram efetivos em mimetizar os sintomas negativos (como o embotamento afetivo e retraimento emocional), e déficits cognitivos (dificuldades na memória semântica e fluência verbal) (Krystal et al., 1994; Bressan and pilowski, 2003). Interessante ressaltar que esses sintomas não só foram suscitados em voluntários saudáveis como também pioraram quadros de pacientes com esquizofrenia (Itil et al. 1967; Malhotra et al., 1997).

Embora a hipofunção do receptor NMDA tenha sido associada aos sintomas da esquizofrenia, os mecanismos subjacentes precisos ainda não são claros. Como mencionado no item 1.5. a hipótese mais aceitável seria uma disfunção dos receptores NMDA nos interneurônios GABAérgicos, levando ao distúrbio glutamatérgico e dopaminérgico, que culminaria com o aparecimento dos sintomas (Cohen et al., 2015; Vinakkota et al., 2023).

Especificamente os interneurônios GABAérgicos são estimulados pela ativação dos receptores NMDA que, por sua vez, fazem sinapses com células piramidais glutamatérgicas excitatórias em um ciclo de *feedback* negativo. Os interneurônios GABA conectam-se dessa maneira a centenas de células piramidais, permitindo-lhes coordenar a atividade da rede sincronizada em todo o cérebro. A atividade dessas células piramidais glutamatérgicas, por sua vez, impulsiona os neurônios dopaminérgicos do estriado (Nakao et al., 2019).

Alinhados com estes achados, estudos realizados em humanos saudáveis e em modelos de roedores mostraram que a administração de antagonistas NMDA resultaram de forma contra intuitiva na hiperatividade dos neurônios piramidais corticais e na liberação aumentada de glutamato (Homayoun et al., 2007). Isto é corroborado por dados de imagens cerebrais mostrando que a administração de um antagonista NMDA resulta em excitação com conseqüente diminuição

do disparo coordenado no córtex pré-frontal, o que está ligado a déficits cognitivos (Jackson et al., 2004; Vollenweider et al., 1997; Duncan et al., 1998).

O estado hiperdopaminérgico do estriado tem sido associado aos sintomas positivos da esquizofrenia. Desta forma, a hipofunção dos receptores NMDA pode estar resultando em um desequilíbrio excitatório/inibitório (E/I) cortical, afetando a capacidade dos interneurônios de sincronizar o disparo através das redes neurais e conduzindo as mudanças no sistema dopaminérgico, culminando em última análise nos sintomas clínicos de esquizofrenia (Nakazawa et al., 2020; Homayoun et al., 2007).

Portanto, considerando o exposto acima, um dos fármacos mais utilizados como modelo de esquizofrenia é a cetamina (Bialon and Wasic, 2022).

1.6.1. Cetamina

A cetamina é um composto derivado da fenciclidina e amplamente utilizada como agente anestésico na medicina humana e veterinária (Rowland, 2005; Bialon and Wasic, 2022). Sua história com a esquizofrenia começou quando alguns autores relataram deterioração do estado mental, após uma injeção aguda, em pacientes acometidos pelo transtorno (Malhotra et al., 1997; Lahti, et al., 2001). Além disso, quando injetada em voluntários saudáveis também produziu sintomas do tipo da esquizofrenia (Beck et al., 2020). Com essas observações em humanos foi a vez de testar em animais, iniciando assim a trajetória da cetamina como modelo de esquizofrenia não só em roedores, mas também em peixes (Riehl et al., 2011) e em primatas (Blackman et al., 2013). Estudos clínicos e pré-clínicos relataram que as injeções do anestésico levam a comprometimentos da memória (Ranganathan et al., 2017; Roussy et al., 2021), além de provocar comportamento do tipo ansioso (Wasik et al., 2019; Azimi et al., 2022), socialmente alterado (Xu et al., 2019), hiperlocomoção (Sedky et al., 2021) e comprometimento do controle sensório-motor (Fujikawa et

al., 2021). Com isso acredita-se que o modelo da esquizofrenia induzida por cetamina seja um dos mais adequados para estudar os sintomas positivos, negativos e cognitivos; distúrbio de neurotransmissores; e outras características do transtorno (Frohlich et al., 2014).

Descobriu-se que o tratamento subcrônico com cetamina diminui o número de células positivas para BDNF no córtex cingulado posterior (Xu et al., 2019), córtex frontal, hipocampo e estriado (Ben-Azu et al., 2018) e os níveis de proteína BDNF na amígdala, hipocampo, córtex frontal e corpo estriado (Canever, et al., 2018; de Araújo et al., 2021). Além disso, as injeções de cetamina em animais causaram distúrbios em outros fatores neurotróficos, como NGF ou neurotrofina-3 (NT-3) (Becker et al., 2008). A administração do fármaco também foi responsável pelo desequilíbrio inflamatório de citocinas em modelos animais de esquizofrenia. Alguns autores demonstraram níveis aumentados de IL-6 pró-inflamatória no hipocampo e tecido cerebral inteiro em camundongos (de Araújo et al., 2021) e níveis aumentados de mRNA de IL-6, IL-1beta e TNF-alfa no tecido do hipocampo após injeção aguda [88]. Estes fatores contribuem com o valor de constructo para modelos animais de doenças degenerativas, como é o caso da esquizofrenia (Li et al., 2017; Bialon and Wasic, 2022).

Os outros efeitos produzidos pela administração de cetamina incluem deficiências nos neurônios GABAérgicos. Fujikawa e colaboradores mostraram expressão reduzida de interneurônios PV+ na região CA1 do hipocampo em camundongos após o tratamento com cetamina (Fujikawa et al., 2021). Outro estudo apresentou uma densidade mais baixa de internêuronios GABAérgicos nas regiões CA1-CA3 e no giro denteado (Pérez et al., 2019). Que é outro fator que se relaciona com a fisiopatologia do transtorno (Bialon and Wasic, 2022).

1.6.2. Vantagens e limitações do modelo de esquizofrenia com cetamina

A geração de um modelo animal de esquizofrenia com cetamina é um processo relativamente simples, barato, rápido e baseia-se apenas em injeções intraperitoneais do fármaco. Como citado anteriormente o anestésico mimetiza fatores comportamentais e moleculares do transtorno (Howes et al., 2023). Entretanto, o modelo apresenta limitações também. Pesquisadores utilizam diferentes doses e regimes de administração da cetamina; assim, às vezes obtêm resultados divergentes (por exemplo, em algumas publicações, a cetamina melhora a memória; em outras faz justamente o contrário), e os resultados obtidos podem ser mal interpretados. Além disso este modelo fornece valor limitado na compreensão da etiopatologia da esquizofrenia (Howes et al., 2023; Vinnakota et al., 2023).

A cetamina é composta por uma mistura racêmica; logo, existem dois isômeros ópticos: R (-) e S (+). Esses dois enantiômeros foram purificados usando o método de recristalização, então instituiu-se cetamina como mistura racêmica, arcetamina como apenas o isômero R (-) e escetamina com o S(+) (Hashimoto, 2019; Zhang et al., 2014). A escetamina possui mais afinidade pelo receptor NMDA e recentemente foi aprovada pelo FDA como tratamento para pacientes com depressão refratária e/ou ideação suicida (Zhang et al., 2022). Estudos sugerem que a arcetamina produz um efeito do tipo antidepressivo mais robusto em modelos animais enquanto a escetamina mimetiza melhor os sintomas do tipo psicóticos (Hashimoto, 2020).

Acredita-se que a busca por modelos de esquizofrenia mais fidedignos seria a chave para desenvolver tratamentos mais eficazes (Coyle et al., 2020; Howes et al., 2023).

1.7. TRATAMENTOS ATUAIS

Os agentes antipsicóticos em utilização na clínica incluem as fenotiazinas e compostos estruturalmente semelhantes, tais como tioxantenos, benzepinas, butirofenonas, e difenilbutilpiperidinas. Estes medicamentos são geralmente classificados em agentes típicos e

atípicos, com base em seu mecanismo de ação e nos riscos relativos de efeitos adversos motores e metabólicos. Eles também podem ser classificados como agentes antipsicóticos de primeira ou segunda geração (Howes et al., 2023).

Os antipsicóticos típicos ou de primeira geração possuem como principal mecanismo de ação o bloqueio de receptores dopaminérgicos, mais especificamente D2 e D3. Os primeiros a serem introduzidos na clínica foram as fenotiazinas. Atualmente, os antipsicóticos de primeira geração em uso clínico nos Estados Unidos incluem a clorpromazina (Thorazine: 1957, o protótipo inicial de antipsicótico), flufenazina (Prolixin: 1972), perfenazina (Trilafon: 1957), proclorperazina (Compazine: 1956, usado principalmente como terapia de náusea em vez de psicose), tioridazina (Mellaril: 1978) e trifluoperazina (Stelazine: 1959). E ainda, o haloperidol (Haldol: 1967), loxapina (Loxitane: 1976), molindona (Moban: 1974) e pimozida (Orap: 1984, usada principalmente para a síndrome de Tourette). O lítio também é frequentemente discutido no contexto das terapias antipsicóticas, embora a sua principal utilização seja para a estabilização da doença bipolar e seu mecanismo de ação mais obscuro (Correl et al., 2022; Howes et al., 2023).

Em contrapartida os fármacos mais modernos apresentam mecanismos mais abrangentes e são classificados como atípicos ou de segunda geração. Além da interação fraca com os receptores dopaminérgicos ainda bloqueiam receptores serotoninérgicos 5HT2A e funcionam como agonistas de receptores 5HT1A. Devido a essa característica os antipsicóticos atípicos são medicamentos que apresentam menos efeitos adversos extrapiramidais, todavia seu efeito terapêutico não é unânime (Chow et al., 2023). Atualmente, os antipsicóticos de segunda geração em uso na clínica incluem o aripiprazol (*Abilify*: 2002), asenapina (*Saphris*: 2007), brexpiprazol (*Rexulti*: 2015), cariprazina (*Vraylar*: 2016), clozapina (*Clozaril*: 1975-79, 1989), iloperidona (*Fanapt*: 2010), lurasidona (*Latuda*: 2010), olanzapina (*Zyprexa*: 1996), paliperidona (*Invega*: 2006), pimavanserina (*Nuplazid*: 2016), quetiapina (*Seroquel*: 1997), risperidona (*Risperdal*: 1993) e

ziprasidona (*Geodon*: 2001). Alguns desses fármacos também são usados para tratar doenças bipolares e depressão grave (Correl et al., 2022; Chow et al., 2023; Howes et al., 2023).

1.7.1. Efeitos adversos e a necessidade de novos tratamentos

A eficácia clínica dos antipsicóticos atuais é extremamente limitada devido não só a abrangência deficitária dos sintomas, mas também a uma série de potenciais efeitos adversos (Chow et al., 2023). A divisão tradicional é que, para os antipsicóticos de primeira geração, a ocupação dos receptores de dopamina nas vias nigrostriatais está associada a efeitos músculo-esqueléticos, enquanto o perfil multirreceptor dos antipsicóticos de segunda geração está associado a efeitos metabólicos. Uma série de outros efeitos potenciais, desde sintomas cardíacos até hiperprolactinemia, podem se fazer presente nos indivíduos. Fatores como ação anticolinérgica e anti-histaminérgica também demonstraram influenciar os perfis de efeitos adversos dos antipsicóticos (Howes et al., 2023). No entanto, em contraste com a investigação dos perfis de eficácia, os efeitos adversos dos antipsicóticos não são relatados de forma contundente para se estabelecer guidelines robustos no âmbito da estrutura “Dose, Tempo e Suscetibilidade” (Correl et al., 2022).

Devido ao exposto acima a procura por tratamentos mais abrangentes e com menor potencial de efeitos adversos se faz urgente nas pesquisas da esquizofrenia, neste cenário surge a investigação de fármacos com mecanismos de ação diferentes dos antipsicóticos atuais, como é o caso do canabidiol e do nitroprussiato de sódio (Crippa et al., 2015).

1.8.CANABIDIOL

1.8.1. Um pouco de história

O canabidiol (CBD) foi isolado pela primeira vez na década de 1940 por Adams e colaboradores (Adams et al., 1940), mas só começou a receber atenção e ter sua exata estrutura química elucidada em 1963, pelo grupo do professor Raphael Mechoulam da Universidade de Israel (Mechoulam et al. 1963). No início da década de 1970 vários estudos reportavam que o CBD não reproduzia os efeitos psicotomiméticos da cannabis sativa o que levou a crença do CBD ser um canabinóide inativo. Isso só foi de fato combatido quando o grupo brasileiro do professor Elisaldo Carlini realizou diversos estudos e comprovou que o CBD possuía seus próprios efeitos (Zuardi, 2008; Crippa et al., 2018).

Estudos posteriores sobre a interação entre canabinóides mostravam que o CBD bloqueava ou potenciava os efeitos do THC em testes em animais, dependendo da proporção e da relação de dose entre os dois (Takahashi et al., 1970; Zuardi et al., 1984). Estudando a interação entre o CBD e o tetrahydrocannabinol (THC) em humanos saudáveis, altas doses orais de THC provocavam ansiedade e sintomas psicóticos, que eram atenuados quando o CBD era administrado junto (Zuardi et al., 1982). Estes resultados contribuíram para apoiar a associação dos dois canabinóides no SativexR (*GW-Pharm*, Reino Unido), um medicamento utilizado mundialmente para o tratamento da dor e espasticidade em condições como a esclerose múltipla. Além disso, estas observações contribuíram para a compreensão dos efeitos distintos da *cannabis sativa* em diferentes populações, explicados pelas concentrações variadas dos constituintes da planta. Ao mesmo tempo, estas descobertas sugeriam que o CBD poderia ter propriedades ansiolíticas e antipsicóticas e deram origem a linhas de investigação que continuam até hoje (Crippa et al., 2018).

1.8.2. Introdução

A planta *Cannabis sativa* contém mais de 100 compostos químicos que partilham uma estrutura química semelhante, conhecidos como canabinóides. O principal composto psicoativo

da cannabis é o THC, responsável pelos principais efeitos associados ao uso da planta. Nosso grupo se concentrou em estudar os efeitos do CBD, justamente pelo seu potencial terapêutico e por não reproduzir os efeitos psicomiméticos (Carlini, 2010). Então, desde a década de 1970 publicamos uma série de artigos científicos mostrando os potenciais efeitos terapêuticos do CBD em diferentes modelos animais de distúrbios neuropsiquiátricos, e em ensaios clínicos com humanos. Fomos os primeiros a demonstrar os efeitos ansiolíticos e antipsicóticos do CBD em animais, nas décadas de 1970 e 1980, e mais tarde em humanos, com resultados bastante promissores (Zuardi, 2008). Além da ansiedade e da psicose, foram realizadas pesquisas básicas e clínicas sobre outras possibilidades do CBD. Com isso, análogos sintéticos patenteáveis do CBD com forte potencial de transferência de conhecimento para o setor produtivo foram recentemente desenvolvidos (Crippa et al., 2018).

1.8.3. Estudos em humanos

Especificamente a década de 1990 foi marcada pela entrada do CBD no estudo da esquizofrenia. Tudo começou com um relato de caso do professor Antônio Zuardi sobre uma jovem com o transtorno que apresentava efeitos adversos graves ao ser tratada com os antipsicóticos disponíveis. Então durante 4 semanas ela foi tratada com CBD e teve uma redução significativa de sintomas positivos e negativos (Zuardi et al., 1995). Após esse feito, novo ensaio com três pacientes do sexo masculino refratários ao tratamento foi realizado, porém apenas um apresentou uma melhora parcial (Zuardi et al., 2006).

Nenhum dos pacientes apresentou efeitos adversos durante o tratamento com CBD (76). Mais tarde, três ensaios clínicos duplo-cegos e controlados que investigaram a eficácia e tolerabilidade do CBD em pacientes com esquizofrenia confirmaram as nossas descobertas preliminares. Um estudo comparou os efeitos do CBD com a amisulprida em pacientes com esquizofrenia tratados durante 4 semanas (Hallak et al., 2010). Ambos os tratamentos reduziram

significativamente os sintomas psicóticos, sem diferenças entre eles. No entanto, o CBD induziu efeitos adversos mais brandos comparado ao antipsicótico. Os efeitos do CBD também foram investigados em pacientes com primeiro episódio de psicose em um ensaio cruzado controlado por placebo (Leweke et al., 2011). O CBD diminuiu significativamente os sintomas psicóticos após 2 semanas em comparação com o valor basal, embora as diferenças em relação ao placebo não tenham alcançado significância estatística (Crippa et al., 2018).

A visão de que o CBD poderia ter efeitos antipsicóticos foi ainda apoiada pelos nossos estudos em seres humanos saudáveis com psicose induzida artificialmente (Hallak et al., 2011, Martin-Santos et al., 2012). Num ensaio duplo-cego, controlado por placebo, o CBD demonstrou atenuar os sintomas de despersonalização induzidos pela cetamina (Hallak et al., 2011). Este efeito nos sintomas dissociativos também levantou hipóteses de potenciais utilizações terapêuticas do CBD em condições como transtorno de estresse pós-traumático (TEPT), intoxicação por cannabis e alguns transtornos de personalidade.

Mais recentemente, pacientes com esquizofrenia foram randomizados para receber CBD ou placebo durante 6 semanas, adicionado aos seus medicamentos antipsicóticos existentes (McGuire et al., 2018). Após o tratamento, o grupo CBD apresentou menores escores psicóticos e melhora dos sintomas cognitivos. Os efeitos adversos do dois foram equivalentes entre os grupos. Com isso, uma vez que os efeitos antipsicóticos do CBD não parecem depender do antagonismo dos receptores de dopamina, este composto pode de fato representar uma nova classe de tratamento para os transtornos psicóticos.

1.8.4. Estudos em animais

Utilizando modelos animais o professor Zuardi e colaboradores constataram que o CBD diminuiu o comportamento do tipo psicótico induzido por agonistas dopaminérgicos, efeito

comparado ao haloperidol (Zuardi et al., 1991). Entretanto, diferente do haloperidol o CBD não apresentou os efeitos adversos motores, apenas aumentou os níveis de prolactina quando a dose era superior a 120 mg/kg. Este perfil terapêutico impulsionou sua comparação com os antipsicóticos atípicos (Zuardi et al., 1991).

Além disso, nosso grupo também mostrou que o CBD foi capaz de diminuir a hiperlocomoção e o comprometimento da inibição pré-pulso induzidos por anfetamina (Moreira et al., 2005; Pedrazzi et al., 2015). Posteriormente esse perfil foi replicado nos modelos de cetamina e MK801 (Moreira et al., 2005; Gomes et al., 2015). Mais uma vez seu padrão terapêutico foi comparado ao da clozapina. Interessante ressaltar que as doses para o CBD produzir efeito do tipo antipsicótico são maiores que as doses necessárias para o efeito ansiolítico (Crippa et al., 2018).

O canabinóide foi testado não apenas como tratamento, mas também como prevenção. Nosso grupo publicou trabalhos indicando que o pré tratamento peripuberal com o CBD inibiu o desenvolvimento da hiperlocomoção na idade adulta no modelo de poli I:C; indicando uma característica duradoura que se apresenta como ferramenta promissora no estudo de indivíduos UHR e em fases prodrômicas (Peres et al., 2016). Interessante ressaltar que em nenhum estudo o CBD apresentou efeito adverso motor e nem de catalepsia (Crippa et al., 2018).

1.9. NITROPRUSSIATO DE SÓDIO

Já o nitroprussiato de sódio (NPS) quimicamente pertence a família dos prussiatos. Consiste em um núcleo de ferro cercado por cinco moléculas de íons cianeto e uma molécula de íon nitrosônio (NO⁺) (Miller and Megson, 2017). Devido a essa característica acaba sendo um liberador de óxido nítrico como resultado da sua metabolização, o que confere sua função terapêutica de potente vasodilatador. Por essas propriedades o NPS é amplamente utilizado como medicamento para o tratamento de hipertensão e insuficiência cardíaca (Scatena et al., 2010). Além disso, uma

série de estudos apontou o perfil antioxidante do NPS. Assim, a sua utilidade como potencial agente neuroprotetor para acidente vascular cerebral e doenças neurodegenerativas, como Parkinson ou doença de Alzheimer, também está sob avaliação (Godinez-Rubi et al., 2013).

1.9.1 Óxido nítrico e esquizofrenia

O óxido nítrico é um gás solúvel, instável, altamente difusível e com meia vida muito curta (em torno de 4 segundos). Ele é formado pela conversão de L-arginina em L-citrulina por uma enzima dependente de cálcio, a calmodulina NO sintase (Garthwaite et al., 1988). A ativação dessa cascata química produz monofosfato de guanosina cíclico (cGMP) que, por sua vez, ativa proteínas quinase dependente de cGMP (PKG), fosforila diversas outras proteínas e participa de inúmeros processos fisiológicos [14]. A ação do NO é encerrada pela enzima fosfodiesterase que neutraliza o cGMP (Kleppisch, 2009).

O óxido nítrico está envolvido em processos complexos que incluem desde a imunidade celular (Hibbs et al., 1988), tônus vascular (Palmer et al., 1987), até a neurotransmissão (Garthwaite, 1988). No que diz respeito ao sistema nervoso central, está bem documentado que este gás desempenha um papel crucial na plasticidade sináptica, aprendizagem e memória (Pitsikas, 2015) e parece exercer uma ação moduladora na liberação de vários neurotransmissores como acetilcolina, GABA, glutamato, dopamina e serotonina (Trabace et al., 2004; Zoupa and Pitsikas, 2021). Além disso, evidências recentes apontam que baixas concentrações de óxido nítrico podem conferir neuroproteção e regular a sinalização fisiológica (como neurotransmissão ou vasodilatação), ao passo que níveis elevados promovem efeitos imunológicos/inflamatórios que são neurotóxicos (Calabrese et al., 2007).

Com tantos pontos em comum com protagonistas da fisiopatologia da esquizofrenia, estudos em humanos e em animais vêm investigando o potencial antipsicótico do NPS (Zoupa and Pitsikas, 2021).

1.9.2. Estudos em humanos

Nossos trabalhos foram pioneiros no estudo do NPS enquanto antipsicótico. Mais especificamente em 2013 publicamos estudo randomizado, duplo-cego, controlado por placebo, em 20 pacientes com esquizofrenia que estavam nos primeiros 5 anos do transtorno. Eles tinham idades em torno de 19 e 40 anos. Observamos que uma única infusão de NPS (0,5 mg/min) durante 4 horas foi o suficiente para atenuar os sintomas positivos, negativos, de ansiedade e de depressão que os pacientes apresentavam. Curiosamente esses efeitos duraram até 4 semanas após a administração do fármaco (Hallak et al., 2013). Outro estudo subsequente confirmou a eficácia do NPS. Quando administrado no mesmo esquema de tratamento do estudo 1, reduziu distúrbios cognitivos, como atenção seletiva e memória de trabalho; algo extremamente relevante nos estudos de tratamentos da esquizofrenia por ser um grupo de sintomas que os antipsicóticos clássicos não englobam (Maia-de-Oliveira et al., 2015).

Apesar dos resultados promissores, trabalhos subsequentes falharam em reproduzir esses achados. O NPS foi utilizado no mesmo regime de tratamento (Stone et al., 2016), aplicado em duplicata com 1 semana de intervalo (Wang et al., 2018), de forma aguda (Brown et al., 2019) e de forma repetida (Adelino et al., 2021), mas sem os resultados em sintomas negativos e déficits cognitivos observados nos estudos anteriores.

Essa inconstância de resultados parece estar ligada a fatores como tempo e curso do transtorno, presença de sintomas negativos significativos e o fato dos pacientes serem fumantes ou não. Pois nos primeiros estudos (Hallak et al., 2013; Maia-de-Oliveira et al., 2015) os pacientes

possuíam pouco tempo de transtorno, apresentavam bastante sintomas negativos e não fumavam. Já nos estudos subsequentes o oposto ocorreu (Stone et al., 2018; Wang et al., 2018; Brown et al., 2019; Adelino et al., 2021). Importante ressaltar o papel da nicotina como fator crítico da ineficácia do NPS, uma vez que esta interfere no metabolismo de óxido nítrico (Kocygit et al., 2018). Ademais, outro fator digno de nota é que em todos os estudos clínicos mencionados, o NPS foi bem tolerado e não foram relatados efeitos secundários adversos (Hallak et al., 2013; Maia-de-Oliveira et al., 2015; Stone et al., 2018; Wang et al., 2018; Brown et al., 2019; Adelino et al., 2021).

1.9.3. Estudos em animais

Trabalhos pré-clínicos mostraram que o tratamento e o pré tratamento intraperitoneal com o NPS, de forma aguda ou subcrônica, reduziu a hiperlocomoção, expressão de c-fos (marcador de atividade neuronal), déficits de memória de reconhecimento e prejuízos na atenção ocasionadas pelos modelos de esquizofrenia com fenciclidina (Bujas-Bobanovic et al., 2000), apomorfina (agonista dopaminérgico) (Gourgiotiset al., 2012), anfetamina (Issy et al., 2014), cetamina (Trevlopoulou et al., 2016) e MK801 (Hurtubise et al., 2017). Mas mais estudos são necessários para confirmar e investigar esse efeito do tipo antipsicótico.

2. OBJETIVOS

2.1. Objetivo geral

O objetivo do presente trabalho foi investigar a influência do sexo nos modelos animais da cetamina e na eficácia terapêutica do CBD e do NPS em diferentes regimes de administração.

2.2. Objetivos específicos:

I- Avaliar o efeito na adolescência do pré tratamento crônico com CBD e NPS durante fase de neurodesenvolvimento, em ratos machos e fêmeas (artigo 1).

II- Investigar as diferenças comportamentais após administração de cetamina racêmica e esketamina em ratos adolescentes machos e fêmeas (artigo 2).

III- Avaliar o efeito na adolescência do tratamento agudo com CBD e NPS em ratos machos e fêmeas (short communication, artigo 3).

3. ARTIGOS

3.1. ARTIGO 1: **Eficácia profilática do canabidiol e do nitroprussiato de sódio em um modelo de esquizofrenia com cetamina: efeitos dependentes do sexo em déficits cognitivos e sintomas do tipo-positivo**

Os tratamentos atuais da esquizofrenia (SZ) permanecem insatisfatórios em abranger todos os sintomas do transtorno. Além disso, os antipsicóticos produzem vários efeitos adversos e apresentam respostas dependentes do sexo. Recentemente, novas estratégias foram propostas para prevenir o aparecimento de sintomas, como o canabidiol (CBD) e o nitroprussiato de sódio (NPS). No entanto, os efeitos destes compostos dependentes do sexo ainda não foi estudado. Nós investigamos a eficácia profilática do CBD e do NPS em comportamentos positivos e negativos, bem como deficiências cognitivas no modelo de esquizofrenia com cetamina S+(do inglês, ketamina - KET) em ambos os sexos. Especificamente, pré-tratamos ratos Wistar com CBD e NPS (sozinhos e em combinação) durante 21 dias durante o desenvolvimento do cérebro (12 a 32 dias pós-natais). Após um intervalo de 10 dias, induzimos deficiências semelhantes à esquizofrenia por meio de 5 injeções diárias de KET. As alterações comportamentais foram avaliadas pela avaliação da locomoção no teste de campo aberto (CA), teste de preferência de sacarose (PS) e teste de reconhecimento de novos objetos (RO). Mostramos pela primeira vez os efeitos sexo-dependentes destes fármacos em modelo de SZ. As ratas fêmeas foram mais sensíveis que os ratos machos à hiperlocomoção induzida por KET e aos comprometimentos da memória a longo prazo no RO; enquanto nos ratos, o tratamento com KET diminuiu o consumo de sacarose e reduziu o desempenho da memória de curto prazo no RO. O pré-tratamento isolado com CBD ou NPS por si só teve eficácia profilática limitada. No entanto, a combinação CBD+NPS diminuiu a

hiperlocomoção e preveniu os défices encontrado no RO em ambos os sexos. Notavelmente, a análise multivariada indicou que a eficácia profilática do CBD+NPS é superior nas ratas. Além disso, a clusterização não supervisionada indicou fenótipos de comportamento distintos em ambos os sexos.

Conclusão: Nossos dados fornecem a primeira evidência pré-clínica da eficácia profilática dependente do sexo do CBD e NPS em um modelo animal de esquizofrenia.

Palavras-chave: esquizofrenia, sexo, cetamina, profilaxia, canabidiol, nitroprussiato de sódio

ESTE ARTIGO ESTÁ SUBMETIDO AO PERIÓDICO *CNS Neuroscience & Therapeutics* (fator de impacto: 5.5). COMPROVANTE DE SUBMISSÃO APÓS MATERIAL SUPLEMENTAR

TITLE: Prophylactic Efficacy of Cannabidiol and Sodium Nitroprusside in a Ketamine-Model of Schizophrenia: Sex-Dependent Effects on Positive-Like and Cognitive Impairments

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ABSTRACT

Introduction: The current schizophrenia (SZ) treatments remain unsatisfactory for treating all the disorder symptoms. Moreover, antipsychotics produce several adverse effects and show sex-dependent responses. Recently, new strategies have been proposed to prevent the emergence of symptoms, such as cannabidiol (CBD) and sodium nitroprusside (SNP). Nevertheless, the sex-dependent effects of these compounds are poorly addressed.

Materials and methods: We investigated the prophylactic efficacy of CBD and SNP in positive- and negative-like behaviors, as well as cognitive impairments in a ketamine (KET) rodent model of schizophrenia in both sexes. Specifically, we pretreated Wistar rats with CBD and SNP (alone or in combination) for 21 days during brain development (12 to 32 postnatal days). After an interval of 10 days, we induced schizophrenia-like impairments by KET injections. Behavioral alterations were assessed by the evaluation of locomotion in the open field test (OFT), sucrose preference test (SPT), and novel object recognition (NOR) test.

Results: We show for the first time KET sex-dependent effects. Females were more sensitive than males to KET-induced hyperlocomotion and NOR long-term memory impairments, while in males, KET treatment decreased sucrose consumption and reduced NOR short-term memory performance. CBD or SNP pretreatment alone had limited prophylactic efficacy. However, the CBD-SNP combination decreased hyperlocomotion and prevented NOR deficits in both sexes. Notably, multivariate analysis associated with machine learning classification indicated that CBD-SNP prophylactic efficacy is superior in females. Also, unsupervised clusterization indicated distinct behavior phenotypes in both sexes.

Conclusion: Our data provide the first preclinical evidence of CBD-SNP sex-dependent prophylactic efficacy in an animal model of schizophrenia.

Keywords: schizophrenia, sex, ketamine, prophylactic, cannabidiol, sodium nitroprusside

INTRODUCTION

Schizophrenia (SZ) is a debilitating mental disorder characterized by positive and negative symptoms, as well as cognitive deficits^{1,2}. Even though antipsychotic medication ameliorates mainly positive symptoms, antipsychotics have poor efficacy in treating cognitive impairments and generally exhibit adverse effects^{3,4}. In addition, the symptoms of schizophrenia and the efficiency of antipsychotics are sex-dependent; men have more negative symptoms and high rates of treatment non-responders, while women have a higher occurrence of positive symptoms and are more susceptible to developing side effects with antipsychotics^{5,6}. Interestingly, healthy women volunteers are also more affected by KET-induced psychotic symptoms than men^{7,8}. However, few studies have considered sex differences in the effectiveness of antipsychotics^{9,10}.

Recently, pharmacological intervention during brain development has been proposed as a new strategy to prevent SZ symptoms¹¹. These approaches comprehend SZ as a neurodevelopmental disorder with late symptoms potentially preventable^{12,13}. Indeed, subjects with high clinical risk for psychosis, when treated during the prodromal stage with atypical antipsychotics, had decreased transition rates to psychosis and improved positive symptoms^{14,15}. However, earlier brain development stages could be an even more interesting time window to prevent later symptoms of SZ^{16,17}. Unfortunately, early antipsychotic treatment is discouraged due to tolerability and safety issues^{17,18}. Besides, there still a lack of evidence that pharmacological interventions during brain development prevent later SZ symptoms.

In rodents, the KET model is typically used as a translational pharmacological model of SZ^{19,20}. Subchronic KET injections produce positive-like symptoms and cognitive deficits, such as hyperlocomotion and impairments in the novel object recognition (NOR) task respectively²¹⁻²⁴. The KET model is also sensitive to typical and atypical antipsychotics, making it a valuable tool for investigating the antipsychotic potential of new drugs²⁵. Nevertheless, similar to clinical studies, there

is a paucity of preclinical work with females, although some evidence indicates that female rats are more sensitive than males to KET effects^{8,26}.

Cannabidiol (CBD) and sodium nitroprusside (SNP) have been proposed as new pharmacological treatments during the development of SZ^{27,28}. CBD is the major non-psychotomimetic compound of *Cannabis sativa* and presents safe antipsychotic effects in humans and rodents²⁹. In patients with high clinical risk for psychosis, CBD partially normalizes blood oxygen level-dependent (BOLD) signals in mesolimbic structures³⁰. Furthermore, chronic CBD treatment during adolescence decreases hyperlocomotion and memory impairments in SZ animal models^{31–33}. SNP is a donor of nitric oxide capable of reverting positive symptoms in humans and hyperlocomotion in rodents^{34–36}. During adolescence, SNP treatment prevented the development of SZ-like behaviors, such as hyperlocomotion, social interaction deficits, and memory impairments in an animal model³⁷. Interestingly, in clinical studies, it seems that the efficacy of CBD and SNP as antipsychotics is higher in younger subjects^{38,39}. Moreover, due to their different mechanisms of action, the combination of the two drugs becomes an interesting alternative to be investigated²⁷. However, the possible sex-specific prophylactic effects of CBD and SNP, alone or combined, during development in SZ animal models are still unknown.

In the present study, we evaluated whether pretreatment with CBD and SNP, alone or in combination, would decrease the emergence of SZ-like behavior in the KET model. To test our hypothesis, rats were pretreated with CBD and/or SNP during development, and their behaviors were evaluated at adulthood after KET-induced impairments. We analyzed the ambulatory and exploratory activity in the OFT, the anhedonia state using the SPT and the recognition memory in the NOR test. All the experiments were performed with male and female rats to investigate the sex-dependent effects of the drugs.

MATERIALS AND METHODS

Animals

Male (n = 75) and female (n = 71) Wistar rats were delivered to the local animal facility on a postnatal day (P)10, and the treatments started at P12. On P21, the rats were weaned and housed. The pups stayed with their mother for breastfeeding during the 21 days of pretreatment, then were separated into four animals per cage until the beginning of the experiments. Animals were housed in a 12-h light-dark cycle (lights on at 7 a.m.) at a constant temperature of the colony room of 21±2°C. Water and food were provided *ad libitum*. All the procedures followed the National Council for the Control of Animal Experimentation guidelines and were approved by the local ethics committee (Ribeirão Preto Medical School, University of São Paulo; protocol: 62/2016).

Experimental Design

The experimental design was the same for both sexes, and is summarized in Figure 1-A. First, P12 rats were submitted to a pretreatment once daily for 21 days with vehicle (VEH), CBD, SNP, or both (CBD-SNP). After a 10-day interval, the animals were submitted to saline 0.9% (SAL) or KET treatment once daily for 5 days, followed by the behavioral tests.

Drugs

CBD (BSPG-Pharm, United Kingdom; 1 mg/kg i.p.) was diluted in Tween 80 and in saline (2% of Tween 80 and 98% of NaCl 0.9% solution). SNP (*Cristalia*, Brazil; 2.5 mg/kg i.p.) was freshly dissolved in 5% glucose. All the pretreatment solutions were injected in a volume of 1 ml/kg/day. VEH pretreatment consisted of saline (0.9% w/v, *Isofarma*, *Eusébio*, Brazil) administered for 21 days, except every 5 days, when we administered a glucose injection (5% w/v, *Isofarma*, *Eusébio*, Brazil) instead of saline. During the treatment, subjects received saline or KET (*Cristalia*, Brazil; 30 mg/kg i.p.) diluted in 0.9% NaCl in a volume of 10 ml/kg/day. The care and doses chosen were based on previous studies^{31,36,39,40}.

Behavioral tests

Open Field Test

Locomotor activity was evaluated in the OFT on the last day of treatment. Thirty minutes after the last SAL or KET injection, rats were placed in the OFT apparatus center for 20 min (Figure 1-B). The OFT was performed and analyzed as described previously by our group³⁴.

Sucrose Preference Test

The SPT was conducted over 3 days (P48-P50) (Figure 1-C) as previously published by our group³⁴. The subjects were placed alone in cages with free access to one bottle of water and another one of 1% w/v sucrose solution. SPT was measured as the ratio of sucrose consumed to total fluid consumed and values converted to a percentage.

Novel Object Recognition Test

The NOR test was also based on a protocol performed previously by our group^{33,41} and was applied on the same days as the SPT (Figure 1-D). Briefly, the NOR protocol consisted of three phases: habituation on the first day (P48), training and test 1 on day 2 (P49), and test 2 on the third day (P50). In the training session, rats were allowed to explore two equal objects. Thirty minutes (test 1) and 24 H after (test 2) the training session, a new object replaced one of the previous objects and the animals were allowed to explore them. In the NOR task, object exploration was defined by the time spent sniffing or touching the object (t), and NOR performance was evaluated by the Discrimination Index (DI)⁴². Based on previous work which considered individual differences in the NOR protocol^{43,44}, DI was calculated as: $DI = (t_{test} - t_{habit})$, where $t_{test} = (t_{novel} - t_{familiar} / t_{total time})$ and $t_{habituation} = (t_{object A} - t_{object A'} / t_{total time})$.

Data analysis

Radar chart

All behavioral measures were used to compose radar charts, which were constructed performing a Min-Max normalization between each behavioral variable for all the experimental groups: $Z = [x - \min(x) / \max(x) - \min(x)]$. Radar charts are useful for presenting multivariate data and assessing the profile of treatments, both in clinical or basic research⁴⁵⁻⁴⁷.

Multivariate analysis

Multivariate analysis was performed using Matlab (2019, The MathWorks Inc.), Scikit-learn⁴⁸ and Yellow Brick⁴⁹ packages in Python. Pearson's correlation was used to infer a bivariate linear relationship between behavioral attributes. For correlograms, the behavioral features were sorted by hierarchical agglomerative clustering using the average linkage method and correlations as metrics. Specifically, we eliminated multivariate outliers for correlation analysis that could indicate spurious correlations by applying the Local Outlier Factor (LOF)⁵⁰. The LOF indicated two outliers for the female group, while in the males, no outlier was detected. For the rest of the multivariate analysis, no outliers were excluded.

Linear Discriminant Analysis

We used linear discriminant analysis (LDA) for dimensionality reduction while maintaining class-discriminatory information. The objective of the LDA is to find a projection - linear combinations of original space features - that maximizes differences between different class means, while minimizing the within-class variation^{51,52}.

The behavioral features were z-scored, separating males from females, using the Control, KET, CBD-KET, SNP-KET and CBD-SNP-KET groups and eliminating redundant features (such as distance and velocity). We performed a multivariate normality test as indicated in Trujillo-Ortiz et al.,

2002⁵³. Female and male groups did not reject the null hypothesis that the sample came from a multivariate normal distribution ($p > 0.05$).

We used the Scikit-Learn algorithm⁴⁸ with a singular value decomposition solver and three components for LDA dimensionality reduction. Original data were projected in the first two LDA dimensions (LD1 and LD2). For each feature, we calculated the LDA loadings as the scaled eigenvectors and explained variance as the scaled eigenvalues. Distances between groups in the two dimensional LDA were measured by Euclidean's distance between every observation of a treatment group and all the observations of the KET group. This calculation gave us a distribution of distances that were compared using the Kolmogorov-Smirnov test.

Classification

We used logistic regression to build a classification model to estimate treatment efficacy. We constructed a simple binary model in which subjects of different treatments would be classified as control-like or ketamine-like based on discriminant components from LDA. We used logistic regression as a classifier (since LDA as a classifier makes more restricted assumptions about the data) using LASSO, 'L1' regularization avoiding overfitting and providing a more parsimonious decision boundary than a LDA algorithm⁵⁴. The logistic regression model was performed via the scikit-learn function using a bilinear solver. We also tested other classification algorithms such as Support Vector Machines and k-Nearest Neighbors that produced similar results to the one of the logistic regression (Figure S1).

To compare the probabilities of animals being classified as control or KET, we calculated the Jensen-Shannon divergence between the averaged control probability distribution (calculate from the logistic regression model) and the probability distribution of the remaining groups. Jensen-Shannon divergence is based on the Kullback-Leibler divergence formally described in Endres et al., 2003⁵⁵. Jensen-Shannon divergence was calculated for each animal about the probability distribution of the control group, and group results were compared using one-way ANOVA.

Clusterization

We performed unsupervised clusterization analysis using an agglomerative hierarchical algorithm to establish the behavioral relationship between subjects. We used the ward method to define clusters, and the metric used was Euclidean distance. We used this approach for LDA scores and original variables. An optimal number of clusters was estimated using the Elbow method⁵⁶. However, final definition was based on parsimonious interpretability of the results (Figure S2).

Statistical Analysis

Levene's and Lilliefors tests were used to evaluate whether the data were parametric. Data from OFT and SPT were analyzed by two-way analysis of variance (ANOVA) (treatment vs sex or pretreatment vs treatment). NOR data were analyzed by repeated-measures three-way ANOVA (pretreatment vs treatment vs time). Multiple comparisons were performed using Newman-Keuls *post hoc* test (NK). The Kruskal-Wallis test following Dunn's test analyzed the divergence of the probability distribution of each sex. All results are expressed as mean \pm SEM (standard error of mean), and statistical significance was set at $p < 0.05$.

RESULTS

Distinctive sex-dependent effects of ketamine

To evaluate sex-dependent effects of KET on behavioral assessments, we investigated the difference between control and experimental groups of both sexes (Control-M, Control-F, KET-M, and KET F, $n = 8-10$ per group). Our data show that KET treatment induces hyperlocomotion in both sexes; however, hyperlocomotion is higher in females (Treatment-Sex effect: $F_{(1,34)} = 5.553$, $p < 0.05$; NK significance: Control-M vs KET-M, Control-F vs KET-F, KET-M vs KET-F, Control-M vs KET-F, Control-F vs KET-M) without significant differences in percentage of time spent in the center (Sex effect: $F_{(1,34)} = 4.145$, $p < 0.05$; no NK significance). In males, KET treatment decreases the sucrose

consumption (Treatment-Sex effect: $F_{(1,34)} = 22.26$, $p < 0.01$; NK significance: Control-M vs KET-M, Control-F vs KET-M, KET-F vs KET-M). In addition, males submitted to KET treatment decreased DI in the NOR 30 min test, while in females, KET decreased DI in the NOR 24 H test (Treatment-Sex-Time effect: $F_{(1,34)} = 6.006$, $p < 0.05$; NK significance: Control-M NOR 30 vs KET-M NOR 30, Control-F NOR 24 vs KET-F NOR 24, Control-F NOR 30 vs KET-M NOR 30; Control-M NOR 24 vs KET-F NOR 24) (Figure 1-E).

Interestingly, we observed in males and females a tendency to show opposite correlations between total distance and sucrose consumption; whereas males showed a negative correlation ($r = -0.3676$, $p = 0.13$), females presented a positive correlation ($r = 0.4346$, $p = 0.07$) (Figure 1-F). Indeed, bivariate correlations, using all the different groups, indicated a sex differential relationship between all behavioral features, despite not being statistically significant (Figure 1-G).

Prophylactic effects of CBD associated with SNP decreased positive-like and cognitive impairments in males

After investigating the sex-dependent effects of the KET-model, we assessed in male rats the prophylactic efficacy of CBD and/or SNP on KET-induced impairments ($n = 8-12$ per group) (Figure 2). CBD pretreatment did not prevent hyperlocomotion (Treatment effect: $F_{(1,36)} = 17.50$, $p < 0.01$; NK significance: Control vs KET, Control vs CBD-KET, CBD vs KET, CBD vs CBD-KET). However, CBD pretreatment alone increased the proportion time in the center (Pretreatment effect: $F_{(1,36)} = 5.343$, $p < 0.05$; Treatment effect: $F_{(1,36)} = 6.974$, $P < 0.05$; NK significance: Control vs CBD; CBD vs KET, CBD vs CBD-KET). CBD did not modify sucrose consumption. However, CBD prevented KET-induced deficits in the 30 min NOR test (Time-Treatment effect: $F_{(1,38)} = 4.124$, $p < 0.05$; Time-Pretreatment effect: $F_{(1,38)} = 8.082$, $p < 0.01$; NK significance: Control NOR 30 vs KET NOR 30, CBD NOR 30 vs KET NOR 30, CBD-KET NOR 30 vs KET NOR 30) (Figure 2-A).

Similar to CBD pretreatment, SNP did not prevent KET-induced hyperlocomotion (Pretreatment-Treatment effect: $F_{(1,31)} = 4.164$, $p < 0.05$; NK significance: Control vs KET, SNP vs KET) and did not

affect the proportion in the time spent in the center. However, SNP pretreatment alone decreased the sucrose consumption (Pretreatment effect: $F_{(1,31)} = 6.238$, $p < 0.05$; Treatment effect: $F_{(1,31)} = 9.314$, $p < 0.01$; NK significance: Control vs SNP, Control vs KET, Control vs SNP-KET). Regarding cognitive effects, SNP presented prophylactic efficacy in the 30 min NOR test (Time-Pretreatment effect: $F_{(1,30)} = 11.53$, $p < 0.05$; Time-Treatment effect: $F_{(1,30)} = 5.174$, $p < 0.05$; NK significance: Control NOR 30 vs KET NOR 30; SNP NOR 30 vs KET NOR 30, SNP-KET NOR 30 vs KET NOR 30) (Figure 2-B).

When we explored the prophylactic effects of CBD-SNP pretreatment, CBD-SNP decreased KET-induced hyperlocomotion (Pretreatment-Treatment effect: $F_{(1,32)} = 4.817$, $p < 0.05$; NK significance: Control vs KET, KET vs CBD-SNP-KET, Control vs CBD-SNP-KET, CBD-SNP vs KET) without significant effects in the proportion center. In SPT, CBD-SNP pretreatment did not decrease sucrose consumption like SNP pretreatment, but did not prevent KET effects either (Treatment effect: $F_{(1,32)} = 10.93$, $p < 0.01$; NK significance: Control vs KET, Control vs CBD-SNP-KET). However, CBD-SNP pretreatment prevented KET effects in 30 min in the NOR test (Pretreatment-Treatment-Time effect: $F_{(1,32)} = 5.422$, $p < 0.05$; NK significance: Control NOR 30 vs KET NOR 30, CBD-SNP NOR 30 vs KET NOR 30, CBD-SNP-KET NOR 30 vs KET NOR 30) (Figure 2-C).

Prophylactic effects of CBD associated with SNP decreased positive-like and cognitive impairments in females

In females, as seen before, KET behavior impairments were even more robust than in males. Pretreatment with CBD and SNP, particularly in combination, was more effective as well (Figure 3). CBD pretreatment in females decreased hyperlocomotion (Pretreatment-Treatment effect: $F_{(1,37)} = 11.28$, $p < 0.01$; NK significance: Control vs KET, Control vs CBD-KET, CBD vs KET, CBD vs CBD-KET, KET vs CBD-KET), but in females CBD alone did not increase the proportion of time spent in the center. CBD pretreatment decreased sucrose preference after KET treatment (Pretreatment-Treatment effect: $F_{(1,37)} = 5.565$, $p < 0.05$; NK significance: Control vs CBD-KET, CBD vs KET, KET vs CBD-KET) and did not prevent 24 H NOR test deficits (Treatment effect: $F_{(1,36)} = 15.22$, $p < 0.01$;

Pretreatment-Treatment effect: $F_{(1,36)} = 8.052$, $p < 0.01$; NK significance: Control NOR 30 vs KET NOR 30, Control NOR 24 vs KET NOR 24, Control NOR 24 vs CBD NOR 24, Control NOR 24 vs CBD-KET 24) (Figure 3-A).

SNP pretreatment in females decreased hyperlocomotion (Pretreatment-Treatment effect: $F_{(1,32)} = 8.474$, $p < 0.01$; NK significance: Control vs KET, SNP vs KET, KET vs SNP-KET) without effects in the proportion of time spent in the center. Similar to males, SNP pretreatment decreased sucrose consumption (Pretreatment effect: $F_{(1,32)} = 22.98$, $p < 0.01$; NK significance: Control vs SNP, Control vs SNP-KET, SNP vs KET, KET vs SNP-KET) and did not prevent KET deficits in the NOR test (Pretreatment-Treatment effect: $F_{(1,32)} = 6.634$, $p < 0.05$; NK significance: Control NOR 30 vs KET NOR 30, SNP NOR 30 vs KET NOR 30, Control NOR 24 vs KET NOR 24) (Figure 3-B).

In combination, CBD and SNP pretreatment in females decreased hyperlocomotion (Pretreatment-Treatment effect: $F_{(1,30)} = 6.524$, $p < 0.05$; NK significance: Control vs KET, Control vs CBD-SNP-KET, CBD-SNP vs KET, CBD-SNP vs CBD-SNP-KET, KET vs CBD-SNP-KET) without affecting the proportion of time spent in the center. Improved cognitive parameters evaluated by the NOR test (Pretreatment-Treatment effect: $F_{(1,30)} = 30.09$, $p < 0.01$; NK significance: Control NOR 30 vs KET NOR 30, CBD-SNP NOR 30 vs KET NOR 30, CBD-SNP-KET NOR 30 vs KET NOR 30, Control NOR 24 vs KET NOR 24, CBD-SNP NOR 24 vs KET NOR 24, CBD-SNP-KET NOR 24 vs KET NOR 24) and decreased sucrose preference after KET treatment (Pretreatment-Treatment effect: $F_{(1,30)} = 5.844$, $p < 0.05$; NK significance: KET vs CBD-SNP-KET) (Figure 3-C).

Sex-dependent efficacy of CBD-SNP prophylactic effects in KET model

To evaluate and compare the efficacy of different treatments, we initially applied LDA for dimensionality reduction and identified behavioural features that better distinguish each group in males and females (Figure 4). LDA in males revealed that the LD1 component discriminated control and KET groups and is associated with total distance (Pearson's correlation with original variables, $R^2 = 0.63$, $p < 0.01$), reduced sucrose consumption ($R^2 = -0.55$, $p < 0.01$) and cognitive deficits in

NOR 30 min ($R^2 = -0.63$, $p < 0.01$). LD2 is mainly correlated with decreased performance in the 30 min NOR cognitive test ($R^2 = -0.66$, $p < 0.01$) and discriminates part of the treated animals (Figure 4-A). The discriminant analysis classifier based on LD1 and LD2 explained variance of 90.38% in males (Figure S2-A). We observed a similar efficacy in the different treatment groups to be classified as control-like animals (41.66% CBD-KET, 75% SNP-KET and 55.55% CBD-SNP-KET, Figure 4-B). LD1 and LD2 scores were similar in treatment groups (Figure S2-B). Also, there was no difference in the divergence of probability distribution between the KET group and treatment groups ($H(4,37) = 7.904$, $p > 0.05$) (Figure 4-C). Likewise, the cumulative distribution function of the Euclidean distance of treated animals and KET subjects was not different (Kolmogorov-Smirnov test, $p > 0.05$) (Figure S2-B).

In females, LDA clearly distinguished KET from the control group by LD1 that is associated with low values in locomotion ($R^2 = -0.72$, $p < 0.01$) and sucrose consumption ($R^2 = -0.45$, $p < 0.01$) and higher values in NOR 30 min ($R^2 = -0.58$, $p < 0.01$) and NOR 24 H test performance ($R^2 = -0.76$, $p < 0.01$). LD2 was correlated with low sucrose consumption ($R^2 = -0.77$, $p < 0.01$) and increased time in the center ($R^2 = 0.49$, $p < 0.01$) and discriminated mainly treated animals (Figure 4-D). LD1 and LD2 explained variance of 88.59% in females (Figure S2-A). The logistic regression model with the LDA in females showed an increased number of animals in the CBD-SNP-KET classified as control (44.44% CBD-KET, 50% SNP-KET and 100% CBD-SNP-KET, Figure 4-E). In females, the CBD-SNP-KET group had a higher LD1 score compared to other groups, reflecting a distinct behavior feature (Figure S2-B). The divergence of probability distribution of all treatment groups is distinct from the KET group ($H(4,33) = 21.96$, $p < 0.05$), and, remarkably, the CBD-SNP-KET group ($H(4,33) = 21.96$, $p = 0.0001$, KET vs CBD-SNP-KET). Indeed, CBD-SNP-KET presented a higher distance of KET subjects than the SNP-KET and CBD-KET groups, although SNP presented a higher distance than the CBD group (Kolmogorov-Smirnov test, $p < 0.01$) (Figure S2-C).

Sex-dependence of distinct behavior phenotypes

Next, we used unsupervised hierarchical clustering to reveal different subpopulations of treated animals. Since the classification model produces a binary response (control-like or KET-like), we aimed at a more descriptive approach that could identify different clusters based on behavioral phenotype independently of group labels.

In males, we identified 4 clusters of behavioral phenotype based on LDA score (Figure 5-A) (Figure S3-A). Cluster 1 is described by low locomotion and cognitive score, being composed of a mixture of groups. Cluster 2 presents an entirely control-like phenotype and is composed of control animals and treated animals. Cluster 3 comprises treated animals, mainly CBD, and is characterized by a marked improvement in cognitive measure. Lastly, cluster 4 presents behavioral features of a psychotic-like phenotype: increased locomotion and reduction of sucrose consumption and NOR 1 scores. Consistently, cluster 4 is mainly formed by KET animals. The confidence intervals for each male cluster are summarized in table S1.

In females, cluster analysis produced a conspicuous picture with 3 distinct phenotypes based on LDA score (Figure 5-E) (Figure S3-B). Cluster 1 comprises KET animals presenting: increased locomotion, increased sucrose consumption, and cognitive deficits. Cluster 2 comprises control animals and part of the CBD-SNP-KET group, presenting a control-like phenotype. As an intermediary phenotype cluster, cluster 3 is composed mainly of the CBD and SNP treatment, and despite presenting an improvement in the NOR test and decreased hyperlocomotion, it appears that sucrose consumption is reduced. The entire female confidence intervals cluster is summarized in table S2.

Finally, we conducted an unsupervised clustering with all groups of both sexes (Figure S4). We observed the formation of three distinct clusters in which positive- and anxiety-like behaviors are more closely correlated, while negative- and cognitive-like behaviors describe another feature cluster. In cluster 1, we found the behavioral phenotype of the typical control animal, composed of the majority of the Control animals (80% for both sexes) and the CBD-SNP-KET female group (50%). Cluster 2 can be interpreted as an intermediate phenotype between clusters 1 and 3 and has, on average, good cognitive performance and reduced locomotion but reduced sucrose consumption. This cluster is the most mixed, having most animals of the KET-male group and part of treatment groups. Cluster 3 is

defined by a behavioral phenotype typical of KET impairments, formed by the majority of the KET-F (90%) and CBD-KET-F (66.66%), besides part of the KET-M and SNP-KET-F group (37.5% for both groups).

DISCUSSION

Considering preliminary data in the literature, we hypothesized that the combination of CBD and SNP would be an interesting prophylactic treatment to be investigated in a KET model of SZ in both sexes. In general, KET elicited sex-dependent impairments, but both male and female rats presented hyperlocomotion and memory impairments. Females had a better pharmacological response profile than males to CBD-SNP pretreatment. To our knowledge, this is the first evidence of sex-dependent KET response and prophylactic efficacy of CBD and SNP in combination in an animal model of SZ.

Basic research studies pointed to sex differences in animal models^{7,57,58}. Similar to our results, other studies show that females rats presented higher locomotor activity^{24,57,59} and male mice presented decreased sucrose consumption⁶⁰, but it was not investigated in a KET model of SZ. Females showed a positive correlation between total distance and sucrose preference, while in males, the opposite occurred, a negative correlation (Figure 1). This difference suggests that females have more addictive-like behavior, increasing hyperlocomotion and increasing sucrose consumption, while males present with the typical KET symptoms, with psychosis and anhedonia-like behavior. In general, females responded better to the pretreatments tested, which is interesting from a translational perspective, considering that women benefit more from traditional antipsychotics than men^{6,57}.

There is long-standing evidence of CBD antipsychotic effects^{61,62}. When CBD is chronically administered in younger subjects, both positive-like symptoms and cognitive impairments improve substantially. Peres et al. described that CBD treatment for 30 days during peri-adolescence decreased hyperlocomotion induced by the maternal immune activation with poly I:C³³. In addition, this same CBD treatment prevented hyperlocomotion, prepulse inhibition, and contextual fear

conditioning deficits observed in the SHR strain model of schizophrenia³¹. Osborne et al. reported that twice-daily CBD administered for three weeks reversed cognitive and working memory deficits, reflected in NOR and rewarded T-maze tests, respectively³². However, our results show that CBD pretreatment alone diminished hyperlocomotion exclusively in females, while cognitive impairments were prevented only in male rats. Curiously, the first clinical evidence of the antipsychotic effect with CBD was in a young woman⁶³.

Several studies have described SNP antipsychotic-like effects as well^{34,37,64}. Our group conducted the first clinical work reporting the antipsychotic effects of SNP, demonstrating that a single dose infusion was sufficient to improve SZ positive, negative and cognitive symptoms^{36,64}. In SZ animal models, Maia de Oliveira et al. described that pretreatment with SNP reversed KET-induced hyperlocomotion for up to 1 week after administration³⁵. In addition, Diana et al. demonstrated that treatment with SNP during the periadolescence could prevent behavioral abnormalities that model positive, negative, and cognitive symptoms in the SHR model³⁷. Other studies showed that both pre- and treatments with SNP were sufficient to reduce hyperlocomotion. However, it was not enough to reverse cognitive deficits³⁴. In opposition, Wang and colleagues. in a recent work also described that SNP pretreatment was insufficient to decrease psychotic-like behavior in a model induced by MK-801, another NMDA antagonist⁴⁰. Accordingly, our data showed that SNP pretreatment alone prevented hyperlocomotion only in females, but the cognitive impairments were reduced in both sexes.

The CBD and SNP mechanisms of action are not completely known, but there is evidence in the literature about their antipsychotic mechanism. Briefly, synaptic plasticity impairments induced by KET could be reversed or prevented by CBD affecting neuronal activation and synaptic plasticity in cortico-mesolimbic circuits⁶⁵⁻⁶⁹. Also, CBD showed an ability to increase hippocampal neurogenesis, activate 5HT1A receptors, and inhibit anandamide reuptake⁷⁰⁻⁷². The antipsychotic effect exerted by SNP is mainly related to its capacity to donate NO^{27,37}. Due to this characteristic, SNP can ultimately modulate molecular events that act directly on NMDA receptors, possibly correcting their

dysfunction⁷³. Further studies are required to investigate physiological and molecular changes associated with the prophylactic effects of CBD and SNP.

Our multivariate analysis possesses an exploratory character since, for more robust conclusions, it would be necessary to investigate a more significant number of animals per group. However, these analyses are interesting because they show how the variables interact despite the mean value. Thereby, we were able to identify groups that were not obvious previously and set up behavioral clusters with the same characteristics. Furthermore, it indicates some important patterns between treatments and sex differences. For example, the findings in females suggest that control behavior phenotype is close to CBD-SNP treatment, while in males, CBD-SNP indicates a milder effect (Figure 4). On the other hand, it also shows significant variability between animals, even in the same regimen treatment (Figures 4 and 5). Ketamine injections seem to produce markedly different behavior phenotypes in males and females (Figure 5). Nevertheless, they corroborate that females were more affected by KET, while the results are more heterogeneous in males. It is interesting to note that this could explain the diminished therapeutic response to pre-treatments in males. These data corroborate the need for multivariate analyses in basic research to highlight the individuality of animals and aspects that do not appear in univariate analyses⁷⁴.

In conclusion, we can state that a different therapeutic approach was proposed for SZ-like symptoms in both sexes for the first time, which is interesting from a translational point of view since men and women show differences in SZ. We revealed optimistic results with the combination of CBD and SNP pretreatment, two drugs different from current antipsychotics and with a safer profile of side effects. We have also shown that pretreatment after pregnancy and before periadolescence could be an exciting approach for investigating new proposals for SZ treatment. However, our findings may be a consequence only due to pharmacokinetics, an interaction between CBD-NPS. Without the plasma dosage and their correlations, doubts in this regard remain, and further studies are needed.

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CONFLICT OF INTEREST

JAC, AWZ, and JEH are coinventors of the patent “Cannabinoid-containing oral pharmaceutical composition, method for preparing and using same,” INPI on September 16, 2016 (BR 112018005423-2). JAC, AWZ, and JEH are coinventors of the patent “Fluorinated CBD compounds, compositions and uses thereof. Pub. No.: WO/2014/108899. International Application No.: PCT/IL2014/050023,” Def. US number Reg. 62193296; July 29, 2015; INPI on August 19, 2015 (BR1120150164927; Mechoulam R, Zuardi AW, Kapczinski F, Hallak JEC, Guimarães FS, Crippa JAS, Breuer A). Universidade de São Paulo (USP) has licensed this patent to Phytects Pharm (USP Resolution No. 15.1.130002.1.1) and has an agreement with Prati-Donaduzzi to “develop a pharmaceutical product containing synthetic CBD and prove its safety and therapeutic efficacy in the treatment of epilepsy, schizophrenia, Parkinson’s disease, and anxiety disorders”. JAC is a member of the International Advisory Board of the Australian Centre for Cannabinoid Clinical and Research Excellence (ACRE) – National Health and Medical Research Council (NHMRC). JAC and JEH have received travel support to attend scientific meetings and personal consultation fees from BSPG-Pharm. The other authors declare that they have no conflicts of interest.

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FIGURES LEGENDS

Figure 1 – Experimental design and ketamine sex-dependent effects. (A) Experimental design. (B) Open field test (OFT). After the last SAL or KET injection, locomotor activity was evaluated in the OFT by total distance travelled and proportion of time in the center of the arena. (C) Sucrose preference test (SPT). Rats were individualized in cages containing two bottles, one with water and one with sucrose solution, for 3 days. (D) Novel object recognition (NOR). The NOR test consisted of three phases: habituation, training following test 1, and test 2. (E) Sex-dependent effects of KET treatment. (F) Behavioral features correlations in male and females after KET treatment. Pearson's correlation between total distance and sucrose consumption in male rats (left) and female rats (right). (G) Correlation between behavioral features including all experimental groups. Right: Correlogram of behavioral features in all-male groups. Left: Correlogram of behavioral features in all-female groups. * $p < 0.05$.

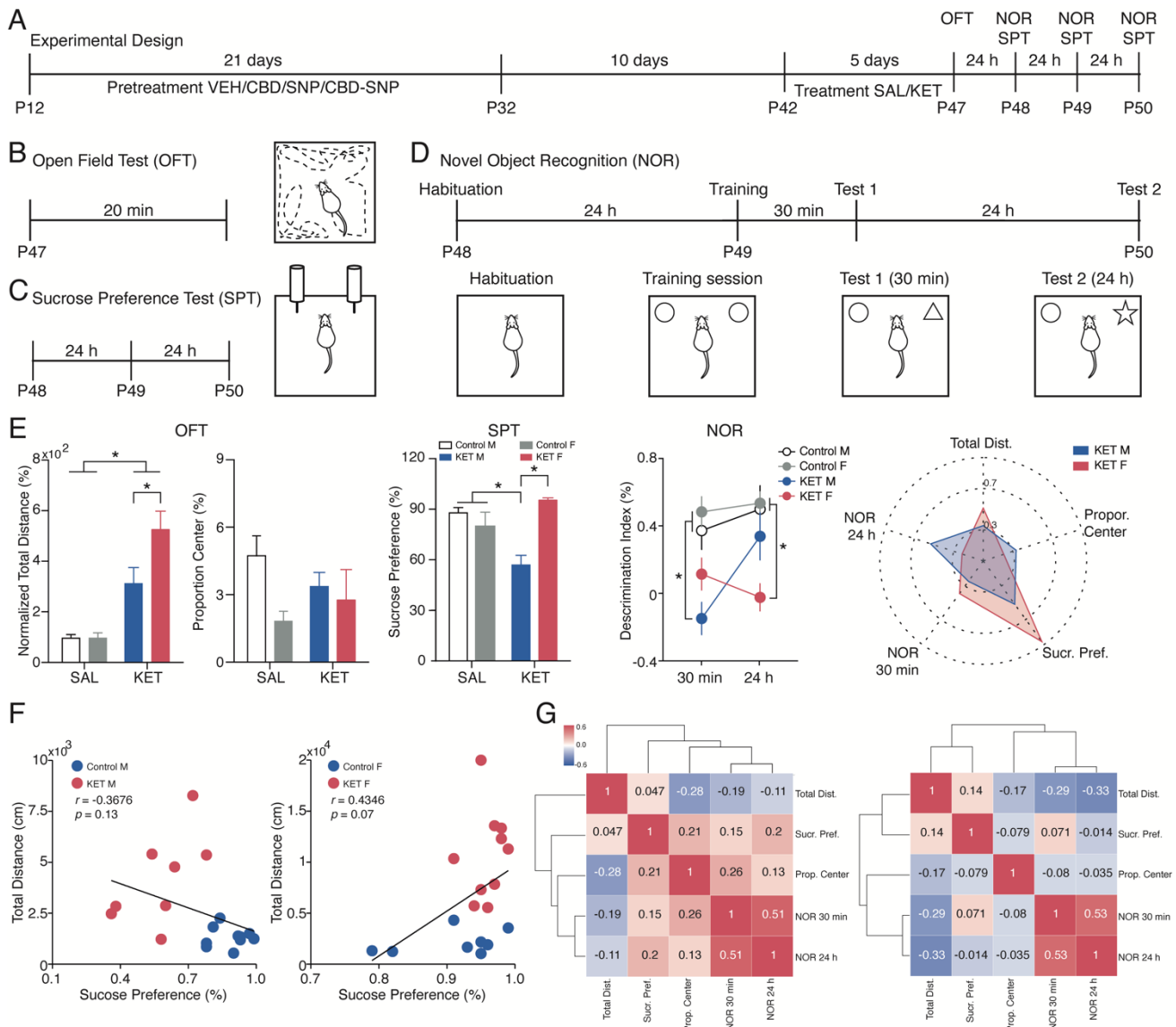
Figure 2 – Prophylactic effects of cannabidiol and sodium nitroprusside pretreatment on schizophrenia-like impairments in males. (A) CBD pretreatment prevented ketamine-induced deficits in the 30 min NOR test. (B) SNP pretreatment prevented deficits in the 30 min NOR test, although it decreased sucrose consumption when administered alone. (C) Pretreatment with CBD and SNP decreased positive-like and cognitive impairments KET-induced. * $p < 0.05$; ** $p < 0.01$.

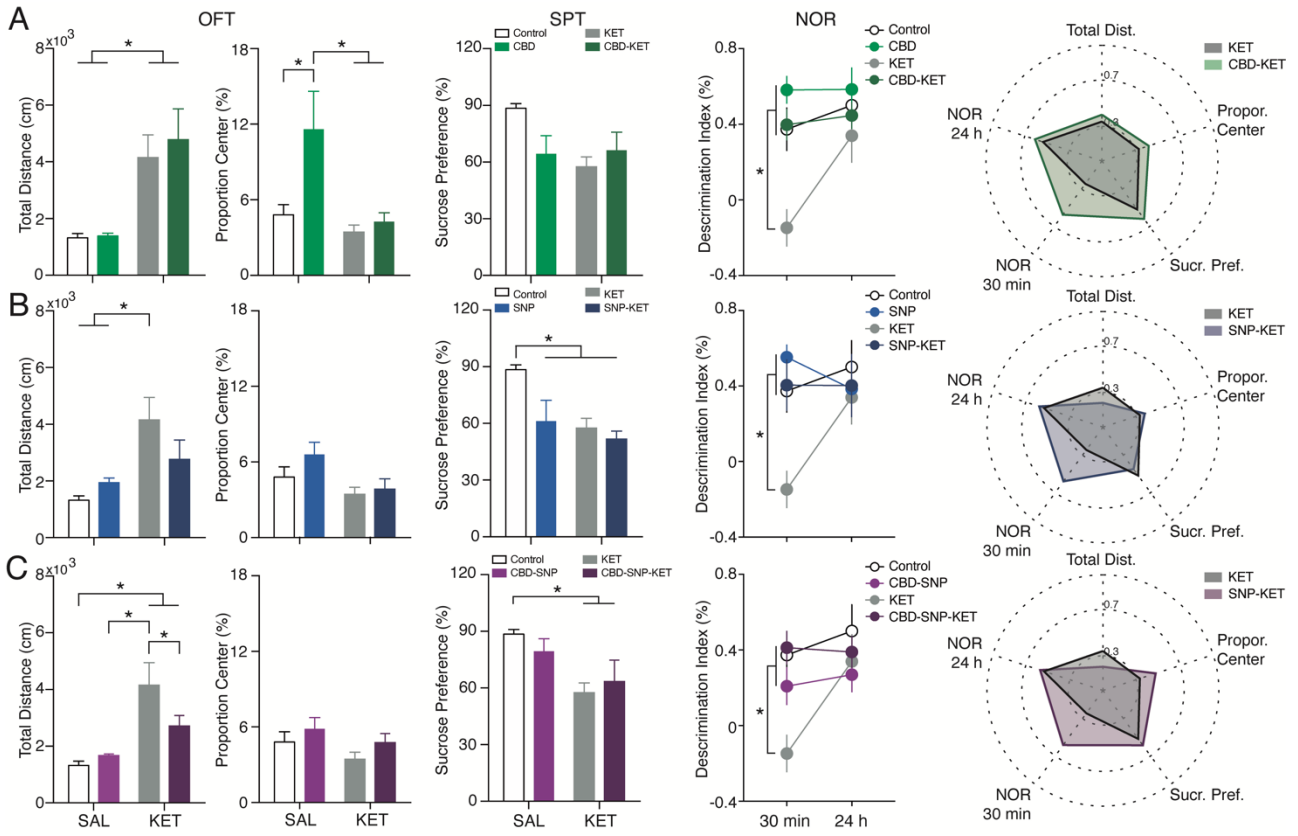
Figure 3 – Prophylactic effects of cannabidiol and sodium nitroprusside pretreatment on schizophrenia-like impairments in females (A) CBD pretreatment prevented KET-induced deficits in the OFT and in the 30 min NOR test. (B) SNP prevented KET-induced deficits in the OFT, although it decreased sucrose consumption when administered alone (C) Pretreatment with CBD and SNP prevented KET-induced positive-like symptoms, sucrose preference and cognitive impairments. * $p < 0.05$; ** $p < 0.01$.

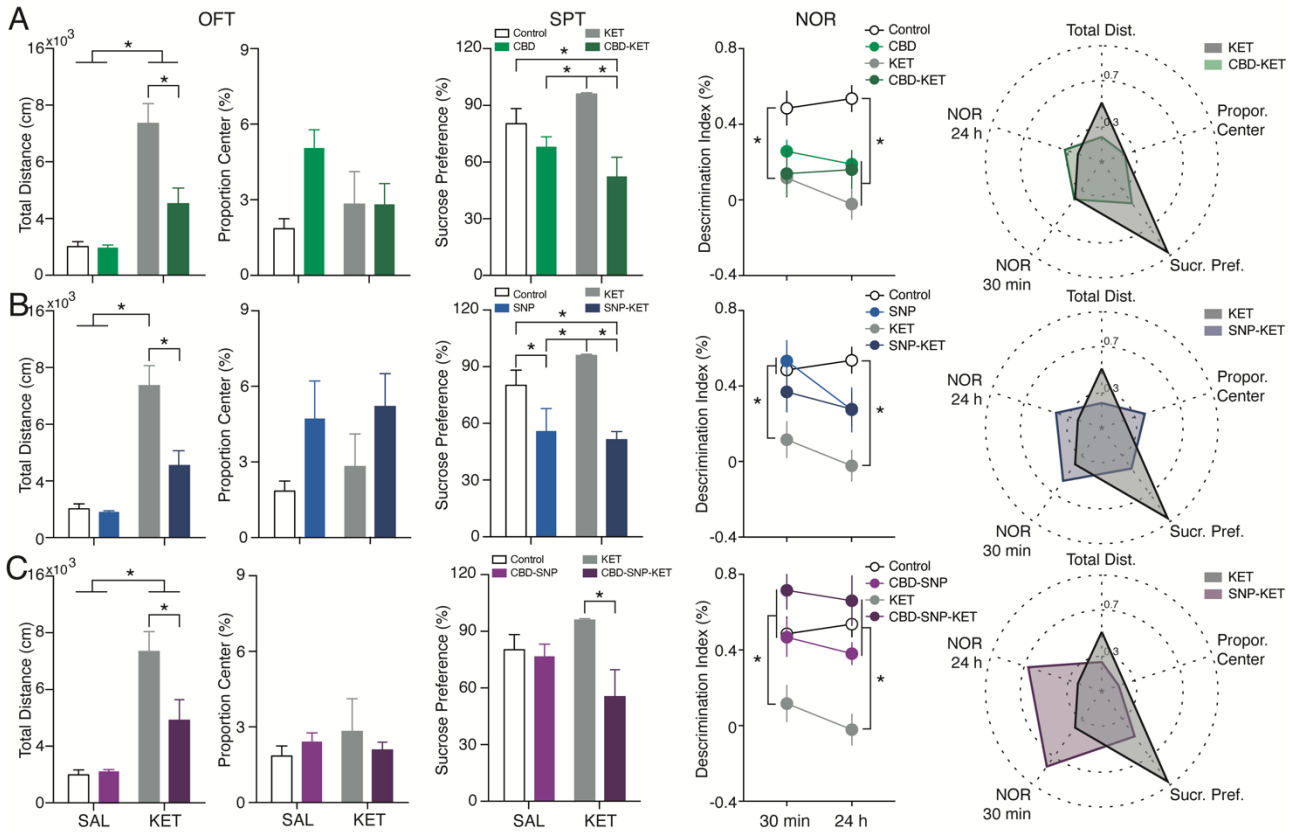
Figure 4 – Multivariate effects of treatment in males and females. (A) Linear discriminant analysis in males. **(B)** Percentage of male animals classified as control. **(C)** The comparative divergence between control probability distribution suggests proximity of behavioral phenotype of KET and pretreatments group. **(D)** Linear discriminant analysis in females. **(E)** Percentage of female animals classified as control. All females in the CBD-SNP-KET group are classified as control. **(F)** Pretreatment female groups present a distinct comparative divergence from the control group, especially the CBD-SNP-KET group. * $p < 0.05$; ** $p < 0.01$.

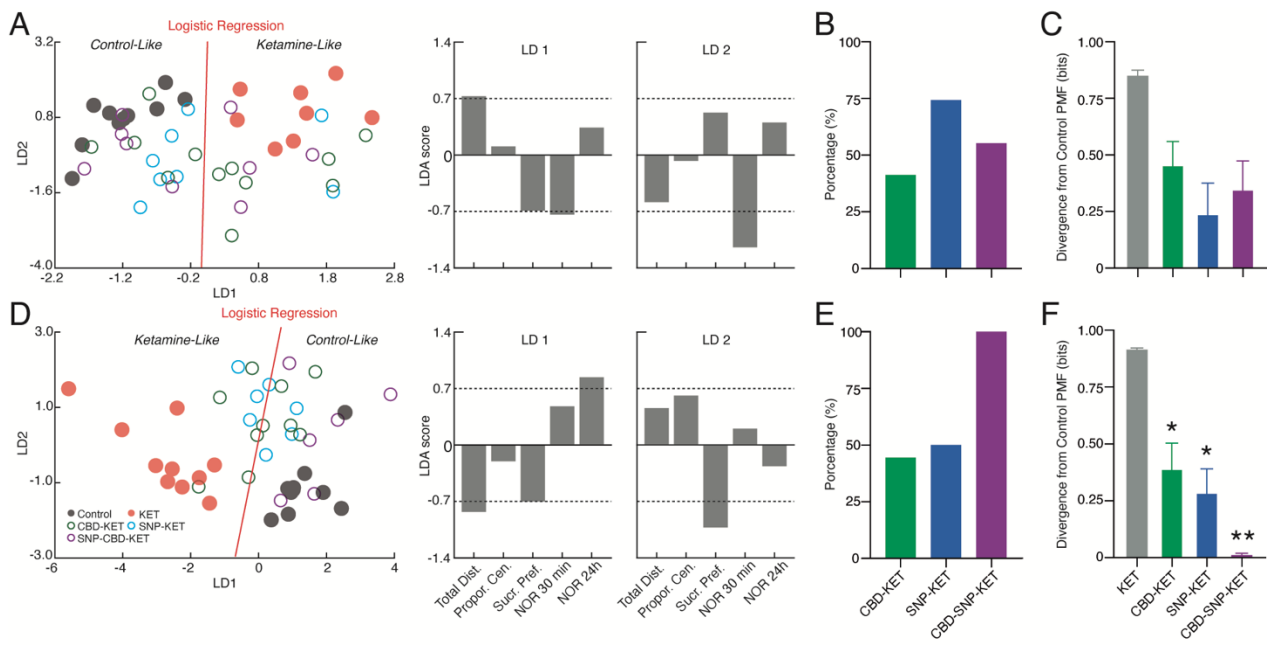
Figure 5 – Identification of clusters using behavioral features. (A) Dendrogram of the 4-cluster formation in males and their LDA analysis components followed by their stacked bars and radar plot. **(B)** Dendrogram of the 3-cluster formation in females and their LDA analysis components followed by their stacked bars and radar plot. Stacked bar plot, representing the percentage of animals from each group on the identified clusters. Radar plot representing the mean values of the normalized behavioral features by cluster in males.

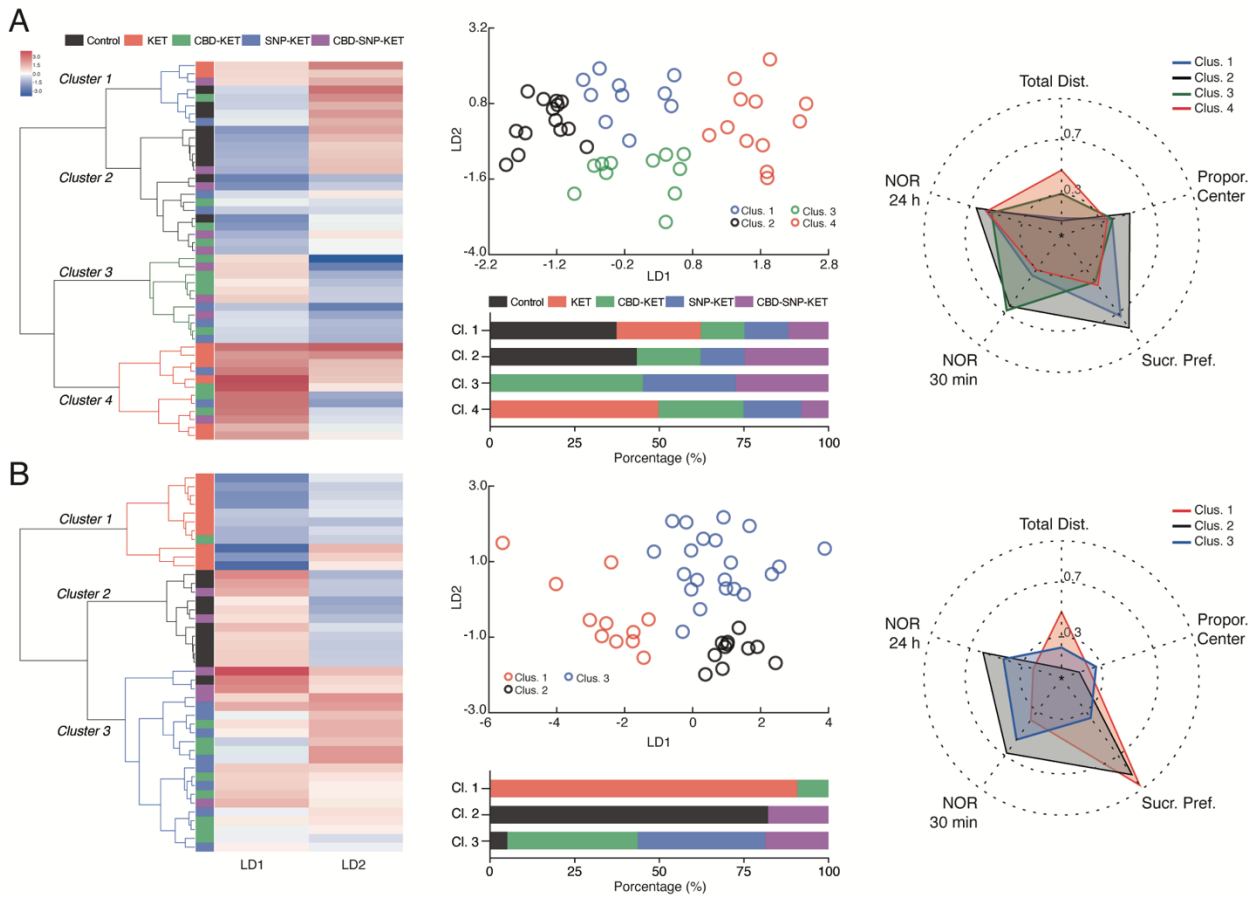
FIGURES











SUPPLEMENTARY MATERIAL

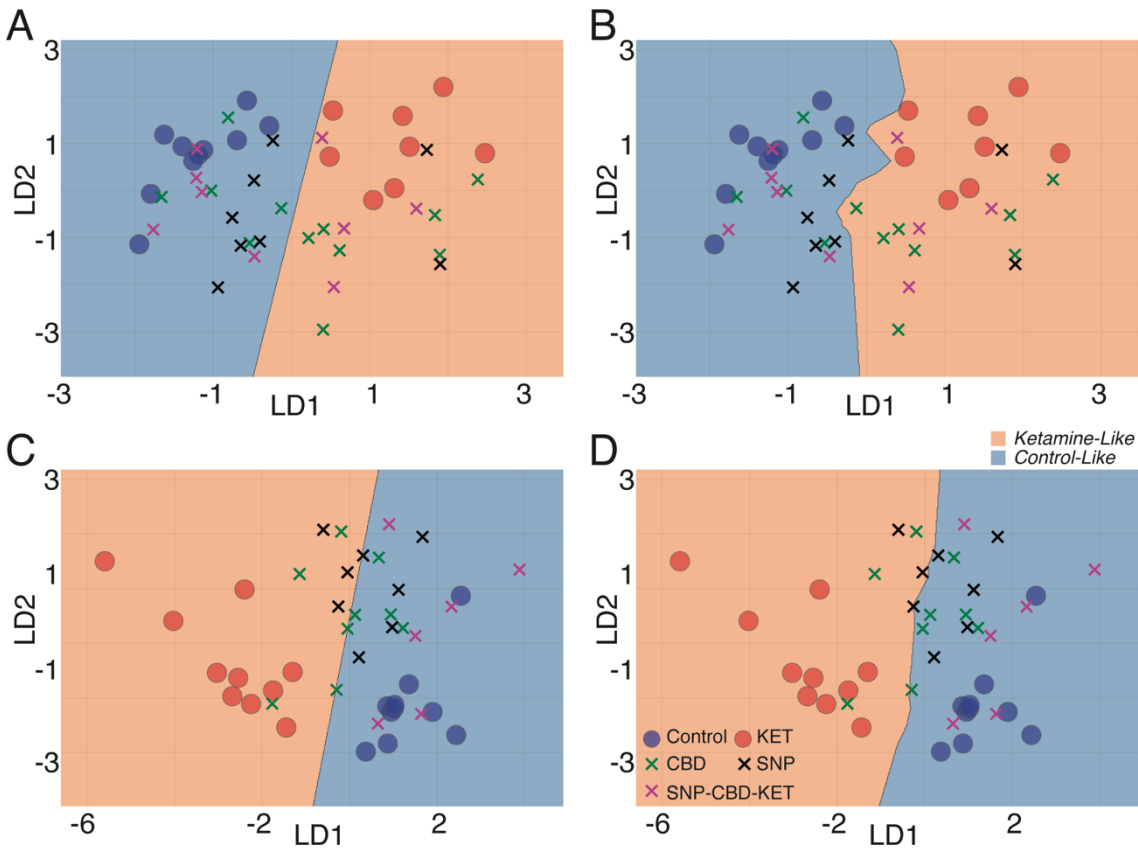


Figure S1 - Classification algorithms to estimate treatment efficacy produced similar results. (A-B) Support Vector Machines and *k*-Nearest Neighbors in male rats. **(C-D)** Support Vector Machines and *k*-Nearest Neighbors in female rats.

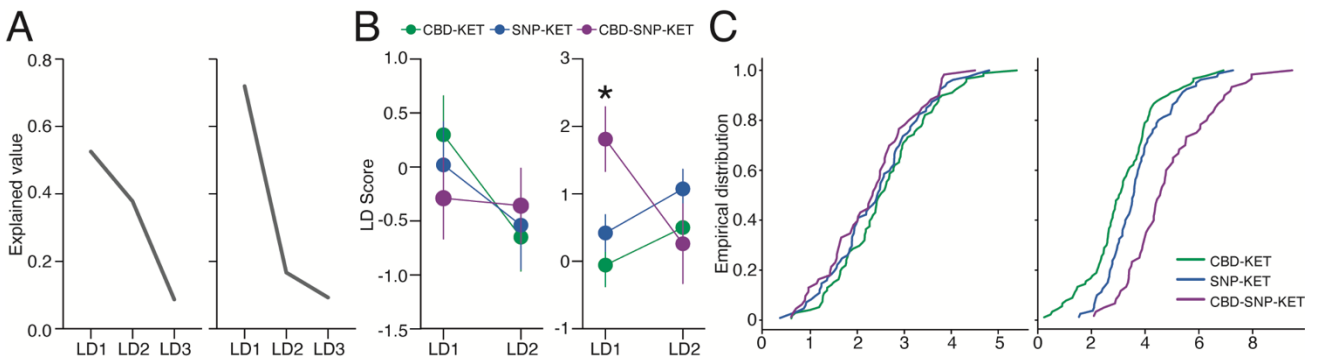


Figure S2 - Linear discriminant scores and the empirical distribution of treated groups. (A) Explained variance of the male (right) and female (left) linear discrimination. **(B)** LD1 and LD2 score of treatment groups in males (right) and females (left) (LD Score-Treatment effect: $F_{(2,20)} = 6.675$, $p <$

0.01; NK *post hoc* significance: CBD-SNP-KET LD1 vs CBD-KET LD1, CBD-SNP-KET LD1 vs SNP-KET LD1). **(C)** The empirical cumulative distribution function for the distances of treated subjects and KET animals in males (right) and females (left).

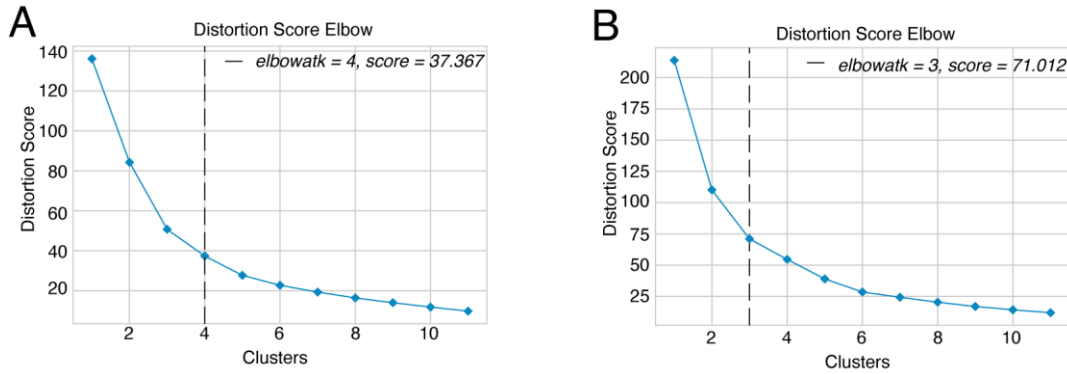


Figure S3 - Distortion score using the elbow method for male and female clusterization. (A) Distortion scores indicating 4 clusters in male groups. **(B)** Distortion scores indicating 3 cluster formations in female groups.

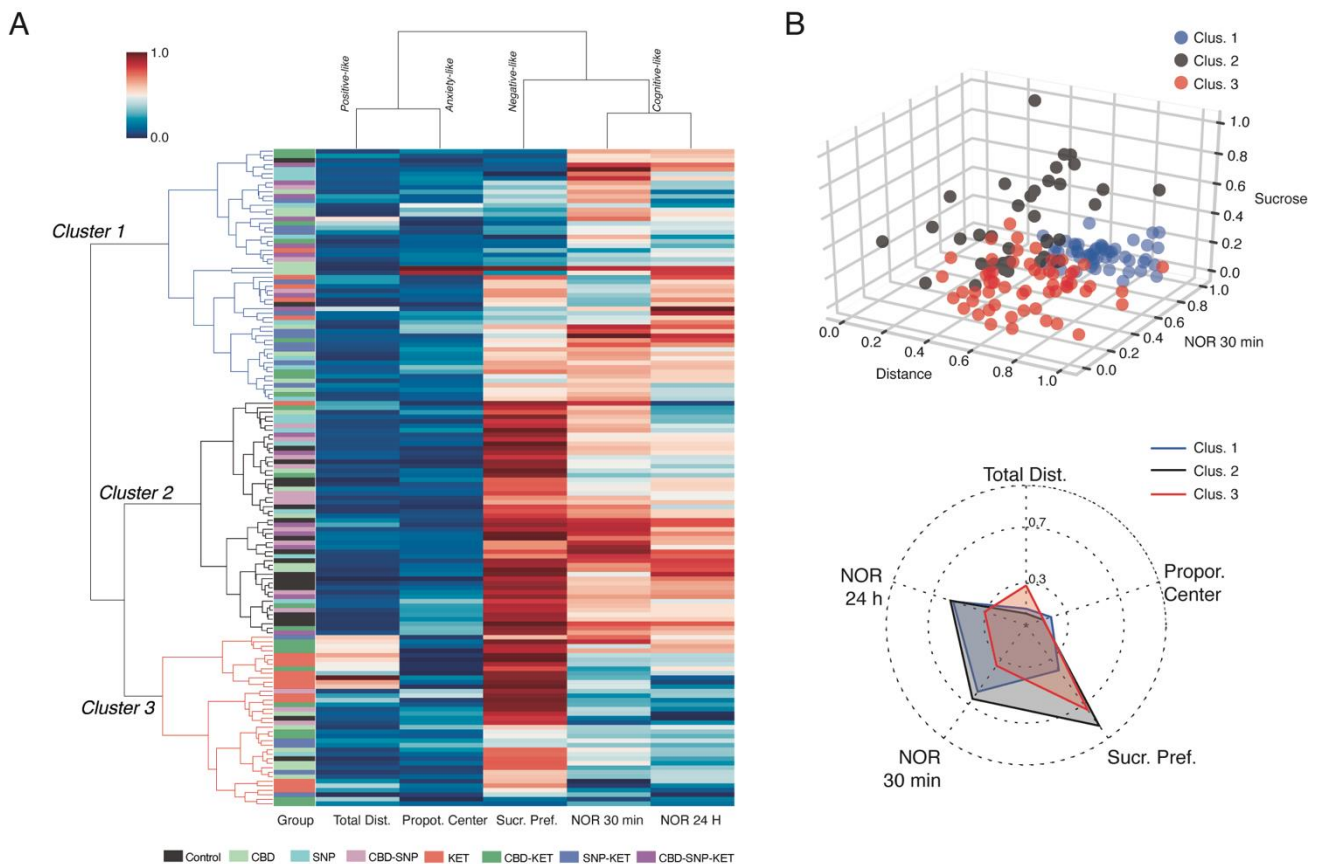


Figure S4 – Behavioral characteristics describe marked differences between all experimental groups. (A) Dendrogram of the agglomerative cluster and normalized behavioral features for each individual. Results indicate the formation of three distinct and interpretable clusters. **(B)** Cluster formation on a three-dimensional plot of the most descriptive behavioral features followed by radar plot.

Table 1

Males Mean and Confidence Intervals Clusters

Cluster	Total Distance	Proportion Center Time	Sucrose Consumption	DI NOR 30 min	DI NOR 24 H
1	5587.94 (4298.77 - 7190.38)	3.56 (2.53 - 4.65)	0.51 (0.37 - 0.66)	-0.04 (-0.19 - 0.11)	0.39 (0.18 - 0.59)
2	1680.56 (1338.52 - 2050.28)	5.16 (3.75 - 6.55)	0.85 (0.76 - 0.91)	0.50 (0.38 - 0.64)	0.51 (0.32 - 0.67)
3	3759.85 (2306.11 - 5528.59)	3.95 (3.34 - 4.64)	0.49 (0.34 - 0.63)	0.57 (0.43 - 0.71)	0.34 (0.16 - 0.53)
4	1882.79 (1418.07 - 2426.20)	3.91 (2.90 - 4.89)	0.76 (0.66 - 0.86)	0.05 (-0.06 - 0.14)	0.40 (0.12 - 0.67)

Table 2

Females Mean and Confidence Intervals Clusters

Cluster	Total Distance	Proportion Center Time	Sucrose Consumption	DI NOR 30 min	DI NOR 24 H
1	5259.61 (4031.10 - 6722.72)	3.56 (2.44 - 4.88)	0.45 (0.36 - 0.54)	0.35 (0.20 - 0.50)	0.32 (0.19 - 0.46)
2	10166.61 (7649.48 - 12684.31)	2.83 (1.05 - 5.38)	0.96 (0.95 - 0.97)	0.10 (-0.04 - 0.28)	-0.02 (-0.15 - 0.11)
3	2424.99 (1637.24 - 3362.96)	1.87 (1.26 - 2.53)	0.88 (0.79 - 0.94)	0.53 (0.37 - 0.69)	0.56 (-0.43 - 0.69)

Tables – Descriptive values of the data in male clustered groups presented in figure 5 A (**Table 1**) and female clustered groups shown in figure 5 B (**Table 2**).

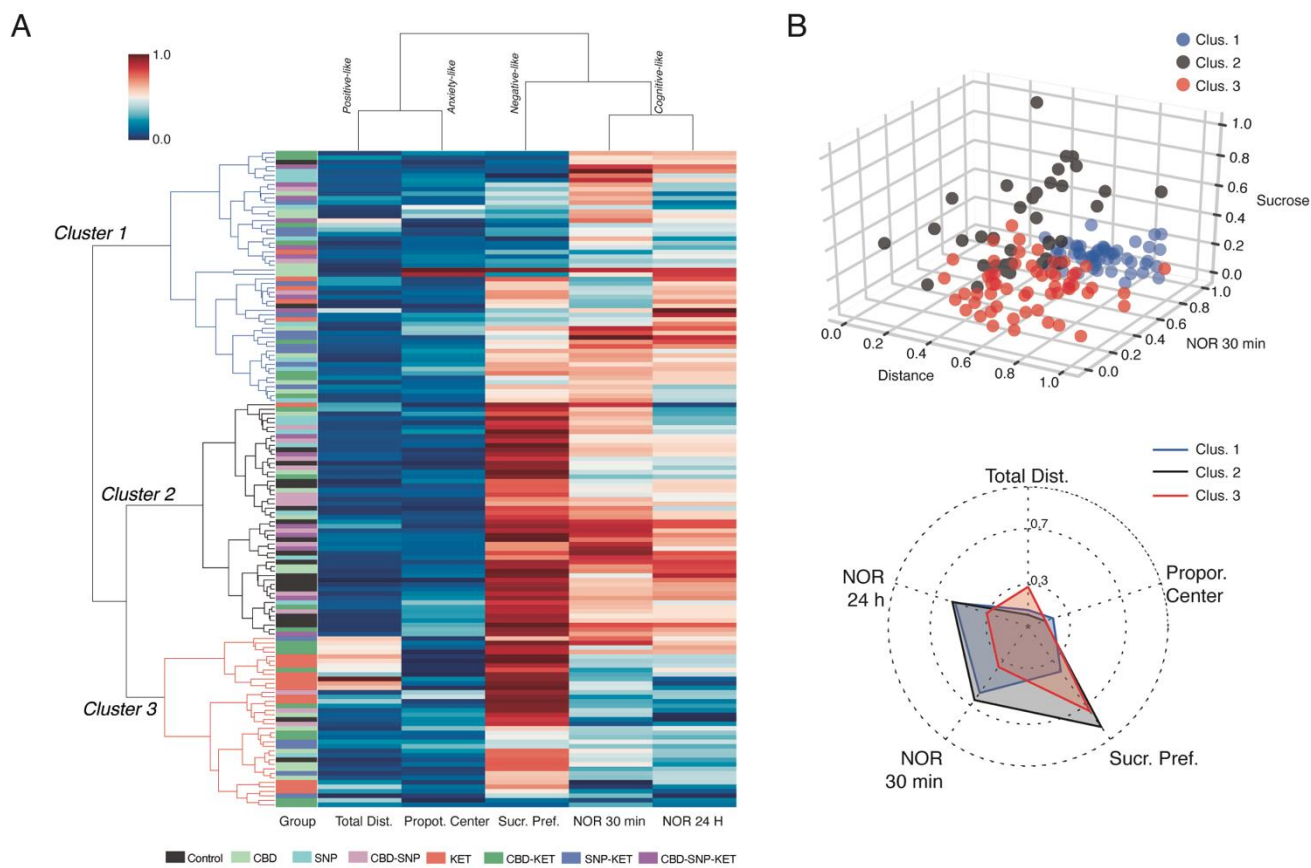


Figure S4 – Behavioral characteristics describe marked differences between all experimental groups. (A) Dendrogram of the agglomerative cluster and normalized behavioral features for each individual. Results indicate the formation of three distinct and interpretable clusters. **(B)** Cluster formation on a three-dimensional plot of the most descriptive behavioral features followed by radar plot.

COMPROVANTE SUBMISSÃO

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3.2. ARTIGO 2: **Efeitos dependentes do sexo da cetamina racêmica e da escetamina em ratos adolescentes**

A esquizofrenia (SZ) é um dos transtornos mentais mais debilitantes que existe. Os sintomas são geralmente classificados em três grandes categorias; sintomas positivos e negativos e déficits cognitivos. Segundo a hipótese dopaminérgica, a mais clássica e popular para explicar a fisiopatologia da SZ, os sintomas positivos ocorrem devido à hiperativação dopaminérgica nas regiões límbicas, e os negativos e cognitivos ocorrem devido à hipoativação dopaminérgica nas regiões corticais. Uma das principais características do transtorno é a diferença entre os sexos. Pesquisas indicam que a SZ se manifesta de forma diferente em homens e mulheres em termos de fisiopatologia, epidemiologia, resposta ao tratamento e prognóstico. O conhecimento da fisiopatologia do distúrbio é o principal aliado na busca por tratamentos mais eficazes, portanto, a pesquisa em animais torna-se uma ferramenta valiosa. Nesse sentido, o modelo de cetamina (KET) é um dos mais utilizados, pois consegue mimetizar os três tipos de sintomas em roedores. A KET possui dois isômeros ópticos, R(-) e S(+) (KET S). O isômero S tem mais afinidade pelos receptores NMDA, por isso acredita-se que pode produzir mais sintomas semelhantes aos da SZ do que os outros tipos de KET. Quando misturados produz-se a forma mais popular de KET, a racêmica (KET R). Considerando o exposto, neste trabalho investigamos as diferenças sexuais entre ratos machos e fêmeas da linhagem Wistar Sprague Dawley, no modelo KET R (30 mg/kg) e KET S (30 mg/kg). Para tanto, após cinco dias de tratamento, foi realizada uma bateria de testes comportamentais para avaliar sintomas do tipo positivos (teste de campo aberto - CA e inibição pré-pulso - PPI), do tipo negativos (teste de preferência de sacarose - PS) e déficits cognitivos (teste de localização de objetos - LO). Nossos resultados mostraram que a KET R e a KET S não apresentaram diferenças nos comportamentos estudados, nem em ratos

machos nem em ratas fêmeas. Além disso, mostra os efeitos das cetaminas dependentes do sexo. As ratas caminharam mais e mais rápido no CA além de explorarem mais os objetos no LO. Tomados em conjunto, nossos dados corroboram a necessidade de investigar o dimorfismo sexual como uma variável considerável no estudo da SZ.

Palavras-chave: Diferenças sexuais. Esquizofrenia. Cetamina. Esketamina. Modelo animal.

Title

Sex-dependent effects of Racemic Ketamine and Esketamine on adolescents rats

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ABSTRACT

Schizophrenia (SZ) is one of the most debilitating mental disorders that exists. Symptoms are generally classified into three broad categories; positive and negative symptoms and cognitive deficits. According to the dopaminergic hypothesis, the most classic and popular to explain the pathophysiology of SZ, positive symptoms occur due to dopaminergic hyperactivation in limbic regions, and negative and cognitive ones occur due to dopaminergic hypoactivation in cortical regions. One of the main characteristics of the disorder is the difference between the sexes. Research indicates that SZ manifests itself differently in men and women in terms of pathophysiology, epidemiology, response to treatment, and prognosis. The knowledge of the pathophysiology of the disorder is the main ally in the search for more effective treatments, therefore, research on animals becomes a valuable tool. In this sense, the ketamine (KET) model of SZ is one of the most used because it can mimic the three types of symptoms in rodents. KET has two optical isomers, R(-) and S(+) (KET S). The S isomer has more affinity for NMDA receptors, so it is believed that it can produce more SZ-like symptoms than the other types of KET. When they are mixed produced the most popular form of KET, the racemic one (KET R). Considering the above, in this work we propose the investigation of sexual differences between male and female rats of the Wistar Sprague Dawley lineage, in the KET R (30 mg/kg) and KET S (30 mg/kg) model. To this end, after five days of treatment, a battery of behavioral tests was carried out to evaluate positive-like symptoms (open field test - OFT and prepulse inhibition - PPI), negative (sucrose preference test - SPT) and cognitive deficits (object location test - OLT). Our results show that KET R and KET S did not present differences in SZ-like behaviors, neither in males nor in females. Also, it shows the sex-dependent effects of the ketamines. Females walked more and faster in the OFT; and explored more the objects in the OLT. Taken together our data corroborates the need to investigate sexual dimorphism as a considerable variable when studying SZ.

Keywords: Sex-differences. Schizophrenia. Ketamine. Esketamine. Animal model.

INTRODUCTION

Understanding sexual differences in responses to pharmacological treatments is crucial for the development of more effective and personalized therapeutic approaches, when considering the importance of such differences is necessary for the causal investigation of mental disorders, such as Schizophrenia (SZ). SZ is one of the most debilitating mental disorders, ranking as the 12th most disabling disorder among 310 diseases in 2016 (Charlson et al., 2018). It has serious consequences on the quality of life not only for affected patients but also for their socio-family circle (Tandon et al., 2009; Harvey et al., 2012; Maibing et al., 2015). The causality for this condition is still unknown (Perez et al., 2014), characterized by heterogeneity in etiology, prognosis, and clinical presentation (Seaton et al., 2001; Xiao et al., 2022; Rantala et al., 2022).

The pathophysiology of SZ is mainly based on two theories attempting to explain the positive and negative symptoms and cognitive deficits present in SZ. The first hypothesis is the dysfunctionality of dopaminergic neurotransmission (Meltzer; Stahl; 1976), which comprises positive symptoms as a result of dopaminergic hyperactivation in limbic regions, while negative and cognitive symptoms occur due to dopaminergic hypoactivation in cortical regions (Toda; Abi-Dargham, 2007; Frohlich and Van Horn, 2013). The second hypothesis is based on glutamatergic neurotransmission, a glutamatergic dysfunction that would later result in dopaminergic dysfunction, explaining the triad of symptoms (Frohlich and Van Horn, 2013; Howes et al., 2015, Uno et al., 2019).

In addition to all the complexity of the pathophysiology of the disorder, the manifestation of the condition presents differences related to sex (Riecher-Rössler et al., 2018), with a generally greater incidence in men than in women. Estimated ratios are approximately 1.5 (Abel et al., 2010; Van der Werf et al., 2014), a fact observed and described since the early 20th century by Kraepelin, indicating that hospitalization for SZ occurred more frequently in young men (Kraepelin, 1909).

However, this discrepancy varies, increasing or decreasing according to age (Mendrek and Mancini-Marie, 2016). One of the possible explanations for such variations is the high physiological levels of estradiol in women of reproductive age, as the early age of menarche was associated with the later age of onset of SZ in females (Cohen et al., 1999; Häfner, 2003; Hill, 2016).

Sexual differences are evident in the expressions of the three classes of symptoms, indicating that women tend to present more positive symptoms, while men consistently express more negative symptoms (Abel et al., 2010) and cognitive deficits (Leung and Chue, 2000). Furthermore, impairments related to SZ indicate that there is worse performance in males in attention, language, and executive function compared to females (Goldstein et al., 1994, 1998; Seidman et al., 1997; Hoff et al., 1998), and that female individuals excel in verbal learning and memory tasks (Bozikas et al., 2010; Hill, 2016). The severity of the disorder appears to be greater in men, possibly due to earlier onset (Abel et al., 2010). On the other hand, women tend to respond better to available antipsychotic treatments and require lower doses for therapeutic effectiveness; however, this depends on the type of antipsychotic, menopausal status, and progression of SZ (Abel et al., 2010).

The question of the mechanisms of SZ includes the variable of sex, which indicates that it contributes to changes in various aspects: behavioral, emotional, and cognitive expressions, with influences of neurotransmitters, hormones, brain connectivity, for example. For these reasons, animal models become a valuable research tool, and in the context of SZ, the ketamine (KET) model is one of the most used tools to study SZ-like behaviors (Nikiforuk and Popik, 2012; Frohlich and Van Horn, 2013; Ram et al., 2013; Kekesi et al., 2015; Radford et al., 2020), as it can mimic positive and negative symptoms and cognitive deficits (Frohlich and Van Horn, 2013; Pitsikas, 2018). KET is characterized as an anesthetic that antagonizes NMDA-type glutamatergic receptors, demonstrating efficiency in mimicking psychosis both in clinical research (Nagels et al., 2018; Beck

et al., 2020) and in basic research (Pitsikas et al., 2008; Frohlich and Van Horn, 2013; Bieber et al., 2022). However, the current scientific literature presents a substantial gap regarding the assessment of gender disparities in response to racemic ketamine (KET R) and its S enantiomeric form, esketamine (KET S). KET S has more affinity for NMDA receptors, has more anesthetic potency and is generally more related to the psychotomimetic effects of the drug compared to KET R (Pitsikas, 2018). Due to its ability to mimic the symptoms of SZ, the discussion has been reignited on the importance of the glutamatergic system in the pathophysiology of the disorder.

In this study, we propose to comprehensively investigate the behavioral differences between male and female adolescent rats subjected to subchronic treatment with KET R and KET S. The aim is to shed light on the possible sexual effects of these psychoactive agents, evaluating behavioral changes linked to sex, age, and the type of treatment.

METHODS

Animals

Sprague Dawley rats were housed in transparent acrylic boxes (45x32x17cm) that were kept in ventilated racks (Insight, Brazil) with controlled temperature (24 ± 2 °C) and humidity ($60\pm 5\%$), 12h light-dark cycle, and free access to water and food. Females and males were maintained in separate racks. All the procedures were approved by the local Committee on Ethics in the Use of Animals (Ribeirão Preto Medical School, University of São Paulo; protocol: 1141/2022).

Experimental procedure

Animals were treated with saline (SAL), KET R or KET S from postnatal day (PD) 42 to PD47. After five days of pharmacological treatment, experimental groups were submitted to behavioral

paradigms to measure locomotor activity (open field test – OFT), anhedonic and hedonic behavior (sucrose preference test - SPT), spatial memory (object location test – OLT) and sensorimotor gating (prepulse inhibition – PPI). The experimental procedure is summarized in figure 1.

Pharmacological treatment

Intraperitoneal (i.p.) injections of SAL 0.9% (1ml/kg), KET R (30mg/kg) or KET S (30mg/kg).

Behavioral tasks

OFT, OLT, SPT, PPI

Locomotor activity was evaluated for 20 minutes in the open field apparatus (46 x 46 x 46 cm) 30min after the last i.p. injection.

In the following day, animals were habituated for 20 minutes to the object location apparatus (42,5 x 39,5 x 83 cm). For the training in the object location task, animals were exposed during 10 minutes to two identical objects that were located in top corners of the arena. For the test session, one object was moved to a new location (right bottom corner). Animal was facing the wall during the release in the arena, which was always in the same location (middle of the bottom wall). The exploration rates of the objects were quantified during training and test.

Concomitantly with the OL task, the SPT was performed for 3 consecutive days. On the first two days, the animals were single housed and received one bottle containing 200 ml of water and other bottle containing 200 ml of sucrose solution at 15% concentration. On the third day of the task, the sucrose concentration was reduced to 2%. Over the 3 days, the side of the box where the bottles were placed were alternated so that there was no preference for the side, but rather for the solution. The consumption was calculated every 24h and expressed using the rate between the

volume of sucrose consumed (VSac) divided by the sum of the consumed volume of water (VW) and sucrose (%Sac= [VSac/ VSac+VW]*100).

In the next day, rats were examined for PPI of the acoustic startle. The PPI test consisted of 60 trials (intertrial interval: 15 ± 7 s) divided in 10 blocks with 6 semi-randomized trials each. At the beginning and end of each session, animals were exposed to 6 pulse trials to evaluate habituation to the startle reflex during the experiment. The acoustic stimuli consisted of 18 prepulse alone (6 of each intensity: 71dB, 77dB and 83dBb with 20 ms duration), 6 startling pulse alone (120 dB intensity and 40 ms duration), 18 prepulse followed by a startling pulse (71dB, 77dB and 83dB intensity and 20 ms duration, prepulse 100 ms before the pulse) and no stimulus, all of which accompanied by background noise (65 dB white noise). PPI was calculated by the equation: %PPI = $(100 - 100 \times [\text{mean startle amplitude at PP + P}]/[\text{mean startle amplitude at P}])$. Habituation was quantified by the equation: % HAB=100-[100*(HAB1/HAB0)] (Valsamis and Schmid, 2011). 24 hours after the PPI task, locomotor activity were re-evaluated for 20min in the open field apparatus (Kislal and Blizard, 2016).

Statistical Analysis

Levene's and Shapiro-wilk tests were used to evaluate whether the data were parametric. Data from OFT, SPT and PPI were analyzed by two-way analysis of variance (ANOVA) (treatment vs sex), or one-way ANOVA considering the independent groups (M SAL x F SAL x M S+ x F S+ X M R x F R). Multiple comparisons were performed using Bonferroni *post hoc* test. All results are expressed as mean \pm SEM (standard error of mean), and statistical significance was set at $p < 0.05$.

RESULTS

KET R and KET S do not present differences in showing SZ-like behaviors, neither in males nor in females

None of the evaluated parameters of psychotic-like behavior presented differences between KET R and KET S, neither in males (M SAL n=14, M S+ n=14, M R n=14) nor in females (F SAL n=12, F S+ n=15, F R n=14). When we analyzed the velocity in the open field 1, the 2-WAY ANOVA (sex x treatment; M SAL x F SAL x M S+ x F S+ X M R x F R) showed a difference in the treatment factor [$F(2,77) = 24.521$; $p < 0.001$], but Bonferroni post hoc pointed that this difference was that KET groups (M S+, FS+, M R F R) walked faster than the ketamines ones (M SAL and F SAL). At the same time, there was no difference between ketamines in this parameter (figure 2). When velocity was analyzed over time (every 5 minutes) it demonstrated the same pattern. The 1-WAY ANOVA repeated measures (independent groups, M SAL x F SAL x M S+ x F S+ [$F(9.582,147.558) = 2.216$; $p = 0.021$]) post hoc showed that KET groups do not differ from each other, neither in males nor in females, and that M SAL does not differ from M S+. In contrast, it differs from M R, while in females F SAL was different from F S+ and F R (figure 4).

When we analyzed the total distance walked the open field 1, the same pattern of results occurred. The 2-WAY ANOVA (sex x treatment; M SAL x F SAL x M S+ x F S+ X M R x F R) showed difference in the treatment factor [$F(2,77) = 24.794$; $p < 0.001$]. Post hoc Bonferroni pointed that this difference was that the KET groups (M S+, FS+, M R F R) walked more than the SAL ones (M SAL and F SAL). At the same time, there was no difference between ketamines (figure 2). When total distance was analyzed over time, it demonstrated the same result. The 1-WAY ANOVA repeated measures (independent groups, M SAL x F SAL x M S+ x F S+, [$F(9.580,147.533) = 2.217$; $p = 0.021$]) and post hoc test showed that KET groups do not differ from each other, neither in males nor in females. Also, M SAL does not differ from M S+ while it differs from M R, while in females F SAL was different of F S+ and F R (figure 4).

The last analyzed parameter in the open field 1 test was how much the animals remained in the center of the arena. The 2-way ANOVA showed a difference in the sex variable [$F(1,77) = 11.347$;

$p=0.001$], post hoc shows that males remained more in the center than females. In the treatment variable [$F(2,77) = 3.230$; $p=0.045$], the SAL groups (M SAL and F SAL) only differ from the KET R groups (M KET R and F KET R), where KET R stayed less in the center (figure 4).

In the object location test, the 2-way ANOVA showed that there was no difference between the treatments tested [$F(2,77) = 0.671$; $p=0.514$] (figure 5); nor in the PPI71 test [$F(2,77) = 0.995$; $p=0.380$], nor PPI77 [$F(2,77) = 0.584$; $p=0.563$] nor in PPI83 [$F(2,77) = 0.383$; $p=0.685$], figure 7.

Sex-dependent dependent effects of the ketamines

The sex-dependent effects of the ketamines was observed in several measured parameters. In the open field test 1, the 2-WAY ANOVA (sex x treatment; M SAL x F SAL x M S+ x F S+ X M R x F R) showed that females walked faster than males [$F(1,77) = 20.737$; $p<0.001$], walked more [$F(1,77) = 21.001$; $p<0.001$] and were less in the center [$F(1,77) = 11.347$; $p=0.001$] (figure 2).

In addition to the psychotic-like parameters, we also observed differences in the cognitive-like responses. The post hoc of the 2-way ANOVA (sex x treatment) showed that females explored more the objects in the object location test; M SAL x F SAL x M S+ x F S+ X M R x F R; [$F(1,77) = 20,766$; $p<0.001$] (figure 5).

Ketamine psychotic-like effects do not remained over time

The ketamines deficits observed in the open field test 1 (mentioned above, item 1) did not remain after 5 days in the open field test 2. The animals did not show hyperlocomotion (2-WAY ANOVA (sex x treatment; M SAL x F SAL x M S+ x F S+ F(2,77) = 1.450; $p=0.241$) nor increase in their velocity [$F(2,77) = 1.450$; $p=0.241$] (figure 7).

Antidepressant-like effect of the KET R

The analysis of the last day of the sucrose preference test showed that the KET R groups (M KET R and F KET R) ingested more the sweet solution than the KET S+ groups (M KET S and F KET S) (2-WAY ANOVA (sex x treatment, treatment factor; M SAL x F SAL x M S+ x F S+ X M R x F R [F(2,77) = 3.134; p=0.049] (figure 6).

Furthermore, the KET R groups had less startle than the SAL (M SAL and F SAL) and KET S ones in the PPI startle parameter ([F(2,77) = 3.507; p=0.041] - 2-WAY ANOVA; sex x treatment, treatment factor; M SAL x F SAL x M S+ x F S+ X M R x F R) (figure 7).

DISCUSSION

KET is an anesthetic NMDA receptor antagonist that had its use expanded in recent decades. When typing KET on scientific websites we can see its effect being performed on pathologies such as major depressive disorder, bipolar disorder, schizophrenia, and addiction problems. Interestingly, it can serve as a treatment or as a cause of the symptoms depending on the psychiatric illnesses (Wright and Kabbaj, 2018; Strong and Kabbaj, 2018; Hashimoto, 2020). In this sense, KET was approved by the FDA as a treatment for treatment-resistant depression in the United States and is one of the most popular pharmacological schizophrenia models (Strong and Kabbaj, 2018). KET is a racemic mixture containing equal parts of the two enantiomers (R) and (S) ketamine, in which the latter presents approximately 3 to 4 times more affinity by the NMDA receptor. Due to this characteristic, (S) KET has more psychotomimetic effects (Krystal et al., 2023). Considering the difficulty in isolating only the R isomer, in general, most of the aforementioned

studies investigated the effect of racemic KET, both in humans and in animal models (Hashimoto, 2020). The group of Hashimoto et al from Japan was one of the only ones to isolate the R isomer and increasingly present promising data on its antidepressant-like effect (Hashimoto, 2020; Zhang and Hashimoto, 2018). Here, we used racemic KET and the isolated S isomer in the open field test, object localization test, sucrose preference test, and PPI. In most tests, there was no statistical difference between the KETs.

In the open field test, the KETs showed no differences between them in producing hyperlocomotion or in increasing velocity. The biggest difference found was between sexes, females traveled more and faster than males. One study treated 10-week-old Sprague-Dawley rats with 2 and 5 mg/kg of KET and observed their behavior 10 minutes after injection. Specifically, they quantified stereotyped behavior (head weaving, ataxia, and circling). They observed that after 5 minutes of injection, ataxia was greater in females while head weaving was greater in male rats (Radford et al., 2020). Here, the female's behavior was recorded 30 minutes after the injection, considering this is the moment recommended to reach the peak of psychotic-like behavior (refs). However, in males, we observed that the peak of behavior occurred shortly after the injection. It is interesting to note that studies that used other models of schizophrenia also observed that females walked more and/or faster than males (Frye et al., 2000; Neill et al., 2010; Hill, 2016).

Aiming to investigate cognitive-like symptoms, we subjected the animals to the object localization test 24 hours after the last injection of the ketamines. We observed that females explored objects for a longer time than males, regardless of the treatment (KET R, KETS, or SAL). Using the KET chronic model of schizophrenia, Hauser and colleagues subjected mice to several spatial memory tasks. They reported that KET induced significant deficits in the recognition of new objects and that was positively correlated with the reduction of parvalbumin expression and c-fos interneurons (measure of neuronal activity) in the prefrontal cortex of C57Bl6N mice. They

suggested that this result indicates that adult mice chronically treated with KET exhibit a combined impairment of novelty exploration and discrimination, which may represent both negative (motivational) and cognitive-like symptoms (Hauser et al., 2017).

To improve the therapeutic strategy for schizophrenia treatment concerning cognitive symptoms, Leger and Neil carried out a systematic review of schizophrenic patients and animal models considering sex as a variable. From this bibliographic survey, some important conclusions were reached, such as sexual dimorphism in cognition exists both in the clinic and animal models, females and women have an advantage in social cognition, man and male rats perform better in working memory tasks while female rats and women in visual memory ones (Leger and Neil, 2016).

Several studies point to the antidepressant-like effect of both the racemic mixture and the isolated R isomer in comparison to S. These effects appeared in the neonatal dexamethasone exposure model, the chronic social defeat stress, and the learned helplessness model of depression. In addition to distinct administration routes such as intracerebral and intraperitoneal (Hashimoto, 2020; Hill, 2016).

In the search for a schizophrenia model that addresses negative and cognitive symptoms, Hill and collaborators proposed one called "two hit". In this model, animals were separated from their mother in the prenatal phase, treated with corticosterone during adolescence, and had their behavior evaluated in tests such as the Y-maze and sucrose preference in adulthood. They observed that males showed a significant deficit in short-term memory, something that did not happen with females. On the other hand, females showed anhedonic-like signs in the sucrose preference test while males did not (Hill et al., 2014).

The prepulse inhibition test is widely used in basic research to screen new antipsychotic drugs or to validate models of schizophrenia (Kahn and Powell, 2018). In our work, we found that

there was no difference between the sexes in any parameter analyzed, only that the KET R groups had less startle than the control groups. A study using the same KET dosage used here observed effect only at a 77 dB pulse, and despite testing on males and females, it also found no difference between sexes in this parameter (Fodor et al., 2016). Another study aimed to investigate the sex variable for the sensorimotor control of acoustic stimulation, spatial memory, pain sensitivity, and motor control. They found differences between the sexes: males showed less sensitivity to pain while females showed greater impairment in sensory control and increased acoustic startle reaction. From this, they stated that the results indicate the same sexual selectivity presented in humans, with a higher incidence of the risk rate in men than in women (Kekesi et al., 2015).

Conclusion

Ketamine is a very important drug in the study of psychiatric illnesses. Despite its variations, it did not show any difference in effect in the behavioral tests tested. Here it was investigated in female and male rats and showed significant differences between the sexes. This emphasizes the need to include females and women in the screening study of new drugs as well as in the search for more reliable models. Also, more studies are needed to investigate the types of ketamine and its isomers in animal models.

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CONFLICT OF INTEREST

The authors declare no interest conflict.

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FIGURES LEGENDS

Figure 1 – Experimental design. The animals were pre-treated for five days with KET S or KET R and then underwent a battery of behavioral tests.

Figure 2 – Open field test 1 results. Both KET treatments showed hyperlocomotion. Females traveled more and faster than males (* $p < 0.05$).

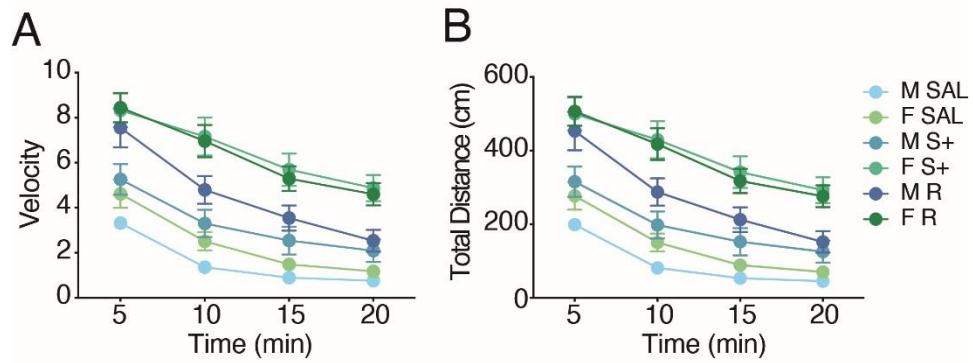
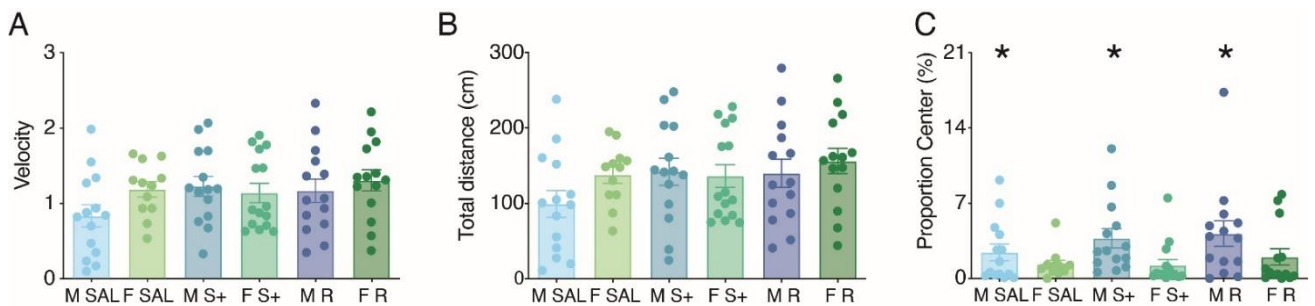
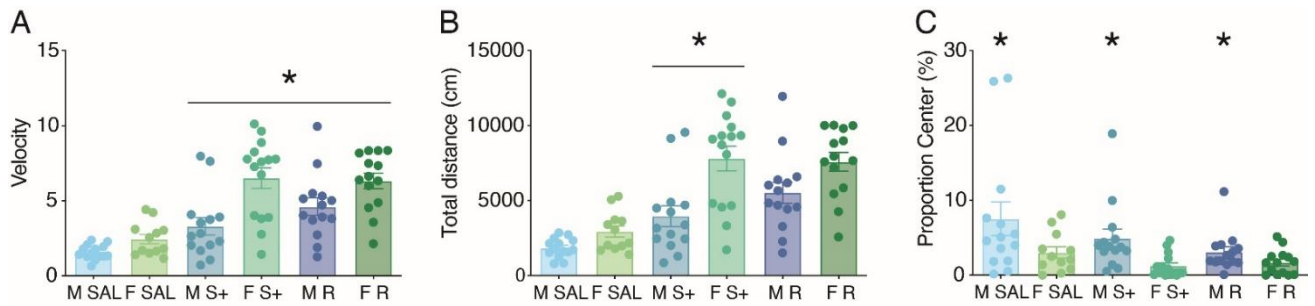
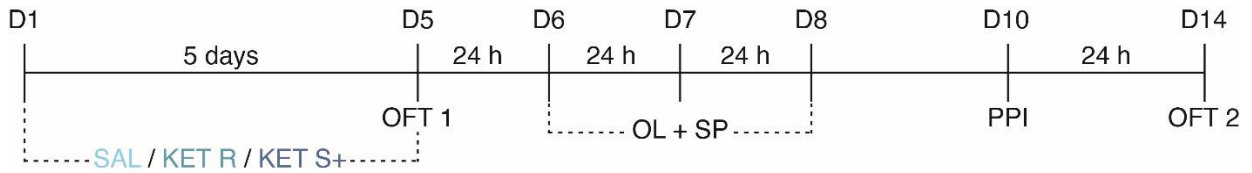
Figure 3 - Open field test 2 results. Lack of KET effect nine days after the end of treatment.

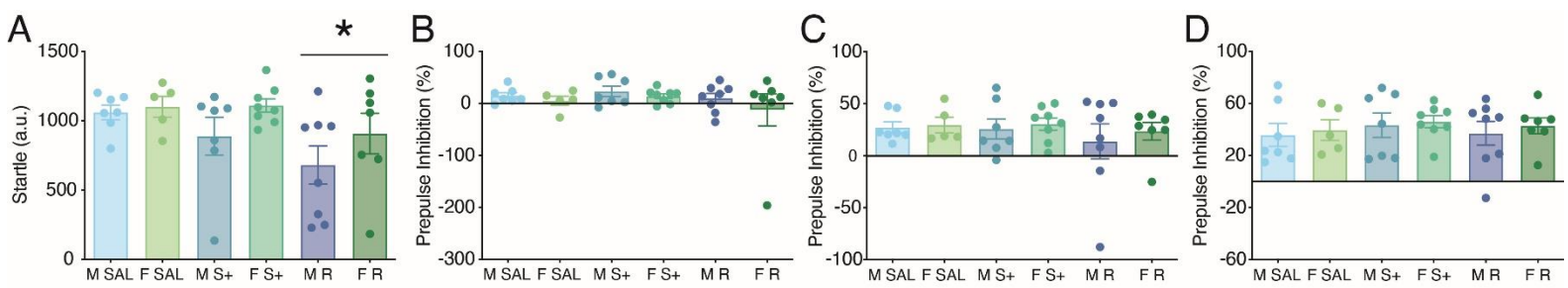
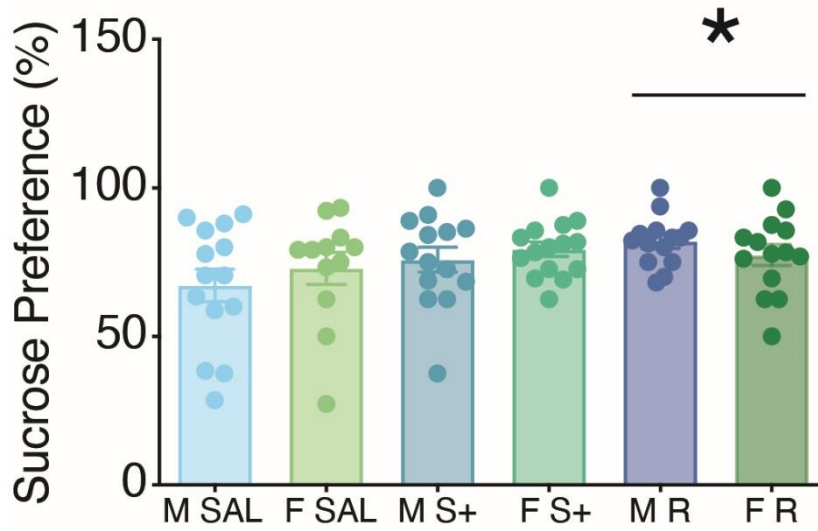
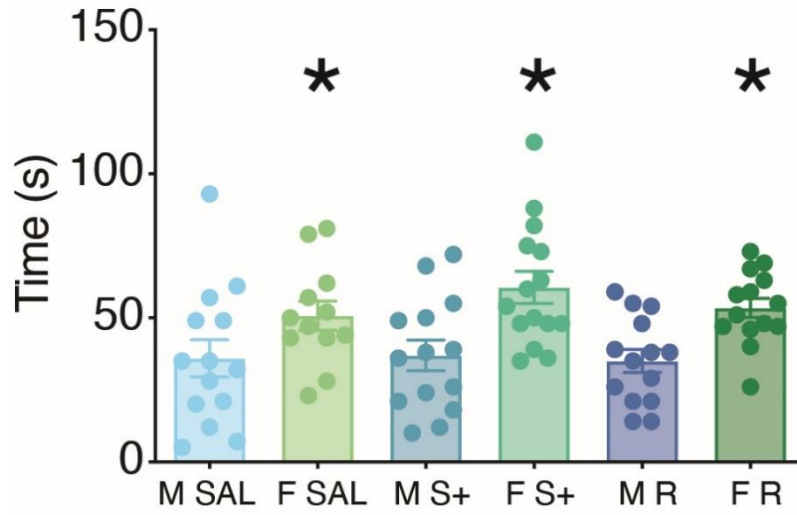
Figure 4 – Velocity and total distance of open field test 1 over time. All groups walked less over time.

Figure 5 - Object location test results. Females explored more objects than males (* $p < 0.05$).

Figure 6 – Sucrose preference test results. KET R groups drank more sugar solution than other groups (* $p < 0.05$).

Figure 7 - Prepulse Inhibition. KET R reduced startle in PPI test (* $p < 0.05$).





3.3. ARTIGO 3: **O tratamento com nitroprussiato de sódio diminui o comportamento do tipo-psicótico em ratas fêmeas, mas não machos, em modelo de esquizofrenia com cetamina**

A esquizofrenia (SZ) é um transtorno mental debilitante grave. A causa da SZ é desconhecida, mas a principal hipótese aponta para anomalias ocorridas durante o neurodesenvolvimento que podem culminar no aparecimento de sintomas durante a adolescência. Os sintomas são geralmente classificados em três grandes categorias: sintomas positivos, sintomas negativos e déficits cognitivos. Um dos fatores mais significativos do transtorno é o sexo, pois apresenta diferenças na epidemiologia, fisiopatologia, resposta ao tratamento e prognóstico entre homens e mulheres. No entanto, poucas pesquisas têm sido destinadas a investigar esta questão. O tratamento atual da SZ é ineficiente, não cobre todos os tipos de sintomas e causa efeitos adversos significativos. Nesse sentido, o canabidiol (CBD) e o nitroprussiato de sódio (NPS) têm demonstrado efeitos interessantes. Aqui, investigamos o tratamento agudo de CBD (30 mg/kg) e NPS (2,5 mg/kg), isoladamente e em combinação, em ratos adolescentes machos e fêmeas em um modelo de psicose com cetamina S+ (KET – 30 mg/kg). Os animais foram testados no teste de campo aberto (CA) e os resultados foram dependentes do sexo. As ratas andaram mais e mais rápido. Além disso, responderam melhor à abordagem terapêutica. O tratamento com NPS e CBD + NPS diminuiu o comportamento psicótico em fêmeas, mas não em ratos machos. Esses resultados vão ao encontro com necessidade de investigar o sexo como variável significativa nos estudos de SZ.

Palavras-chave: Esquizofrenia, cetamina, sexo, canabidiol, nitroprussiato de sódio

Title

Sodium nitroprusside treatment decreases psychotic-like behavior in female but not male rats in a ketamine schizophrenia-model

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ABSTRACT

Schizophrenia (SZ) is a severe debilitating mental disorder. The cause of the SZ is unknown, but the main hypothesis points to anomalies occurring during neurodevelopment that may culminate in the appearance of symptoms during adolescence. Symptoms are generally classified into three broad categories: positive symptoms, negative symptoms, and cognitive deficits. One of the most significant factors of the disorder is sex, as it presents differences in epidemiology, pathophysiology, response to treatment, and prognosis between men and women. However little research has been aimed at investigating this question. The current treatment of SZ is inefficient, does not cover all kinds of symptoms, and causes significant adverse effects. In this sense, cannabidiol (CBD) and sodium nitroprusside (SNP) have demonstrated interesting effects. Here, we investigated the acute treatment of CBD (30 mg/kg) and SNP (2.5 mg/kg), alone and in combination, in adolescent male and female rats in a ketamine (KET – 30 mg/kg) psychosis model. The animals were tested in the open field test (OFT) and the results were sex-dependents. Female rats traveled more and faster. Also, they responded better to the therapeutic approach. SNP and CBD + SNP treatment decreased psychotic-like behavior in females but not in male rats. Those results are in line with the need to investigate sex as a significant variable in SZ studies.

Keywords: Schizophrenia, ketamine, sex, cannabidiol, sodium nitroprusside

INTRODUCTION

Schizophrenia (SZ) causes serious consequences for the quality of life not only of affected patients but also in their socio-family circle (Maibing et al., 2015; Tandon et al., 2009). Furthermore, due to society's inability to incorporate these individuals into income-generating tasks, SZ has a direct impact on the global economy (Hoseinipalangi et al., 2021). The cause of the disorder is still unknown, but the main hypothesis points to anomalies occurring during neurodevelopment that generally culminate in the appearance of first symptoms during adolescence (Eyles, 2021; Fatemi & Folsom, 2009). In fact, adolescent individuals with a high probability of developing SZ form a specific clinical group called the ultra-high risk (UHR) (Fusar-Poli et al., 2017; Sommer et al., 2016). Symptoms are generally classified into three broad categories: positive symptoms, negative symptoms, and cognitive deficits. The first type of symptom includes hallucinations, delusions, and altered perceptions in general. Negative symptoms include social dullness, avolition, and poverty of speech. In the last category, cognitive symptoms include difficulty concentrating, disorientation in time and space, difficulties in executive functions, and mnemonic deficits (Green et al., 2004). Another significant factor of the disorder is sex, as it presents differences in epidemiology, pathophysiology, response to treatment, and prognosis between men and women (Beck et al., 2020; Hill, 2016; Perez et al., 2014).

According to the dopaminergic hypothesis, the most popular to explain SZ cause, positive symptoms occur due to dopaminergic hyperactivation in limbic regions, and negative and cognitive symptoms occur due to dopaminergic hypoactivation in cortical regions. Despite being corroborated by some works, the dopaminergic hypothesis is insufficient to explain the entire disorder (Gomes & Grace, 2021). In this sense, other hypotheses arise in an attempt to complement, such as the glutamatergic hypothesis which states that a disturbance in glutamatergic transmission occurs even before the dopaminergic disturbance (Coyle et al., 2020; Eskenazi et al.,

2021; Frohlich & Van Horn, 2014). The nitrenergic hypothesis, despite being controversial, links the symptoms of SZ with the dysfunction of nitric oxide (NO) neurotransmission (Morales-Medina et al., 2021; Oh & Fan, 2020). Research indicates that the gaps left in explaining the pathophysiology of the disorder are mainly responsible for the failure of current treatments (Gomes & Grace, 2021; Perez et al., 2014).

In general, antipsychotics are currently divided into two large classes, typical (or first generation) and atypical (or second generation). This division occurs due to the differences between the mechanism of action (Kimura et al., 2021; Perju-Dumbrava & Kempster, 2020). In addition to the adverse effects, treatment with antipsychotics is insufficient to act on the triad of SZ symptoms, often being exclusive only to psychotic effects (Carli et al., 2021; Kimura et al., 2021). In this sense, other drugs with different mechanisms of action have demonstrated more comprehensive and relatively safer antipsychotic effects, such as cannabidiol (CBD) and sodium nitroprusside (SNP) (Chesney et al., 2021; Hallak et al., 2013).

CBD is the main non-psychotomimetic compound of *Cannabis sativa* and has shown antipsychotic effects with a safe profile in humans and rodents (Chesney et al., 2021). SNP is known to be a nitric oxide donor used in hypertensive emergencies (Zoupa & Pitsikas, 2021). Despite concerns about the drug due to its high probability of causing toxicity at high doses, NPS has demonstrated antipsychotic efficacy in reversing symptoms in both humans and animals (Hallak et al., 2013; Kandratavicius et al., 2015; Maia- de-Oliveira et al., 2015; Zoupa & Pitsikas, 2021). Interestingly, in clinical studies, it appears that the effectiveness of CBD and SNP as antipsychotics is greater in younger individuals (Stone et al., 2017). Furthermore, due to their different mechanisms of action, the combination of the two drugs becomes an interesting alternative to be investigated (Crippa et al., 2015). Although the isolated effects of the two drugs are promising, little

is known about their efficiency when administered together, and in this sense, animal models become a valuable research tool.

Among the various models to study SZ, the ketamine model (Ketamine - KET) is one of the most used precisely because it can mimic positive and negative symptoms and cognitive deficits (Frohlich & Van Horn, 2014; Pitsikas, 2018). Although the popularity of the KET model (Frohlich & Van Horn, 2014; Kekesi et al., 2015; Nikiforuk & Popik, 2012; Radford et al., 2020; Ram et al., 2013), few studies have proposed to investigate the sex variation on the SZ model, being exclusively for studies in depression and addiction being exclusively for studies in depression and addiction (denBuuse et al., 2015; McDougall et al., 2017; Radford et al., 2020).

Considering the above, here we investigated the effect of KET as a SZ model in male and female rats, and compared the effect of the CBD and SNP administration, alone and in combination, in the open field test (OFT).

METHODS

Animals

All experiments were conducted on male and female adolescent rats (7 to 8 weeks old) of the Wistar Hannover lineage, weighing between 200 and 300 g, from the Medicine of Ribeirão Preto – FMRP/USP. They were housed in a vivarium with a controlled temperature of 22 ± 2 °C and subjected to a 12/12 hour light/dark cycle, with the lights on at 7 am.

Experimental groups and treatments

CBD (BSPG-Pharm, United Kingdom; 30 mg/kg i.p.) was diluted in corn oil). SNP (Cristalia, Brazil; 2.5 mg/kg i.p.) was freshly dissolved in 5% glucose. Esketamine (Cristália; KET - 30 mg/kg, i.p) was dissolved in saline (0.9% sodium chloride). The total volume of all drugs was 1ml/kg. All doses

and experimental protocols were established based on previous work (Kandratavicius et al., 2015; Maia-de-Oliveira et al., 2015; Peres et al., 2018) and pilot experiments.

According to the work proposal and considering the vehicle for the drugs, experimental groups were divided into 8 by sex (KET – CBD+SNP; SAL – CBD+SNP; KET- VEH (oil+glucose5%); SAL- VEH (oil+glucose5%); KET- CBD; SAL – VEH (oil); KET – NPS; SAL – VEH (glucose5%).

Estrous cycle assessment through vaginal cytology

The vaginal smear was collected (8 am-11 am) by dispensing 10 uL of sodium phosphate buffer (PBS; 0.1 M) with a plastic pipette tip into the vaginal cavity and subsequently aspirating the secretion. The smear was pipetted in a glass slide for further identification of the estrous cycle in a bright field microscope (10x and 20x objective lenses). Predominance of nucleated epithelial cells indicated the proestrus phase, while the presence of anucleated cells defined the estrus phase. Metaestrus was recognized by the same proportion among leukocytes, anucleated and nucleated cells, and diestrus by predominance of leukocytes (Marcondes et al., 2002).

Behavioral testing

Open Field Test

The animals were placed in an acrylic arena for 30 minutes in which they moved freely, both before (CA1) and after the injection of KET 5 and CBD NPS (CA2). The acrylic apparatus measures 46 x 46 x 46 cm (height x width x length; Insight- Brazil) and is coupled to 6 bars, with 16 infrared sensors each, which detects and quantifies the animal's locomotion during the 30 minutes test (Seibenhener & Wooten, 2015).

Experimental procedure

We recorded the naïve animal's behavior in OFT 1. Immediately after, they received an injection of KET, 10 minutes later of CBD, and 10 minutes after SNP. After 10 minutes of the last treatment injection (totaling 30 minutes after KET), the animals in the pilot experiment and the females underwent OFT 2. To capture the psychotic-like behavior in males, they were subjected to OFT 2 immediately after the injection of KET. The vaginal cytology of female rats was verified every day (figure 1).

Statistical Analysis

Shapiro-Wilk and Levene's tests were used to evaluate whether the data were parametric. Data from pilot study were analyzed by Independent t-test or Mann-Whitney. OFT data were analyzed by one-way ANOVA or one-way repeated measures ANOVA (independent groups). Multiple comparisons were performed using Bonferroni post hoc test. All results are expressed as mean \pm SEM (standard error of mean), and statistical significance was set at $p < 0.05$.

RESULTS

Sex and administration time differences of the ketamine treatment

To assess whether the psychotic-like effect of the KET had a difference between the sexes, we administered saline to females (F SAL n=16), to males (M SAL (1) n=7; M SAL (2) n=8), ketamine in females (F KET n=16) and in males (M KET (1) n=7; MKET (2) n=12). The drug showed a difference between the sexes, both in the time of action and in the final effect.

When measuring the action of KET 30 minutes after its administration in males, it did not result in hyperlocomotion in the open field test, on the contrary, the group treated with SAL walked more than the group treated with KET (pilot 1, M SAL (1) x M KET (1), Independent t-test ($t(12) =$

3.144; $p= 0.008$). Only when we evaluated locomotion immediately after the injection, did the hyperlocomotion appear (pilot 2, M SAL (2)), Mann-Whitney ($U = 18$; $p=0.021$); figure 2).

In contrast, females not only showed hyperlocomotion behavior 30 minutes after KET injection but also walked more than those males who showed hyperlocomotion (independent groups, F SAL X F KET X M SAL(2)), 1-WAY ANOVA [$F(3,48) = 13.257$; $p<0.001$], Bonferroni post hoc points out that the F KET group walked the most, figure 3).

Due to the results mentioned above of the difference in response between the control groups of males and females, we choose to separate them to evaluate the effect of treatments with CBD and/or SNP.

SNP and CBD+SNP acute treatment decreased positive-like behaviors in females

Treatment with SNP and treatment with CBD+SNP reversed the deficits caused by KET in the open field test in females (SAL VEH $n=16$, SAL CBD $n=8$, SAL SNP $n=8$, SAL CBD+SNP $n=8$, KET VEH $n=16$, KET CBD $n=9$, KET SNP $n=8$, KET CBD+SNP $n=10$).

This effect was observed when analyzing the total distance traveled (independent groups, SAL VEH X SAL CBD X SAL SNP X SAL CBD+SNP X KET VEH X KET CBD X KET SNP X KET CBD+SNP; One-way ANOVA [$F(7,75) = 9.178$; $p<0.001$]. Post hoc points out that the SAL VEH group was only different from the KET VEH and KET CBD groups, while the KET VEH group was different from all other groups except KET CBD, figure 4). When we analyzed the distance traveled over time (every 5 minutes), the same result was observed (independent groups SAL VEH X SAL CBD X SAL SNP X SAL CBD+SNP X KET VEH X KET CBD X KET SNP X KET CBD+SNP, One-way ANOVA repeated measures [$F(13.324, 142.761) = 2.321$; $p=0.007$]. Bonferroni show that the SAL VEH group was different only from the KET VEH e KET CBD groups. KET VEH was different from all of them except

KET CBD group, figura 4). Over time, all groups decreased locomotion (1-way ANOVA repeated measures [F(1,903,142,761) = 11,444; p<0.001], figure 4).

The same pattern was observed when we analyzed velocity. Both total velocity (SAL VEH X SAL CBD X SAL SNP X SAL CBD+SNP X KET VEH X KET CBD X KET SNP X KET CBD+SNP, ANOVA 1 VIA [F(7,75) = 9.178; p<0.001]; post hoc pointing out that the SAL VEH group was only different from the KET VEH and KET CBD groups; while the KET VEH group was different from all other groups except KET CBD, figure 5), as well as velocity over time. The one-way ANOVA repeated measures (SAL VEH X SAL CBD X SAL SNP X SAL CBD+SNP X KET VEH X KET CBD X KET SNP X KET CBD+SNP, [F(13.325, 142,768) = 2.322; p=0.007], Bonferroni points out that the SAL VEH group was only different from the KET VEH and KET CBD groups, while the KET VEH group was different from all other groups except KET CBD, figure 5). Over time, all groups decreased velocity (one-way ANOVA repeated measures [F(1,904,142,768) = 11,445; p<0.001], figure 4).

SNP and CBD+SNP acute treatment did not decrease positive-like behaviors in males

In contrast, none of the proposed treatment types was enough to reduce the KET deficits in males (SAL VEH (2) n=8, SAL CBD n=10, SAL SNP n=10, SAL CBD+SNP n=11, KET VEH (2) n=12, KET CBD n=11, KET SNP n=11, KET CBD+SNP n=12).

The absence effect was observed when analyzing the total distance traveled SAL VEH X SAL CBD X SAL SNP X SAL CBD+SNP X KET VEH X KET CBD X KET SNP X KET CBD+SNP, ANOVA 1 VIA [F(7,77) = 5,881; p<0,001]; post hoc showed that KET groups showed hyperlocomotion in comparison to SAL groups, figura 6). When we analyzed the distance traveled over time, the same lack of effect was observed SAL VEH X SAL CBD X SAL SNP X SAL CBD+SNP X KET VEH X KET CBD X

KET SNP X KET CBD+SNP, one-way ANOVA repeated measures [$F(20.608,226,689) = 1.661; p=0.039$]; post hoc showed that KET groups traveled more than SAL ones, figura 6).

The same response pattern was observed when we analyzed velocity. In the total velocity parameter (SAL VEH X SAL CBD X SAL SNP X SAL CBD+SNP X KET VEH X KET CBD X KET SNP X KET CBD+SNP, one-way ANOVA [$F(7,77) = 5,640; p<0,001$]; post hoc Bonferroni points that KET groups traveled faster than the SAL ones figura 6). As well as in the velocity over time parameter ($[F(20.874,229,616) = 1.451; p=0.097$, figure 6). In general, all groups decreased the velocity over time ($[F(2.982,229,616) = 26.319; p<0.001]$) figure 6.

The reproduction hormones interfere with locomotion in females

The sexual hormones by themselves altered the locomotion of females in open field 1 (F proestrus n=16, F estrus n=17, F metestrus n=17, F diestrus n=23), without treatment yet. We observed that naïve females that were during the estrous cycle phase of metestrus and diestrus walked more than naïve females that were in proestrus and estrus (F proestrus + F estrus x F metestrus + F diestrus, independent t-test ($t(71) = -2.561; p=0.013$). Likewise, the analysis showed that only females in the proestrus group walked more than those in the diestrus group (F proestrus x F diestrus, independent t-test ($t(37) = -2.426; p=0.020$) figure 8.

DISCUSSION

Considering the relevance of searching new treatments for SZ as well as taking into account sex differences, we investigated the acute effect of CBD and/or SNP injections in a KET SZ-model in male and female rats. Their behavior was performed in the open field test. We noticed significant differences between male and female behaviors, not only in the chosen model itself but also in the proposed treatments.

In literature, studies proposed to investigate the effect of the sex variable in the KET antidepressive therapeutic approach (Zhang and Hashimoto, 2018; Hashimoto, 2020) as well as its addictive potential (McDougall et al., 2017; 2019). Despite being a very popular SZ model, few studies have included female subjects in their research (Hill, 2016). Abelaira and collaborators found that after the maternal deprivation stress for 10 days, significant deficits appeared in the open field and forced swimming tests. Also, a significant increase in oxidative stress meters. Acute treatment of 15 mg/kg KET reversed these deficits in males, but not in females (Abelaira et al., 2021). Even with KET subchronic treatment (for 7 days - 5 mg/kg), Ladesma-Corvi et al observed clear differences between the sexes in the antidepressant-like response, which occurred only in adult male rats and was practically ineffective in female rats (Ladesma-Corvi et al., 2023). Similar to our results, other studies show that females rats presented higher locomotor activity (Frye et al., 2000; Neill et al., 2010; Hill, 2016), but it was not investigated in a KET model of SZ.

Here SNP and CBD + SNP treatments reversed psychotic-like behavior in females but not in males. Interestingly, from a translational point of view, women respond better to available pharmacological treatments than men (Hill et al., 2014; Hill, 2016). As the SNP is present in both administration regimes, it is probably the main responsible for the therapeutic effect.

Considering the damage that occurred in the nitric oxide (NO) metabolism in the pathophysiology of SZ, hypotheses have emerged about the therapeutic effect of NO donors, such as SNP (Morales-Medina et al., 2021; Oh & Fan, 2020). Our group was the first to test this hypothesis. In 2013, we published a study in which we found that just a single infusion of SNP was enough to reduce the three types of SZ symptoms (Hallak et al., 2013). Later studies corroborated this finding (Maia-de-Oliveira et al., 2014; 2015), others did not (Brown et al., 2019). It is currently believed that the therapeutic effect found with the SNP depends on variables such as duration of disorder, smoking, and age (Zoupa and Pitsikas, 2021). Precisely because there was no consensus, studies in

animal models were performed to help elucidate its mechanism of action (Maia-de-Oliveira et al., 2015; Kandratavicius et al., 2015).

Maia-de-Oliveira and collaborators observed that pretreatment with SNP reversed KET hyperlocomotion for up to one week after administration (Maia-de-Oliveira et al., 2015). Another study demonstrated that treatment with SNP during adolescence managed to prevent behavioral abnormalities in adulthood elicited by the SHR model. Parameters that mimicked positive, negative and cognitive symptoms were reversed (Diana et al., 2018). Other studies demonstrated that both pre- and treatment reduced hyperlocomotion behavior but not cognitive deficits (Kandratavicius et al., 2015). Using another NMDA receptor antagonist, MK 801, Wang and colleagues reported that SNP pretreatment decreased positive-like behaviors (Wang et al., 2019). It is interesting to highlight that our unpublished data also demonstrate the SNP antipsychotic-like effect on hyperlocomotion in females and on cognitive-like symptoms in both sexes (submitted manuscript).

The amount of sexual hormones itself interferes with behavior (Hill, 2016). Here, in the estrous cycle phases that have more estrogen (proestrus and estrus), naïve females traveled less compared to females that were in metestrus and diestrus. Interestingly, Radford and colleagues also observed during proestrus and estrus that females showed more psychotic-like behavior, but were under the effect of 5 mg/kg of KET and were adult females (here we used adolescent animals) (Radford et al., 2020). In contrast, other work observed an increase in the dopaminergic neuron activity during the estrus phase and a decrease in metestrus in females subjected to the MAM model. They believed that this effect may be mediated in part by progesterone in the ventral hippocampus, as increases in the activity of the dopaminergic neuron population (observed in estrus) were normalized by intrahippocampal administration of the progesterone receptor antagonist mifepristone (but not of estrogen receptor antagonists, fulvestrant) (Perez et al., 2014).

Conclusion

Our data corroborate the importance of including female subjects in the search for new SZ treatments, considering that there was a difference not only in the proposed model (KET) but also in the treatments studied (CBD and SNP). Also, the sex parameter could be included in the discussion of SNP therapeutic efficacy. More studies are needed.

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CONFLICT OF INTEREST

JEH is coinventor of the patent “Cannabinoid-containing oral pharmaceutical composition, method for preparing and using same,” INPI on September 16, 2016 (BR 112018005423-2). JEH is coinventor of the patent “Fluorinated CBD compounds, compositions and uses thereof. Pub. No.: WO/2014/108899. International Application No.: PCT/IL2014/050023,” Def. US number Reg. 62193296; July 29, 2015; INPI on August 19, 2015 (BR1120150164927; Mechoulam R, Zuardi AW, Kapczinski F, Hallak JEC, Guimarães FS, Crippa JAS, Breuer A). Universidade de São Paulo (USP) has licensed this patent to Phytects Pharm (USP Resolution No. 15.1.130002.1.1) and has an agreement with Prati-Donaduzzi to “develop a pharmaceutical product containing synthetic CBD and prove its safety and therapeutic efficacy in the treatment of epilepsy, schizophrenia, Parkinson’s disease, and anxiety disorders”.

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FIGURES LEGENDS

Figure 1 – Experimental design.

Figure 2 – Pilot experiment in males. A. 30 minutes after injection SAL group traveled more than KET group. **B.** KET showed hyperlocomotion immediately after injection. * $p < 0.05$.

Figure 3 – Comparison between male and female pilot experiment. Females KET group traveled more than males and females SAL group. * $p < 0.05$.

Figure 4 – Total distance in females. SNP and CBD+SNP decreased hyperlocomotion in females. * $p < 0.05$.

Figure 5 – Velocity in females. SNP and CBD+SNP decreased velocity in females. * $p < 0.05$.

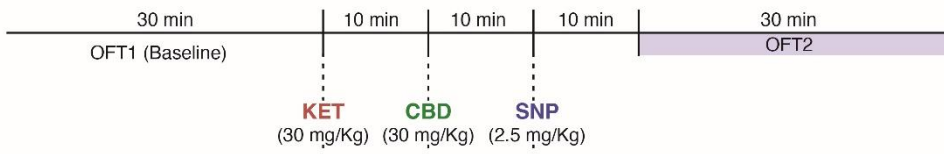
Figure 6 – Total distance in males. KET groups traveled more than other groups. ** $p < 0.05$.

Figure 7 - Velocity in males. KET groups traveled faster than other groups. ** $p < 0.05$.

Figure 8 – Estrous cycle in locomotion. Naïve females in metestrus and diestrus phase walked more than females in proestrus and estrus.

FIGURES

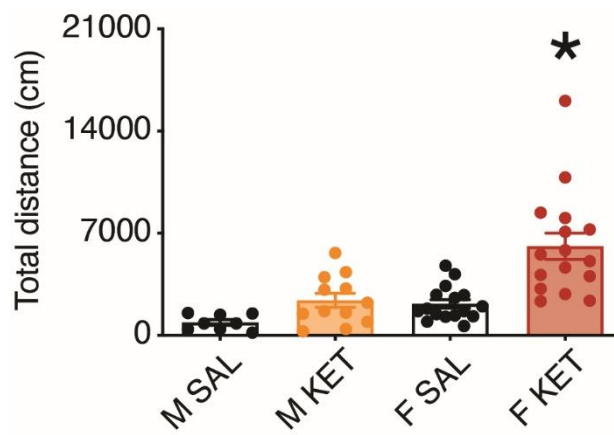
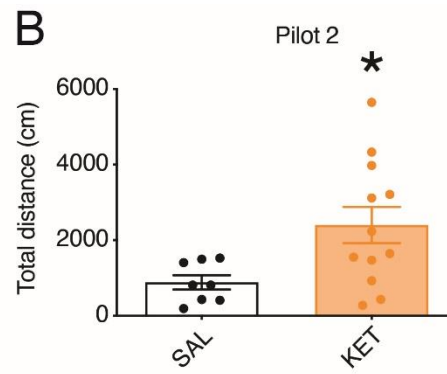
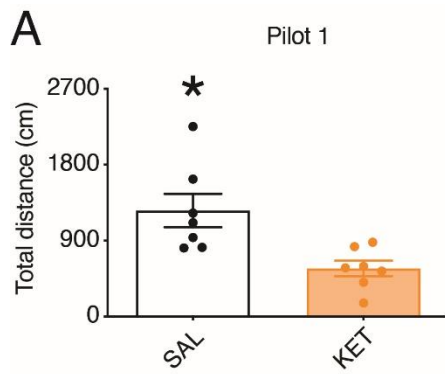
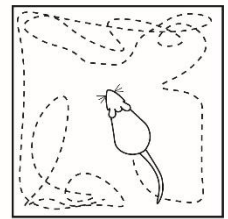
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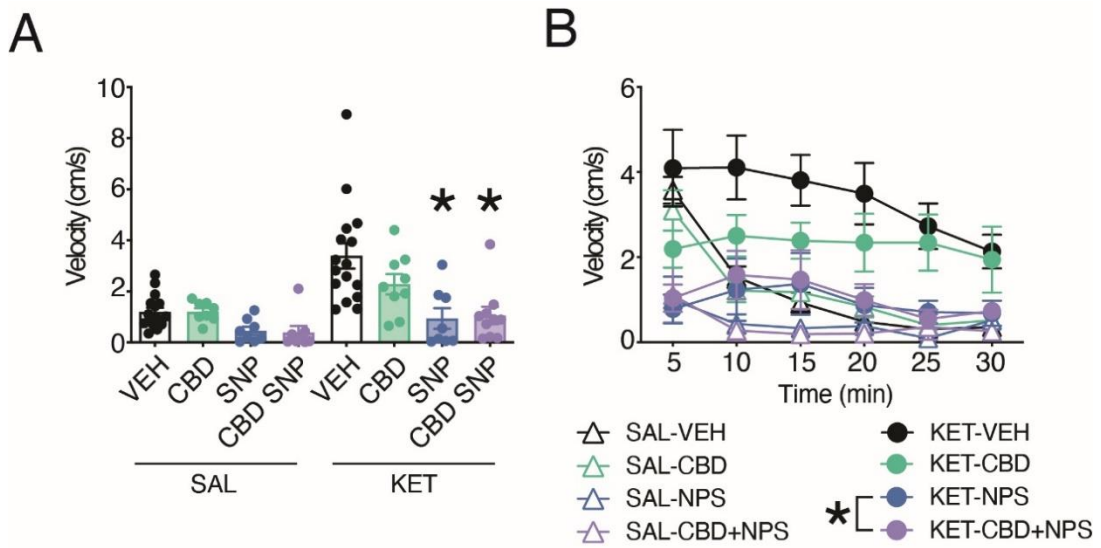
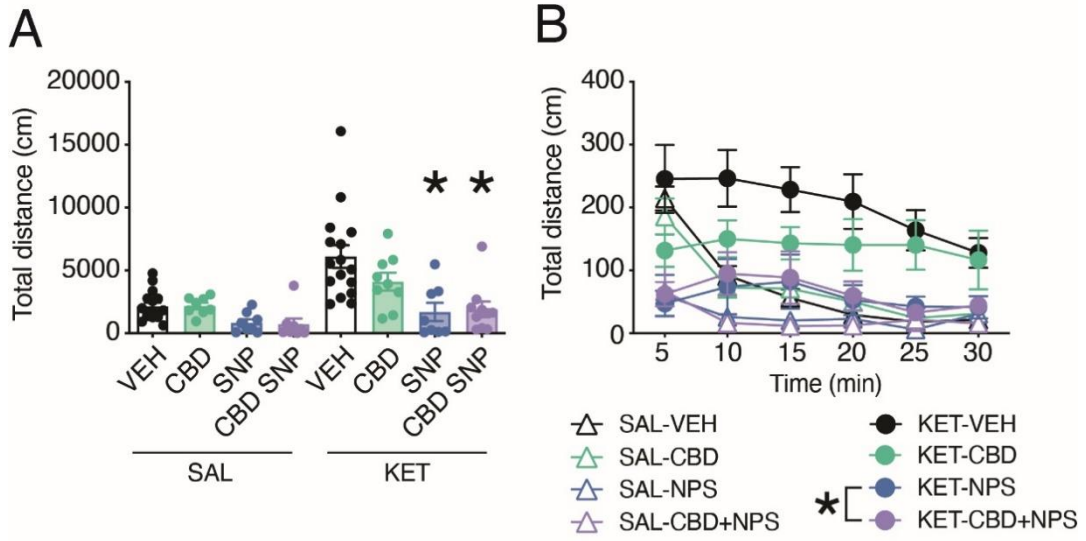


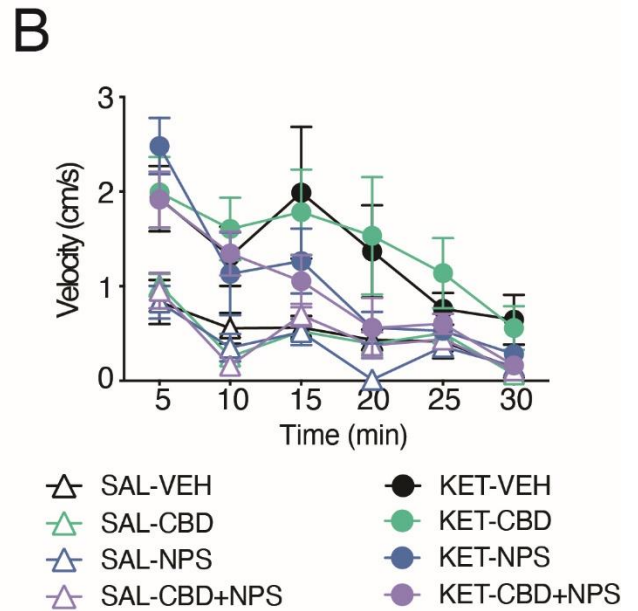
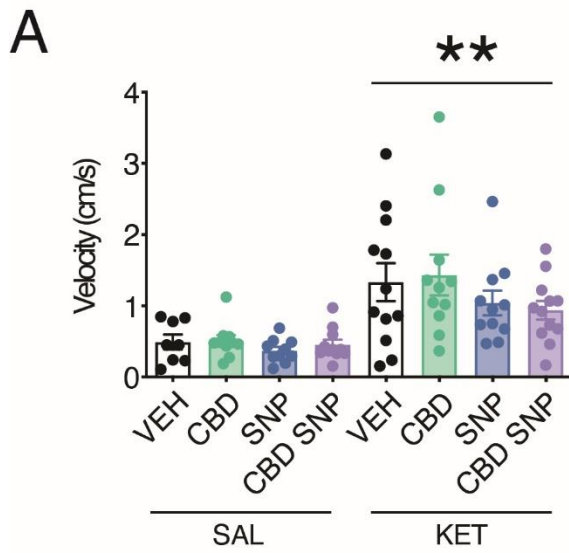
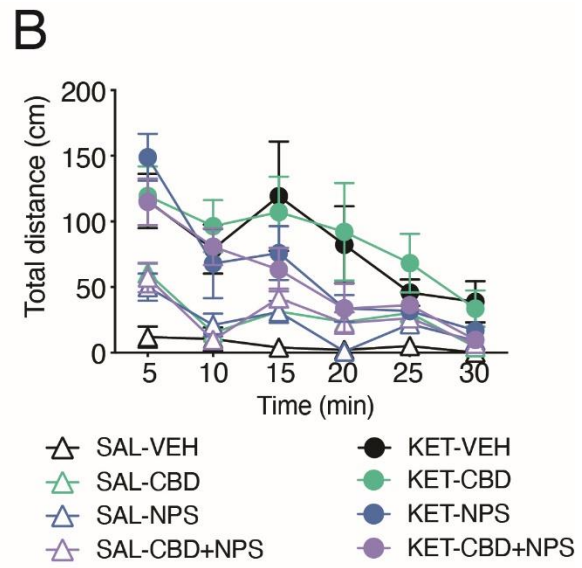
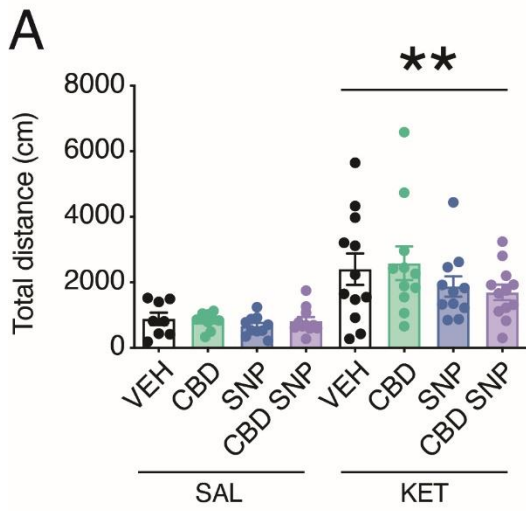
Protocol 2

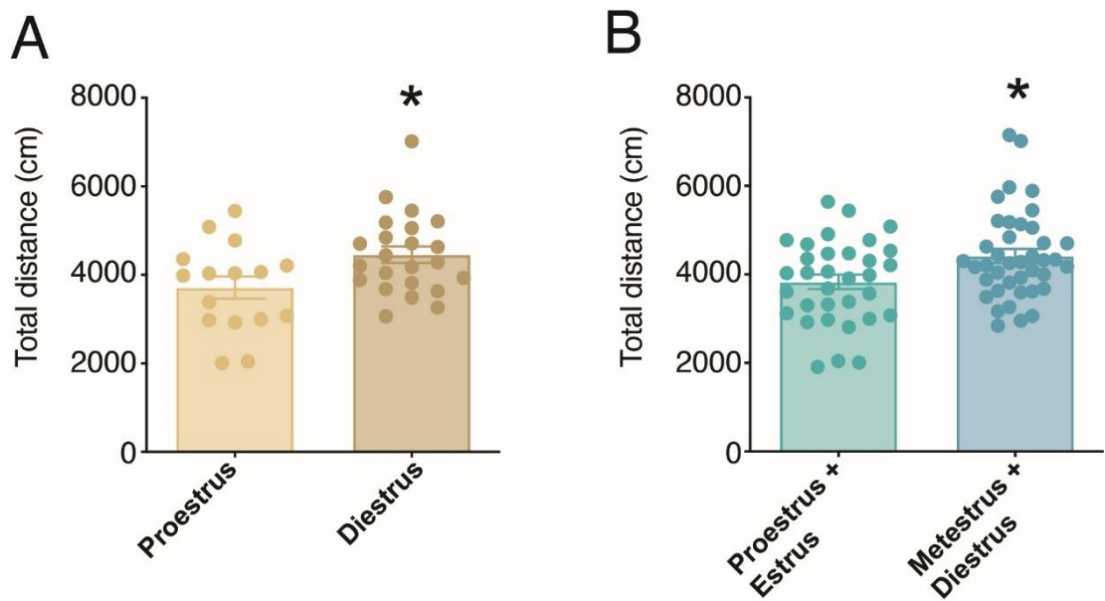


Open Field Test









4. CONCLUSÕES

- Existem diferenças comportamentais significativas entre ratos machos e fêmeas. Desde o modelo de SZ proposto (KET) até os pré e tratamentos estudados (CBD e NPS).
- O regime de pré-tratamento com CBD e NPS durante o neurodesenvolvimento proposto no primeiro artigo, é uma ferramenta promissora nos estudos de modelos animais de SZ.
- A junção de CBD + NPS diminuiu comportamento do tipo-cognitivo além do comportamento do tipo-positivo, o que confirma seu potencial terapêutico mais abrangente que os antipsicóticos disponíveis.

- Apesar da teoria, na prática não houve diferença entre a KET racêmica e a KET S em mimetizar comportamento do tipo-SZ.
- Considerando o pioneirismo do trabalho, bem como suas limitações, mais estudos são necessários.

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6. ANEXO

A FAPESP destina um espaço chamado “dificuldades do período” em seus relatórios semestrais. Com a liberdade concedida a mim neste anexo, vou fazer o mesmo por aqui.

Ninguém prometeu que seria fácil...

Confesso que me pego um pouco amarrotada saindo deste processo e o mais bizarro é perceber que não sou exceção. Os níveis de transtornos mentais na pós-graduação são em média três vezes maiores que na população em geral. Se você está saindo da *stricto sensu* sem ao menos um ISRS, você não viveu a pós-graduação direito. Desvalorização financeira e insegurança com o futuro próximo é rotina para o pesquisador brasileiro. Vivemos com o pior dos dois mundos, nem o direito trabalhista do CLT nem a liberdade do liberal. Assinamos um contrato de dedicação exclusiva e é isso, trabalho de domingo a domingo, inglês em dia e ser mais *multitask* que a Alexa. Tirando a experiência de conviver em um dos meios mais egocêntricos do mundo profissional, “inteligência atrai vaidade” diz o poeta, então pisamos em ovos o tempo todo para não ofender os “Deuses do Olimpo”. Ainda que eu tive o privilégio do privilégio de um orientador que confia e respeita o meu trabalho, mas com a grande maioria acontece justamente o contrário.

Para além dos desafios universais tiveram também as provações individuais. Iniciei meu doutorado em 2018 com o projeto intitulado “Avaliação comportamental, eletrofisiológica e molecular do óxido nítrico no modelo do desamparo aprendido em ratos”. Cheios de entusiasmo fizemos milhões de planos para o projeto. Técnicas moleculares e eletrofisiológicas que só a FMRP poderia proporcionar (afinal vim para USP para aprendê-las) estavam no pacote. Durante quase 1 ano realizamos os experimentos piloto que foram suficientes para garantir uma bolsa de doutorado FAPESP. Perfeito, até estourar a fatídica bomba da ração adulterada dos animais provenientes do biotério central. Então, na impossibilidade de confiar nos dados obtidos em ratos

subnutridos, nos propomos a reproduzir os resultados encontrados nos experimentos piloto, mas em outra linhagem de ratos. Se tem uma lição que aprendi na pós-graduação, é que reproduzir dados é mais difícil que ganhar na mega da virada. Como previsto, apesar dos nossos esforços, os novos *hannover* não aprenderam a tarefa e não reproduziram o comportamento antes obtido. No meio dessas mudanças de protocolos e idas e vindas na literatura, estourou a Pandemia da COVID-19 e ficamos meses afastados do laboratório. Concomitante ao caos universal que enfrentávamos minha vida virou de cabeça para baixo com o adoecimento da minha Mãe.

Diabética, fazia um tempo que reclamava de uma ferida no dedo mindinho do pé, que doía e não cicatrizava. Com a anuência e o apoio do Jaime me afastei de Ribeirão e fui para Ilha Solteira cuidar dela, pois passaria por cirurgia de desobstrução dos vasos sanguíneos. A primeira cirurgia não deu o resultado que ansiávamos. Nem a primeira, nem a segunda, nem a terceira e nem a quarta. Após duas amputações na perna esquerda a infecção do coto foi para a corrente sanguínea. Septicemia foi a *causa mortis*, diz a certidão de óbito. O que seria dias de afastamento virou meses e o luto é um companheiro incômodo desde então.

Ao retornar para Ribeirão o aparato que utilizávamos para verificar o comportamento dos animais havia quebrado. Além do que outras pessoas estavam na fila para utilizá-lo e tinham prioridade devido a seus prazos que estavam mais próximos que o meu. Foi o momento em que decidimos “trocar de projeto”. Baseado no trabalho que eu havia participado tivemos a ideia do “Avaliação do tratamento agudo com canabidiol e nitroprussiato de sódio em ratos machos e fêmeas em modelo de psicose induzida por cetamina S+: caracterização comportamental e molecular”. Com a aprovação da FAPESP iniciamos outra vez um experimento piloto. De novo a ausência da reprodução dos dados (pleonasma) nos tirou o sono. Ratos machos não apresentaram hiperlocomoção 30 minutos após administração da cetamina, como afirmava a literatura. Mas

dessa vez a incapacidade em reproduzir os dados acabou sendo algo providencial, pois enfatizou a importância da nossa questão da diferença entre sexos para estudar modelos de esquizofrenia.

Quando tudo corria relativamente dentro da normalidade, alguns fatores me pegaram de surpresa. A morte repentina do meu tio, junto com um término de relacionamento conjugal, mais duas desocupações inesperadas de imóveis, e encerramento da bolsa FAPESP; resultaram no diagnóstico que tardei a buscar: transtorno depressivo maior com crises do pânico. Experimentei a angústia de saber na teoria o que estava passando, mas não conseguir fazer nada com esse conhecimento. Decorar as indicações do DSM-5 e o mecanismo de ação dos psicotrópicos teve pouco efeito terapêutico. Não diminuiu a culpa paralisante nem o retardo psicomotor que me impediu de frequentar o laboratório durante meses. Interessante que durante anos briguei contra o preconceito com os transtornos da mente, mas quando chegou a minha vez, quase demorei tempo demais para buscar ajuda.

Sei que aqui não é o tópico de agradecimento mas preciso encerrar, exaltando a importância que a minha família, amigos, Jaime, Dr. José Augusto, Lígia Zampronio, Marie Curie (canina), exercícios físicos e os óleos essenciais tiveram na minha recuperação. Graças a eles e a mim mesma uso o pretérito perfeito para descrever o que vivi, *"the dogs days are over"*. Percebi que tirar ensinamentos das tragédias não só nos deixa mais fortes como também mais sábios. Por isso saio mais sábia dessa experiência grandiosa que foi o doutorado. Hoje eu concordo com o Pessoa: ***valeu a pena pois minha alma nunca foi pequena.***