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The pharmacotherapy of depression - searching for new mechanisms and drug interactions. Basic and clinical research.

Edited by
Katarzyna Stachowicz, Anna Tabęcka-Łonczynska and Magdalena Sowa-Kucma

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The pharmacotherapy of depression - searching for new mechanisms and drug interactions. Basic and clinical research.

Topic editors

Katarzyna Stachowicz — Maj Institute of Pharmacology, Polish Academy of Sciences, Poland

Anna Tabęcka-Łonczynska — University of Information Technology and Management in Rzeszow, Poland

Magdalena Sowa-Kucma — University of Rzeszow, Poland

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Nicholas M Barnes,
University of Birmingham,
United Kingdom

*CORRESPONDENCE
Katarzyna Stachowicz,
stachow@if-pan.krakow.pl

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Editorial: The pharmacotherapy of depression—searching for new mechanisms and drug interactions basic and clinical research

Katarzyna Stachowicz[1]*, Magdalena Sowa-Kućma[2] and Anna Tabecka-Łonczyńska[3]

[1]Department of Neurobiology, Maj Institute of Pharmacology, Polish Academy of Sciences, Kraków, Poland, [2]Department of Human Physiology, Institute of Medical Sciences, Medical College of Rzeszow University, Rzeszow, Poland, [3]Department of Biotechnology and Cell Biology, Medical College, University of Information Technology and Management in Rzeszow, Rzeszow, Poland

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Editorial on the Research Topic
The pharmacotherapy of depression—searching for new mechanisms and drug interactions basic and clinical research

Recognizing the importance of depression in this particular volume of Frontiers in Pharmacology, we invited the authors to highlight new concepts in the pharmacotherapy of depression. According to the WHO, the COVID-19 pandemic has caused a massive increase in the global prevalence of anxiety and depression. Pharmacotherapy of depression is mainly based on substances first discovered and synthesized in the 1960s and 1970s; hence there is a great need to define new molecular pharmacological targets and interactions between administered drugs to increase treatment efficacy and reduce side effects characteristic of currently available antidepressants. This problem was described by Stachowicz and Sowa-Kućma. In addition, a structured state of knowledge of what is happening at the preclinical and clinical levels is presented in a two-part review by (Vasiliu). The first part of this review focuses on monoaminergic, orexinergic, GABA-ergic, and anti-inflammatory agents with antidepressant activity. Many of the antidepressants described are currently being marketed. In addition, new drugs are being developed, and psychoactive substances and drugs previously introduced to the pharmaceutical market are also being used. Orexin receptor modulators and unused medications from the group modulating the properties of GABA-A receptors/neurosteroid analogs are showing promising results. Anti-cytokine therapies and COX-2 inhibitors also demonstrate antidepressant properties, and the benefits of biological treatments should not be overlooked (Vasiliu). The second part of Vasiliu's review deals with clinical trials of antidepressants, among which are non-

monoaminergic substances—esketamine for treatment-resistant major depression and brexandone for postpartum depression. The best-known group are glutamatergic agents: antagonists of NMDA receptors or their GluN2B subunits, AMPA receptor enhancers, and metabotropic receptor ligands. Combinations of pharmacological agents are also being studied. On this basis, it can be concluded that the development of new therapies offers high hopes for the effective treatment of depression shortly (Vasiliu). Another review paper also provides no less exciting data by Zhao et al., which details the current state of research on the molecular, cellular, and neuronal mechanisms of dopamine (DA) receptors involved in depression, including the types of receptors and their differential distribution in the brain. Importantly, research progress on the role of D1-D2 heterodimers in depression is also presented (Zhao et al.).

High hopes for finding new, better, and more effective antidepressant therapies are offered primarily by the original studies presented in this issue, and they often explore completely new areas. For example, Meng et al. demonstrated the antidepressant properties of LPM570065 (a potent triple 5-HT/NE/DA reuptake inhibitor) in mice that experienced "two-hit" stress and described the mechanisms involved in this effect. In addition to an increase in the density of dendritic spines in hippocampal CA1 neurons after LPM570065 administration, interesting epigenetic mechanisms were described. Two-hit stress-induced changes in the mouse hippocampus, such as hypermethylation and downregulation of the OXTR (oxytocin receptor) gene along with increased levels of DNA methyltransferases proteins (Dnmt1 and Dnmt3a), were reversed by LPM570065 (Meng et al.). In addition, Lebeau et al. detected electroconvulsive seizure-responsive proteins in a rodent model unresponsive to chronic fluoxetine. These included cell adhesion, cytoskeletal, coagulation, and those involved in regulating immune responses. Similarly, studies in a rat model of chronic unpredictable mild stress (CUMS) using honokiol as the biologically active substance extracted from *Magnolia Officinalis* demonstrated its antidepressant effects (Fan et al.). These effects were attributed to VEGFR2-mediated activation of HIF-1α-VEGF and PI13K/AKT/mTOR signaling and increased expression of proteins associated with synaptic plasticity: SYN1 and PSD95. These results were also confirmed by *in vitro* studies in which honokiol increases synaptic plasticity in PC12 cells through activation of the HIF-1α-VEGF signaling pathway (Fan et al.).

Amylin receptors (AMYRs) are a novel target under investigation for depression (Jiang et al.). AMYRs are dimers of calcitonin receptors (CTRs) with receptor activity modifying proteins (RAMPs). Their potential in depression has been verified using agonists (salmon calcitonin) and antagonists (AC187) of AMYRs in mice.

Herbs and folk medicine preparations (in this case, Chinese medicine) interest researchers. In this case, Yang et al. described the antidepressant activity of an ancient Chinese formula called Xiaoyaosan. Its antidepressant activity, among many, involves the GLUT4 pathway and autophagy mechanisms.

Monitoring side effects and drug-drug interactions is essential in the search for new antidepressants. Such a significant problem of antidepressant-drug interactions with over-the-counter (OTC) drugs is described by Woroń et al. More than four percent of antidepressant side effects were associated with OTC, particularly omeprazole, diphenhydramine, ginkgo Biloba, ibuprofen, and diclofenac. In addition, Miziak et al. discussed the co-occurrence of epilepsy and depression and how to successively treat both diseases, excluding side effects and drug interactions.

As can be seen, the problem under discussion was treated very comprehensively, and thanks to the efforts of the authors, reviewers, and editors, it was possible to offer a passionate and novel compendium on depression.

Author contributions

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Conflict of interest

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Salmon Calcitonin Exerts an Antidepressant Effect by Activating Amylin Receptors

Jian Jiang[1†], Jun Ju[1†], Liang Luo[2†], Ze Song[3], Huanquan Liao[4], Xiuyan Yang[1], Shoupeng Wei[1], Dilong Wang[1], Wenhui Zhu[1], Jinlong Chang[1], Junzhe Ma[1,5], Hao Hu[6], Jiezhong Yu[7], Huiqing Wang[8], Sheng-Tao Hou[9], Shupeng Li[10], Huiliang Li[6]* and Ningning Li[1,5]**

[1]Tomas Lindahl Nobel Laureate Laboratory, The Seventh Affiliated Hospital, Sun Yat-Sen University, Shenzhen, China, [2]Department of Critical Care Medicine, The Seventh Affiliated Hospital, Sun Yat-sen University, Shenzhen, China, [3]Oncology Department, The Seventh Affiliated Hospital, Sun Yat-sen University, Shenzhen, China, [4]The Clinical Neuroscience Center, The Seventh Affiliated Hospital, Sun Yat-sen University, Shenzhen, China, [5]China-UK Institute for Frontier Science, Shenzhen, China, [6]Wolfson Institute for Biomedical Research, Division of Medicine, Faculty of Medical Sciences, University College London, London, United Kingdom, [7]The Fourth People's Hospital of Datong City, Datong, China, [8]The Fifth People's Hospital of Datong City, Datong, China, [9]Brain Research Centre and Department of Biology, School of Life Sciences, Southern University of Science and Technology, Shenzhen, China, [10]State Key Laboratory of Oncogenomics, School of Chemical Biology and Biotechnology, Peking University Shenzhen Graduate School, Shenzhen, China

Depressive disorder is defined as a psychiatric disease characterized by the core symptoms of anhedonia and learned helplessness. Currently, the treatment of depression still calls for medications with high effectiveness, rapid action, and few side effects, although many drugs, including fluoxetine and ketamine, have been approved for clinical usage by the Food and Drug Administration (FDA). In this study, we focused on calcitonin as an amylin receptor polypeptide, of which the antidepressant effect has not been reported, even if calcitonin gene-related peptides have been previously demonstrated to improve depressive-like behaviors in rodents. Here, the antidepressant potential of salmon calcitonin (sCT) was first evaluated in a chronic restraint stress (CRS) mouse model of depression. We observed that the immobility duration in CRS mice was significantly increased during the tail suspension test and forced swimming test. Furthermore, a single administration of sCT was found to successfully rescue depressive-like behaviors in CRS mice. Lastly, AC187 as a potent amylin receptor antagonist was applied to investigate the roles of amylin receptors in depression. We found that AC187 significantly eliminated the antidepressant effects of sCT. Taken together, our data revealed that sCT could ameliorate a depressive-like phenotype probably *via* the amylin signaling pathway. sCT should be considered as a potential therapeutic candidate for depressive disorder in the future.

Keywords: behavior test, depression, salmon calcitonin, chronic restraint stress, amylin receptor, AC187

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Katarzyna Stachowicz, Maj Institute of Pharmacology (PAN), Poland

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Agnieszka Nikiforuk, Maj Institute of Pharmacology (PAN), Poland
Narumi Hashikawa-Hobara, Okayama University of Science, Japan

****Correspondence:***
Shupeng Li
lisp@pku.edu.cn
Huiliang Li
huiliang.li@ucl.ac.uk
Ningning Li
linn29@mail.sysu.edu.cn

[†]These authors have contributed equally to this work

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INTRODUCTION

Major depressive disorder is a life-threatening chronic mental disease affecting more than 20% of the population worldwide (Yan et al., 2010). Depression is a psychiatric disorder characterized by low mood and lack of interest in work, life, and social activities, which often leads to suicidal attempts and suicides (Marinova et al., 2014). Unfortunately, the range of available medications for depression treatment in the clinic is relatively limited and traditionally restricted to fluoxetine and ketamine. However, fluoxetine works usually after a long treatment period of 2–4 weeks and is ineffective in patients with severe depression, *via* selectively inhibiting 5-hydroxytryptamine (5-HT) reuptake to enhance the extracellular 5-HT level (Hirschfeld, 2000; Al-Harbi, 2012). Ketamine exerts an antidepressant effect *via* different mechanisms, including but not limited to blocking *N*-methyl-D-aspartate (NMDA) receptor, activating α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor, and regulating synaptic plasticity (Newport et al., 2015; Zanos et al., 2018). Meanwhile, ketamine has certain severe side effects, e.g., hallucinogenic, addictive, and neurotoxic responses, preventing its widespread application in the clinic (Short et al., 2018). Therefore, there is an increasing demand to develop new medications with high antidepressant capacity and safety.

Recently, the potential of peptides, e.g., neuropeptide Y (NPY), vasopressin, and calcitonin-related peptides, to treat neuropsychiatric disorders has been reported. The latest study has shown that the mRNA level of NPY in the prefrontal cortex (PFC) and hippocampus regions was significantly decreased in the postmortem brain of depressed and suicide patients (Sharma et al., 2022). Moreover, another study reported that NPY exerts an antidepressant effect when administered intranasally in rats (Nahvi et al., 2021). Besides, vasopressin can be involved in the regulation of the hypothalamic–pituitary–adrenal (HPA) axis to play an antidepressant effect (Rana et al., 2021). Calcitonin gene-related peptide (CGRP) has proven efficacy for the preventive treatment of migraine that is associated with depression and has improved depressive-like behavior in mice with 15-day chronic restraint stress (CRS) by intracerebroventricular administration (Hashikawa-Hobara et al., 2015; Spierings et al., 2021) Among them, calcitonin and calcitonin-related peptides as small molecular peptides have been widely used in the treatment of neuropsychological diseases. Calcitonins, including calcitonin, CGRP, amylin, and adrenomedullin, are a class of peptide hormones generated in vertebrates. The coding sequences of calcitonin (32 amino acids) and CGRP (37 amino acids) are located in the same gene and formed into two mature peptides by alternative splicing (Amara et al., 1982; Rosenfeld et al., 1983). A previous report has indicated that CGRP treatment increased the swimming time in mice during a forced swimming test (FST) (Schorscher-Petcu et al., 2009). Concurrently, another study has demonstrated that an injection of CGRP prior to a period of 15-day chronic stress could reduce the immobility time, suggesting the antidepressant potential of CGRP (Hashikawa-Hobara et al., 2015). Furthermore, the antidepressant effects of CGRP disappeared after blocking the CGRP receptor (Hashikawa-Hobara et al., 2015). Conversely, the levels of CGRP were increased in the hippocampus, frontal cortex, and amygdala in the rat model of depression established by maternal deprivation (Angelucci et al., 2019). It is noted that in the clinical trial of depressive patients, calcitonin in the serum is reduced (Mathe et al., 2002). However, whether calcitonin could exert an antidepressant effect remains unclear.

Amylin receptors (AMYRs) consist of calcitonin receptor (CTR) dimerized with receptor activity-modifying proteins (RAMPs) (Hay et al., 2018). *Kalafateli* et al. reported that AMYRs are expressed in the whole brain including reward-processing brain areas (Kalafateli et al., 2021). AMYRs are involved in motivated ingestive behavior and alcohol drinking (Mietlicki-Baase et al., 2017; Kalafateli et al., 2021). Small-molecule AMYR agonists are considered effective treatment candidates, especially because they can handle crossing the blood–brain barrier and perform with high specificity (Sonne et al., 2021). Salmon calcitonin (sCT) is a therapeutic agent known for the treatment of osteoporosis and Paget's disease. The efficacy and favorable safety have been verified after several decades of clinical practice (McLaughlin and Jialal, 2021). Recent studies have shown that sCT affects reward-related areas of the brain such as the laterodorsal tegmental area (LDTg), ventral tegmental area (VTA), and nucleus accumbens (NAc) shell to modulate alcohol-related behaviors in rodents (Zakariassen et al., 2020; Kalafateli et al., 2021). Furthermore, sCT can attenuate certain nicotine-induced behaviors by modulating AMYRs (Aranas et al., 2021). sCT is an agonist of AMYRs (i.e., AMYR polypeptide), and its role in depression remains to be elucidated.

In the present study, we aimed to verify the antidepressant potential of sCT. First, we evaluated the effectiveness of the mouse model with CRS, and we detected the level of calcitonin in the serum and cerebral cortex taken from the depressive-like mice. Second, we showed that the depressive phenotype was alleviated by an acute application of sCT. Finally, to validate the receptor-dependent antidepressant potential of sCT, we harnessed AC187, an antagonist of AMYR, to block the antidepressant effects of sCT.

METHODS AND MATERIALS

Animals

Mice were housed in a pathogen-free SPFII animal facility in a condition-controlled room (23°C ± 1°C, 50% ± 10% humidity) at the Southern University of Science and Technology (SUSTech), Shenzhen, China. A 12-h light/dark cycle was automatically imposed. Mice were maintained in a group of six in each ventilated cage and given access to food and water *ad libitum*. All animal experiments were conducted according to the protocols approved by the Animal Care Committee at SUSTech. The ARRIVE (Animal Research: Reporting of *In Vivo* Experiments) guidelines were followed when animal experimentation was designed and performed. Male C57BL/6J mice were imported from Guangdong Laboratory Animal Center (Guangzhou, China).

Chronic Restraint Stress Model

After being acclimated to the facility for a week, mice were subjected to CRS as previously described, with minor modifications (Seo et al., 2012). Briefly, mice were restrained in flexible cylindrical plastic tubes fitted to allow them to breathe. Going through CRS, mice were immobilized in the tube once for 2 h for 14 consecutive days. Mice assigned to a non-stressed group stayed in their own home cages.

Open-Field Test

The open-field test (OFT) was used to measure the level of voluntary movement and anxiety (Choleris et al., 2001). Mice were put in the test room for 1 h to habituate to the environment. The open box (40 × 40 × 40 cm, L × W × H) was divided equally into 16 smaller grids, and the central four grids were set as the central area (20 × 20 cm). The distance that the mice traveled and the time that they spent in the central area were recorded for 10 min by EthoVision XT software (Noldus Information Technology, Leesburg, VA, USA).

Elevated Plus Maze

The elevated plus maze (EPM) was formed with four arms, with open and closed arms crossed with each other, standing 1 m above the floor. The test mice were placed in the center facing the open arm, and their activity was measured for 5 min. The total time spent in open arms, center area, and closed arms was recorded with a video tracking system and analyzed by Noldus EthoVision XT10 software.

Tail Suspension Test

Tail suspension test (TST) was performed according to the previously reported protocols (Ali et al., 2020; Li W et al., 2021). Briefly, mice were placed in the test room for 1 h to be familiarized with the environment. After that, the mouse tails were taped to the iron hook in the tail suspension box. EthoVision XT software was used to record the immobility time of mice within 6 min upon tail suspension.

Forced Swimming Test

FST was performed according to previously reported protocols (Ali et al., 2020; Lee et al., 2021). Mice were placed in the test room for 1 h to be familiarized with the environment. The apparatus was a diaphanous cylindrical plexiglass container with a diameter of 11.5 cm and a height of 30 cm. Mice were individually placed in the water cylinder (water temperature: 22°C–24°C) for a 6 min test session, and the cumulative immobility time was counted and analyzed for the last 5 min. The immobility time was recorded by the EthoVision XT software. Mice were thought to be stationary when they floated motionless in the water or moved to keep their nose above the surface of the water.

Three-Chamber Test

Social behaviors were adapted from experiments previously described (Sgritta et al., 2019). The three-chamber apparatus (60 × 40 × 20 cm, L × W × H) was divided into three interconnected chambers. Mice were first habituated for a 10-min period in three-chamber apparatus divided by transparent plexiglass. Sociability was evaluated during a second 10-min period in which the test mice could interact either with a small cage (Empty) in one chamber or a genotype, age, sex-matched stranger mouse (Mouse 1) that was placed in a cage on the other chamber. Subsequently, preference for social novelty was assayed, in a third 10-min period, by introducing a second stranger mouse (Mouse 2) into the previously empty cage. The time spent interacting with the empty cage or Mouse one or Mouse two was recorded and measured using the EthoVision XT 10 software package.

Novel Object Recognition Test

The novel object recognition (NOR) task was adapted from experiments previously described (Leonzino et al., 2019). Mice were habituated to a box (40 × 40 × 40 cm, L × W × H) for 10 min and then returned to their home cage. Twenty-four hours after habituation, animals were exposed to two identical objects for 10 min exploration sessions in the same box. Two hours after object exploration, one object was replaced with a novel object, and the animals were then allowed to explore the two objects (i.e., novel vs. old) for 10 min. The time the mice spent sniffing within 2 cm of each object or directly touching the objects was recorded.

Drug Administration

sCT (Tocris Bioscience, Bristol, United Kingdom) was diluted in 0.9% sterile saline and injected subcutaneously at 50 IU/kg (12.5 ug/kg) bodyweight 1 h before the behavioral test. Mice in the control group were injected with saline at the same volume. The AMYR antagonist AC187 (250 μg/kg, Cohesion Biosciences, London, United Kingdom) was injected intraperitoneally 10 min before the sCT administration. One hour after the sCT injection, the above behavioral tests were performed. The dosage and timing of the drug administration were determined based on previous studies (Kalafateli et al., 2019b).

Mouse Calcitonin Measurement

Frozen cerebral cortex tissues were lysed with radioimmunoprecipitation assay (RIPA) buffer (Beyotime, Shanghai, China) and homogenized on ice. The supernatants were collected after centrifugation (Li W et al., 2021). mCT was measured by a sandwich ELISA (Jianglai Biological Technology Co., Ltd., Shanghai, China) in 100 μl of serum or supernatant of cortical homogenate according to the manufacturer's protocols. Briefly, after the 96-well plate was cleaned 3 times using wash buffer, 100 μl of standard or sample was added to each well and incubated for 2 h at 37°C. Subsequently, the plate was washed 3 times using wash buffer and incubated using a biotin-conjugated antibody for 1 h at 37°C. Streptavidin–horseradish peroxidase (HRP) was added for a 30-min incubation at 37°C. Optical density was measured under 450 nm with an ELISA microplate reader.

Western Blotting Assay

The brain tissues used to detect CTR expression were taken from the mice of the CRS and control groups that went through

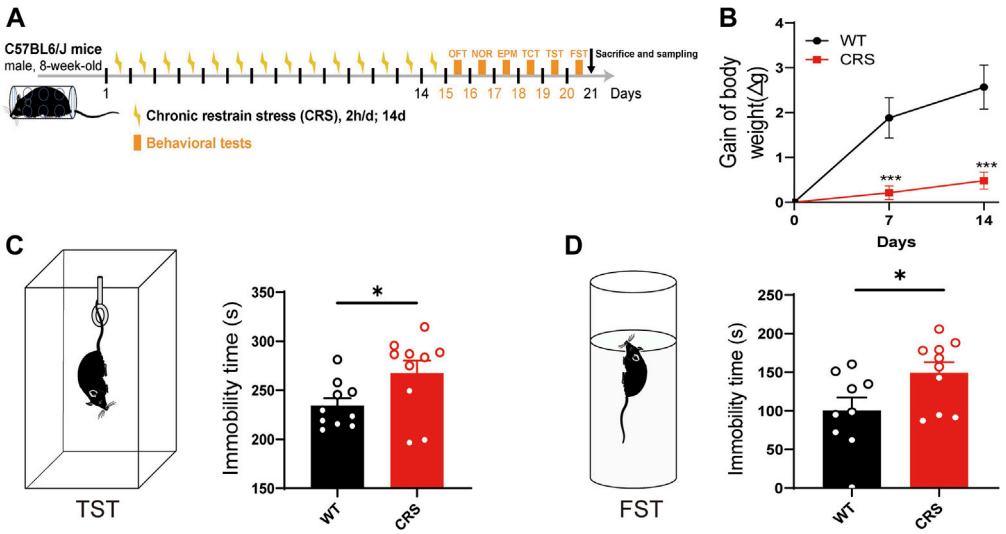

FIGURE 1 | CRS mice showed a depressive-like phenotype. Schematic diagram showing experimental procedures of CRS mouse model **(A)**. Compared with WT controls, the weight of mice relative decreased after 7 or 14 days of CRS (WT: $n = 10$; CRS: $n = 10$) **(B)**. In the tail suspension experiment, the immobility time of CRS mice was significantly increased (WT: $n = 10$; CRS: $n = 10$) **(C)**. In FST, the immobility time of CRS mice was also increased (WT: $n = 9$; CRS: $n = 10$) **(D)**. The data were analyzed by unpaired *t*-test. $^{*}p < 0.05$, $^{***}p < 0.001$. CRS, chronic restraint stress; WT, wild type; FST, forced swimming test.

behavioral tests. Total protein was isolated from the dissected tissues using moderate-intensity RIPA buffer (Beyotime, Shanghai, China). After being centrifuged at 12,000 rpm at 4°C for 15 min, supernatants were collected, and protein concentration was determined using Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, MA, United States). Proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), transferred to a polyvinylidene fluoride (PVDF) membrane (Merck Millipore, Guangzhou, China), and blocked in 5% fat-free milk in 1× TBST (0.1% Tween20 in Tris-buffered saline) for 1 h at room temperature. The blots were incubated with primary antibodies of CTR (Abcam, Cambridge, United Kingdom) in a 5% bovine serum albumin (BSA) solution overnight at 4°C. On the second day, the blots were washed with 1× TBST 3 times and incubated with HRP-conjugated secondary antibodies (anti-rabbit IgG, ProteinTech Group, Inc., Wuhan, China). Immunodetection was performed using a super ECL detection reagent (Yeasen Biotech, Shanghai, China), and the signal was detected with a ChemiDoc™ Touch Imaging System (Bio-Rad, Shanghai, China).

Statistical Analysis

All data are represented as the mean ± SEM. Serous and cerebral cortical mCT levels and behavioral tests between the wild-type (WT) and CRS groups were analyzed by independent-samples *t*-test. The data of behavioral tests, among three groups, were analyzed with one-way ANOVA followed by Tukey's multiple-comparisons test. Two-way ANOVA followed by Bonferroni's multiple-comparisons test was used to analyze the results of the three-chamber social test and NOR test. Statistical analysis was performed using GraphPad Prism 8 (GraphPad Software, La Jolla, CA, United States), and $p < 0.05$ was considered statistically significant. The sample size calculation in this study was based on "AEEC animal experimentation sample size calculator" (http://www.lasec.cuhk.edu.hk/sample-size-calculation.html).

RESULTS

Mice With CRS Showed Significant Depressive-Like Behaviors

To explore the therapeutic effects on depression, we established an animal model of CRS, which is widely used to screen for antidepressants (Campos et al., 2013; Li Y et al., 2021; Lee et al., 2021). In the present study, we treated male C57BL/6J mice with CRS for 2 weeks starting at the age of 8 weeks (**Figure 1A**). Compared to the control group, the body weight of mice was relatively reduced after 7 and 14 days of CRS [$t_{(18)} = 4.072$, $p < 0.001$; $t_{(18)} = 4.462$, $p < 0.001$, **Figure 1B**)], and thus the CRS model was primarily established, as the weight loss is often as a key indicator of depression. TST and FST were then performed in mice after 14 days of CRS. We found that the immobility time of CRS mice was significantly increased in both TST and FST [$t_{(18)} = 2.268$, $p < 0.05$, **Figure 1C**; $t_{(17)} = 2.266$, $p < 0.05$, **Figure 1D**], suggesting that our model of depression was successfully established. In addition, no significant deficits in social ability [$F_{(1,\ 36)} = 0.740$, $p > 0.05$, **Supplementary Figure S1B**] and cognitive memory [$F_{(1,\ 36)} = 0.036$, $p > 0.05$, **Supplementary Figure S1E**] were observed in this mouse model of depression.

Locomotion and Anxiety Level did not Change in CRS Mice

Depressive and anxiety disorders are often associated, but the relationship between them has been controversial. In TST and

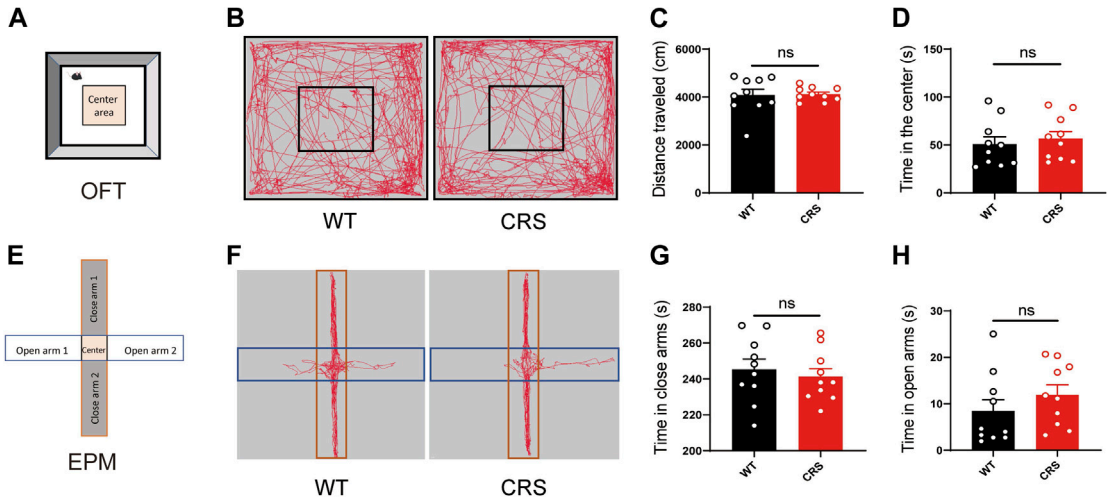

FIGURE 2 | Locomotion and anxiety did not change in CRS mice. Scheme of the OFT **(A)**. Representative images of movement trace in OFT **(B)**. The distance traveled in OFT did not change in CRS mice (WT: $n = 10$; CRS: $n = 10$) **(C)**. Time in the center did not change compared with WT controls **(D)**. Scheme of the EPM **(E)**. Representative images of movement trace in EPM **(F)**. Time in closed arms did not change in CRS mice (WT: $n = 10$; CRS: $n = 10$) **(G)**. Time in the open arms did not change compared with WT controls **(H)**. The data were analyzed by unpaired *t*-test. CRS, chronic restraint stress; OFT, open field test; WT, wild-type; EPM, elevated plus maze.

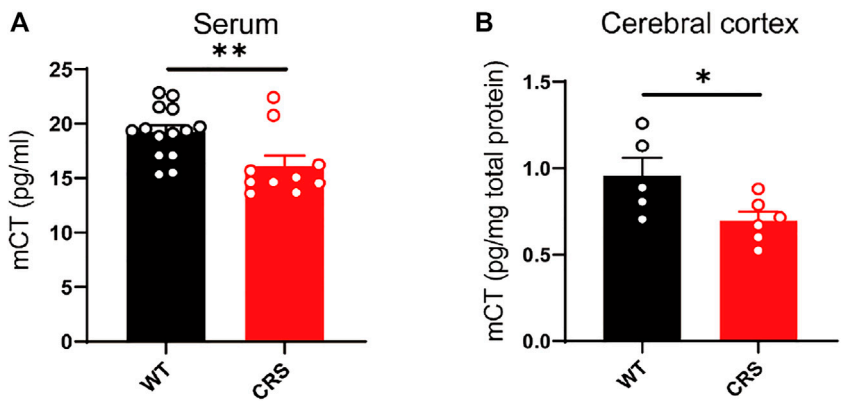

FIGURE 3 | The level of mCT was reduced in the serum and cerebral cortex of CRS mice. mCT was reduced in the serum of CRS model (WT: $n = 14$; CRS: $n = 10$) **(A)**. mCT was reduced in the cerebral cortex of CRS model (WT: $n = 5$; CRS: $n = 6$) **(B)**. The data were analyzed by unpaired *t*-test. $^{*}p < 0.05$, $^{**}p < 0.01$. CRS, chronic restraint stress; WT, wild type.

FST, the immobility time may be highly linked to the ability of spontaneous movement in mice. To demonstrate that the increased immobility time of the CRS mice was attributed to depression but not to the effect of locomotion, we conducted the OFT to monitor the locomotion in the mice. We showed that there was no significant change in the distance traveled [$t_{(18)} = 0.143$, $p > 0.05$, **Figures 2A–C**]. The time that the CRS mice spent in the middle area, usually reflecting anxiety, did not alter either [$t_{(18)} = 0.097$, $p > 0.05$, **Figure 2D**]. In addition, we examined whether the CRS mice were accompanied by anxiety in the EPM. Of interest, there was no profound change in the time spent in the open and closed arms between the WT mice and the CRS mice [$t_{(18)} = 0.550$, $p > 0.05$, **Figures 2E–G**; $t_{(18)} = 1.089$, $p > 0.05$, **Figure 2H**].

The Levels of Serous and Cortical Calcitonin were Decreased in CRS Mice

In the clinical trial of depressive patients, calcitonin in the serum is reduced (Mathe et al., 2002). Here, we measured the mouse calcitonin (mCT) level in the serum and found its decline in the CRS mice compared to the control group [$t_{(22)} = 2.832$, $p < 0.01$, **Figure 3A**]. As the calcitonin is mainly produced in the

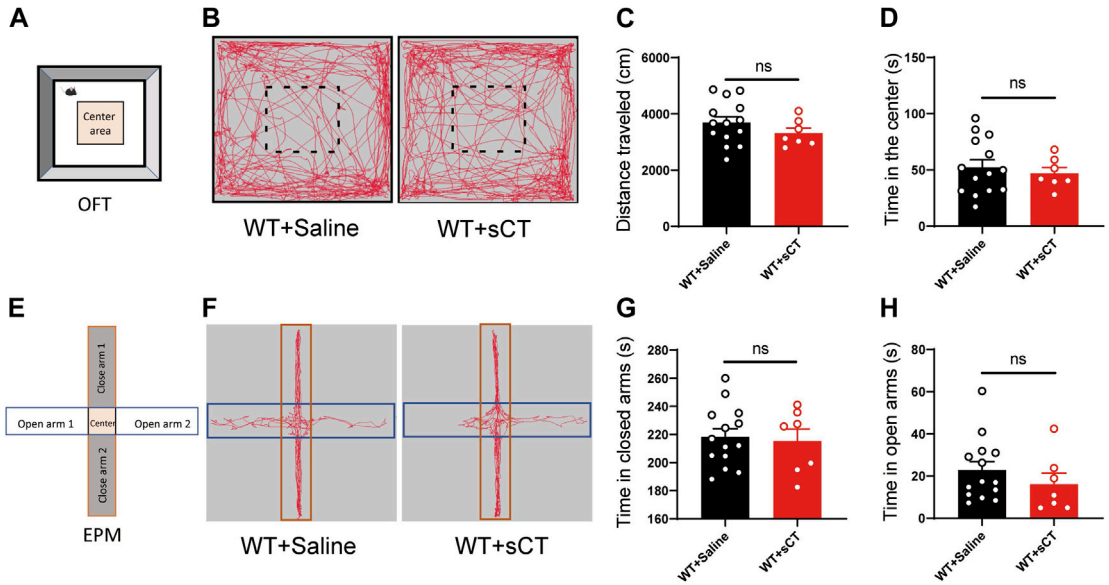

FIGURE 4 | Salmon calcitonin (sCT) did not affect locomotion and anxiety level in WT mice. Scheme of the OFT **(A)**. Representative images of movement trace in OFT **(B)**. The distance travelled in OFT did not change with application of sCT (WT + saline: $n = 14$; WT + sCT: $n = 7$) **(C)**. Time in the center did not change with the application of sCT **(D)**. Scheme of the EPM **(E)**. Representative images of movement trace in EPM **(F)**. Time in closed arms did not change with application of sCT (WT + saline: $n = 14$; WT + sCT: $n = 7$) **(G)**. Time in open arms did not change with the application of sCT **(H)**. The data were analyzed by unpaired *t*-test. WT, wild type; OFT, open field test; EPM, elevated plus maze.

parathyroid gland and could cross the blood–brain barrier, we also assessed the mCT level and found a decline in the cerebral cortex of the CRS mice [$t_{(9)} = 2.39$, $p < 0.05$, **Figure 3B**].

Salmon Calcitonin did not Affect Locomotion and Anxiety of Wild-Type and CRS Mice

To exclude the possibility of sCT interference on the mouse locomotion, we injected sCT into the WT mice (i.e., non-stressed control) 1 h before the OFT to investigate whether it could affect spontaneous locomotion. We found that the distance that the WT mice traveled after the administration of sCT was not changed as compared to the control group injected with saline [$t_{(19)} = 1.162$, $p > 0.05$, **Figures 4A–C**]. At the same time, the time of WT mice into the center was not altered either [$t_{(19)} = 0.541$, $p > 0.05$, **Figure 4D**]. On the next day, we performed an EPM test on the same groups of mice 1 h after subcutaneous injection of sCT or saline. In the EPM test, the time that the mice stayed in the open arms or closed arms was not changed [$t_{(19)} = 0.302$, $p > 0.05$, **Figures 4E–G**; $t_{(19)} = 0.984$, $p > 0.05$, **Figure 4H**]. Similarly, we found that sCT did not affect locomotion and anxiety levels in CRS mice (**Supplementary Figure S2**).

sCT Exerts Antidepressant Effects in CRS Mice

Previous studies have recorded that the elimination half-life of sCT could persist for about 1–1.5 h (Chin et al., 2004; Bhandari et al., 2015). Thus, we applied a subcutaneous administration of sCT and did the behavior test 1 h afterward. **Figure 5A** shows the schematic illustration of the experimental design. The antidepressant effects of sCT were indicated by the responses in TST and FST. In the TST, the immobility time of the depressive-like mice was profoundly increased compared to that of the WT mice, while sCT administration significantly reduced such immobility time [$F_{(2,\ 38)} = 0.324$, $p < 0.001$, **Figure 5B**]. Similarly, in the FST, the immobility time of the CRS mice was increased, while the sCT administration rescued it significantly [$F_{(2,\ 44)} = 0.444$, $p < 0.01$, **Figure 5C**]. These results suggested that the sCT has antidepressant effects in CRS mice, while not affecting the locomotion and anxiety levels.

Expression of CTR was not Altered in the Prefrontal Cortex, Cerebral Cortex (Without PFC), and Hippocampus of CRS Mice

Considering the antidepressant effects of sCT, we sought to investigate if the expression of AMYR plays a role in the CRS condition. Hence, we performed Western blotting of CTR, one core component of AMYR, in PFC, cerebral cortex (without PFC), and hippocampus of the CRS mice vs. the WT controls. The expression of CTR was detected in all these three regions, and CTR protein levels were normalized to those of GAPDH. As illustrated in **Figure 6**, the CTR protein levels were not significantly changed in the PFC [$t_{(8)} = 0.842$, $p > 0.05$], cerebral cortex (without PFC) [$t_{(8)} = 0.601$, $p > 0.05$], and hippocampus [$t_{(8)} = 1.052$, $p > 0.05$] of CRS mice compared with unstressed mice.

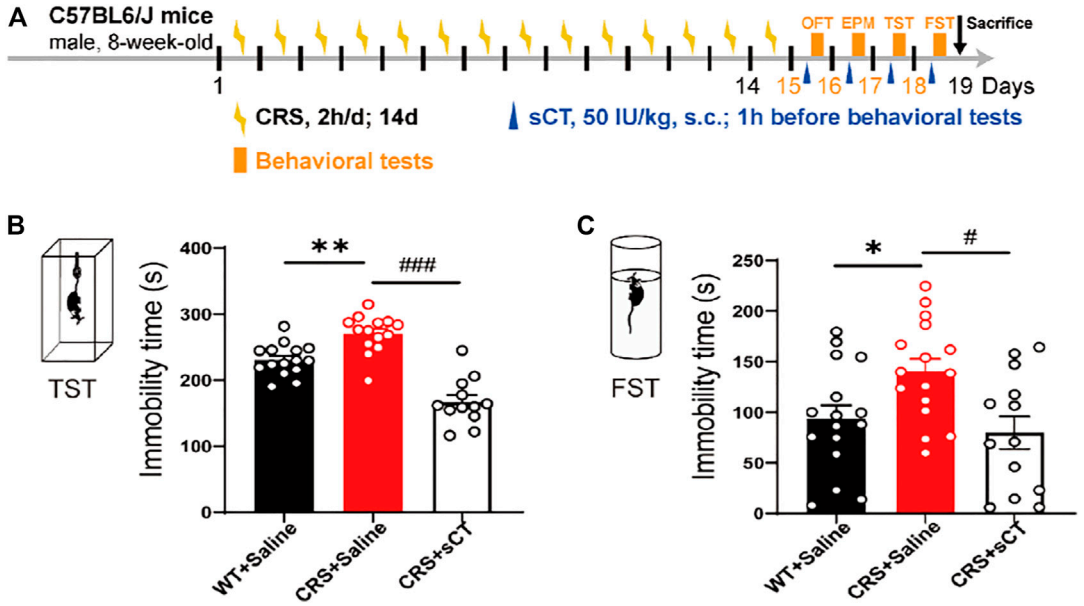

FIGURE 5 | sCT exerted an antidepressant effect in CRS mice. Schematic illustration of the experimental design **(A)**. In TST, the immobility time of CRS mice was increased, while sCT could decrease the immobility time of CRS mice (WT + saline: $n = 15$; CRS + saline: $n = 14$; CRS + sCT: $n = 12$) **(B)**. In FST, the immobility time of CRS mice was increased, while sCT shortened immobility time in CRS mice (WT + saline: $n = 16$; CRS + saline: $n = 16$; CRS + sCT: $n = 13$) **(C)**. The data were analyzed by one-way ANOVA with Tukey's multiple-comparisons test. $^{*}p < 0.05$, $^{**}p < 0.01$ vs. WT + saline; $^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$ vs. CRS + saline. sCT, salmon calcitonin; CRS, chronic restraint stress; TST, tail suspension test; FST, forced swimming test.

sCT Exerts Antidepressant Effects in the CRS Model by Activating the Amylin Receptor

AMYRs can be activated by their agonist peptides, such as sCT, or suppressed by the antagonists including AC187, AC66, and CGRP. Among them, the AC187 has the most antagonistic effect (Hay et al., 2005). As was observed in **Figure 5**, sCT could decrease the immobility time of the depressive-like mice. When AMYRs were inhibited by AC187, such an antidepressant effect of sCT was potently stymied [$t_{(23)} = 2.353$, $p < 0.05$, **Figures 7A,B**]. Similarly, in the FST, when AMYRs were inhibited by AC187, the antidepressant effect of sCT was significantly blocked [$t_{(25)} = 3.744$, $p < 0.001$, **Figure 7C**]. Collectively, these results delineated that sCT exerted antidepressant effects *via* AMYRs (**Figures 8A,B**).

DISCUSSION

Depressive disorders are a complex of neuropsychiatric diseases that affect a large population, while the current pharmacotherapies have a major limitation, a long working latency. In the present study, to evaluate the antidepressant potential of acute sCT injection, we first reproduced an animal model of CRS (**Figure 1**) with no locomotion and anxiety phenotypes (**Figure 2**) but with a significantly declined level of endogenous calcitonin in the serum and cerebral cortex (**Figure 3**). Further, we showed that the sCT treatment did not alter locomotor activity and anxiety in the non-stressed mice (**Figure 4**), nor in the CRS group (**Supplementary Figure S2**). Interestingly enough, we found that sCT did reduce the immobility time of the CRS mice during FST and TST analyses (**Figure 5**), demonstrating its antidepressant potential. Although no changes of CTR expression have been observed in various depression-associated brain regions in the stressed mice (**Figure 6**), the AMYR antagonist, AC187, potently reversed the antidepressant effect of sCT, suggesting the AMYR-function-dependent role of sCT (**Figure 7**). Of note, our results showed that AC187 did not affect the immobility time of CRS mice in TST and FST, suggesting that AC187 alone did not induce depressive-like behavior changes in CRS mice (**Supplementary Figure S3G, H**). Furthermore, the locomotion and anxiety levels in CRS mice were not affected by AC187 (**Supplementary Figure S3B, C, E, F**). Similarly, AC187 did not affect locomotion, anxiety level, and depressive-like behavior in WT mice (**Supplementary Figure S4**).

Calcitonin is a single peptide hormone secreted by parafollicular or C cells of the thyroid gland and composed of 32 amino acids (Srinivasan et al., 2020). sCT derived from salmon has a greater affinity for AMYRs than calcitonin from other species and has longer half-life elimination, which is widely used in the clinical treatment of bone diseases (Andreotti et al., 2006). To this end, this study investigated the potential antidepressant effects of sCT, known as a hormone for the regulation of serum calcium concentration. Furthermore, it has been reported that calcium level in serum is increased in people with depression (Joffe et al., 1996). This may be due to deficiencies in calcium's

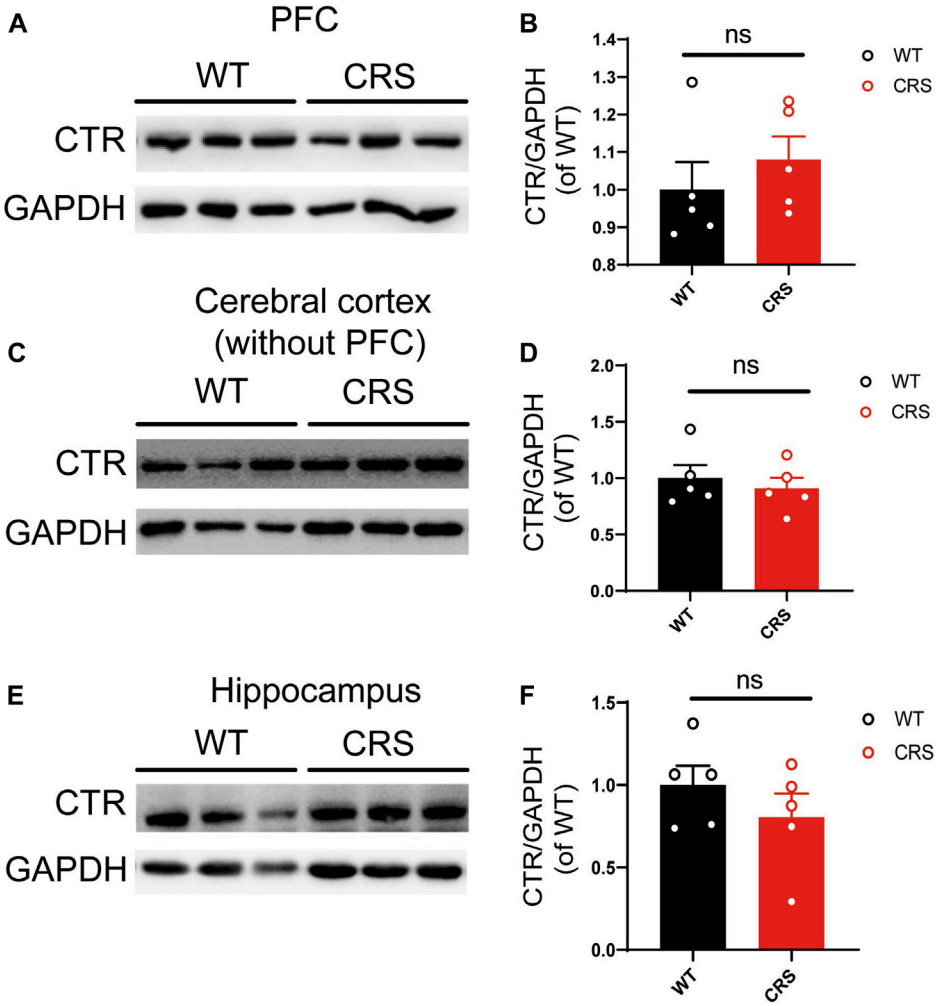

FIGURE 6 | The level of CTR was not altered in PFC, cerebral cortex (without PFC), and hippocampus. The Western blotting bands in PFC **(A)**. Relative CTR expression was not altered in PFC (WT: $n = 5$; CRS: $n = 5$) **(B)**. The Western blotting bands in the cerebral cortex (without PFC) **(C)**. Relative CTR expression was not altered in the cerebral cortex (without PFC) **(D)**. The Western blotting bands in the hippocampus **(E)**. Relative CTR expression was not altered in the hippocampus **(F)**. The data were analyzed by unpaired *t*-test. CTR, calcitonin receptor; PFC, prefrontal cortex; WT, wild type; CRS, chronic restraint stress.

regulatory ability in depressed patients. Our study found that calcitonin level was decreased in both the serum and the cerebral cortex after CRS (**Figure 3**). Similar to the results of our study, in depressed patients, the calcitonin level is decreased in the cerebrospinal fluid (Mathe et al., 2002). Therefore, it is reasonable to postulate that dysregulation of the calcitonin level may lead to hypercalcemia in depressed patients.

Calcitonin has a profound effect on the physiological function of the central nervous system. For instance, sCT can affect food intake through its effects on the NAc, VTA, and lateral dorsal tegmental area (Mietlicki-Baase et al., 2013; Mietlicki-Baase et al., 2015; Reiner et al., 2017). Moreover, sCT regulates alcohol intake by activating the mesolimbic dopamine system (Kalafateli et al., 2019a; Kalafateli et al., 2021). CGRP, as a member of the calcitonin family, has significant antidepressant effects in the depression model (Hashikawa-Hobara et al., 2015). Calcitonin and the CGRP are amino acid peptides encoded by the same gene and share a similar structure. Calcitonin mRNA is expressed in thyroid C cells, but CGRP mRNA is mainly produced in neurons, while they can bind to the same type of receptors (Coleman et al., 2003). These data support our hypothesis on calcitonin/CGRP against stress-induced depressive-like behaviors. Indeed, we found that acute sCT administration alleviated depressive-like phenotypes in CRS mice (**Figure 5**). Particularly remarkable, sCT did not affect the locomotion and anxiety level in the stressed and non-stressed mice (**Figure 4**). In keeping with our observation, *Kalafateli* et al. found that sCT treatment for 5 days did not affect the locomotion of WT mice (Kalafateli et al., 2020). In addition, injections of sCT to activate the VTA AMYRs did not reduce the time of animals spent in the inner zone or the number of entries to the inner zone (Mietlicki-Baase et al., 2017). Thus, it is thought that sCT or its receptor is not involved in the anxiety-related neural circuits.

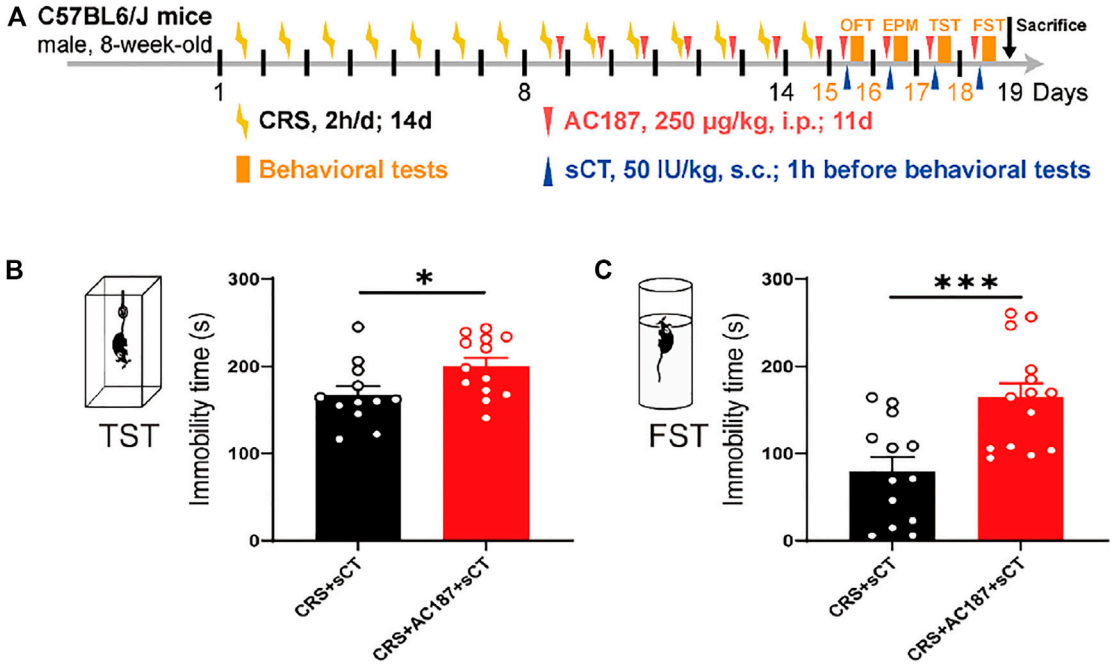

FIGURE 7 | sCT exerted an antidepressant effect in CRS mice by activating AMYR. Timeline of CRS exposure, sCT, AC187 administration, and behavioral tests **(A)**. In TST, when AMYR was inhibited by AC187, the antidepressant effect of sCT was blocked (CRS + sCT: $n = 12$; CRS + sCT + AC187: $n = 13$) **(B)**. In FST, the antidepressant effect of sCT was blocked when the AMYR inhibitor AC187 was administered (CRS + sCT: $n = 13$; CRS + sCT + AC187: $n = 14$) **(C)**. The data were analyzed by unpaired *t*-test. $^{*}p < 0.05$, $^{***}p < 0.001$. sCT, salmon calcitonin; CRS, chronic restraint stress; AMYR, amylin receptor; TST, tail suspension test; FST, forced swimming test.

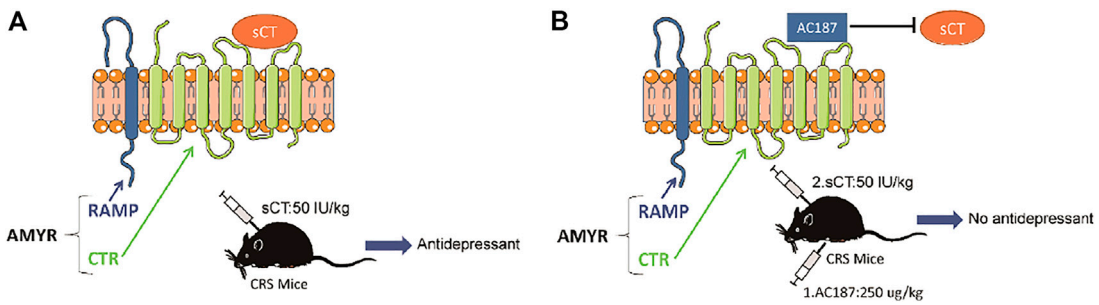

FIGURE 8 | Working model of the sCT antidepressant effect by activating amylin receptors in CRS mice. sCT can activate AMYRs to play an antidepressant role **(A)**. AC187 eliminates the antidepressant effect of sCT **(B)**. sCT, salmon calcitonin; CRS, chronic restraint stress; AMYRs, amylin receptors.

The CTR family is extremely complex, including CTR and CTR-like receptors and three RAMPs (Garelja et al., 2021). AMYR consists of a core CTR and one out of three RAMPs and is expressed in the whole brain including reward-related regions (Kalafateli et al., 2021). Similarly, we found that CTR was universally expressed in the brain including PFC, cerebral cortex (without PFC), and hippocampus, but the levels of its expression were not changed in the CRS mice (**Figure 6**), suggesting that the depressive disorder is not significantly related to or dependent on the expression of CTR in the brain of CRS mice. In addition, agonists mainly work through enhancing the intrinsic activity *via* changing the conformation of the protein molecule, instead of modulating the expression level of the receptor, if it is applied in a relatively short period and limited time. Up until now, sCT is one of the most potent AMYR agonists, and compared to calcitonin in humans and rats, sCT (i.e., from salmon) activates the AMYR more effectively and consistently (Andreassen et al., 2014; Gydesen et al., 2017). Nowadays, small-molecule peptide ligands have been widely studied in depression, owing to the ability to cross the blood–brain barrier and specifically bind to their receptors (Holmes et al., 2003; Nwokafor et al., 2020). They could be therapeutic candidates for the treatment of depression. In this study, we delineated the potential role of the calcitonin–AMYR axis in depression by exploiting both the agonist (e.g., sCT) and the inhibitor/antagonist of AMYR (e.g., AC187) (Hay et al., 2005). We have demonstrated that the AC187 abolished the antidepressant effects of sCT (**Figure 7**), indicating that AMYRs play a functional

role in the treatment of depression, although the expression level of AMYRs remained unchanged in major brain regions.

In summary, our study reveals a new role of sCT in the antidepressant effect by interacting with its receptors AMYRs. Our results may pave the way for future studies on determining the downstream mechanism of the AMYR, as well as for clinical research into potential targeted therapeutic strategies for the treatment of depression.

CONCLUSION

The present study demonstrated that sCT rescued the depressive-like behaviors in the CRS mouse model of depression; however, the AC187, a potent antagonist of AMYRs, significantly eliminated the antidepressant effects of sCT. Therefore, our results indicated that sCT may exert rapid antidepressant effects by activating AMYRs.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The animal study was reviewed and approved by The Animal Care Committee in the Southern University of Science and Technology (Shenzhen, China).

AUTHOR CONTRIBUTIONS

JJi, JJu, and NL conceived and designed the experiments. Material preparation and animal experiments were performed by JJi, JJu, XY, LL, ZS, DW, WZ, and JC. Data collection and analysis were performed by JJi, JJu, LL, SW, HL, JM, HH, JY, and HW. The first draft of the manuscript was written by JJu and JJi and was revised by NL, HLL, SL, S-TH, and SW. All authors contributed to and have approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2022.826055/full#supplementary-material

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Epigenetic Mechanism of 5-HT/NE/DA Triple Reuptake Inhibitor on Adult Depression Susceptibility in Early Stress Mice

Ping Meng†, Chunmei Li†, Sijin Duan, Shengmin Ji, Yangyang Xu, Yutong Mao, Hongbo Wang and Jingwei Tian**

School of Pharmacy, Key Laboratory of Molecular Pharmacology and Drug Evaluation (Yantai University), Ministry of Education, Collaborative Innovation Center of Advanced Drug Delivery System and Biotech Drugs in Universities of Shandong, Yantai University, Yantai, China

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***Correspondence:**
Chunmei Li
lichunmei@luye.cn
Jingwei Tian
Tianjingwei618@163.com

†*These authors share first authorship*

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Major depressive disorder (MDD) is a chronic, remitting and debilitating disease and the etiology of MDD is highly complicated that involves genetic and environmental interactions. Despite many pharmacotherapeutic options, many patients remain poorly treated and the development of effective treatments remains a high priority in the field. LPM570065 is a potent 5-hydroxytryptamine (5-HT), norepinephrine (NE) and dopamine (DA) triple reuptake inhibitor and both preclinical and clinical results demonstrate significant efficacy against MDD. This study extends previous findings to examine the effects and underlying mechanisms of LPM570065 on stress vulnerability using a "two-hit" stress mouse model. The "two-hit" stress model used adult mice that had experienced early life maternal separation (MS) stress for social defeat stress (SDS) and then they were evaluated in three behavioral assays: sucrose preference test, tail suspension test and forced swimming test. For the mechanistic studies, methylation-specific differentially expressed genes in mouse hippocampal tissue and ventral tegmental area (VTA) were analyzed by whole-genome transcriptome analysis along with next-generation bisulfite sequencing analysis, followed by RT-PCR and pyrophosphate sequencing to confirm gene expression and methylation. LPM570065 significantly reversed depressive-like behaviors in the mice in the sucrose preference test, the tail suspension test, and the forced swimming test. Morphologically, LPM570065 increased the density of dendritic spines in hippocampal CA1 neurons. Hypermethylation and downregulation of oxytocin receptor (*Oxtr*) in the hippocampal tissues along with increased protein expression of Dnmt1 and Dnmt3a in mice that experienced the "two-hit" stress compared to those that only experienced adulthood social defeat stress, and LPM570065 could reverse these changes. Combined, these results suggest that methylation specificity of the gene *Oxtr* in the hippocampus may play an important role in early life stress-induced susceptibility to depression and that the5-HT/NE/DA triple reuptake inhibitor LPM570065 may reduce depression susceptibility via the reversal of the methylation of the gene *Oxtr*.

Keywords: DNA methylation, triple reuptake inhibitors, second stress, depression susceptibility, epigenetics

INTRODUCTION

Major depressive disorder is an important cause of human suffering, illness, and disability worldwide (Bromet et al., 2011; Gaebel et al., 2017). The World Health Organization ranks depression as the third leading cause of the global burden of disease and predicts that the disorder will rank first by 2030, with surveys showing that almost one in five people will experience a depressive episode at some point in their lives. The complex etiology of depression has not yet been fully elucidated and may involve genetic, environmental factors and their interactions. Abundant evidence suggests that exposure to early life stress (ELS) increases the risk of depression and may also lead to persistent changes in neural structure and depression-like behavior in adulthood. (Caspi et al., 2003; de Lima et al., 2011; Nemeroff and Binder, 2014). The experience of adversities during this critical period has lifelong impacts on the brain and behavior (Doherty et al., 2017).

Increasing evidence shows that early life stress increases susceptibility to acquired social failure and a "two-hit" model can mimic this adversity where mice first experienced early-life stress such as maternal separation ("first hit") and then mice were exposed to repeated social defeat stress ("second hit") (Peña et al., 2017). This manipulation causes long-lasting transcriptional and epigenetic alterations that prime the ventral tegmental area (VTA) to be in a depression-like state (Barnett Burns et al., 2018; Reshetnikov et al., 2021). So far, many epigenetic modification mechanisms have been discovered and DNA methylation is one of the most well-studied mechanisms that is believed to be involved in depression (Uddin et al., 2013; Bakusic et al., 2016; Chen et al., 2017). Early life stress can epigenetically modify depression-related genes by affecting DNA methylation, which in turn could cause structural and functional changes in the brain (Ding and Dai, 2019). Additionally, some antidepressants can epigenetically alter certain signaling molecules beyond their traditionally-believed pharmacological mechanisms to contribute to their antidepressant efficacies. For example, the antidepressant fluoxetine can epigenetically alter the CaMKIIα promotor in nucleus accumbens to regulate ΔFosB binding, which represents a new epigenetic mechanism of antidepressant action independent of its serotonin reuptake inhibition (Robison et al., 2014).

LPM570065 (also known as LY03005, ansofaxine, and toludesvenlafaxine) (**Figure 1**) is a new chemical entity and a 5-HT/NE/DA triple reuptake inhibitor. LPM570065 exhibits high binding affinity to serotonin transporter (SERT), norepinephrine transporter (NET) and dopamine transporter (DAT), and increases the release of 5-HT, NE and DA in the striatum after oral administration (Zhu et al., 2021). In several preclinical models including the forced swimming test, the chronic unpredictable mild stress model and the olfactory bulbectomized model, LPM570065 demonstrated significant antidepressant-like effects (Zhang et al., 2014; Zhu et al., 2021). In a phase II clinical study, LPM570065 extended-release tablet was safe, well-tolerated, and effective in improving depression symptoms in MDD patients (Mi et al., 2021), suggesting that LPM570065 could be a useful treatment option for MDD patients.

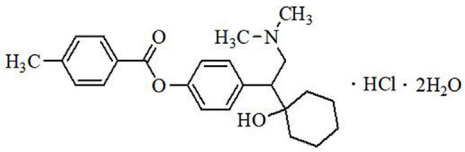

FIGURE 1 | Chemical structure of LPM570065.

This study was designed to examine the antidepressant-like effects of LPM570065 in a mouse "two-hit" model, a model that is known to induce extensive transcriptional and epigenetic changes in the brain (Peña et al., 2017; Reshetnikov, et al., 2021), and further examine the underlying epigenetic mechanism of antidepressant action.

MATERIALS AND METHODS

Animals

Adult male and female C57BL/6J mice (20–22 g) were purchased from Jinan Pengyue Experimental Animal Center (license number: SCXK20190003). All animals were acclimated to the laboratory environment for at least 5 days before the start of the experiments. Animals were housed in sterile cages under standard conditions (21°C, 50 ± 10% relative humidity, 12/12 h light/dark cycle, food and water ad libitum). The standard protocol was followed for animal mating, and breeding to generate litters. All behavioral experiments were conducted during the animals' light cycle and in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (8th edition, 2011) and approved by Yantai University Laboratory Animal Care and Use Committee. Experimenters were blind to experimental groups and drug treatments. Protocol was approved by the Animal Ethics Committee of Yantai University (registration number is YTU20200226) (Yantai, China).

Drugs

LPM570065 (> 99.93% pure, white powder) was provided by State Key Laboratory of Long-acting and Targeting Drug Delivery Technologies (Yantai, China). The purity of the compound was verified by HPLC. Fluoxetine hydrochloride was purchased from Sigma-Aldrich (St. Louis, MO, United States). LPM570065 was suspended with 0.5% sodium carboxymethylcellulose (SCMC). Fluoxetine hydrochloride was suspended with 0.5% SCMC.

Experimental Design

A schematic description of the experimental timeline was shown in **Figure 2A**. Male mice were randomly assigned into 5 groups (n = 24 per group): Control group [mice subjected to neither stress and later received vehicle (1 ml/kg) treatment], Single-stress group (mice only subjected to social defeat stress in adulthood and later received vehicle treatment), Double-stress group [mice subjected to double stress (both maternal separation stress and social defeat stress) and later received vehicle treatment], LPM570065 group [mice subjected to double stress

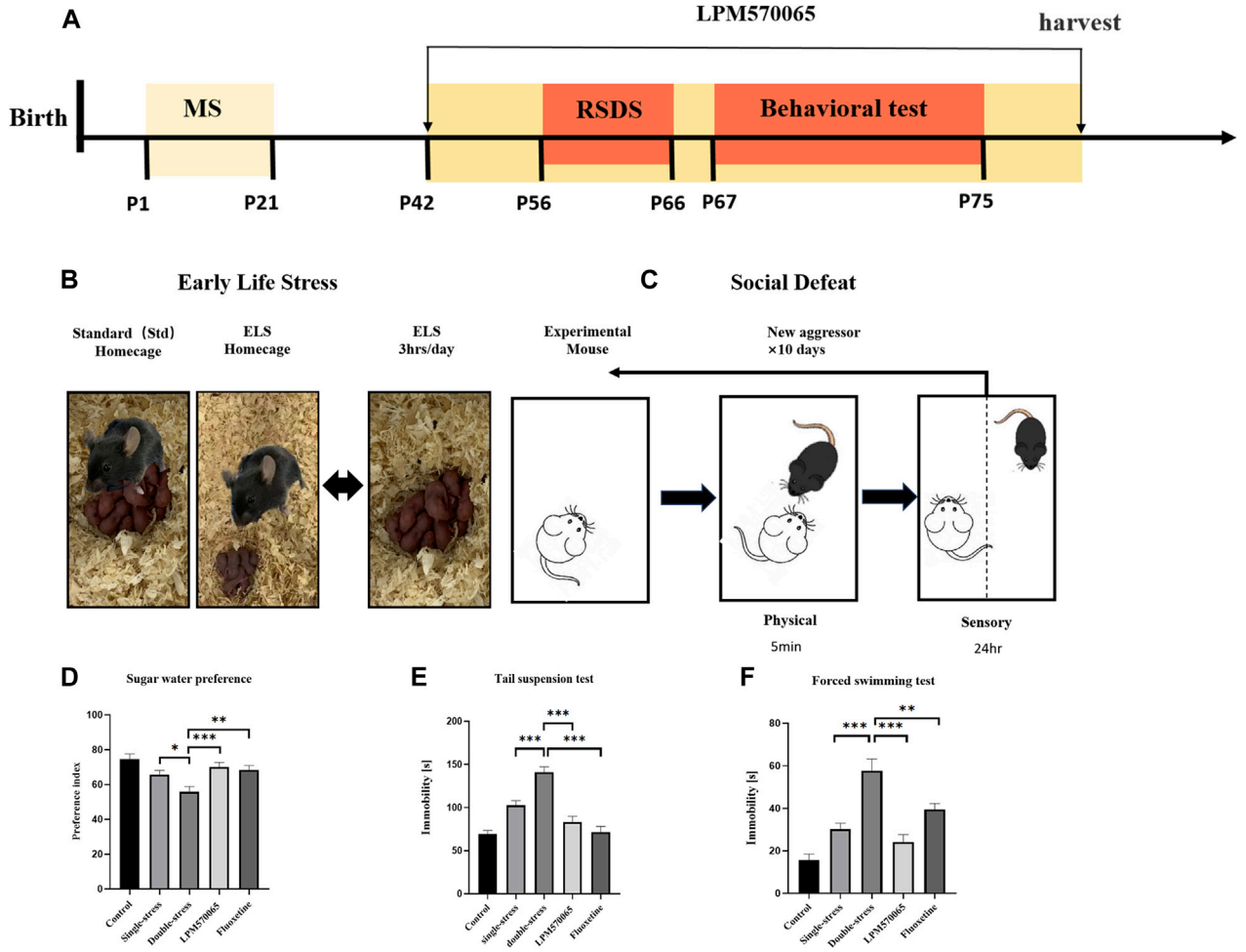

FIGURE 2 | LPM570065 reversed the increased susceptibility to depression in mice experiencing "two-hit" stress. **(A)** A schematic of mother-infant separation. **(B)** A schematic of repeated social defeat stress. **(C)** Experimental timeline. **(D)** Results of sucrose preference test. **(E)** Results of tail suspension test. **(F)** Results of forced swimming test. One-way ANOVA with Bonferroni multiple comparison correction and Student's *t* test. Data are mean ± SEM (n = 24 mouse per group), and statistical analysis is shown in **Supplementary Data**. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. Double stress group.

and later received LPM570065 (64 mg/kg) treatment], fluoxetine group [mice subjected to double stress and later received fluoxetine (12 mg/kg) treatment]. The vehicle or drug was given by intragastrical administration (i.g.), starting from postnatal day [PND] 42, mice would receive vehicle (0.5% SCMC, i.g.), LPM570065 (64 mg/kg, i.g.) or fluoxetine (12 mg/kg, i.g.) twice daily for the duration until harvest (**Figure 2A**). The dose of fluoxetine (12 mg/kg) was chosen based on previous studies (Sun et al., 2019), which showed significant behavioral effects.

MATERNAL SEPARATION STRESS

The maternal separation protocol was as previously described (El Khoury et al., 2006; Musazzi et al., 2010). Briefly, the mouse pups were separated from their dams and moved to separate home cages for 3 h per day during PNDs 1–21 days (**Figure 2B**). Other than the separation period, the pups were cared for by their dams all the time.

Repeated Social Defeat Stress

A standard protocol for repeated social defeat stress was followed as described (Golden et al., 2011). Briefly, male CD-1 mice were screened for aggressiveness according to the established criteria (Golden et al., 2011). Adult male C57BL/6J (8 weeks old) mice were subjected to 10 daily, 10 min defeats by a male CD-1 aggressor mouse and were then housed across a plexiglass divider to allow for sensory contact for the remainder of the day. Attack latency, duration, and frequency were not different among groups. Control mice were housed in cages separated from other control mice by a plexiglass divider and were rotated to a different cage daily (**Figure 2**).

Behavioral Tests

Sucrose Preference Test

The sucrose preference test was performed as previously described with minor modifications (Liu et al., 2018). Briefly, before the test, the individually housed mouse was habituated to consume 1% sucrose solution for 72 h. Animals were deprived of food and water for 12 h and then provided with leakproof bottles containing 1% sucrose or water, randomly placed on the left or right side of the cage. The volume of liquid before and after the 12-h test was weighed to evaluate sucrose and water consumption (n = 24). Sucrose preference (%) was calculated as.

Sucrose preference (%) = sucrose consumption (g)/[sucrose consumption (g) + water consumption (g)] × 100%.

Tail Suspension Test

The TST was performed in a quiet test room according to published literature with minor modifications (Cryan et al., 2005). Briefly, a four-compartment tail suspension chamber was used, and mice were suspended at 1–2 cm from the tail tip on iron rings with adhesive tape at a height of 35 cm from the table in the middle of the compartment, without contact with the wall. Mice were videotaped for 6 min and the videos were later analyzed by trained experimenters blind to drug treatments wherein the immobility times of the mice during the final 4 min of the 6-min test were recorded and analyzed.

Forced Swimming Test

The FST was performed in a quiet test room as described previously with minor modifications (Petit-Demouliere et al., 2005; Dang et al., 2009). Briefly, each mouse was individually placed in a vertical Plexiglas cylinder (40 cm high, 20 cm diameter) containing warm water (25 ± 1°C) at a depth of 20 cm. On the first day of the experiment, the mice were placed in the cylinder for 15 min, and 24 h later they were placed back into the cylinder for a 6 min test. During the test, the mice were videotaped for later scoring by two experimenters blind to the treatment conditions. The immobility times of the mice during the final 4 min of the 6-min test were recorded and analyzed. The inter-rater reliability was 0.93.

Golgi Staining

Golgi staining was performed according to the manufacturer's instructions for the FD Rapid Golgi Staining Kit (FD NeuroTechnologies, MD, United States) (Li et al., 2019). Briefly, fresh mouse brains were treated with impregnating solutions (A and B) and stored in total darkness for 2 weeks. The brains were transferred to solution C and stored for 72 h. Brains were cut to 150 μm thickness in a freezing microtome (Thermo Fisher Scientific, United States) and the hippocampal sections were mounted on gelatin-coated microscope slides (Hitobiotec Group, Kingsport, United States). The sections were then immersed in a mixture of solutions D and E. After elution and dehydration, the sections were coated with resin mounting medium. The number of spines of hippocampal CA1 neurons was counted every 10 μm on 40 μm dendritic segments in hippocampal slices using a German ZEISS microscope. Three mice per group were used for brain area sectioning, and three neurons per brain section per mouse were selected for quantification using a double-blind method.

RNA-Seq and Data Analysis

The high-throughput RNA sequencing analysis for this study was provided by a commercial service (Biotech Biotechnology Inc, Shanghai, China). First, total RNA was extracted from the hippocampal and the VTA tissue of four groups of mice (three samples per group): control group, single-stress group, double-stress group, and LPM570065 group, respectively. The RNA quality and quantity were then analyzed using the Quantum Bit RNA Detection Kit and the Quantum Bit 2.0 Fluorometer (Life Technologies, CA, United States).

Reduced Representation Bisulfite Sequencing

For the control group, single-stress group, double-stress group, and LPM570065 group (two samples per group), RRBS library creation and heavy sodium sulfite transformation was performed, followed by sequencing analysis. Briefly, total DNA from mouse hippocampal and VTA tissue were extracted separately using the QIAamp Fast DNA Tissue Kit (Qiagen, Düsseldorf, Germany) according to manufacturer's instructions, followed by sodium sulfite transformation and sequencing using an Illumina Novaseq™ 6000 instrument. Differentially methylated regions (DMRs) were identified using default parameters (sliding window analysis, size 1,000 bp, 500 bp overlap, $p < 0.05$).

Validation of Methylation Using Pyrophosphate Sequencing Technology

We performed pyrophosphate sequencing based on the results of previous RNA-seq and RRBS sequencing results from the hippocampal tissue to further validate the results, and analyzed the methylation levels of differential genes in the promoter regions of depression susceptibility-related genes between the single- and double-stress groups, and between the double-stress group and the LPM570065 group. The VTA tissue was not analyzed here as previously combined sequencing (RNA-seq and RRBS sequencing) failed to identify genes that met the screening criteria. The kit (B518251, Sangon, Shanghai, China) was used to extract gDNA from the mouse hippocampal samples, followed by the bisulfite transformation step according to the EZ DNA methylation-gold™ kit instructions (D5005, Zymo Research, CA, United States). The DNA was then subjected to PCR and the corresponding genes were sequenced by pyrophosphate using the PyroMark Q48 System (Qiagen) following the instructions of the PyroMark Q48 Advanced CpG reagent (974022, Qiagen). The following genes were analyzed: CLIP associating protein 1 (*Clasp1*), potassium large conductance calcium-activated channel, subfamily M, alpha member 1 (*Kcnma1*), Kruppel-like factor 4 (gut) (*Klf4*), oxytocin receptor (*Oxtr*), adhesion G protein-coupled receptor A2 (*Adgra2*), adhesion G protein-coupled receptor A2 (*Sgms1*), adhesion G protein-coupled receptor A2 (*Kcna1*), and

primers specific for zinc finger CCCH type containing 12C (*Zc3h12c*), as shown in Primer **Supplementary Table S1**.

Western Blotting

Western blotting experiments were performed as previously described (Pillai-Kastoori et al., 2020; Wang et al., 2020). Briefly, mouse hippocampal tissues (n = 8 per group) were collected and homogenized in RIPA buffer containing PMSF (1:100). Lysates were then spun at 12,000 rpm for 20 min at 4°C, and supernatant protein levels were assessed via BCA assay (Beyotime, Shanghai, China). Equal protein quantities (50 μg/sample) were separated *via* SDS-PAGE (GenScript, Nanjing, China) prior to transfer to PVDF membranes (Millipore, MA, United States) blocked with 5% milk or 5% bovine serum albumin, and incubated with the primary antibodies β-actin (AF0003, Beyotime), Dnmt1 (D63A6-5032S, Cell Signaling Technology), Dnmt3a (ab188470, Abcam) and were incubated overnight at 4°C. The membranes were washed three times with TBST and protein bands were detected by ECL after incubation with horseradish 20 peroxidase (HRP)-conjugated secondary antibody (#A0216, Beyotime Institute of Biotechnology). Protein bands were quantified using ImageJ, and normalized using β-actin.

Real-Time PCR

RNA was extracted from mouse hippocampal tissue in each group (n = 6 per group) using TRIzol (Invitrogen, CA, United States) according to the manufacturer's protocol. the quantity and the quality of eluted RNA samples were verified using a spectrophotometer (Nano Drop 2000, Applied Biosystems, California, United States). The quality of all samples of RNA mass (A260/A280) was 1.8–2.0. cDNA was obtained using SPARK script II RT Plus Kit (Spark Jade, Shandong, China). RT-qPCR analysis was then performed using an ABI 7500 RT-PCR instrument. Normalization was performed with β-actin and three replicate operations were performed for each gene, after which the relative quantitative expression of genes was calculated using the $2^{-\Delta\Delta CT}$ method. All primers in this study were purchased from Biotech Biotechnology Inc (Biotech, Shanghai, China).

The Primers Used Are as Follows

β-actin forward:5′-GTA AAG ACC TCT A TG CCA ACA-3′ and β-actin reverse:5′-GGA CTC A TC GTA CTC CTG CT-3';

Oxtr forward:5′-TGGCGGTCCTGTGTCTCATACTG-3′and OXTR reverse:5′-CGACATCAGCAACAGCAGGTAGG-3'

Statistical Analysis

Data were expressed as means ± SEM. One-way ANOVA with Bonferroni multiple comparison correction, or by Student's t test were used for data analysis. If ANOVA revealed significant group differences, post hoc-Bonferroni tests were performed to evaluate group differences. Data were analyzed using SPSS for Windows® version 21.0 (IBM, NY, United States) and GraphPad Prism v 9.0 (GraphPad Software, CA, United States). Results showing $p < 0.05$ were considered statistically significant for all analyses.

RESULTS

LPM570065 reduced susceptibility to depression-like behaviors in adult mice subjected to maternal separation.

To evaluate depression-like behaviors in mice, including anhedonia and behavioral despair, we performed SPT, TST and FST in mice on PD 67~75. In SPT, the consumption preference for 1% sucrose solution was significantly lower in the double-stress group compared to the single-stress group ($p < 0.05$), and the preference for 1% sucrose solution was significantly higher in mice treated with LPM570065 (64 mg/kg) and fluoxetine (12 mg/kg) ($p < 0.01$, $p < 0.001$, **Figure 2D**). In TST, the duration of immobility was significantly increased in mice of the double-stress group compared to the single-stress group ($p < 0.001$) and this was significantly lower in mice treated with LPM570065 (64 mg/kg) and fluoxetine (12 mg/kg) ($p < 0.001$, **Figure 2E**). In FST, the immobility time was significantly increased in the mice from the double-stress group compared with the single-stress group ($p < 0.001$), while the mice treated with LPM570065 (64 mg/kg) and fluoxetine (12 mg/kg) had a significantly shorter immobility time, suggesting the alleviation of behavioral despair ($p < 0.01$, $p < 0.001$, **Figure 2F**).

LPM570065 protected against the reduced number of dendritic spines in the hippocampal CA1 of mice subjected to stress.

Dysregulation of synaptic plasticity in the hippocampal CA1 area is associated with major depression (Golden et al., 2013). As expected, the number of dendritic spines in the CA1 area was significantly lower in the single-stress group compared to the vehicle group and the number was even significantly lower in the double-stress group as compared to both the vehicle group and the single-stress group ($p < 0.001$). Interestingly, LPM570065 (64 mg/kg) treatment significantly protected against the stress-induced reduction of dendritic spines, which was not only higher than the double-stress group, it was similar to the vehicle group ($p < 0.001$, **Figure 3**), suggesting significant protection. These results suggest that maternal separation stress and repeated social defeat stress were significant adversary events that led to dramatic dysregulation of synaptic plasticity in the hippocampal CA1 area and the triple reuptake inhibitor LPM570065 was able to provide significant protection against neuronal insults related to these adversary events.

RNA-Seq Analysis of Hippocampal and VTA Tissue in Mice

To investigate the potential epigenetic mechanism of LPM570065 to prevent early-life stress-induced depression susceptibility, we performed RNA-seq analysis on hippocampal and VTA tissue from mice.

The heat map showed clear clustering between samples of different groups (**Figure 4A**). Transcriptome analysis revealed that repeated social defeat stress altered the expression of 637 genes and double-stress (MS + RSDS) altered the expression of 906 genes as compared to the control group. Cluster analysis showed 604 differentially expressed susceptible genes between

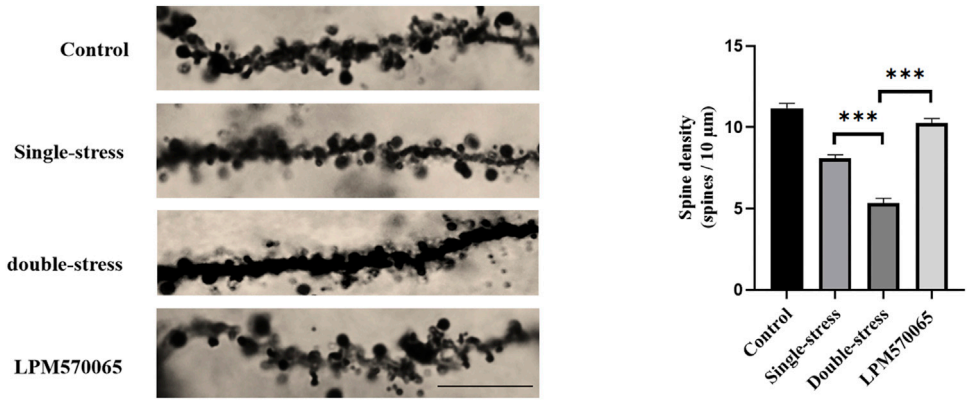

FIGURE 3 | LPM570065 increased the density of dendritic spines in the hippocampus. Dendritic spine density in the hippocampus of mice that experienced early life stress further decreased after a second stressful event in adulthood ($p < 0.001$). The density of dendritic spines in the hippocampus of mice treated with LPM570065 increased ($p < 0.001$). Histograms showed number of dendritic spines per 10 μm of dendrite length for hippocampal pyramidal neuron. One-way ANOVA with Bonferroni multiple comparison correction. Data are mean ± SEM ($n = 9$ neurons). ***$p < 0.001$ vs. Double stress group.

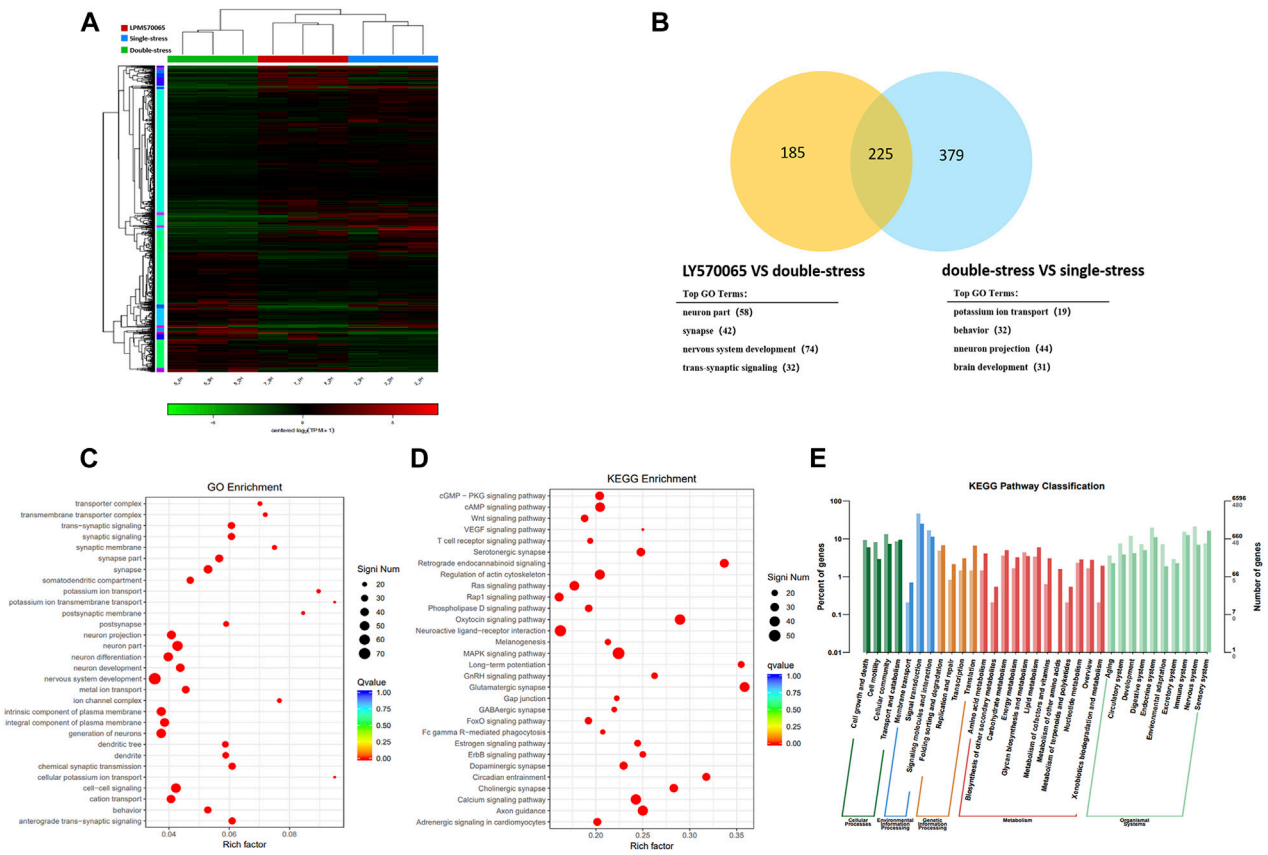

FIGURE 4 | Transcriptome sequencing analysis of the mouse hippocampus and cluster analysis of mouse hippocampal DEGs. For the overall analysis of each group of genes, **(A)** Venn diagram of the clustering analysis of the two compared groups, and gene enrichment GO analysis. **(B)** Clustering heat map of each sample illustrated good inter-group homogeneity and clustering repeatability between samples. **(C)** go function enrichment. **(D,E)** KEGG analysis of differential gene enrichment pathways. ($n = 3$/group, two mice were mixed in each sample, |Fold-change| > 1.5, $p < 0.05$).

double-stress (MS + RSDS) and single-stress (CSDS) groups, and 410 differentially expressed genes (DEGs) between the LPM570065 group and double stress (MS + RSDS) group. The clustering analysis of double stress-single stress *versus* LPM570065-double stress obtained 225 differentially expressed genes (|Fold-change| > 1.5, $p < 0.05$, **Figure 4B**). All of these 225 DEGs were downregulated in the double-stress (MS + CSDS) group and upregulated in the LPM570065 group (**Supplementary Table S2**).

Transcriptome analysis of the mouse VTA tissue showed clear clustering between the groups of samples in the heat map (**Supplementary Figure S1B**). Transcriptome analysis showed that repeated social defeat stress altered the expression of 331 genes and double stress (MS + RSDS) altered the expression of 892 genes as compared to the control group. Cluster analysis yielded 63 differentially expressed susceptibility genes between the double-stress group and the single-stress group, and 778 DEGs between the LPM570065 group and the double-stress group. Cluster analysis of double stress—single stress *versus* LPM570065—double stress identified 29 differentially DEGs. All of these 29 DEGs were downregulated in the double-stress group and upregulated in the LPM570065 group (|Fold-change| > 1.5, $p < 0.05$, **Supplementary Figure S1A** and **Supplementary Table S3**).

We next performed enrichment analysis of DEGs in the hippocampal CA1 tissue by the top GO algorithm and functional annotation of the genes revealed that they were associated with synaptic signaling, nervous system development, and other related functions (**Figure 4C**). KEGG pathway enrichment further revealed that these genes were enriched in endocannabinoid signaling, regulation of oxytocin signaling pathway, and glutamatergic synaptic pathway (**Figure 4D**), and the annotated classification of the KEGG pathway showed that DEGs were associated with developmental, environmental adaptation, neurological, and other related pathways (**Figure 4E**).

Enrichment analysis of DEGs in mouse VTA by top GO algorithm and functional annotation of these genes revealed that these genes are associated with developmental and other related functions (**Supplementary Figure S1C**). KEGG pathway enrichment further revealed that most genes were enriched in Neuroactive ligand-receptor interaction and Rap1 signaling pathway, among other pathways (**Supplementary Figure S1D**), and the annotated classification of KEGG pathways showed relevance to environmental adaptation, neurological, and other related pathways (**Supplementary Figure S1E**).

DNA Methylation Analysis of the Hippocampus and VTA in Mice

In the DNA methylation analysis, by comparing hippocampal tissue samples from mice in double-stress and single-stress groups, we found that 64% (66,028/102,521) of promoter DMRs exhibited hypermethylation. Comparing the hippocampal tissue samples from mice in the LPM570065 group and double-stress group, we found that 68% (63,578/93,002) of the promoter DMRs exhibited hypomethylation (**Figures 5A,B**). By cluster analysis of double stress-single stress *versus* LPM570065-double stress, we found that 4,178 promoter DMRs were hypermethylated in the double-stress group and hypomethylated in the LPM570065 group ($p<0.05$, **Figure 5C** and **Supplementary Table S4**).

Using the same analysis, by comparing mouse VTA tissue samples from the double-stress group and the single-stress group, we found that 14% (18,486/128,300) of the promoter DMRs exhibited hypermethylation. Comparing mouse VTA tissue samples from the LPM570065 group and the double-stress group, we found that 51% (23,723/46,094) of the promoter DMRs exhibited hypermethylation (**Supplementary Figures S2A,B**). By cluster analysis of double stress-single stress vs. LPM570065-double stress, we found that 839 promoter DMRs were hypermethylated in the double-stress group and hypomethylated in the LPM570065 group (**Supplementary Figure S2C** and **Supplementary Table S5**). KEGG enrichment analysis of genes related to promoter DMRs methylated in double stress-single stress and methylated in LPM570065-double stress was performed in mouse hippocampal CA1 and VTA tissue, respectively (**Figure 5D** and **Supplementary Figure S2D**), and both analyses found more genes enriched in signaling (17%) and neurological aspects (10%).

Changes in Methylation Levels of DEGs in the Hippocampus and VTA

In the combined analysis of differentially expressed genes and methylation genes in the mouse hippocampal tissue, among the 225 DEGs obtained in the double stress-single stress *versus* LPM570065-double stress clustering analysis, 41 of them had DMRs in the promoter region (|Fold-change| > 1.5, $p < 0.05$, **Figure 6A**), and their methylation levels were negatively correlated with the gene expression in the double-stress group, i.e., hypermethylation and down-regulation in the double-stress group and hypomethylation and up-regulation in the LPM570065 group. Interestingly, these 41 genes were largely associated with the regulation of potassium channels, G proteins, synapses, synaptic transmission, and oxytocin (**Supplementary Table S6**). However, with the samples from VTA tissue, no gene met the screening criteria after co-analysis, as such further studies were performed only with hippocampal tissue from these mice.

Validation of gene expression and methylation of depression susceptibility-related genes in the mouse hippocampus.

Among the 41 related genes, there were 8 depression-related genes (*Clasp1, Klf4, Oxtr, Adgra2, Sgms1, Kcna1, Zc3h12c*) (**Table 1**) with which genetic validation was performed in the double-stress group with low expression of hypermethylation and LPM570065 group with high expression of hypomethylation by RT-PCR and pyrophosphate sequencing. The results showed that the gene *OXTR* was hypo-expressed but hypermethylated in the double-stress group and hyper-expressed but hypomethylated in the LPM570065 group ($p < 0.05$, $p < 0.01$, $p < 0.001$, **Figures 6B,C**).

LPM570065 regulated the expression of DNA methyltransferases (DNMTs) in the mouse hippocampus.

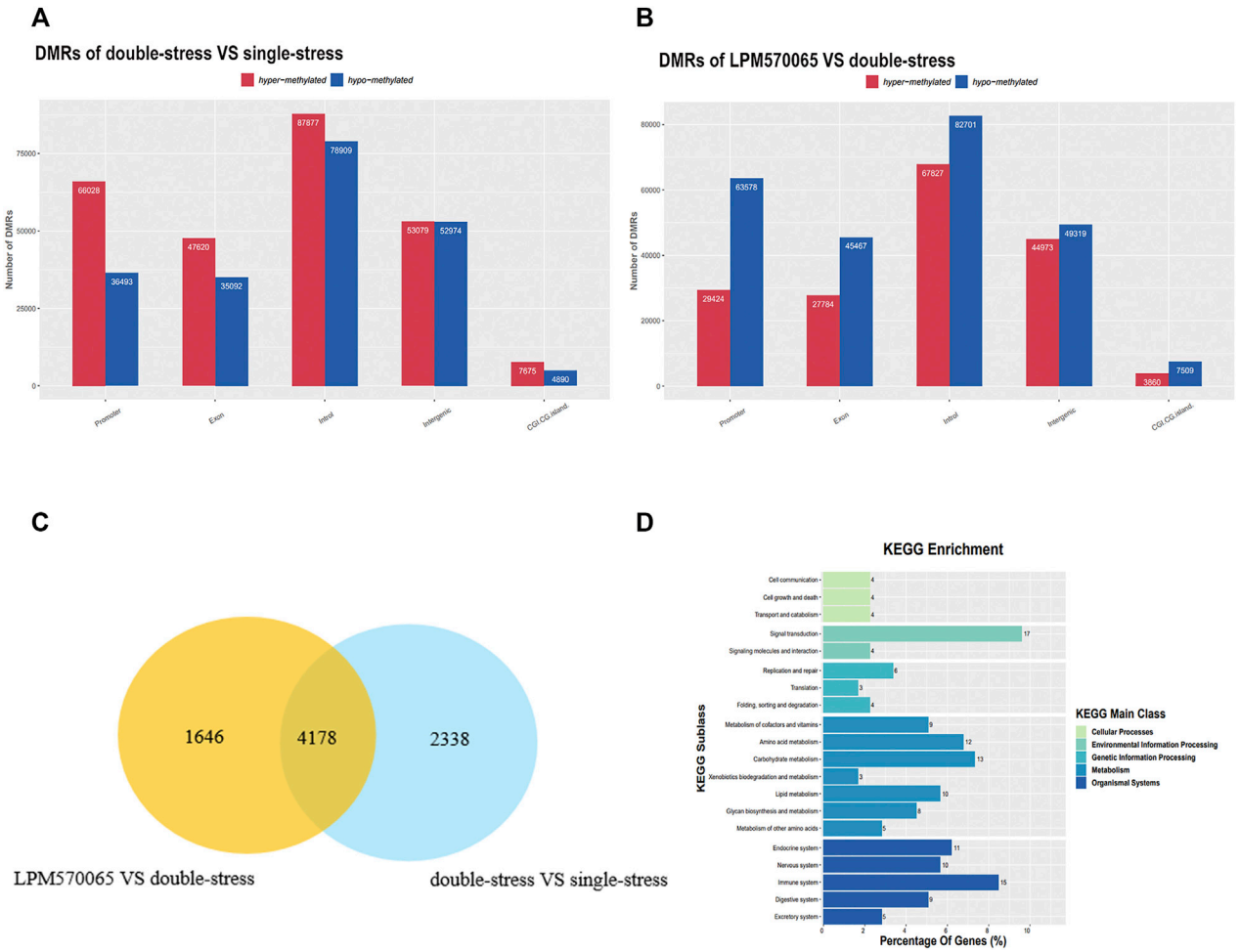

FIGURE 5 | RRBS sequencing for further screening of differential genes for methylation. **(A,B)** Representation in bar graphs of DMRs hypermethylation or hypomethylation in the hippocampus of double stress vs. single stress and LPM570065 vs. double stress mice. (n = 2/group, each two mice mixed as one sample, |Fold-change| > 1.5, $p < 0.05$). **(C)** Venn diagram of cluster analysis of the two compared groups identified DMRs between the two compared groups. **(D)** KEGG enrichment analysis, 17% of the genes were found to be enriched in the signaling pathway.

Methyltransferases (DNMT) play an important role in the maintenance of methylation in DNA replication and repair. To evaluate the effects of early life stress and LPM570065 on DNMTs, the protein expression levels of DNMT1 and DNMT3a were studied. The expression of both DNMT1 and DNMT3a proteins was significantly higher in the double-stress group compared to the single-stress group ($p < 0.001$) while the expression of both DNMT1 and DNMT3a proteins was significantly reduced after LPM570065 treatment ($p < 0.001$) (**Figure 7**).

DISCUSSION

Environmental factors following early traumatic stressful experiences are thought to be important triggers of behavioral abnormalities and psychiatric disorders. Early life stressors (especially ELS) can leave "scars" on the brain, leading to increased susceptibility to depression in later life through epigenetic mechanisms (Torres-Berrío et al., 2019). Although the effects of 5-HT reuptake inhibitors such as fluoxetine, escitalopram and other antidepressants have been studied in relation to their epigenetic mechanisms of antidepressant actions (Chmielewska et al., 2019), little is known of the epigenetic mechanisms of 5-HT/NE/DA triple reuptake inhibitor antidepressants in mediating their antidepressant actions. Results from this study show that when mice that experienced early life stress events (maternal-infant separation in this case) are exposed to social failure (repeated social defeat stress) in adulthood, their depression susceptibility was significantly increased, along with impaired neurogenesis and reduced hippocampal dendritic spine density. Importantly, we found that treatment with the triple reuptake inhibitor LPM570065 significantly improved the depression-related behavioral and morphological changes. Through epigenetic mechanistic analysis, we identified a differentially expressed

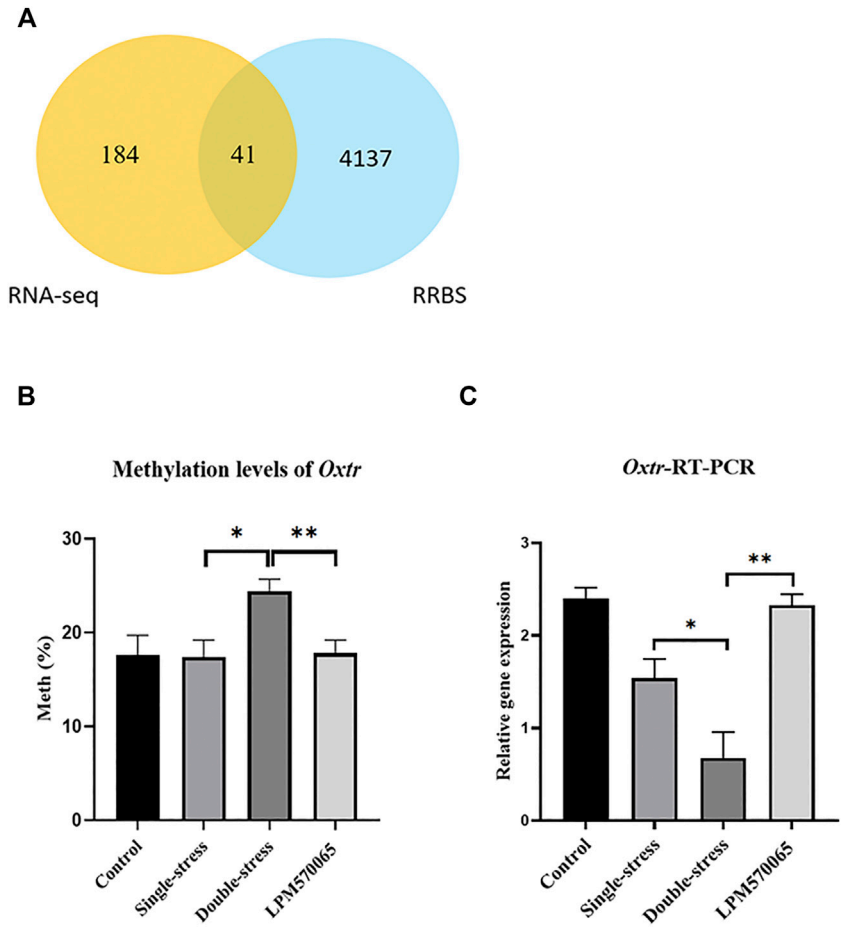

FIGURE 6 | Methylation and validation of DEGs. **(A)** Venn diagram of joint analysis of differential genes screened by RNA-seq and clustered with genes that underwent methylation in RRBS. **(B)** Methylation of gene-specific hippocampal *Oxtr* was assessed by pyrophosphate sequencing in each group of hippocampal tissues. **(C)** Validation of *Oxtr* expression in each group of hippocampal tissues by RT-qPCR. One-way ANOVA with Bonferroni multiple comparison correction. Data expressed as mean ± SEM, $n = 5–6$. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. Double stress group.

TABLE 1 | Eight genes exhibiting concordance between patterns of promoter hypermethylation or hypomethylation and gene downregulation or upregulation, respectively.

Gene id	GeneName	Sequencing	Double-stress VS single-stress			LPM570065 VS double-stress		
			log2FC	pValue	result	log2FC	pValue	result
ENSMUSG00000064302	Clasp1	RNA-seq	−1.11	0.0002	down	1.10	0.0032	up
		RRBS	2.28	5.09E-11	hyper-methylated	−0.606	0	hypo-methylated
ENSMUSG00000063142	Kcnma1	RNA-seq	−1.13	0.0006	down	0.93	0.0014	up
		RRBS	1.58	0.0002	hyper-methylated	−1.631	1.78E-07	hypo-methylated
ENSMUSG00000003032	Klf4	RNA-seq	−1.20	0.0005	down	0.75	0.0372	up
		RRBS	3.62	4.55E-49	hyper-methylated	−4.616	2.26E-67	hypo-methylated
ENSMUSG00000049112	Oxtr	RNA-seq	-0.92	0.0032	down	0.84	0.0123	up
		RRBS	2.21	1.05E-14	hyper-methylated	−2.087	8.27E-12	hypo-methylated
ENSMUSG00000031486	Adgra2	RNA-seq	−1.00	4.93E-6	down	0.84	0.0003	up
		RRBS	0.807	0	hyper-methylated	−0.921	0.0037	hypo-methylated
ENSMUSG00000040451	Sgms1	RNA-seq	−0.79	0.0281	down	0.76	0.0204	up
		RRBS	3.59	1.14E-71	hyper-methylated	−2.920	2.59E-62	hypo-methylated
ENSMUSG00000047976	Kcna1	RNA-seq	−1.01	0.0026	down	0.81	0.0098	up
		RRBS	1.17	3.37E-10	hyper-methylated	−0.599	0.0000747718836970886	hypo-methylated
ENSMUSG00000035164	Zc3h12c	RNA-seq	−0.80	0.0136	down	0.72	0.0079	up
		RRBS	0.84	0	hyper-methylated	−1.420	3.56E-06	hypo-methylated

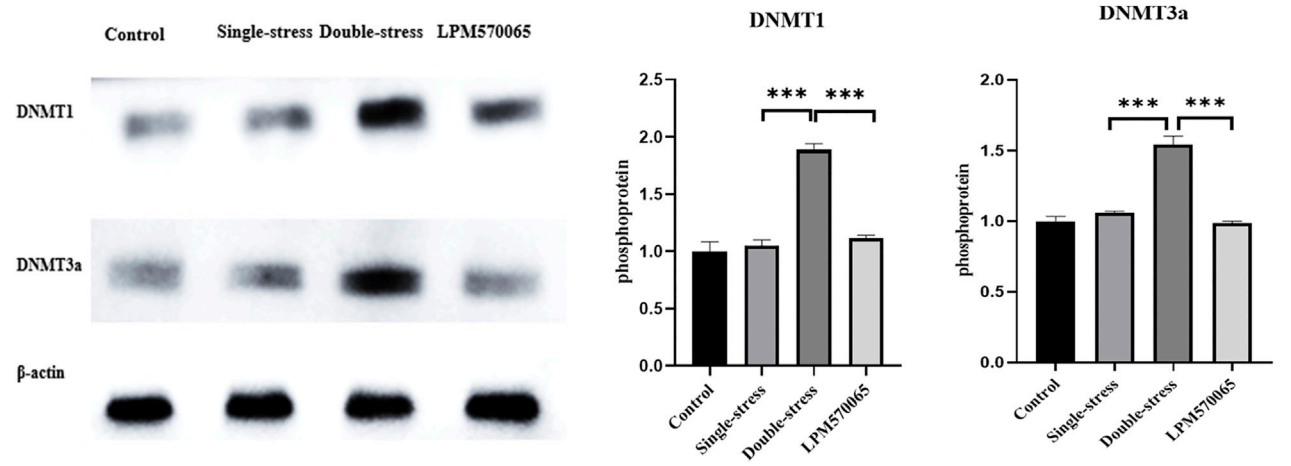

FIGURE 7 | DNMT1 and DNMT3a protein expression in hippocampus. One-way ANOVA with Bonferroni multiple comparison correction. Data are normalized to controls and are expressed as means ± SEM (n = 8/group) ***p < 0.001 vs. Double stress group.

methylation gene, *Oxtr*, in the mouse hippocampus. *Oxtr* expression was reduced in mice experiencing double stress and this was negatively correlated with the degree of DNA methylation. Importantly, LPM570065 was able to significantly ameliorate depression susceptibility in mice experiencing double stress and this effect was at least partially mediated by reversing the methylation of *Oxtr*. Together, this study extended previous results of the antidepressant-like activity of the triple reuptake inhibitor LPM570065 by revealing a novel epigenetic mechanism of antidepressant actions of this new and potentially important antidepressant drug.

Clinical studies have shown that early life stress increases the incidence of depression later in life (Williams et al., 2016). Preclinical studies have used the combination of early life stress with secondary stress event in adulthood to create a behavioral phenotype that mimics human depression vulnerability (Han et al., 2019). For example, experiencing the "first hit" of maternal separation stress during early life makes an individual more vulnerable to the "second hit" of stressful events in adulthood such as repeated social defeat stress (Peña et al., 2017). In the present study, we adapted this "two-hit" model of depression vulnerability to evaluate the antidepressant-like effects of the triple reuptake inhibitor LPM570065. This model incorporated the early life stress event of maternal separation with the later life stressful event of repeated social defeat and this created a behavioral phenotype that mimics many aspects of human depression symptoms such as anhedonia and social despair (Peña et al., 2017). Our previous studies have shown that LPM570065 did not produce statistically significant changes in voluntary locomotor activity in unstressed mice and rats. (**Supplementary Tables S7, S8**). However, in TST and FST, LPM570065 administration decreased the immobility time in unstressed mice and rats (**Supplementary Tables S9, S10**). Indeed, the stressed mice demonstrated significantly reduced sucrose preference and increased immobility in the SPT, FST and TST assays. Under this situation, LPM570065 treatment demonstrated significant protective efficacy such that for all the behavioral measures the mice subjected to double stress were not different from the control mice that never experienced the stress. This suggests that LPM570065 was able to protect against impact of stressful events on behavioral normality and ameliorate depression vulnerability. This finding adds to the previous preclinical and clinical studies supporting the antidepressant efficacy of LPM570065 and suggests that triple reuptake inhibitors in general, and LPM570065 in particular, could be useful new tools to combat against depression.

In an effort to interrogate the potential mechanisms of antidepressant actions of LPM570065beyond its direct monoaminergic reuptake inhibition effects, we examined its effects on neuroplastic and epigenetic changes induced by stressful events. Neuroplasticity is a fundamental mechanism of neuronal adaptation, and chronic stress can induce or exacerbate depression and disrupt neuroplasticity (Pittenger and Duman, 2008). Altered dendritic spines are strongly associated with depression (Ménard et al., 2016; Ammala et al., 2019; Huang et al., 2019). The CA1 region in the hippocampus has been one of the most extensively studied brain regions in depression research (Ma et al., 2021). Patients with MDD show a marked reduction in left CA1 volume (Roddy et al., 2018). Our results show that dendritic spine density in the hippocampal CA1 region is markedly reduced in the double stress model compared to the single stress model, which may be due to the early maternal-infant separation stress prior to the exposure of social defeat stress in adulthood. Most importantly, we found that LPM570065 treatment prevented the dendritic spine density decrease. These results suggest that LPM570065 may be able to prevent deleterious synaptic plasticity maladaptation and subsequently reduce the development and demonstration of depressive-like behaviors.

RNA-seq technology provides crucial information on depression-related pathways and regulatory mechanisms, and RBSS is able to identify DNA methylation patterns associated

with specific genes in the brain. Here we adopted a combination of RNA-seq and RRBS to search for DEGs in the mouse hippocampus. This effort led to the identification of *Oxtr*, a gene known to be closely associated with early parental care, depression and their interactions (Cataldo et al., 2018), the promoter methylation status of which was negatively correlated with the observed gene expression pattern. Here we found that *Oxtr* was a DEG in the hippocampus between mice experiencing double stress (maternal separation and repeated social defeat) and those only experienced single stress (repeated social defeat). This is consistent with the established relationship between *Oxtr* expression and early parental care (Cataldo et al., 2018). Importantly, LPM570065 was able to reverse this methylation status, suggesting that the antidepressant efficacy of LPM570065 may be associated with its effect on *Oxtr* gene alterations.

Oxtr contains seven transmembrane domains and belongs to the class 1 family of G protein-coupled receptors. The oxytocinergic system plays a key role not only in shaping social behaviors (e.g., trust, social support) but also in regulating responses to stressors (Olff et al., 2013). There is growing evidence that the central 5-HT and oxytocin (OT) systems are closely related and that 5-HT may affect social behavior (e.g., socialization, aggression, depression) through OT release. Studies also show that DA induces OT release (Melis et al., 1989) and that OT-DA interactions are mediated by specific types of DA receptors (D2-DA receptor) in the regulation of social bonding (Liu and Wang, 2003). There is also evidence supporting the interaction between NE and oxytocin, with oxytocin release being regulated by NE in the hypothalamic-neurophysical system (Salmina et al., 2010). Collectively, here we hypothesize that increased *Oxtr* methylation may contribute to the maintenance of early life stress-induced depression susceptibility in adult mice, and that LPM570065, a novel and potent 5-HT/NE/DA triple reuptake inhibitor, may exert its antidepressant effects by reducing *Oxtr* methylation to reverse depression susceptibility.

Methyltransferases (DNMT) play an important role in maintaining methylation in DNA replication and repair (Lyko, 2018). Changes in DNMT expression may be mechanistically related to the changes in DNA methylation in the promoter regions of stress- and depression-related genes (Boersma et al., 2014) such as P11 (Melas et al., 2012). Here we detected a significant increase in the protein expression levels of DNMT1 and DNMT3a in the mice from double stress group, which showed a positive correlation with the methylation levels of the gene *Oxtr*, and, interestingly, LPM570065 treatment reduced the expression of DNMT1 and DNMT3a. These results suggest that LPM570065 might reverse the methylation of *Oxtr* and reduce depression susceptibility in the mice by modulating the expression of DNMT1 and DNMT3a.

In conclusion, our findings suggest that methylation specificity of the gene *Oxtr* may play an important role in early life stress-induced susceptibility to depression in adult mice. LPM570065, a novel 5-HT/NE/DA triple reuptake inhibitor, can reverse the methylation of gene *Oxtr*, thus reducing the susceptibility to depression in mice with experience of early life stress. These results extend previous preclinical and clinical studies of the demonstrated antidepressant efficacy and further support that LPM570065 could be a useful therapy for MDD.

DATA AVAILABILITY STATEMENT

The data presented in the study are deposited in the NCBI SAR repository, accession number PRJNA801464.

ETHICS STATEMENT

The animal study was reviewed and approved by All animal protocols were approved by the Laboratory Animals Care and Use Committee of Yantai University.

AUTHOR CONTRIBUTIONS

PM: Methodology, Software, Validation, Formal analysis, Investigation, Data curation, Writing—original draft, Writing—review and editing, Visualization. CL: Conceptualization, Methodology, Formal analysis, Investigation, Writing—review and editing, Supervision. SD: Methodology, Investigation, Data curation. SJ: Methodology, Data curation. YX: Methodology, Data curation. YM: Methodology, Data curation. HW: Formal analysis, Resources, Writing—review and editing, Supervision. JT: Conceptualization, Resources, Project administration.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2022.848251/full#supplementary-material

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SYSTEMATIC REVIEW
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Investigational Drugs for the Treatment of Depression (Part 1): Monoaminergic, Orexinergic, GABA-Ergic, and Anti-Inflammatory Agents

Octavian Vasiliu *

Department of Psychiatry, Dr.Carol Davila University Emergency Central Military Hospital, Bucharest, Romania

Therapeutic management of depression has currently important limitations, and its low efficacy is reflected in high rates of non-response even after multiple trials of antidepressants. Almost two-thirds of the patients diagnosed with major depression who received a 4–6 weeks trial of antidepressant could not reach remission, and more than 30% of these patients are considered treatment-resistant. In bipolar depression, the situation is also discouraging if we analyze the high suicide rate, the risk for the treatment-emergent affective switch when antidepressants are added, the high rate of treatment resistance (up to 25%), and the severe functional impairments associated with these episodes. Therefore, new therapeutic agents are needed, as well as new pathogenetic models for depression. The vast majority of the currently approved antidepressants are based on the monoamine hypothesis, although new drugs exploiting different neurotransmitter pathways have been recently approved by FDA. Brexanolone, an allopregnanolone analog, is an example of such new antidepressants, and its approval for post-partum depression inspired the search for a new generation of neurosteroids and GABA-ergic modulators, with an easier way of administration and superior tolerability profile. Orexin receptors antagonists are also extensively studied for different psychiatric disorders, depression included, in phase II trials. Antiinflammatory drugs, both cyclo-oxygenase 2 inhibitors and biological therapy, are investigated in patients with depressive disorders based on the proven correlation between inflammation and mood disorders in preclinical and clinical studies. Also, a new generation of monoamine-based investigational drugs is explored, ranging from triple reuptake inhibitors to atypical antipsychotics, in patients with major depression. In conclusion, there is hope for new treatments in uni- and bipolar depression, as it became clear, after almost seven decades, that new pathogenetic pathways should be targeted to increase these patients' response rate.

Keywords: treatment-resistant depression, brexanolone, immunomodulators, orexin receptors antagonists, triple monoamines reuptake inhibitors, atypical antipsychotics

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Anna Tabęcka-Łonczynska, University of Information Technology and Management in Rzeszow, Poland

Reviewed by:
Mauricio Schuler Nin, Federal University of Rio Grande, Brazil
Najah Leslie Walton, Tufts University School of Medicine, United States

***Correspondence:**
Octavian Vasiliu
octavvasiliu@yahoo.com

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INTRODUCTION

The exploration of therapeutic options for major depressive disorder (MDD) is very important for clinicians, due to the significant functional impairment, high rate of relapse, and treatment resistance associated with this pathology (de Sousa et al., 2015; Lacerda, 2020). The worldwide prevalence of MDD is estimated to be around 16%, and remission is obtained by only one-third of these patients (de Sousa et al., 2015; Lacerda, 2020). The pathophysiology of MDD is still largely unknown, and the monoamine hypothesis remains the most explored explanation supported by data from animal models and human trials (de Sousa et al., 2015). Although large efforts have been invested in the research of neurobiologically oriented treatments for MDD, only a few products have been FDA-approved outside the conceptual framework of the monoamine hypothesis. These exceptions are brexanolone, for post-partum depression, and esketamine for treatment-resistant MDD. However, multiple pathogenetic mechanisms have been investigated, from dysfunctions in the orexinergic or γ-aminobutyric acid (GABA) neurotransmission to glutamatergic, opioidergic, or sestrin modulators (Schüle et al., 2014; Poleszak et al., 2016; Fogaça et al., 2019; Sengupta et al., 2019; Han et al., 2020). A new generation of monoaminergic agents has been studied, e.g. new triple monoaminergic inhibitors and new atypical antipsychotics (Fava et al., 2019; Mi et al., 2021). Old drugs have been repurposed as antidepressants or adjuvants to current antidepressant treatment, in the hope of finding new ways to mitigate residual symptoms or to increase the chance of reaching a response/remission in treatment-resistant MDD patients (Vasiliu et al., 2017; Kalmoe et al., 2020; Papakostas et al., 2020). Combining different classes of pharmacological agents in one formulation is another explored strategy to increase the potency of the antidepressant treatment. This approach is based on reciprocal augmentation of different drugs' pharmacodynamical properties, mitigating the risk of certain adverse events to one drug by adding another, or exploiting their distinct pharmacokinetic properties to increase the plasma concentration of a specific agent (Thase et al., 2019; Sakurai et al., 2022).

The limited efficacy of currently marketed antidepressants is only one of the challenges that clinicians are facing, another important aspect of the therapeutic management being the low tolerability profile of several drugs, pharmacokinetic interactions at CYP450 isoenzymes with concomitantly administered medications for comorbid disorders, long duration until the antidepressant effect onset, the necessity of long-term drugs administration, *etc.* (Vasile et al., 2011; Vasiliu, 2019).

Therefore, this review has as its main objective to explore new investigational products with antidepressant properties, considering their phase of development, their reported efficacy and tolerability, and their contribution to the construction of a new pathogenetic model of depression.

METHODOLOGY

A systematic review of the papers referring to new drugs in different phases of clinical research was conducted through the main electronic databases (PubMed, MEDLINE, Cochrane, Web of Science (Core Collection), PsychINFO, Scopus, and EMBASE using the paradigm "investigational antidepressants/products" OR "new antidepressants/agents" AND "clinical trial" AND "major depressive disorder" OR "bipolar disorder" OR "depression." Lists of references for every article corresponding to the search paradigm were investigated, and they were added to the review if they were not detected through the previously mentioned paradigm.

A broad search was chosen to include the widest variety of molecules corresponding to the review's objective. For this purpose, a supplementary search was added, targeting investigational products for depression explored in the clinical trials repositories run by the United States National Library of Medicine and the National Institutes of Health (clinicaltrials.gov), World Health Organisation (International Clinical Trials Registry Platform), and European Union (EU Clinical Trial Register). The search within the clinical trial databases was structured by the disorder- "depression" (both unipolar and bipolar), type- "interventional," population- "adults" and "adolescents," and trial phase from I to III, but all statuses of recruitment were allowed. If the outcome of a registered trial for an investigational product was not mentioned in any of the explored repositories, the respective drug manufacturer's site was explored, to verify if any results are available.

All papers and references from the previously mentioned electronic databases were allowed in the primary search, if they were published between January 2000 and February 2022. Data regarding clinical studies found in the repositories were also included in the primary search.

This systematic review is based on the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) statement, and all the data collection, review, reporting, and discussion were conducted according to this statement (**Figure 1**) (Moher et al., 2009). The inclusion and exclusion criteria are presented in **Table 1**. The PRIMA-P Checklist (Moher et al., 2015) is presented in **Table 2**.

All pharmacological agents included in the collected data were grouped into nine categories: monoamine-based drugs, orexin receptors modulators, GABA-A receptors modulators and neurosteroid analogs, anti-inflammatory therapies, glutamatergic antidepressants, sestrin modulators, cholinergic agents, combinations of agents, and a residual category for all other molecules with distinct mechanisms of action. In this part of the review, only the first four categories will be analyzed.

RESULTS

Seven investigational products with a monoamine-based mechanism of action were found in 25 references, including one phase I study, 13 phase II, one phase II/III, and eight phase III trials. One orexin receptor-modulator had been identified in three references, including two phase I studies and one phase II trial. Four neurosteroid analogs or GABA-A receptor modulators have been identified in 13 references, including one phase I study, six phase II, one phase II/III, four

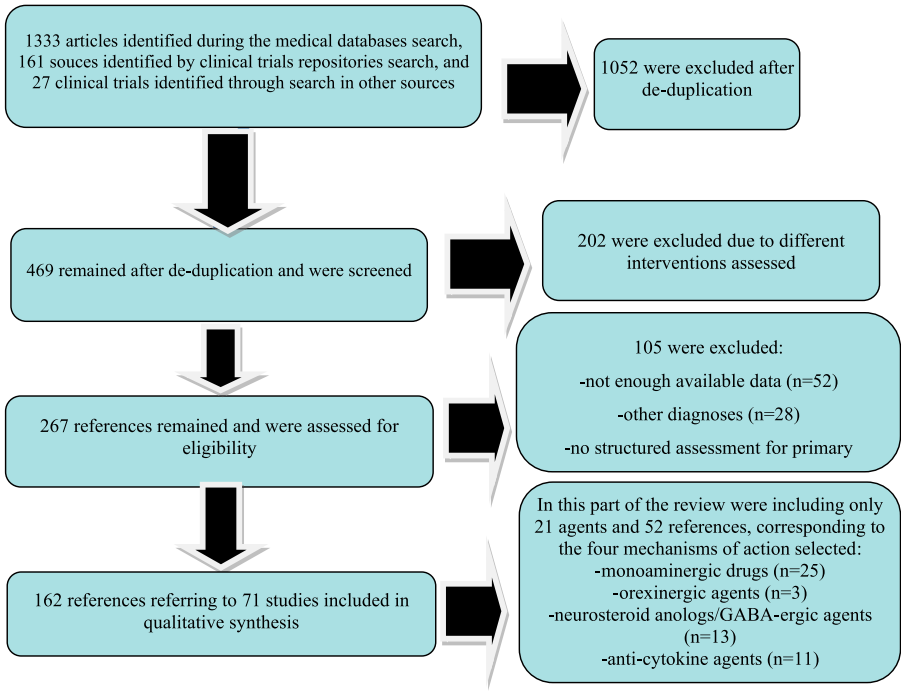

FIGURE 1 | Results of the PRISMA-based search paradigm.

TABLE 1 | Inclusion and exclusion criteria.

Operational criteria	Inclusion criteria	Exclusion criteria
Population	Selected population groups were allowed—adolescents and adults. No superior age limit was specified. The main diagnoses were major depressive disorder and bipolar depression. Treatment-resistant forms, first mood episodes, or chronic depression were included. Chronic organic co-morbidities were allowed. Diagnoses should be based on criteria specified by the authors of that paper/sponsors of the trial. Both ICD10 and DSM (IV, IV-TR, or 5) diagnosis criteria were allowed	Studies that did not specify age limits for their samples, and studies that enrolled children. The presence of psychiatric comorbidities with significant impact on cognition, mood, behavior, and overall functionality (e.g., psychotic disorders, severe neurocognitive disorders, substance use disorders)
Intervention	Pharmacological, or combined, pharmacological and psychotherapeutic interventions. New investigational drugs, or repurposed drugs for antidepressant use, were included. Only monoaminergic, orexinergic, GABA-ergic/neurosteroids, and anti-inflammatory agents are included in this part of the review	Psychotherapy as monotherapy for MDD/bipolar depression. Already marketed antidepressants, FDA-approved for all the indications specified in the "population" section of this table, if they were the main intervention. These types of agents were allowed only as active comparators
Environment	Both in-patient and out-patient regimens	Unspecified environment
Primary and secondary variables	Evaluation of the efficacy, safety, and tolerability of new investigational drugs with antidepressant properties	All research with unspecified variables. Reviews without predefined quantifiable objectives, or poorly defined primary outcome measures
Study design	Any phase of clinical investigation from I to III was admitted if it corresponded to the predefined objective of this review. Phase IV studies were permitted, if specific variables related to depression were included, for drugs not approved for this indication	Studies with unspecified or poorly defined design. Studies with unclearly defined population/statistical methods. Case reports, case series
Language	Any language of publication was admitted if the *in-extenso* published paper was available. The same language criteria were applied for clinical trials identified in metadata repositories	—

phase III, and one not assessed for a clinical phase trial. Eight anti-cytokine therapy and one COX-2 inhibitor have been identified in 11 sources, and their anti-depressive properties have been explored in one phase I study, five phase II, seven phase III, and two phase IV trials. All these agents and their mechanisms of action are presented in **Figure 2**.

TABLE 2 | PRISMA-P 2015 Checklist (Moher et al., 2015). This checklist has been adapted for use with protocol submissions to Systematic Reviews from **Table 3** in Moher D et al: Preferred reporting items for systematic review and meta-analysis protocols (PRISMA-P) 2015 statement. Systematic Reviews 2015 4:1.

Section/topic	#	Checklist item	Information reported		Line number(s)
			Yes	No	
Administrative information					
Title					
Identification	1a	Identify the report as a protocol of a systematic review	☒	☐	68–74
Update	1b	If the protocol is for an update of a previous systematic review, identify it as such	☐	☐	Not applicable
Registration	2	If registered, provide the name of the registry (e.g., PROSPERO) and registration number in the Abstract	☐	☐	Not applicable
Authors					
Contact	3a	Provide the name, institutional affiliation, and e-mail address of all protocol authors; provide the physical mailing address of the corresponding author	☒	☐	4–9
Contributions	3b	Describe contributions of protocol authors and identify the guarantor of the review	☐	☐	Not applicable, only one author
Amendments	4	If the protocol represents an amendment of a previously completed or published protocol, identify it as such and list changes; otherwise, state a plan for documenting important protocol amendments	☐	☐	Not applicable
Support					
Sources	5a	Indicate sources of financial or other support for the review	☒	☐	755
Sponsor	5b	Provide a name for the review funder and/or sponsor	☐	☐	Not applicable
Role of sponsor/funder	5c	Describe roles of funder(s), sponsor(s), and/or institution(s), if any, in developing the protocol	☐	☐	Not applicable
Introduction					
Rationale	6	Describe the rationale for the review in the context of what is already known	☒	☐	14–63
Objectives	7	Provide an explicit statement of the question(s) the review will address concerning participants, interventions, comparators, and outcomes (PICO)	☒	☐	64–66, **Table 1**
METHODS					
Eligibility criteria	8	Specify the study characteristics (e.g., PICO, study design, setting, time frame) and report characteristics (e.g., years considered, language, publication status) to be used as criteria for eligibility for the review	☒	☐	68–96, **Table 1**
Information sources	9	Describe all intended information sources (e.g., electronic databases, contact with study authors, trial registers, or other grey literature sources) with planned dates of coverage	☒	☐	69–70, 77–80
Search strategy	10	The present draft of the search strategy is to be used for at least one electronic database, including planned limits, such that it could be repeated	☒	☐	68–96
Study records					
Data management	11a	Describe the mechanism(s) that will be used to manage records and data throughout the review	☒	☐	92–96
Selection process	11b	State the process that will be used for selecting studies (e.g., two independent reviewers) through each phase of the review (i.e., screening, eligibility, and inclusion in meta-analysis)	☒	☐	68–74, **Table 1**
Data collection process	11c	Describe the planned method of extracting data from reports (e.g., piloting forms, done independently, in duplicate), and processes for obtaining and confirming data from investigators	☒	☐	82–84
Data items	12	List and define all variables for which data will be sought (e.g., PICO items, funding sources), any pre-planned data assumptions, and simplifications	☒	☐	**Table 1**
Outcomes and prioritization	13	List and define all outcomes for which data will be sought, including prioritization of main and additional outcomes, with rationale	☒	☐	**Table 1**
Risk of bias in individual studies	14	Describe anticipated methods for assessing the risk of bias of individual studies, including whether this will be done at the outcome or study level, or both; state how this information will be used in data synthesis	☒	☐	82–84
DATA					
Synthesis	15a	Describe criteria under which study data will be quantitatively synthesized	☐	☒	—
	15b	If data are appropriate for quantitative synthesis, describe planned summary measures, methods of handling data, and methods of combining data from studies, including any planned exploration of consistency (e.g., I^2, Kendall's tau)			—
	15c	Describe any proposed additional analyses (e.g., sensitivity or subgroup analyses, meta-regression)	☐	☒	—
	15d	If quantitative synthesis is not appropriate, describe the type of summary planned	☒	☐	92–96
Meta-bias(es)	16	Specify any planned assessment of meta-bias(es) (e.g., publication bias across studies, selective reporting within studies)	☐	☒	—
Confidence in cumulative evidence	17	Describe how the strength of the body of evidence will be assessed (e.g., GRADE)	☐	☒	—

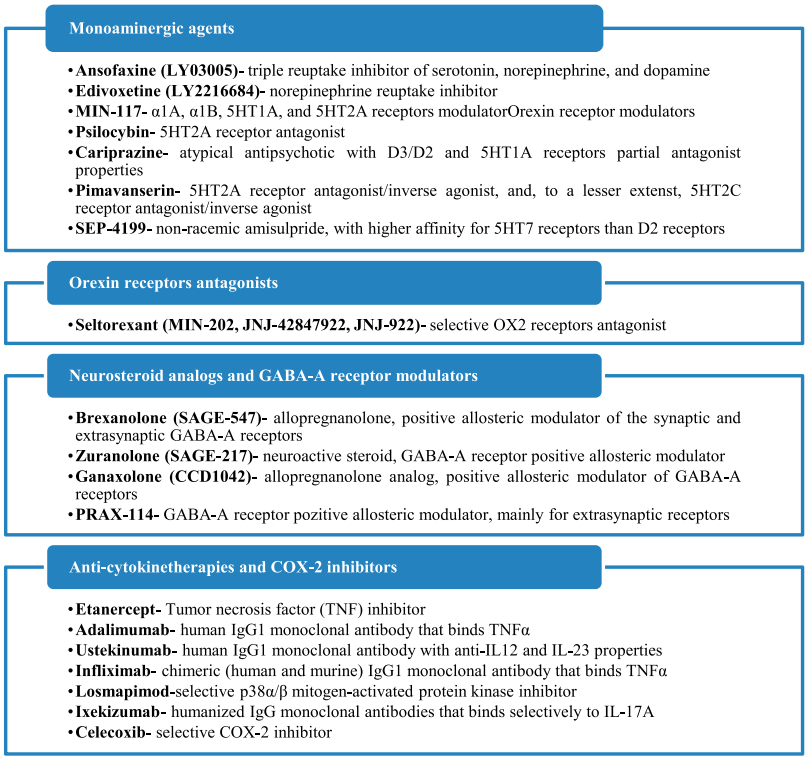

FIGURE 2 | Mechanisms of action of the identified antidepressants in the pipeline, which are presented in this review.

INVESTIGATIONAL DRUGS WITH MONOAMINERGIC MECHANISM OF ACTION

In this section different molecules have been included, based on their common property of modulating one or more monoamine systems, classically involved in the pathogenesis of mood disorders (serotonin, dopamine, noradrenaline). *New antidepressants* that possess the ability to inhibit monoamine reuptake, *psilocybin* (which antagonizes 5HT2A receptors), *new atypical antipsychotics* (cariprazine, lumateperone), and *non-racemic amisulpride* (SEP-4199) are analyzed from their efficacy and tolerability perspective in patients with depressive disorders (**Table 3**).

Ansofaxine (LY03005) is a potential triple reuptake inhibitor of serotonin, norepinephrine, and dopamine, orally administered as an extended-release tablet (Mi et al., 2021). A multicenter, randomized, double-blind, placebo-controlled, dose-finding, phase II clinical trial, conducted in China, enrolled adult MDD patients (N = 255) who were randomly assigned to receive fixed dose of ansofaxine (40, 80, 120, or 160 mg/day) or placebo for 6 weeks (Mi et al., 2021). Significant differences were found in the mean HAMD-17 total score changes at week 6 in all the active intervention groups vs placebo, and the overall tolerability of the drug was good (Mi et al., 2021). Treatment-related adverse events occurred in 141 patients, with an incidence of 52, 65.4, 56.8, 62.7, and 38.7% in the 40, 80, 120, 160 mg and placebo groups, respectively (Mi et al., 2021).

Another randomized, multicenter, double-blind, placebo-controlled, phase III study evaluated the efficacy and safety of ansofaxine hydrochloride extended-release tablets in 558 Chinese adult patients diagnosed with MDD (National Library of Medicine [NLM], NCT04853407). According to the manufacturer's site, the results of this trial showed that LY03005 (80 mg or 160 mg/day) was safe and effective at week 8, with statistically significant improvements in both primary (Montgomery Asberg Depression Rating Scale- MADRS total score) and secondary (Hamilton Depression Rating Scale-HAMD-17, Clinical Global Impression- CGI, Hamilton Anxiety Rating Scale- HAM-A, HAMD-17 Anxiety/Somatization factor, HAMD-17 Cognitive Impairment Factor, HAMD-17 Blocking factor, MADRS Anhedonia Factor Score and Sheehan Disability Scale total score) endpoints vs placebo (Luye Pharma, 2021). No serious adverse events occurred during this trial, and common adverse events (>5% incidence) in the LY03005 group were nausea, vomiting, headache, and drowsiness (NLM, NCT04853407).

Edivoxetine (LY2216684) is a highly selective norepinephrine reuptake inhibitor investigated for the treatment of MDD, as an adjunctive agent to the current antidepressant (Ball et al., 2016). Analysis of data derived from three randomized, phase III, 8-week, placebo-controlled trials, with a 3-week double-blind placebo lead-in phase, that evaluated the efficacy of edivoxetine (6–18 mg/day) as an adjunctive treatment for patients with MDD and partial response to SSRIs did not support a significant improvement in the clinical status of these patients (701, 689, and 449 participants) (Ball et al.,

TABLE 3 | Monoaminergic modulators with antidepressant properties in the pipeline.

Authors/Trial sponsor	Methodology	Results	Clinical trial phase, trial identifier (if available)
Mi et al. (2021)	Ansofaxine (LY03005), DBRCT, *N* = 255, MDD, 6 weeks	HAMD-17 total score changes at week 6 were significant vs placebo. The overall tolerability was good	Phase II, NCT03785652
Luye Pharma (2021), NLM (2021a)	Ansofaxine, DBRCT, *N* = 58, MDD, 8 weeks	MADRS total score, HAMD-17 total score, CGI, HAMA, HAMD-17 Anxiety/Somatization factor, Cognitive Impairment factor, Blocking factor, MADRS Anhedonia factor, SDS total score—all were statistically significant improved vs placebo at week 8. No SAE occurred during this trial. Nausea, vomiting, headache, and drowsiness were the most commonly reported adverse events	Phase III, NCT04853407
Ball et al. (2016)	Edivoxetine (LY2216684) adjunctive to the ongoing antidepressant regimen, three DBRCT, *N* = 701, 689, and 449, MDD with partial response to SSRI, 8 weeks	The mean outcome was the mean change from baseline to week 8 in the MADRS total score. This outcome was not reached by any of these three trials. Most of the secondary objectives were not reached, either	Phase III, NCT01173601 Phase III, NCT01187407 Phase III, NCT01185340
Oakes et al. (2015)	Edivoxetine, *N* = 1249, MDD, 8 weeks open-label (edivoxetine + SSRI) + open-label 12 weeks stabilization period + DBRCT 24 weeks	No significant difference between edivoxetine and placebo was detected at the end of the trial (evaluated by MADRS total score)	Phase III, NCT01299272
Ball et al. (2014)	Edivoxetine /placebo adjunctive to SSRI, DBRCT, *N* = 131, MDD partial responsive to SSRI, 10 weeks	No significant differences in efficacy between groups at the end of the trial, based on the MADRS total score	Phase II, NCT00840034
Pangallo et al. (2011)	Edivoxetine, DBRCT, *N* = 495, MDD, 10 weeks	MADRS scores were improved significantly by edivoxetine vs placebo at week 10. Higher rates of response and remission were higher with edivoxetine. SDS scores also were significantly improved vs placebo	Phase II/III, NCT00795821
Ball et al. (2015)	Edivoxetine as adjunctive to SSRI, open-label, *N* = 328, MDD with partial response to SSRI, 54 weeks	The study discontinuation rate due to adverse events was 17%, 13 SAE (1 death). Most commonly reported adverse events: nausea, hyperhidrosis, constipation, headache, dry mouth, dizziness, vomiting, insomnia, upper respiratory tract infection. Mean MADRS score improvements were −17.0 at week 54	Phase III, NCT01155661
Davidson et al. (2016)	MIN-117 vs placebo vs paroxetine, DBRCT, *N* = 84, moderate-to-severe MDD, 6 weeks	MADRS total score was improved by MIN-117 vs placebo at week 6. Remission with MIN-117 was achieved by 24% of patients (2.5 mg investigational product). The overall tolerability was good	Phase II, EudraCT 2015-000306-18
National Library of Medicine (2018)	MIN-117, DBRCT, *N* = 360, adult MDD patients, 6 weeks	No significant differences between active drug and placebo were detected by MADRS, HAMA, and CGI-S scores evolution	Phase II, NCT03446846
Carhart-Harris et al. (2021)	Psilocybin vs escitalopram, DBRCT, *N* = 59, moderate-to-severe MDD, 6 weeks	QIDS-SR scores at week 6 were not significantly changed vs placebo. Response rate 70% (psilocybin) vs 48% (placebo)	Phase II, NCT03429075
Griffiths et al. (2016)	Psilocybin, DBRCT, cross-over trial, *N* = 51 cancer patients + depression + anxiety, 5 weeks + 6 months follow-up	GRID-HAMD-17 and HAM-A scores were decreased by high-dose psilocybin. Quality of life, life meaning, and optimism scores improved, and	Phase II, NCT00465595

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TABLE 3 | (*Continued*) Monoaminergic modulators with antidepressant properties in the pipeline.

Authors/Trial sponsor	Methodology	Results	Clinical trial phase, trial identifier (if available)
		death anxiety decreased under psilocybin treatment. At 6 months these changes persisted, 80% of these patients presented clinically significant decreases in depressed mood and anxiety scores	
Ross et al. (2016)	Psilocybin vs niacin + psychotherapy, DBRCT, *N* = 29 patients with cancer-related anxiety and depression, 7 weeks, cross-over design	Rapid and sustained improvements in anxiety and depression before crossover, plus decreases in cancer-related demoralization and hopelessness, improvements in spiritual well-being, and quality of life. At the follow-up visit (6.5 months) consistent anxiolytic and antidepressant effects were present in the psilocybin group	Phase I, NCT00957359
Carhart-Harris et al. (2016)	Psilocybin + psychological support, open-label, *N* = 12, moderate-to-severe, treatment-resistant MDD, 3 months	The mean self-rated intensity of psilocybin effects was dose-related, and the drug was well tolerated by all patients. Depressive symptoms were markedly reduced at 1 week and 3 months compared to baseline, after high-dose treatment. Anhedonia and anxiety were markedly improved, also	Phase II, ISRCTN14426797
Davis et al. (2021)	Psilocybin, DBRCT, *N* = 24, MDD + psychotherapy, 4 weeks	The mean GRID-HAMD scores were significantly lower in the immediate treatment group, and the QIDS-SR scores reflected a rapid decrease in mean depression score after the first session, which remained significant up to week 4. In the overall sample, 71% of the participants had week 1 and week 4 clinically significant responses to the intervention. The remission rate was 58% at week 1 and 54% at week 4	Phase II, NCT03181529
COMPASS, (2021)	Psilocybin + psychological support, DBRCT, *N* = 233, treatment-resistant MDD, 4 weeks	The high dose drug (25 mg) induced a significant decrease in MADRS scores vs inactive dose after day 1, and these improvements persisted after week 3, but the difference between the low dose (10 mg) group and the control group was not significant	Phase IIb, NCT03775200
Fava et al. (2018)	Cariprazine (low doses/high doses) adjunctive to antidepressant, DBRCT, *N* = 231, treatment-resistant MDD, 19 weeks	No differences were reported on any measures between low doses of cariprazine and placebo, and higher doses led to numerically greater mean change in MADRS and CGI-I scores. MADRS response and remission rates were higher vs placebo, but without reaching statistical significance. The overall tolerability was good	Phase II, NCT00854100
Durgam et al. (2016)	Cariprazine (low doses/high doses) adjunctive to antidepressants, DBRCT, *N* = 269, treatment-resistant MDD, 8 weeks	Reductions in MADRS total score at week 8 were significantly greater for the high dose of cariprazine vs placebo, but not for the low dose. Treatment-emergent adverse events most commonly reported were akathisia, insomnia, and nausea	Phase II, NCT01469377
Earley et al. (2018)	Cariprazine adjunctive to antidepressants, DBRCT, *N* = 530, 8 weeks	Cariprazine did not significantly improve MADRS total score or SDS score vs placebo. A nonsignificant decrease in depressive symptoms	Phase III, NCT01715805

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TABLE 3 | (*Continued*) Monoaminergic modulators with antidepressant properties in the pipeline.

Authors/Trial sponsor	Methodology	Results	Clinical trial phase, trial identifier (if available)
		was, however, recorded in the cariprazine-treated patients vs placebo group. Cariprazine improved significantly CGI-I score vs placebo, and a significantly higher proportion of patients achieved MADRS response with cariprazine vs placebo (but not significant). The overall tolerability of cariprazine was good	
Fava et al., 2019	Pimavanserin as an adjunctive agent, DBRCT, *N* = 207, MDD with inadequate response to SSRI/SNRI, 10 weeks	Pimavanserin + ongoing SSRI/SNRI treatment significantly improved depressive symptoms (reflected in HAMD-17 total score change). Dry mouth, nausea, and headache were the most common adverse events in pimavanserin-treated patients. In patients with anxious depression, the response rate was 55.2 vs 22.4% (pimavanserin vs placebo) and the remission rate was 24.1 vs 5.3% (pimavanserin vs placebo), among patients with a baseline Anxiety/ Somatization factor ≥7	Phase II, NCT03018340
National Library of Medicine (2019a)	Pimavanserin as adjunctive agent DBRCT, *N* = 298, MDD with inadequate response to antidepressant treatment, 5 weeks	Recruitment incomplete due to COVID-19-related problems. A 9-point HAMD total score decline at week 5 for pimavanserin treatment was reported vs 8.1 points for placebo ($p = 0.295$). A CGI-S change at week 5 of −1.4 vs −1.1 (pimavanserin vs placebo) was also reported. Response and remission rates were 31.1 and 18.2% vs 30.9 and 16.8% (pimavanserin vs placebo)	Phase III, NCT03968159
National Library of Medicine (2019b)	Pimavanserin as an adjunctive agent, *N* = 236, MDD and inadequate response to antidepressant treatment, 52 weeks	The trial was prematurely terminated "for business reasons and not due to safety concerns"	Phase III, NCT04000009
Loebel et al. (2022)	SEP-4199, DBRCT, *N* = 289/ 337 patients, BD type I, 6 weeks	Endpoint improvement in MADRS total score was observed on both the primary analysis (*N* = 289 participants) for SEP-4199 200 mg/day and 400 mg/day and the secondary, full ITT, analysis (*N* = 337 participants) for both regimens. Median increases in prolactin were +83.6 μg/L for the 200 mg/day dosage, +95.2 μg/L for 400 mg/day	Phase II, NCT03543410
National Library of Medicine (2021b)	SEP-4199, DBRCT, *N* = 522 (estimated), BD type I, 6 weeks	The trial is ongoing	Phase III, NCT05169710

BD, bipolar depression; CGI-I, Clinical Global Impression-Improvement; CGI-S, Clinical Global Improvement-Severity; DBRCT, double-blind randomized controlled trial; HAMA, Hamilton Anxiety Rating Scale; HAMD-17, Hamilton Depression Rating Scale; MADRS, Montgomery-Asberg Depression Rating Scale; QIDS-SR, Quick Inventory of Depressive Symptomatology - Self-rated; MDD, major depressive disorder; NLM, National Library of Medicine; SAE, severe adverse event; SDS, Sheehan Disability Scale; SNRI, Serotonin and norepinephrine reuptake inhibitor; SSRI, Selective serotonin reuptake inhibitor.

2016). The primary outcome was the mean change from baseline to week 8 in the MADRS total score, but each trial failed to meet this primary objective and most of the secondary objectives (Ball et al., 2016).

A phase III trial included an 8-week, open-label phase that evaluated edivoxetine (12–18 mg/day, flexible-dose regimen) as adjunctive to selective serotonin reuptake inhibitors (SSRI) treatment in 1249 MDD patients (Oakes et al., 2015). This

first phase was followed by a 12-week open-label stabilization if participants were in remission at the end of week 8, followed by a randomized, double-blind, placebo-controlled period of 24 weeks (Oakes et al., 2015). In the last phase of the trial, 294 patients were randomized to continue adjunctive edivoxetine, and 292 were switched to adjunctive placebo (Oakes et al., 2015). The comparison of the two groups at the end of the study, based on the MADRS total score change, did not show the presence of significant differences in time to re-emergence of symptoms, rates of symptom re-emergence, or rates of sustained remission (Oakes et al., 2015).

A phase II, double-blind, placebo-controlled, 10-week therapy of adjunctive edivoxetine (6–18 mg/day) or adjunctive placebo with SSRI, which enrolled 131 participants, did not report a significant difference in the primary outcome change from baseline to week 8 in the MADRS total score, with a 0.26 effect size (Ball et al., 2014). Significant treatment differences favoring edivoxetine were shown only in the role functioning and the functional impact of the fatigue (Ball et al., 2014).

In another randomized, double-blind, placebo-controlled trial, LY2216684 (6–18 mg/day) was evaluated in 495 adult MDD patients for 10 weeks (Pangallo et al., 2011). The investigational product improved significantly MADRS scores vs placebo from baseline to week 10, and it was also associated with a higher probability of achieving response (49.5%) and remission (29.7%) compared with placebo (29.3 and 18.8%, respectively) (Pangallo et al., 2011). For Sheehan Disability Scale (SDS) global functional impairment score, LY2216684 administration resulted in significantly greater improvement compared with placebo, and more edivoxetine-treated patients discontinued the study due to adverse events or death (Pangallo et al., 2011).

A multicenter, 54-week, open-label trial of adjunctive edivoxetine 12 or 18 mg once daily in MDD patients with partial response to the current SSRI therapy (N = 328 participants completed the trial) showed mean improvements of −17 points on MADRS from baseline to week 54, and a rate of study discontinuation due to adverse events of 17% (Ball et al., 2015). Treatment-emergent adverse events most commonly reported were nausea, hyperhidrosis, constipation, headache, dry mouth, dizziness, vomiting, insomnia, and upper respiratory tract infection (Ball et al., 2015).

MIN-117 is a potential antidepressant agent with α1A, α1B, 5HT1A, and 5HT2A receptors modulator properties; this product also possesses serotonin and dopamine transporter reuptake inhibition activity (Davidson et al., 2016). A four-arm, parallel-group, multicentric, randomized, double-blind, placebo- and positive-controlled trial evaluated two doses (0.5 and 2.5 mg) of MIN-117 in 84 patients with moderate-to-severe MDD, to detect a signal and to estimate the effect size (Davidson et al., 2016). A dose-dependent superiority of MIN-117 over placebo, determined by MADRS scores change at week 6, was demonstrated (Davidson et al., 2016). The effect size for the 2.5 mg dose of MIN-117 was 0.33 compared with placebo, and 0.23 for the lower dose (Davidson et al., 2016). Remission was achieved by 24% of the patients treated with 2.5 mg MIN-117, and both doses of the investigational product were well tolerated, without differences in the incidence and types of adverse events between MIN-117 and placebo (Davidson et al., 2016). Another randomized, double-blind, parallel-group, placebo-controlled phase II study investigated the efficacy and safety of MIN-117 in 360 adult patients diagnosed with MDD, monitored for 6 weeks (NLM, NCT03446846). The results posted on clinicaltrials.gov did not support the existence of significant differences between active drug and placebo in either the primary outcome (MADRS score change) or secondary outcomes (HAMA and CGI-S score changes) for any of the tested doses (NLM, NCT03446846).

The psychedelic molecule **psilocybin** (4-phosphoryloxy-N,N-dimethyltryptamine) has been associated with positive results in clinical trials dedicated to depression and anxiety treatment (Carhart-Harris et al., 2021). This compound has 5HT2A receptor antagonism, a pathway exploited by other products with antidepressant properties, e.g. trazodone, nefazodone, or mirtazapine (Celada et al., 2004; Carhart-Harris et al., 2021). In a phase II, double-blind, randomized, controlled trial, patients with a long history of moderate-to-severe depression (N = 59) received either psilocybin or escitalopram, over 6 weeks of treatment (Carhart-Harris et al., 2021). Psilocybin was administered as 25 mg doses separated by 3 weeks, plus 6 weeks of daily placebo, or as two distinct doses of 1 mg 3 weeks apart plus 6 weeks of daily oral escitalopram treatment (Carhart-Harris et al., 2021). After 6 weeks, the difference between groups in terms of QIDS-SR (Quick Inventory of Depressive Symptomatology- Self-reported) scores was not significant (p = 0.17), with a response being detected in 70% of the patients treated with psilocybin+placebo vs 48% in the group receiving psilocybin+escitalopram treatment (Carhart-Harris et al., 2021). The remission rates, based also on QIDS-SR scores, were 57 and 28%, respectively, while other secondary outcomes generally favored psilocybin vs escitalopram, and the incidence of adverse events was similar in both trial groups (Carhart-Harris et al., 2021). In conclusion, this trial did not support the efficacy of psilocybin in comparison with escitalopram, at least in the main outcome treatment (Carhart-Harris et al., 2021).

A randomized, double-blind, cross-over trial investigated the effects of a very low dose (1 or 3 mg/70 kg)-equivalent to placebo vs a high dose (22 or 30 mg/70 kg) of psilocybin administered in a counterbalanced sequence with 5 weeks between sessions and a 6-month follow-up, in 51 cancer patients with life-threatening diagnoses and symptoms of depression and/or anxiety treatment (Griffiths et al., 2016). The two primary outcome measures were clinician-rated GRID-HAMD-17 and HAM-A scores (Griffiths et al., 2016). Other scales evaluated general psychiatric symptoms, quality of life, self-rated optimism concerning own illness, anxiety about death, attitudes toward death, and life meaningfulness (Griffiths et al., 2016). The subjective effect measures were assessed 7 h after psilocybin, using the Hallucinogen Rating Scale, Mysticism Scale, 5-Dimension Altered States of Consciousness, and the States of Consciousness Questionnaire (Griffiths et al., 2016). High-dose psilocybin decreased clinician- and self-rated measures of depressed mood and anxiety, increased quality of life, life meaning, and optimism scores, and also decreased death anxiety

(Griffiths et al., 2016). At 6-month follow-up, these changes persisted, as 80% of the patients continued to show clinically significant decreases in depressed mood and anxiety (Griffiths et al., 2016).

In a double-blind, 7-week, cross-over, placebo-controlled trial, 29 patients with cancer-related anxiety and depression were randomized to treatment with single-dose psilocybin (0.3 mg/kg) or niacin, both in conjunction with psychotherapy (Ross et al., 2016). Before the crossover, psilocybin induced rapid and sustained improvements in anxiety and depression and led to decreases in cancer-related demoralization and hopelessness, improved spiritual well-being, and quality of life (Ross et al., 2016). At the follow-up visit (6.5 months), psilocybin was associated with consistent anxiolytic and antidepressant effects (60–80% of the participants still benefitted from the intervention), sustained existential distress reductions, quality of life amelioration, and improvements in attitude toward death (Ross et al., 2016). The psilocybin-induced mystical experience is assumed to have mediated the therapeutic effect of psilocybin on mood and anxiety symptoms (Ross et al., 2016).

An open-label, feasibility trial enrolled 12 patients with moderate-to-severe, unipolar, treatment-resistant major depression, who received two oral doses of psilocybin (10 and 25 mg, 7 days apart) in conjunction with psychological support (Carhart-Harris et al., 2016). The mean self-rated intensity of psilocybin effects was dose-related, and the drug was well tolerated by all the patients (Carhart-Harris et al., 2016). The adverse reactions were transient anxiety during drug onset, transient confusion or thought disorder, mild and transient nausea, and transient headache (Carhart-Harris et al., 2016). Depressive symptoms were markedly reduced at 1 week and 3 months, compared with baseline, after high-dose treatment (Carhart-Harris et al., 2016). Anhedonia and anxiety were markedly improved and these changes were maintained for long periods (Carhart-Harris et al., 2016).

In a randomized clinical trial, 24 participants with MDD who received immediate psilocybin-assisted therapy or delayed treatment were compared using clinician-rated assessments of depression severity (GRID-HAMD-17) and self-reported (QIDS-SR) for 1 month (Davis et al., 2021). Two psilocybin sessions (20 mg/70 kg—first session, 30 mg/70 kg—second session) were given, in the context of supportive psychotherapy (approximately 11 h), and patients were randomized to begin treatment immediately or after an 8-week delay (Davis et al., 2021). The mean GRID-HAMD scores were significantly lower in the immediate treatment group, and the QIDS-SR scores reflected a rapid decrease in the mean depression score after the first session, which remained significant up to week 4 (Davis et al., 2021). In the overall sample, 71% of the participants had at week 1 and week 4 a clinically significant response to the intervention (≥50% reduction in GRID-HAMD score) (Davis et al., 2021). The remission rate was 58% at week 1 and 54% at week 4 (score ≤7 on GRID-HAMD) (Davis et al., 2021).

In a randomized, multicenter, controlled, double-blind, phase IIb clinical trial, a single dose of COMP360 (psilocybin) was given to 233 patients with treatment-resistant depression, in conjunction with psychological support (COMPASS, 2021). All patients discontinued antidepressants before participation in this trial (COMPASS, 2021). Psilocybin was administered as 10 mg, 25 mg, or a comparator dose of 1 mg (COMPASS, 2021). The high-dose drug led to a significant decrease in MADRS scores vs inactive dose after day 1, and these improvements persisted after week 3, but the difference between the low-dose (10 mg) group and the control group was not significant (COMPASS, 2021). At least twice the number of patients in the high-dose group showed response and remission at week 3 and week 12, compared with the 1-mg group (COMPASS, 2021). The overall tolerability of COMP360 was good, with more than 90% of treatment-emergent adverse events being mild or moderate in intensity (COMPASS, 2021).

Cariprazine is an atypical antipsychotic under investigation as an adjuvant agent to antidepressants in patients diagnosed with MDD. Cariprazine is an orally active agent that possesses a 10-fold higher affinity for dopamine D3 receptors than for D2 receptors (partial agonist) and potent serotonin 5HT1A receptor partial agonist properties; its active metabolite, didesmethyl-cariprazine, has a half-life of 1–3 weeks (Earley et al., 2018; Citrome, 2019). This antipsychotic is FDA-approved for the treatment of schizophrenia, manic/mixed episodes in bipolar I disorder, and bipolar depression (Citrome, 2019; Vasiliu, 2021). Its antidepressive potential is attributed to the high affinity for and occupancy of D3 receptors, which are localized in motivation and reward-related brain areas (Earley et al., 2018). Based on the analysis of pivotal registration trials with cariprazine in bipolar depression (n = 4 studies), rates of treatment response (≥50% reduction of MADRS total score at endpoint) under cariprazine 1.5–3 mg/day treatment vs placebo were 46.3 vs 35.9% (NNT = 10) (Citrome, 2019). Based on the same analysis, the rates for remission (≤10 MADRS final total score) were 30.2 vs 20.9% (cariprazine vs placebo), leading to an NNT value of 11. The discontinuation rates due to adverse events were 6.7% for cariprazine vs 4.8% for placebo (NNH = 51) (Citrome, 2019).

A double-blind, placebo-controlled, randomized, 19-week phase II study evaluated the efficacy, safety, and tolerability of adjunctive cariprazine (0.1–0.3 mg and 1.0–2.0 mg/day) as an antidepressant treatment for adults with treatment-resistant MDD (N = 231) (Fava et al., 2018). No differences were reported on any measures between low doses of cariprazine and placebo. Higher doses led to numerically greater mean change in MADRS and CGI-I scores, and MADRS response and remission rates vs placebo, but without reaching statistical significance (Fava et al., 2018). The overall tolerability was good and adverse events in both dosage groups included headache, arthralgia, restlessness, fatigue, increased appetite, insomnia, dry mouth, and constipation (Fava et al., 2018). Another randomized, double-blind, placebo-controlled, flexible-dose study evaluated adult patients diagnosed with MDD with an inadequate antidepressant response, who were randomized to 8-week adjunctive treatment with placebo (N = 269), cariprazine 1–2 mg/day (N = 274), or cariprazine 2–4.5 mg/day (N = 276) (Durgam et al., 2016). Reductions in MADRS total score at week 8 were significantly greater for the high dose of cariprazine vs placebo, but not for the low dose (Durgam et al., 2016). The

favorable effect was detected early, at weeks 2, 4, and 6 for the 2–4.5 mg/day regimen, and at weeks 2 and 4 for the 1–2 mg/day regimen (Durgam et al., 2016). Treatment-emergent adverse events most commonly reported were akathisia, insomnia, and nausea (Durgam et al., 2016).

The results of a double-blind, randomized, placebo-controlled, phase III study evaluating the efficacy of adjunctive cariprazine (1.5–4.5 mg/day) added to tricyclics in patients with previous inadequate response to monotherapy with antidepressants (N = 530 participants) showed that cariprazine did not significantly improve MADRS total score or SDS score vs placebo (Earley et al., 2018). A nonsignificant decrease in depressive symptoms was, however, recorded in the cariprazine-treated patients vs the placebo group (Earley et al., 2018). Cariprazine improved significantly CGI-I score vs placebo, and a significantly higher proportion of patients achieved MADRS response with cariprazine vs placebo (but not significant) (Earley et al., 2018). The overall tolerability of cariprazine was good, with metabolic parameters and body weight changes not being different from placebo (Earley et al., 2018). Akathisia and restlessness were the most commonly reported adverse events (Earley et al., 2018).

Pimavanserin is an approved drug for the treatment of Parkinson's disease psychosis, and it possesses potent 5HT2A receptor antagonist/inverse agonist properties, with lesser activity as a 5HT2C antagonist/inverse agonist, and no interaction with adrenergic, dopaminergic, histaminergic, or muscarinic receptors (Fava et al., 2019). The results of a multicenter, randomized, double-blind, placebo-controlled, phase II study (the CLARITY trial) in patients with MDD and inadequate response to an SSRI or serotonin and norepinephrine reuptake inhibitor (SNRI) (N = 207 participants) showed that the addition of pimavanserin to ongoing SSRI/SNRI treatment may lead to significant improvements in depressive symptoms (reflected in HAMD-17 total score change, p = 0.004) (Fava et al., 2019). Early and sustained separation of pimavanserin from placebo at a statistically significant level occurred at week 1 visit (Fava et al., 2019). Dry mouth, nausea, and headache were the most common adverse events in pimavanserin-treated patients (Fava et al., 2019).

The effect of pimavanserin on anxious depression was determined from the data collected in the CLARITY trial, and the anxiety/somatization (AS) factor, derived from the HAMD items, decreased significantly in patients with initially high AS scores (≥7) (Papakostas et al., 2020). The response rate (≥50% reduction in HAMD-17 from baseline) was 55.2 vs 22.4% (pimavanserin vs placebo) and the remission rate (HAMD final score <7) was 24.1 vs 5.3% (pimavanserin vs placebo), among patients with a baseline AS factor ≥7 (Papakostas et al., 2020). Therefore, adjunctive pimavanserin to the current antidepressant treatment seems efficient in patients with anxious MDD (Papakostas et al., 2020).

A phase III, randomized, multicenter, double-blind, placebo-controlled study enrolled 298 participants diagnosed with MDD and inadequate response to antidepressant treatment to evaluate the efficacy and safety of adjunctive pimavanserin 34 mg/day, with the main outcome measure being the change from baseline to week 5 in HAMD-17 total score (NLM, NCT03968159). The recruitment was halted by the sponsor due to the COVID outbreak, but the data posted on clinicaltrials.gov show a nine-point HAMD total score decline at week 5 for pimavanserin treatment and 8.1 for placebo (p = 0.295), and a CGI-S change at week 5 of −1.4 vs. −1.1 (pimavanserin vs placebo) (NLM, NCT03968159). The response and remission rates were 31.1 and 18.2% vs 30.9 and 16.8% (pimavanserin vs placebo) (NLM, NCT03968159). Therefore, no significant difference seems to exist between active treatment and placebo in the main variables. The rate of serious adverse events was similar in the two groups (NLM, NCT03968159).

A phase III, 52-week extension study to assess the safety and tolerability of adjunctive pimavanserin in patients with MDD and inadequate response to antidepressant treatment was prematurely terminated "for business reasons and not due to safety concerns," according to the sponsor's announcement posted on clinicaltrials.gov (NLM, NCT04000009). No results have been made publicly available as of February 2022.

Atypical antipsychotics with antagonist activity at the serotonin **5-HT7 receptors have been associated with antidepressant efficacy** (Loebel et al., 2022). SEP-4199 is a non-racemic amisulpride represented by an 85:15 ratio of R-amisulpride:S-amisulpride (Loebel et al., 2022). The investigational oral drug is described pharmacodynamically as possessing increased potency for 5-HT7 receptors vs dopamine D2 receptors (because of different affinity for these receptors by enantiomers), which is expected to be beneficial for the treatment of bipolar depression (Loebel et al., 2022). In a randomized, 6-week, double-blind, placebo-controlled trial, endpoint improvement in the MADRS total score was observed on both the primary analysis (N = 289 participants) for SEP-4199 200 mg/day and 400 mg/day, and on the secondary, full ITT, analysis (N = 337 participants) for both regimens, in patients with bipolar I depression (Loebel et al., 2022). Low rates of individual adverse events were reported (under 8%) and minimal effects on weight and lipids were detected. Median increases in prolactin were +83.6 μg/L for the 200 mg/day dosage, +95.2 μg/L for 400 mg/day vs no increase on placebo (Loebel et al., 2022). Another phase 3, randomized, double-blind, placebo-controlled, parallel-group study is ongoing, and it has as objective the evaluation of the efficacy, safety, and tolerability of SEP-4199 CR at a fixed dose of 200 mg/day or 400 mg/day in patients diagnosed with bipolar I depression, during 6 weeks, with an estimated enrollment of 522 participants (NLM, NCT05169710).

In conclusion, antidepressants targeting the monoaminergic system are still actively researched, although the available data are mixed. Ansofaxine, MIN-117, cariprazine, pimavanserin, and SEP-4199 are the most promising molecules in this category. Also, it should be mentioned that many investigational products within this class have been discontinued from clinical research (Perez-Caballero et al., 2019). Therefore, the attention of the researchers has been focused on a different, non-monoaminergic mechanism for future antidepressants.

TABLE 4 | Orexinergic agents with antidepressant properties in the pipeline.

Authors/Trial sponsor	Methodology	Results	Clinical trial phase, trial identifier (if available)
Recourt et al. (2019)	Seltorexant (MIN-202, JNJ-42847922, JNJ-922) vs diphenhydramine vs placebo, DBRCT, *N* = 47, MDD, 4 weeks	Core symptoms of depression were improved after 10 days with seltorexant vs placebo and its efficacy persisted up to day 28	Phase Ib, NCT02476058
Savitz et al. (2021)	Seltorexant + ongoing antidepressant, DBRCT, *N* = 287, MDD with insufficient response to 1–3 SSRI/SNRI, 6 weeks	MADRS scores improved more in the seltorexant (20 mg) vs placebo at weeks 3 and 6. If baseline ISI≥15 the efficacy of seltorexant 20 mg/day was higher vs placebo	Phase IIb, NCT03227224
National Library of Medicine (2021c)	Seltorexant + ongoing antidepressant, DBRCT, *N* = 52 (estimated), MDD with inadequate response to SSRI/ psychotherapy	The outcomes will be related to tolerability, depression severity, clinical global impression, sleep quality, cognitive performance, and pharmacokinetic parameters	Phase I, NCT04951609

DBRCT, double-blind randomized controlled trial; ISI, Insomnia Severity Index; MADRS, Montgomery-Asberg Depression Rating Scale; MDD, major depressive disorder; NLM, National Library of Medicine; SNRI, Serotonin and norepinephrine reuptake inhibitor; SSRI, Selective serotonin reuptake inhibitor.

OREXIN RECEPTORS ANTAGONISTS

Orexins (hypocretins) are produced from the same precursor peptide, prepro-orexin, in the lateral and posterior hypothalamus (Han et al., 2020). Type A and type B orexins (or hypocretin-1 and -2) are ligands for type 1 and type 2 receptors (OX1R,2R) which are protein G-coupled, and modulate functions such as feeding, sleep, and motivated behaviors (Sakurai, 2014; Han et al., 2020). OX1R have a higher affinity for orexin-A and OX2R present a similar affinity for both orexin-A and B (Jha, 2021). Selective OX1R antagonists (SORA1) may be useful in the treatment of anxiety and drug addiction, selective OX2R antagonists (SORA2) are investigated in animal models for the therapy of sleep disorders, while dual OX1R and OX2R antagonists (DORA) are already marketed for the treatment of insomnia (Staton et al., 2018; Han et al., 2020). **Seltorexant (MIN-202, JNJ-42847922, JNJ-922)** is a SORA2 agent studied for the treatment of insomnia and MDD (Recourt et al., 2019). This agent may normalize excessive arousal and attenuate depressive symptoms, and in a randomized, double-blind, diphenhydramine-, and placebo-controlled study (*N* = 47 MDD patients) it significantly improved after 10 days' core depressive symptoms compared with placebo (Recourt et al., 2019). The antidepressant efficacy of seltorexant was maintained with continued treatment for up to 28 days, and this effect coincided with a relative increase in delta- and decreased theta-, alpha-, and beta power during stage 2 sleep (Recourt et al., 2019).

In a phase IIb, randomized, placebo-controlled, adaptive dose-finding study, 287 patients with MDD who had an insufficient response to 1-3 SSRI/SNRI during the current episode were randomized to placebo or seltorexant (20 or 40 mg) as add-ons to the currently administered antidepressant (Savitz et al., 2021). A significant reduction in depressive symptoms (MADRS scores) was observed for seltorexant (20 mg), and in the subset of patients with sleep disturbance, the difference between seltorexant 20 mg and placebo was larger (Savitz et al., 2021).

Another phase I, randomized, 6-week, double-blind, placebo-controlled trial is currently exploring the efficacy of seltorexant as adjunctive therapy to antidepressants in adolescents with MDD who have an inadequate response to SSRIs and psychotherapy (NLM, NCT04951609). The estimated enrollment is reported to be 52 patients, and the outcomes will be related to tolerability, depression severity, clinical global impression, sleep quality, cognitive performance, and pharmacokinetic parameters (NLM, NCT04951609).

A synthesis of the data regarding the clinical trials focused on the orexinergic modulators with antidepressant properties in the pipeline is presented in **Table 4**. Based on the reviewed data, seltorexant may be useful as monotherapy for MDD, and as an add-on agent for treatment-resistant depression, but more trials with a longer duration of monitoring are needed.

GABA-A RECEPTOR MODULATORS AND NEUROSTEROID ANALOGS

One of the extensively explored pathophysiological mechanisms for MDD is the dysfunction of the GABA neurotransmission and the downregulation of neurosteroid biosynthesis (Schüle et al., 2014; Hoffmann et al., 2020). It is presumed that alteration of the transmembrane channels that make up GABA-A receptors can induce anxiety and neurodevelopmental disorders (Edinoff et al., 2021; Zhu et al., 2018). Reduced levels of allopregnanolone in the peripheral circulation or cerebrospinal fluid were associated not only with major depression, anxiety disorders, premenstrual dysphoric disorder, but also with negative symptoms of schizophrenia or impulsive aggression (Schüle et al., 2014). Based on the observation that allopregnanolone, an endogenous neuroactive steroid that possesses GABA-A receptor-positive allosteric modulating properties, may improve symptoms of depression and anxiety by intensifying GABA-ergic signaling in the central nervous system (Schüle et al., 2014); a series of these steroid analogs have been tested for the treatment of MDD. Besides the role of GABA-ergic

neurotransmission regulation, allopregnanolone may have positive effects on mood disorders by the enhancement of neurogenesis, myelination, neuroprotection, and regulatory effects on the hypothalamus-pituitary-adrenocortical (HPA) axis (Schüle et al., 2014).

Brexanolone (SAGE-547) is the first FDA-approved intravenous treatment for postpartum depression and represents a soluble, β-cyclodextrin-based form of the neuroactive steroid allopregnanolone (Cooper et al., 2019). Due to its binding to GABA-A receptors, brexanolone enhances the inhibitory effects of GABA when it occupies these receptors, leading to an acute decrease in anxiety levels and depression symptoms (Schüle et al., 2014). Brexanolone is not recommended for patients engaging in activities that require high levels of alertness, and it should be avoided in end-stage renal disease (eGFR<15 ml/min/m^2) (Edinoff et al., 2021).

In a proof-of-concept, phase II, label study of brexanolone, which included four women with severe post-partum depression (HAMD≥20), titrated to a dose similar to third-trimester allopregnanolone levels, this antidepressant was associated with 14 adverse events, but none of them was severe (Kanes SJ. et al., 2017). The mean HAMD score decreased to levels suggesting remission of symptoms, after 84 h of monitoring (Kanes SJ. et al., 2017).

In a phase II trial, brexanolone decreased at 60 h the total HAMD score with a significantly greater impact than placebo (Kanes S. et al., 2017). The overall tolerability of brexanolone was good, with no serious adverse events or discontinuations due to adverse events (Kanes S. et al., 2017). The most frequently reported adverse effects were dizziness, somnolence, and sinus tachycardia (Kanes S. et al., 2017).

In two double-blind, multicentric, phase III trials, women with post-partum depression (N_1 = 138, and N_2 = 108, respectively), of severe intensity (HAMD≥26 for one trial, and 20–25 for the other trial) received a single i.v. injection of brexanolone 90 or 60 μg/kg vs placebo for 60 h (first trial), or brexanolone 90 μg/kg vs placebo (trial 2) for the same duration (Meltzer-Brody et al., 2018). Patients who received brexanolone in both trials presented significant clinical improvement, according to the HAMD scores, after 60 h vs placebo, with rapid onset of the therapeutic action and long-lasting treatment response (evaluated up to 30 days) (Meltzer-Brody et al., 2018). The most frequently reported adverse events in the brexanolone groups were headache, dizziness, and somnolence (Meltzer-Brody et al., 2018).

A *posthoc* analysis of three trials conducted with brexanolone (N = 299 women with post-partum depression) showed a superior effect for the active drug vs placebo after 60 h and at day 30 (Meltzer-Gerbasi et al., 2021). Significantly more patients treated with brexanolone than those who received placebo achieved minimal, moderate, and large HAMD-17 score change at hour 60, as well as a large meaningful response at day 30 (Meltzer-Gerbasi et al., 2021). Also, patients treated with brexanolone had a higher probability to sustain HAMD-17 remission and CGI-I response until day 30 vs the placebo-treated group (Meltzer-Gerbasi et al., 2021).

A review that extracted data from 26 studies dedicated to pharmacological and pharmacological/nonpharmacological combination therapies in postpartum depression concluded that matching-adjusted indirect comparisons between brexanolone and placebo arms of comparator studies, on one side, and between SSRIs vs placebo, on the other side, lead to larger differences in the change from baseline scores in HAMD and Edinburgh Postnatal Depression Scale (EPDS) in favor of brexanolone vs SSRIs (Cooper et al., 2019). The differences in HAMD scores were between 12.79 (day 3) and 0.97 (last observation), whereas the EPDS score difference varied between 7.98 (day 3) and 4.05 (last observation) (Cooper et al., 2019).

Zuranolone (SAGE-217) **is a neuroactive steroid and GABA-A receptor-positive allosteric modulator that shares a similar pharmacodynamic profile as brexanolone injection** (Martinez Botella et al., 2017). Both zuranolone and brexanolone have an affinity for synaptic (γ subunit-containing) as well as for extrasynaptic (δ subunit-containing) GABA-A receptors, but the first agent has been formulated for oral administration and once-daily dosing (Althaus et al., 2020). In preclinical models, zuranolone potentiated GABA currents synergistically with diazepam, in a noncompetitive manner (Althaus et al., 2020).

In two phase I studies of SAGE-217, which included 108 healthy volunteers, the investigational product was well tolerated and its pharmacokinetic profile was well characterized (Hoffmann et al., 2020). In a double-blind, phase II trial, 89 patients with MDD were randomized on 30 mg SAGE-217 or placebo once daily, and they were monitored using change in the HAMD scores as the main outcome (Gunduz-Bruce et al., 2019). Administration of SAGE-217 for 14 days resulted in significant improvements in depressive symptoms compared with placebo (Gunduz-Bruce et al., 2019). There were reported no serious adverse events, and the most common adverse events in the active substance group were headache, dizziness, nausea, and somnolence (Gunduz-Bruce et al., 2019).

In a phase III, double-blind, randomized, outpatient, placebo-controlled trial, 153 patients diagnosed with post-partum depression were assigned to treatment with zuranolone (30 mg) or placebo for 14 days and were monitored using HAMD-17 scores as the primary outcome (Deligiannidis et al., 2021). On day 15, zuranolone improved HAMD scores from baseline vs placebo, and this trend to superiority was observed from day 3 and persisted until day 45 (end of study visit) (Deligiannidis et al., 2021). Significant differences between zuranolone and placebo were observed in the therapeutic response rate, remission rate, and MADRS score improvement, whereas HAMA scores also improved significantly (Deligiannidis et al., 2021). The treatment was generally well tolerated; one patient experienced a serious adverse event (confusional state) and one was discontinued because of an adverse event (Deligiannidis et al., 2021).

A phase III trial evaluating the efficacy of SAGE-217 in adults with severe post-partum depression is ongoing, with an estimated

completion date of September 2022 (NLM, NCT04442503). A number of 192 patients are expected to be enrolled in this trial and monitored for 14 days, with the primary outcome being the severity of depression, determined by the HAMD-17 score on day 15 (NLM, NCT04442503).

Ganaxolone (CCD1042) is an allopregnanolone analog explored mainly as an anticonvulsant, but also as an adjunctive agent for the treatment of persistent depression in postmenopausal women and as monotherapy in postpartum depression (Dichtel et al., 2020).

In an open-label clinical trial (N = 10 participants, mean age 62.8 ± 6.3 years) ganaxolone (225 mg b.i.d., increased to 450 mg b.i.d. if tolerated) was administered orally for 8 weeks in cases where an adequately dosed antidepressant did not lead to response after ≥6 weeks (Dichtel et al., 2020). Ganaxolone was associated with a favorable evolution, with MADRS scores decreasing after 8 weeks, and this decrease persisted over a 2-week taper, with 44% of the subjects who completed the 8-week treatment period experiencing response (MADRS score decrease ≥50%) and remission (final MADRS<10) (Dichtel et al., 2020). The response and remission rates persisted in 100 and 50% of subjects at 10 weeks, and the secondary endpoints showed also significant improvement (sleep quality, changes in appetite, and weight) (Dichtel et al., 2020). Sleepiness, fatigue, and dizziness were reported as adverse events during this trial, without significant effects on quality of life or sexual function (Dichtel et al., 2020).

In a phase 2 trial, ganaxolone was administered i.v. at median doses of 60, 90, and 140 μg/kg/h as a 60-h infusion in patients with severe postpartum depression (N = 58, HAMD≥26) (Marinus Pharmaceuticals, 2018). Ganaxolone was efficient, with the most robust results being reported in the highest dose group. A clinically meaningful reduction in the HAMD-17 total score vs placebo was reported at 48 h and this improvement was maintained until the last visit, on day 34 (Marinus Pharmaceuticals, 2018). The rate of response was high on day 34 and after 60 h (75%, and 67%, respectively), and also the rate of remission was important (54 and 33%, respectively) (Marinus Pharmaceuticals, 2018). Sedation and dizziness were the most frequently reported adverse events, but no serious adverse event/discontinuation due to adverse events was observed (Marinus Pharmaceuticals, 2018). In the second part of this trial, 33 patients with postpartum depression received a 6-h infusion with ganaxolone (20 mg/h), and then oral ganaxolone 900 mg daily or placebo for 28 days (Marinus Pharmaceuticals, 2019). The HAMD-17 scores decreased rapidly at 6 h, but on day 28 there was no significant difference between the active drug and placebo (Marinus Pharmaceuticals, 2019). Sedation, dizziness, and somnolence lasted between 2 and 10 days, except for one case where sedation lasted throughout the treatment period (Marinus Pharmaceuticals, 2019). The secondary outcomes (CGI, EPDS, and Spielberger State-Trait Anxiety 6- STAI-6) showed similar trends with the HAMD-17 scores (Marinus Pharmaceuticals, 2019).

In another, open-label study, 25 patients diagnosed with postpartum depression received 675 mg of oral ganaxolone for 28 days, and 43 patients received 675 mg of oral ganaxolone for 2 days, followed by 1125 mg once daily for the remainder of the study (Marinus Pharmaceuticals, 2019). The high-dose regimen was superior as reflected by the evolution of the HAMD-17 scores, and this trend was maintained over the treatment regimen (Marinus Pharmaceuticals, 2019). The onset of the favorable effect was detected at 24 h, and the treatment was generally safe and well-tolerated, with no serious adverse events/discontinuation due to adverse events being reported (Marinus Pharmaceuticals, 2019).

PRAX-114 is a GABA-A receptor-positive allosteric modulator that achieves 10.5-fold greater potentiation of extrasynaptic receptors vs synaptic receptors in animal models (Hughes et al., 2021). Two clinical trials dedicated to the efficacy and tolerability of oral PRAX-114 in MDD are ongoing: the first one is a phase II/III trial, randomized, double-blind, placebo-controlled, which will compare the effects of 40 mg active drug in 200 participants, using HAMD as a primary outcome measure; the second trial will evaluate the effects of PRAX-114 as adjunctive treatment (10, 20, 40, or 60 mg/day) vs placebo over HAMD scores in a phase II, randomized, double-blind design, with an expected enrollment of 125 participants with MDD and inadequate response to antidepressant treatment (NLM, NCT04832425; NLM, NCT04969510).

In conclusion, neurosteroid analogs are interesting therapeutic options for the treatment of depressive disorders (postpartum depression, MDD, postmenopausal depression, treatment-resistant depression), with brexanolone being already marketed for post-partum depression. It is expected that the inconvenience of i.v. administration in the case of brexanolone is to be overcome by the other pregnanolone analogs (**Table 5**).

ANTI-INFLAMMATORY THERAPIES AS POTENTIAL TREATMENTS FOR DEPRESSIVE DISORDERS

Anti-cytokine therapies have been recently cornered as potential strategies for decreasing MDD symptoms severity, although they have been mostly investigated in patients with severe, chronic organic diseases, where the mood manifestations were not the center of clinical attention, but associated features (Drevets et al., 2022). There is overwhelming evidence that immune dysregulation is frequently associated with depression, and MDD has been associated with elevated levels of pro-inflammatory cytokines and acute-phase proteins both in the central nervous system and in the blood, but also with decreased adaptive immune response, a bias toward autoimmunity, and other immune changes (Drevets et al., 2022). The effects of anti-cytokine therapies as adjunctive agents in patients with treatment-resistant MDD or bipolar depression have been investigated in clinical trials, with mixed results (Drevets et al., 2022).

A meta-analysis of the trials investigating the effects of anti-inflammatory cytokines interventions in patients with chronic inflammatory conditions, where depressive symptoms severity was measured as a secondary outcome,

TABLE 5 | Neurosteroid analogs and GABA-A receptor modulators with antidepressant properties in the pipeline.

Authors/Trial sponsor	Methodology	Results	Clinical trial phase, trial identifier (if available)
Kanes et al. (2017a)	Brexanolone (SAGE-547), open-label, *N* = 4, PPD, 84 h	Mean HAMD and CGI-I scores had favorable evolution; 14 adverse events were reported, but no SAE	Phase II, NCT02285504
Kanes et al. (2017b)	Brexanolone, DBRCT, *N* = 21, severe PPD, 60 h	HAMD total scores decreased significantly vs placebo at 60 h. Dizziness and somnolence were the most frequently reported adverse events	Phase II, NCT02614547
Meltzer-Brody et al. (2018)	Brexanolone, two DBRCT, *N* = 138 and 108, severe PPD, 60 h	HAMD scores evolution supported the existence of a significant clinical improvement vs placebo, which persisted up to 30 days. Headache, dizziness, somnolence were the most commonly reported adverse events	Phase III, NCT02942004 Phase III, NCT02942017
Gerbasi et al. (2021)	Brexanolone, post-hoc analysis of three trials, *N* = 299, PPD, 30 days	Brexanolone was superior to placebo after 60 h and 30 days. Higher probability to sustain HAMD-defined remission and CGI-I response vs placebo at day 30	Phase II, NCT02614547 Phase III, NCT02942004 Phase III, NCT02942017
Hoffmann et al. (2020)	Zuranolone (SAGE-217), two trials, DBRCT, *N* = 108 healthy volunteers (72 and 36, respectively), single ascending dose study and multiple ascending dose study	Safety, tolerability, and pharmacokinetics of SAGE-217. Mild and transient sedation was observed. Most adverse events were reported as mild/moderate intensity. No SAE was reported	Phase I
Gunduz-Bruce et al. (2019)	Zuranolone, DBRCT, *N* = 89, MDD, 14 days	HAMD scores improved significantly vs placebo, no SAE was reported. Dizziness, headache, nausea, and somnolence were the most common adverse events	Phase II, NCT03000530
Deligiannidis et al. (2021)	Zuranolone, DBRCT, *N* = 153, PPD, 45 days	HAMD scores were improved by zuranolone vs placebo from day 3, up to day 45. HAMA and MADRS also improved under zuranolone treatment vs placebo. The overall tolerability of zuranolone was good, with one SAE (confusional state)	Phase III, NCT02978326
National Library of Medicine (2020)	Zuranolone, DBRCT, *N* = 192, severe PPD, 14 days	HAMD-17 at day 15 is the main outcome measure, the study is ongoing (as of February 2022)	Phase III, NCT04442503
Dichtel et al. (2020)	Ganaxolone (CCD1042) as augmentation strategy, open-label, pilot study, *N* = 10, MDD with insufficient response, 8 weeks	MADRS scores decreased during 7 weeks, 44% response rate at week 8. Sleep quality, appetite changes, and body weight also improved. Sleepiness, fatigue, and dizziness were the most common adverse events	N/A, NCT02900092
Marinus Pharmaceuticals (2018)	Ganaxolone i.v., *N* = 58, severe PPD, 34 days	HAMD-17 total score decreased vs placebo at 48 h and the decrease was maintained until day 34. Sedation, dizziness were the most commonly reported adverse events	Phase II, NCT03228394
National Library of Medicine (2021d)	Ganaxolone i.v. 6 h, followed by oral administration 28 days, *N* = 33, PPD	HAMD-17 scores decreased rapidly at 6 h but did not separate zuranolone from placebo at day 28	Phase II, NCT03460756
National Library of Medicine (2021e)	PRAX-114 in MDD patients, DBRCT, *N* = 200 and 125, respectively, 43 days	The change in the HAMD total score at day 15 is the main outcome measure; studies are ongoing (as of February 2022)	Phase II/III, NCT04832425 Phase II, NCT04969510

CGI-I, Clinical Global Impression- Improvement; DBRCT, double-blind randomized controlled trial; HAMD-17, Hamilton Depression Rating Scale; MADRS, Montgomery-Asberg Depression Rating Scale; MDD, major depressive disorder; NLM, National Library of Medicine; PPD, post-partum depression; SAE, severe adverse event.

showed a significant antidepressant effect vs placebo (based on data from seven randomized controlled studies, with 2370 participants) (Kappelmann et al., 2018). Antitumor necrosis factor (TNF) drugs were the most investigated interventions (*n* = 5 trials) in this meta-analysis, and adalimumab, etanercept, infliximab, and tocilizumab all showed statistically significant improvements in depressive symptoms (Kappelmann et al., 2018). In separate meta-analyses (*n* = 2 randomized controlled trials and eight nonrandomized and/or placebo studies) the results were similar, with small-to-medium effect estimates favoring anti-cytokine therapy (Kappelmann et al., 2018). The baseline symptom severity was associated with predictive value for antidepressant effect, but other variables, like the severity of the physical illness, sex, age, or study duration, did not have predictive value (Kappelmann et al., 2018).

According to a mega-analysis of randomized, placebo-controlled trials (*n* = 18) for one of nine disorders (*N* = 10,743 patients diagnosed with ulcerative colitis, rheumatoid arthritis, psoriasis, asthma, ankylosing spondylitis, multicentric Castleman's disease, osteoarthritis, lupus, neuropathic pain), patients with high severity scores had modest, but significant effects on core symptoms and quality of life-related measures (mental health and vitality) under immune therapy targeting one

of seven mechanisms (IL-6, TNF-α, IL-12/23, CD20, COX2, BLγS, p38/MAPK14) (Wittenberg et al., 2020). Anti-IL6 antibodies and anti-IL-12/23 antibodies had larger effects on depressive symptoms than other drug classes (Wittenberg et al., 2020). Effects of anti-IL-12/23 remained significant and anti-IL-6 antibodies remained only at a trend level of efficacy after controlling for physical response to treatment (Wittenberg et al., 2020).

Etanercept was evaluated in a double-blind, randomized trial, with 618 patients diagnosed with moderate to severe psoriasis, in a 50-mg twice-weekly regimen vs placebo (Tyring et al., 2006). After 12 weeks of monitoring, 47% of patients in the active group achieved the primary outcome, i.e. ≥75% improvement from baseline in Psoriasis Area and Severity Index (PASI) score vs 5% in the placebo group (Tyring et al., 2006). Also, a higher proportion of the patients receiving etanercept had ≥50% improvement in HAMD or BDI at week 12 compared to placebo, and the improvement in the fatigue was significant and clinically meaningful at the endpoint (Tyring et al., 2006). Improvements in depressive symptoms were weakly correlated with objective measures of skin clearance or joint pain (Tyring et al., 2006).

In a phase III, randomized, double-blind clinical trial, patients with moderate to severe Crohn's disease (N = 499) **adalimumab** was administered every other week or weekly (two maintenance groups) and was compared to adalimumab induction-only group, using measurements for quality of life, depression severity (self-evaluated), fatigue, pain severity, and inflammatory bowel questionnaires, during 56 weeks (Loftus et al., 2008). After 4-week adalimumab induction therapy, patients experienced significant improvements in all measures related to their quality of life (HR-QOL) (Loftus et al., 2008). Patients who continued active treatment after the induction period therapy in a 40 mg every 2 weeks regimen reported less depression, fewer fatigue symptoms, greater improvements in their irritable bowel symptoms, greater SF-36 physical summary scores, and less abdominal pain from weeks 12 to 56 (Loftus et al., 2008). They also presented a greater SF-36 mental component summary score at week 56 (Loftus et al., 2008). The 40-mg adalimumab weekly regimen also was associated with less depression and fewer fatigue symptoms at week 56 (Loftus et al., 2008).

Ustekinumab was evaluated in a randomized trial with patients presenting moderate to severe psoriasis (N = 1230) who were monitored for their anxiety, depression, and skin-related quality of life for 12 weeks (Langley et al., 2010). Greater improvements at the last study visit were reported for patients receiving ustekinumab (either 45 or 90 mg) vs placebo on all outcomes, determined by the Hospital Anxiety and Depression Scale (HADS)- Anxiety and Depression subscales, and Dermatology Life Quality Index, all of these changes being statistically significant (Langley et al., 2010).

In a 12-week, randomized, double-blind, placebo-controlled, parallel-group trial, 60 participants diagnosed with bipolar I or II depression, presenting also pretreatment biochemical and/or phenotypic evidence of inflammatory activation, were randomized to receive three intravenous infusions of **infliximab** or placebo, as adjunctive treatment, at baseline and weeks 2 and 6 (McIntyre et al., 2019). The primary efficacy outcome was the change at week 12 compared with the baseline of the MADRS scores (McIntyre et al., 2019). Overall baseline-to-end change in the MADRS total score was observed across treatment × time interaction, but the reduction of symptom severity was not significant at week 12 (McIntyre et al., 2019). Infliximab-treated patients with a childhood history of physical abuse exhibited greater reductions in MADRS scores and higher response rates (McIntyre et al., 2019). Therefore, it seems that although the therapeutic benefit of infliximab is minor in patients with bipolar depression, a subpopulation (i.e. those with physical and/or sexual abuse) may have a significant reduction in depressive symptoms during this treatment vs placebo (McIntyre et al., 2019).

In a double-blind, placebo-controlled, randomized clinical trial, 60 medically stable outpatients with MDD, who were either on a consistent antidepressant treatment regimen (N = 37) or medication-free (N = 23) for 4 weeks or more, and who were moderately resistant to treatment, received three infusions with infliximab (5 mg/kg) or placebo at baseline, weeks 2 and 6 (Raison et al., 2013). No overall difference in change of HAMD scores between treatment groups across time was detected, but there was a significant interaction between treatment × time × hs-CRP concentration (Raison et al., 2013). Changes in HAMD scores (baseline to week 12) favored infliximab vs placebo if the baseline hs-CRP concentration was greater than 5 mg/L, and favored placebo if this concentration was ≤5 mg/L (Raison et al., 2013). A higher rate of response was also detected in patients with baseline hs-CRP>5 mg/L who received infliximab vs placebo (62 vs 33%), without reaching a statistically significant level (Raison et al., 2013). Baseline concentrations of TNF and its soluble receptors were significantly higher in infliximab-treated responders vs nonresponders, and hs-CRP concentrations decreased significantly from baseline to week 12 in the active treatment group compared to placebo (Raison et al., 2013). Again, immune therapy seems to have efficacy in a certain sub-population, namely patients with high baseline inflammatory biomarkers (Raison et al., 2013).

Losmapimod (GW856553) is a p38MAPK inhibitor that was administered in a 7.5-mg b.i.d. dosage for 6 weeks in two randomized, placebo-controlled trials in subjects with MDD and prominent symptoms of loss of energy/interest and psychomotor retardation, who also had rheumatoid arthritis (Inamdar et al., 2014). In one of these studies (N = 24 patients), prematurely terminated due to variables related to rheumatoid arthritis, the Bech 6-item depression subscale of HAMD-17 favored losmapimod, but in the other study (N = 128) no advantage for losmapimod was detected on the same scale (Inamdar et al., 2014). No significant biomarkers (key cytokines) changes were detected during treatment (Inamdar et al., 2014). Based on the combined data of these two trials, 7.5 mg losmapimod was not effective in patients with MDD and rheumatoid arthritis.

TABLE 6 | Anti-cytokine therapies and COX-2 inhibitors in the pipeline as add-on agents to antidepressants.

Authors/Trial sponsor	Methodology	Results	Clinical trial phase, trial identifier (if available)
Tyring et al. (2006)	Etanercept, DBRT, *N* = 618, psoriasis + depressive symptoms, 12 weeks	HAMD and BDI improvements in the active group vs placebo	Phase III, NCT00111449
Loftus et al. (2008)	Adalimumab, DBRCT, *N* = 499, Crohn's disease + depressive symptoms, 56 weeks	HR-QOL improvement (SF-36), depressive symptoms, and fatigue improvements	Phase III, NCT00077779
Langley et al. (2010)	Ustekinumab, DBRCT, *N* = 1230, psoriasis + depressive/anxiety symptoms, 12 weeks	HADS- Anxiety and Depression subscales scores significantly improved	Phase III, NCT00307437
McIntyre et al. (2019)	Infliximab as adjunctive treatment, DBRCT, *N* = 60, BD + inflammatory activation, 12 weeks	MADRS's total score baseline-to-end change was not significant. A higher response rate under infliximab was observed if a childhood history of physical abuse was present	Phase II, NCT02363738
Raison et al. (2013)	Infliximab ± antidepressant, DBRCT, *N* = 60 outpatients, MDD, 12 weeks	HAMD did not record significant changes, but baseline hs-CRP>5 mg/L improved more under infliximab vs placebo	Phase IV, NCT00463580
Inamdar et al. (2014)	Losmapimod (GW856553), DBRCT, *N* = 24 MDD or 128 MDD (two studies), 6 weeks	The first study Bech 6-item subscale of HAMD-17 score evolution favored losmapimod. Study prematurely terminated. The second study no advantage of losmapimod, using the same main outcome measure	Phase II, NCT00569062 Phase II, NCT00976560
Sun et al. (2017)	Sirukumab (CNTO136) and siltuximab (CNTO328), two DBRCT, *N* = 176 methotrexate-resistant rheumatoid arthritis, and 79 multicentric Castleman's disease, respectively, plus prevalent depressed mood and anhedonia, 12 weeks (sirukumab) or 15 weeks (siltuximab)	SF-36 items for depressive symptoms showed significant improvement only during siltuximab treatment. These improvements were correlated with baseline soluble IL-6 receptor levels	Phase II, NCT00718718 Phase II, NCT01024036
Griffiths et al. (2017)	Ixekizumab, DBRCT, three studies, psoriasis + depressive symptoms, 12 weeks	QIDS-SR scores reflected a greater improvement in their depression severity score vs placebo. Higher remission rates and significant hsCRP reduction in active groups vs placebo	Phase III, NCT01474512 Phase III, NCT01597245 Phase III, NCT01646177
Müller et al. (2006)	Celecoxib + reboxetine/placebo, DBRCT, *N* = 40, MDD, 6 weeks	HAMD scores improved in both groups, but celecoxib outperformed the placebo	Phase IV
Majd et al. (2015)	Celecoxib + sertraline/placebo, DBRCT, *N* = 30, outpatients with first episode of depression, 8 weeks	HAMD scores improved in both groups, with a trend to superiority for celecoxib at week 4, but not at week 8	Phase III, IRCT201009043106N3
Abbasi et al. (2012)	Celecoxib + sertraline/placebo, *N* = 40, MDD, 6 weeks	Celecoxib decreased significantly more IL-6 serum concentrations and HAMD scores vs placebo	Phase I, IRCT138903124090N1

BD, bipolar depression; BDI, Beck Depression Inventory; DBRCT, double-blind randomized controlled trial; HAMD-17, Hamilton Depression Rating Scale; HR-QOL, Health-related quality of life; HADS, Hospital Anxiety Depression Scale; MADRS, Montgomery-Asberg Depression Rating Scale; MDD, major depressive disorder; QIDS-SR, Quick Inventory of Depressive Symptomatology – Self-rated.

According to a systematic review of preclinical and clinical studies, **sirukumab,** an anti-IL-6 human monoclonal antibody, may have potential benefits in patients with inflammatory disorders and neuropsychiatric disorders (Zhou et al., 2017). In individuals with complex psychiatric disorders, e.g. mood disorders, the most likely to benefit domains with sirukumab are negative valence disturbances (anxiety, depression, ruminations), positive valence disturbances (anhedonia), and general cognitive processes (Zhou et al., 2017). Sirukumab (*N* = 176) and **siltuximab** (*N* = 65), both anti-IL-6 antibodies, have also been shown to be effective in reducing depressive symptoms severity in patients with multicentric Castleman disease or rheumatoid arthritis, even after controlling for symptom severity of primary illness, based on two phase 2, double-blind, placebo-controlled trials (Sun et al., 2017). The improvement in depressive symptoms by siltuximab was positively correlated with the baseline soluble IL-6 receptor level (Sun et al., 2017). The improvement in depressive symptoms was significant over placebo only in the siltuximab study (Sun et al., 2017).

An integrated analysis of three randomized, double-blind, controlled, phase 3 trials focused on the efficacy of **ixekizumab** (a high-affinity monoclonal antibody targeting IL-17A) in patients diagnosed with psoriasis and moderately severe depressive symptoms at baseline (QIDS-SR16 total score ≥11) evidenced at week 12 a significantly greater improvement in their depression severity score (80 mg every 2 weeks vs placebo, or 80 mg every 4 weeks vs placebo) (Griffiths et al., 2017). Higher rates of depressive symptoms remission, and significant hsCRP and PASI (Psoriasis Area and Severity Index) reductions were also reported in patients treated with ixekizumab vs placebo (Griffiths et al., 2017).

Celecoxib is a cyclooxygenase-2 (COX-2) inhibitor investigated as an adjuvant treatment in patients with MDD, based on the high levels of prostaglandin E2 (PGE2) levels detected in this disorder (Müller et al., 2006). In a prospective, double-blind, add-on study, 40 patients diagnosed with MDD were randomized to either reboxetine + celecoxib, or to reboxetine + placebo (Müller et al., 2006). After 6 weeks, both groups of patients showed significant improvement in HAMD scores, but celecoxib was associated with significantly greater

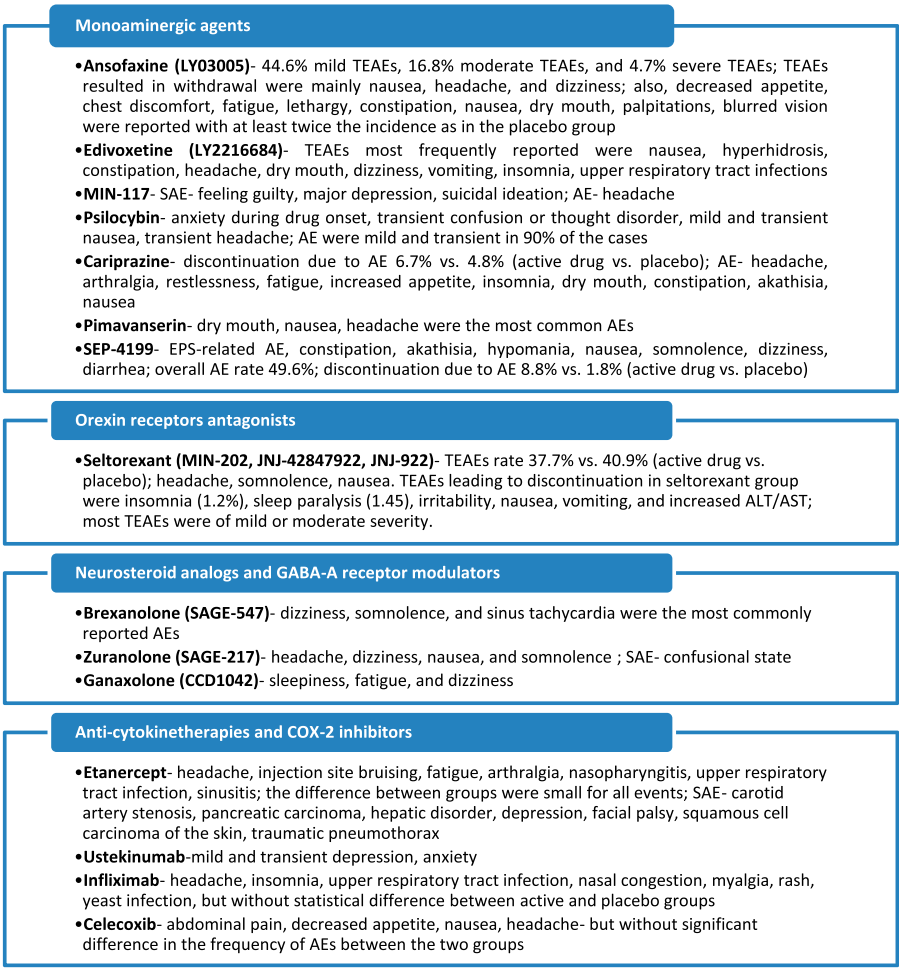

FIGURE 3 | Main adverse events reported in clinical trials for investigational antidepressants. TEAE, treatment-emergent adverse events; AE, adverse events; SAE, severe adverse events; EPS, extrapyramidal symptoms. Based on the data from Mi et al., 2021; Ball et al., 2015; Carhart-Harris et al., 2016; COMPASS, 2021; Citrome, 2019; Fava et al., 2018; Durgam et al., 2016; Fava et al., 2019; Loebel et al., 2022; Savitz et al., 2021; Kanes S. et al., 2017; Gunduz-Bruce et al., 2019; Deligiannidis et al., 2021; Dichtel et al., 2020; Tyring et al., 2006; Langley et al., 2010; Raison et al., 2013; Abbasi et al., 2012.

improvement compared to placebo (Müller et al., 2006). In another trial, 30 female outpatients, diagnosed with first episode of depression, were randomized into two groups, one receiving sertraline + celecoxib 100 mg b.i.d, and the other sertraline + placebo twice daily (Majd et al., 2015). Both groups showed improvement in their depressive symptoms from baseline, but celecoxib was associated with a greater decrease in HAMD scores vs placebo after 4 weeks of treatment (Majd et al., 2015). Response rates were also found to be significantly higher in patients who received celecoxib vs placebo, at week 4 (Majd et al., 2015). At week 8, the differences between the two groups were not significant (Majd et al., 2015). This study suggests that celecoxib may hasten the onset of therapeutic action of sertraline, but the differences in efficacy vs placebo are not persistent.

In yet another trial, randomized, double-blind, placebo-controlled, 40 patients with MDD and HAMD baseline score ≥18 were randomized to celecoxib (200 mg b.i.d) or placebo in addition to sertraline, for 6 weeks (Abbasi et al., 2012). Patients who received celecoxib showed a significantly higher reduction of IL-6 serum concentrations and HAMD scores than the placebo group, and also more response and remission (95 and 35% vs 50 and 5%, respectively) (Abbasi et al., 2012). Baseline serum IL-6 levels were significantly correlated with baseline HAMD scores, and also a significant correlation was observed between the reduction of HAMD scores and the reduction of IL-6 serum levels at week 6 (Abbasi et al., 2012).

Anti-inflammatory agents, both immuno-modulators and COX-2 inhibitors, may represent adjuvant strategies to antidepressants in depressive disorders, as the results of clinical trials seem promising until now (**Table 6**). Larger trials with MDD patients, and not only depressive associated features in chronic organic diseases, are needed, to validate the efficacy of this approach.

A synthesis of the safety and tolerability profile of the investigational products reviewed in this article is presented in **Figure 3**.

CONCLUSION

A large number of investigational products with antidepressant properties exist in the pipeline. The monoaminergic hypothesis of depression is still able to generate new drug research, and seven new molecules have been found in phase I to III clinical trials. Besides new drugs (i.e., edivoxetine, ansofaxine, MIN-117, SEP-4199), there are also several already marketed agents that are repurposed for MDD treatment (i.e., cariprazine, pimavanserin), or old psychoactive substances (i.e., psilocybin) tested as add-ons to current antidepressant therapy. Orexin receptor modulators are also investigated for MDD treatment (i.e., seltorexant), with promising results in phase IIb trials (NLM, NCT04951609). Fueled by the success of brexanolone, approved by the FDA for post-partum depression, four new drugs with GABA-A receptors modulating properties/neurosteroids analogs are investigated in phase I to III clinical trials. Anti-cytokine therapies and COX2-inhibitors have been proven to possess antidepressant properties in phase I to IV clinical trials, although not all these studies had positive results. Also, the tolerability of biological therapies should be weighed against their potential benefits. In conclusion, there are promising molecules that had been associated with favorable results in clinical research, but it is difficult to predict which of these agents will be approved in the next few years.

A second part of this review will extend the knowledge regarding new antidepressants in the pipeline, by including drugs with glutamatergic, cholinergic, sestrinergic, and other mechanisms of action.

Regarding the limitations of this review, it should be mentioned that due to the inclusion and exclusion criteria there is a possibility that not all investigational drugs with antidepressant properties were analyzed. Also, the current status of the development for most of the reviewed products was not assessed, but this is related to the lack of this kind of information in the searched databases. Even when manufacturers' websites were included in the search for new antidepressant drugs, this type of information was generally not available; therefore, it was preferred not to include it in this review.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

As the only author, I assume the accuracy of collecting, processing, and presenting data within this review.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2022.884143/full#supplementary-material

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Xiaoyaosan Exerts Antidepressant-Like Effect by Regulating Autophagy Involves the Expression of GLUT4 in the Mice Hypothalamic Neurons

Fu-Rong Yang[1,2], Xiao-Xu Zhu[1], Ming-Wang Kong[1], Xiao-Juan Zou[1], Qing-Yu Ma[3], Xiao-Juan Li[3] and Jia-Xu Chen[3,4]**

[1]School of Basic Medical Science, Hubei University of Chinese Medicine, Wuhan, China, [2]Hubei Provincial Key Laboratory of Occurrence and Intervention of Rheumatic Diseases, Hubei Minzu University, Enshi, China, [3]Formula-Pattern Research Center, School of Traditional Chinese Medicine, Jinan University, Guangzhou, China, [4]School of Traditional Chinese Medicine, Beijing University of Chinese Medicine, Beijing, China

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***Correspondence:**
Xiao-Juan Li
lixiaojuan@jnu.edu.cn
Jia-Xu Chen
chenjiaxu@hotmail.com

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Many studies have proven that autophagy plays a pivotal role in the development of depression and it also affects the expression of GLUT4 in the hypothalamus. Xiaoyaosan has been shown to exert antidepressant effects in a variety of ways, but its underlying mechanism by which Xiaoyaosan regulates autophagy as well as GLUT4 in the hypothalamus remains unclear. Thus, in this study, we established a mouse model of depression induced by chronic unpredictable mild stress (CUMS), and set up autophagy blockade as a control to explore whether Xiaoyaosan exerts antidepressant effect by affecting autophagy. We examined the effects of Xiaoyaosan on behaviors exhibited during the open field test, tail suspension test and sucrose preference test, and the changes in autophagy in hypothalamic neurons as well as changes in GLUT4 and the related indicators of glucose metabolism in CUMS-induced depressive mouse model. We found that CUMS- and 3-MA-induced mice exhibited depressive-like behavioral changes, with decreased LC3 expression and increased p62 expression, suggesting decreased levels of autophagy in the mouse hypothalamus. The expression of GLUT4 was also decreased, and it was closely related to the level of autophagy through Rab8 and Rab10. Nevertheless, after the intervention of Xiaoyaosan, the above changes were effectively reversed. These results show that Xiaoyaosan can regulate the autophagy in hypothalamic neurons and the expression of GLUT4 in depressed mice.

Keywords: depression, autophagy, GLUT4, hypothalamus, xiaoyaosan

INTRODUCTION

Depression is a common mental and psychological disorder whose main clinical features are feeling down, anorexia, fatigue, and cognitive impairment. Depression is also accompanied by insomnia, autonomic nervous system and gastrointestinal tract disorders, a strong sense of self-blame and inferiority, and even suicidal tendency. The WHO listed major depressive disorder (MDD) as the third leading cause of the global burden of disease, and it is expected that the disease will rank first by

2030 (WHO, 2008). The economic burden caused by MDD is approximately $2.5 trillion in the US, accounting for 10% of the total global disease burden (Bach et al., 2020). Depression is also a major independent risk factor for other diseases, such as cardiovascular disease, dementia, diabetes, and osteoporosis (Knol et al., 2006). Depression can also severely restrict patients' social and psychological functions, reduce their quality of life, and bring misfortune upon them (Pan et al., 2019). The high proportion of suicides among patients also has some direct adverse effects on social stability. Due to the diverse and complex causes and mechanisms of the disease, coupled with the high incidence of disease, depression is becoming a serious social, economic and medical problem threatening the world that has not yet been clarified.

Recent studies have shown that autophagy is mainly involved in the biological process of energy metabolism (Yang et al., 2019). Autophagy is a key mechanism for eukaryotes to maintain cell renewal and homeostasis based on the intracellular lysosomal degradation pathway (Luo et al., 2019). LC3 is a reliable marker of autophagosome, and p62 is the most characteristic molecule and degradation product of autophagy, and the expressionls of both can reflect the autophagic activity of cells. LC3 II/LC3 I can reflect the degree of autophagy, the ratio is positively correlated with the degree of autophagy, and p62 is inversely proportional to the degree of autophagy. Many studies have shown that the onset of depression is related to the degree of autophagy. Some studies have shown that enhanced autophagy can alleviate depression-like behaviors to some extent (Shu et al., 2019), while others have found the opposite (Liao et al., 2021). Previous research has found that mice with depression induced by chronic restraint stress exhibit increased fasting and postprandial blood glucose levels, and reduced insulin levels (Pan et al., 2019) and these symptoms of glucose metabolism disorders are related to central neuron autophagy.

Glucose transporter-4 is the main regulatory protein in the body to take up glucose from peripheral tissues, and it plays an important role in maintaining the body's glucose homeostasis (Govers, 2014; Amira et al., 2019). GLUT4 plays a role in regulating systemic glucose homeostasis in hypothalamic neurons (Hongxia et al., 2015), and autophagy plays an important role in regulating the translocation of GLUT4 (Safa et al., 2018). Rab protein, a member of the Ras GTPase superfamily, is closely related to autophagy in regulating the maturation, transportation and fusion of GLUT4 vesicles (Ao et al., 2014). Rab8 and Rab10 jointly regulate the maturation of autophagy and the transport of GLUT4 vesicles and are an important regulatory mechanism by which autophagy participates in the regulation of GLUT4 translocation (Jaldin-Fincati et al., 2017). We screened and preliminarily verified that GLUT4 is the target molecule of hypothalamic neurons involved in glucose metabolism through high-throughput sequencing of whole-genome DNA methylation (Li, X. J, 2018). However, the involvement of GLUT4 in hypothalamic neurons during the biological process of glucose metabolism and its relationship with autophagy are currently unclear.

Xiaoyaosan was originally described in Taiping Huimin Heji Jufang, a chinese materia medica officially compiled in the Song Dynasty of China (960–1127 AD). The Xiaoyaosan formula contains eight herbs, including Radix Bupleuri: Radix Paeoniae Alba: Angelica Sinensis:Poria Cocos: Rhizoma Atractylodis Macrocephalae: Rhizoma Zingiberis Recens: Radix Glycyrrhizae: Herba Menthae = 5:5:5:5:5:5:4:1. We have been conducting researches on the antidepressant effects of Xiaoyaosan for many years, and we have found that Xiaoyaosan has a modulating effect on depression-like behaviors caused by CUMS (Yan et al., 2018; Ding et al., 2020). However, whether Xiaoyaosan can regulate the autophagy of hypothalamic neurons and further affect the glucose metabolism in a mouse model of depression through GLUT4 is not yet known. CUMS is known to be risk factor for psychiatric disorders, and stress-induced animal models of mood disorders have therefore been widely investigated. Since it effectively induces the pathophysiology of depression, CUMS is considered as an appropriate paradigm for imitating psychiatric-related illnesses in rodents. In view of this, we established a mouse model of depression induced by CUMS for 6 weeks, evaluated the model, and related indicators of autophagy and energy metabolism were detected. After verifying that the level of autophagy decreased in depressed mice, we then injected the autophagy inhibitor 3-Methyladenine (3-MA) into the control mice, and tested the behavioral and related indicators of autophagy and energy metabolism. Hence, we explored the regulatory effect of Xiaoyaosan on the autophagy and GLUT4 mediated by it.

MATERIALS AND METHODS

Animals

One hundred and eight specific pathogen-free (SPF), 6-8-week-old male C57BL/6J mice weighed 18–25 g. The animals were provided by Beijing Sibeifu Biotechnology Co., Ltd.(SYXK (yue)2019–0010) and were housed in an animal room with a barrier system under standard experimental conditions (The permit number of the local Ethics Committee is 20210512–05). The mice were bred in the SPF animal room of Jinan University, at room temperature (21 ± 2)°C, relative humidity of 30%–40%, and light and dark conditions for 12 h (light, 7:00~~19:00, dark, 19:00~7:00). There were two batches of mice and all of them were given free access to distilled water and fed a standard rodent diet.

After 7 days of habituation, the first batch of 48 mice were randomly divided into four groups: the control group, CUMS group, Xiaoyaosan group and fluoxetine group, twelve mice in each group, were fed in three cages. The protocols were approved by the Institutional Animal Care and Use Committee of Jinan University and strictly abided by the Beijing Experimental Animal Ethics and Welfare Guidelines (released on 2006-01-01) to minimize animal suffering. Except for the control group, the other three groups of mice received CUMS for three consecutive weeks. The CUMS paradigm consists of various mild stressors, such as food and water deprivation for 24 h, 45° tilted cage for 24 h, inversion of day/night light cycle for 24 h, odor (glacial acetic acid) stimulusfor 24 h, wet bedding for

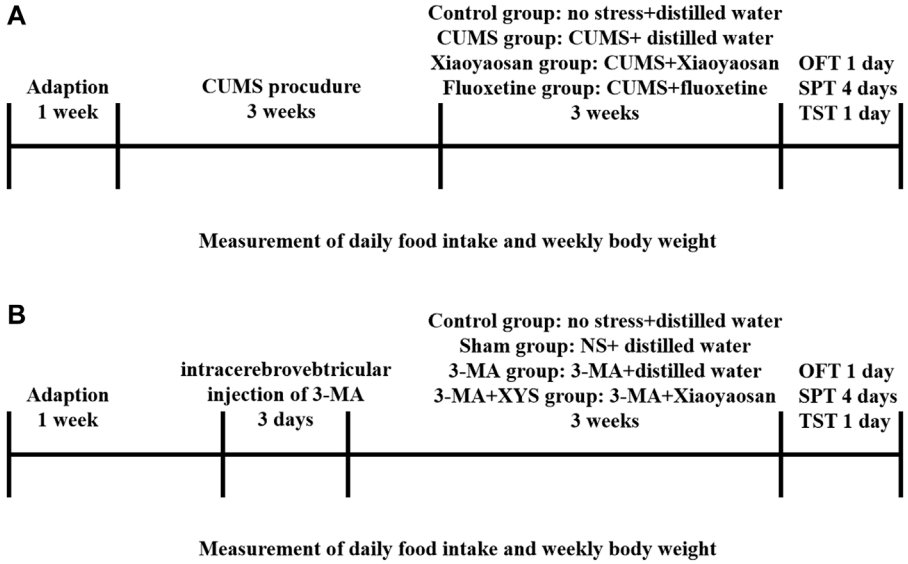

FIGURE 1 | Experimental schedule in this study. **(A)** After 1 week of habituation, except for those in the control group, the first mice in the three other groups were subjected to chronic unpredictable mild stress (CUMS) stress for 3 weeks. The mice in each group received the corresponding treatment for 3 weeks after the stress ended. Daily food intake and weekly body weight were recorded from the first day of stress until the end of treatment. The open field test (OFT), sucrose preference test (SPT) and tail suspension test (TST) were performed. Then, the animals were sacrificed, and tissue was collected and cryopreserved. **(B)** After 1 week of habituation, except for those in the control group, the first mice in the three other groups were injected with NS and 3-MA respectively. The mice in each group received the corresponding treatment for 3 weeks after the injection. Daily food intake and weekly body weight were recorded from the first day of wound recovery until the end of treatment. The open field test (OFT), sucrose preference test (SPT) and tail suspension test (TST) were performed. Then, the animals were sacrificed, and tissue was collected and cryopreserved.

24 h, 45°C oven heat drying for 5 min, restraint for 2 h, etc. We implemented a stressor on 1 day, and each of these stressors was guaranteed not to be applied on consecutive days (**Figure 1A**).

As shown in **Figure 1B**, the second batch of 60 mice were randomly divided into four groups after 7 days of habituation: the control group, Sham group, 3-MA group and 3-MA + XYS group, fifteen mice in each group, were fed in three cages. Except for the mice in control group, the others were deeply anesthetized with sodium pentobarbital and fixed in a stereotaxic apparatus with a pair of ear bars and an incisor bar. A small incision was made to expose the skull, and the bregma was labelled to orient the coordinates. The coordinates of the lateral ventricle were 1.4 mm anteroposterior (AP), 1.1 mm mediolateral (ML), and 4.9 mm dorsoventral (DV) to the bregma. After marking the position, first drill the skull with a suitable size electric drill, then fix the micro syringe, and start the injection procedure in the system (RWD Life Science, Shenzhen, China). We injected normal saline (NS) and 3-MA (Sigma-Aldrich, United States) (dissolved with normal saline) into the hypothalamus of the Sham group and 3-MA group, 3-MA + XYS group, respectively. One-time injection of 3-MA into mouse hypothalamus at a concentration of 12 μg/μl, 30 μg per mouse. The mice were immediately removed from the stereotaxic apparatus and placed in an incubator to maintain their basal body temperature after surgery. Mice were given intramuscular injections of penicillin for three consecutive days after surgery to prevent infection. After the treatment of the two batches of mice was finished, the open field test (OFT), sucrose preference test (SPT) and tail suspension test (TST) were performed. After one of behavioral tests finished, the mice were allowed to rest for a day before proceeding to the next one.

Preparation of Drugs

The Xiaoyaosan dry extract (provided by Jiuzhitang Co., Ltd. (Changsha, China)) was produced based on the procedure described in the Chinese Pharmacopoeia 2015 Edition (National Pharmacopoeia Commission, 2015). We completed the study on the drug properties of Xiaoyaosan compounds using DrugBank and a gene chip combined with cMap (Yuan et al., 2020). According to the 60-kg/d dosage conversion from human to animal (Ao et al., 2014), the mice in the Xiaoyaosan group were given Xiaoyaosan powder dissolved in distilled water at a dose of 0.658 g/kg/d and 0.1 ml/kg bodyweight via gavage. Fluoxetine hydrochloride tablets (20 mg/tablet) were obtained from Patheon France (packaged by Lilly Suzhou Pharmaceutical Co., Ltd, Suzhou, China), and the fluoxetine group was given fluoxetine dissolved in distilled water at a dose of 2.6 mg/kg/d and 0.1 ml/kg body weight via gavage every day. The control group and CUMS group were given an equal volume of distilled water.

Starting from the fourth week of CUMS, intragastric gavage was administered to mice in each group 1 h after the completion of CUMS treatment every day, and those requiring 24 h for stress were given intragastric gavage at 21:00. The gavage treatment was continued for 3 weeks.

Body Weight and Food Intake

To evaluate whether CUMS will affect the physical conditions of the mice, the body weight and the food intake of mice in each group were weighed every week from the first day of the experiment. The data were continuously monitored and recorded for 6 weeks. The formula for calculating the daily food intake of mice is daily food intake = total daily food weight (Gram)-daily remaining food weight (Gram).

Open Field Test

1 h later after finishing CUMS stress and administration, all C56BL/6J mice were subjected to an open field test to evaluate the behavioral characteristics of mice in each group, such as autonomous activities and space exploration. We placed the open field box (50 × 50 × 50 cm) with a central zone (40 × 40 cm) in a quiet operation room, installed a camera directly above the open field box and connected the camera to a computer for real-time observation and recording. Thirty minutes before the start of the test, the mice of each group were put into the quiet operating room for habituation. We gently placed the mouse into the center of the open field box and started timing and video recording. Observer 5.0 and EthoVision XT (Noldus Information Technology, Netherlands) software were used to analyze behavioral parameters such as the total distance traveled and the time spent in the open area of each group of mice within 5 min. After the test, we first cleaned the urine and feces left by the test mouse with a wet towel, and then sprayed 75% ethanol to remove the residual odor. After the smell dissipated, the next mouse was put into the box for testing. The observer remained quiet throughout the experiment.

Tail Suspension Test

The tail suspension test was used to evaluate the activity and the desperate behavior of mice. Thirty minutes before the start of the test, the mice were put into a quiet operating room to habituate to the environment. A special tail suspension box was placed in the quiet operating room. A camera was installed directly in front of the tail suspension box and connected to a computer for real-time observation and video recording. We hung the mice above the suspension box with tape at a height of approximately 30 cm from the bottom of the box and started timing and video recording. Observer 5.0 and EthoVision XT software were used to analyze the immobility time of mice in each group within 5 min.

Sucrose Preference Test

After the test was over, we assessed the degree of anhedonia in mice to determine the severity of depressive state by a sucrose preference test. All mice were trained to habituate to the sucrose water for 72 h. First, we put two bottles of 1% sucrose water in each cage at the same time (within the first 24 h). Then, we placed a bottle of distilled water and a bottle of 1% sucrose water in each cage (the second 24 h). Finally, after the mice were fasted for 24 h, we performed SPT. Each mouse was tested in a cage. A bottle of 1% sucrose in water and a bottle of distilled water was simultaneously placed in each cage. The mice were allowed to drink freely for 1 h, and then the two bottles were removed and weighed to calculate the sucrose preference rate of the mice. The preference for sucrose was calculated according to the following formula: sucrose consumption (g)/[water consumption (g) + sucrose consumption (g)]×100%.

Intraperitoneal Injection Glucose Tolerance Test (IPGTT)

After finishing the SPT, and 24 h after the mice resumed their diet, they were fasted again for 12 h. We intraperitoneally injected 2 mg/g glucose prepared as 0.2 g/ml glucose solution into each mouse, collected blood from the tail of the mice at different time points at 0, 15, 30, 60, and 120 min after being injected, and then detected and recorded blood glucose levels. We used a fully automatic biochemical detector (Roche, Switzerland) to test blood glucose in accordance with the instructions.

Enzyme-Linked Immunosorbent Assay

Twenty-four hours after the IPGTT, the mice were deeply anesthetized with sodium pentobarbital, and retro-orbital blood was collected. The supernatant was collected after centrifugation, and the serum insulin concentration was assayed with an ELISA kit (Solarbio Life Science, Beijing, China) according to the instructions. Wash buffer and antibody-HRP conjugate working solutions were prepared after the reagents were warmed, and the standards were serially diluted. Set up blank control wells and add samples and reagents to the corresponding well plates. We measured the OD 450 value immediately after the operation, drew a standard curve, and calculated the sample concentration.

Transmission Electron Microscopic Analysis

A small portion (~1 mm^3) of the hypothalamus from one mouse in each group was sectioned and incubated for 2 h at 4°C in 2.5% glutaraldehyde (Solarbio Life Science, P1126, Beijing, China). The specimens were rinsed with 0.1 M phosphoric acid, postfixed in 1% osmium tetroxide for 2–3 h, and then rinsed with 0.1 M phosphoric acid again for 15 min × 3 times. The specimens were dehydrated with different concentrations of ethanol at 4°C, and put into acetone three times at room temperature, then embedded in epoxy resin. After curing in the oven, the specimen is cut into ultrathin sections of 50–60 nm which were stained with lead citrate and examined by transmission electron microscopy (JEM-1010, Japan). Then we randomly selected any cell in the hypothalamus tissue for observation, with 10 visual fields for each cell and counted the number of autophagosomes in the 10 visual fields of each group by observing the electron microscope pictures.

Immunofluorescent Staining

Mice brain tissue was fixed in 4% paraformaldehyde (Solarbio Life Science, P1110, Beijing, China) for 48 h, embedded in conventional paraffin and then sliced into 5 μm coronal sections. After numbering the slices, they were placed on a slide warmer for more than 2 h and then they were deparaffinized with xylene and gradient alcohol. Antigen

TABLE 1 | Primer sequences used in RT-qPCR analysis.

Gene symbol	Forward primer	Reverse primer
LC3	ATCATCGAGCGCTACAAGGG	AGCCGAAGGTTTCTTGGGAG
p62	GACAAGAGTAACACTCAGCCAAGCA	CTCCATCTGTTCCTCTGGCTGTC
GLUT4	GCTGAAGGATGAGAAACGGAAGT	TTCTACTAAGAGCACCGAGACCAA
Rab8	TGGCACTCGACTATGGGATCA	AGGAGACTGCACCGGAAGAA
Rab10	TCGGACGATGCCTTCAATACC	TGTAGTAGGAGGTTGTGATGGTGTG
β-actin	GTGACGTTGACATCCGTAAAGA	GTAACAGTCCGCCTAGAAGCAC

retrieval was performed on the slices: The slices were placed in a container containing sodium citrate buffer, and antigen retrieval was performed in a pressure cooker. After the solution in the pressure cooker boiled for 3 min, the container was taken out and cooled naturally. Do not take out the slices. After cooling to room temperature, the slides were rinsed in PBS (pH 7.4) for 5 min × 3 times. Then they were placed in 0.5% Triton X-100(Solarbio Life Science, P1080, Beijing, China) (prepared in phosphate-buffered saline) for 30 min at room temperature. The slices were blocked in goat serum working solution for 30 min, then the blocking solution was removed, and incubated the slices in GLUT4 primary antibody (1; 500, Bioss Antibodies, bs-0384R, Beijing, China) at 4°C overnight. Then we incubated the slices with a fluorescent secondary antibody (1:200, Alexa Fluor® 594, ab150116) in the dark, stained the nucleus (DAPI: Solarbio, S2110, Beijing, China) and mounted the slices. Finally, the slices were covered with cover glass, observed and photographed under an upright fluorescence microscope (Olympus, BX53/53 M, Japan). The immunofluorescence intensity of GLUT4 in the hypothalamus of mice in each group was analyzed by ImageJ.

Western Blot Analysis

We observed the expression of LC3 (1:500, Cell Signaling Technology, #2775), p62 (1:500, Cell Signaling Technology, #5114), GLUT4 (1:500, Cell Signaling Technology, #2213), Rab8 (1:500, Cell Signaling Technology, #6975), and Rab10 (1: 500, Cell Signaling Technology, #8127) by WB. Protein was extracted from hypothalamic tissue using the Tissue Protein Extraction Kit (Shanghai Beibo Biotechnology Co., Ltd, BB-3101–2, China) and the protein concentration was measured with a BCA Protein Quantitative Kit (Shanghai Biyuntian Biotechnology Co., Ltd, P0012, China). We loaded 20 μg of protein extract per well based on protein concentration and performed gel electrophoresis. Then we transfered the protein to a PVDF membrane (activated by soaking in methanol in advance). The molecular weight of the target protein is the basis for our selection of membrane specifications. For molecular weights less than 20 kDa, we used the 0.22 μm pore size membrane, and for molecular weights greater than 20 kDa, we used the 0.45 μm pore size membrane. After blocking the membrane with 5% nonfat milk in TBST for 30 min, incubate with corresponding specific antibodies against LC3B, p62, GLUT4, Rab8, and Rab10 overnight at 4°C. After incubation with the appropriate HRP-conjugated secondary antibodies. (goat anti-mouse IgG-HRP: 1:2,000, Asbio Technology, As006; goat anti-rabbit IgG-HRP: 1:2,000, Asbio Technology, As006) for 1 h and rinsing with tris buffered saline tween (TBST), The bands were visualized with an enhanced chemiluminescence reagent (Millipore, Billerica, MA) and then scanned and analyzed by an image analyser (Bio–Rad, California, United States). The intensity of the protein bands was normalized to GAPDH (1: 1,000, Cell Signaling Technology, #5174).

Real-Time Fluorescence Quantitative Polymerase Chain Reaction

The mRNA expression of LC3, p62, GLUT4, Rab8 and Rab10 mRNA in the hypothalamus was detected by RT–qPCR. Total RNA in the hypothalamus was extracted with TRIzol reagent (Solarbio Life Science, Beijing, China), and then the concentration was measured with a spectrophotometer (Eppendorf, Germany). First-strand cDNA was synthesized using a RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, United States) on a C1000 TouchTM Thermal Cycler (Bio-Rad, California, United States) according to the manufacturer's instructions in a total volume of 20 μl. Primers were designed based on published mRNA sequences using Primer three primer selection software, and then synthesized by a professional biotechnology company (Sangon Biotech Co., Ltd, Shanghai, China), and the sequences are shown in **Table 1**. PCR was used to amplify the cDNA with a Power SYBR® Green PCR Master Mix kit (Thermo Fisher Scientific, United States) in a total volume of 25 μl on a CFX96 Real-time PCR System (Bio-Rad, California, United States) consisted of the following: template cDNA (2 ng/1 μl, equal to 100 ng of total RNA template), 2 × SYBR Green PCR Premix HS Taq mM dNTPs (12.5 μl), 10 μM forward primer (0.5 μl), 10 μM reverse primer (0.5 μl), complement deionized water to 25 μl. RT-qPCR reaction with the following cycling parameters: 94°C for 3 min; 40 cycles of 94°C for 30 s, 63°C for 30 s and 72°C for 30 s. The amplification reactions were performed in triplicate. In this study, β-actin was used as the reference gene and the results were calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

Statistical Analysis

SPSS (Statistic Package for Social Science) 21.0 software (IBM, Chicago, IL, United States) was used for statistical analysis of the data expressed as the mean ± standard error of the mean (SEM). We first performed a normality test on the data. When the data were normally distributed, one-way ANOVA was used for

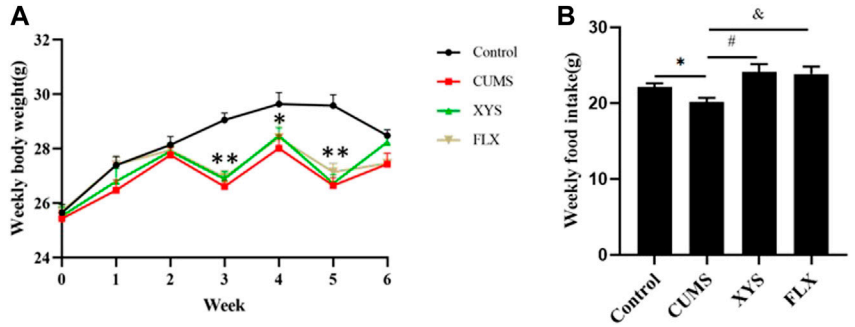

FIGURE 2 | Effects of CUMS, Xiaoyaosan, and fluoxetine on the weekly body weight gain and daily food intake of the mice.**(A)** Weekly body weight ($F(3, 44) = 20.310$, $p < 0.0001$, n = 12) **(B)** Weekly food intake ($F(3, 20) = 7.885$, $p = 0.0092$, n = 12). The values shown represent the mean ± SEM. $^{*}p < 0.05$ or $^{**}p < 0.01$ compared with the control group.

multiple group analysis, and LSD was further used for pairwise comparisons. When the variances were not uniform, a nonparametric test was used, and then a pairwise comparison was performed. When the data did not conform to a normal distribution, Kruskal–Wallis H was used to compare among K independent samples in the nonparametric test. When pairwise comparison was required, two independent samples were used for analysis. The body weight and food intake of mice in each group were analyzed by repeated measures analysis of variance. $p < 0.05$ was defined as significant.

RESULTS

Xiaoyaosan Increased the Body Weight and Food Intake of Mice Induced by CUMS

During the study, the daily food intake and weekly body weight of the mice were monitored to verify the influence of CUMS on these parameters. As shown in **Figure 2A**, before the CUMS started, the weight of the mice in each group was the same. During the first and second weeks, the weight of the mice in each group increased gradually, and the increase in the control group was greater than that of the other groups. At the end of the third week, due to the food and water restriction of the mice on the day before the weighing, the body weight of the mice in the CUMS group, fluoxetine group and Xiaoyaosan group decreased ($p < 0.01$), except for the control group. After administration began, during the fourth week, the weight of the mice in the fluoxetine group and the Xiaoyaosan group was slightly higher than that of the CUMS group. The weight of the mice in all the groups was higher than that at any previous time point, but it was still lower than the control group ($p < 0.05$). At the end of the fifth week, due to the food and water restriction, the weight of the mice the CUMS group, fluoxetine group and Xiaoyaosan group decreased and was significantly lower than that of the control group ($p < 0.01$). By the end of the sixth week, the body weight of mice in the CUMS group, fluoxetine group and Xiaoyaosan group increased again, while the weight of the mice in the control group decreased due to external interference factors, and there was no significant difference compared with the other three groups ($p > 0.05$).

Due to the stress of food and water restriction, the food intake on that day was not weighed. The food intake results of the mice in each group at different time points are shown in **Figure 2B**. The weekly food intake of the control group was higher than that of the CUMS group ($p < 0.05$). After intervention with Xiaoyaosan and fluoxetine, the weekly food intake of mice increased correspondingly. ($p < 0.05$).

Xiaoyaosan Ameliorates CUMS-Induced Depression-Like Behaviors in Mice

During OFT, the total distance traveled by the mice in OFT of each group is shown in **Figures 3A,B**. Compared with the control group, the total distance traveled in the open field by the mice in the CUMS group is significantly reduced ($p < 0.05$). The mice in the Xiaoyaosan group and fluoxetine group improved their mobility to varying degrees after treatment, and the data in the Xiaoyaosan group were statistically different from those of the CUMS group ($p < 0.05$). The total distance traveled in the fluoxetine group was significantly greater than that of the CUMS group ($p < 0.01$). The results of the time spent in the open area in the OFT are shown in **Figure 3C**. The time spent in the open area in the CUMS group was largely less than that in the control group ($p < 0.01$). Compared with the CUMS group, the mice in the Xiaoyaosan group and fluoxetine group spent significantly more time in the open area ($p < 0.05$). These indicated that exposure to the CUMS procedure decreased the exploratory behavior of mice, but Xiaoyaosan can change the behavior of mice.

The immobility time in the TST is shown in **Figure 3D**. The immobility time of mice in the CUMS group was significantly longer than that of the control group ($p < 0.01$). The immobility time of mice in the Xiaoyaosan group was less than that in the CUMS group ($p < 0.05$). Compared with the CUMS group, the immobility time of the mice in the fluoxetine group was also significantly reduced ($p < 0.01$).

As shown in **Figure 3E**, CUMS reduced the sucrose consumption of mice in the CUMS group compared with the

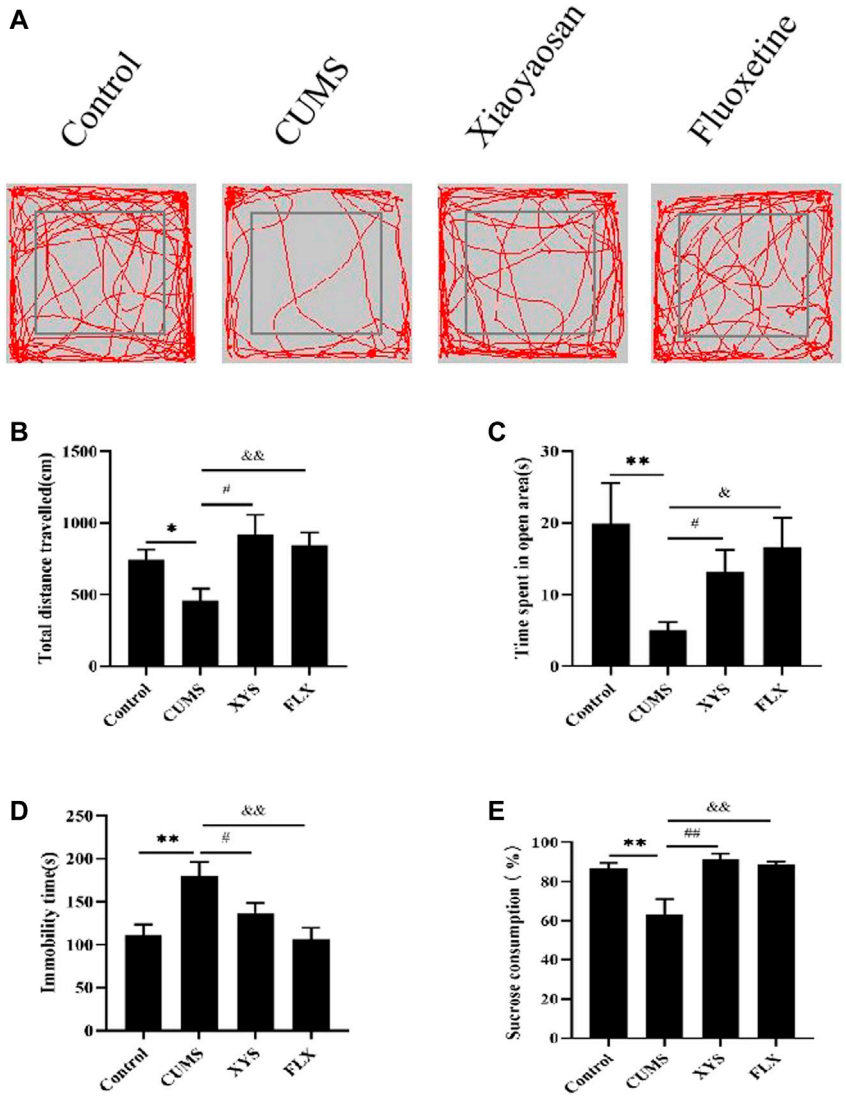

FIGURE 3 | Xiaoyaosan ameliorate depression-like behaviors in mice induced by CUMS. **(A)** Map of the trajectory taken by mice in each group in the OFT assessed by video tracking software. **(B)** Total distance traveled in the OFT ($F(3, 44) = 4.017$, $p = 0.0130$, n = 12). **(C)** Time spent in the open area in the OFT ($F(3, 44) = 2.723$, $p = 0.0556$, n = 12). **(D)** Immobility time spent in the TST ($F(3, 44) = 6.056$, $p = 0.0015$, n = 12). **(E)** Sucrose consumption in the SPT ($F(3, 44) = 8.602$, $p = 0.0001$, n = 12). The values shown represent the mean ± SEM. The values represent the mean ± SEM.$^{*}p < 0.05$ or $^{**}p < 0.01$ compared with the control group. $\#p < 0.05$, $\#\#p < 0.01$ compared with the CUMS group. $\&p < 0.05$, $\&p < 0.01$ compared with the CUMS group.

control group ($p < 0.01$). The sucrose consumption of the two treatment groups was significantly higher than that of the CUMS group ($p < 0.01$), indicating that both Xiaoyaosan and fluoxetine can effectively ameliorate the depression-like state in mice.

Xiaoyaosan Promotes Hypothalamic Autophagy in CUMS-Induced Mice

We monitored autophagy in the hypothalamus of each group of mice by TEM, and the results are shown in **Figure 4A** and **Figure 4B** (the yellow arrow in the figure shows the autophagosome, and the red arrow shows the damaged mitochondaria). We found that the hypothalamic mitochondrial morphology of the CUMS group was damaged and that the number of autophagosomes was significantly reduced compared with that of the control group ($p < 0.01$). The Xiaoyaosan and fluoxetine groups had more complete mitochondrial morphology and more autophagosomes ($p < 0.01$).

As shown in **Figure 4C** and **Figure 4D**, the protein levels of LC3 especially LC3II in the hypothalamus of the CUMS group were lower than those of the control group, and the LC3II/LC3I ratio was also decreased ($p < 0.05$). Regarding the protein expression of p62 in the hypothalamus, the expression in the mice of CUMS group was higher than that of the control group ($p < 0.05$) (**Figure 4E**). Xiaoyaosan and fluoxetine reduced the

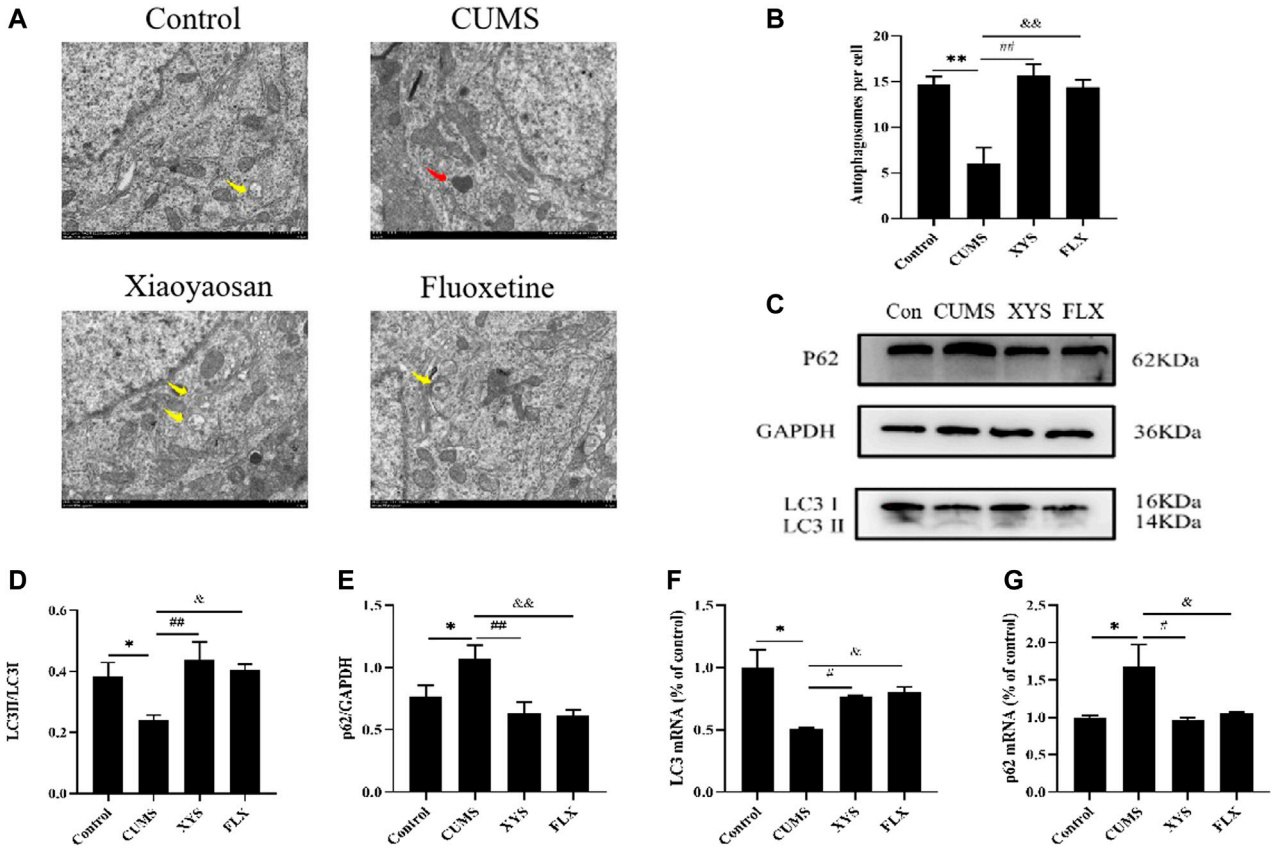

FIGURE 4 | Xiaoyaosan promote hypothalamic autophagy in CUMS-induced mice. **(A)** Transmission electron microscope showed the situation of autophagosomes and mitochondria in the hypothalamus of the mice (scale bar = 1 μm). Mitochondria were disrupted in CMS group (the red arrow). Autophagosomes were observed in control, Xiaoyaosan and fluoxetine group (yellow arrows). **(B)** The number of autophagosomes in the hypothalamus of the mice ($F(3, 8) = 13.383$, $p = 0.0017$, n = 1). **(C)** Representative western blotting of LC3 and p62 expressions. **(D)** Quantitative analysis of protein levels of LC3($F(3, 16) = 4.904$, $p = 0.0133$, n = 5). **(E)** Quantitative analysis of protein levels of p62($F(3, 16) = 5.931$, $p = 0.0064$, n = 5). **(F)** The expression of LC3 mRNA ($F(3, 12) = 7.326$, $p = 0.0047$, n = 4). **(G)** The expression of p62 mRNA ($F(3, 12) = 5.160$, $p = 0.0161$, n = 4). The values represent the mean ± SEM. $^{*}p < 0.05$ or $^{**}p < 0.01$ compared with the control group. #$p < 0.05$, ##$p < 0.01$ compared with the CUMS group. &$p < 0.05$, &$p < 0.01$ compared with the CUMS group.

expression of this protein in the hypothalamus of these two groups ($p < 0.01$ or $p < 0.05$). Changes in mRNA expression, shown in **Figures 4F,G**, exhibited the same trend ($p < 0.05$).

Effect of Xiaoyaosan on Glucose Metabolism in the Hypothalamus of Mice Induced by CUMS

Based on the results of GLUT4 immunofluorescence in each group of mice (**Figures 5A,B**), the expression of GLUT4 in the dorsal medial nucleus and paraventricular nucleus of the hypothalamus in the mice of CUMS group was significantly lower than that in the control group ($p < 0.01$). Compared with the CUMS group, the expression of GLUT4 in the dorsal medial nucleus and paraventricular nucleus of the hypothalamus in the two treatment groups was drastically increased ($p < 0.01$).

In terms of mouse serum insulin results (**Figure 5C**), the serum insulin level of mice in the CUMS group was significantly less than that of the control group ($p < 0.01$). After administering Xiaoyaosan and fluoxetine, compared with the CUMS group, the decreased serum insulin levels in the two groups were effectively reversed and improved by a large margin ($p < 0.01$).

By testing the blood glucose of mice at 0, 15, 30, 60, and 120 min, we found that the blood glucose value of each group reached its peak at 15 min. The blood glucose level of the mice in the CUMS group was higher than that of the control group and the two treatment groups at each time point, and was drastically higher than that of the Xiaoyaosan group when at 60 min ($p < 0.01$). At 120 min, the blood glucose of the other three groups was statistically significantly lower than that of the CUMS group ($p < 0.01$ or $p < 0.05$) (**Figure 5D**).

As shown in **Figures 5E–H**, the GLUT4 protein expression in the hypothalamus of the CUMS group was significantly lower than that of the control group ($p < 0.01$), but GLUT4 expression increased after administion of Xiaoyaosan and fluoxetine to the mice, and there was a significant difference ($p < 0.05$). Regarding Rab8 and Rab10 protein expression in the hypothalamus, the expression in the CUMS group was lower than that of the control

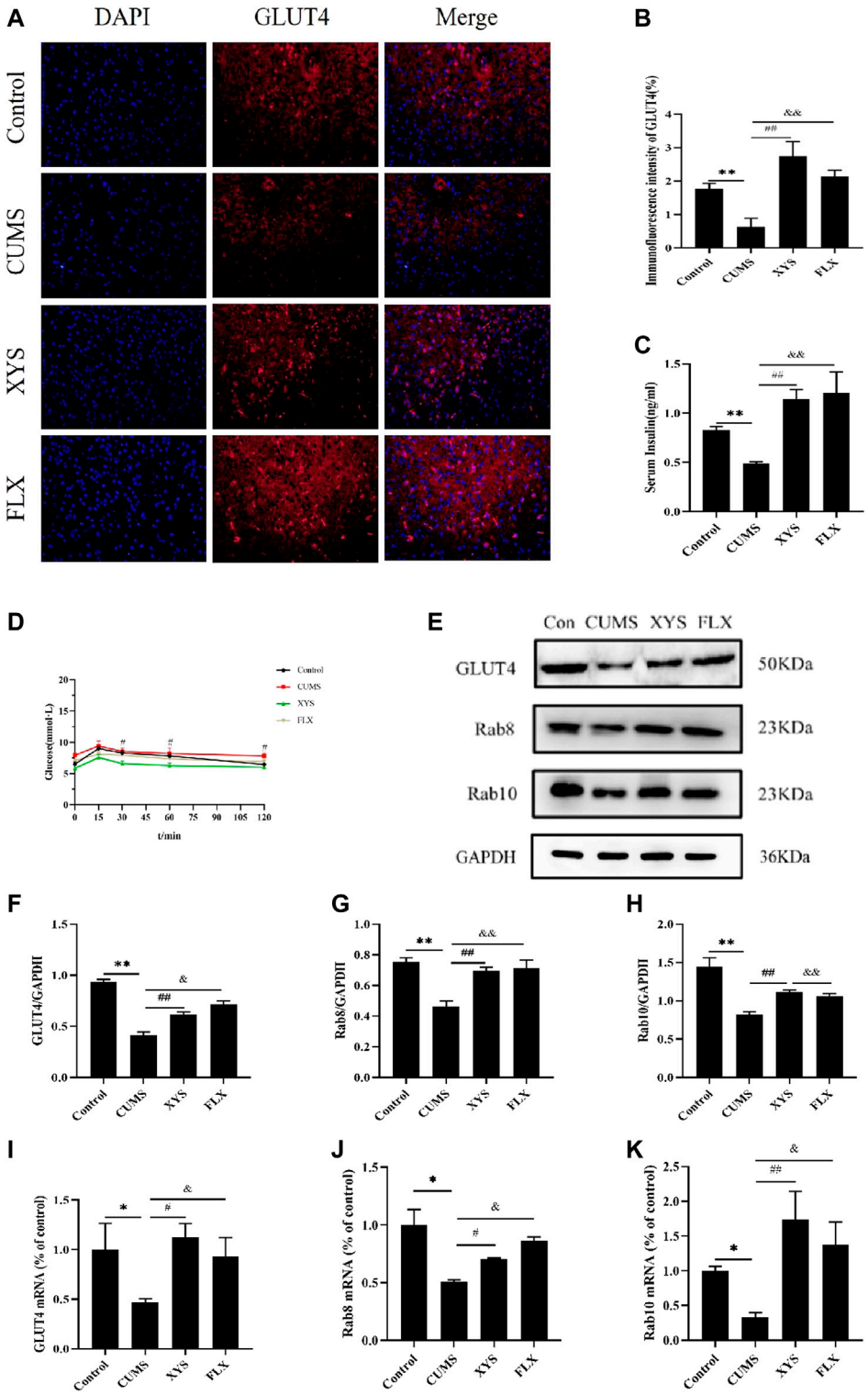

FIGURE 5 | Effect of Xiaoyaosan on glucose metabolism in hypothalamus of CUMS-induced mice. **(A)** Immunofluorescence expression of GLUT4 in hypothalamus of the mice (scale bar = 20 μm). **(B)** Immunofluorescence intensity of GLUT4 (%) ($F(3, 8) = 9.938$, $p = 0.0045$, n = 3). **(C)** Serum insulin level of the mice ($F(3, 16) = 7.973$, $p = 0.0018$, n = 5). **(D)** The blood glucose of the mice ($F(3, 36) = 2.542$, $p = 0.0716$, n = 10). **(E)** Representative western blotting of GLUT4, Rab8 and Rab10 expressions. **(F)** Quantitative analysis of protein levels of GLUT4 ($F(3, 16) = 57.780$, $p < 0.0001$, n = 5). **(G)** Quantitative analysis of protein levels of Rab8 ($F(3, 16) = 13.041$, $p = 0.0001$, n = 5). **(H)** Quantitative analysis of protein levels of Rab10 ($F(3, 16) = 14.085$, $p < 0.0001$, n = 5). **(I)** The expression of GLUT4 mRNA ($F(3, 12) = 2.542$, $p = 0.1054$, n = 4). **(J)** The expression of Rab8 mRNA ($F(3, 12) = 9.332$, $p = 0.0018$, n = 4). **(K)** The expression of Rab10 mRNA ($F(3, 12) = 5.218$, $p = 0.0155$, n = 4). The values represent the mean ± SEM.$^{*}p < 0.05$ or $^{**}p < 0.01$ compared with the control group. #$p < 0.05$, ##$p < 0.01$ compared with the CUMS group. &$p < 0.05$, &$p < 0.01$ compared with the CUMS group.

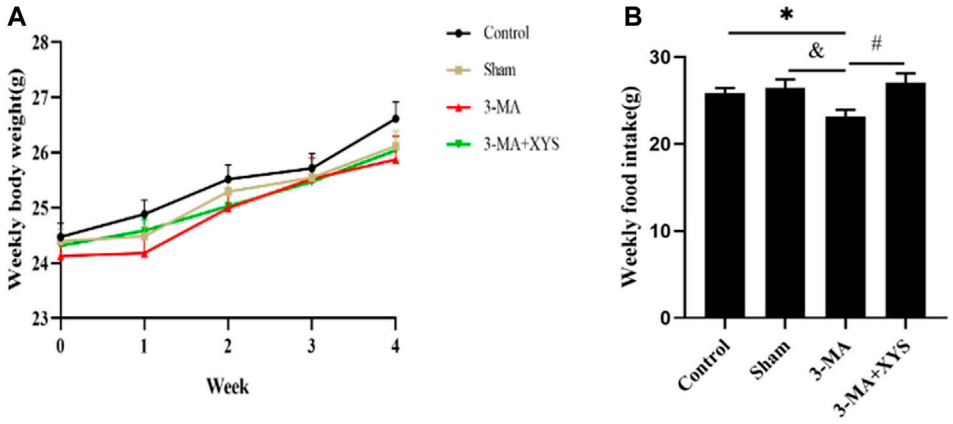

FIGURE 6 | Effects of 3-MA and Xiaoyaosan on the weekly body weight gain and daily food intake of the mice. **(A)** Weekly body weight ($F(3, 56) = 1.689$, $p = 0.1797$, $n = 15$). **(B)** Weekly food intake ($F(3, 8) = 4.002$, $p = 0.0518$, $n = 15$). The values shown represent the mean ± SEM. $*p < 0.05$ compared with the control group. $\&p < 0.05$ compared with the 3-MA group. $\#p < 0.05$ compared with the 3-MA group.

group ($p < 0.01$), and the expression in the Xiaoyaosan group and the fluoxetine group were higher than that in the CUMS group ($p < 0.01$).

The RT-qPCR results demonstrate the GLUT4 mRNA in the hypothalamus of CUMS group was lower than that of the control group ($p < 0.05$). After the mice received Xiaoyaosan and fluoxetine intervention, the GLUT4 mRNA expression increased ($p < 0.05$) (**Figure 5I**). The mRNA expression levels of Rab8 and Rab10 in the hypothalamus of the CUMS group were lower than those of the control group ($p < 0.05$); the expression levels of these two parameters in the mice of the Xiaoyaosan group and fluoxetine group were higher than those in the CUMS group, with significant differences ($p < 0.01$ or $p < 0.05$) (**Figures 5J,K**).

Effects of Xiaoyaosan on the Body Weight and Food Intake of Mice Injected With 3-MA

To explore the role of autophagy in this study, we injected mice with 3-MA, an autophagy inhibitor, to observe the body weight changes of mice in each group (**Figure 6A**). Before the experiment, the body weight of the mice in each group was the same. While the experiment was in progress, the body weight of the mice was steadily increasing every week, and the weight of the control group increased slightly in the first and fourth weeks compared with the other groups. The body weight of mice in the sham group increased slightly in the second week compared with the other groups and mice in the 3-MA + XYS group showed a steady increasing trend. In the last week, the weight of the mice in the control group was the highest. The mice in the sham group and 3-MA + XYS group had the similar body weights but lower body weights than those in the control group. The weight of the mice in the 3-MA group was slightly lower than that of the other three groups ($p > 0.05$).

The changes in food intake of mice in each group at different time points after 3-MA injection are shown in **Figure 6B**. The weekly food intake of the control group and sham operation group was greater than that of the 3-MA group ($p < 0.05$). The mice in the 3-MA + XYS group had the largest food intake, while the mice in 3-MA group had the lowest food intake ($p < 0.05$).

Effect of Xiaoyaosan on the Behavior of Mice Injected With 3-MA

We evaluated the behavior of mice after they were injected with 3-MA. Catching sight of the results of OFT, we found that the total distance traveled by mice in the sham group did not change significantly ($p > 0.05$), while that of mice in the 3-MA group was significantly reduced ($p < 0.01$)compared with that of the control group. The total distance traceled of mice in sham group and 3-MA + XYS group was significantly greater than that in the 3-MA group ($p < 0.01$) (**Figures 7A,B**). Mice in the 3-MA group spent less time in the open area than that of the control group ($p < 0.01$), but there was no significant difference between the sham and the control group ($p > 0.05$). However, compared with the 3-MA group, the time spent in open area of the mice in sham group and 3-MA + XYS group was significantly increased, and the results were statistically different ($p < 0.01$, $p < 0.05$) (**Figure 7C**).

The immobility time of the mice in the 3-MA group during the TST was significantly longer than that of the control group ($p < 0.05$), while no significant difference existed between the sham group and the control group ($p > 0.05$). Compared with the 3-MA group,the immobility time of the mice in the sham group was significantly shorter ($p < 0.05$), and it was effectively reduced in the 3-MA + XYS group when compared with the 3-MA group ($p < 0.05$ (**Figure 7D**).

Regarding sucrose consumption, mice injected with 3-MA reduced sucrose consumption compared with the control group ($p < 0.01$). Data of the mice injected with saline were not much different from the control group ($p > 0.05$) but obviously higher than that of 3-MA group ($p < 0.01$), and the

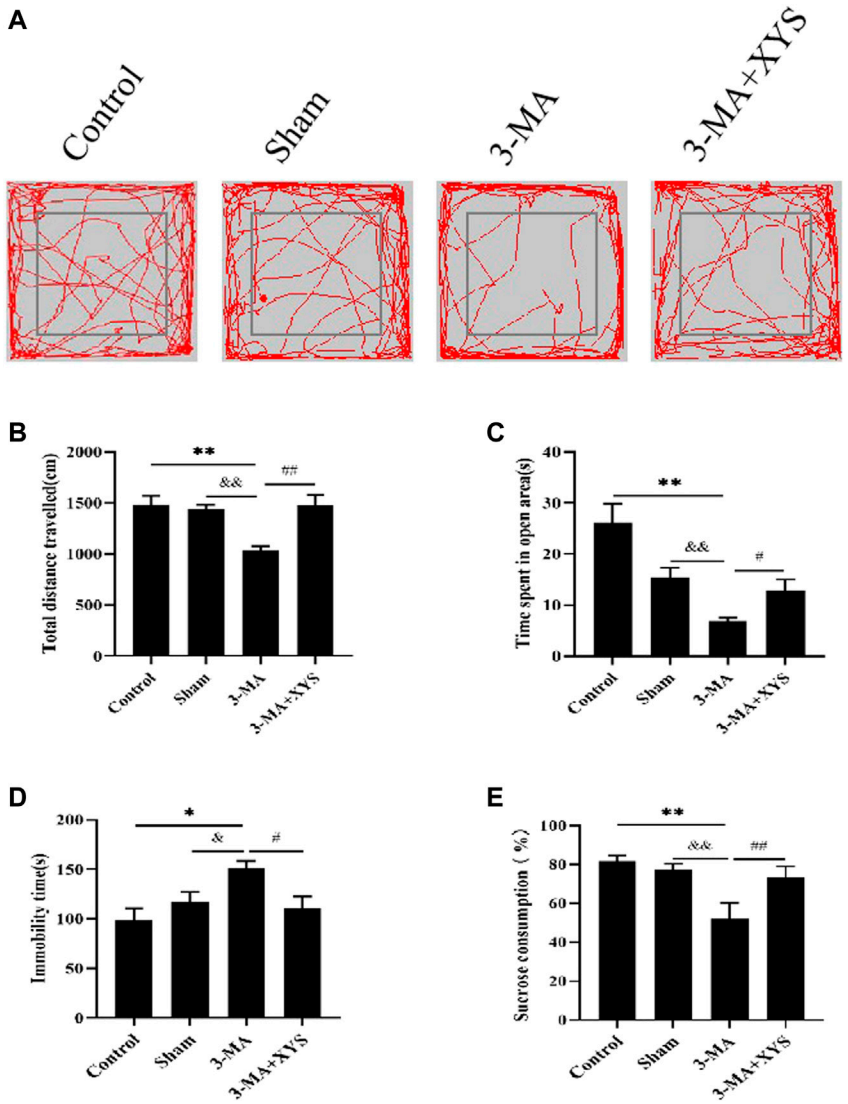

FIGURE 7 | Xiaoyaosan ameliorate depression-like behaviors in mice Injected with 3-MA. **(A)** Map of the trajectory taken by mice in each group in the OFT assessed by video tracking software. **(B)** Total distance traveled in the OFT ($F(3, 56) = 8.933$, $p < 0.0001$, n = 15). **(C)** Time spent in open area in the OFT ($F(3, 56) = 11.767$, $p < 0.0001$, n = 15). **(D)** Immobility time spent in the TST ($F(3, 56) = 4.534$, $p = 0.0065$, n = 15). **(E)** Sucrose consumption in the SPT ($F(3, 56) = 29.444$, $p < 0.0001$, n = 15). The values shown represent the mean ± SEM. The values represent the mean ± SEM. $^{*}p < 0.05$ or $^{**}p < 0.01$ compared with the control group. &$p < 0.05$, &$p < 0.01$ compared with the 3-MA group. #$p < 0.05$, ##$p < 0.01$ compared with the 3-MA group.

same was true for the 3-MA + XYS group ($p < 0.01$) (**Figure 7E**).

Xiaoyaosan Increased Autophagy Expression in Mice Injected With 3-MA

As shown in **Figures 8A,B**, by taking a TEM image of the mouse hypothalamus, we found that the mitochondrial morphology of the mice in the 3-MA group was damaged, and the number of autophagosomes was obviously reduced compared with that in the control group ($p < 0.01$). The sham group had more mitochondrial damage than the control group, but there was little difference in the number of autophagosomes ($p > 0.05$). The number of autophagosomes in the hypothalamus of mice in sham group was significantly higher than that in the 3-MA group ($p < 0.01$), whereas after the mice injected with 3-MA were treated with Xiaoyaosan, their mitochondria were more complete, and the number of autophagosomes in the hypothalamus also increased significantly ($p < 0.01$).

Next we observed the expression of LC3 and p62 in the mouse hypothalamus (**Figures 8C–E**). Compared with the control group and sham group, the expression of LC3II was significantly lower in the mice injected with 3-MA, and the expression of p62 was increased, both of which were significantly different ($p < 0.01$ or $p < 0.05$). These two values between the sham group and the control group showed no differences. After treatment with Xiaoyaosan, the expression of LC3II

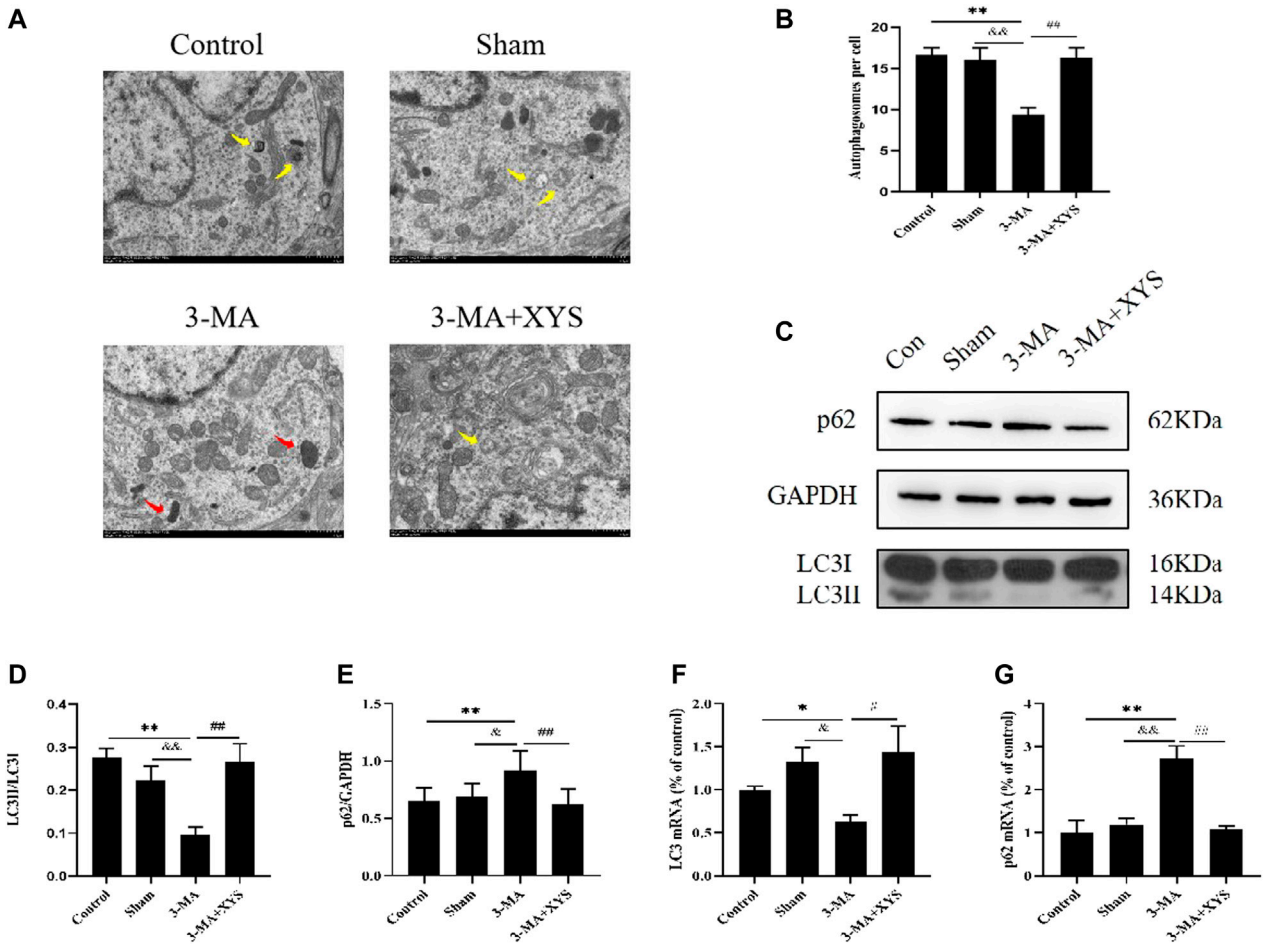

FIGURE 8 | Xiaoyaosan Increased Autophagy Expression in Mice Injected with 3-MA. **(A)** Transmission electron microscope showed the situation of autophagosomes and mitochondria in the hypothalamus of the mice (scale bar = 1 μm). Mitochondria were disrupted in 3-MA group (the red arrow). Autophagosomes were observed in control, Sham and 3-MA + XYS group (yellow arrows). **(B)** The number of autophagosomes in the hypothalamus of the mice ($F(3, 8) = 9.243$, $p = 0.0056$, n = 1). **(C)** Representative western blotting of LC3 and p62 expressions. **(D)** Quantitative analysis of protein levels of LC3 ($F(3, 16 = 7.689$, $p = 0.0021$, n = 5). **(E)** Quantitative analysis of protein levels of p62 ($F(3, 16 = 4.772$, $p = 0.0146$, n = 5). **(F)** The expression of LC3 mRNA ($F(3, 12 = 3.998$, $p = 0.0346$, n = 4). **(G)** The expression of p62 mRNA ($F(3, 12 = 14.176$, $p = 0.0003$, n = 4). The values represent the mean ± SEM. $^{*}p < 0.05$ or $^{**}p < 0.01$ compared with the control group. $\&p < 0.05$, $\&p < 0.01$ compared with the 3-MA group. $\#p < 0.05$, $\#\#p < 0.01$ compared with the 3-MA group.

in mice increased considerably, while the opposite trend was observed for p62 ($p < 0.01$).

LC3 mRNA expression in the hypothalamus was lower in the 3-MA group compared with the control group ($p < 0.05$), and the expression was slightly higher in sham group but not statistically significant ($p > 0.05$). After administration of Xiaoyaosan, the inhibitory effect of 3-MA on LC3 mRNA in the hypothalamus was effectively reversed compared with that of the 3-MA group ($p < 0.05$) (**Figure 8F**). The expression of p62 mRNA in the hypothalamus was just the opposite. The 3-MA group was significantly higher than the control and sham groups ($p < 0.01$). There was no difference between the sham group and the control group ($p > 0.05$), and p62 mRNA in the 3-MA + XYS group was effectively reduced by comparation with that in the 3-MA group ($p < 0.01$) (**Figure 8G**).

Xiaoyaosan Improved Glucose Metabolism in Mice Injected With 3-MA

Figure 9A shows the immunofluorescence results of GLUT4 in mice. The expression of GLUT4 in the dorsal medial nucleus and paraventricular nucleus of the hypothalamus in the control group mice and in the paraventricular nucleus of the hypothalamus in sham group mice were both greater than that in the 3-MA group ($p < 0.01$). The sham group had relatively lower expression in the paraventricular nucleus and there was no significant difference in the dorsal medial nucleus compared with the control group with no significance level ($p > 0.05$). The expression of GLUT4 in the dorsal medial nucleus and paraventricular nucleus of the mice in the 3-MA + XYS group was increased compared with that in the 3-MA group ($p < 0.01$).

The results of serum insulin are shown in **Figure 9B**. The serum insulin levels of the mice in the 3-MA group were

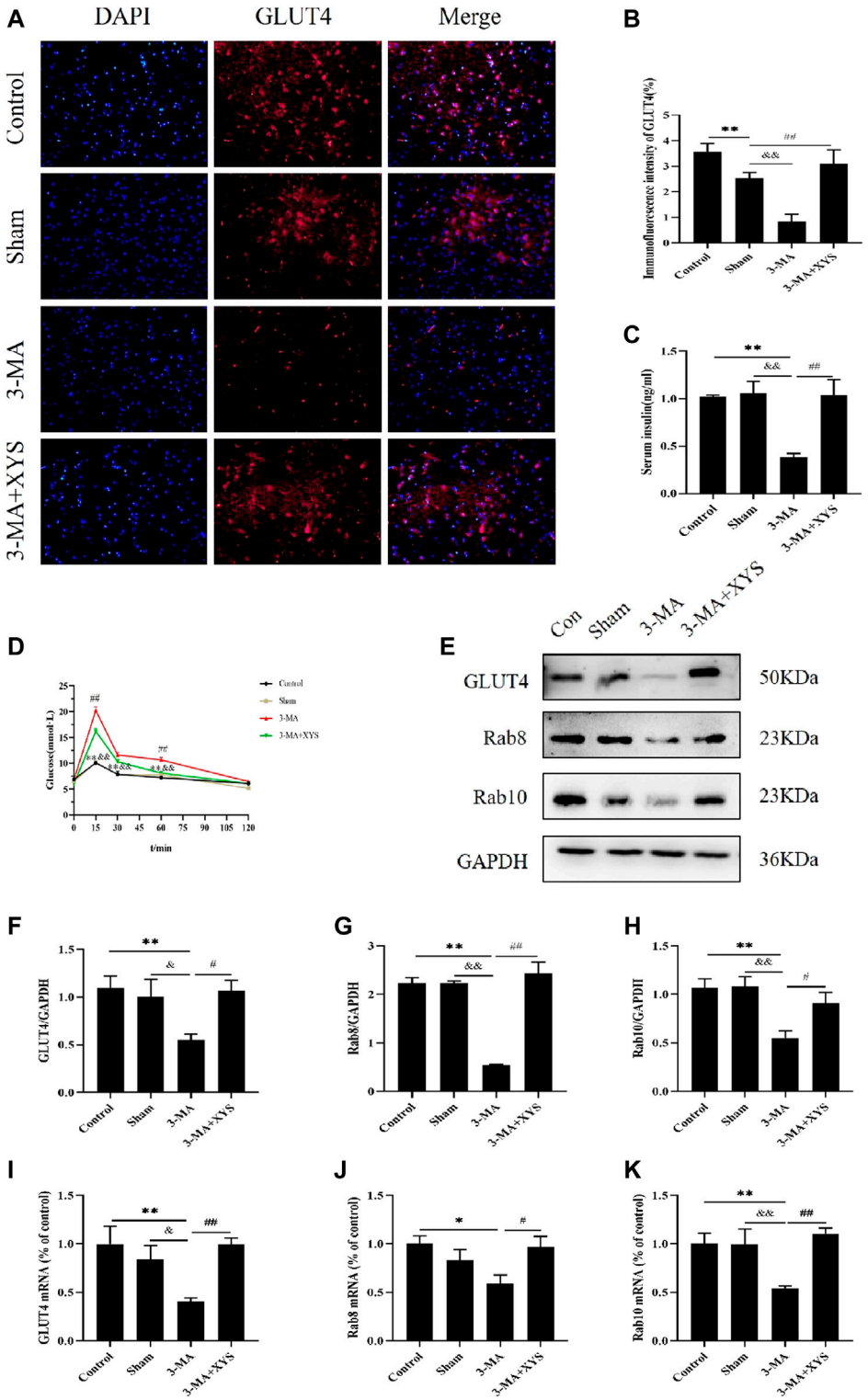

FIGURE 9 | Effect of Xiaoyaosan on glucose metabolism in hypothalamus of mice injected with 3-MA. **(A)** Immunofluorescence expression of GLUT4 in hypothalamus of the mice (scale bar = 20 μm). **(B)** Immunofluorescence intensity of GLUT4 (%) ($F(3, 8) = 10.900$, $p = 0.0034$, n = 3). **(C)** Serum insulin level of the mice ($F(3, 16) = 9.543$, $p = 0.0008$, n = 5). **(D)** The blood glucose of the mice ($F(3, 56) = 22.165$, $p < 0.0001$, n = 15). **(E)** Representative western blotting of GLUT4, Rab8 and Rab10 expressions. **(F)** Quantitative analysis of protein levels of GLUT4 ($F(3, 16) = 4.001$, $p = 0.0266$, n = 5). **(G)** Quantitative analysis of protein levels of Rab8 ($F(3, 16) = 23.664$, $p < 0.0001$, n = 5). **(H)** Quantitative analysis of protein levels of Rab10 ($F(3, 16) = 6.453$, $p = 0.0045$, n = 5). **(I)** The expression of GLUT4 mRNA ($F(3, 12) = 5.221$, $p = 0.0155$, n = 4). **(J)** The expression of Rab8 mRNA ($F(3, 12) = 3.655$, $p = 0.0443$, n = 4). **(K)** The expression of Rab10 mRNA ($F(3, 12) = 6.112$, $p = 0.0091$, n = 4). The values represent the mean ± SEM. $^{*}p < 0.05$ or $^{**}p < 0.01$ compared with the control group. &$p < 0.05$, &$p < 0.01$ compared with the 3-MA group. #$p < 0.05$, ##$p < 0.01$ compared with the 3-MA group.

significantly lower than that of the normal group and sham group ($p < 0.01$). No difference was found between the control group and sham group ($p > 0.05$). After administration of Xiaoyaosan in mice injected with 3-MA, the decrease in serum insulin was distinctly improved ($p < 0.01$).

By monitoring the blood glucose at 0, 15, 30, 60, and 120 min, we found that the blood glucose values of mice in each group reached their peak at 15 min, and the 3-MA group exceeded the values of the other three groups at each time point. The blood glucose level of mice in the 3-MA group exceeded that in the control group and sham group at 15, 30, and 60 min ($p < 0.01$). After administration of Xiaoyaosan, the blood glucose level of mice injected with 3-MA decreased significantly at 15 and 60 min ($p < 0.05$). The blood glucose values of mice in all groups basically returned to the same level at 120 min (**Figure 9C**).

The reduction of GLUT4 protein expression in the hypothalamus of mice in the3-MA group was greater than that in the control and sham groups ($p < 0.01$ or $p < 0.05$). After administering Xiaoyaosan to the mice injected with 3-MA, the GLUT4 protein expression recoveried significantly ($p < 0.05$) (**Figures 9D,E**). Regarding the protein expression of Rab8 and Rab10 in the hypothalamus, both were significantly lower in the 3-MA group than that in the control group and sham group ($p < 0.01$). The expression of these two proteins in the hypothalamus of the 3-MA + XYS group was in sharp contrast with the 3-MA group ($p < 0.01$ or $p < 0.05$) (**Figures 9D,F,G**).

GLUT4 mRNA expression remained consistent with that of protein expression (**Figure 9H**). The levels of Rab8 and Rab10 mRNA in the 3-MA group were significantly lower than those in the control group and sham group ($p < 0.05$ or $p < 0.01$), but the expression of Rab8 was not much different from the latter ($p > 0.05$). There was no significant difference in the expression of the two genes between the control and sham groups ($p > 0.05$). The expression levels of Rab8 and Rab10 in the 3-MA + XYS group both increased sharply ($p < 0.05$ or $p < 0.01$) (**Figures 9I,J**).

DISCUSSION

The clinical application of Xiaoyaosan is very extensive and involves internal medicine, surgery, gynecology, pediatrics, etc. Xiaoyaosan has a relatively high probability of application in psychiatric and nervous system diseases, and depressive psychosis is the most common disease (Liu et al., 2020). Our team has also conducted in-depth research on Xiaoyaosan for treatment of depression for many years, and its mechanism involves many aspects (Yan et al., 2018; Ma et al., 2019; Hou et al., 2020). Xiaoyaosan regulates HPA axis dysregulation in depressed rats and inhibits its hyperactivity (Chen et al., 2008a; Song et al., 2020). Xiaoyaosan can be effective by regulating 5-HT metabolism disorder and promoting its synthesis (Jiao et al., 2018), can promote the recovery of synaptic structure and function in depression (Meng et al., 2013), and can reverse the structural damage of mitochondria and neurons, and increase the expression of neurotrophic factors (Chen et al., 2008b; Jiang et al., 2016). Xiaoyaosan can also have an antidepressant effect by improving intestinal microbes and regulating the gut-brain axis (Zhu et al., 2019; Hao et al., 2021). The antidepressant effect of Xiaoyaosan is not achieved through a certain way, but exerts its clinical efficacy through a combination of aspects, dimensions, levels, and targets. In this study, we explored the mechanism of Xiaoyaosan on depression through other ways.

Chronic unpredictable mild stress is known to be risk factor for psychiatric disorders, and it can effectively induces the pathophysiology of depression, so it has been widely used to establish modeling depression in rodents (Svitlana et al., 2019; Ma et al., 2021). Currently, the widely used assays for depression-related behaviours include the OFT, the SPT, the TST and so on. The OFT is based on the nature of rodents who simultaneously fear open spaces and wish to explore novel environments (Ferreira et al., 2018). In this study, the total distance travelled and the time spent in open area of CUMS-induced mice were decreased simultaneously, which means the miice are less eager to explore. The SPT utilizes rodents' preferences for the taste of sugar. When animals show depressive-like behavior, the pleasure of drinking sweet water partly disappears, hence, the consumption of sucrose is significantly reduced (Wang et al., 2017). That's exactly what we found in our study. The TST is based on the fact that animals subjected to the short-term, inescapable stress of being suspended by their tail, will develop an immobile posture to estimate the helpless emotion of the mice. In our study, the immobility time of the mice induced by CUMS was longer than the other mice, indicating the depressive state of mice to some extent. The results ot the above three tests all indicate that CUMS-induced mice exhibit depressive-like behaviors.

Many pathogeneses account for the onset of depression. In recent years, the relationship between autophagy and depression has received much attention. Studies have shown that reduced autophagy is found in animal models of depression (Zhao et al., 2017; Gulbins et al., 2018; Huang et al., 2018). LC3 is a reliable marker of autophagosomes (Feng et al., 2016), p62 is a characteristic molecule and degradation product of autophagy (Glick et al., 2010), and the expression levels of both can reflect the autophagy activity of cells. The amount of LC3II in the cell is positively correlated with the number of autophagosomes (Tanida, 2011). Autophagosomes and lysosomes are fused to form autophagolysosomes and then degraded by lysosomal enzymes (Tanida et al., 2008). p62 is a typical autophagy receptor, a multifunctional protein distributed in cells, and is involved in the proteasomal degradation of ubiquitinated proteins. When Atg7 (A protein associated with autophagy) and p62 are knocked out in combination, the accumulation of polyubiquitinated aggregates in cells can be reversed (Komatsu et al., 2007). Overexpression of p62 delays the delivery of proteasome substrates to the proteasome, thereby affecting its degradation. In addition, p62 and the proteasome can regulate the activity of HDAC6 deacetylase, thereby affecting autophagy degradation (Liu et al., 2016). In this study, we learned from the TEM, WB and RT-qPCR results that the number of autophagosomes in mice induced by CUMS was less than that in the control group, and this was changed after treatment. The

mRNA and protein levels of the autophagy-related markers LC3 and p62 in the hypothalamus of mice incuded by CUMS were quite different from those in the control group and the two treatment groups: the LC3II/LC3I ratiodecreased, especially the expression of LC3II ratio, which decreased significantly, while the expression of p62 increased. These results suggested that the level of autophagy in the hypothalamus of mice induced by CUMS was significantly reduced, but after Xiaoyaosan intervention of, the levels of LC3 and p62 were effectively reversed, indicating that Xiaoyaosan had a regulatory effect on autophagy in the hypothalamus of those mice.

GLUT4 is the primary regulatory mechanism by which glucose uptake occurs in the periphery and plays an important role in maintaining glucose homeostasis. In recent years, it has been discovered that in the central nervous system, GLUT4 is widely present in neurons in the cortex, olfactory bulb, hippocampus and hypothalamus. Due to the regulation of glucose sensing in the hypothalamus, GLUT4 in the hypothalamus can affect the whole body glucose metabolism. In general, insulin can promote the translocation of GLUT4 from the cell to the cell membrane in the form of vesicles in general. Insulin in the body can affect glucose metabolism by GLUT4 translocation, and can especially make an important contribution in regulating systemic glucose homeostasis in hypothalamic neurons. Autophagy plays an important regulatory role in the translocation of GLUT4 and can be used as a carrier for GLUT4 translocation. By regulating autophagy as a target for GLUT4, it is possible to control the translocation and circulation of GLUT4 to regulate insulin (Elhassan et al., 2018). Many proteins in the Ras GTPase superfamily are involved in the regulation of autophagy (Szatmari et al., 2014). Studies have confirmed that upregulation of Rab8 and Rab10 can increase the expression of GLUT4 in skeletal muscle cell membranes, thereby increasing glucose uptake in skeletal muscle (Samad et al., 2017), and Rab8 and Rab10 can colocalize with GLUT4 and autophagosomes (Zhang et al., 2017). Rab8 and Rab10 jointly participate in the process of regulating the maturation of autophagy and the transport of GLUT4 vesicles. At the same time, autophagy regulates the translocation and expression of GLUT4 through Rab8 and Rab10, which are closely related. Therefore, this study investigated glucose metabolism from the perspective of the effects of autophagy on the expression of GLUT4 mediated by Rab8 and Rab10. In this study, the results of immunofluorescence, WB and RT-qPCR showed that the changes in GLUT4, Rab8 and Rab10 expression in the hypothalamus of mice induced by CUMS were consistent with the changes in autophagy levels. At the same time, the insulin and blood glucose levels of depressed mice showed abnormalities. After Xiaoyaosan and fluoxetine intervention, the protein and mRNA levels of GLUT4, Rab8 and Rab10 were effectively increased, and the insulin and blood glucose levels of the mice in the treatment group were also significantly improved. The above results clarified that Xiaoyaosan regulates the expression of GLUT4 in the hypothalamus of mice induced by CUMS by regulating autophagy and affects the glucose metabolism throughout the body.

We found that the changes in glucose metabolism and autophagy have a causal relationship. Autophagy mediates Rab8 and Rab10 to affect the expression of GLUT4. To confirm this conclusion, we injected 3-MA, an autophagy inhibitor, into the cerebral ventricle of C57BL/6J mice to further study the regulatory effect of 3-MA on autophagy in the mice hypothalamus and observed the changes in Rab8, Rab10 and GLUT4, as well as the regulatory effect of Xiaoyaosan on them. 3-MA is a chemical drug that has been found to inhibit the expression of autophagy and is often used as an autophagy inhibitor in scientific research to study the effects of changes in autophagy levels on subjects. In recent years, as research on autophagy has gradually increased, studies on the relationship between autophagy and depression have also risen. To explore the effect of changes in autophagy on depression, 3-MA has been used as a control in many studies (Chen et al., 2019; Shih et al., 2019; Ali et al., 2020).

We found that after injection of 3-MA into the brain ventricle of mice, the results of OFT, TST and SPT showed that the mice in the 3-MA group exhibited depression with anxiety-like behavior change, indicating from another perspective that reduced levels of autophagy can induce depressive-like behavior. The number of autophagosomes in the hypothalamus decreased, indicating that autophagy was inhibited. The WB and RT-qPCR results were consistent, and both showed a decrease in autophagy levels. After treatment with Xiaoyaosan, the above phenomena were improved, indicating that Xiaoyaosan increased the autophagy level in the 3-MA group. The immunofluorescence results showed that Xiaoyaosan significantly improved the depression with anxiety-lik behavior of mice in the 3-MA group and increased the expression of GLUT4 in the hypothalamus of mice injected with 3-MA. At the same time, the expression of Rab8 and Rab10 showed the same trend as GLUT4. Therefore, the expression levels of Rab8, Rab10 and GLUT4 were consistent with the changes in autophagy, indicating that autophagy plays a nonnegligible role. The serum insulin level of the mice in the 3-MA group decreased, accompanied by an increase in blood glucose. Xiaoyaosan improved the disorder, and the mechanism may be related to the regulation of GLUT4. These results demonstrate that 3-MA effectively reduced the expression of autophagy and glucose metabolism in the mouse hypothalamus, and Xiaoyaosan ameliorated these effects. These results indicate that the mechanism of Xiaoyaosan's antidepressant effect may be achieved by regulating the level of autophagy.

In addition, this study also selected fluoxetine as a positive control for the study of autophagy because fluoxetine is a commonly used clinical antidepressant with stable efficacy. Second, studies have shown that fluoxetine can promote the formation of autophagosomes in CUMS mice and exert its antidepressant effect by enhancing the level of autophagy (Shu et al., 2019). By comparison with fluoxetine, we can further determine the effect of Xiaoyaosan on depression.

CONCLUSION

In general, our work provides new insights for revealing the biological mechanism of Xiaoyaosan's antidepressant properties. Xiaoyaosan can improve glucose metabolism and its associated indicator GLUT4 in hypothalamus in the CUMS induced depressive behavior in mice, the mechanism of which may involve improving the hypothalamic autophagy. This study helps us to further understand the mechanisms of XYS as a potential antidepressant.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Materials**, further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The animal study was reviewed and approved by the Institutional Animal Care and Use Committee of Jinan University.

AUTHOR CONTRIBUTIONS

The experiments were conceived and designed by F-RY, X-JL, and J-XC. The animal experiments were carried out by F-RY. The data were analyzed by F-RY and X-JL. The manuscript was written by F-RY and X-JL. All authors read and approved the final manuscript. J-XC is primarily responsible for the final content.

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Investigational Drugs for the Treatment of Depression (Part 2): Glutamatergic, Cholinergic, Sestrin Modulators, and Other Agents

Octavian Vasiliu *

Department of Psychiatry, Dr. Carol Davila University Emergency Central Military Hospital, Bucharest, Romania

Many investigational drugs with antidepressant activity are currently explored in different phases of clinical research, with indications such as major depressive disorder, treatment-resistant major depression, bipolar depression, post-partum depression, and late-life depression. Although the vast majority of the antidepressants in clinical use are based on the monoaminergic hypothesis of depression, recent data supported the launching on the market of two new, non-monoamine-modulating drugs. Esketamine for treatment-resistant major depression and brexanolone for post-partum depression are two exceptions from the monoaminergic model, although their use is still limited by high costs, unique way of administration (only intravenously for brexanolone), physicians' reluctance to prescribe new drugs, and patients' reticence to use them. Glutamatergic neurotransmission is explored based on the positive results obtained by intranasal esketamine, with subanesthetic intravenous doses of ketamine, and D-cycloserine, traxoprodil, MK-0657, AXS-05, AVP-786, combinations of cycloserine and lurasidone, or dextromethorphan and quinidine, explored as therapeutic options for mono- or bipolar depression. Sestrin modulators, cholinergic receptor modulators, or onabotulinumtoxinA have also been investigated for potential antidepressant activity. In conclusion, there is hope for new treatments in uni- and bipolar depression, as it became clear, after almost 7 decades of monoamine-modulating antidepressants, that new pathogenetic pathways should be targeted to increase the response rate in this population.

Keywords: treatment-resistant depression, bipolar depression, esketamine, brexanolone, glutamate, onabotulinumtoxinA

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Anna Tabęcka-Łonczynska, University of Information Technology and Management in Rzeszow, Poland

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Shupeng Li, Peking University, China
Ashish Dhir, University of California, Davis, United States
Dominik Strzelecki, Medical University of Lodz, Poland

***Correspondence:**
Octavian Vasiliu
octavvasiliu@yahoo.com

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INTRODUCTION

Major depressive disorder (MDD) has a significant functional impact on patients' psychosocial functioning and quality of life (Fried and Nesse, 2014). Also, individual symptoms of depression, especially sad moods and concentration problems, are associated with high levels of dysfunction in daily activities, based on an analysis of data from the STAR*D trial (Sequenced Treatment Alternatives to Relieve Depression) (Fried and Nesse, 2014). Almost 60% of individuals diagnosed with MDD report severe or very severe impairment of functioning (Kessler et al., 2003). A significant proportion of patients diagnosed with MDD will have treatment-resistant forms (TRD), which associate high direct and indirect costs, and those patients who could not reach

remission have considerable healthcare resource utilization, with significant economic impact (Petrescu et al., 2014; Heerlein et al., 2022).

Patients diagnosed with bipolar disorder also may develop significant functional impairment (due to direct effects of illness severity, cognitive impairments, psychiatric comorbidities, etc.), and they spend a large duration of their lives in depressive episodes or recovering from these episodes (Levy and Manive, 2012; Solomon et al., 2016).

Postpartum depression affects up to 15% of mothers, and its short-term and long-term negative consequences on child development are well-established (Pearlstein et al., 2009). Few therapeutic options are validated for this specific pathology, and fear in mothers related to breastfeeding during antidepressant administration is a significant obstacle to efficient therapeutic management (Pearlstein et al., 2009).

Another difficult-to-treat type of mood disorder is late-life depression, where vascular factors and psychological and social factors are intertwined, and a significant risk of completed suicide is also a major threat (Vasiliu and Vasile, 2016; Alexopoulos, 2019).

New antidepressants that could be administered either as monotherapy or as an add-on to the ongoing treatment in the case of partial/inadequate response are urgently needed in clinical practice. Glutamatergic and cholinergic drugs targeting components of the hypothalamic-pituitary-adrenal axis and other non-monoaminergic systems are currently under investigation in clinical research. The main objective of this review is to explore new investigational products with antidepressant properties and their reported efficacy and tolerability in depressive disorders.

METHODOLOGY

A systematic review of the articles referring to new drugs in phases I to III of clinical studies was conducted through the main electronic databases (PubMed, MEDLINE, Cochrane, Web of Science (Core Collection), PsychINFO, Scopus, and EMBASE using the paradigm "investigational antidepressants/products" OR "new antidepressants/agents" AND "clinical trial" AND "major depressive disorder" OR "bipolar disorder" OR "depression." Lists of references for every article corresponding to the search paradigm were investigated, and they were added to the review if they were not detected through the previously mentioned paradigm.

A broad search was chosen to include the widest variety of molecules. For this purpose, a supplementary search was added, targeting investigational products for depression explored in the clinical trials repositories run by the United States National Library of Medicine and the National Institutes of Health (clinicaltrials.gov), World Health Organization (International Clinical Trials Registry Platform), and European Union (EU Clinical Trial Register). The search within the clinical trial databases was structured by the disorder, "depression"; type, "interventional"; population, "adults"; and "adolescents," and trial phases I to III, but all statuses of recruitment were allowed. If the outcome of a registered trial for an investigational product was not mentioned in any of the mentioned repositories, the respective drug manufacturer's site was explored to verify if any results were available.

All articles and references from electronic databases and clinical studies repositories included were allowed in the primary search if they were published between January 2000 and February 2022.

This systematic review is based on the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) statement, and all the data collection, review, reporting, and discussion were conducted according to this statement (**Figure 1**) (Moher et al., 2015). Inclusion and exclusion criteria are mentioned in **Figure 4**.

All pharmacological agents included in the collected data were grouped into nine categories: monoamine-based drugs, orexin receptor modulators, GABA-A receptor modulators, neurosteroid analogs, anti-inflammatory therapies, glutamatergic antidepressants, sestrin modulators, cholinergic agents, combinations of agents, and a residual category for all other molecules with distinct mechanisms of action. The first four categories of agents have been described in the first part of this review.

RESULTS

The results of the PRISMA-based search paradigm are presented in **Figure 2**. Glutamatergic agents are the most extensively researched category of antidepressants, and 29 different molecules have been found in 72 distinct sources (**Table 1**). Thirteen phase I studies, two phase I/II trials, 30 phase II trials, one phase II/III trial, seven phase III trials, five phase IV trials, and eight not assessed for clinical phase trials were reviewed in this category.

Sestrin modulators were identified in two sources referring to one phase I and one phase II trials, assessing a single agent from this category. Four different combinations of pharmacological agents were identified in 13 sources, referring to 5 phase II trials, 8 phase III trials, and one not assessed for a clinical phase trial.

Cholinergic antidepressants have been identified in 10 distinct sources, referring to three investigational products, explored in two phase I trials, four phase II trials, two phase IV trials, and two not assessed for clinical phase trials. Eight other antidepressants with distinct mechanisms of action have been identified in 13 sources, referring to one phase I trial, seven phase II trials, two phase IV trials, and three not assessed for clinical phase trials.

All agents identified through this database search are presented in **Figure 3**.

Glutamatergic Agents

Traxoprodil (CP-101,606) is a potent, selective antagonist of the GluN2B subunit within the NMDA receptor, with the capacity to

Section/topic	#	Checklist item	Information reported		Line number(s)
			Yes	No	
ADMINISTRATIVE INFORMATION					
Title					
Identification	1a	Identify the report as a protocol of a systematic review	☒	☐	60-66
Update	1b	If the protocol is for an update of a previous systematic review, identify it as such	☐	☒	
Registration	2	If registered, provide the name of the registry (e.g., PROSPERO) and registration number in the Abstract	☐	☒	
Authors					
Contact	3a	Provide the name, institutional affiliation, and e-mail address of all protocol authors; provide the physical mailing address of the corresponding author	☒	☐	4-9
Contributions	3b	Describe contributions of protocol authors and identify the guarantor of the review	☐	☐	Not applicable, one author only
Amendments	4	If the protocol represents an amendment of a previously completed or published protocol, identify it as such and list changes; otherwise, state a plan for documenting important protocol amendments	☐	☒	
Support					
Sources	5a	Indicate sources of financial or other support for the review	☒	☐	1139
Sponsor	5b	Provide a name for the review funder and/or sponsor	☐	☐	Not applicable
Role of sponsor/funder	5c	Describe roles of funder(s), sponsor(s), and/or institution(s), if any, in developing the protocol	☐	☐	Not applicable
INTRODUCTION					
Rationale	6	Describe the rationale for the review in the context of what is already known	☒	☐	53-58
Objectives	7	Provide an explicit statement of the question(s) the review will address concerning participants, interventions, comparators, and outcomes (PICO)	☒	☐	83-88, Table 1
METHODS					
Eligibility criteria	8	Specify the study characteristics (e.g., PICO, study design, setting, time frame) and report characteristics (e.g., years considered, language, publication status) to be used as criteria for eligibility for the review	☒	☐	Table 1
Information sources	9	Describe all intended information sources (e.g., electronic databases, contact with study authors, trial registers, or other grey literature sources) with planned dates of coverage	☒	☐	60-88
Search strategy	10	The present draft of the search strategy is to be used for at least one electronic database, including planned limits, such that it could be repeated	☒	☐	60-66
STUDY RECORDS					
Data management	11a	Describe the mechanism(s) that will be used to manage records and data throughout the review	☒	☐	60-78
Selection process	11b	State the process that will be used for selecting studies (e.g., two independent reviewers) through each phase of the review (i.e., screening, eligibility, and inclusion in meta-analysis)	☒	☐	60-78, Table 1
Data collection process	11c	Describe the planned method of extracting data from reports (e.g., piloting forms, done independently, in duplicate), and processes for obtaining and confirming data from investigators	☒	☐	74-76
Data items	12	List and define all variables for which data will be sought (e.g., PICO items, funding sources), any pre-planned data assumptions, and simplifications	☒	☐	Table 1
Outcomes and prioritization	13	List and define all outcomes for which data will be sought, including prioritization of main and additional outcomes, with rationale	☒	☐	Table 1
Risk of bias in individual studies	14	Describe anticipated methods for assessing the risk of bias of individual studies, including whether this will be done at the outcome or study level, or both; state how this information will be used in data synthesis	☐	☒	
DATA					
Synthesis	15a	Describe criteria under which study data will be quantitatively synthesized	☐	☒	
	15b	If data are appropriate for quantitative synthesis, describe planned summary measures, methods of handling data, and methods of combining data from studies, including any planned exploration of consistency (e.g., I^2, Kendall's tau)	☐	☒	
	15c	Describe any proposed additional analyses (e.g., sensitivity or subgroup analyses, meta-regression)	☐	☒	
	15d	If quantitative synthesis is not appropriate, describe the type of summary planned	☒	☐	83-88
Meta-bias(es)	16	Specify any planned assessment of meta-bias(es) (e.g., publication bias across studies, selective reporting within studies)	☐	☒	
Confidence in cumulative evidence	17	Describe how the strength of the body of evidence will be assessed (e.g., GRADE)	☐	☒	

FIGURE 1 | PRISMA-P 2015 Checklist (Moher et al., 2015). This checklist has been adapted for use with protocol submissions to systematic reviews from **Table 3** in Moher D et al.: preferred reporting items for systematic review and meta-analysis protocols (PRISMA-P) 2015 statement. Systematic Reviews 2015 4:1.

potentiate the antidepressant-like effects of certain drugs in animal models (Poleszak et al., 2016). Traxoprodil inhibits the channel activity of subunits GluN1/GluN2B and reduces the time and frequency of its opening, thus preventing an excessive influx of calcium ions into neurons and secondary damage (Poleszak et al., 2016). Traxoprodil exhibited antidepressant activity in the forced swim test in rats (an animal model to screen molecules with antidepressant effect), and co-administration of traxoprodil with imipramine, fluoxetine, or escitalopram, each in subtherapeutic doses, affected at a significant level the pseudo-depressive behavior in this model (Poleszak et al., 2016).

In patients with TRD (defined by lack of response to at least one adequate trial of a selective serotonin reuptake inhibitor, SSRI), CP-101,606 was administered in a randomized, placebo-controlled, double-blind study (Preskorn et al., 2008). During the first phase of the study, subjects received a 6-week open-label administration of paroxetine and single-blind i.v. placebo infusion, with non-responders being randomized in the second phase to a double-blind single infusion of CP-101,106 or placebo plus treatment with paroxetine for up to an additional 4 weeks (Preskorn et al., 2008). The main outcome (Montgomery Asberg Depression Rating Scale, MADRS score on day 5 during the second phase) differentiated the active drug from the placebo (Preskorn et al., 2008). The response rate on Hamilton Depression Rating Scale (HAMD) was 60% *versus* 20% for traxoprodil *versus* placebo, and 78% of these active drug responders maintained their response for at least 1 week after the infusion (Preskorn et al., 2008). The antidepressant response was possible without producing significant dissociative reactions, with overall good tolerability (Preskorn et al., 2008).

A randomized, placebo-controlled, crossover pilot trial evaluated the efficacy and tolerability of the orally administered, selective GluN2B antagonist **rislenemdaz** (**MK-0657)** in patients with TRD ($N = 5$ participants) (Ibrahim et al., 2012). After 1 week drug-free period, subjects were randomized to receive either MK-0657 monotherapy (4–8 mg/day) or placebo for 12 days (Ibrahim et al., 2012). Significant antidepressant effects were reported as early as day 5 in patients receiving active drug *versus* placebo, as reflected by the evolution of the HAMD and Beck Depression Inventory (BDI) scores, but no improvement was observed on the MADRS, the primary efficacy measure (Ibrahim et al., 2012). The tolerability was good, without dissociative adverse events in patients receiving MK-0657 (Ibrahim et al., 2012).

EVT-101 is another orally administered, potent, and selective glutamate GluN2B antagonist (Strobel et al., 2016). A phase II, randomized, double-blind, parallel-group, 4-week study was designed to evaluate the efficacy of EVT-101 in patients with TRD (after the confirmation of treatment resistance in a prospective treatment period with citalopram) but was prematurely terminated because a clinical hold was issued by FDA (NLM, NCT01128452).

AGN-241751 is an orally active, NMDA-receptor positive allosteric modulator, currently tested as an antidepressant in clinical trials, although its precise mechanism of action and specific NMDA subunit for which it is ligand is still unknown (Pothula et al., 2021). AGN-241751 reverses behavioral deficits induced by chronic unpredictable stress in mice and possesses antidepressant-like properties in animal models (Pothula et al., 2021). Explored mechanisms of action, based on animal models, are represented by the enhancement of the NMDA-receptor activity in excitatory and parvalbumin-inhibitory neurons in the medial prefrontal cortex, activation of the Akt/mTOR

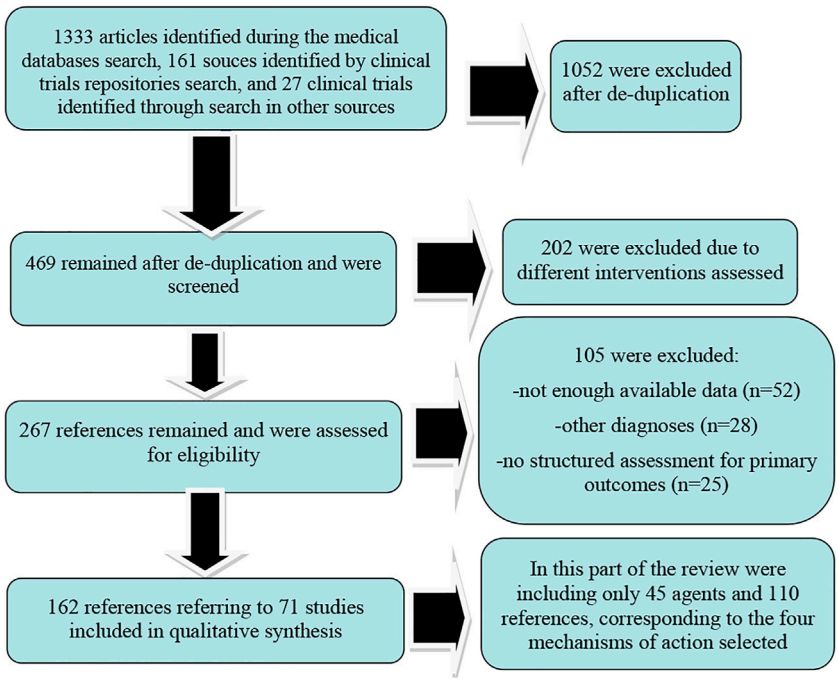

FIGURE 2 | Results of the PRISMA-based search paradigm.

signaling, and increased level of the synaptic proteins responsible for synaptic plasticity in the prefrontal cortex (Pothula et al., 2021). Also, according to the same study on mice, GluN2B subunits from the excitatory neurons in the prefrontal cortex are the initial cellular trigger underlying antidepressant effects of AGN-241751 (Pothula et al., 2021).

A two-part, double-blind, placebo-controlled, single and multiple-dose (part A) or twice-daily dose (part B), phase I/II trial conducted with adult participants (N = 223) diagnosed with MDD was completed in 2019 (NLM, NCT03726658). Both parts of the trial used an efficacy measure, the MADRS score, and the primary outcome was the change in this score on day 1 and day 7 after the administration of AGN-241751 (NLM, NCT03726658). No results have yet been posted as of February 2022. Another randomized, double-blind, placebo-controlled, fixed-dose, phase II trial included 251 adult participants diagnosed with MDD and evaluated the efficacy at day 1 after the initial dose of AGN-241751, defined by MADRS score change (NLM, NCT03586427). No results have been published from this trial, either.

MIJ821 is a glutamate GluN2B antagonist investigated in a proof-of-concept, randomized, subject and investigator-blinded, parallel-group, placebo-controlled study on patients with TRD (N = 70 participants) (Ghaemi et al., 2021). Low dose and high dose infusions of MIJ821 (0.16 mg/kg weekly or bi-weekly *versus* 0.32 mg/kg weekly or bi-weekly) were compared to placebo (weekly) and ketamine infusion (0.5 mg/kg weekly) at 24 h, 48 h, and 6 weeks, the primary outcome measure being the change in the MADRS scores (Ghaemi et al., 2021). The adjusted mean differences *versus* placebo were significant for all MIJ821 dosing regimens and ketamine at 24 and 48 h (Ghaemi et al., 2021). At 6 weeks, none of the active interventions retained their statistical significance by comparison to placebo (Ghaemi et al., 2021).

Another double-blind, randomized, placebo-controlled, dose-ranging, phase II trial is ongoing, its objective being the investigation of efficacy and safety of intravenous MIJ821 infusion in addition to comprehensive standard of care (SOC) in patients with MDD and suicidal ideation with intent (NLM, NCT04722666). This study consists of three periods: a screening phase (up to 48 h), a double-blind core period (6 weeks), and an extension period (up to 52 weeks). It will enroll an estimate of 195 patients (NLM, NCT04722666).

Dextromethadone (d-methadone, esmethadone, REL-1017) has low micromolar affinity at GluN2 subunits (2A-2D) of the NMDA receptors, with a slightly superior affinity for GluN2B subunit (Callahan et al., 2004; Fogaça et al., 2019). Dextromethadone also has a very low affinity for the μ and δ-opioid receptors and does not produce opioid-like effects in humans at doses predicted to induce antidepressant activity (Fogaça et al., 2019). In a multicenter, randomized, double-blind, placebo-controlled, phase IIa trial, two dosages of REL-1017 (25 or 50 mg orally daily) were compared to placebo (N = 21, 19, and 22 participants, respectively) to assess the efficacy and tolerability of this product in patients with MDD who did not improve after 1–3 standard antidepressant treatments (Fava et al., 2022). Patients experienced mild or moderate adverse events during the 7 days of the trial, with no evidence of dissociative or psychotomimetic effects, opioid effects, or withdrawal signs

TABLE 1 | Glutamatergic agents with antidepressant properties in the pipeline.

Authors	Methodology	Results	Clinical trial phase, trial identifier (if available)
GluN2B antagonists			
Preskorn et al. (2008)	Traxoprodil (CP-101,606), 6-week open-label + 4-week DBRCT, *N* = 30 MDD non-responders to open-label phase	MADRS score on day 5 (main outcome) significantly differentiated the active drug from the placebo. The response rate on HAMD was 60 *vs*. 20% for traxoprodil *vs*. placebo. The overall tolerability was good	Phase II, NCT00163059
Ibrahim et al. (2012)	Rislenemdaz (MK-0657), *N* = 5 TRD patients, 12 days	Significant antidepressant effects were reported as early as day 5 in patients receiving active drug *vs*. placebo (HAMD and BDI scores), but no improvement was observed on the MADRS (the primary efficacy measure). The tolerability was good, without dissociative AE in patients receiving MK-0657	Phase I, NCT00472576
NLM (2010a)	EVT-101, DBRCT, *N* = 8 TRD patients, 4 weeks	The primary outcome measure is the safety and tolerability profile of EVT-101. The study was prematurely discontinued due to a clinical hold issued by FDA.	Phase II, NCT01128452
NLM (2018a)	AGN-241751, two-part DBRCT, *N* = 233 MDD patients, 7 days	The primary outcome was the change in this score on day 1 and day 7 after the administration of AGN-241751. No results were posted as of February 2022	Phases I/II, NCT03726658
NLM (2018b)	AGN-241751, DBRCT, *N* = 251 MDD patients, 24 h	The efficacy at day 1 after the initial dose of AGN-241751, defined by MADRS score change, was the primary outcome. No results have yet been posted	Phase II, NCT03586427
Ghaemi et al. (2021)	Low dose/ high dose MIJ821 *vs*. placebo *vs*. ketamine, DBRCT, *N* = 70, TRD patients, 6 weeks	The adjusted mean differences *vs*. placebo were significant for all MIJ821 dosing regimens and ketamine at 24 and 48 h. At 6 weeks, none of the active interventions retained their statistical significance *vs*. placebo	Phase II, NCT03756129
NLM (2021a)	MIJ821 + comprehensive SOC, dose-ranging, *N* = 195 patients MDD + suicidal ideation/intent (estimated), 52 weeks	Primary outcome measure, MADRS total score at 24 after the first infusion and up to 52 weeks. Secondary outcomes, treatment-emergent AE (number and severity), pharmacokinetics, response rate, sustained response rate, remission rate, sustained remission rate	Phase II, NCT04722666
Fava et al. (2022)	High-dose/low-dose dextromethadone (REL-1017) adjunctive to ongoing antidepressant treatment, DBRCT, *N* = 62 TRD patients, 7 days	Patients experienced mild or moderate AE during the 7 days of the trial, with no evidence of dissociative or psychotomimetic/ opioid/withdrawal signs. MADRS scores improved on day 4 in both REL-1017 groups and persisted up to 14 days	Phase IIa, NCT03051256
NLM (2021b)	REL-1017 adjunctive to antidepressant treatment, two DBRCT, *N* = 400 MDD patients for each trial (estimated enrollment), 28 days	The primary outcome measure is MADRS total score change from baseline to day 28. These trials are ongoing as of February 2022	Phase III, NCT04688164 Phase III, NCT04855747
NLM (2021c)	REL-1017 as monotherapy, DBRCT, *N* = 400, MDD, 28 days	The primary outcome measure is MADRS total score change from baseline to day 28. The trial is ongoing	Phase III, NCT05081167
NLM (2021d)	REL-1017 as adjunctive to current antidepressant treatment, open-label, *N* = 600 MDD patients (estimated enrollment), 52 weeks	MADRS total score change from baseline to week 52 is the primary outcome. This trial is ongoing as of February 2022	Phase III, NCT04855760
Agbo et al. (2017)	AZD6765 (lanicemine), open-label and DBRCT, respectively, *N* = 46 and 40, respectively, healthy subjects, 6 days	Pharmacokinetic analysis was performed by non-linear mixed-effects modeling. The population pharmacokinetic model adequately described the clinical observation of lanicemine in healthy volunteers	Phase I, NCT01069822 Phase I, NCT00785915
Agbo et al. (2017)	AZD6765, DBRCT, single dose or multiple infusion, respectively, *N* = 34 and 152, respectively, treatment-resistant MDD patients, 24 h and 3 weeks, respectively	Pharmacokinetics parameters were already mentioned above. The overall tolerability of 100 mg lanicemine was good, and an antidepressant effect was detected after single-dose infusion, peaked at 72 h, and dissipated *vs*. placebo by 10–13 days. In the multiple-dose trial, 100 and 150 mg lanicemine were compared to placebo, and MADRS total score changed significantly at week 3 in the active drug groups. Most secondary outcomes (HAMA, QIDS-SR, Q-LES-Q) supported the significant improvement in MADRS at week 3 in the 100 mg lanicemine group	Phase IIa, NCT00491686 Phase IIb, NCT00781742
Sanacora et al. (2017)	AZD6765 (50/100 mg) adjunctive to ongoing antidepressant treatment, DBRCT, *N* = 302 MDD patients with inadequate response to treatment, 12 weeks	Lanicemine was generally well-tolerated, but neither dose was superior to placebo in decreasing the severity of the depressive symptoms (MADRS total score, QIDS-SR, SDS, CGI)	Phase IIb, NCT01482221
Zarate et al. (2013)	AZD6765 (150 mg), DBRCT, *N* = 22 TRD patients, 7 days	MADRS score significantly improved, within 80 min, in subjects receiving AZD6765 compared to placebo, and this improvement remained significant only through 110 min. The HAMD scores reflected a difference between groups at 80 and 110 min and also on day 2. The response rate was 32% in the AZD6765-treated group *vs*. 15% in placebo-treated patients. No difference between groups was reported in the rate of psychotomimetic and dissociative AE	Phase II, NCT00986479
AMPA receptor potentiators			
O'Donnell et al. (2021)	TAK-653, five escalating doses *vs*. placebo, three-crossover phases, DBRCT, *N* = 24 healthy volunteers, three phases of 1 day each, separated by wash-out periods of 10–15 days	This investigational product did not affect resting motor threshold or paired-pulse responses in humans, determined by cortical sp/ ppTMS	Phase I, NCT03792672
NLM (2015a)	TAK-653, escalating single and multiple doses *vs*. placebo, *N* = 88 healthy volunteers, 14 + 31 days	The overall tolerability of the investigational product was good; no SAE were reported	Phase I, NCT02561156

(Continued on following page)

TABLE 1 | (*Continued*) Glutamatergic agents with antidepressant properties in the pipeline.

Authors	Methodology	Results	Clinical trial phase, trial identifier (if available)
GluN2B antagonists			
NLM (2017a)	TAK-653, DBRCT, TRD patients, 57 days	The primary outcome was time to relapse (MADRS total score). The study was withdrawn (business decision). No subject has been reported as enrolled in this trial	Phase II, NCT03312894
NLM (2021e)	(2R,6R)-Hydroxy- norketamine, DBRCT, SAD, and MAD, *N* = 48 (estimates) healthy volunteers, 8 or 19 days (SAD, and MAD, respectively)	Primary outcome measures are related to safety and tolerability. The trial is ongoing as of February 2022	Phase I, NCT04711005
NMDA-receptor antagonist			
NLM (2016)	NRX-101 *vs*. lurasidone after stabilization on ketamine, DBRCT, four-arm trial, *N* = 22 BD patients + suicidal ideation/behavior, 42 days	The results support the superior efficacy of the ketamine followed by NRX-101 *vs*. ketamine followed by lurasidone treatment, as reflected by the BDM scores. No SAE were reported in any of these trial arms, and no significant difference in the rate of AE was observed between NRX-101 and lurasidone-treated groups	Phase II, NCT02974010
NLM (2018c)	NRX-101 *vs*. lurasidone after stabilization on ketamine, DBRCT, *N* = 72 (estimated) BD patients + suicidal ideation/behavior, 6 weeks	The main outcome of this trial is the improvement in the depressive symptoms between NRX-101 and lurasidone as measured by the MADRS total score. This trial is ongoing	Phase II, NCT03396068
NLM (2018d)	NRX-101 *vs*. lurasidone, *N* = 24 (estimated) moderate severity BD patients + suicidal ideation, 6 months	This trial is active. The main outcome is the improvement of depressive symptoms severity as measured by the MADRS for 6 months	Phases II/III, NCT03402152
NLM (2018e)	NRX-100 *vs*. placebo, DBRCT, *N* = 150 (estimated) BD patients + suicidal ideation/ behavior, 24 h	The primary outcome is the C-SSRS score. This trial is ongoing	Phase III, NCT03396601
Park et al. (2020)	AV-101 *vs*. placebo, cross-over DBRCT, *N* = 19 TRD patients, multiple time frames	No treatment effects were detected using linear mixed models, as determined by primary (HAMD score) or secondary (C-SSRS, response/remission rate) outcome measures. No differences for AE were reported at any time between groups	Phase II, NCT02484456
Murphy et al. (2021)	AV-101 9720/1440 mg) *vs*. placebo, cross-over DBRCT, 4/5 h	Only the high dose (1440 mg) of AV-101 in humans succeeded in engaging brain targets in humans	Phases I/II, NCT03583554
Preskorn et al. (2015)	Rapastinel (GLYX-13), single-dose, 1, 5, 10, or 30 mg/kg *vs*. placebo, DBRCT, *N* = 116 MDD patients with inadequate/partial response to antidepressants, 16 weeks	The effect of GLYX-13 was significant *vs*. placebo on day 7, but not different on day 14 on HAMD-17. Reductions in HAMD were most important for 5 and 10 mg/kg. No treatment-related SAE occurred during the study	Phase II, NCT01234558
Moskal et al. (2014), Preskorn et al. (2015)	GLYX-13 *vs*. placebo, DBRCT, *N* = 53, healthy volunteers, 4 weeks	Pharmacokinetic parameters were described after a single i.v. dose administration (0.5–2.5 mg/kg)	Phase I, NCT01014650
NLM (2012a)	GLYX-13 (5 or 10 mg/kg) *vs*. placebo, DBRCT, *N* = 369 MDD patients with inadequate/partial response to antidepressants, 16 weeks	The primary outcome measure is HAMD total score change. The study was completed, but the results are not disclosed	Phase II, NCT01684163
NLM (2019a)	GLYX-13 (225/450 mg i.v.), open-label extension, *N* = 61 MDD patients with inadequate/partial response to antidepressants, 48 months	The primary outcome was the number of participants who experienced an AE during the trial. The study was terminated by the sponsor in 32 cases, and 11 participants withdrew. Patients were rolled in NCT03668600, but this trial was also terminated (business decision)	Phase II, NCT02192099
NLM (2013)	Apimostinel (NRX-1074) *vs*. placebo, DBRCT, MAD, *N* = 100 healthy volunteers, 28 days	The primary outcome was observed and laboratory-confirmed safety. Undisclosed results	Phase I, NCT01856556
NLM (2015b)	NRX-1074 375/500/750 mg orally administered *vs*. placebo, DBRCT, MAD, *N* = 15 healthy volunteers, 28 days	The primary outcomes were related to safety and tolerability. Undisclosed results	Phase I, NCT02366364
Brooks (2015)	NRX-1074 *vs*. placebo, DBRCT, *N* = 140 MDD patients, 14 days	The primary outcome was the HAMD-17 total score change. Improvement reported after one dose of NRX-1074 infusion had an effect size of 0.88. It was also observed that 72% of the patients receiving the highest of the three tested doses demonstrated a clinically meaningful response at 24 h *vs*. 39% in the placebo group	Phase II, NCT02067793
NLM (2015c)	Ketamine i.v (single infusion) 0.1/0.25/0.5 mg/kg *vs*. midazolam 0.03 mg/kg (active placebo), DBRCT, *N* = 33 late-life TRD patients, 28 days	The rate of response (50% reduction on MADRS total score) at day 7 was 72.7% for 0.5 mg/kg ketamine i.v. *vs*. 46.2% for midazolam (active placebo) and 87.5 *vs*. 66.7% at day 28	Phase III, NCT02556606
NLM (2017b)	Ketamine 0.5 mg/kg *vs*. placebo, DBRCT, *N* = 64 prenatal depression patients, 48 h	EPDS score at 48 h after delivery is the main outcome measure. Undisclosed results	Phase IV, NCT03336541
Lapidus et al. (2014)	Intranasal ketamine (SLS-002) *vs*. placebo, cross-over DBRCT, *N* = 20 TRD patients, 24 h	Patients treated with SLS-002 significantly improved their depressive symptoms 24 h after drug administration *vs*. placebo (MADRS total score change), and the overall tolerability was good, with minimal AE. Response criteria were met by 8 out of the 18 patients treated with ketamine 24 h after drug administration *vs*. 1 out of 8 patients on placebo	Phase II, NCT01304147
NLM (2020), PRNewswire (2021a)	SLS-002 + SOC, *N* = 236 (estimated) MDD patients with imminent risk of suicide, two phases: the first phase is open-label, while the second is double-blind, 24 h and 16 days, respectively	Analysis of the first 17 patients enrolled in this trial demonstrated a rapid onset of antidepressant action from the first dose. Mean MADRS scores met the remission criteria on day 6. The trial is ongoing	Phase II, NCT04669665
Leal et al. (2021)	R-Ketamine (PCN-101), open-label, pilot trial, *N* = 7 TRD patients, 24 h	The mean MADRS score changed significantly, with 20.3 points in 24 h, and no clear dissociative symptoms were reported	Phase N/A

(Continued on following page)

TABLE 1 | (*Continued*) Glutamatergic agents with antidepressant properties in the pipeline.

Authors	Methodology	Results	Clinical trial phase, trial identifier (if available)
GluN2B antagonists			
PRNewswire (2021b)	PCN-101 *vs*. placebo, DBRCT, *N* = 58 healthy volunteers	PCN-101 was safe and well-tolerated at all doses up to 150 mg, and no SAE were reported, according to the manufacturer's press release. In the second stage of the study, the relative safety and tolerability of PCN-101 were compared to that of S-ketamine, and the results demonstrated that PCN-101 required a substantially higher dose to obtain similar perceptual changes to S-ketamine	Phase I, ACTRN12620000226909
Huang et al. (2013), NLM (2009a)	N-Methylglycine (sarcosine) *vs*. citalopram, DBRCT, *N* = 40 MDD patients, 6 weeks	Sarcosine significantly improved HAMD, CGI, and GAF scores more than citalopram treatment. Sarcosine was associated with a higher probability of symptom remission, quicker response, and less risk for dropout. The overall tolerability of sarcosine was good, without significant AE	Phase II, NCT00977353
NLM (2021f)	Sarcosine *vs*. placebo as add-on to SSRI, *N* = 60 MDD patients, 8 weeks	The primary outcome measure is the change in the severity of depressive symptoms from baseline (MADRS total score change). The trial is ongoing	Phase IV, NCT04975100
Heresco-Levy et al. (2006)	D-Cycloserine *vs*. placebo as add-on to ongoing antidepressant, cross-over DBRCT, *N* = 22 TRD patients, 6 weeks	D-Cycloserine induced symptoms reduction and was well tolerated, but the efficacy did not reach statistically significant levels in patients with D-cycloserine *vs*. placebo adjuvant treatment	Phase I
Heresco-Levy et al. (2013)	D-Cycloserine *vs*. placebo as add-on to ongoing antidepressant, DBRCT, *N* = 26 TRD patients, 6 weeks	D-Cycloserine was well tolerated, had no psychotomimetic effects, and improved depressive symptoms, as measured by HAMD and BDI at a significantly level *vs*. placebo	Phase II, NCT00408031
Chen et al. (2019)	D-Cycloserine, *N* = 32 MDD or BD patients who responded to ketamine i.v. in an open-label first phase, DBRCT, 6 weeks	Final total HAMD scores did not differ between the two groups, but a potential effect of D-cycloserine over suicide ideation/ behavior was identified by mixed model analysis throughout the follow-up period	Phase II
NLM (2018f)	D-Cycloserine *vs*. modafinil + CBT, DBRCT, *N* = 36 MDD patients, 3 weeks	The primary outcome measures were the recall of CBT content, the delayed recall of emotional story items, and the delayed recall of logical memory after 2 and 3 weeks. The results have not yet been published	Phase II, NCT02376257
Chen et al. (2014)	Dextromethorphan/ placebo + valproic acid, DBRCT, *N* = 309 BD patients, 12 weeks	Plasma cytokine levels declined in all groups, and changes in BDNF levels were significantly higher in the valproic acid + dextromethorphan 60 mg/day group than in the valproic acid + placebo group	Phase N/A
Nagele et al. (2015)	Nitrous oxide *vs*. placebo, cross-over DBRCT, *N* = 21 TRD and non-TRD patients, 24 h	Depressive symptoms improved significantly at 2 and 24 h after nitrous oxide administration *vs*. placebo (according to HAMD-21 scores). Treatment response was observed in four patients (20%), and three patients had a full remission after nitrous oxide *vs*. one patient (5%) and none after placebo. No SAE occurred, and all AE were brief and of mild-to-moderate severity	Phase II, NCT02139540
NLM (2017c)	Nitrous oxide *vs*. placebo, DBRCT, *N* = 34, 24 h	The primary outcome is HAMD-21 scores at 2 and 24 h after treatment. Undisclosed results	Phase II, NCT03283670
Zarate et al. (2004)	Riluzole 168.8 mg/day, open-label trial, *N* = 19 TRD patients, 6 weeks	Significant improvement in MADRS scores occurred in weeks 3–6, in trial completers, and CGI-S and HAMA also improved significantly during weeks 3–6. The most common adverse events during the trial were headache, gastrointestinal distress, tension, or inner unrest	Phase N/A
Brennan et al. (2010)	Riluzole 100–200 mg/day, open-label trial, *N* = 14 BD patients, 6 weeks	Riluzole led to a significant reduction of HAMD scores, while the glutamine/glutamate (Gln/Glu) ratios increased significantly by day 2 of the treatment	Phase N/A, NCT00544544
Sanacora et al. (2007)	Riluzole 100 mg/day + ongoing antidepressant, open-label trial, *N* = 10 TRD patients, 6 + 6 weeks	HAMD and HAMA scores declined significantly following the initiation of riluzole augmentation treatment, and the effect of riluzole became significant at the end of the first week of the trial and persisted for the 12-week duration of monitoring	Phase N/A
NLM (2010b)	Riluzole/placebo + ongoing SSRI/SNRI, DBRCT, *N* = 104 TRD patients, three-phase study (24 weeks, in total)	Rough, unpublished data did not support a large difference between groups in the MADRS scores, while the response rate at week 8 (secondary outcome) was higher for placebo than for any of the active groups	Phase II, NCT01204918
NLM (2012b)	Riluzole + sertraline *vs*. placebo + sertraline, DBRCT, *N* = 21 MDD outpatients, 8 weeks	The primary outcome measures were the mean change in HAMD scores from baseline to endpoint and the number of patients with antidepressant response or remission at week 8. This study was prematurely terminated due to administrative reasons	Phase II, NCT01703039
NLM (2001)	Riluzole 50–200 mg/day, single-arm, single-blind, *N* = 31 MDD patients, 6 weeks	No results were posted or published	Phase II, NCT00026052
Mathew et al. (2010)	Lamotrigine *vs*. placebo pre-treatment, followed by ketamine infusion, responders were randomized on riluzole 100–200 mg/ day or placebo, DBRCT; *N* = 26 recurrent or chronic MDD, 24–72 h after i.v. ketamine	An interim analysis did not find any significant differences between riluzole and placebo regarding the main outcome (time-to-relapse). The trial was discontinued for futility	Phase IV, NCT00419003

(Continued on following page)

TABLE 1 | (*Continued*) Glutamatergic agents with antidepressant properties in the pipeline.

Authors	Methodology	Results	Clinical trial phase, trial identifier (if available)
GluN2B antagonists			
NLM (2006a)	Riluzole 100–200 mg/day *vs*. placebo, DBRCT, *N* = 94 BD patients, 8 weeks	The main outcome measure was the mean change in the MADRS score. The rough, unpublished results did not support the superior efficacy of riluzole *vs*. placebo	Phase II, NCT00376220
Zarate et al. (2005)	Riluzole 50–200 mg/day + lithium, open-label study, *N* = 14 BD patients, 8 weeks	The linear mixed model for total MADRS score showed a significant treatment effect at week 8, without cases of switch into hypomania or mania	Phase N/A
NLM (2003)	Riluzole 50–200 mg/day *vs*. placebo, DBRCT, *N* = 19 BD patients, 8 weeks	The study was terminated due to the superior efficacy of placebo in an interim analysis	Phase II, NCT00054704
Zarate et al. (2006)	Memantine 5–20 mg/day *vs*. placebo, DBRCT, *N* = 32 MDD patients, 8 weeks	The results of this trial (MADRS scores change from baseline to week 8) were negative	Phase N/A
Smith et al. (2013)	Memantine 5–20 mg/day *vs*. placebo + antidepressant, DBRCT, *N* = 31 patients with partial or non-responsive MDD, 8 weeks	No statistical differences were observed between groups on primary or secondary efficacy outcomes or safety outcomes	Phase N/A
NLM (2006b)	Memantine 5–20 mg/day + lamotrigine, DBRCT, *N* = 29 BD patients, 8 weeks	The primary outcome was the change in HAMD-17 from baseline to week 8. Unpublished results show a decrease of 9 *vs*. 7 points in patients treated with memantine *vs*. placebo. The most frequently reported adverse events in the memantine group were somnolence, indigestion, diarrhea, headache, and coughing	Phase IV, NCT00305578
NLM (2002)	Memantine 5–20 mg/day *vs*. placebo, DBRCT, *N* = 112 MDD outpatients, three-phase study (2, 8, and 16 weeks)	No results of this trial have been released	Phase III, NCT00040261
NLM (2006c)	Memantine 5–20 mg *vs*. placebo as add-on to antidepressants, DBRCT, *N* = 31 MDD patients with incomplete response/ non-response to antidepressants	The main outcome was the change in MADRS scores at week 8. Unpublished results did not support a significant difference between groups (–7.13 *vs*. –7.25 points in memantine *vs*. placebo). The rate of serious adverse events was similar in the two groups	Phase IV, NCT00344682
Metabotropic glutamate receptors antagonists			
Watanabe et al. (2021)	TP0473292 (TS-161) *vs*. placebo, DBRCT, SAD, and MAD, *N* = 70 healthy subjects, 10 days	The investigational product penetrated the brain–blood barrier, and the most frequently reported AE were nausea, vomiting, and dizziness, with an exposure-related incidence	Phase I, NCT03919409
NLM (2021g)	TS-161 *vs*. placebo, DBRCT, *N* = 25 (estimated), TRD patients, 21 days	The main outcome is the change from baseline to day 21 on MADRS total scores. The trial is ongoing	Phase II, NCT04821271
Umbricht et al. (2020)	Decoglurant (RO4995819) *vs*. placebo, DBRCT, *N* = 357 TRD patients, 6 weeks	At week 6, no significant differences were observed between any active treatment arms and placebo in decreasing MADRS scores, response, or remission rates. No effects of decoglurant were observed on CANTAB. A high rate of placebo response was observed	Phase II, NCT01457677
NLM (2012c)	RO4995819 *vs*. placebo as adjunctive therapy, DBRCT, TRD patients, 6 weeks	The main outcome measure was MADRS total score change. The trial was withdrawn by the sponsor. No subject was enrolled	Phase II, NCT01733654
Quiroz et al. (2016)	Basimglurant (RG-7090) *vs*. placebo as an adjunctive agent to SSRI/SNRI, DBRCT, *N* = 333 MDD patients, 6 weeks	No difference was observed in the primary outcome, MADRS change from baseline to the endpoint, between basimglurant MR and placebo. Secondary endpoints were modified by adjunctive basimglurant MR 1.5 mg daily, especially in patient-rated measures. The most frequently reported AE was dizziness, but it was of mild intensity and transient	Phase IIb, NCT01437657
NLM (2015d)	RG-7090 *vs*. placebo, DBRCT, MAD, *N* = 56 healthy subjects + MDD patients, 10 weeks	The primary outcomes were tolerability and safety of the investigational product. The results of this trial are undisclosed as of February 2022	Phase I, NCT02433093
NLM (2010c)	AZD-2066 *vs*. placebo *vs*. duloxetine, DBRCT, *N* = 131 MDD patients, 6 weeks	The primary outcome was MADRS totals core change from baseline to week 6. The improvement was –13.1 (AZD 2066), –14 (duloxetine), and –14.1 (placebo). The response rate was the same in all three groups	Phase IIa, NCT01145755

AE, adverse events; AMPA, alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid; BD, bipolar depression; BDI, Beck Depression Inventory; BDM, Bipolar Inventory of Symptoms Scale-derived MADRS; BDNF, brain-derived neurotrophic factor; CANTAB, Cambridge Neuropsychological Test Automated Battery; CBT, cognitive-behavioral therapy; CGI, Clinical Global Impression; C-SSRS, Columbia Suicidality Severity Scale; DBRCT, double-blind randomized controlled trial; FDA, Food and Drug Administration; GAF, Global Assessment of Functioning; HAMD, Hamilton Depression Rating Scale; MAD, multiple ascending dose; MADRS, Montgomery Asberg Depression Rating Scale; MDD, major depressive disorder; N/A, not applicable; NIMH, National Institute of Mental Health; NMDA, N-methyl-D-aspartate; QIDS-SR, Quick Inventory of Depressive Symptomatology-Self-Report; Q-LES-Q, Quality of Life Enjoyment and Satisfaction Questionnaire-Short Form; SAD, single ascending dose; SAE, severe adverse events; SDS, Sheehan Disability Score; SNRI, serotonin and norepinephrine reuptake inhibitor; SOC, standard of care; SSRI, selective serotonin reuptake inhibitors; sp/ppTMS, single-pulse/paired-pulse transcranial magnetic stimulation; TRD, treatment-resistant MDD.

and symptoms (Fava et al., 2022). MADRS scores improved on day 4 in both REL-1017 dosage groups, and this change persisted through the follow-up visit (day 14) (Fava et al., 2022).

Two phase III, multicenter, double-blind, placebo-controlled outpatient trials are ongoing, and they have as objective the assessment of the efficacy and safety of REL-1017 as an adjunctive treatment of MDD (RELIANCE-I, II) (NLM, NCT04688164). The estimated enrollment in these trials is estimated to be 400 participants, who will be monitored for 28 days, with changes in MADRS total score as the main outcome (NLM, NCT04855747). REL-1017 will also be evaluated as monotherapy in MDD patients in a randomized, placebo-controlled, phase III trial (RELIANCE-III) with a duration of 28 days (NLM, NCT05081167). However, another phase III trial is dedicated to the open-label evaluation of the long-term safety of REL-1017 as adjunctive treatment of MDD

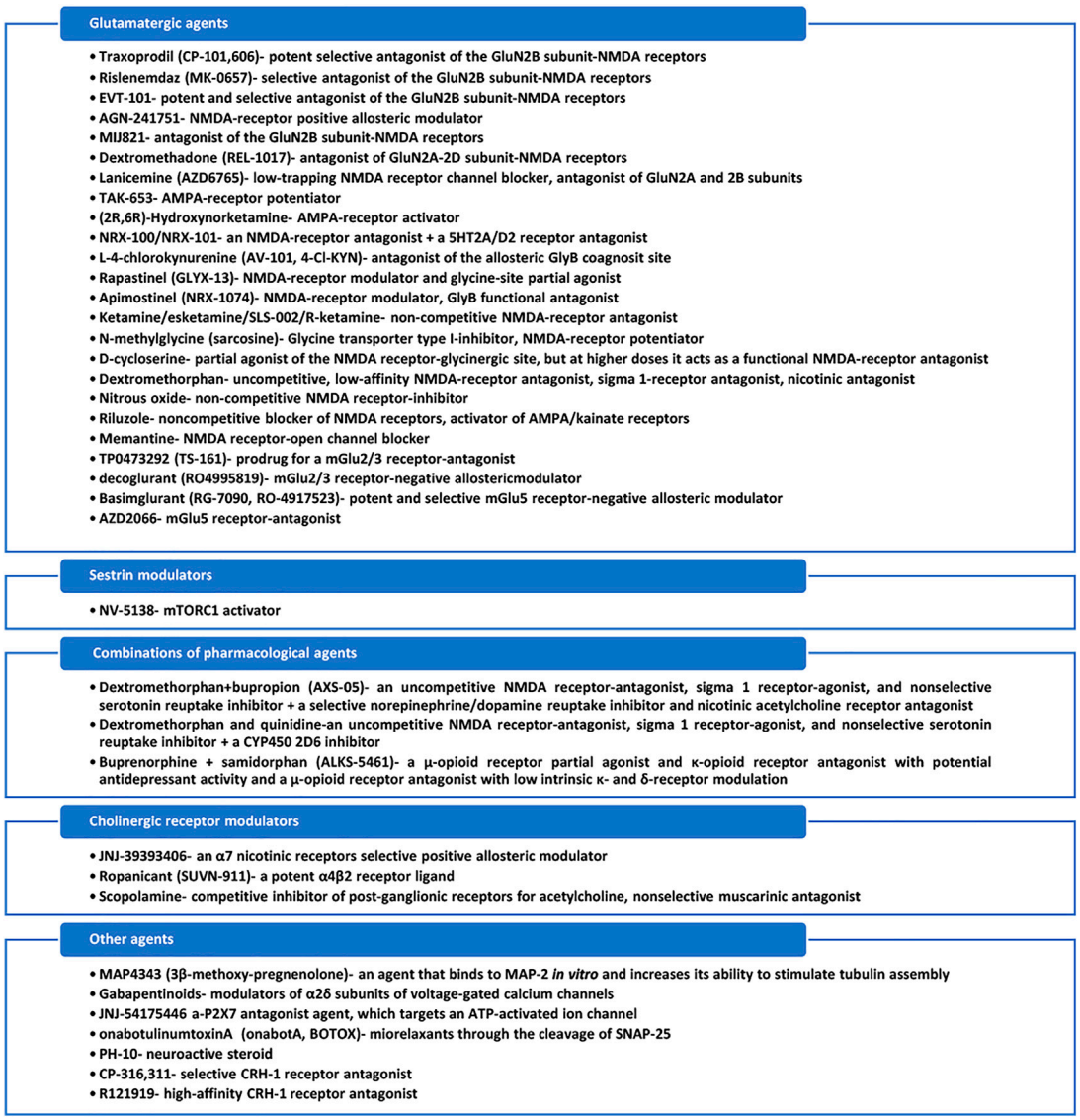

FIGURE 3 | Mechanisms of action of the identified antidepressants in the pipeline, which are presented in this review.

and is expected to recruit 600 participants for a monitoring period of 52 weeks (NLM, NCT04855760).

AZD6765 (lanicemine) is a low-trapping NMDA-receptor channel blocker, with an affinity for GluN2A and GluN2B complexes, with antidepressant efficacy demonstrated in three out of four clinical studies (Agbo et al., 2017; Sengupta et al., 2019). Lanicemine has a fast off-rate and is a low-trapping NMDA-receptor antagonist, unlike ketamine and MK-801 properties that lead to a favorable safety profile (Sengupta et al., 2019). This drug also acts over opiate, sigma, and muscarinic receptors (Sengupta et al., 2019). The results of two phase I studies in healthy subjects and two phase II trials in MDD patients were integrated into a pharmacokinetic analysis, and the model developed adequately described lanicemine properties in both clinical and non-clinical samples (Sanacora et al., 2014; Agbo et al., 2017). In both phase II trials, 100 mg lanicemine was efficient in decreasing the MADRS total score, and most of the secondary outcome measurements were up to 3 weeks (Sanacora et al., 2014).

In a randomized, multicenter, parallel-arm, double-blind, placebo-controlled, phase IIb trial, 302 adult patients with MDD and inadequate treatment response received 15 double-blind i.v. infusions of adjunctive lanicemine 50 mg, lanicemine 100 mg, or saline over a 12-week course, in addition to ongoing antidepressants (Sanacora et al., 2017). Lanicemine was generally well-tolerated, but neither dose was superior to placebo in decreasing the severity of the depressive symptoms (Sanacora et al., 2017).

In another double-blind, randomized, crossover, placebo-controlled trial 22 subjects diagnosed with TRD were enrolled, and they received a single infusion of AZD6765 (150 mg) or

placebo on two test days, 1 week apart (Zarate et al., 2013). The MADRS score significantly improved, within 80 min, in subjects receiving AZD6765 compared to placebo, but this improvement remained significant only for 110 min (Zarate et al., 2013). The HAMD scores reflected a difference between groups at 80 and 110 min and also on day 2 (Zarate et al., 2013). The response rate was 32% in the AZD6765-treated group *versus* 15% in placebo-treated patients (Zarate et al., 2013). No difference between groups was reported in the rate of psychotomimetic and dissociative adverse effects (Zarate et al., 2013).

The contradictory results regarding the efficacy of lanicemine in phase II trials raise important questions about the drug dosage, the relevance of the placebo effect, and the potential factors that may influence treatment response in MDD patients.

TAK-653 is an AMPA receptor potentiator with virtual no agonistic activity in animal models (Hara et al., 2021). Both acute and sub-chronic administration of TAK-653 in rats produced significant antidepressant-like effects on the reduction of the submissive behavior model but did not induce a hyper locomotor response, which is a behavioral index associated with psychotomimetic side effects in humans (Hara et al., 2021).

A phase I, randomized, crossover, double-blind, placebo-controlled study enrolled 24 healthy volunteers to evaluate the central nervous system pharmacodynamic activity of TAK-653 in healthy volunteers using transcranial magnetic stimulation (TMS) (O'Donnell et al., 2021). Doses of 0.5 and 6 mg of TAK-653 or placebo were administered, and single-pulse or paired-pulse motor cortex TMS (spTMS and ppTMS) coupled with electromyography as evidence of cortical excitability change under treatment were monitored (O'Donnell et al., 2021). TAK-653 increased the amplitude of motor-evoked potentials in study participants but did not affect resting motor threshold or paired-pulse responses (O'Donnell et al., 2021). Another phase I, randomized study recruited 88 healthy subjects in order to evaluate the safety, tolerability, and pharmacokinetics of escalating single and multiple doses of TAK-653 (NLM, NCT02561156). The overall tolerability of the investigational product was good, with no severe adverse events being reported (NLM, NCT02561156).

A phase II clinical trial assessing the efficacy and safety of TAK-653 in TRD was withdrawn by the sponsor (NLM, NCT03312894).

(2R,6R)-Hydroxynorketamine is a metabolite of ketamine/esketamine, which does not bind to the NMDA receptors and does not cause dissociative effects or abuse potential in mice (Zanos et al., 2016). The antidepressant actions of hydroxynorketamine involve early and sustained AMPA-receptor activation, according to a preclinical model of depression (Zanos et al., 2016). A double-blind, placebo-controlled, phase I, single ascending dose and multiple ascending dose study focusing on the safety, pharmacokinetics, and pharmacodynamics of (2R,6R)-hydroxynorketamine in healthy volunteers is ongoing, with a total of 48 subjects planned to be enrolled (NLM, NCT04711005).

NRX-100/NRX-101 consists of an initial single dose of ketamine (NRX-100) administered intravenously for clinical stabilization, followed by oral D-cycloserine plus lurasidone (NRX-101), and this sequential treatment regimen has as its main indication the control of suicidal ideation/behavior in bipolar depression (Hecking et al., 2021). Ketamine is an NMDA-receptor antagonist, and lurasidone is an atypical antipsychotic with 5HT2A/D2 receptor antagonist properties (Hecking et al., 2021). D-Cycloserine component of the NRX-101 is included in this combination because of its effects on inhibiting NMDA receptors and raising levels of glutamate/glutamine (Glx) in the anterior cingulate cortex (NLM, NCT03396068). Increased Glx has been reported to correlate with clinical improvement following electroconvulsive therapy and following i.v. the administration of ketamine, according to magnetic resonance spectroscopy studies (NLM, NCT03396068).

The efficacy of the sequential administration of NRX-101 has been explored in a randomized, active-comparator, phase II trial, with the main outcome being the BDM (Bipolar Inventory of Symptoms Scale-derived MADRS) score change from baseline to day 42 (NLM, NCT02974010). This trial had four arms: ketamine followed by oral NRX-101, ketamine followed by oral lurasidone, saline solution followed by oral NRX-101, and saline solution followed by oral lurasidone (NLM, NCT02974010). Many 22 adult subjects diagnosed with bipolar depression and suicidal ideation or behavior were randomized in this trial (NLM, NCT02974010). The results (yet unpublished in a peer-reviewed journal) support the superior efficacy of ketamine followed by NRX-101 *versus* ketamine followed by lurasidone, as reflected by the Bipolar Inventory of Symptoms Scale-derived MADRS (BDM) scores at day 42 (NLM, NCT02974010). No significant difference in the rate of adverse events was observed between NRX-101 and lurasidone-treated groups (NLM, NCT02974010).

NRX-101 is currently undergoing a randomized, active comparator (lurasidone), phase II trial on patients diagnosed with bipolar depression and suicidal ideation, following initial stabilization with ketamine (NLM, NCT02974010). The main outcome of this trial is the improvement of depressive symptoms as measured by MADRS total score, and the expected enrollment is 72 participants (NLM, NCT02974010). Another randomized, active comparator (lurasidone), phase II/III trial focused on the efficacy of NRX-101 in patients diagnosed with moderate bipolar depression and suicidal ideation is expected to begin recruitment, and its primary outcome will be the improvement of depressive symptoms severity measured by MADRS during 6 months (NLM, NCT03395392). A randomized, phase II/III, Glx biomarker validation study is planned to recruit 24 participants diagnosed with bipolar depression who will receive either NRX-101 *versus* placebo or NRX-101 *versus* lurasidone (NLM, NCT03402152). In this trial, the main outcome will be the mean change in the Glx area under the curve (AUC) measured after the administration of the investigational product *versus* the active comparator (NLM, NCT03402152).

The efficacy of NRX-100 (0.5 mg/kg over 40 min) is investigated in an ongoing, randomized, placebo-controlled, phase III trial, in which the primary outcome is the Columbia Suicidality Severity Scale (C-SSRS) score (NLM, NCT03396601). The main objective of this trial is to determine if NRX-100 is superior to placebo infusion in the

rapid stabilization of patients with severe bipolar depression and active suicidal ideation and behavior, determined after 24 h by the percentage of participants who achieve response (C-SSRS score ≤3) (NLM, NCT03396601). Subjects who respond to NRX-100 will be offered enrollment in a 6-week follow-up study of NRX-101 *versus* SOC to validate the maintenance effect of ketamine (NLM, NCT03396601).

L-4-Chlorokynurenine (AV-101, 4-Cl-KYN) is an antagonist of the allosteric glycine B (GlyB) coagonist site, and this mechanism of glutamatergic modulation is considered better tolerated and safer than NMDA-receptor antagonism (Wallace et al., 2017). AV-101 is the prodrug of 7-chlorokynurenic acid, one of the most potent GlyB antagonists currently known, which possesses ketamine-like antidepressant properties in animal models of depression and efficacy in animal models of neuropathic pain (Zanos et al., 2015; Wallace et al., 2017). When the behavioral responses in animal models, measured on the 24 h forced swim test, learned helplessness test, and novelty-suppressed feeding test, were evaluated, AV-101 induced rapid, dose-dependent, and persistent antidepressant-like effects following a single dose (Zanos et al., 2015). The antidepressant effects of AV-101 were prevented by pretreatment with glycine or alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptor antagonists (Zanos et al., 2015). AV-101 was not associated with the rewarding or psychotomimetic effects of ketamine, and it did not lead to locomotor sensitization or stereotypic behaviors (Zanos et al., 2015).

In a randomized, controlled, double-blind, cross-over trial, the effects of AV-101 in patients with TRD were investigated ($N = 19$ participants) by the administration of 4-Cl-KYN oral monotherapy (1080 mg/day, 7 days, then 1440 mg/day, 7 days) or placebo (14 days) (Park et al., 2020). The administration of AV-101 was preceded by a period of 2 week drug-free regimen (Park et al., 2020). No treatment effects were detected using linear mixed models, as determined by primary (HAMD score) or secondary outcome measures (Park et al., 2020). No difference between groups for any peripheral or central biological indices or adverse effects was reported (Park et al., 2020). These negative results raise doubts related to the capacity of AV-101 to penetrate the brain and engage the NMDA receptors and the kynurenine pathway effectively (Murphy et al., 2021). To verify this aspect, another randomized, double-blind, placebo-controlled, crossover, phase I study ($N = 10$ healthy volunteers) explored the dose-related effects of AV-101 (720 and 1440 mg) on the engagement of the NMDA receptors (Murphy et al., 2021). The results showed that only the high dose (1440 mg) of AV-101 in humans succeeded in engaging brain targets in humans, suggesting the necessity of testing these doses in depression (Murphy et al., 2021).

Rapastinel (GLYX-13) is an NMDA-receptor modulator with glycine-site partial agonist properties, which possesses cognitive enhancement properties and rapid and long-lasting antidepressant activity in both animal models and humans (Burgdorf et al., 2015a). In clinical trials, rapastinel produced marked antidepressant effects that last for at least 1 week after a single dose (Moskal et al., 2014; Burgdorf et al., 2015b). Animal models of depression support the existence of a hippocampal long-term potentiation effect of rapastinel that persisted up to 2 weeks after a single dose (2 mg/kg i.v.), supposedly *via* triggering NMDA-receptor-dependent processes and increasing the mature spine density in the hippocampus and medial prefrontal cortex in rats (Burgdorf et al., 2015b).

Of the three trials identified in the clinicaltrials.gov archive, which refers to the effects of rapastinel in MDD patients, only two have results. The first proof-of-concept trial was double-blind, placebo-controlled, randomized, phase II, single i.v. GLYX-13 (1, 5, 10, or 30 mg/kg) and enrolled 116 participants with MDD who had not benefited from at least one monoaminergic antidepressant for their current episode (Preskorn et al., 2015). GLYX-13, administered at a 5 or 10 mg/kg i.v. dose reduced depressive symptoms (measured by HAMD-17) on days 1–7 (Preskorn et al., 2015). The antidepressant effect had its onset within 2 h and persisted for 7 days on average (Preskorn et al., 2015). No psychotomimetic or other significant adverse events were reported (Preskorn et al., 2015).

The second trial, with undisclosed results, included 369 participants with MDD and inadequate/partial response to antidepressants, and it had a double-blind, placebo-controlled, randomized withdrawal design (NLM, NCT01684163). A phase II, open-label extension trial investigated the safety of long-term repeat exposure to GLYX-13 in subjects who participated in the previously mentioned trial (NLM, NCT02192099; NLM, NCT01684163). In the extension, rapastinel (250/450 mg i.v.) was administered to 61 participants with completed eight or more weeks of treatment in the previous study and were willing to continue treatment (NLM, NCT02192099). Patients who were originally assigned to 5 mg/kg received 225 mg rapastinel, and those assigned to 10 mg/kg in the first trial received 450 mg active drug (NLM, NCT02192099). Unpublished results posted on the clinicaltrials.gov site show a high rate of severe adverse events (SAE) (23%) and adverse events (98%) collected during 48 months (NLM, NCT02192099). Therefore, this study was terminated by the sponsor in 32 cases, and 11 participants withdrew.

Apimostinel (NRX-1074) is a compound with NMDA-receptor modulating properties, more specifically, a functional antagonist at the GlyB site of the NMDA receptors (Wilkinson and Sanacora, 2019). This product was investigated in phase I trials as i.v. formulation and an orally bioavailable drug candidate (NLM, NCT02366364; NLM, NCT01856556). A phase I trial investigated the safety and tolerability of multiple oral ascending doses of NRX-1074 (375, 500, and 750 mg) in 15 healthy volunteers, but the results have not yet been released (NLM, NCT02366364). The phase I trial investigating i.v. and oral formulae also has undisclosed results (NLM, NCT01856556).

NRX-1074 led to statistically significant improvement in MDD 24 h after intravenous administration (1, 5, or 10 mg) in a randomized, double-blind, placebo-controlled phase II study (Brooks, 2015). The improvement reported after one dose of NRX-1074 infusion had an effect size (0.88), more than double the average effect size typically seen with most antidepressants after 4–6 weeks of a repeated dose, according to the company release note (Brooks, 2015). This trial recruited 140 patients with

MDD, and the primary outcome measure was HAMD-17 (Brooks, 2015). It was also observed that 72% of the patients receiving the highest of the three tested doses demonstrated a clinically meaningful response at 24 h *versus* 39% in the placebo group (Brooks., 2015).

The antidepressant effects of **ketamine** are supported by randomized clinical trials, with a fast onset of action, high response rates in TRD, and efficacy against suicidality (Lacerda, 2020). While intranasal esketamine was approved by FDA in 2019 for TRD, when added to a traditional oral antidepressant, the racemic mixture of ketamine is currently investigated for MDD (Bahr et al., 2019). Ketamine/esketamine are non-competitive NMDA-glutamate receptor antagonists, with a higher affinity for these receptors in the case of S-ketamine enantiomer (Bahr et al., 2019). The mechanisms of action underlying their positive effects on MDD are unclear, but they probably involve improvement of brain plasticity *via* stimulation of BDNF (brain-derived neurotrophic factor) production and activation of the mammalian target of rapamycin (mTOR) (Sattar et al., 2018; Bahr et al., 2019). Ketamine and esketamine actions over the mTOR pathway are responsible for additional stimulation of BDNF, thus increasing brain plasticity through dendritic growth and improving synaptic transmission (Ignacio et al., 2016; Bahr et al., 2019).

A meta-analysis (n = 14 clinical trials) showed that a single infusion of (R,S)-ketamine (0.5 mg/g, 40 min) induces a response rate of 50%–70% in TRD (Kishimoto et al., 2016). According to a meta-analysis that evaluated the efficacy of ketamine for the treatment of MDD, the treatment effects may last up to 6 weeks after drug administration (Conley et al., 2021). Another meta-analysis that compared racemic ketamine and esketamine (n = 24 trials, N = 1877 participants) used as primary outcomes the response and remission from depression, change in depression severity, suicidality, retention in treatment, drop-out rate, and drop-outs due to adverse events, concluding that ketamine was associated with greater overall response and remission rates, as well as lower dropouts (Bahji et al., 2021).

A randomized, phase III trial evaluated the effects of i.v. ketamine (0.1, 0.25, or 0.5 mg/kg) *versus* midazolam (0.03 mg/kg) in 33 military veterans with late-life TRD (NLM, NCT02556606). The rate of response (50% reduction on MADRS total score) at day 7 was 72.7% for 0.5 mg/kg ketamine i.v. *versus* 46.2% for midazolam (active placebo) and 87.5% *versus* 66.7% at day 28 (NLM, NCT02556606).

Another interesting study evaluated the efficacy of low-dose ketamine administered during cesarean delivery as a method to decrease the incidence of postpartum depression in parturients with prenatal depression (NLM, NCT03336541). This phase IV trial was completed, but its results are not available.

SLS-002 is the racemic mixture of ketamine with intranasal administration, currently undergoing phase II clinical trials (NLM, NCT04669665). In a randomized, double-blind, crossover study, 20 TRD participants received intranasal ketamine hydrochloride (50 mg) or saline solution and were monitored for 7 days (Lapidus et al., 2014). Patients treated with ketamine had significant improvements in their depressive symptoms 24 h after drug administration, and the overall tolerability was good, with minimal adverse effects (Lapidus et al., 2014).

A phase II, randomized, initial open-label sequence and a double-blind, randomized, placebo-controlled second sequence will evaluate the efficacy, safety, and tolerability of SLS-002 in addition to SOC on symptoms of MDD and suicidality, in participants at imminent risk for suicide as determined by change in MADRS total score at 24 h after the first dose (NLM, NCT04669665). In the first part of the study, 17 patients were enrolled, and SLS-002 demonstrated a rapid onset of action from the first dose through the last visit, with the mean MADRS scores meeting the remission criteria on day 6 (PRNewswire, 2021a).

R-Ketamine (PCN-101), or arketamine, has been associated with a longer-lasting and more potent antidepressant effect than ketamine and esketamine in animal studies (Zhang et al., 2014). Because it proved to have weaker hypnotic and analgesic actions than the racemate and esketamine in humans, arketamine did not become commercially available for anesthesiology use (Leal et al., 2021). Unlike S-ketamine, arketamine can elicit a sustained antidepressant effect in mice, which appears to be mediated by increased BDNF-TrkB signaling and synaptogenesis in the prefrontal cortex, dentate gyrus, and CA3 hippocampal region (Yang et al., 2015). Arketamine was not associated with abuse or psychotomimetic activity (Yang et al., 2015).

In an open-label pilot trial, seven subjects with TRD received a single intravenous infusion of arketamine (0.5 mg/kg), and the MADRS score at 24 h after administration was defined as the primary outcome (Leal et al., 2021). The mean MADRS score changed significantly, with 20.3 points in 24 h, and no clear dissociative symptoms were reported (Leal et al., 2021).

A phase I, two-stage, single-center, randomized, placebo-controlled, double-blind study evaluated first the safety, tolerability, and pharmacokinetics of single PCN-101 ascending doses in 58 healthy adult volunteers, administered *via* intravenous infusion (PRNewswire, 2021b). PCN-101 was safe and well-tolerated at all doses up to 150 mg, and no SAE were reported, according to the manufacturer's press release (PRNewswire, 2021b). In the second stage of the study, the relative safety and tolerability of PCN-101 were compared to that of S-ketamine, and the results showed that substantially higher doses of PCN-101 are required to obtain similar perceptual changes with S-ketamine (PRNewswire, 2021b).

N-Methylglycine (sarcosine) inhibits glycine transporter-I and thus potentiates the NMDA function, improving depression-like behavior in rodent models and depression in humans (Chen et al., 2017). A single dose of sarcosine produced an antidepressant-like effect with rapid concomitant increases in the mTOR signaling pathway activation and enhancement of the AMPA receptor membrane insertion in rats (Chen et al., 2017). Long-term administration of sarcosine had favorable effects in

rats exposed to chronic unpredictable stress but not in stress-naive rats (Chen et al., 2017).

In a complex study, which explored the efficacy of sarcosine in animal models and depressed patients, the results were favorable: 1) sarcosine decreased immobility in the forced swim test and tail suspension test, reduced the latency to feed in the novelty-suppressed feeding test, and reversed behavioral deficits caused by chronic unpredictable stress test in an animal model of depression; 2) in MDD patients (N = 40), sarcosine (500–1500 mg/day sarcosine) improved significantly HAMD, Clinical Global Impression (CGI), and GAF scores more than citalopram (20–60 mg/day) treatment, and it was associated with a higher probability of symptom remission, quicker response, and less risk for drop out (Huang et al., 2013; NLM, NCT04975100).

A phase IV clinical trial designed to evaluate the efficacy of sarcosine as an add-on to currently administered antidepressants in patients with MDD is ongoing and is estimated to recruit 60 adult participants who will be randomized on sarcosine + SSRI or placebo + SSRI (NLM, NCT04975100). The primary outcome measure is the change in depressive symptoms severity from baseline, assessed with MADRS, during 8 weeks (NLM, NCT04975100).

D-Cycloserine is an antibiotic that also possesses partial agonistic properties at the NMDA-receptor-associated modulatory glycine site, and at dosages ≥100 mg/day, it acts as a functional NMDA-receptor antagonist with antidepressant effects (Heresco-Levy et al., 2006). In a double-blind, placebo-controlled 6-week crossover trial, 22 TRD patients received 250 mg/day of D-cycloserine added to their ongoing antidepressant (Heresco-Levy et al., 2006). D-Cycloserine induced symptoms reduction and was well tolerated, but the efficacy did not reach statistically significant levels in patients with D-cycloserine *versus* placebo adjuvant treatment (Heresco-Levy et al., 2006). In another double-blind, placebo-controlled, 6-week, parallel-group trial, 26 TRD patients received a gradually titrated high dose (1000 mg/day) of D-cycloserine added to their current antidepressant (Heresco-Levy et al., 2013). D-Cycloserine was well tolerated, had no psychotomimetic effects, and improved significantly depressive symptoms *versus* placebo, as measured by HAMD and BDI scores (Heresco-Levy et al., 2013). Also, pretreatment glycine serum was considered a relevant variable that interacted with the treatment outcome (Heresco-Levy et al., 2013). This second trial suggested that the antagonistic properties of D-cycloserine begin at a higher dose than expected in the first trial, probably above the level of 500 mg/day.

In another trial, 32 patients with TRD (17 with MDD and 15 with bipolar depression) who responded to ketamine infusion with an average 9.47 ± 4.11 HAMD score at baseline were randomly divided into 6-week D-cycloserine treatment *versus* placebo (Chen et al., 2019). During the 6-week treatment, the total HAMD scores did not differ between the two groups, but a potential effect of D-cycloserine over suicide ideation/behavior was identified by mixed model analysis throughout the follow-up period (Chen et al., 2019).

The administration of D-cycloserine as a pre-treatment before computer-based cognitive-behavioral therapy (CBT) sessions for depression to assess the impact of this approach on therapeutic learning has been explored in a randomized, phase II trial of 36 participants (NLM, NCT02376257). D-Cycloserine (250 mg/day) was compared in this trial with modafinil (100 mg/day) and placebo, and the primary outcome measures were the recall of CBT content, the delayed recall of emotional story items, and the delayed recall of logical memory after 2 and 3 weeks (NLM, NCT02376257). The results of this trial have not yet been published in a peer-review journal.

Dextromethorphan has uncompetitive, low-affinity NMDA-receptor antagonist properties and σ-1 receptor-agonist and nicotinic antagonist effects (Nguyen et al., 2016). Dextromethorphan inhibits the serotonin transporter and the norepinephrine transporter to a lesser extent (Nguyen et al., 2016). It also inhibits voltage-gated calcium channels (Nguyen et al., 2016). According to a review of the clinical and preclinical studies referring to the efficacy and tolerability of dextromethorphan, this agent is well tolerated and exerts clinically significant antidepressant effects, especially in adults with bipolar depression (Majeed et al., 2021). In a randomized, double-blind, 12-week clinical trial, 309 patients with bipolar disorder received either valproic acid and low-dose (30 or 60 mg/day) dextromethorphan or valproic acid plus placebo (Chen et al., 2014). Before treatment, patients with bipolar disorder had significantly higher plasma cytokine and lower plasma BDNF levels than healthy controls, and after treatment, HAMD and Young Mania Rating Scale (YMRS) scores in each treatment group showed significant improvement (Chen et al., 2014). Plasma cytokine levels declined in all groups, and changes in BDNF levels were significantly greater in the valproic acid + dextromethorphan 60 mg/day group than in the valproic acid + placebo group (Chen et al., 2014).

Nitrous oxide has a largely unknown mechanism of action, but it is considered a non-competitive inhibitor of NMDA-glutamate receptors (Kalmoe et al., 2020). Its main clinical use is inhalational general anesthesia and analgesia for short procedures, but it is also used recreationally by adolescents and young adults (Kalmoe et al., 2020). The euphoria-inducing effects of nitrous oxide have been hypothesized to have clinical benefits in patients with MDD (Kalmoe et al., 2020). In a proof-of-concept, placebo-controlled crossover trial, 20 patients with TRD were randomized to 1 h inhalation of 50% nitrous oxide/50% oxygen or 50% nitrogen/50% oxygen (the last one being equivalent to placebo) (Nagele et al., 2015). Depressive symptoms improved significantly at 2 and 24 h after nitrous oxide administration *versus* placebo (according to HAMD-21 scores) (Nagele et al., 2015). Treatment response was observed in four patients (20%), and three patients had a full remission after nitrous oxide *versus* one patient (5%) and none after placebo (Nagele et al., 2015). No SAE occurred, and all adverse events were brief and of mild-to-moderate severity (Nagele et al., 2015). Another phase II, randomized, double-blind trial that evaluated the efficacy of inhaled nitrous oxide for TRD investigated the impact of nitrous oxide 25% or 50% *versus* placebo over HAMD-21 scores at 2 and 24 h after inhalation in 34 patients, but results have not been disclosed (NLM, NCT03283670).

Riluzole is a neuroprotective agent which inhibits the voltage-dependent sodium channels on glutamatergic nerve terminals and activates AMPA/kainate receptors, but it may induce a noncompetitive blockade of NMDA receptors (Doble, 1996; Zarate et al., 2004).

In an open-label trial, 19 patients diagnosed with treatment-resistant depression received riluzole 168.8 mg/day (mean dose) for 6 weeks (Zarate et al., 2004). Significant improvement in MADRS scores occurred in weeks 3–6, in trial completers, and CGI-S and HAMA also improved significantly during weeks 3–6 (Zarate et al., 2004). The response rate for completers at week 6 was 46%, and the remission rate was 31% (Zarate et al., 2004). The most common adverse events during the trial were headache (58%), gastrointestinal distress (43%), tension, or inner unrest (26%) (Zarate et al., 2004).

In an open-label trial, 100–200 mg riluzole was administered for 6 weeks to 14 patients with bipolar depression, and it led to a significant reduction of HAMD scores, while the glutamine/glutamate (Gln/Glu) ratios increased significantly by day 2 of the treatment (Brennan et al., 2010). N-Acetyl aspartate (NAA) levels increased in NAA from baseline to week 6 (Brennan et al., 2010). Therefore, riluzole seems to rapidly increase the Gln/Glu ratios, suggesting increased glutamate-glutamine cycling, which may lead to enhanced neuronal plasticity and reduced depressive symptoms (Brennan et al., 2010).

Riluzole was added to ongoing medication for 6 weeks, followed by an optional 6-week continuation phase in 10 patients diagnosed with treatment-resistant depression (Sanacora et al., 2007). HAMD and HAMA scores declined significantly following the initiation of riluzole augmentation treatment, and the effect of riluzole became significant at the end of the first week of the trial and persisted for the 12-week duration of monitoring (Sanacora et al., 2007).

A phase II, randomized, double-blind, placebo-controlled, adjunctive trial on treatment-resistant MDD enrolled 104 participants who received 1) 100 mg riluzole added to ongoing SSRI/SNRI for 8 weeks, 2) riluzole/placebo added to SSRI/SNRI for 4 weeks and placebo added to the same agents for another 4 weeks, or 3) placebo added to SSRI/SNRI for 8 weeks (NLM, NCT01204918). The main outcome measures were the change in MADRS scores after 4 and 8 weeks (NLM, NCT01204918). The final results of this trial were not published in a journal, but the rough data available on the clinicaltrials.gov site did not support a large difference between groups, while the response rate at week 8 (secondary outcome) was higher for placebo than for any of the active groups (NLM, NCT01204918).

Another randomized, double-blind, phase II trial evaluated the efficacy of riluzole (50 mg b.i.d) *versus* placebo as an add-on to sertraline (100 mg/day) in 21 outpatients diagnosed with MDD during 8 weeks, and the primary outcome measures were the mean change in HAMD scores from baseline to endpoint and the number of patients with antidepressant response or remission at week 8 (NLM, NCT01703039). This study was prematurely terminated due to administrative reasons.

Another 6-week, single-arm, single-blind phase II study enrolled 31 patients with MDD without psychotic features and evaluated the efficacy of riluzole (NLM, NCT00026052). The study was completed, but no results were posted or published.

A randomized, placebo-controlled, double-blind, continuation-phase IV study evaluated the safety and effectiveness of ketamine and riluzole in patients with treatment-resistant MDD (Mathew et al., 2010). A total of 26 medication-free patients received open-label i.v. ketamine (0.5 mg/kg over 40 min), and before infusion, they were randomized to lamotrigine (300 mg) or placebo (Mathew et al., 2010). The response rate was 65% (17 patients), according to the MADRS scores at 24 h following ketamine, while lamotrigine failed to attenuate the mild, transient side effects associated with ketamine and did not enhance its antidepressant effects (Mathew et al., 2010). After 72 h of infusion, the response was obtained by 14 patients (54%), and they were randomized to continue with riluzole (100–200 mg/day) or placebo (Mathew et al., 2010). An interim analysis did not find any significant differences between riluzole and placebo regarding the main outcome (time-to-relapse), with 80% of patients relapsing on riluzole *versus* 50% on placebo (Mathew et al., 2010). Therefore, the trial was discontinued for futility.

A randomized, placebo-controlled, double-blind, phase II trial evaluated the efficacy and safety of riluzole (50–200 mg/day) in 94 participants diagnosed with bipolar depression for 8 weeks, and the main outcome measure was the mean change in MADRS score (NLM, NCT00376220). The results were posted on clinicaltrials.gov and did not support the superior efficacy of riluzole *versus* placebo (NLM, NCT00376220).

Another 8-week, open-label study of riluzole (50–200 mg/day) in combination with lithium recruited 14 acutely depressed bipolar patients (MADRS score ≥20) who first followed 4 weeks of lithium treatment (Zarate et al., 2013). The linear mixed model for total MADRS score showed a significant treatment effect at week 8, without cases of switch into hypomania or mania (Zarate et al., 2013).

An 8-week, double-blind, placebo-controlled, phase II trial evaluated the efficacy and safety of riluzole (50–200 mg/day) in 19 participants diagnosed with bipolar depression, but the study was terminated due to the superior efficacy of placebo in an interim analysis (NLM, NCT00054704).

Memantine is classified as an NMDA-receptor-open channel blocker because it can enter these channels and block current flow only after they are opened (Johnson and Kotermanski, 2006). A double-blind, placebo-controlled trial enrolled 32 patients diagnosed with MDD, randomized on memantine (5–20 mg/day) or placebo for 8 weeks (Zarate et al., 2006). The results of this trial did not support the efficacy of memantine based on the linear mixed models for total MADRS scores (Zarate et al., 2006). Another randomized, double-blind, placebo-controlled trial evaluated the efficacy of memantine (5–20 mg/day) as an add-on to antidepressant treatment in 31 participants with partial or non-responsive MDD for 8 weeks (Smith et al., 2013). No significant change in MADRS scores was detected in patients who received memantine *versus* those on placebo, either over the entire study or at study completion (Smith et al., 2013). A minimal-to-small effect size was observed, favoring placebo (d = 0.19) (Smith et al., 2013). No statistical differences were

observed between groups on secondary efficacy outcomes or safety outcomes (Smith et al., 2013).

A phase IV, randomized, placebo-controlled trial investigated the efficacy and safety of memantine (5–20 mg) augmentation administered for 8 weeks in 29 adult patients diagnosed with bipolar depression and incomplete response to lamotrigine (NLM, NCT00305578). The primary outcome was the change in HAMD-17 from baseline to week 8, and the posted results on clinicaltrials.gov show a decrease of 9 *versus* 7 points in patients treated with memantine *versus* placebo (NLM, NCT00305578). The most frequently reported adverse events in the memantine group were somnolence, indigestion, diarrhea, headache, and coughing (NLM, NCT00305578).

A double-blind, randomized, phase III trial evaluating the safety and effectiveness of memantine (5–20 mg/day) included three phases: during the first stage, adult outpatients with MDD without psychotic features (N = 112) have tapered off all psychiatric medications over 2 weeks (washout period); in the second phase, participants were randomized on memantine or placebo three times a day for 8 weeks; and participants who responded well to the treatment entered phase III, a 16-week continuation period of either memantine or placebo (NLM, NCT00040261). No results of this trial have been released.

However, another single-site, double-blind, placebo-controlled, parallel-group, phase IV trial enrolled 31 participants diagnosed with MDD and non-response or incomplete response to antidepressants were randomized on either memantine (5–20 mg/day) or placebo as an add-on for 8 weeks (NLM, NCT00344682). The main outcome was the change in MADRS scores at week 8, and the results posted on clinicaltrials.gov did not support a significant difference between groups (−7.13 *vs*. −7.25 points in memantine *vs*. placebo) (NLM, NCT00344682). The rate of serious adverse events was similar in the two groups (20 *vs*. 18.75% in memantine *vs*. placebo) (NLM, NCT00344682).

The blockade of metabotropic glutamate 2/3 (mGlu2/3) receptors is considered a potentially interesting approach in the treatment of MDD, based on several preclinical studies (Sanacora et al., 2008; Watanabe et al., 2021). **TP0473292 (TS-161)** is the prodrug of a novel mGlu2/3 receptor antagonist, investigated in trials for MDD treatment (Watanabe et al., 2021). In a first-in-human, randomized, double-blind, single ascending dose (15–400 mg) and 10-day-multiple-ascending dose (50–150 mg), phase I trial on healthy subjects (N = 70), the pharmacokinetic profile of TS-101 was described (Watanabe et al., 2021). The prodrug was extensively converted into its active metabolite, and plasma exposure to this metabolite increased with the dose administered (Watanabe et al., 2021). The investigational product penetrated the brain–blood barrier, and the most frequently reported adverse events were nausea, vomiting, and dizziness, with an exposure-related incidence (Watanabe et al., 2021). An ongoing, placebo-controlled, phase II study is dedicated to the evaluation of TS-161 efficacy in TRD, with the main outcome being the change from baseline to day 21 on MADRS total scores and an estimated enrollment of 25 participants (NLM, NCT04821271).

On the contrary, **decoglurant (RO4995819)**, a mGlu2/3 receptor negative allosteric modulator, failed in a phase II trial to exert any antidepressant or procognitive effects (Umbricht et al., 2020). During this 6-week, double-blind, multicenter, randomized trial, 357 participants diagnosed with MDD who did not respond to two adequate trials of an SSRI/SNRI received decoglurant 5 mg (N = 101), 15 mg (N = 102), or 30 mg (N = 55) daily, or placebo (N = 99) although their adherence was confirmed through positive drug levels (Umbricht et al., 2020). At week 6, no significant differences were observed between any active treatment arms and placebo in decreasing MADRS scores, in response, or in remission rates (Umbricht et al., 2020). No effects of decoglurant were observed on Cambridge Neuropsychological Test Automated Battery (CANTAB)—cognitive accuracy and cognitive speed composite scores (Umbricht et al., 2020). High placebo response was observed, which may impair the ability of this trial to detect an efficacy signal. Another phase II trial that was intended to evaluate the efficacy of decoglurant *versus* placebo as adjunctive therapy in patients with MDD and inadequate response to their ongoing antidepressant was withdrawn by the sponsor (NLM, NCT01733654).

Metabotropic glutamate type 5 (mGlu5) receptors are ubiquitously expressed throughout the brain, and their dysfunction is involved in the pathogenesis of several diseases, for example, Alzheimer's disease, Parkinson's disease, and MDD (NLM, NCT01145755). Despite the success of the negative allosteric mGlu5 receptor modulators in preclinical studies, no such agent has been associated with favorable results in clinical trials with MDD patients (NLM, NCT01145755). **Basimglurant (RG-7090, RO-4917523)** is a potent and selective mGlu5 receptor negative allosteric modulator with good oral availability and a long half-life, which allows for once-daily administration (Lindemann et al., 2015; Quiroz et al., 2016). It has antidepressant properties and anxiolytic-like and antinociceptive effects (Lindemann et al., 2015). In a phase IIb, multicenter, double-blind, randomized clinical trial, basimglurant MR (0.5 or 1.5 mg) was compared with placebo in 333 adult patients with MDD, as adjunctive to ongoing antidepressant medication (an SSRI or SNRI agent), for 6 weeks (Quiroz et al., 2016). No difference was observed in the primary outcome, MADRS change from baseline to the endpoint, between basimglurant MR and placebo (Quiroz et al., 2016). Secondary endpoints were modified by adjunctive basimglurant MR 1.5 mg daily, especially in patient-rated measures (Quiroz et al., 2016). The most frequently reported adverse event was dizziness, but it was of mild intensity and transient (Quiroz et al., 2016). Another phase I, single-center, randomized, multiple-ascending dose trial, evaluated the safety of basimglurant *versus* placebo (N = 56 participants with MDD or healthy subjects) (NLM, NCT02433093). No results of this trial were posted as of February 2022.

AZD2066 is a mGlu5 receptor antagonist that was assessed in a phase IIa, multicenter, randomized, double-blind, double-dummy, active (duloxetine), placebo-controlled, and parallel-group study on 131 patients diagnosed with MDD, and the results (posted on clinicaltrials.gov) were negative (MADRS

TABLE 2 | Sestrin modulators with antidepressant properties in the pipeline.

Authors	Methodology	Results	Clinical trial phase, trial identifier (if available)
NLM (2018g)	NV-5138 *vs.* placebo, SAD, DBRCT, N_1 = 48 (estimated) healthy volunteers and N_2 = 40 treatment-resistant MDD, 9 days	The primary outcome was the incidence of treatment-related AE. The results of this trial have not yet been disclosed	Phase I, NCT03606395
NLM (2021h)	NV-5138 *vs.* placebo, DBRCT, *N* = 40 (estimated), TRD patients, 5 weeks	The primary outcome measure was the efficacy measured by MADRS total score. This trial is ongoing	Phase II, NCT05066672

AE, adverse events; DBRCT, double-blind, randomized controlled trial; MADRS, Montgomery Asberg Depression Rating Scale; MDD, major depressive disorder; SAD, single ascending dose; TRD, treatment-resistant MDD.

total score change was the primary outcome) (Arsova et al., 2020; NLM, NCT01145755).

In conclusion, glutamate modulators are a promising class of antidepressant agents, although several molecules have failed in different stages of clinical development. The recent FDA approval of intranasal esketamine as an adjunctive agent for TRD is an argument in favor of glutamatergic neurotransmission importance in the pathophysiology of mood disorders.

Sestrin Modulators

Sestrins are small, stress-induced proteins with multiple roles; for example, they are involved in oxidative stress, DNA damage response, cell growth, metabolic homeostasis, and mTORC1 signaling (Sengupta et al., 2019). The inhibition of mTORC1 by sestrins 1 and 2 can be reversed by the influx of sufficient levels of amino acids, whereas sestrin 3 cannot be regulated by amino acids (Sengupta et al., 2019). Suppressed mTORC1 signaling has been suggested as a possible pathogenic mechanism in MDD, and NMDA receptor modulators such as ketamine are dependent upon the mTORC1 activation in brain areas responsible for mood, for example, the medial prefrontal cortex (Sengupta et al., 2019).

NV-5138 is a novel small molecule activator of mTORC1 signaling, both *in vivo* and *in vitro*, orally available, and can transiently activate mTORC1 in several peripheral tissues and the brain (Sengupta et al., 2019). The impact of NV-5138 on synaptic function and BDNF signaling is similar to ketamine, suggesting a shared mTORC1 signaling-mediated mechanism for their antidepressant effect (Kato et al., 2019). A single dose of NV-5138 produced a rapid and long-lasting antidepressant effect and rapidly reversed anhedonia caused by chronic stress exposure in animal models of depression (Kato et al., 2019). The antidepressant action of NV-5138 required BDNF release, as the behavioral responses were blocked by infusion of BDNF-neutralizing antibodies into the medial prefrontal cortex (Kato et al., 2019).

A randomized, placebo-controlled, phase I trial explored the effects of a single ascending dosage level of NV-5138 in healthy volunteers and a single dose of NV-5138 in subjects with TRD, but no results of this trial have yet been disclosed (NLM, NCT03606395).

Another placebo-controlled, randomized, phase II trial is planned to evaluate the efficacy and tolerability of NV-5138 in adults with TRD (estimated enrollment: 40 participants), with a monitoring period of 5 weeks, and MADRS change to baseline as the primary outcome measure (NLM, NCT05066672).

In conclusion, the modulation of sestrins as a pharmacodynamic substrate for a new class of antidepressants is still in the early phases of research (**Table 2**) and is supported mostly by animal studies.

Combinations of Pharmacological Agents

The combination of dextromethorphan and bupropion (AXS-05) is currently explored as an orally administered therapy for patients with MDD, based on the pharmacodynamic properties of an uncompetitive NMDA-glutamate antagonist, σ1 agonist, and nonselective serotonin reuptake inhibitor (dextromethorphan), and a selective norepinephrine/dopamine reuptake inhibitor with nicotinic acetylcholine receptor antagonist properties (bupropion) (Sakurai et al., 2022). Beside its antidepressant properties, bupropion is credited with the protection of dextromethorphan from rapid metabolization *via* CYP450 2D6 because of this antidepressant potent inhibition of these hepatic isoenzymes (Sakurai et al., 2022).

In a randomized, double-blind, active-controlled, multicenter phase II trial (ASCEND), 80 patients diagnosed with moderate-to-severe MDD were treated for 6 weeks with AXS-05 (45 mg dextromethorphan/105 mg bupropion twice daily) or bupropion (105 mg twice daily) (Axsome Therapeuticals, 2019). Change in MADRS score was the primary outcome, and the rate of remission and response was superior for the AXS-05 group at the end-point, with early separation from the bupropion-treated group (Axsome Therapeuticals, 2019). The pharmacological combination was safe and well-tolerated, with similar rates of adverse events in the AXS-05 and bupropion arms (Axsome Therapeuticals, 2019). In the AXS-05 group, the most frequent adverse events were nausea, dizziness, dry mouth, decreased appetite, and anxiety (Axsome Therapeuticals, 2019). No psychotomimetic effects, weight gain, or increased sexual dysfunction were reported in the AXS-05 group (Axsome Therapeuticals, 2019).

Another phase II, randomized, double-blind, placebo-controlled, relapse prevention, multicenter trial (MERIT) explored the efficacy of AXS-05 in patients with TRD (*N* = 44 participants, who presented ongoing symptoms of depression despite receiving treatment with two or more prior antidepressants during the current major depressive episode) (Axsome Therapeuticals, 2021). Patients who achieved stable

remission under AXS-05 in a previous trial (MADRS scores ≤ 12 at two or more consecutive visits, separated by at least 4 weeks) were randomized to continue the same treatment or to discontinue it and switch to placebo (Axsome Therapeuticals, 2021). AXS-05 met the primary endpoint by significantly delaying the time to relapse of depressive symptoms compared to placebo, with no relapse over ≥6 months (Axsome Therapeuticals, 2021). Also, AXS-05 met the key secondary endpoint of relapse prevention, according to the relapse rates during the double-blind period (Axsome Therapeuticals, 2021).

In a phase III randomized, double-blind, placebo-controlled, multicenter trial (GEMINI), 327 adult patients diagnosed with moderate-to-severe MDD were randomized to treatment with either AXS-05 or placebo once daily for the first 3 days and twice daily thereafter for a total of 6 weeks (Axsome Therapeuticals, 2020). AXS-05 demonstrated a significant reduction in patient-reported depressive symptoms, evaluated by QIDS-SR-16 and PGI-I (Patient Global Impression of Improvement), compared to placebo at week 6 (Axsome Therapeuticals, 2020). The response on QIDS-SR-16 total score (at least 50% improvement) was significantly greater for AXS-05 starting from week 1 and at every time point thereafter, with 53.4% of patients achieving response compared to 33% of placebo patients at week 6 (Axsome Therapeuticals, 2020). On the PGI-I, AXS-05 demonstrated efficacy *versus* placebo, with 47.2% of patients achieving the level of "very much" or "much" improvement *versus* 31.3% of placebo patients at week 6 (Axsome Therapeuticals, 2020). The evolution of symptoms measured with clinician-rated scales (i.e., MADRS and CGI-I) supported the favorable results observed on self-reported scales, the difference from placebo being consistent at week 6 (Axsome Therapeuticals, 2020). The most commonly reported adverse events associated with AXS-05 were dizziness, nausea, headache, diarrhea, somnolence, and dry mouth (Axsome Therapeuticals, 2020).

Another phase III, randomized, double-blind, active-controlled, multicenter trial (STRIDE-1) evaluated the efficacy of AXS-05 in TRD ($N = 312$ adult participants who had failed two or three prior treatments) but did not show a statistically significant difference between the investigational product (45 mg dextromethorphan/105 mg bupropion, twice daily) and active control (150 mg bupropion, twice daily) after 6 weeks, according to MADRS total score (Biospace, 2020). The secondary outcomes favored, however, AXS-05 *versus* active control, with significantly higher rates of remission from depression (defined by QIDS-SR-16 ≤ 5) at week 1 and at every time point thereafter (Biospace, 2020). Also, AXS-05 improved cognitive function (monitored by the Massachusetts General Hospital Cognitive and Physical Functioning Questionnaire, CPFQ) and reduced anxiety symptoms (Hamilton Anxiety Rating Scale, HAM-A) (Biospace, 2020).

The fixed-dose combination of dextromethorphan hydrobromide and quinidine (DXMQ) was created based on the CYP450 2D6 enzyme inhibition induced by quinidine and the previously mentioned pharmacodynamic properties of dextromethorphan, which recommend this combination as a potential antidepressant therapy (Murrough et al., 2017; Majeed et al., 2021). DXMQ was approved by FDA in 2010 for the treatment of the pseudobulbar affect (Murrough et al., 2017). In an open-label, phase IIa clinical trial examining the efficacy and tolerability of orally administered DXMQ (45 mg/10 mg every 12 h) in 20 patients with TRD during 10 weeks, the MADRS score (primary outcome) significantly decreased from baseline to endpoint (Murrough et al., 2017). The QIDS-SR score also decreased significantly during the DXMQ treatment, and the response and remission rates in the intent-to-treat sample were 45% and 35%, respectively (Murrough et al., 2017).

A retrospective chart review included depressed patients ($N = 77$) diagnosed with treatment-resistant bipolar disorder type II or NOS, who received treatment with DXMQ 20 mg/10 mg twice daily in addition to their current treatment (Kelly and Lieberman, 2014).On day 90, the CGI-I score was 1.66, and some patients improved their clinical status within 1–2 days after the beginning of DXMQ administration (Kelly and Lieberman, 2014). A significant number of patients ($N = 19$) discontinued treatment due to adverse events, mainly nausea (Kelly and Lieberman, 2014).

Deudextromethorphan/quinidine (AVP-786) combines d6-dextromethorphan and quinidine sulfate in an oral formulation, with deuterium in the dextromethorphan molecule, a heavier and stable isotope of hydrogen, in order to increase this drug's half-life (Gant, 2014). A phase II, multicenter, randomized, double-blind, placebo-controlled study assessed the efficacy, safety, and tolerability of DXMQ as adjunctive therapy in patients with MDD and inadequate response to antidepressant treatment ($N = 206$ participants) (NLM, NCT02153502). The primary outcome was the change in the MADRS score during the 10 weeks of the trial (NLM, NCT02153502). No results have been yet posted as of February 2022 (**Figure 4**).

The co-formulation of **buprenorphine and samidorphan (ALKS-5461)** associates a μ-opioid receptor partial agonist and κ-opioid receptor antagonist with potential antidepressant activity (buprenorphine) and a μ-opioid receptor antagonist with low intrinsic κ- and δ-receptor modulation (samidorphan), which is intended to decrease the risk of buprenorphine abuse and dependence (Thase et al., 2019).

A phase II, multicenter, randomized, double-blind, placebo-controlled, two-stage sequential parallel comparison trial enrolled adults with MDD who had an inadequate response to 1–2 courses of antidepressant treatment (Fava et al., 2016). Participants ($N = 142$) were randomized to adjunctive treatment with 2/2 mg BUP/SAM, 8/8 mg BUP/SAM, or placebo, and they were monitored using HAMD, MADRS, and the CGI-S for 4 weeks, then they followed a 1-week taper (Fava et al., 2016). Compared to the placebo group, significant improvements were reported in patients treated with 2/2 mg BUP/SAM across the three depression outcome measures, and evidence of improvement was also found in the 8/8 mg BUP/SAM group, but without achieving statistical significance (Fava et al., 2016). The overall tolerability was good, and there was no evidence of opioid withdrawal when treatment was discontinued (Fava et al., 2016).

In the FORWARD-3 trial, adult patients with MDD and inadequate response to antidepressant therapy ($N = 399$ participants in group 1 and 30 in group 2) were randomized in a double-blind manner to 2/2 mg bupropion/samidorphan

Operational criteria	Inclusion criteria	Exclusion criteria
Population	Selected population groups were allowed-adolescents and adults. No superior age limit was specified. The main diagnoses were major depressive disorder and bipolar depression. Treatment-resistant forms, first mood episodes, or chronic depression were included. Chronic organic co-morbidities were allowed. Diagnoses should be based on criteria specified by the authors of that paper/sponsors of the trial. Both ICD10 and DSM (IV, IV-TR, or 5) diagnosis criteria were allowed.	Studies that did not specify age limits for their samples, and studies that enrolled children. The presence of psychiatric comorbidities with significant impact on cognition, mood, behavior, and overall functionality (e.g., psychotic disorders, severe neurocognitive disorders, substance use disorders).
Intervention	Pharmacological, or combined, pharmacological and psychotherapeutic interventions. New investigational drugs, or repurposed drugs for antidepressant use were included.	Psychotherapy as monotherapy for MDD/bipolar depression. Already marketed antidepressants, FDA-approved for all the indications specified in the „population" section of this table. Investigational products with monoaminergic, orexinergic, GABA-ergic, neurosteroid and anti-inflammatory mechanisms of action were not allowed (they are part of the first section of this review, presented elsewhere).
Environment	Both in-patient and out-patient regimens.	Unspecified environment.
Primary and secondary variables	Evaluation of the efficacy, safety, and tolerability of new investigational drugs with antidepressant properties.	All research using unspecified variables. Reviews without pre-defined quantifiable objectives, or poorly defined primary outcome measures.
Study design	Any phase of clinical investigation, from I to III was admitted if it corresponded to the pre-defined objective of this review. Phase IV studies were permitted, if specific variables related to depression were included, for drugs not approved for this indication.	Studies with unspecified or poorly defined design. Studies with unclearly defined populations or statistical methods used. Case reports, case series.
Language	Any language of publication was admitted if the *in-extenso* published paper was available. For clinical trials identified in metadata repositories, the same language criteria were applied.	

FIGURE 4 | Inclusion and exclusion criteria.

(BUP/SAM) or placebo for 6 weeks (Zajecka et al., 2019). There were no differences in the MADRS-based response or remission rates between groups, and the least-square mean change in the MADRS total score at the end of treatment was not significantly different from placebo, although BUP/SAM did improve the overall depressive symptoms severity (Zajecka et al., 2019). Adverse events were mild or moderate in severity, and no evidence of abuse potential during treatment was detected (Zajecka et al., 2019).

Two global, multicenter, randomized, double-blind, placebo-controlled, sequential parallel-comparison design studies (FORWARD-4 and FORWARD-5) evaluated the safety and tolerability of 2/2 mg ALKS-5461 as an adjunctive treatment for MDD in adults who did not present an adequate response to antidepressant therapy (N_1 = 385 participants, and N_2 = 407 participants) (Fava et al., 2020). FORWARD-4 also evaluated a 0.5/0.5 mg dose and FORWARD-5 a 1/1 mg dose for 5 weeks during the first stage and 6 weeks during the second stage (Fava et al., 2020). FORWARD-5 achieved the primary endpoint because 2/2 mg BUP/SAM was superior to placebo, according to the MADRS total score and MADRS-6 (Bech) score change from baseline to the last visit (Fava et al., 2020). However, FORWARD-4 did not achieve the primary endpoint, although separate analyses showed significant differences between groups at other time points (Fava et al., 2020). The pooled analysis of these two trials demonstrated a greater reduction in MADRS total scores from baseline for 2/2 mg BUP/SAM *versus* placebo at multiple time points, including the last visit, and a significant average change from baseline to week 3 to the end of the study (Fava et al., 2020). The overall tolerability of BUP/SAM was good,

with most adverse events being of mild or moderate severity. There was minimal evidence of abuse and no evidence of dependence or opioid withdrawal by adverse events report or objective measures (Fava et al., 2020). FORWARD-1 was a phase III, randomized, double-blind trial that evaluated the safety and tolerability of two titration regimens for ALKS-5641 as adjunctive treatment in MDD adults with inadequate response to antidepressant therapy (N = 66 patients) (NLM, NCT02085135). No results were published, but according to the raw data presented on the clinicaltrial.gov archive, no SAE were recorded in either group, while 67.65% of the subjects who received 1-week titration and 87.5% of those with 2-week titration had adverse events during the 8 weeks of monitoring (NLM, NCT02085135).

FORWARD-2 was an open-label, 52-week study to evaluate the long-term safety and tolerability of BUP/SAM 2/2 mg as adjunctive therapy to ongoing antidepressant treatment for MDD patients unresponsive to prior antidepressant therapy (N = 1486 participants) (Thase et al., 2019).

Adverse events were reported by 75.7% of the patients, but the majority were of mild or moderate intensity (Thase et al., 2019). The most common adverse events were nausea, headache, constipation, and dizziness (Thase et al., 2019). Discontinuation due to adverse events was recorded in 10.4% of the cases, and SAE were reported in 3.2% of the patients (Thase et al., 2019). Following abrupt BUP/SAM discontinuation, the incidence of opioid withdrawal symptoms was low (6.5%) (Thase et al., 2019). Improvements in MADRS scores were maintained until the last visit, suggesting durability of antidepressant effect in patients receiving continuous treatment (Thase et al., 2019).

Another randomized, placebo-controlled, double-blind, phase IIIb trial evaluated the efficacy, safety, and tolerability of adjunctive ALKS-5461 in patients with treatment-refractory MDD (N = 278 participants) (NLM, NCT03188185). This study had a sequential parallel comparison design: in stage 1, subjects were randomized to ALKS-5461 or placebo, and in stage 2, only placebo non-responders from stage 1 were re-randomized to active drug or placebo (NLM, NCT03188185). The results posted on clinicaltrials.gov show non-significant differences between groups according to the main outcome measure, MADRS score (p = 0.128) (NLM, NCT03188185). The overall tolerability was good, with no SAE recorded in either stage of this trial, while the most reported adverse events within the ALKS-5461-treated patients were nausea, constipation, vomiting, fatigue, dizziness, somnolence, headache, and sedation (NLM, NCT03188185).

In conclusion, various formulations of combined pharmacological agents have been investigated for MDD or TRD, with positive results for AXS-05 (although a negative phase III trial also exists) and DXMQ (a single-phase IIa trial) but controversial results for ALKS-5461 (phase II and III trials) (**Table 3**).

Cholinergic Receptor Modulators

Pharmacological interventions targeting nicotinic receptors have been explored in multiple psychiatric disorders, for example, MDD, neurocognitive disorders, nicotine use disorder, or schizophrenia (Davidson et al., 2021). **JNJ-39393406** is an investigational product with properties of α7 nicotinic receptors selective positive allosteric modulator, and it can lower agonist and nicotine threshold for activation of these receptors 10–20-fold while increasing the maximum agonist response 17–20-fold (Davidson et al., 2021). In a randomized, double-blind, placebo-controlled, add-on to psychotropics, parallel-group trial, 71 patients diagnosed with MDD were monitored for 2 weeks (Davidson et al., 2021). The primary outcome measures were the Brief Assessment of Cognition in Schizophrenia (BACS) composite score and the MADRS scores (Davidson et al., 2021). No significant difference for the primary outcomes was detected at the end of the study, nor for the secondary outcomes (Davidson et al., 2021). The overall tolerability of JNJ-39393406 was good, without differences in the adverse events rate between active drug and placebo groups (Davidson et al., 2021).

Ropanicant (SUVN-911) is a potent α4β2 receptor ligand with oral bioavailability, good brain penetration, and marked antidepressant activity in animal models of depression (Nirogi et al., 2020). A phase I, single-center, open-label, single-dose study evaluated the effect of food, gender, and age on the safety and pharmacokinetic profile of SUVN-911, administered orally in healthy subjects (N = 28 participants), but results are not available (NLM, NCT03551288). Another phase I, double-blind, placebo-controlled, single-center clinical study explored the safety, tolerability, and pharmacokinetic profile of single and multiple doses of orally administered SUVN-911 or placebo to healthy male subjects (N = 64), but no results are available for this study either (NLM, NCT03155503).

Scopolamine is a competitive inhibitor of post-ganglionic muscarinic receptors for acetylcholine, and it acts as a nonselective muscarinic antagonist (Zhang et al., 2017). The effects of scopolamine hydrobromide administration (4 μg/kg i.v.) were evaluated in two trials, a double-blind, placebo-controlled, dose-finding study followed by a double-blind, placebo-controlled, crossover clinical trial (Furey and Drevets, 2006). Adult outpatients diagnosed with MDD or bipolar disorder (N = 19) received multiple sessions of i.v. infusions of placebo or scopolamine hydrobromide, and these sessions were 3–5 days apart (Furey and Drevets, 2006). Patients who received a placebo followed by scopolamine showed no significant change in the main outcomes (MADRS and HAMA scores) during the placebo phase, but significant reductions in both depression and anxiety rating scores were observed after scopolamine administration (Furey and Drevets, 2006). Patients who received scopolamine first and placebo second also showed significant reductions in depression and anxiety rating scale scores after scopolamine i.v., relative to baseline, and these effects persisted during the placebo phase (Furey and Drevets, 2006).

Outpatients with MDD (N = 23) were enrolled in a double-blind, placebo-controlled, crossover trial, and they were randomized into either a placebo-scopolamine or a scopolamine-placebo sequence (Drevets and Furey, 2010). Scopolamine was administered in 4 μg/kg i.v dose, in repeated

TABLE 3 | Combinations of pharmacological agents with antidepressant properties in the pipeline.

Authors	Methodology	Results	Clinical trial phase, trial identifier (if available)
NLM (2019b)	AXS-05 *vs*. bupropion, DBRCT, *N* = 80 moderate-to-severe MDD patients, 6 weeks	Change in the MADRS score was the primary outcome, and the rate of remission and response was superior for the AXS-05 group at the end-point, with early separation from the bupropion-treated group. The pharmacological combination was safe and well-tolerated. The most frequent treatment-related AE were nausea, dizziness, dry mouth, decreased appetite, and anxiety	Phase II, NCT03595579
NLM (2021i)	AXS-05 *vs*. placebo, DBRCT, *N* = 44, TRD patients, 52 weeks	AXS-05 met the primary endpoint by significantly delaying the time to relapse of depressive symptoms compared to placebo, with no relapse over at least 6 months Also, the active medication met the key secondary endpoint of relapse prevention	Phase II, NCT04608396
NLM (2020)	AXS-05 *vs*. placebo, DBRCT, *N* = 327 moderate-to-severe MDD patients, 6 weeks	AXS-05 significantly decreased patient-reported depressive symptoms, evaluated by QIDS-SR-16 and PGI-I, compared to placebo at week 6. The response on QIDS-SR-16 total score was significantly greater for AXS-05 starting from week 1 and at every time point thereafter, with 53.4% of patients achieving response compared to 33% of placebo patients at week 6	Phase III, NCT04019704
Biospace (2020)	AXS-05 *vs*. bupropion, DBRCT, *N* = 312 TRD patients, 6 weeks	The change in MADRS total score was not significantly different between the two groups. The secondary outcomes favored, however, AXS-05 *vs*. active control, with significantly higher rates of remission from depression at week 1 and at every time point thereafter. AXS-05 improved cognitive function and reduced anxiety symptoms	Phase III, NCT02741791
Murrough et al. (2017)	DXMQ, open-label, *N* = 20 TRD patients, 10 weeks	MADRS score (primary outcome) significantly decreased from baseline to endpoint. The QIDS-SR score also decreased significantly during DXMQ treatment, and the response and remission rates in the intent-to-treat sample were 45% and 35%, respectively	Phase IIa, NCT01882829
Kelly and Lieberman (2014)	DXMQ + ongoing antidepressant, retrospective, *N* = 77 treatment-resistant BD type II/NOS patients	On day 90, the CGI-I score was 1.66, and some patients improved their clinical status within 1–2 days after the beginning of DXMQ administration. An important number of patients (*N* = 19) discontinued treatment due to AE, mainly nausea	Phase N/A
NLM (2014a)	AVP-786 *vs*. placebo as adjunctive to current therapy, DBRCT, *N* = 206 MDD patients with inadequate response to antidepressants, 10 weeks	The primary outcome was MADRS total score. Undisclosed results	Phase II, NCT02153502
Fava et al. (2016)	ALKS-5461 *vs*. placebo, DBRCT, *N* = 142 MDD patients with inadequate response to antidepressant therapy, 5 weeks	Significant improvements were reported in patients treated with 2/2 mg BUP/SAM in HAMD, MADRS, and CGI-S, and evidence of improvement was also found in the 8/8 mg BUP/SAM group, but without achieving statistical significance. The overall tolerability was good	Phase II, NCT01500200
Zajecka et al. (2019)	ALKS-5461 *vs*. placebo as adjunctive therapy, *N* = 399 + 30, MDD patients with inadequate response to antidepressant treatment, 6 weeks	There were no differences in MADRS-based response or remission rates between groups, and the LSM change in MADRS total score at the end of treatment was not significantly different from placebo, although BUP/SAM did improve the overall depressive symptoms severity. Treatment-related AE were mild or moderate in severity	Phase III, NCT02158546
Fava et al. (2020)	ALKS-5461 as adjunctive therapy to ongoing antidepressant, *N* = 385, and 407 MDD patients, respectively, 5 + 6 weeks (two stages)	One of these trials achieved its primary outcome (MADRS total score and Bech-6 score change from baseline to week 6), while the other did not. The pooled analysis of these two trials demonstrated a greater reduction in MADRS total scores from baseline for 2/2 mg BUP/SAM *vs*. placebo from baseline at multiple time points, including the last visit, and a significant average change from baseline to week 3 to the end of study. The overall tolerability was good	Phase III, NCT02158533 Phase III, NCT02218008
NLM (2014b)	ALKS-5461 two titration doses adjunctive to ongoing treatment, *N* = 66 MDD patients with inadequate response to treatment, 8 weeks	No SAE were recorded in either group of titration, while 67.65% of the subjects who received 1-week titration and	Phase III, NCT02085135

(Continued on following page)

TABLE 3 | (*Continued*) Combinations of pharmacological agents with antidepressant properties in the pipeline.

Authors	Methodology	Results	Clinical trial phase, trial identifier (if available)
		87.5% of those with 2-week titration had AE during the study	
Thase et al. (2019)	ALKS-5461 as adjunctive therapy, open-label, *N* = 1486 MDD patients unresponsive to prior antidepressant therapy, 52 weeks	AE were reported by 75.7% of the patients, but the majority were of mild or moderate intensity. Discontinuation due to AE was recorded in 10.4% of the cases. SAE were reported in 3.2% of the patients. Improvements in MADRS scores were maintained until the last visit, suggesting durability of antidepressant effect in patients receiving continuous treatment	Phase III, NCT02141399
NLM (2017d)	ALKS-5461 *vs*. placebo adjunctive to current treatment, *N* = 278 TRD patients, 5 + 6 weeks (two stages)	Non-significant differences between groups were reported according to the main outcome measure, MADRS total score. The overall tolerability was good, with no SAE recorded in either stage of this trial	Phase IIIb, NCT03188185

AE, adverse events; DBRCT, double-blind, randomized controlled trial; DXMQ, dextromethorphan hydrobromide and quinidine; HAMD, Hamilton Depression Rating Scale; MADRS, Montgomery Asberg Depression Rating Scale; MDD, major depressive disorder; PGI-I, Patient Global Impression of Improvement; QIDS-SR, Quick Inventory of Depressive Symptomatology-Self-Report; SAE, severe adverse events; TRD, treatment-resistant MDD.

sessions, 3–5 days apart (Drevets and Furey, 2010). MADRS scores decreased by 32% in patients who received first scopolamine ($p < 0.001$) *versus* those who received the placebo first, and improvement was significant at the first evaluation that followed scopolamine administration (Drevets and Furey, 2010). Scopolamine administration was associated with drowsiness, blurred vision, dry mouth, lightheadedness, and reduced blood pressure, and no participant dropped out due to side effects (Drevets and Furey, 2010).

In a double-blind, randomized, controlled, phase IV trial, 14 MDD participants received either scopolamine 0.15 mg b.i.d and naltrexone 1 mg b.i.d for 4 weeks or placebo, and they were monitored for 4 weeks (NLM, NCT03386448). According to the results posted by the sponsor, the change of MADRS scores from baseline to the end of the study visit (the primary outcome) was significant in favor of the scopolamine and naltrexone group ($p = 0.03$), and the rate of adverse events in the active group was 25% (mainly nausea) *versus* 0% in the placebo group (NLM, NCT03386448).

A randomized, double-blind, placebo-controlled, phase II trial focused on the evaluation of i.v. scopolamine in patients with bipolar disorder who experience a depressive episode of at least moderate severity is ongoing as of April 2022 (NLM, NCT04211961). The main outcome is the change in the HAMD score at 2 weeks, and the recruitment target is 50 participants (NLM, NCT04211961).

A randomized, controlled trial had the objective of comparing the effects of ketamine + placebo, scopolamine + placebo, and ketamine + scopolamine in patients with treatment-resistant MDD, monitored for up to 4 months, but this study was withdrawn due to lack of funding (NLM, NCT01613820).

A randomized, placebo-controlled, phase II study evaluated the efficacy of i.v. scopolamine (4 μg/kg) in seven patients diagnosed with MDD who are receiving electroconvulsive therapy (ECT) (NLM, NCT01312844). According to the unpublished results posted on clinicaltrials.gov, scopolamine was not significantly superior to placebo in any of the primary outcomes: the change in the HAMD-17 scores was −17.5 *versus* −14.0 (scopolamine *vs*. placebo) at the time of ECT completion (about 2 weeks), the mean time to response for patients receiving ECT was 8.33 *vs*. 5.0 days, and the mean number of ECT sessions to achieve response/remission were 2.33 *versus* 2.5/10 *versus* 6.5 (scopolamine *vs*. placebo) (NLM, NCT01312844).

In another double-blind, placebo-controlled, phase IV clinical trial, 66 adult outpatients with severe MDD were randomized on 1) scopolamine 0.3 mg/ml i.m. in the morning and placebo i.m. in the afternoon, 2) scopolamine 0.3 mg/ml i.m. twice daily, or 3) placebo i.m. (0.9% saline) twice daily (NLM, NCT03131050). All patients also received 10 mg/day of escitalopram for 4 weeks of treatment (NLM, NCT03131050). No results of this trial have been yet released.

In conclusion, targeting cholinergic neurotransmission as a key mechanism for antidepressant activity is supported by several trials, but most of the research on this pharmacodynamic mechanism is still in its early phase (**Table 4**).

Other Agents

Animal studies suggest a possible role of microtubule-associated protein type-2 (MAP-2) as a target for the treatment of depressive disorders due to the association of this pathology with neuronal abnormalities in brain microtubule function, including changes in α-tubulin isoforms (Bianchi and Baulieu, 2012). The synthetic pregnenolone-derivative **MAP4343 (3β-methoxy-pregnenolone)** binds to MAP-2 *in vitro* and increases its ability to stimulate tubulin assembly (Bianchi and Baulieu, 2012). The effects of a single injection of MAP4343 and fluoxetine in naïve Sprague Dawley rats were compared, with positive results for the investigational product (Bianchi and Baulieu, 2012). The MAP4343 had efficacy in the rat forced swimming test, the most widely used model of depression, by decreasing immobility behavior (Bianchi and Baulieu, 2012). These antidepressant effects were accompanied by

TABLE 4 | Cholinergic receptor modulators and other agents with antidepressant properties in the pipeline.

Authors	Methodology	Results	Clinical trial phase, trial identifier (if available)
Cholinergic agents			
Davidson et al. (2021)	JNJ-39393406 *vs.* placebo as add-on, DBRCT, *N* = 71 MDD patients, 2 weeks	No significant differences between groups in BACS composite score or MADRS total score were reported at week 2. The overall tolerability was good	Phase II, NCT02677207
NLM (2018h)	Ropanicant (SUVN-911), open-label, single-dose study, *N* = 28 healthy subjects	Primary outcome measure is AUC. Results have not been yet disclosed	Phase I, NCT03551288
NLM (2017e)	SUVN-911 *vs.* placebo, single/multiple doses, DBRCT, *N* = 64 healthy male subjects	Primary outcome measures-ECG, vital signs, C-SSRS. Results have not yet been disclosed	Phase I, NCT03155503
Furey and Drevets (2006)	Scopolamine 4 μg/kg i.v. *vs.* placebo, two DBRCTs, *N* = 19 MDD/BD patients, repeated sessions 3–5 days apart	Patients who received placebo followed by scopolamine showed no significant change in the main outcomes (MADRS and HAMA scores) during the placebo phase, but significant reductions in both outcomes were observed after scopolamine administration. Patients who received scopolamine first and placebo second also showed significant reductions in depression and anxiety rating scale scores after scopolamine i.v., relative to baseline, and these effects persisted during the placebo phase	Phase N/A
Drevets and Furey (2010)	Scopolamine 4 μg/kg i.v. *vs.* placebo, DBRCT, *N* = 23 MDD outpatients, repeated sessions 3–5 days apart	MADRS scores decreased by 32% in patients who received first scopolamine ($p < 0.001$) *vs*. those who received the placebo first, and improvement was significant at the first evaluation that followed scopolamine administration. Scopolamine administration was associated with drowsiness, blurred vision, dry mouth, lightheadedness, and reduced blood pressure, and no participant dropped out due to side effects	Phase II, NCT00369915
NLM (2017f)	Scopolamine 0.15 mg b.i.d. + naltrexone 1 mg b.i.d. *vs.* placebo, DBRCT, *N* = 14 MDD patients, 4 weeks	Unpublished results support a significant change of the MADRS scores from baseline to the end of the study visit (the primary outcome) in favor of the scopolamine and naltrexone group ($p = 0.03$), and the rate of adverse events in the active group was 25% (mainly nausea) *vs*. 0% in the placebo group	Phase IV, NCT03386448
NLM (2019c)	Scopolamine i.v. *vs.* placebo, DBRCT, *N* = 50 (recruitment target), 2 weeks	The main outcome is the change in HAMD score at 2 weeks. The trial is ongoing	Phase II, NCT04211961
NLM (2012d)	Ketamine + placebo, scopolamine + placebo, and ketamine + scopolamine, DBRCT, *N* = 0 MDD participants, 4 months	This study was withdrawn due to a lack of funding	Phase N/A, NCT01613820
NLM (2011a)	Scopolamine 4 μg/kg i.v. *vs.* placebo + ECT, DBRCT, *N* = 7 MDD patients, 2 weeks	Unpublished results suggest that scopolamine was not significantly superior to placebo in any of the primary outcomes: the change in the HAMD-17 scores was −17.5 *vs*. −14.0 (scopolamine *vs*. placebo) at the time of ECT completion (about 2 weeks); the meantime for response for patients receiving ECT was 8.33 *vs*. 5.0 days, and the mean number of ECT sessions to achieve response/remission was 2.33 *vs*. 2.5/10 *vs*. 6.5 (scopolamine *vs*. placebo)	Phase II, NCT01312844
NLM (2017g)	Scopolamine 0.3 mg/ml or 0.6 mg/ml i.m. *vs.* placebo + escitalopram 10 mg/day, DBRCT, N = 66 outpatients with severe MDD, 4 weeks	No results of this trial have been yet released	Phase IV, NCT03131050
Other agents			
NLM (2019d)	MAP4343 *vs.* placebo, DBRCT, *N* = 110 (estimated), TRD patients, 42 days	The primary outcome measure is HAMD's total score change. The results of this trial have not yet been disclosed	Phase II, NCT03870776
Frye et al. (2000)	Gabapentin *vs.* lamotrigine *vs.* placebo, *N* = 31 treatment-resistant MDD and BD patients, 6 weeks	Response rates (based on CGI ratings of much or very much improved) were 26% for gabapentin, 52% for lamotrigine, and 23% for placebo. The overall tolerability of gabapentin was good	Phase N/A
Arnold et al. (2015)	Pregabalin *vs.* placebo + SSRI/SNRI, cross-over DBRCT, *N* = 197 fibromyalgia + depression patients, periods of 2 × 6 week	Pregabalin significantly improved HADS score, both anxiety and depression scale scores, Fibromyalgia Impact Questionnaire total score, but not EuroQoL 5-dimensions scores	Phase N/A
Timmers et al. (2018)	JNJ-54175446 *vs.* placebo, SAD, N = 77 healthy participants	AE were reported in 56% of the participants, and the most frequently reported was headache (18.6%)	Phase I, NCT02475148
NLM (2019d)	JNJ-54175446 *vs.* placebo, DBRCT, *N* = 142 (estimated), MDD patients with incomplete response to antidepressants, 8 weeks	The primary outcome measure is MADRS total score change. The trial is ongoing	Phase II, NCT04116606

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TABLE 4 | (*Continued*) Cholinergic receptor modulators and other agents with antidepressant properties in the pipeline.

Authors	Methodology	Results	Clinical trial phase, trial identifier (if available)
Cholinergic agents			
Brin et al. (2020)	OnabotulinumtoxinA (onabotA) 30 U/50U *vs.* placebo, DBRCT, *N* = 255 MDD patients, 24 weeks	Onabot 30U approached significance *vs*. placebo, according to the MADRS scores change, and reached significance at weeks 3 and 9, with secondary endpoints also reaching significance at several time points. The overall tolerability was good	Phase II, NCT02116361
NLM (2011b)	OnabotA (29–40 U) *vs.* placebo, DBRCT, *N* = 30 MDD patients, 6 weeks	Patients were monitored using HAMD-21, and the change in the active drug followed by placebo group was significant *vs*. placebo followed by active drug (−12.7 *vs*. −0.4) at 12 weeks ($p < 0.001$). No SAE were recorded in either group	Phase II, NCT01392963
Finzi and Rosenthal (2014)	OnabotA (29U/40U), *vs.* placebo, DBRCT, *N* = 85 MDD patients, 6 weeks	Response rates (based on MADRS scores) at 6 weeks from the injection date were 52% and 15% in the onabotA *vs*. placebo groups ($p < 0.001$). The remission rate (also based on MADRS score) was 27% *vs*. 7% in the onabotA *vs*. placebo	Phase IV, NCT01556971
NLM (2018i)	OnabotA *vs.* placebo, DBRCT, *N* = 58 (estimated), TRD patients, 6 weeks	The main outcome measure is the proportion of patients with improvement of depressive symptoms based on the MADRS scale at 6 weeks after injection. The trial is ongoing	Phase N/A, NCT03484754
NLM (2009b)	OnabotA (20–50 U), open-label, *N* = 50, MDD and non-depressed individuals, 12 weeks	BDI score change was −14.9 in the MDD group at week 12 *vs*. −2.7 in the healthy volunteers, while the self-esteem improved by three points on RSES *vs*. −0.4 in the healthy participants at the endpoint. The quality of life (WHOQOL-BREF) improved in the MDD group with 0.5 points at week 12 compared to baseline, and 0.2 points in the comparator group	Phase IV, NCT01004042
Monti et al. (2019)	PH-10 low-dose/high-dose *vs.* placebo, DBRCT, *N* = 30 MDD patients, 9 weeks	HAMD-17 total score change at endpoint *vs*. baseline showed a trend for difference ($p = 0.07$), with a greater reduction of depression severity scores for high dose PH10 *vs*. placebo. The positive effects of PH10 were recorded from week 1 for the high dose ($p = 0.03$). No SAE were reported, and the overall tolerability was good	Phase IIa
Binneman et al. (2008)	CP-316,311 *vs.* placebo *vs.* sertraline, DBRCT, *N* = 167 recurrent MDD patients, 6 weeks	The change from baseline in the HAMD score at the final visit was not significantly different between the investigational product group and placebo group, although sertraline did differentiate itself from the placebo	Phase II, NCT00143091
Zobel et al. (2000)	R121919, open-label, *N* = 24 MDD patients, 30 days	The drug was safe and well-tolerated within the 30-day observation period. It induced reductions in depression and anxiety scores using clinician-rated and patient-scored instruments (HAMD, BDI, HAMA, CGI, STAI)	Phase IIa

AE, adverse events; BD, bipolar depression; BDI, Beck Depression Inventory; CGI, Clinical Global Impression; DBRCT, double-blind, randomized controlled trial; ECT, electroconvulsive therapy; HADS, Hospital Anxiety and Depression Scale; HAMA, Hamilton Anxiety Rating Scale; HAMD, Hamilton Depression Rating Scale; MADRS, Montgomery Asberg Depression Rating Scale; MDD, major depressive disorder; PGI-I, Patient Global Impression of Improvement; QIDS-SR, Quick Inventory of Depressive Symptomatology-Self-Report; RSES, Rosenberg Self-Esteem Scale; SAD, single ascending dose; SAE, severe adverse events; SNRI, serotonin and norepinephrine reuptake inhibitor; SSRI, selective serotonin reuptake inhibitors; STAI, State-Trait Anxiety Inventory; TRD, treatment-resistant MDD; WHOQOL-BREF, World Health Organization Quality of Life-BREF.

modifications of α-tubulin isoforms in the hippocampus, amygdala, and prefrontal cortex (Bianchi and Baulieu, 2012). A phase II, double-blind, randomized, placebo-controlled, parallel, multicentric trial was planned to enroll 110 adult patients with TRD (NLM, NCT03870776).

Gabapentinoids modulate α2δ subunits of voltage-gated calcium channels and have been explored in numerous psychiatric disorders, MDD included (Vasiliu et al., 2017). A double-blind, randomized, crossover, placebo-controlled study compared the efficacy of gabapentin and lamotrigine monotherapy *versus* placebo in 31 patients with refractory unipolar or bipolar mood disorders who were monitored for 6 weeks (Frye et al., 2000). Response rates (based on CGI ratings of much or very much improved) were 26% for gabapentin, 52% for lamotrigine, and 23% for placebo, with a Cochran's Q *post hoc* difference between gabapentin and placebo of 0.08 ($p = 0.70$) (Frye et al., 2000). The overall tolerability of gabapentin was good (Frye et al., 2000).

A randomized, placebo-controlled, double-blind, 2-period, 2-way crossover study was composed of two 6-week treatment periods, separated by 2 weeks of taper/wash-out phases, and recruited 197 patients diagnosed with fibromyalgia taking a stable dose of SSRI or serotonin and norepinephrine reuptake inhibitor (SNRI) for comorbid depression (Arnold et al., 2015). These patients were randomized on pregabalin/placebo or placebo/pregabalin (300–450 mg/day) as an adjuvant to the

current antidepressant treatment (Arnold et al., 2015). Pregabalin significantly improved Hospital Anxiety Depression Scale (HADS) score, both anxiety and depression scales scores, and Fibromyalgia Impact Questionnaire total score but not EuroQoL 5-dimensions scores (Arnold et al., 2015).

Microglial cells within the central nervous system are presumedly involved in the neuroinflammation that has been associated with multiple neuropsychiatric disorders (Bhattacharya and Ceusters, 2020). Neuroinflammatory drug targets on microglia cells within the central nervous system have been of interest, especially in the last decades. **JNJ-54175446** is a brain penetrant-P2X7 antagonist agent which targets an ATP-activated ion channel, abundantly expressed on microglia and peripheral immune cells (Bhattacharya and Ceusters, 2020). In a first-in-human, placebo-controlled, single ascending dose study, JNJ-54175446 demonstrated in healthy participants (N = 77) dose-dependent increases in plasma exposure, cerebrospinal fluid exposure, and *ex vivo* inhibition of IL-1β from human blood (Timmers et al., 2018). Adverse events were reported in 56% of the participants, of which the most frequently reported was headache (18.6%) (Timmers et al., 2018). A phase II, randomized, placebo-controlled, double-blind trial of the antidepressant efficacy of JNJ-54165446 in patients with MDD and incomplete response to monoaminergic antidepressants is currently ongoing, and as the main outcome, it has measured the change in the MADRS score from baseline to week 8 (NLM., NCT04116606).

Another approach to MDD treatment involves nonsystemic options, with the main benefit of avoiding the onset of adverse events and potentially dangerous pharmacokinetic interactions frequently associated with orally administered classical antidepressants. Local injections of **onabotulinumtoxinA (onabotA, BOTOX)** may determine muscle relaxation through a complex process involving the cleaving of SNAP-25 (synaptosomal-associated protein-25 kD) (Brin et al., 2020). The consequence of this process is a lack of neurotransmitter content released from the vesicles in the synaptic cleft, including acetylcholine from motor neurons (Brin et al., 2020).

A 24-week multicenter, randomized, double-blind, placebo-controlled, two-cohort, parallel-group, phase II clinical trial evaluated the effects of 30 and 50 U onabotA in outpatients female patients (N = 255) with MDD (Brin et al., 2020). The investigational product or placebo was divided into six or eight glabellar injections (Brin et al., 2020). Onabot 30U approached significance *versus* placebo, according to the MADRS scores change, and reached significance at weeks 3 and 9, with secondary endpoints also reaching significance at several time points (Brin et al., 2020). Onabot 50U did not separate at week 6 from placebo in any variables (Brin et al., 2020). The overall tolerability was good, and the most commonly reported adverse events (5% in either of the active treatment groups) were headache, upper respiratory infections, and eyelid ptosis (Brin et al., 2020). OnabotA 30U administered in a single injection during a unique session had a consistent efficacy signal across multiple depression symptom scales for at least 12 weeks (Brin et al., 2020).

In a phase II, randomized, double-blind, cross-over trial, 30 patients diagnosed with MDD received an injection of clostridium botulinum toxin type A neurotoxin complex (29–40 U total injection) in the glabella region or placebo, and after 3 months, they received a placebo injection or Botox to the same region (NLM, NCT01392963). Patients were monitored using HAMD-21, and the change in the active drug followed by placebo group was significant *versus* placebo followed by active drug (−12.7 *vs*. −0.4) at 12 weeks (p < 0.001) (NLM, NCT01392963). No SAE were recorded in either group (NLM, NCT01392963).

In a phase IV, randomized trial, a single dose of onambotulinumtoxinA (29 U for females or 40 U for males) or placebo injections was administered into corrugator and procerus frown muscles in 85 patients diagnosed with MDD (Finzi and Rosenthal, 2014). Response rates (MADRS scores decreased by≥50%) at 6 weeks from the injection date were 52% and 15% in the onabotA *versus* placebo groups (p < 0.001) (Finzi and Rosenthal, 2014). The remission rate (MADRS score < 10) was 27% *versus* 7% in the onabotA *versus* placebo (Finzi and Rosenthal, 2014).

Another ongoing trial is investigating the effect of onabotA injection in the corrugator and procerus muscle in patients with TRD in comparison to the infiltration in the crow's feet area, in addition to the current antidepressant treatment (NLM, NCT03484754). The estimated enrollment is 58 participants, and the main outcome measure is the proportion of patients with improvement of depressive symptoms based on the MADRS scale at 6 weeks after injection (NLM, NCT03484754).

Quality of life, depressive symptoms, and self-esteem were the main outcomes in a phase IV, non-randomized, open-label trial, which enrolled 50 patients diagnosed with MDD *versus* non-depressed subjects who received onabotA injections of 20–40 U in five points of the glabellar area (NLM, NCT01004042). The change in BDI scores was −14.9 in the MDD group at week 12 *versus* −2.7 in the healthy volunteers, while the self-esteem improved by three points on the Rosenberg Self-Esteem Scale *versus* −0.4 in the healthy participants at endpoint (NLM, NCT01004042). The quality of life (WHOQOL-BREF score) improved in the MDD group with 0.5 points at week 12 compared to baseline and 0.2 points in the comparator group (NLM, NCT01004042).

PH-10 is a synthetic, odorless neuroactive steroid from the family of pherines, formulated for intranasal administration (Monti et al., 2019). It engages nasal chemosensory receptors and modulates neural circuits in the limbic amygdala and other basal forebrain structures, inducing antidepressant-like effects (Monti et al., 2019). In a single site exploratory phase IIa study, 30 patients with MDD were randomized to 8 weeks of self-administered intranasal PH10 low dose (3.2 μg), high dose (6.4 μg), or placebo (Monti et al., 2019). The analysis of HAMD-17 changes at endpoint *versus* baseline showed a trend for difference (p = 0.07), with a greater reduction of depression severity scores for high dose PH10 *versus* placebo (Monti et al., 2019). The positive effects of PH10 were recorded starting from week 1 for the high dose (p = 0.03) (Monti et al., 2019). No serious adverse effects were reported, and the overall tolerability was good (Monti et al., 2019).

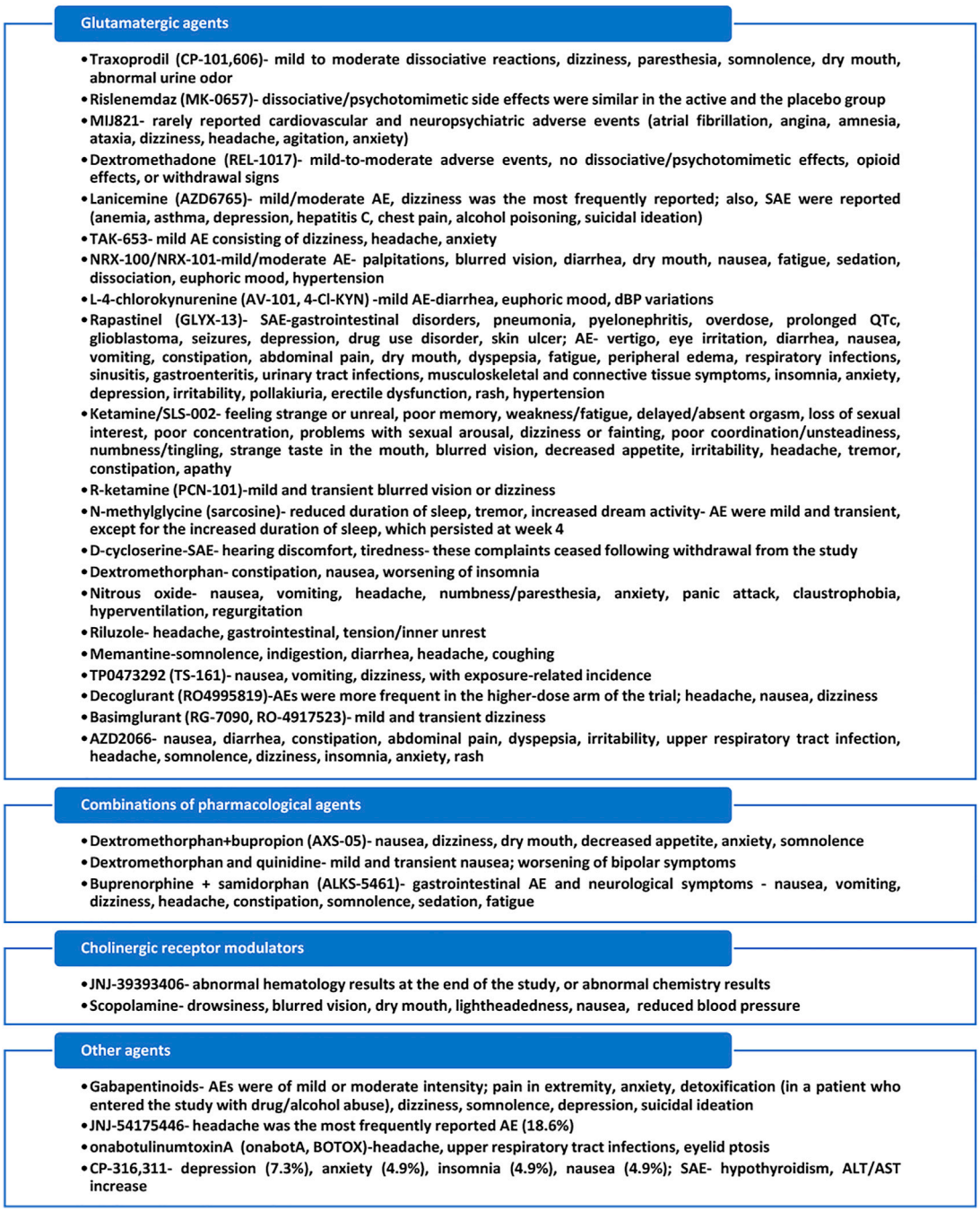

FIGURE 5 | Main adverse events identified in clinical trials exploring new antidepressant agents. AE, adverse events; dBP, diastolic blood pressure; SAE, severe adverse events. Based on data from Preskorn et al. (2008); Ibrahim et al. (2012); Ghaemi et al. (2021); Fava et al. (2022); Sanacora et al. (2017); O'Donnell et al. (2021); NLM, NCT02974010; Park et al. (2020); NLM, NCT02192099; Lapidus et al. (2014); Leal et al. (2021); Huang et al. (2013); Heresco-Levy et al. (2013); Majeed et al. (2021); Nagele et al. (2015); Zarate et al. (2004); NLM, NCT00305578; Umbricht et al. (2020); Quiroz et al. (2016); NLM, NCT01145755; Axsome Therapeuticals (2019); Axsome Therapeuticals (2020); Kelly and Lieberman (2014); Fava et al. (2016); NLM, NCT03188185; Davidson et al. (2021); Drevets and Furey (2010); NLM, NCT03386448; Arnold et al. (2015); Timmers et al. (2018); Brin et al. (2020); Binneman et al. (2008).

Dysregulation of the hypothalamic-pituitary-adrenal (HPA) axis has been suggested as an important pathogenetic mechanism in MDD, but up to date, there are no drugs targeting this system approved for use in patients with mood disorders (Menke, 2019).

CRH-1 receptor antagonists have been investigated in patients with MDD or anxiety disorders without significant results, but several authors consider that a more homogenous group of participants in clinical trials (i.e., those with significant CRH signaling dysfunction)

may help detect a signal for these molecules (Menke, 2019). A phase II, randomized, parallel assignment, 6-week, fixed-dose, double-blind, double-dummy, placebo and sertraline controlled, multicenter trial evaluated the safety and efficacy of CP-316,311 (a selective CRH-1 antagonist) in outpatients with recurrent MDD (N = 167 participants) (Binneman et al., 2008). The efficacy of 400 mg of CP-316,311 twice daily was compared with 100 mg sertraline daily or placebo, and the interim analysis led to the trial termination (Binneman et al., 2008). The change from baseline in the HAMD score at the final visit was not significantly different between the investigational product group and placebo group, although sertraline did differentiate itself from the placebo (Binneman et al., 2008).

Another high-affinity, CRH-1 receptor antagonist, R121919, was investigated in a clinical trial (N = 24 patients with MDD), and it was safe and well-tolerated within the 30-day observation period (Zobel et al., 2000). The CRH-1 blockade did not impair the baseline corticotropin/cortisol activity, and it did not have such an effect following an exogenous CRH challenge (Zobel et al., 2000). R121919 induced reductions in depression and anxiety scores using clinician-rated and patient-scored instruments (Zobel et al., 2000).

In conclusion, these molecules with distinct pharmacodynamic properties indicate, besides the complexity of the MDD pathogenesis, the need for further exploration of different central and peripheric ways (e.g., steroid hormones, ion channel modulators, or locally administered exoproteins) to decrease the severity of depressive symptoms (**Table 4**).

A synthesis of the safety and tolerability data available for investigational products reviewed in this study is presented in **Figure 5**.

CONCLUSION

Multiple non-monoaminergic pathways are considered of interest in clinical research in the treatment of depressive disorders. Glutamatergic agents are by far the most extensively researched, and several sub-categories have been identified: antagonists of GluN2B subunits of NMDA receptors (seven investigational products), NMDA-receptor antagonists (14 agents), AMPA receptor potentiators (two agents), and metabotropic receptor antagonists (four agents). These agents were investigated in phases I–III of clinical trials. One sestrin modulator is investigated in phases I and II of clinical trials. Combinations of pharmacological agents (i.e., AXS-05, DXMQ, AVP-786, and ALKS-5461) are investigated for their antidepressant properties in phases II and III of clinical trials. Two cholinergic agents (i.e., JNJ-39393406 and SUVN-911) are explored in phases I and Ii of clinical trials, but phase Ii trials have undisclosed results. Other agents (i.e., MAP4343, gabapentin, pregabalin, JNJ-54175446, onabotA, PH10, CP-316, 311, and R1219191) have been studied in phases I–IV clinical trials. In conclusion, there is an abundance of investigational products that reached phases II and III of clinical research, although it is too early to formulate a prognosis if any of these agents will be approved any time soon for MDD or bipolar depression.

Limitations of this review refer to the possible exclusion of antidepressants in the pipeline due to the pre-formulated criteria of this search and the lack of availability of relevant data regarding the current status of investigation for these products in the explored references.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

As the only author, I assume the entire responsibility for collecting, processing, and presenting data within this review.

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Magdalena Sowa-Kucma, University of Rzeszow, Poland

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César Augusto Brüning, Federal University of Pelotas, Brazil
Mojtaba Madadi Asl, Institute for Research in Fundamental Sciences (IPM), Iran

***Correspondence:**
Bingjin Li
libingjin@jlu.edu.cn

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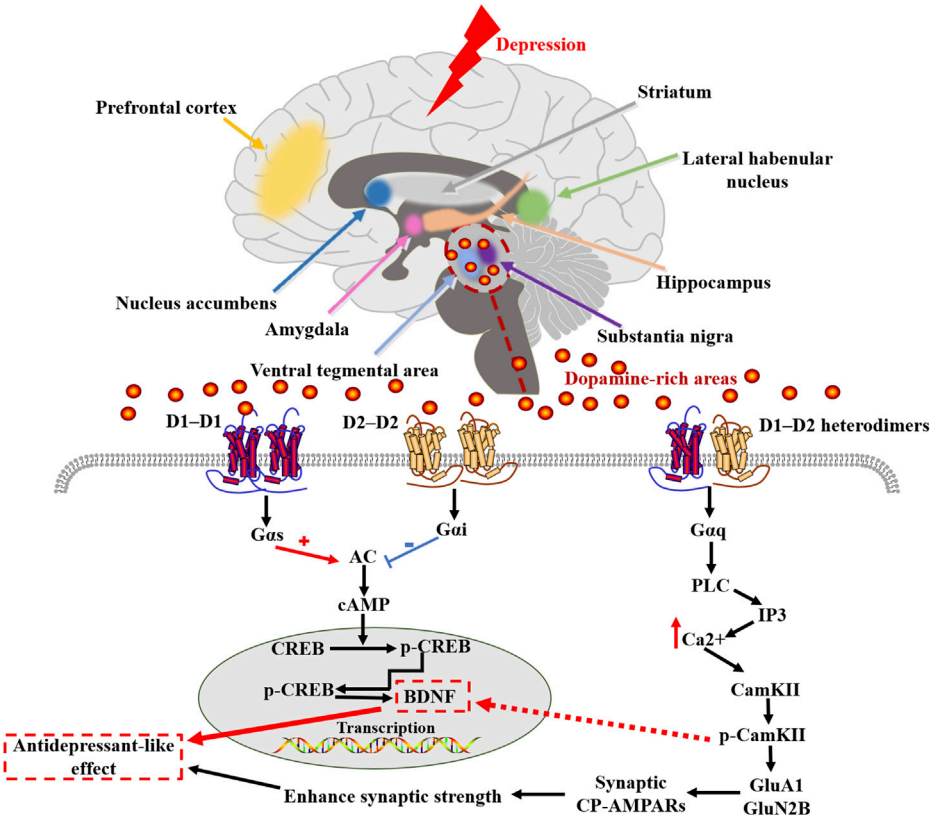
Dopamine Receptors: Is It Possible to Become a Therapeutic Target for Depression?

*Fangyi Zhao[1,2], Ziqian Cheng[1,2], Jingjing Piao[1,2], Ranji Cui[1,2] and Bingjin Li[1,2]**

[1]Jilin Provincial Key Laboratory on Molecular and Chemical Genetic, The Second Hospital of Jilin University, Changchun, China, [2]Engineering Laboratory for Screening of Antidepressant Drugs, Jilin Province Development and Reform Commission, Changchun, China

Dopamine and its receptors are currently recognized targets for the treatment of several neuropsychiatric disorders, including Parkinson's disease, schizophrenia, some drug use addictions, as well as depression. Dopamine receptors are widely distributed in various regions of the brain, but their role and exact contribution to neuropsychiatric diseases has not yet been thoroughly studied. Based on the types of dopamine receptors and their distribution in different brain regions, this paper reviews the current research status of the molecular, cellular and circuit mechanisms of dopamine and its receptors involved in depression. Multiple lines of investigation of these mechanisms provide a new future direction for understanding the etiology and treatment of depression and potential new targets for antidepressant treatments.

Keywords: dopamine receptors, pathogenesis of depression, neural circuits, signaling pathway, brain regions



GRAPHICAL ABSTRACT | The pathogenesis of depression cannot be separated from the involvement of various brain regions. Dopamine and its dopamine receptors are widely distributed in various regions of the brain, but the role and exact mechanisms of dopamine receptors in psychiatric disorders such as depression have not been thoroughly investigated. In this paper, we review the current status of research on the molecular pathways and mechanisms of dopamine receptor involvement in depression from the perspective of the types of dopamine receptors and their distribution in different regions of the brain, including the progress of research on the role of dopamine receptor D1-D2 heterodimers, which provides potential new targets for antidepressant drug therapy.

INTRODUCTION

Depression is one of the most common chronic psychiatric disorders with a high morbidity and recurrence rate, which is mainly characterized by low mood, cognitive dysfunction, the inability to experience pleasure from normally rewarding stimuli (anhedonia), despair, sleep disturbance, etc. (Malhi and Mann, 2018; Rehm and Shield, 2019; Rice et al., 2019). According to the World Health Organization, by 2030, depression will be the leading cause of disability worldwide (Mathers and Loncar, 2006; Brhlikova et al., 2011). Depression is a public health problem that needs to be addressed urgently, but its etiology and pathophysiology remain to be fully understood despite many advances in the understanding of this disease (Fox and Lobo, 2019). Several hypotheses have been proposed to explain the causes of depression. These include the monoamine hypothesis (Schildkraut, 1965), the monoamine receptor hypothesis (Sulser et al., 1978; Charney et al., 1981), inflammation hypothesis (Normann and Cornelius, 1978), neural immunity hypothesis (Andreoli et al., 1989), neurotrophic factor hypothesis (Altar, 1999), hypothalamic-pituitary-adrenal (HPA) axis hypothesis (Kathol et al., 1989; Nestler et al., 2002), neurogenesis hypothesis (Malberg et al., 2000), neuronal and synaptic plasticity hypothesis (Schinder and Poo, 2000) as well as neural circuit hypothesis (López et al., 1999). Each hypothesis is related to specific cellular pathways and mechanisms of interaction between parts of the neural circuits underlying emotional, motivational, mnemonic, and cognitive deficits in depression (Peng et al., 2015). There is a certain degree of evidence for cellular and circuit level changes underlying each of these hypotheses, but many questions remain unanswered. The monoamine neurotransmitter hypothesis is the most widely studied hypothesis at present, which suggests that the occurrence of depression is related to reduced function of the monoamine neurotransmitters such as norepinephrine (NE) and serotonin (5-HT), and in more modern conceptions of the hypothesis, also dopamine (DA) (Nutt, 2008). Currently, the first-line drugs clinically used for the treatment of depression are all developed under the classical monoamine hypothesis, among which the most common antidepressants include tricyclic antidepressants (TCAs), selective 5-HT reuptake inhibitors (SSRIs), dual 5-HT and NE reuptake inhibitors

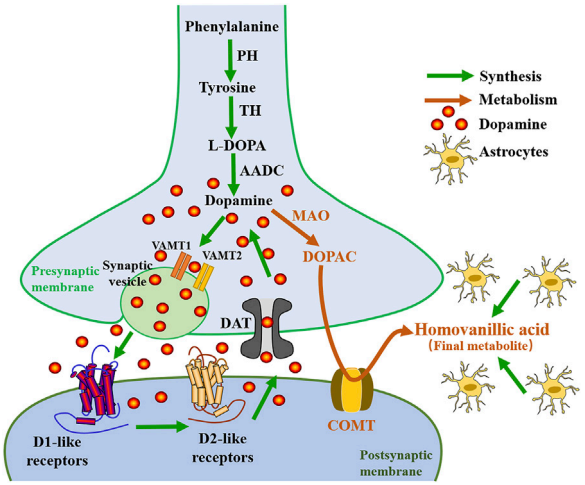


FIGURE1 | Diagram of the relationship between the synthesis and metabolism of dopamine. PH, phenylalanine hydroxylase; TH, tyrosine hydroxylase; l-DOPA, levo-dopa; AADC, aromatic L-amino acid decarboxylase; VMAT, vesicular monoamine transporter; LTP, Long-term potentiation; MAO, monoamine oxidase; DOPAC, 3,4-dihydroxyphenylacetic acid; COMT, catechol-o-methyltransferase.

(SNRIs), noradrenergic and specific serotonergic antidepressants (NaSSA), and other types (Schatzberg, 1998; Qin et al., 2014; McCormack, 2015; Blier, 2016). Nevertheless, these antidepressants generally have many disadvantages, such as slow onset, high rates of side effects, high recurrence rate, a high rate of interactions with other drugs, heterogeneous therapeutic responses, and other limitations (Peretti et al., 2000). These limitations suggest the need for additional research on the pathogenesis of depression to develop new antidepressant drugs based on a better understanding of the underlying mechanisms. One approach in this search for additional mechanisms is to study the receptors that specifically bind to the monoamine neurotransmitters.

In recent years, clinical research has shown that the function of the DA systems and DA receptors is involved in depression (David et al., 2020). This is not surprising because DA affects a variety of functions relevant to depression: emotion, perception, behavior, and motivation. However, recent research has shown that DA receptors and their heterodimers play a crucial role in the communication and connection of various neural circuits that may be involved in depression. Previously, DA receptors were thought to be primarily monomeric receptors, but now there is increasing evidence that multiple DA receptors can exist in the form of oligomers, forming homomeric and heteromeric receptor complexes, including ionotropic glutamate receptors (George et al., 2014; Guitart et al., 2014; Marsango et al., 2015). Some of these interactions may be regulated through signal transduction mechanisms, particularly through adenylate cyclase (AC) and cyclic adenosine monophosphate (cAMP) signaling (Beaulieu et al., 2015; Yeom et al., 2020). In recent studies, these complexes have been suggested to be a new direction for the study of the etiology of depression (Hasbi et al., 2020b; Hasbi et al., 2020c; Noori et al., 2020). Different DA receptor subtypes act on different neuronal pathways, which are likely to be the focus of research on the pathogenesis of depression (de Kwaasteniet et al., 2014).

THE SYNTHESIS AND METABOLISM OF DOPAMINE AND ITS RELATIONSHIP WITH DEPRESSION

Several animal models of depression are associated with decreased dopaminergic (DAergic) activity and anhedonia-like behavior. Anhedonia is thought to be a core feature of depression, and DA plays a key role in the perception of pleasure and reward, as well as motivated behavior, so it is important to discuss the normal roles of DA in behavior before exploring the relationship between DA receptors and depression. DA is the most abundant catecholamine neurotransmitter in the brain (Drozak and Bryla, 2005), which plays an important role in regulating rapid glutamate- and gamma-aminobutyric acid (GABA) -mediated neurotransmission in many brain regions, and is involved in many physiological and behavioral processes, including aspects of reward valuation and motivation, motor control and behavioral selection, attention and certain aspects of cognition, and some types of hormone secretion (Gainetdinov et al., 2002). There are five DAergic receptor subtypes in the G protein-coupled receptor (GPCR) superfamily (D1R, D2R, D3R, D4R, and D5R) (Gurevich et al., 2016). DA is synthesized directly from tyrosine by the enzyme tyrosine hydroxylase (TH), or indirectly from the essential amino acid phenylalanine (Franco et al., 2021), which is transformed into tyrosine by phenylalanine hydroxylase (PH) (Klein et al., 2019). As shown in **Figure 1**, when tyrosine enters the neuron, it is transformed into L-3,4-dihydroxyphenyl-L-alanine (l-DOPA) catalyzed by TH in the cytoplasm. l-DOPA is absorbed by large neutral amino-acid (LNAA) transporters and decarboxylated to DA by aromatic L-amino acid decarboxylase (AADC) present in neurons and glial cells. Studies have shown that l-DOPA plays a neuroprotective role on DAergic neurons through astrocytes (Asanuma and Miyazaki, 2016). As a metabolic precursor of DA, l-DOPA plays an important role in DAergic neurotransmission. Acute l-DOPA treatment enhances the transmission of DA in the substantia nigra and is one of the standard treatments for Parkinson's disease (Cao et al., 2020). In addition, clinical studies have shown that l-DOPA improves the cognitive processing and gait speeds of elderly patients with depression (Rutherford et al., 2019). These improvements were associated with reduced binding of labeled raclopride in selected striatal subregions, indicative of increased DAergic neurotransmission. DA and glutamate released by midbrain DA neurons have different properties, which are reflected in different synaptic vesicle mechanisms (Silm et al., 2019). Vesicular monoamine transporters (VMAT) mediate the packaging and storage of the monoamines (5-HT, DA, histamine, adrenaline, and NE) (Yaffe et al., 2018). VMAT is responsible for the transport of cytoplasmic monoamines into synaptic vesicles for storage and subsequent extracellular release in the CNS (central nervous system) (Wimalasena, 2011). VMAT is responsible for the packaging and transport of

TABLE1 | Classification, function, and localization of dopamine receptors in the brain as well as the relevant signaling pathway.

	Dopamine receptor subtypes				
	D1-like receptor		D2-like receptor		
	D1 receptor	D5 receptor	D2 receptor	D3 receptor	D4 receptor
Second messenger effect	Increase AC	Increase AC	Decrease AC	Decrease AC	Decrease AC
Cognate G protein	Gαs/olf	Gαs/olf	Gαi/o	Gαi/o	Gαi/o
cAMP production	Stimulate	Stimulate	Inhibit	Inhibit	Inhibit
Localization	Striatum	Ventral tegmental area	Nucleus accumbens	Olfactory nodules	Prefrontal cortex
	Nucleus accumbens	Striatum	Olfactory nodules	Nucleus accumbens	Anterior motor cortex
	Prefrontal cortex	Thalamus	Striatum	Striatum	Cingulate cortex
	Substantia nigra	Hippocampus	Islands of Calleja	Amygdala	Substantia nigra
	Amygdala	Olfactory nodule	Substantia nigra	Hypothalamus	Hypothalamus
	Hippocampus	Substantia nigra	Ventral tegmental area	Islands of Calleja	Hippocampus
	Thalamus		Hippocampus	Ventral tegmental area	Caudate nucleus
			Pituitary	Basal ganglia	Nucleus accumbens
				Prefrontal cortex	Ventral tegmental area
Relevant pathway	cAMP/PKA signaling	cAMP/PKA signaling	cAMP/PKA signaling	cAMP/PKA signaling	cAMP/PKA signaling
	DARPP-32 signaling	DARPP-32 signaling	DARPP-32 signaling	DARPP-32 signaling	DARPP-32 signaling
	ERK signaling	ERK signaling	GSK-3β signaling	GSK-3β signaling	GSK-3β signaling
	MAPK signaling	MAPK signaling	PI3K/AKT signaling	PI3K/AKT signaling	PI3K/AKT signaling

Note: AC, Adenylyl cyclase; cAMP, cyclic adenosine monophosphate; PKA, protein kinase A; DARPP-32, dopamine- and cAMP-regulated neuronal phosphoprotein; ERK, Extracellular signal-regulated kinases; GSK-3β, Glycogen synthase kinase-3beta; MAPK, mitogen-activated protein kinases; PI3K, phosphoinositide 3-kinase; Akt, protein kinase B.

neurotransmitter molecules into presynaptic storage vesicles prior to release into the synaptic cleft when an action potential or other signal leads to increased synaptic calcium levels (Gantz et al., 2015). Two closely related VMATs, VMAT-1 and VMAT-2 have been cloned, expressed, and characterized, and both have distinct pharmacological properties and tissue distribution characteristics (Wimalasena, 2011).

VMAT-1 is expressed preferentially in neuroendocrine cells and the peripheral nervous system, while VMAT-2 is mainly expressed in the CNS (Wimalasena, 2011). Although most research on DAergic mechanisms in the brain has focused on VMAT-2, a recent study by Lohoff et al. suggests that significant changes in DAergic signaling in the frontal cortex occur in VMAT-1 null-mutant mice (Lohoff et al., 2019). This suggests that the role of VMAT-1 in CNS function may be underappreciated and that it may be involved in functions relevant to the pathogenesis and/or treatment of psychiatric disorders. Genetic variation in the VMAT-1 gene (*SLC18A1*) has been implicated in the activity of neural circuits associated with emotion, it plays an important role in brain structural changes in patients with depression (Vaht et al., 2016; Won et al., 2017). DA signaling and distribution are mainly regulated by VMAT-2 and DA transporter (DAT) proteins, which transport DA to synaptic vesicles and presynaptic terminals, respectively, and are regulated by complex processes such as phosphorylation and protein-protein interactions (German et al., 2015). Conditional deletion of VMAT-2 in astrocytes leads to loss of prefrontal cortex (PFC) DA homeostasis, resulting in impaired synaptic transmission and plasticity as well as impaired executive function (Petrelli et al., 2020). Petrelli et al. concluded that the lack of VMAT-2-dependent DA stores in astrocytes causes an abnormal increase in mitochondrial enzyme monoamine oxidase B (MAOB) and the plasma membrane organic cation transporter 3 (OCT3) activity, which leads to a decrease in extracellular DA levels (Petrelli et al., 2020). Clinical pharmacological studies have shown that the uptake of DA by monoaminergic neurons mediated by VMAT-2 can prevent the oxidation of DA, and the overexpression of VMAT-2 may provide a potential target for neuroprotective therapy in various psychiatric diseases (Segura et al., 2019). DAT is a plasma membrane glycoprotein selectively expressed in the presynaptic membrane of central DAergic neurons (Mortensen and Amara, 2003). It belongs to the Na+ CI– dependent membrane transporter gene family and is most densely distributed in the basal ganglia (Wang et al., 2015). Accurate regulation of synaptic DA levels by DAT ensures the phasic nature of the DA signal, which underlies the ability of DA to encode reward prediction errors. The spatial and temporal strength of DA signaling is largely dependent on the role of DAT, which regulates both extracellular and intracellular DA levels (Giros et al., 1996). Pharmacological changes in DAT function not only modulate DA reuptake, but also induce rapid alterations in the plasmalemmal expression of the transporter (Kahlig and Galli, 2003). DAT is regulated by different presynaptic proteins, including DRD2 and DRD3 (acting as sensors of extracellular DA concentration, regulating the synthesis and release of DA), and abnormal DAT function is closely associated with several neurodegenerative diseases and psychiatric disorders (Rouge-Pont et al., 2002; Sokoloff et al., 2006). Research by Condon et al. (2019) shows that DAT is the primary regulator of DA short-term plasticity, controlling the balance between release-dependent and release-independent mechanisms. Some studies suggest that decreased DAT availability may be a hallmark of anhedonic depression, suggesting that DAT may serve as a specific therapeutic target for patients with high levels of anhedonia (Camardese et al., 2014). Degradation of DA occurs via two enzymatic processes catalyzed by MAO and cathecholamine O methyltransferase (COMT), which produces

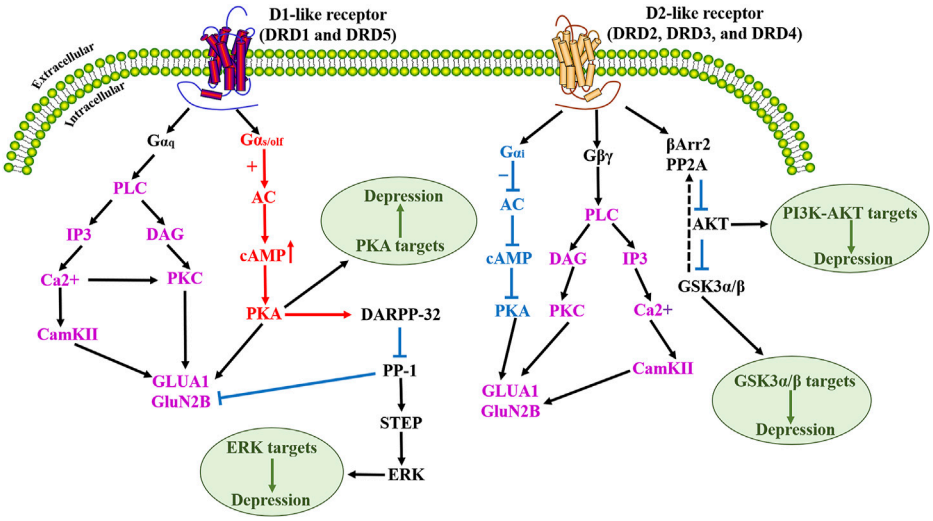


FIGURE 2 | Schematic diagram of D1-like dopamine receptor and D2-like dopamine receptor activation signaling cascade. Upon activation of the dopamine D1-like receptor, activated PKA mediates the phosphorylation of DARPP-32, which acts as an effective inhibitor of PP-1, which in turn dephosphorylates another phosphatase STEP. Dopamine D1-like receptor activation mediates dopamine-dependent inhibitory cascades by increasing ERK phosphorylation by blocking the dephosphorylation of STEP. Numerous studies have demonstrated that modulation of the cAMP/PKA signaling pathway can improve depression. D2-like receptor activation promotes phosphorylation/activation of Akt and phosphorylation/inactivation of its substrate GSK-3β. The physiological significance of this D2 receptor-activated Akt/GSK3 signaling has been extensively discussed in terms of neuroprotection against oxidative stress in depression. In addition, numerous studies have also shown that the PI3K/Akt pathway has an integral role in the treatment of depression. PLC, Phospholipase C; IP3, Inositol triphosphate; CamKII, calmodulin-dependent protein kinase II; DAG, diacylglycerol; PKC, protein kinase C; GluA1, glutamate A1; GluN2B, Glutamate Receptor Ionotropic, NMDA 2B; AC, Adenylyl cyclase; cAMP, cyclic adenosine monophosphate; PKA, Protein Kinase A; DARPP-32, Dopamine- and cAMP-regulated phosphoprotein; PP-1, protein phosphatase-1; STEP, striatal-enriched tyrosine phosphatase; ERK, extracellular-signal-regulated kinases; βArr2, β-arrestin-2; PP2A, protein phosphatase 2A; Akt, protein kinase B; PI3K, phosphatidylinositol-3; GSK3, glycogen synthase kinase 3.

homovanillic acid (HVA, a primary DA metabolite) (Franco et al., 2021) (see **Figure 1**).

MAO is a mitochondrial enzyme that inactivates DA in the brain. It was concluded that the MAOB and COMT are mainly expressed in astrocytes (Cahoy et al., 2008; Petrelli et al., 2020). In fact, the HVA is made in glial cells (astrocytes). Astrocytes can coordinate neural development by orchestrating synapse formation and function, which may be closely related to the pathogenesis of neurodevelopmental abnormalities common in psychiatric disorders (Chung et al., 2015). Reduced COMT activity in the PFC predicts a decrease in midbrain DA synthesis (Meyer-Lindenberg et al., 2005). COMT variants that alter DA function also affect prefrontal cortical connectivity, and these differences are associated with depression (Na et al., 2018). The COMT Val158Met polymorphism affects levels of DA, which plays an important role in depression (Camardese et al., 2014; Otsuka et al., 2019). Inoue et al. have shown that transmembrane protein 132D (TMEM132D), COMT, and GABA receptor alpha 6 subunits ($GABR_{A6}$) genotypes are associated with emotional processing in the cingulate, frontal cortex, and hippocampus in panic disorder and major depressive disorder (MDD) (Papaleonidopoulos et al., 2018). Results have shown that the levels of HVA in the cerebrospinal fluid (CSF), are decreased in patients with depression (Reddy et al., 1992; Saloner et al., 2020). Antidepressant treatments reversed DAergic hypoactivity and anhedonia-like behavior, as well as increased HVA levels in CSF (Horikoshi et al., 2019), which suggests an important role of DA in the pathophysiology of depression.

CLASSIFICATION AND DISTRIBUTION OF DOPAMINE RECEPTORS AND THEIR SIGNALING PATHWAYS

Based on their ligand recognition properties and their effect on cAMP, DA receptors were initially divided into two pharmacological families: D1-like receptors and D2-like receptors. D1-like receptors are coupled to Gs and Golf proteins, whose binding activates adenylate cyclase (AC), increasing the activation of the cAMP/PKA cascade response, and intracellular events resulting modification of cortico-striatal glutamatergic synapses (Beninger and Miller, 1998). Signaling cascades activated by D1-like receptors can also have long-term effects on cellular function by regulating transcription. For example, D1-like receptor agonists increase cAMP levels and the phosphorylation of the cAMP-response element binding protein (CREB) at Ser133, which subsequently regulates the transcription of many genes that are important for a variety of psychiatric disorders (Zhang et al., 2016). Related studies have suggested that the behavioral effects of some D1 agonists are not related to cAMP/PKA signaling, but rather involve non-cAMP-mediated signaling, including phospholipase C (PLC)-mediated

calcium elevation (O'Sullivan et al., 2004). SKF-83959 is a highly D1-biased ligand with a full agonistic effect (*via* Gαq) on D1-mediated activation of PLC signaling and an antagonistic effect on D1-mediated AC signaling (O'Sullivan et al., 2004). In contrast, D2-like receptors are coupled to Go and Gi proteins, which are involved in the inactivation of AC, resulting in a decrease in cytosolic cAMP levels (Beaulieu and Gainetdinov, 2011; Alexander et al., 2019). Binding of DA to D2-like receptors inhibit the cAMP/PKA signaling pathway ultimately affecting the CREB phosphorylation. Later, five DA receptor subtypes were cloned by molecular biology techniques: the D1-like receptors included the D1R and D5R subtypes, while the D2-like receptors included the D2R, D3R, and DRD4 subtypes (**Table 1**) (Undieh, 2010). There are two splice variants of the DRD2 gene that result in receptors of different lengths (number of amino acids), we were termed DRD2L (long) and DRD2S (short)]. DA receptors are mainly distributed in the CNS and peripheral nervous system. Among them, D1R and D2R are the most abundant subtypes in the CNS (Wang et al., 2008). D1-like receptors are mainly present postsynaptically, whereas D2-like receptors are present in postsynaptic DAergic target neurons and also act presynaptically as autoreceptors on DA neurons. The activity of DA receptors is extremely complex and is regulated by a variety of factors in different brain regions, including the ventral tegmental area (VTA), nucleus accumbens (NAc), Substantia nigra, PFC, hippocampus, amygdala, striatum, and lateral habenular nucleus (LHb), and ventral pallidum (VP). The localization of D1-like and D2-like receptors is different.

D1-like receptors are highly expressed in the striatum, NAc, substantia nigra, olfactory bulb, amygdala, and PFC, with lower levels of expression in the hippocampus, cerebellum, thalamus, and hypothalamus (Beaulieu et al., 2007; Dunlop and Nemeroff, 2007). In NAc, plasticity-related signaling of Ca^{2+}/calmodulin-dependent protein kinase II (CamKII) and adenosine A2A receptors (A2ARs) are required for discrimination learning (Iino et al., 2020). Recent evidence suggests that all of these G-protein-mediated signaling cascades converge on, the phosphorylation of two ionotropic glutamate receptor subunits, GluA1 and GluN2B, which play a key role in glutamatergic transmission (Koutsokera et al., 2014). D1-like receptors are expressed in striatal GABAergic medium spiny neurons (MSNs) that project to the medial globus pallidus and the substantia nigra reticulata (SNr) (i.e., the direct nigrostriatal pathway), while DRD2 is expressed on the MSNs that project to the lateral globus pallidus (i.e., the indirect pathway). D1-like receptors influence the function of multiple voltage-gated ion channels, as well as N-Methyl-D-Aspartate (NMDA) and $GABA_A$ receptors, by directly or indirectly acting on DARPP-32, the MAPK signaling pathway (such as ERK, JNK, P38), and other kinases and phosphatases (Chen et al., 2004). DRD2 receptor is mainly distributed in the hippocampus, striatum, thalamus, pituitary olfactory nodule, substantia nigra, and VTA (Missale et al., 1998). It has become increasingly clear that D2R acts through protein kinase B (Akt)-GSK-3 (glycogen synthase kinase 3) signaling cascade, and this signaling pathway involves the multifunctional scaffold protein β-arrestin2 (βArr2), which plays a role in GPCR desensitization (Beaulieu et al., 2007). The expression of D3R is relatively low in the central nervous system and is mainly distributed in the limbic system, including the NAc shell and olfactory tubercle (Missale et al., 1998). It also has a lower level of expression in other portions of the striatum, basal ganglia, the NAc core, islands of Calleja, substantia nigra, the VTA, hippocampus, septum, and various cortical regions (Le Moine and Bloch, 1996; Gurevich et al., 2016; Solís et al., 2017). DRD4 receptor is expressed at low levels in the basal ganglia and high expression in the striatum, frontal cortex, medulla, amygdala, hypothalamus, midbrain, and islands of Calleja, however, these levels are much lower than other DA receptors. DRD5 receptor also has lower levels of expression in other brain regions, including the PFC, anterior motor cortex, cingulate cortex, substantia nigra, hypothalamus, and hippocampus (Dunlop and Nemeroff, 2007). DRD5 also has a low level of expression overall, but this does include MSNs of the caudate nucleus and the VTA (Hernández-Echeagaray et al., 2007). However, the contribution of these receptors to circuit-level functional connections between brain regions remains poorly understood. Previous studies have shown that several subtypes of DA receptors may colocalize on some cells, but the receptors are largely segregated. The signaling pathway diagram for D1-like receptors and D2-like receptors is summarized in **Figure 2**, and a summary of all 5 DA receptors is given in **Table 1**.

The ultimate actions of DA receptor stimulation are also affected by dimerization. DA D1-D2 heterodimers are expressed in key cerebral cortical and subcortical regions in all species, and the differences in their expression in the striatum of different species suggest an evolutionary role of D1-D2 heterodimers in higher CNS function (Hasbi et al., 2020c). D1-D2 receptor heterodimers in subsets of neurons were first found in the rat striatum and are coupled with Gαq proteins to regulate intracellular calcium signaling (Perreault et al., 2012a), directly linking DA and calcium signaling (Perreault et al., 2012a; Perreault et al., 2014). Related studies have shown that the expression of D1-D2 receptor heterodimers in the striatum of juvenile rats is lower than in adult rats, and as result juvenile rats are less sensitive to D1-D2 receptor combined stimulation (Perreault et al., 2012b). This suggests that there may be significant age-dependent neurotransmission differences in the D1-D2 receptor heteromeric pathway combined with an *in situ* proximity ligation assay (PLA) technique with different neuronal markers to characterize the neurons expressing D1-D2 receptor heterodimers in the striatum (including the caudate nucleus, the putamen, and the NAc core and shell of the), finding heterodimers in all striatal regions and projection neurons of the direct and indirect basal ganglia pathways (Rico et al., 2017).

D1-D2 heterodimers induce calcium release via a Gαq-dependent pathway, distinct from G_s/G_{olf}- or G_i/G_o- dependent pathways activated by the D1 receptor or D2 receptor independently (Rashid et al., 2007; Hasbi et al., 2009). The increase in intracellular calcium content is rapid and transient, independent of extracellular calcium influx, and involves activation of Gq protein and phospholipase C (PLC) (Hasbi et al., 2009). D1-D2 heterodimers trigger calcium signaling by activating Gαq and PLC, leading to the activation of calmodulin

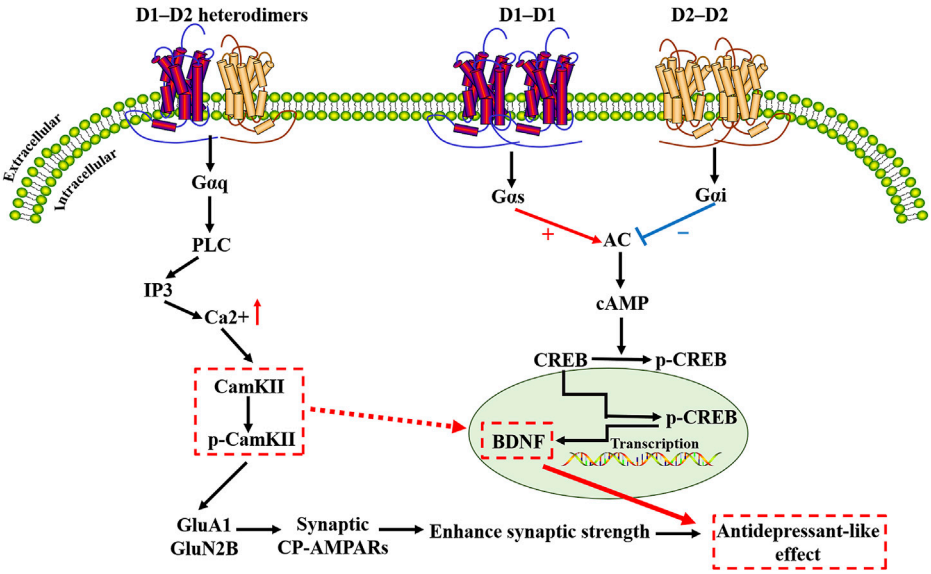


FIGURE 3 | Schematic diagram of the dopamine receptor complex signaling cascade. D1-D2 heterodimers induce calcium release via a Gαq-dependent pathway, distinct from Gs/Golf- or Gi/Go- dependent pathways activated by D1 receptor heterodimers or D2 receptor heterodimers. D1-D2 heterodimers trigger calcium signaling by activating Gαq and PLC, leading to the activation of calmodulin kinase II-α(CaMKIIα). When calcium ions enter cells through various ion channels on the membrane, CaMKIIα is activated. Activation of CaMKIIα promotes its autophosphorylation, which binds to GluN2B and phosphorylates GluN2B at the S1303 site (p-GluN2B). Studies have shown that the interaction between GluN2B and CaMKIIα is significant for the synaptic CaMKIIα localization and activity. Phosphorylation of GluA1 and GluN2B plays a key role in the glutamatergic transmission and is regulated by the D1-D2 heterodimers signaling pathway. Inhibition of the recruitment, deletion, or activity of Ca(2+)-permeable AMPA receptors (CP-AMPARs) would interfere with long-term depression. CaMKIIα/BDNF/CREB-dependent neural plasticity pathways may be an important target for dopamine receptor complex research. D1R, dopamine D1-like receptor; D2R, dopamine D2-like receptor; PLC, Phospholipase C; IP3, Inositol triphosphate; CamKII, Calmodulin kinase II; p-CamKII, Phosphorylated CamKII; GluA1, glutamate A1; GluN2B, Glutamate Receptor Ionotropic, NMDA 2B; CP-AMPARs, calcium-permeable AMPA receptors; AC, Adenylyl cyclase; cAMP, cyclic adenosine monophosphate; CREB, cAMP-response element-binding protein; p-CREB, Phosphorylated-CREB; BDNF, Brain-Derived Neurotrophic Factor.

kinase II-α (CaMKIIα) (Hasbi et al., 2009; Ng et al., 2010; Perreault et al., 2012a). Specific activation of D1-D2 receptor heterodimers in striatal neurons and the cellular co-expression of DRD1 and DRD2 leads to the intracellular release of calcium from stores sensitive to activation of inositol triphosphate receptors (IP3-R) (So et al., 2005). This calcium signaling results in an increased form of phosphorylation-activated form of CaMKIIα in striatal neurons and rat striatum (So et al., 2005). Phosphorylation of GluA1 and GluN2B plays a key role in the glutamatergic transmission and is regulated by the D1-D2 heterodimers signaling pathway. Expression of the GluA1 subunit of the AMPA receptor is associated with anhedonia. Studies have shown that mice lacking GluA1 (mice with *Gria1* knockout) show a reduction in licking cluster size, a measure of palatability of feeding behavior, and GluA1 is necessary for hedonic responding (Strickland et al., 2021).

Information processing in the brain requires multiple forms of synaptic plasticity involving NMDA-type glutamate receptors (NMDAR) and AMPA-type glutamate receptors (AMPAR), including long-term potentiation (LTP) and long-term depression (LTD), and homeostatic scaling, potentially mediated by DA (Madadi Asl et al., 2018; Madadi Asl et al., 2022). PKA can anchor the scaffold protein AKAP150 to regulate GluA1 phosphorylation and plays a role in controlling Ca2+ -permeable AMPA receptor (CP-AMPAR) synaptic binding in NMDAR-dependent LTD (Sanderson et al., 2016). Inhibition of the recruitment, deletion, or activity of CP-AMPAR, would interfere with LTD, therefore, synaptic recruitment of CP-AMPAR is required to transiently increase NMDAR Ca (2+) signaling during LTD induction (Sanderson et al., 2016). On this basis, D1-D2 heterodimers-mediated signal transduction pathways may be thought to play an important role in other forms of synaptic plasticity as well, especially in LTP (Hasbi et al., 2009). Long-term synaptic plasticity is an essential form of brain plasticity. Inhibition of facilitated synaptic transmission may impair the function and structure of brain circuits implicated in the pathophysiology of depression, and antidepressants may counteract these alterations (Holderbach et al., 2007). Relevant findings suggest that activation of the D1-receptor complex, raises intracellular levels of cAMP, while the D2-receptor complex, inhibits intracellular levels of cAMP. The cAMP-response element binding protein (CREB) activates protein kinases in different DA receptors, such as protein kinase A (PKA), calmodulin-dependent protein kinase (CaMK) after phosphorylation at Ser133, and binds to the cAMP response element (CRE) of the target gene promoter (Santanavanich et al., 2005). This unique intracellular calcium signaling pathway links DA and brain-derived neurotrophic factor (BDNF) through a rapid increase in calcium signaling and CaMKIIα activation (Hasbi et al., 2009). Experimental studies have shown that basal levels of p-CAMKII, total CaMKII and BDNF are reduced in the CA1 region of the hippocampus of stressed rats

(Alzoubi et al., 2013). BDNF, which is synthesized and released at glutamate nerve terminals, plays an important role in neuronal development by regulating protein synthesis and has been shown to increase the translation of hundreds of proteins isolated from synaptoneurosomes. In this review, we summarize recent studies on the etiology and pathogenesis of depression that involve different DA receptor mechanisms and different brain regions. The signaling pathway diagram summary of the DA receptor complex is shown in **Figure 3**.

DOPAMINE RECEPTORS AND DEPRESSION

Dopamine D1-Like Receptors and Depression

Positron emission tomography (PET) and functional magnetic resonance imaging (fMRI) can be used to study receptor binding potentials in the human brain *in vivo* (Hamilton et al., 2018). PET studies using radioligands for DRD1 have shown some promise as a means of researching the DA system in psychiatric diseases, ideally with higher selectivity radioligands, so that DRD1 can be evaluated as a candidate biomarker for disease and ultimately for treatment (Cervenka, 2019; Stenkrona et al., 2019; Yokokura et al., 2020). Currently, mice lacking DA D1 receptors are widely used to study the involvement of DA receptors D1-like class (D1 and D5) in motor and cortical striatal LTD and LTP, and endogenous DA stimulation of different subtypes of striatal neurons D1 and D5 receptors induces LTP and LTD, respectively (Kerr and Wickens, 2001; Rivera et al., 2002). Centonze et al. concluded that D1 and D5 receptors have different effects on the dependence of activity on both synaptic plasticity and spontaneous motor activity differently (Centonze et al., 2003). It has long been known that DRD1/DRD5 mechanisms regulate long-term plasticity and memory in the hippocampus (Hansen and Manahan-Vaughan, 2014). The mossy fiber (MF) synapse in the hippocampal CA3 region plays an important role in the molecular mechanisms of synaptic plasticity. Hagena et al. suggest that D1/D5 receptors are critical in regulating synaptic plasticity in MF-CA3 synapses, especially as a modulator of candidate processes for long-term memory (Hagena and Manahan-Vaughan, 2016). Lazenka et al. have shown that DA D1 receptor signal transduction is involved in behavioral pain-related depression in rats, suggesting that indirect and/or direct D1 receptor agonists might alleviate pain-related behavioral depression (Lazenka et al., 2017). In addition, Desormeaux et al. showed that modulation of selective DA D1-like receptor agonist A77636 induced antidepressant-like effects in rats (Desormeaux et al., 2020). Quetiapine is an atypical antipsychotic that is effective in treating depression and anxiety disorders. Male BALB/c mice injected with quetiapine every other day and pretreated with the D1 receptor antagonist SKF-35866 in the following experiments found a significant increase in the preference to the quetiapine-paired chamber in mice treated with 120 mg/kg quetiapine, and this effect was blocked by pretreatment with SKF-35866, suggesting that the antidepressant-like effects of quetiapine may be modulated by D1 receptors (Althobaiti, 2021). Recent experimental studies have shown that activation of the DRD1 receptor and PKA is involved in the memory-improving effect of acute physical exercise (Ramires Lima et al., 2021).

A large number of studies have shown that the impaired function of the medial prefrontal cortex (mPFC) is involved in depression. Hare et al. used optogenetics to stimulate the pyramidal cells expressing DRD1 in mPFC and found that the activation of the pyramidal cells expressing DRD1 could produce rapid and long-lasting antidepressant and anti-anxiety responses (Hare et al., 2019). The application of optogenetics techniques has made it possible to perform a more precise anatomical and cellular dissection of the role of specific DA receptors in DA-related functions (Beaulieu et al., 2015). Numa et al. showed that downregulation of D1R in mPFC reduces c-Fos expression in the interstitial nucleus of the posterior limb of the anterior commissure (IPAC) induced by social defeat stress. However, contrary to the above findings, Fedotova et al. showed that the D1 receptor antagonist SCH-23390 produced antidepressant-like effects in ovariectomized rats, where repeated administration of SCH-23390 greatly enhanced the antidepressant-like effects (Fedotova and Ordyan, 2011). However, the D1 receptor agonist SKF-38393 failed to alter depressi-like behavior in ovariectomized rats in FST but blocked the antidepressant-like effects of 17β-estradiol (17β-E2) (Fedotova and Ordyan, 2011). This suggests that D1 receptors may be activated by subthreshold social defeat stress in the mPFC. However, the specific mechanisms by which D1 receptor agonists and D1 receptor antagonists affect DAergic properties need to be further investigated.

Dopamine D2-Like Receptors and Depression

With the development of novel radioligands, *in vivo* imaging can provide a new perspective on the pathophysiology of depression. An approach using PET has suggested that deep brain stimulation (DBS) of the medial forebrain bundle (MFB) partially reverses depression-like phenotypes associated with DRD2 blockade (Thiele et al., 2020). This effect appears to be related to increased levels of both DRD2 and DRD1. A growing body of evidence suggests that the direct D2-like receptor agonist pramipexole has antidepressant effects, particularly in electroconvulsive treatment (ECT) resistant depression (Gauthier et al., 2017) or in patients with deficits in baseline reward processing (Whitton et al., 2020). The effect of traumatic brain injury (TBI) on DA receptor binding was examined in patients with post-injury MDD (TBI-MDD) and patients without post-injury MDD (TBI-NON), as well as non-TBI control patients (Jolly et al., 2019). [11C]PHNO PET imaging was used to assess DRD2/DRD3 binding ratios (Le Foll et al., 2016). TBI was associated with reduced binding ratios overall, and these values were even lower in MDD patients, although this difference was not statistically significant. Given the small number of subjects, the finding is worthy of adding to determine whether DAergic mechanisms may be involved in post-traumatic depression. Fatima et al. (2020) suggested that

selective D2 receptor agonists Ropinirole (ROPI) alleviate depression by upregulating tyrosine hydroxylase and increasing neurogenesis in the hippocampal region of prenatally stressed rats. Papp et al. (2019) found that both Wistar and Wistar-Kyoto rats (which have been validated as an animal model for treatment-resistant depression) exposed to chronic mild stress had reduced sucrose intake and impaired memory consolidation. Chronic treatment with serotonin and norepinephrine reuptake inhibitor venlafaxine reversed these effects in Wistar rats, while DBS reversed depression-like effects in Wistar-Kyoto rats (Papp et al., 2019). Venlafaxine reversed the effect of the DRD2 agonist L-742,626 on memory consolidation in unstressed, but not stressed, Wistar rats, while in Wistar-Kyoto rats, DBS reversed the effects of L-742,626, or the DRD3 agonist 7-hydroxy-N, N-di-N-propyl-2-aminotetralin (7-OH-DPAT) in both the stressed and unstressed rats (Papp et al., 2019). These results suggest that the effect of stress on memory consolidation impairment in rats can involve both DRD2 and DRD3 receptors in the ventral medial prefrontal cortex and that DBS effects on depressive symptoms may act in part through effects on DA function. The use of tractography for more refined deep brain stimulation electrode targeting and closed-loop deep brain stimulation approaches are the future trends in the treatment of depression (Dougherty, 2018).

A study in DRD3−/− mice demonstrated that the elimination of DRD3 receptors induces chronic depressive symptoms (Moraga-Amaro et al., 2014). Moreover, DRD3 expression and function are down-regulated during stress and depression, and antidepressant therapy can reverse these changes, suggesting that enhanced DAergic neurotransmission mediated by DRD3 down-regulation is involved in the adaptive changes underlying antidepressant activity. BDNF regulates the expression of DRD3 in certain brain regions, and the induction of BDNF by antidepressant therapy is related to behavioral outcomes (Leggio et al., 2013). Wang et al. (2020) used DRD3 KO mice to further demonstrate that DRD3 deficiency-induced depressive-like behavior involves neuroinflammation in mesolimbic brain regions, which helps us to understand DRD3 KO-induced depressive-like behavior and provides potential molecular and cellular targets for the treatment of depressive phenotypes. Experimental studies suggest that exposure to neonatal maternal separation (MS) and chronic mild stress (CMS) in adulthood completely inhibits reward-induced intra-NAc DA release, which is a useful indicator of depression severity and various therapeutic efficacy (Minami et al., 2017). Inflammation plays an important role in the pathophysiology of depression, and the peripheral administration of lipopolysaccharide (LPS) is one of the most common models of inflammation-induced depression. LPS results in a significant decrease in DRD3 in the VTA, mPFC, and NAc, key structures within the mesolimbic DAergic system (Wang J. et al., 2018). Pre-treatment stratification in depressed patients may be beneficial by taking into account the role of multiple anti-inflammatory agents in depression. DRD4 is also associated with the pathophysiology of several psychiatric disorders characterized by cognitive deficits, including depression (Rondou et al., 2010; Navakkode et al., 2017). Previous studies have shown increased expression of DRD4 in the basal amygdala of depressed patients compared to control subjects, and *in vivo* imaging studies of depressed patients show results consistent with those *post mortem* findings (Xiang et al., 2008). DRD4 appears to have a homeostatic role on synapses that stabilizes neural network activity. Navakkode et al. demonstrated that DRD4 plays a bidirectional role in the CA1 region of the hippocampus (Navakkode et al., 2017). Blocking DRD4 affects late-LTP and transforms early-LTP into late LTP (Navakkode et al., 2017). This enhanced LTP was dependent on protein synthesis, NMDAR activation, and CaMKII phosphorylation, as well as $GABA_A$-receptor, mediated mechanisms (Navakkode et al., 2017).

Dopamine D1-D2 Receptor Heterodimers and Depression

DA receptors are involved in homomeric and heteromeric complexes, which provide new targets for antidepressant drug discovery and are important for a deeper understanding of the complex physiological roles of these receptors in the brain (Perreault et al., 2014). It is necessary to further understand the role of DA receptor changes in specific brain regions of depressed patients and to determine the specific DA receptor mechanisms and other molecular complexes that underlie these functional changes that lead to depressive symptomatology. This will help to elucidate the pathophysiology of depression and aid in the development of new drugs with greater efficacy and fewer side effects. Recently, an increasing number of articles have highlighted the ability of both DRD1 and DRD2 to form heterodimers, and a growing body of evidence has linked D1-D2 heterodimers to drug addiction, Parkinson's disease, schizophrenia, depression, and anhedonia (Shen et al., 2015; Hasbi et al., 2020b; Hasbi et al., 2020c; Noori et al., 2020). D1 and D2 receptors can form a heterodimeric complex that is present in a heterologous system and primary striatal neurons, as well as in the rodent brain *in vivo* (Perreault et al., 2014). However, most studies carried out to date stem from observations in heterologous systems and the biological significance of DA receptor heterodimers *in vivo* is only beginning to emerge. Recent data from *in situ* assessment of mRNA expression using RNA-FISH techniques revealed significant co-localization of DRD1 and DRD2 receptor mRNAs in the NAc, amygdala, piriform cortex, olfactory tubercle, claustrum, prelimbic cortex, and orbitofrontal cortex (Hasbi et al., 2020c). Perreault et al. (2014) showed that D1-D2 heterodimers may differentially regulate c-fos expression in a region-dependent manner either through its activation or through tonic inhibition of neuronal activity.

Studies have demonstrated that D1-D2 receptor heterodimers are upregulated in the postmortem brain of patients with depression and have identified an interfering peptide that disrupts D1-D2 receptor interactions (Pei et al., 2010). Pei et al. (2010) used the interfering peptide Tat-$D2L_{IL3\text{-}29\text{-}2}$ to block the D1-D2 receptor heterodimers, significantly reducing immobility time in the forced swimming test without affecting locomotor performance and reducing escape failure in the learned helplessness tests in rats. This implies that the D1-D2 receptor heterodimers play an important role in the pathology of

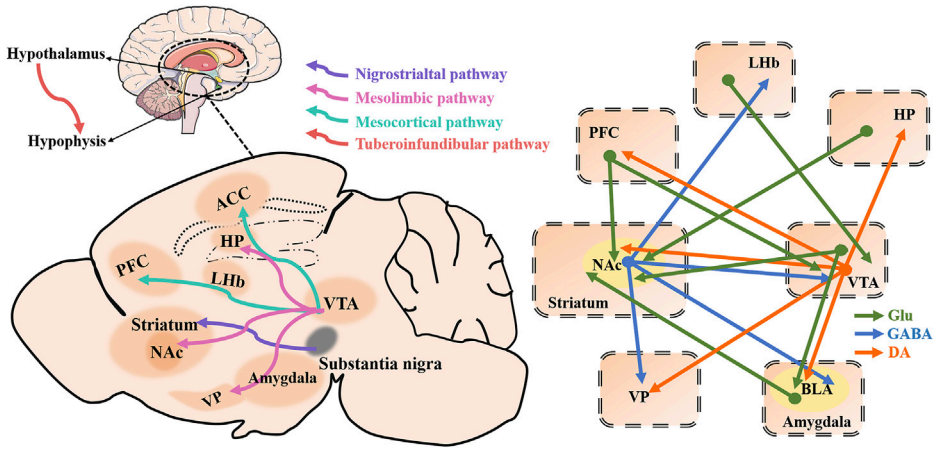


FIGURE 4 | Four major brain dopaminergic pathways as well as depression-related brain regions and a schematic of brain circuitry implicated in depression. Dopaminergic (DAergic; orange) and glutamatergic (Gluergic; green) direct inputs converge on γ-aminobutyric acid (GABA)ergic (blue) neurons in the NAc to regulate behaviors of depression. The dopaminergic pathway from the VTA to the NAc plays a key role in reward-related behavior. Direct dopaminergic outputs of the VTA arise from the NAc, PFC, VP, BLA, and Hp, while the VTA, VP, BLA, and LHb receive NAc GABAergic inputs as well VTA, PFC, BLA, and Hp glutamatergic inputs. Besides, VTA receives PFC and LHb glutamatergic inputs. ACC, anterior cingulate cortex; HP, hippocampus; LHb, lateral habenula; PFC, prefrontal cortex; NAc, nucleus accumbens; VTA, ventral tegmental area; VP, ventral pallidum.

depression. Regulation of D1-D2 receptor heterodimers may be a novel pharmacological target for the treatment of depression and anxiety disorders, particularly addressing the high incidence of these conditions in females (Hasbi et al., 2020c). The coupling between DR1 and DR2 receptors in the brain is significantly increased in MDD patients (Hasbi et al., 2020c). A new study suggests that genetic interactions between DA receptor regulatory regions may influence the level of depressive symptoms through epistatic interactions between DRD1 and DRD2 regulatory elements that may affect D1-D2 heterodimers function (Corrales et al., 2016). Both DRD1 and DRD2 can form homomers and heterodimers, and the receptor configurations in the homomeric and heteromeric states appear to involve changes in their respective intracellular conformations, producing different G-protein coupling and subsequent activation of different signaling pathways (Hasbi et al., 2014). There are currently no selective antagonists targeting the D1-D2 heterodimers, but serial deletions and point mutations have been used to identify the amino acids involved in the interaction interface between the receptors. Residues in the DRD1 receptor located in the carboxylic tail interact with the DRD2 receptor to form D1-D2 receptor heterodimers (Hasbi et al., 2014). Interfering peptides block the formation of D1-D2 heterodimers and block the calcium signaling pathways activated by D1-D2 heterodimers, revealing a role of the D1-D2 complex in regulating behavioral despair *in vivo* (Hasbi et al., 2014). This interfering peptide may represent a new pharmacological tool to selectively disrupt GPCR heterodimers activity without affecting the function of the constituent receptors to elucidate the functional and behavioral consequences of D1-D2 heterodimers activity (Hasbi et al., 2014), and perhaps a potential therapeutic avenue for affecting heterodimer function in the absence of effects on the individual receptors. Identification of an interfering peptide that interferes with D1-D2 receptor heterodimers and has antidepressant-like effects may provide a new therapeutic strategy for the treatment of major depressive disorder (Pei et al., 2010; Duan et al., 2013; Brown and Liu, 2014).

CIRCUIT MECHANISMS OF DEPRESSION RELATED TO DOPAMINE RECEPTORS (INTERESTING NEW TARGETS)

Four major brain DAergic pathways are involved in mammalian brain function: the nigrostriatal pathway (from cells in the A9 region), the mesolimbic and the mesocortical pathways (often collectively termed the mesocorticolimbic pathway, from cells in the A10 region, and the thalamic-tuberoinfundibular pathway (from cells in the A12 region) (Beaulieu and Gainetdinov, 2011). The nigrostriatal pathway projects from the substantia nigra pars compacta to the dorsal striatum (caudate and putamen) (Beaulieu and Gainetdinov, 2011). The mesocorticolimbic DAergic pathway is the most thoroughly studied DA pathway at present. The mesolimbic DA system, composed of DAergic projections from the VTA to the NAc, amygdala, hippocampus, and olfactory tubercle, plays a key role in reward-related learning, cognition, motivation, and decision-making processes (Polter et al., 2018; Heymann et al., 2020). The mesocortical pathway also originates in the VTA and projects into the frontal and temporal cortices, particularly the anterior cingulate cortex (ACC), entorhinal cortex, and PFC, which is thought to be important for attention and executive function (Dunlop and Nemeroff, 2007; Beaulieu and Gainetdinov, 2011). Some aspects of anterior pituitary function are also controlled by DAergic activity. The thalamic-tuberoinfundibular pathway originates from the arcuate nucleus of the hypothalamus (A12) and projects onto the median hypothalamic eminence (Ben-Jonathan and Hnasko, 2001). The details are shown in **Figure 4**.

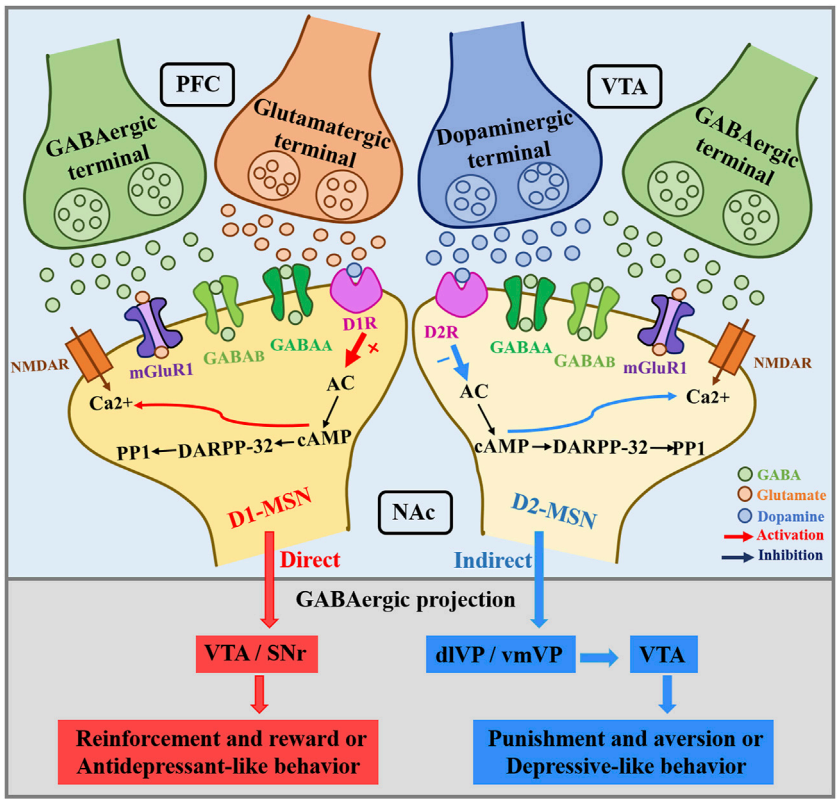


FIGURE 5 | NAc dopamine receptors in D1-MSNs and D2-MSNs receive PFC glutamatergic and GABAergic inputs, and VTA dopaminergic and GABAergic inputs. The direct NAc innervation of the VTA/SNr is mainly mediated by D1-MSNs. In general, NAc core projects to dlVP, NAc shell innervates the vmVP, and this process is mainly mediated by D2-MSNs.Whereas D1-MSNs mediate reinforcement and reward or antidepressant-like behavior, D2-MSNs have been associated with punishment and aversion or depressant-like behavior. VTA, ventral tegmental area; SNr, substantia nigra pars reticulata; VP, ventral pallidum; dlVP, dorsolateral pallidum; vmVP, ventromedial pallidum.

Ventral Tegmental Area

The VTA is a heterogeneous brain structure that plays a central role in reward-seeking and processing, learning, motivation, and neuropsychiatric disorders that involves alterations in these functions such as depression and addiction (Lüscher and Malenka, 2011; Cohen et al., 2012; Barker et al., 2016). Numerous studies have shown that DA neuronal activity of VTA plays a role in the pathophysiology of depressive symptoms, and regulating the activity of DA neurons in VTA has great potential as an antidepressant strategy (Bai et al., 2017). The mesocorticolimbic DA system originates from the VTA and mainly projects to the PFC, NAc, hippocampus, BLA, dorsal striatum, ventral striatum, and the OT, where DA transmission is partially regulated through negative feedback mechanisms via DA D2 autoreceptors located on cell bodies and terminals of VTA DAergic neurons (Gasbarri et al., 1994; Chaudhury et al., 2013) (**Figure 4**). The DAergic pathway from the VTA to the NAc plays a key role in reward-related phenomena and plays an important role in aberrant motivational and emotional processes involved in psychiatric disorders (Nielsen et al., 2016). DAergic neurons have been the main focus of VTA research. DAergic neurons in the VTA are key components of the reward pathway, but their activity is tightly controlled by several types of inhibitory GABAergic inputs (Polter et al., 2018) (**Figure 4**). VTA GABA interneurons bi-directionally regulate the activity of local DA neurons, which is the basis of reward or aversion at the behavioral level (Creed et al., 2014). The VTA contains a mixture of DAergic (~65%) and GABAergic (~30%) neurons, and their interactions coordinate reward-seeking behavior and influence depressive-like behavior (Margolis et al., 2006; Nair-Roberts et al., 2008; Yamaguchi et al., 2011) (**Figure 4**). Although DA projection neurons are usually emphasized, VTA projection neurons can express DA, glutamate, or GABA, and are capable of multiplexing combinations of these neurotransmitters as well as neuropeptides (Barker et al., 2016). VTA GABA projection neurons project to areas such as the NAc, the VP, the PFC, the LHb, the central amygdala (CeA), and the dorsal raphe nucleus (DRN), among other regions (Juarez and Han, 2016; Bouarab et al., 2019) (**Figure 4**). The *in vivo* discharge pattern of DA neurons in the VTA is controlled by GABA afferents mainly from the NAc and local GABA interneurons (Simmons et al., 2017). VTA GABA neurons have a variety of functions, both affecting DAergic activity through local inhibitory control and exerting DA-independent effects (Bouarab et al., 2019). For example, the optogenetic strategies of selective stimulation of VTA GABA neurons as well as their projection fibers to the NAc, suggest that

the dynamic interaction between VTA DA and GABA neurons can control the initiation and termination of reward-related behaviors (van Zessen et al., 2012). In addition to the populations of VTA DA and GABA neurons, that have been studied for a long time, glutamatergic neurons have been identified in the VTA (Yamaguchi et al., 2007). Indeed, approximately 5% of the total neuronal content of the VTA) were recently discovered in the VTA, expressing the vesicular glutamate transporter 2, VGluT2 (Barbano et al., 2020). Papathanou et al. (2018) suggested that VGluT2 in mature DA neurons actively promotes glutamate neurotransmission in the NAc, and highlighted the co-release of VGluT2-mediated glutamate in the complex mechanisms of synaptic plasticity in drug addiction.

Optogenetic stimulation of the NAc lateral shell inputs to the VTA produced a robust real-time place preference and positive reinforcement of intracranial self-stimulation (Pignatelli and Bonci, 2018). Indeed, the inputs of information from the NAc subnuclei to specific VTA microcircuits will be important for a deeper understanding of the mechanisms of neuropsychiatric diseases that involve altered motivational function (Pignatelli and Bonci, 2018). The activation of D1-D2 receptor heteromers in NAc induced the enhanced expression of GABA-related proteins in NAc and VTA (Perreault et al., 2012a). Activation of the D1-D2 receptor heteromers increases GABAergic tone in the NAc and perhaps by NAc efferent inhibition of the VTA (Hasbi et al., 2017). In optogenetic experiments of DA neurons in VTA, staged optogenetic activation of these neurons can alleviate chronic stress-induced depressive-like behavior within a few seconds, a phenomenon that requires DA receptors in the NAc to function, although the specific type of receptor is not known (Zhang et al., 2019). DRD2 receptor activation enhances Kv7.4 currents through a Gi/o and redox-dependent cellular pathway, and Kv7.4 facilitates DA-induced inhibition of spontaneous firing of VTA DA neurons (Su et al., 2019). DRD2 receptor-mediated auto-inhibition may be involved in the development of depressive-like behaviors induced by stress, and thus the selective targeting of Kv7.4 is considered a promising antidepressant treatment strategy (Su et al., 2019).

Nucleus Accumbens

The NAc is part of the striatum, which together with the olfactory tubercles makes up the ventral striatum (Marcott et al., 2018). The NAc is one of the key regions of the brain reward circuit, and in some neuropsychiatric disorders, such as depression, there is an aberrant response to rewarding and aversive signals. The NAc receives glutamatergic projections from the PFC, hippocampus, and amygdala, as well as DAergic innervation from the VTA (Groenewegen et al., 1999). As an important part of the midbrain VTA-NAc-PFC reward circuit, critical afferent projections to the NAc arise from a direct projection from the midbrain VTA (Zhou et al., 2018; Castro and Bruchas, 2019; Soares-Cunha et al., 2020). Glutamatergic synaptic transmission is mainly mediated by AMPAR and NMDAR, and the MSNs in the core and shell of the NAc receive glutamatergic input from PFC, hippocampus, and amygdala. In rodents, more than 95% of the cells in NAc are MSNs, which receive excitatory input from four major brain regions, namely the PFC, hippocampus, basolateral amygdala, and the thalamus (Sesack and Grace, 2010). Morphological evidence suggests that DA D1 and D2 receptors form complexes in the dorsal striatum and NAc of mammalian species (including mice, rats, non-human primates, and humans), and in all of these species, a higher number of MSNs expressing the D1-D2 heteromers was observed in the NAc than in the dorsal striatum (caudate and putamen) (Hasbi et al., 2020a).

GABA MSNs subtypes that co-express DRD1 and DRD2 also exhibit glutamatergic phenotype, thus showing a combined GABAergic/glutamatergic phenotype (Perreault et al., 2012a). The NAc mainly contains GABA-expressing MSNs divided into subtypes based on the expression of DA receptors: DRD1-containing MSNs (D1-MSNs) and DRD2-containing MSNs (D2-MSNs) (Francis et al., 2019). These two populations of MSNs constitute the main NAc output projections, which have different functional roles in stress and reward-mediated behavior (Soares-Cunha et al., 2020). Since cells expressing both receptors appear to have glutamatergic expression as well, this would suggest that this pathway acts separately from these other well-characterized GABAergic output pathways. DAergic signaling mainly acts through D1-MSNs and D2-MSNs. D1-MSNs project primarily to the VTA/SNr (the direct pathway) (Perreault et al., 2010). D2-MSNs project indirectly to the VTA/SNr *via* the dlVP/vmVP (Soares-Cunha et al., 2016; McCutcheon et al., 2019). These striatal projections are summarized in **Figure 5**. D1-MSNs are involved in mediating responses to reward signals, while D2-MSNs are involved in mediating responses to aversive signals (Soares-Cunha et al., 2016). The classical view of striatal D1R signaling as pre-reward/reinforcement and D2R signaling as pre-aversive is too simplistic, and it is premature to assume that neurons expressing D1R and D2R play completely independent (and opposite) roles (Soares-Cunha et al., 2016). This relationship is clearer for the dorsal striatum than for the ventral striatum, where the relationship to reward/aversion may be less distinct and dependent on the duration of stimulation (Soares-Cunha et al., 2020). The positive enhancement that is mediated by midbrain DA neurons entails the activation of D1 and D2 receptors in the NAc. Targeting D1-MSN activity may provide new therapeutic strategies for depression or other affective disorders. The mesolimbic DAergic system role in the pathophysiology of depression is more and more obvious. BDNF is elevated in the NAc of depressed patients and contributes positively to depressive-like behavior in rodents. BDNF is widely considered to be critical for neural and synaptic plasticity throughout the nervous system, and recent studies have shown that BDNF in the mesolimbic DA circuit originates from DA neurons in the VTA that project into the NAc (Koo et al., 2019). Koo et al. concluded that chronic social defeat stress (CSDS) mice exacerbate failure-induced behavioral symptoms during repeated optogenetic stimulation of the mesolimbic VTA-NAc circuit and that these behavioral symptoms can be normalized by BDNF-TrkB blockade in the NAc (Wook Koo et al., 2016). Staged stimulation of the VTA-NAc pathway promotes the release of BDNF and DA from VTA DA terminals (Bass et al., 2013). D1-

D2 receptor heteromers are highly expressed in NAc and have been shown to enhance BDNF expression and signal transduction in NAc (Shen et al., 2015). Research by Rahman et al. has shown that the simultaneous activation of DA D1- and D2-like receptors in the NAc stimulates long-loop negative feedback pathway from the NAc to the VTA reducing somatodendritic DA release, while the sole activation of D1- or D2-like receptors in the NAc reduces DA terminal release but without any effect in the VTA (Rahman and McBride, 2001).

Recent research has shown that the chemokine receptor CCR2 contributes to depression associated with neuropathic pain by increasing NR2B-mediated currents in both D1- and D2- MSNs in the NAc shell (Wu et al., 2018). Further experiments showed that inhibition of CCR2 in D1R-MSN and D2R-MSN reduced SNL-induced neuropathic pain and depressive-like behavior (Wu et al., 2018). Using whole-cell patch-clamp electrophysiology, Francis et al. (2015) found that the excitatory synaptic input frequency of D1-MSNs decreased while that of D2-MSNs increased in mice that exhibited depressive-like behavior after experiencing CSDS. Notably, bidirectional alterations in D1-MSN activity promoted the opposite behavioral outcome of chronic social stress, while bidirectional modulation of D2-MSN did not alter the behavioral response to CSDS (Francis et al., 2015). The relationship of NAc neurons in rats that co-express DRD1 and DRD2, forming D1-D2 heterodimers, with depression is unclear. MSNs have been shown to have the unique property of expressing D1-D2 receptor heterodimers. The NAc exhibits relatively abundant D1–D2 heterodimers (Perreault et al., 2010), and activation of D1–D2 heterodimers in the NAc shell can alter the expression of proteins involved in GABA and glutamate activity in VTA and the SNr (Fatima et al., 2020). The polylactic acid (PLA), fluorescence resonance energy transfer (FRET), and immunoprecipitation techniques were used to establish the presence of D1-D2 heterodimers in the striatum of rats and monkeys. Perreault et al. (2010) found that in NAc cell bodies, the energy transfer between fluorescent-labeled D1R and D2R was very high, indicating a stronger receptor-receptor interaction and higher densities of heterodimers. Those authors subsequently showed that MSNs co-expressing DRD1 and DRD2 showed a unique dual GABA/glutamate phenotype and activation of the D1R–D2R heterodimers altered the expression of proteins involved in GABA and glutamate activity in regions of the mesolimbic and nigrostriatal pathways (Fatima et al., 2020). A novel mechanism that modulates depressive-like and anxiety-like behavior in rats through the DA system involves D1-D2 receptor heterodimers (Shen et al., 2015). Recent research suggests that higher D1-D2 heterodimer expression in female animals may significantly increase susceptibility to depressive-like and anxiety-like behavior (Hasbi et al., 2020c). Specifically, compared with male rats, activation of D1-D2 heterodimers in the NAc of female rats resulted in greater activation of BDNF/TrkB and Akt/GSK3/β-catenin, two important depression-related signaling pathways, and this difference may explain the greater predisposition of female rats to depressive and anxiety behaviors (Hasbi et al., 2020c). In Sprague-Dawley rats, selective activation of D1-D2 heterodimer increased grooming behavior and reduced AMPA receptor GluA1 phosphorylation via calcium/calmodulin kinase II-α, suggesting that D1-D2 heterodimer play a role in modulating the sensitivity of the reward pathway (Perreault et al., 2010). Therefore, targeting D1-MSN/D2-MSN activity may provide novel treatment strategies for depression or other affective disorders (Francis et al., 2015).

The Prefrontal Cortex

DA regulation in the PFC plays a key role in the modulation of stress responsiveness, cognition, motivation, and emotional behavior, and DA regulation mediates a variety of effects on neuronal physiology and function in the PFC (Cohen et al., 2012). Previous studies have shown that decreased DAergic transmission in the medial PFC is associated with the pathophysiology of depression. All subtypes of D1-like receptors and D2-like receptors are present in PFC, but DRD1 receptors are the most abundant (Santana et al., 2009). The DRD1 receptor is highly expressed in the glutamatergic pyramidal cells of the PFC (Arnsten et al., 1994), while the DRD2 receptor is most commonly found in GABAergic interneurons in the PFC and plays a role in inhibiting NMDA receptor-mediated excitatory neurotransmission. In cortical regions, DA modulates excitatory postsynaptic currents (EPSCs) and inhibitory postsynaptic currents (IPSCs) through DRD1 and DRD2 receptors (Zheng et al., 1999; Trantham-Davidson et al., 2004). Activation of the D1 receptor enhances the firing of DRD1+ pyramidal cells and VIP-positive (VIP+) interneurons, which indicates that the DRD1 receptor enhances both excitatory and inhibitory microcircuits in the PFC (Anastasiades et al., 2019). Alterations in DRD1 density are associated with cognitive dysfunction in psychiatric disorders, and in the PFC DRD1 also plays a key role in the regulation of working memory (McCarthy et al., 2020). Infusions of a DRD1 DA receptor agonist directly into the mPFC and infusion of DRD2 receptor antagonist into the NAc shell, reduced stress-induced behavioral changes in DA-deficient rats, indicating that DAergic transmission via DRD1 in the mPFC modulates DRD2 -mediated stress responsiveness in the NAc (Scornaiencki et al., 2009). Pyramidal neurons (PYR), as the main output neurons in the mPFC, play an important role in stress-induced cortical dysfunction. Recent evidence has shown that PYR neurons expressing DA DRD1 (D1-PYR) or DRD2 (D2-PYR) exhibit differences in ion channel expression, inhibitory synaptic innervation, and subcortical projection targets (Anastasiades et al., 2019). DRD1 and DRD2 are expressed on glutamatergic PYR neurons in the PFC, but the role of D1 and D2 receptors expressed in PFC PYR in depression and antidepressant responses is largely unknown.

DA activates DRD1 and DRD2 receptors in PFC, signaling by stimulating Gαs or inhibiting Gαi proteins respectively, as well as β-arrestins, to regulate the activity of pyramidal neurons and interneurons (Beaulieu et al., 2007). Although several antidepressant drugs can affect the DA system of the mPFC, the role of the D1-like or D2-like receptors in the PFC region in the antidepressant process is still unclear. l-SPD, which has a unique pharmacological profile of DRD1 agonism and DRD2 antagonism exerted antidepressant-like effects on the CMS model of depression (Zhang et al., 2017). Specifically, l-SPD activates the

downstream signaling of the PKA/mTOR pathway, leading to an increase in expression of the synaptogenesis-related proteins PSD 95 and synapsin I. Additionally, l-SPD also triggers long-term potentiation in the mPFC, suggesting that the D1R/PKA/mTOR signaling cascade plays a key role in l-SPD-mediated antidepressant responses (Zhang et al., 2017). Recent studies have shown that elevated mPFC DA levels may further enhance excitatory synaptic transmission through activation of the D1/PKA/DARPP32 intracellular signaling pathways, which may be the underlying mechanism of antidepressant-like effects. Recently, the mechanism of the antidepressant-like action of ketamine in the PFC region has become increasingly clear (Wohleb et al., 2017). Ketamine infusions in the ilPFC are sufficient to produce long-lasting antidepressant-like responses in rats (Fuchikami et al., 2015). Similar effects could be produced by optogenetic stimulation of ilPFC neurons, while the effects of systemic ketamine could be blocked by optogenetic inactivation. Further study demonstrated that the antidepressant effect of optogenetic stimulation was mediated by DRD1-expressing, but not DRD2-expressing mPFC neurons (Hare et al., 2019). Some of these effects were associated with structural changes in mPFC neurons. D1 receptor and its associated signaling pathways in mPFC neurons mediate acute stress-induced dendritic plasticity and contribute to the suppression of stress susceptibility (Shinohara et al., 2018).

By contrast, specific layer V pyramidal neuron subtypes in PFC selectively express DRD2, triggering post-depolarization of Ca^{2+} dependent channels, which can effectively regulate the activity of specific PFC neurons (Gee et al., 2012). The effect of the antidepressant venlafaxine on memory consolidation impairment in Wistar rats with chronic mild stress (an animal model of treatment-resistant depression) is related to the D2-like receptor inhibition in the ventromedial prefrontal cortex, suggesting an important relationship between depression and D2-like activity (Papp et al., 2019). Studies have shown that altered expression and function of DRD3 in patients and animal models of depression correlate with the severity of depression or depressive-like behavior. DRD3 has been extensively studied in animal models of LPS-induced inflammatory depressive-like behavior. LPS significantly reduces DRD3 in the VTA, mPFC, and NAc, key regions within the mesolimbic DAergic system. LPS reduces DRD3 in the VTA, mPFC, and NAc (Wang J. et al., 2018). The DRD3 agonist pramipexole had antidepressant effects in the LPS model, while the DRD3 antagonist NGB2904 induced depressive-like behavior by preventing the induction of pro-inflammatory cytokines and BDNF and ERK1/2-CREB signaling pathways. These findings provide a mechanism for the role of DRD3 in LPS-induced depressive-like behavior by mediating potential cross-effects between pro-inflammatory cytokines (tumor necrosis factor-α, interleukin-1β, and interleukin-6), BDNF, and changes in the ERK1/2-CREB signaling in the VTA and NAc. This indicates that DRD3 is a potential target for the treatment of depression.

Hippocampus

Hp is a complex structure in the temporal lobe associated with memory, cognition, and stress. Hp has functional differences along its dorso-ventral axis reflected in differences in gene expression and anatomical connectivity (Castro-Hernández et al., 2017). The dorsal Hp (dHp) is associated with different types of memory and cognitive function, while the ventral Hp (vHp) is associated with the emotional and motivational consequences of stress, including depression and anxiety (Bagot et al., 2015). The Hp contains high levels of glucocorticoid receptors and mediates feedback inhibition to the HPA axis. Chronic activation of this system produces changes in stress responses that contribute to the development of depression. The Hp is the most frequently studied brain region in depression research, along with other areas of the brain that are associated with stress, memory formation/consolidation, and emotion, such as the PFC and amygdala (Liu et al., 2017). Numerous studies have shown that DA receptors regulate long-term synaptic plasticity and memory function in the Hp, and also play a key role in imparting novelty and reward signals that influence memory formation (Hansen and Manahan-Vaughan, 2014; Rocchetti et al., 2015; Palacios-Filardo and Mellor, 2019; Park et al., 2021). In animal models of depression, chronic and severe stress impairs Hp-dependent explicit memory formation, and this effect can be explained by changes in hippocampal synaptic plasticity, e.g., alterations in LTP and LTD (Kim and Diamond, 2002). In the dentate gyrus (DG) of the Hp, the D1-like receptor antagonists block the LTD induced by afferent stimulation (Wiescholleck and Manahan-Vaughan, 2014). Pharmacological, genetic, biochemical, and imaging methods have been used to show that activation of DRD1 in the hippocampus, but not DRD2, increases calcium inflow through NMDA receptors, which enhances the MEK-ERK and mTOR pathways (David et al., 2020). Both pathways inhibit eEF2K activity by phosphorylation of eEF2K on e366, resulting in dephosphorization of eEF2 Thr56, suggesting that eEF2 may be a promising therapeutic target for the treatment of depression (David et al., 2020). In models of desperate behavior induced by prenatal stress (PNS) and chronic unpredictable mild stress (CUMS), the selective non-ergoline DA D2-like receptor agonist ropinirole (ROPI) upregulates Hp and PFC developmental gene expression, possibly because D2 receptor agonists increase the levels of the rate-limiting enzyme TH in the Hp and PFC (Fatima et al., 2020). At the same time, downregulation of GSK-3β and enhanced BDNF and TH expression are observed, thereby promoting adult hippocampal neurogenesis and alleviating symptoms of depression (Fatima et al., 2020). In a model of LPS-induced peripheral inflammation, the DA DRD2/DRD3 agonist pramipexole (PPX) inhibited the increase in LPS-induced IL-1 expression and eliminated the increase in 3-nitrotyrosine (3-nitrotyrosine, 3-NT) in the Hp (Lieberknecht et al., 2017). However, the authors of this study also concluded that the antidepressant-like effects observed with PPX in LPS-treated mice may be dependent on its anti-inflammatory properties and may not be related to the activation of DAD2 receptors (Lieberknecht et al., 2017). Therefore, the role of dopamine D2 receptors in Hp needs to be further investigated. In rodents, the projection to the NAc from the vHp is associated with stress susceptibility, and stress-induced increases in vHp-NAc activity are consistent with the increase of spontaneous

excitatory postsynaptic current frequency (Muir et al., 2020). Different subpopulations of D1+ MSNs in the NAc medial shell (NAcMS) project to the VTA(D1+^{VTA} MSNs) and the VP (D1+^{VP} MSNs) and receive inputs from the vHp and basolateral amygdala (BLA) (Baimel et al., 2019). Although the vHp and BLA inputs target D1+^{VTA} and D1+^{VP} MSNs, those vHPC inputs are stronger on D1+^{VTA} MSNs. Through optogenetic manipulation to bidirectional control of afferent-specific synaptic function, a unique role for vHp-NAc in driving depressive-like behavioral phenotypes was shown (Bagot et al., 2015). These studies suggest that circuit-level therapeutic interventions that inhibit the overactivation of presynaptic vHp may constitute an effective strategy for the treatment of depression.

Lateral Habenular Nucleus

It has recently emerged that the LHb is a critical brain region in the pathophysiology of depression. There is growing evidence from rodent and human studies that abnormal activity in the LHb is associated with depressive symptoms, such as helplessness and lack of pleasure (Yang et al., 2018). Pharmacological and optogenetic manipulation of the LHb activity alters DAergic regulation of mPFC neuronal activity, which controls multiple brain processes that are relevant to depression. Chronic stress-induced hyperactivity VTA-projecting LHb neurons is associated with increased passive coping response, and the neurons show increased burst and tonic firing (Cerniauskas et al., 2019). Hyperactivity of LHb is found in both rodent models of depression and human patients with depression. In LHb neurons, p11 is a multifunctional protein associated with depression and is significantly upregulated under chronic restraint stress. That is, downregulation of P11 expression in LHb can reduce stress-induced depressive-like behavior (Seo et al., 2018). Moreover, overexpression of p11 in D2 receptor-containing LHb neurons induces depressive-like behaviors, suggesting that p11 in LHb may be a key molecule in the regulation of negative emotions (Seo et al., 2018). Quantitative proteomic screens show that LHb expression of the β form of calcium/calmodulin-dependent protein kinase type II (βCaMKII) is up-regulated in animal models of depression and down-regulated by antidepressants (Li et al., 2013).

Additional evidence shows that DA function in the habenula regulates these outputs and depressive-like behavior. DRD2 has functionally important expression in LHb, especially in the middle portion of LHb, which regulates the effect of aversive stimuli on behavioral output influenced by DAergic activity (Aizawa et al., 2012). LHb DRD2+ neurons regulate emotional behavior via negative reward signals and participate in stress-induced depressive-like behavior (Matsumoto and Hikosaka, 2007; Stamatakis and Stuber, 2012). Injecting DA antagonists into mPFC blocks aversive emotions induced by activation of the LHb-VTA pathway, and increased DA neuronal activity in the mesocortical projections, either via direct LHb inputs or indirectly *via* the rostromedial tegmental nucleus (RMTg)) (Stamatakis and Stuber, 2012). Both DRD1 and DRD2 are expressed in the LHb (Chan et al., 2017). Injection of DA receptor agonists or antagonists into LHb showed that activation or inhibition of DRD1 but not DRD2 in LHb increased anxiety-like behavior, but decreased depressive-like behavior in rats (Chan et al., 2017). The above results confirm that the DRD1+ and DRD2+ LHb neurons are important molecular and cellular determinants of depressive-like behavior. DRD1 dysfunction in the LHb increases anxiety-like behavior but decreases depressive-like behavior, and impairs aversive learning in rats, suggesting that proper activation of DRD1 in LHb is important for this processing, and manipulation of LHb neurons through DRD1 may be a target for the treatment of depression (Proulx et al., 2014). Studies report that unilateral 6-hydroxydopamine lesions of the substantia nigra pars compacta (SNc) in rats induces depressive-like behavior and hyperactivity of the LHb. Intra-LHb injection of the DRD4 receptor agonist A412997 and antagonist L-741,742 increase depressive-like behavior and produce antidepressant-like effects in SNc-lesioned rats (Hui et al., 2020). These studies suggest roles for DAergic receptor in the modulation of habenula circuits involved in depression. The role of DAergic receptor heterodimers in habenula function have not yet been explored.

Ventral Pallidum

The VP is an important node in the medial limbic network, being the primary output of the NAc and projecting to the LHb and the VTA (Wulff et al., 2019). VP is the central structure of the reward system, receiving intensive innervation from NAc, and consists mainly of GABAergic neurons, with roles in cognition and addiction (Root et al., 2015). The VP is a significant convergence point at the interface of the motivational and reward circuitry associated with depression (Smith et al., 2009). The VP mainly contains GABAergic neurons, but also contains a smaller proportion of cholinergic and glutamatergic neurons (Geisler et al., 2007; Faget et al., 2018; Tooley et al., 2018). VP neurons send projections to different areas of the brain, some associated with reward (e.g., DAergic VTA neurons) and some associated with aversion (e.g., LHb). Glutamatergic VP neurons increase the activity of neurons in the LHb, medial tegmental nucleus, and GABAergic VTA neurons and adaptively limit reward-seeking (Tooley et al., 2018). It has been shown that the VP is the convergence point of MSN expressing DA receptor type 1 (D1-MSNs) and type 2 (D2-MSNs) of the NAc (Creed et al., 2016), and targeting VP may provide a new therapeutic strategy for depression. There are two discrete circuits of parvalbumin-positive (PV) neurons in the VP, which project to either the LHb or the VTA, with consequently different potential roles in the pathogenesis of depression (Knowland et al., 2017). Optogenetic techniques have revealed that both excitatory and inhibitory VP cells drive motivational behavior, and fine-tuning these inhibitory/excitatory signaling pathways is critical for normal hedonic and motivational processes (Faget et al., 2018).

POTENTIAL ANTIDEPRESSANT-LIKE EFFECTS OF DOPAMINE RECEPTOR AGONISTS/ANTAGONISTS

The role of DA receptors in depression has attracted increasing attention recently. DA receptors have been identified in many brain

TABLE 2 | Representative dopamine receptor agonists and antagonists and their role in the treatment of depression.

Dopamine receptor agonist	Representative substance	Subjects (Methodology)	Effects on depressive-like behavior	References
D1R Agonist	Pergolide	41 non-demented patients suffering from mild or moderate depression and Parkinson's disease	Demonstrated antidepressant effects in PD patients	Rektorová et al. (2013)
	SKF-81297	Chronically stressed rats	100 ng of SKF 81297 significantly ameliorate depressive-like behavior	Mizoguchi et al. (2002)
	SKF-38393	Adult ovariectomized female rats	Blocked the antidepressant-like effect	Fedotova and Ordyan (2011)
D2R Agonist	Cabergoline	Male Wistar and Wistar-Kyoto rats	Antidepressant-like property	Chiba et al. (2010)
	Piribedil	The placebo-controlled, randomized, double-blinded trial was conducted in 37 patients with Parkinson's disease presenting with apathy	Dopamine agonist piribedil improves apathy in Parkinson's disease	Thobois et al. (2013)
	Quinpirole	Sprague Dawley rats	Delivery of quinpirole into the NAc of control animals induced depressive-like behaviors	Qiao et al. (2020)
	Pramipexole	five RCTs, three open-label trials, and five observational studies, with 504 participants	Patients treated with Pramipexole showed improvement in depressive symptoms	Tundo et al. (2019)
	Ropinirole	32 unipolar and bipolar patients who remained depressed	No difference in primary or secondary outcome measures was detected between the treatment and control groups	Gershon et al. (2019)
	L-742,626	Wistar or Wistar-Kyoto rats	Venlafaxine reversed the effect of L-742,626 in controls	Papp et al. (2019)
D3R Agonist	Rotigotine	48 PD patients	Rotigotine improves apathy, depression, and anxiety in PD patients	Castrioto et al. (2020)
D4R Agonist	PD-168,077	174 transverse hippocampal slices (400 μm) prepared from 87 male Wistar rats	D4R activation induces synaptic depression	Navakkode et al. (2017)
D1/D5R Agonist	SKF-38393	Adult zebrafish (AB wild-type; ~50:50 male: female ratio at 4-month of age)	Pretreatment with the agonist SKF-38393 protects subjects from conspecific alarm substance (CAS, a natural stressor)	Fontana et al. (2021)
D1R Antagonist	SKF-83566	Forty-nine male Long–Evans rats	Administration of SKF 83566 blocked LTP in mPFC and resulted in long-term depression induced by high-frequency stimulation	Coppa-Hopman et al. (2009)
	Haloperidol	seven patients (five men and two women; mean age = 36.7 years, SD = 13.8, range = 26–61)	The first description of the efficacy and safety of the SSRI citalopram in combination with haloperidol in the treatment of psychotic depression	Bonomo et al. (2002)
D2R Antagonist	Raclopride	Adult outpatients with depression >59 years old	Depression status is associated with lower [[11C] raclopride binding	Rutherford et al. (2019)
	Sulpiride	ten patients and ten age-matched male volunteers	Improve depressive-like behavior and may have the effect of increasing dopamine turnover	Verbeeck et al. (2001)
	Risperidone	16-week randomized placebo-controlled trial for participants concurrently treated with risperidone	A combination of Risperidone and Omega-3 improves depressive-like behavior	Robinson et al. (2019)
D3R Antagonist	7-OH-DPAT	Wistar or Wistar-Kyoto rats	Potential anti-anxiety and antidepressant effects	Rogóz et al. (2004)
D4R Antagonist	L-741,742	Male Sprague Dawley rats	Electrophysiological currents were inhibited by DA-D4-receptor antagonist L-741,742 and it was observed in LHb neurons when DA uptake or degradation was blocked	Root et al. (2015)
D1/D5R Antagonist	SCH-23390	Gerbil (*Meriones unguiculatus*, *n* = 130) pups	Decreasing dopamine receptor signaling diminishes social learning	Paraouty et al. (2021)

regions associated with the development of depression. Unfortunately, traditional approaches that directly manipulate DA receptors cannot be used in clinical practice because of their effects on blood pressure (Sassano-Higgins et al., 2011; Wang S. et al., 2018). An increasing number of studies have shown that the action of DA receptor modulators may be a potential treatment for depression if these peripheral DA effects can be overcome. In recent years, PET imaging with [11C]-(+)-PHNO has enabled researchers to assess *in vivo* the occupancy of DA receptors and/or their down- or up-regulation by given drug treatment (Leggio et al., 2016). Today, the search for useful molecular determinants of DA selectivity seems achievable. The advances in D1- and D2-like receptor agonists and antagonists have provided more selective compounds that may be able to selectively target different DA receptors and perhaps address depression-related symptoms. D1R is a promising drug target, where its selective activation may provide a new approach for the treatment of depression. In female rats, repeated injections of the D1 receptor partial agonists SKF 83959 increase BDNF expression and TrkB activation, thereby affecting depressive and anxiety-like behavior (Hasbi et al., 2020c). Pergolide targets DRD1 and DRD2 receptors and improves visual-spatial working memory, verbal learning and memory, and executive function in schizotypal patients (McClure et al., 2010), indicating that DA agonists may be beneficial for cognitive abnormalities in

schizophrenia spectrum disorders. These effects may extend to antidepressant effects. Studies have shown that depressive-like behavior induced by chronic unpredictable stress (CUS) is accompanied by a significant decrease in both DA levels and DRD2 expression in NAc. Before CUS in Sprague Dawley rats, infusion of the DRD2 agonist Quinpirole and DRD2 antagonist eticlopride to NAc reversed depressive-like behavior induced by CUS and normalized DA levels in NAc (Qiao et al., 2020). Interestingly, the most recent studies found that Partial agonist activity of the DRD2 is a key feature of third-generation antipsychotics (TGAs), which have antidepressant-like effects and improved cognitive performance (Chen et al., 2022). The unique and selective DRD2-selective partial agonist (−)-IHCH7041 may provide the medical community with chemical tools for exploring signaling pathways that counteract the efficacy and side effects of psychiatric disorders such as depression (Chen et al., 2022). A growing body of evidence also suggests that DRD3 receptor antagonists may be effective antidepressants (Maramai et al., 2016). Cariprazine is a partial agonist of D2/D3 receptors that has recently been approved in the United States for the treatment of psychiatric disorders (Findlay et al., 2017). Cariprazine reduces anhedonia resulting from chronic unpredictable stress and shows an effective antidepressant effect comparable to aripiprazole and the tricyclic antidepressant imipramine (Duric et al., 2017). Furthermore, the antianhedonic-like effect of Cariprazine was not observed in D3 knockout mice, suggesting that the cariprazine antidepressant-like activity is mediated by DRD3 (Duric et al., 2017). Aripiprazole, Blonanserin, and the D2/D3 receptors partial agonist Cariprazine play an important role in the treatment of depression, and this may not be possible without the role of D3R (Leggio et al., 2016). Therefore, the development of novel, more selective chemical scaffolds for D3R ligands may be essential. Injection of the DRD4 agonist A-412,997 or the antagonist L-741,742 into the LHb affects the expression of depressive-like behaviors and produces antidepressant-like effects in SNc-lesioned rats (Hui et al., 2020). In general, DA receptor agonists/antagonists will be a new option for the treatment of depression. Representative DA receptor agonists and antagonists and their role in the treatment of depression are summarized in **Table 2**.

CONCLUSION

According to the types of DA receptors and their distribution in different brain regions, this paper reviews the current research status of the molecular, cellular and neural circuit mechanisms of DA receptors involved in depression, including the research progress into the role of DA receptor D1-D2 heterodimers. Understanding the function and localization of DA and its receptors in the brain and the complexity of their signaling mechanisms as well as pharmacological strategies based on receptor complexes may have potential new applications in the depression pathogenesis. Multidimensional analysis of DA receptors and DA receptor-related mechanisms or post-receptor signaling cascades will provide an exciting opportunity for depression treatment, which will minimize the side effects of depression, and these approaches may be closely related to the metabolic targeting of DA receptors and heterodimers and their downstream intracellular signaling events.

AUTHOR CONTRIBUTIONS

FZ wrote the manuscript. ZC and JP made the critical revisions. RC reviewed editing and funding acquisition. BL completed conception, design, editing, and funding acquisition. All authors approved the final version of the manuscript for submission.

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GLOSSARY

5-HT serotonin
A2ARs adenosine A2A receptors
AADC aromatic L-amino acid decarboxylase
AC adenylate cyclase
ACC anterior cingulate cortex
Akt protein kinase B
AMPAR AMPA-type glutamate receptors
BDNF brain-derived neurotrophic factor
BLA basolateral amygdala
CamKII Ca^{2+}/calmodulin-dependent protein kinase II
cAMP cyclic adenosine monophosphate
CNS central nervous system
COMT cathecholamine O methyltransferase
CP-AMPAR Ca^{2+} -permeable AMPA receptor
CREB cAMP-response element binding protein
CSDS chronic social defeat stress
CSF cerebrospinal fluid
CUMS chronic unpredictable mild stress
D1/2/3/4/5R dopamine receptor subtype 1/2/3/4/5
D1-MSNs DRD1-containing MSNs
D2-MSNs DRD2-containing MSNs
DA dopamine
DAergic dopaminergic
DARPP-32 dopamine- and cAMP-regulated phosphoprotein
DAT DA transporter
DBS deep brain stimulation
DG dentate gyrus
dlVP dorsolateral subcompartment of the ventral pallidum
DRD1 dopamine receptor D1
DRD2 dopamine receptor D2
DRD3 dopamine receptor D3
DRD4 dopamine receptor D4
DRD5 dopamine receptor D5
ECT electroconvulsive treatment
EPSCs excitatory postsynaptic currents
ERK extracellular signal-regulated kinase
fMRI functional magnetic resonance imaging
FRET fluorescence resonance energy transfer
GABA gamma-aminobutyric acid
$GABR_{A6}$ GABA receptor alpha 6 subunits
GPCR G protein-coupled receptorG protein-coupled receptors
GPCR G protein-coupled receptorG protein-coupled receptors
GSK-3 glycogen synthase kinase 3
HVA homovanillic acid
ilPFC inferolateral prefrontal cortex
IP3-R inositol triphosphate receptors
IPSCs inhibitory postsynaptic currents
JNK c-Jun N-terminal kinase
l-DOPA L-3,4-dihydroxyphenyl-L-alanine
LHb lateral habenular nucleus
LNAA large neutral amino-acid
LPS lipopolysaccharide
LTD Long-term depression
LTP Long-term potentiation
MAO monoamine oxidase
MAPK mitogen-activated protein kinases
MDD major depressive disorder
mPFC medial prefrontal cortex
MSNs medium spiny neurons
NAc nucleus accumbens\
NaSSA noradrenergic and specific serotonergic antidepressants
NE norepinephrine
NMDAR NMDA-type glutamate receptors
OCT3 organic cation transporter 3
PET Positron emission tomography
PFC prefrontal cortex
PH phenylalanine hydroxylase
PKA protein kinase A
PLA proximity ligation assay
PLC phospholipase C
SNr substantia nigra reticulata
SNRIs dual 5-HT and NE reuptake inhibitors
SSRIs selective 5-HT reuptake inhibitors
TCAs tricyclic antidepressants
TH tyrosine hydroxylase
TrkB tropomysin related kinase B
VMAT vesicular monoamine transporters
vmVP ventral medial of the ventral pallidum
VP ventral pallidum
VTA ventral tegmental area

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EDITED BY
Anna Tabęcka-Łonczynska,
University of Information Technology and Management in Rzeszow, Poland

REVIEWED BY
Alicja Kowalczyk,
Wroclaw University of Environmental and Life Sciences, Poland
Kamila Rybczyńska-Tkaczyk,
University of Life Sciences of Lublin, Poland

*CORRESPONDENCE
Hong-Sheng Chang,
chs1971@sina.com
Xiang-Qing Xu,
happyxiangqing@163.com

†These authors have contributed equally to this work

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Honokiol improves depression-like behaviors in rats by HIF-1α- VEGF signaling pathway activation

Xiao-Xu Fan[1†], Wen-Yan Sun[1†], Yu Li[1], Qin Tang[1], Li-Na Li[2], Xue Yu[2], Shu-Yan Wang[2], Ang-Ran Fan[2], Xiang-Qing Xu[3*] and Hong-Sheng Chang[1*]

[1]School of Chinese Materia Medica, Beijing University of Chinese Medicine, Beijing, China, [2]School of Traditional Chinese Medicine, Beijing University of Chinese Medicine, Beijing, China, [3]Experiment Center, Encephalopathy Department, Affiliated Hospital of Shandong University of Chinese Medicine, Jinan, China

Increasing evidence indicates that the pathogenesis of depression is closely linked to impairments in neuronal synaptic plasticity. Honokiol, a biologically active substance extracted from *Magnolia Officinalis*, has been proven to exert significant antidepressant effects. However, the specific mechanism of action remains unclear. In this study, PC12 cells and chronic unpredictable mild stress (CUMS) model rats were used to explore the antidepressant effects and potential mechanisms of honokiol *in vitro* and in rats. *In vitro* experiment, a cell viability detection kit was used to screen the concentration and time of honokiol administration. PC12 cells were administered with hypoxia-inducible factor-1α (HIF-1α) blocker, 2-methoxyestradiol (2-ME), and vascular endothelial growth factor receptor 2 (VEGFR-2) blocker, SU5416, to detect the expression of HIF-1α, VEGF, synaptic protein 1 (SYN 1), and postsynaptic density protein 95 (PSD 95) by western blotting. In effect, we investigated whether the synaptic plasticity action of honokiol was dependent on the HIF-1α-VEGF pathway. *In vivo*, behavioral tests were used to evaluate the reproducibility of the CUMS depression model and depression-like behaviors. Molecular biology techniques were used to examine mRNA and protein expression of the HIF-1α-VEGF signaling pathway and synaptic plasticity-related regulators. Additionally, molecular docking techniques were used to study the interaction between honokiol and target proteins, and predict their binding patterns and affinities. Experimental results showed that honokiol significantly reversed CUMS-induced depression-like behaviors. Mechanically, honokiol exerted a significant antidepressant effect by enhancing synaptic plasticity. At the

Abbreviations: 2-ME, 2-methoxyestradiol; CNS, central nervous system; CUMS, chronic unpredictable mild stress; HIF-1α, hypoxia-inducible factor-1α; HP, hypoxic preconditioning; HPA, hypothalamus-pituitary-adrenal; HREs, hypoxia response elements; IH, intermittent hypoxia; MAOIs, monoamine oxidase inhibitors; OFT, open field tests; PSD 95, postsynaptic density protein 95; SPT, sucrose preference tests; SSRIs, selective serotonin reuptake inhibitors; SYN 1, synapsin 1; TCAs, tricyclic antidepressants; VEGF, vascular endothelial growth factor; VEGFR-2, vascular endothelial growth factor receptor 2.

molecular level, honokiol can activate the HIF-1α-VEGF signaling pathway *in vitro* and *in vivo*, as well as promote the protein expression levels of SYN 1 and PSD 95. Taken together, the results do not only provide an experimental basis for honokiol in the clinical treatment of depression but also suggest that the HIF-1α-VEGF pathway may be a potential target for the treatment of depression.

KEYWORDS

honokiol, antidepressant effect, HIF-1α-VEGF signaling pathway, synapse plasticity, molecular docking

Introduction

Depression is a severe psychiatric disorder characterized by a significant and persistent low mood (Niu and Snyder, 2022). Common symptoms include slow thinking, impaired cognitive function, diminished volitional activity, sleep disturbances, and appetite loss (LeMoult and Gotlib, 2019). With the development of society, the incidence rate of depression is increasing due to several factors, such as social, environmental, psychological, and genetic factors. Depression, one of the main causes of diseases globally, severely endangers the physical and mental health of individuals. No effective drug is available to treat depression, which exhibits a disturbingly complex pathogenesis. Most classical antidepressants currently used in clinical practice, such as tricyclic antidepressants (TCAs), monoamine oxidase inhibitors (MAOIs), and selective serotonin (5-HT) reuptake inhibitors (SSRIs), are only effective in 30%–40% of patients. Besides, they have drug resistance and, high recurrence rate. Long-term use can cause sleep disorders, gastrointestinal disorders, cardiotoxicity, sexual dysfunction, and other side effects (Racagni and Popoli, 2010; Correll et al., 2015; Blackburn, 2019). Therefore, due to the urgent need to discover safer and more effective drugs for treating depression, in-depth research on the pathogenesis of depression is becoming increasingly important.

Honokiol is a polyphenol compound with few toxic side effects and several pharmacological effects. In recent years, honokiol has been found to have an obvious protective effect on the nervous system by regulating neurotrophic factors and promoting nerve regeneration (Woodbury et al., 2013; Talarek et al., 2017). Additionally, it has other pharmacological effects, such as free radical scavenging, anti-inflammatory, analgesic, antioxidant, antibacterial, and antitumor effects (Wang et al., 2020; Chen et al., 2021; Trifan et al., 2021). Honokiol has potent antidepressant activity when it is used alone or in combintion. Studies have shown that honokiol combined with ginger oil has an antidepressant effect in CUMS model rats (Qiang et al., 2009). The alcohol extract of Banxia Houpu Decoction is rich in honokiol, which can reduce the changes in brain neurotransmitters and prevent immune and inflammatory reactions to exert antidepressant properties on rodent depression models (Wang et al., 2005). Honokiol has protective effects on brain regions, such as the hippocampus and cortical neurons (Borgonetti et al., 2021). Furthermore, it can block hippocampal endoplasmic reticulum stress to eliminate cognitive impairment and depression-like behaviors induced by chronic restraint stress (Jangra et al., 2016). Other studies have reported that honokiol normalized the function of the hypothalamus-pituitary-adrenal (HPA) axis and increased the level of brain-derived neurotrophic factor in the hippocampus, which has an antidepressant effect on CUMS rat models (Wang et al., 2018). In addition to the above studies, numerous experimental studies have shown that honokiol has therapeutic effects in acute and chronic stress models, lipopolysaccharide depression model, and corticosterone-induced depression model (Xu et al., 2008; Pitta et al., 2013; Sulakhiya et al., 2014). Honokiol is a small molecule that readily enters the central nervous system through the blood-brain barrier to have a direct effect on nerve cells (Lin et al., 2012). It has wide potential to develop into a drug for neurological diseases. Therefore, clarifying the antidepressant mechanism of honokiol has a reference role in the development of novel antidepressants.

Hypoxia-inducible factor-1 (HIF-1) is a transcription factor of hypoxia response. As a major regulator of cell oxygen homeostasis in mammalian cells, HIF-1 is composed of the active subunit, HIF-1α, and the constitutive subunit, HIF-1β (Kaelin and Ratcliffe, 2008). Increasing the level of hypoxia-inducible factors may become a potential target for depression therapy (Kang et al., 2021). Research has proven that hypoxic preconditioning (HP) produces significant antidepressant effects by increasing the expression of HIF-1α in the hippocampus, hypothalamic paraventricular nucleus, and neocortex of rats (Baranova et al., 2010). The target genes of HIF-1, erythropoietin (EPO), and vascular endothelial growth factor (VEGF), have been proven to elicit antidepressant effects in animal models (Deyama et al., 2019). Indeed, VEGF plays an essential role in the antidepressant effects. Peripheral administration of EPO produces a robust antidepressant-like effect (Girgenti et al., 2009). More importantly, studies have found that activating the HIF-1α-VEGF signaling pathway can effectively reverse CUMS depression-like behaviors and memory impairment and promote synaptic growth in primary hippocampal neurons. The HIF-1α-VEGF pathway plays an

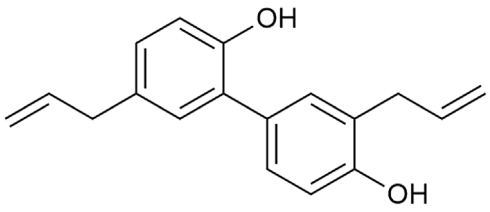

FIGURE 1
The structure of honokiol (Chemical formula: $C_{18}H_{18}O_2$; molecular weight: 266.34).

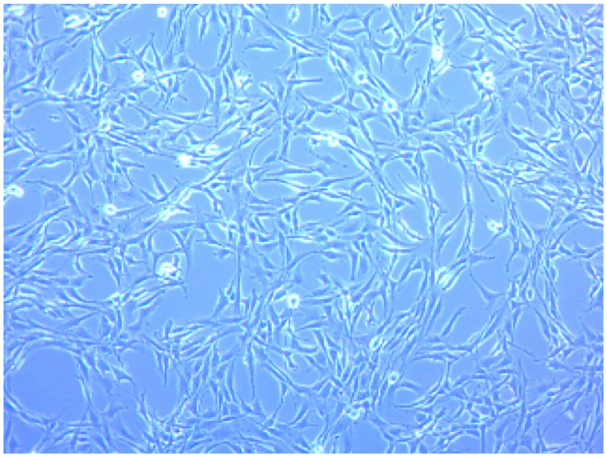

FIGURE 2
Morphology of normally cultured PC12 cells under the light microscope (×100).

important role in promoting hippocampal neurogenesis and synaptic plasticity *in vivo* and *in vitro* (Li G. et al., 2020). Collectively, activation of the HIF-1α-VEGF signaling pathway is a promising strategy to improve depression-like behaviors.

Our previous study found that 10 mg/kg of honokiol can significantly improve depression-like behaviors in acute and chronic stress mouse models (Wang et al., 2017). The aim of the present study was to investigate the necessity of the HIF-1α-VEGF pathway in enhancing neuronal synaptic plasticity with honokiol and to clarify the antidepressant effect of honokiol on CUMS depression rat models by activating the HIF-1α-VEGF pathway.

Materials and methods

Animals and cells

Male Sprague Dawley rats, specific pathogen-free (SPF) grade, weighing 200–220 g, were obtained from SiPeiFu Biotechnology Co., Ltd., [Animal license number: SYXK (Beijing) 2020-0033]. All animal procedures were approved by the Experimental Animal Ethics Committee of the Academic Committee of Beijing University of Chinese Medicine (Project identification code: BUCM-4-2021090301–3049). Rat pheochromocytoma PC12 cells were generously provided by the Research and Experiment Center, School of Traditional Chinese Medicine, Beijing University of Chinese Medicine.

Drugs and reagents

Drugs used included paroxetine (catalog number: H13M10Z82647, Yuanye, Shanghai, China), SU5416 (catalog number: ab145056, Abcam, Cambridge, United States), 2-methoxyestradiol (catalog number: A4188, APE×BIO, Houston, United States), and honokiol (catalog number: SH8140, Solarbio, Beijing, China). The structure of honokiol is shown in Figure 1.

All reagents for cell culture were purchased from Gibco (Carlsbad, CA, United States). Protease inhibitor (catalog number: KGP603, Kaiji Biology, Jiangsu, China); phosphatase inhibitor (catalog number: KGP602, Kaiji Biology, Jiangsu, China). The primary antibodies and their dilution ratios in western blot were as follows: anti-HIF-1α (1:1,000, Abcam Cat# ab179483, RRID:AB_2732807), anti-phospho-PI3K (1:500, Abcam Cat# ab182651, RRID:AB_2756407), anti-VEGF (1:3000, Proteintech Cat#19003-1-AP, RRID:AB_2212657), anti-VEGFR-2 (1:800, Proteintech Cat# 26415-1-AP, RRID:AB_2756527), anti-PI3K (1:10000, Proteintech Cat# 60225-1-Ig, RRID:AB_11042594), anti-AKT (1:5,000, Proteintech Cat# 60203-2-Ig, RRID:AB_10912803), anti-phospho-AKT (1:5,000, Proteintech Cat# 66444-1-Ig, RRID:AB_2782958), anti-mTOR (1:25,000, Proteintech Cat# 66888-1-Ig, RRID:AB_2882219), anti-SYN 1 (1:6,000, Proteintech Cat# 20258-1-AP, RRID:AB_2800493), anti-PSD 95 (1:3,000, Proteintech Cat# 20665-1-AP, RRID:AB_2687961), anti-α-Tubulin (1:5,000, Proteintech Cat# 11224-1-AP, RRID:AB_2210206), anti-phospho-mTOR (1:1,000, Cell Signaling Technology Cat# 5536, RRID:AB_10691552). The secondary antibodies and their dilution ratios in western blot were as follows: HRP-conjugated affinipure goat anti-rabbit IgG (1:5,000, Proteintech Cat# SA00001-2, RRID: AB_2722564), and HRP-conjugated affinipure goat anti-mouse IgG (1:5,000, Proteintech Cat# SA00001-1, RRID: AB_2722565).

Cell study design

Cell culture

PC12 cells were cultured in Dulbecco Modified Eagle Medium (DMEM; high glucose), which contained 10% fetal bovine serum and 1% penicillin/streptomycin at 37°C with 5%

CO_2. The medium was changed every 1 or 2 days. Generally, cell passage was performed when the cell fusion was approximately 80%. Cells in the logarithmic phase were used for subsequent experiments. Under suitable conditions, the cells adhered to the wall in the form of clusters with clearly visible edges. Their specific shape was shuttle-shaped with a strong refractive index, as shown in Figure 2.

Cell viability assay

The viability of PC12 cells was evaluated using Cell Counting Kit-8 (catalog number: CK04, Tongren, Japan). PC12 cells were seeded at a density of 5×10^3 cells in one well of a 96-well plate. After culturing for 24 h, the cells were washed three times with 1 × PBS. Subsequently, PC12 cells were treated with 2, 5, 8, 10, and 16 μM honokiol at 37°C under 5% CO_2 for 24 or 48 h. PC12 cells were incubated with CCK-8 working solution at 37°C for 1 h in the dark. Finally, the absorbance of each well was measured using a 96-well microplate reader (Shanghai Thermo Fisher Scientific, Inc.) at 450 nm. Relative cell viability was defined and calculated using the following formula: [A (experimental group)—A (blank)]/[A (control group)—A (blank)].

HIF-1α and VEGFR-2 blocking experiments

Cell experiments were divided into six groups: Control (Con), Con + Honokiol, Con + Honokiol + SU5416, Con + Honokiol + 2-ME, Con + SU5416, and Con + 2-ME groups. PC12 cells were cultured in 6-well plates at a density of 5×10^5 cells per well for 24 h. After culturing, the cells were treated accordingly with honokiol (2 μM), 2-ME (5 μM), and SU5416 (4 μM). Specifically, cells in the Con + Honokiol group were treated with 2 μM honokiol for 24 h; cells in the Con + SU5416 group were pretreated with 4 μM SU5416 for 30 min and then replaced with new DMEM for 24 h; cells in the Con + Honokiol + SU5416 group were pretreated with 4 μM SU5416 for 30 min before honokiol treatment for 24 h; cells in the Con+2-ME group were pretreated with 5 μM 2-ME for 30 min and then replaced with new DMEM for 24 h; cells in the Con + Honokiol+2-ME group were pretreated with 5 μM 2-ME for 30 min before honokiol treatment for 24 h. After incubation, HIF-1α, VEGF, SYN 1, and PSD 95 protein expressions were detected by western blotting. SU5416, 2-ME, and honokiol were diluted in DMSO to corresponding concentrations, and the final concentration of DMSO was fixed at 0.1%. The dosages of these blockers have been previously reported by other studies (Fournier et al., 2012; Khan et al., 2015).

Animal study design

Establishment of chronic unpredictable mild stress depression model

Before the experiment, all rats were fed normally for 7 days under standard laboratory conditions, including constant temperature (20–24°C), humidity (50–55%), standard ventilation system, and standard drinking water and food, to ensure that the rats adapt to the environment.

Rats were randomly divided into four groups (n = 8 in each group): Control (Con), CUMS (CUMS + saline), CUMS + Par (CUMS + 4.8 mg/kg paroxetine, i.p.), and CUMS + Honokiol (CUMS+10 mg/kg Honokiol, i.g.) groups. All rats, except those in the control group, were exposed to chronic unpredictable mild stress for 28 days. Stressors are shown in Table 1. The same stressor was not repeated within 7 days; otherwise, the rats would predict the stressors. Honokiol was suspended in 0.5% sodium carboxymethylcellulose (CMC-Na) at a certain concentration before use. The control and CUMS groups were given the same amount of saline. The CUMS + Par group received 4.8 mg/kg honokiol intraperitoneally. The CUMS + Honokiol group was given 10 mg/kg honokiol by intragastric administration. Each group of rats received the corresponding drug for 21 days after modeling. After the last dose on day 21, sucrose preference test and open field test were performed, respectively. After one of behavioral tests finished, rats were allowed to rest for a day before proceeding to the next one. The rats were weighed and anesthetized by intraperitoneal injection of 1% pentobarbital (2.75 ml/kg) before being sacrificed. In preparing the 1% pentobarbital, 0.9% saline was used. Finally, the hippocampus was taken for the detection of related indicators.

Open field test

The experiment was conducted in a quiet and stable light environment. Rats were brought to the testing room to adapt for 1 h before the OFT. The experimental setup consisted of two parts: the open field reaction chamber and the recording and analysis system. The open box with a circular bottom was 40 cm high, and 100 cm long in diameter. The bottom of the open box was divided equally into 25 small squares of equal areas. A digital camera was set up 2 m above the open field, which could cover the entire interior of the open field. A rat was placed in the center of an open box to adapt for 1 min, and then the small animal behavior recording analysis system (Etho-Vision XT9, Noldus, Netherlands) was used to record the distance and velocity of movement within 5 min. Before each rat was tested, the open box was cleaned with alcohol to remove all feces and odor left by previous rats.

Sucrose preference test

SPT was used to assess anhedonia in rats. Each rat was reared in a single cage during the test. The test consisted of two stages: 1) Adaptation stage: each rat was simultaneously given a bottle of 1% sucrose water and pure water for 48 h; 2) Test stage: after the adaptation phase, the rats were deprived of water for 24 h. Subsequently, each rat was given a bottle of 1% sucrose water and one bottle of pure water. Rats were free to drink during the test for 3 h. The consumption of sucrose water and pure water

TABLE 1 Chronic unpredictable mild stress (CUMS) procedure.

Stressors	Details
Food and water deprivation	Rats were subjected to 24 h of food and water deprivation
Tail pinching	Tail pinch 1 cm from the beginning of the tail for 2 min
Physical restrain	Confinement in a tube for 2 h
Cold water swimming	Rats were placed in a cylindrical clear glass jar filled with 4°C cold water for 5 min
Wet bedding	Wet bedding for 24 h
Continuous illumination	Continuous illumination for 24 h
Cage tilt	45°cage inclined for 12 h

TABLE 2 Primer sequences.

Name of gene	Primer	Sequence (5′–3′)
HIF-1α	Forward	GTCAGCAACGTGGAAGGTGC
	Reverse	GCACCAAGCACGTCATAGGC
VEGF	Forward	GCAGTGCTCCCCATCCGCTG
	Reverse	TGCTCGTCCGACAGCTGGGA
VEGFR-2	Forward	TGGCAATTCCCGTCCTCAAAGC
	Reverse	CTTGGTCACTCTTGGTCACACTGTC
PI3K	Forward	GCAACTCCTGGACTGCAACT
	Reverse	CAGCGCACTGTCATGGTATG
AKT	Forward	TAGCCATTGTGAAGGAGGGC
	Reverse	CCTGAGGCCGTTCCTTGTAG
mTOR	Forward	GCTCCAGCACTATGTCACCA
	Reverse	CGTCTGAGCTGGAAACCAGT
β-actin	Forward	CATCCTGCGTCTGGACCTGG
	Reverse	TAATGTCACGCACGATTTCC

during the test was weighed. The sucrose preference was calculated as the percentage of sucrose consumption in the total consumption of sucrose water and pure water.

Quantitative real-time PCR assay

Total RNA was extracted from the hippocampi of rats using the Hipure Total RNA Mini Kit (catalog number: R4111-02, MAGEN). RNA concentration of every sample was measured using an ultraviolet spectrophotometer (UV-2000, Unico). Samples were placed on ice during the operation to prevent RNA degradation. The RevertAid First Strand cDNA Synthesis Kit (catalog number: K1622, Thermo Scientific) and the Power SYBR Green PCR Master Mix (catalog number: 4367659, Invitrogen) were used to reverse transcription. Thereafter, the following scheme was adopted: initial denaturation at 95°C for 10 min, denaturation at 95°C for 10 s, annealing at 60°C, and extension for 30 s. Fifty amplification cycles were completed. Amplification and quantification were performed using a Real-Time PCR device (Bio-Rad, USA). β-Actin was considered as the internal reference. The results were analyzed using the $2^{-\Delta\Delta Ct}$ method for relative quantification. The sequences of involved primers are shown in Table 2.

Western blotting

Total protein was extracted from rat hippocampi using RIPA lysis buffer containing protease- and phosphatase inhibitors. After homogenization, the solution was allowed to stand on ice for 20 min before centrifugation at 13,000 rpm at 4°C for 15 min. After the supernatant was collected, protein concentration was measured using a BCA protein assay kit (catalog number: KGP902, Kaiji Biology, Jiangsu, China). Thereafter, 20 μg protein was separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to PVDF membranes (0.45 μm, Millipore, United States) at 300 mA for 1.5 h. The membranes were blocked with 5% skim milk for 1.5 h and then incubated with the primary antibodies at 4°C overnight. After washing with TBST thrice, the membranes were then incubated with secondary antibodies at room temperature for 1 h. Subsequently, the membranes were developed using the Ultrasensitive ECL Chemiluminescence Kit (catalog number: P10100, NCM, Jiangsu, China). The labeled proteins were detected using a fully automated chemiluminescence image analysis system (Tanon-5200, Shanghai, China). Gray value analysis was performed using the ImageJ software (The National Institutes of Health, USA).

Molecular docking

Three-dimensional structures of target proteins associated with depression, including HIF-1α, VEGF, and VEGFR-2, were downloaded from the RCSB PDB database (https://www.rcsb.org/). The crystal structures were selected from the species "Homo sapiens", with a resolution of < 3 and intact binding sites. This was then saved as a PDB format. Water molecules and small molecule ligands were removed from the protein structures using the PyMol 2.4.1 software (Delano Scientific LLC, Italy). The AutoDockTools software was used to add hydrogen and finally convert to the PDBQT

format. The two-dimensional structure of honokiol was obtained from the PubChem database (https://pubchem.ncbi.nlm.nih.gov/) and saved in the SDF format. Afterwards, the SDF format was converted to the Mol2 format using the Open Bable 2.4.1 software. Finally, the AutoDockTools software was used to assign charges, detect rotatable keys, and save them as PDBQT format files. The AutoDock 4.2.6 software (The Scripps Research Institute, USA) was used to perform molecular docking analysis on targets and active ingredients. Visualization was performed using the PyMol 2.4.1 software.

Statistical analysis

The experimental data are expressed as the mean ± standard error of the mean (SEM). GraphPad Prism 9 (GraphPad Software Inc, San Diego, CA, United States) and SPSS 20 (SPSS, Chicago, Illinois, United States) were used for statistical analysis and graphing. Data with normal distribution and homogeneity of variance were analyzed using one-way ANOVA followed by an LSD post hoc test. $p > 0.05$ was considered normally distributed. The Kruskal-Wallis test was used for data with non-normal distribution. Differences were statistically significant when $p < 0.05$.

Results

Effects of honokiol on PC12 cells

Effects of honokiol on the viability of normal PC12 cells

First, this study screened the concentration and time of honokiol administration. PC12 cells were exposed to different concentrations of honokiol for 24 and 48 h. After honokiol treatment for 24 h, PC12 cell viability was observed to have a significant increase at 2 and 5 μM and a marked decrease at 16 μM, compared to the control group (Figure 3; $F_{5,12} = 12.678$; $^{*}p < 0.05$; $^{**}p < 0.01$). Additionally, the cell viability of 5 μM honokiol was not significantly different from than of the control group at 48 h. Cell viability began to decrease significantly when the concentration of honokiol was > 8 μM (Figure 3; $F_{5,12} = 11.497$; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$). Furthermore, the cell viability at 16 μM honokiol for 24 and 48 h were 51.0 ± 16.9% ($F_{5,12} = 12.678$, $^{**}p < 0.01$) and 44.3 ± 12.9% ($F_{5,12} = 11.497$, $^{***}p < 0.001$). Additionally, the cell viability was as high as 135.4 ± 10.6% for 24 h at 2 μM honokiol, which showed a significant pro-proliferative effect, and showed no significant difference from that of 5 and 8 μM honokiol. Therefore, 2 μM honokiol for 24 h incubation was chosen as the concentration and time of honokiol administration.

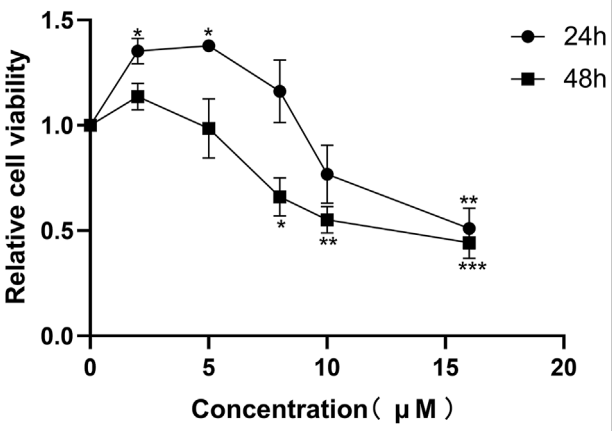

FIGURE 3
Effects of honokiol on the viability of normal PC12 cells. Data are expressed as mean ± SEM ($n = 3$). $^{*}p < 0.05$ vs. Con group. $^{**}p < 0.01$ vs. Con group. $^{***}p < 0.001$ vs. Con group.

Effects of SU5416 and 2-ME on synaptic plasticity of neurons in PC12 cells

To investigate the mechanism of the antidepressant-like effect of honokiol, we used 2-ME and SU5416 to explore whether the neuronal synaptic plasticity effect of honokiol was dependent on the HIF-1α-VEGF signaling pathway. HIF-1α ($F_{5,12} = 4.05$, $p = 0.002$), VEGF ($F_{5,12} = 3.121$, $p = 0.044$), PSD 95 ($F_{5,12} = 3.121$, $p = 0.011$), and SYN-1($F_{5,12} = 3.317$, $p = 0.045$) protein expressions were significantly higher after honokiol treatment, compared to the control group (Figure 4). Compared with the Con + Honokiol group, HIF-1α ($F_{5,12} = 4.05$, $p = 0.012$), VEGF ($F_{5,12} = 3.121$, $p = 0.004$), PSD 95 ($F_{5,12} = 3.121$, $p = 0.007$), and SYN 1 ($F_{5,12} = 3.317$, $p = 0.005$) protein expressions were significantly lower after 2-ME treatment (Figure 4). Additionally, HIF-1α ($F_{5,12} = 4.05$, $p = 0.027$), VEGF ($F_{5,12} = 3.121$, $p = 0.03$), and SYN 1 ($F_{5,12} = 3.317$, $p = 0.038$) protein expressions in the Con + Honokiol + SU5416 group were significantly decreased (Figure 4), without any significant difference in PSD 95 protein expression. Based on these results, honokiol was shown to enhance synaptic plasticity in PC12 cells by activating the HIF-1α-VEGF signaling pathway. The blockers played an inhibitory role. Moreover, HIF-1α, VEGF, PSD 95, and SYN 1 protein expressions in cells treated with 2-ME and SU5416 only did not differ from those of the control group, which excluded the possibility that 2-ME and SU5416 mediated interference.

Effects of honokiol in chronic unpredictable mild stress depression rat models

Effects of honokiol in open field test

The OFT was used to evaluate the spontaneous activity of rats. The distance and velocity of movement of the CUMS group were

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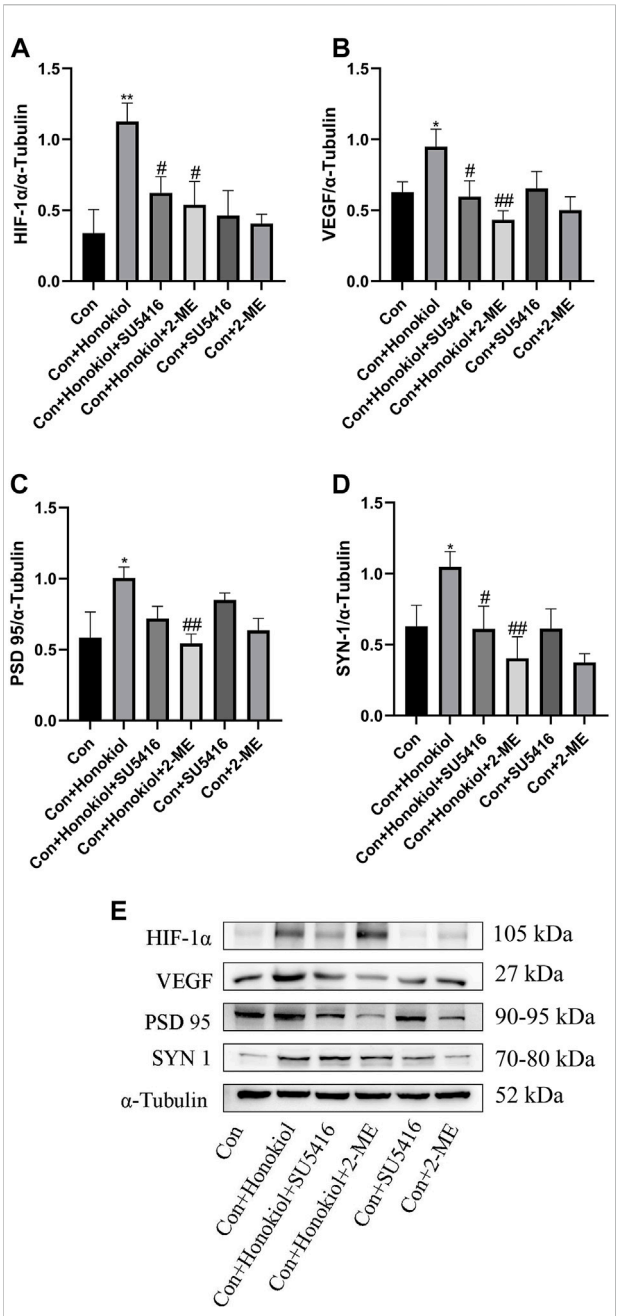

FIGURE 4
Effects of SU5416 and 2-ME on synaptic plasticity of neurons in PC12 cells. **(A)** HIF-1α protein expression. **(B)** VEGF protein expression. **(C)** PSD 95 protein expression. **(D)** SYN 1 protein expression. **(E)** The Western blot bands. Data are expressed as mean ± SEM (n = 3). $^{*}p < 0.05$ vs. Con group. $^{**}p < 0.01$ vs. Con group. $^{\#}p < 0.05$ vs. Con + Honokiol group. $^{\#\#}p < 0.01$ vs. Con + Honokiol group.

lower than those of the control group (Figure 5. $F_{3,28} = 39.532$, $^{***}p < 0.001$). However, the distance and velocity of movement of the honokiol treatment group were markedly higher than those of the CUMS group (Figure 5. $F_{3,28} = 39.532$, $^{\#\#\#}p < 0.001$).

Effects of honokiol in sucrose preference test

The sucrose preference test was used to assess motivation, emotional state, and pleasure-deficit behavior in rats. Compared with the control group, the CUMS group had a decreased sucrose preference rate (Figure 6. $F_{3,28} = 15.638$, $^{***}p < 0.001$). By contrast, the sucrose preference rate was elevated after treatment with honokiol (Figure 6. $F_{3,28} = 15.638$, $^{\#}p < 0.05$). No significant difference in the rate of sucrose preference was observed between the honokiol and paroxetine groups. The results suggest that honokiol reversed CUMS-induced anhedonia.

Effects of honokiol on the HIF-1α-VEGF signaling pathway in the hippocampus

The mRNA and protein expression levels of HIF-1α and VEGF were detected by real-time PCR and western blot technologies. HIF-1α and VEGF mRNA and protein expression were obviously lower in the CUMS group than in the control group (Figure 7. HIF-1α protein: $F_{3,8} = 5.127$, $p = 0.009$; VEGF protein: $F_{3,8} = 4.055$, $p = 0.018$; HIF-1α mRNA: $F_{3,20} = 5.286$, $p = 0.01$; VEGF mRNA: $F_{3,20} = 10.945$, $p = 0.016$). Contrarily, honokiol significantly increased the expression levels of HIF-1α and VEGF mRNA and protein in the CUMS rat models (Figure 7. HIF-1α protein: $F_{3,8} = 5.127$, $p = 0.01$; VEGF protein: $F_{3,8} = 4.055$, $p = 0.03$; HIF-1α mRNA: $F_{3,20} = 5.286$, $p = 0.001$; VEGF mRNA: $F_{3,20} = 10.945$, $p < 0.001$).

Effects of honokiol on mRNA and protein expression of VEGFR-2 in the hippocampus

By detecting the expression of VEGFR-2 mRNA and protein using Real-time PCR and western blot, it was explored whether honokiol exerted an antidepressant-like effect by acting on VEGFR-2. Data demonstrated that VEGFR-2 mRNA and the protein expression level were remarkably reduced in the CUMS group compared to the control group (Figure 8. VEGFR-2 protein: $F_{3,8} = 4.234$, $p = 0.014$; VEGFR-2 mRNA: $F_{3,20} = 13.855$, $p = 0.001$). However, VEGFR-2 mRNA and protein expression were significantly higher after honokiol treatment (Figure 8. VEGFR-2 protein: $F_{3,8} = 4.234$, $p = 0.024$; VEGFR-2 mRNA: $F_{3,20} = 13.855$, $p < 0.001$).

Effects of honokiol on mRNA and protein expression of PI3K/AKT/mTOR pathway in the hippocampus

This experiment was conducted to explore whether the PI3K/Akt/mTOR signaling pathway was involved in the antidepressant-like effects of honokiol. PI3K, AKT, mTOR mRNA, and p-PI3K, p-AKT, and p-mTOR protein expressions were significantly downregulated in the hippocampus of CUMS rat models, compared with the control group (Figure 9. p-PI3K protein: $F_{3,8} = 6.226$, $p = 0.011$; p-AKT protein: $F_{3,8} = 35.534$, $p < 0.001$; p-mTOR

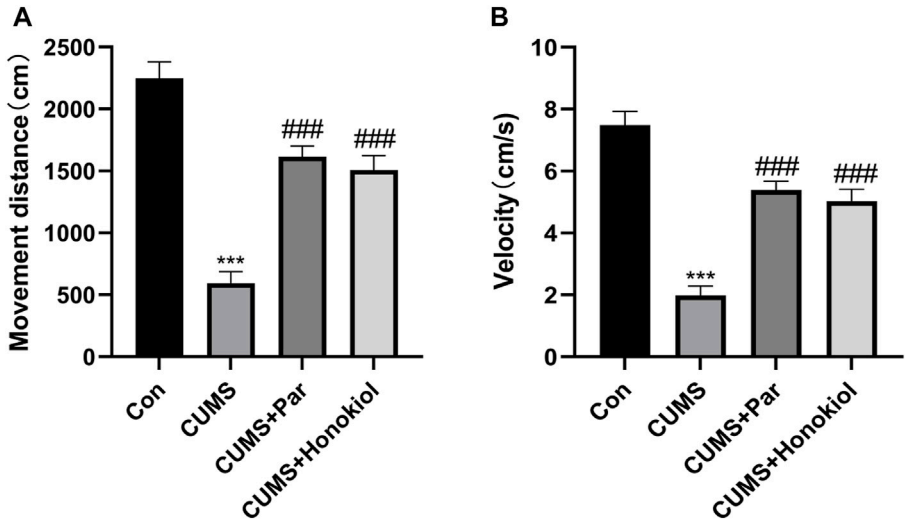

FIGURE 5
Effects of honokiol in OFT. **(A)** The movement distance. **(B)** The movement velocity. Data are expressed as mean ± SEM (n = 8/group). ***p < 0.001 vs. Con group. $^{###}p$ < 0.001 vs. CUMS group.

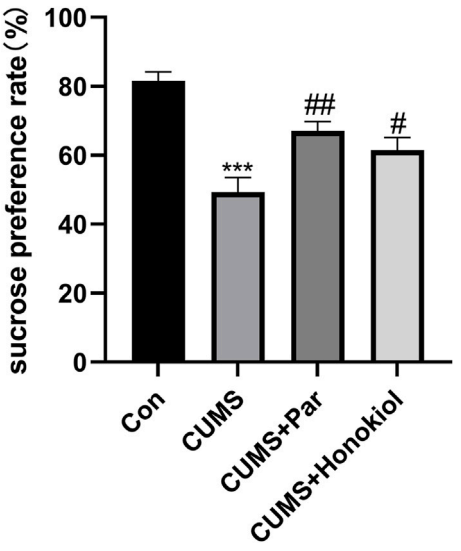

FIGURE 6
Effects of honokiol in SPT. Data are expressed as mean ± SEM (n = 8/group). ***p < 0.001 vs. Con group. $^{#}p$ < 0.05 vs. CUMS group.

protein: $F_{3,8}$ = 9.638, p = 0.004; PI3K mRNA: $F_{3,20}$ = 12.077, p = 0.038; AKT mRNA: $F_{3,20}$ = 6.717, p = 0.002; mTOR mRNA: $F_{3,20}$ = 11.456, p = 0.004). Additionally, honokiol significantly elevated PI3K, AKT, and mTOR mRNA and p-PI3K, p-AKT, and p-mTOR protein expression in CUMS rat models (Figure 9. p-PI3K protein: $F_{3,8}$ = 6.226, p = 0.026; p-AKT protein: $F_{3,8}$ = 35.534, p < 0.001; p-mTOR protein: $F_{3,8}$ = 9.638, p = 0.001; PI3K mRNA: $F_{3,20}$ = 12.077, p < 0.001; AKT mRNA: $F_{3,20}$ = 6.717, p = 0.001; mTOR mRNA: $F_{3,20}$ = 11.456, p < 0.001). Therefore, the results confirmed that honokiol improved CUMS-induced depressive behaviors in rats *via* the PI3K/AKT/mTOR pathway.

Effects of honokiol on regulators related to synaptic plasticity in the hippocampus

In addition to studying the synaptic plasticity action of honokiol on PC12 cells, the experiment explored whether honokiol exerted an antidepressant effect in CUMS rat models by modulating synaptic plasticity-related regulators. Data indicated that PSD 95 ($F_{3,8}$ = 4.936, p = 0.009) and SYN 1 ($F_{3,8}$ = 5.893, p = 0.003) protein expressions were significantly lower in the CUMS group than those of the control group (Figure 10). PSD 95 ($F_{3,8}$ = 4.936, p = 0.026) and SYN 1($F_{3,8}$ = 5.893, p = 0.036) protein expression levels were elevated after honokiol treatment (Figure 10).

Molecular docking results

Generally, lower binding energy between ligand and receptor is considered to result in more stable binding conformation and greater possibility of interaction. The binding energies between core target protein receptors (HIF-1α, VEGF, and VEGFR-2) and a small molecule ligand (honokiol) were −6.01, −6.49, and −6.1 kcal/mol, respectively. The molecular docking binding energies were all < −5 kcal/mol, which indicated high binding activity and stable docking. Hydrogen bonding is the

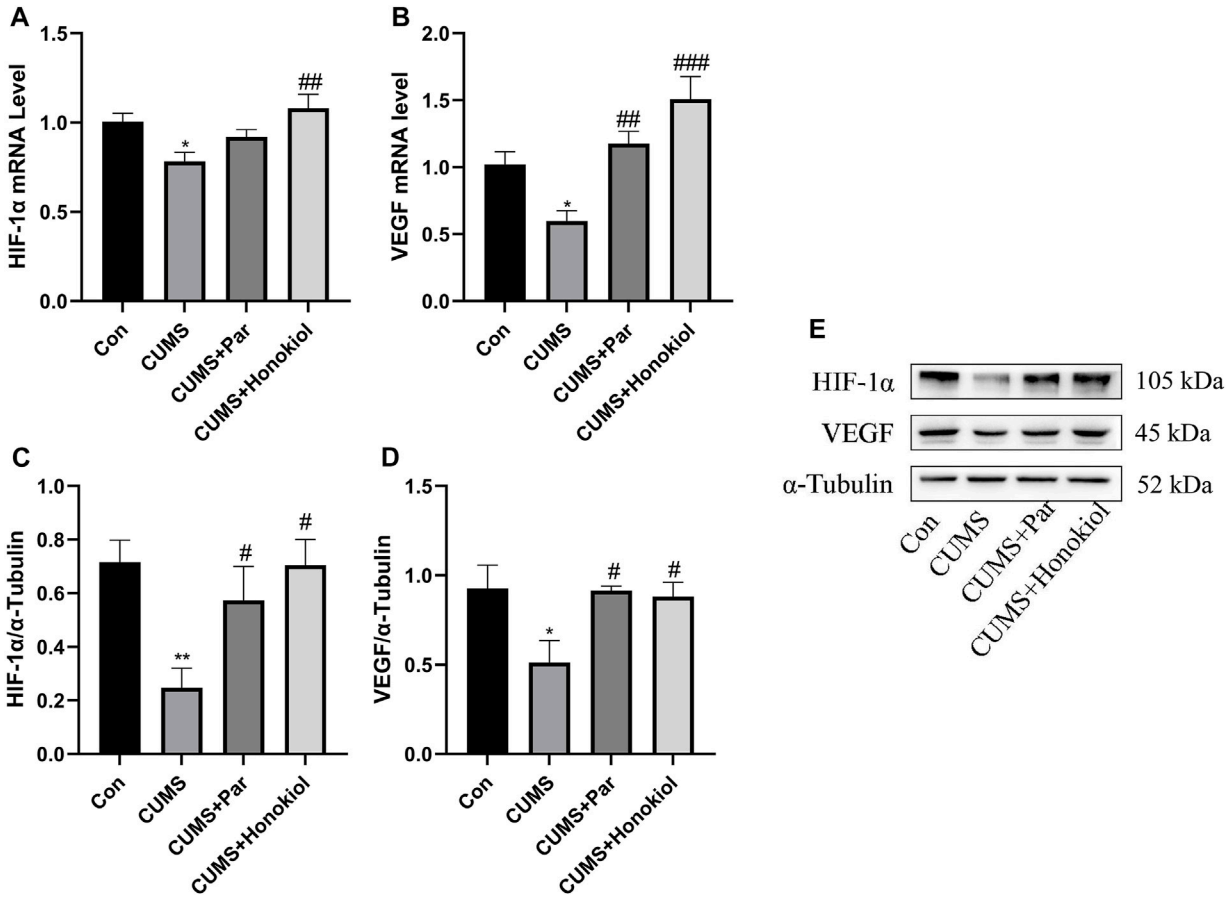

FIGURE 7
Effects of honokiol on the HIF-1α-VEGF signaling pathway in the hippocampus. **(A)** HIF-1α mRNA expression. **(B)** VEGF mRNA expression. Data are expressed as mean ± SEM (n = 6/group). **(C)** HIF-1α protein expression. **(D)** VEGF protein expression. **(E)** The Western blot bands. Data are expressed as mean ± SEM (n = 3/group). $^{*}p < 0.05$ vs. Con group. $^{**}p < 0.01$ vs. Con group. $^{\#}p < 0.05$ vs. CUMS group. $^{\#\#}p < 0.01$ vs. CUMS group. $^{\#\#\#}p < 0.001$ vs. CUMS group.

main force that drives ligand binding to the active site. Honokiol can form hydrogen bonds with active sites, ASP-100, LYS-21, VAL-148, and ALA-102, of the HIF-1α gene encoding protein (PDB:1L8C). Furthermore, it can form hydrogen bonds with active sites, CYS-26, CYS-104, and HIS-4, of the VEGF gene encoding protein (PDB:6z3f). It can form hydrogen bonds with active sites, ASP-814 and, ILE-1025, of the VEGFR-2 gene encoding protein (PDB:3VHE). The molecular docking schematics of honokiol and core targets are shown in Figures 11–13.

Discussion

In this study, we aimed to demonstrate whether honokiol has antidepressant-like properties by activation of the HIF-1α-VEGF signaling pathway. The study of antidepressant mechanisms using 2-ME and SU5416 *in vitro* showed that honokiol enhanced synaptic plasticity in PC12 cells, which relied on the activation of the HIF-1α-VEGF signaling pathway. *In vivo*, honokiol could reverse CUMS-induced depression-like behaviors. Honokiol activated the HIF-1α-VEGF signaling pathway, regulated the PI3K-AKT-mTOR signaling pathway mediated by VEGFR-2, and increased the expression of synaptic plasticity regulators. We believe these findings collectively demonstrate that honokiol is a promising and effective drug for the treatment of depression, and HIF-1α-VEGF pathway is a promising target.

The PC12 cells used in this study, a common neural cell strain, were derived from rat adrenal pheochromocytoma. PC12 cells were differentiated into neurons. As shown in Figure 2, morphologically differentiated PC12 cells have multiple neurites and long synapses. Additionally, MAP2 expression can be observed by immunofluorescence to identify whether PC12 cells differentiated into neurons (Yi et al., 2021). PC12 cells are close to nerve cells in terms of morphology,

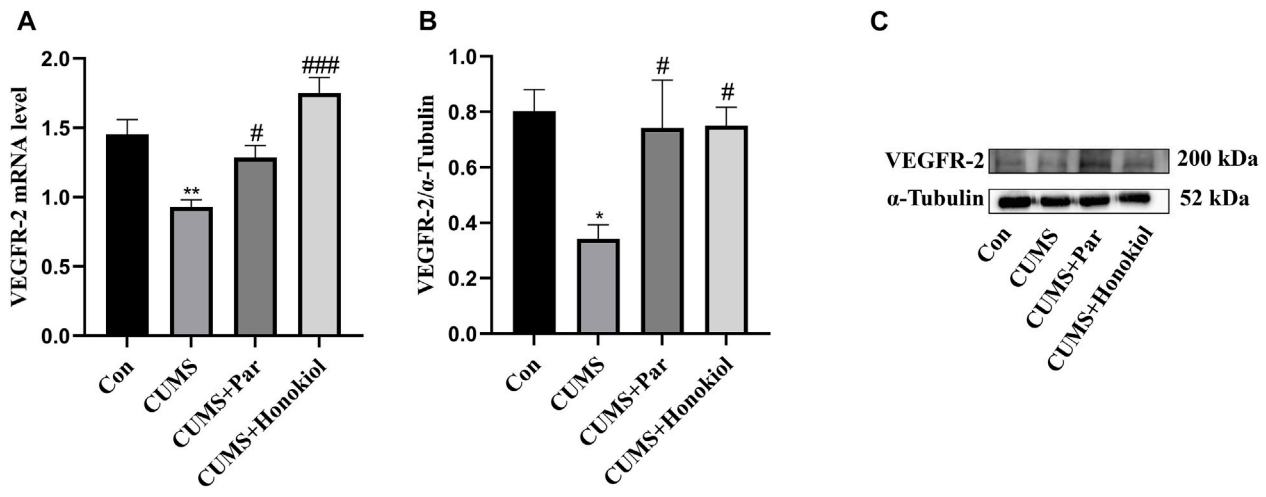

FIGURE 8
Effects of honokiol on mRNA and protein expression of VEGFR-2 in the hippocampus. **(A)** VEGFR-2 mRNA expression. Data are expressed as mean ± SEM (n = 6/group). **(B)** VEGFR-2 protein expression. **(C)** The Western blot bands. Data are expressed as mean ± SEM (n = 3/group). $^{*}p < 0.05$ vs. Con group. $^{**}p < 0.01$ vs. Con group. $^{\#}p < 0.05$ vs. CUMS group. $^{\#\#\#}p < 0.001$ vs. CUMS group.

physiology, and biochemical function, and have the characteristics of passage (Tischler and Greene, 1975). PC12 cells have become a tool cell for studying neurophysiology and neuropharmacology *in vitro*. Using various techniques to study differentiated PC12 cells is a direction needed for future studies.

The CUMS depression model is a reliable and effective rodent model of depression (Antoniuk et al., 2019). Various environmental stimuli are applied to animals for a long time under unpredictable conditions, inducing animals to produce various long-term physiological, behavioral, neuroendocrine, and other changes, which can effectively simulate all kinds of psychological pressures in patients with depression (Leonard and Maes, 2012). The results of the SPT and OFT showed that the CUMS model was successfully established. Honokiol improved autonomic activity and reversed the loss of pleasure in CUMS rats, indicating an effective antidepressant action.

HIF-1, a cellular oxygen sensor, is upregulated during tissue hypoxia to protect cells from hypoxia-induced dysfunction, whereas it is rapidly degraded under normoxic conditions (Ke and Costa, 2006; Kaelin and Ratcliffe, 2008). Many target genes are regulated by HIF-1 and mediate protein synthesis through HIF-1 binding to hypoxia response elements (HREs) of the target genes (Semenza, 2012). Intermittent hypoxia (IH) has been reported to stimulate hippocampal angiogenesis and neurogenesis, enhance nerve cell proliferation, and improve brain memory impairment (Bouslama et al., 2015). Additionally, IH has antidepressant-like effects in various animal models of depression, such as behavioral despair models and chronic mild stress models (Zhu et al., 2010). Specifically, this effect is achieved by improving the expression of HIF-1 and its target genes, EPO and VEGF. Most importantly, EPO and VEGF are sufficient to produce robust antidepressant effects in animal models (Deyama et al., 2019).

VEGF is a physiologically powerful mitogen of endothelial cells (Kim et al., 2008). In addition to its pro-angiogenic activity, recent studies have revealed that VEGF has neurotrophic and neuroprotective potential in the central nervous system (CNS) (Rosenstein et al., 2003). VEGF affects neuronal plasticity in the CNS and promotes axonal growth and neurogenesis (Tillo et al., 2012). Long-term cognitive deficits and a decrease in VEGF level have been observed in patients with depression (Viikki et al., 2010). In the treatment of depression, some antidepressants increase VEGF levels and promote nerve cell proliferation (Warner-Schmidt and Duman, 2007). As a neurotrophic factor, VEGF has become a hot spot in the study of psychiatric disorders and the effects of psychotropic drugs (Howell and Armstrong, 2017). VEGFR-2, a cell surface receptor for VEGF, can exert antidepressant-like effects by interacting with VEGF (De Rossi et al., 2016). VEGF plays a significant role in learning and memory, which specifically reflects on long-term enhancement, plasticity improvement, and cognitive function improvement mediated by its receptor VEGFR-2 (Yang et al., 2014). Additionally, chronic stress in the dentate gyrus of the adult hippocampus reduces cell proliferation near blood vessels and the expression of VEGF and VEGFR-2 proteins (Heine et al., 2005). Based on the complexity of the pathogenesis of depression, VEGF and VEGFR-2 signals deserve further investigation as potential targets for antidepressant therapy (Kenwood et al., 2019). In summary, the HIF-1α-VEGF signaling pathway is closely related to the treatment of depression, which is consistent with our experimental results.

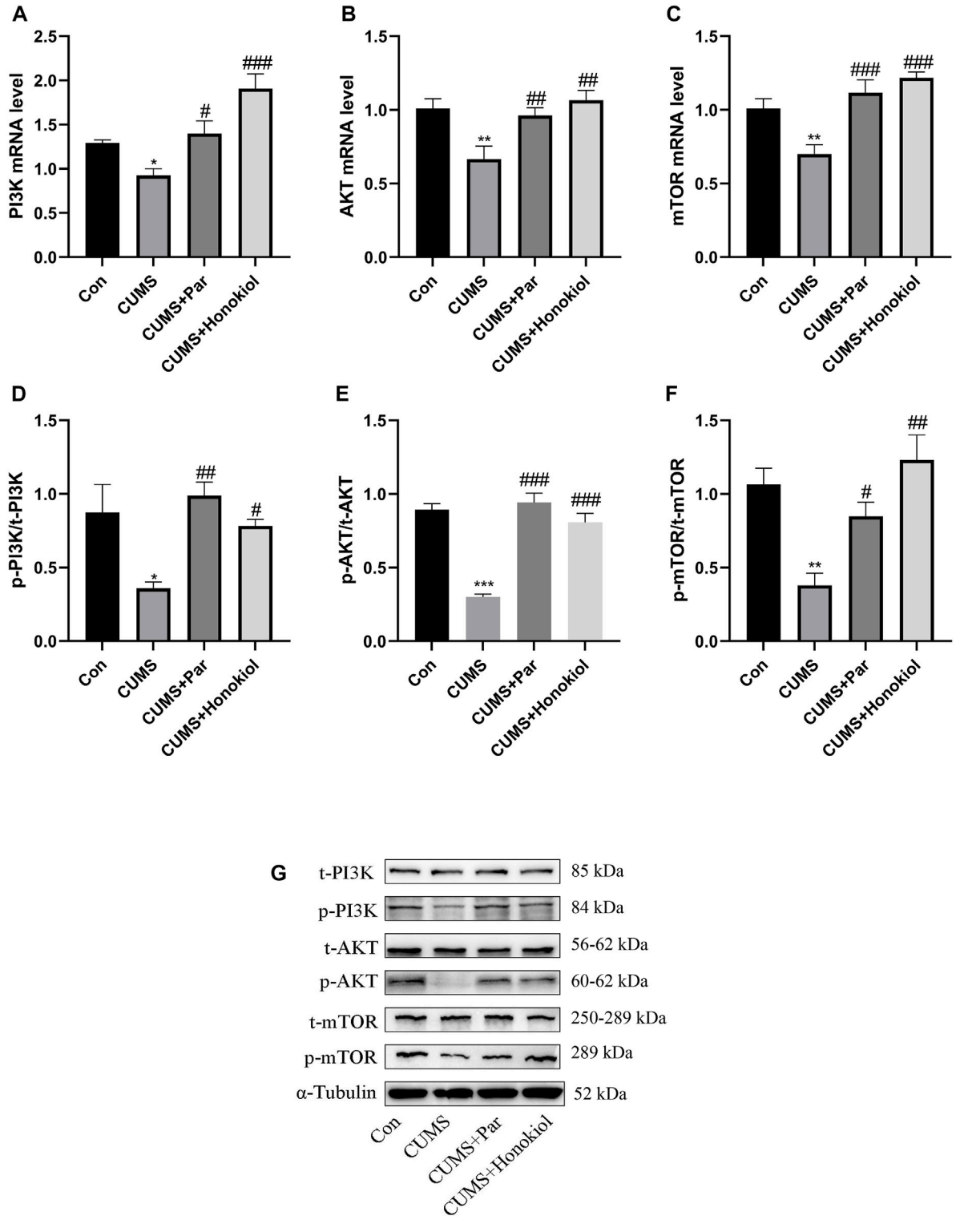

FIGURE 9
The effects of honokiol on PI3K/AKT/mTOR signaling pathway in the hippocampus. **(A)** PI3K mRNA expression. **(B)** AKT mRNA expression. **(C)** mTOR mRNA expression. Data are expressed as mean ± SEM (n = 6/group). **(D)** p-PI3K protein expression. **(E)** p-AKT protein expression. **(F)** p-mTOR protein expression. **(G)**The Western blot bands. Data are expressed as mean ± SEM (n = 3/group). $^{*}p < 0.05$ vs. Con group. $^{**}p < 0.01$ vs. Con group. $^{***}p < 0.001$ vs. Con group. $^{\#}p < 0.05$ vs. CUMS group. $^{\#\#}p < 0.01$ vs. CUMS group. $^{\#\#\#}p < 0.001$ vs. CUMS group.

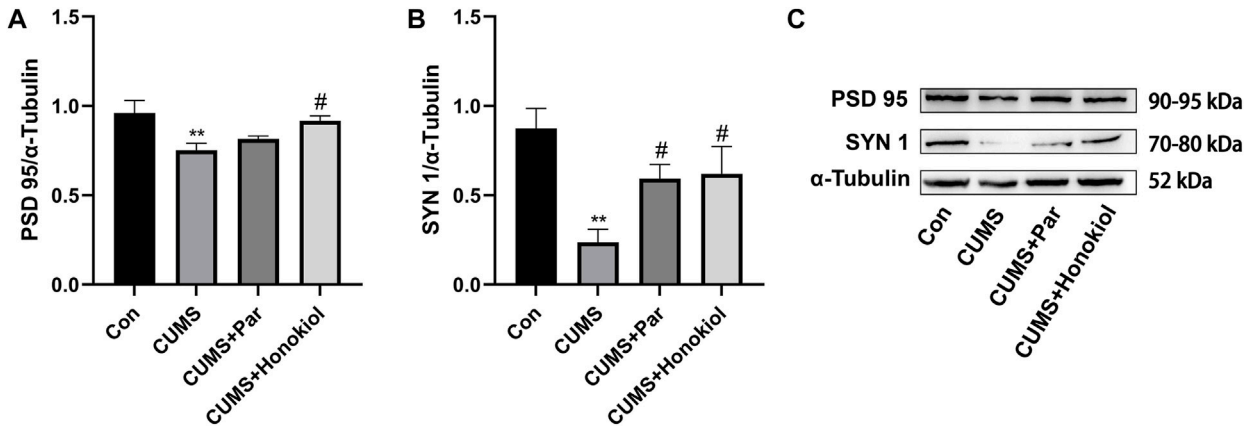

FIGURE 10
Effects of honokiol on regulators related to synaptic plasticity in the hippocampus. **(A)** PSD 95 protein expression. **(B)** SYN 1 protein expression. **(C)** The Western blot bands. Data are expressed as mean ± SEM (n = 3/group). $^{**}p < 0.01$ vs. Con group. $^{\#}p < 0.05$ vs. CUMS group.

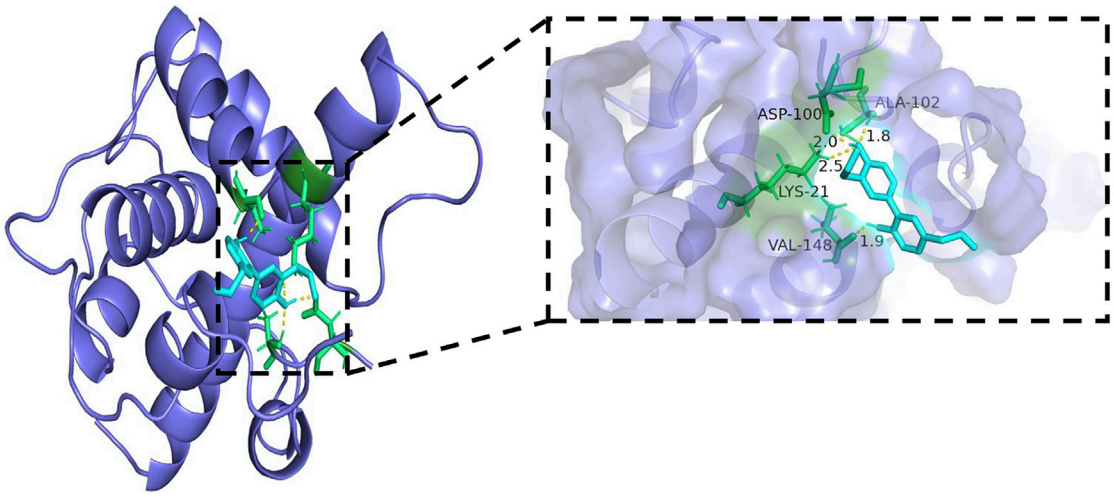

FIGURE 11
Molecular docking schema of Honokiol and HIF-1α.

Honokiol significantly increased mRNA and protein expression of HIF-1α, VEGF, and VEGFR-2 in the CUMS model group.

2-ME, an anti-angiogenic, antiproliferative, and pro-apoptotic agent, is a natural metabolite of estradiol and an effective HIF-1α inhibitor (Aquino-Gálvez et al., 2016). 2-ME inhibits the transcriptional activity and protein expression of HIF-1α by inducing microtubule depolymerization and the production of reactive oxygen species (Khan et al., 2015; Khan et al., 2018). SU5416 is a potent and selective inhibitor of VEGFR-2. Angiogenesis inhibitor, SU5416, inhibits VEGFR-2 expression by inhibiting endothelial cell mitosis, tyrosine kinase catalysis, and microcirculation (Vajkoczy et al., 1999; Zagorski et al., 2022). Besides, *in vivo* and *in vitro* experiments have shown that VEGF activates Erk1/2 and AKT signaling pathways in adult rat hippocampi and hippocampal neuronal precursor cells, and this effect is blocked by SU5416 (Fournier et al., 2012). SU5416 has been widely used in hippocampal nerve-related diseases. Our experimental findings demonstrated that the neuroplasticity effect of honokiol was blocked by 2-ME and SU5416, supporting the conclusion that the beneficial effect of honokiol was dependent on the HIF-1α-VEGF pathway.

Currently, numerous studies have been conducted on the downstream pathways of VEGFR-2. Studies have shown that the PI3K-AKT-mTOR signaling pathway mediated by VEGFR-2 can

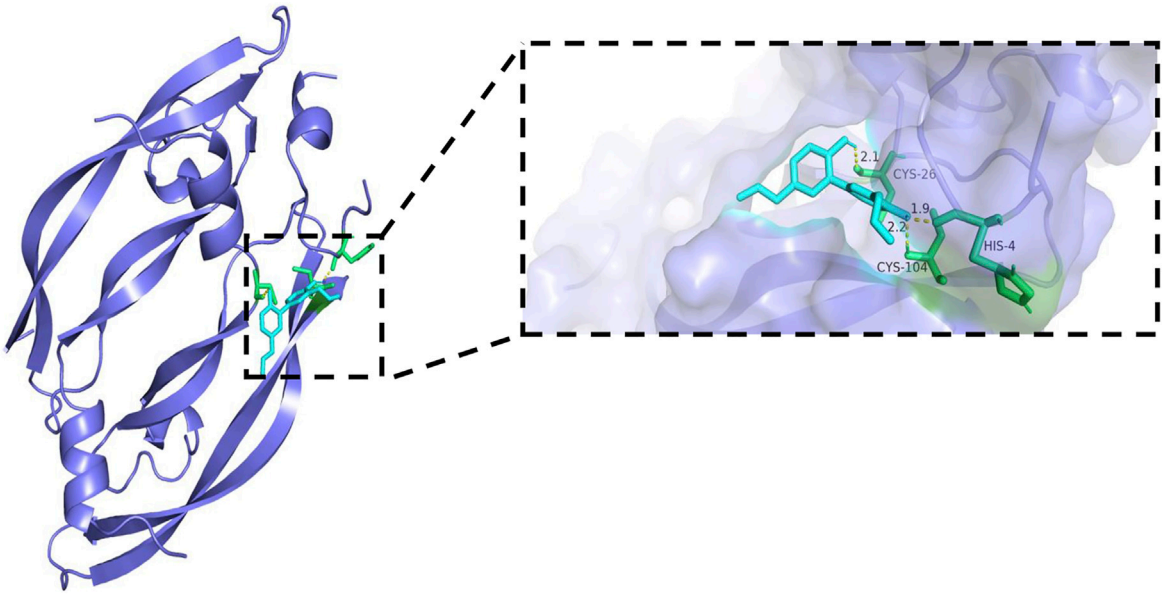

FIGURE 12
Molecular docking schema of Honokiol and VEGF.

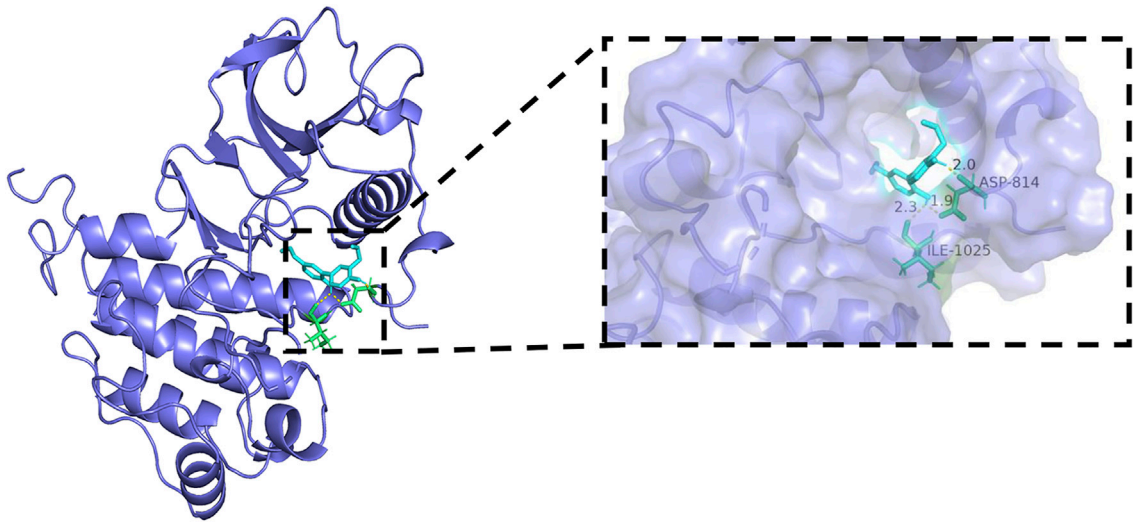

FIGURE 13
Molecular docking schema of Honokiol and VEGFR-2.

regulate neuroplasticity (Cao et al., 2017). PI3K receives extracellular stimulation to initiate downstream AKT/mTOR, which promotes neurogenesis, neural cell proliferation, and synaptic plasticity (Fakhri et al., 2021). The activity of this pathway was significantly reduced in depressed states. Consistent with our experimental results, the expression levels of PI3K-AKT-mTOR pathway-related genes and proteins decreased in CUMS depression rat models.

Mammalian target of rapamycin (mTOR), a serine-threonine kinase, regulates various cellular functions in mammals. mTOR signaling is closely related to synaptic plasticity and can improve impairment of synaptic plasticity in multiple ways (Xia et al.,

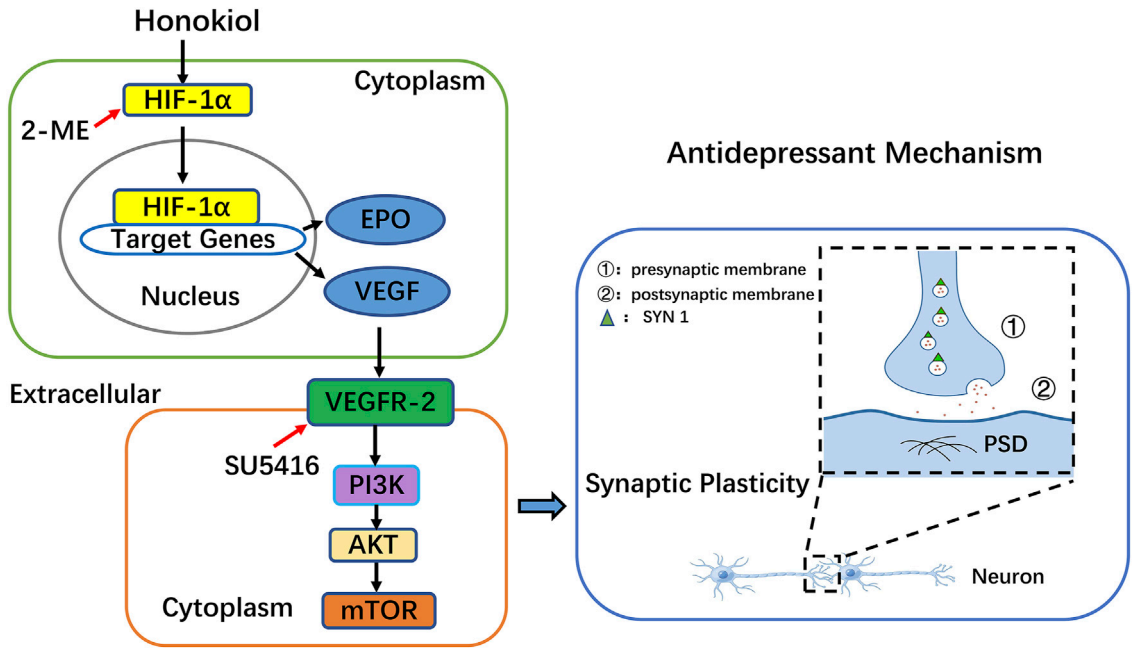

FIGURE 14
The antidepressant mechanism of Honokiol. Honokiol activates HIF-1α and translocates HIF-1α into the nucleus. Target genes, such as vascular endothelial growth factor (VEGF) and erythropoietin (EPO), are regulated by hypoxia-inducible factor-1α (HIF-1α). HIF-1α can bind to the hypoxia response elements of many target genes to mediate protein synthesis. Ultimately, these growth factors are secreted into the extracellular space between cells. VEGF binds to and activates its receptor VEGFR-2 on adjacent cells. Activation of the VEGFR-2 receptor leads to activation of the PI3K-AKT-mTOR signaling pathway in adjacent cells. The PI3K-AKT-mTOR signaling pathway plays an important role in nerve cell proliferation, survival, and synaptic plasticity, and finally improves depression. 2-ME and SU5416 were applied to investigate whether synaptic plasticity in neurons is dependent on the HIF-1α-VEGF pathway.

2021). Nuclear factor-kappa B (NF-κB), an important transcription factor, regulates the expression of many immune and inflammatory factors (Hoffmann and Baltimore, 2006). The NF-κB signaling pathway plays an important role in depression-like behaviors induced by acute and chronic stress and lipopolysaccharides (Munhoz et al., 2006; Koo et al., 2010). The mTOR pathway and its downstream factor, NF-κB, have been found to play a crucial role in maintaining normal physiological functions of the nervous system, especially for learning and cognitive functions (Dan et al., 2008; Lu et al., 2011). Additionally, mTOR is activated to regulate its target gene, NF-κB, under hypoxia, thus affecting cell growth, metabolism, proliferation, and differentiation (Jiang et al., 2010; Dhingra et al., 2013). Studies have shown that the pathogenesis of depression is closely related to the inflammatory response (Raison et al., 2006). Honokiol can improve inflammation-induced depression-like behaviors. Our previous study found that honokiol improved depression-like behaviors in LPS-induced depression mouse models by inhibiting the NF-κB signaling pathway and reducing the levels of pro-inflammatory cytokines (Zhang et al., 2019). Besides, honokiol can exert anti-inflammatory effects through various pathways, such as inhibition of the PI3K/Akt pathway (Kim and Cho, 2008) and; inhibition of the activation of the NF-κB signaling pathway through the inhibition of IκB kinase (IKK) activities (Tse et al., 2005).

Synaptic plasticity is a characteristic of synapses that undergo more lasting changes in morphology and function. Additionally, it is the basis for the recovery of learning, memory, and sensory dysfunction (Ruggiero et al., 2021). The pathogenesis of depression was previously reported to be associated with synaptic plasticity disorders (Pilar-Cuéllar et al., 2013; Li et al., 2020b). Our previous studies have shown that honokiol exhibits significant antidepressant-like effects by affecting tryptophan metabolism and reducing the serum levels of corticosterone to improve neuronal plasticity (Zhang et al., 2019; Zhang et al., 2020). Furthermore, honokiol was shown to bind to neural cell adhesion molecules to enhance neuronal survival and synaptic plasticity (Xu et al., 2017). The common mechanism of antidepressants in the treatment of depression is to enhance the synaptic plasticity of neurons (Mitra et al., 2006; Jayatissa et al., 2008). Synaptic plasticity changes are closely bound up with the expression of synaptic proteins. Postsynaptic PSD 95 and presynaptic SYN 1 are the main protein markers of synapses. PSD 95, one of the postsynaptic dense protein family and a key marker of the postsynaptic membrane, regulates synaptic transmission and synaptic function (Sheng and Hoogenraad, 2007). SYN 1, a

specifically labeled protein of synaptic vesicles, reflects the synaptic number, density, and distribution (Rajappa et al., 2016). PSD 95 and SYN 1 play a vital role in promoting signaling and synaptic plasticity. Damaged hippocampal neurons and disturbed synaptic plasticity have been found in CUMS model rats, and depression-like behaviors can be significantly improved after treatment with some antidepressants (Feyissa et al., 2009; Zhang et al., 2018; Lu et al., 2021). Our study findings indicated that PSD 95 and SYN 1 protein levels were significantly reduced in the model group. Both were remarkably elevated after honokiol treatment, confirming that honokiol can improve synaptic plasticity in CUMS depression rat models. The antidepressant mechanism of honokiol is illustrated in Figure 14.

Molecular docking is an important technology for predicting affinity through the interaction between ligand and receptor to realize structure-based drug design (Xiao et al., 2022). The results showed that all core targets had good docking activity with honokiol (binding energy < −5.0 kcal/mol), further confirming that honokiol can play a therapeutic role in depression by modulating the HIF-1α-VEGF pathway.

Currently, significant differences have been observed in testing protein expression on the basis of $n = 3$ per group. The western blot results presented were treated as observations due to the number of animals tested. In future studies, samples size should be increased to further confirm the reliability of the results.

Conclusion

Our results suggest that honokiol has an antidepressant effect in CUMS model rats. The observed beneficial effects may be attributed to the activation of the HIF-1α-VEGF signaling pathway, VEGFR-2-mediated PI3K/AKT/mTOR signaling pathway, and increased expression of the synaptic plasticity-related proteins, SYN 1 and PSD 95. The results also show that *in vitro*, honokiol enhances synaptic plasticity in PC12 cells by activating the HIF-1α-VEGF pathway.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding authors.

Ethics statement

The animal study was reviewed and approved by the Experimental Animal Ethics Committee of the Academic Committee of Beijing University of Chinese Medicine. Written informed consent was obtained from the owners for the participation of their animals in this study.

Author contributions

X-XF and W-YS: formal analysis, writing-original draft, conceived and designed the research, performed the experiments, analyzed, interpretated the data and drafted the manuscript; YL and QT: performed the experiments and analyzed; XY, A-RF, S-YW and L-NL: analyzed, revised the manuscript, writing-review and editing; H-SC and X-QX: conceived and designed the research, revised the manuscript, Writing-review and editing. All the above authors have read and approved the final manuscript.

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Conflict of interest

The 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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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2022.968124/full#supplementary-material

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EDITED BY
Katarzyna Stachowicz,
Polish Academy of Sciences (IF PAS), Poland

REVIEWED BY
Marta Jóźwiak-Bębenista,
Medical University of Lodz, Poland
Przemyslaw Jan Danek,
Polish Academy of Sciences (IF PAS), Poland

*CORRESPONDENCE
Marcin Siwek,
marcin.siwek@uj.edu.pl

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Unprescribed and unnoticed: Retrospective chart review of adverse events of interactions between antidepressants and over-the-counter drugs

Jarosław Woroń [1,2,3], Adrian Andrzej Chrobak [4], Daniel Ślęzak [5] and Marcin Siwek [6]*

[1]Department of Clinical Pharmacology, Chair of Pharmacology, Faculty of Medicine, Jagiellonian University Medical College, Kraków, Poland, [2]Department of Anesthesiology and Intensive Care No. 1, Department of Internal Medicine and Geriatrics, University Hospital in Cracow, Kraków, Poland, [3]University Center for Monitoring and Research on Adverse Drug Effects in Krakow, Kraków, Poland, [4]Department of Adult Psychiatry, Chair of Psychiatry, Jagiellonian University Medical College, Kraków, Poland, [5]Division of Medical Rescue, Faculty of Health Sciences with the Institute of Maritime and Tropical Medicine, Medical University of Gdańsk, Gdańsk, Poland, [6]Department of Affective Disorders, Chair of Psychiatry, Jagiellonian University Medical College, Kraków, Poland

Aim: To systematically evaluate prevalence and clinical characteristics of adverse effects of antidepressants and OTC drugs interactions in a retrospective chart review.

Methodology: Dataset of 1,145 registered adverse events were evaluated. Reports were selected for further analysis if pharmacoepidemiological evaluation indicated the presence of high probability of a causal relationship between antidepressants and OTC interaction and the occurrence of side effect. Following variables were extracted from the records: sex, age, medical comorbidities, antidepressant and other concomitant medications, clinical consequences ant the possible interaction mechanisms.

Results: 368 showed causal relationship with the simultaneous use of antidepressant with another drug. 15 adverse events (4%) were related to the use of OTC medicine, particularly omeprazole, diphenhydramine, Japanese ginkgo biloba, ibuprofen, diclofenac and sildenafil. All of the analysed side effects were categorized as the result of pharmacokinetic interactions. Here we report identified OTC drugs with corresponding antidepressants and clinical manifestations of DDI. Omeprazole: agomelatine (nausea, abnormal dreams), fluoxetine (extrapyramidal symptoms, paresthesias), sertraline (vertigo, yawning), escitalopram (oral vesiculation). Diphenhydramine: sertraline (diaphoresis, insomnia, vertigo), paroxetine (pruritus, headache), duloxetine (oropharyngeal pain). Japanese ginkgo biloba: citalopram (bradycardia), trazodone (vertigo, taste pervesion), mianserine (restless legs syndrome). Diclofenac: escitalopram (oral vesiculation), and fluoxetine (restless legs syndrome). Ibuprofen: agomelatine (anxiety and nausea), sertraline and omeprazole (QTc prolongation). Sildenafil: fluoxetine (genital oedema) and sertraline (myocardial infarction).

Conclusion: The use of OTC drugs by the patients should be monitored. Pharmacokinetic interactions between nonprescribed medicines and antidepressants may increase concentration and severity of side effects of latter ones.

KEYWORDS

antidepressants, drug-drug interactions, over-the-counter drugs, adverse effects, depression

Introduction

The use of over-the-counter (OTC) drugs is an ubiquitous phenomena, and the number of patients undertaking non-prescribed medication is increasing. Studies show that the rate of those practices in developing countries may reach up to 90% (Sánchez-Sánchez et al., 2021). Important role of OTC drugs is to promote self-care and simultaneously decreasing the burden of health care systems (Nomura et al., 2016). However, significant problems may arise when the use of those medications is not properly controlled, and physicians are unaware their patients take them. In up to 46% of cases, medics are not informed about the use of OTC drugs (Albert et al., 2014). Patients may not report and underestimate negative consequences associated with those medicines. Reports indicate that patients consider OTC drugs as safe and notice only their positive effects (Ngo et al., 2010; Eickhoff et al., 2012; Wawruch et al., 2013). The use of those medicines is higher in the group of patients with chronic diseases (Kim et al., 2018) and elderly individuals (Sihvo et al., 2000; Junius-Walker et al., 2007; Albert et al., 2014; Sheikh-Taha and Dimassi, 2018). The lack of information on the use of OTC drugs, especially in the abovementioned groups of patients, carries a significant risk of uncontrolled drug-drug interactions (DDI) leading to harmful effects.

The group of patients particularly vulnerable to the occurrence of side events are those receiving psychopharmacological treatment (Woroń and Siwek, 2018; Woroń et al., 2019; Siwek et al., 2020). Psychotropic medication is commonly used. According to National Health Interview Survey, 15.8% adults were under psychopharmacological treatment in the past 12 months (Terlizzi and Zablotsky, 2019). A group of drugs that are particularly frequently prescribed are antidepressants. In addition to treating major depressive disorder, these medicines are commonly used in therapy of anxiety disorders, eating disorders, insomnia or chronic pain. In the USA during 2015–2018, 13.2% of adults used antidepressants in the past 30 days (Brody and Gu, 2015). In some countries those drugs are also available without prescription from online shops, and they can be purchased from some conventional pharmacies (Sánchez-Sánchez et al., 2021).

Polytherapy, defined as the use of two or more drugs at the same time, is a common phenomenon in clinical psychiatry. In the USA, up to one third of the patients received at least three psychotropic drugs and over the time this proportion is rising (Mojtabai and Olfson, 2010). Simultaneous use of even two medications poses the risk of adverse interactions, and if seven drugs are used at the same time, the occurrence of such an interaction is certain (Vickers et al., 2006; McIntyre et al., 2016; Schatzberg and Nemeroff, 2017; Woroń and Siwek, 2018). This results in the drugs toxicity effects, increased number of adverse reactions and importantly, significant risk of non-compliance (Kukreja et al., 2013). Polytherapy will naturally lead to polypharmacy (polypragmasia) which is defined as the use of multiple concurrent medications, varying from two to eleven drugs at once according to the different definitions (Masnoon et al., 2017). This phenomenon will lead to inadequate and insufficient use of medications what will be associated with the lack of expected efficacy (Woroń and Siwek, 2018). Clinically important group of DDI consists of cytochromes-450(CYPs)-mediated pharmacokinetic interactions. CYPs comprise a large group of enzymes responsible for catalyzing the oxidative biotransformation of most of the drugs. Medicines differ in their interaction profile with CYP enzymes. Through the inhibition of those protein complexes, some drugs can lead to the significant increase of other medicines concentration and their side effects severity (Wienkers and Heath, 2005; Bibi, 2014; Danek et al., 2020).

Given the popularity of both antidepressants and OTC drugs, DDI between those two groups of medicines should be a common phenomenon. However, this topic has not been extensively researched. The aim of our study is to evaluate the incidence and characteristics of adverse interactions of antidepressants and OTC drugs in a retrospective chart review.

Materials and methods

A retrospective chart review was performed to evaluate the prevalence and clinical characteristics of DDI of antidepressants and OTC drugs. Analysis was performed by all the authors. The dataset involved reports on the occurrence of adverse reactions being the consequence of adverse interactions between simultaneously used drugs. They were evaluated at the University Center for Monitoring and Research on Adverse Drug Effects, Department of Clinical

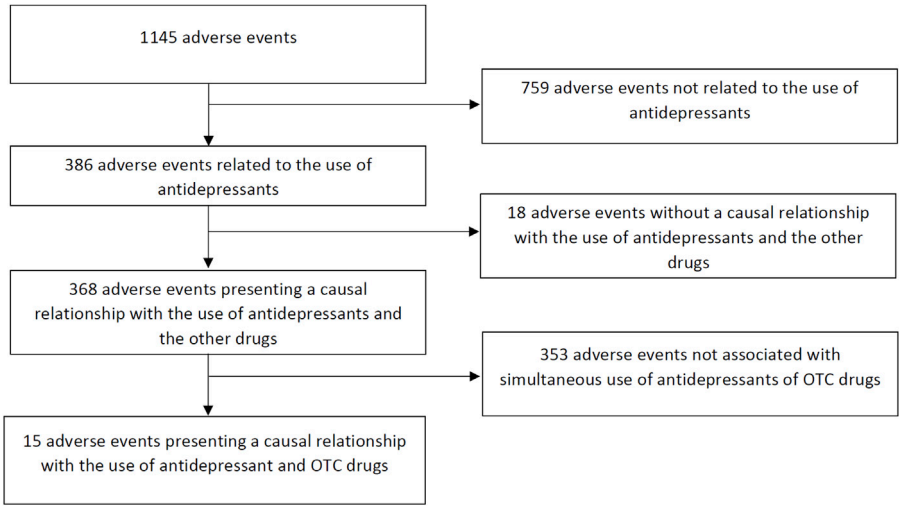

FIGURE 1
Flow chart of retrospective chart review. OTC—over-the-counter.

Pharmacology at Jagiellonian University Medical College in Cracow. It is one of the Regional Centers that, in accordance with the legal acts in Poland, formally monitor and report complications related to the pharmacotherapy. Additionally, this organisation provides specialist consultations for both hospitals and clinics from the Lesser Poland, Silesian, Holy Cross and Subcarpathian regions. The Center collaborates closely with the Department of Affective Disorders of the Jagiellonian University Medical College due to the significant increase of the side events associated with the use of psychotropic drugs. Annually, the Center makes approximately 850–1,100 consultations.

In this study we have analysed reports that came from all over the Poland in the period between January 2017 till March 2018. They were selected for further analysis when the following criteria have been met: 1) patient received at least one antidepressant drug, 2) patient used at least one OTC medicine, 3) the presence of a high probability of a causal relationship in terms of pharmacokinetic, pharmacodynamic interactions or the interactions associated with aggregation of side effects associated with the simultaneous use of antidepressant and OTC drugs indicated by pharmacoepidemiological analysis. Figure 1 shows a flow chart of our retrospective chart review. Dataset of 1,145 registered adverse events were analysed. 386 of those were related to the use of antidepressants, from which 368 showed causal relationship with the simultaneous use of antidepressant with another drug. 15 of those adverse events (4.08%) were related to the use of OTC medicine.

Results

Data extracted from 15 adverse events presenting a causal relationship with the use of antidepressants and OTC drugs is presented in Table 1. The mean age of the patients presented in the reports was 57 ± 10.9. They were seven men and eight women. The most common group of antidepressants associated with the occurrence of adverse events were selective serotonin reuptake inhibitors (SSRIs, 10 cases, 66% cases). Those involved sertraline (seven patients, 47%), fluoxetine (three patients, 20%), paroxetine (one patient, 6%), escitalopram (one patient, 6%), citalopram (one patient, 6%). Other antidepressants related to the occurrence of adverse events were: agomelatine (two patients, 13%), mianserine (one patient, 6%), trazodone (one patient, 6%), duloxetine (one patient, 6%). In case of OTC drugs, interactions involved omeprazole (five patients, 33% - in two cases there was significant interaction of omeprazole with another OTC drug: ibuprofen or diclofenac), diphenhydramine (three patients, 20%), Japanese ginkgo biloba (three cases, 20%), diclofenac (two patients, 13%), sildenafil (two patients, 13%), ibuprofen (two patients, 13%). All of the patients had at least two medical comorbidities. Two patients did not take other medication than antidepressant and OTC drug. All of the analysed side effects were the result of pharmacokinetic interactions. Detailed description of their proposed mechanisms and clinical consequences are presented in Tab.1.

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TABLE 1 Interactions between antidepressants and over-the-counter drugs in the analyzed group and possible interaction mechanisms.

Antidepressant medication	OTC drug	Sex and age of the patient	Medical comorbidities	Other concomitant medications	Clinical consequences of the interaction	Possible interaction mechanism
Sertraline	Diphenhydramine	F/51	Insomnia, major depressive disorder	-	Diaphoresis, insomnia, vertigo	Pharmacokinetic: inhibition of CYP2D6 by diphenhydramine → increased concentration and side effects of sertraline (metabolized by CYP2D6)
Paroxetine	Diphenhydramine	F/48	Insomnia, major depressive disorder	-	Pruritus, headache	Pharmacokinetic: inhibition of CYP2D6 by diphenhydramine → increased concentration and side effects of paroxetine (metabolized by CYP2D6)
Duloxetine	Diphenhydramine	M/58	Insomnia, major depressive disorder, hypertension	zofenopril, indapamide	Oropharyngeal pain	Pharmacokinetic: inhibition of CYP2D6 by diphenhydramine → increased concentration and side effects of duloxetine (metabolized by CYP2D6)
Citalopram	Japanese ginkgo biloba	M/64	Major depressive disorder, type 2 diabetes, dyslipidemia, vertigo	rosuvastatin, metformin	Bradycardia	Pharmacokinetic: Japanese ginkgo biloba extracts inhibit the activity of CYP3A4 → increased concentration and side effects of citalopram (metabolised by CYP3A4)
Trazodone	Japanese ginkgo biloba	F/62	Insomnia, tinnitus, vertigo, type 2 diabetes	petformin, dapagliflozin	*Vertigo*, taste perversion	Pharmacokinetic: Japanese ginkgo biloba extracts inhibit the activity of CYP3A4 → increased concentration and side effects of trazodone (metabolised by CYP3A4). This is of clinical significance if trazodone is used in daily doses above 300 mg
Mianserine	Japanese ginkgo biloba	F/70	Insomnia, vertigo, hypertension	perindopril, indapamide	Restless legs syndrome	Pharmacokinetic: Japanese ginkgo biloba extracts inhibit the activity of CYP3A4 → increased concentration and side effects of mianserine (metabolised by CYP3A4)
Agomelatine	Omeprazole	M/32	Major depressive disorder, heartburn	antacid on demand	Nausea, abnormal dreams	Pharmacokinetic:omeprazole inhibits CYP2C9 and CYP2C19 → increased concentration and side effects of agomelatine (metabolised by CYP2C9 and CYP2C19)
Fluoxetine	Omeprazole	M/37	Major depressive disorder, gastroesophageal reflux disease	ranitidine	Extrapyramidal symptoms, paresthesias	Pharmacokinetic:omeprazole inhibits CYP2C9 and CYP2C19 → increased concentration and side effects of fluoxetine (metabolised by CYP2C9 and CYP2C19)
Sertraline	Omeprazole	F/64	Major depressive disorder, osteoarthritis of the knee	chondroitin sulfate, diclofenac topical	*Vertigo*, yawning	Pharmacokinetic: omeprazole inhibits CYP2C9 and CYP2C19 → increased concentration and side effects of K/67sertraline (metabolised by CYP2C9 and CYP2C19)
Escitalopram	Diclofenac + Omeprazole	F/67	Major depressive disorder, lower back pain syndrome	pregabaline, etofenamate topical	Oral vesiculation	Pharmacokinetic: omeprazole inhibits CYP2C9 and CYP2C19 → increased concentration of diclofenac, which inhibits CYP2C9 and CYP3A4 → increased concentration and side

(Continued on following page)

TABLE 1 (*Continued*) Interactions between antidepressants and over-the-counter drugs in the analyzed group and possible interaction mechanisms.

Antidepressant medication	OTC drug	Sex and age of the patient	Medical comorbidities	Other concomitant medications	Clinical consequences of the interaction	Possible interaction mechanism
						effects of escitalopram (metabolized by CYP3A4)
Fluoxetine	Diclofenac	F/65	Major depressive disorder, osteoarthritis	paracetamol	Restless legs syndrom	Pharmacokinetic: diclofenac inhibits CYP2C9 and CYP3A4 → increased concentration and side effects of fluoxetine (metabolized by CYP2C9 and CYP3A4)
Agomelatine	Ibuprofen	M/54	Insomnia, lower back pain	pregabaline, buprenorphine	Anxiety, nausea	Pharmacokinetic: ibuprofen inhibits CYP2C9 → increased concentration and side effects of agomelatine (metabolized by CYP2C9)
Sertraline	Ibuprofen + omeprazole	F/59	Major depressive disorder, painful shoulder syndrome, gastroesophageal reflux disease	itopride	QTc prolongation (560 msec)	Pharmacokinetic: omeprazole inhibits CYP2C9 → increased concentration of ibuprofen, which inhibits CYP2C9 → increased concentration and side effects of sertraline (metabolized by CYP2C9)
Fluoxetine	Sildenafil	M/59	Major depressive disorder, dyslipidemia, type 2 diabetes, erectile dysfunctions	atorvastatine, Metformin, pentoxifylline	Genital oedema	Pharmacokinetic: fluoxetine inhibits CYP3A4 → increased concentration and side effects of sildenafil (metabolized by CYP3A4)
Sertraline	Sildenafil	M/64	Major depressive disorder, hypertension, erectile dysfunctions	ramipril lercanidipine	Myocardial infarction	Pharmacokinetic: sertraline inhibits CYP3A4 → increased concentration and side effects of sildenafil (metabolized by CYP3A4)

Discussion

In this study we have performed the first retrospective chart review of the adverse events caused by the simultaneous use of antidepressants and OTC drugs, based on a thorough analysis of the 1,145 reports. 4% of the adverse events caused by interaction of antidepressants with other drugs, were caused by their simultaneous use with OTC medication, particularly omeprazole, diphenhydramine, Japanese ginkgo biloba, ibuprofen, diclofenac and sildenafil. In this restrospective chart-review, as in our previous studies (Woroń and Siwek, 2018; Woroń et al., 2019; Siwek et al., 2020), the mean age of patients is noteworthy, indicating that the higher risk of DDI is probably age-related.

Omeprazole was the most commonly used OTC drug associated with adverse events evaluated in our study. Proposed mechanisms of those interactions involve interaction with cytochrome 450 isoenzymes responsible for antidepressants metabolism. Particularly, through inhibition of CYP2C9 and CYPC19 omeprazole increases the concentration and side effects of agomelatine (nausea and abnormal dreams), sertraline (vertigo and yawning), fluoxetine (extrapyramidal symptoms and paresthesias) (Karam et al., 1996; Schatzberg and Nemeroff, 2017). In two cases omeprazole was used in conjunction with another OTC drug, which likely contributed to the manifestation of the adverse effects. The first of those patients received escitalopram with omeprazole and diclofenac what was associated with the occurrence of oral vesiculation. This adverse event may be related to the fact that omeprazole inhibits CYP2C9 and CYP2C19 what results in increased concentration of diclofenac. The latter one inhibits CYP3A4 what would lead to increased concentration and side effects of escitalopram, metabolised by this isoenzyme (Schatzberg and Nemeroff, 2017). It has been shown that omeprazole can lead to 93.9% increase of escitalopram concentration, and esomeprazole causes 38,5% increase of sertraline concentration (Gjestad et al., 2015). In cases where it is necessary to combine an antidepressant with a proton pump inhibitor, pantoprazole and lansoprazole will be more favorable. It has been shown that both of those drugs were associated with significantly less pronounced increase of

escitalopram and sertraline concentrations (Gjestad et al., 2015).

Diphenhydramine is a first-generation antihistamine drug that acts as an inverse agonist on the H1 receptor with a serotonin reuptake inhibitor property (Khan et al., 2018), which was the root molecule from which fluoxetine was synthesized (Wong et al., 1995). It is most commonly used as a treatment for cold, allergic reactions, as well as insomnia (Simons, 1994). Study has shown that this drug may significantly influence antidepressants metabolism. Lessard et al., 2001 showed that through the inhibition of CYP2D6, diphenhydramine may lead to the more than 2-fold increase in plasma concentration of venlafaxine in the group of extensive metabolizers. This effect could be related to a significantly increased risk of adverse effects (Lessard et al., 2001). In our study, for the first time we have shown increased side effects of other antidepressant drugs, that were simultaneously used with diphenhydramine: sertraline (diaphoresis, insomnia and vertigo), paroxetine (pruritus and headache) and duloxetine (oropharyngeal pain). All of those drugs are metabolized by CYP2D6 (Schatzberg and Nemeroff, 2017), thus it is likely that the mechanisms of their interactions with diphenhydramine will be similar to that for venlafaxine.

Flavones and flavonols contained in the raw Japanese ginkgo biloba material may be potentially used by patients as a self-management aimed to improve brain blood supply, mental performance, memory and to decrease severity of depressive symptoms (Woroń and Siwek, 2018). Drug interactions with herbal OTC drugs are an important problem in psychopharmacotherapy (Woroń and Siwek, 2018). Studies point out that ginkgo biloba have a significant antiplatelet activity, which may add to the antiplatelet effects of SSRIs and SNRIs that may lead to the increased risk of haemorrhagic complications (Ryu et al., 2014; Woroń and Siwek, 2018). Ginkgo biloba may also induce CYP2C19 leading to the accelerated metabolism of omeprazole leading to reduction of its efficacy in prevention of upper gastrointestinal bleeding and may increase the risk of bleeding during SSRI or SNRI therapy (Woroń and Siwek, 2018). Few studies present side effects associated with the use of antidepressants and ginkgo biloba, other than an increased risk of haemorrhage. There is a single case report of coma when this drug was combined with trazodone (Feakes et al., 2000). In our previous study we have shown that simultaneous us of this herbal medicine with fluoxetine was associated with dizziness and hypotension (Woroń and Siwek, 2018). In this retrospective chart review we report three cases of patients with adverse events related to the use of ginkgo biloba, combined with the use of following antidepressants: citalopram (bradycardia), trazodone (vertigo and taste perversion), mianserine (restless legs syndrome). All of those drugs are metabolised by CYP3A4 (Schatzberg and Nemeroff, 2017) which is inhibited by ginkgo biloba (Wang et al., 2022). This may result in the increased concentration and side effects of those antidepressants, involving abovementioned symptoms.

Most of the studies evaluating interactions between antidepressant drugs and NSAIDs are focused on SSRI. Main groups of those interactions involve: 1) inhibition of platelet aggregation and function through the different mechanisms; 2) independent effect of SSRIs without direct pharmacokinetic interaction, e.g. increase of gastric acid secretion; 3) pharmacokinetic interaction through the inhibition of the CYP2C9, leading to the increased concentration of antidepressants metabolised by this isoenzyme (Moore et al., 2015). Most of the studies are focused on the interactions related to the increased risk of heamorrhage. In our chart review, side events associated with the use of NSAIDs were examples of the third group - pharmacokinetic interactions. The use of ibuprofen was associated with an increase in the severity of antidepressants side effects in case of two patients. Combination of this drug with sertraline and omeprazole was related to the significant QTc prolongation (560 msec). To our best knowledge, no cases of a similar interaction have been reported so far. Literature shows that the use of sertraline is associated with the low risk for QTc prolongation (Funk and Bostwick, 2013). Also, studies point out that this SSRI is often recommended as a safe and effective antidepressant in the group of patients with cardiovascular diseases (Mohapatra et al., 2005; Funk and Bostwick, 2013; Woroń et al., 2019). Due to the widespread use of NSAID in this population, special attention should be taken because their use may increase cardiotoxic capacity of the SSRIs. Another adverse event was observed in the case of patient under fluoxetine and diclofenac. Interaction between those two drugs most likely contributed to the manifestation of the restless legs syndrome. Mechanism of exacerbation of this disorder is not fully known yet. It has been hypothesized that fluoxetine, through selective enhancement of serotonin transmission leads to the inhibition of dopaminergic transmission that is related to the restless legs syndrome pathophysiology (Bakshi, 1996; Hoque and Chesson, 2010). We suggest that the occurrence of this side effect in our study was related to the inhibition of CYP2C9 and CYP3A4 by diclofenac. As both of those isoenzymes are involved in the metabolism of fluoxetine, this will increase its concentration and side effects. Finally, we have identified interaction between ibuprofen and agomelatine that resulted in anxiety and nausea. To our best knowledge, this interaction was not described in literature so far. Mechanism of this phenomenon most likely is associated with the inhibition of CYP2C9 involved in agomelatine metabolism (Saiz-Rodríguez et al., 2019).

Erectile impotence is a well-documented, common symptom of depression as well as a side effect of SSRI (Damis et al., 1999; Seidman et al., 2001; Farre et al., 2004). Sildenafil, a phosphodiesterase five inhibitor, is a commonly used drug to treat erectile dysfunctions available as OTC drug in Poland and United Kingdom. It has been shown that this drug may help to ameliorate SSRI-induced sexual dysfunctions as well as those related to the disorder (Damis et al., 1999; Seidman et al., 2001; Nurnberg and Hensley, 2003; Farre et al., 2004). A randomized

controlled trial has shown that sildenafil, combined with selective and nonselective serotonin reuptake inhibitors, was well tolerated. The most common adverse event was headache and less frequently flushing, dyspepsia, nasal congestion, and transient visual disturbances. No serious adverse events were reported (Seidman et al., 2001)0. In our study we have identified one case of a patient with myocardial infarction associated with the use of sildenafil combined with sertraline. Sildenafil is considered a safe drug that, when used appropriately, does not seem to increase to risk of myocardial infarction or sudden cardiac death (Kontaras et al., 2008). However, there are case reports indicating occurrence of acute myocardial infarction after sildenafil ingestion in a nitrate free patient without known cardiac history (Feenstra et al., 1998; Kekilli et al., 2005; Hayat et al., 2007). It has been hypothesized that the mechanism of this adverse event may be related to the increased levels of cyclic guanosine monophosphate levels, which mediates the relaxation of vascular smooth muscle, resulting in redistribution of arterial blood flow leading to inadequate coronary perfusion (Feenstra et al., 1998; Kekilli et al., 2005; Hayat et al., 2007). In our case, patient had no previous history of coronary artery disease, and his medical comorbidities involved major depressive disorder, hypertension and erectile dysfunction. We hypothesized that the risk of myocardial infarction could have been increased by the inhibition of CYP3A4 by sertraline, that leaded to increased concentration and side effects of sildenafil. Physicians should take into consideration the occurrence of this rare and serious adverse event related to sildenafil and be aware of pharmacokinetic interactions occurring with sertraline. Another case of adverse event identified in our chart review was a patient that revealed genital oedema after simultaneous use of fluoxetine and sildenafil. We have not found any case of a patient presenting similar clinical consequence of DDI. Most probable mechanism of this adverse event is similar to the previously described patient, and involves increased vasodilatation caused by increase of sildenafil levels due to inhibition of CYP3A4 by fluoxetine.

The most common group of antidepressants associated with the occurrence of adverse events in our retrospective chart review were SSRIs. All of the DDI described in our study represented pharmacokinetic mechanisms related to the inhibition of cytochrome P450 and the increase of those drugs concentrations and side effects. However, it should be noted that the use of SRRIs may associated with occurrence o the serious adverse effect in form serotonin syndrome. Many OTC drugs, such as dextromethorphan, purple echinacea, ginseng or ginkgo biloba (Khan et al., 2018; Woroń and Siwek, 2018) may increase the concentration of serotonin and worsen this life-threatening condition. Thus, doctors should be aware that, apart from pharmacokinetic interactions, concominant use of OTC drugs and SSRIs may be associated with the occurrence of serious adverse events associated with pharmacodynamic reactions.

In this restrospective chart-review, as in our previous studies (Woroń and Siwek, 2018; Woroń et al., 2019; Siwek et al., 2020), the mean age of patients (57 ± 10.9) is noteworthy, indicating that the higher risk of DDI is probably age-related. Studies indicate that older adults are major OTC consumers. Moreover, this group is particularly often affected by the problem of polypharmacy which significantly increases the risk of DDI. National Health and Social Life survey showed that 81% of older adults took at least one prescribed medication, 29% used five ore more drugs. Among them 42% of patients used at least one OTC medicine (Qato et al., 2008; Albert et al., 2014). More frequent use of OTC drugs and more common occurrence of the related DDI in this clinical group may be also associated with the lower average healthy literacy (Albert et al., 2014), presence of medical comorbidities (Sheikh-Taha and Dimassi, 2018), decreased hepatic and prehepatic drug metabolizing efficiency, decreased renal excretory ability, higher sensivity of receptors in central nervous system and deterioration of general homeostatic mechanisms (Turnheim, 2004; Hersh et al., 2007). We have shown, that only 4% antidepressant drug interactions were related to OTC medication. This low detection rate is most likely associated with lack of awareness, rather than rare occurrence of those interactions, what has been also pointed out in the case of studies evaluating prevalence of such drug interactions (Hämmerlein et al., 2007; Scherf-Clavel, 2022). Nationwide survey in Germany showed that only 8.6% of drug-related problems were associated with the use of OTC drugs (Hämmerlein et al., 2007). Another study showed that drug-drug interactions were related to only 4.1% of all drug related problems associated with the use of OTC medicines (Eickhoff et al., 2012). One study showed that even one-third of observed drug-drug interactions may be caused by OTC products (Fiss et al., 2010). Significant number of those interactions may have gone unnoticed because of the lack of documentation of OTC use (Olesen et al., 2013). Considering the frequent use of both antidepressants and OTC drugs, it can be assumed that in clinical practise occurrence of the significant interactions between those two groups of medicines is more common. In order to minimize the observed problem of DDI of antidepressants and OTC drugs, primary care physicians and psychiatrists should ask patients about the use of non-prescribed medications. Also pharmacists may play a role of a strong support group for doctors in reducing the risk of described potential side effects by asking patients about the use of OTC drugs and informing them about possible interactions.

Conclusion and recommendations

- The pharmacokinetic profile of the patients medications should be investigated in order to evaluate whether there is

overlap between cytochrome P450 isoenzymes involved in the metabolism of the drugs used, what may affect their concentration.

- OTC drugs can interact with each other, which may cumulatively increase the concentration and side effects of antidepressants.

- Particular attention should be paid in situations where an antidepressant is used in the maximum dose or the dose is titrated rapidly, because pharmacokinetic interaction with OTC drug may lead to exceeding the therapeutic concentration.

- Interactions of antidepressants and OTC drugs may result of life-threatening adverse events, e.g. myocardial infarction described in our study.

- Patients should be asked by doctors (primary care physician or psychiatrist) as well as the pharmacist about the usage of OTC drugs and informed about possible side effects caused by their simultaneous use with antidepressants.

- The use of OTC drugs by the patient should be described in the medical records in order to be able to monitor the adverse events associated with the use of these drugs.

Data availability statement

The raw data supporting the conclusion of this article will be made available by the authors, without undue reservation.

Author contributions

Conceptualization, JW, MS, and AC.; methodology, JW, MS, and AC; formal analysis, JW, MS, and AC: investigation, JW, MS, and AC; data curation: JW and MS.; writing—original draft preparation, AC and DS; writing—review and editing, JW, MS, AC, and DS.; visualization, AC.; supervision, JW, MS, DS. All authors have read and agreed to the published version of the manuscript.

Conflict of interest

The 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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EDITED BY
Maria Grazia Morgese,
University of Foggia, Italy

REVIEWED BY
Adrian Andrzej Chrobak,
Jagiellonian University Medical College, Poland
Cheng Jiang,
Yale University, United States
Luc Maroteaux,
inserm U1270, France
Raquel Romay-Tallon,
University of Illinois at Chicago, United States

*CORRESPONDENCE
Magdalena Sowa-Kućma,
msowa@ur.edu.pl

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The treatment of depression — searching for new ideas

Katarzyna Stachowicz[1] and Magdalena Sowa-Kućma [2,3]*

[1]Department of Neurobiology, Maj Institute of Pharmacology, Polish Academy of Sciences, Kraków, Poland, [2]Department of Human Physiology, Institute of Medical Sciences, Medical College of Rzeszow University, Rzeszow, Poland, [3]Centre for Innovative Research in Medical and Natural Sciences, Medical College of Rzeszow University, Rzeszow, Poland

Depression is a severe mental health problem that affects people regardless of social status or education, is associated with changes in mood and behavior, and can result in a suicide attempt. Therapy of depressive disorders is based mainly on drugs discovered in the 1960s and early 1970s. Selective serotonin reuptake inhibitors (SSRIs) and serotonin-norepinephrine reuptake inhibitors (SNRIs) are frontline pharmacological strategies for the medical treatment of depression. In addition, approved by FDA in 2019, esketamine [as nasal spray; N-methyl-D-aspartate (NMDA) receptors antagonist with additional effects on α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors, hyperpolarization-activated cyclic nucleotide-gated (HCN) channels, L-type voltage-dependent calcium channel (L-VDCC), opioid receptors, and monoaminergic receptors] is an essential compound in suicide and drug-resistant depression. However, the treatment of depression is burdened with severe side effects, and in many cases, it is ineffective. An equally important issue is the choice of antidepressant therapy in people with comorbid somatic diseases, for example, due to possible interactions with the patient's other drugs. Therefore, there is a great need for new antidepressants with different mechanisms of action and the need to refine the search for new substances. The purpose of this review was to discuss new research directions and new trends that dominate laboratories worldwide. We have reviewed the literature to present new points on the pharmacological target of substances with antidepressant activity. In addition, we propose a new perspective on depressive therapies.

KEYWORDS

depression, antidepressants, new target, glutamate receptors, DSCAM

Background to depression

The disease of emotions and motivation

Following World Health Organization (WHO) data from the Institute of Health Metrics and Evaluation, the Global Health Data Exchange (GHDx) indicated that 280 million people in the world suffer from depression (Institute of Health Metrics and Evaluation Global Health Data Exchange, 2021). Depression is a very complex mental illness. Criteria for major depressive disorder (MDD) are one or more major depressive

episodes (the lifetime absence of mania and hypermania) with five symptoms present during 2 weeks (DSM-5, 2013; Uher et al., 2014; Abdel-Bakky et al., 2021). The symptoms of depression can be divided into emotional and physical. The emotional symptoms of depression are stress, sadness, loss of interest, anxiety, hopelessness, difficulties with concentration, feeling of guilt, and suicidal thoughts (Abdel-Bakky et al., 2021). Physical symptoms include lack of energy, fatigue, pain, sleep disturbances, headaches, and psychomotor activity changes (Abdel-Bakky et al., 2021). The complexity of depression is evidenced by the classification of this illness proposed by the (American Psychiatric Association (APA), 2013) shown in Figure 1.

MDD can be divided into 14 subcategories, including that: "with anxious distress," "with mixed features," "with melancholic features," "with mood-congruent psychotic features," "with mood-incongruent psychotic features," "with catatonia," "with peripartum onset" categories (DSM-5, 2013).

As you can see, depression is a complex disease, which makes it challenging to diagnose unequivocally. According to statistics, women suffer from depression more often. It has been documented that depression in women occurs three times more often than in men. Hormonal aspects significantly impact the course of depression and treatment, which is particularly evident in postpartum depression (Kroska and Stowe, 2020). However, recent events related to the COVID-19 pandemic have shown increased incidence in all gender and age groups, including children (Bueno-Notivol et al., 2021; Hawes et al., 2021). Symptoms such as depression, anxiety, and cognitive impairment are considered to be the main symptoms of the post-acute COVID-19 syndrome (Mazza et al., 2022).

The etiology of depression has not yet been fully established and may involve genetic and environmental factors (Gaebel et al., 2017). Depression often coexists with other mental disorders (e.g., anxiety disorders and substance use disorders) and various somatic illnesses, including cardiovascular disease (e.g., hypertension, coronary artery disease), metabolic syndromes (e.g., diabetes), respiratory diseases (e.g., chronic obstructive pulmonary disease), various deficiencies (e.g., severe anemia), infections (e.g., tuberculosis, AIDS, influenza), collagen disorders, endocrine diseases (e.g., hypothyroidism, Cushing's disease), and others (Dornquast et al., 2017; Abdel-Bakky et al., 2021). It's known that depression may induce somatic disorders and *vice versa*—the presence of chronic somatic conditions may lead to the development of mental diseases. Patients with somatic diseases have a higher risk of developing mental illness. On the other hand, in people with severe mental illness, the risk of developing somatic disorders is twice as high as in patients without psychiatric disorders. The data indicate that nearly 50% of patients experiencing mental disorders have clinically significant comorbid physical illnesses, which often go undiagnosed for extended periods (Dornquast et al., 2017; Steffen et al., 2020). Importantly, it is indicated that

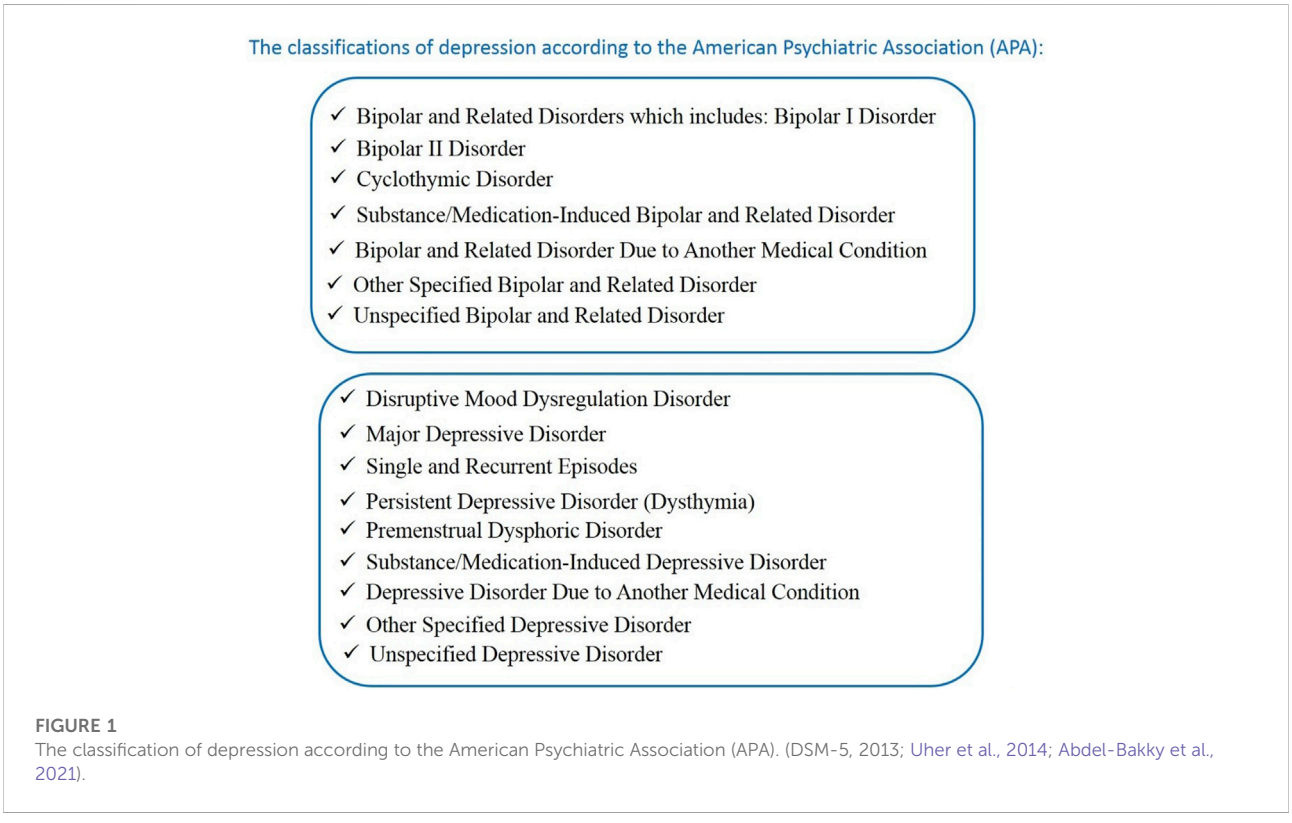

FIGURE 1
The classification of depression according to the American Psychiatric Association (APA). (DSM-5, 2013; Uher et al., 2014; Abdel-Bakky et al., 2021).

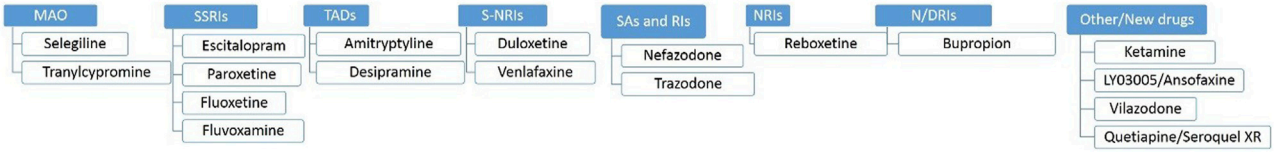

FIGURE 2
Selected examples of antidepressants by groups. MAO, Mono Amino Oxidase; SSRIs, Selective Serotonin Reuptake Inhibitors; TADs, Tricyclic Antidepressants; S-NRIs, Serotonin-Norepinephrine Reuptake Inhibitors; SAs and RIs, Serotonin Antagonist and Reuptake Inhibitors; NRIs, Norepinephrine Reuptake Inhibitors; N/DRIs, Noradrenaline and Dopamine Reuptake Inhibitors.

comorbidities are the most critical factor influencing the economic burden of depression. Hence, it is necessary to consider them in the treatment of depression (Steffen et al., 2020).

Between 2003 and 2005, in the European Union (EU), about 27% of adults were affected by a mental disorder (Wittchen et al., 2011). The following survey in 2011 detected 164.7 million people affected by mental health problems, which was about 38.2% of people (Wittchen et al., 2011). 2011 survey included 14 new disorders not included in the 2005 study (Wittchen et al., 2011) hence differences; but the number of affected people is enormous. Recent events related to the COVID-19 pandemic caused by the SARS-COV-2 virus have resulted in a significant increase in the incidence of depression in the population (Bueno-Notivol et al., 2021). A study by Bueno-Notivol et al. (2021) found a global estimated prevalence of depression in 2021 was seven times higher than in 2017, which is about a 25% increase. Authors searched for cross-sectional, community-based studies listed on PubMed or Web of Science from 1 January 2020, to 8 May 2020, that reported prevalence of depression (Bueno-Notivol et al., 2021). A random-effects model was used to estimate the pooled proportion of depression (Bueno-Notivol et al., 2021). Interestingly, the meta-regression observation showed that the prevalence of depression was independent of the percentage of women, mean age at baseline, response rate, or methodological quality (Bueno-Notivol et al., 2021), which suggests a severe global problem. Similar observations were documented in the United States after the pandemic of COVID-19 (Hawes et al., 2021). Another increase in depression can be expected in connection with the outbreak of the war in Ukraine in 2022 (Jain et al., 2022).

The antidepressants used in the clinic

Today, the treatment of depression is primarily based on drugs discovered in the 1960s and 1970s. It should be mentioned here that Monoamine Oxidase Inhibitors (MAOIs) as selegiline, tranylcypromine, and phenelzine; Selective Serotonin Reuptake Inhibitors (SSRIs) as escitalopram, paroxetine, fluoxetine, fluvoxamine; Tricyclic Antidepressants (TADs) as amitriptyline, desipramine, iprindol; Serotonin-Norepinephrine Reuptake Inhibitors (S-NRIs) as venlafaxine, duloxetine, desvenlafaxine; Serotonin Antagonist and Reuptake Inhibitors (SAs and RIs) as nefazodone and trazodone; Norepinephrine Reuptake Inhibitors (NRIs) as reboxetine, viloxazine; Noradrenaline and Dopamine Reuptake Inhibitors (N/DRIs) as bupropion; and other drugs as esketamine, ansofaxine, vilazodone or quetiapine; see Figure 2. Moreover, just recently the FDA approved Zulresso (brexanolone) for postpartum depression (Powell et al., 2020).

The number and variety of antidepressants available today seem pretty large, but their use causes many problems. First, these drugs require a long administration time (except for esketmine) to obtain a therapeutic effect (Abdel-Bakky et al., 2021; McCarron et al., 2021). In addition, their use is associated with many side effects, such as weight gain, sexual dysfunction, dizziness, headache, anxiety, psychosis, cognitive dysfunctions, etc. (Abdel-Bakky et al., 2021; McCarron et al., 2021). Considering SSRIs, prolonged bleeding time, and, when used during pregnancy, heart defects and pulmonary hypertension in newborns have been observed (Berard et al., 2017; Calvi et al., 2021). For newer medications, such as those approved by the FDA for treatment of MDD, ansofaxine (potential triple reuptake inhibitor of serotonin, norepinephrine, and dopamine, approved in 2019) or vilazodone (a selective serotonin reuptake inhibitor and serotonin 5-HT1A receptor partial agonist, approved in 2021), the side effects are very similar to traditional antidepressants. They are mild to moderate, with a slightly different frequency than, for example, SSRIs or NRIs. Interestingly, sexual dysfunctions typical for conventional antidepressants are not observed after using ansofaxine (Mi et al., 2022; Chauhan et al., 2022). Also, esketamine, recommended for patients with drug-resistant depression, may have many unpleasant consequences, including dissociation, anxiety, nausea, increased blood pressure, and headache. These side effects are mild, transient, and dose-related and will disappear with subsequent treatments. It is also indicated that the frequency of their occurrence is twice as high in patients receiving simultaneously nasal ketamine and orally another antidepressant than esketemine alone (Ceban et al., 2021). Many of the drugs mentioned have not been approved for

pregnant women (Abdel-Bakky et al., 2021; McCarron et al., 2021).

On the other hand, in patients with comorbidities, there are severe contraindications to combining antidepressants with other drugs due to possible drug-drug interactions. The risk of their occurrence is exacerbated by complex polypharmacy regimens and extended treatment periods which result in tolerance problems to ineffectiveness and serious adverse events (Low et al., 2018; Woron et al., 2019). Pharmacokinetic interactions of antidepressants and cardiac drugs seem particularly dangerous because cytochrome 450 isoenzymes metabolize both in the liver. Significantly, both groups of medications can affect the activity of these enzymes. For example, fluoxetine and paroxetine are inhibitors of CYP2D6, and calcium channel blockers inhibit the activity of CYP3A4. Consequently, combined drugs from both groups may lead to hypotension or an increased risk of gastrointestinal bleeding (Woron et al., 2019). Hence, when using polypharmacy, careful selection of drugs with a low interaction potential is necessary. Unfortunately, this is not always possible due to the limited availability of antidepressants with a simple metabolic profile. Moreover, in many cases (especially patients with the severe clinical condition), the possibility of oral administration of antidepressants is severely limited. The exception is esketamine, which the FDA has approved for treating drug-resistant depression as a nasal spray; its action is fast, but also be careful when using it due to the potential risk of drug-drug interactions (Turner, 2019).

All of this necessitates the search for new, more effective treatments for depression and mental health. First, there is a need for drugs that will give a quick therapeutic response, few side effects, and a limited amount of interactions with other medications. Therefore, in the next chapter, we will consider new strategies for treating depression and identify potential pharmacological targets for new active substances designed to treat depression effectively.

Towards better treatment of depression

The promising pharmacological targets

When looking for a new way to treat depression, it is essential to define the main cellular/molecular targets for these findings. At present, the most important new targets for antidepressants seem to be glutamate receptors (GluRs) (Stachowicz 2021a; Stachowicz, 2021; Vasiliu 2022a) or gamma-aminobutyric acid (GABA)-ergic modulators; paying attention to both intra- and extra-synaptic GABA-A receptors (Brickley and Mody, 2012; Luo et al., 2013; Vasiliu, 2022). Although the use of GABA-ergic ligands seems to be effective in the treatment of depression, direct interference with the GABA pathway has side effects in the form of drowsiness or sedation, which may hinder daily activities (Vasiliu, 2022); for this reason, the weight appears to be tipping in favor of the GluRs ligands. These agents seem to be an auspicious new point in the pharmacological treatment of depression (Stachowicz 2021a; Stachowicz, 2021; Vasiliu 2022a), which is confirmed by the scale of both preclinical phase I and III clinical trials, where we can find as many as 27 active substances following the last finding (Stachowicz 2021a; Stachowicz, 2021; Vasiliu 2022a). Among GluRs ligands considered in clinical use were NMDA receptor (NMDAR) or its GluN2A/GluN2B subunits antagonists (e.g., NRX-101 or AZD6765, respectively), NMDAR positive allosteric modulators (e.g., AGN-241751), NMDAR-glycine site agonists (e.g., GLYX-13), AMPA receptor potentiators (e.g., TAK-653) and metabotropic glutamate receptors (mGluRs) antagonists (e.g., TS-161) (Stachowicz 2021a; Vasiliu 2022a). Among them, AZD6765, GLYX-13, and TAK-653 did not reach clinical use (Kadriu et al., 2020). However, compounds like GLYX-13 became the prototype for the synthesis of the next generation of compounds with similar mechanisms of action, including apimostinel (GATE-202, NRX-1074), a second-generation analog with increased potency, and zelquistinel (GATE-251, AGN-241751), a third-generation small molecule with increased power and high oral availability (Neurosciences Gate, 2022). The central hypothesis in using these compounds in depression is based on the idea that excessive excitatory transmission related to Glu and so-called neurotoxicity lead to impairment of brain functions, transmission, and plasticity, manifested by mental disorders such as depression (Skolnick, 1999; Stachowicz 2021a).

Other groups of active substances considered in the pharmacotherapy of depression are not apparent compounds acting as anti-cytokines and COX-2 inhibitors (Stachowicz 2021a; Vasiliu 2022a). This group of active substances is aimed at patients with immunological disorders coexisting with depression, where it achieves impressive results both as monotherapy and in combination with classical antidepressants (Stachowicz 2021a; Vasiliu 2022a). Importantly, these compounds could be effective in patients after COVID-19 infection because the psychopathological mechanisms underlying the symptoms of depression after COVID-19 are mainly related to inflammation caused by the peripheral immune response to viral infection and persistent psychological stress during and after an illness. Currently, eight active substances are in phase I to IV clinical trials (Vasiliu 2022). However, clinical reports are already showing promise with combination therapy for COX-2. Following Sethi et al. (2019), the favored use of celecoxib with reboxetine, fluoxetine, and sertraline was observed in depressed patients. In the latter, improvements in HRDS and HAM-D scales were observed if antidepressants were combined with celecoxib. Our preclinical studies are also optimistic, as positive antidepressant effects were observed in the animal model after co-administration of COX-2 inhibitor (NS398) with mGluR

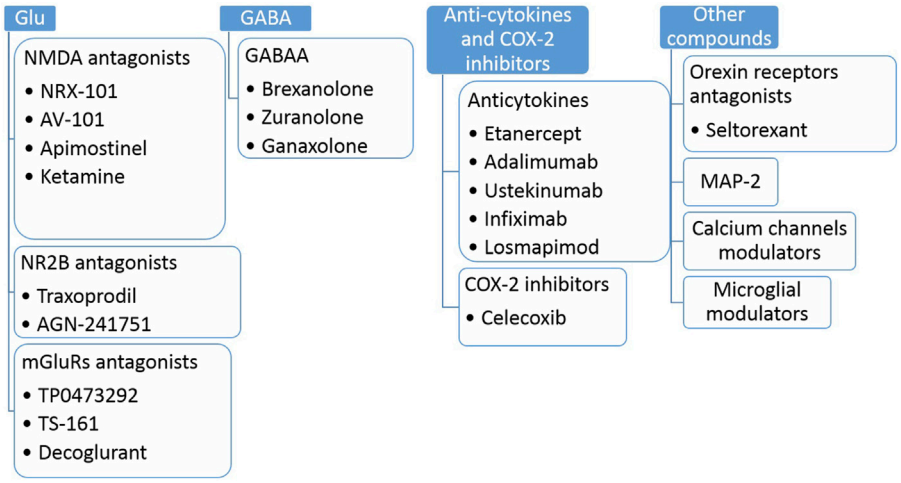

FIGURE 3
Selected examples of antidepressant compounds in clinical trials (Phase I-IV). Glu, glutamate; NMDA, N-methyl-D-aspartate; NR2B, NMDA receptor subunit 2B; mGluRs, metabotropic glutamate receptors, GABAA, gamma-aminobutyric acid receptors A; COX-2, cyclooxygenase 2; MAP-2, microtubule-associated protein 2.

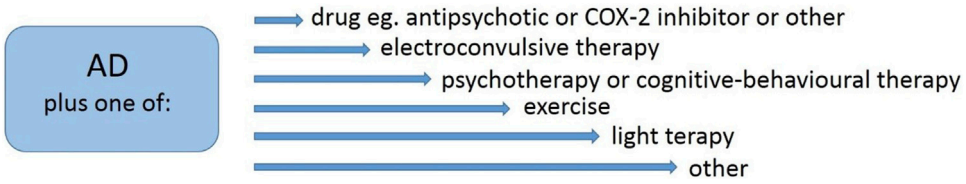

FIGURE 4
Examples of combined therapy in the treatment of depression. AD, antidepressant; COX-2, cyclooxygenase 2.

ligand and imipramine (Stachowicz 2021a). We have also started to decipher the mechanisms of the influence of antidepressants on fertility parameters in rodents, which may be crucial when looking for active substances without side effects (Solek et al., 2021; Tabecka-Lonczynska et al., 2021).

Quite a new group of antidepressants are orexin receptor antagonists, or compounds acting through microtubule-associated protein type-2 (MAP-2) *via* calcium channels or microglial mechanisms (for a more extensive review on the topic, please refer to Vasiliu 2022; Vasiliu 2022a). As far as orexins are concerned, ligands for type 1 and 2 receptors (OX1R and OX2R) can modulate feeding, sleep, motivated behavior, anxiety, and addiction; hence they have a vast potential to regulate many aspects of depression (Vasiliu 2022). For details described in this section, see Figure 3.

Of course, we must not forget the substances that affect the 5-HT system. Intensive work is underway in this field to implement new compounds acting through new receptors such as 5-HT7 or safer compounds directed towards 5-HT1A or 5-HT2A (e.g., MIN-117 or psilocybin, respectively) (Vasiliu 2022); the topic will be expanded in the following subsections.

Combination therapy

Because it was estimated that 15%–20% of depressed patients do not respond to the treatment (Rush et al., 2006; Kalmoe et al., 2020), there are ongoing attempts to use combination therapy to improve mental health (Figure 4).

One way to improve the outcome of the treatment is a combination of known antidepressants (ADs) with the substance directed to a different target, e.g., combination with antipsychotics (Davis et al., 2021; Vázquez et al., 2021; Kishi et al., 2021) or COX-2 inhibitors (Muller et al., 2006; Muller, 2019; Sethi et al., 2019; Stachowicz, 2021). Second-generation antipsychotics (e.g., olanzapine, risperidone, or aripirazole) are

effective in combination therapy; however, they still have not overcome the effectiveness of combination therapy with lithium (Vázquez et al., 2021; Kishi et al., 2021). Some hope is linked with combination therapy with COX-2 inhibitors, while much evidence is that this path is effective (Muller et al., 2006; Muller, 2019; Sethi et al., 2019).

A combination of electroconvulsive therapy with ADs is known, and the recent discovery regarding its use with esketamine seems very promising and demonstrates high efficacy in drug-resistant depression (Kavakbasi et al., 2021). Subsequent directions of combining treatments focus on ADs administration and application of psychotherapy (Guidi and Fava, 2021), cognitive-behavioral therapy with virtual reality (Stamou et al., 2021), exercise (Xie et al., 2021), or light therapy (Even et al., 2008) and others. These methods enrich the treatments that benefit and require specialists' involvement and the patient's engagement.

The last decade's new finding is a microbiota-gut axis in depression (Cryan et al., 2019; Stachowicz, 2019; Simpson et al., 2021). Following Cryan et al. (2019), there is a new concept: "the concept of psychobiotics for treating various neurological and psychiatric disorders through targeting the gut microbiota." The idea of enriching therapy of depression by psychobiotics directed research into entirely new paths; hence a vast amount of research in laboratories around the world is moving in this direction (Cryan et al., 2019; Stachowicz, 2019; Simpson et al., 2021). In connection with the above, mutual regulation between host microbiota and the effectiveness of ADs was recently described (Cryan et al., 2019; Stachowicz, 2019; Duan and Xie, 2020; Simpson et al., 2021); the topic will be expanded in the last subsection.

The latest findings of the COVID-19 pandemic and social isolation documented the positive effects of gardening and physical activity on mental health (Bu et al., 2021). The benefits of gentle exercises for mental health may be connected with reducing blood pressure, regulating neuroendocrine and neuroimmune systems, and giving psychological benefits (Bu et al., 2021).

A new look at old drugs

When presenting new ideas for treating depression, one cannot ignore the idea of a "new look at old drugs." Compounds that affect the 5-HT system are a large group of AD in the clinic, including 5-HT1A agonists, e.g., vilazodone and vortioxetine (Wróbel et al., 2019). Searching for new antidepressant compounds is focused on that directed to dual agonist 5-HT1A activity and SSRI (Herold et al., 2011; Wróbel et al., 2019) as a compound that both can accelerate desensitization and downregulation of autoreceptors and directly stimulate postsynaptically localized 5-HT neurons (Herold et al., 2011; Wróbel et al., 2019). The idea is that kind of stimulation has a faster antidepressant potential. Among dually acting compounds, molecular targets are 5-HT2A, 5-HT6, 5-HT7, and D2 (Wróbel et al., 2019). Analogs of gepirone, novel pyridol/pyrimidine derivatives, alkylnitroquipazines, and others are synthesized (Paluchowska et al., 2005; Herold et al., 2011; Wróbel et al., 2019; Ślifirski et al., 2019; Król et al., 2021).

Inhibitors of the serotonin transporter (SERT) have long been in clinical use. The "new look at old drugs" regarding SERT compounds is based on the physicochemical properties of SERT ligands. Understanding of physicochemical properties of interactions with targeted sites may be beneficial in designing new compounds with antidepressant properties. Does conformational rearrangement or ligand flexibility play a role in binding reaction and efficacy (Martin et al., 2008)? Martin et al. (2008) documented increased enthalpy with a polar surface area of S-citalopram, duloxetine, fluoxetine, indatraline, paroxetine, sertraline, venlafaxine, but not fluvoxamine; what suggests a SERT inhibitor binding site is polar and allows hydrated ligands to bind without imposing the enthalpic penalty expected from ligand dehydration (Martin et al., 2008). Furthermore, following Martin et al. (2008), entropy/enthalpy compensation in ligand-protein interactions is counteracted by the following regulation (conformational flexibility or hydrophobic properties are regulators of entropy H-bonds or van der Waals interactions in enthalpy). Martin's group found SERT inhibitors bind to 5-HT transporter in a competitive manner (Apparsundaram et al., 2008), further concluding that 5-HT pore may allow for more than one set of interactions with antidepressants (Apparsundaram et al., 2008).

Antipsychotics have attracted some attention in the last decade; the more it has been observed that discontinuation of antidepressants correlates with hypomania or mania (Kassm and Naja, 2018). Second-generation antipsychotics, e.g., aripiprazole, quetiapine, and olanzapine in combination with fluoxetine, have been approved to treat depression (Kato and Chang, 2013). The target audience is patients who failed to respond to monotherapy with ADs (Kato and Chang, 2013). However, Second-generation antipsychotics were more potent than placebo; esketamine or lithium use trumped the antipsychotic results (Vázquez et al., 2021). The research is ongoing, so there is no conclusion.

The new ideas and directions of (preclinical) research

This subsection would not be presented with accepted and established trends, e.g., the antidepressant effects when targeting trophic factors like brain-derived neurotrophic factor (BDNF) or results of drugs directed to tropomyosin receptor kinase B (TrkB). Here, there will be many new ideas born in the depression field.

One fresh concept is described in Stachowicz (2018) and Wong et al. (2013). Rearrangement of the cytoskeleton of dendrites and spines and adhesion between spines as a predictor of mental health is an entirely new research direction. Following Wong et al. (2013), cytoskeletal abnormalities cause dendritic regression and decrease and are common in depression. In cytoskeletal rearrangement, actin filaments are engaged (F-actin, G-actin) and also actin-binding proteins (ABPs) and postsynaptic density (PSD) proteins, creating an interactive dendritic spine scaffold (Wong et al., 2013). The interplay of actin filaments with microtubules is responsible for organelles' circulation in the dendritic spine and thus for the rotation of the receptor components and anchoring them in the cell membrane (Wong et al., 2013). Various types of receptors are present in the PSD. Still, the ionotropic glutamate receptors (iGluRs) seem particularly important in depressive disorders and the search for new anti-depressants. For a long time, it was thought that a sufficient explanation of the functional changes in the excitatory transmission is post-translational modifications (e.g., phosphorylation) located in the postsynaptic membrane receptors. However, the studies from the last 30 years have destabilized the static image of excitatory synapses and revealed its highly dynamic structure. It is known that glutamate receptors show lateral mobility along the cell surface between synaptic and extrasynaptic regions. They undergo constitutive trafficking to and from the cell surface with a surface half-life measured in 10 min (Nishimune et al., 1998). They are delivered to and removed from the synaptic membranes regulated by neural activity or the degree of electrical stimulation of the neuron. These mechanisms control the number of receptors and the receptors' subunit composition, which determines the proper functioning of excitatory synapses. Regulation of the movement of receptors to synapses is multistep and includes transport from the endoplasmic reticulum, trafficking along dendrites, and local transport in a synaptic bulb. This process is controlled by numerous PSD proteins interacting with receptors *via* PDZ domains (e.g., PSD-95, Shank3/ProSAP2, SAP-97, PICK-1), and other scaffolding proteins (e.g., Homer, CaMKII) and the same receptors, which include many sites undergoing (not only) phosphorylation. Significantly, PSD proteins interact with many intracellular proteins (e.g., PSD-95 with synaptic Ras GTPase activating protein and guanylate kinase-related protein) (Dutta et al., 2021; Shaw and Koleske, 2021).

In addition to the above-discussed processes and molecules, adhesion is essential for synaptic formation by associating pre- and post-synaptic partners in a specific neuro space (Stachowicz, 2018). The importance of the problem was noted by Stachowicz (2018) in the review of the DSCAM protein. DSCAM is only an example, but the adhesive mechanisms involving other adhesive proteins are fundamental in synaptic plasticity and, thus, neural conduction and communication (Stachowicz, 2018; Jiang et al., 2021). Abnormal synaptic connection is associated with learning and memory disturbances and neuropsychiatric and neurodevelopmental disorders (Dutta et al., 2021).

An entirely new finding in the field of depression is the discovery of a new signal pathway involved in the disease—that is, engagement of the antioxidant pathway with nuclear factor erythroid-derived 2-like 2 (Nrf2) (Kansanen et al., 2013; Bouvier et al., 2017; Nakayama et al., 2020). Nrf2 was discovered in 1994 as a member of the human cap"n"collar (CNC) basic-region leucine zipper transcription factor family (Cuadrado et al., 2019). As a product of the NFE2L2 gene, Nrf2 forms heterodimers with other bZip proteins; and regulates the expression of about 250 human genes participating in inflammation, redox metabolism, or proteostasis (Robledinos-Anton et al., 2019). Activators of the Nrf2 pathway are under clinical investigation in Phase I-IV in multiple sclerosis, autism spectrum disorder, Alzheimer's disease, major depression, and others (Robledinos-Anton et al., 2019). In a depression field, activation of Nrf2 translocation restores redox homeostasis and reverses vulnerability to depression (Bouvier et al., 2016). Furthermore, Nrf2-null mice show depressive-like behavior, and treatment with Nrf2 agonists possesses antidepressant-like potential (Nakayama et al., 2020).

In recent years another discovery in the field of depression is the already mentioned microbiota-gut axis (Cryan et al., 2019; Stachowicz, 2019; Simpson et al., 2021). Our results with the *E. coli* lipopolysaccharide (LPS) use suggest the engagement of this mechanism in synaptic plasticity with the involvement of excitatory amino acid transporters (EAATs)/COX-2/metabotropic glutamate receptors (mGluRs) (Stachowicz et al., 2021). However, more sophisticated research documented the gut microbiota may regulate the hypothalamic-pituitary-adrenal (HPA) axis, producing neurotransmitters (Sirisinha 2016). Probiotics containing appropriate species of bacteria can lower cortisol; microbiota transplanted from a healthy animal can change animal behavior (Sirisinha 2016). This search for mechanisms of depression, thus related to the immune system, also includes recent reports on the occurrence of depression after COVID-19. Following Mohammadkhanizadeh and Nikbakht (2021), central mechanisms involved in COVID-19-induced depression are inflammation, including uncontrolled activation of microglia, and following the release of inflammatory cytokines (TNF-alpha, IL-6, IL-1beta), nitric oxide, prostaglandin E2. Furthermore, damage to mitochondria directly by reaching them for transcription of the virus genome and indirectly by devasting properties of pro-inflammatory cytokines and ROS (Mohammadkhanizadeh and Nikbakht, 2021). Damage to the hippocampus observed after COVID-19 as a structure involved in depression has drawn the particular attention of researchers to the mechanisms linking respiratory viral infections and depression (Mohammadkhanizadeh and Nikbakht, 2021). Impairment of hippocampal synaptic plasticity and neurogenesis, followed by

stress and dysregulation of the HPA axis, contributes to the progression of symptoms of depression (Mohammadkhanizadeh and Nikbakht, 2021). Next, vitamin D deficiency, Zinc, and magnesium are related to depression in COVID-19 (Mohammadkhanizadeh and Nikbakht, 2021).

Inflammatory processes in depression have long been a focus of research. Elevated levels of pro-inflammatory cytokines such as IL-1b, IL-6, and TNFa are observed in the serum of patients with depression (Iwata et al., 2013). The cytokines above are associated with somatic symptoms, described as illness behaviors, including fatigue and loss of appetite (Iwata et al., 2013), which overlap with typical symptoms of major depression. Subsequently, depression has been well described as a common complication of interferon treatment for malignant melanoma and chronic hepatitis (Musselman et al., 2001), and interferon-induced depression has been linked to a complex pathophysiological substrate involving serotonergic and dopaminergic neurotransmission as well as glucocorticoid and neurotrophic factors (Udina et al., 2016).

Concluding remarks

Summing up, as it can be seen based on this study, there is an intense search for new substances with an antidepressant profile. Researchers have focused on the hunt for compounds that directly interfere with the functioning of the Glu system through its various receptors, both NMDA, AMPA, and mGluRs. In addition, the next direction of research is still the 5-HT system. Still, in a new perspective, the game includes new receptors, such as 5-HT7, and multidirectional therapy, such as, e.g., triple reuptake inhibitors of 5-HT/norepinephrine/dopamine. These are, of course, the main directions of the search. Still, there are also attempts to search for active substances among compounds acting by orexin receptors, COX-2 inhibitors, incorporation of phagocytic, microglial, epigenetic mechanisms, or combination therapies. Personalized antidepressant treatment should also be considered in the future, considering gender differences or genetics, among other things. This problem was described in 2012 by Gvozdic et al. (2012) Undoubtedly, the development of new antidepressants based on new mechanisms of action is necessary due to the increasing number of patients, the unsatisfactory effectiveness of existing pharmacotherapy, or possible side effects and interactions with drugs used for other diseases.

Author contributions

Conceptualization: KS and MS-K; formal analysis: KS and MS-K; funding acquisition: KS and MS-K; investigation (literature review and collection): KS and MS-K; writing—original draft: KS; writing—review and editing: KS and MS-K Both authors have read and agreed to the published version of the manuscript.

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Conflict of interest

The 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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EDITED BY
Katarzyna Stachowicz,
Maj Institute of Pharmacology (IF PAS), Poland

REVIEWED BY
Przemyslaw Jan Danek,
Maj Institute of Pharmacology (IF PAS), Poland
Lei Chen,
Sichuan University, China

*CORRESPONDENCE
Stanisław J. Czuczwar,
stanislaw.czuczwar@umlub.pl
Ryszard Pluta,
pluta@imdik.pan.pl

†These authors share senior authorship

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Comorbid epilepsy and depression—pharmacokinetic and pharmacodynamic drug interactions

Barbara Miziak[1], Stanisław J. Czuczwar[1]*† and Ryszard Pluta[2]*†

[1]Department of Pathophysiology, Medical University, Lublin, Poland, [2]Laboratory of Ischemic and Neurodegenerative Brain Research, Mossakowski Medical Research Institute, Polish Academy of Sciences, Warsaw, Poland

Background: Major depressive disorder may be encountered in 17% of patients with epilepsy and in patients with drug-resistant epilepsy its prevalence may reach 30%. This indicates that patients with epilepsy may require antidepressant treatment.

Purpose: Both pharmacodynamic and pharmacokinetic interactions between antiepileptic (antiseizure) and antidepressant drugs have been reviewed. Also, data on the adverse effects of co-administration of antiepileptic with antidepressant drugs have been added. This article was submitted to Neuropharmacology, a section of the journal Frontiers in Pharmacology.

Methods: The review of relevant literature was confined to English-language publications in PUBMED databases. Table data show effects of antidepressants on the seizure susceptibility in experimental animals, results of pharmacodynamic interactions between antiepileptic and antidepressant drugs mainly derived from electroconvulsions in mice, as well as results concerning pharmacokinetic interactions between these drugs in clinical conditions.

Conclusion: Antidepressant drugs may exert differentiated effects upon the convulsive threshold which may differ in their acute and chronic administration. Animal data indicate that chronic administration of antidepressants could reduce (mianserin, trazodone) or potentiate the anticonvulsant activity of some antiepileptics (fluoxetine, reboxetine, venlafaxine). There are also examples of neutral interactions (milnacipran).

KEYWORDS

antiepileptic drugs (AEDs), antidepressant drugs, pharmacodynamic interactions, phrmacokinetic interactions, seizures, epilepsy, depression

1 Introduction

The prevalence for major depressive disorder (MDD) is in the range of 10% in the general adult population but as a comorbid condition in epilepsy, MDD is observed more frequently. For instance, it is observed in around 17% of patients with epilepsy and in patients with drug-resistant epilepsy, its prevalence may reach 30% or even more. Interestingly, in patients with remissions, MDD is noted in 6–9% of cases (Wiegartz et al., 1999; Jackson and Turkington, 2005; Tellez-Zenteno et al., 2007; Piedad et al., 2012). Depression is evidently responsible for the worsening of life quality in patients with epilepsy and may additionally increase seizure frequency and reduce the anticonvulsant efficacy of antiepileptic drugs (Hitiris et al., 2007).

A question arises whether depression may be a significant risk factor for the development of epilepsy. The existing evidence seems to confirm this possibility and there are even opinions available, pointing to common pathophysiological mechanisms in depression and epilepsy (Kanner, 2008). Both, in patients with temporal lobe epilepsy or depression, an enhanced hippocampal interleukin-1β signaling was evident (Kondziella et al., 2007). The involvement of reduced GABA-mediated inhibition and excessive glutamatergic neurotransmission are, no doubt, involved in generation of seizure activity (Łukawski et al., 2016). Disturbed GABA-ergic and glutamatergic neurotransmissions have been also observed in depression, for instance proton magnetic resonance spectroscopy revealed abnormal GABA and glutamate concentrations in the cortex and glutamate receptor antagonists were shown to exert antidepressant effects in animal models (Brambilla et al., 2003; Kugaya and Sanacora, 2005). After all, both epilepsy and depression are accompanied by atrophy of the temporal lobe (amygdalar nuclei, hippocampus, entorhinal cortex), frontal lobe, and temporal lateral neocortex. Also, reduced binding of serotonin in the frontal and temporal lobes, clear cut dysfunction of the hypothalamic-pituitary-adrenal axis, altered tryptophan metabolism, dysregulated neurogenesis, and neuroinflammation were found (Kanner, 2008; Singh and Goel, 2021).

As already mentioned, the prevalence of MDD in patients with epilepsy is high and surprisingly, in many cases depression is not diagnosed and remains untreated, possibly because of fear of adverse effects that might be induced by antidepressant drugs and their probable negative influence upon the convulsive threshold (Kanner et al., 2012; Bosak et al., 2015; Gangar and Bhatt, 2020; Singh and Goel, 2021). There are factors, evidently increasing the risk of depression in patients with epilepsy–older age, female gender, low level of education, unemployment, poor pharmacological control of seizures, polytherapy, stigma, and anxiety (Yang et al., 2020). The female gender faces a higher risk of depression in patients with epilepsy, preferably during the reduction of estrogenic activity encountered in the post-partum and late luteal phase. The estrogenic reduction may be associated with up-regulation of single nucleotide polymorphisms, and down-regulation of serotonin- and GABA-mediated events (Zarcone and Corbetta, 2017).

Below, the influence of a number of antidepressant drugs upon the seizure susceptibility of experimental animals in a wide range of seizure models as well as pharmacodynamic interactions between antiepileptic and antidepressant drugs are reviewed. Review of clinical data will be focused on pharmacokinetic interactions between these drugs. Literature search was performed basing mainly on PUBMED databases and English-language publications with no time frame. Relevant references were also considered from extracted publications.

2 Antidepressant drugs and seizure susceptibility

In many cases, the influence antidepressant drugs on the seizure threshold refers to either acute or chronic dosing. The results are presented in Table 1 on the basis of data reviewed by Banach et al. (2016).

3 Interactions between antiepileptic and antidepressant drugs in animal seizure models

3.1 Selective serotonin reuptake inhibitors

Fluoxetine (given acutely at subprotective doses against electroconvulsions in mice) potentiated the anticonvulsant activity of a number of conventional AEDs (carbamazepine, phenobarbital or phenytoin) against maximal electroshock-induced seizures (MES) in mice (Leander, 1992; Borowicz et al., 2006). In the same seizure test, fluoxetine (but at higher dose of 15–25 mg/kg) was also able to increase the protective action of valproate (Borowicz et al., 2006). Pharmacokinetic verification revealed that the brain concentrations of carbamazepine and phenobarbital were elevated (Borowicz et al., 2006). When administered chronically, this SSRI (at 15–20 mg/kg) was effective in enhancing the antiseizure efficacy of carbamazepine, phenytoin, and valproate whilst the protective activity of phenobarbital was increased only at 20 mg/kg of fluoxetine (Borowicz et al., 2007b). The brain concentrations of all conventional antiepileptic drugs studied were significantly increased by fluoxetine (Borowicz et al., 2007b).

Considering pentylenetetrazol (PTZ)-induced seizures in mice, acute fluoxetine (10 mg/kg) potentiated the anticonvulsant action of valproate whilst it was ineffective when combined with ethosuximide. Brain concentration of valproate was not affected and co-administered fluoxetine did

TABLE 1 Influence of various antidepressant drugs on the seizure threshold and epileptiform activity.

Class of antidepressants	Antidepressant	Dose of antidepressant (mg/kg)	Treatment		Method of administration	Animal model	Effect on seizures	
			Acute	Chronic			Epileptiform activity	Seizure threshold
Tricyclic antidepressants	Amitriptyline	20–50	+		i.p. or i.v	Rabbits	↑	
						Cats		
						Rats (implanted with intracerebral electrodes/kindling)		
						Pilocarpine-induced seizures in rats	↑	
		20–30	+		i.p	Mice (electrically induced convulsions)		↑
	Imipramine	1–25	+		i.v	Rabbits	↑	
						Cats implanted with intracerebral electrodes freely moving rats		
		10–50	+		i.p	Mice		
						Photosensitive baboons (papio papio)		
						Sham and mygdale-kindled rats		
		≥20–25	+		i.p	Cats	↑	
						Mice		
		30–40	+		i.p	Mice (electrically induced convulsions))		↑
	Desipramine	NNo data	No data	No data	No data	Freely moving rats implanted with intracerebral electrodes	↑	
						Mice (flurothyl-induced seizures)		↓
						Rats (kindling)	↓	
						Rats (audiogenic seizures in genetically epilepsy-prone	↓	
		up to 40				Mice		0
Selective serotonin reuptake inhibitors	Fluoxetine	5–20	+		No data	Mice	protective effect	
		10	+			Rats	↑	
		2,5-20	+			Rats	0	
		10	+			Rats (PTZ-induced seizures)		0
		10–20	+		i.p	Rats (seizures induced by pilocarpine)	↑ or ↓	
		ED_{50} = 15.9 or 8.2	+		No data	Wistar rats with high predisposition to audiogenic seizures (sound-induced seizures in (geprs))	↓	
		2.5–10	+		i.p	Rat model of ethanol withdrawal syndrome	↓	

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10.3389/fphar.2022.988716

TABLE 1 (*Continued*) Influence of various antidepressant drugs on the seizure threshold and epileptiform activity.

Class of antidepressants	Antidepressant	Dose of antidepressant (mg/kg)	Treatment		Method of administration	Animal model	Effect on seizures	
			Acute	Chronic			Epileptiform activity	Seizure threshold
		5–20	+		applied into substantia nigra or i.p	Rats (seizures evoked by injection of bicuculline into the area tempestas)	↓	
		15–25	+		No data	Mice (MES induced seizures)		↑
		10 (chronic treatment, 7 days)		+	No data	Rats (PTZ-induced seizures)		↓
				+	s.c	Amygdala-kindled rats	↑	
		10		+	Dietary	Mouse model of genetically predisposed/ handling-triggered epilepsy	↓	
		10		+	No data	Rats (electrically induced hippocampal seizures)		↑
		10	+			Mice (MES induced seizures)		0
				+				
	Fluoxetine + 5-Hydroxytrypto-phan	15	+		i.p	Rats (sound-induced seizures in (geprs))	↓	
	Paroxetine	1	+		No data	Mice (picrotoxin induced Seizures)	↓	
		50	+		No data	Rats (sham and amygdala-kindled)	No effect in behavior or EEG	
	Sertraline	40/24 h		+	via osmotic minipump	Mice (flurothyl-induced seizures)	0	
		40	+					↓
		2.5	+		No data	Rats (4-aminopyridine seizures)	↓	
		0.75/day		+				
		>5	+		No data	Rats (PTZ-induced seizures)	↓	
Serotonin and norepinephrine reuptake inhibitors	Venlafaxine	20–40	+		i.p	Audiogenic seizures in the rat model of ethanol withdrawal syndrome	↓	
			+			Mice (flurothylinduced seizures)		
		75–100	+			Rats (PTZ-induced seizures)	↑	
		150	+			Rats (spontaneous convulsions)	↑	
		12.5–25	+	+		Mice (electrically induced convulsions)		↑
	Milnacipran	10–40	+		i.p	Mice (electrically induced convulsions)		↑
Noradrenaline reuptake inhibitor	Reboxetine	20		+	via osmotic minipumps	Mice (flurothyl-induced seizures)	↑ ↓	↓

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10.3389/fphar.2022.988716

TABLE 1 (*Continued*) Influence of various antidepressant drugs on the seizure threshold and epileptiform activity.

Class of antidepressants	Antidepressant	Dose of antidepressant (mg/kg)	Treatment		Method of administration	Animal model	Effect on seizures	
			Acute	Chronic			Epileptiform activity	Seizure threshold
		20–30	+		No data	Kainic acid-induced post-status epilepticus rat model for temporal lobe epilepsy	↓	
			+		No data	Zebrafish larva (PTZ-induced seizures)	↓	
		10–20	+		No data	Mice (PTZ-induced seizures)		↑
		30	+		No data	Mice (pilocarpine-induced limbic seizures)	↑	
		8–16	+		No data	Mice (electrically induced convulsions)		↑
				+				0
Norepinephrine and dopamine reuptake inhibitor	Bupropion	ED_{50}–19.4	+		No data	Mice (MES-induced seizures)	↓	
		CD97–139.5	+		No data	Seizure activity per se	↑	
		7.5–10 twice daily		+	No data	Mice (electrically induced convulsions)		↑
		10 and 50	+		No data	Rats (kainic acid-induced seizures)	↓	
Reversible inhibitor of monoamine oxidase A	Moclobemide	62.5–75	+		No data	Mice (electrically induced convulsions)		↑
Other antidepressants	Mianserin	81.2	+		i.v	Rabbits	↑	
		30	+		i.v	Rats (implanted with intracerebral electrodes	↑	
		30–40	+		No data	Mice (electrically induced convulsions)		↑
	Trazodone	10–40	+		No data	Mice (electrically induced convulsions)		0
		40		+	No data			↑
		up to 80	+		No data	PTZ-, strychnine-, imipramine- and MES-induced seizures	0	
		100					↑	
	Tianeptine	1.25–10	+		No data	Rats (PTZ-induced seizures)	↓	
		40–80	+		No data	Mice (PTZ-induced seizures)	↓	
		5–20	+		i.p	Audiogenic seizures in the rat model of ethanol withdrawal syndrome	↓	
				+				
		25–50	+		No data	Mice (electrically induced convulsions)		0
				+				

↑—Increase in convulsive activity/increase of the convulsion threshold; ↓—decrease in convulsive activity/decrease of the convulsion threshold; 0–no impact; MES, maximal electroshock test; PTZ, pentylenetetrazol; i.p, intraperitoneal injection; i.v.–intravenous injection; s.c.–subcutaneous administration. Data taken from Banach et al. (2016).

10.3389/fphar.2022.988716

not augment the neurotoxic potential of either valproate or ethosuximide (Borowicz et al., 2012b).

Sertraline (given orally at 10 mg/kg to mice) significantly reduced the protective efficacy of gabapentin against PTZ which manifested in a considerable increase in seizure severity (Rizwan et al., 2003). Interestingly, sertraline at this dose also exacerbated PTZ-induced convulsions (Rizwan et al., 2003).

As regards acute paroxetine, this SSRI (1 mg/kg) enhanced the anticonvulsant activity of valproate (50 mg/kg) reflected by the prolonged latency to onset of seizures induced by picrotoxin (3.5 mg/kg) in mice. The evident reduction of the seizure score by valproate was not further potentiated by paroxetine (Kamal, 2012). The author of this study evaluated some more parameters. The results indicate that valproate-produced elevation of GABA within nucleus accumbens was further significantly increased by paroxetine (Kamal, 2012).

3.2 Serotonin and noradrenaline reuptake inhibitors

In the MES test, acute milnacipran (at 10 mg/kg, which elevated the convulsive threshold) reduced the ED_{50} values of conventional antiepileptic drugs (carbamazepine, phenobarbital, phenytoin, valproate) whilst at a subthreshold dose of 5 mg/kg, only the antiseizure activity of phenobarbital and carbamazepine was enhanced (Borowicz et al., 2010). Interestingly, chronic milnacipran (5–40 mg/kg) remained ineffective upon the protection offered by these antiepileptic drugs. No pharmacokinetic interactions with antiepileptic drugs, regarding acute or chronic milnacipran, were noted. Milnacipran did not also potentiate the neurotoxic potential of the conventional antiepileptics (Borowicz et al., 2010).

Venlafaxine was evaluated in the same convulsive test. At the subthreshold single or chronic dose of 6.25 mg/kg, this SNRI elevated the protective action of valproate. At 12.5 mg/kg (the dose increasing the convulsive threshold), venlafaxine was also able to potentiate the antiseizure activity of carbamazepine and phenobarbital but not that of phenytoin (Borowicz et al., 2011). The brain concentration of phenytoin was lowered by venlafaxine so the enhancing effect of venlafaxine could be masked by a pharmacokinetic interaction (Borowicz et al., 2011).

3.3 Noradrenaline reuptake inhibitor

Reboxetine (in acute doses of 2–8 mg/kg) combined with carbamazepine, phenobarbital and valproate led to reductions in their ED_{50} against MES in mice. Potentiation of the anticonvulsant action of phenytoin was observed for reboxetine at the dose range of 8–12 mg/kg. Importantly, this antidepressant drug elevated the electroconvulsive threshold starting from 8 mg/kg (Borowicz et al., 2014). When given chronically, this NRI (8–12 mg/kg) enhanced the protection by CBZ against MES, being, however, ineffective when combined with phenobarbital, phenytoin or valproate. The antidepressant drug, following chronic administration, did not affect the electroconvulsive threshold. In neither effective combination of reboxetine with antiepileptic drugs pharmacokinetic interactions were observed in terms of the brain concentration of these drugs (Borowicz et al., 2014).

Regarding PTZ-induced convulsions in mice, acute reboxetine, in the subthreshold doses of 6–8 mg/kg, did not affect the protective efficacy of clonazepam, ethosuximide, phenobarbital and valproate (Popławska et al., 2015).

3.4 Noradrenaline and dopamine reuptake inhibitor

Bupropion, similarly to some antiepileptic drugs, was acutely effective against MES in mice with an ED_{50} value of 19.4 mg/kg. In doses exceeding 100 mg/kg, this antidepressant proved as a convulsive agent (Tutka et al., 2004). When given chronically to mice at 5 mg/kg, the NDRI significantly enhanced the anticonvulsant effect of felbamate, lamotrigine and topiramate against MES. However, the combined treatment with lamotrigine resulted in a pharmacokinetic interaction as both, the plasma and brain level of this antiepileptic drug were significantly elevated. Although the plasma concentration of topiramate was reduced, its brain concentration was unaffected by bupropion. Neurotoxicity of antiepileptic drugs was not influenced by the antidepressant drug (Barczyński et al., 2011).

3.5 Reversible inhibitor of monoamine oxidase A

Acute but not chronic moclobemide (62.5 and 75 mg/kg) raised the threshold for electroconvulsions in mice. When given acutely, moclobemide (up to the subthreshold dose of 50 mg/kg) potentiated the anti-MES efficacy of carbamazepine, phenobarbital and valproate. Chronic RIMA (37.5–75 mg/kg) enhanced the protection offered by carbamazepine, phenobarbital, phenytoin and valproate against MES in mice and only its combination with phenobarbital was of pharmacodynamic nature. Its other combinations with antiepileptic drugs resulted in significant elevations of their brain concentrations. However, neither acute nor chronic moclobemide co-administered with antiepileptic drugs produced any impairment of motor performance or deficit of long-term memory (Borowicz-Reutt and Banach, 2021).

3.6 Tricyclic antidepressant drugs

Amitriptyline (at a single dose of 20 or 30 mg/kg) elevated the electroconvulsive threshold in mice and at the subthreshold dose of 10 mg/kg, it potentiated the anticonvulsant action of valproate against MES in mice. The ED_{50} value of valproate was diminished from 255 to 150 mg/kg (Kleinrok et al., 1991), Desipramine (acutely up to 40 mg/kg) was ineffective upon electroconvulsions in mice but at 20 mg/kg, the antidepressant significantly reduced the ED_{50} of valproate against MES from 255 to 135 mg/kg (Kleinrok et al., 1991).

As regards imipramine, the antidepressant given acutely at 30 or 40 mg/kg proved effective against electroconvulsions and at 20 mg/kg potentiated the protective action of valproate against MES in mice reflected by the reduction of its ED_{50} from 255 to 128 mg/kg (Kleinrok et al., 1991). No pharmacokinetic interactions were evident in combinations of valproate with desipramine. Other combinations of valproate with TCAs were not verified in terms of pharmacokinetics. Also, data on the interactions of chronic TCAs and AEDs are not available.

3.7 Other antidepressant drugs

Mianserin administered acutely (30 and 40 mg/kg) significantly raised the electronvulsive threshold in mice but when given chronically at 30 mg/kg, it was proconvulsant in the threshold electoconvulsive test (Borowicz et al., 2007a). Combinations of acute mianserin (up to 20 mg/kg) with antiepileptic drugs resulted in the reductions of ED_{50} values of carbamazepine, phenytoin and valproate against MES in mice. In contrast, mianserin given chronically (up to 20 mg/kg) decreased the anticonvulsant activity of phenytoin and valproate against MES whilst the protective action of carbamazepine against MES was unaffected. Modifications of the anticonvulsant efficacy of antiepileptic drugs by mianserin was of pharmacodynamic nature. Neither combination was associated with neurotoxicity evaluated in the chimney test (motor coordination) and passive avoidance task (long-term memory) (Borowicz et al., 2007a).

Tianeptine (25–50 mg/kg) neither acutely nor chronically affected the threshold for electroconvulsions in mice. In combinations with antiepileptic drugs, acute and chronic tianeptine (up to 50 mg/kg) potentiated the protective action of carbamazepine, phenobarbital and valproate, but not that of phenytoin, in the mouse MES test (Borowicz et al., 2013). Only the brain concentration of phenobarbital was reduced by tianeptine, other combinations showing pharmacodynamic profile. Motor coordination and long-term memory were not impaired in mice by combinations of tianeptine with antiepileptic drugs (Borowicz et al., 2013).

Trazodone, given singly at 10–40 mg/kg did not modify the electroconvulsive threshold in mice whilst on a chronic basis at 40 mg/kg it raised the threshold (Borowicz et al., 2012a). The acute antidepressant (up to 40 mg/kg), significantly reduced the anticonvulsant efficacy of carbamazepine and phenytoin against mouse MES. The protective action of phenobarbital and valproate in this test was not changed when combined with trazodone (up to 40 mg/kg). Combinations of chronic trazodone (up to 40 mg/kg) with antiepileptic drugs in the above test, yielded comparable results to its acute administration. Combined treatments of trazodone with antiepileptic drugs resulted in numerous pharmacokinetic interactions. The brain level of phenytoin was decreased and that of valproate elevated when co-administered with acute or chronic trazodone. Only chronic trazodone reduced the brain concentration of carbamazepine and phenobarbital. Finally, acute and chronic trazodone significantly augmented the neurotoxicity of phenytoin but not that of other antiepileptic drugs (Borowicz et al., 2012a). Detailed results of interactions between AEDs and antidepressants in seizure models have been presented in Table 2.

4 Pharmacokinetic interactions between antiepielptic and antidepressant drugs in clinical conditions

There are many inducers of hepatic microsomal enzymes or uridine diphosphate glucuronosyltransferase (UGT) among conventional antiepileptic drugs. The hepatic cytochrome P450 system (CYP) plays a more important role in these interactions (for review, Spina et al., 2016). Apart from the liver cytochrome P450 (CYP) enzymes, also brain CYP enzymes may participate in the local metabolism of a variety of drugs and neuroactive endogenous substances (for instance, neurosteroids), being engaged in biosynthesis of neurotranmitters as well (Daniel et al., 2022). Further, the central nervous system is apparently involved, via neuroendocrine and neuroimmune mechanisms, in the adjustment of liver CYP activity. Some antidepressant drugs have been documented to affect liver and brain CYP enzymes. A good example is fluoxetine which inhibits CYP2D in the liver, striatum and nucleus accumbens whilst stimulating its activity in the cerebellum. These complex interrelations clearly indicate that *in vitro* data on CYP enzyme inhibition (Danek et al., 2020) or stimulation by a psychotropic drug (Danek et al., 2021) may be not sufficient to predict pharmacokinetic interactions with concomitant drugs. *In vivo* data may comprise all mechanisms involved in the final effect of a drug upon the activity of CYP enzymes (Daniel et al., 2022).

Potent inducers of liver CYP encompass for example, carbamazepine, phenobarbital or phenytoin which activate a number of CYP isoforms—CYP1A2, CYP2C9, CYP2C19, and

CYP3A4 (Spina et al., 2016). Consequently, chronic administration of these antiepileptic drugs may be associated with significant pharmacokinetic interactions resulting in reducing the plasma concentration of co-administered antidepressants. This predicted interaction was actually confirmed in terms of TCAs and many others–for instance, bupropion, citalopram, escitalopram, paroxetine, reboxetine or venlafaxine (for review, Italiano et al., 2014). For instance, carbamazepine at low daily doses of 200 or 400 mg led to a significant reduction in plasma concentrations of S-citalopram and R-citalopram, by 27 and 31%, respectively (Spina et al., 2016). A comparable effect was observed for paroxetine whose plasma level was decreased by 25% in patients receiving carbamazepine, phenobarbital or phenytoin (Spina et al., 2016). A case-report study (2 patients) even provided evidence that carbamazepine led to a sharp reduction in plasma concentration of sertraline, which was associated with loss of sertraline's efficacy (Spina et al., 2016). Newer (or second-generation) antiepileptic drugs generally cause less pharmacokinetic interactions with concomitant medications, however, some of them are week enzyme inducers. That is why pharmacokinetic interactions may accompany combinations of clobazam, eslicarbazepine, oxcarbazepine, perampanel, rufinamide, and topiramate combined with antidepressants (Patsalos and Perucca, 2003; Spina et al., 2016). These newer antiepileptics may activate CYP3A4, however, some of them may also behave as weak inhibitors of CYP2C19 (eslicarbazepine, felbamate, oxcarbazepine, topiramate). A possibility of pharmacokinetic interactions with antidepressants is thus much lower than in the case of conventional antiepileptic drugs. The data on this issue are scarce. In 2021, in the single-center, open-label, randomized, 5-period cross-over trial, an interaction between the newer antiepileptic drug, gabapentin (25–300 mg), and trazodone (2.5–10 mg) was evaluated in healthy volunteers following single dose fasted administration. In no case pharmacokinetic interactions were evident in terms of the plasma AUC or C_{max} (Ruggieri et al., 2021).

In contrast to other conventional antiepileptic drugs, valproate is an inhibitor of microsomal liver enzymes, CYP2C9 being distinctly affected. Also, the antiepileptic inhibits UGTs and epoxide hydrolase. It may be thus predicted that valproate can cause increases in concomitant plasma concentrations of antidepressant drugs. This was in fact confirmed in patients taking amitriptyline or venlafaxine. Regarding the latter, the mean, dose-corrected, plasma concentration of its active metabolite, O-desmethylvenlafaxine was elevated by 27% (Italiano et al., 2014; Spina et al., 2016). Among newer antiepileptic drugs, stiripentol inhibits CYP1A2, CYP2C19, and CYP3A4 isoforms (Spina et al., 2016) so potentially, it could affect the pharmacokinetic parameters of antidepressant drugs, however, no data are available on this issue.

5 Pharmacokinetic interactions between antidepressant and antiepileptic drugs in clinical conditions

Numerous antidepressants have been documented to inhibit various isoforms of microsomal liver enzymes so these drugs are likely to affect the plasma concentrations of antiepileptics. Theoretically, increases in plasma concentrations of antiepileptic drugs may be expected following concomitant antidepressant drugs inhibiting P450 cytochrome enzymes.

The available evidence points to the fact that this assumption may not necessarily occur. For instance, fluvoxamine (100 mg daily, given for 3 weeks in seven patients), as an inhibitor of CYP1A2 and CYP2C19, did not modify carbamazepine's (800–1,600 mg daily) plasma concentration. Initial case report studies revealed the existence of a pharmacokinetic interaction between both drugs. Only a case report study is available on the interaction of fluvoxamine with phenytoin, indicating a significant rise in the plasma phenytoin level (Spina et al., 2016). However, fluoxetine which is an inhibitor of CYP2D6 and to a lesser degree inhibiting CYP2C19, caused significant elevations in the plasma levels of a number of antiepileptic drugs—for instance, phenytoin and valproate. In the case of the latter, a probable increase in its toxicity was observed (Monaco and Cicolin, 1999; Spina et al., 2016). There are unequivocal data as regards the influence of fluoxetine on the plasma concentration of carbamazepine. Some case reports point to significant elevations of carbamazepine's plasma levels but a study conducted on eight patients taking carbamazepine (800–1,600 mg daily revealed no impact of fluoxetine (co-administered for 3 weeks with this AED) on its steady-state concentration (Spina et al., 2016). However, in one case of combined treatment with fluoxetine and carbamazepine, even a Parkinson-like syndrome was reported (Monaco and Cicolin, 1999). Moreover, fluoxetine may elevate the concentration of the carbamazepine's toxic metabolite, carbamazepine-10,11-epoxide although there are also data reporting no such effect (Monaco and Cicolin, 1999). Equivocal effects were obtained for fluvoxamine and carbamazepine whilst results on fluvoxamine-induced considerable elevation of phenytoin plasma level are only available (Spina et al., 2016; Zaccara and Franco 2022).

Viloxazine co-administered with carbamazepine may lead not only to the rise in the plasma concentration of this AED but also to the increased level of carbamazepine-10,11-epoxide. Whilst the plasma elevation of the AED was in the range of 50% of basal plasma concentration, the metabolite level was increased by 16% (Monaco and Cicolin, 1999). Viloxazine can also affect the metabolism of oxcarbazepine, leading to a 15% rise in the ADE's metabolite—hydroxycarbazepine and elevate the concentration of phenytoin (Monaco and Cicolin, 1999).

TABLE 2 Interactions between AEDs and antidepressant drugs in experimental seizure models.

Class of antidepressants	Antidepressant	Dose of antidepressant [mg/kg]	Treatment		Method of administration	Antiepileptic drug	Dose of AEDs/ED_{50}^{c} ED_{50}^{e} (mg/kg)	Method of administration	Seizure model	Anticonvulsant activity of AEDs	Neurotoxic effects of AEDs				References
			Acute	Chronic							Chimney test	Passive-avoidance task	Rotarod test	Spontaneous alternation behavior (SAB) models	
Tricyclic antidepressants	Amitriptyline	10	+		i. p	VPA	ED_{50}^{c}–255/ ED_{50}^{e}–150	i. p	MES	↑	-	-	-	-	Kleinrok et al. (1991)
	Imipramine	20	+		i. p	VPA	ED_{50}^{c}–255/ ED_{50}^{e}–128	i. p	MES	↑	-	-	-	-	
	Desipramine	20	+		i. p	VPA	ED_{50}^{c}–255/ ED_{50}^{e}–135	i. p	MES	↑	-	-	-	-	Kleinrok et al. (1991)
Selective serotonin reuptake inhibitors	Fluoxetine	15–20		+	i. p	CBZ	ED_{50}^{c}–9.8/ ED_{50}^{e}–4.1	i.p	MES	↑	0	0	-	-	Borowicz et al. (2007a)
	Fluoxetine	15–20		+	i.p	PHT	ED_{50}^{c}–12.2/ ED_{50}^{e}–7.6	i.p	MES	↑	0	0	-	-	Borowicz et al. (2007b)
	Fluoxetine	15–20		+	i.p	VPA	ED_{50}^{c}–211/ ED_{50}^{e}–130.6	i.p	MES	↑	0	0	-	-	Borowicz et al. (2007a)
	Fluoxetine	20		+	i.p	PB	ED_{50}^{c}–25.5/ ED_{50}^{e}–18.5	i.p	MES	↑	0	0	-	-	Borowicz et al. (2007b)
	Fluoxetine	10	+		i.p	VPA	ED_{50}^{c} –117.2/ ED_{50}^{e}–78.2	i.p	PTZ	↑	0	0	-	-	Borowicz et al. (2012a)
	Fluoxetine	10	+		i.p	ETX	ED_{50}^{c}–137.7/ ED_{50}^{e}–122.1	i.p	PTZ	0	0	0	-	-	Borowicz et al. (2012b)
	Fluoxetine	15–25	+		i.p	VPA	ED_{50}^{c}–212.5/ ED_{50}^{e}– 151.1–110.3	i.p	MES	↑	0	0	-	-	Borowicz et al. (2006)
	Paroxetine	1	+		i.p	VPA	50	i.p	seizures induced by picrotoxin	↑	-	-	-	-	Kamal (2012)
	Sertraline	10	+		p.o	GBP	312	p.o	PTZ	↓	-	-	-	↓	Rizwan et al. (2003)
Serotonin and norepinephrine reuptake inhibitors	Venlafaxine	6.25	+		i.p	PHT	ED_{50}–10	i.p	MES	0	0	0	-	-	Borowicz et al. (2011)
	Venlafaxine	6.25	+		i.p	VPA	ED50c –253.5/ ED50e–199.6	i.p	MES	↑	0	0	-	-	Borowicz et al. (2011)
	Venlafaxine	6.25		+	i.p	VPA	ED_{50}^{c}–210.7/ ED_{50}^{e}–142.6	i.p	MES	↑	0	0	-	-	Borowicz et al. (2011)
	Venlafaxine	12.5	+		i.p	CBZ	ED_{50}^{c}–10.3/ ED_{50}^{e}–6.7	i.p	MES	↑	0	0	-	-	Borowicz et al. (2011)
	Venlafaxine	12.5	+		i.p	PB	ED_{50}^{c}–15.4/ ED_{50}^{e}–10.7	i.p	MES	↑	0	0	-	-	Borowicz et al. (2011)
	Venlafaxine	12.5	+		i.p	PHT	ED_{50}^{c}–12.4/ ED_{50}^{e}–6.4	i.p	MES	0	0	0	-	-	Borowicz et al. (2011)
	Milnacipran	10	+		i.p	CBZ	ED_{50}^{c}–11.2/ ED_{50}^{e}–5.2	i.p	MES	↑	0	0	-	-	Borowicz et al. (2010)

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10.3389/fphar.2022.988716

TABLE 2 (*Continued*) Interactions between AEDs and antidepressant drugs in experimental seizure models.

Class of antidepressants	Antidepressant	Dose of antidepressant [mg/kg]	Treatment		Method of administration	Antiepileptic drug	Dose of AEDs/ED_{50}^{c} ED_{50}^{e} (mg/kg)	Method of administration	Seizure model	Anticonvulsant activity of AEDs	Neurotoxic effects of AEDs				References
			Acute	Chronic							Chimney test	Passive-avoidance task	Rotarod test	Spontaneous alternation behavior (SAB) models	
	Milnacipran	10	+		i.p	PB	ED_{50}^{c}–18.4/ ED_{50}^{e}–14.4	i.p	MES	↑	0	0	-	-	Borowicz et al. (2010)
	Milnacipran	10	+		i.p	PHT	ED_{50}^{c}–8.5/ ED_{50}^{e}–5.1	i.p	MES	↑	0	0	-	-	Borowicz et al. (2010)
	Milnacipran	10	+		i.p	VPA	ED_{50}^{c}–213/ ED_{50}^{e}–157.1	i.p	MES	↑	0	0	-	-	Borowicz et al. (2010)
	Milnacipran	5	+		i.p	CBZ	ED_{50}^{c}–11.2/ ED_{50}^{e}–6.2	i.p	MES	↑	0	0	-	-	Borowicz et al. (2010)
	Milnacipran	5	+		i.p	PB	ED_{50}^{c}–18.4/ ED_{50}^{e}–11.9	i.p	MES	↑	0	0	-	-	Borowicz et al. (2010)
	Milnacipran	5–40		+	i.p	CBZ	ED_{50}^{c}–11.2/ ED_{50}^{e}–10.1	i.p	MES	0	0	0	-	-	Borowicz et al. (2010)
	Milnacipran	5–40		+	i.p	PB	ED_{50}^{c}–18.4/ ED_{50}^{e}–15.1	i.p	MES	0	0	0	-	-	Borowicz et al. (2010)
	Milnacipran	5–40		+	i.p	PHT	ED_{50}^{c}–8.5/ ED_{50}^{e}–10.6	i.p	MES	0	0	0	-	-	Borowicz et al. (2010)
	Milnacipran	5–40		+	i.p	VPA	ED_{50}^{c}–213/ ED_{50}^{e}–210.7	i.p	MES	0	0	0	-	-	Borowicz et al. (2010)
Noradrenaline reuptake inhibitor	Reboxetine	6–8	+		i.p	CLZ	ED_{50}^{c}–0.0019/ ED_{50}^{e}–0.014	i.p	PTZ	0	0	0	-	-	Popławska et al. (2015)
		6–8	+		i.p	ETX	ED_{50}^{c}–140.9/ ED_{50}^{e}–139.6	i.p	PTZ	0	0	0	-	-	Popławska et al. (2015)
		6–8	+		i.p	PB	ED_{50}^{c}–18/ ED_{50}^{e}–15.3	i.p	PTZ	0	0	0	-	-	Popławska et al. (2015)
		6–8	+		i.p	VPA	ED_{50}^{c}–115.3/ ED_{50}^{e}–101.6	i.p	PTZ	0	0	0	-	-	Popławska et al. (2015)
		2–8	+		i.p	CBZ	ED_{50}^{c}–11.3/ ED_{50}^{e}–7.2	i.p	MES	↑	0	0	-	-	Borowicz et al. (2014)
		2–8	+		i.p	PB	ED_{50}^{c}–18.7/ ED_{50}^{e}–11.9	i.p	MES	↑	0	0	-	-	Borowicz et al. (2014)
		2–8	+		i.p	VPA	ED_{50}^{c}–244.3/ ED_{50}^{e}–181	i.p	MES	↑	0	0	-	-	Borowicz et al. (2014)
		8–12	+		i.p	PHT	No data	i.p	MES	↑	0	0	-	-	Borowicz et al. (2014)
		8–12		+	i.p	CBZ	ED_{50}^{c} –11.9/ ED_{50}^{e}–8.7	i.p	MES	↑	0	0	-	-	Borowicz et al. (2014)
		8–12		+	i.p	PB	ED_{50}^{c}–24.1/ ED_{50}^{e}–22.7	i.p	MES	0	0	0	-	-	Borowicz et al. (2014)
		8–12		+	i.p	PHT	ED_{50}^{c}–9.9/ ED_{50}^{e}–9.4	i.p	MES	0	0	0	-	-	Borowicz et al. (2014)
		8–12		+	i.p	VPA	ED_{50}^{c}–240.2/ ED_{50}^{e}–203.1	i.p	MES	-	0	0	-	-	Borowicz et al. (2014)

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10.3389/fphar.2022.988716

TABLE 2 (*Continued*) Interactions between AEDs and antidepressant drugs in experimental seizure models.

Class of antidepressants	Antidepressant	Dose of antidepressant [mg/kg]	Treatment		Method of administration	Antiepileptic drug	Dose of AEDs/ED_{50}^{c} ED_{50}^{e} (mg/kg)	Method of administration	Seizure model	Anticonvulsant activity of AEDs	Neurotoxic effects of AEDs				References
			Acute	Chronic							Chimney test	Passive-avoidance task	Rotarod test	Spontaneous alternation behavior (SAB) models	
Norepinephrine and dopamine reuptake inhibitor	Bupropion	5		+	i.p	Felbamate	ED_{50}^{c}–48.79/ ED_{50}^{e}–37.28	i.p	MES	↑	-	-	0	-	Barczyński et al. (2011)
				+	i.p	LTG	ED_{50}^{c}–4.58/ ED_{50}^{e}–3.01	i.p	MES	↑	-	-	0	-	
				+	i.p	TPM	ED_{50}^{c}–60.95/ ED_{50}^{e}–41.58	i.p	MES	↑	-	-	0	-	
Reversible inhibitor of monoamine oxidase A	Moclobemide	50	+		i.p	CBZ	ED_{50}^{c}–9.9/ ED_{50}^{e}–2.9	i.p	MES	↑	0	0	-	-	Borowicz-Reutt and Banach (2021)
		50	+		i.p	PB	ED_{50}^{c}–16.4/ ED_{50}^{e}–7.2	i.p	MES	↑	0	0	-	-	Borowicz-Reutt and Banach (2021)
		50	+		i.p	VPA	ED_{50}^{c}–211.9/ ED_{50}^{e}–148.5	i.p	MES	↑	0	0	-	-	Borowicz-Reutt and Banach (2021)
		37.5–75		+	i.p	PHT	ED_{50}^{c}–9.9/ ED_{50}^{e}–5.8	i.p	MES	↑	0	0	-	-	Borowicz-Reutt and Banach (2021)
Other antidepressants	Mianserin	up to 20	+		i.p	CBZ	ED_{50}^{c} –13.2/ ED_{50}^{e}–6.2	i.p	MES	↑	0	0	-	-	Borowicz et al. (2007a)
	Mianserin	up to 20	+		i.p	PHT	ED_{50}^{c}–8.3/ ED_{50}^{e}–5.2	i.p	MES	↑	0	0	-	-	Borowicz et al. (2007b)
	Mianserin	up to 20	+		i.p	VPA	ED_{50}^{c}–221/ ED_{50}^{e}–168.6	i.p	MES	↑	0	0	-	-	Borowicz et al. (2007a)
	Mianserin	up to 20	+		i.p	PHT	ED_{50}^{c}–7.8/ ED_{50}^{e}–11.5	i.p	MES	↓	0	0	-	-	Borowicz et al. (2007a)
	Mianserin	up to 20	+		i.p	VPA	ED_{50}^{c}–244.3/ ED_{50}^{e}–324.2	i.p	MES	↓	0	0	-	-	Borowicz et al. (2007b)
	Trazodone	20		+	i.p	CBZ	ED50c–12.5/ ED50e–17.2	i.p	MES	↓	0	0	-	-	Borowicz et al. (2012a)
	Trazodone	up to 40	+		i.p	CBZ	ED50c–11.4/ ED50e–15.4	i.p	MES	↓	0	0	-	-	Borowicz et al. (2012b)
	Trazodone	20		+	i.p	PHT	ED50c–9.9/ ED50e–16.2	i.p	MES	↓	↑	↑	-	-	Borowicz et al. (2012a)
	Trazodone	up to 40	+		i.p	PHT	ED50c–8.2/ ED50e–12.5	i.p	MES	↓	↑	↑	-	-	Borowicz et al. (2012b)
	Trazodone	20		+	i.p	PB	ED50c–25.0	i.p	MES	0	0	0	-	-	Borowicz et al. (2012a)
	Trazodone	up to 40	+		i.p	PB	ED50c–17.9	i.p	MES	0	0	0	-	-	Borowicz et al. (2012b)
	Trazodone	20		+	i.p	VPA	ED50c–245.0	i.p	MES	0	0	0	-	-	Borowicz et al. (2012a)

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10.3389/fphar.2022.988716

TABLE 2 (*Continued*) Interactions between AEDs and antidepressant drugs in experimental seizure models.

Class of antidepressants	Antidepressant	Dose of antidepressant [mg/kg]	Treatment		Method of administration	Antiepileptic drug	Dose of AEDs/ED_{50}^{c} ED_{50}^{e} (mg/kg)	Method of administration	Seizure model	Anticonvulsant activity of AEDs	Neurotoxic effects of AEDs				References
			Acute	Chronic							Chimney test	Passive-avoidance task	Rotarod test	Spontaneous alternation behavior (SAB) models	
	Trazodone	up to 40	+		i.p	VPA	ED50c–234.0	i.p	MES	0	0	0	-	-	Borowicz et al. (2012b)
	Tianeptine	up to 50	+		i.p	CBZ	ED_{50}^{c}–16.6/ ED_{50}^{e}–10.2	i.p	MES	↑	0	0	-	-	Borowicz et al. (2013)
	Tianeptine	up to 50	+		i.p	PB	ED_{50}^{c}–24.7/ ED_{50}^{e}–14.2	i.p	MES	↑	0	0	-	-	Borowicz et al. (2013)
	Tianeptine	up to 50	+		i.p	VPA	ED_{50}^{c}–265.4/ ED_{50}^{e}–192	i.p	MES	↑	0	0	-	-	Borowicz et al. (2013)
	Tianeptine	up to 50	+		i.p	PHT	no data	i.p	MES	0	0	0	-	-	Borowicz et al. (2013)
	Tianeptine	up to 50		+	i.p	CBZ	ED_{50}^{c}–19.9/ ED_{50}^{e}–9.2	i.p	MES	↑	0	0	-	-	Borowicz et al. (2013)
	Tianeptine	up to 50		+	i.p	PB	ED_{50}^{c}–21.4/ ED_{50}^{e}–16.7	i.p	MES	↑	0	0	-	-	Borowicz et al. (2013)
	Tianeptine	up to 50		+	i.p	VPA	ED_{50}^{c}–274.1/ ED_{50}^{e}–194.4	i.p	MES	↑	0	0	-	-	Borowicz et al. (2013)
	Tianeptine	up to 50		+	i.p	PHT	no data	i.p	MES	0	0	0	-	-	Borowicz et al. (2013)

↑- increased; ↓—decreased;—no test was carried out (in neurotoxic effects of AEDs); 0–no effect; AEDs, antiepileptic drugs; CLZ, clonazepam; CBZ, carbamazepine; ED_{50}^{c}–ED_{50} in control group; ED_{50}^{e}–ED_{50} in experimental group; ETX, ethosuximide; GBP, gabapentin; i.p.–intraperitoneal injection; p.o.–oral administration; LTG, lamotrigine; MES, maximal electroshock-induced convulsions; PB, phenobarbital; PHT, phenytoin; PTZ, pentylenetetrazol-induced seizures; TPM, topiramate VPA, valproate.

10.3389/fphar.2022.988716

A number of other antidepressant drugs, TCAs (imipramine or nortriptyline) or trazodone may lead to an increase in concentration of phenytoin (Monaco and Cicolin, 1999). Also, trazodone (a CYP3A4 inhibitor) has been shown to precipitate carbamazepine's neurotoxicity which was evidently associated with the elevated plasma concentration of this AED. Remarkably, the signs of toxicity tended to disappear when the antidepressant drug was withdrawn (Spina et al., 2016).

Interestingly, sertraline (an inhibitor of CYP2D6), although displayed no potential to affect the plasma concentrations of either carbamazepine or phenytoin, it distinctly increases the plasma levels of valproate and lamotrigine, and these combinations were actually associated with toxicity. However, another study did not confirm the interaction of sertraline with lamotrigine (Spina et al., 2016).

Examples of co-administrations of paroxetine and mirtazapine which do not inhibit CYP enzymes involved in the metabolism of AEDs may suggest that the reduced activity of CYP enzymes is generally responsible for the observed interactions between antidepressant drugs and AEDs. Actually, none of these two antidepressants have been reported to significantly affect the plasma levels of AEDs (Monaco and Cicolin, 1999; Spina et al., 2016). Citalopram and escitalopram (as inhibitors of CYP2D6) have been also found not to interact with carbamazepine following chronic administration of both groups of drugs in healthy volunteers (Spina et al., 2016). Pharmacokinetic interactions have been summarized in Tables 3, 4.

6 Possibly pharmacodynamic interactions between antiepileptic and antidepressant drugs in clinical conditions

Data on possibly pharmacodynamic interactions between these two groups of drugs are scarce. Kagawa et al. (2014) evaluated the efficacy of lamotrigine in patients with treatment-resistant depressive disorder who were taking 3 psychotropic drugs (antidepressants, mood stabilizers, atypical antipsychotics). Lamotrigine was given at 100 mg daily for 18 patients (without valproate) and 75 mg daily for those taking valproate (N = 16). Percentage improvements at week eight was clearly dependent on plasma lamotrigine concentrations of ≥12.7 μmol/L. At this lamotrigine concentration 73% of patients were improved whilst in patients with lower lamotrigine plasma concentrations only 23% benefitted from lamotrigine augmentation. Interestingly, neither serum brain-derived neurotrophic factor nor interleukin-6 were not affected by lamotrigine combined with other antipsychotropic drugs (Kagawa et al., 2017).

Also topiramate and pregabalin were documented to potentiate the antidepressant efficacy of antidepressants (for instance, SSRIs) (Elger et al., 2017).

7 Adverse effects associated with co-administration of antiepileptics and antidepressants in patients with epilepsy and depression

One of the adverse effects, in patients taking AEDs and antidepressant drugs, may be associated with body weight gain. Reduced weight gain has been shown to accompany combinations of zonisamide or topiramate with amitryptiline, bupropion, mirtazapine or paroxetine whilst increased weight gain—combinations of carbamazepine, gabapentin, pregabaline or VPA with amitryptiline, mirtazapine or paroxetine (Italiano et al., 2014).

AEDs have been documented to exert a negative impact on bone health and in case, antidepressants possess a similar activity, the combined treatment may eventually lead to additive or even synergistic effects (Miziak et al., 2019). Although, the experimental data do not seem to support such a possibility, the results from two meta-analyses are not that optimistic. Actually, TCAs and SSRIs have been shown to significantly increase the risk fractures (Miziak et al., 2019).

Both some AEDs and antidepressants have been documented to stimulate the activity of CYP enzymes (Italiano et al., 2014). Consequently, it is advised to avoid combined treatments with liver enzyme enhancers among these groups of drugs. This restriction may apply to carbamazepine, felbamate or phenytoin and bupropion, duloxetine, TCAs or trazodone, on the other. These combinations may result in subsequent liver injury (Italiano et al., 2014).

Patients with the presence of long QTc syndrome and family history of sudden death are prone to lethal ventricular arrhythmias (torsade de pointes). Some antidepressants (citalopram, fluoxetine, sertraline) and the AEDs (felbamate and probably carbamazepine, lamotrigine, and phenytoin) (Auerbach et al., 2018) predispose patients to arrhythmias so there combined use is not recommended (Italiano et al., 2014).

Some other adverse effects may accompany combined treatment with AEDs and antidepressants—reduced production of sweat and hypohydrosis, increased risk for hyponatremia (especially when carbamazepine is co-administered with antidepressants) (Banach et al., 2016).

TABLE 3 Pharmacokinetic interactions between antiepileptic and antidepressant drugs.

Antiepileptic drug	Dose of antiepileptic drugs (mg/kg/day)	Antidepressant; dosage (mg/kg/day)	Plasma concentration of the antidepressant (%)	References
CBZ	200 or 400	Citalopram; 40-60	↓ (21) S-citalopram	Spina et al. (2016)
CBZ	200 or 400	Citalopram; 40-60	↓ (31) R-citalopram	Spina et al. (2016)
CBZ	ND	Paroxetine	↓ (25)	Spina et al. (2016)
CBZ	ND	Sertraline	↓ (ND)	Spina et al. (2016)
GBP	25–300	Trazodone; 2.5-10	0	Ruggieri et al. (2021)
PB	ND	Paroxetine	↓ (25)	Spina et al. (2016)
PHT	ND	Paroxetine	↓ (25)	Spina et al. (2016)
VPA	ND	Amitriptyline	↑ (50-60)	Italiano et al. (2014)
VPA	ND	Venlafaxine	↑ (27)	Spina et al. (2016)

↑- increased; ↓ - decreased; ND, no data; 0–no effect; CBZ, carbamazepine; GBP, gabapentin; PB, phenobarbital; PHT, phenytoin; VPA, valproate.

TABLE 4 Pharmacokinetic interactions between antidepressant and antiepileptic drugs.

Antidepressant	Dose of an antidepressant (mg/kg/day)	Duration of treatment with an antidepressant	Antiepileptic drug; dosage (mg/kg/day)	Duration of treatment with an antiepileptic drug	Plasma concentration of antiepileptic drugs (% increase where applicable)	References
Citalopram	40	14 days	CBZ; 400	35 days	0	Spina et al. (2016)
Escitalopram	ND	ND	CBZ; 400	ND	0	Spina et al. (2016)
Fluoxetine	ND	ND	PHT and VPA	ND	↑	Monaco and Cicolin, (1999); Spina et al. (2016)
Fluoxetine	20	3 weeks	CBZ; 800-1,600	3 weeks	0	Spina et al. (2016)
Fluvoxamine	100	3 weeks	CBZ; 800-1,600	3 weeks	0	Spina et al. (2016)
	100	3 weeks	PHT	ND	↑	Spina et al. (2016); Zaccara and Franco (2022)
Imipramine Nortriptyline Trazodone	ND	ND	PHT	ND	↑	Monaco and Cicolin, (1999)
Sertraline	200	17 days	PHT; 300	24 days	0	Spina et al. (2016)
Sertraline	200	17 days	CBZ; 400	32 days	0	Spina et al. (2016)
Sertraline	100	ND	VPA	ND	↑ (3-fold elevation in serum concentration)	Spina et al. (2016)
Sertraline	25	ND	LTG	ND	↑ (2-fold elevation in plasma)	
Viloxazine	ND	ND	CBZ	ND	↑ (up to 50%)	Monaco and Cicolin, (1999)

↑- increased; ↓—decreased; ND, no data; 0–no effect; CBZ, carbamazepine;; LTG, lamotrigine; PHT, phenytoin; VPA, valproate.

7 What is the significance of animal data pointing to the fact that some antidepressants may reduce the anticonvulsant activity of some antiepileptic drugs?

One of reasons for co-administration of AEDs with antidepressants is co-morbid depression in patients with epilepsy although, as already mentioned, it may be frequently underdiagnosed or untreated because of fear of untoward drug interactions (Kanner et al., 2012; Bosak et al., 2015). The existing experimental evidence on pharmacodynamic interactions between AEDs and antidepressants may give possible clues on which drug combinations in depressed patients with epilepsy need to be avoided. The importance of pharmacokinetic interactions between these groups of drugs in experimental animals possess rather no clinical implications due to different metabolic pathways in rodents and humans. However, their estimation in experimental studies could help delineate pure pharmacodynamic or mixed pharmacodynamic/pharmacokinetic interactions. Certainly, pure pharmacodynamic interactions would have more predictive value when transferring the experimental data to clinical conditions.

Theoretically, antidepressant drugs raising the convulsive threshold would seem safe to be combined with AEDs in patients with epilepsy. However, antidepressants start to exert therapeutic effects following chronic administration. That is why they were evaluated in terms of their influence upon the convulsive threshold both after acute and chronic treatment. Mianserin is a good example that acute vs. chronic administration may yield opposite effects—whilst given acutely, it increased the anticonvulsive threshold in mice but its chronic administration resulted in an opposite effect. Comparably, its acute combinations with phenytoin or valproate led to the potentiation of their protective efficacy against maximal electroshock-induced convulsions in mice and chronic ones resulted in significant reductions in their anticonvulsant activities (Borowicz et al., 2007). Chronic trazodone significantly elevated the electroconvulsive threshold in mice and no effect on the threshold was observed after its acute injection (Borowicz et al., 2012a). Interestingly, following its chronic combinations with phenobarbital or carbamazepine, reductions in the anticonvulsant effects of these AEDs against maximal electroshock in mice were evident. Nevertheless these reductions were associated with significant decreases in the brain concentration of these AEDs so, probably, a possibility of pharmacodynamic interactions is minimal. On the other hand, chronic trazodone was neutral upon the protective effect of valproate although its brain concentration was elevated by more than 30%. A possibility arises that the negative impact of trazodone on the anticonvulsant potential of valproate was actually masked by the pharmacokinetic interaction (Borowicz et al. 2012b). Tianeptine (acutely or chronically), although ineffective upon the electroconvulsive threshold, it significantly enhanced the protection offered by carbamazepine and valproate in the chronic protocol, without affecting their brain concentrations (Borowicz et al., 2013). The last example concerns venlafaxine which acutely and chronically raised the electroconvulsive threshold and following its chronic administration, also potentiated the anticonvulsant activity of valproate against maximal electroshock. The protection of other AEDs (carbamazepine, phenobarbital, phenytoin) was not modified (Borowicz et al., 2011).

To the degree, the experimental data may be transferred to clinical conditions, interactions between AEDs and antidepressants resulting in neutral outcomes or in the potentiation of anticonvulsant activity of an AED can be considered safe as regards the seizure control. In contrast, when an antidepressant leads to reduced anticonvulsant effects of AEDs then its clinical use may be not recommended in patients with epilepsy and depression. A neutral effect of an antidepressant masked by a pharmacokinetic interaction, showing a significant rise in the brain concentration of an AED, must be considered negative. When a neutral effect of the combined treatment is associated with a reduced brain concentration of an AED then probably no reduction in seizure control can be expected.

8 Conclusion

Mesial temporal lobe epilepsy with hippocampal sclerosis is frequently associated with a prevalence of major depression in the range of up to 25%, which further points to a close pathophysiological relationship between these two disorders (da Nobrega Marinho et al., 2022). The patients with either epilepsy or epilepsy with major depression exhibited a reduced expression of 5-HT_{1A} receptors in comparison with the control patients (da Nobrega Marinho et al., 2022). Possibly, SSRIs would be a good choice to treat major depression in these patients considering that these drugs did not negatively modify the anticonvulsant activity of a number of AEDs in experimental conditions.

Chronic venlafaxine, both elevated the threshold and enhanced the anticonvulsant activity of valproate although other conventional AEDs were not affected. Mianserin reduced the threshold and the protective activity of valproate and phenytoin. Anyway, on the basis of the effects of antidepressant drugs upon the convulsive threshold, it is not possible to predict their interactions with AEDs. Actually, although chronic trazodone increased the convulsive threshold, its interactions with AEDs were assumed negative. Another example also shows that a chronic antidepressant without effect on the threshold, can effectively potentiate the anticonvulsant activity of AEDs (tianeptine and carbamazepine

or valproate). Even though, experimental data may yield significant clues to the clinicians on the pharmacodynamic interactions between AEDs and antidepressants, patients with epilepsy and depression require careful monitoring for seizure frequency and adverse effects. Preclinical findings (mainly from studies with maximal electroshock-induced convulsions in mice) indicate that safe antidepressants for patients with epilepsy might be bupropion, fluoxetine, milnacipran, moclobemide, paroxetine, reboxetine, tianeptine, and venlafaxine. On the other hand, results of experimental studies may discourage using mianserin (especially in patients with epilepsy on phenytoin or valproate) and trazodone (in patients receiving valproate). The clinical evidence is generally in accordance with experimental data on safe antidepressants in patients with epilepsy (Harden and Goldstein, 2002), the exception being bupropion reported as a drug with a greater risk of seizures (Harden and Goldstein, 2002; Pisani et al., 2022). Other antidepressants to be used with caution include clomipramine and maprotiline (Harden and Goldstein, 2002; Pisani et al., 2022). No clinical support exists for mianserin and trazodone as antidepressants not recommended in patients with epilepsy.

In an elegant review on many experimental aspects of the interactions between AEDs and antidepressant drugs, Borowicz-Reutt (2021) has analyzed probable mechanisms involved in the potentiation of the anticonvulsant activity of some AEDs by antidepressant co-treatment. One of such mechanisms might be reduced synaptic glutamate release (fluoxetine, reboxetine, venlafaxine). Probably, the brain-derived neurotrophic factor (BDNF) could be involved, considering that some antidepressants were documented to increase its brain expression (for example, moclobemide or fluoxetine) and BDNF itself exhibited its own anticonvulsant activity (Borowicz-Reutt, 2021). Also, enhanced serotonergic, noradrenergic or dopaminergic neurotransmission by a number of antidepressants could be involved in the positive interactions between these groups of drugs as generally, stimulation of at least some serotonergic, noradrenergic or D-2 dopaminergic receptors was found anticonvulsant in some experimental models of seizures (Löscher and Czuczwar, 1985; Löscher and Czuczwar, 1986; Löscher and Czuczwar, 1987).

Mianserin, as a drug increasing the noradrenergic action (Dell'Osso et al., 2011), was found to reduce the anticonvulsant activity of phenytoin and valproate which is hard to explain at the moment. Probably, chronic administration of this antidepressant resulted in a number of events leading eventually to negative pharmacodynamic interactions with these AEDs. A similar situation was encountered when analyzing the effect of calcium channel inhibitors upon the anticonvulsant activity of AEDs against maximal electroshock-induced seizures in mice. Although calcium channel inhibitors generally enhanced the anticonvulsant activity of AEDs (Kułak et al., 2004), niguldipine was an exception to the rule. Whilst it elevated the electroconvulsive threshold, but when combined with AEDs, it led to the reduction of the anticonvulsant action of carbamazepine and phenobarbital (Borowicz et al., 1997).

As already mentioned, AEDs and antidepressants may interact via pharmacokinetic or pharmacodynamic mechanisms. The available data on the efficacy of antidepressants in alleviating depressive symptoms associated with epilepsy, however, seems limited. According to Maguire et al. (2021), who have reviewed randomized controlled trials and non-randomized studies of interventions, responses to antidepressants in patients with epilepsy were highly variable. Possibly, better results may be achieved when a patient is prescribed carbamazepine, gabapentin, lamotrigine, topiramate or valproate. These antiepileptics have been documented to improve mood (Alhashimi et al., 2022; Shamabadi, 2022).

Finally, as already mentioned, polytherapy in epilepsy is a recognized risk factor for the development of depression. The existing experimental data on interactions between AEDs and antidepressants refer to one drug from each group so no experimental clues are available on this issue. Therefore, management of depression in patients with epilepsy on polytherapy seems more challenging and may result in more negative drug interactions.

Author contributions

BM: Literature search, table preparation. SC: Conceptualization, writing. RP: Preparing and editing MS. All authors approved the submitted version.

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Conflict of interest

The 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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EDITED BY
Katarzyna Stachowicz,
Maj Institute of Pharmacology (PAS), Poland

REVIEWED BY
Bruno Pierre Guiard,
Université de Toulouse, France
David S. Middlemas,
A. T. Still University, United States

*CORRESPONDENCE
Jean-Philippe Guilloux,
jean-philippe.guilloux@universite-paris-saclay.fr

†These authors have contributed equally to this work and share last authorship

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Peripheral proteomic changes after electroconvulsive seizures in a rodent model of non-response to chronic fluoxetine

Rodolphe H. Lebeau[1], Indira Mendez-David[1], Laura Kucynski-Noyau[1], Céline Henry[2], David Attali[3], Marion Plaze[3], Romain Colle[4,5], Emmanuelle Corruble[4,5], Alain M. Gardier[1], Raphaël Gaillard[3], Jean-Philippe Guilloux[1*†] and Denis J. David[1†]

[1]Batiment Henri Moissan, CESP-Inserm, MOODS Team, Université Paris-Saclay, Orsay, France, [2]PAPPSO, Micalis Institute, INRAE, AgroParisTech, Université Paris-Saclay, Jouy-en-Josas, France, [3]Centre Hospitalier Sainte Anne, Service Hospitalo-Universitaire, Paris, France, [4]Université Paris-Saclay, Faculté de Médecine, CESP-Inserm, MOODS Team, Le Kremlin Bicêtre, France, [5]Service Hospitalo-Universitaire de Psychiatrie de Bicêtre, Hôpitaux Universitaires Paris-Saclay, Assistance Publique-Hôpitaux de Paris, Hôpital de Bicêtre, Le Kremlin Bicêtre, France

Major depressive disorder (MDD) is the psychiatric disorder with the highest prevalence in the world. Pharmacological antidepressant treatment (AD), such as selective serotonin reuptake inhibitors [SSRI, i.e., fluoxetine (Flx)] is the first line of treatment for MDD. Despite its efficacy, lack of AD response occurs in numerous patients characterizing Difficult-to-treat Depression. ElectroConvulsive Therapy (ECT) is a highly effective treatment inducing rapid improvement in depressive symptoms and high remission rates of ~50–63% in patients with pharmaco-resistant depression. Nevertheless, the need to develop reliable treatment response predictors to guide personalized AD strategies and supplement clinical observation is becoming a pressing clinical objective. Here, we propose to establish a proteomic peripheral biomarkers signature of ECT response in an anxio/depressive animal model of non-response to AD. Using an emotionality score based on the analysis complementary behavioral tests of anxiety/depression (Elevated Plus Maze, Novelty Suppressed Feeding, Splash Test), we showed that a 4-week corticosterone treatment (35 µg/ml, Cort model) in C57BL/6JRj male mice induced an anxiety/depressive-like behavior. A 28-day chronic fluoxetine treatment (Flx, 18 mg/kg/day) reduced corticosterone-induced increase in emotional behavior. A 50% decrease in emotionality score threshold before and after Flx, was used to separate Flx-responding mice (Flx-R, n = 18), or Flx non-responder mice (Flx-NR, n = 7). Then, Flx-NR mice received seven sessions of electroconvulsive seizure (ECS, equivalent to ECT in humans) and blood was collected before and after ECS treatment. Chronic ECS normalized the elevated emotionality observed in Flx-NR mice. Then, proteins were extracted from peripheral blood mononuclear cells (PBMCs) and isolated for proteomic analysis using a high-resolution MS Orbitrap. Data are available via

ProteomeXchange with identifier PXD037392. The proteomic analysis revealed a signature of 33 peripheral proteins associated with response to ECS (7 down and 26 upregulated). These proteins were previously associated with mental disorders and involved in regulating pathways which participate to the depressive disorder etiology.

KEYWORDS

electroconvulsive therapy, fluoxetine, non-response, peripheral biomarkers, major depressive disorder, difficult to treat depression

1 Introduction

Major depressive disorder (MDD) is the psychiatric disorder with the highest prevalence in the world according to World Health Organization (Liu et al., 2020). MDD can lead to significant mortality, morbidity, reductions in quality of life, and have considerable costs for the society (American Psychiatric Association 2013). The main treatments for moderate to severe MDD are based on antidepressant drugs (AD), such as selective serotonin reuptake inhibitor (SSRI). However, the pharmacotherapeutic strategy is partially efficient. Indeed, the STAR*D study (Rush et al., 2006; Trivedi et al., 2006) revealed that the remission is obtained only in one-third of treated patients with a first antidepressant family and this rate can be increased up to 70% using various pharmacotherapeutic approaches. Non-response and/or resistance occurs in a large subset of patients, constituting difficult-to treat depression, that can lead to Treatment Resistant Depression (TRD).

In order to treat these patients, many strategies have been investigated, including fast-acting therapy such as esketamine nasal spray (Bahji et al., 2021) or brain stimulation therapies (Voineskos et al., 2020). Electroconvulsive therapy (ECT), a therapeutic protocol known since 1930s for treat psychiatric disorders (Pagnin et al., 2004), is the most used and the most effective approach with a remission rate ≈80% (Group 2003) and can be applied as a second line of treatment (Milev et al., 2016).

The potential of peripheral proteome (the panel of detectable proteins) for monitoring health and disease states was investigated for brain diseases (Htike et al., 2019) including mood disorders (Preece et al., 2018). Moreover, detecting peripheral mRNA or proteins whose expression is specifically regulated by a treatment allow the identification of potential biomarkers of response to treatment that can have a predictive value (Guilloux et al., 2015; Mendez-David et al., 2017).

Several clinical studies tried to identify novel biomarkers predicting ECT outcomes and responses to targeted treatments [for review, (Maffioletti et al., 2021)]. Thus, ECT modulates the expression of 10 and ≈40 proteins in the blood of patients after either acute or chronic ECT, respectively (Stelzhammer et al., 2013). Moreover, AD treatment adjuncts with chronic ECT changes proteins profile compared to patients receiving only ECT. However, most of the studies are concentrated on one or few markers and many studies are relatively old, with small sample sizes and methodological biases (Maffioletti et al., 2021).

Many studies led during the last 30 years have shown that electroconvulsive seizure (ECS), the preclinical version of ECT, induces the expression modulation of a large variety of genes in a tissue-dependent manner (Sakaida et al., 2013; Kobayashi and Segi-Nishida 2019; Rimmerman et al., 2021). However, very few studies looked at the protein expression modulating effect of ECS. Some studies have described protein changes in the brain parenchyma (Pinna et al., 2018) or in naïve animals (Glaviano et al., 2014), which limits the use of these proteins as biomarkers of ECS and their translational utility. Thus, it will be of interest to study the effects of ECS on peripheral proteome as a surrogate for assessing treatment efficacy in a mouse model of AD non-response.

The purpose of our study will be to identify proteome variations induced by ECS in the peripheral blood mononuclear cells (PBMC), in the context of non-response to an antidepressant drug in an animal model of anxio-depressive disorders. To model the pathology, we used a pharmacological-induced model of depressive-like behavior previously developed by our team (David et al., 2009; Mendez-David et al., 2017). Concretely, mice were treated first with corticosterone (Cort) to induce a depressive-like phenotype, then with a combination of Cort and fluoxetine (Flx) for 4 weeks. After each step of treatment, an emotionality score (equivalent to clinical scores assessing the severity of depression) was established (Guilloux et al., 2011) to evaluate changes in emotional behavior and treatment response. After Flx treatment, mice that failed to recover a score similar to non-depressed mice receive a 2-weeks ECS treatment. A blood draw was performed in animals before and after ECS treatment for proteomic analysis, revealing a signature of 33 peripheral proteins associated with response to ECS (7 down- and 26 upregulated), with one priorly found to be associated to antidepressant response (Mendez-David et al., 2017).

2 Materials and methods

2.1 Animals

Adult C57BL/6JRj male mice were purchased from Janvier Farms (Le Genest St Isle, France). All mice were 7–8 weeks old, weighed 23–25 g at the beginning of the treatment and were

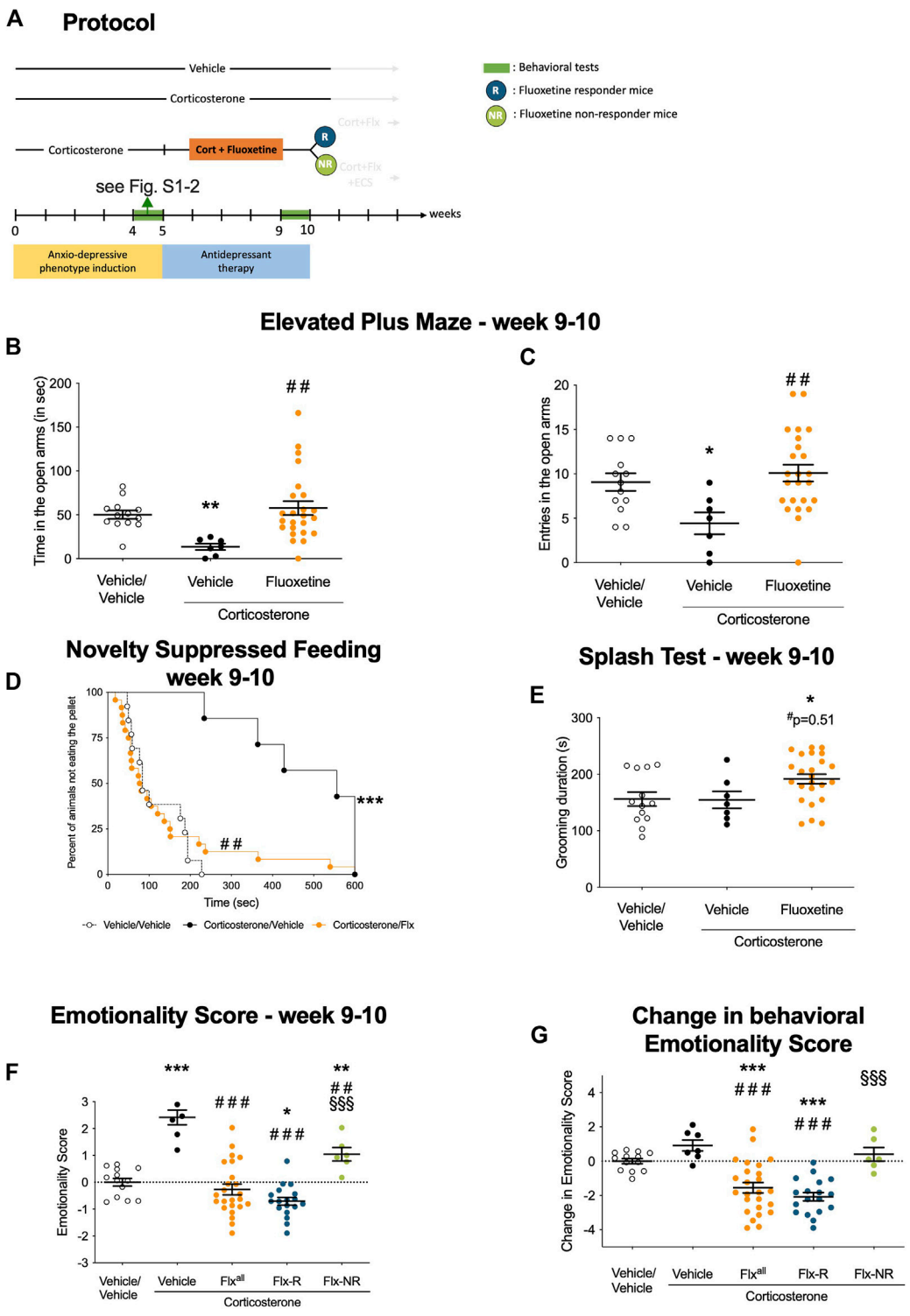

FIGURE 1
Chronic fluoxetine treatment produces reversed anxiety and depression-like phenotype in a mouse model of anxiety/depression. **(A)** Timeline of experiments: In place of normal drinking water, grouped-housed male C57BL/6JRj mice were presented during 10 weeks with vehicle (0.45% hydroxypropyl-β-cyclodextrin) or corticosterone (35 μg/ml) in the presence or absence of an antidepressant (fluoxetine, 18 mg/kg/day) during the last 5 weeks of the corticosterone regimen. Emotionality z-score was calculated after each behavioral session. Then, we investigated whether the behavioral changes induced after chronic corticosterone (week 4–5, see Supplementary Figure S2) were reversed by fluoxetine treatment (week 9–10, Figures 1B–G). The same animal was successively tested in the Elevated Plus Maze (EPM), the Novelty Suppressed Feeding (NSF), the Splash
(*Continued*)

FIGURE 1 (Continued)
Test (ST) during both behavioral sessions **(B–C)** Effects of corticosterone (35 µg/ml, Cort) regimen given alone or in combination with fluoxetine (18 mg/kg/day) on anxiety behaviors measured at Week 9–10 in the Elevated Plus Maze (EPM). Anxiety, measured for various parameters is expressed as mean total time in seconds **(B)** or entries **(C)** in open arms of EPM paradigm.**(D)** Effects of 4 weeks of corticosterone regimen (35 µg/ml) alone or in combination with fluoxetine (18 mg/kg/day) on anxiety- and depression related behaviors measured at Week 9–10 in the Novelty Suppressed Feeding paradigm. Results are expressed as cumulative survival with percentage of animals that have not eaten over 10-min. **(E)** Effects of 4 weeks of corticosterone regimen (35 µg/ml) alone or in combination with fluoxetine (18 mg/kg/day) on depression related behaviors in the Splash Test (ST) measured at Week 9–10. Results are expressed as mean of grooming duration (in seconds). **(F-G)** Effects of 4 weeks of corticosterone regimen (35 µg/ml, Cort) alone or in combination with fluoxetine (18 mg/kg/day) on anxiety/depression-like behaviors on the emotionality score measured at Week 9–10. Test Z-values (elevated plus maze, novelty-suppressed feeding and splash test) are calculated by averaging individual Z-scores to obtain emotionality Z-scores **(F)**. Change in behavioral emotionality score between W4 and W10 **(G)**.Values plotted are mean ± SEM [n = 7–21 animals for vehicle (VEH, open circle), corticosterone (Cort, black dot), corticosterone-fluoxetine mice (Fluoxetine or Flx^{all}, orange dot) corticosterone-fluoxetine responding mice (Flx-R, blue circles), corticosterone-fluoxetine non-responding mice with ECS treatment (Flx-NR ECS, green circles)]. One way ANOVA with post-hoc tests or Kaplan–Meier survival analysis followed by Mantel–Cox log-rank test were applied (*$p < 0.05$, **$p < 0.01$ versus Vehicle/vehicle group, #$p < 0.05$, #$p < 0.01$ versus Cort/vehicle group, §$p < 0.05$, §§$p < 0.01$ versus Flx-R mice).

maintained on a 12L:12 D schedule (lights on at 06:00). Mice were housed in groups of five. Food and water were provided *ad libitum*. The protocols involving animals and their care were conducted in conformity with the institutional guidelines in compliance with national and international laws and policies (Council directive #87–848, 19 October 1987, Ministère de l'Agriculture et de la Forêt, Service Vétérinaire de la Santé et de la Protection Animale, permissions # 92–256B to DJD) and in compliance with protocols approved by the Institutional Animal Care and Use Committee (CEE26 authorization #4747).

2.2 Treatments

Corticosterone (4-pregnen-11b-DIOL-3 20-DIONE 21-hemisuccinate from Sigma (Sigma-Aldrich Saint-Quentin Fallavier, France) was dissolved in vehicle (0.45% hydroxypropyl-β-cyclodextrin, Sigma-Aldrich Saint-Quentin Fallavier, France). Fluoxetine hydrochloride (18 mg/kg per day in the drinking water) was purchased from Anawa Trading (Zurich, Switzerland).

2.3 Protocol

The dose and duration of corticosterone treatment were selected based on previous study [Cort model (David et al., 2009; Mendez-David et al., 2013; Mendez-David et al., 2014),]. Corticosterone (35 µg/ml, equivalent to about 5 mg/kg/day, n = 31) or vehicle (0.45% β-cyclodextrine, β-CD, n = 13) were available *ad libitum* in the drinking water during the first 4 weeks of the protocol (Supplementary Figure S2A).

Chronic corticosterone consumption in mice dramatically inhibit the HPA axis function and response to stress with a resulting low concentration of serum corticosterone (See Supplementary Figure S3E, David et al., 2009) During the following 4 weeks of the protocol, corticosterone was delivered alone (n = 7 animals) or in the presence of fluoxetine (18 mg/kg/day, n = 24 animals, Figure 1A). Treatments were maintained until the end of the experiments. Behavioral sessions to assess anxiety/depression-like phenotype, the antidepressant response to fluoxetine and to chronic ECS occurred on week 5, 10, and 12, respectively. Thus, each animal underwent three behavioral sessions aiming at evaluating emotional behavior.

2.4 Electroconvulsive seizure

The ECS paradigm consisted of seven ECS sessions across a 15-days period (once every 2 days, Figure 2A) delivered with an Ugo Basile pulse generator (model #57800–001, shock parameters: 100 pulse/s frequency, 3 ms pulse width, 1 s shock duration and 50 mA current). Mice were administered inhaled isoflurane anesthesia (2%) prior to ECS sessions, and they remained anesthetized throughout the procedure. The stimulation parameters were chosen because they reliably induced tonic-clonic convulsions (Schloesser et al., 2015). Fluoxetine treatment was still administered during the 2 weeks of ECS treatment.

2.5 Behavioral experiment evaluation

2.5.1 Elevate maze plus

The elevated plus maze (EPM) is a widely used behavioral assay for rodents and it has been validated to assess the anti-anxiety effects of pharmacological agents [for review (Walf and Frye 2007)]. This test was performed as described previously (Mendez-David et al., 2014). The maze is a plus-cross-shaped apparatus, with two open arms and two arms closed by walls linked by a central platform 50 cm above the floor. Mice were individually put in the center of the maze facing an open arm and were allowed to explore the maze for a duration of 5 min. The time spent in and the numbers of entries into the open arms were used as an anxiety index. All parameters were measured using a videotracker (EPM3C, Bioseb, Vitrolles, France).

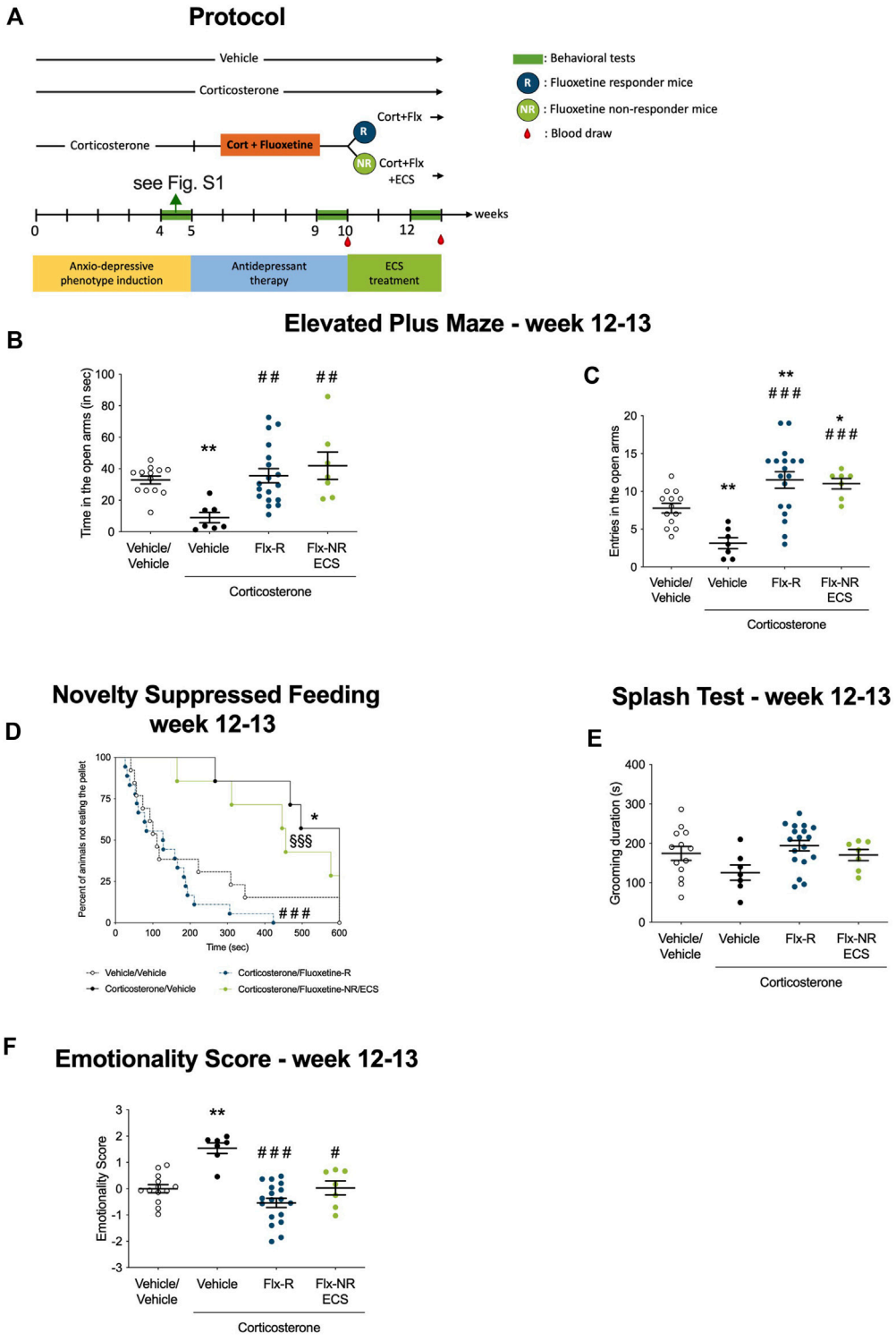

FIGURE 2
Fluoxetine non-responding mice emotional behavior was rescued after chronic electroconvulsive seizures (ECS administration). **(A)** Timeline of experiments: Following protocol presented in Figure 1A, Responders and non-responders animals were selected and Peripheral Blood Mononuclear Cells were isolated from whole blood after W10. Chronic ECS treatment was applied in NR-mice while R underwent sham treatment. Emotional behavior was tested at W12 after ECS administration and PBMC extracted from whole blood. **(B–C)** Effects of chronic ECS on anxiety behaviors in the Elevated Plus Maze (EPM) in Flx-NR mice. Anxiety, measured for various parameters is expressed as mean total time in seconds **(B)** or entries **(C)** in open arms of EPM paradigm. **(D)** Effects of chronic ECS on anxiety- and depression related behaviors in the Novelty Suppressed Feeding paradigm
(*Continued*)

FIGURE 2 (Continued)
in Flx-NR mice. Results are expressed as cumulative survival with percentage of animals that have not eaten over 10-min. **(E)** Effects of 4 weeks of chronic ECS on depression related behaviors in the Splash Test (ST). Results are expressed as mean of grooming duration (in seconds). **(F)** Effects of 4 weeks of chronic ECS on the emotionality score. Test Z-values (elevated plus maze, novelty-suppressed feeding and splash test) are calculated by averaging individual Z-scores to obtain emotionality Z-scores **(F)**.Values plotted are mean ± SEM [n = 7–21 animals for vehicle (VEH, open circle), corticosterone (Cort, black dot), corticosterone-fluoxetine responding mice (FLX-R, blue dots), corticosterone-fluoxetine non-responding mice with ECS treatment (FLX-NR ECS, green dots)]. One way ANOVA with post-hoc tests or Kaplan–Meier survival analysis followed by Mantel–Cox log-rank test were applied (*p < 0.05, **p < 0.01 versus Vehicle/vehicle group, #p < 0.05, ##p < 0.01 versus Cort/vehicle group, §p < 0.05, §§p < 0.01 versus Flx-R mice).

2.5.2 Novelty suppressed feeding test

The NSF is a conflict test that elicits competing motivations: the drive to eat and the fear of venturing into the center of a brightly lit arena. The latency to begin eating is used as an index of anxiety/depression-like behavior, because classical anxiolytic drugs as well as chronic antidepressants decrease this measure. The NSF test was carried out during a 10 min period as previously described (David et al., 2009). Briefly, the testing apparatus consisted of a plastic box (50 × 40 × 20 cm), the floor of which was covered with approximately 2 cm of wooden bedding. 24 h prior to behavioral testing, all food was removed from the home cage. At the time of testing, a single pellet of food (regular chow) was placed on a white paper platform positioned in the center of the box. Each animal was placed in a corner of the box, and a stopwatch was immediately started. The latency to eat (defined as the mouse sitting on its haunches and biting the pellet with the use of forepaws) was timed. Immediately afterwards, the animal was transferred to its home cage, and the amount of food consumed by the mouse in the subsequent 5 min was measured serving as a control for change in appetite as a possible confounding factor.

2.5.3 Splash test

This test consisted of squirting a 10% sucrose solution on the mouse's snout. This procedure induces grooming behaviors, due to the viscosity and palatability of the sucrose. The grooming behavior is sensitive to chronic stress or chronic Cort exposure and antidepressant treatment (Mendez-David et al., 2014). The total time spent in different grooming behaviors (i.e., face, paws, hindquarter, and shoulders) was directly recorded for 5 min in the home cage of the animals.

2.5.4 Behavioral emotionality measurement

Three behavioral tests (i.e., EPM, NSF and ST) were used to measure components of animal behavioral emotionality. Z-score methodology was used to investigate the potential of combining results within and across the different behavior tests for depressive/anxious-like behaviors and investigate the treatment effects in the Cort model. The emotionality-related data was normalized as previously described (Guilloux et al., 2011; Mekiri et al., 2017). Briefly, z scores are standardized scores (by the group mean and group standard deviation). They indicate how many standard deviations (σ) an observation (x) is above or below the mean of a control group (μ).

$$z = \frac{X - \mu}{\sigma}$$

Z scores for behavioral measures were first averaged within the test, and then across the test for equal weighting of the three tests comprising the final emotionality score. The increased behavioral emotionality was defined as decreased activity in the open arms in the EPM, increased NSF latency and decreased grooming in the splash test compared with control group means. The vehicle group was defined as the control. Emotionality score was calculated after each behavioral round.

2.6 Isolation of mouse peripheral blood mononuclear cells

To determine a biological signature of response to ECS, representative animals of each group were used for proteomics analysis. The procedure was performed in unanesthetized mice as previously described (Mendez-David et al., 2013). In compliance with the laboratory animal care guidelines, about 0.4 ml of blood per mice was collected in K_3EDTA tubes using the submandibular bleeding method. The punctures were performed with 5 mm point size sterile lancets (MediPoint, Mineola, NY) where the orbital vein and the submandibular vein join to form the jugular vein (Joslin 2009). A light pressure with dry gauze was applied to the punctured area for hemostasis. Separation and extractions of PBMCs were done using the iodixanol mixer technique (Ford and Rickwood 1990). Separations of mouse PBMCs were purified of mouse whole blood through density centrifugation (1,000 rpm at 20°C for 30 min) using solution B with the OptiPrep™ gradient solution (Sigma-Aldrich Saint-Quentin Fallavier, France). After centrifugation, OptiPrep™ gradient solution separated layers of blood, with PBMCs under a layer of plasma. The PBMCs layers were carefully removed from the tube and transferred to a new 50 ml conical tube and were washed twice with solution B. After centrifugations (1,200 rpm at 20°C for 7 min) and several washing steps, mouse PBMCs were recovered with a last centrifugation (3,000 rpm at 4°C for 5 min) and stored at 80°C before subsequent assay.

2.7 Proteomics analysis

2.7.1 Protein separation

Protein extracts from PBMCs were homogenized in solution solubilization (Urea 7M, Thiourea 2M, CHAPS 3%, Nonidet P-40 1%, DTT 1%). Protein concentration was measured using 2D-Quant kit (GE Healthcare, France) and 15 µg of proteins were loaded and separated by 12% SDS-PAGE. A short migration was then performed (7 min, 80 V, 25 W followed by 4 min, 200 V, 25 W) and gels were stained with Coomassie colloidal blue (EZblue, Sigma-Aldrich, France).

2.7.2 Protein in-gel digestion

Portions of gel that contain all proteins were cut and digested as followed: pieces of gel were successively washed and de-stained with water, acetonitrile (ACN) and 25 mM ammonium bicarbonate (NH_4HCO_3). A reduction/alkylation step was performed with dithiothreitol (DTT) 10 mM and iodoacetamide 55 mM. Gels were dehydrated with acetonitrile and rehydrated at 4 °C in 12 ng/µl sequencing grade modified trypsin (Promega, France) solubilized in 25 mM NH_4HCO_3 in 1 h and then digested at 37°C overnight. After tryptic digestion, peptides were extracted by incubating gel pieces in extraction solvent (0.5% trifluoroacetic acid (TFA)/50% ACN) for 15 min and in ACN for 15 min at room temperature. Supernatants were vacuum dried. The dried extract peptides were dissolved in 50 µl of loading buffer (0.08% TFA/2% ACN) just before mass spectrometry analysis.

2.7.3 Mass spectrometry analysis

Four microliters of sample were loaded on the nano-UPLC Ultimate 3000 RSLCnano (Thermo). Sample was loaded at 20 µL/min on the pre-column cartridge (PepMap 100 C18, 5 µm; 300 µm i.d., 5 mm, Thermo Scientific) and peptides were then separated with a gradient of acetonitrile on the reverse phase column PepMap 100 C18 (stationary phase: C18, 3 µm; column: 75 µm i.d., 500 mm; nanoViper, Thermo Scientific, France). Buffers were 0.1% formic acid in 2% acetonitrile (A) and 0.1% formic acid in 80% acetonitrile (B). The peptide separation was realized during 64 min at 300 nL/min with a linear gradient from 0 to 45% B for 55 min followed by a gradient from 45% to 98% B for 5 min. Eluted peptides were analyzed on-line with a high resolution mass spectrometer Orbitrap Fusion Lumos Tribrid (Thermo Scientific, France) using a nanoelectrospray interface in positive polarity mode, on PAPPSO platform (http://pappso.inra.fr). Peptide ions were analyzed using Xcalibur 3.0 (Thermo Scientific, France) with following data-dependent acquisition steps: 1) full MS scan in orbitrap (mass-to-charge ratio [m/z] = 400–1500; mass tolerance, ± 10 ppm) and 2) MS/MS in Ion Trap with CID activation (collision energy, 35%; activation time, 30 ms; centroid mode). Dynamic exclusion time was set to 60 s.

2.8 Statistical analysis

2.8.1 Behavioral analysis

To assess the behavioral consequences of a chronic corticosterone treatment in the EPM, NSF, and ST tests or emotionality scores, results were expressed as mean ± SEM values. Normality of data were checked using a Shapiro-Wilk normality test. Depending on the normality of the data, the parametric or non-parametric procedure was performed using a Student *t*-test or a Mann and Whitney test for two groups' comparison. For analysis with >2 groups, a one-way ANOVAs or a Kruskal–Wallis test were applied to the data as appropriate. Significant main effects were followed by Fisher's *post-hoc* or Dunn's multiple comparison tests. Regarding the NSF test, we used the Kaplan–Meier survival analysis owing to the lack of normal distribution of the data. Mantel–Cox log rank test was used to evaluate differences between experimental groups. Statistical significance was set at $p < 0.05$. Data were analyzed using Prism 8.4.3 software (GraphPad, La Jolla, United States).

2.8.2 Data processing and bioinformatics analysis

Peak lists were generated as mzXML files using the converter MSConvert (ProteoWizard). A database search was performed using X!TandemPipeline software developed by PAPPSO facility (version 3.4.3; http://pappso.inra.fr/bioinfo/xtandempipeline/.3; http://pappso.inra.fr/bioinfo/xtandempipeline/) (Langella et al., 2017) with search parameters as followed: enzymatic cleavage by trypsin digestion with one possible miscleavage, fixed carbamidomethylation modification on cysteine and variable oxidation on methionine; precursor mass tolerance of ± 10 ppm and fragment mass tolerance of 0.5 Da. Several databases were used: the Uniprot KB/SwissProt Mus musculus database (24,977 entries, version January 2017) and a homemade contaminant database (trypsin, keratine, *etc.*). The identified proteins were filtered with a minimum of two different peptides required with a peptide *E-value* < 0.01, and a protein *E-value* (product of unique peptide *E values*) < 10^{-4}. Combine analysis mode with all samples was performed and results were grouping proteins: proteins which have at least one peptide in common. This allowed to group proteins with similar functions. Within each group, proteins with at least one specific peptide relatively to other members of the group were reported as subgroups. One subgroup represents one specific protein. Proteins are characterized with their spectral number. Label free quantification of proteins were achieved with spectral counting approach (SC), which is a strategy to determine a relative quantification of proteins from their number of spectra obtained with tryptic peptides in MS. This quantification relies on the more of a particular protein is present in a sample, the more MS spectra are detected for peptides of that protein. Statistical analysis was performed using MassChroqR package developed by PAPPSO team (http://pappso.inra.fr/bioinfo/masschroq/) (R version 3.3.2). A generalized linear mixed

model (GLM) with a Poisson distribution was applied. This model suits in the case of a counting like SC. The principal component analysis was obtained by simulating the kernel densities from group's means and variances assuming bivariate normal distributions. This distribution was generated using protein abundances as variables. Hierarchical bivariate clustering was performed using Euclidean distances and unweighted pair group averages as the aggregation method. All data analyses and graphical representations were performed using the R package MassChroq. Significant changes in protein abundance was determined by analysis of variance (ANOVA) using a Chi-square test. Treatment effect was considered with an adjusted p value for multiple testing by a Benjamini–Hochberg procedure (Benjamini and Hochberg 1995). Student t tests were performed to identify proteins which showed significant differences expressed between groups with the following criteria: p-value was set at <0.05. The resulting exploratory list may carry a higher rate of false positives at the protein level but allowed investigation of cumulative effects over larger sets of proteins and pathways.

2.8.3 Ingenuity pathway analysis

Selected proteins were overlaid on the global molecular network of Ingenuity Pathway Analysis (Ingenuity® Systems, www.ingenuity.com) allowing for a generation of gene networks based on their connectivity. Their score takes into account the relative numbers of network eligible molecules, of molecules analyzed and the total number of molecules in Ingenuity's knowledge base. Disease links are generated on the literature-based association with illness by IPA.

3 Results

Detailed statistical results are provided in Supplementary Table S1.

3.1 Physiological response to treatments

As previously observed (David et al., 2009), a 4-week treatment with Cort increased mouse body weight in comparison to controls (Supplementary Figure S1A, $p < 0.01$). Chronic Flx treatment decreased weight gain compared to Cort or vehicle-treated mice (Supplementary Figure S1B, $p < 0.001$ and $p < 0.01$, respectively). At the end of the protocol (W12), the weight gain in the Vehicle/Vehicle mice was bigger than that in Cort/Vehicle and Cort + Flx-R mice (Supplementary Figure S1C, $p < 0.05$ and $p < 0.01$ respectively). Flx-NR mice treated with ECS showed a greater weight gain compared to Cort/Veh and Cort/Flx-R mice ($p < 0.05$ and $p < 0.01$ respectively).

3.2 Chronic corticosterone induced higher emotionality

A 4-week treatment with Cort (Supplementary Figure S2A) induced an anxiety/depression-like phenotype in C57BL/6JRj mice as previously shown: we confirm here that the higher emotionality induced by chronic corticosterone administration is stable over time throughout the entire protocol and allow for longitudinal studies (David et al., 2009; Mendez-David et al., 2014; Siopi et al., 2016; Mendez-David et al., 2017; Cabeza et al., 2021). In the EPM, Cort-treated mice displayed a decrease in the time spent and number of entries in the open arms (Supplementary Figures S2B–C, $p < 0.01$). In the NSF tests, a decrease in latency to feed was observed after chronic Cort treatment (Supplementary Figure S2D, $p < 0.0001$) that was not correlated with food consumption in the homecage (Supplementary Table S1), as previously described (David et al., 2009; Mendez-David et al., 2017; Pinna et al., 2018). No significant effect was observed in the Splash test (Supplementary Figure S2E), but the behavioral emotionality score was significantly increased after chronic Cort treatment (Supplementary Figure S2F, $p < 0.0001$) compared to the Vehicle/Vehicle treatment.

3.3 Selection of responders/non-responders to chronic fluoxetine treatment

A 4-week treatment with fluoxetine (18 mg/kg/d, protocol in Figure 1A) or vehicle was administered to Cort-treated mice and antidepressant response was monitored with the same battery of tests as in Supplementary Figure S2.

In the EPM, Cort/Flx mice showed an increase in the time spent (Figure 1B, $p < 0.01$) and the number of entries in the open arms of the EPM (Figure 1C, $p < 0.01$) compared to Cort/Veh mice. In the NSF, a 4-week Flx treatment reduced the increase in the latency to feed after Cort (Figure 1D, $p < 0.01$). A non-significant ($p = 0.51$) increase in grooming duration was observed in Cort/Flx *versus* Cort/Veh mice in the splash test (Figure 1E). Applying z-normalization across tests after the second round of behavior showed that chronic Flx decreased Cort-induced emotionality score (Figure 1F, $p < 0.0001$ *versus* Veh/Veh group; $p < 0.0001$ *versus* Cort/Veh group). Thus, chronic Flx decreased anxiety/depression-like phenotype in Cort-treated mice, confirming prior observations (Guilloux et al., 2011; Mendez-David et al., 2015). Overall, chronic Flx treatment significantly reduced behavioral emotionality in Cort-treated mice, as shown in measuring the change in emotionality score value between Week 10 and Week 5 (Figure 1G, $p < 0.001$).

As previously shown (Mendez-David et al., 2017), phenotypic variability was observed after chronic Flx

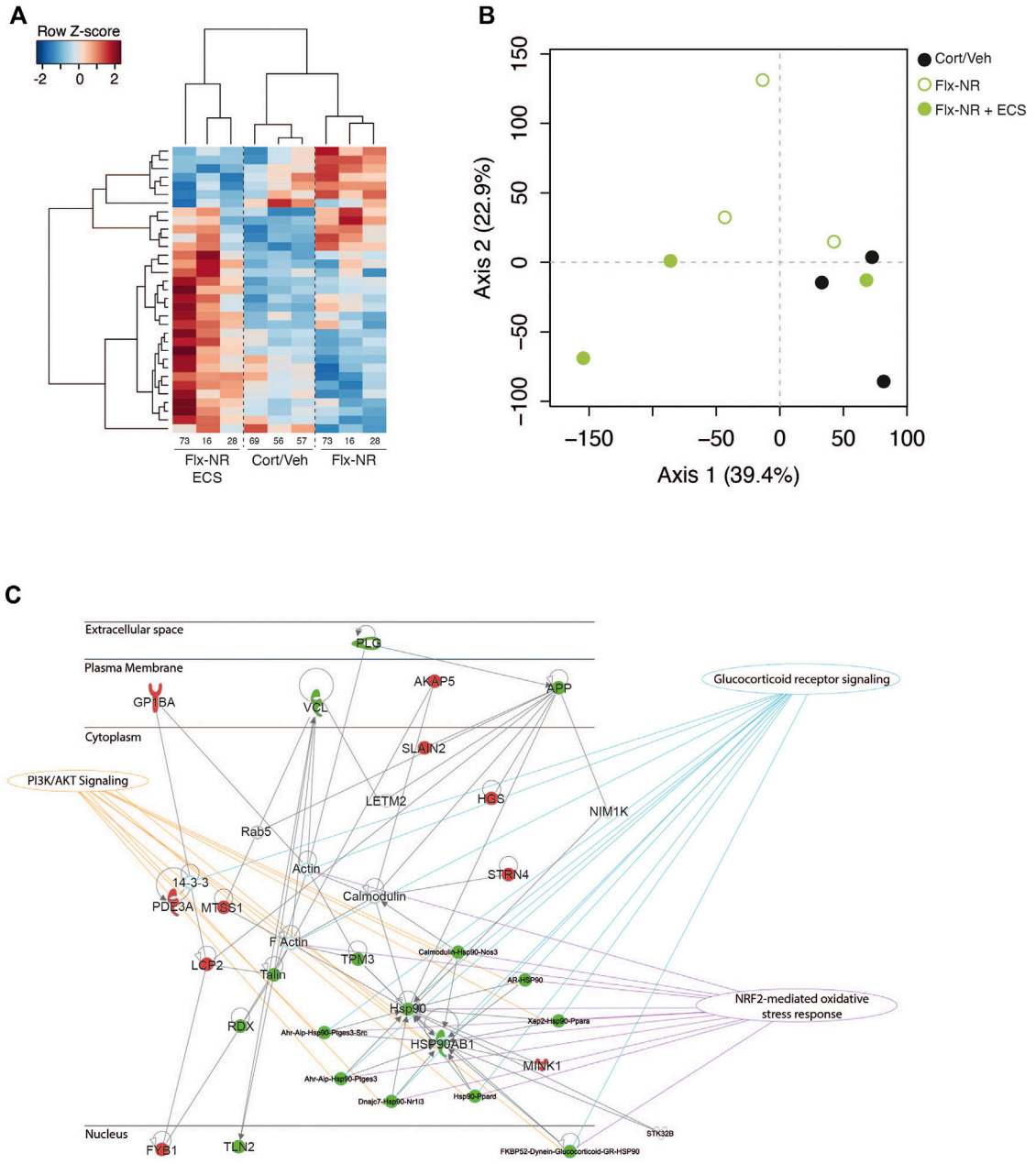

FIGURE 3
Peripheral proteomic changes after fluoxetine exposure in responders and non-responders **(A)** Hierarchical bivariate clustering of expression profiles of animals (column) and proteins (rows) depicts the differences between Cort/Veh, Flx-NR, and Flx-NR-ECS groups. An animal's expression is red for above-average values, and blue for below-average values **(B)** Principal Component Analysis of expression profiles revealed two main axis separating results. **(C)** Ingenuity Pathway Analysis revealed a molecular interaction network based on differentially expressed proteins. This protein network was significantly connected to the following canonical pathways: glucocorticoid receptor signaling, PI3K/Akt signaling and NRF2-mediated oxidative stress response.

administration. Two subgroups of mice could be defined based on the reduction of their emotionality score: a 50% decrease in emotionality score threshold was used to separate Flx-responding mice (Flx-R, $n = 18$), or Flx non-responders mice (Flx-NR, $n = 7$). The 50% threshold was defined for each animal individually, by comparing their individual score at W10 vs. the score obtained at W5. This threshold was based on the clinical human criterion (Nierenberg and DeCecco 2001) and previously used in

TABLE 1 Ingenuity pathway analysis results.

Top Networks		Score
1) Cell Morphology, Hematological System Development and Function, Inflammatory Response		43
2) Cellular Development, Cellular Growth and Proliferation, Hematological System Development and Function		31
3) Cancer, Cell Morphology, Cellular Movement		3
Top diseases and Disorders	# Molecules	*p-value*
Inflammatory Response	13	4.01E-03 - 4.55E-08
Cardiovascular Disease	8	4.01E-03 - 6.15E-07
Hematological Disease	18	4.01E-03 - 6.15E-07
Organismal Injury and abnormalities	29	4.01E-03 - 6.15E-07
Connective Tissue Disorders	13	4.01E-03 - 1.26E-05
Top canonical pathways	–log (*p-value*)	Ratio
Fc Receptor-mediated Phagocytosis in Macrophages and Monocytes	3.6	0.032 (3/94)
Neuroprotective Role of THOP1 in Alzheimer's Disease	3.3	0.025 (3/118)
Leukocyte Extravasation Signaling	2.7	0.016 (3/193)
Remodeling of Epithelial Adherens Junctions	2.4	0.029 (2/68)
Actin Cytoskeleton Signaling	2.4	0.012 (3/245)

Results show top biological networks, diseases and disorders and canonical pathways obtained by Ingenuity Pathway Analysis (IPA). Network scores represent negative log-values of right-tailed Fisher's Exact Tests for network consistence. Pathways and disease *p*-values represent significance of over-representation of candidate proteins within respective protein groups. *p*-value ranges indicate values for various disease sub-classifications.

preclinical studies (Mendez-David et al., 2017). These two groups displayed a significant difference in behavioral emotionality levels (Figure 1G, $p < 0.0001$).

3.4 Effect of chronic electroconvulsive seizures treatment in fluoxetine non-responding mice

We tested the effects of chronic ECS in Flx-NR mice who received seven sessions of ECS over 14 days, while Flx treatment was maintained in the two groups of Flx-treated mice (R and NR) as described in the protocol (Figure 2A).

In the EPM, ECS in Flx-NR mice increased time spent and number of entries in open arms compared to Cort/Veh mice (Figures 2B,C, $p < 0.01$ and $p < 0.001$, respectively). By contrast, EPM parameters in Flx-NR mice treated with ECS were not significantly different from those measured in Flx-R mice (Figures 2B,C, $p = 0.387$ and $p = 0.742$, respectively). In the NSF test, chronic Flx treatment in responder animals was still effective in comparison to Cort/Veh mice (Figure 2D, $p < 0.0001$). Non significant decrease in the latency to feed in the NSF and increase the grooming behavior in the splash test were observed in the Cort/Flx-ECS group in comparison to the Cort/Veh group (Figure 2D, $p = 0.26$; Figure 2E, $p = 0.14$). Overall, we observed that ECS reduced the emotionality score in Flx-NR mice as indicated by its significant decreased value compared to Cort/Veh (Figure 2F, $p < 0.05$). Moreover, no relevant difference between the Flx-R and Flx-NR-ECS groups was observed (Figure 2F, $p = 0.14$).

3.5 Protein changes in Flx-NR mice after chronic electroconvulsive seizures treatment

Using a high resolution mass spectrometry analysis by X! TandemPipeline, specific proteins in PBMCs ($n = 3$ samples per group) were detected (Supplementary Tables S2). Characterized proteins with less than two peptides were excluded. Hierarchical clustering of the expressed proteins distinguished Cort/Veh treated mice from Cort/Flx-NR and Cort/Flx-ECS mice (Figure 3A) and revealed 33 proteins showing differential changes. This aggregate behavior of this large-scale systemic response was quantified with Principal Components Analysis (PCA, Figure 3B), which confirmed hierarchical clustering analysis. According to PCA, proteins' abundance separated Cort/Veh, Cort/Flx-NR and Cort/Flx-ECS mice.

A group effect was observed for 33 proteins ($p < 0.05$, Supplementary Table S2). The levels of expression of 14 proteins were significantly modified by chronic fluoxetine treatment in Cort/Flx-NR mice, and 17 proteins in Cort/Flx-ECS mice compared to Cort/Veh mice ($p < 0.05$, Figure 3A and Supplementary Table S2). ECS induced significant changes in expression of 18 proteins (7 down-regulated and 11 upregulated).

Ingenuity Pathway analysis revealed three main networks associated with the 33 differentially expressed proteins (Figure 3C; Table 1), which relates to broad diseases and disorders such as "Inflammatory response," "Cardiovascular or Hematological Diseases." Canonical pathways linked to these DE proteins evidently relates to circulating components or process found in blood.

4 Discussion

4.1 Electroconvulsive seizures corrected anxiety/depression phenotype in Flx-NR

Lack of treatment response is one of the major limits of current antidepressant treatment, which can be circumvent using pharmacological combination or treatment. Novel pharmacological approaches including ketamine are also gaining attention, however they yet did not present a superiority of effect compared to electroconvulsive therapy (Veraart et al., 2021). Despite the frequent and widespread use of ECT, possible cognitive impairments and other adverse effects were described and its molecular mechanisms underlying its efficacy remains largely unclear [for review (Maffioletti et al., 2021)]. Thus, before engaging into ECT treatment, the search for predictors of ECT response in animal models of anxiety/depression would benefit to patients with MDD.

This work was performed in a different and new cohort of mice compared to our previous report on peripheral markers associated with non-response to Flx (Mendez-David et al., 2017). Here, we confirmed our previous report showing non-response to chronic fluoxetine (Flx-NR) treatment in a pharmacological model of anxiety/depression (Mendez-David et al., 2017). We found a ≈72% response rate to chronic fluoxetine (18 responders out of 25 Flx-treated mice). This response rate is very similar to our prior work (65%, Mendez-David et al., 2017), performed with the same experimental protocol, with similar behavioral evaluation and threshold for defining treatment response. These preclinical results are also in line with clinical observation from the STAR*D study (Rush et al., 2006; Trivedi et al., 2006) showing ineffective antidepressant drug treatment despite a mean of several weeks of SSRI therapy.

Interestingly, we reveal that chronic ECS treatment in Flx-NR mice rescued the behavioral emotionality score. While numerous studies explored the effect of chronic effects of ECS in naïve rodents (Duman and Vaidya 1998), few of them explored its benefits and its mechanism of action in animal model of anxiety/depression (Schloesser et al., 2015; Jonckheere et al., 2018). To our knowledge, the present study is the first examining ECS effects in the context of non-response to an antidepressant drug treatment. Prior works in the Cort model of depressive disorder showed that chronic ECS reverses Cort-induced depressive-like phenotype (Schloesser et al., 2015), an effect that may be supported by both rescuing Cort-induced deficit in dendritic spine morphology and increasing expression of *Bdnf* activity-dependent exons 1 and 6 (Maynard et al., 2018). Recently, increased synaptic connectivity and extended neuronal survival were confirmed as key factors for ECS efficacy in a genetic animal model relevant to some aspects of depression (Jonckheere et al., 2018). Here, a 14-day protocol of ECS alleviated the non-response to chronic fluoxetine treatment in a neuroendocrine-based rodent model of MDD and antidepressant treatment resistance. Our preclinical ECS results confirm that the use of ECT in the context of non-response to classic antidepressant treatment could benefit to patients with MDD.

4.2 Peripheral proteomic changes after electroconvulsive seizures

According to a recent review on molecular biomarkers of ECT effects, most of the studies are concentrated on one or few markers. Expression studies on gene transcripts and microRNAs are rare and genetic studies are sparse. To date, no conclusive evidence regarding ECT molecular markers of clinical response has been reached (Maffioletti et al., 2021).

Our study is also the first report of ECS-induced proteome variations in the blood, and most especially in peripheral blood mononuclear cells (PBMC), samples easy to transpose into future clinical trials. The identification of protein biomarkers indicates that these proteins are involved in cell adhesion/motility (FYB1, TLN2), cell structure and cytoskeleton (CRAD, MTSS1, and SMTN), coagulation (F11R, PLG) and immune system (LCP2 and PPP6R3), signaling pathway (AKAP5, MINK1, and TMP3), vesicle trafficking (i.e., SH3GLB2, CC2D1B, and CAVIN1) or in cell metabolism (ADSS, GK, HSP90AB1, and HSG) and cell cycle (FYB1 and PPP6R3). At a single molecular level, some of these proteins have been prior associated with mental health. For example, ADSS polymorphism or increased blood levels of ADSS were associated with schizophrenia and bipolar disorder (Zhang et al., 2008a; Zhang et al., 2008b) and (Tsuang et al., 2005), respectively.

The scaffolding protein AKAP5 (also called AKAP150 in rodent species and AKAP79 on human) have a dual role in depression. Indeed, genetic blockade of AKAP5 expression in the basolateral nuclei of the amygdala prevents chronic stress -induced depressive like disorders in mice (Zhou et al., 2019). Conversely, the disruption of AKAP5 in E18 embryonic cortical neurons (Mari et al., 2015) (Mari et al., 2015) negatively affect the inhibition of ASIC1a -a ion channel known to be involved in neuropathologies including depression and whose pharmacologic inhibition has antidepressant-like effects (Coryell et al., 2009). These opposite roles in the biological processes of depression could be explain by the protein partner bound by AKAP5: the complex of AKAP5 with calcineurin inhibit the ASIC1a functions (Mari et al., 2015) while the association with PKA promote ASIC1a activity (Chai et al., 2007).

Overexpression of APP in mice has been associated with anxious and depressive-like phenotype in APP knock-in mice (Locci et al., 2021). However, conflicting reports in mouse models (Sakakibara et al., 2018; Latif-Hernandez et al., 2019) suggest a dynamic emotional response depending on the test performed (Pervolaraki et al., 2019), the age of the animal but independent

of the Aβ plaque load. This last, formed by the cleavage of APP, was identified recently in cerebrospinal fluid, with other protein biomarkers, as an hypothetic biomarker of the ECT efficacy (Kranaster et al., 2019).

CC2D1B participate in a dual repressor element with Freud2, both molecules acting as strong repressors of the expression of serotonin 1A receptor, a key receptor involved in pathophysiology of depression and antidepressant drug response (Samuels et al., 2016).

While no direct link between plasminogen (PLMN) and depression has been observed, its activation into plasmin is controlled by tissue-type plasminogen activator (tPA) which cleave PLMN into plasmin. One of the role of plasmin is to convert pro-BDNF into mature BDNF in the brain, and thus this pathway has been implicated in depression and treatment response (Tsai 2017). Here, we found a reduction in PLMN levels after ECS treatment: this result correlates with prior observations showing an increase in tPa expression after ECS exposure (Segawa et al., 2013) suggesting an increase in plasminogen cleavage and increase in plasmin levels.

Prolyl endopeptidase (PPCE or PREP) peripheral levels have been observed decreased in MDD subjects (Maes et al., 1994), and antidepressant treatment has been shown to increase PREP serum level (Maes et al., 1995). Interestingly, here ECS treatment reduced PPCE levels in fluoxetine non-responding mice.

Finally, TALIN2, a protein involved in the cytoskeleton and the adhesion as well as in signaling pathway (by production of PIP2) increased in the serum of MDD patients (Al-Hakeim et al., 2020). Interestingly, we prior found TALIN2 to be downregulated in animals that respond to chronic fluoxetine vs. non-responding mice (Mendez-David et al., 2017). We confirmed here this association as ECS reduced TLN2 expression compared in Flx-NR mice.

A preclinical study looking at a proteomic peripheral profile of ECS effects in naïve male Sprague-Dawley rats found a biosignature that differs from what we obtained here (Glaviano et al., 2014). These discrepancies could be due to a difference in the experimental models used (naïve vs. animal model of non-response to SSRI), but also difference in the peripheral tissue studied (plasma vs. PBMC). Whether these changes observed in PBMC can correlate to similar changes in the brain remains to be tested. However, some studies shown similar expression changes between in PBMCs and the hippocampus after stress in mice (van Heerden et al., 2009), and convergent signaling pathways between amygdala and blood (Daskalakis et al., 2014). More recently, Hervé et al. (2017) have shown peripheral gene expression changes allowing for the identification of biomarkers predictive of treatment response in mice and depressed subjects. While correlation in proteomic expression between selective brain regions and PBMC has yet not been explored using high-throughput techniques, however the use of PBMCs as a surrogate for proteomic studies in psychiatric disorders has been proposed (Rahmoune and Guest 2017).

At the single molecule level, prior association of these different markers have been mentioned in the literature. However, MDD and treatment response cannot be resumed by individual factors. Thus, only association study of protein network would benefit to patients with MDD. Here, we described a main protein network based on 33 proteins involved in three regulatory pathways known to participate in the etiology of mood disorders: glucocorticoid receptor signaling (Cattaneo and Riva 2016; McEwen and Akil 2020), PI3K/Akt signaling (Fujiki et al., 2010; Matsuda et al., 2019) and NRF2-mediated oxidative stress response (Bakunina et al., 2015; Mendez-David et al., 2015). A recent review underlined biological systems related to neurotrophic and inflammatory/immune systems in the effects of ECT (Maffioletti et al., 2021), the involvement of this last system being also observed and assess in the cerebrospinal fluid in patients (Kranaster et al., 2019).

5 Limitations and conclusion

One of the strengths of this study is that ECS were applied in mice that did not respond to chronic fluoxetine administration. Whether this biosignature, specific to non-response to fluoxetine vs other antidepressant drug treatment, remains to be further tested. Indeed, MDD is more prevalent in women (Albert 2015). Our study was performed in male mice because we previously showed that chronic corticosterone administration in female C57BL/6 mice did not affect the emotionality score (Mekiri et al., 2017). It could be interesting to confirm whether such a biosignature could be reproduced in another animal model of anxiety/depression, e.g., chronic social defeat stress model or chronic mild stress.

Here, we tested the effects of ECS in Flx-NR animals while Flx treamtent was maintained. Thus, an interaction of ECS with Flx cannot be ruled out and the effects of ECS alone -without Flx treatment-has not be tested. However, as some clinical studies suggest, concomitant pharmacotherapy may increased ECT efficacy and decrease the risk of relapse (Sackeim et al., 2009; Haskett and Loo 2010). Similarly, we did not test whether the changes in proteins expression are state-dependent and would be similar in naïve mice, as electroconvulsive treatment are of interest in a pathological situation.

While the present study was performed in a low number of animals, we believe that its longitudinal trajectory with proteomic profiling within the same animal before and after ECS strengthen its scientific validity. This study paves the way for a greater definition of peripheral biomarkers related to antidepressant treatment response, with the goal of a better prediction and translational validation of these methods. Yet, whether or not these proteomic changes observed in PBMCs from mice mirror biological changes in brain tissues remains to be tested.

Data availability statement

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (Perez-Riverol et al., 2022) partner repository with the dataset identifier PXD037392.

Ethics statement

The animal study was reviewed and approved by The Institutional Animal Care and Use Committee (CEE26 authorization 4747).

Author contributions

RL, IM-D, LK-N, CH, DD, and J-PG designed and performed research, drew figures, and analyzed the data. All authors contributed to the writing of the manuscript.

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Conflict of interest

The 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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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2022.993449/full#supplementary-material

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