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NEUROESTEROIDES E MICRORNAS NA DEPRESSÃO: marcadores comportamentais e biológicos em uma abordagem pré-clínica

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NEUROESTEROIDES E MICRORNAS NA DEPRESSÃO:
marcadores comportamentais e biológicos em uma abordagem pré-clínica

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RESUMO

A depressão é um transtorno altamente prevalente e incapacitante de etiologia tanto ambiental quanto hereditária. Devido ao seu diagnóstico se basear na comunicação subjetiva de sintomas, há um grande esforço na busca por biomarcadores que auxiliem no diagnóstico deste e de outros transtornos associados, como o transtorno do estresse pós-traumático (TEPT). Esta busca se concentra em moléculas que participem da neurobiologia dos transtornos depressivos, que certamente envolve sistemas que vão além da teoria serotonérgica proposta a partir do mecanismo de ação dos agentes antidepressivos mais utilizados atualmente. Neuroesteroides como a alopregnanolona, proteínas neurotróficas como o BDNF e microRNAs como o miR-144-3p são algumas das classes de moléculas com relevância na neurobiologia da depressão. Este trabalho teve como objetivo avaliar o potencial de neuroesteroides, proteínas neurotróficas e microRNAs como biomarcadores de transtornos depressivos. Para isto, foram desenvolvidas três revisões de literatura, um capítulo metodológico e um manuscrito experimental apresentados neste trabalho. As revisões de literatura demonstram que a) neuroesteroides como a alopregnanolona e proteínas neurotróficas como o BDNF encontram-se reduzidos em modelos animais de depressão, mediando o aparecimento de comportamentos tipo-depressivos; b) neuroesteroides e proteínas neurotróficas são promissores candidatos a biomarcadores de transtornos depressivos e TEPT; e c) os níveis reduzidos de alopregnanolona encontrados na depressão e TEPT fazem parte da desregulação da resposta ao estresse mediada pelo eixo HPA. Subsequentemente, o processo de implementação da metodologia de cromatografia líquida acoplada à espectrometria de massas em tandem para a detecção da alopregnanolona e seus isômeros é descrito e os resultados parciais são apresentados. Por fim, ratos machos e fêmeas foram cruzados seletivamente com base em alta ou baixa expressão de comportamento tipo-depressivo (imobilidade no teste do nado forçado) por três gerações, recebendo na última geração tratamento com diferentes doses de fluoxetina e tendo a expressão do miR-144-3p quantificada no sangue. Os resultados encontrados demonstram um efeito tipo-antidepressivo em todos os animais, além de apontar que os níveis do microRNA 144-3p no sangue de ratos está correlacionado com comportamentos tipo-depressivos, particularmente em machos não tratados da linhagem de baixa imobilidade. Os resultados deste trabalho permitem concluir que neuroesteroides, proteínas neurotróficas e microRNAs são biomarcadores promissores de transtornos depressivos. Estudos subsequentes de validação clínica são necessários para sua eventual implementação na prática clínica.

Palavras-chave: depressão, biomarcadores, neuroesteroides, fator neurotrófico derivado do encéfalo, microRNAs, inibidores seletivos de recaptação de serotonina

ABSTRACT

Depression is a highly prevalent and incapacitating disorder with environmental and hereditary etiology. Because its diagnosis is based on the subjective communication of symptoms, there is a great effort in the pursuit of biomarkers that can aid in the diagnosis of this and associated disorders such as the posttraumatic-stress disorder (PTSD). This search is focused on molecules that participate in the neurobiology of depressive disorders, which certainly involves systems beyond the serotonergic theory proposed based on the mechanism of action of the most currently prescribed antidepressive agents. Neurosteroids such as allopregnanolone, neurotrophic proteins such as BDNF, and microRNAs such as miR-144-3p are some of the classes of molecules with relevance in the neurobiology of depression. This work aimed to evaluate the potential of neurosteroids, neurotrophic proteins, and microRNAs as biomarkers of depressive disorders. To achieve this goal, three literature reviews, one methodological chapter and one experimental manuscript were developed and are presented in this work. The literature reviews show that a) neurosteroids such as allopregnanolone and neurotrophic proteins such as BDNF are reduced in animal models of depression, mediating the emergence of depressive-like behaviors; b) neurosteroids and neurotrophic proteins are promising candidate biomarkers for depressive disorders and PTSD; and c) reduced allopregnanolone levels found in depression and in PTSD are part of the dysregulation of the stress response mediated by the HPA axis. Subsequently, implementation of the liquid chromatography coupled with tandem mass spectrometry methodology for the detection of allopregnanolone and its isomers is described, and partial results are presented. Finally, male and female rats were selectively bred based on high or low expression of depressive-like behavior (immobility in the forced swim test) for three generations, being then treated with fluoxetine and having the blood expression of miR-144-3p quantified. Results found show an antidepressant-like effect in all animals and point that blood levels of the microRNA 144-3p are correlated with depressive-like behaviors, particularly in untreated males from the low immobility strain. The results of this work allow to conclude that neurosteroids, neurotrophic proteins, and microRNAs are promising biomarkers for depressive disorders. Subsequent studies of clinical validation are needed for their eventual implementation in the clinical practice.

Key words: depression, biomarkers, neurosteroids, brain-derived neurotrophic factor, microRNAs, selective serotonin reuptake inhibitors

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LISTA DE ABREVIATURAS E SIGLAS

ANOVA	Análise de variância
BDNF	*Brain-derived neurotrophic factor*
CEUA	Comissão em Ética no Uso de Animais em Pesquisa
DSM	*Diagnostic and Statistical Manual of Mental Disorders*
HPLC	*High performance liquid chromatography*
HSD	Hidroxiesteroide desidrogenase
ISN	*International Society for Neurochemistry*
ISRS	Inibidores seletivos da recaptação da serotonina
ISSN	*International Standard Serial Number*
LC-MS/MS	Cromatografia líquida com espectrometria de massas em tandem
miRNA	microRNA
mRNA	RNA mensageiro
MTBE	Éter metil-terc-butílico
TEPT	Transtorno do estresse pós-traumático
UHPLC	*Ultra-high-performance liquid chromatography*
UPLC	*Ultra performance liquid chromatography*

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PARTE I – INTRODUÇÃO E OBJETIVOS

I.1 INTRODUÇÃO

I.1.1 DEPRESSÃO

A saúde mental é uma preocupação crescente no Brasil e no mundo, com altos investimentos em políticas de saúde pública sendo alocados para a prevenção e o tratamento de transtornos mentais nas últimas décadas. A depressão é um dos principais transtornos mentais que acometem a população mundial, tendo a maior prevalência entre os transtornos do humor e gerando grande perda de qualidade de vida e funcionalidade (FERRARI et al., 2022). Ainda, estima-se que tenha havido um aumento significativo na incidência de transtornos mentais nos últimos anos em função da pandemia de COVID-19 (SANTOMAURO et al., 2021). No Brasil, há evidência epidemiológica indicando uma alta taxa de prevalência de ao menos um episódio depressivo ao longo da vida, especialmente comparado com outros países em desenvolvimento (BROMET et al., 2011). Acomete indivíduos principalmente entre os 20 e 40 anos de idade, e a taxa de prevalência é quase duas vezes maior em mulheres do que em homens (KESSLER; BROMET, 2013). A depressão reduz significativamente a expectativa de vida dos indivíduos acometidos principalmente devido ao risco de suicídio (CASSANO; FAVA, 2002), além de contribuir para a piora da saúde em outros sistemas, aumentando, por exemplo, o risco do desenvolvimento de doenças cardiovasculares (SWARDFAGER et al., 2010).

A depressão é caracterizada por um sentimento muito forte de tristeza e desesperança que pode durar meses ou anos, potencialmente levando indivíduos deprimidos a sentir que a vida não vale a pena ser vivida (FERRARI et al., 2013). A manifestação clínica da depressão, diagnosticada a partir de um conjunto de critérios estruturados com duração bem definida e realizada por profissional capacitado é denominada “transtorno depressivo maior” —ou, alternativamente, simplesmente “depressão maior” (BENTLEY; PAGALILAUAN; SIMPSON, 2014). O transtorno depressivo maior é provavelmente o mais conhecido e prevalente dos “transtornos depressivos”, grupo de condições clínicas distintas que consistem em manifestações depressivas diagnosticáveis com diferente intensidade, sintomatologia específica, etiologia e/ou contexto temporal. Alguns destes transtornos depressivos incluem o “transtorno depressivo persistente” (anteriormente conhecido como “distimia”) e o “transtorno disfórico pré-menstrual” (UHER et al., 2014).

I.1.1.1 Diagnóstico dos transtornos depressivos

A quinta e mais recente edição do Manual Diagnóstico e Estatístico de Transtornos Mentais (*Diagnostic and Statistical Manual of Mental Disorders*; DSM) reúne os critérios clínicos mais atualizados para o diagnóstico de transtornos depressivos. O transtorno depressivo maior é diagnosticado quando um indivíduo apresenta, durante um período contínuo de no mínimo duas semanas, um dos dois sintomas centrais (humor depressivo e anedonia) acompanhado de pelo menos mais três sinais ou sintomas secundários que incluem mudanças no apetite ou peso, alterações no sono, retardo ou agitação psicomotora, fadiga, sentimento de culpa ou desvalorização e dificuldade de concentração (BENTLEY; PAGALILAUAN; SIMPSON, 2014). O transtorno depressivo maior possui subtipos clinicamente definidos, como o “transtorno depressivo maior com início perinatal”, mais conhecido como “depressão pós-parto”. O DSM-5 ainda especifica o diagnóstico do transtorno depressivo persistente como a presença de humor deprimido na maior parte do dia, presente na maioria dos dias durante um período de dois anos ininterruptos (sem ausência de sintomas por mais de 2 meses) ou mais acompanhado de ao menos mais dois sintomas secundários (UHER et al., 2014).

I.1.1.1.1 Diagnóstico diferencial com outros transtornos

Apesar destes critérios diagnósticos terem sido clinicamente validados e nortearem a prática clínica na psiquiatria, é notável que a sintomatologia do transtorno depressivo maior se confunde com a de outros transtornos depressivos, além de possuir ampla sobreposição com o conjunto de sintomas característicos dos transtornos bipolares (HIRSCHFELD, 2014), transtornos da ansiedade (SHEN et al., 2022) e com o transtorno do estresse pós-traumático (TEPT) (GROS et al., 2012). O caso do TEPT é de particular importância pois apresenta alta comorbidade com o transtorno depressivo maior (THAIPISUTTIKUL et al., 2014). Apesar de o TEPT estar classificado em uma categoria diferente no DSM-5 e possuir diversos sintomas em grupos relacionados a pensamentos intrusivos e comportamentos de evitação, outros grupamentos sintomáticos relacionados a alterações negativas na cognição e humor

(MIAO et al., 2018) se assemelham a sintomas presentes no transtorno depressivo maior—conforme pode ser observado na Figura I.1.

Figura I.1 – Esquema representando a sobreposição sintomática entre o transtorno depressivo maior e o transtorno do estresse pós-traumático

Fonte: adaptado de ASPESI; PINNA, 2018.

Devido a esta considerável semelhança entre os sintomas de diversos transtornos psiquiátricos, o diagnóstico incorreto destas é frequente na prática clínica (AYANO et al., 2021; VERMANI; MARCUS; KATZMAN, 2011). No mesmo sentido, o fato de que o diagnóstico primário e diferencial do transtorno depressivo maior e de outros distúrbios neuropsiquiátricos é realizado através da comunicação de sintomas subjetivos também contribui para uma menor eficácia preditiva na detecção e caracterização destas desordens. Desta forma, a busca de critérios objetivos na forma de marcadores biológicos para o diagnóstico de transtornos do humor tem se intensificado nos últimos anos, com o objetivo de aumentar sua sensibilidade e especificidade (ASPESI; PINNA, 2018).

I.1.1.2 Biomarcadores na depressão

O conceito de biomarcadores (também ocasionalmente referidos como “marcadores biológicos”) se aplica a medidas objetivas que podem ser confiavelmente quantificadas e que informem sobre a existência e gravidade de alguma condição clínica. Este é um conceito naturalmente amplo, que inclui desde medidas corporais físicas (como pressão arterial ou peso corporal), obtenção de imagens de características anatômicas de interesse, ou também da quantificação de moléculas de interesse no sangue ou em outros tecidos biológicos. A utilidade diagnóstica de um biomarcador depende de diversos fatores, como a viabilidade e confiabilidade de sua mensuração (p. ex., presença em uma matriz biológica de razoável obtenção e existência de metodologias analíticas capazes de realizar sua quantificação em precisão suficiente), bem como da sua capacidade preditiva em relação à condição clínica investigada e capacidade de direcionar a escolha terapêutica mais adequada (STRIMBU; TAVEL, 2010).

No âmbito dos transtornos depressivos, diferentes tipos de biomarcadores foram propostos para o seu diagnóstico, com base em diferentes aspectos do que é conhecido a respeito da sua neurobiologia.

I.1.1.3 Etiologia da depressão

Apesar de muitas décadas de estudo, as causas que levam um indivíduo saudável a desenvolver o Transtorno Depressivo Maior ainda não foram completamente elucidadas. De forma geral, o conhecimento atualmente estabelecido nos informa que a depressão é um transtorno multifatorial, no qual aspectos relacionados ao ambiente interagem com fatores inerentes aos indivíduos de forma a resultar no aparecimento do transtorno (ENGLAND; SIM, 2009). Os fatores ambientais que colaboram para o desenvolvimento da depressão são, em geral, estressores ocasionais ao longo da vida dos indivíduos, como luto, perda de status social ou econômico, adoecimento por causas diversas, entre outros (HAMMEN, 2005; HAMMEN et al., 2009). Tais estressores, quando ocorrem esporadicamente, tendem a desencadear uma resposta adaptativa ao estresse, que pode incluir períodos curtos mas autolimitantes de humor deprimido e outros sintomas relacionados. Acredita-se que, quando presentes em grande número e/ou por período prolongado, estes

estressores são os responsáveis por uma possível resposta mal adaptativa ao estresse, que pode levar a condições neuropsiquiátricas como o transtorno depressivo maior (GOLD, 2015). Vale notar que estressores de grande intensidade e agudos em natureza podem caracterizar um evento traumático e levar a condições distintas, como o TEPT.

Os fatores individuais que interferem no desenvolvimento da depressão estão majoritariamente relacionados ao constructo genético de cada indivíduo. Este papel é demonstrado através do estudo da herdabilidade de transtornos depressivos com a investigação de incidência e prevalência de transtornos depressivos em parentes próximos. Uma meta-análise que analisou estudos familiares, incluindo estudos em gêmeos monozigóticos e dizigóticos, estimou a herdabilidade da depressão em 37% (SULLIVAN; NEALE; KENDLER, 2000). Estudos subsequentes realizados em gêmeos encontraram resultados semelhantes, observando que a herdabilidade da depressão se torna mais evidente em indivíduos que apresentam casos recorrentes do transtorno depressivo maior em comparação com aqueles que tiveram um único episódio (FERNANDEZ-PUJALS et al., 2015; KENDLER et al., 2006; POLDERMAN et al., 2015).

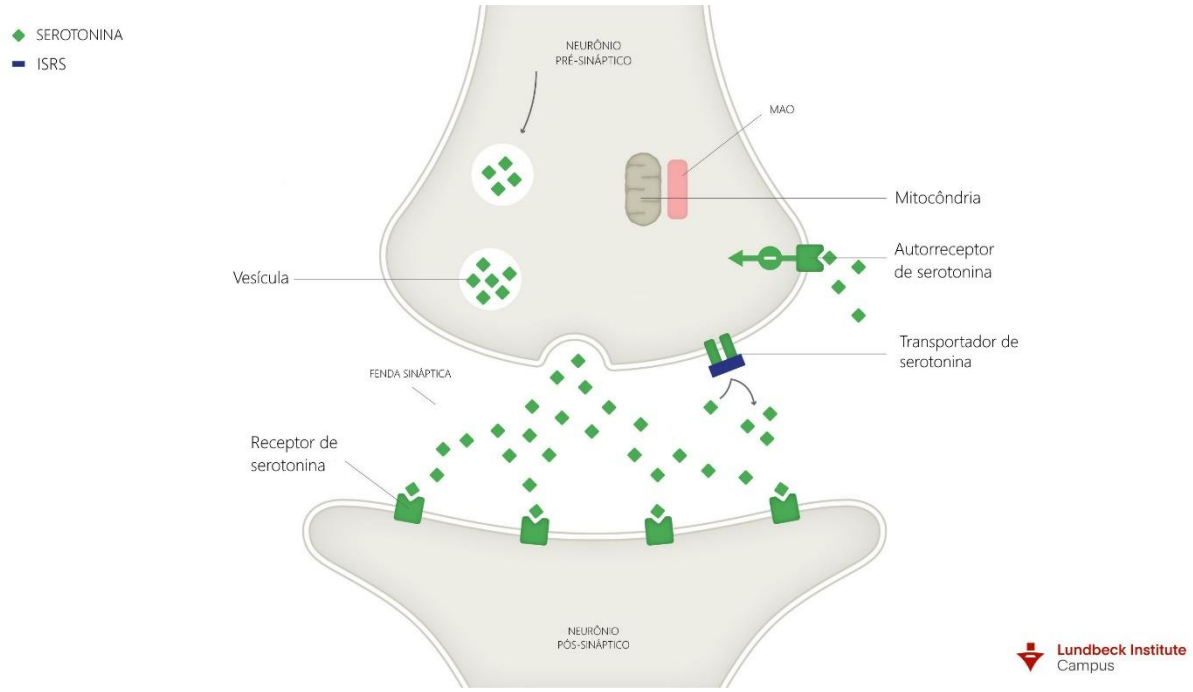
I.1.1.4 Antidepressivos e hipóteses neurobiológicas resultantes

Os primeiros antidepressivos foram descobertos nos anos 1950 durante ensaios clínicos para o tratamento da tuberculose, sendo investigados neste sentido após a observação de que os fármacos em investigação exerciam um efeito inesperado e positivo sobre o humor dos indivíduos de pesquisa. Mais tarde, estes fármacos foram caracterizados como inibidores da enzima monoamina oxidase (MAO), responsável pela degradação de neurotransmissores monoaminérgicos. Ao diminuir a degradação destas monoaminas, suas concentrações na fenda sinápticas se elevam, sendo pressuposto este o mecanismo de ação pelo qual os efeitos clínicos eram observados (SANDLER, 1990). Neste contexto foi que surgiram os antidepressivos tricíclicos, que aumentam a concentração de monoaminas (particularmente serotonina e noradrenalina) na fenda sináptica ao inibir sua recaptação. Clinicamente, no entanto, estas classes de antidepressivos apresentam uma alta ocorrência de efeitos colaterais, provavelmente devido aos diversos efeitos não pretendidos resultantes da inibição da MAO e da baixa seletividade dos

antidepressivos tricíclicos, que também atuam sobre outros receptores que incluem os histamínicos e muscarínicos (FANGMANN et al., 2008). Ainda assim, este conjunto de farmacoterapias antidepressivas e seus propostos mecanismos de ação deram origem à teoria monoaminérgica da depressão, que postula que sintomas depressivos são ocasionados por deficiências na neurotransmissão mediada por estas monoaminas (HIRSCHFELD, 2000). Nesta época, esta teoria também foi fundamentada em supostos efeitos pró-depressivos observados após o tratamento com reserpina, um fármaco anti-hipertensivo que reduz os níveis de monoaminas na fenda sináptica (BAUMEISTER; HAWKINS; UZELAC, 2003; GOVINDARAJULU et al., 2021). Atualmente, no entanto, a evidência clínica aponta para um efeito incerto da reserpina sobre a depressão, com parcelas semelhantes de estudos apontando para um efeito pró-depressão, antidepressivo ou neutro em indivíduos deprimidos (STRAWBRIDGE et al., 2023).

Posteriormente, uma nova geração de antidepressivos foi desenvolvida com base em evidências que a serotonina possuiria um papel de importância superior à outras monoaminas. A partir disto, surgiram os inibidores seletivos da recaptação da serotonina (ISRS), dentre os quais o primeiro fármaco aprovado desta classe foi a fluoxetina (comercializada originalmente sob o nome Prozac®). O perfil drasticamente reduzido de efeitos adversos provocado por fármacos desta classe os caracterizou como pertencentes à segunda geração de antidepressivos, sustentada pela teoria serotonérgica da depressão—essencialmente uma versão mais restrita da teoria monoaminérgica, destacando o papel da serotonina (e, em menor magnitude, da noradrenalina) no desenvolvimento de transtornos depressivos (PEREZ-CABALLERO et al., 2014). O mecanismo de ação dos ISRS encontra-se representado na Figura I.2

Figura I.2 – Representação do mecanismo de ação de inibidores seletivos da recaptação da serotonina na sinapse neuronal



Fonte: adaptado da biblioteca do Instituto Lundbeck (disponível em: <https://us-institute.progress.im/en/content/mechanism-action-selective-serotonin-re-uptake-inhibitors-ssris-0>; acesso em: 11 de fevereiro de 2024)

No entanto, mesmo com décadas de avanço e grande quantidade de recursos alocados no desenvolvimento de novos fármacos antidepressivos, a eficácia dos fármacos de segunda geração ainda se encontra longe de atingir um patamar terapêutico elevado. Apesar de elevarem rapidamente os níveis de serotonina na fenda sináptica, sua eficácia clínica somente começa a ser observada após semanas de tratamento contínuo (COMMONS; LINNROS, 2019). Além disso, estes fármacos somente mostram-se superiores ao placebo em 50% de todos os ensaios clínicos randomizados—incluindo aqueles que não são publicados ou são publicados com distorções relacionadas aos desfechos primários (VRIES et al., 2018). Esta lacuna de eficácia motivou a busca de novas farmacoterapias antidepressivas que utilizam agentes moduladores de outros sistemas de neurotransmissão como o glutamatérgico e o GABAérgico, resultando em antidepressivos de ação rápida como a escetamina e a brexanolona (CHEN et al., 2023; KRYSTAL; KAVALALI; MONTEGGIA, 2024). Resultados de uma recente meta-análise guarda-chuva sugerem que não há relação entre alterações no sistema serotonérgico e o desenvolvimento do transtorno

depressivo maior, questionando a hipótese de que a depressão é causada primariamente por uma menor atividade ou concentração de serotonina (MONCRIEFF et al., 2023). Assim, a existência de outros mecanismos neurobiológicos envolvidos em magnitude similar, talvez superior à do sistema serotonérgico torna-se plausível, inclusive podendo explicar parte dos efeitos antidepressivos observados com alguns destes fármacos. Uma das evidências neste sentido é o achado de que a fluoxetina age como um estimulante de enzimas esteroidogênicas, mesmo em concentrações inferiores àquelas nas quais seu efeito inibidor sobre a recaptação da serotonina está presente (PINNA; COSTA; GUIDOTTI, 2009). Além disso, descobriu-se que o tratamento antidepressivo é dependente da modulação de proteínas neurotróficas, que também são apontadas como um importante mediador dos transtornos depressivos (DUMAN; LI, 2012). Muitos destes processos são regulados epigeneticamente por agentes como os microRNAs, que também têm sido apontados como potenciais marcadores no estudo da depressão (FRIES et al., 2019). As próximas sessões abordam estas três classes e seu potencial papel na neurobiologia da depressão.

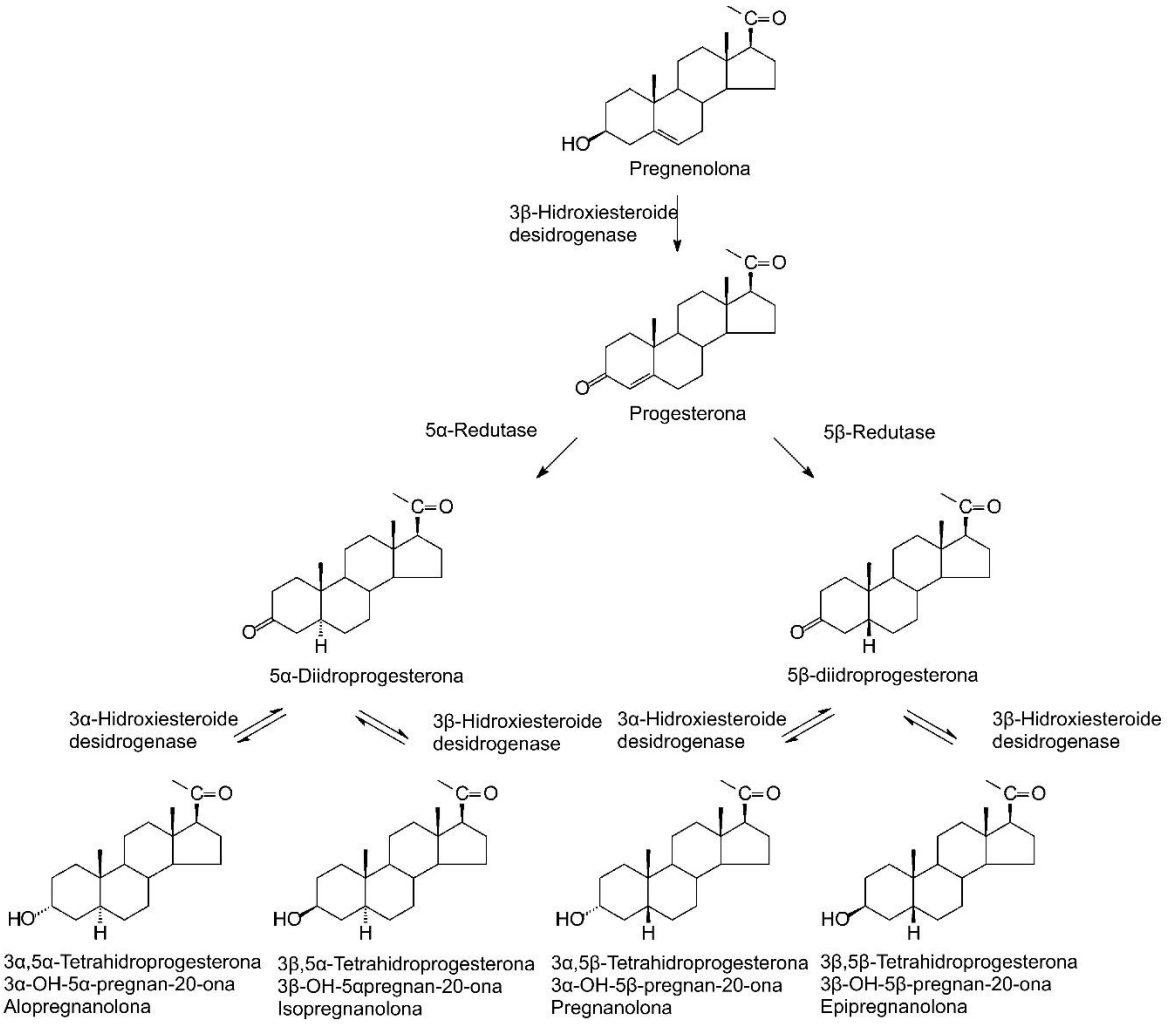
I.1.2 NEUROESTEROIDES

Os hormônios esteroides desempenham diversos papeis no funcionamento do organismo, regulando funções endócrinas relacionadas ao metabolismo, imunidade, osmorregulação, função sexual, entre outras. A maior parte destes hormônios atua sobre receptores nucleares, possuem ação lenta e ocasionam efeitos de natureza somática (ou periférica). No entanto, alguns destes hormônios esteroides endógenos são capazes de modular diretamente a ação neuronal de forma rápida, atuando sobre receptores presentes em neurônios e células gliais e sendo chamados de “esteroides neuroativos” (PAUL; PURDY, 1992). Alguns destes esteroides neuroativos são sintetizados *de novo* no sistema nervoso (ou seja, independentemente de fontes periféricas como as glândulas adrenais ou gônadas) a partir do colesterol ou de precursores como a progesterona, sendo então chamados de “neuroesteroides” (ROBEL; BAULIEU, 1994).

I.1.2.1 Síntese dos neuroesteroides

A síntese de neuroesteroides (representada na Figura I.3) ocorre majoritariamente em células da glia, (principalmente astrócitos e oligodendrócitos), mas também em neurônios hipocampais e cerebelares e na glândula pineal. O primeiro passo é o transporte do colesterol para o interior da mitocôndria, onde através de clivagem por enzimas do tipo p450 ocorre a conversão para o precursor neuroesteroide pregnenolona. Após sair da mitocôndria por difusão passiva, a pregnenolona sofre ação da enzima 3β-hidroxiesteroide desidrogenase (3β-HSD) e é convertida para progesterona, que por sua vez pode ser reduzida pela enzima 5α-redutase (dando origem à 5α-diidroprogesterona) ou 5β-redutase (dando origem à 5β-diidroprogesterona). Tanto a 5α- quanto a 5β-diidroprogesterona podem ser metabolizadas pelas enzimas 3α- ou 3β-HSD, dando origem a quatro diferentes neuroesteroides isoméricos: alopregnanolona (3α,5α-tetrahidroprogesterona), pregnanolona (3α,5β-tetrahidroprogesterona), isopregnanolona (3β,5α-tetrahidroprogesterona) e epipregnanolona (3β,5β-tetrahidroprogesterona) (LLOYD-EVANS; WALLER-EVANS, 2020). Referente à nomenclatura destes isômeros, é importante ressaltar o caso da isopregnanolona (3β,5α), que também é conhecida pelos nomes isoalopregnanolona e mesmo epialopregnanolona, o que pode gerar confusão devido à similaridade com os prefixos utilizados para referir-se a outros isômeros. Esclarece-se que, neste trabalho, será utilizada a nomenclatura mais aceita “isopregnanolona”.

Figura I.3 – Síntese da alopregnanolona e seus isômeros



Fonte: adaptado de HILL et al., 2007.

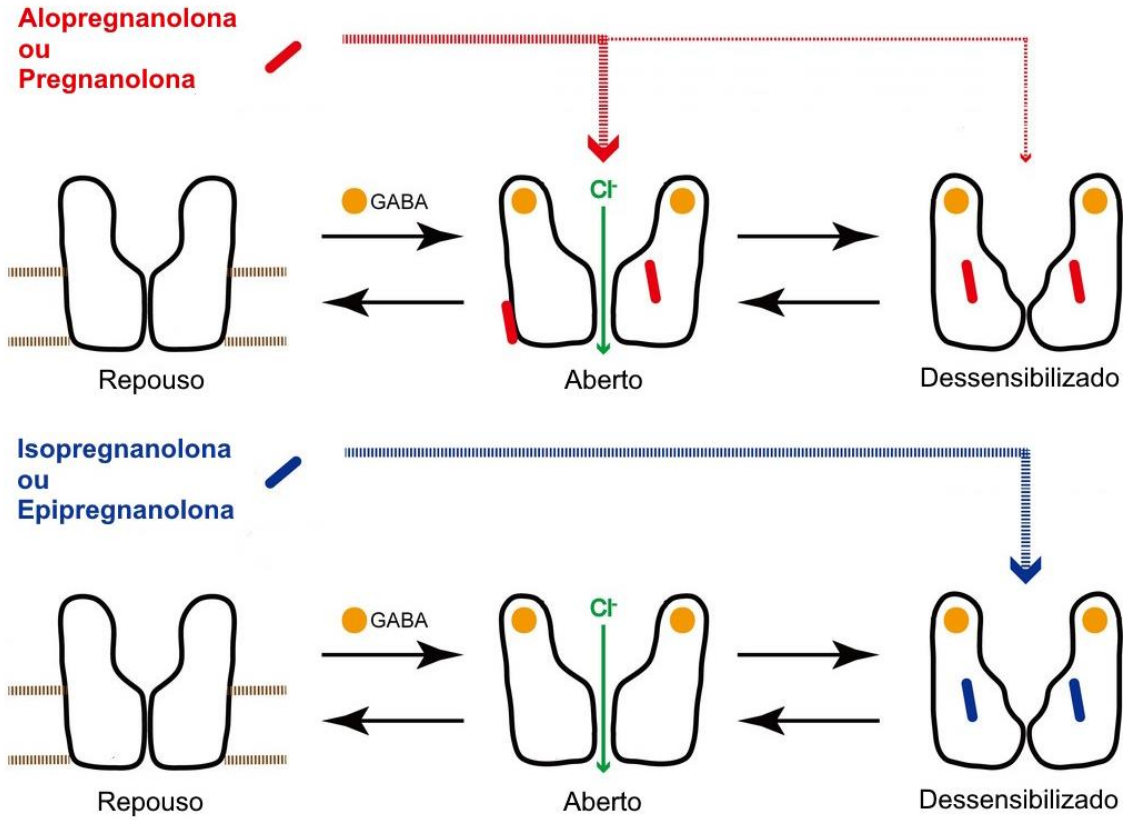
I.1.2.2 Isômeros da pregnanolona

Os isômeros da pregnanolona atuam sobre o sistema GABAérgico, que é o principal mediador da neurotransmissão inibitória no sistema nervoso (Figura I.4). Estes quatro neuroesteroides isoméricos possuem ação moduladora sobre o receptor $GABA_A$, que consiste em um canal iônico pentamérico dependente de ligante. Ao ser ativado pelo GABA, ocorre a abertura do canal de Cl^- ao redor do qual as cinco subunidades se localizam, hiperpolarizando o neurônio pós-sináptico e dificultando a propagação de um potencial de ação. Importantemente, os isômeros da pregnanolona exercem uma modulação alostérica sobre os receptores $GABA_A$, ou seja, não competem pelo sítio de ligação do GABA na estrutura do receptor (CHUA; CHEBIB, 2017).

A pregnanolona e a alopregnanolona possuem ação modulatória positiva equipotente sobre receptores $GABA_A$, apresentando maior afinidade por receptores com uma subunidade δ em sua estrutura, além das duas subunidades α e duas subunidades β que compõem o conjunto pentamérico do receptor. Estes receptores contendo uma subunidade δ são extrassinápticos e responsáveis pela inibição tônica (lenta) no sistema nervoso central, enquanto receptores que contém uma subunidade do tipo γ tendem localizar-se na área da fenda sináptica e mediar a inibição fásica (rápida). Conforme mencionado anteriormente, a ligação ao receptor ocorre em sítio distinto do ortostático, na interface entre as subunidades α e β dentro do domínio transmembrana (LEGESSE et al., 2023). Em concentrações nanomolares de alopregnanolona e a pregnanolona tipicamente presentes no espaço extracelular, há a potencialização do efeito do GABA através da modulação alostérica. No entanto, quando presentes em níveis micromolares, a alopregnanolona e a pregnanolona tem o potencial de ativar diretamente o receptor $GABA_A$ independentemente de ligação com GABA (BELELLI; LAMBERT, 2005).

A isopregnanolona (JOHANSSON et al., 2016) e a epipregnanolona (PRINCE; SIMMONDS, 1992) exercem uma ação modulatória negativa sobre os receptores $GABA_A$. Acreditava-se que esta era uma ação modulatória negativa funcional, ou seja, que estes dois isômeros 3β-reduzidos diminuíam a ação do receptor $GABA_A$ através da competição pelo mesmo sítio alostérico sobre o qual a pregnanolona e a alopregnanolona se ligam (PRINCE; SIMMONDS, 1992). No entanto, posteriormente se observou que a isopregnanolona e a epipregnanolona agem como antagonistas não-competitivos diretos no receptor $GABA_A$, particularmente na presença de concentrações elevadas de GABA (WANG et al., 2002). Ainda assim, o papel neurofisiológico destes dois isômeros ainda não é completamente compreendido, sendo que já foi demonstrado que a epipregnanolona pode apresentar uma fraca ação potenciadora sobre receptores $GABA_A$ (BUKANOVA et al., 2021).

Figura I.4 – Ligação e ação dos isômeros da pregnanolona sobre o receptor $GABA_A$



Fonte: adaptado de SUGASAWA et al., 2020.

A maior parte dos estudos que comparam os níveis circulantes destes hormônios foram conduzidos no contexto da gestação, onde os níveis alopregnanolona se encontram mais elevados (50–100 nmol/L), seguido pela pregnanolona (20–30 nmol/L), isopregnanolona (15–30 nmol/L) e por último pela epipregnanolona (2,5–5 nmol/L) (HILL et al., 2007; KLAK et al., 2003; PAŘÍZEK et al., 2005). Em mulheres não gestantes em idade fértil, os níveis observados são significativamente mais baixos (aproximadamente 100 vezes menores), mas as proporções entre os isômeros são mantidas tanto na fase folicular quanto luteal do ciclo menstrual (HAVLÍKOVÁ et al., 2006). Em homens, os níveis circulantes destes neuroesteroides tem magnitude semelhante a mulheres não-gestantes mas com proporções diferentes, com níveis de isopregnanolona superiores aos níveis de alopregnanolona (BICIKOVA et al., 2011). Levando em consideração que estes isômeros possuem ações modulatória distintas sobre o receptor $GABA_A$ e que seus níveis estão presentes em proporções distintas mas comparáveis, a separação eficaz destes compostos em experimentos que visem mensurar um ou mais destes isômeros

se faz fundamental para que a quantificação seja específica e não abranja mais de um composto que possui efeitos neurofisiológicos distintos.

O envolvimento dos neuroesteroides na depressão foi primeiramente descrito de forma simultânea por dois grupos de pesquisa independentes que observaram níveis reduzidos de alopregnanolona em pacientes com transtorno depressivo maior, com subsequente normalização após tratamento bem-sucedido com antidepressivos (ROMEO et al., 1998; UZUNOVA et al., 1998). Diversos estudos subsequentes aprofundaram o papel dos neuroesteroides—particularmente da alopregnanolona—em transtornos depressivos, instigando a investigação desta classe de esteroides neuroativos tanto como um potencial biomarcador quanto como um eventual tratamento para transtornos depressivos (revisado em PINNA, 2020).

I.1.3 NEUROTROFINAS

O neurotrofismo é o processo responsável pela sobrevivência, desenvolvimento e função dos neurônios componentes do sistema nervoso central e periférico. Tais processos são mediados por proteínas conhecidas como fatores neurotróficos. Os principais fatores neurotróficos responsáveis pelos processos de neurotrofismo em mamíferos usualmente recebem o nome de neurotrofinas, englobando o fator de crescimento neural, a neurotrofina-3, a neurotrofina-4 e o fator neurotrófico derivado do encéfalo (*brain-derived neurotrophic factor*, BDNF) (HUANG; REICHARDT, 2001). Estas quatro neurotrofinas são proteínas de alto peso molecular com sequência e estrutura semelhante devido ao fato de serem derivadas de um gene ancestral comum (HALLBÖÖK, 1999).

Evidências clínicas e pré-clínicas demonstram que a atrofia neuronal, incluindo a perda de neurônios e células gliais são fatores que contribuem para o desenvolvimento de transtornos depressivos. Além disso, o tratamento com antidepressivos—incluindo fármacos da classe dos ISRS—é capaz de reverter este processo. Desta forma, foi originada uma hipótese neurotrófica da depressão e resposta antidepressiva (DUMAN; LI, 2012).

A atrofia neuronal observada em transtornos depressivos está relacionada com uma redução na expressão de neurotrofinas, dentre as quais o BDNF tem sido a mais estudada. Análises post-mortem demonstram que os níveis plasmáticos de BDNF encontram-se reduzidos em comparação com controles (GADAD et al., 2021). Em

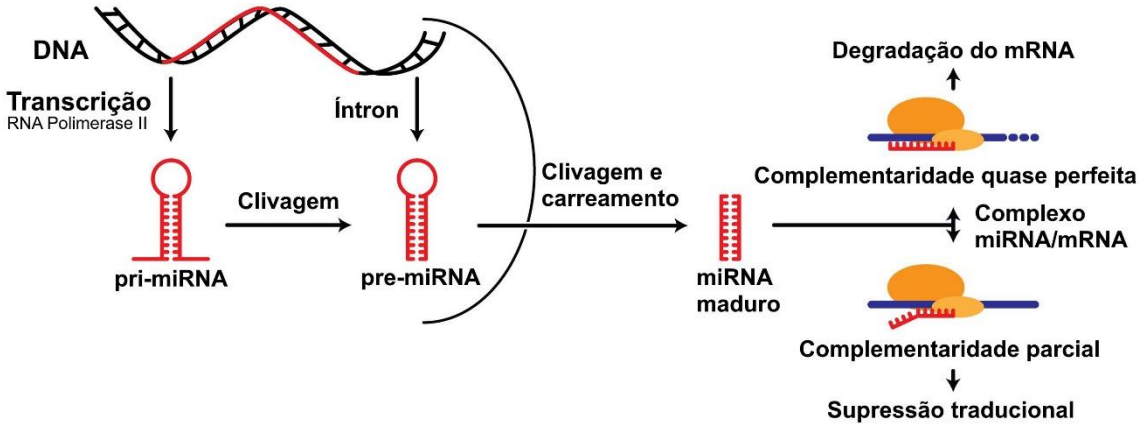
camundongos, a infusão intra-hipocampal de BDNF potencializou o efeito tipo-antidepressivo da paroxetina, um fármaco da classe dos ISRS (DELTHEIL et al., 2009). Além disso, o BDNF parece não apenas facilitar, mas também ser indispensável para o aparecimento do efeito antidepressivo de fármacos (CASTRÉN; RANTAMÄKI, 2010). Importantemente, a sobrerregulação da expressão de BDNF responsável por viabilizar efeitos antidepressivos pode ser mediada pela alopregnanolona (NIN et al., 2011).

I.1.5 MICRORNAS

MicroRNAs (miRNAs) são moléculas endógenas pequenas e não-codificantes que consistem em uma fita única de RNA contendo de 18 a 25 nucleotídeos. A principal função dos miRNAs é suprimir ou silenciar a expressão gênica através da sua ligação por complementaridade de pares de base a moléculas de RNA mensageiro (mRNA) (HO; CLARK; LE, 2022). Um dos mecanismos moleculares de silenciamento por miRNAs é a clivagem direta da fita alvo de mRNA, mas este mecanismo ocorre majoritariamente em plantas, onde há um pareamento completo ou quase completo com a molécula de mRNA (ZHANG et al., 2022). A supressão da expressão gênica também pode ocorrer através do encurtamento da cauda poli-A da molécula de mRNA (o que resulta em sua desestabilização) ou da conjugação estável ao mRNA (que inibe a tradução proteica ao truncar os mecanismos ribossômicos, mecanismos que dependem de um pareamento menor com o mRNA (sequência de 6 a 8 nucleotídeos) e que é predominante em animais (JONAS; IZAURRALDE, 2015). Importantemente, um único miRNA comummente exerce ação silenciadora sobre diversos mRNA-alvos diferentes, e qualquer determinada molécula de mRNA pode ser silenciada por múltiplos miRNAs distintos. Esta complexa rede regulatória é majoritariamente explorada através de modelos preditivos computacionais de bioinformática, com os mecanismos de maior interesse podendo ser validados experimentalmente (RIOLO et al., 2021). A síntese dos miRNAs (representada na Figura I.5) tem início no núcleo celular, onde uma molécula-grampo de miRNA primária (pri-miRNA) é transcrita pela enzima polimerase II. Ainda no núcleo, esta molécula de pri-miRNA tem suas extremidades e 5’ e 3’ clivadas por um complexo enzimático para dar origem a uma molécula de miRNA precursor (pre-miRNA). O pre-miRNA é transportado para o citoplasma celular, onde será processado em duas

etapas (remoção do grampo e remoção da dupla fita) para dar origem à molécula madura de miRNA (O'BRIEN et al., 2018).

Figura I.5 – Biogênese das moléculas de miRNA



Fonte: adaptado de RYAN; JOILIN; WILLIAMS, 2015.

Os miRNAs apresentam um altíssimo grau de conservação mesmo entre espécies evolutivamente distantes (LEE; RISOM; STRAUSS, 2007), tornando-os alvos interessantes para pesquisas translacionais. Em seres humanos, pouco mais de 500 miRNAs diferentes já foram propriamente descritos, validados, e indexados em repositórios específicos (FROMM et al., 2020). No entanto, este processo é significativamente longo e certamente encontra-se incompleto, de modo que o número real de miRNAs humanos certamente é maior. Uma estimativa baseada em abordagens *in silico* aponta que 2300 miRNAs diferentes possam estar presentes no genoma humano (ALLES et al., 2019), número que pode ou não se confirmar a depender de subsequente validação experimental.

Diversos miRNAs têm sido propostos como biomarcadores promissores de diferentes doenças como câncer, doenças cardiovasculares e distúrbios neurológicos (CONDRAT et al., 2020). Recentemente, miRNAs também têm sido propostos como biomarcadores de transtornos neuropsiquiátricos, incluindo a depressão (FRIES et al., 2019). Estas evidências são oriundas tanto de estudos clínicos que verificaram sua expressão post-mortem no cérebro ou periférica no soro, sangue ou outros tecidos. Além disso, estudos pré-clínicos em roedores também avaliaram a expressão de diversos miRNAs em um modelo animal de depressão baseado em estresse, com mensurações ocorrendo em regiões cerebrais como o córtex pré-frontal e o hipocampo (DING et al., 2023). Portanto, alterações nos perfis de miRNAs podem

desempenhar um papel fundamental na patogênese da depressão, influenciando processos biológicos e vias de sinalização neural. Dentre os numerosos microRNAs investigados no contexto da depressão e seus modelos animais, há evidências promissoras a respeito dos miRNAs miR-124-3p (ROY et al., 2017), miR-135a-5p (ŻURAWEK; TURECKI, 2021) e miR-144-3p (VAN DER ZEE et al., 2022).

I.1.5 MODELOS ANIMAIS DE DEPRESSÃO E TESTES COMPORTAMENTAIS

Na pesquisa pré-clínica, há diversos modelos animais de depressão e testes comportamentais utilizados para desenvolver a pesquisa de base em relação à neurobiologia dos transtornos depressivos. Nesta seção, destacam-se os de maior relevância para este trabalho. No entanto, antes de expor cada um destes modelos e testes, é relevante ressaltar que os conceitos de “modelo de depressão” e de “teste comportamental” frequentemente se confundem, apesar de se tratar de paradigmas diferentes—ainda que relacionados. Aqui, esclarece-se que um modelo é composto por uma variável independente (caracterizada pela manipulação responsável por induzir um estado tipo-depressivo) e por uma variável independente (consistente em uma quantificação de uma variável comportamental ou neuroquímica); um teste comportamental, portanto, constitui-se apenas pela variável independente (SLATTERY; CRYAN, 2014).

I.1.5.1 Testes comportamentais

I.1.5.1.1 Teste do nado forçado

O teste do nado forçado (também chamado de “teste da natação forçada”) foi originalmente proposto por Porsolt e colaboradores em 1977 como um teste de eficácia antidepressiva em ratos. O teste se baseia em um conceito de “desespero comportamental” (*“behavioral despair”*), evidenciado pelo fato de que após algum tempo de natação forçada os animais tendem a desistir dos comportamentos de escape e permanecem imóveis. Ao demonstrar que a administração de antidepressivos eficazes na prática clínica são capazes de exercer alterações comportamentais específicas e facilmente mensuráveis neste teste (duração da imobilidade), o mesmo foi proposto como uma ferramenta de varredura na busca de

novos fármacos antidepressivos. Neste teste, ratos são colocados durante 15 minutos em tanques cilíndricos transparentes com altura de 40 cm e diâmetro de 18 cm, sendo preenchidos com água na temperatura de 25 °C a uma profundidade de 15 cm. Vinte e quatro horas depois, os ratos são recolocados no cilindro e o tempo em segundos durante o qual os animais permanecem imóveis durante uma sessão de 5 minutos é contabilizado. O fármaco de interesse é administrado por injeções intraperitoneais em três momentos: 24 horas, 5 horas e 1 hora antes do início da segunda sessão de nado forçado (PORSOLT; LE PICHON; JALFRE, 1977).

Modificações no protocolo do teste foram propostas ao longo do tempo, com destaque para as propostas por Detke e Lucki em 1995. O aumento da profundidade da água de 15 cm para 30 cm melhorou significativamente a sensibilidade do teste, especialmente para a detecção de ISRS. Além disso, a diferenciação e contabilização dos comportamentos de mobilidade apresentados durante a sessão de teste (nadar e escalar) foi proposta como uma ferramenta para investigar o mecanismo neurobiológico principal através do qual o fármaco em análise exerce um eventual efeito antidepressivo observado no teste: nadar como um marcador serotonérgico e escalar como um marcador noradrenérgico. Outra modificação proposta foi uma análise fracionada do teste, no qual o comportamento presente em incrementos de 5 segundos é registrado pelo observador (configurando uma contagem de determinado comportamento), de um total de 60 contagens (DETKE; LUCKI, 1995). Atualmente, protocolos semelhantes a este são amplamente utilizados em experimentos pré-clínicos para a verificação da eficácia antidepressiva de compostos clássicos ou inovadores (SLATTERY; CRYAN, 2012; YANKELEVITCH-YAHAV et al., 2015).

Além do seu emprego estrito como um teste de eficácia antidepressiva, o nado forçado também é proposto como um teste capaz de mensurar comportamentos tipo-depressivos, ou seja, alterações comportamentais que podem ser utilizadas como marcadores comportamentais específicos de um estado tipo-depressivo induzido nos roedores por alguma outra intervenção. Ainda, comportamentos tipo-depressivos podem ser análogos a sintomas depressivos em humanos, estando presentes—ainda que em diferentes magnitudes—em animais tipo-deprimidos ou não (LEITE-ALMEIDA; CASTELHANO-CARLOS; SOUSA, 2022; YANKELEVITCH-YAHAV et al., 2015).

I.1.5.1.2 Teste da preferência pela sacarose

O teste da preferência pela sacarose foi desenvolvido com o objetivo de medir um comportamento análogo à anedonia em seres humanos (PRIMO et al., 2023), que por sua vez é um dos dois sintomas centrais do transtorno depressivo maior juntamente com a presença de humor deprimido. A anedonia é definida como a diminuição ou incapacidade de sentir prazer oriundo de estímulos positivos, bem como manifestar interesse pelos mesmos (BENTLEY; PAGALILAUAN; SIMPSON, 2014). A possibilidade de modelar a manifestação de um dos sintomas centrais da depressão torna o teste de preferência pela sacarose um protocolo atrativo para estudos pré-clínicos relacionados a transtornos depressivos. Em roedores, o teste baseia-se na preferência natural destes animais por líquidos e alimentos palatáveis, dentro dos quais uma solução doce é um potencial reforçador da busca por este estímulo (SCHEGGI; DE MONTIS; GAMBARANA, 2018).

A base deste protocolo consiste na apresentação de duas garrafas—uma contendo água e outra contendo sacarose—para os ratos ou camundongos, e o consumo de cada uma delas é aferido após um tempo determinado. A preferência pela sacarose é calculada dividindo o consumo da solução de sacarose pelo consumo total de líquidos (ou seja, a soma do consumo da solução de sacarose e do consumo da solução de água), geralmente sendo expresso em porcentagem (LIU et al., 2018). Após a exposição a um modelo animal de depressão, principalmente o estresse crônico moderado e imprevisível, a preferência pela sacarose diminui em animais estressados quando comparados os níveis basais e com controles não estressados (WILLNER, 2017). Os protocolos utilizados neste teste são variados, com diferenças significativas em relação ao período do teste, concentração de sacarose, duração do teste, habituação prévia à sacarose e privação de água e/ou comida. Esta alta heterogenia metodológica na aplicação deste teste leva a uma falta de padronização que pode explicar resultados conflitantes encontrados na literatura (PRIMO et al., 2023).

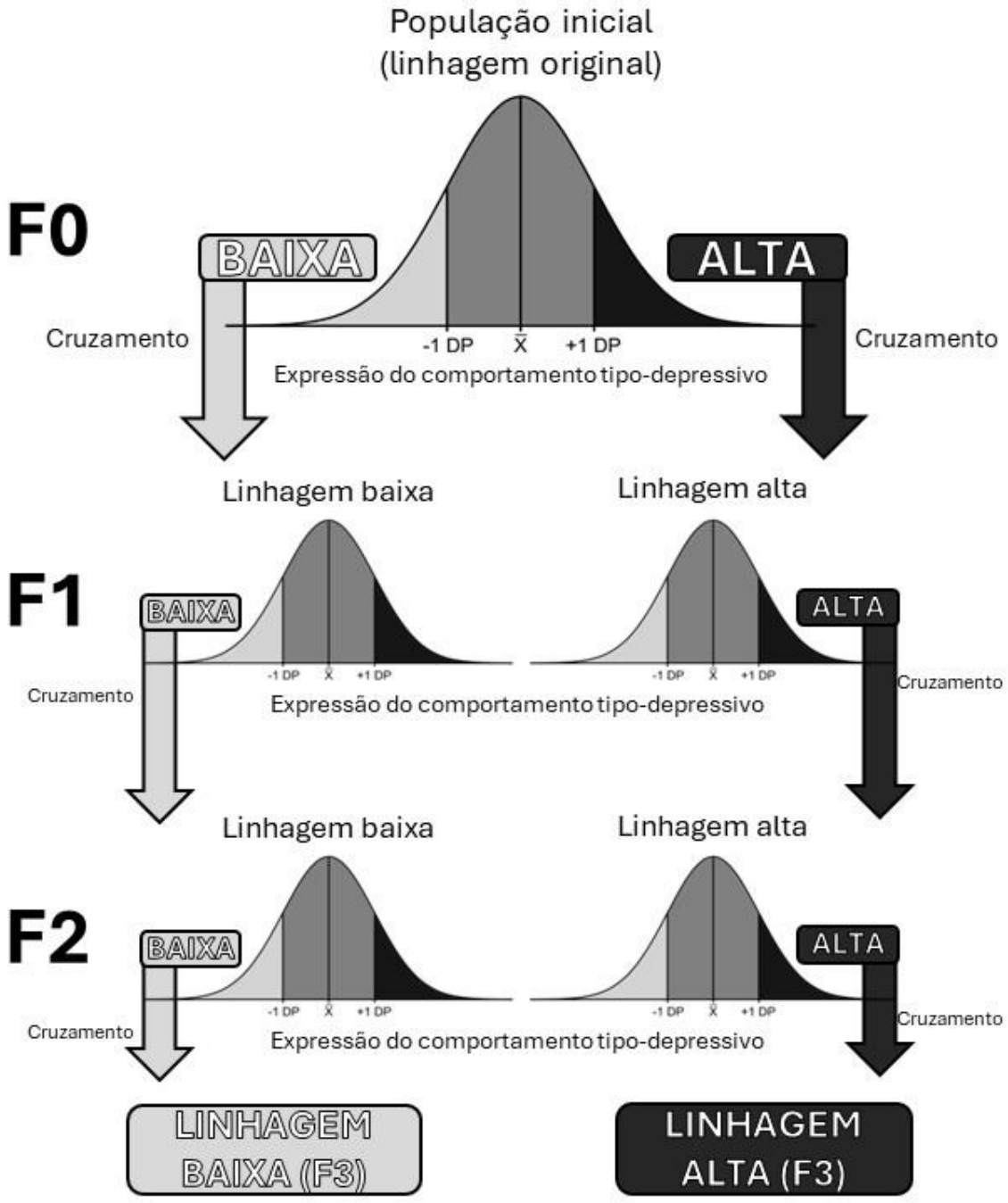
I.1.5.2 Modelos animais de depressão

Na pesquisa pré-clínica, diferentes modelos animais de depressão são empregados para investigar os possíveis mecanismos neurobiológicos subjacentes

aos transtornos depressivos. Tais modelos buscam obter uma validade de face ao mimetizar os fatores de risco conhecidos para o desenvolvimento do transtorno depressivo maior, sendo estes predominantemente de natureza ambiental e estressora conforme discutido anteriormente. Neste contexto encontram-se modelos animais de depressão como a bulbectomia olfatória, o isolamento social, separação maternal, derrota social, e estresse crônico moderado e imprevisível, entre outros. Animais submetidos a estes modelos apresentam alterações comportamentais e neurobiológicas que se assemelham àquelas observadas em transtornos depressivos, frequentemente atingindo validades de face e de constructo satisfatórias (ABELAIRA; RÉUS; QUEVEDO, 2013; CZÉH et al., 2016).

Outros modelos buscam reproduzir os aspectos genéticos ou hereditários da depressão através do cruzamento seletivo. Nestes estudos, uma população inicial de animais é submetida a um ou mais testes comportamentais para determinar a distribuição basal de comportamentos tipo-depressivos. Todos os animais que apresentam níveis extremos em relação a estes comportamentos (geralmente tanto para mais quanto para menos) são cruzados por algumas gerações até atingir uma separação fenotípica comportamental, possibilitando avaliar a potencial eficácia antidepressiva de fármacos e investigar mecanismos neurobiológicos subjacentes nestas linhagens (SLATTERY; CRYAN, 2014). O teste do nado forçado foi utilizado como característica do fenótipo tipo-depressivo para este fim em múltiplos trabalhos (GERSNER et al., 2014; WEISS; CIERPIAL; WEST, 1998; WILL; AIRD; REDEI, 2003), inclusive em nosso laboratório, onde ratos foram cruzados seletivamente por duas gerações (F0-F2) para dar origem a linhagens mais ou menos tipo-deprimidas (ALMEIDA, 2017; ALMEIDA et al., 2018). A Figura I.6 demonstra um protocolo para o cruzamento seletivo baseado na seleção comportamental.

Figura I.6 – Representação esquemática do cruzamento seletivo por meio de seleção comportamental



Fonte: autores.

I.2 JUSTIFICATIVA

A depressão caracteriza um urgente problema de saúde pública, contribuindo significativamente para o aumento da incapacidade e sofrimento em uma grande parcela da população. Muita atenção é direcionada para a parte ambiental da sua etiologia, mas sua carga genética/hereditária é considerável e também deve ser levada em conta em estudos experimentais. Além disto, o diagnóstico da depressão e a monitorização dos efeitos dos tratamentos é baseado unicamente na comunicação subjetiva de sinais e sintomas psíquicos. Esta subjetividade associada à similaridade sintomática com outros transtornos como o TEPT pode dificultar o diagnóstico da depressão, de forma que o desenvolvimento de marcadores biológicos objetivos se faz necessário para ajudar na detecção, diferenciação e escolha farmacoterapêutica deste transtorno.

Apesar da alta oferta de fármacos antidepressivos no mercado, sua eficácia, quando comparada com placebo em ensaios clínicos randomizados, permanece baixa. Portanto, fica claro que a transmissão monoaminérgica, sobre a qual virtualmente todos estes antidepressivos atuam, não é o único sistema implicado na psicobiologia da depressão. Neste sentido, outros alvos neurobiológicos devem ser buscados para o desenvolvimento de terapias mais eficazes e para a determinação de potenciais biomarcadores diagnósticos e de eficácia dos tratamentos. Assim, uma revisão detalhada a respeito de diversos mecanismos neurobiológicos alternativos da depressão se faz urgente e relevante.

Neuroesteroides como a alopregnanolona e seus isômeros possuem ação modulatória sobre sistemas de neurotransmissão com implicação na depressão, sendo que seus níveis tendem a acompanhar a sintomatologia depressiva e estão relacionados com processos neurotróficos. Importantemente, a quantificação da alopregnanolona e seus isômeros não constitui tarefa trivial, já que e a similaridade química impõe desafios analíticos importantes e a busca de metodologias confiáveis para verificar a relevância neuromoduladora diferencial destes compostos traz um obstáculo que deve ser superado para o uso destas moléculas como biomarcadores. Outras moléculas, dentre as quais se destacam os miRNAs, tem recebido espaço no estudo da neurobiologia da depressão e propostos como biomarcadores em função da regulação epigenética que exercem.

Modelos animais que possam levar em conta a influência de fatores individuais (linhagens mais ou menos tipo-depressivas) podem esclarecer questões ainda pouco exploradas a respeito da farmacoterapia antidepressiva clássica, particularmente no âmbito da mensuração de marcadores biológicos associados com comportamentos depressivos e com a eficácia do tratamento com antidepressivos. Portanto, a mensuração sanguínea de marcadores neuroesteroidais periféricos pode ser um arsenal diagnóstico útil e comum a outros transtornos, melhorando não somente a identificação da entidade patológica, mas principalmente seu tratamento. Além disso, alterações de ordem epigenética podem contribuir nesta abordagem bioquímica do diagnóstico, seja de maneira isolada ou compondo um portfólio de métodos analíticos que indicam com maior especificidade o diagnóstico da depressão.

I.3 OBJETIVOS

I.3.1 OBJETIVO GERAL

Avaliar o potencial de neuroesteroides, neurotrofinas e microRNAs como biomarcadores de transtornos depressivos através de um modelo geracional de depressão em ratos.

I.3.2 OBJETIVOS ESPECÍFICOS

1) Avaliar o papel da alopregnanolona e de proteínas neurotróficas sobre comportamentos tipo-depressivos em modelos animais de depressão através de uma revisão de literatura;

2) Investigar o potencial de neuroesteroides e proteínas neurotróficas como biomarcadores do transtorno depressivo maior e TEPT através de uma revisão de literatura;

3) Descrever o papel da alopregnanolona sobre o eixo HPA e seu papel na neurobiologia de transtornos depressivos e TEPT regulação da resposta ao estresse através de uma revisão de literatura;

4) Descrever a implementação da metodologia de cromatografia líquida acoplada à espectrometria de massas em tandem para a quantificação da alopregnanolona e seus isômeros;

5) Avaliar o efeito do tratamento antidepressivo com fluoxetina, da susceptibilidade individual a um estado tipo-depressivo, e do sexo sobre os níveis do miRNA 144-3p no sangue de ratos.

PARTE II – ARTIGOS CIENTÍFICOS E RESULTADOS ADICIONAIS

II.1 CAPÍTULO I – O papel da alopregnanolona sobre comportamentos tipo-depressivos: foco em proteínas neurotróficas

O artigo apresentado a seguir está relacionado com o objetivo específico 1 e busca responder as seguintes perguntas:

1) O que ocorre com os níveis cerebrais de alopregnanolona nos modelos animais de depressão mais utilizados na literatura científica?
2) O tratamento com antidepressivos é capaz de restaurar os níveis cerebrais de alopregnanolona para patamares normais nestes modelos de depressão?
3) Qual é o efeito da administração exógena direta de alopregnanolona sobre comportamentos tipo-depressivos em diferentes testes comportamentais?
4) Há associação de processos neurogênicos com a regulação neuroesteroidogênica provocada em modelos de depressão e restauração dos níveis causados pelo tratamento com antidepressivos clássicos ou com alopregnanolona?

Trata-se de um artigo de revisão narrativa ampla, com sistematização dos resultados encontrados na literatura através de grandes tabelas explanatórias e comparativas, além de dissertação textual sobre os temas de interesse da revisão. Neste trabalho, fica demonstrado que modelos animais de depressão baseados em estresse diminuem consideravelmente os níveis cerebrais de alopregnanolona em regiões de relevância para a neurobiologia dos transtornos depressivos. Além disso, o tratamento com antidepressivos é capaz de reverter estes níveis, sendo que a alopregnanolona exógena exerce efeitos tipo-antidepressivos robustos e reprodutíveis. Marcadores de neurogênese, inclusive o BDNF, também estão associados com uma redução nos níveis cerebrais de alopregnanolona e o tratamento com este neuroesteroide é capaz de aumentar a expressão de BDNF no cérebro de roedores. Inclusive, intervenções ambientais de cunho ‘positivo’ como o enriquecimento ambiental promovem neurogênese que pode estar acompanhada de um aumento na neuroesteroidogênese cerebral.

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The role of allopregnanolone in depressive-like behaviors: Focus on neurotrophic proteins

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ABSTRACT

Allopregnanolone (3α,5α-tetrahydroprogesterone; pharmaceutical formulation: brexanolone) is a neurosteroid that has recently been approved for the treatment of postpartum depression, promising to fill part of a long-lasting gap in the effectiveness of pharmacotherapies for depressive disorders. In this review, we explore the experimental research that characterized the antidepressant-like effects of allopregnanolone, with a particular focus on the neurotrophic adaptations induced by this neurosteroid in preclinical studies. We demonstrate that there is a consistent decrease in allopregnanolone levels in limbic brain areas in rodents submitted to stress-induced models of depression, such as social isolation and chronic unpredictable stress. Further, both the drug-induced upregulation of allopregnanolone or its direct administration reduce depressive-like behaviors in models such as the forced swim test. The main drugs of interest that upregulate allopregnanolone levels are selective serotonin reuptake inhibitors (SSRIs), which present the neurosteroidogenic property even in lower, non-SSRI doses. Finally, we explore how these antidepressant-like behaviors are related to neurogenesis, particularly in the hippocampus. The protagonist in this mechanism is likely the brain-derived neurotrophic factor (BFNF), which is decreased in animal models of depression and may be restored by the normalization of allopregnanolone levels. The role of an interaction between GABA and the neurotrophic mechanisms needs to be further investigated.

1. Introduction

Depression (also referred to as 'major depression' or 'major depressive disorder') is a highly prevalent mental illness that is estimated to affect up to nearly four percent of the world population and is the leading cause of disability worldwide (Rehm and Shield, 2019). It is clinically characterized mainly by its core symptoms of depressed mood and anhedonia, but a wide array of accompanying secondary symptoms render depression a rather heterogeneous disorder regarding its phenotype (Bentley et al., 2014). This heterogeneity is also reflected in its etiology, which is likely responsible for the disappointingly low success rate (around 50%) of widely prescribed antidepressants that act by increasing brain monoamine levels when these drugs are confronted against placebo in clinical trials (Vries et al., 2018). Thus, it becomes evident that additional neurotransmitter systems are deeply involved with depression, and that molecular targets other than monoamine modulators must be pursued in order to achieve a more complete efficacy in the pharmacological treatment for depression.

Evidence of the relationship of the GABAergic system with mood disorders dates as far back as 1980 when treatment with valproic acid was reported to show positive effects for bipolar disorder (Emrich et al., 1980). In fact, GABA levels are diminished in the brain of depressed patients (Sanacora et al., 2004), and the stimulation of GABAergic transmission has been proposed as a novel strategy for the treatment of depression, particularly through stimulation of the GABA type A receptor ($GABA_AR$) (Lüscher and Möhler, 2019). The $GABA_AR$ is one of the main receptors for GABA and consists of an ion-gated channel that hyperpolarizes the postsynaptic neuron when activated (Chua and

Abbreviations: BDNF, brain-derived neurotrophic factor; CSF, cerebrospinal fluid; CUS, chronic unpredictable stress; EKR, extracellular signal-regulated kinase; FST, forced swim test; GABA, γ-aminobutyric acid; $GABA_AR$, GABA type A receptor; HSD, hydroxysteroid dehydrogenase; NGF, nerve growth factor; PTSD, post-traumatic stress disorder; PXR, pregnane xenobiotic receptor; SBSS, selective brain steroidogenic stimulant; SSRI, selective serotonin reuptake inhibitor; THP, tetrahydroprogesterone; TrkB, tropomyosin receptor kinase B; TSPO, 18 kDa translocator protein; USV, ultrasonic vocalization

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Table 1
Brain allopregnanolone levels in animal models of depression.

Published in	Spe-cies	Sex (es)	Model(s)		Behavioral test (s)	Brain areas(s)	Main findings	
			Name(s)	Dura-tion			Behavioral changes	Brain allopregnanolone levels
Social isolation								
Matsumoto et al. (1999)	Mice	♂	SI	2–10 wks	None	• FC	N/A	• SI (6–10 wks): ↓
Serra et al. (2000)	Rats	♂	SI SI + HDL SI→FSS	1–30 d 30 d 30 d	None	• CTX	N/A	• SI (1 or 2 d): ≅ • SI (7 or 30 d): ↓ • SI + HDL: ≅ • FSS: ↑ ($\uparrow_{SI}$ > $\uparrow_{GH}$)
Pinna et al. (2003)	Mice	♂	SI	1 d–8 wks	RIT	• OB	• SI (4–8 wks): $\uparrow_{aggression}$	• SI (4–8 wks): ↓
Pinna et al. (2004b)	Mice	♂	SI	4–6 wks	None	• OB • FC	N/A	• ↓
Pinna et al. (2005)	Mice	♂ + ♀	SI	3 wks	RIT	• OB	• SI: $\uparrow_{aggression}$ in $♂_{sham}$ • ORX: $\cong_{aggression}$ • ORX + TP: $\uparrow_{aggression}$ • SI: $\cong_{aggression}$ in $♀_{sham}$ • SI + TP: $\uparrow_{aggression}$ in $♀_{sham}$ • SI + OVX: $\cong_{aggression}$ • SI + OVX + TP: $\uparrow_{aggression}$	• SI: ↓ in $♂_{sham}$ • ORX: ≅ • SI + ORX: ↓ • SI: ≅ in $♀_{sham}$ • SI + TP: ↓ in $♀_{sham}$ • SI + OVX: ≅ • SI + OVX + TP: ↓
Pibiri et al. (2006)	Mice	♂ + ♀	SI	3 wks	RIT	• OB • FC	• SI $\uparrow_{aggression}$ in ♂ • SI $\cong_{aggression}$ in ♀ • SI + TP: $\uparrow_{aggression}$ in ♀	• SI: ≅ • SI + TP: ↓
Nelson and Pinna (2011)	Mice	♂	SI	4 wks	RIT	• OB • FC • BLA • HPC • STR	• Aggression (SI vs. GH): N.I.	• SI: ↓ in OB, FC, BLA and HPC • SI: ≅ in STR
Evans et al. (2012)	Rats	♂	$SI_{maint.}$ SI_{rescue}	6 wks 10 wks	FST NSFT OFT	• HPC	• SI (6 and 10 wks): $\uparrow_{immobility}$ • SI: $\uparrow_{novel\ food\ remaining}$ • No changes in the OFT	• SI (6 and 10 wks): ↓
Pibiri et al. (2008)	Mice	♂	SI	3–4 wks	CFCT	• OB • FC • AMY • STR • HPC • CBL	• SI: $\uparrow_{freezing}$	• ↓ in OB, FC, AMY and HPC • ≅ in STR and CBL
Pisu et al. (2016)	Rats	♂ + ♀	SI SI→FSS	30 d 30 d	SPT	• CTX	• SI $\downarrow_{preference}$ in ♂ • SI $\cong_{preference}$ in ♀	• SI: ↓ ($\downarrow_{♂}$ > $\downarrow_{♀}$) • FSS: ↑ in ♂ ($\uparrow_{GH}$ < $\uparrow_{SI}$) • FSS: ≅ in ♀ (GH = SI)
Other animal models								
Uzunova et al. (2003)	Rats	♂	OBX	N/A	None	• WB • FC • HPC • CTX • AMY	N/A	• ↑ WB at 7 and 28 d • ↓ in AMY and FC at 7 and 14 d • ↑ in CTX at 7 and 14 d • $\downarrow_{tend.}$ in HPC at 14 d
Uzunova et al. (2004)	Rats	♂	OBX	5 wks	None	• CTX	N/A	• ↓
Zimmerberg et al. (2005)	Rats	♂ + ♀	H-UVL L-UVL	N/A	FST SIT DBT OFT	• HPC + AMY	• Immobility: L-UVL < H-UVL • SIT: L-UVL > H-UVL in $♀_{proestrus}$ • Center area: L-UVL > H-UVL • No changes in the OFT	• L-UVL > H-UVL in ♂ • L-UVL > H-UVL in $♀_{diestrus}$ • L-UVL = H-UVL in $♀_{proestrus}$
Zhang et al., 2014b	Rats	♂	TDS	Single event	CFCT EPM SCT	• PFC	• $\uparrow_{freezing}$ • $\downarrow_{time\ in\ open\ arms}$ • $\uparrow_{rearings}$	• ↓
Qiu et al. (2016)	Rats	♂	DM1	2 wks	FST SPT NSFT OFT	• HPC • PFC	• $\uparrow_{immobility}$ • $\downarrow_{preference}$ • $\uparrow_{latency\ to\ feed}$ • No changes in the OFT	• ↓
Zhang et al. (2017)	Rats	♂	CUS	4 wks	SPT NSFT OFT	• HPC • PFC	• $\downarrow_{preference}$ • $\uparrow_{latency\ to\ feed}$ • $\downarrow_{rearings}$ • $\downarrow_{crossings}$	• ↓
Qiu et al. (2017)	Rats	♂	CUS	4 wks	FST SPT NSFT OFT	• HPC • PFC	• $\uparrow_{immobility}$ • $\downarrow_{preference}$ • $\uparrow_{latency\ to\ feed}$ • No changes in the OFT	• ↓
Guo et al. (2017)	Rats	♂	CUS	3 wks	SPT NSFT OFT	• HPC • AMY	• $\downarrow_{preference}$ • $\uparrow_{latency\ to\ feed}$ • $\downarrow_{perm.\ center}$ • $\downarrow_{distance}$	• ↓ in HPC • ↓ in AMY
Lee et al. (2018)	Rats	♂	SPS	Single event	CFCT OFT	• HPC • PFC	• $\uparrow_{freezing}$ • No changes in the OFT	• ↓

(*continued on next page*)

Table 1 (*continued*)

Published in	Spe-cies	Sex (es)	Model(s)		Behavioral test (s)	Brain areas(s)	Main findings	
			Name(s)	Dura-tion			Behavioral changes	Brain allopregnanolone levels
Xu et al. (2018a)	Rats	♂	CUS	4 wks	EPM NSFT OFT	• HPC • PFC	• ↓time in open arms • ↑latency to feed • No changes in the OFT	• ↓
Xu et al. (2018b)	Rats	♂	SPS	Single event	CFCT EPM OFT	• HPC • PFC • AMY	• ↑freezing • ↓time in open arms • No changes in the OFT	• ↓
Zhang et al. (2018)	Mice	♂	TDS	Single event	CFCT EPM OFT	• DG	• ↑freezing • ↓time in open arms • No changes in the OFT	• ↓
Su et al. (2019)	Rats	♂	SPS	Single event	CFCT EPM OFT	• HPC • PFC	• ↑freezing • ↓time in open arms • No changes in the OFT	• ↓

Abbreviations and legends: increases (↑); decreases (↓); does not change (≅); followed by (→); tendency (tend.); whole brain (WB); cerebral cortex (CTX); hippocampus (HPC); dentate gyrus (DG); prefrontal cortex (PFC); frontal cortex (FC); amygdala (AMY); basolateral amygdala (BLA); striatum (STR); cerebellum (CBL); olfactory bulb (OB); olfactory bulbectomy (OBX); ovariectomy (OVX); orchiectomy (ORX); testosterone propionate (TP); diabetes mellitus type 1 (DM1); chronic unpredictable stress (CUS); high ultrasonic vocalization line (H-UVL); low ultrasonic vocalization line (L-UVL); social isolation (SI); group housed (GH); foot-shock stress (FSS); single prolonged stress (SPS); time-dependent sensitization (TDS); handling (HDL); maintenance (maint.); contextual fear conditioning test (CFCT); elevated plus maze (EPM); sucrose preference test (SPT); novelty-suppressed feeding test (NSFT); forced swim test (FST); open field test (OFT); resident-intruder test (RIT); defensive burying test (DBT); social interaction test (SIT); staircase test (SCT); not applicable (N/A); not informed (N.I.); *except if noted otherwise.
Unless otherwise specified, changes in allopregnanolone levels refer to all interventions used (model or drugs), doses, or brain areas analyzed in each study.

Chebib, 2017). Post-mortem studies in suicidal individuals have demonstrated epigenetic alterations in the expression and resulting composition of $GABA_A$Rs in suicidal individuals (Poulter et al., 2008; Yin et al., 2016), while *in vivo* imaging experiments have revealed functional dysfunctions in $GABA_A$Rs in the brain of depressed individuals (Klumpers et al., 2010).

Neurosteroids — endogenous molecules synthesized in the central nervous system from cholesterol — act as positive allosteric modulators of $GABA_A$Rs (Baulieu et al., 2001), placing this group of substances in a prominent position regarding the development of novel pharmacotherapies for depression. Extensive research has been conducted in this field for the last 20 years and has recently culminated with the approval of brexanolone, an intravenous formulation of allopregnanolone, as a new strategy for the treatment of severe postpartum depression by the United States Food and Drug Administration (Meltzer-Brody et al., 2018; Scott, 2019). The neurosteroid allopregnanolone (3α,5α-tetrahydroprogesterone, often abbreviated as 3α,5α-THP) presents a particularly high potency of positively modulating both synaptic and extrasynaptic $GABA_A$Rs (Carver and Reddy, 2013). Like other neurosteroids, its synthesis from cholesterol begins in the mitochondria with the cleavage of its side-chain, which gives origin to the neurosteroid precursor pregnenolone. In the cytoplasm, the action of the 3β-hydroxysteroid dehydrogenase (HSD) makes the conversion of pregnenolone to the widely distributed steroid hormone progesterone, which can then be metabolized to allopregnanolone by the successive action of two enzymes: 5α-reductase and 3α-HSD (Mellon et al., 2001). Importantly, the synthesis of allopregnanolone is downregulated in depressed individuals, as evidenced by its diminished levels in the cerebrospinal fluid (CSF) (Uzunova et al., 1998) and plasma (Schüle et al., 2006).

A significant portion of the research regarding the antidepressant effects of allopregnanolone has been conducted in experimental animals. More importantly, these preclinical studies allowed the exploration of specific mechanisms of action by which allopregnanolone might exert its antidepressant effects. In addition to detailing its interaction with $GABA_A$Rs and to which subunits it binds with higher affinity, many studies provide valuable insights into the mechanisms by which neurogenesis is related to depressive manifestations and to the antidepressant effects of allopregnanolone and other antidepressants, with the brain-derived neurotrophic factor (BDNF) as the main agent (Nin et al., 2011). These studies in animals took advantage of the possibility of measuring or infusing allopregnanolone in key regions of the limbic system and generated an extensively rich literature on the physiopathological and therapeutic role played by allopregnanolone in depressive-like behaviors across several experimental models of depression.

Taking this rationale into account, this review presents and discusses studies that explore the role of allopregnanolone on depressive-like behaviors in rodents. We examined reports of antidepressant-like effects of exogenous allopregnanolone or its regulation in several animal models of depression. Furthermore, we explore the evidence that links the depression modulating properties of allopregnanolone with neurogenesis, particularly mediated by the neurotrophic protein BDNF.

1.1. Brain allopregnanolone levels in animal models of depression

Several animal models of psychiatric disorders have used rodents to study the role of allopregnanolone in emerging depressive-like behaviors. A common strategy to reach this goal has been to induce a depression-like state in laboratory animals and quantify the levels of allopregnanolone in brain regions of interest (i.e., that integrate the neurocircuit known to be involved in the regulation of mood), comparing them to non-intervened controls. These models are based on what is known of the etiological aspects of depression, namely internal susceptibility (genetic construct) and external agents (environmental stressors). Though some models have been generated based on the genetic/heritable aspect of depression, most are based on the induction of a depression-like state through the application of stressors. The successful induction of this depression-like state is frequently confirmed by applying behavioral tests that measure ethological manifestations analogous to depressive symptoms. In this section, we review (Table 1) and discuss the most common models used to these ends and what they reveal about the role of allopregnanolone in the neurobiology of depression.

1.2. Forced swim test

Most of the behavioral data that will be presented in the following sections come from the forced swim test (FST), an animal model widely used to detect antidepressant-like activity across different classes of both potential and well-established antidepressant agents. The FST is based on the quantification of the time spent immobile by the rodent while being forced to swim (in rats, 24 h after a previous, longer exposure), which is interpreted as a depressive-like behavior (Detke and

Lucki, 1995; Porsolt et al., 1977). It has excellent predictive validity and reproducibility, as well as a significant translation between the clinical potency and the potency of antidepressants detected in the test (Slattery and Cryan, 2012). Although this model fails in aspects of face validity (e.g.: the detection of acute antidepressant effects of monoamine modulators seldom translate to what is observed in the clinical setting) (Nestler and Hyman, 2010), longer immobile behaviors are seen in animal models with concomitant depression-like states such as diabetes (Gomez and Barros, 2000) and a heritable genetic component has been proposed to influence depressive-like manifestations (Almeida et al., 2018).

Interestingly, the initial stress induced by the forced-swim session is accompanied by an acute increase in brain allopregnanolone that lasts from 10 min to 2 h after a 10-min exposure, as measured in whole or frontal cortex of male rats (Purdy et al., 1991; Vallée et al., 2000). Further experiments corroborated this finding by reporting an increase in the 5α-reductase enzyme in the prefrontal cortex of male rats (Sánchez et al., 2008). Brain allopregnanolone is known to surge around 30 min after exposure to acute stressors including CO_2 inhalation (Barbaccia et al., 1996), fixation stress (Higashi et al., 2005) and foot-shock (Pisu et al., 2013; Serra et al., 2002), which is likely what drives the observations in the FST. Though the aforementioned findings refer to male rats only, some studies indicate that there seems to be a sex influence, but they point to contradictory conclusions. Pisu et al. (2016) were able to replicate the foot-shock findings in male but not female rats, while Sze et al. (2018) found the opposite, namely a lack of effect in males and an increase in brain allopregnanolone in females after a 2-min FST. Finally, the stress-induced increase in allopregnanolone levels was not replicated in the brains of male mice after exposure to the FST, being that its levels were actually decreased in selective limbic brain areas of these animals (Maldonado-Devincci et al., 2014). Taken together, these results demonstrate that allopregnanolone levels rise in the brain of male rats after exposure to the forced swim test, though this is significantly less certain for female rats or mice. After the initial surge, allopregnanolone levels tend to return to those of unstressed controls, at least in those studies with longer endpoints of up to 2 h (Barbaccia et al., 1996; Purdy et al., 1991), though any further modulations remain unknown.

Thus, the FST is a reliable tool to study the antidepressant-like effects of allopregnanolone in the preclinical setting (as reviewed in Section 1.8). However, it is essential to point out that the FST is not a model of depression *per se*, but rather a model to quantify behaviors that could be considered as being analogous to symptoms of depression in humans, particularly in the context of assessing the effectiveness of antidepressant drug therapies (Nestler and Hyman, 2010). Therefore, even though its application to this end has been successfully established, it is not immediately possible to determine the mechanisms of action by using this model due to some limitations regarding construct validity. Furthermore, several anxiolytic drugs have been long been shown to reduce immobility in the FST (Flugy et al., 1992; Gomez and Barros, 2000) and, since allopregnanolone also presents anxiolytic-like effects (as reviewed in Schüle et al., 2011), it is difficult to distinguish between anxiolytic- and antidepressant-like effects by observing a decrease in immobility.

One other important point to consider is that the findings reported with the FST are mostly obtained in naïve rodents — that is, the animals were not submitted to a long-term protocol aiming to induce a lasting state analogous to depression —, which ultimately does not translate to the target population in humans (i.e., clinically depressed patients). Despite the fact that the detection of antidepressant-like effects in non-depressed-like rodents does not necessarily contradict clinical observations (Serretti et al., 2010), it is reasonable to postulate that the induction of a depression-like state would grant a higher translational value to studies that investigate the role of allopregnanolone on depressive-like behaviors.

1.3. Social isolation

One prominent model used to induce a depression-like state is the social isolation paradigm, which is a protocol typically used to model post-traumatic stress disorder (PTSD). In this model, the long-term deprivation of social interaction acts as a powerful stressor that results in a robust and consistently reproducible PTSD-like state in rodents, mainly characterized by the emergence of anxiety-like and aggressive behaviors (Guidotti et al., 2001). In fact, several studies have shown a social isolation-induced increase in aggression against a same-sex intruder in male mice (Pibiri et al., 2006; Pinna et al., 2005, 2003). As recently reviewed by Pinna (2019), the social isolation model may also reflect in some typical depressive-like behaviors due to the overlap between PTSD and depressive disorders. Though a review by Bogdanova et al. (2013) has reported mixed evidence on the effects of social isolation in the FST, it failed to mention contemporary studies that showed a clear immobility-inducing effect by social isolation in rats (Djordjevic et al., 2012; Evans et al., 2012) — which certainly tips the scales in favor of the use of social isolation within a depression-like paradigm. Additionally, social isolation has been shown to induce other depressive-like behaviors in rats, namely a decreased preference for sucrose and an increased ejaculation latency (Wallace et al., 2009).

Importantly, social isolation has also consistently been associated with a significant decrease in allopregnanolone levels in different brain regions of male rats (Evans et al., 2012; Pisu et al., 2016; Serra et al., 2000) and mice (Matsumoto et al., 1999; Nelson and Pinna, 2011; Pibiri et al., 2008; Pinna et al., 2004b, 2005). Allopregnanolone is downregulated in the cerebral cortex (Pisu et al., 2016; Serra et al., 2000) and hippocampus (Evans et al., 2012) of rats, as well as in the olfactory bulb (Nelson and Pinna, 2011; Pibiri et al., 2008; Pinna et al., 2004b, 2005), frontal cortex (Matsumoto et al., 1999; Nelson and Pinna, 2011; Pibiri et al., 2008; Pinna et al., 2004b), amygdala and hippocampus of socially isolated mice, remaining unchanged in the cerebellum and in the striatum (Nelson and Pinna, 2011; Pibiri et al., 2008).

There are significantly fewer studies investigating these parameters in female rodents, but the extant literature is complete enough to demonstrate that their response to social isolation is rather distinct from males. A study by Pinna et al. (2005) specifically compared the effects of social isolation in both sexes, showing that this model failed to increase aggression and to reduce allopregnanolone in the olfactory bulb of females. Interestingly, concomitant daily treatment with testosterone propionate in females resulted in increased aggression and reduced olfactory bulb allopregnanolone levels similar to what was observed in males. These findings in females were later replicated and the same modulatory effect was observed in the frontal cortex (Pibiri et al., 2006). In rats, a more recent study showed that, even though social isolation reduced cerebrocortical allopregnanolone in both sexes, the decrease was greater in males than in females (Pisu et al., 2016). These differences are likely related to the distinct dynamic hormonal profile of females, which is intimately involved with progesterone metabolism and behavioral manifestations related to mood and emotions (reviewed by Frye, 2009).

The downregulation of allopregnanolone in brain areas involved with the corticolimbic system after social isolation strongly suggests that this neurosteroid plays an important role in mood disorders and in the emergence of associated depression-like behaviors. Moreover, it indicates that the fluctuations of other hormones, and thus of other neurosteroids in the brain, may exert complementary mood regulation in rodents.

1.4. Chronic unpredictable stress

Another model that stands out because of its long history as a classical animal model of depression is the chronic unpredictable stress paradigm (CUS; also called “chronic mild stress”, “chronic variable stress” and other variations). Originally proposed by Paul Willner in

1987, this model consists of the application of a series of variable, unpredictable stressors for a long period of time (5–9 weeks) that results in a depression-like state characterized mainly by anhedonia-like behaviors (Willner, 2017a). There are reports of decreased preference for sucrose (Guo et al., 2017; Qiu et al., 2017; Zhang et al., 2017) and of increased immobility in the FST (Qiu et al., 2017) in male rats submitted to CUS. Anxiety-like behaviors measured in tests such as the elevated plus-maze and of novelty suppressed feeding were also present in animals submitted to CUS (Guo et al., 2017; Qiu et al., 2017; Xu et al., 2018b; Zhang et al., 2017). Notably, all of these behavioral findings were associated with allopregnanolone downregulation in the hippocampus (Guo et al., 2017; Qiu et al., 2017; Xu et al., 2018b; Zhang et al., 2017), prefrontal cortex (Qiu et al., 2017; Xu et al., 2018b; Zhang et al., 2017) and amygdala (Guo et al., 2017). These reductions are further explained by the downregulation of hippocampal and amygdalar mRNA expression of neurosteroidogenic enzymes of importance to the biosynthesis of brain allopregnanolone, such as the 3α-HSD, 3β-HSD and 5α-reductase (Guo et al., 2017).

Though some uncertainty regarding the reproducibility of the CUS model has been frequently raised over the years, a significant portion of this apparent problem might derive from factors such as excessively short exposures (two weeks or less) (Willner, 2017b). In fact, the aforementioned studies used three to four week protocols of unpredictable stressors to observe depressive-like behaviors associated with brain allopregnanolone downregulation. The above findings provide additional neurobiological mechanisms for this model, namely allopregnanolone downregulation in key limbic regions associated to behavioral changes induced by several stressors, and highlight the need to further investigate the role of allopregnanolone and other neurosteroids in this paradigm.

1.5. Other rodent models

Reports of changes in brain neurosteroid levels in other animal models of depression date as far back as 2003, when Uzunova and colleagues provided the initial evidence that the rat olfactory bulbectomy modulates brain allopregnanolone levels depending on the brain region and the time after the intervention (Uzunova et al., 2003). Later, a model in which rats were selectively bred for high or low infantile ultrasonic vocalizations after maternal separation showed a line-dependent modulation of brain allopregnanolone that was associated with depression-like behaviors in males and proestrus females (Zimmerberg et al., 2005). Other studies using procedures such as the single prolonged stress (2 h-restraint + 20 min-forced swim + loss of consciousness by ether vapor) (Lee et al., 2018; Su et al., 2019; Xu et al., 2018a), time-dependent sensitization (single sequence of 15 inescapable foot-shocks) (Zhang et al., 2014, 2018) and even streptozotocin-induced type 1 diabetes combined with a high fat diet (Qiu et al., 2016), have consistently demonstrated depression-like states in male rats and decreased allopregnanolone levels in the prefrontal cortex and hippocampus.

All of the studies aggregated in this section demonstrated that there is a consistent decrease in brain allopregnanolone levels in corticolimbic regions associated with a plethora of long term stress-based animal models of psychiatric disorders that induce a depression-like state in rodents. Taking all these behavioral results associated with allopregnanolone fluctuations into account, the next stages in experimental research have been to investigate potential therapies/interventions targeted to increase allopregnanolone in the brain regions of interest in order to elicit antidepressant-like effects.

1.6. Antidepressant-like action of allopregnanolone

In the next sections of our review, we gather the evidence that demonstrates the capability of allopregnanolone to decrease depressive-like behaviors in rodents when submitted to preclinical screening tests used to assess antidepressant activity, such as the FST. We begin by exploring the increase in brain allopregnanolone elicited by certain classical antidepressants believed to exert their effects through a different mechanism: the upregulation of brain neurosteroidogenesis.

1.7. Stimulation of allopregnanolone biosynthesis by antidepressants

The selective serotonin reuptake inhibitor (SSRI) fluoxetine has widespread use in medicine and is one of the antidepressant drugs that are most commonly used as a positive control during preclinical experiments. This is because it consistently decreases immobility time by increasing swimming in the FST (as originally described by Detke and Lucki, 1995), which is a parameter related to serotonergic mechanisms. Interestingly, the first evidence of a drug-induced modulation of brain allopregnanolone in the context of depression was provided by Uzunov and colleagues in the following year, and also had fluoxetine as the main comparable substance (Uzunov et al., 1996). In this report, a single injection of fluoxetine or paroxetine increased allopregnanolone levels in corticolimbic brain regions of rats for at least 2 h after treatment. These observations sparked interest regarding the capability of other SSRIs to act as selective brain steroidogenic stimulants (SBSSs), a possibility that was further supported by subsequent *in vitro* experiments that demonstrated the increased activity of neurosteroidogenic enzymes associated with fluoxetine, paroxetine and sertraline (Griffin and Mellon, 1999). Furthermore, *in vivo* increases in cerebrocortical allopregnanolone levels induced by numerous SSRIs were later reported after a three-week treatment with paroxetine in male mice (Nechmad et al., 2003) and with fluoxetine, desipramine, sertraline and venlafaxine in olfactory bulbectomized male rats (Uzunova et al., 2004). It is worth mentioning that other classes of drugs have also been reported to increase brain allopregnanolone levels, namely the atypical antipsychotics clozapine and olanzapine (Marx et al., 2006, 2003) and the benzodiazepines midazolam (Qiu et al., 2015) and estazolam (Xu et al., 2018b). On the other hand, a single injection of the tricyclic antidepressant imipramine at behaviorally active doses has consistently been shown not to change brain allopregnanolone levels after 30 min (Pinna et al., 2003, 2004b; Uzunov et al., 1996), probably due to its reported lack of effect on neurosteroidogenic enzymes (Griffin and Mellon, 1999). This is relevant because it is one of the lines of evidence that indicates that not all antidepressants act by this mechanism and that allopregnanolone upregulation is not simply a side effect of monoamine reuptake, but rather a direct effect of SSRIs like fluoxetine. The precise mechanism by which fluoxetine increases brain allopregnanolone levels has not yet been fully determined, but early evidence pointed to the direct activation of the 3α-HSD enzyme which converts 5α-dihydroprogesterone into allopregnanolone (Griffin and Mellon, 1999). This finding could not be replicated, however (Trauger et al., 2002), and more recent evidence indicates that, at least in female rats, the inhibition of a steroid microsomal dehidrogenase may consist in a more robust mechanism by avoiding allopregnanolone oxidation to 5α-dihydroprogesterone (Fry et al., 2014).

Importantly, fluoxetine has been shown to increase brain neurosteroid content at doses remarkably lower than those needed to inhibit serotonin reuptake, as determined by *ex vivo* uptake measurements on brain slices or *in vivo* serotonin detection by microdialysis (Devall et al., 2015; Pinna et al., 2003, 2004b). Thus, these findings granted further support for a direct action of fluoxetine on steroidogenesis, as outlined above. Additionally, they raised the question of whether the administration of fluoxetine at such low doses is capable of eliciting a similar antidepressant-like action by upregulating brain allopregnanolone while being devoid of any significant serotonergic action. Early studies have failed to detect an antidepressant-like effect in the mice FST with low-range doses (1 and 5 mg/kg) (Khisti et al., 2000; Khisti and Chopde, 2000), but a later work achieved this goal in rats using doses as low as 1 and 2 mg/kg (Molina-Hernández et al., 2005). This indicates that a possible antidepressant-like effect of non-SSRI doses of fluoxetine

hovers just over the detectable threshold in the FST, and different species or subtle modifications in the protocol might affect its results.

Given the difficulty generated by such a low and narrow dose window, most of the studies that investigated the antidepressant-like action of allopregnanolone opted to directly administer the neurosteroid instead of upregulating its levels by using drugs that act indirectly and might exert the same target effect by confounding mechanisms.

1.8. Exogenous allopregnanolone administration

The seminal studies on the antidepressant-like effect of exogenous allopregnanolone were conducted by Khisti and colleagues in 2000, in which immobility in the FST was reduced 30–60 min after a single intraperitoneal administration in male mice, in doses that ranged between 0.5 and 2 mg/kg (Khisti et al., 2000; Khisti and Chopde, 2000). The same effect was also detected in ovariectomized female mice in a dose range of 0.5–5 mg/kg (Rodríguez-Landa et al., 2007). To the best of our knowledge, only one study reported a lack of behavioral effects after systemic allopregnanolone injection in male rats, in spite of its neurochemical effects (Naert et al., 2007). However, it is important to point out that even though this study evaluated a comprehensive timeframe after treatment, the only dose tested (0.05 mg/kg) is ten times lower than the lowest dose ever reported to elicit antidepressant-like effects after systemic acute treatment. Chronic treatment regimens with subcutaneous allopregnanolone, over several weeks, have also shown antidepressant-like activity in rats, whether in ovariectomized females (Molina-Hernández et al., 2005) or in males (Evans et al., 2012). It must be mentioned that allopregnanolone easily crosses the blood-brain barrier (Hellgren et al., 2014), and the systemic administrations of the neurosteroid allow the assessment of its whole-brain effect. However, a common and more cost-effectively approach has been to infuse microdoses of the neurosteroid directly into the cerebral ventricles, from where it diffuses across the brain with similar distribution and antidepressant-like effects to the systemic injection (Almeida et al., 2018; Khisti et al., 2000; Khisti and Chopde, 2000; Shirayama et al., 2011).

Other studies directed these microinjections to specific areas of the limbic system, with the ultimate goal of assembling a more detailed map of the probable specific brain sites of action where allopregnanolone evokes its antidepressant-like effect. These studies targeted areas where allopregnanolone levels were downregulated in animal models of depression (see Sections 1.3, 1.4 and 1.5) and used the same dose range as in the intracerebroventricular protocols. The brain area that presents the most replicated results is the hippocampus (Nin et al., 2008; Rodríguez-Landa et al., 2009; Shirayama et al., 2011), with the amygdala (Shirayama et al., 2011) and the nucleus accumbens (Molina-Hernández et al., 2005; Nin et al., 2012) contributing as important regions for the antidepressant-like effects in the FST. However, in contrast with the well-reported allopregnanolone downregulation in the prefrontal cortex associated with animal models of depression (see Sections 1.3, 1.4 and 1.5), the few studies that have infused allopregnanolone in this brain area did not detect important behavioral changes in the FST (Almeida et al., 2019; Shirayama et al., 2011). All of these results (compiled in Table 2) support the hypothesis that allopregnanolone, in a similar manner as some neurotransmitters involved with depression, exerts its antidepressant role through pathways associated with the limbic system, probably acting on all the main brain sites responsible for the balance that promotes depressive/antidepressive regulation.

A few studies have reported behavioral changes following exogenous allopregnanolone administration to rodents submitted to animal models of depression. In mice, a single intraperitoneal injection of allopregnanolone reduced the social isolation-induced aggressive behavior in males and females (Pibiri et al., 2006). Additionally, in socially isolated rats, a subcutaneous insertion of allopregnanolone-containing pellets normalized immobility time in the FST, both when treatment was started at the onset of isolation or six weeks into the protocol (Evans et al., 2012). More recently, the intracerebroventricular infusion of allopregnanolone has been reported to reduce depressive-like behaviors in a line of rats that were selectively bred to present high immobility in the FST, while having no effect on the line bred to present low immobility in the FST (Almeida et al., 2018). Apart from these studies, researchers have generally preferred to treat depressed-like animals with neurosteroidogenesis-modulating drugs, verifying their behavioral effects, and then quantifying allopregnanolone in brain regions of interest.

2. Pharmacological allopregnanolone upregulation: behavioral effects in animal models of depression

SSRIs — specially fluoxetine and norfluoxetine — have been the most frequently used drugs to attempt a reversion of brain allopregnanolone downregulation in animal models of depression. A particularly relevant subset of these studies has tested the effect of a single administration of these drugs at non-SSRI doses (Table 3). In socially isolated male mice, for instance, low doses of fluoxetine have been shown to normalize allopregnanolone levels in the frontal cortex of mice without eliciting the same effect in group-housed animals (Matsumoto et al., 1999). Posterior studies have detailed the stereotypic-specific neurosteroidogenic action of fluoxetine and its active metabolite norfluoxetine in this model, demonstrating that the S-isomers of both drugs — but especially norfluoxetine — showed much higher potency in reducing aggressive behavior (Pinna et al., 2004b) and increasing allopregnanolone levels in the frontal cortex and olfactory bulb (Pinna et al., 2003, 2004b). S-norfluoxetine at similar non-SSRI doses exerted the same effect in female mice treated with testosterone propionate (Nelson and Pinna, 2011), and in male mice when infused directly into the basolateral amygdala (Pibiri et al., 2006). The presence of a depression-like state seems to be important for this neurosteroidogenic effect to take place, since low doses of fluoxetine have been reported not to increase whole brain allopregnanolone in naïve male rats (Fry et al., 2014).

Regarding the classic SSRI doses (Table S1), long-term oral treatment with fluoxetine has also successfully restored brain allopregnanolone levels in models of social isolation (Evans et al., 2012), chronic unpredictable stress (Guo et al., 2017), single prolonged stress (Lee et al., 2018), and streptozotocin-induced diabetes combined with high fat diet (Qiu et al., 2016), in regions such as the prefrontal cortex and hippocampus. This normalization was accompanied by antidepressant- (Evans et al., 2012; Qiu et al., 2016) and anxiolytic-like (Lee et al., 2018) effects, but the doses applied in these studies were high enough to also inhibit serotonin reuptake. Chronic treatment (10–30 days) with sertraline (15 mg/kg) has also normalized (Qiu et al., 2017; Su et al., 2019; Xu et al., 2018b, 2018a; Zhang et al., 2018) or at least shown a tendency to increase (Zhang et al., 2014) brain allopregnanolone levels in stress-induced animal models of depression while exerting antidepressant-like (Qiu et al., 2017) or anxiolytic-like (Qiu et al., 2017; Su et al., 2019; Xu et al., 2018b, 2018a; Zhang et al., 2018) effects. These reports come mainly in the context of positive controls from studies that demonstrated that some biologically active compounds purified from natural extracts are able to increase brain allopregnanolone (Guo et al., 2017; Lee et al., 2018; Qiu et al., 2017; Su et al., 2019; Xu et al., 2018b), suggesting that the group of SBSSs may be larger than currently estimated (Table S2). However, it is important to point out that these latter findings lack independent replication and their neurosteroidogenic mechanism of action remains even more elusive than that of SSRIs.

A specific molecular target unrelated to SSRIs that has received crescent attention in the past years has been the 18 kDa translocator protein (TSPO), a five transmembrane domain protein that is mainly located in the mitochondria of glial cells (Costa et al., 2012). This

Table 2
Behavioral effects of exogenous allopregnanolone administration in rodents.

Published in	Spe-cies	Sex	Dose(s)	Route	Time before test	Region(s)	Behavioral test (s)	Main findings
Khisti and Chopde (2000)	mice	♂	1 × 0.5–2 mg/kg 1 × 0.5–2 μg/mouse	i.p. m.i.	30 min 15 min	• Syst. • CV	• FST	• 0.5; 1; 2: ↓immobility • 1; 2: ↓immobility
Khisti et al. (2000)	mice	♂	1 × 0.5–2 mg/kg 1 × 0.5–2 μg/mouse	i.p. m.i.	30 min 15 min	• Syst. • CV	• FST	• 0.5; 1; 2: ↓immobility • 1; 2: ↓immobility
Molina-Hernández et al. (2005)	rats	♀	21 × 0.5–2 mg/kg/d 1 × 1–4 μg/rat	s.c. m.i.	30 min 15 min	• Syst. • NAcc	• FST	• 1.5; 2: ↓immobility (↑climbing) • 3; 4: ↓immobility (↑climbing)
Rodríguez-Landa et al. (2007)	mice	$♀_{OVX}$	1 × 0.5–3 mg/kg	i.p.	1 h	• Syst.	• FST • OFT	• 1; 2; 3: ↓immobility
Pibiri et al. (2006)	mice	♂ + ♀	1 × 1.25–5 mg/kg	i.p.	30 min	• Syst.	• RIT	• ALLO (2.5; 5): ↓aggression
Naert et al. (2007)	rats	♂	1 × 0.05 mg/kg	i.p.	15–300 min	• Syst.	• FST	• No changes in the FST
Nin et al. (2008)	rats	♂	3 × 1.25–5 μg/rat	m.i.	24, 5, 1 h	• HPC	• FST	• 2.5: ↓immobility
Rodríguez-Landa et al. (2009)	rats	♂	1 × 2 μg/rat	m.i.	immedia-tely	• HPC_{LS} • HPC_{SF} • HPC_{D}	• FST • OFT	• HPC_{LS} and HPC_{D}: ↓immobility
Shirayama et al. (2011)	rats	♂	1 × 0.1 or 1 μg/rat	m.i.	4 d	• CV • HPC_{CA3} • HPC_{DG} • PFC • NAcc • BLA • CeA	• LHT • OFT	• 1: ↓lat./failure to escape ○ ICV, HPC_{CA3} and CeA • HPC_{DG}, PFC and BLA: no effect • No changes in the OFT
Evans et al. (2012)	rats	♂	50 mg released over 50 days	s.c.		• Syst.	• FST • NSFT	• ↓immobility
Nin et al. (2012)	rats	♂	3 × 1.25–5 μg/rat	m.i.	24, 5, 1 h	• NAcc	• FST • M-Groom	• 5: ↓immobility (↑climbing) • 1.25; 5: ↓% correct transitions
Almeida et al. (2018)	rats	♂	3 × 5 μg/rat	m.i.	24, 5, 1 h	• CV	• FST	• ↓immobility (↑swimming)
Almeida et al. (2019)	rats	♂	3 × 1.25–5 μg/rat	m.i.	24, 5, 1 h	• PFC	• FST • M-Groom	• No changes in the FST and M-Groom

Abbreviations and legends: increases (↑); decreases (↓); does not change (≗); ovariectomy (OVX); systemic (syst.); intraperitoneally (i.p.); subcutaneously (s.c.); microinjection (m.i.); hippocampus (HPC); dentate gyrus (DG); dorsal (D); lateral septum (LS); septofimbrial (SF); prefrontal cortex (PFC); central amygdala (CeA); basolateral amygdala (BLA); cerebral ventricle (CV); nucleus accumbens (NAcc); forced swim test (FST); open field test (OFT); resident-intruder test (RIT); learned helplessness test (LHT); novelty-suppressed feeding test (NSFT); microstructured grooming (M-Groom); latency (lat.).

protein is believed to play a major role in the transport of cholesterol to the inner mitochondrial membrane (Schüle et al., 2011), though this has been disputed given that TSPO-knockout mice present normal steroidogenesis, at least in non-cerebral tissue (Morohaku et al., 2014; Tu et al., 2014). What is known is that once cholesterol enters the mitochondria, it can then be converted to neurosteroid precursor pregnenolone by P450scc, making its transport a putative rate-limiting step for brain neurosteroidogenesis (Schüle et al., 2011). In fact, lentivirus-induced overexpression of this protein in the dentate gyrus has been shown to increase hippocampal allopregnanolone concentrations in mice (Li et al., 2017) and to normalize its levels in the dentate gyrus of mice exposed to a foot-shock-induced PTSD model (Zhang et al., 2018). These modulations were accompanied by reductions in depressive-like behaviors (Li et al., 2017) and anxiolytic-like effects (Zhang et al., 2018), making TSPO a promising therapeutic target for psychiatric disorders, especially in depression. Because TSPO expression is also increased in damaged neural tissue and is involved with inflammatory responses associated with neurodegenerative disorders (Dupont et al., 2017; McNeela et al., 2018), the specificity of this target must be further investigated and its role in other disorders must be considered as well.

In pharmacological approaches, long-term treatment (18 days–6 weeks) with TSPO agonist YL-IPA08 at a dose of 0.3 mg/kg/day has been shown to normalize brain allopregnanolone levels while abolishing depressive- (Zhang et al., 2017) and anxiety-like behaviors (Zhang et al., 2017, 2014) induced by stress-induced models of depression. Similar protocols using another TSPO ligand, AC-5216, have also reversed depressive- (Qiu et al., 2016; Zhang et al., 2017) and anxiety-like (Zhang et al., 2017) behaviors by increasing allopregnanolone in the prefrontal cortex and hippocampus. TSPO is also a molecular target for benzodiazepines (hence its previous denomination of "peripheral-type benzodiazepine receptor"), explaining why midazolam, for instance, decreases immobility in the FST while increasing brain allopregnanolone levels (Qiu et al., 2015).

Taken together, all these data suggest that fluoxetine and norfluoxetine, in doses which only act via neurosteroidogenic pathways (that is, as SBSSs agents), but also in the classical posology that elicits serotonin reuptake inhibition, exert antiaggression, antidepressant, and anxiolytic-like effects. The aggressive-like induction is a specific indicator of stress and is highly associated with the PTSD-like behaviors induced by social isolation, which can also evoke depressive and anxiety-like states. Also, despite the relatively small number of articles addressing the antidepressant effect of TSPO manipulation, the results seem to be highly replicable and robust (synthesized in Table 3), indicating that pharmacological induction of this neurosteroids synthesis show satisfactory results regarding its antidepressant-like effects in rodents.

3. Allopregnanolone and neurotrophic pathways

The protagonism regarding the mechanism of action by which allopregnanolone elicits its antidepressant effects has classically been attributed mainly to the GABAergic system. Like several other similar neuroactive steroids, allopregnanolone acts as a positive allosteric modulator on the main inhibitory receptor of the nervous system, the $GABA_AR$ (for a more detailed review on this topic, see the paper by Zorumski et al., 2019). Other neurotransmitter systems have also been implicated — though with remarkably less evidence — in that behavioral response, either directly or indirectly. Serotonin, as the main neurotransmitter involved with mood regulation, has been shown to modulate antidepressant allopregnanolone effect (Khisti and Chopde, 2000), as well as dopamine (D';Aquila et al., 2010; Frye et al., 2004), and some neurosteroidogenic enzymes (Espallergues et al., 2012; Khisti and Chopde, 2000; Pinna et al., 2005).

Table 3
Behavioral effects of the pharmacological normalization of brain allopregnanolone in animal models of depression.

Published in	Spe-cies	Sex (es)	Model		Treatment(s)				Behavio-ral tests	Measu-red after	Brain areas(s)	Main findings	
			Name	Dura-tion	Drug(s)	Dose(s) in mg/kg*	Ro-ute	Dura-tion				Brain allopregnanolone levels	Behavioral changes
Fluoxetine (FLX) and norfluoxetine (NFLX) at selective brain steroidogenic stimulant doses													
Matsumoto et al. (1999)	Mice	♂	SI	2–10 wks	● FLX	0.45; 0.9	i.p.	Single	None	0.5 h	● FC	● FLX: ↑ ($\uparrow_{0.9}$ > $\uparrow_{0.45}$)	N/A
Pinna et al. (2003)	Mice	♂	SI	1 d–8 wks	● R-FLX ● S-FLX ● R-NFLX ● S-NFLX	0.37; 0.45 0.14–0.45 0.27 0.07; 0.27	i.p. i.p. i.p. i.p.	Single Single Single Single	RIT	0.5 h 0.5 h 0.5 h 0.5 h	• OB	● R-FLX (0.45): ↑ ● S-FLX (0.37; 0.45): ↑ ● R-NFLX: ≦ ● S-NFLX (0.07; 0.27): ↑	● R-FLX (0.45–1.1): $\downarrow_{aggression}$ ● S-FLX (0.28–1.1): $\downarrow_{aggression}$ ● R-NFLX: (0.53–1): $\downarrow_{aggression}$ ● S-NFLX (0.09–1): $\downarrow_{aggression}$
Pinna et al. (2004b)	Mice	♂	SI	4–6 wks	● R-FLX ● S-FLX ● R-NFLX ● S-NFLX	0.37; 0.6 0.14–0.6 0.04–0.27 0.07–0.27	i.p. i.p. i.p. i.p.	Single Single Single Single	None	0.5 h 0.5 h 0.5 h 0.5 h	● OB ● FC	● R-FLX (0.6): ↑ • S-FLX (0.37–0.6): ↑ • R-NFLX: ≦ • S-NFLX (0.07–0.27): ↑	N/A
Pibiri et al. (2006)	Mice	♀	SI + TP	3 wks	● S-NFLX	0.53; 1.1	i.p.	Single	RIT	N.I.	● OB ● FC	● S-NFLX: ↑	● S-NFLX: $\downarrow_{aggression}$
Nelson and Pinna (2011)	Mice	♂	SI	4 wks	● S-NFLX (in BLA)	1.1 μg/mouse	m.i.	Single	RIT	< 1 h	● OB ● FC ● BLA ● HPC ● STR	● S-NFLX: ↑ in BLA and HPC	● S-NFLX: $\downarrow_{aggression}$
TSPO modulators													
Zhang et al., 2014b	Rats	♂	TDS	Single event	● YL-IPA08 • PK11195	0.1–1 1; 3	i.g. i.p.	18 d 18 d	CFCT EPM	1 h 1 h	• PFC	● YL-IPA08 (0.3): ↑ ○ PK11195 ⊣ ↑	● YL-IPA08 (0.1–0.3): $\downarrow_{freezing}$ • YL-IPA08 (0.1–0.3): $\uparrow_{open\ arms\ time}$
Qiu et al. (2016)	Rats	♂	DM1	2 wks	● AC-5216 ● PK11195	0.1–1 1; 3	i.g. i.p.	13 d 13 d	FST SPT NSFT OFT	24 h 24 h	● HPC ● PFC	● AC-5216 (0.3; 1): ↑ in PFC • AC-5216 (1): ↑ in HPC ○ PK11195 ⊣ $\uparrow_{(1)}$ • PK11195: ≦	● AC-5216 (0.3; 1): $\downarrow_{immobility}$ • AC-5216 (1): $\uparrow_{sucrose\ preference}$ • AC-5216 (1): $\downarrow_{latency\ to\ feed}$ • No changes in the OFT
Zhang et al. (2017)	Rats	♂	CUS	4 wks	● AC-5216 ● YL-IPA08	0.3 0.1–1	i.g. i.g.	6 wks 6 wks	SPT NSFT OFT	N.I. N.I.	● HPC ● PFC	● AC-5216: ↑ • YL-IPA08 (0.1; 0.3): ↑	● AC-5216: $\uparrow_{sucrose\ preference}$ • AC-5216: $\downarrow_{latency\ to\ feed}$ • AC-5216: $\uparrow_{rearings}$ • YL-IPA08 (0.1–0.3): $\uparrow_{sucrose\ preference}$ • YL-IPA08 (0.1–1): $\downarrow_{latency\ to\ feed}$ • YL-IPA08 (0.1–0.3): $\uparrow_{rearings}$

(*continued on next page*)

Table 3 (*continued*)

Published in	Spe-cies	Sex (es)	Model		Treatment(s)				Behavio-ral tests		Measu-red after	Brain areas(s)	Main findings	
			Name	Dura-tion	Drug(s)	Dose(s) in mg/kg*		Ro-ute	Dura-tion				Brain allopregnanolone levels	Behavioral changes
Zhang et al. (2018)	Mice	♂	TDS	Single event	• TSPO-LV (in HPC_{DG}) • PK11195	4×10^8 (TU/μl) 3	m.i. i.g.		30 d 30 d	CFCT EPM OFT	N.I.	• DG	• $TSPO_{OX}$: ↑ ○ PK11195 ⊣ ↑	• ↓freezing (PK11195 ⊣ effect) • ↑open arms time (PK11195 ⊣ effect) • No changes in the OFT

Abbreviations and legends: increases (↑); decreases (↓); does not change (≅); blocks (⊣); hippocampus (HPC); dentate gyrus (DG); prefrontal cortex (PFC); frontal cortex (FC); basolateral amygdala (BLA); striatum (STR); olfactory bulb (OB); fluoxetine (FLX); norfluoxetine (NFLX); testosterone propionate (TP); intraperitoneally (i.p.); intragastric gavage (i.g.); microinjection (m.i.); diabetes mellitus type 1 (DM1); chronic unpredictable stress (CUS); social isolation (SI); time-dependent sensitization (TDS); 18 kDa translocator protein (TSPO); lentivirus (LV); overexpression (OX); contextual fear conditioning test (CFCT); elevated plus maze (EPM); sucrose preference test (SPT); novelty-suppressed feeding test (NSFT); forced swim test (FST); open field test (OFT); resident-intruder test (RIT); not applicable (N/A); not informed (N.I.); *except if noted otherwise. Unless otherwise specified, changes in allopregnanolone levels refer to all interventions used (model or drugs), doses, or brain areas analyzed in each study.

Going beyond the neurotransmitter systems, animal models of depression induced by chronic stress, such as those explored in Sections 1.3, 1.4 and 1.5, have been shown to modify a distinct aspect of neurobiology, namely the brain neurogenesis. This is evidenced by the reduction of both the production and survival of adult-born hippocampal granule cell neurons in animals submitted to these models. Because the effectiveness of antidepressants is largely dependent on these cells, neurogenesis has been considered an important mechanism by which depressive behaviors are modulated (reviewed by Samuels and Hen, 2011). In fact, hippocampal allopregnanolone downregulation is associated with impaired neurogenesis in the same brain region (Evans et al., 2012). In the same direction, robust evidence has been presented to support a role for neurotrophic agents in the antidepressant effect of allopregnanolone and SBSSs. Here, we gather the data showing that the treatment with allopregnanolone or SBSSs alters neurotrophic protein levels in the limbic system of depressed-like rodents by interacting directly with or in parallel to GABAergic modulation, discussing the participation of neurogenesis in depressive-like states.

3.1. BDNF: a key neurotrophic protein involved in the behavioral effects of allopregnanolone

Complex interactions between genetics, hormones, neurotransmitters, and environmental factors are involved in depression. BDNF is a crucial mediator of neuronal plasticity, which regulates synaptic composition, neuronal maturation, neurotransmitter release, survival and excitability in the adult nervous system (Huang and Reichardt, 2001). The pro and mature isoforms of BDNF can be synthesized by and released from neurons, being widely distributed in the limbic system. They bind to the tropomyosin receptor kinase B (TrkB), which has a higher affinity for the mature isoform of the neuropeptide (Nagahara and Tuszynski, 2011). Recently, the BDNF-TrkB signaling has been pointed out as a likely mediator between antidepressant agents and the improvement of depressive symptoms (Björkholm and Monteggia, 2016). Stress and depression have been widely documented to reduce the expression of BDNF in both animal and clinical studies. Two meta-analyses have shown that serum BDNF concentrations are low in untreated depressed patients and normalized by antidepressant treatment, and the greater decrease in symptom alleviation was accompanied by a greater increase in serum BDNF concentrations (Molendijk et al., 2014; Sen et al., 2008).

Such observations are more likely to be seen in women than in men (Huang et al., 2008), but the lack of experimental studies in females makes it difficult to verify if these findings are replicable in the brain. One study found that social isolation reduced BDNF in the cerebral cortex of male but not female rats (Pisu et al., 2016), which points to the opposite direction of clinical findings. In fact, though not assessing BDNF specifically, other studies in rodents that used social isolation have revealed a sex-dependent response in aggression-like behavior and brain allopregnanolone levels (being lower in females), as discussed in Section 1.3. However, studies that took a different approach by knocking out the BDNF gene in mice found pro-depressant effects in females but not males, when matched to wild-type controls (Autry et al., 2009; Monteggia et al., 2007). Given the contradicting findings in the literature, the need for more studies that investigate sex-dependent differences on the role of BDNF on depression becomes even more evident.

In humans, these BDNF modulations become especially apparent in the context of pregnancy, since its serum levels decline considerably throughout pregnancy with a subsequent postpartum increase. Moreover, an inverse relationship between depressive symptoms and serum BDNF during the 3rd trimester (Christian et al., 2016) and postpartum (Gazal et al., 2012) is observed. This supports the role of this neurotrophin in the development of postpartum depression, a very serious complication following delivery that may affect 10–15% of

women within the first 3 months postpartum with important consequences to both mother and child (Christian et al., 2016; Gazal et al., 2012). Due to their apparent superior efficacy, SSRIs are the first line of treatment for severe cases of postpartum depression (Kim et al., 2014).

As reviewed by Nin and colleagues in 2011, "the pharmacological actions of SSRIs are induced by their ability to act as SBSSs, which suggests a novel and more selective mechanism for the behavioral action of this class of drugs". In fact, this review summarizes the association of depression and decreased cerebral and systemic BDNF, and also that SBSSs succeed to reverse these BDNF decreased levels (Nin et al., 2011). In a more recent review, Kojima et al. (2019) offered a possible explanation for the decreased BDNF expression in patients with major depressive disorder and in animal models of depression. Considering that BDNF expression is controlled by neuronal activity, low BDNF pro-peptide levels in the CSF may be the result of lower neuronal activity in the brain of depressed individuals. In fact, there seems to be an important connection between BDNF (both in its pro and mature isoforms) and GABAergic activity, though the specific mechanisms by which this interaction takes place are still being elucidated. Some evidence points to a net excitatory effect in the superior colliculus by postsynaptic inhibition of the GABAergic currents (Henneberger et al., 2002), but since this is not seen in the visual cortex (Abidin et al., 2008) or amygdala (Meis et al., 2019), it seems to be a region-dependent effect. In the hippocampus, BDNF is thought to increase cell surface expression of $GABA_A$Rs by TrkB activation-induced inhibition of receptor endocytosis, enhancing GABAergic inhibition (Porcher et al., 2018).

Several studies have demonstrated a general downregulation of BDNF in the hippocampus and frontal cortex in stress-based animal models of depression (Phillips, 2017). As already discussed in this review, such animal models have been shown to decrease allopregnanolone levels in these and other brain areas relevant to the neurobiology of depression (see Sections 1.3, 1.4 and 1.5). In the social isolation protocol, for instance, the reduction in cerebrocortical allopregnanolone is accompanied by decreased hippocampal BDNF in male rats, though not in females (Pisu et al., 2016). Similar effects on BDNF have been observed after exposure to CUS (Rudyk et al., 2019), with a greater magnitude in those animals that present more accentuated depressive-like behaviors (Tornese et al., 2019). This stress-induced downregulation appears to have long-lasting effects since hippocampal BDNF is decreased until seven days after a single prolonged stress protocol (Lee et al., 2018). Additionally, long-term treatment with allopregnanolone (Evans et al., 2012), fluoxetine (Evans et al., 2012; Lee et al., 2018), or other potential SBSSs (Lee et al., 2018) restores the low hippocampal BDNF levels back to normal.

Indeed, the hippocampus appears to be the main region involved with the neurotrophic regulation of allopregnanolone. When the pregnane xenobiotic receptor (PXR) — a protein involved in cholesterol metabolism — is knocked down in rats, a downregulation in hippocampal allopregnanolone and in BDNF is observed, suggesting that PXR may influence the allopregnanolone synthesis by neuroplasticity mechanisms (Frye et al., 2014). Conversely, BDNF levels in that region are increased 3 h following a single low-dose of allopregnanolone i.p. administration (Naert et al., 2007). Moreover, 1 h after sub-acute intra-prefrontal cortex infusion, it is increased both in the left and right hippocampus, with a tendency to be higher in the right hemisphere (Almeida et al., 2019). Interestingly, these rapid hippocampal BDNF regulations were observed even in the absence of an associated antidepressant-like effect in the FST.

There is some evidence indicating that the hippocampus is not the only relevant brain region implicated in the BDNF mediation of antidepressant effects. The prefrontal cortex, for instance, has been associated with BDNF reduction after depressant manipulations (Lee et al., 2018; Zhang et al., 2017) and the infusion of allopregnanolone in this area increases BDNF mRNA expression in the left hemisphere of the same region (Almeida et al., 2019). However, given that intra-prefrontal cortex allopregnanolone infusion is without antidepressant-like effects in rats (Almeida et al., 2019; Shirayama et al., 2011), the role of BDNF in this region is significantly less clear. It is possible that these frontal BDNF alterations in depressed animals are consequences of hippocampal effects, and that local (frontal) allopregnanolone-induced BDNF increases play no role in its antidepressant effect. However, the lack of studies investigating the direct infusion of BDNF in the prefrontal cortex renders these ideas merely speculative.

Nevertheless, BDNF is not the only protein that is likely involved with depressive-like states and neurogenesis in brain limbic areas. Other growth factors participate in cell proliferation, migration, and differentiation, especially in the nervous system. Besides BDNF, some other neurotrophic proteins have been associated with depression and the antidepressant effect of classical antidepressants, especially the nerve growth factor (NGF) (reviewed by Mondal and Fatima, 2019). To our knowledge, there is a single *in vitro* experiment that demonstrated, after exposure to moderate concentrations of allopregnanolone, a decrease in the toxicity of NGF-treated cells (Afrazi et al., 2014). Another neurotrophic protein studied was reelin, which was proposed by Pinna and colleagues as another potential neurogenic protein involved with neurosteroid behavioral attenuation (Pinna et al., 2004a). In that paper, it is shown that there is an increase in aggression in male and female mice associated with a decrease of brain allopregnanolone, and this behavior is reversed with concomitant reelin modulation. Furthermore, in socially isolated male mice, aggression can be prevented by treatment with L-methionine, which has also been shown to decrease reelin (Pinna et al., 2004a).

Moreover, other markers of neurogenesis have provided evidence concerning allopregnanolone's role in neurotrophy. The changes in hippocampal allopregnanolone induced by time-dependent sensitization and TSPO overexpression in the dentate gyrus — discussed in Section 2 — were directly associated with the proliferation of progenitor cells as shown by bromodeoxyuridine immunohistochemistry (Zhang et al., 2018). This effect is robust enough to be also observed in a neurodegenerative model induced by chronic treatment with lipopolysaccharide, where an increase in newborn neurons by TSPO overexpression is additionally reported (Wang et al., 2016). The administration of exogenous allopregnanolone has also been shown to restore cell proliferation and rescue cell survival in the subgranular zone of the dentate gyrus after social isolation (Evans et al., 2012), probably through its BDNF-mediated neurogenic effects. These findings indicate that this particular structure, the dentate gyrus, is probably the main functional area within the hippocampus responsible for the neurogenesis mediated by allopregnanolone. And, in addition to influencing neurogenesis, allopregnanolone apparently also inhibits neurodegeneration by suppressing extracellular signal-regulated kinases (EKRs) phosphorylation *in vitro* (Mendell et al., 2018). On the other hand, chronic exposure to exogenous allopregnanolone may evoke the opposite effect, since a regimen of three times/week subcutaneous injection has been shown to decrease recruitment of hippocampal progenitor cells — though one injection/week did increase neurogenesis (Chen et al., 2011). This, in association with the observation that long-term continuous allopregnanolone administration leads to memory decline and hippocampus shrinkage (Bengtsson et al., 2016), demonstrates that the effects of allopregnanolone on neurogenesis is likely dependent on treatment duration and frequency.

All these data suggest that BDNF participates as an important player in the antidepressant effect induced by allopregnanolone, and that its manipulation arises as a promising alternative for the pharmacological approach of depression. In addition, the papers reviewed suggest a wide field to be explored regarding the relationship between allopregnanolone and other neurotrophic proteins, regarding their role in the neurotrophic antidepressant-like effect.

3.2. Environmental interventions to increase neurotrophy: what role do neurosteroids play?

Because neurogenesis is a process that is intimately linked to a wide array of external factors, animal models of depression represent only a small fraction of the environmental conditions that importantly modify this aspect of brain biology. The input of adequate or inadequate stimuli, particularly during the developmental phases in life, may significantly contribute to a higher or lower pattern of neurogenesis, respectively. One example is maternal care, a complex set of nursing actions that, if executed poorly, may reflect in neurochemical and behavioral deficits in adulthood (Nephew and Murgatroyd, 2013). In fact, one of the conditions that may result in poor maternal care (mainly characterized by grooming and licking of the pups) is the early age social isolation of the mothers, which is also associated with low circulating allopregnanolone levels (Pisu et al., 2017). This factor exerts an important effect on the dams since rats from low licking/grooming dams present more anxiety-like behaviors and lower hippocampal allopregnanolone levels in adulthood (Borrow and Cameron, 2017). An interesting observation is that these rats were compared to animals from high licking/grooming dams, which presented comparatively higher brain allopregnanolone levels. Thus, it suggests that "positive" life experiences might also exert an effect in neurosteroidogenesis, perhaps mediated by the action of neurotrophic agents.

One strategy to model positive stimuli is to expose the animals — preferably at a young age, generally just after weaning — to an enriched environment. In the laboratory setting, this means to provide a richer housing condition that normally focuses on three main pillars: greater social interaction, diversified sensory input, and incentive to voluntary exercise (van Praag et al., 2000). Environmental enrichment has been associated with neurotrophic changes in the brain, which is supported by a recently published systematic review of animal studies that demonstrated a robust neurogenic effect associated with this paradigm, having BDNF as one of its main regulating agents (Barros et al., 2019). Thus, this paradigm becomes a provocative possibility to investigate the neurogenesis-related upregulation in brain steroid synthesis. Munetsuna et al. (2011) provided the first evidence of modulation in brain steroidogenesis by environmental enrichment in a comprehensive experiment that quantified the hippocampal mRNA expression of 19 different enzymes involved with brain steroid metabolism. They showed that male rats reared in an enriched environment for eight weeks presented a higher expression of the neurosteroidogenic enzymes 5α-reductase type 1 and 3α-HSD when compared to standard housing-reared animals. Of particular note, these two enzymes are the main ones involved in allopregnanolone biosynthesis, strongly suggesting its participation in the neuroprotective effects induced by this paradigm. In aged females, environmental enrichment also increased these and other neurosteroidogenic enzymes in the hippocampus (Rossetti et al., 2015). A recent experiment studied the sensory and motor aspects of environmental enrichment separately in a 10-day protocol, showing similar results in young female adults with a much more modest effect in aged rats (Rossetti et al., 2019). In males, four weeks of environmental enrichment reverses the deficits in neuronal plasticity induced by previous social isolation for the same amount of time by restoring hippocampal BDNF, NGF and activity-regulated cytoskeletal associated protein levels, as well as dendritic spine density and other markers of neurotrophy (Biggio et al., 2019). Also, some studies have reported an antidepressant-like effect in the FST elicited by environmental enrichment in male rats (Ashokan et al., 2018; Possamai et al., 2015).

Similar neurotrophic changes have been associated with the emergence of resilient phenotypes against chronic stress, preventing the establishment of a depression-like state. In this case, these changes were induced by knocking out a proapoptotic gene (Bax) in mice, which increased the survival of hippocampal cells (Anacker et al., 2018). A resilient phenotype may also be induced by environmental enrichment, which prevents the emergence of depressive-like behaviors induced by social defeat stress, presumably by also increasing hippocampal neurogenesis (Lehmann and Herkenham, 2011). However, this resilience has not yet been experimentally linked to brain allopregnanolone levels, though this hypothesis has been raised by Biggio et al. (2014). Other studies have attempted to generate resilient rats by selective breeding rats that presented high or low traits related to depression. A study conducted by Frye's research group that bred rats with high or low rates of infantile ultrasonic vocalization (USV) after maternal separation found that the low USV line presented higher brain allopregnanolone levels associated with lower depressive-like behaviors, which suggests an associated resilient profile (Zimmerberg et al., 2005). More recently, we bred rats in our laboratory that presented high or low immobility in the FST and showed that allopregnanolone was only able to reduce immobility in the high immobility line (Almeida et al., 2018).

This limited but important assortment of data suggests that neurosteroids play a crucial role on environmental stimulus during early ages in animals. It remains to be established whether the stress resilience achieved in these studies is dependent on the neuroprotective effects of allopregnanolone, which — if proven true — will broaden the importance of this neurosteroid in the neurobiology of depression.

4. Conclusion

All of the aforementioned evidence discussed in this review demonstrates that allopregnanolone plays a major role in depressive-like manifestations in humans and in animal models. The systemic downregulation of allopregnanolone seen in depressed individuals is well-reproduced across several animal models of depression, which provide an additional level of detail regarding its brain regulation. In depressed-like animals, allopregnanolone levels are consistently downregulated in areas of the corticolimbic system that are responsible for mood regulation. Moreover, its infusion in these areas exerts antidepressant-like effects, which evidences its importance in the neurobiology of depression. These preclinical observations led to the development of a formulation fit for intravenous infusions in humans, brexanalone, that demonstrated efficacy for the treatment of postpartum depression and is currently approved for clinical use.

Also, the amelioration of symptoms observed after treatment with widely prescribed antidepressants, particularly SSRIs, is at least partially due to the capacity of these substances to increase brain allopregnanolone content, as largely demonstrated in animal studies. The main drugs that upregulate allopregnanolone levels are SSRIs, which present this neurosteroidogenic property even in lower non-serotonergic doses, which are known to exert an SBSS action. This drug-induced upregulation of allopregnanolone reduces depressive-like behaviors in models such as the FST, which is also achieved with other agents that increase brain neurosteroidogenesis levels by different mechanisms.

Furthermore, among the varied mechanisms by which allopregnanolone might exert its antidepressant effects, the increase in hippocampal neurogenesis by the upregulation of neurotrophic proteins is proving to be a relevant pathway for this antidepressant action, giving origin to a crescent and vibrant field of research. This rationale is associated to the fact that hippocampal neurogenesis is lower in depressed-like animals, and reversed predominantly by increases in BDNF in antidepressant-treated animals. There is plenty of evidence pointing to the role of altered GABAergic function and of altered BDNF in major depressive disorder and in allopregnanolone effects. It is still needed to understand if and how these two mechanisms might be related to the quick, effective and lasting antidepressant effects of neurosteroid antidepressants such as brexanolone. Is one of these mechanisms more important for the clinical effects of brexanolone than the other, or is there a synergism or potentiation between the GABAergic and neurotrophic systems that better explain the effects seen in the clinical setting?

An interplay between allopregnanolone's effects on GABA and on neurogenesis might bring a dual response that has to be investigated regarding brexanolone's rapid and long lasting clinical effects. Also, one may assume that the increase in neurosteroidogenesis by interventions such as the environmental enrichment points to a mechanism through which allopregnanolone is involved with stress resilience. Future studies should further investigate if and how allopregnanolone is able to improve resilience and whether genetic factors play a significant role in this particular pathway of neuroprotection. With the very recent authorization for the use of brexanalone for the treatment of postpartum depression, it becomes evident that allopregnanolone and other neurosteroidogenic agents may be an important tool for the treatment of affective disorders, and may prove to be effective for the treatment of major depression disorder and bipolar disorders in areas were other, more classical antidepressants have failed.

CRediT authorship contribution statement

Felipe Borges Almeida: Conceptualization, Investigation, Writing - original draft. **Maurício Schüler Nin:** Conceptualization, Investigation, Writing - review & editing. **Helena Maria Tannhauser Barros:** Conceptualization, Writing - review & editing, Supervision.

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Appendix A. Supplementary data

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II.2 CAPÍTULO II – O potencial dos neuroesteroides e proteínas neurotróficas como biomarcadores para o transtorno depressivo maior e TEPT

O artigo apresentado a seguir está relacionado com o objetivo específico 2 e busca responder as seguintes perguntas:

1) Quais são as evidências clínicas e pré-clínicas que embasam um potencial uso de neuroesteroides e proteínas neurotróficas como biomarcadores no transtorno depressivo maior e TEPT?
2) Quais são os mecanismos neurobiológicos relacionados ao papel destas duas classes de potenciais biomarcadores?

Este artigo constitui-se em uma revisão narrativa da literatura na qual abordamos alterações nos níveis de neuroesteroides (com destaque para a alopregnanolona) e proteínas neurotróficas (com destaque para o BDNF) em pacientes com transtorno depressivo maior e TEPT. Tendo em vista a sobreposição sintomatológica entre estes dois transtornos e a necessidade do desenvolvimento de marcadores biológicos objetivos para seu diagnóstico diferencial, discute-se o potencial uso destas moléculas como biomarcadores para estes transtornos. Neste sentido, os níveis de alopregnanolona encontram-se reduzidos tanto no transtorno depressivo maior quando no TEPT. Em relação ao BDNF, seus níveis encontram-se reduzidos na depressão enquanto sua regulação no TEPT é complexa e aparentemente dependente de fatores relacionados ao evento traumático, podendo ser uma importante chave como marcador diferencial. A possibilidade de mensuração da alopregnanolona em diferentes matrizes é apresentada, com a dosagem salivar sendo uma alternativa promissora à aferição sérica devido à maior praticidade e ausência de efeitos artefatuais relacionados ao estresse da coleta. Por fim, são apresentados estudos proeminentemente realizados *in vitro* que sugerem uma regulação direta do BDNF através da ação da alopregnanolona.

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MDPI

Review

Neurosteroids and Neurotrophic Factors: What Is Their Promise as Biomarkers for Major Depression and PTSD?

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Abstract: Even though major depressive disorder (MDD) and post-traumatic stress disorder (PTSD) are among the most prevalent and incapacitating mental illnesses in the world, their diagnosis still relies solely on the characterization of subjective symptoms (many of which are shared by multiple disorders) self-reported by patients. Thus, the need for objective measures that aid in the detection of and differentiation between psychiatric disorders becomes urgent. In this paper, we explore the potential of neurosteroids and neurotrophic proteins as biomarkers for MDD and PTSD. Circulating levels of the GABAergic neuroactive steroid, allopregnanolone, are diminished in MDD and PTSD patients, which corroborates the finding of depleted neurosteroid levels observed in animal models of these disorders. The neurotrophic protein, brain-derived neurotropic factor (BDNF), is also reduced in the periphery and in the brain of MDD patients and depressed-like animals that express lower neurosteroid levels. Although the role of BDNF in PTSD psychopathology seems less clear and merits more research, we propose a causal link between allopregnanolone levels and BDNF expression that could function as a *biomarker axis* for the diagnosis of both MDD and PTSD.

Keywords: allopregnanolone; BDNF; PTSD; MDD; rodent models; biomarkers

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1. Introduction

Major depressive disorder (MDD) and post-traumatic stress disorder (PTSD) are among the most prevalent, debilitating, and incapacitating mental illnesses that pose a significant disease burden and loss of adjusted life years [1]. Both disorders are believed to emerge as a result of a maladaptive response to stressful events in individuals who fail to develop resilience. MDD and PTSD have similar etiology, manifest overlapping symptoms, and MDD is often seen as a worsening condition in PTSD, which can be accompanied by suicide. The shared symptomatology is relevant because the clinical diagnosis of MDD and PTSD is still based on subjective rather than objective measures that are entirely based on symptom evaluation, following criteria defined by the Diagnostic and Statistical Manual of Mental Disorders (DSM)-V. Even though the DSM-V offers a structured set of symptoms for each disorder, the over reliance on subjective self-reports of symptoms contributes to diagnoses that are misleading at times, mainly in the first medical visits, and in cases characterized by a mild to moderate depression and distress [2]. For this reason, the addition of an array of objective measures of neurobiological parameters would warrant a significant boost in the diagnosis accuracy of psychiatric disorders, including PTSD and MDD. Great progress has been achieved in the study of neurochemical deficits that underlie the manifestation of psychiatric disorders, and several novel biomarker candidates to help in the diagnosis of PTSD and MDD have been proposed.

In this review, we focus on two promising biomarker candidates that may offer a valid biosignature for a better diagnosis and predict occurrence of psychiatric disorders: neuros-

teroid biosynthesis, such as allopregnanolone and GABAergic congeners, and expression of neurotrophic proteins, including brain-derived neurotrophic factor (BDNF).

2. Biomarkers for Psychiatric Disorders: An Unmet Need

The term "biomarker" refers to a broad category of measurable indicators of the existence or the severity of a disease/disorder, being objective indications of medical state observed from the patient, which can be measured in an accurate and reproducible manner. Thus, biomarkers objectively indicate the pathological state of a subject that correlate to assessed medical symptoms, which are limited to indications perceived by the patient on their mental health disorders. The Biomarkers Definitions Working Group provided an international definition of biomarker for the field of pharmacological clinical trials: "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention" [3].

The development of reliable biomarkers for psychiatric disorders depends on a large understanding of the pathological processes that underlie the specific neuropathology under examination. This can be particularly challenging for psychiatric disorders because of their broad symptomatic characterization and the lack of a sufficiently detailed understanding of the functional abnormalities, either neural or somatic, associated with psychiatric disorders. Nevertheless, significant progress in this field has been made in the last decades, and the development of reliable biomarkers for MDD and PTSD is becoming a realistic possibility.

There are several kinds of biomarkers that have been proposed for the diagnosis and prognosis of MDD and PTSD, including genetic and epigenetic markers, proteins, and neurohormones [4]. Each of these parameters attempts to reflect part of a theoretical axis of biological alterations that gives origin to symptoms or to the targeted disorders. The origin of such alterations is believed to be largely dependent on the exposure to stressful events, and a complex combination of factors that include the nature of the stressor and the individual's construct may result in one disorder instead of another. Exposure to stress in a chronic, repeated fashion is believed to be an important risk factor for the development of MDD, while exposure to acute, yet intense, traumatic events, often in individuals that experience chronic stress conditions, may precipitate the development of PTSD. Given that protracted and acute stress play an important role in the etiology of these disorders, the body's physiological regulation of the stress response becomes an important point of study to contextualize biochemical alterations in MDD and PTSD.

3. Neurosteroids

Neurosteroids are endogenous steroids synthesized in the central and peripheral nervous system from cholesterol [5] and belong to the broader category of neuroactive steroids, which also include peripherally or artificially synthesized steroids with activity in the brain [6]. The synthesis of neurosteroids, or neurosteroidogenesis, follows a few distinct steps: (1) cholesterol is trafficked to the outer mitochondrial membrane by action of the steroidogenic acute regulatory (StAR) protein; (2) cholesterol is then transported into the inner mitochondrial membrane by the 18 kDa translocator protein (TSPO); (3) its side-chain is cleaved by the CYP11A1 enzyme, which produces the neurosteroid precursor, pregnenolone. Pregnenolone can then be converted to progesterone, which originates a wide array of steroid-derived molecules that include sex and stress hormones, as well as endogenous neuroactive steroids [7]. In this review, we will focus on the neurosteroid pathway (represented in Figure 1) that begins with the reduction of progesterone by the action of the 5α-reductase type I (5α-RI) enzyme, into 5α-dihydroprogesterone, which is subsequently converted by the 3α-hydroxysteroid dehydrogenase (3α-HSD) type III enzyme to 3α,5α-tetrahydroprogesterone (commonly known as allopregnanolone).

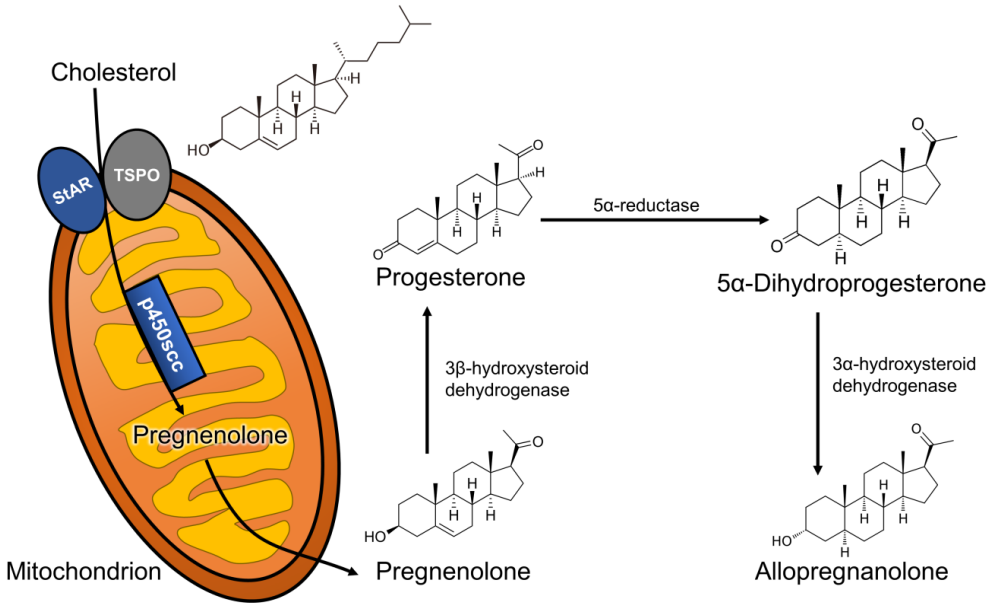

Figure 1. Allopregnanolone biosynthetic pathway starting from cholesterol metabolism. Abbreviations: StAR: steroidogenic acute regulatory protein; TSPO: 18 kDa translocator protein; scc: side-chain cleavage.

Neurosteroids rapidly modulate neuronal excitability due to their affinity to ligand-gated ion channels and other receptors expressed in the synaptosomal membranes of brain cells and neurons. Arguably, the most important neuronal action exerted by neurosteroids is related to the modulation of the inhibitory activity mediated by the neurotransmitter GABA through the allosteric potentiation of the $GABA_A$ receptor [8–10]. Allopregnanolone and its stereoisomer, pregnanolone, are among the most potent neurosteroids in positively and allosterically modulating $GABA_A$ receptors. These GABAergic steroids are found diminished in the cerebrospinal fluid (CSF) [11], serum [12], and plasma [13] of depressed individuals. The expression of the 5α-RI enzyme, which is essential for allopregnanolone synthesis, is reduced in the postmortem prefrontal cortex (Brodman area 9) of depressed individuals [14]. Exogenous administration of allopregnanolone and other GABAergic synthetic neurosteroid analogs resulted in antidepressant and anxiolytic effects, which is believed to be partly due to the role neurosteroids play in the modulation of GABAergic inhibition, thereby also reducing depressive symptoms [15]. The reduced allopregnanolone in the CSF and plasma of depressed individuals [11–13] can be increased after antidepressant treatment with selective serotonin reuptake inhibitors (SSRIs), including fluoxetine and fluvoxamine [12].

Affective disorders, including MDD and PTSD, show a strong sex-bias being more prevalent in women than in men, which suggests that sex steroids may play a role. Both progesterone and its metabolite, allopregnanolone, have been the focus of several investigations looking at sex-specific roles of neurosteroids in depressive disorders. Lower serum allopregnanolone levels have also been observed in women suffering from postpartum depression (PPD) [16]. Significant fluctuations in progesterone and allopregnanolone levels during pregnancy, which rise considerably before abruptly decreasing following delivery, may be responsible for the development of PPD pathophysiology [17,18]. From a predictive perspective, the second trimester of pregnancy seems to play an important factor and should be considered in the development of PPD symptoms during the last days of pregnancy and in the post-partum period [19,20].

In female patients with PTSD, CSF, serum, and plasma allopregnanolone levels are also lower than in healthy age-matched controls, which has been associated with enzymatic dysfunction at the levels of 3α-HSD [21,22]. Additionally, women with PTSD show a lower capacity for allopregnanolone synthesis from its precursor, which was confirmed in serum samples [23,24]. Similarly, in males with PTSD, the concentrations of allopregnanolone

in the CSF were found lower than in healthy controls [25]. Ratios with allopregnanolone precursors supported a 5α-RI expression/function abnormality. In these patients, the allopregnanolone level decrease was inversely correlated with increased PTSD and depression symptoms. These findings align with previous results that observed a 5α-RI expression downregulation in the human post-mortem brain (Brodmann area 9) of male depressed subjects. Intriguingly, the enzymatic deficits in the allopregnanolone pathway may underlay a biosignature relevant for psychopathology related to dysfunction in reproductive steroid biosynthesis. Allopregnanolone levels were also found to be reduced in the medial orbital frontal cortex of individuals with PTSD in comparison to controls, using postmortem brain tissue samples [26]. A recent study showed that PTSD symptoms were inversely correlated with combined CSF levels of allopregnanolone and pregnanolone, which was not observed in matched trauma-exposed controls [25]. These results are believed to correlate with decreased brain allopregnanolone levels, which are observed in the limbic system areas that are relevant to affect regulation in several animal models of depression and of PTSD (for an in-depth review on this topic, see [27–29]). Even though MDD and PTSD share some neurobiological aspects, it is timely to investigate if allopregnanolone downregulation follows a distinct trajectory between the two disorders. Further, it is conceivable that allopregnanolone and pregnanolone levels are compared to the levels of their precursors, calculating ratios that estimate their synthesis rate and also investigating the function and/or expression of the biosynthetic enzymes.

In humans, CSF measurements of neurosteroids are expected to reflect brain levels. However, a study conducted in male and female rats showed a poor correlation of CSF allopregnanolone levels with its content in relevant brain areas, such as the hippocampus and cerebral cortex [30]. Plasma levels were surprisingly predictive of cerebral cortex levels [30], encouraging the extrapolation of peripheral neurosteroid findings to the CNS. Another study in humans found a significantly more robust correlation between CSF and serum free allopregnanolone levels [31]. In human studies in males, however, correlation of steroid levels in the CSF and plasma were either weak or very weak [32]. On the other hand, regardless of the precise correlation within subjects, plasma allopregnanolone levels have been shown to reflect the directional changes predicted in the brain. In addition to plasma, the non-invasive sampling of saliva could offer an alternative to blood draws for the quantification of neurosteroids in that it would reduce the stress associated with blood collection that could affect circulating neuroactive steroid levels, and thereby confounding interpretation of the experimental results. To our knowledge, there have not yet been studies comparing allopregnanolone levels in saliva with blood or CSF levels, but the reliable use of cortisol measurement in saliva [33], suggests that this may be an appropriate direction to focus in future research. Similarly, salivary progesterone levels successfully reflect plasma levels, as confirmed also in studies conducted during pregnancy [34].

Altogether these summaries suggest that more studies should be conducted to establish clear correlation among central neurosteroid biosynthesis changes with those occurring in the periphery, including blood and saliva. These investigations will be crucial in assessing a putative biomarker role of neurosteroids in psychiatric disorders.

4. Neurotrophic Proteins

Exposure to chronic stress can alter BDNF expression, which has also been found abnormal in psychiatric disorders, such as MDD and PTSD. Anxiety and depressive behaviors in humans have been linked with social stress that, when modeled in animals, is associated with changes both in HPA axis function and a down-regulation of BDNF protein and mRNA expression in the hippocampus [35].

MDD and PTSD are associated with poor ability to maintain and generate functional neurons in key brain regions and circuitry, particularly in the hippocampus, prefrontal cortical regions, and nuclei of the amygdala. Consistently, impaired neurogenesis has been observed in animal models of several psychiatric disorders [36,37]. Neurogenesis and other neurotrophic events, such as maintenance and dendritic spine proliferation and matura-

tion, are generally mediated by neurotrophic proteins, which include the nerve growth factor (NGF), neurotrophin-3 (NT-3), neurotrophin-4 (NT-4), as well as other mediators represented by the steroid dehydroepiandrosterone (DHEA) and its sulfate ester (DHEA-S), among others. However, one of the most important mediators of neurogenesis is undoubtedly BDNF, a neurotrophic protein synthesized by neurons and abundantly expressed in the limbic system, where it plays a role in mood regulation [38]. Like other neurotrophins, BDNF is found in a precursor (proBDNF) and mature (mBDNF) form, and each isoform binds with high affinity to distinct receptors in the brain. While proBDNF binds to the neurotrophin receptor p75 ($p75^{NTR}$), mBDNF exerts its action mainly through activation of the tropomyosin receptor kinase B (TrkB). Although there is some cross-activation of $p75^{NTR}$ by mBDNF and of TrkB by proBDNF that can play a role in some physiological processes, the affinity of this cross-activation remains low and generally does not result in significant overall functional effects. Importantly, the activation of each receptor results in distinct downstream effects that are functionally opposite in nature (Figure 2). Thus, while activation of TrkB leads to neurite outgrowth, stimulates cell migration, increases branching of the dendritic tree, and induces long-term potentiation (LTP), activation of p75NTR reduces neurite length, suppresses cell migration, diminishes branching of the dendritic tree, and induces long-term depression (LTD). Both LTP and LTD are processes that increase or decrease parameters related to synaptic plasticity, respectively [39,40].

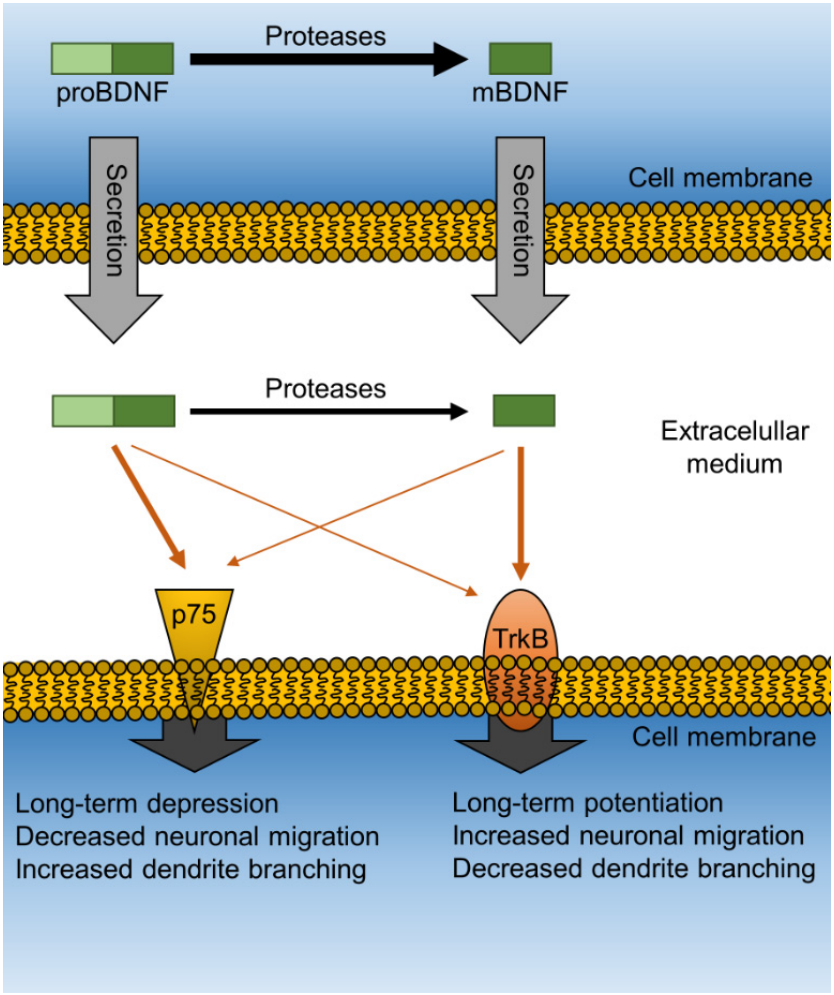

Figure 2. Diagram of the functional role of BDNF isoforms. Conversion of the precursor proBDNF to the mature form mBDNF occurs mainly intracellularly. Both isoforms are secreted and exert their actions on neurotrophin receptor p75 ($p75^{NTR}$) or the tropomyosin receptor kinase B (TrkB). Downstream effects of proBDNF and mBDNF are functionally opposite.

There are naturally occurring variations in the BDNF gene that significantly alter its function. Perhaps the most relevant of such variations is a single nucleotide polymorphism (SNP) that occurs in the pro-coding region of the *bdnf* gene (rs6265) called Val66Met (or V66M). Even though this variation does not occur in the mBDNF peptide sequence, its presence leads to inadequate folding and loss of function of the mature neurotrophin. Being highly prevalent (30–50% of carriers in the U.S. and some European countries), this SNP likely plays an important role in BDNF function when related to the development of several neurodegenerative and psychiatric disorders, including MDD [41]. A meta-analysis demonstrated that the V66M polymorphism modulated the relationship between early-life stress and the development of MDD [42]. The role of V66M in PTSD is less clear and less investigated, given that no correlation was found between Val66Met or serum BDNF with the development of PTSD in road traffic accident survivors [43]. On the other hand, a more recent study in U.S. military veterans showed that V66M carriers (i.e., heterozygous for the SNP) exposed to trauma experienced PTSD symptoms with higher severity compared to non-carriers [44].

Like allopregnanolone content, BDNF levels are decreased in depressed patients and respond to antidepressant treatments. For instance, leukocyte BDNF mRNA expression was found to be reduced in depressed patients and restored by antidepressant treatment with escitalopram [45]. Accordingly, peripheral BDNF appears to be reduced in acute depressive episodes and returns to normal during remission [46]. However, a lack of correlation between serum BDNF and severity of MDD symptoms was also reported [47]. In the same direction, animal models of depression such as the chronic unpredictable/mild stress have shown that vulnerable animals that develop depression-like behaviors after exposure to the stressor show a significant reduction in hippocampal BDNF expression, as opposed to resilient animals [48]. Moreover, BDNF overexpression or its knockdown in the hippocampus mediates resilience to chronic stress as measured by behavioral outcomes, granting further support to its central role in the development of MDD symptoms [49].

In PTSD, however, clinical evidence shows changes in BDNF levels are less clear and often contradictory. Some studies suggest that subjects who developed PTSD after exposure to traumatic events present lower blood BDNF levels when compared to those who did not develop the disorder [50] or to healthy controls [51,52]. Another study showed an increase in BDNF levels after antidepressant treatment in PTSD patients, but their baseline levels did not differ from non-PTSD trauma survivors [53]. In another study, however, BDNF tended to be higher in PTSD patients and reached statistical significance when measured in the early stages after trauma [54]. Thus, the clinical data point to a rather complex role of peripheral BDNF levels in PTSD, which are likely dependent on the nature of the trauma and the time after exposure to the traumatic event.

Some preclinical studies have modeled PTSD in rodents and measured brain BDNF to elucidate its central role in this disorder. In the social isolation paradigm, hippocampal BDNF levels have been shown to be decreased in male (but not female) rats [36,55,56]. Because the social isolation model might mimic many of the overlapping characteristics of PTSD and depression [29], it is important to consider expanding this investigation to other stress-based PTSD rodent models in order to provide further support to these findings. Indeed, more recent studies that have examined this topic have found that rats exposed to single prolonged stress (SPS) also present reduced hippocampal BDNF levels [57,58]. These results in animals point to a pivotal, central role of BDNF underlying behavioral dysfunction related to PTSD neurobiology, although the correlation with peripheral BDNF levels is less clear and merits further investigation.

In a complementary strategy to study the role of BDNF in the development of MDD or PTSD after exposure to chronic stress, weaning in an enriched environment has also been studied to verify whether BDNF could drive resilience against the development of these disorders. Post-weaning environmental enrichment has been shown to increase BDNF in the hippocampus when compared to rats reared in standard or isolated conditions [59]. Furthermore, exposure to a socially enriched environment was able to restore the reduction in

hippocampal BDNF induced by post-weaning social isolation [60] or by maternal separation [61]. Pre-weaning enrichment was also able to rescue animals from anxiety-like behaviors in adulthood induced by post-weaning maternal separation stress, while increasing BDNF content in the basolateral amygdala [62]. Thus, these findings grant further support for the role of BDNF in driving resilience and contrasting the development of MDD or PTSD, highlighting its potential as a biomarker for the characterization of these disorders.

5. The Allopregnanolone and BDNF Link

In preclinical in vivo studies in rodents, a correlation between hippocampal BDNF and allopregnanolone levels is supported by studies that aimed to model depression using a variety of protocols (for review see [27]). In vitro data showing that allopregnanolone promotes proliferation of neural progenitor cells [63], cell survival [64], and neuronal differentiation [65] demonstrate allopregnanolone's capacity in inducing neurotrophic modifications, thus supporting the hypothesis of a causal—rather than simply correlational—link between these two agents [55]. Many in vivo studies put forth the hypothesis that allopregnanolone can regulate the expression of BDNF in the brain. In male mice, social isolation reduced both allopregnanolone and BDNF in the hippocampus, and long-term treatment with allopregnanolone or SSRIs, administered at low, neurosteroidogenic doses, restored endogenous allopregnanolone and BDNF expression to normal levels quantified in control group-housed rodents [55]. These data were later confirmed by studies conducted in socially isolated male rats administered with allopregnanolone [36]. In female rats, knocking out the pregnane xenobiotic receptor (important for steroidogenesis) reduced hippocampal allopregnanolone concomitantly with a decrease in BDNF expression in the same brain area examined [66]. Chronic treatment with allopregnanolone restored impaired BDNF levels found in the spinal cord of a mouse model of motoneuron degeneration [67]. Low to moderate concentrations of allopregnanolone increased BDNF expression in vitro in response to neuronal damage induced by 6-hydroxydopamine (6-OHDA) administration [68].

The precise mechanism, by which allopregnanolone may have induced BDNF expression upregulation, is still elusive, but a recent investigation proposed that allopregnanolone binding at $GABA_AR$ leads to voltage-gated L-type Ca^{2+} channel opening and subsequent phosphorylation of the calcium/calmodulin-dependent protein kinase II δ3 subunit (CaMKIIδ3), which then increases BDNF mRNA expression [69]. Further studies should determine potential transcription factors/regions for the BDNF gene that allopregnanolone could regulate directly or indirectly.

Altogether, the finding that allopregnanolone biosynthesis and regulation of BDNF expression could be part of the same mechanism (Figure 3) potentially offers a biomarker profile at the interface of the neurosteroid system and neurotrophic factor expression and function suitable to be proposed in the development of precise diagnosis and treatment for MDD and PTSD. Allopregnanolone was recently approved by the US FDA for the treatment of PPD in an intravenous formulation named brexanolone and marketed as Zulresso™ [70]. Its pharmacological action was fast, required only a short course administration, and appeared to be long-lasting. These groundbreaking findings have recently posed allopregnanolone/brexanolone among only two recently US FDA-approved fast-acting antidepressants. The hypothesis of whether allopregnanolone, which is found decreased in several neuropsychiatric disorders, could entail predictions as biomarker of psychiatric disorders deserves further investigation and characterization in preclinical and clinical studies. The angle of looking at a biomarker profiling including allopregnanolone biosynthesis in relation with expression of BDNF is fascinating but remains a topic to be further clarified in future investigations.

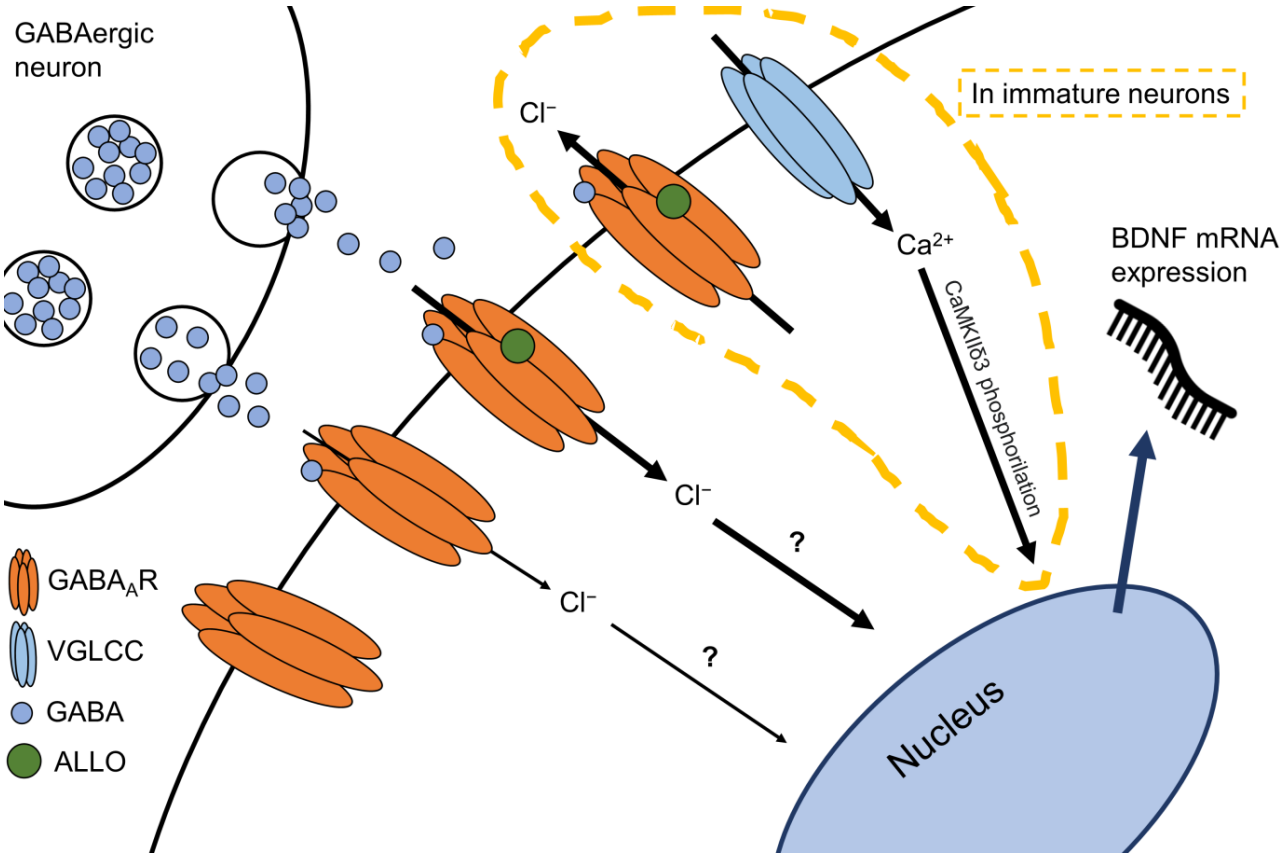

Figure 3. Schematic representation of a putative functional link between allopregnanolone and BDNF. Allopregnanolone (ALLO) potentiation of GABAergic neurotransmission is thought to be involved in BDNF mRNA expression upregulation, though the precise mechanism still needs to be elucidated. One proposed pathway, in immature neurons, is the phosphorylation of the calcium/calmodulin-dependent protein kinase II δ3 subunit (CaMKIIδ3) as a response to allopregnanolone-induced voltage-gated L-type Ca^{2+} channel (VGLCC), which upregulates BDNF gene expression [68].

6. Conclusions

Notwithstanding recent progress in the field, neuropsychiatry still remains a field lacking development of reliable biomarkers to objectively help in the diagnosis and treatment of several debilitating conditions that affect the lives of millions of patients worldwide. Recent investigations in rodent stress models of PTSD and MDD suggest that neurosteroid biosynthesis and expression of BDNF are linked with allopregnanolone regulating the expression of neurotrophic factors [27,36,55]. Given the role of both these neurobiological factors in psychiatric disorders, including MDD and PTSD, the hypothesis that they may serve in biomarker profiling of these conditions is encouraging, and their role in developing individual-tailored treatment to improve response to antidepressants will constitute an enormous advance in the field of neuropsychopharmacology.

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II.3 CAPÍTULO III – O papel do eixo HPA e da alopregnanolona na neurobiologia de transtornos depressivos maiores e TEPT

O artigo apresentado a seguir está relacionado com o objetivo específico 3 e busca responder as seguintes perguntas:

1) De que maneira o eixo HPA encontra-se desregulado em transtornos depressivos maiores e no TEPT?
2) Qual é o papel da alopregnanolona no sistema regulatório do eixo HPA?
3) A suplementação exógena de alopregnanolona pode ser uma opção viável no tratamento destes transtornos?

Trata-se de uma revisão de literatura que busca verificar a conexão do papel de neuroesteroides como a alopregnanolona na resposta ao estresse mediada pelo eixo HPA, dado que estressores ambientais são contribuidores majoritários para o desenvolvimento de transtornos depressivos e TEPT. Os níveis de cortisol—um importante hormônio do eixo HPA—parecem estar diferencialmente desregulados nestes transtornos, com uma parcela dos indivíduos deprimidos apresentando níveis elevados de cortisol enquanto pacientes acometidos pelo TEPT tendem a exibir níveis reduzidos de cortisol. Dentre os mecanismos regulatórios do eixo HPA, destaca-se a regulação GABAérgica sobre a qual a alopregnanolona exerce efeito modulatório. A duração do estímulo estressor (agudo e crônico) afeta os níveis de alopregnanolona de forma distinta, podendo estar relacionada com o aparecimento de transtornos depressivos ou do TEPT. Demonstra-se também que a biossíntese alterada de alopregnanolona em transtornos do humor participa de um sistema complexo de retroalimentação regulatória sobre o eixo HPA que contribui para a cronificação destes transtornos. Por fim, tratamentos baseados em alopregnanolona ou seus análogos são propostos como uma alternativa para o tratamento da depressão e TEPT à luz da aprovação recente deste tratamento na depressão pós-parto.

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Review

The Role of HPA Axis and Allopregnanolone on the Neurobiology of Major Depressive Disorders and PTSD

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Abstract: Under stressful conditions, the hypothalamic-pituitary-adrenal (HPA) axis acts to promote transitory physiological adaptations that are often resolved after the stressful stimulus is no longer present. In addition to corticosteroids (e.g., cortisol), the neurosteroid allopregnanolone (3α,5α-tetrahydroprogesterone, 3α-hydroxy-5α-pregnan-20-one) participates in negative feedback mechanisms that restore homeostasis. Chronic, repeated exposure to stress impairs the responsivity of the HPA axis and dampens allopregnanolone levels, participating in the etiopathology of psychiatric disorders, such as major depressive disorder (MDD) and post-traumatic stress disorder (PTSD). MDD and PTSD patients present abnormalities in the HPA axis regulation, such as altered cortisol levels or failure to suppress cortisol release in the dexamethasone suppression test. Herein, we review the neurophysiological role of allopregnanolone both as a potent and positive GABAergic neuromodulator but also in its capacity of inhibiting the HPA axis. The allopregnanolone function in the mechanisms that recapitulate stress-induced pathophysiology, including MDD and PTSD, and its potential as both a treatment target and as a biomarker for these disorders is discussed.

Keywords: stress; hypothalamus-pituitary-adrenal axis; neurosteroids; depression; brexanolone; PTSD

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1. Introduction

The adaptation to adverse conditions is a feature of utmost importance for life preservation in all organisms. Complex systems have evolved to enable a dynamic shifting of biological functions with multiple feedback mechanisms to reset the system back to homeostatic conditions. Abnormal exposure to stressors may disbalance this system and exacerbate neuroendocrine dysfunction [1]. Indeed, maladaptive responses to stress have been implicated in the development of multiple physical and neuropsychiatric disorders. Even though the precise etiology of such disorders remains largely unknown, the concept that both individual susceptibilities (determined by genetics and epigenetic factors) and stressful conditions (determined by the environment) contribute to the onset of psychiatric disorders is well accepted [2,3]. The magnitude and periodicity of stressors, in combination with an individual's genetic construct, are also important factors that may predict what condition may arise after stress exposure. Chronic, repeated stress is believed to be an important risk factor in the development of major depressive disorder (MDD) [4], while exposure to a single, yet extremely intense traumatic event in combination with chronic stressful conditions may lead to anxiety spectrum disorders or precipitate posttraumatic stress disorder (PTSD) [5,6].

Understanding stress-related biologic alterations underlying these conditions is useful not only for a diagnostic perspective, but also to unveil targets for novel pharmacological interventions. In this review, we summarize some pathophysiological mechanisms that are involved in the hypothalamic pituitary adrenal (HPA) stress response and that are altered

in MDD and PTSD. We discuss the benefits and pitfalls of promising markers regulating stress response encompassing the HPA axis. These entail the promise of improving future diagnostic accuracy of psychiatric disorders, as well as providing novel treatments. Even though only a subgroup of PTSD or depressed patients displays alterations of the HPA axis, targeting the HPA axis components might add to the treatment of PTSD or may serve as adjunct treatment in treatment-resistant subjects.

The stress response is highly complex and involves several neurobiological alterations in neurotransmitter systems, peptides, neuro-hormones, endocannabinoids, and other endogenous molecules. Here, our main focus is the intriguing role of allopregnanolone in modulating the HPA response in acute and chronic stress that may bring new diagnostic and treatment options for MDD and PTSD.

2. Stress, the HPA Axis and Mood Disorders

Major life events such as early or adulthood life stress are associated with increased inflammatory processes that can mediate depression and other mood disorders. Two main physiological pathways are involved in converting social-environmental adversity into broad proinflammatory transcriptional modifications that play a role in stress-induced mood disorders: the sympathetic nervous system (SNS), and the HPA axis [7].

In response to a stressor, the paraventricular nucleus (PVN) of the hypothalamus releases corticotropin releasing hormone (CRH) and arginine vasopressin (AVP). While AVP activates the locus ceruleus-norepinephrine (LC-NE) neuromodulatory system that triggers the behavioral "fight or flight" response from the SNS (mediated by epinephrine and norepinephrine), CRH acts on the pituitary gland, which in response secretes adrenocorticotropic hormone (ACTH) into the bloodstream. Once ACTH reaches the adrenal glands, it triggers the release of cortisol (in humans) or corticosterone (in rodents), which acts as anti-inflammatory hormones, and coordinates the physiological behavioral response to stress. Under normal conditions, the negative feedback of cortisol on CRH and ACTH ensures HPA homeostasis through the activation of glucocorticoid receptors (GRs) and mineralocorticoid receptors (MRs), which will act mainly by regulating gene transcription. Occupancy of MRs is generally high even in the lower levels of the circadian rhythm of glucocorticoids, and thus, the GR plays an important role in the stress response, whether this is acute or chronic [8]. This process of dynamic adaptation to reach homeostasis in the face of adverse conditions is termed allostasis, and the accumulation of such changes over time is called the allostatic load [9]. A high allostatic load, due to chronic exposure to stress, may lead to a process known as glucocorticoid insensitivity, and less negative feedback by glucocorticoids may occur when cells become less sensitive to the effects of over-secreted glucocorticoids [7].

Alterations in the HPA axis have been consistently reported in subjects suffering from MDD or PTSD. About 30% of depressed individuals display higher levels of cortisol [10,11], and subjects in remission present levels comparable to healthy, never-depressed controls [12]. A recent meta analysis conducted to determine cortisol level change as a biomarker for depression confirmed that cortisol measured in saliva is a predictor, but with small predictive effect on onset or relapse, and recurrence of subsequent MDD [13]. The high cortisol levels and the lack of diurnal oscillation of cortisol in mood disorders has been associated with lack of suppression to its release induced by chronic stress and inflammation. The dexamethasone (DEX) suppression test was widely studied in the 1970s and 1980s as a diagnostic tool for psychiatric disorders, including MDD, given that about 66% of depressed patients show inability to suppress cortisol release after DEX administration [14,15]. Low diagnostic sensitivity and specificity for MDD was subsequently demonstrated [16], which was later improved by the addition of CRH administration in a refined DEX/CRH test [17,18]. The DEX suppression test was also proposed as a predictor of individual treatment response to antidepressant treatment. An elevated neuroendocrine response to the combined DEX/CRH test can be detected during an acute depressive episode, but it fails to represent specificity in depression given that this response is common to other psychiatric

disorders as well as several medical conditions [19]. The possibility that non-suppression of cortisol in the DEX/CRH test might constitute a biomarker for subpopulations of a given psychiatric disorder and/or might predict a higher antidepressant treatment efficacy is under discussion.

Endocrine alterations pertaining to the HPA axis are also present in PTSD, though they are qualitatively different from those found in MDD. Basal cortisol levels are often found to be reduced in PTSD patients [20]. A recent meta-analysis found lower morning salivary cortisol in PTSD patients compared to healthy subjects [21,22]. The hypoactivity of the HPA axis found in PTSD patients relates to an enhanced responsiveness and expression of GRs, which in turn facilitates the negative feedback mechanism [20]. This imbalance involving low cortisol and high GR-mediated signaling predicts a higher response to DEX. Although there are conflicting data regarding patients with PTSD and DEX-induced cortisol suppression, a majority of studies does in fact point to a higher suppression of cortisol in the DEX suppression test than in trauma-exposed healthy subjects [23]. Additionally, the HPA axis response to stressors seems to be altered in individuals suffering from PTSD. In women, an impaired cortisol response to a stress test was found in PTSD but not in MDD patients [24], which was also replicated in men with PTSD [25].

The HPA axis response to stress can also be regulated by GABAergic signaling, mainly through the activation of GABA type A receptors ($GABA_ARs$) [26]. $GABA_ARs$ are ligand-gated ionotropic receptors that promote the influx of Cl^- and subsequent hyperpolarization of postsynaptic neurons when activated. In light of the heteropentameric structure, a large number of receptor subtypes can be composed with different synaptic $GABA_ARs$ localization that participate both in *phasic* inhibition (fast action mediated by synaptic receptors), as well as *tonic* inhibition (slow and persistent form of inhibition mediated by extrasynaptic receptors) (reviewed in [27]). Brain regions involved in the regulation of the HPA axis make GABAergic connections with the PVN and its surrounding area, and thus CRH neurons receive many GABAergic inputs. These originate from the subparaventricular zone, the anterior hypothalamic area, dorsomedial hypothalamic nucleus, the medial preoptic area, lateral hypothalamic area, and some subnuclei in the bed nucleus of the stria terminalis [28]. Studies in animals have supported this hypothesis, since heterozygous $\gamma2^{+/-}$ mice show overactivity of the HPA axis [29]. Additionally, the infusion of the $GABA_AR$ antagonist, bicuculline, into the PVN accentuates the increase in corticosterone caused by stress, while the infusion of the GABAmimetic, muscimol, in the same region, dampens the corticosterone increase after stress [28]. Thus, endogenous GABAergic modulators, particularly those with action at $GABA_ARs$, have been proposed as prominent treatment strategies for the treatment of MDD and PTSD. One such category of endogenous $GABA_AR$ neuromodulators constitutes the neurosteroids, allopregnanolone and its equipotent isomer, pregnanolone.

Several other possible mechanisms of action of allopregnanolone deserve to be acknowledged due to their importance for the treatment of mood disorders. Beyond the GABAergic system, allopregnanolone also exerts modulatory effects on calcium channels, autophagy mechanisms, expression of nuclear receptors, hippocampal neurogenesis, among other mechanisms [30]. In this review we focus on the GABAergic mechanism of action of allopregnanolone, however, it is relevant to point out that other important molecular pathways may contribute to its antidepressant effect. Its involvement in the glutamatergic neurotransmission and in BDNF signaling was reviewed in previous outstanding contributions to this topic [31,32].

3. Allopregnanolone Biosynthesis Implication in Stress Response

Allopregnanolone is defined as a neuroactive steroid and a neurosteroid [33], in that: (a) it possesses a steroidal structure and that (b) its synthesis takes place, at least partially, in the central nervous system, which defines it as a neurosteroid. It may also be synthesized in the adrenal glands, ovaries and testicles and may act as a neuroactive steroid after reaching and acting in the brain. Regardless of the site of synthesis, the process of

neurosteroidogenesis begins with the trafficking of cholesterol to the outer mitochondrial membrane and its subsequent internalization to the inner mitochondrial membrane by the action of the steroidogenic acute regulatory (StAR) protein and the 18 kDa translocator protein (TSPO). Once in the inner mitochondrial membrane, the crucial step of cholesterol side chain cleavage takes place by action of the CYP11A1 enzyme, and the neurosteroid precursor pregnenolone is produced. By the action of the 3β-hydroxysteroid dehydrogenase (3β-HSD) enzyme, pregnenolone can be converted into progesterone. Beyond being a precursor of allopregnanolone and many other pregnan steroidal molecules, progesterone is also a sex hormone with a large role on the regulation of physiological and behavioral effects. Relevant for the allopregnanolone synthesis pathway (Figure 1) is the action of the 5α-reductase enzyme, which exists in two main isoforms (type 1 and type 2, abbreviated 5α-R1 and 5α-R2, respectively). 5α-R1 is the most abundant isoform in the brain and is the principal agent in converting progesterone to 5α-dihydroprogesterone (5α-DHP), which is the final precursor to allopregnanolone. 5α-DHP is converted to allopregnanolone by action of the 3α-hydroxysteroid dehydrogenase (3α-HSD), a cytosolic enzyme found in the brain and in steroidogenic tissues as well. It is worth highlighting that pregnanolone—the equipotent isomer of allopregnanolone—is also synthesized from progesterone through the action of the 5β-reductase (5β-R) enzyme, which originates 5β-dihydroprogesterone (5β-DHP). 5β-DHP is also transformed by 3α-HSD into pregnanolone [34,35].

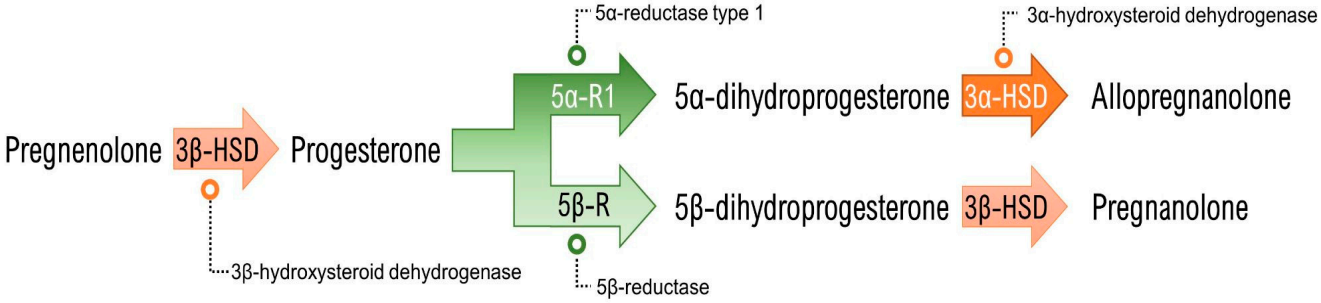

Figure 1. Allopregnanolone (and its isomer pregnanolone) synthesis pathway from pregnenolone.

Allopregnanolone and pregnanolone are capable of modulating neuronal activity, specifically potentiating GABAergic neurotransmission due to their potent, positive and allosteric modulation of the action of GABA at $GABA_A$Rs. Importantly, pretreatment with allopregnanolone attenuates the endocrine response to stress [36,37], probably owing the GABA-mediated inhibition of CRH neurons. This appears to occur following tonic activation of extrasynaptic $GABA_A$Rs containing the δ subunit, which makes them particularly sensitive to neurosteroids [38]. Additionally, allopregnanolone acts through genomic pathways, downregulating the gene expression of CRH [36] and of AVP [37] in the hypothalamus of rats. These findings place allopregnanolone as an important neuromodulator involved in the regulation of stress response and as part of the allostatic mechanisms exerting a negative feedback on the HPA axis (Figure 2).

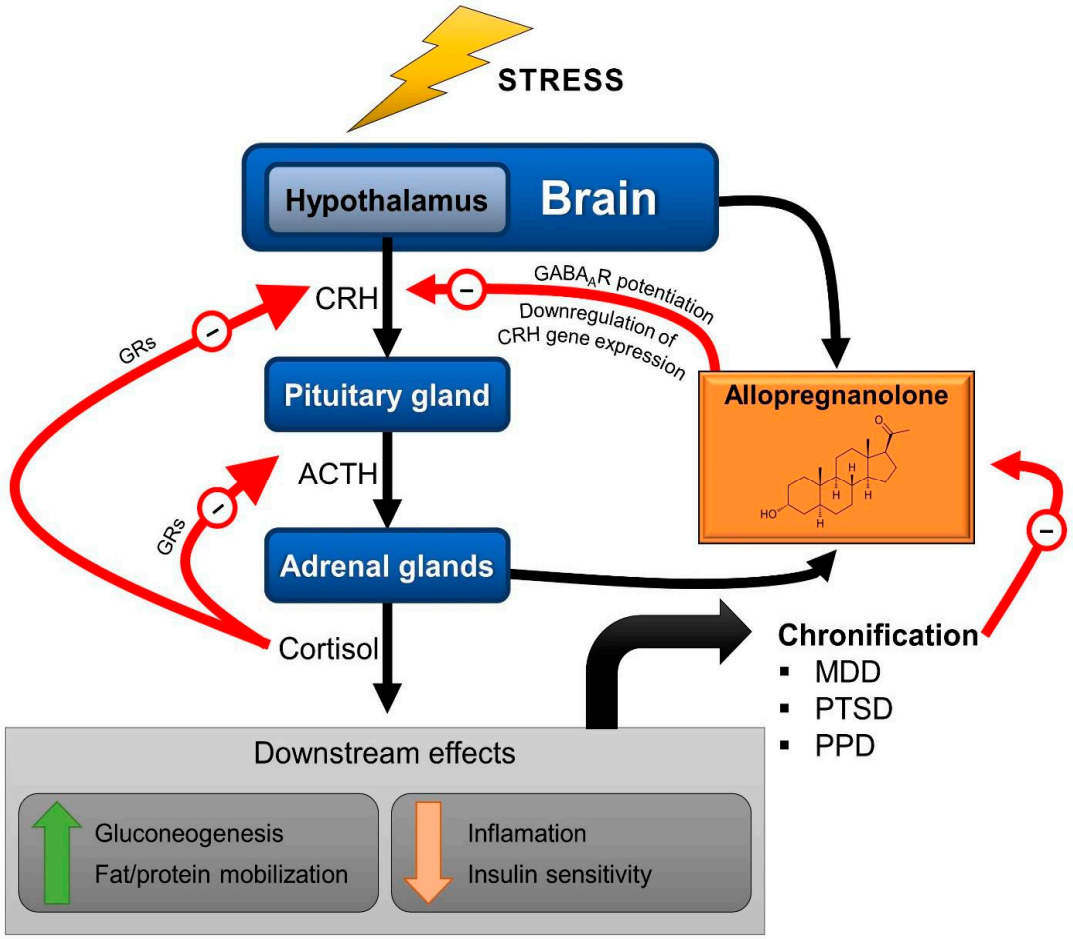

Figure 2. Schematic representation of the HPA axis with the neurosteroid allopregnanolone as a modulator of the stress response. Through positive allosteric modulation of the $GABA_A$ receptors ($GABA_A$Rs) present in corticotropin-releasing hormone (CRH) neurons, allopregnanolone participates in the negative feedback that eventually terminates the acute stress response. ACTH: adrenocorticotropic hormone; GRs: glucocorticoid receptors; MDD; major depressive disorder; PTSD: post-traumatic stress disorder; PPD: postpartum depression. Red arrows (−) indicate negative feedback.

Indeed, as first demonstrated by Purdy and colleagues, exposure to acute stress quickly elevates allopregnanolone levels in both plasma and brain [39]. This stress-induced surge in brain and periphery allopregnanolone levels was further replicated using other stressors, such as CO_2 inhalation [40] or foot shock [41,42]. Even though allopregnanolone surges in both brain and plasma in response to acute stress, intriguingly, each event is characterized by distinct kinetics. Purdy and colleagues also demonstrated in their seminal findings [39] that, although a majority of the allopregnanolone increase in the brain originates from peripheral sources, even in adrenalectomized rats there is a significant cortical increase that demonstrates the importance of its de novo synthesis in the brain. Furthermore, allopregnanolone increases more quickly in the brain compared to plasma [39]. This acute stress-induced increase in allopregnanolone is probably part of a wider mechanism that aims to promote homeostasis and that regulates HPA axis activation due to their action on GABAergic neurotransmission. Studies in handling-habituated rats (i.e., rats accustomed to the manipulation and process involved immediately before sacrifice) as 'unstressed' controls have found a significant reduction in [^{3}H]GABA binding at $GABA_A$Rs after a single foot shock, which indicates a rapid reduction in GABAergic neurotransmission following acute stress [43]. Allopregnanolone then surges in the brain and acts to restore homeostasis by positively potentiating $GABA_A$Rs, restoring GABAergic neurotransmission.

4. Chronic Stress and Its Role in MDD, PTSD and Allopregnanolone Levels

When an individual is subjected to one or more stressors for extended periods of time in a significant frequency during a given time period of significant length, long-term effects on physiological parameters begin to appear. Such effects are mostly demonstrated by increased levels of stress hormones, such as cortisol. These modifications define the allostatic load, which can manifest in the incapacity of adaptation to a stressor or the inability to properly terminate the stress response [9]. The repeated activation of the HPA axis leads to neuroendocrine adaptations that impair the secretion of glucocorticoids and throw off the balance that these stress mediators exert over other neural systems involved in stress response.

Importantly, chronic stress has been implicated in the development of MDD, particularly in the context of precipitating depressive episodes [44,45]. Chronic stress is also believed to play a role in the exacerbation of PTSD symptomatology, with MDD frequently being comorbid with PTSD. This finding is in kind with the observation that an impaired HPA axis response is frequently observed in MDD and PTSD (as discussed in Section 3, above), strengthening the link between the chronification of stress and the appearance of depressive manifestations. In addition to the high circulating cortisol levels and abnormal response in the DEX or DEX/CRH suppression test, depressed patients also present alterations in peripheral allopregnanolone levels, which have been found to be decreased in serum [46], plasma [47,48] and cerebrospinal fluid [49]. The mechanisms orchestrating this complex pathophysiological response that contrasts acute and chronic levels of allopregnanolone are not fully understood. Some studies show that increased allopregnanolone levels due to persistent or repetitive stress may induce tolerance probably by altering the sensitivity of $GABA_AR$s [50,51], particularly through changes in subunit composition. Chronic administration of allopregnanolone decreased the mRNA levels of β2, β3, α2 and α3 subunits in the mammalian cortex, without changing γ2 expression [52]. It is hypothesized that chronic stress, through yet unknown mechanisms, may attenuate the upregulation of steroidogenic enzymes in order to reduce allopregnanolone levels and regain $GABA_AR$ sensitivity. However, experimental data are still needed to elucidate whether this is in fact the mechanism behind chronic stress-induced allopregnanolone downregulation (as reviewed in [53]).

Interestingly, sex dimorphism appears to be an important factor for the alterations in allopregnanolone levels in PTSD patients. Women with PTSD show decreased cerebrospinal fluid levels of allopregnanolone with unchanged levels of progesterone and 5α-DHP, which indicated an impairment in the 3α-HSD enzyme function [54]. Male PTSD patients also present lower allopregnanolone levels in the cerebrospinal fluid that correlated inversely with depressive symptoms, but the results point to a deficit in the 5α-R1 enzyme function or expression [55]. The deficit in 3α-HSD function in women with PTSD was also demonstrated using much less invasive methods than the lumbar puncture. Allopregnanolone measured in the plasma of female patients with PTSD confirmed a conversion deficit of progesterone to allopregnanolone compared to trauma-exposed women without PTSD [56,57]. These findings, together with the increasing ability to consistently and more reliably measure neurosteroids, potentially even in easy-access specimens, including saliva, lends an interesting perspective on their use as biomarkers of MDD and PTSD [35].

Indeed, many of the protocols designed for animal studies that are frequently used to model MDD involve the application of chronic stressors that are accompanied by similar alterations in the HPA axis [4]. In the same direction, chronic administration of corticosterone is used as a rodent model of depression that is characterized by high immobility in the forced swim test and by impaired hippocampal neurogenesis. The latter can be mediated by reelin, a neurotrophin characteristic of GABAergic interneurons [58]. Additionally, low allopregnanolone levels are also observed in the brain of rodents submitted to animal models of depression based on chronic stress protocols, such as the social isolation [59] and chronic unpredictable stress (reviewed in [32]). Another interesting finding is that, in socially isolated male rats, the acute stress-induced increase in allopregnanolone is even

greater than in group-housed animals, even though their basal levels are lower, indicating a dysregulation in allopregnanolone biosynthesis caused by chronic stress [60].

Less clear is the role of chronic stress in the pathophysiology of PTSD. The efforts to model PTSD in animals have largely focused on the induction of a traumatic event of sufficient magnitude to induce behavioral alterations reproducing the symptoms of the disorder in humans. This paradigm is in line with the understanding that acute/unique—rather than chronic/repeated—stress is in the center of the pathophysiology of PTSD. In humans, PTSD is a protracted and persisting disorder with post-trauma symptoms that fail to be extinguished. Thus, the best models should be those that are able to distinguish alterations in fear extinction and fear extinction retention after trauma exposure [57,61]. Some models that feature longer exposures to stress (e.g., social defeat stress) or even chronic stress exposure (e.g., social isolation) have also been proposed to be relevant for the study of the vulnerability to PTSD, presenting several of the neurobiological characteristics of the disorder [62]. Though less directly translatable to the clinical manifestation of PTSD, these models may be useful to investigate several facets of this highly heterogeneous disorder.

5. Allopregnanolone-Based Therapeutics as a Treatment Option for MDD and PTSD

The regulatory capacity that allopregnanolone exerts on the HPA axis indicates a potential target for the normalization of the neuroendocrine alterations observed following protracted stressful conditions, potentially contributing to the alleviation of symptoms and perhaps leading to remission of MDD and PTSD. The potentiation of GABAergic neurotransmission on CRH neurons via exogenous allopregnanolone administration could therefore be a possible strategy for the normalization of the HPA axis responsiveness in PVN neurons, at the same time that this neuromodulator improves behavioral alterations by facilitating corticolimbic GABAergic signaling.

Such a therapeutic approach has also shown clinical efficacy with the intravenous administration of brexanolone (a pharmaceutical preparation of allopregnanolone marketed as Zulresso™) for the treatment of postpartum depression (PPD) [63,64]. Being the first neurosteroid to receive F.D.A. approval for the treatment of depressive disorders [65], brexanolone may very well be the starting point of a whole novel field of neurosteroid-based pharmacotherapies for the treatment of psychiatric disorders. Even though PPD is a specific subtype of depressive disorders that affects only a particular population of subjects (i.e., women in the perinatal period), alterations in the HPA axis have been implicated in its pathophysiology as well [66]. Perhaps due to the dynamic and intense nature of the changes in endocrine regulation during pregnancy [67], there are conflicting findings regarding the specific nature of HPA axis alterations in PPD. According to a recent systematic review, the best evidence points to an attenuated response to either physiological or non-physiological stimuli in women suffering from PPD [68]. Both the clinical success of brexanolone for the treatment of PPD and the data from animal studies that show allopregnanolone-induced positive behavioral responses associated with the normalization of the HPA axis function [66] provide an encouraging setting for its therapeutic application in related psychiatric disorders, particularly MDD. The HPA axis and allopregnanolone alterations found in PTSD (albeit distinct from those found in MDD) suggest a promising prospect for allopregnanolone-based treatments for this condition as well, but more research is needed to clarify this topic.

Clinical data on antidepressant effects of allopregnanolone are mostly restricted to PPD, however, several clinical trials are ongoing to study the efficacy of similar molecules in the treatment of MDD. One such example is the allopregnanolone analog SAGE-217, which, despite its recent clinical trial failure due to the absence of detectable blood levels in 10% of patients, has shown positive effects in patients that presented significant drug levels. In these patients, a statistically significant decrease in symptoms measured by the Hamilton Depression Rating Scale was achieved in days 3, 8, 12, and 15 after treatment with SAGE-217 [69]. Additionally, recent results with ganaxolone, an allopregnanolone

analogue, treatment-resistant depression of post-menopausal women, point out that it may be a useful adjunctive in patients with depression and insomnia [70].

In animal models, allopregnanolone antidepressant-like effects after systemic or intracerebroventricular administration has been largely demonstrated in the forced swim test, a well-validated model for assessing antidepressant action [71–77]. Furthermore, upregulation of brain neurosteroid levels by antidepressant drugs such as the selective serotonin reuptake inhibitors (SSRIs) fluoxetine and norfluoxetine in socially isolated mice has been shown to mediate, at least partially, the amelioration of behaviors with relevance to PTSD, such as aggressive-like behaviors [78–80]. In fact, because of the significant magnitude of this effect, and because the upregulation of neurosteroidogenesis occurs independently of any downregulation of serotonin reuptake, these drugs were considered to also act as selective brain steroidogenic stimulants (SBSSs), underscoring a whole novel antidepressant mechanism for well-established antidepressants [81]. Furthermore, the allopregnanolone analog ganaloxone, when administered in socially isolated mice, has also exerted antiaggressive and anxiolytic-like behaviors in non-sedative doses [82], even when mice were socially isolated from the early adolescent period [83]. Indeed, another allopregnanolone analog, SGE-516, also showed promising results in mouse models of PPD based on absent or reduced expression of the δ $GABA_AR$ subunit or lack of the K^+/Cl^- co-transporter (KCC2). Both δ-containing $GABA_ARs$ and KCC2 are important for effective GABAergic inhibition, specifically in the regulation of CRH neurons. SGE-516 was able to prevent the stress-induced increase in corticosterone and the dephosphorylation and downregulation of KCC2, while decreasing depression-like behaviors observed during the postpartum period [84].

Overall, the neuromodulatory role of allopregnanolone and its analog molecules on GABAergic neurotransmission, particularly acting on the regulation of the HPA axis, may represent a main mechanism underlying the treatment of MDD and PTSD. Stimulation of allopregnanolone biosynthesis by the development of novel, highly selective neurosteroidogenic drugs may become a reality in the near future and provide a much-needed diversification in the pharmacological treatment of these debilitating disorders [30,33,69].

6. Conclusions

Allopregnanolone plays a regulatory role on the neuroendocrine alterations resulting from the stress response, particularly in conjunction with the feedback mechanisms regulating the HPA axis, mediated by $GABA_ARs$. Both preclinical and clinical studies have observed allopregnanolone's rapid increase in acute stress and persistently diminished levels in chronic stress. Future studies are required to determine the role of GABAergic dysfunction, altered neurosteroid signaling in stress, and HPA axis dysregulation in MDD and PTSD as well as the usefulness of HPA biomarkers associated with neurosteroid biosynthesis in the diagnosis and treatment of these disorders.

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II.4 CAPÍTULO IV – Quantificação de neuroesteroides por cromatografia líquida acoplada à espectrometria de massas em tandem

Estes são resultados relacionados ao objetivo específico 4, abordando a implementação da metodologia de cromatografia líquida acoplada à espectrometria de massas em tandem para a quantificação de neuroesteroides e outros hormônios esteroides em amostras de soro e tecido cerebral de ratos. Este trabalho foi desenvolvido na Central Analítica da UFCSPA em parceria com o Prof. Dr. Tiago Franco de Oliveira e a Dra. Sarah Eller, além de contar com a mentoria do Prof. Dr. Graziano Pinna.

Aqui, o processo de implementação da metodologia referida é descrito nas etapas de otimização da extração líquido-líquido em amostras de soro e da separação cromatográfica dos analitos de interesse, particularmente entre os isômeros da pregnanolona. Para isto, foi necessário primeiramente determinar as condições ideais para a extração dos principais analitos de interesse, observando-se que uma mistura 7:3 de acetato de etila:hexano produz os melhores resultados. Também foram realizados testes para separação isomérica da pregnanolona, com dois dos seus isômeros sendo separados com sucesso em concentrações altas de soluções-padrão. Destacam-se os desafios encontrados para a separação isomérica em concentrações próximas àquelas encontradas *in natura* nas amostras de interesse, com propostas de estratégias subsequentes para aprimoramento da separação cromatográfica e detecção dos compostos por espectrometria de massas, como possível derivatização dos compostos.

Estes resultados foram apresentados em forma de pôster no congresso da Sociedade Internacional de Neuroquímica (*International Society for Neurochemistry*, ISN) em agosto de 2023 em Porto, Portugal e premiados com a participação e apresentação na mesma modalidade na Escola Avançada do congresso que ocorreu anteriormente ao evento. Deste prêmio e apresentações resultou um resumo publicado no periódico *Journal of Neurochemistry* (ISSN:1471-4159; Fator de impacto: 4,7; Qualis A1), apresentado ao final deste capítulo.

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ABSTRACT

Advanced school awardees

A 02-11 | The developmental trajectory of synaptic vesicle endocytosis in ASD models

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Brain communication is dependent on the evoked fusion of neurotransmitter-containing synaptic vesicles (SV) at the presynapse and their subsequent reformation and refilling, termed SV recycling. Central neurons perform different modes of endocytosis to replenish the SV pool and sustain neurotransmission. The three modes of SV endocytosis are: clathrin-mediated endocytosis (CME), activity-dependent bulk endocytosis (ADBE), and ultrafast endocytosis (UFE). Whereas it is posited that the synaptogenesis and synaptic maturation requires neurotransmitter release, it is unclear which endocytosis modes are present in early development to provide SVs for this process. To determine this, we established a developmental timeline of SV recycling in wild-type hippocampal neurons in vitro via live cell imaging of calcium influx, SV exocytosis and CME (using the genetically-encoded reporter synaptophysin-pHluorin) and ADBE (using imaging of large fluorescent dextran uptake, and morphological assays of horse-radish peroxidase uptake). We discovered that while most presynaptic events increased linearly until a plateau at 14 days in vitro (DIV), ADBE displayed a pronounced increase between 10 to 14 DIV. Therefore the triggering of ADBE occurs co-incident to the process of synapse maturation. Based on our previous finding of defective ADBE in neurons derived from multiple autism spectrum disorder (ASD) models, we monitored ADBE in $Fmr1^{-/y}$ neurons through early development. We found that that neurons from this model of fragile X-syndrome display a delayed onset of ADBE when compared to wild-type. This finding could therefore be a starting point for understanding how the developmental shifts in presynaptic mechanisms perturbs brain development and circuitry in ASD.

A 03-17 | *Cola acuminata* extract against neuroinflammation in Parkinson's disease by inhibiting NLRP3 inflammasome components in THP-1 cells

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Neuroinflammation is a common hallmark of Parkinson's disease with NLRP3 inflammasome proven to be activated in microglia of PD patient's brain. In this study, Cola acuminatabarks extract (CA) was investigated for its inhibitory effect on inflammasome activation. In preliminary experiments, CA showed no cytotoxicity on THP-1 cells and anti-inflammatory effect against nitric oxide produced following lipopolysaccharides stimulation. Furthermore, CA inhibited the production of IL-1β and IL18 cytokines released in the culture supernatant as shown by ELISA assays. At the gene level, RTqPCR showed that gene expression of the main inflammasome components including NFkB, NLRP3, caspase-1, IL1-β and IL18 were significantly reduced when the cells were pretreated with CA followed by inflammasome activation. The same observation was made at the protein level where NFkB, NLRP3, caspase-1, IL1-β and IL18 proteins were significantly reduced in western blot experiments. Confocal images detected NLRP3 and ASC proteins colocalized in cell cytoplasm; confirming inflammasome activation. However, pretreatment with CA showed no colocalization suggesting a prevention of inflammasome activation. In addition, experiments on Aβ phagocytosis showed that CA pretreatment can restore the phagocytic activity of THP-1 cells which was impaired following inflammasome activation. Altogether, our findings for the first time describe a promising role of cola acuminatabarks extract in preventing inflammasome activation and protection against neuroinflammation which is a key factor in PD development.

A 13-01 | "The role of LPA6 receptor signaling in CNS myelination"

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Myelination allows fast and efficient saltatory propagation in the central nervous system (CNS) through the finely tuned transcriptional program of oligodendrocyte differentiation. In this vein, lysophosphatidic acid (LPA) is an important endogenous phospholipid that exerts its signaling actions through the activation of its cognate G protein-coupled receptors (GPCRs) mediating multiple biological functions. Dysregulation of LPA signaling has been implicated in the pathology of the inflammatory demyelinating disease Multiple Sclerosis (MS). Among the six identified LPA receptors, the LPA_6 receptor is the most recently identified. Interestingly, LPA_6can be found in oligodendrocytes, however, its role in oligodendrocyte maturation and CNS myelination has only recently been explored by us.

Here, we present data demonstrating that LPA_6 is expressed at all the stages of the oligodendrocyte lineage in enriched cultures of rat brain-derived oligodendrocytes. However, surface localization of this receptor appears to be limited to maturating stages of differentiating oligodendrocytes. Functionally, our data reveal a negative modulatory role of LPA_6 in the regulation of oligodendrocyte maturation. To gain insight into the possible roles of LPA_6 signaling under pathological conditions, we analyzed the expression of LPA_6 in tissue samples of human MS white matter lesions, finding increased protein levels of LPA_6. Moreover, treatment with N-glycosidase F suggests the possibility of post-translational N-glycosylation modifications.

Taken together, these findings reveal a novel molecular mechanism that modulates oligodendrocyte maturation and may represent a potential novel therapeutic target for stimulating myelin repair under inflammatory demyelinating conditions as seen in MS.

A 14-03 | Quantification of pregnanolone isomers and progesterone by liquid chromatography–tandem mass spectrometry in serum and brain

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Pregnanolone and its isomers (allopregnanolone, epipregnanolone and isopregnanolone) are progesterone metabolites known as neurosteroids due to its de novo synthesis in the brain. These molecules are also called neuroactive steroids because of their important role in modulating neurotransmission, particularly through modulation of the GABA-A receptor. Being implicated in the neurobiology of several neuropsychiatric disorders, the need for measurement of these neurosteroids in biological tissue of clinical and preclinical samples has increased in recent times. The low circulating levels of these hormones in clinical samples and small amount of brain tissue obtained from laboratory animals require a method with very high sensitivity; furthermore, the chemical similarity between pregnanolone isomers—and even their precursor progesterone—demands a very high specificity. Commonly available methods such as ELISA or RIA, however, do not fulfill these requirements. Thus, this work reports on the ongoing implementation of a liquid chromatography–tandem mass spectrometry (LC–MS/MS) methodology for the quantification of neurosteroids in serum and brain. For this experiment, blood and brain of Wistar rats were collected and processed by centrifugation and pooling (blood) or homogenization in a 0.1% formic acid solution (brain). The optimal extraction solvent for each biological matrix was determined using a simplex-centroid design with the solvents ethyl acetate (EAc), hexane and methyl tert-butyl ether (MTBE). Further extraction optimization includes factors such as number of cycles and optimal pH. Determination of the lower limit of quantification (LLOQ), linearity, accuracy, intra and inter-day precision, matrix effect, selectivity and carry-over for this methodology are being carried out according to the Guidelines on Bioanalytical Method Validation. Results so far have revealed that, in serum, allopregnanolone yields are optimal with a 7:3 EAc:Hexane solution ($p < 0.001$), while progesterone shows no difference between solvents ($p = 0.363$). Solvents tested in brain tissue seem to be equivalent for both allopregnanolone and progesterone ($p > 0.1$). These initial findings provide the first base for the implementation of the advanced LC–MS/MS methodology, with excellent specificity and sensitivity to confidently and rapidly quantify neurosteroids in the brain.

II.5 CAPÍTULO V - O efeito do tratamento antidepressivo com fluoxetina, da susceptibilidade individual a um estado tipo-depressivo, e do sexo sobre os níveis do miRNA 144-3p no sangue de ratos

O artigo está relacionado ao objetivo específico 5 e pretende responder às seguintes perguntas:

1) Ratos cruzados seletivamente para apresentar comportamentos mais ou menos tipo-depressivos no teste do nado forçado apresentam diferente preferência pela sacarose e alterações nos níveis de miR-144-3p?
2) A fluoxetina é capaz de exercer seus efeitos antidepressivos nestas linhagens de animais acompanhados de alterações na expressão de miR144-3p?
3) Há efeito do sexo sobre comportamentos tipo-depressivos e expressão de miR-144-3p?
4) Existe associação entre as variáveis comportamentais e os níveis periféricos de miR-144-3p?

Trata-se de um artigo experimental realizado em ratos Wistar machos e fêmeas selecionados fenotipicamente no teste do nado forçado e cruzados sucessivamente para obter duas linhagens com perfis distintos de imobilidade no teste. Neste trabalho, demonstramos que há diferença comportamental entre as duas linhagens no teste do nado forçado mas não em outro teste de comportamento tipo-depressivo, o teste de preferência pela sacarose. O tratamento com fluoxetina promoveu efeitos tipo-antidepressivos no teste do nado forçado na dose mais alta administrada. Apesar das condições ou intervenção não terem alterado os níveis periféricos de miR-144-3p, sua expressão está inversamente correlacionada com imobilidade no teste do nado forçado em machos com baixa imobilidade não tratados, sendo que esta associação desaparece após o tratamento com fluoxetina e indica um potencial uso desse marcador. Todos os procedimentos experimentais foram aprovados pela Comissão em Ética no Uso de Animais em Pesquisa (CEUA) da UFCSPA (ver Anexo A).

Este trabalho está preparado na forma de um manuscrito a ser submetido para publicação no periódico *Progress in Neuro-Psychopharmacology and Biological Psychiatry* (ISSN 1878-4216; Fator de impacto 5,6; Qualis A1).

PARTE III – DISCUSSÃO GERAL E CONCLUSÕES

III.1 DISCUSSÃO GERAL

O primeiro capítulo traz um levantamento aprofundado a respeito da regulação da alopregnanolona e de proteínas neurotróficas em modelos animais de depressão, além de descrever efeitos tipo-antidepressivos e neurotróficos da alopregnanolona. Fica evidenciado que diferentes modelos animais de depressão provocam uma redução nos níveis cerebrais de alopregnanolona em áreas encefálicas envolvidas em transtornos depressivos. Além disso, a reversão dos comportamentos tipo-depressivos através do tratamento com antidepressivos é capaz de normalizar estes níveis. Neste sentido, fármacos pertencentes à classe de ISRS como a fluoxetina são capazes de estimular diretamente a biossíntese de alopregnanolona, levantando a possibilidade de que este efeito neuroesteroidogênico contribua significativamente para o aparecimento dos seus efeitos antidepressivos. Esta regulação cerebral promovida por modelos de depressão e tratamento antidepressivo também é observado na expressão da proteína neurotrófica BDNF, que possui um importante papel na neurogênese em áreas como o hipocampo e o córtex pré-frontal. O BDNF pode participar da mediação dos efeitos antidepressivos da alopregnanolona, pois a administração exógena deste neuroesteroide ocasiona uma suprarregulação da expressão local de BDNF. Além disso, estratégias de intervenção com objetivo do desenvolvimento de resiliência como o enriquecimento ambiental são capazes de aumentar a expressão tanto de BDNF quanto de enzimas neuroesteroidogênicas (ALMEIDA; NIN; BARROS, 2020).

O segundo capítulo dá sequência ao racional teórico do que o antecede, explorando a relevância neurobiológica da alopregnanolona e do BDNF em modelos animais de depressão e analisando o potencial uso de neuroesteroides e de proteínas neurotróficas como biomarcadores para o transtorno depressivo maior e para o TEPT. Devido à dependência da comunicação subjetiva de sintomas para o diagnóstico destes transtornos, há uma necessidade urgente para o desenvolvimento de marcadores biológicos objetivos que possam ser fácil e confiavelmente mensurados. Nesta investigação através de uma revisão narrativa observa-se que neuroesteroides como a alopregnanolona encontram-se reduzidos em indivíduos deprimidos e acometidos pelo TEPT, e a influência diferencial do estresse sobre a etiologia destes transtornos pode levar a assinaturas específicas de regulação da alopregnanolona que sejam úteis como biomarcadores. Outro aspecto atrativo é a possibilidade da

quantificação de neuroesteroides na saliva, uma matriz biológica de fácil coleta e que não ocasiona achados artificiais devido ao estresse induzido pela punção venosa. Em relação ao BDNF, há relação inversa dos níveis periféricos desta proteína neurotrófica com sintomas relevantes, e esta associação é mais evidente na depressão do que no TEPT. Ainda, a presença de polimorfismos no gene que codifica esta proteína pode estar relacionada a um maior risco de desenvolver depressão e maior severidade de sintomas em pacientes com TEPT. Estes achados levantam a hipótese de uma ligação causal entre os níveis de alopregnanolona e BDNF, mas estudos mecanísticos que possam embasar esta conclusão são escassos (ALMEIDA; BARROS; PINNA, 2021).

No terceiro capítulo, o papel da alopregnanolona em desordens depressivas e TEPT no contexto da regulação do estresse realizada pelo eixo HPA é explorado através de uma revisão crítica da literatura. O estresse desempenha um papel importante na etiologia desses dois transtornos, seja de natureza cronificada (relevante principalmente na depressão) ou de maneira traumática (de relevância maior no TEPT). De fato, uma parcela dos indivíduos diagnosticados com transtorno depressivo maior apresenta desregulações no eixo HPA como níveis cronicamente elevados de cortisol. Este achado levou à proposição do teste de supressão da dexametasona como um possível teste laboratorial auxiliar para o diagnóstico da depressão, mas sua baixa especificidade levou ao seu abandono na prática clínica. Interessantemente, indivíduos acometidos pelo TEPT tendem a apresentar níveis séricos cronicamente reduzidos de cortisol e uma resposta oposta ao teste de supressão da dexametasona quando comparados com indivíduos deprimidos, podendo ser uma ferramenta no diagnóstico diferencial entre essas entidades patológicas. Este processo de regulação compartilhado entre sistema nervoso e endócrino tem como provável explicação a participação de neurônios responsáveis pela secreção de hormônios regulatórios do eixo HPA que recebem inervação GABAérgica que pode ser positivamente modulada pela ação da alopregnanolona. Desta forma, a alopregnanolona participa do eixo inibitório sobre o eixo HPA. Portanto, a redução nos níveis de alopregnanolona ocasionada pelo estresse crônico associado à depressão contribui para uma hiperexcitabilidade deste sistema, levando aos níveis elevados de cortisol mencionados anteriormente. Tendo em vista o papel da alopregnanolona na neurobiologia dos transtornos depressivos e TEPT, e que ambos são relacionados ao estresse, o tratamento com alopregnanolona pode ser uma

alternativa viável no tratamento destas desordens. A disponibilidade de uma formulação intravenosa da alopregnanolona (conhecida como brexanolona) para o tratamento de depressão pós-parto e resultados promissores oriundos de ensaios clínicos de fase 3 com análogos da alopregnanolona fortalecem a busca de terapias baseadas em neuroesteroides (ALMEIDA; PINNA; BARROS, 2021).

O quarto capítulo aborda os resultados obtidos até o presente momento no processo de implementação da metodologia de cromatografia líquida acoplada à espectrometria de massas em tandem para a detecção de neuroesteroides, com foco principal na quantificação específica da pregnanolona e de seus isômeros. Tendo em vista a base conceitual apresentada nos capítulos I, II e III, que direcionam ao potencial uso de neuroesteroides como biomarcadores para o diagnóstico de depressão e TEPT, a sequência exploratória desse racional aponta para a necessidade de construção e melhoria de técnicas analíticas que contribuam para essa abordagem. A otimização do processo de extração líquido-líquido através da determinação experimental do solvente mais eficaz na recuperação dos analitos demonstrou um melhor desempenho de uma mistura majoritariamente composta por acetato de etila. A separação isomérica se mostrou mais desafiadora, sendo alcançada até o momento apenas em concentração elevada dos padrões analíticos (ou seja, concentrações suprafisiológicas sem relevância clínica ou pré-clínica) e para três dos quatro isômeros—não foi possível separar a epipregnanolona na corrida cromatográfica. A otimização dos parâmetros cromatográficos é um processo sequencial e evolutivo que se encontra em andamento, com o objetivo de realizar a separação dos quatro isômeros em concentrações comparáveis àquelas encontradas em amostras de soro e cérebro provenientes dos experimentos com animais.

O quinto e último capítulo traz dados experimentais obtidos em um modelo animal hereditário da depressão, no qual ratos foram seletivamente cruzados com base em seus comportamentos tipo-depressivos no teste do nado forçado. Na sequência desta construção e consolidação de conhecimentos, este capítulo busca explorar um componente importante da depressão, que é a base hereditária deste transtorno, assim como a responsividade ao tratamento com farmacoterapia consolidada para a mesma. Os principais achados são que o tratamento com fluoxetina reduziu comportamentos tipo-depressivos nestes animais e que ratos de ambas as linhagens apresentam preferência semelhante pela sacarose, demonstrando que este modelo de depressão não exibe comportamentos mais ou

menos tipo-anedônicos associados à seleção no teste do nado forçado. De três miRNAs diferentes testados, somente o miR-144-3p exibiu expressão suficiente no sangue para ser quantificado nestes animais. A expressão de miR-144-3p não diferiu entres grupos de tratamento ou de condição, mas foi correlacionada com a duração do mecanismo de escape pelo qual a fluoxetina provocou seu efeito antidepressivo. Dentro dos subgrupos experimentais, apresentou uma correlação forte e inversa com a imobilidade em machos não tratados da linhagem menos tipo-depressiva. Correlações entre variáveis comportamentais e marcadores bioquímicos são relevantes pois indicam uma relação proporcional que pode se traduzir em um uso futuro como biomarcadores dessa doença. Aqui, os resultados do miR-144-3p instigam futuras investigações em outros modelos e testes comportamentais que possam melhor elucidar seu real potencial como biomarcador para transtornos depressivos.

III.2 CONCLUSÕES

O conjunto de achados reunidos no presente trabalho permite traçar as seguintes conclusões:

1) Neuroesteroides como a alopregnanolona e proteínas neurotróficas como o BDNF encontram-se reduzidos em modelos animais de depressão, mediando o aparecimento de comportamentos tipo-depressivos;
2) Neuroesteroides e proteínas neurotróficas são promissores candidatos a biomarcadores de transtornos depressivos e TEPT;
3) Os níveis reduzidos de alopregnanolona encontrados na depressão e TEPT fazem parte da desregulação da resposta ao estresse crônico mediada pelo eixo HPA;
4) A separação de compostos isoméricos é um desafio considerável em técnicas que utilizam a espectrometria de massas, aplicando-se a isômeros da pregnanolona;
5) Os níveis do miRNA 144-3p no sangue de ratos está correlacionado com comportamentos tipo-depressivos, em um modelo hereditário de depressão, particularmente em machos não tratados da linhagem de baixa imobilidade.

Desta forma, conclui-se que neuroesteroides, proteínas neurotróficas e miRNAs são biomarcadores promissores de transtornos depressivos e que estudos subsequentes de validação pré-clínica e, posteriormente, clínica, são necessários para eventual implementação na prática clínica. O fomento a projetos que busquem estabelecer tais biomarcadores se justifica tendo em vista a necessidade existente na prática clínica em identificar transtornos psiquiátricos, assim como para diferenciá-los, tornando os métodos diagnósticos e seguimento farmacoterapêutico mais precisos como consequência da introdução destes biomarcadores na prática clínica.

III.3 PERSPECTIVAS

Como toda abordagem baseada em metodologia científica que responde sequencialmente perguntas de pesquisa, os resultados alcançados neste trabalho servem como base para a elaboração de novos experimentos que visem testar hipóteses adicionais e desenvolver estratégias capazes de superar desafios encontrados, complementando as conclusões aqui obtidas. Neste sentido, existem diferentes estratégias que podem ser adotadas para alcançar a detecção fidedigna a alopregnanolona e seus isômeros através da espectrometria de massas. Ainda utilizando-se da separação por cromatografia líquida, a sensibilidade e especificidade do método podem ser melhoradas através da adição de uma etapa de derivatização antes da injeção no aparelho de UPLC. Existem múltiplos agentes derivatizantes que podem ser conjugados com os neuroesteroides, sendo que a escolha deve ser feita experimentalmente. Alternativamente, o método de cromatografia pode ser alterado de líquida para gasosa. Esta estratégia implicaria na adição de diversas etapas além da derivatização, como uma extração com múltiplos ciclos, pré-separação por HPLC com detecção das frações de interesse por radioatividade (implicando na aquisição de isótopos radiomarcados dos analitos de interesse) e agrupamento manual das frações selecionadas. Apesar de mais trabalhosa, esta técnica conta com extensivo treinamento do autor em laboratório parceiro no exterior, que possui ampla *expertise* nesta metodologia (ver Apêndice A).

A partir da finalização da implementação da metodologia para quantificação de neuroesteroides, será possível mensurar os níveis séricos e cerebrais dos mesmos nos animais provenientes do experimento abordado no Capítulo V. Da mesma forma, níveis séricos (proteína) e cerebrais (mRNA) de BDNF podem ser quantificados nestes animais. Isto permitirá um conjunto de análises associativas que pode ajudar a elaborar um eixo de biomarcadores para transtornos depressivos. Além disso, a quantificação dos miRNAs que apresentaram baixa expressão pode ser reavaliada e amplificada através de adequações no protocolo de análise que operem fora das especificações do kit utilizado. Por esta razão, estas novas análises teriam que ser devidamente validadas e seu sucesso determinado experimentalmente. No mesmo sentido, percebeu-se a dificuldade de encontrar revisões sistematizadas e completas da literatura que ajudem a guiar a escolha de miRNAs específicos para sua investigação relacionada à modelos de depressão. Assim surge a perspectiva de

realizar uma revisão de escopo no tema que possa auxiliar outros pesquisadores pré-clínicos com interesse de investigar o papel de miRNAs utilizando modelos de depressão.

De maneira geral, abre-se um novo campo de exploração analítica que contribuirá para a precisão diagnóstica de TEPT, de depressão e de quadros mistos ou interrelacionados que carecem de ferramentas e técnicas que permitam melhorias nas tomadas de decisão. Além disto, estes biomarcadores poderão ser analisados quanto a sua capacidade de detectar os indivíduos que terão melhores respostas farmacoterapêuticas com os diferentes tipos de antidepressivos, fortalecendo principalmente as abordagens farmacoterapêuticas.

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APÊNDICE A

Co-autoria em manuscrito resultante de projeto experimental realizado durante estágio de doutorado-sanduíche no Departamento de Psiquiatria da *University of Illinois at Chicago*, no qual a quantificação de neuroesteroides no soro é realizada através da metodologia de cromatografia gasosa acoplada à espectrometria de massas.

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Title: Independent effects of acute estradiol or progesterone on perimenstrual changes in suicidal ideation, affective symptoms, and 3α-reduced progesterone metabolites: A crossover randomized controlled trial

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Abstract

Background: Across several patient cohorts recruited for recent suicidal ideation (SI), current affective disorder, and natural menstrual cycles, we have observed a dimensional (present to varying degrees) perimenstrual (PM) worsening of SI and depressed mood, specifically relative to an early luteal (EL) nadir. In our prior crossover trial, PM administration of combined estradiol (E2) and progesterone (P4) reduced this exacerbation of SI and depressive symptoms versus placebo (PBO). This three-period crossover trial extended this work by examining *independent* effects of E2, P4, and PBO on PM worsening of SI and related depressive symptoms. Further, we examined two neuroactive steroid metabolites of P4 (3α,5α-tetrahydroxyprogesterone or allopregnanolone/ALLO, 3α,5β-tetrahydroxyprogesterone or pregnanolone/PA), since some have hypothesized that NAS withdrawal contributes to PM affective changes.

Methods: A naturally-cycling transdiagnostic sample with affective disorder and past-month SI (*N*(per-protocol)=23; N(intent-to-treat)=44) completed three double-blind, counterbalanced conditions: perimenstrual administration of (1) .1mg/day transdermal E2 (plus PBO pills), (2) 200mg/day oral micronized P4 (plus PBO patch), and (3) PBO patch and pills. From three visits per condition (+7, +14, and +22 days after an LH surge of >= 40 mIU/ml), blood was assayed (via GC/MS) for E2, P4, ALLO, and PA.

Results: Despite the fact that only P4 (vs. PBO) prevented PM withdrawal of ALLO and PA, P4 (vs. PBO) increased PM worsening of SI, but had no other symptom effects. In contrast, E2 (vs. PBO) reduced PM worsening of SI, depressed mood, hopelessness, anxiety, overwhelm, rejection sensitivity, and perceived burdensomeness—even though it did not prevent ALLO or PA withdrawal.

Conclusions: Cyclical worsening of depressive symptoms and SI are improved with perimenstrual E2 administration, implicating E2 withdrawal or depletion as a mechanism.

Cyclical withdrawal or depletion from P4, ALLO, or PA may not contribute to cyclical affective changes, and P4 administration may worsen SI.

Introduction

Suicide is the fourth leading cause of death among individuals assigned female at birth (AFAB) of reproductive age (ages 15-45) in the United States [1]. Despite decades of research, useful methods for predicting acute escalation of suicidal ideation (SI) and acute risk of suicide attempt remain elusive [2]. Accumulating cross-sectional [3], longitudinal [4, 5], and experimental [6] studies point to the ovarian steroid fluctuations of the menstrual cycle as time-varying predictors of acute suicide risk. Hospitalizations for suicide attempt peak in the peri-menstrual phases of the cycle (i.e., around menstrual bleeding) [3, 7]. Consistent with evidence that roughly 60% of female patients with depressive disorder show prominent perimenstrual worsening of at least one symptom [8], our longitudinal studies in naturally-cycling outpatients with SI have consistently demonstrated a dimensional perimenstrual worsening of SI relative to an early luteal nadir [4, 5], a pattern statistically mediated by identical changes in depressed mood and cognition [4]. The impact of these patterns is further underscored by epidemiologic findings that up to three quarters of patients with prospectively-documented perimenstrual affective changes report lifetime SI [9, 10], and one third report a lifetime suicide attempt [9]. The menstrual cycle is therefore a plausible time-varying predictor of acute suicide risk that may also account for the female-biased sex difference in suicidal thoughts and behaviors observed during the reproductive years [11, 12].

In order to develop targeted interventions to address this unique biotype of suicidality, it is critical to understand the steroid pathophysiology of perimenstrual changes in depressive symptoms and SI. Human experiments have focused primarily on elucidating steroid mediators of the luteally-confined affective symptoms that characterize most patients with DSM-5 premenstrual dysphoric disorder (PMDD). These studies demonstrate that periovulatory estradiol (E2) and progesterone (P4) flux are the primary trigger for affective symptoms that emerge in the early-to-midluteal phase and remit in the midfollicular phase [13–15], that these effects are likely mediated by surges in the neuractive steroid metabolites of P4 [16] rather than

P4 itself [17], and that inducing midluteal withdrawal from E2 and P4 does not further trigger symptoms in this population [13]. However, accumulating experimental evidence also demonstrates differing steroid mechanisms in patients with cyclical worsening of chronic affective symptoms—especially depression and SI, and that perimenstrual steroid withdrawal may play a more prominent role in this population. Specifically, experiments have repeatedly failed to demonstrate a benefit of ovarian steroid suppression with GnRHa, a well-supported treatment for PMDD [18], in patients with perimenstrual worsening of depressed mood [19–21], and even suggest that such treatments, which lead to ovarian steroid withdrawal and depletion, may specifically worsen depressed mood [22].

Our recent clinical trial (CLarifying the Endocrinology of Acute Risk-1 or CLEAR-1) in naturally-cycling outpatients recruited for SI further demonstrated that buffering perimenstrual steroid withdrawal through the simultaneous perimenstrual administration of E2 and P4 (vs. placebo) can prevent perimenstrual worsening of SI and associated depressive symptoms. In that randomized crossover trial, administration of .1mg transdermal E2 and 200mg oral micronized P4 (100mg twice daily) in the two weeks around menses onset reduced perimenstrual worsening of SI and depressive symptoms compared to PBO. These effects were observed primarily on the days during and just before menstruation, supporting the hypothesis that steroid supplementation ameliorated symptoms by preventing withdrawal or depletion of ovarian steroids. It remains unknown, however, whether these effects were due to the administration of E2, P4, or both. The present trial (“CLEAR-2”) is a new three-period crossover trial that extends the first trial by testing the <u>independent</u> effects of perimenstrual supplementation of the same doses and routes of E2 or P4 against a placebo (PBO; *i.e.,* E2 administration only vs. P4 administration only vs. placebo).

Previous research focused on pregnancy and menopause provides further evidence for the plausibility of both E2 withdrawal and P4 withdrawal as potential drivers of perimenstrual-emergent changes in affect and SI. Some studies have implicated combined withdrawal [23,

24], while others have pointed to individual withdrawal from E2 [25, 26]. Other research has found P4 and its metabolites to be significantly reduced in association with depressive, anxious, and impulsive behavior [27, 28]. In the case of P4, these effects are mediated by withdrawal from GABAergic neuroactive steroid (NAS) metabolites such as 3α,5α-tetrahydroxyprogesterone (allopregnanolone/ALLO) and 3α,5β-tetrahydroxyprogesterone (pregnanolone/PA) which enhance inhibitory neurotransmission by positively modulating the GABA-A Receptor (GABAAR) [29, 30]; Figure 1 illustrates this biochemical pathway.

Based on the evidence reviewed above, we hypothesized prior to study initiation that P4 withdrawal is the primary steroid trigger for perimenstrual-onset affective symptoms and SI due to the deleterious effects of abrupt withdrawal from ALLO and PA, and that E2 withdrawal may serve as an additional trigger. Therefore, we predicted that administration of P4 (vs. PBO) to buffer withdrawal from ALLO and PA would significantly reduce the perimenstrual worsening of symptoms. Given the beneficial effects of E2 on depression in other populations [31–33], we also hypothesized a secondary benefit of E2. Ultimately, results of the hormone experiment described below uncovered the opposite. Although P4 (vs. PBO) increased both ALLO and PA, it worsened cyclical exacerbation of SI and showed no significant effects on other perimenstrual symptom trajectories. In contrast, E2 (vs. PBO) reduced PME of diverse affective symptoms and SI.

Materials and Methods

The CLEAR-2 clinical trial is registered on clinicaltrials.gov (NCT03498313), with more detailed preregistration of hypotheses and data management/analytic strategies preregistered on the Open Science Framework (https://osf.io/wxsey). The University of Illinois at Chicago Institutional Review Board approved this protocol.

Participants

Participants were recruited from the community via social media advertisements. Ads emphasized studying the biology of depression, stress, and suicidal thoughts, and did not mention sex, gender, hormones, or the menstrual cycle to increase generalizability and minimize demand characteristics. Eligibility criteria (assessed through online survey, phone, and enrollment visits) included: AFAB; past-month suicide *ideation* (no imminent risk at enrollment or attempt within three months); seeing a licensed mental health professional at least once every three months; aged 18-45; BMI 18-34.99 kg/m^2; no serious or chronic nonpsychiatric illness; no personal, familial, or genetic risk factors for blood clots or hormone-related cancers; regular cycles (every 21-35 days); no dysmenorrhea (defined as menstrual physical symptoms causing >=2 days of clinically significant impairment per month); not pregnant, breastfeeding, or within one year postpartum; not using devices/medications containing hormones; not taking risperidone or haloperidol; no current depressive episode with perinatal onset; no ongoing treatment for prospectively-confirmed PMDD; no history of hospitalization for mania; no regular nicotine use; and no other factor expected to interfere with compliance significantly.

Experimental Design

To clarify the independent effects of E2 or P4 on perimenstrual affect and SI changes, we employed a randomized, placebo-controlled, double-blind (investigator, participant, assessor), three-period crossover trial design; Figure 2 provides visuals of the design. Throughout, we use “experimental interval” instead of “study period” to prevent confusion with “menstrual period.” During enrollment, participants were told, “In this study, we are using a hormone experiment to determine whether preventing certain hormone changes could help to reduce suicidal thoughts. In our prior study, many people felt better while taking these hormones, a few felt worse, and others felt no different. Although the hormones may affect your symptoms, they

are not currently considered a treatment." Participants completed a baseline cycle of daily ratings followed by three experimental conditions, trisected by two one-month washout phases. Each experimental interval spanned two weeks surrounding expected menses onset (*i.e.,* medication taken from days +7 to +20 following a positive 40mIU/ml urine luteinizing hormone (LH) test, given that expected menses onset is about 13.3 days after the preovulatory surge in LH (day 0) (40)).

Laboratory visits occurred on days LH +7, +14, and +22 of each experimental interval. Serum collected at each visit was assayed via gas chromatography followed by mass spectrometry (GC/MS, see Supplement) for E2, P4, and NAS. In the **placebo (PBO) condition**, participants received oral and transdermal matched placebos. In the **estradiol (E2) condition**, participants received .1mg/d transdermal E2 (Climara™; dosed as patch placed by staff on upper buttock at +7 and replaced at +14 (following LH+ test = 0) and placebo pills. In the **progesterone (P4) condition**, participants received a placebo patch and 200mg/d oral micronized P4 (Prometrium™; 100mg BID). Hormonal conditions were intended to reduce the downward slope of natural perimenstrual steroid withdrawal (*i.e.,* reducing steepness). The investigational pharmacy handled randomization, blinding, and dispensation. Twice daily, participants received pill reminders via SMS. Study activities occurred at a University outpatient clinic in the midwestern United States. Participants were paid $2,650.

Assessment Schedule

During a 3-hour enrollment visit, the Structured Clinical Interview for DSM-5 [SCID-5; 34], the Borderline Personality Disorder module of the Structured Clinical for DSM-5 Personality Disorder [SCID-5-PD; 35], and Columbia-Suicide Severity Rating Scale [C-SSRS; 36] were administered. From enrollment to study completion, participants rated SI and affective symptoms daily (link delivered via SMS at a participant-elected time after 5 pm). In addition, brief daily phone calls during experimental intervals monitored adverse events, suicide risk, and compliance.

Outcomes

Primary outcomes included daily SI (passive and active) and planning**.** Daily severity of passive and active SI was measured as the mean of multiple items from the Adult Suicidal Ideation Questionnaire [ASIQ; 37] with a scale from 1=Not at All to 5=Extremely based on the past 24 hours. Passive SI items included "I wished I were dead," "I thought that life was not worth living," "I wished I could go to sleep and not wake up," and, "I thought it would be better if I was not alive" (Multilevel Reliabilities: RkF=.99, R1R=.63, RkR=.99, Rc=.86, RkRn=.99, Rcn=.77). Items assessing active SI included, "I thought about killing myself," "I thought about killing myself, but would not do it," "I thought that if things did not get better, I would kill myself," "I wanted to kill myself" (Multilevel Reliabilities: RkF=.99, R1R=.66, RkR=.99, Rc=.72, RkRn=.99, Rcn=.61). Suicidal planning items included ("I thought about how I might kill myself," "I thought about when I might kill myself"'). Due to limited reports of suicidal planning, this variable was recoded as a dichotomous variable, with any response other than 1 (Not at All) on either item coded as 1 (planning present) versus 0 (absent). **Secondary outcomes** included five symptom families linked to suicide risk, measured using the daily record of severity of problems [DRSP; 38]. These five families included (1) *Depressed Mood and Cognition*: depressed mood (DRSP1: "felt depressed, sad, "down," or "blue"), hopelessness (DRSP2: "felt hopeless"); (2) *Threat Perception*: anxiety (DRSP4: "felt anxious, tense, 'keyed up,' or 'on edge'"), overwhelm (DRSP16: "I felt overwhelmed, that I could not cope"); *Social Cognitive Bias:* rejection sensitivity (DRSP6: "I was more sensitive to rejection or my feelings were easily hurt"), perceived burdensomeness (Interpersonal Needs Questionnaire item, "I felt that I was a burden to other people, or that they would be better off without me"); and (4) *Executive Dysfunction*: difficulty concentrating (DRSP9: "had difficulty concentrating"), impulsivity (Adapted item from UPPS-P, "I did something impulsive that I might later regret "), and (5) *Frustrative non-reward*: anger/irritability (DRSP8: "felt angry or irritable"). All secondary items were rated from 1=Not at All to 6=Extreme.

Covariates

Pain was measured using a single item ("Please rate your experiences of physical pain in the past 24 hours on a scale of 0-10"). Days since enrollment was calculated as the current date minus the enrollment date. Dose of serotonergic antidepressants (citalopram, escitalopram, fluoxetine, fluvoxamine, sertraline, desvenlafaxine, duloxetine, venlafaxine, vilazodone, nortriptyline) was monitored daily via self-report and used to compute a standardized variable (standardized serotonergic antidepressant dose (SSAD; 0=None, 1=Standard Low Dose, 2=Standard Moderate or High Dose).

Condition and Cycle Phase Coding

Condition and cycle phase coding procedures, which Figure 2 depicts in detail, are identical to those described in the CLEAR-1 Trial [39] and are included in the supplement. The cycle phase codings in the experimental cycles were coded such that (1) the early luteal phase, when we have observed the nadir of depressive symptoms and SI in our longitudinal studies of this population [4], was coded as the pretreatment reference phase, and (2) the perimenstrual and early follicular phases, when we have observed the peak of depressive symptoms and SI in the same studies, were coded among our cycle phases so as to test whether specific increases under PBO in these phases could be prevented by steroid administration.

Statistical Analyses and Power

Because this is a mechanistic experiment, we focused on per-protocol analyses (PP); however, exploratory intent-to-treat (ITT) analyses are reported. Descriptive analyses utilized indices from the Carolina Premenstrual Assessment Scoring System [C-PASS; 40]. Outcome analyses were carried out in three-level models (daily or laboratory observations at level 1, nested within experimental interval at level 2, nested within participants at level 3). We expected a symptom increase from the early luteal to the perimenstrual (E2 and P4 withdrawal) or early follicular (recent E2 and P4 withdrawal, low levels) phases under PBO that would be prevented in the E2 or P4 conditions. We predicted an interaction of experimental condition (at

level 2) with cycle phase contrast (between early luteal and perimenstrual, for example, at level 1) predicting daily outcomes. Daily physical symptoms were centered around the individual's mean and covaried in models, along with days since enrollment and daily SSAD. Analyses proceeded in R in *lme4*. Random intercepts were included at person and condition levels. Due to singularity arising from highly correlated random phase contrast slopes, random phase slopes were combined in each model based on the pattern of correlations among the initial random slope estimates. The Benjamini-Hochberg procedure was utilized to control the familywise false discovery rate at .05; all perimenstrual and early follicular interactions with conditions listed in bold in Tables 3-5 survived this correction.

A priori power calculations based on the expectation of a conventionally medium (f = .25) within-person experimental effect and significant outcome clustering (ICC ~ .60) in a repeated measures model indicated that just 12 three-condition completers (corresponding to ~864 daily observations, given three experimental conditions with 24 daily ratings apiece) would be required to achieve 80% power for the simple experimental effect. To increase generalizability and power, we aimed for at least 24 completers.

Results

Participant Flow

Figure 3 (CONSORT) illustrates participant flow. Recruitment and data collection began July 2018 and ended May 2021. Of 139 screened, 60 enrolled, and 49 were randomized. PP analyses included 23 participants (63 experimental intervals, 1344 surveys). ITT analyses included 44 participants (104 experimental intervals, 1940 surveys).

Baseline Characteristics

Table 1 depicts baseline characteristics in the PP sample by first condition completed (E2, P4, or PBO; no differences observed). Supplemental Table 1 compares the ITT and PP samples. Intraclass correlation coefficients (ICC) indicated a substantial degree of clustering in outcomes (~.3-.7). Supplemental Figures depict repeated measure correlations among daily

symptoms. As noted in the introduction, analyses in the baseline daily ratings of patients recruited using these methods across all of our clinical trials demonstrates a nadir of depressive symptoms and SI in the early luteal phase and a peak of these symptoms in the perimenstrual and early follicular phases, concurrent with menstrual ovarian steroid withdrawal or depletion (see Figure 2) [4]. Of note, these symptoms did not follow the typical pattern of symptoms associated with PMDD, in which symptoms arise in the early-to-midluteal phase and remit fully by the end of menses, with a typical nadir in the midfollicular phase.

Adherence and Missingness

Study adherence was high in the PP sample, with 181 missing surveys (10%) and 15 incomplete surveys (0.8%) out of 1767 expected. On average, participants reported missing less than one pill per condition, and all patches that fell off prematurely were replaced within 24 hours. In the E2 condition (N=580 surveys), 9.8% of surveys were missing, and 1% of surveys were incomplete; in the P4 condition (N=590), 11.7% of surveys were missing, and 0.7% were incomplete, and in the PBO condition (N=597), 9.2% were missing, and 1.2% were incomplete. Supplemental Table 2 presents a model predicting survey missingness from condition, indicating no significant differences.

E2 and P4 Levels

Models predicting condition-by-phase effects on E2 and P4 are presented in Table 2 and depicted in Figure 4. In all conditions, there were significant main effects of cycle phase on P4, such that perimenstrual and late follicular P4 levels were significantly lower than midluteal P4. E2 was nominally lower in the perimenstrual phase. P4 administration did not significantly alter trajectories of P4 from the midluteal to either the perimenstrual (medication) or late follicular (medication withdrawal) visit, despite pill compliance, ovulation confirmation, and precise visit timing; this is likely due to rapid first-pass metabolism to NAS. E2 administration nominally prevented the drop in E2 from the midluteal to perimenstrual phases (p=.055). E2 also caused an unexpectedly greater midluteal-to-perimenstrual reduction in P4.

Luteal Phase Length, Menstrual Bleeding, and Physical Symptoms

Supplemental Table 3 and Supplemental Figures present condition effects on luteal phase length and menstrual bleeding; no significant differences were observed. P4 (vs. PBO) led to a larger early-luteal-to-perimenstrual increase in physical pain (see Table 4).

Participant Blinding

Participants rated their agreement with the statement, “In the past week, I thought I was taking a placebo” on a visual analog scale (0-10) at the second visit of each condition (7 days into the 14-day experimental interval). Supplemental Table 4 presents results, which revealed no significant differences, consistent with successful blinding.

Hypothesis Tests

The primary hypothesis tests focus on the statistical interaction of Condition and Experimental Cycle Phase (Early Luteal to Perimenstrual, Early Luteal to Early Follicular) predicting daily symptoms. CONSORT guidelines advise against statistical tests for crossover effects [41].

Impact of Covariates on Symptoms: Time, Physical Pain, and SSAD

The passage of time did not predict the severity of any symptom, highlighting the chronicity of symptoms and the appropriateness of the crossover design. Daily increases in physical pain were associated with increases in overwhelm and impulsivity. Relative to no use, low doses of serotonergic antidepressants were associated with slightly greater passive SI; moderate to high doses were associated with greater passive SI but lower depressed mood.

Primary Suicide-Related Outcomes

Table 3 presents primary hypothesis tests. Contrary to hypotheses, P4 (relative to PBO) caused a greater early-luteal-to-perimenstrual *increase* in passive SI, and late follicular withdrawal from P4 (vs. PBO) led to a more rapid reduction in passive SI during the late follicular phase (see Figure 5, bottom panel). P4 (vs. PBO) did not influence the trajectory of active SI.

In contrast, E2 (vs. PBO) reduced PME of both passive and active SI throughout the experimental period (see Figure 5). Of note, there was a higher baseline level of active SI in the E2 condition relative to PBO; however, this effect was covaried in all models.

No condition effects on the likelihood of suicidal planning were observed.

Secondary Outcomes

Tables 4 and 5 present the results of models predicting secondary outcomes.

Depressed Mood and Cognition. Relative to PBO, E2 reduced early-luteal-to-early follicular increases in depressed mood and hopelessness (see Figure 5, top panel), whereas there were no significant effects of P4.

Threat Perception. Relative to PBO, E2 prevented early-luteal-to-perimenstrual increases in anxiety and feelings of overwhelm (see Figure 6), whereas there were no significant effects of P4.

Social Cognitive Bias. Relative to PBO, E2 prevented early-luteal-to-perimenstrual and early-luteal-to-early follicular increases in both rejection sensitivity (see Figure 6) and perceived burdensomeness. For perceived burdensomeness, E2 (v PBO) also prevented earlier early-luteal-to-midluteal increases. P4 showed no differences from PBO.

Executive Dysfunction. Neither E2 nor P4 significantly affected cyclic trajectories of difficulty concentrating or impulsivity. Of note, impulsivity was generally reduced across the entire experimental phase, regardless of condition.

Frustrative Non-Reward. Neither E2 nor P4 influenced cyclical changes in anger/irritability.

Neuroactive Steroid Outcomes

Table 2 presents models predicting NAS. Relative to PBO, P4 administration increased both ALLO and PA at the perimenstrual (vs. midluteal) visit, and the increase in ALLO remained the day after discontinuation of P4. No significant effects of E2 were observed. Figure 4 depicts all NAS trajectories.

Exploratory Intent-to-Treat Analyses

For completeness, primary hypotheses were also tested in the ITT sample, which included experimental intervals characterized by anovulatory P4, luteal phases ending prior to medication start, medication noncompliance, and significant health or healthcare changes (hospitalizations, medication changes). As expected given the mechanistic nature of the study, no significant experimental effects on SI or other self-reported symptoms were observed (all p's<.05).

Adverse Events

A variety of mild-to-moderate, expected side effects were reported throughout the PBO (12; 33% of exposed), E2 (15; 39% of exposed), and P4 (10; 27% of exposed) conditions.

Discussion

Summary

Based on the prior CLEAR-1 trial (E2+P4 vs. PBO), we expected that P4—and, to some extent, E2—would reduce PME of SI and related symptoms. We further predicted that P4 metabolism to ALLO and PA would explain the benefits of P4. Contrary to predictions, we observed that PME of SI and physical pain worsened during P4, with no effects on other symptoms. We also observed that E2 reduced PME of SI, depressed mood, hopelessness, anxiety, overwhelm, rejection sensitivity, and perceived burdensomeness with similar estimate sizes to the CLEAR-1 trial. Consistent with CLEAR-1, no effects on difficulty concentrating, impulsivity, or anger/irritability emerged. In contrast, we did not observe greater symptom recapitulation under E2 or P4 withdrawal relative to PBO withdrawal, although symptoms increased to some degree at the end of all conditions as the placebo effect dissipated. We conclude that perimenstrual E2 withdrawal or deprivation contributes to PME of SI and related symptoms for susceptible individuals.

Possible Mechanisms of E2-Withdrawal-Related Depression and Suicidality

E2 is a highly conserved molecule influencing many neurobiological risk pathways (see Schiller et al., [42]). E2 regulates neurotransmitters implicated in depression (i.e., serotonin, dopamine, and norepinephrine) and affects neuroplasticity (via increases in brain-derived neurotrophic factor) and neuroprotection (mediated partially by G-protein-coupled receptor 30) [42]. E2 also affects stress and immune functioning; it modulates the hypothalamic-pituitary-adrenal (HPA) axis by regulating basal and stress-induced adrenocorticotropic hormone and cortisol, and inhibits inflammation (e.g., via regulation of cytokine production) [42]. Our results dovetail with previous studies demonstrating that depressive symptoms developing in response to Gonadotropin-releasing hormone agonists (GnRHa) depend partly on the degree of E2 withdrawal [22]. In this paradigm, GnRHa-induced depressive symptoms were also linked to increased serotonin transporter (SERT) binding, which reduces synaptic serotonin [43]. E2 has been suggested to increase serotonin synthesis [44] and decrease serotonin degradation [45]. Additionally, E2 withdrawal can reduce dopamine signaling in prefrontal regions [46, 47], reducing the capacity for affective and behavioral regulation [48–50]. Reduced dopamine response in mesolimbic reward circuitry during E2 withdrawal may also reduce reward sensitivity [51–54] with implications for anhedonia [55, 56].

Lack of P4 Benefit Despite Elevated Neuroactive Steroids

Our findings replicate evidence that oral micronized P4 increases ALLO and PA. We investigated the time course of experimental condition effects on NAS across a midluteal baseline draw, a perimenstrual draw at which participants were taking study medication, and a midfollicular draw after withdrawal from medication. Peripheral changes in P4 levels did not differ between the P4 and PBO, likely due to the long delay between past-evening pill ingestion and the morning blood draw, as well as rapid metabolism of oral micronized P4 into ALLO and PA [57]. Supraphysiologic increases in ALLO and PA, induced by P4, normalized within 24 hours, returning to baseline during the midfollicular visit. Perimenstrual administration of E2 did not alter ALLO or PA compared to PBO. Given the failure of P4 to show symptom benefit

despite raising NAS levels, we conclude that perimenstrual withdrawal from these steroids is unlikely to account for perimenstrual worsening of depression and SI.

Comparisons with Steroid Mechanisms in Other Reproductive Mood Disorders

Experimental studies investigating the steroid mechanisms of affective symptoms in pregnancy and menopause have also found that E2 withdrawal can trigger depressive symptoms. For example, one trial found that individuals with past perimenopausal depression experienced symptom increases upon experimental withdrawal from (vs. continuation of) transdermal E2 [26]. Pilot trials in postpartum depression indicate that transdermal E2 may provide symptom relief compared to placebo [31–33], although one trial was halted due to insufficient elevation of E2 serum concentrations [58]. Considering our finding of a benefit of transdermal E2 observed despite limited elevations in peripheral blood, additional work may be needed to clarify how transdermal metabolism of E2 impacts therapeutic benefit.

In contrast to perimenopausal and postpartum depression, periovulatory surges in ALLO, rather than steroid withdrawal, are the primary trigger for the typical luteally-confined symptoms of PMDD [13, 15, 16]. However, PMDD is a heterogeneous condition; some individuals experience perimenstrual-emergent symptoms with delayed menstrual offset [59]. In these cases, perimenstrual withdrawal from E2 may serve as an alternative or additional trigger for symptoms of PMDD. Further, in patients for which some symptoms are triggered by periovulatory ALLO surges (*e.g.,* irritability and mood swings during the luteal phase) and others are later triggered by perimenstrual E2 withdrawal, the risk of developing chronic affective disorders and suicidality may be especially high.

Although the cyclical affective changes observed in our sample may not meet criteria for PMDD due to elevated baseline symptoms (*i.e.,* lack of “absolute clearance” [40]), the current evidence further supports the study of hormone sensitivity in patients with chronic psychiatric disorders. Further, the different steroid mechanisms of cyclical hormone sensitivity observed across research programs (e.g., ALLO surges, E2 withdrawal) do not appear confined to DSM-5

PMDD (vs. cyclical changes that do not meet strict criteria for number or clearance of symptoms). More work is needed to characterize heterogeneity of hormone sensitivity in patients with significant cyclical changes in their symptoms, regardless of baseline levels.

Strengths, Limitations, and Future Directions

Strengths of this trial include the rare use of a biological experiment in a clinically severe sample with SI, the use of a three-condition design to disentangle effects of E2 and P4, and the use of a crossover design to increase power and reduce noise associated with clinical heterogeneity. The within-person approach allowed us to clarify the time course of suicide risk and associated symptoms in relation to neuroactive steroid levels. Finally, our protocol included gold-standard methods of urine LH-surge testing to determine menstrual cycle phases and GC-MS methods with unsurpassed structure selectivity to quantify neuroactive steroids.

Limitations should be noted. This sample was not specifically recruited for PME or PMDD, limiting our power to observe effects on cyclical symptom change. There was substantial attrition due to clinical instability, COVID-19, and burden of participation. Since we did not include a control group, we cannot yet conclude that the effects observed here arise from a differential sensitivity to E2 withdrawal. Finally, consistent with prior trials, we did not observe substantial elevations in E2 in that condition (as in Wisner et al., 44).

Conclusions

Cycling patients with suicidality exhibit an elevated risk of perimenstrual symptom exacerbation relative to the general population, and the current study points to E2 withdrawal as the primary mechanism of this acute risk. While P4 and ALLO withdrawal feature heavily in longstanding theories of premenstrual emotional change, the current experiment provides some of the most definitive contradictory evidence regarding this hypothesis. Future experiments should probe the mechanisms by which E2 withdrawal influences SI and related symptoms around the onset of menses so that safe, targeted treatments can be developed.

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Disclosures

GP is a paid consultant to PureTech Health, GABA Therapeutics, and NeuroTrauma Sciences. He has two patent applications, one on PEA and PPAR-a agonists (US20180369171A1, granted on May 16, 2023) and one on allopregnanolone analogs (US11266663B2, granted on March 8, 2022) in the treatment of neuropsychiatric disorders.

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Legends for Figures

Figure 1. Simplified synthetic pathway of ALLO and PA. Enzymes are italicized. Acronym: DHP= dihydroprogesterone

Figure 2. Crossover Experimental Design

Figure 3. CONSORT Diagram

Figure 4. Person-Centered Values Illustrating Effects of E2 or P4 on Perimenstrual Trajectories of Neuroactive Steroids

Figure 5. Person-Centered Values Illustrating Effects of E2 or P4 (vs. PBO) on Perimenstrual Exacerbation of Depressed Mood (top) and Suicidal Ideation (bottom)

Figure 6. Person-Centered Values Illustrating Effects of E2 or P4 (vs. PBO) on Perimenstrual Exacerbation of Threat Perception (Overwhelm, top) and Social Cognitive Bias (Rejection Sensitivity, bottom)

Figures

Figure 1. Mechanistic pathway underlying NAS hypotheses.

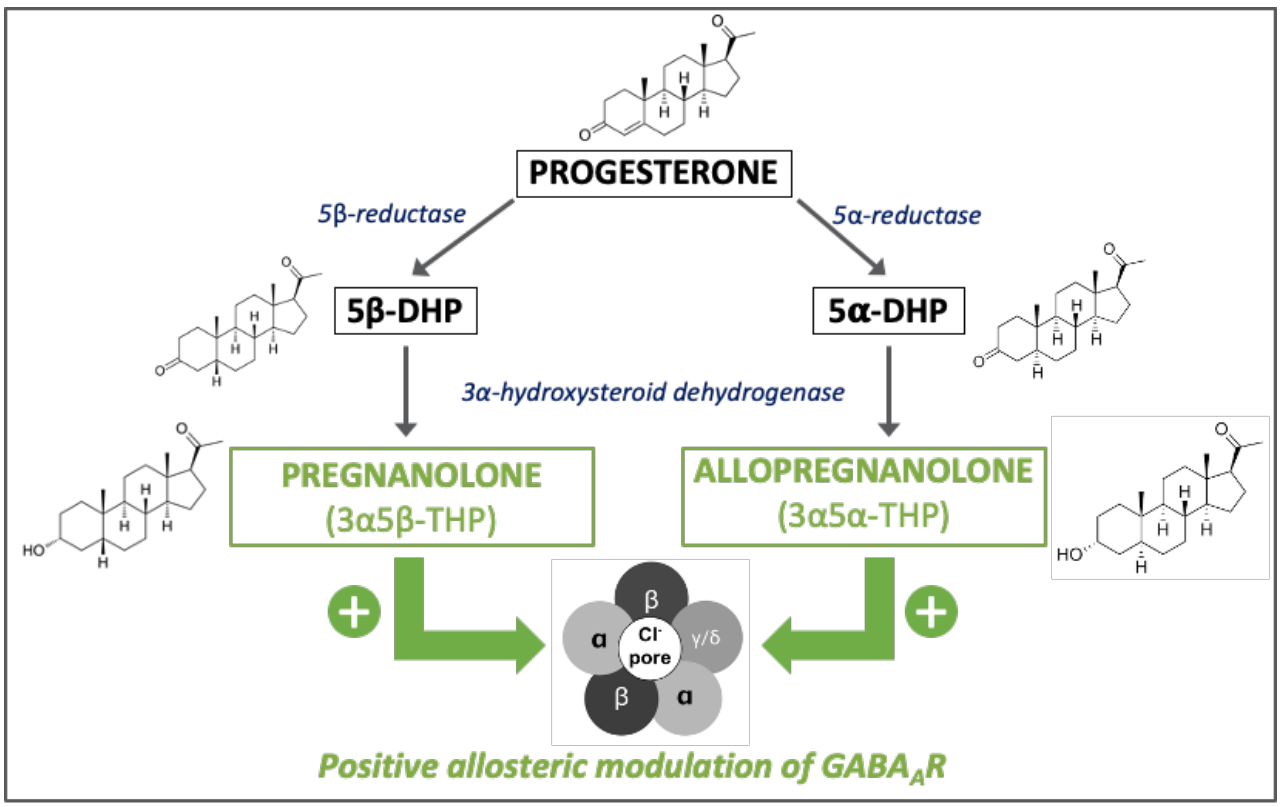


Figure 2. Crossover Experimental Design

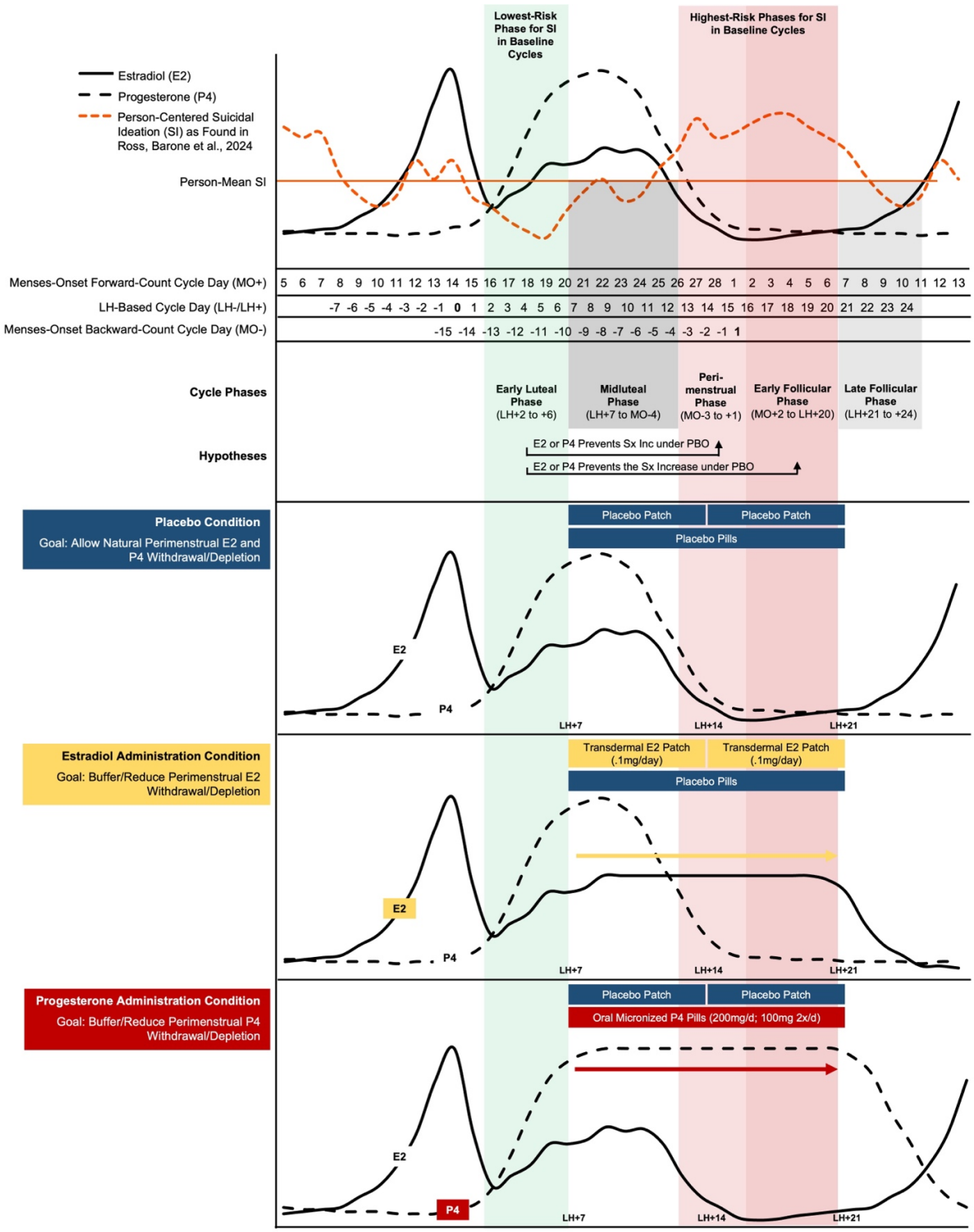


Figure 3. CONSORT Diagram

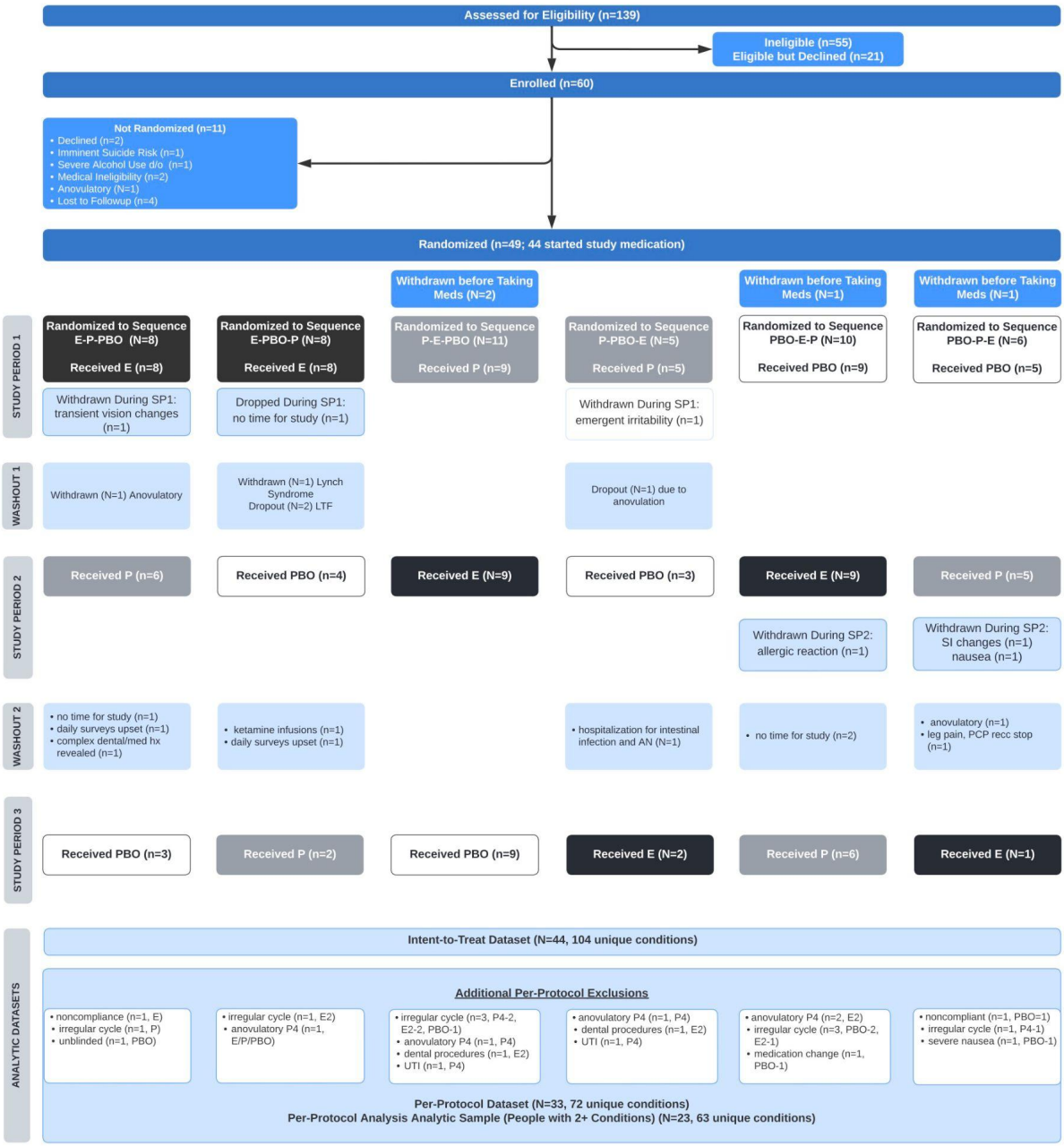


Figure 4. Model-implied steroid values across the experiment.

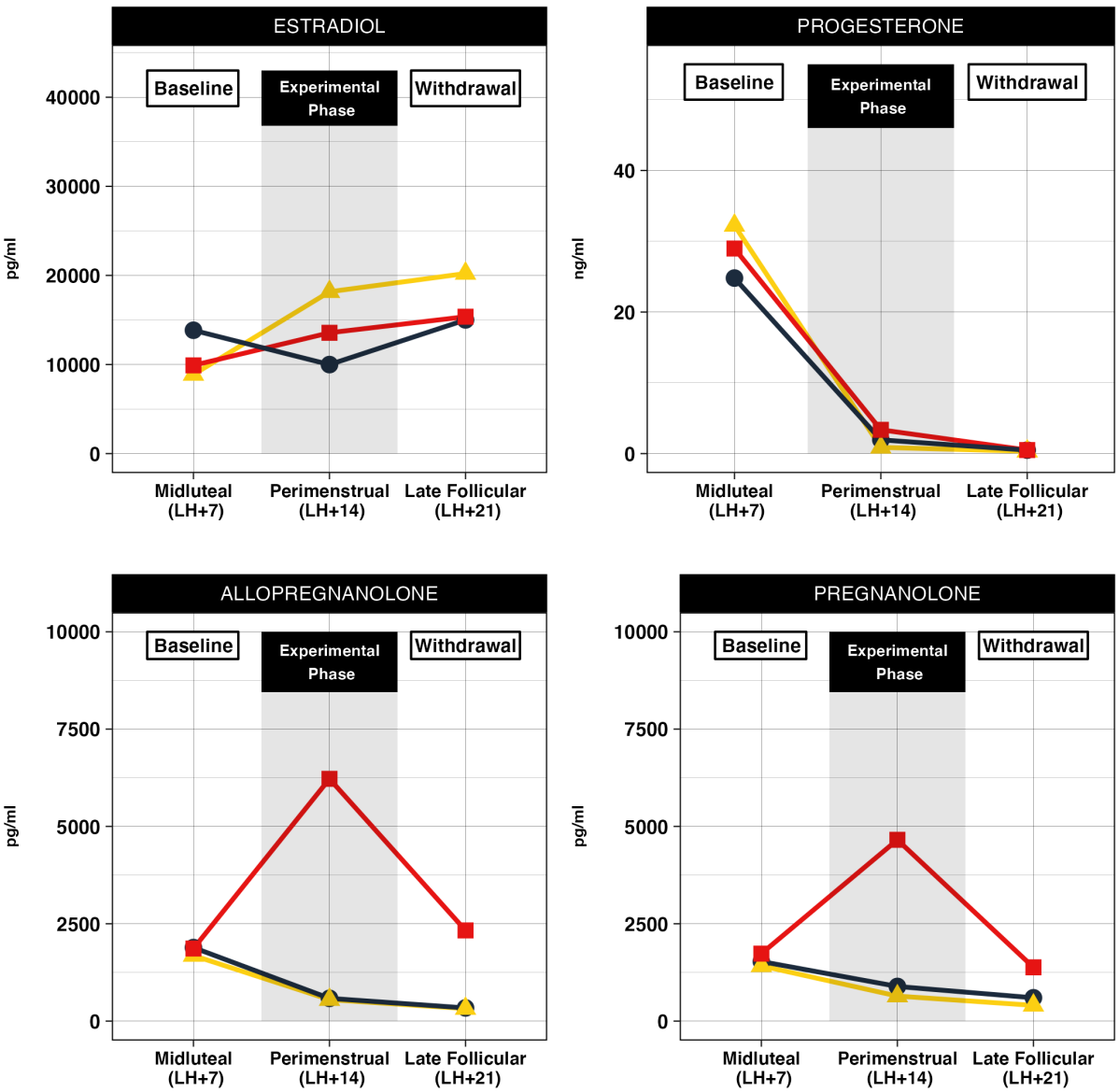


Figure 5. Person-Centered Values Illustrating Effects of E2 or P4 (vs. PBO) on Perimenstrual Exacerbation of Depressed Mood (top) and Suicidal Ideation (bottom)

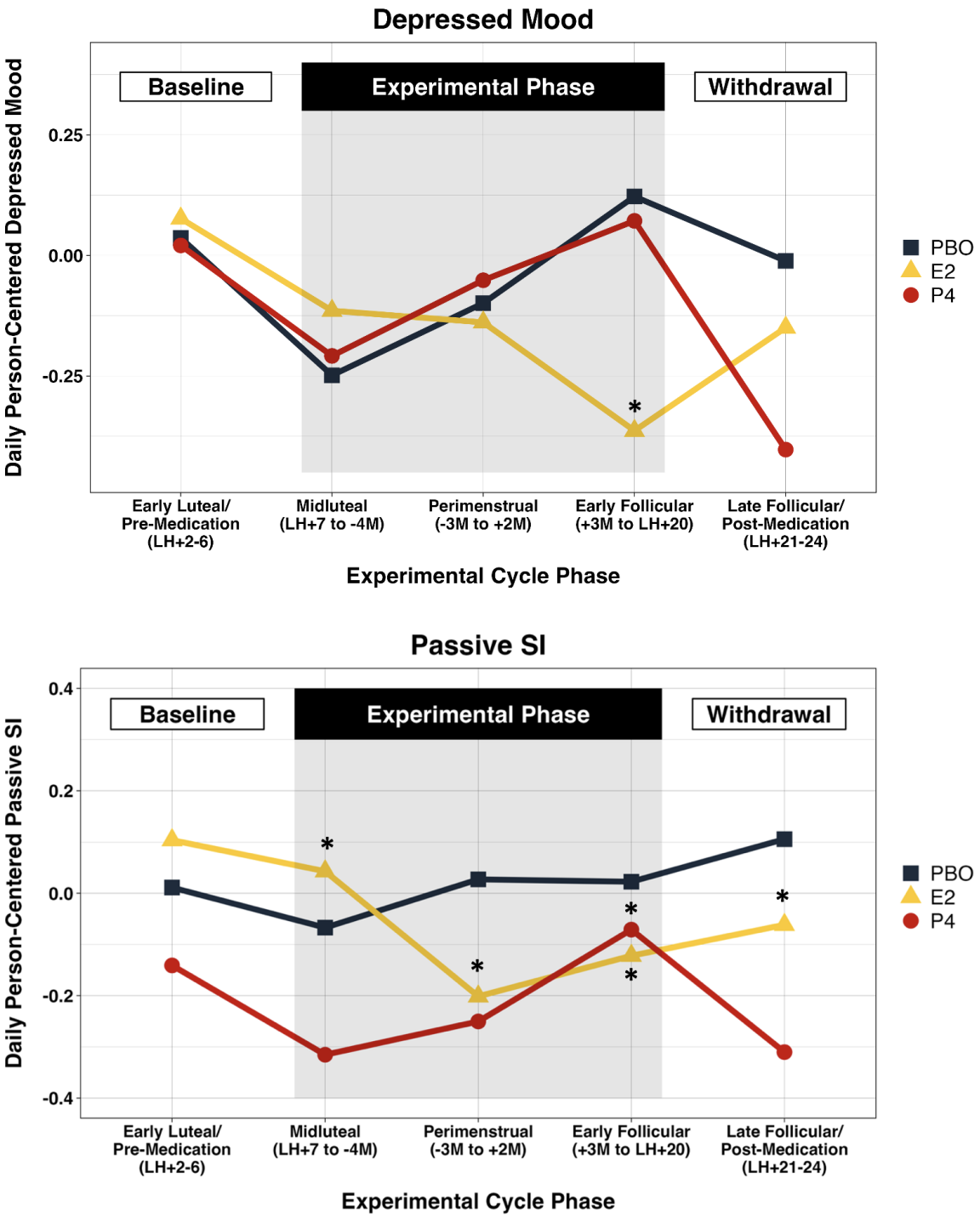
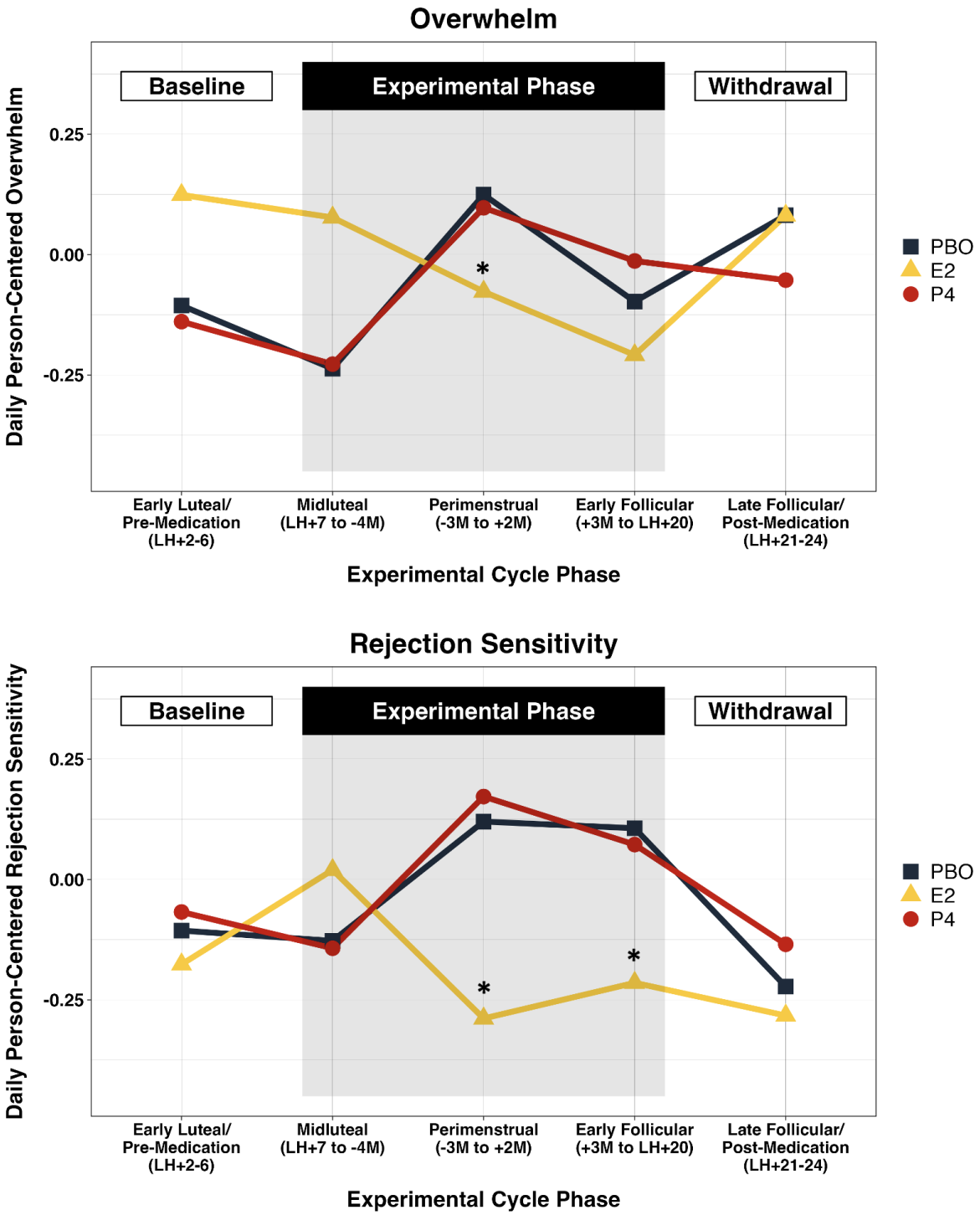


Figure 6. Person-Centered Values Illustrating Effects of E2 or P4 (vs. PBO) on Perimenstrual Exacerbation of Threat Perception (Overwhelm, top) and Social Cognitive Bias (Rejection Sensitivity, bottom)



Tables

For Tables, see attached Excel File.

Table 1. Participant Characteristics in the Per-Protocol Sample and by First Experimental Condition

Table 2. Multilevel models predicting GC/MS steroids (Estradiol, Progesterone, Allopregnanolone, and Pregnanolone)

Table 3. *Multilevel Models Testing Primary Outcomes*

Table 4. *Multilevel Models Testing Secondary Depressed Mood and Cognition and Threat Perception Outcomes*

Table 5. *Multilevel Models Testing Secondary Social Cognitive Bias, Executive Dysfunction, and Frustrative Non-Reward Outcomes*

APÊNDICE B

Co-autoria em artigo de revisão narrativa produzido durante estágio de doutorado-sanduíche no Departamento de Psiquiatria da *University of Illinois at Chicago* sobre o papel da alopregnanolona na depressão pós-parto. Publicado em 2022 no periódico *Frontiers in Global Women's Health* (ISSN: 2673-5059).

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Allopregnanolone in Postpartum Depression

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Postpartum depression (PPD) is a debilitating psychiatric disorder characterized by a high worldwide prevalence and serious long-term negative outcomes for both mothers and children. The lack of a specific treatment and overreliance on pharmacotherapy with limited efficacy and delayed treatment response has constituted a complication in the management of PPD. Recently, the Food and Drug Administration (FDA) in the USA approved a synthetic formulation of the GABAergic neurosteroid allopregnanolone, administered intravenously (brexanolone) for the rapid, long-lasting and effective treatment of PPD. Hereinafter, we review findings on allopregnanolone biosynthesis and $GABA_A$ receptor plasticity in the pathophysiology of PPD. We also discuss evidence supporting the efficacy of brexanolone for the treatment of PPD, which opens a promising new horizon for neurosteroid-based therapeutics for mood disorders.

Keywords: allopregnanolone, post-partum depression, brexanolone, $GABA_A$ receptors, neurosteroid-based therapeutics, rapid-acting antidepressants

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INTRODUCTION

Postpartum depression (PPD) is a subtype of major depressive disorder that affects around 6.5–12.9% of puerperal women every year (1). The precipitating causes can be similar to major depression and include chronic and acute stress exposure, frequently related to the perinatal period (e.g., gestational diabetes, cesarean section, preterm delivery, teenage pregnancy, lack of social support, sleep disorders, and multiparity). Additionally, past traumatic experiences and stress play a role in the late postpartum onset of PPD (2). PPD is characterized by the emergence, during the postpartum period, of at least one of the core depression symptoms (depressed mood and anhedonia) accompanied by at least five other symptoms, including weight loss, sleep disturbances (insomnia or hypersomnia), psychomotor alterations (agitation or retardation), fatigue, feelings of worthlessness or guilt, impaired concentration, and recurring suicidal thoughts or ideation, for a continuous period of at least 2 weeks and that may last for months and even years. Though the symptomatology of PPD is not differentiable from major depressive disorder, aspects such as symptom severity, heritability, and genetic and epigenetic data suggest that PPD is a distinct condition, particularly when occurring in the early postpartum period (3). In addition to the life-threatening risks imposed on the mother and child (e.g., suicide attempts and infanticide, respectively), the negative impact of PPD on the mother-infant relationship can also be severely disruptive to the newborn in the long term, leading to impaired cognitive and

emotional function in adulthood. Frequent risks and outcomes related to major depressive disorder and PPD are represented in **Figure 1**. For many decades, there was no specific pharmacological therapy approved for PPD treatment. The first-line treatment for this condition includes the antidepressants used in the treatment of the major depressive disorder, such as selective serotonin reuptake inhibitors (SSRIs) (4). Though proven to be effective, SSRIs take several weeks to elicit pharmacological effects and the response rate rarely exceeds 50% (5). Considering that the potential grave consequences of PPD may occur abruptly, fast resolution of symptoms is highly desired. Fortunately, as of 2019, the rapid-acting antidepressant brexanolone (marketed as Zulresso^{TM}) has received approval by the United States Food and Drug Administration (FDA) for the treatment of PPD after showing rapid and long-lasting antidepressant effect in the pivotal phase-3 clinical trials (6). Brexanolone is a proprietary pharmaceutical preparation for intravenous (IV) administration of the neurosteroid allopregnanolone. Given allopregnanolone's pleiotropic effects arising from preclinical and clinical studies in the treatment of a large array of neuropsychiatric disorders (7), its approval for the treatment of PPD has generated elevated interest in drug research and development of a new class of therapeutics (8).

This article will focus on the promising new horizon opened by neurosteroid-based treatment for depressive disorders by discussing the role of neurosteroids interfacing with $GABA_A$ receptor function in the pathophysiology and treatment of PPD.

NEUROSTEROIDS AND $GABA_A$ RECEPTOR PLASTICITY IN PPD

Stress plays a major role in both the presentation and severity of PPD and the inability to shut down the stress-induced hypothalamus–pituitary–adrenal (HPA) axis activation has been traditionally suggested as an underlying neurobiological mark of PPD. The GABAergic signaling modulated by neurosteroids, including allopregnanolone and its equipotent GABAergic isomer, pregnanolone, may play a role in some of this disorder manifestation (6, 9, 10). Allopregnanolone also plays a pivotal physiological role by protecting the maternal and fetal brain from harmful levels of maternal glucocorticoids resulting from stress exposure during pregnancy and prevents premature secretion of oxytocin associated with preterm birth. Allopregnanolone is also neuroprotective and promotes the development of the fetal brain (11).

The biosynthesis of allopregnanolone and pregnanolone has been associated with the emergence of depressive disorders and post-traumatic stress disorder (PTSD) (12–14). Allopregnanolone and pregnanolone show remarkable anxiolytic and antidepressant effects, both in humans and in preclinical models (15–17). In 1998, Uzunova et al. (13) and Romeo et al. (12) and respective collaborators simultaneously published that the cerebrospinal fluid (CSF) and serum levels of allopregnanolone were decreased in depressed patients and could be upregulated by SSRI antidepressant treatment. Specifically, allopregnanolone concentration in the CSF correlated with the severity of depressive symptoms, and treatment with fluoxetine and fluvoxamine increased its content only in patients who responded to the treatment with remission of depressive symptoms (12, 13). These studies were followed by findings that allopregnanolone levels decreased similarly in women and men with PTSD, with the lowest levels found in subjects exhibiting PTSD with comorbid depression (14, 18, 19). Ratios of allopregnanolone with precursors and enzymatic expression analysis have suggested a sex-dependent dimorphism in the enzymes that regulate neurosteroid biosynthesis (20). In the post-mortem brain of depressed subjects, allopregnanolone appeared to be decreased by a 5α-reductase type I (5α-RI) expression deficit in the prefrontal cortex Brodmann's area 9 (BA9) (21). Ratio analysis of progesterone to 5α-dihydroprogesterone, and from this to allopregnanolone in PTSD subjects, indicated that while in men, allopregnanolone decreased from a 5α-RI deficit, in women, the enzyme 3α-hydroxysteroid dehydrogenase appeared to be affected (22). Preclinical studies in rodent models of PTSD and depression have confirmed these clinical findings showing altered neurobiology in tests reproducing affective symptoms in humans (23, 24). These findings have implicated allopregnanolone in the pathophysiology of PTSD and depressive disorders, which led to the proposal of its potential biomarker role for subtypes of mood disorders [reviewed in (20, 24, 25)].

There has been limited investigation on neurobiological mechanisms underlying behavioral predictors of PPD during pregnancy and the postpartum period. Although a disorder characterized by a distinct phenotype from major depressive disorder, PPD is also characterized by changes in $GABA_A$ receptor neurotransmission, including altered expression of the receptor subunits and impaired neurosteroid biosynthesis (26–28). However, transient postpartum hypothalamic corticotropin-releasing hormone (CRH) suppression (26), in conjunction with the steroid withdrawal in the aftermath of parturition, is often regarded with the affective instability observed during the postpartum period (6). Even though there is a coincidental timing of abrupt neuroactive steroid decline—including progesterone and allopregnanolone—after parturition and the onset of PPD symptoms, investigations that have attempted to demonstrate lower allopregnanolone levels in the postpartum period in subjects with PPD have often produced mixes results.

Luisi et al. (29) investigated allopregnanolone and progesterone concentrations in maternal and cord serum by radioimmunoassay (RIA). Their concentrations steadily increased throughout the gestation period. At delivery, their serum levels were significantly lower in women who underwent emergency cesarean section. Similarly, umbilical cord serum allopregnanolone and progesterone levels were decreased in an emergency cesarean than in vaginal delivery. Intriguingly, in subjects with chronic hypertension, serum allopregnanolone concentration was significantly increased when compared with the levels in healthy women. In another study conducted in healthy volunteers with low and high psychological scores assessed by the SCL-90 psychometric scale, neuroactive steroids were also measured by RIA during the follicular phase (FP), the luteal phase (LP), and at four time points during pregnancy.

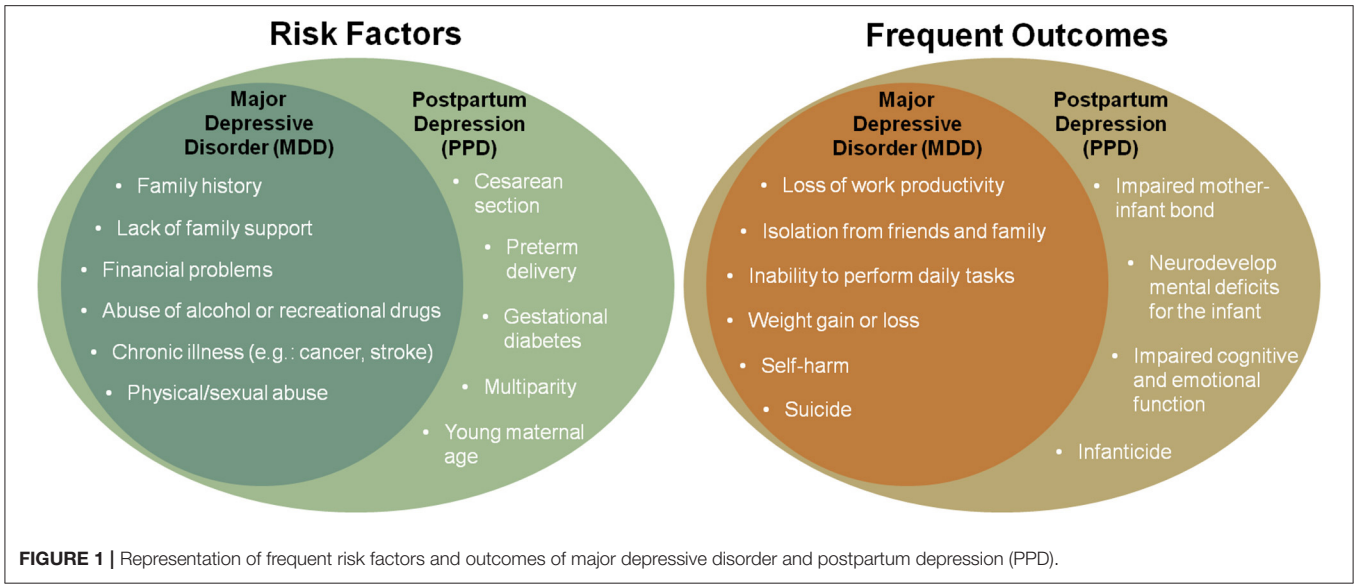

FIGURE 1 | Representation of frequent risk factors and outcomes of major depressive disorder and postpartum depression (PPD).

Progesterone and allopregnanolone levels were higher in LP than in FP and they consistently increased with the progression of pregnancy, however, without differences between low and high psychological score groups (30). Peripartum plasma levels of neuroactive steroids and GABA were quantified by liquid chromatography-mass spectrometry (LC-MS) in healthy subjects and subjects at-risk for PPD established by a prior history of depression or who showed mild depressive or anxiety symptoms. Peripartum GABA levels were lower and progesterone and pregnanolone levels were higher in at-risk PPD vs. healthy subjects. Trait-anxiety scores were positively associated with pregnanolone and allopregnanolone (31). In another study, the authors hypothesized that peripartum neuroactive steroids are related to resting-state functional connectivity in PPD compared to healthy subjects. Plasma allopregnanolone was elevated in subjects with PPD and positively correlated with dorsomedial prefrontal cortex (DMPFC) connectivity in women at risk for PPD (32). In a prospective, nested, case-control study in low-income women of color in early pregnancy, Wenzel et al. (33) examined the concentrations of progesterone, as well as allopregnanolone and pregnanolone and the levels of the allopregnanolone isomers, isoallopregnanolone and epipregnanolone, which act as negative allosteric modulators of the $GABA_A$ receptor. Pregnant women manifested with depression at either or both first and second trimesters. Prenatal depression cases showed higher ratios of both allopregnanolone and pregnanolone to progesterone compared to controls. Subjects with depression at both first and second trimesters showed an increase in epipregnanolone to progesterone ratios from the first to the second trimester, while control subjects showed a decrease in these ratios. Isoallopregnanolone was found to increase in the second trimester alone. Although associated with an increase of allopregnanolone levels, the increase of allopregnanolone isomers with antagonistic function at $GABA_A$ receptors is intriguing and deserves further investigation.

In contrast to these investigations, other studies successfully observed lower allopregnanolone levels in association with anxiety and depression symptoms. Mood and anxiety and allopregnanolone were examined across the peripartum by ELISA at the second and third trimesters and week 6 postpartum in women with a history of mood and/or anxiety disorders and healthy controls. Lower allopregnanolone levels at the postpartum period were associated with higher depression and anxiety scores. This exploratory finding suggests that the relationship between allopregnanolone and mood and anxiety symptoms may change across the peripartum (9). Serum allopregnanolone levels were found significantly lower in women manifesting postpartum "blues" when compared to euthymic women, while progesterone levels did not differ significantly. A significant negative correlation was observed between the Hamilton score and levels of serum allopregnanolone and progesterone (34).

A study that quantified serum allopregnanolone by Celite chromatography and RIA found that women who had elevated depression scores also had significantly lower allopregnanolone levels compared to healthy subjects. Furthermore, a significant negative correlation was observed between self-rated depression scores and allopregnanolone serum concentrations. Self-rated anxiety was not associated with allopregnanolone serum concentrations during pregnancy. This study supports that high allopregnanolone concentrations may underlie depressed mood during pregnancy (35). In another study that examined second and third trimester progesterone and allopregnanolone levels by ELISA, while PPD was diagnosed by clinician interview in pregnant women who had prior diagnosis of mood disorders, it was observed that every additional ng/ml of second trimester allopregnanolone resulted in a 63% reduction in the risk of developing PPD (9).

Association among stress-related neurobiological factors (GABAergic neurosteroids) and indices of anxiety during

TABLE 1 | Summary of studies investigating allopregnanolone levels during pregnancy and its relationship with postpartum depression (PPD).

References	Allopregnanolone levels during pregnancy (except where otherwise stated)	Matrix	Method of measurement
Luisi et al. (29)	Increased progressively; at delivery, levels were significantly lower in women who underwent emergency cesarean section		RIA
Paoletti et al. (30)	Increased progressively in both women with high and low psychological score assessed by the SCL-90 psychometric scale	Serum	RIA
Deligiannidis et al. (31)	Higher in women at risk for PPD	Plasma	LC-MS
Deligiannidis et al. (32)	Higher in women with PPD	Plasma	LC-MS
Wenzel et al. (33)	Higher ratio of allopregnanolone to progesterone in prenatal depressed women	Serum	GC-MS
Osborne et al. (9)	Lower allopregnanolone in the 2nd trimester correlated with higher risk of developing PPD	Plasma	ELISA
Nappi et al. (34)	Levels were lower after delivery in women manifesting postpartum "blues"	Serum	RIA
Hellgren et al. (35)	Lower in women with elevated depression scores	Serum	RIA
Crowley et al. (36)	Lower levels were associated with greater negative emotional responses to stress	Serum	GC-MS

RIA, radioimmunoassay; LC-MS, liquid chromatography-mass spectrometry; GC-MS, gas chromatography-mass spectrometry; ELISA, enzyme-linked immunoassay.

pregnancy showed that lower progesterone and combined measures of allopregnanolone + pregnanolone were associated with greater negative emotional responses to stress, and lower cortisol was associated with worse sleep quality. These data suggest that progesterone and allopregnanolone + pregnanolone levels in the second trimester of pregnancy are inversely related to negative emotional symptoms, and acute stress challenges appear to reduce these steroids to promote negative emotional responses (36).

Finally, a study demonstrated altered sensitivity to neuroactive steroids specifically in patients presenting a history of PPD (37). This investigation supports that while neuroactive steroid levels may not be abnormal, sensitivity to neuroactive steroids may provide a better explanation for the increased susceptibility to develop risk to PPD. Virtually no studies have investigated this topic in clinical studies, however, several basic research investigations backed this assumption [reviewed in (26)].

These studies (summarized in **Table 1**) suggest that while exogenous administration of allopregnanolone is an effective treatment to relieve PPD symptoms, the comprehension of the mechanisms linking neuroactive steroid levels with the onset of symptoms remain elusive. Most of these studies have used an array of different technologies to quantify neuroactive steroids. Hence, procedural methodologies in computing neuroactive steroid analyses may also play a role in explaining the discrepancy in these results. It is also important to note that deficits in allopregnanolone biosynthesis play an important role in major depressive disorder pathophysiology, in addition to their role in the manifestation of depression symptoms during pregnancy [reviewed in (24)].

Changes in $GABA_A$ receptor subunit expression have been demonstrated during protracted stress conditions and in the pathophysiology of PPD in both preclinical and clinical studies. In peripheral blood mononuclear cells, the expression of δ and ρ2 subunits was upregulated during pregnancy in a clinical study (27). Maguire and Mody (28) observed a decrease of both δ and γ2 subunits in a mouse model of PPD, which resulted in decreased tonic and phasic inhibition in pregnant mice. Specifically, the δ subunit expression changes were associated with depressive-like phenotypes and abnormal maternal behaviors. In a rat pregnancy model, the cerebral cortex and hippocampus expression of the $GABA_A$ receptor γ2 subunit decreased during pregnancy, before returning to baseline levels 2 days after delivery. These data were further validated in a model of 5α-reductase (the rate-limiting step-enzyme in allopregnanolone biosynthesis) blockade in pregnant rats, which reduced both plasma and brain allopregnanolone content and prevented the decrease of γ2 mRNA expression observed during pregnancy. Furthermore, these subunit changes resulted in structural and functional changes in the $GABA_A$ receptor demonstrated by decreased stimulatory effect of the GABAmimetic drug muscimol on Cl^- uptake by cerebrocortical membranes. These observations support a role for allopregnanolone in regulating the plasticity of $GABA_A$ receptor-containing γ2 subunit during pregnancy and after delivery (38). Of note, changes in $GABA_A$ receptor subunit expression are also affected by protracted stress in rodent models of anxiety and depression. Decreased γ2 and increased α4 $GABA_A$ receptor subunit expression were observed in the hippocampus and frontal cortex, which correlated with altered pharmacological response to sedative and anxiolytic effects of benzodiazepines (39, 40). Another study in stressed rats also showed upregulation of hippocampal α4 and δ subunits (41). A switch among extrasynaptic δ subunit and synaptic γ2 subunits was observed during pregnancy and across the estrous cycle (38, 42).

Collectively, these studies suggest that impaired dynamic reconfiguration of $GABA_A$ receptor subunit subtypes, their sensitivity to neuroactive steroids, and neuroactive steroid biosynthesis during the perinatal period deserve further investigation.

BREXANOLONE EFFECTS IN THE TREATMENT OF POSTPARTUM DEPRESSION

The finding that allopregnanolone is decreased in subjects with depression and that SSRIs restore allopregnanolone to

normal concentrations in treatment responders has stimulated studies to understand the underlying mechanisms of the neurosteroidogenic effects of these compounds in relieving symptoms of depression and led to exploit neurosteroidogenic targets as novel paths to treat mood disorders (43). In rodent stress models of neurosteroid biosynthesis downregulation and behavioral traits of mood disorders (anxiety-like, aggressive, depression-like behavior, deficits in fear extinction), Pinna et al. investigated the steroidogenic action of SSRIs (44). They found that SSRIs upregulate allopregnanolone by a mechanism independent of serotonin reuptake inhibition, suggesting SSRIs act specifically as selective brain steroidogenic stimulants (SBSSs) at effective doses that are one level of magnitude lower than the active SSRI doses. Intriguingly, behavioral improvement occurred very rapidly (hours) and was long-lasting (45). Clinical trials testing the hypothesis that IV allopregnanolone supplementation could offer a strategy to improve PPD showed improvement in symptoms within hours of active versus placebo infusion (46–48). Four clinical trials assessed the antidepressant efficacy of brexanolone infused over 60 h in women recruited between 6 weeks and 6 months postpartum. In a proof-of-concept study, open-label clinical trial, four women who developed severe PPD received 60 mcg brexanolone infusions. Safety, efficacy, and tolerability assessed by this study showed that all patients were able to complete the infusions, and after 60 h, the depression symptoms dramatically decreased. Sedation accounted for the most severe adverse effects accompanied by pain, rash, dizziness, and flushing. Elevated cost also contributes to an important limitation of brexanolone IV treatment, and an oral formulation of allopregnanolone could abate drug cost and avoid hospitalization required during the IV administration. In phase 3, a double-blind, randomized, outpatient, placebo-controlled clinical trial, an oral formulation of allopregnanolone (e.g., zuranolone) was investigated in women with PPD (49). Zuranolone administered at the dose of 30 mg for 2 weeks significantly improved the Hamilton depression rating scale (HAMD-17) scores 3 days after administration, and this effect was maintained for a 2-week treatment and 45-day follow-up vs. placebo. While a clinical study to investigate the efficacy of zuranolone for major depression showed mixed results, this randomized clinical trial showed improvement of depression with minimal side effects, thereby supporting the development of zuranolone in the treatment of PPD and major depression. Importantly, even though a direct comparison between the effectiveness of the treatment with allopregnanolone vs. that of SSRIs has not been evaluated, the clinical efficacy of brexanolone appears superior to that of widely prescribed traditional antidepressants (22).

While several mechanisms remain to be further investigated, collectively, these studies provide strong support for allopregnanolone biosynthesis and $GABA_A$ receptor sensitivity disturbances in underlying PPD pathophysiology and support the development of neurosteroid-based treatment for rapid improvement of mood disorders (50).

AUTHOR CONTRIBUTIONS

GP conceptualized and wrote the manuscript. GP, FA, and JD revised the manuscript draft. All authors contributed to the article and approved the submitted version.

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Conflict of Interest: GP is a paid consultant to PureTech Health (Boston, MA, USA), GABA Therapeutics, and NeuroTrauma Sciences (Alpharetta, GA, USA). He has two patent applications, one on N-palmitoylethanolamine (PEA) and peroxisome proliferator-activated receptor alpha (PPAR-α) agonists US20180369171A1, pending, and one on allopregnanolone analogs US11266663B2 granted on March 8, 2022 in the treatment of neuropsychiatric disorders.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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ANEXO A

Carta de aprovação do projeto de pesquisa e do adendo de extensão na Comissão em Ética no Uso de Animais em Pesquisa (CEUA) da UFCSPA.

REPÚBLICA FEDERATIVA DO BRASIL
MINISTÉRIO DA EDUCAÇÃO
UNIVERSIDADE FEDERAL DE CIÊNCIAS DA SAÚDE DE PORTO ALEGRE

Pró-Reitoria de Pesquisa e Pós-Graduação

Comissão de Ética no Uso de Animais - CEUA

CARTA DE APROVAÇÃO

A Comissão de Ética no Uso de Animais analisou o projeto:

Número: **213/2022**
Título: **O PAPEL DOS NEUROESTEROIDES NOS EFEITOS COMPORTAMENTAIS CAUSADOS POR FÁRMACOS, CONDIÇÃO AMBIENTAL E LINHAGENS TIPO-DEPRESSIVAS EM RATOS**
Pesquisador(a) Responsável: **Helena Maria Tannhauser Barros**
Vigência: **04/03/2019** a **28/02/2023**

Pesquisadores:

Equipe UFCSPA:

- Helena Maria Tannhauser Barros
- Felipe Borges Almeida

Equipe Externa:

- Maurício Schüler Nin - FURG

A Comissão de Ética no Uso de Animais aprovou o mesmo em reunião.

Parecer: *Projeto de Pesquisa aprovado no novo sistema.*

Porto Alegre, 08 de fevereiro de 2023

FERNANDA BASTOS DE MELLO
Coordenadora Da Comissão De Ética No Uso De Animais

REPÚBLICA FEDERATIVA DO BRASIL
MINISTÉRIO DA EDUCAÇÃO
UNIVERSIDADE FEDERAL DE CIÊNCIAS DA SAÚDE DE PORTO ALEGRE

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MINISTÉRIO DA EDUCAÇÃO
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Pró-Reitoria de Pesquisa e Pós-Graduação

Comissão de Ética no Uso de Animais - CEUA

CARTA DE APROVAÇÃO DE ADENDO

A Comissão de Ética no Uso de Animais analisou o adendo ao projeto:

Número: **213/2022**
Título: **O PAPEL DOS NEUROESTEROIDES NOS EFEITOS COMPORTAMENTAIS CAUSADOS POR FÁRMACOS, CONDIÇÃO AMBIENTAL E LINHAGENS TIPO-DEPRESSIVAS EM RATOS**
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- Felipe Borges Almeida

Equipe Externa:

- Maurício Schüler Nin - FURG

A Comissão de Ética no Uso de Animais aprovou o mesmo em reunião.

Parecer: *Adendo aprovado, alteração do término para 02/2024.*

Porto Alegre, 13 de junho de 2023

FERNANDA BASTOS DE MELLO
Coordenadora Da Comissão De Ética No Uso De Animais

REPÚBLICA FEDERATIVA DO BRASIL
MINISTÉRIO DA EDUCAÇÃO
UNIVERSIDADE FEDERAL DE CIÊNCIAS DA SAÚDE DE PORTO ALEGRE

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