THE BURDEN OF STRESS AND DEPRESSION – NEW INSIGHT INTO FASTER AND EFFICIENT TREATMENT

EDITED BY: Ravid Doron, Gang Chen, Matthew O. Parker and Alon Shamir

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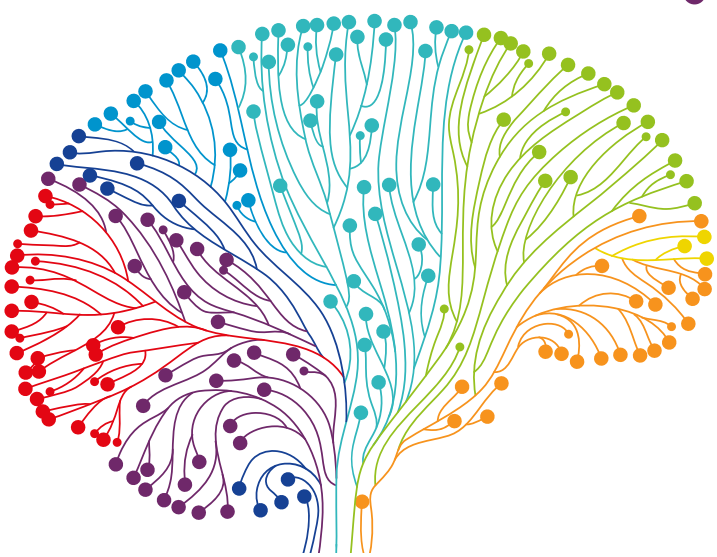

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THE BURDEN OF STRESS AND DEPRESSION – NEW INSIGHT INTO FASTER AND EFFICIENT TREATMENT

Topic Editors:
Ravid Doron, Open University of Israel, Israel
Gang Chen, Jinan University, China
Matthew O. Parker, University of Portsmouth, United Kingdom
Alon Shamir, Mazor Mental Health Center, Israel

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Nuno Sousa,
University of Minho, Portugal

*CORRESPONDENCE
Ravid Doron
raviddor@gmail.com

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Editorial: The burden of stress and depression – new insight into faster and efficient treatment

Keren Nitzan[1], Matthew O. Parker[2], Alon Shamir[3,4], Gang Chen[5] and Ravid Doron[1]*

[1]Department of Education and Psychology, The Open University, Ra'anana, Israel, [2]Institute for Biological and Biomedical Sciences, School of Pharmacy and Biomedical Science, University of Portsmouth, Portsmouth, United Kingdom, [3]Faculty of Medicine, Technion–Israel Institute of Technology, Haifa, Israel, [4]Mazor Mental Health Center, Akko, Israel, [5]Interdisciplinary Institute for Personalized Medicine in Brain Disorders, Jinan University, Guangzhou, China

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Editorial on the Research Topic
The burden of stress and depression – new insight into faster and efficient treatment

Depression and anxiety are prevalent disorders and a major public health concern worldwide. Although current treatments for depression and anxiety have some pronounced limitations—the development of new drugs for mood disorders has been at a standstill for the last decade. Moreover, there is currently no reliable biomarker for the early detection of mood disorders.

This Research Topic presents theoretical and experimental work of unique strategies to diagnose and treat anxiety and depression. It includes 13 articles: 9 original research, one review, two mini-reviews, and one opinion article. Most of the research presented here tries to tackle one of the more severe limitations of currently available treatments: prolonged onset time. Others explore non-conventional therapies for the treatment of depression and anxiety, such as Chinese traditional medicine, including herbs and acupuncture. The section also presents novel research regarding possible biomarkers and risk factors for depression, as well as an interesting view on the importance of DNA methylation in depression.

Current anti-depressants drugs enhance monoamine neurotransmitters, either by enzyme inhibition such as in Monoamine oxidase inhibitors (MAOIs) drugs, reuptake inhibition of both serotonin and adrenaline such as Tricyclic antidepressants (TCAs) and serotonin–noradrenaline reuptake inhibitors (SNRIs) or specifically blocking just the serotonin transporter such as Selective serotonin reuptake inhibitors (SSRIs).

The special topic review paper by Fitzgerald urges researchers to remember that although most of the drug-research industry is focused on drugs that boost synaptic

monoamines, a significant body of studies suggests that noradrenergic transmission reducing drugs can be effective anti-depressants. Fitzgerald emphasizes the importance of the noradrenergic pathways as a target for new drugs, focusing on three major classes of noradrenergic transmission reducing drugs (alpha2 agonists, beta-blockers, alpha1 antagonists), and supports the hypothesis that they have antidepressant-like properties.

In their special topic paper, Bareli et al., give much-needed attention to depression and anxiety associated with substance use disorders (SUDs). They suggest a novel candidate for pharmacological treatment of patients with SUD and comorbid mood/anxiety disorders that may facilitate their rehabilitation. The authors tested, in both animals and patients, a novel combination of opipramol and baclofen (O/B), which is known to attenuate anxiety and depression, for the facilitation of recovery from SUDs. Their findings indicate a beneficial effect of O/B treatment.

Fast onset drugs

One major limitation of current treatment for depression and anxiety is the slow onset of therapeutic action, with several weeks of therapy required before achieving a therapeutic response. Due to the slow-onset nature of current drugs, many patients experience long periods of depressive symptoms without being beneficially treated, and the delayed therapeutic effects lead to discontinuation of treatment (Srimongkon et al., 2018), and potentially putting patients at higher risk of suicide (Valenstein et al., 2009). Finding an accelerating agent for the current anti-depressant drugs will help to improve adherence, quality of life, productivity, and wellbeing of many patients.

Ketamine, a glutamate NMDA receptor channel blocker, can produce a fast and sustained anti-depressant response. The discovery of Ketamine is considered one of the most significant breakthroughs in the field of depression since the 1950s. The special topic paper by Colla et al. reviews mechanisms that may relate to Ketamine's anti-depressant effect. Colla et al. describe current theories of anti-depressant drug action, including monoaminergic signaling, disinhibition of glutamatergic neurotransmission, neurotrophic and neuroplastic effects, and discuss how these different mechanisms might relate to ketamine action. While Chen et al., in their research article, provide insight into the role of glutamate transporter 1 (GLT1) as the critical presynaptic molecule participating in the pathophysiological mechanism of depression and contributing to the antidepressant-like effect of Ketamine. They show that GLT1 expression levels in the Prefrontal Cortex significantly decrease in stressed mice and return to normal by ketamine treatment. Moreover, pretreatment with the GLT1 inhibitor DHK significantly alleviated the rapid antidepressant-like effect of ketamine infusion. Using specific inhibitors, Chen et al., confirm that both AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) receptor and L-type voltage-dependent calcium channels (L-VDCC) are crucial factors in the immediate antidepressant-like effect of Ketamine.

While the central nervous system no doubt plays an integral role in developing anxiety and depression—it is not the only player. Recent evidence suggests a tight connection between the gut, the brain and psychiatric disorders (Mitrea et al., 2022). The special topic paper by Wilkowska et al. reviews the importance of the gut microbiome and gives special attention to the effect of Ketamine on the microbiome in animal models of depression. They present preliminary studies indicating that Ketamine restores bacteria-producing anti-inflammatory substances, reduces the number of bacteria associated with inflammatory processes in the gut, reduces the number of bacteria, previously reported as increased in depression, and increases the abundance of probiotic bacteria known to produce an antidepressant effect. Wilkowska et al. conclude by emphasizing the need for further studies on the effect of ketamine and its enantiomers on individual bacterial species.

While Ketamine is a promising candidate for treating depression and anxiety, it has some acute side effects, and more importantly, its chronic use is associated with potentially severe and possibly persistent toxic effects (Short et al., 2018). Thus, the search for a safe and side-effect-free treatment is still ongoing. Traditional Chinese medicines have a long history of treating mood disorders, some of which are still actively used today; thus, it has the potential to serve as a safe and effective alternative to conventional drugs and can be an alternative option for treatment (Burstein et al., 2021).

Alternative and Chinese medicines

Kim R. Y. et al. demonstrated the anti-depressant effects of Fraxinus rhynchophylla Hance (*F. rhynchophylla* Hance, FX) in a reserpine-induced mouse model of depression. Ten-day treatment alleviated anxiety and depression like-behaviors, attenuated plasma corticosterone concentrations, decreased pro-inflammatory cytokines mRNA levels, and increased hippocampal phosphorylated cAMP response element-binding protein (pCREB) and brain-derived neurotrophic factor (BDNF). Kim R. Y. et al. findings serve as a preclinical basis to confirm the potential of FX as an anti-depressant drug; although, further studies are needed to establish its mechanisms of action.

Exploring a different Chinese herb, Zhang et al. show the beneficial effect of the Yueju pill in clinical trials. Yueju, a herbal medicine, has been shown to promote anti-depressant effects in many preclinical studies. In the special topic paper here, Zhang et al. present the beneficial effect of the Yueju

pill, compared to either placebo or Escitalopram in two separate clinical trials. In a preliminary open-labeled trial on major depressive disorder (MDD) patients, they found symptom alleviation as early as 1 week post a conventional low dose of Yueju. In the confirmatory random controlled double-blinded clinical trial, they found both Escitalopram and Yueju pill resulted in early improvement of depression symptoms, and comparable antidepressant outcomes after 4 weeks of treatment.

Aside from pharmacological interventions, some patients with depression may prefer non-pharmacological options. A unique study, by Sakurai et al., explores for the first time difference in brain activation associated with relaxation effects of Autonomous sensory meridian response (ASMR) videos compared to classical music therapy. Their results show that classical music and the ASMR auditory stimulus produced a pleasant and relaxed state but that ASMR involves more complex brain functions than classical music, especially the activation of the medial prefrontal cortex.

Another alternative Chinese treatment that has emerged as a promising non-pharmacological treatment for reducing depressive symptoms is Acupuncture (Yang et al., 2022).

A meta-analysis by Jiang et al., compares acupuncture's effectiveness to other non-pharmacological treatments such as cognitive-behavioral treatment, mindfulness, behavioral activation program, brain electrical biofeedback therapy, tai chi, and bright light therapy (among others) on Sub-threshold depression (SD). Their results suggest that electroacupuncture and bright light therapy appear to be the better choices in the treatment of SD.

In agreement with this view, the special topic paper by Kawanokuchi et al., demonstrated acupuncture's effectiveness in preventing and treating the symptoms of social defeat stress (SDS)-induced depression in mice. Two weeks of acupuncture restored SDS-reduced brain-derived neurotrophic factor (BDNF), neurotrophin (NT)-3, and NT-4/5 expression. In contrast, acupuncture stimulation suppressed nerve growth factor (NGF) expression induced by SDS. The authors conclude that acupuncture treatment could effectively correct the imbalance in the expression of neurotrophic factors.

Biomarkers and risk factors

BDNF is involved in the pathogenesis of mood disorders and has been associated with the action of anti-depressant and anxiolytic drugs (Colucci-D'Amato et al., 2020). Interestingly, in the previously mentioned clinical trial by Zhang et al., serum levels of BDNF, in combination with depression scores, yielded a new possible marker (i.e., "neuroplasticity index") that may serve as a predictor for anti-depressant treatment outcome.

Indeed, there is a vital need for biomarkers that could predict response to anti-depressant drugs as more than one-third of patients (40%) do not respond to the anti-depressant treatment. The special topic paper by Vieira et al., tried to tackle this important issue. They found that alterations in white matter integrity, specifically in forceps minor and the superior longitudinal fasciculus, are associated with paroxetine treatment response. Although the authors acknowledge several limitations to their study, they offer a promising initial step forward in the path to discovering a reliable biomarker.

Many factors may be involved in the susceptibility to anxiety and depression, such as age, gender, early life stress and more (Mofatteh, 2020). The special topic paper by Kim S. et al. tries to determine whether genetic hypersensitivity to stress would alter behaviors in adulthood after limited mild stress during early adolescence and explore sex differences in response to stress in rats. While Lax, in his special topic paper, maintains that environmental factors, such as early-life stress, make individuals prone to major depression. He stipulates that these environmental factors modulate epigenetic signals to reprogram brain gene-expression patterns, ultimately affecting DNA methylation. Thus, DNA methylation may be used as a potential biomarker to predict MDD and its severity in vulnerable populations and treatment outcomes. Furthermore, Lax suggests that as drugs that modify DNA methylation are available and demonstrate significant effects across both preclinical and clinical studies, they have the potential to be used as adjuvants, increasing the efficacy of classic anti-depressant treatments.

In summary, current treatment for depression and anxiety suffers from major limitations—mainly major side effects and prolonged on-set time. Ongoing research is looking for new treatments, focusing on novel mechanisms both in the central nervous system and periphery—specifically the interesting connection between the gut and the brain. Furthermore, when looking for treatments one should also consider environmental factors and risk factors that can modify genetic processes, such as DNA methylation, and cause treatment resistance and increased susceptibility to anxiety and depression.

"Frontiers" Special topics offer a unique platform to explore innovative research on current matters and should be further continued, focusing on other specific issues such as the search for treatment without side effects and causes for treatment resistance. Overall, we believe that the contributions to the Special Topic "*The burden of stress and depression – new insight into faster and efficient treatment*" highlight the importance of exploring new venues for the treatment of depression and anxiety, and points to alternative herbal and non-pharmacological options; and suggest several possibilities for biomarkers and risk-factor to ultimately facilitate better care for patients.

Author contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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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ORIGINAL RESEARCH
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Antidepressant and Anxiolytic-Like Effects of the Stem Bark Extract of *Fraxinus rhynchophylla* Hance and Its Components in a Mouse Model of Depressive-Like Disorder Induced by Reserpine Administration

Yu Ri Kim[1], Bo-Kyung Park[2†], Chang-Seob Seo[1], No Soo Kim[2] and Mi Young Lee[2]*

[1] Herbal Medicine Research Division, Korea Institute of Oriental Medicine, Daejeon, South Korea, [2] Clinical Medicine Division, Korea Institute of Oriental Medicine, Daejeon, South Korea

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Edited by:
Alon Shamir,
Mazor Mental Health Center, Israel

Reviewed by:
Gustavo Provensi,
University of Florence, Italy
Jonathan Cueto-Escobedo,
Universidad Veracruzana, Mexico

***Correspondence:**
Mi Young Lee
mylee@kiom.re.kr

†Present address:
Bo-Kyung Park
Cellicon Lab, Daejeon BioVenture Town, Daejeon, South Korea

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There is an urgent need to find antidepressants that can be administered for long periods without inducing severe side effects to replace conventional antidepressants that control monoamine levels, such as tricyclic antidepressants (TCAs), monoamine oxidase inhibitors (MAOIs), and selective serotonin reuptake inhibitors (SSRI). We sought to determine the antidepressant effects of *Fraxinus rhynchophylla* Hance (*F. rhynchophylla* Hance, FX) and its components on a reserpine-induced mouse model. One hour after oral administration of FX (30, 50, and 100 mg/kg), esculin (50 mg/kg), esculetin (50 mg/kg), fraxin (50 mg/kg), and fluoxetine (20 mg/kg), reserpine was delivered intraperitoneally to mice. Behavioral experiments were conducted to measure anxiety and depressive-like behaviors after 10 days of administration. FX and its components increased the number of entries into the center of an open field as well as distance traveled within it and decreased immobility duration in the forced swim and tail suspension tests. Reserpine-induced increases in plasma corticosterone concentrations were attenuated by the administration of FX and its components, which were also found to decrease the reserpine-induced enhancement of mRNA levels of *interleukin (IL)-12 p40, IL-6*, and *tumor necrosis factor (TNF)*-α, pro-inflammatory cytokines. Finally, the diminished expressions of hippocampal phosphorylated cAMP response element-binding protein (pCREB) and brain-derived neurotrophic factor (BDNF) by reserpine were increased by FX and its components. Our results suggest that FX and its components regulate anxiety and depressive-like behaviors through stress hormones, immune regulation, and the activation of neuroprotective mechanisms, further supporting the potential of FX and its components as antidepressants.

Keywords: reserpine, depressive-like disorder, *F. rhynchophylla* Hance, antidepressant, anxiolytic, neuroinflammation, neuroprotection

INTRODUCTION

Despite having a global prevalence of 350 million people and a long time-course, depression is not being treated effectively due to stigma and lack of effective therapeutic modalities (Smith, 2014). Depression is a complex mood disorder that manifests as despair and helplessness, all of which can further negatively impact health and mental by changes in appetite, abnormality in social behavior, insomnia, fatigue, frequent headaches, etc., if depressive mood conditions are prolonged (World Health Organization, 2009; Otte et al., 2016; Ikram and Haleem, 2017). The difficulty in treating depression is partly attributable to its varied etiology: genetic factors, environmental factors such as endocrine abnormalities, stress, sex differences, disease (stroke, cancer) (Laoutidis and Mathiak, 2013; Robinson and Jorge, 2016; Hammen, 2018), and especially biochemical factors such as neurotransmitter (norepinephrine, serotonin, GABA, etc.) and neural hormone (thyroid, growth, hypothalamus-pituitary-adrenal cortex axis) abnormalities can all increase the risk of the onset of depression (Saveanu and Nemeroff, 2012; Dell'osso et al., 2016). Antidepressants currently treating depression, tricyclic antidepressants (TCAs), monoamine oxidase inhibitors (MAOIs), and selective serotonin reuptake inhibitors (SSRI), which control existing monoamine levels, have side effects such as constipation, decreased vision, high blood pressure, cognitive impairment, and anticholinergic effects (Donoghue and Tylee, 1996; Feighner, 1999; Tarleton et al., 2016). Furthermore, while such early antidepressants are fast-acting and inexpensive, clinical studies have associated their use with low recovery rates, only 22–40% of patients with depression (Anthes, 2014).

Fraxinus rhynchophylla Hance (*F. rhynchophylla* Hance, FX) is a traditional Chinese medicine mainly found in China and Korea. FX contain coumarin-based components such as esculin, esculetin, and fraxin, and the stem bark obtained from FX—also known as fraxini cortex—has demonstrated efficacy in the treatment of diseases such as acute conjunctivitis, diabetes mellitus, diuretic, analgesic, astringent, and acute liver injury (Guo et al., 2017; Seo et al., 2019). Coumarin-based components isolated from *Fraxinus rhynchophylla* have been reported to have anti-inflammatory effects such as inhibiting tumor necrosis factor (TNF)-α release by peritoneal macrophages induced by LPS and attenuating the production of inflammatory mediators in BV2 microglia (Niu et al., 2012; Song et al., 2014). Among them, esculetin attenuates LPS-induced anxiety and depressive-like behavior and plays a role in inhibiting corticosterone and pro-inflammatory cytokines interleukin (IL)-1β, -6, and TNF-α (Sulakhiya et al., 2016). FX and its components improve depressive-like behavior and anti-inflammatory effects have been studied in several studies as above.

Reserpine was used as a first-line treatment for hypertension, but after chronic use, severe side effects leading to depression were observed (Guo et al., 2015). Studies have shown that this side effect is caused by the depletion of monoamines such as serotonin, dopamine, and norepinephrine (Gao et al., 2016). This evidence has been applied to animal models showing depressive symptoms, and reserpine-administered animal models are being used to study the pathological symptoms of depression (Uriguen et al., 2008). Reserpine administration may affect neuroinflammation by damaging rodent nerve tissues and releasing proinflammatory cytokines such as IL-1β, IL-12, IL-6, TNF-α, and IFN-γ from the hippocampus, liver, and serum (Huang et al., 2004; Zhou et al., 2014; Park et al., 2020). In addition, brain-derived neurotrophic factor (BDNF)-Tropomyosin receptor kinase B (TrkB) signaling participates in emotional, learning, and memory regulation in the hippocampus and activates neurotrophic pathways. Reserpine administration reduces BDNF expression and induces phosphorylation of BDNF binding receptor TrkB, leading to brain dysfunction, which plays an important role in depression (Zong et al., 2019). These neuroinflammatory reactions and neurotrophic factors have been proposed as causes of depression and are being studied as therapeutic targets.

Although our previous studies have identified the FX extract to be effective in attenuating stress-induced depression, depression is not considered a single disease; further research of the effect of FX in different models of depression was warranted. The present study specifically considers a reserpine-induced animal model to examine the applicability of prior findings to depression by environmental causes such as stress and dearth of neural monoamines. Hence, the present study first quantified the components by subjecting FX to high-performance liquid chromatography (HPLC) and then measured depression and anxiety-like behaviors and blood stress hormone levels. In addition, we examined the neuroinflammatory and neuroprotective effects of FX and its components on the hippocampal levels of the following elements: IL-12 p40, IL-6, TNF-α, and cAMP response element-binding protein (CREB)/BDNF. Therefore, our findings are expected to help establish FX and its components as natural products that improve depressive and anxiety-like symptoms, as well as mental disorders benefited by anti-inflammation and neuroprotection.

MATERIALS AND METHODS

Chemicals and Antibodies

For HPLC analysis, esculin (EC, purity 98%) and esculetin (ECT, purity 98%) were purchased from Sigma-Aldrich (St. Louis, MO, United States). Fraxin (FR, purity 98.9%) and formic acid (analytical reagent grade) were purchased from Merck KGaA (Darmstadt, Germany). For animal experiments, reserpine (purity 98%), esculin (purity 98%), esculetin (purity 98%), and fluoxetine (FXT, purity 98% in thin layer chromatography) were supplied by Sigma-Aldrich. Fraxin (purity 98%) was supplied by InterPharm Corporation (Koyang-si, Gyeonggi-do, South Korea). For anesthesia, tiletamine/zolazepam was supplied by Virbac (Zoletil 50; Cedex, France). For western blot analysis, actin antibody was supplied by Sigma- Aldrich. BDNF antibody was supplied by Abcam plc. (Cambridge, United Kingdom). CREB antibody and phosphorylated CREB (pCREB) antibody were supplied by Cell Signaling Technology (Danvers, MA, United STates). For immunofluorescence analysis, BDNF antibody was supplied by Abcam plc, pCREB antibody by Cell Signaling Technology, and NeuN antibody by Merck KGaA.

Preparation of the FX Extract

The stem bark obtained from FX, the origin of Gyeongsangbukdo of Korea, was supplied by Omniherb Co., Ltd. (Susung-gu, Deagu, South Korea). One kg of FX stem bark was submitted to reflux extraction for 3 h with 10 L of 70% ethanol solvent. After filtering the primary strainer, it was concentrated by secondary cotton filtration to freeze-dry the extract and prepare a powder. Approximately 171.86 g of ethanol extract was obtained, and the yield of this extract was 11.46%. The extract was stored at −80°C.

HPLC Reagents, and the Analysis of the FX Sample

The phytochemical analysis of FX was performed using a Shimadzu Prominence LC–20A system (Kyoto, Japan) equipped with a photodiode array (PDA) detector. LC solution software (Version 1.24, SP1, Kyoto, Japan) was employed for the acquisition, processing, and conversion of chromatographic data. A Waters SunFire C_{18} column (250 × 4.6 mm, 5 μm, Milford, MA, United States) maintained at 40°C was used to separate the three marker components in the FX sample. The mobile phases consisted of 0.1% aqueous formic acid and 0.1% (v/v) formic acid in acetonitrile. The gradient elution of the mobile phase was as follows: 5–60% B for 0–40 min, 60% B for 40–45 min, and 60–5% B for 45–50 min. The flow-rate and injection volume were 1.0 mL/min and 10 μL, respectively.

Animals

Seven-week-old male c57BL/6 mice were purchased from DBK Co., Ltd. (Eumseong-gun, Chungcheongbuk-do, South Korea). The mice were housed in specific-pathogen-free (SPF) conditions at a constant temperature and underwent a week-long adaptation period in 12/12 h light/dark cycles. The mice were fed a commercial diet (Cargill, Incorporated., Pyengtaek-si, Gyonggi-go, South Korea) and allowed tap water *ad libitum* throughout the study. All experiments were approved by the Committee on Animal Care of KIOM (17-104) and Use Committee in accordance with the National Institutes of Health Guidelines (NIH).

Treatments and Groups

Before the experiment began, reserpine, FX, EC, ECT, FR, and FXT were dissolved in PBS, and dispensed into 1.5 ml tubes with the amount to be used per day. The dispensed drugs were stored at −20°C, and used one by one on the day of administration. Reserpine was intraperitoneally administered to the mice at a concentration of 0.5 mg/kg (in PBS containing 0.1% dimethyl sulfoxide and 0.3% Tween-80) at a 100 μl dose to induce anxiety and depressive-like behaviors. They were randomly divided into the following nine groups: normal (non-reserpine + PBS), reserpine (reserpine + PBS), FX 30 (reserpine + FX 30 mg/kg), FX 50 (reserpine + FX 50 mg/kg), FX 100 (reserpine + FX 100 mg/kg), EC (reserpine + esculin 50 mg/kg), ECT (reserpine + esculetin 50 mg/kg), FR (reserpine + fraxin 50 mg/kg), and FXT (reserpine + fluoxetine 20 mg/kg). Mice received PBS, FX, EC, ECT, FR, and FXT orally at a 100 μl dose, according to their groups, once a day for a total of 10 days. A schematic of the experimental schedules is shown in **Figure 1** (Park et al., 2018; Yu et al., 2019).

Body Weighing, and Anxiety and Depressive-Like Behavior Tests

Body weight was measured before the experiment (basal) and 5 and 10 days after the beginning of the experiment. The measured increase in body weight was calculated as a percentage of the basal measurement. Anxiety and depressive-like behaviors were measured with open field (OFT), forced swim (FST), and tail suspension tests (TST). OFT measures the activity of mice exposed to a new environment to identify anxiety symptoms. The mice were placed in a white acrylic box (30 × 30 × 40 cm) and recorded for 10 min with EthovisionXT9 (Noldus Information Technology, Wageningen, The Netherlands). The degree of anxiety was measured by analyzing the distance traveled and the number of times the center of the arbitrarily-set acrylic box's central zone (10 × 10 cm) was crossed. FST and TST gauged despair symptoms. During FST, mice were placed in a transparent cylindrical cylinder filled with water at 25°C (H: 45 cm, D: 20 cm). Mice were exposed to the water tank for 15 min the day before the experiment to induce despair and lethargy (Marks et al., 2009), and improvement in behaviors such as induced despair produced by FX and its components confirmed with this experiment the next day. TST measures immobile time while the mouse is suspended with a tape attached to the tail from the top of a 50 cm high white acrylic box. In FST and TST experimental trials, the total recording time was 6 min; the immobility time during the last 4 min was measured using a video-tracking software (SMART 3.0; Panlab S.I., Barcelona, Spain).

Enzyme-Linked Immunosorbent Assay (ELISA)

On day 12, the mice were anesthetized with tiletamine/zolazepam (25 mg/kg), and blood was collected from the heart. Blood was centrifuged at 3,000 rpm at 4°C for 10 min. The separated supernatant plasma was transferred to another tube and stored at −70°C. Corticosterone concentration in plasma was determined with the corticosterone ELISA kit (Cayman chemical company, Ann Arbor, MI, United States). All experiments using this kit were performed according to the manufacturer's protocols. Corticosterone concentration was identified with a VersaMax microplate reader (Molecular Devices, Sunnyvale, CA, United States), and the absorbance was measured at the appropriate optical density using SoftMax pro 6.2.2 (Molecular Devices).

Real-Time Polymerase Chain Reaction (qPCR)

In the hippocampus, RNA was isolated with easy-BLUE™ reagent (iNtRON Biotechnology, Seongnam-si, Gyeonggi-do, South Korea), and cDNA synthesized in equal amounts with PrimeScript RT reagent kit (TaKaRa, Shiga, Japan). The base sequences of the primers used in real-time PCR are shown

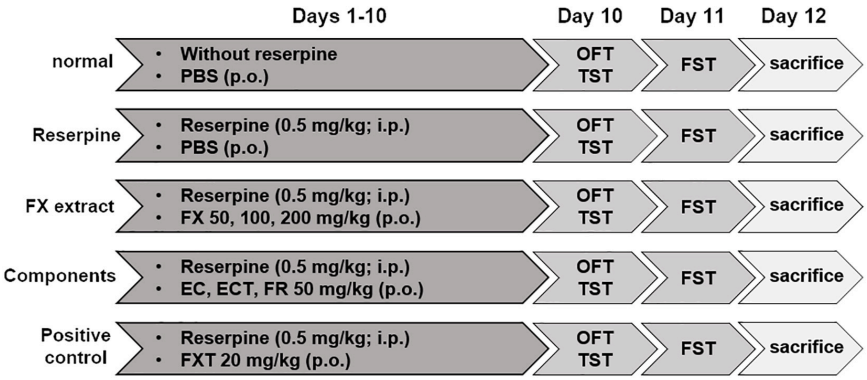


FIGURE 1 | Schematic diagram showing the experimental design. Drugs were orally and intraperitoneally administered from days 1 to 10. The OFT, TST, and pre-FST were performed on day 10. The FST was conducted on day 11. All mice were sacrificed on day 12.

in **Table 1**. The cDNA was loaded onto MicroAmp Fast 96-well reaction plates (Applied Biosystems, CA, United States) with each primer and SYBR Green PCR Master Mix (Applied Biosystems). mRNA was measured with Quantstudio 6 Flex (Applied Biosystems).

Western Blotting

The hippocampus was homogenized in 500 μl RIPA buffer (Thermo Fisher Scientific, Waltham, MA, United States) with Pro-Prep™ (iNtRON Biotechnology) and equalized to the same amount (20 μg) of protein. The equalized samples were separated with 4–20% Mini-PROTEAN TGX Precast Protein Gels (Bio-Rad Laboratories, Inc., Hercules, CA, United States), and separated proteins were transferred to a PVDF (Amersham Biosciences, Piscataway, NJ, United States). Membranes were blocked in 5% skim milk (Bio-Rad Laboratories, Inc.) solution for 1 h at room temperature and incubated with primary antibody overnight at 4°C: actin (Dilution ratio 1:2,000), BDNF (Dilution ratio 1:1,000), CREB (Dilution ratio 1:1,000), and pCREB antibodies (Dilution ratio 1:1,000). Membranes were subsequently incubated with appropriate secondary mouse and rabbit antibodies (Cell Signaling Technology) for 1 h at room temperature. Actin was used as a loading control for all experiments. The density of the protein band was quantified using an ImageQuant LAS 4000 mini (Fujifilm, Tokyo, Japan).

TABLE 1 | Real-time PCR primer sequences.

Gene	Sequence	
	Forward	**Reverse**
Mouse IL-12 p40	5′-AGACATGGAGTCATAG GCTCTG-3′	5′-CCATTTTCCTTCTTGTG GAGCA-3′
Mouse IL-6	5′-GAGGATACCACTCCCAA CAGACC-3′	5′-AAGTGCATCATCGTTG TTCATACA-3′
Mouse TNF-α	5′-AGACCCTCACACTCAGATC ATCTTC-3′	5′-CCACTTGGTGGTTT GCTACGA-3′
Mouse GAPDH	5′-AAGGTGGTGAAGC AGGCAT-3′	5′-GGTCCAGGGTTTCTT ACTCCT-3′

IL, interleukin; TNF, tumor necrosis factor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Immunofluorescence

The whole brain was fixed in 4% paraformaldehyde solution (BIOSESANG, Seongnam, Gyeonggido, South Korea) and dehydrated in 30% sucrose (Samchun chemicals, Gangnam-gu, Seoul, South Korea) solution. The dehydrated brain was modeled with OCT compound (Leica Biosystems, Wetzlar, Germany) and stored at −70°C. Modeled brains were sectioned into 30 μm sections with a cryostat (Leica Biosystems) at −20°C and attached to glass slides (Paul Marienfeld GmbH & Co., Lauda-Königshofen, Germany). Brain sections were post-fixed with 4% paraformaldehyde solution for 15 min and blocked for 1 h in a blocking buffer (1 × PBS/5% normal goat serum/0.3% Triton X-100). BDNF, pCREB, and NeuN antibodies were diluted 1:500 in antibody dilution buffer (1 × PBS/1% BSA/0.3% Triton X-100) and incubated overnight at 4°C. FITC and Texas red-conjugated secondary antibodies (Invitrogen by life technologies, MA, United States) were incubated for 2 h at room temperature, and longitudinal nuclei were stained with VECTASHIELD® Antifade Mounting Medium with DAPI (Vector Laboratories, Inc. CA, United States). Expressions were analyzed in the dentate gyrus of the hippocampus at 20 × (pCREB) and 40 × (BDNF) magnification. Imaging and IOD measurements were performed using a fluorescence microscope (Nikon Instruments Inc., Tokyo, Japan) and the NIS-Elements program (Nikon Instruments Inc.).

Data Analyses

All data are expressed as mean ± standard deviation (*SD*) and analyzed using GraphPad Prism 7 (GraphPad Software, Inc., La Jolla, CA, United States). Statistical analysis was performed using one-way and repeated one-way analyses of variance (ANOVA) with Tukey's *post hoc* comparisons. $P < 0.05$ were considered to indicate statistical significance.

RESULTS

HPLC Analysis of FX Sample

The optimal HPLC analytical method was successfully applied for the quantification of three marker components in the FX sample. All analytes were separated for 20 min with a resolution of ≥ 4.7. Representing the HPLC chromatograms of the FX sample (**Figure 2A**), the retention times of esculin, esculetin, and fraxin were 12.02, 15.11, and 14.36 (**Figure 2B**), respectively. The coefficient of determination (r^2) of the calibration curve of all analytes was 1.0000, indicating that the calibration curve shows excellent linearity. The regression equation, limit of detection (LOD), and quantitation (LOQ) values for the three marker components are presented in **Table 2**. Quantification of these analytes was performed at 335 nm for esculin, 340 nm for fraxin, and 345 nm for esculetin. The amounts of the three marker components (esculin, esculetin, and fraxin) in lyophilized FX sample were detected to be 170.57 ± 0.10, 13.72 ± 0.10, and 47.91 ± 0.36 mg/g, respectively.

Effect of FX Extract and Its Components on Anxiety and Depressive-Like Behaviors

We performed anxiety and depressive-like behavioral tests at 10 days following reserpine (i.p.), FX, components, and FXT (orally) administrations. FX treatment tended to increase the number of entries into the center [$F(8, 45) = 23.84$, $p < 0.0001$]; particularly, ECT and FXT treatment significantly increased the number of entries into the center (Reserpine: 5.571 ± 1.81; ECT: 22.67 ± 11.57, $p < 0.01$; and FXT: 21.5 ± 6.892, $p = 0.033$; **Figure 3A**). The distance traveled in the center [$F(8, 45) = 88.73$, $p < 0.0001$] significantly increased in the FX, ECT, FR, FXT-treated groups (Reserpine: 889.8 ± 73.59; FX 30: 1,405 ± 193.9, $p = 0.0003$; FX 50: 1,313 ± 108.7, $p = 0.0048$; FX 100: 1,241 ± 192.5, $p = 0.0339$; ECT: 1,468 ± 276.2, $p < 0.0001$; FR: 1,377 ± 127.2, $p < 0.0001$; and FXT: 1,579 ± 101, $p < 0.0001$; **Figure 3B**). Immobility time recorded during FST [$F(8, 63) = 8.223$, $p < 0.0001$] was significantly reduced in the FX, EC, ECT, and FR-treated groups (Reserpine:128.9 ± 40.4; FX 30: 40.32 ± 24.11, $p < 0.0001$; FX 50: 52.7 ± 27.68, $p = 0.002$; FX 100: 67.71 ± 36.27, $p = 0.0271$; EC: 37.04 ± 23.94, $p < 0.0001$; ECT: 66.84 ± 32.33, $p = 0.0162$; and FR: 38.74 ± 25.84, $p < 0.0001$; **Figure 3C**), and that recorded during TST [$F(8, 45) = 6.536$, $p < 0.0001$] was significantly reduced in the FX 50 and FR-treated groups (Reserpine: 146.6 ± 25.32; FX 50: 96.19 ± 14.64, $p = 0.049$; and FR: 92.55 ± 22.63, $p = 0.0226$; **Figure 3D**). These results suggest that treatment with FX extract and its components affects anxiety and depressive-like behaviors.

Effect of FX Extract and Its Components on Body Weight and Stress-Related Hormones in Plasma

There were no significant differences between groups in the body weight measured at 5 and 10 days. However, the body weight measured at 10 days tended to increase in the FX 100-treated group relative to the reserpine group (**Figure 4A**). Because reserpine administration increases the concentration of the stress hormone plasma corticosteroid by 3–5 times (Lengvari and Halasz, 1972), the effect of FX extract and its components on the change in plasma corticosterone concentration was confirmed. Corticosterone, a stress-related hormone, was significantly decreased in the plasma of the mice in the FX 50, 100, EC, ECT, FR, and FXT-treated groups (Reserpine: 55.81 ± 44.98; FX 50: 15.95 ± 15.92, $p = 0.0062$; FX 100: 14.01 ± 15.35, $p = 0.0033$; EC: 10.18 ± 5.68, $p < 0.0009$; ECT: 16.81 ± 18.16, $p = 0.008$; FR: 7.457 ± 2.92, $p = 0.0002$; and FXT: 11.51 ± 4.88, $p = 0.0015$; **Figure 4B**). These results suggest that FX extract affects body weight, and FX extract and its component decrease the concentrations of plasma stress-related hormone levels on reserpine-induced mouse model.

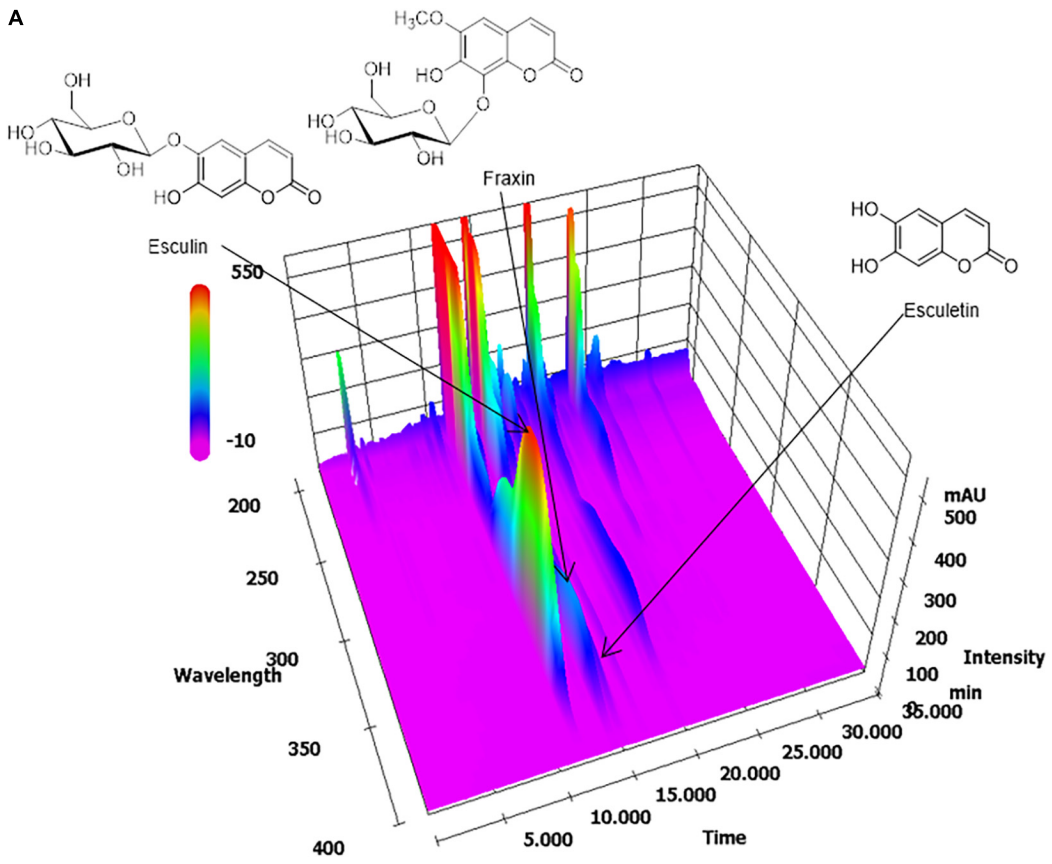
Effect of FX Extract and Its Components on Hippocampal Pro-inflammatory Cytokine mRNA Levels

Antidepressants used in the treatment of depression affect the concentration of pre-inflammatory cytokines and bring anti-inflammatory effects (Kopschina Feltes et al., 2017). The effects of FX extract and its components on the expression of pro-inflammatory cytokines were identified in the hippocampus. Reserpine administration significantly increased the mRNA levels of IL-12 p40 and TNF-α, and tended to increase the mRNA level of IL-6 (IL-12 p40: 319.4 ± 118.1, $p = 0.0063$; TNF-α: 233.6 ± 37.7, $p = 0.0003$; **Figure 5**). The FX 100-treated group attenuated all these increases (IL-12 p40: 33.57 ± 13.68, $p = 0.0008$; IL-6: 82.84 ± 24.05, $p = 0.0037$; TNF-α: 108.4 ± 47.57, $p = 0.0006$). The EC-treated group significantly restored IL-6 mRNA levels (92.71 ± 12.32, $p = 0.0285$), and the ECT-treated group significantly reduced IL-12 p40 (49.05 ± 16.96, $p = 0.0014$), IL-6 (83.01 ± 18, $p = 0.0016$), and TNF-α (130.6 ± 18.2, $p = 0.0062$) mRNA levels. The FR-treated group significantly reduced IL-12 p40 (23.54 ± 9.04, $p = 0.0002$) and TNF-α (114.5 ± 57.67, $p = 0.0006$) mRNA levels. Finally, the FXT-treated group showed significantly reduced IL-12 p40 (22.73 ± 5.222, $p = 0.0005$) and TNF-α (140.1 ± 26.58, $p = 0.0162$) mRNA levels. These results suggest that FX 100 mg/kg and its components regulate the expression of pro-inflammatory cytokines in the hippocampus of mice treated with reserpine.

Effect of FX Extract and Its Components on CREB/BDNF Signaling in the Hippocampus

We measured CREB/BDNF expression to determine the effects of FX extracts and its components on hippocampal neuroprotective mechanisms. Reserpine administration significantly reduced pCREB/CREB expression (64.17 ± 1.60, $p = 0.0344$; **Figure 6**). The decreased expression was restored with the administration of FX extract at doses of FX 30 and 100 mg/kg (FX 30: 106.1 ± 6.33, $p = 0.011$; FX 100: 97.31 ± 3.29, $p = 0.0472$). While BDNF expression decreased with reserpine (78.47 ± 3.35, $p = 0.0314$), FX 30, 50 mg/kg, and esculin significantly increased BDNF expression (FX 30: 99.23 ± 4.26, $p = 0.0385$; FX 50:

A



B

	Retention times	Contents (mg/g)
EC	12.02	170.57±0.10
ECT	15.11	13.71±0.10
FR	14.36	47.91±0.36

FIGURE 2 | FX extract standardization *via* HPLC analysis**. (A)** Three-dimensional HPLC chromatogram of the FX sample. **(B)** Retention times and contents (mg/g) of EC, ECT, and FR.

TABLE 2 | Linear range, regression equations, r^2, LODs, and LOQs of the eight bioactive compounds.

Compound	Linear range (μg/mL)	Regression equation[a]	r^2	LOD[b] (μg/mL)	LOQ[c] (μg/mL)
Esculin	0.78–50.00	y = 21843.50x + 2894.96	1.0000	0.10	0.31
Esculetin	0.78–50.00	y = 36254.96x + 3516.57	1.0000	0.12	0.37
Fraxin	1.56–100.00	y = 18621.50x + 5313.38	1.0000	0.22	0.67

[a] *y: peak area (mAU) of compounds; x: concentration (μg/mL) of compounds.*
[b] *LOD = 3.3σ × S.*
[c] *LOQ = 10σ × S.*
σ is the standard deviation of the y-intercept, and S is the slope of the calibration curve.

103.4 ± 4.99, p = 0.0129; EC: 105.6 ± 1.64, p = 0.0074). The location and cell type expressing pCREB or BDNF were measured using immunofluorescence. Changes in pCREB and BDNF expression were observed in the dentate gyrus of the hippocampus (**Figures 7A,D**). Reserpine administration decreased the pCREB expression (30.33 ± 4.09, p < 0.0001)

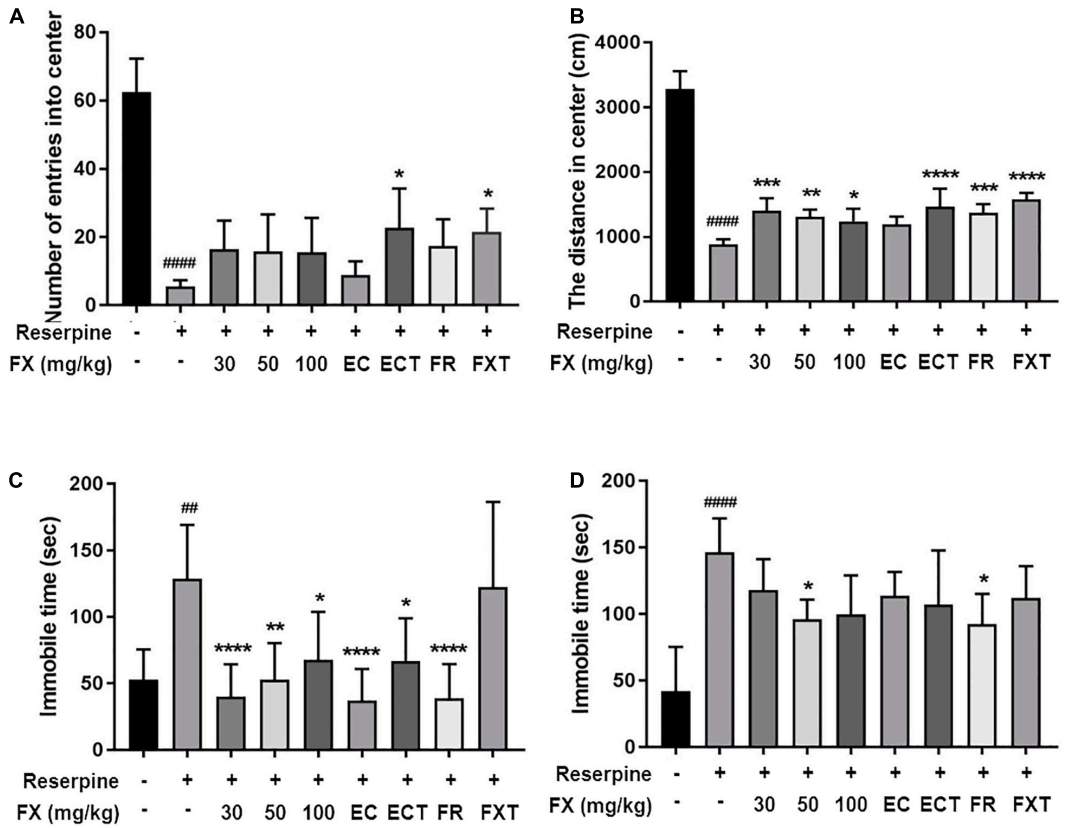


FIGURE 3 | Effect of FX extract and its components on performance in the OFT, TST, and FST. Mice were subjected to the **(A,B)** OFT (n = 6), **(C)** TST (n = 6), and **(D)** FST (n = 8). FX extract, EC, ECT, and FR significantly improved reserpine-induced depressive behaviors. $^{\#\#}P < 0.01$ and $^{\#\#\#\#}P < 0.0001$ vs. normal group; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$ vs. reserpine group.

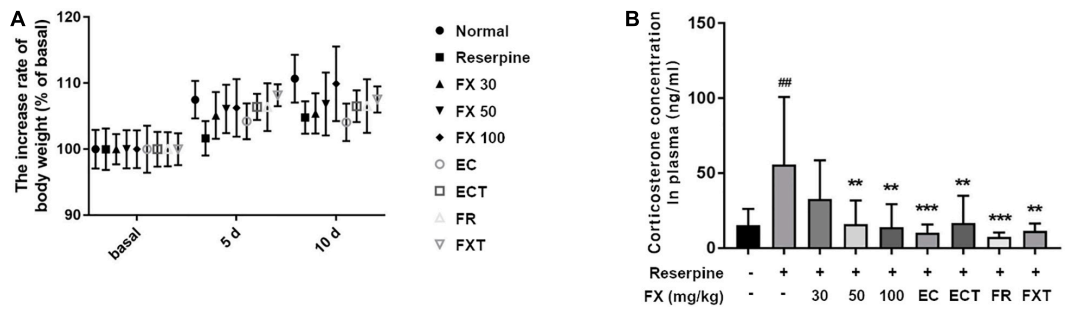


FIGURE 4 | Effect of FX extract and its components on body weight and plasma corticosterone concentration. The **(A)** body weight (n = 8) and **(B)** plasma corticosterone concentrations (n = 8) of the mice were measured. FX extract tended to increase body weight. FX extract, EC, ECT, and FR significantly decreased corticosterone concentration in the plasma. = 8. $^{\#\#}P < 0.01$ vs. normal group; $^{**}P < 0.01$ and $^{****}P < 0.0001$ vs. reserpine group.

and the number of pCREB positive neurons in the hippocampus (32.43 ± 9.27, $p < 0.0001$). However, FX 100, ECT, and FR-treated groups significantly restored the expression of pCREB (FX 100: 72.7 ± 15.41, $p = 0.0024$; ECT: 63.67 ± 23.84, $p = 0.0395$; and FR: 64.82 ± 16.95, $p = 0.0279$; **Figure 7B**) and the number of pCREB positive neurons (FX 100: 116.3 ± 32.05, $p = 0.0231$; ECT: 174.4 ± 94.02, $p < 0.0001$; and FR: 116.6 ± 50.16, $p = 0.0359$; **Figure 7C**). Reserpine administration also reduced BDNF expression (47.56 ± 9.65, $p = 0.0251$) and the number of BDNF positive neurons (99.86 ± 33.92, $p = 0.0018$). FX, EC, ECT, and FXT-treated groups significantly increased BDNF expressions (FX 30: 107.8 ± 30.16, $p = 0.0136$; FX 50: 117.2 ± 35.53, $p = 0.002$; FX 100: 139.3 ± 9.63, $p < 0.0001$; EC: 126 ± 26.46, $p = 0.0001$; ECT: 95.51 ± 26.31, $p = 0.0472$; and FXT: 115.7 ± 36.54, $p = 0.0005$; **Figure 7E**). In particular, FX 100-treated group significantly increased the number of hippocampal

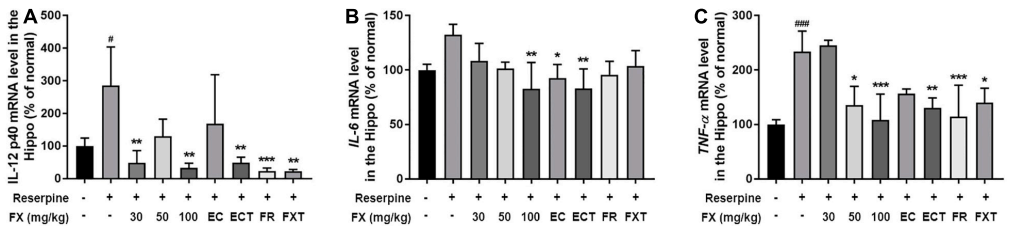


FIGURE 5 | Effect of FX extract and its components on the expression of *IL-12 p40, IL-6,* and *TNF-*α mRNA levels. **(A)** *IL-12 p40*, **(B)** *IL-6,* and **(C)** *TNF-*α mRNA levels were measured in the hippocampus of the mice with real-time PCR (n = 3–4). FX extract, EC, ECT, and FR increased *pro-inflammatory cytokines* mRNA levels. $^{\#}P < 0.05$ and $^{\#\#\#}P < 0.001$ vs. normal group; $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ vs. reserpine group.

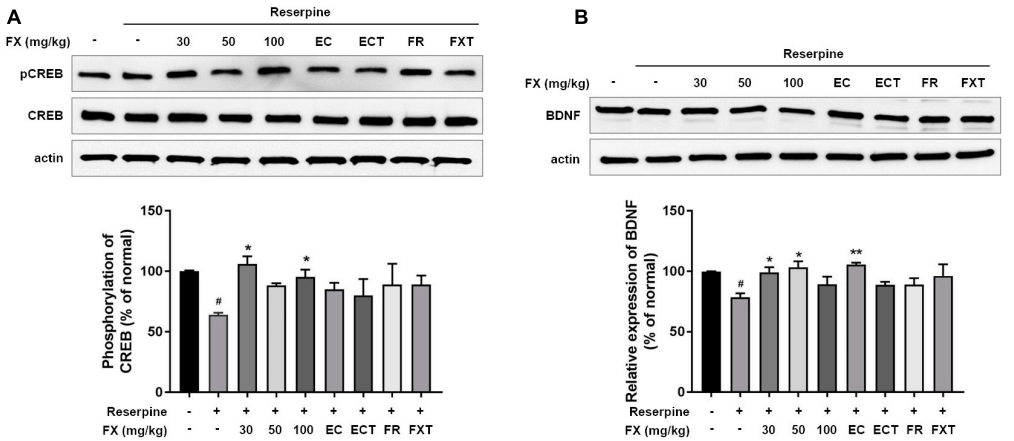


FIGURE 6 | Effect of FX extract and its components on the expression of pCREB/CREB and BDNF. **(A)** pCREB/CREB and **(B)** BDNF levels were measured in the hippocampus. FX extract significantly increased pCREB/CREB levels (n = 3). FX extract EC significantly increased BDNF levels. $^{\#}P < 0.05$ vs. normal group; $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. reserpine group.

BDNF-positive neurons (191.67 ± 24.42, p = 0.0023; **Figure 7F**). These results suggest that the FX extract and its components may exert neuroprotection by increasing the expression of CREB and BDNF in the neurons of hippocampus dentate gyrus.

DISCUSSION

While our previous studies found that FX extract and its components ameliorate chronic stress-induced depression, the present investigation sought to determine whether FX extract and its components are effective when anxiety and depressive-like behaviors can be directly attributed to the biochemical downregulation of monoamine in addition to attendant environmental causes. Specifically, we explored the aforementioned question by administering FX and its components to a reserpine-induced mouse model. FX extract and its components were found to improve anxiety and depression-like behaviors—as measured through OFT, TST, and FST—and affected the expression of stress hormones that contribute to depressive and anxiety symptoms. Furthermore, FX extract and its components decreased pro-inflammatory cytokine mRNA levels elevated by reserpine administration and increased CREB/BDNF signaling. The present findings thus confirm that FX extract and its components have a beneficial effect on depressive states.

Reserpine-induced animal models of depression have been used in many other studies; reserpine has been recognized for over 50 years as an antihypertensive and psychotropic drug. However, its long-term use has been associated with side effects that cause depression by depleting neural concentrations of monoamines. The use of reserpine to emulate depression in animals is thus validated as a correlate of investigating depression in humans (Baumeister et al., 2003; Zhang et al., 2018). Therefore, in this study, we selected an animal model for depressive and anxiety like behavior caused by monoamine depletion by reserpine administration and tried to verify the antidepressant and anxiety relief efficacy of FX extract and its components.

Behavioral tests such as the OFT, TST, and FST are widely used to evaluate antidepressant and anxiolytic activity in animal models. OFT identifies anxiety-like behaviors by measuring search activity in new environments. At the same time, the FST and TST are thought to confirm depressive-like behaviors by stimulating the escape instinct of the mouse and confirming whether an active coping style has been retained. In these tests, increased activity in the center zone and reduced immobility indicates a drug's antidepressant and

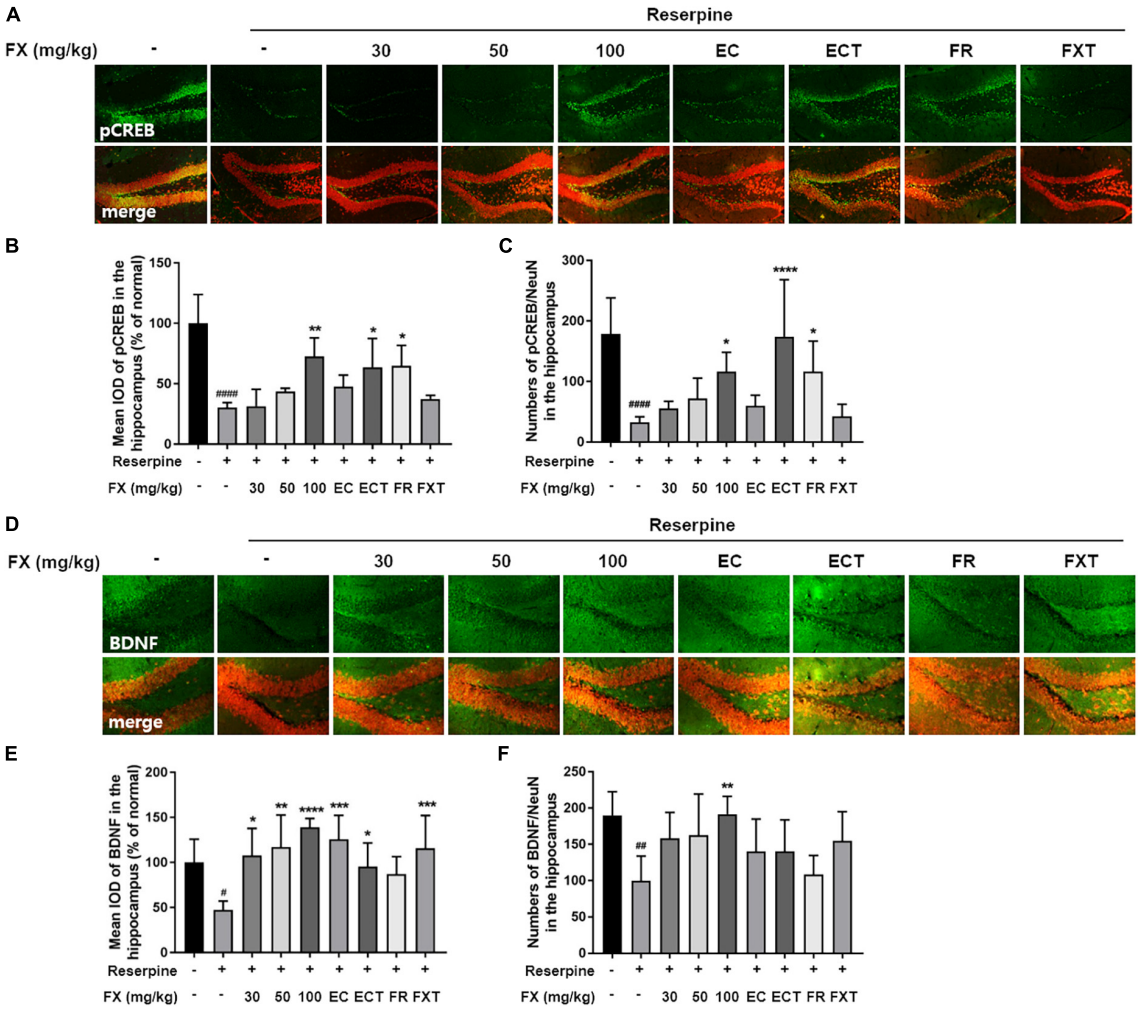


FIGURE 7 | Effect of FX extract and its components on the expression of pCREB and BDNF in the hippocampal neuron. **(A–C)** pCREB and **(D–F)** BDNF level were measured in the dentate gyrus of the hippocampus with immunofluorescence ($n = 5$). FX extract, ECT, and FR significantly increased pCREB levels in the hippocampal neurons. FX extract, EC, and ECT significantly increased BDNF levels. In particular, FX extract significantly increased BDNF levels in the hippocampal neurons. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#\#}P < 0.0001$ vs. normal group; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$ vs. reserpine group.

anxiolytic effect (Chang et al., 2015; Wei et al., 2018). Reserpine administration improves anxiety and depressive-like behaviors shown by mice staying at the edge of the space in OFT and maintaining immobility in TST and FST (Chang et al., 2015). This induced symptom effectively relieves anxiety and depressive symptoms, such as increased crossing and decreased immobility by antidepressant-like PSAP and herbal medicines (Sousa et al., 2018; Park et al., 2020). This study found that reserpine-administered mice exhibited reduced central dwelling time and entry frequency in the OFT as well as increased immobility in the TST and FST. Our results, therefore, suggest that FX and its components attenuate reserpine-induced anxiety and despair, as measured in the OFT and FST/TST, respectively.

Corticosterone is a hormone secreted into the plasma from the adrenal cortex. CRH, secreted by stress, is regulated by the hypothalamus-pituitary-adrenal (HPA) axis. Corticosteroids (ACTH) are secreted by pituitary stimulation, increasing the concentrations of epinephrine and corticoids in the adrenal glands (Petrescu et al., 2018). Stress is thus considered a major cause of depressive symptoms. Stressed animals reportedly feature increased blood concentrations of ACTH (Yang et al., 2015). Reserpine administration has further been observed to increase the level of corticosterone in the blood. The increase in stress hormones due to monoamine reduction is reduced by psychotropic drugs (Mitra et al., 1977; Park et al., 2018). In addition, plasma corticosterone has been shown to increase in LPS induced despair-like mice, another depressive animal model, and be significantly reduced by various antidepressants (Tomaz et al., 2020). Our study further showed that treatment with FX and its components significantly reduced plasma corticosterone concentrations. These results are thought to implicate corticosterone activity and stress elevation in the biochemical effects of reserpine; however, this hypothesis requires further validation.

While the mechanism of action of FX on depressive-like behaviors in the treatment of depression remains unknown, antidepressants have been shown to affect monoamine activity as well as feature anti-inflammatory and neuroprotective effects (Kopschina Feltes et al., 2017); specifically, antidepressants reportedly affect the production of cytokines (Obuchowicz et al., 2014). Several studies have further found that the hippocampal concentrations of the pro-inflammatory cytokines IL-6, IL-1β, and TNF-α are elevated in mice with depression-like behavior (Goshen et al., 2008; Mohamed et al., 2013; Hsieh et al., 2014; Tao et al., 2016). In addition, IL-12 was significantly increased in the plasma of patients with major depressive disorder (Lee and Kim, 2006). Reserpine, which induces anxiety and depression, increased levels of proinflammatory cytokines, TNF-α, IL-6, IL-12, and IFN-γ, mRNA, which induce inflammation and regulate immune cells in serum (Li et al., 2014). In addition, hippocampal pro-inflammatory cytokines in depressive states due to chronic unpredictable mild stress were significantly down-regulated by the administration of FXT, an SSRI antidepressant (Fernandes and Gupta, 2019; Shen et al., 2019). Our study also increased the mRNA levels of IL-12 p40, IL-6, and TNF-α increased by administration of reserpine, thus confirming that FX and components reduced these inflammatory cytokines. These results suggest that the antidepressant effects of FX extract and its components may be involved in neuroinflammation, accompanied by the reduction of IL-12, IL-6, and TNF-α mRNA levels.

The hippocampus plays an important role in learning and memory and is known as a major regulator of stress and mood. However, while the role of hippocampal neurons in depressive and stress disorders has been extensively studied, the exact mechanism underlying their involvement remains unclear (Planchez et al., 2020). The reported influence of antidepressants on synaptic and dendritic remodeling, which underlies antidepressant effects, supports the hypothesis that an effective antidepressant treatment could address neural developmental mechanisms (Segi-Nishida, 2017; Duman et al., 2019). Furthermore, stress-depressed mouse models show that decreased hippocampal levels of BDNF, TrkB, PI3K, and CREB induce neurological damage (Nestler et al., 2002; Wu et al., 2017; Jiang et al., 2019). In addition, the reserpine administration model reduced the levels of BDNF and CREB mRNA in the hippocampus and, in particular, showed a biochemical change of BDNF. Antidepressant administration reduces BDNF and has been shown to affect nerve cell damage recovery (El-Marasy et al., 2021). Our results confirmed that FX extract increased BDNF expression and CREB activity, particularly in hippocampal neurons. These results suggest that the administration of FX extract and its components can modulate BDNF/CREB signaling in hippocampal neurons. However, further validation of the association between BDNF/CREB signaling by FX extract and antidepressant regulation is needed in future studies. Unfortunately, unlike expected, FXT's effect on pCREB and BDNF expression was insignificant. In Pinnock et al. (2010), CREB showed higher activity in hippocampus on day 14 than on day 7 of FXT administration (Pinnock et al., 2010). It was concluded that the duration of FXT administration of 10 days was ambiguous to affect the expression or activity of CREB/BDNF.

CONCLUSION

In conclusion, reserpine administration induces depressive and anxiety-like behaviors, increasing corticosterone and pro-inflammatory cytokines in the plasma and hippocampus, respectively, and decreasing hippocampal pCREB/BDNF expression. These effects were attenuated by FX extract and its components. Our report suggests that FX leads to anti-inflammatory and neuroprotective effects, including a reduction in pro-inflammatory cytokine concentrations and enhancement of pCREB and BDNF expression in hippocampus, as well as antidepressant and anxiolytic-like effects. Our studies may serve as a preclinical basis that confirm the potential of FX and its components as antidepressants capable of addressing depressive and anxiety-like behaviors caused by monoamine changes. Further studies will be needed to establish mechanisms of action that regulate antidepressant and anxiety-relieving effects.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article.

ETHICS STATEMENT

The animal study was reviewed and approved by the Committee on Animal Care of Korea Institute of Oriental Medicine (KIOM 17-104).

AUTHOR CONTRIBUTIONS

YRK performed the experiments, analyzed the data, and wrote the manuscript. B-KP performed the experiments and analyzed the data. C-SS performed the HPLC analysis and wrote the manuscript related to HPLC. NSK reviewed the manuscript and discussed reviewer's comments for manuscript revision. MYL designed the experiments and reviewed the manuscript. All authors have read and approved the manuscript.

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REVIEW
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Gut Microbiota in Depression: A Focus on Ketamine

Alina Wilkowska, Łukasz Piotr Szałach and Wiesław Jerzy Cubała*

Medical University of Gdansk, Gdańsk, Poland

According to the WHO, major depressive disorder is the leading cause of disability worldwide, and it is a major contributor to the overall global burden of disease. The pathophysiology of this common and chronic disease is still not completely understood. The gut microbiome is an increasingly recognized environmental factor that can have a role in depression, acting through the gut–microbiota–brain axis. The available treatment for depression is still insufficient since 30% of patients are treatment-resistant. There is an unquestionable need for novel strategies. Ketamine is an effective antidepressant in treatment-resistant patients. It is suggested that the antidepressant effect of ketamine may be partially mediated by the modification of gut microbiota. In this study, we presented a review of data on gut microbiota in depression with special attention to the effect of ketamine on the microbiome in animal models of depression. Earlier reports are preliminary and are still insufficient to draw firm conclusion, but further studies in this field might help to understand the role of the gut–brain axis in the treatment of depression and might be the ground for developing new effective treatment strategies.

Keywords: ketamine, S-ketamine, R-ketamine, gut microbiota, gut-brain axis, treatment resistance, inflammation, HPA axis

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Edited by:
Ravid Doron,
Open University of Israel, Israel

Reviewed by:
He Liu,
Zhejiang University School of Medicine and Huzhou Central Hospital, China
Chong Chen,
Yamaguchi University Graduate School of Medicine, Japan

****Correspondence:***
Alina Wilkowska
ali.wilkowska@gmail.com

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INTRODUCTION

Major depressive disorder (MDD) is a severe, recurrent disease affecting more than 264 million people of the world population (Smith, 2014; World Health Organization., 2017a). Recent data show that ∼800,000 people with depression commit suicide every year (World Health Organization., 2017b). Depression correlates with disturbances in hypothalamic–pituitary–adrenal (HPA) axis (Barden, 2004) and causes increased inflammatory response (Dantzer et al., 2008; Miller et al., 2009). Increasing evidence suggests that microbiota plays a significant role in development (O'Mahony et al., 2009; Zheng et al., 2016) and possibly in the treatment of depression (Liu et al., 2019). The available treatment strategies for MDD are still insufficient. About 30% of patients are treatment-resistant (Rush et al., 2006). One of the new treatments involves *N*-methyl-D-aspartate receptor (NMDAR) antagonist, namely, ketamine. The mechanism of the antidepressant effect of ketamine is still not fully understood. Studies have indicated that it involves the inhibition of presynaptic and postsynaptic NMDARs in gamma-aminobutyric acidergic (GABAergic) interneurons. The mechanism also activates postsynaptic α-amino-3-hydroxy-5-methyl-4 isoxazole propionic acid receptors (AMPARs) and the brain-derived neurotrophic factor-tyrosine receptor kinase B (BDNF-TrkB) signaling pathway. Ketamine and its enantiomers have a rapid antidepressant and antisuicidal effect as an add-on treatment in unipolar and bipolar treatment-resistant depression (TRD) (Park et al., 2019; Wilkowska et al., 2020). There is an increasing number of evidence that the gut microbiota may play a crucial role in the antidepressant effects of ketamine (Wang et al., 2020).

In this review, first, we introduced the concept of gut–brain axis (GBA) and the studies on which it is based. We presented studies on germ-free (GF) and specific pathogen-free (SPF) mice, using vagotomy, probiotics, and antibiotics. We also discussed the role of the intestinal barrier and blood–brain barrier (BBB) in the GBA. Then, we demonstrated animal and human studies on the effect of depression on the gut microbiome and the functioning of gut–brain barrier. The second part of this review concentrates on ketamine. We started with the antibacterial and anti-inflammatory effects of ketamine and then presented studies on microbiota changes as an effect of ketamine use in animal models of depression. Recently, some reviews that are more informative have been published in this field, but none of them mentions ketamine and its potential role of regulating GBA in major depression (Barandouzi et al., 2020; Yang et al., 2020; Simpson et al., 2021).

GUT–BRAIN AXIS

Human gut microbiota consists of trillions of bacteria that are critical for nutrition and also significantly affect the central nervous system (CNS) (Hooper et al., 2001; Macpherson and Harris, 2004). Studies prove that there is bidirectional communication between brain and gut, and this process is captivated by the term GBA (Cryan et al., 2018). This communication system uses three main pathways, namely, neural—enteric nerves and vagus nerve, inflammatory—cytokines and immune cells, and humoral—HPA axis. Clinical studies suggest that the role of GBA is in the pathophysiology of irritable bowel syndrome (IBS) and of mental disorders such as autism, schizophrenia, Parkinson's disease, Alzheimer's disease, anxiety disorders, and MDD (Cryan et al., 2018).

In order to study pathological microbiota composition, it is necessary to know what defines a healthy gut microbiome. The normal human gut microbiota comprises two major phyla, namely, Bacteroidetes and Firmicutes. The next two most prominent phyla are Actinobacteria and Verrucomicrobia. The composition of microbiota stabilizes by the age of three, and at this point, it resembles the adult microbiome, although it is not constant and can undergo some changes due to the effect of various factors. A healthy colon also hosts primary pathogens such as *Campylobacter jejuni*, *Salmonella enterica*, *Vibrio cholerae*, and *Escherichia coli*, but in very low abundance (Jandhyala et al., 2015).

The current knowledge on GBA comes mainly from animal studies, specifically the ones involving GF mice. This model allows for studying mice in the absence of microbes and for comparing the observed processes in colonized mice, thereby unrevealing the effect of gut microbiota on the brain. There are two models of colonization used in such studies. The first model is SPF environment in which mice are raised with normal functional microbiota, but they are free of pathogens that could disturb their health and research outcomes. The second mice model involves gnotobiotic mice that are simply GF mice inoculated intentionally with known non-pathogenic microorganisms. Sudo et al. compared GF, SPF, and gnotobiotic mice to investigate the effect of microbes on stress response and neuroplasticity. The results have shown increased stress response with higher levels of adrenocorticotropic hormone (ACTH) and cortisol in GF compared with SPF mice. Moreover, the recolonization of the gut with *Bifidobacterium infantis* normalized the HPA axis, but only in young mice, suggesting that the brain is susceptible to microbiota effect at a specific time during development. This observation suggests that commensal microbiota is an environmental determinant that programs the HPA stress response (Sudo et al., 2004). The authors also found that GF male mice had the reduced BDNF and 2A subtype of NMDAR expression in the brain cortex and hippocampus compared with SPF mice (Clarke et al., 2013). Another study has demonstrated that GF male mice have significantly elevated hippocampal serotonin levels compared with conventionally colonized mice, and this change is resistant to the restoration of normal gut flora. It has also been found that mRNA of hippocampal BDNF in GF mice is reduced compared with their conventionally colonized counterparts, but this effect is present only in male animals. Interestingly, this effect is sex-specific, suggesting that the regulation of gut–microbiota–brain axis may be dependent on gender (Clarke et al., 2013). Diaz et al. have observed increased motor activity and reduced anxiety-like behavior in GF mice. This effect was accompanied by increased serotonin, noradrenaline, and dopamine turnover in the striatum of GF mice compared with SPF mice. The authors also found decreased expression of neuroplasticity genes, such as BDNF and nerve growth factor-inducible clone A (NGFI-A), in the brain of GF mice compared with SPF mice. Another noteworthy observation was the decreased expression of synaptic-related proteins (e.g., synaptophysin and PSD-95) in the striatum of SPF and conventionally raised mice compared with GF mice, which suggests that microbiota modulates the sensitive period of synaptogenesis. All these results suggest that gut microbiota is involved in the programming of neuronal circuits and therefore affects behavior (Diaz Heijtz et al., 2011). A study has shown an increased adult hippocampal neurogenesis in GF mice compared with conventionally colonized mice. The recolonization of GF mice did not prevent changes in adult hippocampal neurogenesis, again suggesting that there is a critical window in early life during which gut microbiota influences this process (Ogbonnaya et al., 2015). Another study supporting the role of bacteria in the bidirectional communication of the GBA used *Lactobacillus rhamnosus* (JB-1) as an active agent and compared the expression of GABA receptors in various regions of the brain between treatment group and conventionally fed mice. The authors observed mRNA alterations in region-dependent GABA receptors. Moreover, they also found reduced stress-induced corticosterone and anxiety- and depression-related behaviors in the treatment group. Neurochemical and behavioral effects were not found in vagotomized mice, and this identifies the vagus nerve as one of the main communication pathways between gut microbiota and the brain (Bravo et al., 2011).

Beside humoral and neural pathways, GBA also uses the immune system for this bidirectional communication (Hooper et al., 2012). Microbiota has a critical role in the development of organized lymphoid structures and in the function of immune

system cells. Gut microbes modulate the maturation and function of immune cells in the CNS, such as microglia, and also in peripheral immune cells (Fung et al., 2017).

An alternative way of investigating microbiota and its role in communication with the CNS is to use antibiotics in order to change microbiota composition and observe its effect on brain function and behavior in animals. A study found that the administration of a mixture of oral non-absorbable antimicrobials to SPF mice caused a significant increase in Firmicutes and Actinobacteria and a decrease in Gammaproteobacteria and Bacteroidetes. This change was associated with increased exploratory behavior and increased hippocampal BDNF levels. These effects were reversible after the withdrawal of antibiotics. Oral antibiotic administration did not cause any changes in behavior in GF mice, which suggests that the presence of microbiota is necessary for this effect. The authors performed additional tests and found that the effect of antibiotics was independent of the autonomic nervous system, inflammation, and gastrointestinal neurotransmitters. They suggested that there must be another way of communicating between the gut and the brain, and this way may involve the production of neuroactive substances by bacteria (Bercik et al., 2011).

Evidence from clinical and animal studies shows that enteric microbiota has a significant impact on GBA, interacting directly with enterocytes and enteric neurons (Carabotti et al., 2015). Certain metabolites from gut microbes alter neurotransmitter production in the cells of the colon (Yano et al., 2015; Kiraly et al., 2016). On the other hand, the brain acts on gastrointestinal and immune functions and affects gut microbiota composition in this way (Ha et al., 2014; Carabotti et al., 2015). Furthermore, gut microbes may produce molecules that can act as local neurotransmitters, such as GABA, serotonin, melatonin, histamine, and acetylcholine (Iyer et al., 2004). They can also transform catecholamine substrates to a biologically active form (Asano et al., 2012). It has been reported that binding sites for enteric neurotransmitters are present in bacteria and can influence the function of components of the microbiota (Hughes and Sperandio, 2008).

The interaction of microbiota and GBA might also occur through the release of biologically active peptides from enteroendocrine cells. The digestive tract is a source of regulatory peptides that act locally on the epithelial cells and the enteric nervous system and also have distant targets in the brain (Uribe et al., 1994). For example, galanin stimulates the release of cortisol-releasing factor (CRF) and ACTH, thereby enhancing cortisol secretion. Galanin also seems to stimulate cortisol secretion directly from adrenocortical cells and norepinephrine release from the adrenal medulla in rats (Tortorella et al., 2007). A human study suggests that ghrelin, another psychoactive peptide, has a marked ACTH/CRF effect, and it is probably involved in the modulation of the HPA response to stress (Giordano et al., 2006). Recent studies on GF and conventionalized mice have proved that one of the major human symbionts, *Bacteroides thetaiotaomicron*, promotes neurogenesis in the enteric nervous system and regulates enteroendocrine networks through its major fermentation products, acetate, propionate, and succinate (Aktar et al., 2020; Modasia et al., 2020).

MICROBIOTA AND INTESTINAL BARRIER

The intestinal epithelium acts as a barrier between the host and the commensal bacteria, enabling their symbiotic relationship. Enterocytes form the physical barrier by linking together with various cell junctions such as desmosomes, adherens junctions, and tight junctions (Hiippala et al., 2018). Tight junctions, such as claudins, occluding, and intercellular junctions, control the paracellular permeability and moderate the transepithelial transport (Ulluwishewa et al., 2015). Microbiota dysbiosis can impair the epithelial barrier leading to the so-called "leaky gut," allowing the intestinal content to be in contact with the host periphery, potentially inducing inflammatory response (Walker and Lawley, 2013). There is evidence that the probiotic *Bacteroides fragilis* normalizes increased intestinal epithelial permeability in a mouse model of autism spectrum disorders (Hsiao et al., 2013). Studies suggest that probiotic bacteria enhance the intestinal barrier, causing changes in the tight junction protein expression and distribution. Commensal bacteria, together with intestinal inflammation and dietary components, are the main factors affecting epithelial permeability (Suzuki, 2013).

MICROBIOTA AND BLOOD–BRAIN BARRIER

Blood–brain barrier controls the passage and exchange of molecules and nutrients between the circulatory system and the brain cells. The development of the brain includes the formation of intact BBB, which promises optimal conditions for neuronal growth and cell specification (Engelhardt, 2003). BBB is formed by capillary endothelial cells sealed by tight junctions, astrocytes, and pericytes. Tight junction proteins consist of transmembrane proteins such as claudins, tricellulin, and occludin (Tscheik et al., 2013). There is evidence that lack of gut microbiota is associated with increased BBB permeability and altered expression of tight junction proteins (Braniste et al., 2014). It was also found that fecal transfer from mice with pathogen-free gut flora into GF mice or treatment of GF mice with bacteria that produce short-chain fatty acids (SCFAs) decreases the permeability of the BBB (Thabane et al., 2010). Although the study has not revealed the precise signaling mechanisms through which gut microbiota modulates BBB function, it sheds light on another aspect of microbiota affecting the brain.

Another proof for microbiota regulating GBA was obtained from a population-based study from Walkerton in Canada, according to which, as the consequence of a flood in the year 2000, drinking water was contaminated with *E. coli* and *C. jejuni*. This contamination caused gastrointestinal infections in more than 2,000 inhabitants. Thabane et al. followed over 400 children for 7 years and found that acute bacterial gastroenteritis was associated with more than 4-fold increase in the incidence of IBS

among children exposed to acute gastroenteritis compared with unexposed controls (Thabane et al., 2010).

MICROBIOME IN DEPRESSION

There is a strong connection between chronic stress, microbiota changes, activation of the inflammatory system, and depression. It has been shown that the levels of pro-inflammatory cytokines, mainly the interleukins (IL), such as, IL-1, IL-6, IFN-γ, and TNF-α, are elevated in the serum of patients suffering from depression (Dowlati et al., 2010; Schmidt et al., 2014; Haapakoski et al., 2015). Elevated serum concentrations of other cytokines, such as IL-5, IL-7, IL-8, IL-10, IL-12, and IL-13, have also been reported (Schmidt et al., 2014).

A preclinical study revealed significant changes of the gut microbiome in rats subjected to maternal separation compared with control (O'Mahony et al., 2009). Another study has shown that microbiome composition in mice exposed to the long-term restraint stress was also significantly altered compared with nonstressed mice (Bangsgaard Bendtsen et al., 2012). Bailey et al. have shown that mice exposed to a social stressor called social disruption had lower relative abundance of genus *Bacteroides* and higher relative abundance of genus *Clostridium*. The microbial diversity of stressed mice was significantly reduced. Moreover, stressor-induced increase in levels of circulating IL-6 and monocyte chemoattractant protein-1 (MCP-1) correlated with stressor-induced changes in three members of the microbiota, such as *Dorea* spp., *Coprococcus* spp., and *Pseudobutyrivibrio* spp. (Bailey et al., 2011).

Inflammasome, a protein complex that generates and augments stress-induced immune response *via* activation of caspase-1 and increased IL-1beta and IL-18 secretion, is considered to play an important role in the development of depression. It has been proved that caspase-1 knockout mice, apart from significantly lower depressive-like behaviors and lower IL-1beta and IL-18 levels, presented several microbiota changes in comparison with mice with activated inflammasome (Wong et al., 2016). Zhang et al. tested the link between IL-6 and microbiota in a mouse model of depression. The authors used the anti-mouse IL-6 receptor antibody (MR16-1) and social defeat stress (SDS) model. Susceptible mice presented gut microbiota alterations. MR16-1 had an antidepressant effect, but it also improved decreased Firmicutes/Bacteroidetes ratio at the phylum level and significantly improved the increased levels of *Sutterella* and decreased levels of *Oscillospira* at the genus level in susceptible mice after SDS. These findings suggest that the blockade of IL-6 receptor in the periphery might have an antidepressant effect, achieved through normalizing the altered composition of gut microbiota in susceptible mice after SDS (Zhang et al., 2017).

Zheng et al. demonstrated that the absence of gut microbiota induces depression-like behavior in mice. The human part of the study confirmed that the composition of gut microbiota in patients with MDD was significantly altered compared with control. This effect was characterized by the alterations in Firmicutes, Actinobacteria, and Bacteroidetes at the phylum level. In order to determine whether gut microbiota have a role in developing depression, they colonized GF mice with microbiota from depressed patients. This process caused increased depression-like behaviors in mice (Zhang et al., 2017). Another similar study demonstrated that the oral transplantation of gut microbiota from depressed patients to microbiota-depleted rats induces depressive-like, anhedonic behavioral phenotype with a simultaneous increase in acute phase proteins and altered tryptophan metabolism. The authors suggested that the gut microbiota may play a role in the development of depression most probably through the immunomodulatory pathway and may provide a target in the treatment and prevention of this disorder (Kelly et al., 2016).

Another environmental factor that can modulate GBA in depression is diet; however, this subject is beyond the scope of this review (Aly and Engmann, 2020; Włodarczyk et al., 2021).

PROBIOTICS IN STUDIES ON DEPRESSION

The next set of data on the role of gut microbiota in depression comes from studies with probiotics. Probiotics are microorganisms that contribute to the host gut microbial flora when consumed and produce beneficial effects on health. In animal models of depression, chronic probiotic administration can reduce anxiety and depressive symptoms and correlates with the normalization of biological indicators of depression, such as corticosterone, noradrenaline, BDNF levels, and cytokines (Desbonnet et al., 2010; Bercik et al., 2011; Bravo et al., 2011). Some probiotics such as *L. helveticus* R0052 and *B. longum* R0175 seem to restore tight-junction integrity in the intestinal barrier and also affect hippocampal neurogenesis (Ait-Belgnaoui et al., 2014). One of the studies found that the supplementation of *Bifidobacterium* in mice causes resilience to chronic social defeat stress (CSDS), which is an animal model of depression (Yang et al., 2017a).

A recent systematic meta-analysis including 29 trials of probiotics in humans shows that probiotics have a small, but significant, effect on depression and anxiety, although the number of trials with clinical samples is still not sufficient. Most of the trials investigated *Lactobacilli* alone or in combination with species from other genera, most often *Bifidobacterium* (Liu et al., 2019).

GUT MICROBIOTA IN MDD

The impact of altered microbiota on GBA in depression is the subject of scientific interest (Dinan and Cryan, 2019). The first clinical study investigating gut microbiota in depressed patients vs. controls found no significant differences in diversity between the two groups, but several correlations between depression and the fecal microbiota were observed (Naseribafrouei et al., 2014). Jiang et al. analyzed fecal samples from 46 patients with depression and 30 healthy controls. They found significant differences in microbiota composition of patients with MDD. Bacteroidetes, Proteobacteria, and Actinobacteria were increased,

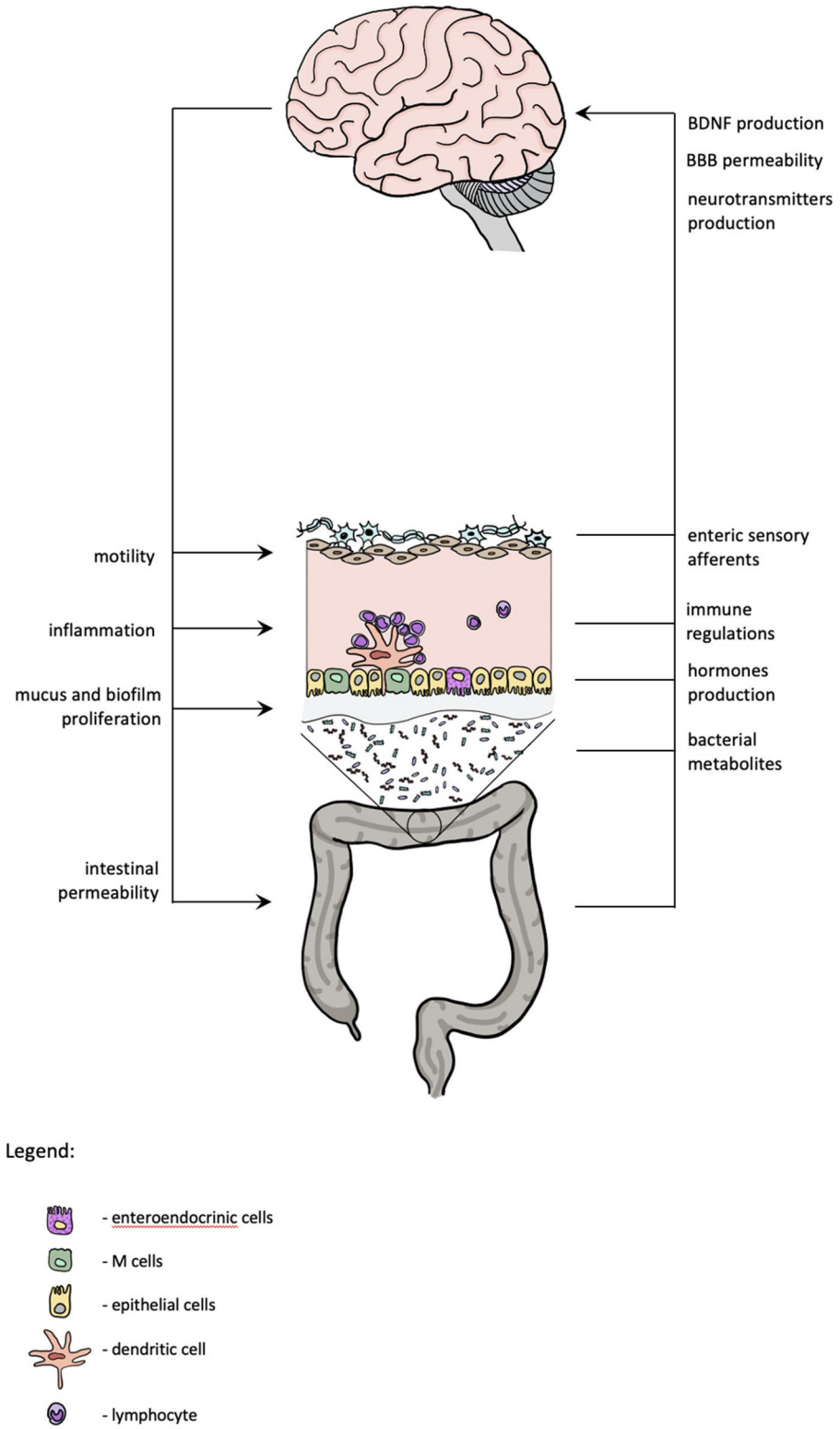

FIGURE 1 | Gut-brain axis and depression. The scheme summarizes the mutual interactions between central nervous system and gastrointestinal system. Functional changes in both might contribute to depression.

while Firmicutes was decreased, in MDD subjects compared with healthy controls. Most notably, the MDD groups had increased levels of Enterobacteriaceae and *Alistipes* but reduced levels of *Faecalibacterium*. A negative correlation was observed between *Faecalibacterium* and the severity of depressive symptoms (Jiang et al., 2015). Another recent study involving 40 patients with practitioner-reported depression and 70 healthy controls (i.e., sample extracted from the cohort of Flemish Gut Flora Project; $n = 1{,}054$) found that two bacterial genera, namely, *Coprococcus* and *Dialister*, were depleted in patients with depression diagnosed by their general practitioner (Valles-Colomer et al., 2019). A recent systematic meta-analysis included two already mentioned reports (Naseribafrouei et al., 2014; Jiang et al., 2015) and additional eight observational studies and found that, at the phylum level, the findings were inconsistent. At the family level, Veillonellaceae, Prevotellaceae, and Sutterellaceae were less abundant in patients with MDD than in non-depressed controls, and Actinomycetaceae was elevated in those with MDD than in controls. At the genus level, *Coprococcus*, *Faecalibacterium*, *Ruminococcus*, *Bifidobacterium*, and *Escherichia* were reduced in patients with MDD than in non-depressed controls, whereas *Paraprevotella* was increased in depressed patients (Sanada et al., 2020).

GUT–BRAIN BARRIER IN MDD

Major depressive disorder is associated with an increased translocation of bacterial products from the gut. According to the "leaky gut hypothesis," increased intestinal permeability in depressed patients may contribute to inflammatory response *via* bacterial translocation across the enterocytes. Maes et al. found that depressed patients have increased IgA and IgM response against lipopolysaccharide (LPS)—part of the wall of gram-negative commensal bacteria (Maes et al., 2012). LPS is recognized by the CD14-Toll-like receptors expressed by peripheral blood mononuclear cells (PBMCs) and also by neurons, microglia, and astrocytes. In depression, bacteria translocate from the epithelium to lamina propria and mesenteric lymph nodes (i.e., site of antigen presentation), which may then activate PBMCs and provoke immunoglobulin production (Maes et al., 2008). It also seems that increased bacterial translocation is related to the level of oxidative and nitrosative stress in depressed patients (Maes et al., 2013). A study on gut permeability in patients with recent suicide attempts in course of MDD found that permeability markers—zonulin and intestinal fatty acid-binding protein (I-FABP)—are altered in patients with recent suicide attempt compared with controls. These markers correlated with the IL-6 levels and I-FABP concentration correlated with the severity of depressive symptoms. The authors suggested that "leaky gut hypothesis" may elucidate the association between inflammation and suicidal behavior (Ohlsson et al., 2019). Increased release of IL-6, IL-1β, and TNFα can lead to alterations in central neurotransmission and cause symptoms called "sickness behavior" (D'Mello and Swain, 2017). Patients with inflammatory disorders, such as chronic liver disease, IBS, and rheumatoid arthritis, have high comorbidity of depression and sickness behavior, although the distinction between both is difficult due to overlap of symptoms such as fatigue, increased anxiety, loss of appetite, sleep disturbances, and loss of social interest (D'Mello and Swain, 2014). The elements of gut-brain axis which might have a role in depression are presented in **Figure 1**.

GUT MICROBIOTA AND ANTIDEPRESSANTS

An increasing number of evidence indicates that drugs affect microbiota composition. Among them, there are psychotropic drugs including antidepressants (Cussotto et al., 2019). According to *in vitro* studies, sertraline is a strong antimicrobial agent which inhibits the growth of *Staphylococcus aureus*, *E. coli*, and *Pseudomonas aeruginosa*, and it also augments the effect of antibiotics (Ayaz et al., 2015). Fluoxetine shows a strong dose-dependent antimicrobial activity *in vitro* against *L. rhamnosus* and *E. coli* (Cussotto et al., 2018). An *in vivo* study on rats on fluoxetine found that this antidepressant completely inhibited the growth of *Succinivibrio* and *Prevotella* taxa (Cussotto et al., 2018). Although the role of its effect on antidepressant properties may be contrary, its side effects are still not clear. A recent study on mice treated with one of five different antidepressants (i.e., fluoxetine, escitalopram, venlafaxine, duloxetine, or desipramine) revealed that all the drugs except desipramine reduced richness (i.e., the variation of microbes in a single sample) of mice gut microbiota, while simultaneously increasing beta diversity (i.e., the variation of microbial communities between samples). This observation can cause concern because it is generally accepted that reduced microbiota richness is more common in conditions such as IBS and obesity. The main aim of this study, however, was to identify bacteria directly influencing the antidepressant mechanism of study drugs. Based on a series of experiments, the authors chose duloxetine and *Ruminococcus flavefaciens* and found that the supplementation of *R. flavefaciens* reduced or even abolished antidepressive and antianhedonic properties of duloxetine. Further investigation has shown that the mechanism of these bacterial actions may involve impairment of mitochondrial oxidative phosphorylation and neural plasticity in medial prefrontal cortex (mPFC). This can be explained by the gene expression changes in synaptic and mitochondrial genes, which are induced by *R. flavefaciens*. Therefore, the dysregulated mitochondrial function and decreased neuroplasticity in mPFC could contribute to *R. flavefaciens* attenuation of antidepressant effects on depressive-like behavior. Most importantly, the authors found reduced levels of serotonin and noradrenaline in mPFC as a result of *R. flavefaciens* supplementation (Lukić et al., 2019).

ANTIBACTERIAL EFFECT OF KETAMINE

The acute effect of ketamine includes inhibition of NMDARs and the activation of AMPARs, as well as molecular signaling

of the mammalian target of rapamycin (mTOR), which results in the enhancement of hippocampal (BDNF) and increased synaptogenesis (Pałucha-Poniewiera, 2018). Apart from these mechanisms, ketamine also has antimicrobial properties, as observed in *in vitro* studies. In an earlier study by Gocmen et al., ketamine was used in high anesthetic doses, and it has shown prominent antibacterial effect against six different strains of bacteria, namely, *S. aureus*, *Staphylococcus epidermidis*, *Enterococcus faecalis*, *Streptococcus pyogenes*, *P. aeruginosa*, and *E. coli*. The authors pointed out that the doses used are not for human or anesthetic purposes, so this antibacterial effect is not possible to observe *in vivo* (Gocmen et al., 2008). In a similar study, the antimicrobial effect of ketamine against *S. aureus*, *E. coli*, and *P. aeruginosa* was confirmed when ketamine was used with propofol [i.e., a mixture called ketofol used for electroconvulsive therapy (ECT) anesthesia] and when ketamine was suggested as safe anesthesia for surgical approaches (Begec et al., 2013). In a more recent study on radish seeds treated with sub-anesthetic doses of ketamine, the authors observed the antibacterial effect on *S. epidermidis* and *Borrelia burgdorferi*. They concluded that the possible mechanism behind this effect could remain in L-glutamate signaling networks and NMDAR ion channels of bacteria (Torres et al., 2018).

ANTI-INFLAMMATORY EFFECT OF KETAMINE

Ketamine has an anti-inflammatory effect in depression (Szałach et al., 2019). The study which included patients with TRD indicated that pro-inflammatory cytokines (mainly IL-6, G-CSF, and IL-1α) reduced 4 h after a single dose of intravenous (IV) ketamine (Kiraly et al., 2017). Another study has shown rapid decreases in levels of IL-6 and TNF-α, as well as a correlation between the decrease in TNF-α and a reduction in the Montgomery–Asberg Depression Rating Scale (MADRS) score (Chen et al., 2018). Kadiru et al. found that ketamine influences the kynurenine pathway by increasing the level of kynurenine and kynurenic acid and decreasing the level of quinolinic acid acting as a rapid anti-inflammatory agent in patients with bipolar depression (Kadriu et al., 2019). In another trial, treatment with 6 ketamine IV infusions in patients with MDD correlated with the elevation of kynurenine and tryptophan; moreover, the downregulation of inflammation was observed and the authors suggested that the anti-inflammatory effect of ketamine may contribute to its rapid antidepressant effect (Kadriu et al., 2019). Moreover, stress-related changes in the gut microbiome affect BDNF concentration and NMDAR activity (Baj et al., 2019), and ketamine may hypothetically reverse these changes. Walker et al. investigated the effect of ketamine in LPS-induced depression and found that ketamine abrogated LPS-induced depressive behaviors by antagonizing NMDA activation (Walker et al., 2013). One of recent studies investigated the relationship between hippocampal volume and inflammatory markers following six ketamine infusions in 44 patients with depression. The authors confirmed antidepressant effect of ketamine and increase in right hippocampal volume, but found no correlation with the change in concentrations of a group of inflammatory markers one day after ketamine treatment. Analyzing cytokine concentrations separately they confirmed significant changes in kynurenic acid, fractalkine, IFN-γ, TNF-α, IL-2, IL-4, IL-6, and IL-10 (Zhou et al., 2020). The abovementioned data suggest that ketamine may reach its antidepressant effect, at least partly, through inhibiting inflammatory reaction, which is one of the main elements of gut–brain interplay.

EFFECT OF KETAMINE ON MICROBIOME IN ANIMAL MODELS OF DEPRESSION

The electronic databases, such as PubMed, MEDLINE, and EBSCO host, were searched for the following keywords and their combinations: ketamine, esketamine, arketamine, and gut microbiota; ketamine and gut microbiome; ketamine and gut bacteria; ketamine and GBA. We have also conducted cross-reference search based on the reports found.

We have identified four studies focusing on the effect of ketamine on gut microbiota in rodents subjected to CSDS—an animal model of depression. This model does not show treatment resistance, which is a significant fact, since most ketamine human studies involve treatment-resistant patients. Such a model has been used in a study on deep brain stimulation (Papp et al., 2018).

Nevertheless, we have found no studies on the ketamine effect on gut microbiota in treatment-resistant rodents. All studies with detailed descriptions are presented in **Table 1**. The first study investigated the role of microbiota in an antidepressant effect of two ketamine enantiomers, S- and R-ketamine (Yang et al., 2017b). The authors investigated both enantiomers since it has been reported that R-ketamine has greater potency and long-lasting antidepressant effects and has fewer adverse effects than S-ketamine (Yang et al., 2015). The results confirmed the antidepressant effect of both enantiomers as the increased immobility time in TST (tail suspension test) and FST (forced swimming test) was reduced after ketamine administration in susceptible mice. R-ketamine had stronger antidepressant and antianhedonic properties than S-ketamine. The observed increased levels of Actinobacteria in CSDS-susceptible mice is in line with the evidence that this phylum is increased in MDD (Jiang et al., 2015; Zheng et al., 2016); interestingly, neither of ketamine enantiomers modified this effect. The authors pointed out the possible role of Deltaproteobacteria and Desulfovibrionaceae in MDD as their presence in the gut correlates with an increased inflammatory response in humans. The number of genus *Butyricimonas* producing butyrate, which is known for anti-inflammatory potential, was restored mainly by R-ketamine after the CSDS, and this observation suggests the role of GBA in the antidepressant effect of R-ketamine (Yang et al., 2017b). In the second study, as a comparator for R-ketamine, the authors used lanicemine, which is also an NMDA antagonist, but it does not show antidepressant effect in humans (Sanacora et al., 2016). In line with this research, lanicemine did not cause any antidepressant effect

TABLE 1 | Studies on ketamine's effect on gut microbiota in rodent model of depression.

References	Animal model and behavioral tests	Intervention	Microbiome after CSDS in susceptible mice	Effect of the intervention on microbiome
Yang et al. (2017b)	Mice CSDS TST FST SPT	R-ketamine S-ketamine 10 mg/kg vs. saline	*Phylum* Tenericutes ↓ Actinobacteria ↑ *Class* Deltaproteobacteria ↑ Mollicutes ↓ *Family* Desulfovibrionaceae ↑ *Genus* Butyricimonas ↓	None None R and S-ketamine ↓ R-ketamine ↑ S-ketamine ↑ R,S-ketamine ↑ R-ketamine more potent
Qu et al. (2017)	Mice CSDS SIT TST FST SPT	R-ketamine 10 mg/kg vs. lanicemine 10 mg/kg vs. saline	*Order* Bacteroidales↓ Clostridiales↓ *Family* Ruminococcaceae↑ Mogibacteriaceae ↓ *Genus* Clostridium↑↑	R-ketamine ↑ R-ketamine ↑ R-ketamine ↓ lanicemine less potent R-ketamine ↓ lanicemine less potent
Getachew et al. (2018)	Rats No model of depression	Ketamine 2.5 mg/kg vs. saline	–	*Phylum* Deferibacteres ↓ Tenericutes↓ *Class* Deferrribacteres↓ Mollicutes↓ *Order* Turicibacterales↑ Desulfuromonadales↓ Deferribacterales↓ Theromonaerobacterales↓ Anaeroplasmatale↓ *Family* Tuberibacteraceae ↑ Clostridiaceae↑ Lactobacillaceae↑ Deferrribacteraceae↓ Ruminococcaceae↓ *Genus* Sarcina ↑ Turicibater↑ Lactobacillus↑ Mucispirillum↓ Ruminococcaceae↓
Huang et al. (2019)	Mice, LPS-induced inflammation model of depression FST Locomotion	Ketamine 10 mg/kg vs. saline	*Phylum* Actinobacteria ↓ Firmicutes ↓ *Class* Coriobacteriia ↓ Clostridia ↓ *Order* Clostridiales ↓ *Family* Prevotellaceae ↑ *Genus* Alloprevotella ↑ Butyricimonas ↑	Ketamine ↑ Correlation with the effect of ketamine on FST in LPS mice: **Negative** *Phylum* Actinobacteria *Class* Coriobacteriia *Order* Clostridiales **Positive** *Family* Prevotellaceae *Genus* Alloprevotella

SIT, social interaction test; TST, tail suspension test; FST, forced swimming test; SPT, Sucrose Preference Test; ↓, decreased number of bacteria; ↑, increased number of bacteria.

contrary to R-ketamine. The authors observed alterations in gut microbiota after CSDS, which were attenuated by R-ketamine and to some extent also by lanicemine, although its effect was less potent. The most interesting effects of R-ketamine in this study were the restoration of Bacteroidales, previously reported as reduced in depression (Naseribafrouei et al., 2014), and Clostridiales (i.e., butyrate-producing bacteria) and reduction in genus *Clostridium*, previously reported as increased in MDD (Jiang et al., 2015; Qu et al., 2017). The third study investigated changes in gut microbiota composition after low-dose ketamine vs. placebo. The authors found a significant increase in *Lactobacillus*, which has been reported as a probiotic with antidepressant properties in animal studies (Getachew et al., 2018; Liu et al., 2019). They also observed the potent reduction of *Mucispirillum* and *Ruminococcus*, which are associated with inflammatory processes in the gut and IBS, and therefore, they suggested that the effect of ketamine may be mediated by these microorganisms (Getachew et al., 2018). High-level *Mucispirillum* can increase gut permeability and be responsible for high LPS concentration, which enhances inflammatory processes (Liu et al., 2019). Ruminococcaceae reduction was also observed in the earlier study (Qu et al., 2017). Another effect was the increase of *Turicibacter*, which is associated with an increase in butyric acid levels in the gut (Zhong et al., 2015). The most recent study investigated the role of gut microbiota in the antidepressive effect of ketamine in an inflammation model of depression. Ketamine had an antidepressant effect, when observed in behavioral tests, as it reduced immobility time, previously increased by LPS administration (Huang et al., 2019). It was found that an increase in the phylum Actinobacteria and class Coriobacteriia and order Clostridiales correlated with reduced immobility time. On the other hand, a decrease in family Prevotellaceae and the genus *Alloprevotella* correlated with the antidepressant effect of ketamine in LPS-treated mice (Huang et al., 2019). The authors also suggested that the phylum Actinobacteria and the class Coriobacteriia are potential biomarkers for the antidepressant effects of ketamine in an inflammation model.

Ketamine seems to restore bacteria producing the anti-inflammatory substance, butyrate, and reduce the number of bacteria associated with inflammatory processes in the gut. It increases the abundance of bacteria, previously reported as reduced in depression, and reduces the amount of bacteria, previously reported as increased in MDD. It also increases the abundance of probiotic bacteria known to produce an antidepressant effect.

These results are preliminary and should be treated with caution, although further investigation of this field may elucidate the mechanism of the antidepressant effect of ketamine and may facilitate further research and lead to the discovery of new treatment strategies in depressive disorders.

CONCLUSION

The gut microbiome and the brain are involved in constant communication. This relationship undergoes specific changes in MDD. Previous studies validated the microbiome as a target for therapeutic intervention in this disorder. Ketamine is a novel rapid-acting antidepressant with antisuicidal properties. The mechanisms behind the therapeutic effect of ketamine are still not fully understood, although its anti-inflammatory properties potentially affect GBA in depression. There is a need for further studies on the effect of ketamine and its enantiomers on individual bacterial species. It is crucial to identify more species engaged in the inflammatory pathway of GBA to study their interactions with mediators of depression and to investigate how ketamine can affect them. There is also a need to reconsider animal models of depression used in these studies to investigate treatment resistance. Apart from animal studies, it will be necessary to conduct clinical studies on patients with MDD treated with ketamine with a detailed examination of microbiota alterations before and after the treatment and correlation with treatment outcome, no such studies have been published so far. Considering the impact of depression on human health, there is an unquestionable need for a better understanding of the role of the GBA in treating depressive symptoms. This understanding will hopefully lead to discovering novel antidepressants acting on microbiota and bring more effective and individualized treatment strategies for depressed patients.

AUTHOR CONTRIBUTIONS

AW: conceptualization, research, writing—original draft preparation, and editing. ŁS: writing, research, and drawing figures. WC: conceptualization, review and editing, and funding acquisition. All authors have read and agreed to the published version of the manuscript.

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White Matter Microstructure Alterations Associated With Paroxetine Treatment Response in Major Depression

*Rita Vieira[1,2,3], Ana Coelho[1,2,3], Joana Reis[1,2,3], Carlos Portugal-Nunes[1,2,3], Ricardo Magalhães[1,2,3,4], Sónia Ferreira[1,2,3], Pedro Silva Moreira[1,2,3,5], Nuno Sousa[1,2,3] and João M. Bessa[1,2,3]**

[1] Life and Health Sciences Research Institute (ICVS), School of Medicine, University of Minho, Braga, Portugal, [2] ICVS/3B's, PT Government Associate Laboratory, Braga/Guimarães, Portugal, [3] Clinical Academic Center – Braga, Braga, Portugal, [4] NeuroSpin, Institut des Sciences du Vivant Frédéric Joliot, Commissariat à l' Énergie Atomique et aux Énergies Alternatives, Université Paris-Saclay, Gif-sur-Yvette, France, [5] Psychological Neuroscience Laboratory, CIPsi, School of Psychology, University of Minho, Braga, Portugal

More than one-third of depressive patients do not achieve remission after the first antidepressant treatment. The "watch and wait" approach used to find the most effective antidepressant leads to an increased personal, social, and economic burden in society. In order to overcome this challenge, there has been a focus on studying neural biomarkers associated with antidepressant response. Diffusion tensor imaging measures have shown a promising role as predictors of antidepressant response by pointing to pretreatment differences in the white matter microstructural integrity between future responders and non-responders to different pharmacotherapies. Therefore, the aim of the present study was to explore whether response to paroxetine treatment was associated with differences in the white matter microstructure at baseline. Twenty drug-naive patients diagnosed with major depressive disorder followed a 6- to 12-week treatment with paroxetine. All patients completed magnetic resonance brain imaging and a clinical assessment at baseline and 6–12 weeks after treatment. Whole-brain tract-based spatial statistics was used to explore differences in white matter microstructural properties estimated from diffusion magnetic resonance imaging. Voxel-wise statistical analysis revealed a significant increase in fractional anisotropy and a decrease in radial diffusivity in forceps minor and superior longitudinal fasciculus in responders compared to non-responders. Thus, alterations in white matter integrity, specifically in forceps minor and the superior longitudinal fasciculus, are associated with paroxetine treatment response. These findings pave the way for personalized treatment strategies in major depression.

Keywords: depression, paroxetine, SSRI, treatment response, diffusion MRI, white-matter, tract-based spatial statistics

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***Correspondence:**
João M. Bessa
joaobessa@med.uminho.pt

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INTRODUCTION

Major depressive disorder (MDD) is one of the major contributors to the overall global burden of disease, affecting nearly 300 million people in 2019 (Vos et al., 2020). According to the American Psychiatric Association (APA) clinical guidelines, there are several approved treatments for MDD, specifically psychotherapy, pharmacotherapy, combination of both psychotherapy

and pharmacotherapy, and other interventions, such as electroconvulsive therapy (ECT) and transcranial magnetic stimulation (TMS) (Gelenberg et al., 2010).

Pharmacotherapy is recommended as an initial treatment for patients with mild to severe MDD symptoms (Gelenberg et al., 2010). Selective serotonin reuptake inhibitors (SSRIs) are usually the first-line treatment choice in clinical practice; however, and despite their clinical relevance, only 60% of MDD patients respond to the first treatment (Gartlehner et al., 2011), and from those, only 36.8% remit (Rush et al., 2006). The remaining patients start a long process of successive trials until finding the most effective treatment, but the remission percentage decreases as the number of treatment trials increase (e.g., 13.7% for the third treatment) (Rush et al., 2006).

The current challenge for clinicians is not the lack of effective treatments, but the choice of the most effective antidepressant for each patient. As there are no objective measures to guide treatment choice, clinicians use the standard approach of "watch and wait" based on close observation of patients for 4–12 weeks (Gelenberg et al., 2010). The period of wait repeats every time there is a new medication trial, extending the length of depressive episodes, consequently enhancing the burden of the disease, and increasing healthcare costs (Leuchter et al., 2009).

Prompted by this context, there has been a focus on identifying neurobiological predictors of pharmacological response by using different magnetic resonance imaging (MRI) modalities, such as structural, functional, and diffusion MRI. These techniques also enable the characterization of brain differences between responders and non-responders, which may lead to better patient prognosis and care.

Alterations in the white matter (WM) microstructure have been linked to antidepressant treatment response and remission in studies using diffusion tensor imaging (DTI). DTI indirectly assesses the WM microstructure properties using simple quantitative measures of the rate and directionality of the water molecule diffusion (Van Hecke et al., 2016). The measures, commonly derived from the DTI tensor, are fractional anisotropy (FA), axial diffusivity (AD), mean diffusivity (MD), and radial diffusivity (RD). FA, the most popular measure, provides information about the degree of anisotropic diffusion. Increased FA values indicate higher WM integrity (Alexander et al., 2011). MD measures the average diffusion rate, and lower values are associated with higher WM integrity (Alexander et al., 2011). AD and RD are defined as the parallel and perpendicular diffusivity to the main direction of the tract, respectively. The former might indicate axonal integrity (Song et al., 2002, 2003; Budde et al., 2007), whereas the latter is associated with the degree of myelination (Song et al., 2002, 2003, 2005; Klawiter et al., 2011).

Davis et al. (2019) characterized the organization and integrity of the WM associated with antidepressant response by describing differences in the FA, MD, AD, and RD between responders and non-responders to escitalopram treatment. It was reported that responders to escitalopram treatment had increased AD in the left external capsule, part of the superior longitudinal fasciculus (SLF), compared to non-responders and controls. Further analysis revealed decreased FA in the corona radiata and sagittal stratum and increased MD and RD in the cingulate portion of the cingulum bundle in non-responders. This comprehensive study suggested a disruption in WM integrity for non-responders to SSRI treatment (Davis et al., 2019). Other studies reported decreased FA of the left hippocampal part of the cingulum bundle in non-responders to citalopram or quetiapine treatment (Tatham et al., 2017), as well as in the WM tracts connecting the raphe nuclei to the amygdala in non-remitters to escitalopram treatment (DeLorenzo et al., 2013), and increased FA in the superior frontal and anterior cingulate cortices associated with non-remission to sertraline (Taylor et al., 2008).

Other studies explored the role of fronto-limbic WM tracts as potential predictors of treatment response and remission in MDD (Korgaonkar et al., 2014; Grieve et al., 2016). Non-remission to antidepressant treatment (escitalopram, sertraline, or venlafaxine-XR) was predicted by a high ratio of FA in the cingulate portion of the cingulum bundle and the stria terminalis. Despite its high specificity (83–88%), it only identified 29% of non-remitters to one of three antidepressant medications (Grieve et al., 2016).

Such findings are promising and represent progress in the identification of imaging biomarkers of treatment response in depression. However, we are still far from having a useful clinical measure to accurately predict response and remission to antidepressant treatments. The heterogeneous findings, which might be a consequence of using different analytical methods, regions of interest, or even antidepressant treatments, call for more studies in order to achieve useful clinical predictors of antidepressant response.

The present study aimed to explore whether response to paroxetine treatment was associated with differences in the WM microstructure at baseline. A sample of drug-naive patients diagnosed with MDD followed a 6- to 12-week treatment with paroxetine, completing an MRI acquisition and clinical assessments pre- and post-treatment.

MATERIALS AND METHODS

Participants

Subjects were recruited at the emergency psychiatry department or the psychiatry outpatient unit of Hospital de Braga. To be eligible for this study, subjects had to be aged between 18 and 65 years, meet the Diagnostic and Statistical Manual of Mental Disorders, 4th edn., Text Revision (DSM-IV-TR) criteria for MDD without psychotic features assessed by an experienced psychiatrist through Structured Clinical Interview for DSM-IV (SCID) (First and Gibbon, 2004), and no prior history of antidepressant treatment (drug-naive). The exclusion criteria were any MRI contradictions, comorbid psychiatric disorders (e.g., bipolar disorder, addictive disorders, and schizophrenia), prior medical history of neurological disorders or traumatic brain injury, and any sign of cognitive impairment defined as Mini-Mental Sate Examination (MMSE) below 24 (Folstein et al., 1975).

Following the aforementioned criteria, 32 patients were enrolled in the study between January 2016 and January 2020. From these, only 20 patients were included in the analysis (**Figure 1**). Information on the demographic and clinical data of these patients is displayed in **Table 1**.

Study Design and Clinical Measures

To reduce any confounds associated with multiple drug targets, we decided to focus on a single SSRI. Paroxetine was chosen because it is one of the most potent and selective SSRIs available (Thomas et al., 1987; Tulloch and Johnson, 1992; Nemeroff, 1994), with proven efficacy and effectiveness to treat MDD (Kroenke et al., 2001; Undurraga and Baldessarini, 2012). Moreover, no study, to our knowledge, has explored whether the response to paroxetine treatment was associated with differences in the WM microstructure at baseline.

All patients were drug-naive and initiated treatment with paroxetine (20 mg/day) after baseline evaluation. Brain MRI and clinical assessments were completed at baseline and 6–12 weeks after the beginning of the treatment. Clinical assessments included the Hamilton Depression Rating Scale (HDRS) (Hamilton, 1960) and the Hamilton Anxiety Rating Scale (HARS) (Hamilton, 1959) to evaluate both depressive and anxiogenic symptomatology, the Beck Scale for Suicidal Ideation (BSSI) (Beck and Steer, 1991) to evaluate suicidal ideation, and the 10-item Perceived Stress Scale (PSS-10) (Cohen and Williamson, 1988) in order to evaluate perceived stress. Response to treatment was defined as $\geq$50% reduction in the HDRS score from baseline to 6–12 weeks after treatment (Cusin et al., 2009).

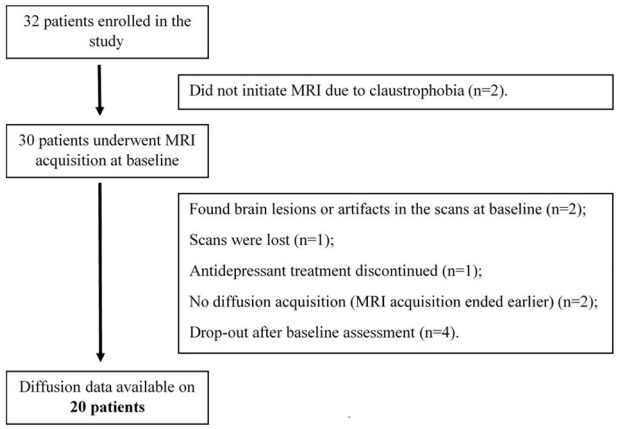

FIGURE 1 | Detailed information on subject recruitment and exclusion. Thirty-two patients were enrolled in the study. From those, 30 patients completed MRI acquisitions and psychological assessment, but only 20 were included in the final analyses.

TABLE 1 | Demographic and clinical characterization of all patients ($N = 20$) included in the data analyses.

	***M* ± SD**
Age (years)	37.75 ± 12.29
Sex (male/female), *n*	6/14
Education (years)	11.40 ± 5.37
Time between assessments (weeks)	8.35 ± 1.69
Pretreatment HDRS score	21.20 ± 8.56
Pretreatment HARS score	23.20 ± 10.25
Pretreatment PSS-10 score	27.45 ± 5.34
Pretreatment BSSI score[a]	5.00 ± 6.99

Data are presented as the mean ± standard deviation.M, mean; SD, standard deviation; HDRS, Hamilton Depression Rating Scale; HARS, Hamilton Anxiety Rating Scale; PSS-10, 10-item Perceived Stress Scale; BSSI, Beck Suicidal Ideation Scale. [a]One participant with missing data.

Diffusion MRI Acquisition

All patients underwent the same acquisition protocol using a clinically approved Siemens MAGNETOM Avanto 1.5T scanner (Siemens Medical Solutions, Erlangen, Germany) equipped with a 12-channel receive-only head coil. The imaging protocol included several different acquisitions, but only the diffusion-weighted imaging (DWI) acquisition was considered for the present study. DWI scans were performed using a spin echo–echo planar imaging (SE-EPI) sequence: TR = 8,800 ms, TE = 99 ms, FoV = 240 mm × 240 mm, acquisition matrix = 120 × 120, 61 two-millimeter axial slices with no gap, 30 non-collinear gradient directions with $b = 1{,}000$ s mm^{-2}, one $b = 0$ s mm^{-2} acquisition, and one repetition.

Before data pre-processing, the raw acquisitions from all participants were visually inspected to discard any brain lesions, critical head motion, or artifacts that could compromise the data.

DWI Image Pre-processing and Tensor Fitting

Diffusion data were pre-processed using the FMRIB Diffusion Toolbox (FDT) provided with the FMRIB Software Library (FSL v6.0.3)[1]. Firstly, DWI images were corrected for motion artifacts and eddy current distortions. The affine transformations were used to register each volume and were applied to rotate gradient vectors. Then, the first b_0 volume of each subject was extracted and skull stripped, creating a brain mask applied to the remaining volumes in order to remove non-brain structures.

Tensor fitting and the scalar map computation steps were performed with DTIFIT, included in the FDT toolbox. In this step, a diffusion tensor model is fitted at each voxel and scalar maps of FA and MD, as well as eigenvector and eigenvalue maps, were generated. AD was defined as the principal diffusion eigenvalue, and RD was computed using the mean of the second and third eigenvalues.

Tract-Based Spatial Statistics

Voxel-wise analyses of the scalar maps between subjects were performed using tract-based spatial statistics (TBSS) procedures (Smith et al., 2006), also part of FSL. To remove potential outliers from the tensor fitting, all FA templates were slightly

[1]https://fsl.fmrib.ox.ac.uk/fsl/

eroded and the end slices were zeroed. Afterward, all the FA templates were non-linearly registered into a 1-mm × 1-mm × 1-mm standard space. This step was performed by non-linearly registering each subject's FA template to each other to find the "most representative one" (i.e., the one that requires the least warping to align all images), subsequently used as the study-specific target image. Next, the selected target image was affine transformed into the Montreal Neurological Institute (MNI) 152 standard space, and each subject's FA template was transformed into this standard space by combining the non-linear transformation to the study-specific target with the affine transformation into the MNI space. Then, the FA templates of all subjects were averaged and the resulting image skeletonized. After visual inspection of the skeletonized image, we thresholded it at 0.35 to remove from the skeleton regions encompassing other tissues, such as gray matter or cerebrospinal fluid (CSF). Finally, all scalar maps (FA, AD, MD, and RD) were projected into this FA skeleton using the same transformation applied to the FA templates.

Statistical Analysis

Demographic and Clinical Data

Statistical analyses of the demographic and clinical data were performed with JASP (version 0.11.1; JASP Team, University of Amsterdam, Netherlands). Comparisons between the groups of responders and non-responders were performed using non-parametric Mann–Whitney tests (U), given the small unpaired number of participants included in each group (Pett, 2016), and chi-squared tests (χ^2) for categorical variables. P-values under 0.05 were considered statistically significant. The effect size was computed using rank-biserial correlation (r_B) and Pearson's phi coefficient (ϕ) for the Mann–Whitney (U) and chi-squared (χ^2) tests, respectively.

Diffusion Data

Non-parametric permutation methods, employed with the *randomize* tool from FSL (Winkler et al., 2014), were used to analyze the skeletonized maps of FA, AD, MD, and RD.

To investigate differences in the WM microstructure at baseline between future responders and non-responders to paroxetine treatment, we performed a two-sample t-test, adjusted for age, sex, and time between assessments (pre- and post-treatment). Five thousand permutations were used for each contrast. Widespread significant differences were detected with threshold-free cluster enhancement (TFCE), and multiple comparisons were corrected using family wise error rate (FWE-R) at $\alpha = 0.05$ and cluster extent threshold of $K > 50$. Clusters showing significant results were identified using the Johns Hopkins University WM Tractography atlas and dilated with the *tbss_fill* tool (included in FSL) for visualization purposes. Additional analyses were performed using IBM® SPSS® Statistics (version 27; IBM Corp., Armonk, NY, United States) to investigate whether the mean global values of the skeletonized maps of FA, AD, MD, and RD predict paroxetine response (see **Supplementary Material**).

RESULTS

Demographic and Clinical Characterization of Groups

Of the 20 participants who completed the posttreatment assessment (6–12 weeks after initiating treatment), 60% ($n = 12$) were classified as responders and 40% ($n = 8$) as non-responders based on the predefined criteria (≥50% reduction in the HDRS score). **Table 2** shows the demographic and clinical characterization for both groups pre- and post-treatment.

No significant differences between groups were found regarding age ($U = 55.50$, $p = 0.589$, $r_B = 0.156$), sex [$\chi^2(1) = 0.159$, $p = 0.690$, $\phi = 0.089$], and education ($U = 39.00$, $p = 0.509$, $r_B = -0.188$) and the HDRS ($U = 48.50$, $p = 1.00$, $r_B = 0.010$), HARS ($U = 53.00$, $p = 0.728$, $r_B = 0.104$), PSS-10 ($U = 49.50$, $p = 0.938$, $r_B = 0.031$), and BSSI ($U = 67.00$, $p = 0.053$, $r_B = 0.523$) scores at baseline. However, time between the pre- and post-treatment assessments was significantly different between groups ($U = 22.00$, $p = 0.044$, $r_B = -0.542$), showing that non-responders (median = 7.00, interquartile range = 1.25) were evaluated earlier than the responders (median = 9.00, interquartile range = 1.50).

After 6–12 weeks of treatment with paroxetine, the responders showed a significant decrease in the HDRS ($U = 84.00$, $p = 0.006$, $r_B = 0.750$), HARS ($U = 77.00$, $p = 0.027$, $r_B = 0.604$), and PSS-10 ($U = 75.00$, $p = 0.041$, $r_B = 0.563$) scores. No significant differences were found in the BSSI scores between groups posttreatment ($U = 70.00$, $p = 0.076$, $r_B = 0.458$).

TABLE 2 | Description of the demographic and clinical data from responders ($n = 12$) and non-responders ($n = 8$) before and after 6–12 weeks of treatment.

	Responders ($n = 12$)		Non-responders ($n = 8$)	
	Pre	**Post**	**Pre**	**Post**
Age (years)	36.50 (14.50)	–	45.50 (27.25)	–
Sex (male/female)	4/8	–	2/6	–
Education (years)	12.00 (8.00)	–	9.00 (7.75)	–
Time between assessments (weeks)	9.00 (1.50)	–	7.00 (1.25)	–
HDRS score	18.00 (19.50)	3.50 (7.75)	24.50 (6.25)	16.00 (8.75)
HARS score	21.00 (21.75)	4.50 (18.50)	24.50 (10.50)	20.50 (12.50)
PSS-10 score	28.50 (6.50)	17.50 (10.75)	29.50 (8.75)	24.50 (6.50)
BSSI score[a]	0.00 (3.50)	0.00 (3.25)	6.00 (8.50)	4.00 (12.25)

Data are presented as median (interquartile range).HDRS, Hamilton Depression Rating Scale; HARS, Hamilton Anxiety Rating Scale; PSS-10, 10-item Perceived Stress Scale; BSSI, Beck Suicidal Ideation Scale. [a]One responder with missing data pretreatment.

Pretreatment Differences in the WM Microstructure Associated With Response

Statistically significant differences between groups were found in the FA and RD maps (**Table 3** and **Figure 2**). Responders showed a significant increase in the FA maps compared with non-responders to paroxetine treatment in clusters including forceps minor, bilateral SLF, and the left fronto-occipital fasciculus. A significant decrease in the RD maps was found in responders when compared to non-responders in the left SLF. Results of the investigation of the predictive value of skeletonized maps suggest that FA is the better measure to discriminate responders from non-responders (see **Supplementary Material**).

DISCUSSION

Our study investigated whether paroxetine treatment response was associated with alterations in the WM integrity at baseline in a sample of drug-naive MDD patients. Response was predefined as a 50% reduction in HDRS after 6–12 weeks of treatment. We showed (**Figure 2** and **Table 3**) that, at baseline, responders had higher FA in the forceps minor and SLF and a decreased RD in SLF than did non-responders to paroxetine treatment after controlling for age, sex, and time between assessments. No significant differences were found between groups regarding any demographic and clinical variables.

More than one-third of patients (40%) did not respond to the antidepressant treatment. The pattern of alterations in the WM microstructure found for this group of patients (decreased FA and increased RD) is consistent with previous studies (Vasavada et al., 2016; Tatham et al., 2017; Davis et al., 2019), pointing to a disruption in WM integrity in non-responders. Differently, other studies have reported higher FA in fronto-limbic WM tracts associated with non-response and remission to antidepressant treatment (Taylor et al., 2008; Korgaonkar et al., 2014). However, depression is a very heterogeneous disorder (Monroe and Anderson, 2015), and these apparent contradictory results could be explained by the nature of depression or the use of different samples of MDD patients (with different ages, treatment choices, or antidepressant washout periods), or even different methodological choices regarding data analysis.

TABLE 3 | White matter tracts with significant differences in FA and RD between future responders and non-responders to paroxetine treatment after controlling for age, sex, and time between assessments.

WM tract	MNI coordinates at signal peak			Cluster size	*p*-values (FWE corrected)
	x	*y*	*z*		
FA: responders > non-responders					
Forceps minor	−15	1	30	2,601	0.015
Forceps minor	20	40	11	1,445	0.024
L superior longitudinal fasciculus	−34	−65	26	2,306	0.018
L superior longitudinal fasciculus	−29	7	40	166	0.042
L inferior fronto-occipital fasciculus	−29	12	−1	190	0.042
R superior longitudinal fasciculus	35	16	28	124	0.048
RD: responders < non-responders					
L superior longitudinal fasciculus	−31	−60	33	269	0.041

The WM tracts were identified using the Johns Hopkins University WM Tractography atlas.L, left; R, right; WM, white matter; MNI, Montreal Neurological Institute; FWE, family wise error, FA, Fractional anisotropy; RD, radial diffusivity. $p < 0.05$ (FWE corrected), $K > 50$.

The forceps minor and SLF were the two major tracts associated with paroxetine treatment response in our study. The forceps minor is a commissural fiber tract that connects the frontal lobes of both hemispheres through the genu of the corpus callosum (Peltier et al., 2010; Voineskos et al., 2010). Decreased FA in this WM tract was previously associated with non-response to ketamine (Vasavada et al., 2016), suggesting (together with our results) that forceps minor disruption might be a potential biomarker for non-response to antidepressant therapy. Interestingly, deep brain stimulation (DBS) of subcallosal cingulate cortex WM, including specific WM tracts, such as the forceps minor, led to a quicker and stable clinical response in treatment-resistant depression (Riva-Posse et al., 2014; Howell et al., 2019). Overall, these findings might indicate that forceps minor disruptions are not only a potential biomarker for antidepressant non-response but also a potential therapeutic target for stimulation therapies.

The SLF is an association fiber tract connecting the frontal, temporal, and parietal lobes (Schmahmann and Pandya, 2009). It has been described as a key component in the pathophysiology of depression (Murphy and Frodl, 2011), and alterations in its WM microstructure have been linked to depression severity (Lai and Wu, 2014). Both reduced FA in the SLF and forceps minor have been associated with treatment-resistant depression (de Diego-Adeliño et al., 2014). Moreover, FA in the SLF together with the other WM tracts predicted non-remission to SSRIs in 15% of patients, with 84% accuracy, suggesting that more than one tract might be required to predict treatment response effectively (Korgaonkar et al., 2015).

Despite the promising findings of this study, there are several limitations that need to be considered in their interpretation. Firstly, replication in a larger sample is warranted to validate our results. Secondly, our findings only point to a possible association between response to paroxetine treatment and alterations in WM microstructures, given the absence of a control group including untreated depressed patients in this study. Future studies should include a control group with no treatment to validate our results and to allow attributing the alterations in the WM microstructure to paroxetine treatment response. Thirdly, the time between assessments was different between the responders and non-responders, but this variable was included in the DTI analyses as a covariate. Moreover, the inclusion of patients only following paroxetine treatment hampers the generalization of our findings to non-response to other antidepressants. It would be interesting to compare different antidepressants in order to establish whether there are common and specific alterations in the WM microstructure associated with response, which could allow better and personalized treatment. Another limitation was the use of a 1.5-T MRI scanner for the DTI acquisitions, which has a lower signal-to-noise ratio compared

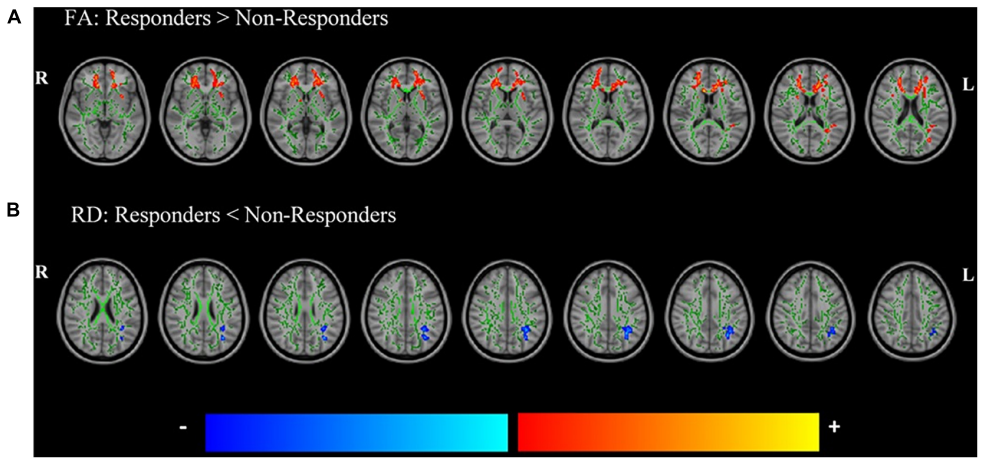

FIGURE 2 | Significant differences in the fractional anisotropy (FA) **(A)** and radial diffusivity (RD) **(B)** maps between future responders and non-responders to paroxetine treatment controlled for time between assessments, age, and sex. Responders had increased FA and decreased RD compared to non-responders. *Red–yellow* voxels indicate a significant increase in FA, whereas *blue–light blue* voxels indicate a significant decrease in RD in future responders compared with non-responders to paroxetine treatment. Significance threshold was set to $p < 0.05$ [family wise error (FWE) corrected for multiple comparisons]. The white matter (WM) skeleton (represented in *green*) is superimposed on a T1-weighted Montreal Neurological Institute (MNI) template.

to 3-T MRI scanners (Lee and Shannon, 2007). Furthermore, tractography analyses of the forceps minor and the SLF could be performed in future studies to characterize them with higher anatomical resolution.

In conclusion, our study showed that responders to antidepressant treatment with paroxetine present statistically significant differences in the WM microstructure in the forceps minor and the SLF tracts when compared to non-responders. These findings, together with previous literature, pave the way for new studies addressing the potential use of these DTI measures as pretreatment markers of antidepressant response.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committees of University of Minho and Hospital de Braga. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

JB and NS conceived the study. JB recruited the participants. RV and CP-N organized the database and schedule and performed the assessments. JR, AC, RM, SF, and PM performed the MRI acquisitions. RV and AC performed the MRI data pre-processing and data analysis. RV wrote the first draft of the manuscript. All authors contributed to the following and final versions of the 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/fnbeh.2021.693109/full#supplementary-material

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Acupuncture Treatment for Social Defeat Stress

Jun Kawanokuchi[1,2], Ken Takagi[1,3], Nobuyuki Tanahashi[4], Teruhisa Yamamoto[3], Nobuyuki Nagaoka[3], Torao Ishida[1] and Ning Ma[1,2]*

[1] Institute of Traditional Chinese Medicine, Suzuka University of Medical Science, Suzuka, Japan, [2] Division of Health Science, Graduate School of Health Science, Suzuka University of Medical Science, Suzuka, Japan, [3] Department of Acupuncture and Moxibustion Science, Faculty of Health Science, Suzuka University of Medical Science, Suzuka, Japan, [4] Department of Clinical Nutrition, Faculty of Health Science, Suzuka University of Medical Science, Suzuka, Japan

Depression is a mood disorder characterized by disordered affect, thoughts, cognition, and behavior. Antidepressant therapy is often the primary treatment for depression. However, antidepressant therapy may cause unwanted side effects, and its effects are slow. Therefore, some patients are seeking alternative treatments for depression, such as acupuncture. However, there are many unclear points regarding the mechanism of the effect of acupuncture on depression. In recent years, we have reported that acupuncture improves the symptoms of mild depression induced by water-immersion stress in a rat model and depression induced by forced swimming in a mouse model. In this study, we examined the effect of acupuncture on the symptoms of social defeat stress (SDS)-induced depression in mice that most closely resemble human symptoms. In this study, we investigated the preventive and therapeutic effects of acupuncture as part of GV20 "Bai-Hui" and Ex-HN3 "Yintang" on model mice with depression induced by SDS. To examine the mechanism of the preventive and therapeutic effects of acupuncture on depression model mice, we examined the expression of neurotrophic factors in the brains of SDS mice. Two weeks of simultaneous acupuncture stimulation as part of GV20 and Ex-HN3 restored SDS-reduced brain-derived neurotrophic factor (BDNF), neurotrophin (NT)-3, and NT-4/5 expression, which was not observed with antidepressants. In contrast, acupuncture stimulation suppressed nerve growth factor (NGF) expression induced by SDS. These results suggest that acupuncture treatment could be effective in correcting the imbalance in the expression of neurotrophic factors. Furthermore, the effects of acupuncture on the expression of neurotrophic factors appear earlier than those of antidepressants, suggesting that it may be a useful treatment for depression.

Keywords: depression, social defeat stress, acupuncture, neurotrophic factors, BDNF, NT-3

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Ravid Doron,
Open University of Israel, Israel

Reviewed by:
Serge Campeau,
University of Colorado Boulder, United States
Keren Nitzan,
Open University of Israel, Israel

****Correspondence:***
Jun Kawanokuchi
kawa-jun@suzuka-u.ac.jp

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INTRODUCTION

In recent years, the number of patients with psychiatric disorders has increased in many countries, including Japan. According to a survey conducted in 2017, depressive disorder is a common mental illness affecting more than 300 million people of all ages worldwide (WHO Fact Sheet 2017 on Depression) (WHO, 2017). Depression is a mood disorder characterized by disordered affect,

thoughts, cognition, and behavior. Social loss due to depression is widespread as it may cause suicide and obstacles in work and school.

Depression is commonly treated with psychotherapy, antidepressant therapy (Kirsch et al., 2008), and/or electroconvulsive therapy (Schlaepfer et al., 2010). Antidepressant therapy is often the primary treatment for depression. However, antidepressant therapy may cause unwanted side effects, and its effects are slow (Khawam et al., 2006; Cipriani et al., 2018). In addition, about one-third of patients are known to have treatment-resistant depression to multiple antidepressants (Al-Harbi, 2012). Therefore, some patients are seeking alternative treatments for depression, such as acupuncture therapy (Qureshi and Al-Bedah, 2013).

Acupuncture is a traditional Chinese method widely used for treating a variety of physical and mental health problems. John Kim (2007) reviewed the biological mechanisms of acupuncture and the effectiveness of electroacupuncture stimulation on depression as part of GV20 "Bai-Hui" and Ex-HN3 "Yintang." It has been reported that acupuncture at the acupoints GV20 and Ex-HN3 can induce sedation and provide relief from stress in animals and humans (Litscher, 2004; Paraskeva et al., 2004; Kim and Nam, 2006). A pilot study by Yeung et al. (2011) suggested that acupuncture is safe, well-tolerated, and effective for partial and non-responders to antidepressants. Discussions on the standardization of acupuncture points began in 2003, and the "WHO Standard Acupuncture Point Locations in the Western Pacific Region" (WHO Standard) containing these acupuncture points were released in 2008 (WHO, 2008). However, there are many unclear points regarding the mechanism of the effect of acupuncture on depression. Animal studies are commonly used in depression research, but few have investigated the effects of acupuncture on depression. Dos Santos et al. (2008) reported that depression model rats induced by forced swimming (FS) stimulated with electroacupuncture at the SP-36 "Zusanli" and Sp-6 "Sanyinjiao" points with electroacupuncture showed antidepressant effects comparable to those of the antidepressant imipramine. In addition, verification of the effect of electroacupuncture at the GV20 and Ex-HN3 points (Liu et al., 2007), GV20 and Sp-6 points (You et al., 2010), or GV20 and EX17 "An-Mian" We have reported that acupuncture, as part of GV20 and Ex-HN3, improves the symptoms of a rat model of mild depression induced by water-immersion stress (Tanahashi et al., 2016; Takagi et al., 2017) and a mouse model of depression induced by FS (Yamamoto et al., 2018). Social defeat stress (SDS) in mice has been developed as a new ethological model of depression that more closely models the psychological stress that humans can experience during antagonistic social interactions (Golden et al., 2011; Toyoda, 2017). Since the SDS-induced depression model is caused by mental stress due to competition and defeat between individuals, it is considered that the SDS model reproduces human depression more than the previous animal models. In this study, we examined the effects of acupuncture on the suppression of depressive-like symptoms in an SDS-induced depression mouse model that is attacked by other animals to mimic the mental stress response that would occur in a more natural environment. Depression-like symptoms were assessed by measuring changes in immobility time using the forced swimming test (FST), which is used as a screening test for antidepressants. Acupuncture was performed on a pair of acupoints of GV20 and EX-HN3 of SDS mice. GV20 is located at inferior border of the occipital protuberance on the vertical midline of posterior of the head. Ex-HN3 is located at the midpoint of glabella between the inner/medial ends of the eyebrows on the face. The classic tricyclic antidepressant, imipramine, was used as a positive control for treatment.

Many studies have been conducted on the mechanisms of depression development in humans and animals. One of the leading hypotheses is the "Neurotrophic factor hypothesis," which explains that the decreased expression of neurotrophic factor, such as a brain-derived neurotrophic factor (BDNF), is related to the development of depression. Neurotrophic factors are composed of four types of secretory proteins: nerve growth factor (NGF), BDNF, neurotrophin-3 (NT-3), and neurotrophin-4/5 (NT-4/5). Focusing on these neurotrophic factors, we investigated the therapeutic mechanism of acupuncture in SDS-induced depression model mice.

MATERIALS AND METHODS

Experimental Animals

All protocols were approved by the Animal Experiment Ethical Review Committee of the Suzuka University of Medical Science (permission number: 189). 6-week-old ICR male mice and C57BL/6J male mice were purchased from CLEA Japan, Inc. (Tokyo, Japan). Animals were maintained at a room temperature of 22 ± 3°C, 55 ± 5% humidity, and a 12-h light/dark cycle. Mice were given free access to commercially available animal feed (CE-2; CLEA Japan, Inc.) and water. Seven-week-old C57BL/6J mice were acclimatized to handling for 1 week before the start of the experiment. In this study, male mice were used to eliminate the effects of the estrous cycle. The number of animals used in the experiment was set to a minimum of eight animals per group. C57BL/6J mice (n = 64) in eight groups and 48 ICR mice (n = 48) in six groups were used. No mice were excluded from the experiment due to death or abnormalities.

Production of Depressive-Like Symptom in Mice

A depressive-like symptom (increased immobility) was induced in C57BL/6J mice using the SDS method (according to the method of Golden) (Golden et al., 2011). An ICR mouse was housed in a compartment separated by a transparent acrylic divider containing many holes. After a few days of habituation, a C57BL/6J mouse is introduced into this compartment, and the ICR mouse would usually severely attack the C57BL/6J mouse. After several minutes of this social conflict, the C57BL/6J mouse was moved to an adjacent compartment for the remainder of the day. The following day, the C57BL/6J mouse was subjected to social conflict with another ICR mouse. This sequence of physical and psychological stress was repeated for 14 days to increase SDS-induced immobility in C57BL/6J mice. The weight of each mouse was measured weekly.

Acupuncture or Pharmacotherapy in SDS-Treated Mice

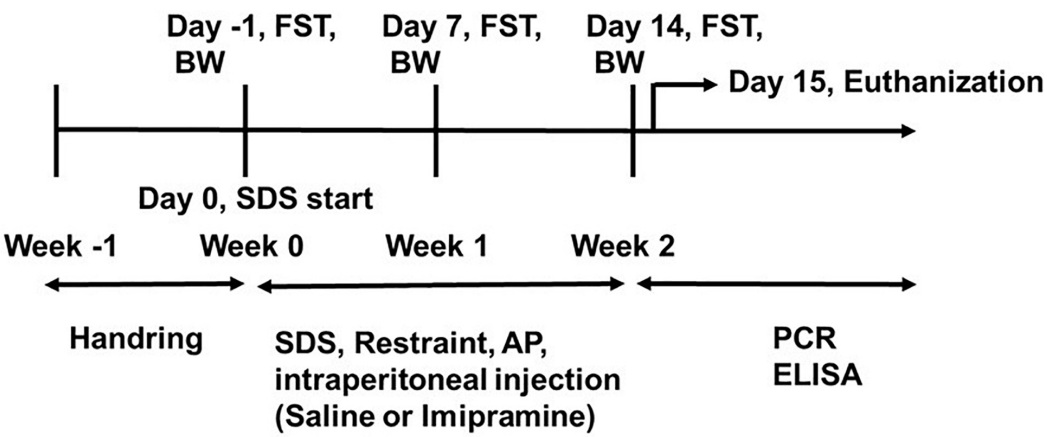
A depressive-like symptom (increased immobility) was induced in mice with 2 weeks of SDS. Acupuncture stimulations or imipramine administration were provided as antidepressant therapies for increased immobility of SDS-treated mice. The classic tricyclic antidepressant imipramine was used as a positive control.

First, the animals were divided into two groups. The first group was an experimental group that examined the effect of acupuncture in preventing the increased immobility of SDS-treated mice, and the treatments were conducted during the preparation of the SDS-treated mice (**Figure 1**). The second group was an experimental group that examined the therapeutic effect of acupuncture on the already developed increased immobility, and the treatments were performed after the SDS-treating period (**Figure 2**).

These two groups of mice were each randomly divided into four experimental groups (n = 8 per group): (1) Control group: SDS (–), tail restraint (+), saline (+); (2) SDS group: SDS (+), tail restraint (+), saline (+); (3) SDS + Acupuncture group: SDS (+), tail restraint (+), acupuncture (GV20 and Ex-HN3), saline (+); and (4) SDS + Imipramine group: SDS (+), tail restraint (+), and imipramine (+).

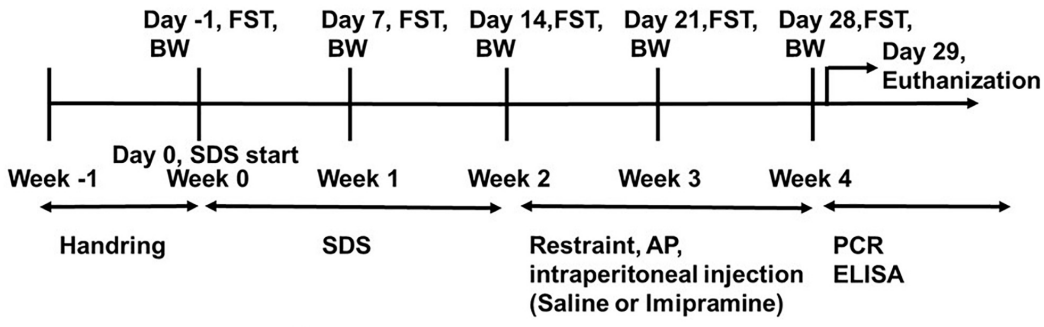
Acupuncture Stimulation

In brief, simultaneous horizontal and inward acupuncture stimuli were continuously given for 20 min with insertion to a depth of 5 mm at the "Bai-Hui" (GV20) and "Yintáng" (Ex-HN3) acupuncture points of mice using stainless steel needles (Acupuncture Needle D-Type, diameter: 0.25 mm; length: 15 mm; SEIRIN Co., Ltd., Shizuoka, Japan). These stimulation points and penetration depths were anatomically analogous to those in rats (**Figure 3**). The method of acupuncture stimulation was based on a previous report (Tanahashi et al., 2016; Takagi et al., 2017; Yamamoto et al., 2018). During acupuncture stimulation, the mouse was fixed with adhesive tape on its tail in an overturned cage. These acupuncture stimuli were performed 10 days out of 2 weeks (20 min daily, Monday through Friday). Groups that did not receive acupuncture stimulation were fixed at the same time as the acupuncture group. All acupuncture treatments and fixations were performed between 10:00 and 14:00. The time of day that acupuncture was performed and the frequency of acupuncture were similar in the "preventive" and "therapeutic" groups. It usually takes 4 weeks for imipramine to work, but our previous studies have shown that imipramine can work in 2 weeks while depressive-like symptoms are increasing (Tanahashi et al., 2016).



Group	SDS	Restraint	AP	Saline	Imipramine
Control	-	+	-	+	-
SDS	+	+	-	+	-
SDS+AP	+	+	+	+	-
SDS+ Imipramine	+	+	-	-	+

FIGURE 1 | The schedule of the "preventive effect" experiment. The timeline of the procedures for the "preventive" group conditions. AP, acupuncture; BW, body weight; ELISA: enzyme-linked immuno sorbent assay; FST, forced swimming test; PCR, polymerase chain reaction; SDS, social defeat stress.



Group	SDS	Restraint	AP	Saline	Imipramine
Control	-	+	-	+	-
SDS	+	+	-	+	-
SDS+AP	+	+	+	+	-
SDS+ Imipramine	+	+	-	-	+

FIGURE 2 | The schedule of the "therapeutic effect" experiment. The timeline of the procedures for the "therapeutic" group conditions. AP, acupuncture; BW, body weight; ELISA, enzyme-linked immuno sorbent assay; FST, forced swimming test; PCR, polymerase chain reaction; SDS, social defeat stress.

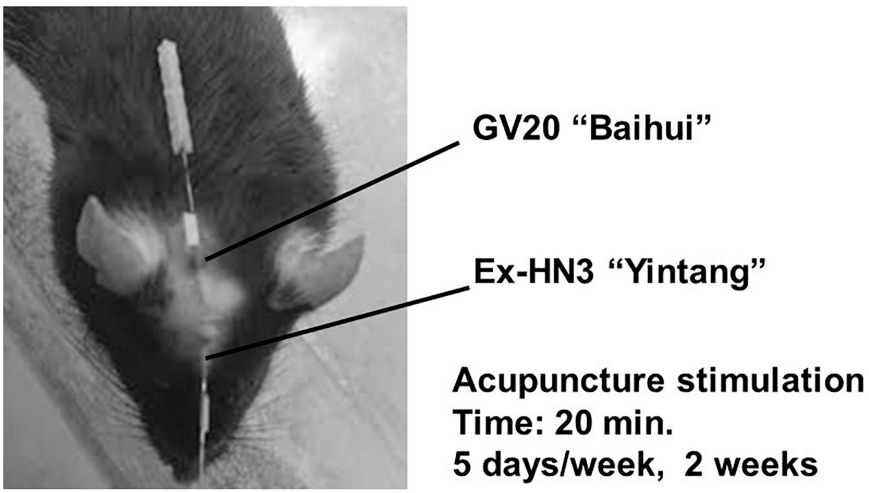


FIGURE 3 | Acupuncture points. Horizontal and inward simultaneous acupuncture stimuli were continued for 20 min with insertion to a depth of 5 mm at the "Bai-Hui" (GV20) and "Yintáng" (Ex-HN3) acupuncture points using stainless steel needles (diameter, 0.25 mm).

Administration of Antidepressant

The dose of antidepressants was set according to previous reports (Porsolt et al., 1977; Adams et al., 2008; Tanahashi et al., 2016). Imipramine (10 mg/kg body weight; Sigma-Aldrich, St. Louis, MO, United States) was dissolved in saline and administered intraperitoneally 5 days/week for 2 weeks. Groups that did not receive pharmacotherapy were injected intraperitoneally with saline. Saline and imipramine injections were administered within 10 min after acupuncture or fixation.

Forced Swimming Test

Forced Swimming Test was performed using the method described by Porsolt et al. (1977). Mice were placed in a plastic beaker (diameter 15 cm, height 22 cm) containing freshwater at 25 ± 2°C to a depth of body height of +5 cm for 6 min. The action of the mouse was recorded with a video camera, and the immobile time for 5 min after excluding the first 1 min from the 6-min recording time was measured. The immobile state is defined as a state in which the mouse suspends most movements

and performs only the limb movements necessary to maintain balance. Brief swimming stops of less than 1 s in stopping time were not counted as immobile time. In all groups, FST was performed between 14:00 and 16:00.

Sample Collection

Mice were euthanized by intraperitoneal injection of barbital sodium salt solution (120 mg/kg) 18–20 h after the last FST. Brains were immediately removed and stored at −80°C, and the right anterior cerebrum was used for molecular biological and biochemical analysis.

RNA Extraction and Reverse Transcription-Polymerase Chain Reaction for Neurotrophic Factors

Total RNA was extracted from brain tissue using the RNeasy Mini kit according to the manufacturer's protocol (Qiagen, Valencia, CA, United States). The amount of RNA was determined spectrophotometrically. cDNAs encoding mouse GAPDH, NGF, BDNF, NT-3, and NT-4/5 were evaluated by reverse transcription-polymerase chain reaction (RT-PCR) using Super Script III (Invitrogen, Carlsbad, CA, United States) and Blend Taq DNA polymerase (Toyobo, Osaka, Japan) in the presence of the specific primers shown in **Table 1**. To quantify the expression, mRNA levels of neurotrophic factors were also analyzed by real-time RT-PCR using PowerUp SYBR Green Master Mix and ABI Prism 7,000 SDS according to the manufacturer's protocol (Thermo Fisher Scientific, Waltham, MA, United States). The specific primers that were used are shown in **Table 2**. To prevent contamination of genomic DNA, primer sequences were set to amplify regions containing many introns. The Standard Curve Method was used as the quantitation method. A calibration curve was prepared for each reaction system, and the quantitative values were calculated.

TABLE 1 | Primer sets for PCR analysis.

Molecule	Forward primer	Reverse primer
GAPDH	ACTCACGGGAAATTCAACG	CCCTGTTGCTGTAGCCGTA
NGF	CATGGGGGAGTTCTCAGTGT	GCACCCACTCTCAACAGGAT
BDNF	AGCCTCCTCTGCTCTTTCTG	TTGTCTATGCCCCTGCAGCC
NT-3	GCCTACGAGTTTGTTGTTTTC	ATGCAGAGCATAAGAGTCAC
NT-4/5	AGCCGGGGAGCAGAGAAG	ACAAGAGGTCCCACTCAGGA

Primer sequences used for reverse transcription-polymerase chain reaction (RT-PCR) analysis. Annealing temperature and PCR cycles: GAPDH (58°C, 28 cycles), NGF (58°C, 35 cycles), BDNF (62°C, 30 cycles), NT-3 (62°C, 35 cycles), and NT-4/5 (65°C, 33 cycles).

TABLE 2 | Primer sets for real-time PCR.

Molecule	Forward primer	Reverse primer
GAPDH	ATGGGAGTTGCTGTTGAAGTCA	CCGAGGGCCCACTAAAGG
NGF	GATCGGCGTACAGGCAGAAC	CAGTGGGCTTCAGGGACAGA
BDNF	CCAAAGGCCAACTGAAGCAGTA	GCAGCCTTCCTTGGTGTAACC
NT-3	TTCTGCCACGATCTTACAGG	GGCAAACTCCTTTGATCCAT
NT-4/5	AGCGTTGCCTAGGAATACAGC	GGTCATGTTGGATGGGAGGTATC

Annealing temperature and PCR cycles: (55°C, 45 cycles).

ELISA

The expression of cerebral neurotrophic factors was assessed using a Multi-Neurotrophin Rapid Screening ELISA kit (Mouse) according to the manufacturer's protocol (Biosensis, SA, Australia).

Statistical Analysis

Statistical significance was assessed using one-way analysis of variance with Tukey–Kramer *post hoc* test using PRISM (version 5.0; GraphPad Software, La Jolla, CA, United States). Statistical significance was set at $p < 0.05$.

RESULTS

Physical and Behavioral Studies on the SDS-Treated Mice

First, we investigated the preventive effects of acupuncture in suppressing the development of immobility induced by SDS. There was no significant difference in body weight between the treatment groups during the 2-week SDS induction [$F(3, 28) = 0.48915$, $p = 0.6926$] (**Figure 4A**). In the FST, we measured whether mice exposed to SDS entered a state of immobility. The immobile time of animals with immobility tended to increase due to stress. In the SDS group without treatment, the immobile time increased significantly [$F(3, 28) = 17.83603$, $p < 0.0001$] (Control vs. SDS, $p < 0.001$) (**Figure 4B**). Simultaneous acupuncture stimulation of GV20 and Ex-HN3 significantly reduced SDS-induced immobility time (SDS vs. SDS+AP, $p < 0.001$) as well as imipramine treatment (SDS vs. SDS+ imipramine, $p < 0.001$).

In addition, we examined the therapeutic effects of acupuncture on immobility induced by SDS. After stopping exposure to SDS, the immobile time of mice increased for 2 weeks [$F(3, 28) = 32.15338$, $p < 0.0001$] (Control vs. SDS, $p < 0.001$). Simultaneous acupuncture stimulation of GV20 and Ex-HN3 significantly reduced SDS-induced immobility time (SDS vs. SDS+AP, $p < 0.001$) as well as imipramine treatment (SDS vs. SDS+ imipramine, $p < 0.001$) (**Figure 5B**). There was no significant difference in body weight between the groups [$F(3, 28) = 0.6794440$, $p = 0.5720$] (**Figure 5A**).

mRNA Expressions of Neurotrophic Factors

To analyze the preventive effects of acupuncture on immobility induced by SDS, we examined the mRNA expression of neurotrophic factors in the brains of SDS mice with ongoing symptoms of depression by RT-PCR and real-time PCR. In the SDS group, mRNA expression of NGF was higher than that in the control group [$F(3, 28) = 6.113497$, $p = 0.0025$] (Control vs. SDS, $p < 0.05$). In the SDS+AP and SDS+ imipramine groups, the mRNA expression of NGF was lower than that in the SDS group (SDS vs. SDS+AP, $p < 0.05$) (SDS vs. SDS+Imipramine, $p < 0.05$) (**Figures 6A**, **7A**). Contrary to NGF expression, mRNA

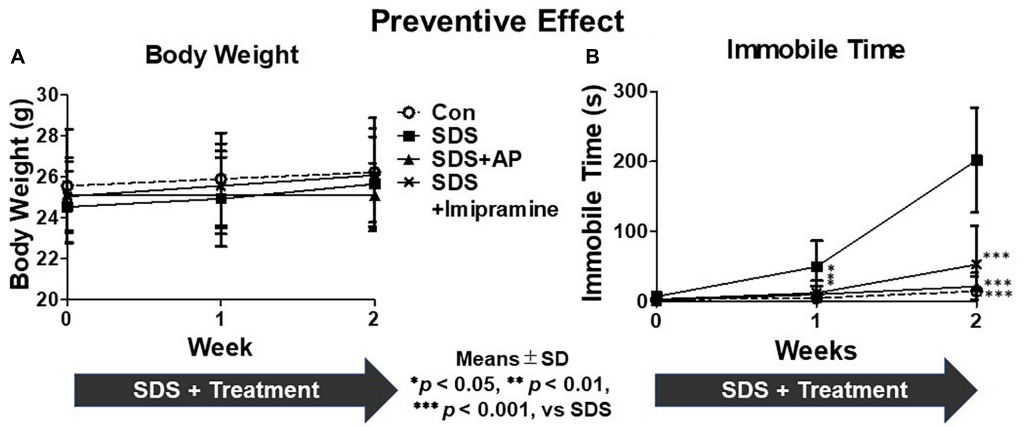


FIGURE 4 | The development of depression symptoms in mice induced by SDS were suppressed by acupuncture. **(A)** Weight changes and **(B)** immobile time in the forced swimming test in mice with SDS stress. Values are means ± SD (n = 8). *p < 0.05, **p < 0.01, and ***p < 0.001 versus SDS group. AP, acupuncture; SD, standard deviation; SDS, social defeat stress.

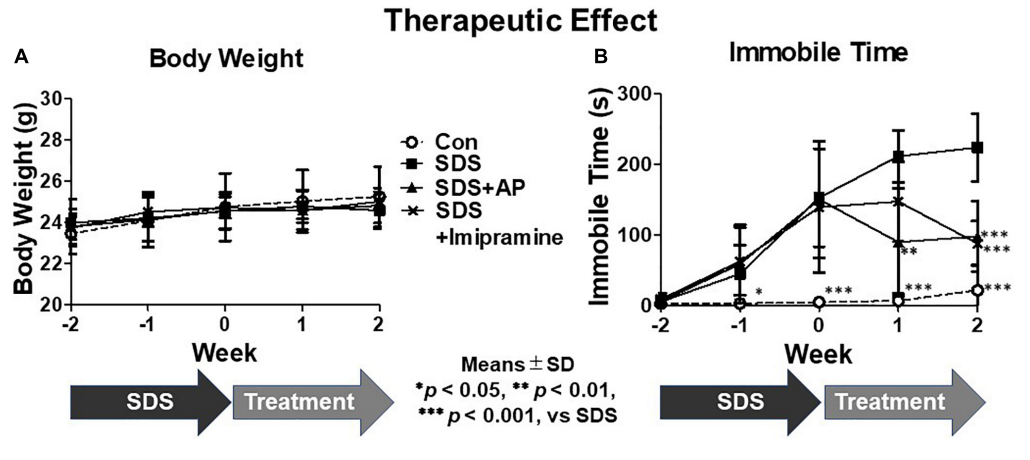


FIGURE 5 | Acupuncture showed therapeutic effect on depressive symptoms in mice induced by SDS. **(A)** Weight changes and **(B)** immobile time in the forced swimming test in mice during treatment of depression induced by SDS stress. Values are means ± SD (n = 8). *p < 0.05, **p < 0.01, and ***p < 0.001 versus SDS group. AP, acupuncture; SD, standard deviation; SDS, social defeat stress.

expression of BDNF [$F(3, 28) = 5.740770$, $p = 0.0034$] (Control vs. SDS, $p < 0.05$), NT-3 [$F(3, 28) = 5.169125$, $p = 0.0057$] (Control vs. SDS, $p < 0.05$), and NT-4/5 [$F(3, 28) = 7.157457$, $p = 0.0010$] (Control vs. SDS, $p < 0.05$) were significantly lower than those of the control in the brain of SDS mice. In the SDS+AP group, SDS-reduced BDNF (SDS vs. SDS+AP, $p < 0.05$), NT-3 (SDS vs. SDS+AP, $p < 0.05$), and NT-4/5 (SDS vs. SDS+AP, $p < 0.05$) mRNA expression were restored but not in the SDS+ imipramine group (**Figures 6A**, **7B,C,D**).

To analyze the therapeutic effects, we examined mRNA expression of neurotrophic factors in the brain of SDS mice, which were treated for 2 weeks after completion of SDS, by RT-PCR and real-time RT-PCR. As with the study of preventive effects, NGF was significantly enhanced in the brain of SDS mice [$F(3, 28) = 7.016273$, $p = 0.0012$] (Control vs. SDS, $p < 0.05$). BDNF [$F(3, 28) = 10.01787$, $p = 0.0001$] (Control vs. SDS, $p < 0.05$), NT-3 [$F(3, 28) = 5.741542$, $p = 0.0034$] (Control vs. SDS, $p < 0.05$), and NT-4/5 [$F(3, 28) = 9.966482$, $p = 0.0001$] (control vs. SDS, $p < 0.05$) were significantly suppressed even 2 weeks after the end of SDS (**Figures 6B**, **8**).

SDS-reduced mRNA expression of BDNF (SDS vs. SDS+AP, $p < 0.05$), NT-3 (SDS vs. SDS+AP, $p < 0.05$), and NT-4/5 (SDS vs. SDS+AP, $p < 0.05$) were significantly enhanced by acupuncture but not by imipramine (**Figures 6B**, **8B,C,D**). Enhanced mRNA expression of NGF by SDS was reduced by acupuncture (SDS vs. SDS+AP, $p < 0.05$) and imipramine (SDS vs. SDS+Imipramine, $p < 0.05$) treatment (**Figures 6B**, **8A**).

Protein Expressions of Neurotrophic Factors

Protein expression of neurotrophic factors was observed in the same way as mRNA expression by ELISA. In the brain of SDS mice with ongoing symptoms of depression, NGF

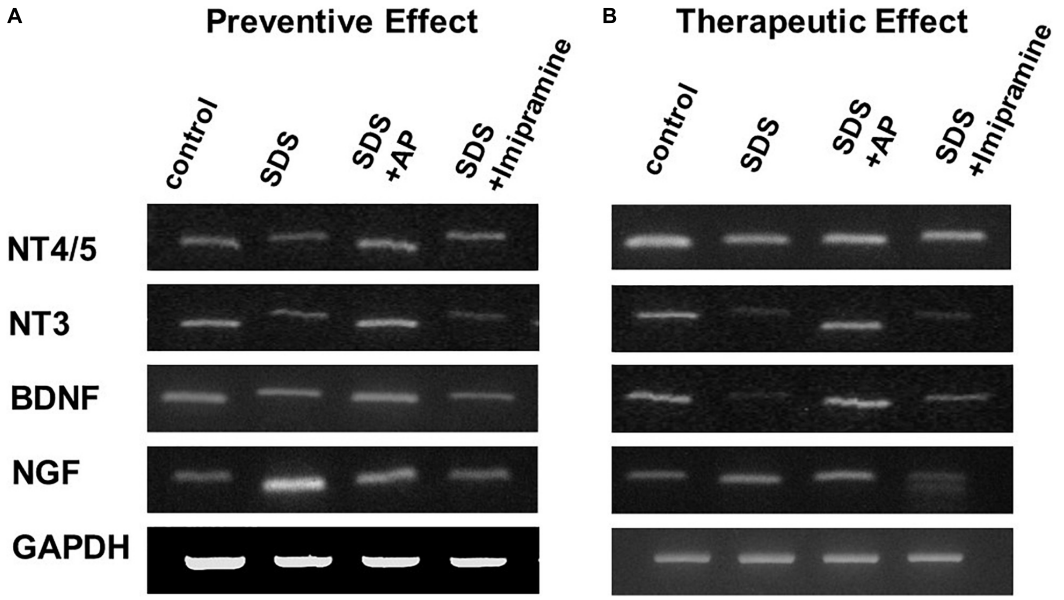


FIGURE 6 | mRNA expressions of neurotrophic factors in the brain of SDS mice. **(A)** mRNA expressions of neurotrophic factors in the brain of SDS mice with ongoing symptoms of depression. **(B)** mRNA expressions of neurotrophic factors in the brain after treatment of depression in SDS mice. AP, acupuncture; BDNF, brain-derived neurotrophic factor; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; NGF, nerve growth factor; NT, neurotrophin; SD, standard deviation; SDS, social defeat stress.

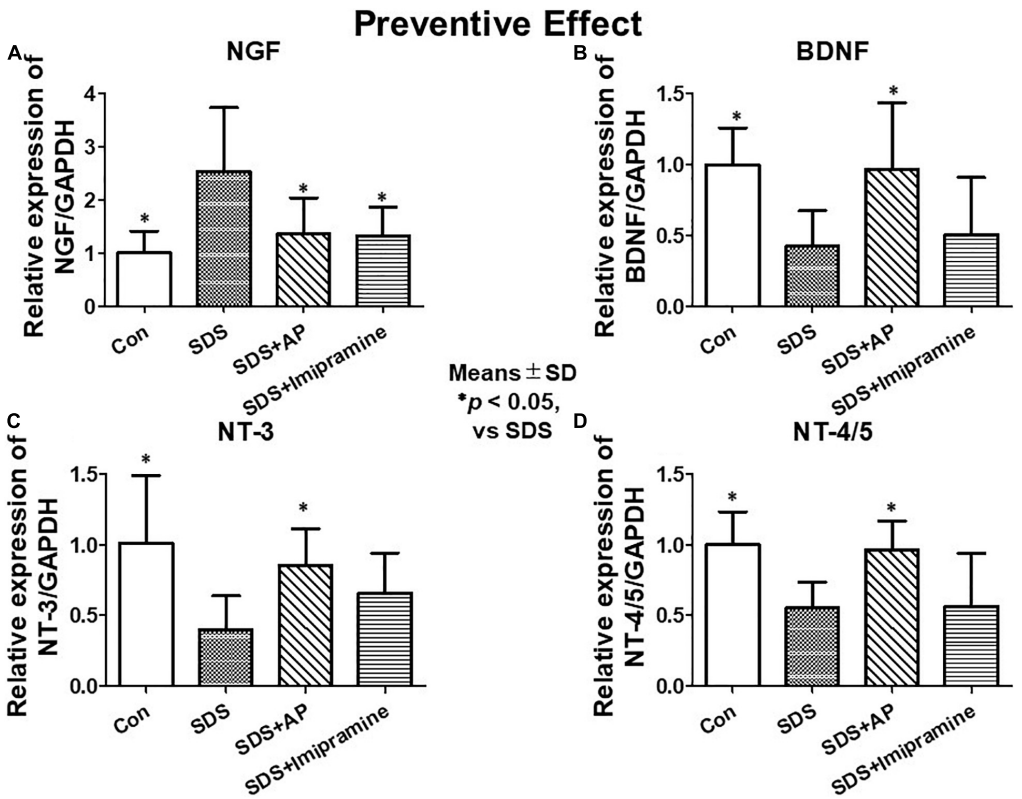


FIGURE 7 | mRNA expressions of neurotrophic factors in the brain of SDS mice with ongoing symptoms of depression quantified by real-time PCR. **(A)** NGF, **(B)** BDNF, **(C)** NT-3, **(D)** NT-4/5. Results are expressed as relative gene expression levels of mRNA. Values are means ± SD ($n = 8$). *$p < 0.05$ versus SDS group. AP, acupuncture; BDNF, brain-derived neurotrophic factor; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; NGF, nerve growth factor; NT, neurotrophin; SD, standard deviation; SDS, social defeat stress.

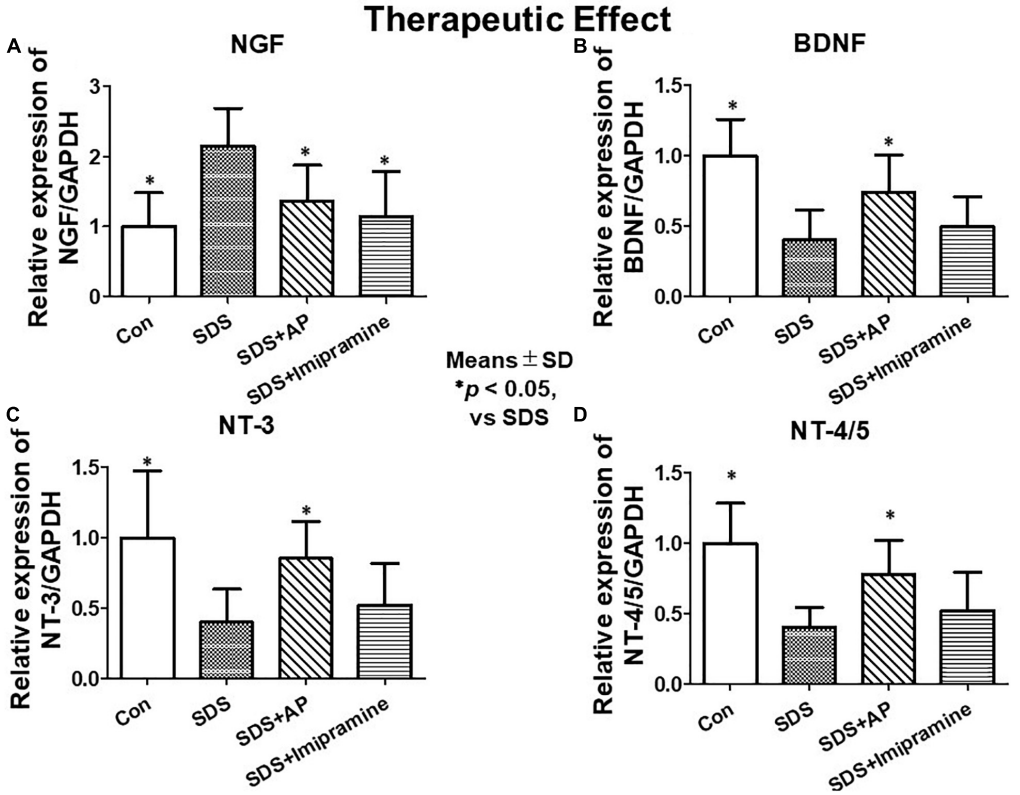


FIGURE 8 | mRNA expressions of neurotrophic factors in the brain after treatment of depression in SDS mice quantified by real-time PCR. **(A)** NGF, **(B)** BDNF, **(C)** NT-3, **(D)** NT-4/5. Results are expressed as relative gene expression levels of mRNA. Values are means ± SD (n = 8). *$p < 0.05$ versus SDS group. AP, acupuncture, BDNF, brain-derived neurotrophic factor; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; NGF, Nerve growth factor; NT, Neurotrophin; SD, standard deviation; SDS, social defeat stress.

protein levels were significantly enhanced [$F(3, 28) = 4.619710$, $p = 0.0095$] (Control vs. SDS, $p < 0.01$), and BDNF [$F(3, 28) = 8.565$, $p = 0.0003$] (Control vs. SDS, $p < 0.01$), NT-3 [$F(3, 28) = 6.331818$, $p = 0.0020$] (Control vs. SDS, $p < 0.01$), and NT-4/5 [$F(3, 28) = 6.155$, $p = 0.0024$] (Control vs. SDS, $p < 0.01$) levels were significantly suppressed (**Figure 9**). The reduced protein levels of BDNF (SDS vs. SDS+AP, $p < 0.01$), NT-3 (SDS vs. SDS+AP, $p < 0.05$), and NT-4/5 (SDS vs. SDS+AP, $p < 0.05$) by SDS were significantly enhanced by acupuncture but not by imipramine (**Figures 9B,C,D**). SDS-enhanced NGF protein was suppressed by acupuncture (SDS vs. SDS+AP, $p < 0.05$) and imipramine (SDS vs. SDS+Imipramine, $p < 0.05$) (**Figure 9A**).

In the investigation of therapeutic effects, we examined the protein levels of neurotrophic factors in the brains of SDS mice treated for 2 weeks after completion of SDS. As with mRNA expression analysis, NGF protein was significantly enhanced [$F(3, 28) = 5.583477$, $p = 0.0039$] (Control vs. SDS, $p < 0.05$) in the brains of mice 2 weeks after the end of SDS. Protein levels of BDNF [$F(3, 28) = 9.429835$, $p = 0.0002$] (Control vs. SDS, $p < 0.01$), NT-3 [$F(3, 28) = 5.188506$, $p = 0.0056$] (Control vs. SDS, $p < 0.05$), and NT-4/5 [$F(3, 28) = 5.890445$, $p = 0.0030$] (Control vs. SDS, $p < 0.05$) were significantly suppressed. SDS-reduced protein levels of BDNF (SDS vs. SDS+AP, $p < 0.01$), NT-3 (SDS vs. SDS+AP, $p < 0.05$), and NT-4/5 (SDS vs. SDS+AP, $p < 0.05$) were significantly enhanced by acupuncture but not by imipramine (**Figures 10B,C,D**). Similar to the study investigating preventive effects, acupuncture and imipramine suppressed SDS-enhanced NGF protein (SDS vs. SDS+AP, $p < 0.05$) (**Figure 10A**).

DISCUSSION

Many animal studies have examined the state of depression (Cryan et al., 2005; Petit-Demouliere et al., 2005; Tsankova et al., 2006). Some researchers have demonstrated the effects of electroacupuncture at the GV20 "Bai-Hui" and Ex-HN3 "Yintáng" points, GV20, and Sp-6 "Sanyinjiao" points, and GV20 and EX17 "An-Mian" points in depression animal models (Liu et al., 2007; Zhu et al., 2009; You et al., 2010). We have reported that simultaneous acupuncture stimulation as part of GV20 and Ex-HN3 improves the symptoms of a rat model of water-immersion-induced depression (Tanahashi et al., 2016; Takagi et al., 2017) and a mouse model of FS-induced depression (Yamamoto et al., 2018).

For many acupuncture researchers, setting a control stimulus is a very challenging problem in experiments. In addition, it is also necessary to consider whether sham needles can be

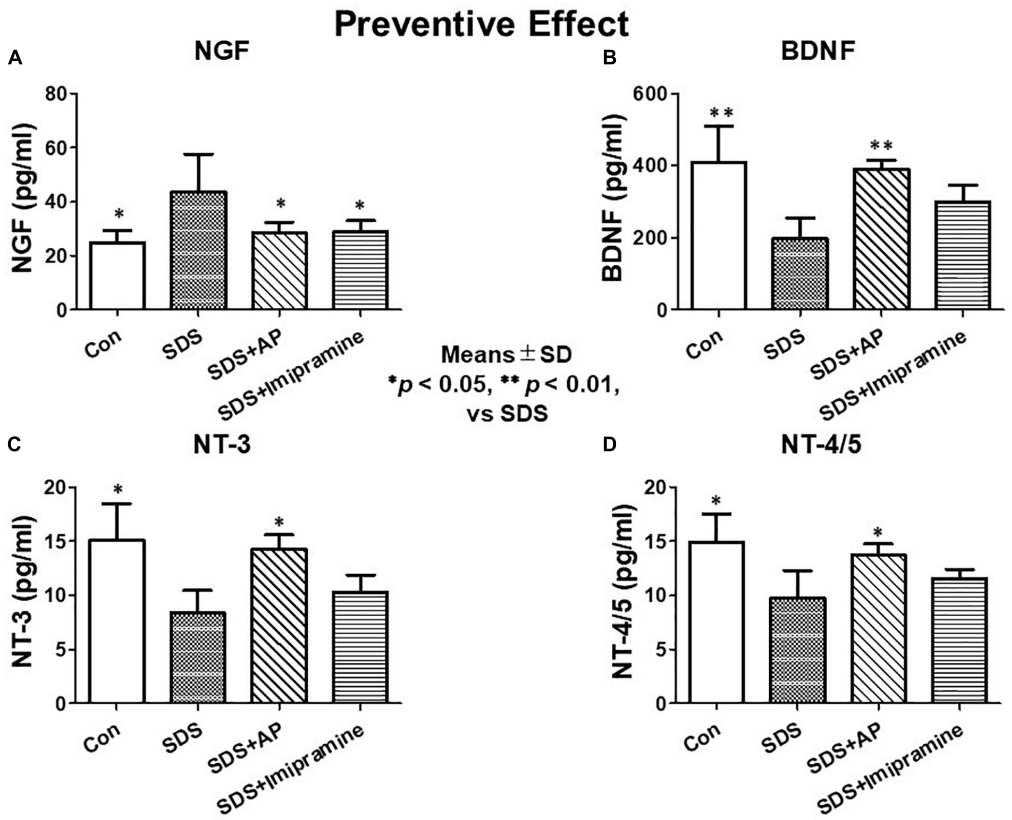


FIGURE 9 | Expressions of neurotrophic factors in the brain of SDS mice with ongoing symptoms of depression. **(A)** NGF, **(B)** BDNF, **(C)** NT-3, **(D)** NT-4/5. Results are expressed as protein content measured by ELISA. Values are means ± SD ($n = 8$). *$p < 0.05$ and **$p < 0.01$ versus SDS group. AP, acupuncture; BDNF, brain-derived neurotrophic factor; NGF, nerve growth factor; NT, neurotrophin, SD, standard deviation; SDS, social defeat stress.

used as a control. There are several types of sham needles available today. However, we were not able to obtain any sham needles applicable to animals that could be used as a control for horizontal subcutaneous acupuncture stimulation of the head as in this study. This is because stimulation of a different part of the body than the target acupuncture point usually has an effect on the body and is not suitable as a control. For these reasons, we set up a group with no acupuncture as a control in this series of studies. Takagi et al. (2017) showed that acupuncture with either GV20 or EX-HN3 alone was not effective in suppressing immobility in water-immersion stressed rats. However, there are few studies on the single use of acupuncture points that show efficacy in combinations of two or more. Additional studies will be required to assess the contribution of specific locations, number of points, subdermal stimulation or other inflammatory factors in the effects of acupuncture.

In this study, we examined the effect of simultaneous acupuncture stimulation as part of GV20 and Ex-HN3 using SDS-treated mice that has been developed as a new ethological model of depression that more closely models the psychological stress that humans can experience during antagonistic social interactions. The experiments were performed in two parts. The first was to verify the effect of acupuncture on preventing SDS-induced immobility, and the second was to verify the effect of acupuncture on SDS-induced immobility that had already developed. 2 weeks of SDS significantly enhanced the immobile time of mice, that is, induced depressive-like symptoms (**Figure 4B**). Simultaneous acupuncture stimulation, as part of GV20 and Ex-HN3, prevented immobility as well as antidepressants. After the end of SDS, an increase in the immobile time of mice was also observed even 2 weeks later (**Figure 5B**), revealing that the effects of SDS persisted long after the end of SDS (**Figure 5B**). Simultaneous acupuncture stimulation as part of GV20 and Ex-HN3 showed a therapeutic effect as well as a suppressive effect on SDS-induced immobility. Previously studies indicated that acupuncture is effective in the neurological system not only as a tool to treat disease, but also as a disease preventive strategy (Li and Wang, 2013). The results of our study showed that simultaneous acupuncture stimulation of GV20 and EX-HN3 can be used not only as a treatment for depression that has already developed, but also as a routine maintenance to avoid depression.

Various theories have been proposed as the causes of depression. The oldest hypothesis is the "monoamine hypothesis," which states that depression may be caused by decreased levels of monoamines in the brain (Nestler et al., 2002; Liu et al., 2007; Schlaepfer et al., 2010). It is also known that acupuncture stimulates monoamines in the brain (Xu et al., 2007;

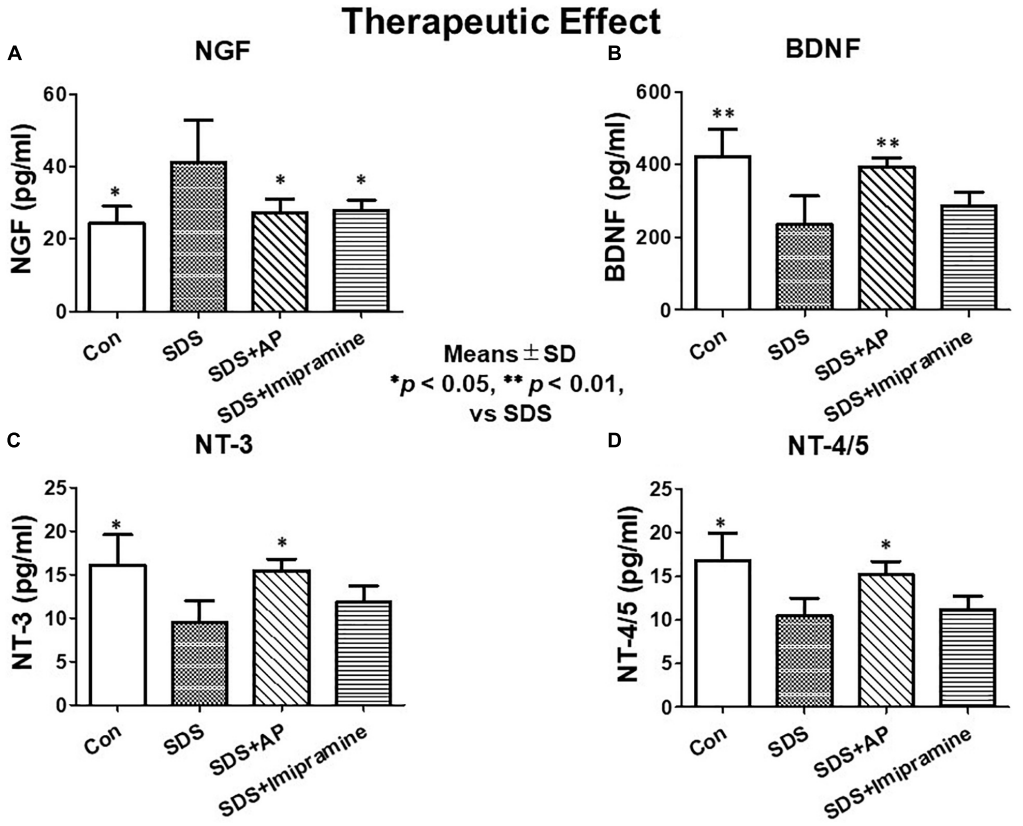


FIGURE 10 | Expressions of neurotrophic factors in the brain after treatment of depression in SDS mice. **(A)** NGF, **(B)** BDNF, **(C)** NT-3, **(D)** NT-4/5. Results are expressed as protein content measured by ELISA. Values are means ± SD ($n = 8$). *$p < 0.05$ and **$p < 0.01$ versus SDS group. AP, acupuncture; BDNF, brain-derived neurotrophic factor; NGF, nerve growth factor; NT, neurotrophin; SD, standard deviation; SDS, social defeat stress.

Murotani et al., 2010). Recently, it has been proposed that decreased neurotrophic factor production, increased glutamate-induced intracellular calcium concentration, and abnormal intracellular signal transduction mechanisms related to neuroplasticity by cortisol via the hypothalamic-pituitary-adrenocortical (HPA) axis are also thought to be the cause of depression (Jacobson and Sapolsky, 1991; Nibuya et al., 1995; Manji et al., 2001; Sanacora et al., 2008). In our previous study, simultaneous acupuncture stimulation of GV20 and Ex-HN3 was shown to reduce blood corticosterone levels in depressed rats (Tanahashi et al., 2016).

In this study, we focused on the expression of neurotrophic factors in the brain, which are key components of depression. To examine the mechanism of preventive and therapeutic effects of acupuncture on depression, we examined the expression of neurotrophic factors in the brains of SDS mice.

The relationship between stress that causes depression and neurotrophic factors has been identified. Stress has been reported to reduce the expression of BDNF mRNA in the hippocampus (Nibuya et al., 1995; Smith et al., 1995). It has also been reported that electroconvulsive stimulation and chronic administration of antidepressants improve the reduction of BDNF mRNA in the rat hippocampus due to stress (Nibuya et al., 1995). Furthermore, chronic administration of tricyclic antidepressants has been reported to increase BDNF and NT-3 protein levels in rodent brains (Okamoto et al., 2003; Tsankova et al., 2006). NT-3 and NT-4/5 are neurotrophic factors that are structurally related to NGF and BDNF. These neurotrophic factors promote the growth and survival of nerve cells, such as BDNF (Barde, 1994; Lindholm, 1994; Zheng et al., 1995; Pérez-Navarro et al., 2000). Unlike NGF and BDNF, there are not many studies currently examining NT-3 and NT-4/5 in depression. Otsuki et al. (2008) examined the expression of NGF, BDNF, NT-3, and NT-4/5 mRNAs in peripheral blood cells of patients with depression and bipolar disorder. They reported that the expression level of NT-3 mRNA decreased in a symptom-dependent manner in these diseases. NT-3 is thought to play a role in the neurobiological processes associated with mood and anxiety disorders. In recent years, it has been proposed that NT-3 is a potential pharmacological target for mood disorders due to its effects on monoamine neurotransmitters, regulation of synaptic plasticity and neurogenesis, enhancement of BDNF signaling, and regulation of the HPA axis (de Miranda et al., 2020). There are even fewer studies on the role of NT-4/5 in depression. Liu et al. (2017) revealed that intranasal administration of recombinant adeno-associated virus (AAV) expressing NT4/5-NAP (Asn-Ala-Pro-Val-Ser-Ile-Pro-Gln, NAPVSIPQ) exerts antidepressant effects in socially isolated mice.

Simultaneous acupuncture stimulation as part of GV20 and Ex-HN3 restored the expression of SDS-reduced BDNF, NT-3,

and NT-4/5. However, imipramine administration suppressed SDS-induced immobility but did not restore these neurotrophic factors. The discrepancy with previous studies is attributed to the effects of treatments on neurotrophic factor recoveries seen in 2 weeks with acupuncture but more time with antidepressants. In addition, differences in animal species or stress types may also be the cause of this discrepancy.

In contrast to neurotrophic factors, such as BDNF, NGF expression was increased in SDS mice. Contrary to our results in SDS, some studies have reported reduced NGF expression in depression (Okamoto et al., 2003). However, increased NGF expression after depressive stress has also been reported (Hadjiconstantinou et al., 2001; de Azevedo Cardoso et al., 2014). Acupuncture stimulation and imipramine suppressed the increase in NGF expression induced by SDS. The reason that shows the behavior only the opposite NGF of neurotrophic factor expression in SDS mouse is still unclear.

As described above, improvements in the production of monoamine and serotonin, the HPA axis, and expression of neurotrophic factors have been proposed as treatments for depression (Jacobson and Sapolsky, 1991; Smith et al., 1995; Manji et al., 2001; Nestler et al., 2002; Okamoto et al., 2003; Adams et al., 2008; Otsuki et al., 2008; Sanacora et al., 2008; Liu et al., 2017; de Miranda et al., 2020). Our studies revealed that acupuncture stimulation ameliorates SDS-induced immobility in mice. The treatment mechanism of acupuncture may be different from that of antidepressants because its effects on neurotrophic factors appear earlier. The therapeutic effects on neurological disorders that have been studied so far have been shown to be related to the mediation of neuroplasticity and regulation of neurotrophic factors and neurotransmitters, but the exact mechanism underlying the effects of acupuncture has not been clarified yet (Xiao et al., 2018). In this study using our SDS-treated mice, it is clarified that simultaneous acupuncture stimulation as part of GV20 and Ex-HN3 strongly restored the expression of neurotrophic factors altered by immobility to their respective normal states, suggesting that this might be a promising therapeutic strategy for depressive disorder.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The animal study was reviewed and approved by Animal Experiment Ethical Review Committee of Suzuka University of Medical Science (Permission number: 189).

AUTHOR CONTRIBUTIONS

JK, TI, NT, and NM contributed to the conception and design of the study. JK, KT, TY, and NN conducted experiments and organized a database. JK performed the statistical analysis and wrote draft of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnbeh.2021.685433/full#supplementary-material

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Are Noradrenergic Transmission Reducing Drugs Antidepressants?

*Paul J. Fitzgerald**

Department of Psychiatry, University of Michigan, Ann Arbor, MI, United States

Major depressive disorder (MDD) remains a significant public health problem worldwide, and revised treatment strategies are therefore urgently needed, including the creation of novel antidepressant compounds or using existing molecular entities in new ways. Etiologic theories of MDD from decades ago have suggested that synaptic deficiencies of monoaminergic neurotransmitters play a causative role in this neuropsychiatric disorder, and that boosting monoamines with drugs such as SSRIs, SNRIs, TCAs, and MAOIs has antidepressant effects and in some individuals can even induce hypomania or mania. While other factors, such as various intracellular molecular pathways and hippocampal neurogenesis, undoubtedly also play a role in MDD, monoaminergic boosting drugs nonetheless have clearly demonstrated antidepressant properties. There is also, however, a body of studies in the preclinical literature suggesting that monoaminergic transmission *reducing* drugs, including noradrenergic ones, also have antidepressant-like behavioral properties in rodents. Given that there is increasing evidence that the monoamines have u-shaped or Janus-faced dose-response properties, in which a mid-range value is "optimal" in a variety of behavioral and physiological processes, it is plausible that either too much or too little synaptic norepinephrine in key circuits may exacerbate MDD in some individuals. Here we briefly review rodent depression-related behavioral data, focusing on the forced swim test, from three major classes of noradrenergic transmission reducing drugs (alpha2 agonists, beta blockers, alpha1 antagonists), and find much support for the hypothesis that they have antidepressant-like properties. Whether these drugs are antidepressants in human subjects remains to be determined.

Keywords: norepinephrine, noradrenaline, serotonin, SSRI (selective serotonergic reuptake inhibitors), propranolol, clonidine, guanfacine, prazosin

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Ravid Doron,
Open University of Israel, Israel

Reviewed by:
Juan Francisco Rodríguez-Landa,
Universidad Veracruzana, Mexico
Keren Nitzan,
Open University of Israel, Israel

***Correspondence:**
Paul J. Fitzgerald
pfitz1940@gmail.com

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INTRODUCTION

Despite intensive efforts by commercial and academic researchers for many decades, major depressive disorder (MDD) remains a significant source of morbidity and mortality throughout the world (Chen et al., 2017; Schmaal et al., 2017; Hasin et al., 2018; Ingram et al., 2020). Many individuals who experience MDD do not respond completely, or in some cases at all, to existing pharmacological or behavioral treatment modalities, leaving a need for new approaches (Mitchell, 2004; Ruhé et al., 2006; Ulrich et al., 2020).

In addition to the demand for improved psychotherapeutic treatments, the field would benefit from the creation of novel pharmacological agents or the repurposing of existing compounds that may, perhaps unexpectedly, have beneficial properties in the treatment of MDD (Ebada, 2017; Demin et al., 2019).

Following the discovery of some of today's widely used antidepressants (MAOIs, TCAs) in the mid-20th century, monoaminergic theories on the etiology of MDD were put forth, suggesting that diminished brain levels of serotonin, norepinephrine (NE), and dopamine are a causative factor in the disorder (Schildkbaut, 1965; Coppen, 1967; Janowsky et al., 1972). In the decades since then, it has become increasingly clear that a number of intracellular molecular pathways (which undoubtedly interact with the extracellular monoamines) also play a role in MDD and the physiological and behavioral responses to antidepressant drugs (Vaidya and Duman, 2001; Tanis and Duman, 2007; Miller et al., 2009; Wohleb et al., 2016), although the continued medical use of antidepressants that boost synaptic monoamines (including SSRIs, SNRIs, NDRIs, TCAs, MAOIs) reinforces the clinical utility of this approach.

For these reasons, it may be surprising to note that in the preclinical literature there is also a significant body of studies suggesting that noradrenergic transmission *reducing* drugs, such as the alpha2 agonist clonidine, exhibit antidepressant-like behavioral properties under a variety of experimental conditions. This may be a surprising finding since a number of the monoaminergic theories of MDD suggest that elevated monoamines should produce mania or hypomania (Schildkbaut, 1965; Coppen, 1967; Janowsky et al., 1972), and by inference transmission reducing drugs may have mood-stabilizing properties but not necessarily be antidepressants. However, a growing body of evidence suggests that endogenous serotonin, NE, and dopamine have u-shaped or Janus-faced dose-response properties for a range of behaviors, wherein too much or too little signaling may be pathological (Baldi and Bucherelli, 2005; Arnsten, 2007; Vijayraghavan et al., 2007; Giustino et al., 2016; Giustino and Maren, 2018; Groft et al., 2019). In this scenario, perhaps a non-optimal (i.e., decreased or elevated) synaptic concentration of each monoamine may result in MDD, at least in some individuals with the disorder.

Below we briefly review rodent preclinical findings on the depression-related behavioral effects of three major classes of noradrenergic transmission reducing drugs: alpha2 agonists, beta blockers, and alpha1 antagonists. We focus on three major behavioral assays: the forced swim test (FST), the tail suspension test (TST), and the sucrose preference test. We conducted a PubMed database search using the following terms (February 7, 2021): clonidine/guanfacine/dexmedetomidine/propranolol/carvedilol/nebivolol/metoprolol/atenolol/prazosin/"beta blocker(s)"/alpha1/alpha2/beta1/beta2/beta3 +"forced swim" /"forced swimming"/"tail suspension"/"sucrose preference" /antidepressant-like/depression-like. This literature search yielded a total of 489 publications. Forty-eight were judged to be relevant articles that included data with at least one of the above types of drugs (alpha2 agonists, beta blockers, alpha1 antagonists), in mice or rats that were exposed to at least one of the above three behavioral assays (FST, TST, sucrose preference). To be included, these papers had to be published in the English language, and the 48 that met these criteria are further described in **Table 1**. There was no limit set on how long ago the papers were published. We did not focus on studies that investigated the interaction between natural products or compounds and these noradrenergic agents.

ALPHA2 AGONISTS

Dating back several decades, there is a body of evidence suggesting that alpha2 adrenergic agonists such as clonidine and guanfacine, which inhibit the presynaptic release of NE and activate alpha2 receptors that are also located postsynaptically, have antidepressant-like properties in rodent models. While there are some opposing data suggesting that alpha2 *antagonists* can have antidepressant-like effects (Muguruza et al., 2013; Uys et al., 2017), a number of studies report that alpha2 agonists such as clonidine are therapeutic when administered acutely. A number of these studies indeed suggest that clonidine, by itself, can produce antidepressant-like effects in tests such as the FST (Malinge et al., 1988, 1989; Cervo and Samanin, 1991; Cervo et al., 1992; Asakura et al., 1993, 1994; Skrebuhhova et al., 1999; Masuda et al., 2001; O'Neill et al., 2001; Malikowska et al., 2017).

Clonidine, in many cases, when given at sub-effective doses, can also potentiate the antidepressant-like effects of a wide range of other drugs that have antidepressant properties such as SSRIs, NDRIs, TCAs, MAOIs, 5HT1A agonists, lithium, lamotrigine, and others (Malinge et al., 1988, 1989; Bourin et al., 1991, 1996, 2002; Hascoät et al., 1991; Hascoet et al., 1994; Redrobe and Bourin, 1997, 1998; Skrebuhhova et al., 1999; Kaster et al., 2007; Zeidan et al., 2007; Taksande et al., 2009; Kotagale et al., 2013). In some cases these effects were shown to be counteracted by alpha2 antagonists such as idazoxan or yohimbine, suggesting clonidine achieves its antidepressant-like properties through activation of the alpha2 receptor (Malinge et al., 1988, 1989; Cervo and Samanin, 1991; Masuda et al., 2001; O'Neill et al., 2001; Zeidan et al., 2007).

In contrast to these potentially therapeutic properties of clonidine, it has also been suggested that this drug can promote depression-like behavior in rodents (Kitada et al., 1983; Parale and Kulkarni, 1986; Ferrari et al., 1991; Rénéric et al., 2002), or under some circumstances has no substantial effect either alone or when co-administered with other putative antidepressants (Kitada et al., 1983; Evangelista et al., 1987; Antkiewicz-Michaluk et al., 2017).

It has also been shown that molecular overexpression of alpha2C adrenoceptors can decrease immobility in the mouse FST (Sallinen et al., 1999), perhaps mimicking the antidepressant-like effects of alpha2 agonists such as clonidine. Antidepressant-like effects of two other alpha2 agonists, guanfacine and dexmedetomidine, have also been reported in rodent models (Stone et al., 2011; Mineur et al., 2015, 2018).

TABLE 1 | Summary of antidepressant-related effects of noradrenergic transmission reducing drugs.

Publication	Species	Strain	Sex	Primary Drug	Secondary Drug	Dose (mg/kg)	Route	Repeats	Time Delay	Stress	Test	Effect
O'Neill et al. (2001)	M	BKTO	F	clonidine	none	0.25	s.c.	0	30 min	none	FS	dec imm
				clonidine	none	0.5	s.c.	0	30 min	none	FS	dec imm
				clonidine	none	1	s.c.	0	30 min	none	FS	dec imm
Malikowska et al. (2017)	M	CD-1	M	clonidine	none	0.1	i.p.	0	60 min	none	FS	dec imm
	M	CD-1	M	clonidine	none	0.1	i.p.	0	60 min	24 h after SPS	FS	n.s.
Asakura et al. (1993)	M	ddY	M	clonidine	none	0.03	i.p.	0	30 min	none	FS	n.s.
				clonidine	none	0.1	i.p.	0	30 min	none	FS	n.s.
				clonidine	none	0.3	i.p.	0	30 min	none	FS	inc swim
				clonidine	none	0.03	i.p.	0	30 min	48 h soc isol	FS	n.s.
				clonidine	none	0.1	i.p.	0	30 min	48 h soc isol	FS	n.s.
				clonidine	none	0.3	i.p.	0	30 min	48 h soc isol	FS	inc swim
Asakura et al. (1994)	M	ddY	M	clonidine	none	0.03	i.p.	0	30 min	none	FS	n.s.
				clonidine	none	0.1	i.p.	0	30 min	none	FS	n.s.
				clonidine	none	0.3	i.p.	0	30 min	none	FS	inc swim
				clonidine	none	1	i.p.	0	30 min	none	FS	inc swim
				clonidine	none	0.03	i.p.	0	30 min	48 h soc isol	FS	n.s.
				clonidine	none	0.1	i.p.	0	30 min	48 h soc isol	FS	n.s.
				clonidine	none	0.3	i.p.	0	30 min	48 h soc isol	FS	inc swim
				clonidine	none	1	i.p.	0	30 min	48 h soc isol	FS	inc swim
Masuda et al. (2001)	M	ddY	M	clonidine	none	0.004	i.p.	0	45 min	none	FS	inc clim
				clonidine	none	0.02	i.p.	0	45 min	none	FS	inc clim
				clonidine	none	0.1	i.p.	0	45 min	none	FS	inc clim
Kaster et al. (2007)	M	Swiss	F	clonidine	none	0.06	i.p.	0	60 min	none	FS	n.s.
Kotagale et al. (2013)	M	Swiss	M	clonidine	none	0.015	i.p.	0	30 min	none	FS	n.s.
				clonidine	bupropion	0.015, 5	i.p.	0	30 min	none	FS	dec imm
Hascoät et al. (1991)	M	Swiss	M	clonidine	none	0.06	i.p.	0	30 min	none	FS	n.s.
				clonidine	none	0.125	i.p.	0	30 min	none	FS	inc mob
				clonidine	none	0.25	i.p.	0	30 min	none	FS	n.s.
				clonidine	none	0.5	i.p.	0	30 min	none	FS	inc mob

(Continued)

TABLE 1 | Continued

Publication	Species	Strain	Sex	Primary Drug	Secondary Drug	Dose (mg/kg)	Route	Repeats	Time Delay	Stress	Test	Effect
				clonidine	none	1	i.p.	0	30 min	none	FS	inc mob
				clonidine	none	0.06	i.p.	0	30 min	none	TS	n.s.
				clonidine	none	0.125	i.p.	0	30 min	none	TS	n.s.
				clonidine	none	0.25	i.p.	0	30 min	none	TS	dec imm
				clonidine	none	0.5	i.p.	0	30 min	none	TS	n.s.
				clonidine	none	1	i.p.	0	30 min	none	TS	n.s.
Malinge et al. (1988)	M	Swiss	M	clonidine	none	0.015	i.p.	0	30 min	none	FS	n.s.
				clonidine	none	0.06	i.p.	0	30 min	none	FS	inc mob
				clonidine	none	0.25	i.p.	0	30 min	none	FS	inc mob
				clonidine	none	1	i.p.	0	30 min	none	FS	inc mob
				clonidine	none	4	i.p.	0	30 min	none	FS	inc mob
				clonidine	none	16	i.p.	0	30 min	none	FS	inc mob
				clonidine	imipramine	0.06, 4	i.p.	0	30 min	none	FS	dec imm
				clonidine	amitriptyline	0.06, 1	i.p.	0	30 min	none	FS	dec imm
				clonidine	maprotiline	0.06, 8	i.p.	0	30 min	none	FS	dec imm
				clonidine	mianserin	0.06, 4	i.p.	0	30 min	none	FS	dec imm
				clonidine	viloxazine	0.06, 2	i.p.	0	30 min	none	FS	dec imm
				clonidine	citalopram	0.06, 2	i.p.	0	30 min	none	FS	dec imm
				clonidine	indalpine	0.06, 4	i.p.	0	30 min	none	FS	dec imm
				clonidine	fluvoxamine	0.06, 4	i.p.	0	30 min	none	FS	dec imm
				clonidine	inprindole	0.06, 32	i.p.	0	30 min	none	FS	dec imm
				clonidine	nialamide	0.06, 32	i.p.	0	30 min	none	FS	dec imm
Bourin et al. (1991)	M	Swiss	M	clonidine	imipramine	0.1, 8	i.p.	0	30 min	none	FS	dec imm
				clonidine	amitriptyline	0.1, 2	i.p.	0	30 min	none	FS	dec imm
				clonidine	maprotiline	0.1, 8	i.p.	0	30 min	none	FS	dec imm
				clonidine	citalopram	0.1, 4	i.p.	0	30 min	none	FS	dec imm
				clonidine	fluvoxamine	0.1, 8	i.p.	0	30 min	none	FS	dec imm
				clonidine	paroxetine	0.1, 8	i.p.	0	30 min	none	FS	dec imm
Bourin et al. (1996)	M	Swiss	M	clonidine	imipramine	0.06, 4	i.p.	0	30 min	none	FS	dec imm
				clonidine	fluoxetine	0.06, 2	i.p.	0	30 min	none	FS	dec imm
				clonidine	trazodone	0.06, 0.5	i.p.	0	30 min	none	FS	dec imm
				clonidine	mianserin	0.06, 4	i.p.	0	30 min	none	FS	dec imm
				clonidine	gepirone	0.06, 4	i.p.	0	30 min	none	FS	dec imm
Bourin et al. (2002)	M	Swiss	M	clonidine	tranylcypromine	0.06, 0.5	i.p.	0	30 min	none	FS	dec imm
				clonidine	phenelzine	0.06, 8	i.p.	0	30 min	none	FS	dec imm

(Continued)

TABLE 1 | Continued

Publication	Species	Strain	Sex	Primary Drug	Secondary Drug	Dose (mg/kg)	Route	Repeats	Time Delay	Stress	Test	Effect
Hascoet et al. (1994)	M	Swiss	M	clonidine	8-OH-DPAT	0.1, 0.5	i.p.	0	30 min	none	FS	n.s.
				clonidine	gepirone	0.1, 4	i.p.	0	30 min	none	FS	dec imm
				clonidine	ipsapirone	0.1, 1	i.p.	0	30 min	none	FS	dec imm
Redrobe and Bourin (1997)	M	Swiss	M	clonidine	imipramine	0.06, 4	i.p.	0	30 min	none	TS	dec imm
				clonidine	fluoxetine	0.06, 2	i.p.	0	30 min	none	TS	dec imm
				clonidine	trazodone	0.06, 0.5	i.p.	0	30 min	none	TS	dec imm
				clonidine	mianserin	0.06, 4	i.p.	0	30 min	none	TS	dec imm
				clonidine	iprindole	0.06, 32	i.p.	0	30 min	none	TS	n.s.
				clonidine	ritanserin	0.06, 0.5	i.p.	0	30 min	none	TS	dec imm
				clonidine	ipsapirone	0.06, 1	i.p.	0	30 min	none	TS	dec imm
Redrobe and Bourin (1998)	M	Swiss	M	clonidine	8-OH-DPAT	0.06, 1	i.p.	0	30 min	none	FS	dec imm
				clonidine	ritanserin	0.06, 4	i.p.	0	30 min	none	FS	dec imm
				clonidine	ketanserin	0.06, 8	i.p.	0	30 min	none	FS	n.s.
Taksande et al. (2009)	M	Swiss	M	clonidine	none	0.015	i.p.	0	30 min	none	FS	n.s.
				clonidine	none	0.03	i.p.	0	30 min	none	FS	n.s.
				clonidine	none	0.06	i.p.	0	30 min	none	FS	dec imm
				clonidine	imipramine	0.015, 2.5	i.p.	0	30 min	none	FS	n.s.
				clonidine	fluoxetine	0.015, 2.5	i.p.	0	30 min	none	FS	dec imm
				clonidine	paroxetine	0.015, 2.5	i.p.	0	30 min	none	FS	dec imm
Zeidan et al. (2007)	M	Swiss	M, F	clonidine	none	0.06	i.p.	0	30 min	none	FS	n.s.
				clonidine	agmatine	0.06, 0.001	i.p.	0	30 min	none	FS	dec imm
Ferrari et al. (1991)	M	Swiss	M	clonidine	none	0.075	i.p.	0	25 min	none	TS	inc imm
				clonidine	none	0.15	i.p.	0	25 min	none	TS	inc imm
Parale and Kulkarni (1986)	M	Wist	M	clonidine	none	0.05	i.p.	0	15 min	none	FS	inc imm
				clonidine	none	0.15	i.p.	0	15 min	none	FS	inc imm
				clonidine	none	0.5	i.p.	0	15 min	none	FS	inc imm
Evangelista et al. (1987)	R	CD-COBS	M	clonidine	none	0.1	i.p.	0	30 min	none	FS	n.s.
Cervo and Samanin (1991)	R	S-D	M	clonidine	none	0.05	i.p.	0	30 min	none	FS	n.s.
				clonidine	none	0.1	i.p.	0	30 min	none	FS	n.s.
				clonidine	none	0.5	i.p.	0	30 min	none	FS	n.s.
				clonidine	none	0.05	i.p.	2	30 min	none	FS	dec imm

(Continued)

TABLE 1 | Continued

Publication	Species	Strain	Sex	Primary Drug	Secondary Drug	Dose (mg/kg)	Route	Repeats	Time Delay	Stress	Test	Effect
				clonidine	none	0.1	i.p.	2	30 min	none	FS	dec imm
				clonidine	none	0.5	i.p.	2	30 min	none	FS	dec imm
				clonidine	none	0.1	i.p.	b.i.d. for 15 days	30 min	none	FS	n.s.
Cervo et al. (1992)	R	S-D	M	clonidine	none	0.1	i.p.	2	30 min	none	FS	dec imm
Kitada et al. (1983)	R	S-D	M	clonidine	none	0.3	s.c.	2	30 min	none	FS	n.s.
				clonidine	desipramine	0.3, 20	s.c., i.p.	2	30 min	none	FS	inc imm
Rénéric et al. (2002)	R	S-D	M	clonidine	none	0.005	i.p.	2	60 min	none	FS	inc swim
				clonidine	none	0.01	i.p.	2	60 min	none	FS	n.s.
				clonidine	none	0.02	i.p.	2	60 min	none	FS	n.s.
				clonidine	none	0.2	i.p.	2	60 min	none	FS	inc clim
Skrebuhhova et al. (1999)	R	Wist	M	clonidine	none	0.1	i.p.	1	30 min	none	FS	n.s.
				clonidine	none	1	i.p.	1	30 min	none	FS	dec imm
				clonidine	desipramine	0.1, 10	i.p.	1	15 min	none	FS	dec imm
Antkiewicz-Michaluk et al. (2017)	R	Wist	M	clonidine	none	0.1	i.p	0	60 min	none	FS	inc clim
Mineur et al. (2015)	M	C57	M, F	guanfacine	none	0.05	i.p.	0	30 min	none	FS	n.s.
				guanfacine	none	0.1	i.p.	0	30 min	none	FS	n.s.
				guanfacine	none	0.15	i.p.	0	30 min	none	FS	dec imm
				guanfacine	none	0.3	i.p.	0	30 min	none	FS	n.s.
				guanfacine	none	0.05	i.p.	q.d. for 15 days	approx 24 h	none	FS	n.s.
				guanfacine	none	0.1	i.p.	q.d. for 15 days	approx 24 h	none	FS	dec imm
				guanfacine	none	0.15	i.p.	q.d. for 15 days	approx 24 h	none	FS	dec imm
				guanfacine	none	0.3	i.p.	q.d. for 15 days	approx 24 h	none	FS	n.s.
Mineur et al. (2018)	M	C57	M, F	guanfacine	none	0.15	i.p.	0	30 min	none	FS	dec imm
				guanfacine	none	0.15	i.p.	0	30 min	none	TS	dec imm
Parale and Kulkarni (1986)	M	Wist	M	guanfacine	none	0.15	i.p.	0	15 min	none	FS	inc imm
Stone et al. (2011)	M	S-W	M	dexmedetomidine	none	0.04 nmol	i.c.v.	0	5 min	none	TS	dec imm
				dexmedetomidine	none	0.1 nmol	i.c.v.	0	5 min	none	TS	dec imm
Al-Tubuly et al. (2008)	M	albino	NS	propranolol	none	1	i.p.	0	60 min	none	FS	dec latency to imm
				propranolol	imipramine	1, 10	i.p.	0	60 min	none	FS	dec latency to imm

(Continued)

TABLE 1 | Continued

Publication	Species	Strain	Sex	Primary Drug	Secondary Drug	Dose (mg/kg)	Route	Repeats	Time Delay	Stress	Test	Effect
Sekio and Seki (2015)	M	CD-1	M	propranolol	LPS	5 µl 400 mM	i.c.v.	0	24 h	none	FS	n.s.
				propranolol	LPS	5 µl 400 mM	i.c.v.	0	24 h	none	TS	n.s.
Zhang et al. (2009)	M	FVB	M	propranolol	none	2.5	i.p.	0	45 min	none	FS	n.s.
				propranolol	desipramine	2.5, 20	i.p.	0	30 min	none	FS	n.s.
Gu et al. (2012)	M	ICR	M	propranolol	none	5	i.p.	0	120 min	none	TS	n.s.
Teste et al. (1990)	M	NMRI	M	propranolol	none	0.12	i.p.	0	60 min	none	TS	n.s.
				propranolol	none	0.5	i.p.	0	60 min	none	TS	n.s.
				propranolol	none	2	i.p.	0	60 min	none	TS	n.s.
				propranolol	none	8	i.p.	0	60 min	none	TS	n.s.
Pesarico et al. (2014)	M	Swiss	M	propranolol	none	2	i.p.	0	45 min	none	FS	n.s.
Evangelista et al. (1987)	R	CD-COBS	M	propranolol	none	5	i.p.	0	120 min	none	FS	n.s.
Abel and Hannigan (1994)	R	F344	M	propranolol	none	1	i.p.	0	60 min	none	FS	n.s.
				propranolol	none	3	i.p.	0	60 min	none	FS	inc imm
				propranolol	none	5	i.p.	0	60 min	none	FS	inc imm
				propranolol	none	1	i.p.	q.d. for 10 days	60 min	none	FS	n.s.
				propranolol	none	3	i.p.	q.d. for 10 days	60 min	none	FS	n.s.
				propranolol	none	5	i.p.	q.d. for 10 days	60 min	none	FS	n.s.
Finnegan et al. (1987)	R	S-D	M	propranolol	none	5	i.p.	q.d. for 7 days	24 h	none	FS	n.s.
Zaidi et al. (2020)	R	S-D	M	propranolol	none	50/day	in water	given for 36 days	9 days	none	FS	n.s.
				propranolol	none	50/day	in water	given for 36 days	9 days	soc defeat	FS	dec imm
Aisa et al. (2008)	R	Wist	F	propranolol	none	2	s.c.	0	60 min	mat sep	FS	dec imm
Zaidi et al. (2020)	R	S-D	M	nadolol	none	18/day	in chow	given for 36 days	9 days	none	FS	n.s.
				nadolol	none	18/day	in chow	given for 36 days	9 days	soc defeat	FS	dec imm
Park et al. (2012)	M	C57	M	butoxamine	none	5	i.p.	1	30 min	none	FS	n.s.
Al-Tubuly et al. (2008)	M	albino	NS	atenolol	none	5	i.p.	0	60 min	none	FS	inc latency to imm
				atenolol	imipramine	5, 10	i.p.	0	60 min	none	FS	n.s.
Stone and Quartermain (1999)	M	S-W	M	betaxolol	none	5	i.p.	0	20 min	none	TS	n.s.
				betaxolol	none	20	i.p.	0	20 min	none	TS	n.s.
Detke et al. (1995)	R	S-D	M	betaxolol	none	10	s.c.	2	60 min	none	FS	n.s.
				betaxolol	8-OH-DPAT	10, 0.5	s.c.	2	60 min	none	FS	n.s.
Zaidi et al. (2020)	R	S-D	M	bisoprolol	none	15/day	in water	given for 36 days	9 days	none	FS	n.s.

(Continued)

TABLE 1 | Continued

Publication	Species	Strain	Sex	Primary Drug	Secondary Drug	Dose (mg/kg)	Route	Repeats	Time Delay	Stress	Test	Effect
				bisoprolol	none	15/day	in water	given for 36 days	9 days	soc defeat	FS	n.s.
Park et al. (2012)	M	C57	M	metoprolol	none	10	i.p.	1	30 min	none	FS	n.s.
Al-Tubuly et al. (2008)	M	albino	NS	prazosin	none	5	i.p.	0	60 min	none	FS	dec latency to imm
			NS	prazosin	imipramine	5, 10	i.p.	0	60 min	none	FS	inc latency to imm
Sekio and Seki (2015)	M	CD-1	M	prazosin	LPS	5 µl 70 mM	i.c.v.	0	24 h	none	FS	dec imm
				prazosin	LPS	5 µl 70 mM	i.c.v.	0	24 h	none	TS	dec imm
Kurosawa et al. (2016)	M	CD-1	M	prazosin	inflammatory cytokines	280 µg	i.c.v.	0	24 h	none	FS	dec imm
				prazosin	inflammatory cytokines	280 µg	i.c.v.	0	24 h	none	TS	dec imm
Sugimoto et al. (2011)	M	DBA/2Cr	M	prazosin	none	1	i.p.	0	60 min	none	FS	n.s.
	M	DBA/2Cr	M	prazosin	none	5	i.p.	0	60 min	none	FS	n.s.
	M	DBA/2Cr	M	prazosin	paroxetine	1, 5	i.p.	0	30 min	none	FS	n.s.
	M	DBA/2Cr	M	prazosin	paroxetine	5, 5	i.p.	0	30 min	none	FS	inc imm
	M	ICR	M	prazosin	none	1	i.p.	0	60 min	none	FS	n.s.
	M	ICR	M	prazosin	none	5	i.p.	0	60 min	none	FS	n.s.
	M	ICR	M	prazosin	paroxetine	1, 5	i.p.	0	30 min	none	FS	n.s.
	M	ICR	M	prazosin	paroxetine	5, 5	i.p.	0	30 min	none	FS	inc imm
Gu et al. (2012)	M	ICR	M	prazosin	none	0.0625	i.p.	0	120 min	none	TS	n.s.
Teste et al. (1990)	M	NMRI	M	prazosin	none	1	i.p.	0	60 min	none	TS	n.s.
				prazosin	none	2	i.p.	0	60 min	none	TS	n.s.
				prazosin	none	4	i.p.	0	60 min	none	TS	n.s.
				prazosin	none	8	i.p.	0	60 min	none	TS	n.s.
Pesarico et al. (2014)	M	Swiss	M	prazosin	none	1	i.p.	0	45 min	none	FS	n.s.
Ribeiro and Pupo (2015)	M	Swiss	M	prazosin	none	0.5	i.p.	0	30 min	none	TS	n.s.
				prazosin	none	1	i.p.	0	30 min	none	TS	inc imm
				prazosin	imipramine	0.5, 32	i.p.	0	30 min	none	TS	inc imm
				prazosin	imipramine	1, 32	i.p.	0	30 min	none	TS	inc imm
Kaster et al. (2007)	M	Swiss	F	prazosin	none	1	i.p.	0	60 min	none	FS	n.s.
Hascoät et al., 1991	M	Swiss	M	prazosin	none	0.25	i.p.	0	30 min	none	FS	n.s.
				prazosin	none	0.5	i.p.	0	30 min	none	FS	n.s.
				prazosin	none	1	i.p.	0	30 min	none	FS	n.s.
				prazosin	none	2	i.p.	0	30 min	none	FS	n.s.
				prazosin	none	4	i.p.	0	30 min	none	FS	n.s.
				prazosin	none	0.25	i.p.	0	30 min	none	TS	n.s.

(Continued)

TABLE 1 | Continued

Publication	Species	Strain	Sex	Primary Drug	Secondary Drug	Dose (mg/kg)	Route	Repeats	Time Delay	Stress	Test	Effect
				prazosin	none	0.5	i.p.	0	30 min	none	TS	n.s.
				prazosin	none	1	i.p.	0	30 min	none	TS	n.s.
				prazosin	none	2	i.p.	0	30 min	none	TS	inc mob
				prazosin	none	4	i.p.	0	30 min	none	TS	inc mob
Stone and Quartermain (1999)	M	S-W	M	prazosin	none	0.5	i.p.	0	20 min	none	TS	inc imm
				prazosin	none	2	i.p.	0	20 min	none	TS	inc imm
Evangelista et al. (1987)	R	CD-COBS	M	prazosin	none	3	s.c.	0	90 min	none	FS	n.s.
Poncelet et al. (1987)	R	S-D	M	prazosin	desipramine	2, 32	i.p.	0	30 min	none	FS	inc imm
Cervo and Samanin (1991)	R	S-D	M	prazosin	none	3	s.c.	0	60 min	none	FS	n.s.
Detke et al. (1995)	R	S-D	M	prazosin	none	1	s.c.	2	60 min	none	FS	n.s.
				prazosin	8-OH-DPAT	1, 0.5	s.c.	2	60 min	none	FS	n.s.
Schreiber and De Vry (1993)	R	Wist	M	prazosin	none	0.1	i.p.	2	60 min	none	FS	n.s.
				prazosin	none	0.3	i.p.	2	60 min	none	FS	dec imm
				prazosin	8-OH-DPAT	0.1, 3	i.p.	2	60 min	none	FS	n.s.
				prazosin	8-OH-DPAT	0.3, 3	i.p.	2	60 min	none	FS	n.s.
Stone et al. (2011)	M	S-W	M	terazosin	none	1 nmol	i.c.v.	0	5 min	none	TS	inc imm
Wu et al. (2017)	R	S-D	M	benoxathian	none	5 μg	prelimbic infusion	0	10 min	none	FS	inc imm
				benoxathian	none	5 μg	prelimbic infusion	0	10 min	none	SP	dec sucr pref

This table comprises mouse and rat studies from our literature search that used these drugs in the forced swim (FS), tail suspension (TS), or sucrose preference (SP) tests. The "Repeats" column indicates how many times a drug treatment was repeated in that group of animals, where zero repeats indicate a single administration of that drug or drug pair. The "Time Delay" column represents the amount of time between the last (or only) administration of the drug or pair of drugs and when the behavioral test was carried out. The "Stress" column indicates whether an acute or chronic stressor was administered prior to testing. The "Effect" column describes the type of statistically significant outcome in the behavioral test or otherwise shows that the result was not statistically significant (n.s.; $p > 0.05$). For single drug administration, the Effect column describes the effect relative to vehicle administration. For pairs of drugs, the Effect column compares the behavioral effect of the pair with when one drug alone was given in that experiment. All experiments that used a prior stressor are marked in red. Experiments that showed a statistically significant antidepressant-like effect are marked in green, whereas those with a depression-like effect are marked in blue. Other abbreviations: [Species: mouse (M), rat (R)], C57BL/6J (C57), Sprague-Dawley (S-D), Swiss Webster (S-W), Wistar (Wist), [Sex: male (M), female (F)], lipopolysaccharide (LPS), intraperitoneal (i.p.), subcutaneous (s.c.), intracerebroventricular (i.c.v.), once a day (q.d.), twice a day (b.i.d.), social defeat (soc defeat), maternal separation (mat sep), social isolation (soc isol), single prolonged stress (SPS), decreased immobility (dec imm), increased immobility (inc imm), increased mobility (inc mob), increased swimming (inc swim), increased climbing (inc clim), decreased sucrose preference (dec sucr pref).

BETA BLOCKERS

Beta blockers such as propranolol and nadolol (non-selective beta1/2 antagonists), metoprolol and atenolol (beta1), and butoxamine (beta2) can exhibit antidepressant-like activity in the FST (Chopra et al., 1988; Beĭer, 1994; Aisa et al., 2008; Park et al., 2012; Zaidi et al., 2020), including potentiation of sub-effective doses of other putative antidepressants such as baclofen (Aley and Kulkarni, 1990), or antagonizing depression-like effects of other agents (Parale et al., 1987). A mouse study of propranolol and nadolol found that whereas these two drugs did not exhibit therapeutic effects in the TST, propranolol did show an antidepressant-like decrease in TST-induced hyperthermia (Liu et al., 2003). The non-selective beta blocker nebivolol has been shown to counteract the depression-like behavioral and pathophysiological effects of the chemotherapeutic agent cisplatin (Abdelkader et al., 2017). An immunocytochemical study of propranolol showed that it could reduce the number of cells that stained for Fos-like immunoreactivity in various subcortical and cortical regions, resembling standard antidepressants such as imipramine and desipramine (Duncan et al., 1996).

In contrast to these potentially therapeutic properties of beta blockers, it has also been suggested that these drugs can promote depression-like behavior in rodents (Abel and Hannigan, 1994; Stone and Quartermain, 1999; Al-Tubuly et al., 2008) including in the presence of other putative antidepressants (Zhang et al., 2009; Gu et al., 2012), or under some circumstances, they have no substantial effect either alone or when co-administered with other putative antidepressants (Danysz et al., 1986; Evangelista et al., 1987; Finnegan et al., 1987; Teste et al., 1990; Beĭer, 1994; Detke et al., 1995; Pesarico et al., 2014; Sekio and Seki, 2015; Zaidi et al., 2020). A number of studies also suggest that the beta3 *agonist* amibegron (also called SR58611A) has antidepressant-like properties in rodents (Consoli et al., 2007; Overstreet et al., 2008; Stemmelin et al., 2008, 2010; Tamburella et al., 2010), and it may achieve these effects by modulating serotonergic and noradrenergic signaling that is triggered by activation of beta3 receptors (Claustre et al., 2008).

ALPHA1 ANTAGONISTS

Alpha1 antagonists such as prazosin and benoxathian can also exhibit antidepressant-like activity in the FST or TST (Sekio and Seki, 2015; Kurosawa et al., 2016; Wu et al., 2017), including potentiation of other putative antidepressants such as imipramine (Al-Tubuly et al., 2008). In contrast, it has also been suggested that alpha1 antagonists can promote depression-like behavior (Stone and Quartermain, 1999; Al-Tubuly et al., 2008), including in the presence of other putative antidepressants or electroconvulsive therapy (ECT; Danysz et al., 1986; Poncelet et al., 1987; Teste et al., 1990; Kaster et al., 2007; Sugimoto et al., 2011; Gu et al., 2012; Ribeiro and Pupo, 2015). Under some circumstances they have no substantial depression-related behavioral effect either alone or when co-administered with other putative antidepressants (Evangelista et al., 1987; Malinge et al., 1988, 1989; Cervo and Samanin, 1991; Schreiber and De Vry, 1993; Detke et al., 1995; Sugimoto et al., 2011; Pesarico et al., 2014). In addition, mice expressing constitutively active mutant alpha1A (but not alpha1B) adrenoceptors exhibit antidepressant-like activity in the FST and TST, that is counteracted by prazosin (Doze et al., 2009).

Table 1 summarizes the results from, and experimental parameters used in the above rodent studies on noradrenergic transmission reducing drugs in the FST, TST, and sucrose preference test. A brief analysis of the table suggests a few prominent themes or findings: (1) clonidine is the drug with the most experimental evidence supporting an antidepressant-like role. Those data support its therapeutic-like role across a variety of both mouse and rat strains, in both the FST and TST, and an amplifying beneficial role when paired with a wide range of established antidepressants; (2) there is less support at this time of an antidepressant-like role for various beta blockers and the alpha1 antagonist prazosin, where a number of studies show depression-like effects for these drugs (although other data are supportive). These drugs appear to not have been studied as extensively in these tests as clonidine; (3) very few of the studies used female mice, which should be a priority in future studies, especially considering that the rate of MDD in women is approximately twice that in men (Baxter et al., 2014; Albert, 2015); (4) only a few of the studies used C57BL/6J mice, which are widely used in behavioral neuroscience, and could be combined with studies of additional strains of mice in further investigations; and (5) prior exposure to chronic stress, which can induce MDD in susceptible human subjects (Hosang et al., 2014; Bonde et al., 2016), was rarely used in these studies and should be further addressed with additional experiments.

DISCUSSION

The preclinical data reviewed above address the issue of whether noradrenergic transmission reducing pharmacological agents have antidepressant-like behavioral properties in rodents. While many of these studies, perhaps most numerously and convincingly for the alpha2 agonist clonidine, suggest that these drugs have therapeutic effects, a number of the publications found no effect or depression-like effects, including for the beta blocker propranolol and the alpha1 antagonist prazosin. How do we reconcile such opposing effects across studies for these drugs? Some possibilities are that they may be due to genetic differences across strains or species of animals, varying responses to acute or chronic stress, or in some cases different behavioral tests that were used. Another explanation is that since there may be a u-shaped or Janus-faced dose-response relationship for noradrenergic signaling (Arnsten, 2007; Giustino et al., 2016; Giustino and Maren, 2018), the different drug doses used in the above studies could have opposing behavioral effects, including through interaction with divergent cortical and subcortical circuits,

which may vary across species and strain. If alpha2 agonists such as clonidine and guanfacine really do have more robust antidepressant-like properties than beta blockers and alpha1 antagonists, this may relate to the more general effect of alpha2 agonists decreasing the presynaptic release of NE (Gresch et al., 1995; Van Gaalen et al., 1997), which would in principle affect signaling at all subtypes of adrenoceptors simultaneously.

A number of the studies reviewed above investigated the interaction of noradrenergic transmission reducing agents with other types of drugs. Several of these studies suggest that these noradrenergic drugs can potentiate the antidepressant-like effects of SSRIs or 5HT1A agonists (Malinge et al., 1988, 1989; Bourin et al., 1991, 1996; Hascoet et al., 1994; Redrobe and Bourin, 1997, 1998; Taksande et al., 2009), although not all studies or data were supportive (Redrobe and Bourin, 1998; Rénéric et al., 2002). Despite these discrepancies, this may be a treatment strategy that has clinical ramifications for the pharmacotherapy of MDD in human subjects. It has been previously suggested (Dremencov et al., 2007a,b; Guiard et al., 2008; Fitzgerald and Watson, 2019) that serotonin and NE may have functionally opposed properties, which is consistent with the hypothesis that noradrenergic transmission reducing drugs can amplify the effects of SSRIs under some conditions. We also suggest here, consistent with a statement in our prior publication (Polis et al., 2019), that noradrenergic transmission reducing drugs may be antidepressants in a subset of humans suffering from MDD, who would also be responsive to the rapidly acting antidepressant ketamine, and to ECT. In this scenario, noradrenergic transmission reducing agents may interact with glutamatergic signaling to chronically suppress neural hyperexcitability associated with some cases of MDD (**Figure 1**), and possibly have rapid therapeutic onset like ketamine (Polis et al., 2019). While the molecular mechanisms through which noradrenergic transmission reducing drugs may achieve antidepressant-like effects are not well understood at this time, one possibility is that they selectively dampen certain intracellular signaling pathways after acting upon alpha and beta-adrenergic G protein-coupled receptors. There is already evidence, for example, that NE modulates the Ras/MAPK, PI3K/Akt, JAK/STAT pathways (Muthalif et al., 1998; Yanagawa et al., 2010; Guo et al., 2013; Maity et al., 2020).

One might argue that noradrenergic transmission reducing drugs are, based on monoaminergic theories of mood disorders, more likely to have mood-stabilizing than antidepressant properties. After all, beta blockers such as propranolol have historically been more associated with induction of MDD or depressive-like symptomatology (Koella, 1985; Rosen and Kostis, 1985) (but also see: Kim et al., 2019; Kessing et al., 2020), or with attenuation of hypomania or mania (Emrich et al., 1979; Nemeth and Mckenzie Chustz, 2020), where the latter property has also been attributed to clonidine (Hardy et al., 1986; Nemeth and Mckenzie Chustz, 2020). One possibility is that if these drugs really are antidepressants under some conditions, they achieve these effects in individuals who exhibit neural "decoupling" of NE with dopamine in mood-related circuits. In such an individual, elevated noradrenergic signaling may result in MDD rather than dopamine-facilitated hypomania or mania (Diehl and Gershon, 1992). Since MDD is also associated with systemic inflammation (Miller et al., 2009), noradrenergic transmission reducing agents may also produce antidepressant effects by counteracting neuroinflammation (Chen et al., 2015; Ding et al., 2019; Apple et al., 2020; **Figure 1**).

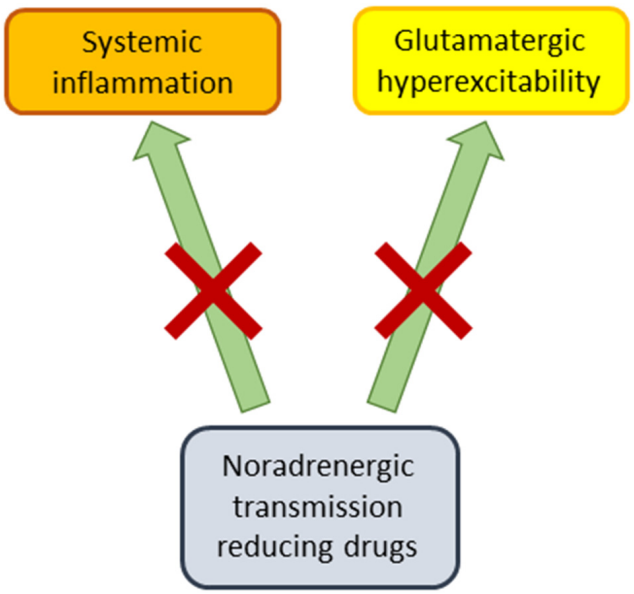

FIGURE 1 | Proposed therapeutic mechanisms of noradrenergic transmission reducing drugs. These pharmacological agents (alpha2 agonists, beta blockers, alpha1 antagonists) may produce antidepressant-like effects by dampening systemic inflammation, while also counteracting glutamatergically-mediated neural hyperexcitability.

In conclusion, while there are conflicting data in rodents as to whether noradrenergic transmission reducing drugs have antidepressant-like properties, a number of studies reviewed above support this hypothesis, at least under some experimental conditions. At present, it is not clear whether neural noradrenergic transmission is elevated or suppressed in MDD (Waldmeier, 1981), where perhaps each state exists in different individuals. For these reasons, additional preclinical, mechanistic studies are needed, including those that induce depression-like behavior in animal models through the use of chronic mild stress. Based on the foundation of preclinical studies reviewed briefly here, further investigation of noradrenergic transmission reducing drugs in human mood disorders also appears warranted.

AUTHOR CONTRIBUTIONS

The author alone conceived of, researched, wrote, and edited this publication.

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Comparative Efficacy of Multiple Therapies for the Treatment of Patients With Subthreshold Depression: A Systematic Review and Network Meta-Analysis

*Xiumin Jiang[1], Yongxin Luo[2], Yiwen Chen[1], Jinglan Yan[1], Yucen Xia[1], Lin Yao[3], Xiaotong Wang[1], Su He[1], Feixue Wang[3], Taiyi Wang[3] and Yongjun Chen[1,3,4]**

[1] South China Research Center for Acupuncture and Moxibustion, Clinical Medical College of Acupuncture, Moxibustion and Rehabilitation, Guangzhou University of Chinese Medicine, Guangzhou, China, [2] Department of Biostatistics and Preventive Medicine, School of Basic Medical Sciences, Guangzhou University of Chinese Medicine, Guangzhou, China, [3] Research Institute of Acupuncture and Moxibustion, Shandong University of Traditional Chinese Medicine, Jinan, China, [4] Guangdong-Hong Kong-Macao Greater Bay Area Center for Brain Science and Brain-Inspired Intelligence, Guangzhou, China

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***Correspondence:**
Yongjun Chen
chyj417@126.com

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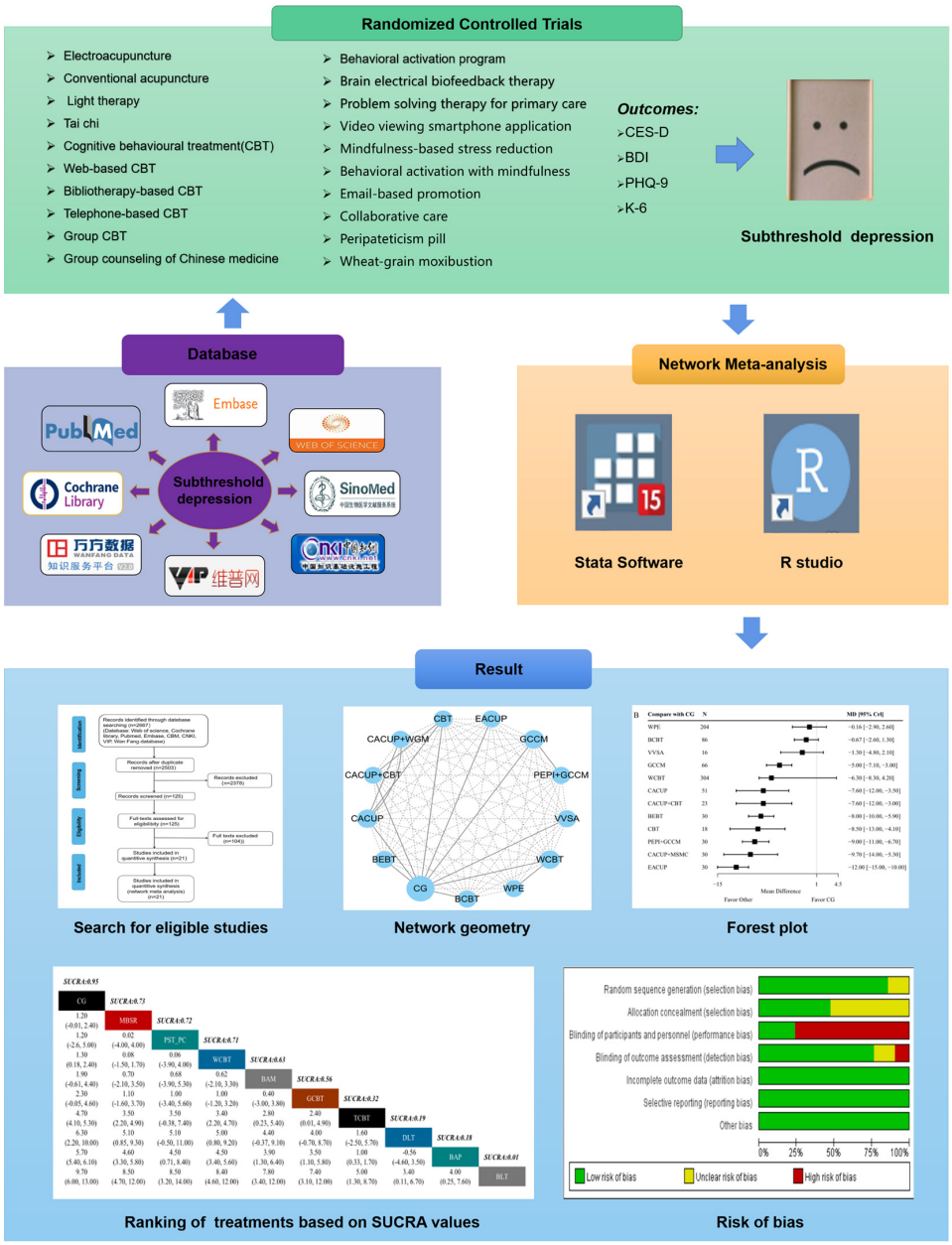
Background: Subthreshold depression (SD) is considered to be the precursor stage of major depression, which is correlated with functional impairment and increased suicide rate. Although there are multiple therapies for the treatment of SD, the comparison and efficacy of various methods has yet to be evaluated. This study aimed to evaluate the efficacy of different therapies by performing a Bayesian network meta-analysis.

Methods: We searched eight databases on April 3, 2021. Center for Epidemiologic Studies Depression Scale (CES-D), Beck Depression Inventory scale (BDI), the Patient Health Questionnaire-9 (PHQ-9), and the Kessler Screening Scale for Psychological Distress (K-6) were used as efficacy outcomes. This Bayesian network meta-analysis used a fixed-effects model.

Findings: Twenty-one randomized controlled trials involving 5,048 participants were included in this study. The results suggested that electroacupuncture (MD −12.00, 95% CrI −15.00, −10.00), conventional acupuncture plus wheat-grain moxibustion (MD −9.70, 95% CrI −14.00, −5.30), and the Chinese traditional peripateticism pill plus group counseling (MD −9.00, 95% CrI −11.00, −6.70) had better efficacy than the control group (CG) in improving CES-D. For BDI outcome, bright light therapy (MD −9.70, 95% CrI −13.00, −6.00), behavioral activation program (MD −5.70, 95% CrI −6.10, −5.40), and dim light therapy (MD −6.30, 95% CrI −10.00, −2.20) were better than the CG. Tai chi (MD −3.00, 95% CrI −4.00, −2.00) was better than CG for PHQ-9 outcomes. Telephone-based cognitive behavioral treatment (MD −2.50 95% CrI −2.70, −2.30) was better than the CG for K-6 scores.

Conclusion: Our results suggest that electroacupuncture or bright light therapy appear to be the better choices in the treatment of SD. This study provide new insights into clinical treatment selection and may aid the development of guidelines for the management of SD.

Keywords: subthreshold depression, multiple therapies, network meta-analysis, systematic review, Bayesian analysis



GRAPHICAL ABSTRACT | Graphical abstract of the network meta-analysis. Note: CES-D, Center for Epidemiologic Studies Depression Scale; BDI, Beck Depression Inventory Scale; PHQ-9, the nine-Item Patient Health Questionnaire; K6, Kessler Screening Scale for Psychological Distress; SUCRA, surface area under the cumulative ranking curves.

INTRODUCTION

Subthreshold depression (SD) is defined in the Diagnostic and Statistical Manual of Mental Disorders as including "dysthymia," "brief recurrent depression," and "minor depressive disorder" (Keller et al., 1995), but not meeting criteria for major depressive disorder (Pincus et al., 1999). Population-based studies have found that SD has a wide prevalence, about 1.4–17.2% (Cuijpers and Smit, 2004), and the prevalence may be higher in the elderly or patients with chronic diseases (Cole and Dendukuri, 2003). Moreover, SD patients not only suffer from reduced quality of life, increased functional disability and mortality rate, but also require more service utilization and economic cost (Cuijpers and Smit, 2002; Cuijpers and Schoevers, 2004; Cuijpers et al., 2013). One third to one half of patients have moderate functional impairment, and at least 10–20% of patients have progressed to severe functional impairment at the 12-month follow-up (Jaffe et al., 1994; Kroenke, 2006; Lyness et al., 2006; Rodríguez et al., 2012). Studies have found that many patients with SD have persistent depressive symptoms, which is considered to be a risk factor for the development of major depressive disorder and other mental disorders (Cuijpers and Smit, 2004; Johnson et al., 2009).

Thus, it is important to determine effective methods for the treatment of SD (Kroenke, 2017).

Early intervention may reduce the risk of symptom progression in patients with SD (Van't Veer-Tazelaar et al., 2011). There are various treatment strategies for SD, including psychotherapy, pharmacotherapy, exercise therapy, and traditional Chinese medicine (TCM) therapy. Previous studies showed that different types of psychotherapy, including cognitive behavior therapy and behavior activation therapy, could reduce the Beck Depression Inventory scale (BDI) score of adults with SD and reduce the incidence of major depressive disorder over 12 months (Furukawa et al., 2012; Buntrock et al., 2015; He et al., 2019). In one meta-analysis of 700 patients, psychotherapy was shown to be more beneficial to patients with SD than care-as-usual approaches and it may prevent the onset of severe depression (Cuijpers et al., 2007). However, psychotherapy is often not readily accessible due to long-cycle and high cost of treatment (Juul et al., 2019). Pharmacotherapy for SD focuses on the use of antidepressants such as tricyclic drugs, 5-hydroxytryptamine, reuptake inhibitors, and others. Antidepressants are one of the main therapies used for moderate-to-severe depressive episodes, but one recent study found that antidepressants are often not better than the placebo for treating SD in randomized trials (Baumeister, 2012; Wang et al., 2019). Therefore, it still unclear whether antidepressants are suitable for the treatment of SD. Meanwhile, studies have confirmed that exercise, TCM psychotherapy, acupuncture, and moxibustion treatments are all effective in the treatment of SD, but there is still a lack of valid and reliable evaluation for these therapies (Tan et al., 2014; Gou et al., 2017; Li et al., 2017a; Xie, 2018). So far, no comparison of the treatment efficacy has been conducted among the above different therapies, which limits decision-making for patients with SD in the clinic and the future research for treatment of SD.

Network meta-analysis of clinical trials involves multivariate and multilevel analysis, which allows clinicians to assess and rank the efficacy of different treatments based on both direct and indirect comparisons (Higgins and Whitehead, 1996; Salanti et al., 2008; Shim et al., 2017). The statistical methods used in network meta-analysis are mainly divided into those with frequentist and Bayesian frameworks (Jansen et al., 2011). The advantages of Bayesian methods over frequentist methods are the use of informative priors and the possibility of hierarchical modeling, which may allow more comprehensive use of the information from historical and vertical data (Yin et al., 2017). Therefore, we conducted a Bayesian network meta-analysis in this study to identify the efficacy of multiple therapies for SD and rank the efficacy of different interventions. The result will help clinicians to choose the optimum prescription from multiple treatments for SD in practice.

MATERIALS AND METHODS

Search Strategy

We searched the following databases: PubMed, Embase, Web of Science, Cochrane Central Register of Controlled Trials (CENTRAL), Chinese Biomedical Literature Database (CBM), China National Knowledge Infrastructure (CNKI), Wan Fang database, and the China Science and Technology Journal database (VIP) that were available by April 3, 2021, using these keywords: "subthreshold depression" OR "subsyndromic depression" AND "randomized" OR "random."

Inclusion and Exclusion Criteria

Studies were included according to the following criteria:

(1) Participants: patients included met the following criteria: they were diagnosed as SD based on the Diagnostic and Statistical Manual of Mental Disorders criteria (Sheehan et al., 1998) or Center for Epidemiologic Studies Depression Scale (CES-D) score $\geq$16 (Shima et al., 1984), BDI score $\geq$14, 7 $\leq$ 17-item Hamilton rating scale for depression score <17, or 8 $\leq$ 24-item Hamilton rating scale for depression score <20.

(2) Intervention: interventions in the treatment groups included acupuncture (electroacupuncture, conventional acupuncture), cognitive behavioral treatment (web-based cognitive behavioral treatment, bibliotherapy-based cognitive behavioral treatment, telephone-based cognitive behavioral therapy), video viewing smartphone application, behavioral activation with mindfulness, behavioral activation program, mindfulness-based stress reduction, problem solving therapy for primary care, email-based promotion, collaborative care, brain electrical biofeedback therapy, tai chi, bright light therapy, dim light therapy, group counseling with Chinese medicine, drug therapy (peripateticism pill), and moxibustion (wheat-grain moxibustion).

(3) Comparators: patients in the control group (CG) were the ones in a waiting list for treatment, under usual care, or having no treatments.

(4) Outcomes: outcomes in this study included at least one of the following evaluation indicators: CES-D, BDI, the 9-item patient health questionnaire (PHQ-9), or the Kessler screening scale for psychological distress (K-6).

(5) Types of studies that were included: randomized controlled trials (RCTs). The following studies were excluded: non-RCTs, single-arm design trials, conference abstracts, systematic reviews, or meta-analyses.

Outcome Measures

We used CES-D as the primary outcome, with higher CES-D scores indicating more severe depressive symptoms. The secondary outcomes were BDI, PHQ-9, and K-6. The higher scores for BDI, PHQ-9 and K-6 indicate more severe depressive symptoms. The effect sizes for all outcomes were mean difference (MD) and 95% credible intervals (CrI).

Data Collection and Quality Assessment

Two investigators independently scanned the titles and abstracts to confirm that the remaining studies met the predefined eligibility criteria, and full-text reviews were conducted for all potentially included studies. The data was extracted using a standard table, including information on the characteristics of the population, intervention(s), comparison(s), and outcome(s). The

quality of each included trial was assessed by two authors based on the Cochrane Risk of Bias tool (Higgins et al., 2011).

Statistical Analysis

We conducted a Bayesian framework network meta-analysis by using R studio 4.0. The research data included in this network meta-analysis were evaluated by the Markov chain Monte Carlo method with 10,000 burn-ins and 50,000 iterations of four each chain and a thinning interval of 10 (Ades et al., 2006). A Brooks Gelman Rubin diagnostic plot was used to assess model convergence and the potential scale reduction parameter (PSRF) was used to evaluate the convergence of the results. When $1.00 \leq \text{PSRF} \leq 1.05$, it indicates that the results have good convergence and high reliability (Van Valkenhoef et al., 2012). I^2 statistic and its 95% CrI were used to measure statistical heterogeneity, which is considered substantial when $I^2 > 50\%$ (Higgins et al., 2003). Node-splitting analysis helps to determine the consistency test with an inconsistency model. In this study, a consistency model was chosen when the p-value of the node-splitting analysis was >0.05. If the p-value of the node-splitting analysis was <0.05, an inconsistency model was selected (Dias et al., 2010). We calculated the surface area under the cumulative ranking curves (SUCRA) to rank the curative effect of various interventions. The value range was 0–1. We used Begg's test and Egger's test to check publication bias, with a p-value of <0.05 indicating publication bias (Higgins et al., 2011). RevMan 5.3 software was used for bias risk assessment.

RESULTS

Research Identification and Selection

The literature search conducted retrieved 2,667 records. After deleting duplicates, 2,503 individual studies were recovered based on titles and abstracts. Among them, 125 studies were further selected for full-text review and 2,378 studies were removed due to the following reasons: non-SD, case report, animal models, republication, or review. Further examination excluded 104 studies according to the following reasons:

(1) The outcome was not the CES-D, BDI, PHQ-9, or K-6. (2) The studies were not RCTs. The remaining 21 studies were used for meta-analysis, including 5,048 patients (**Figure 1**). The characteristics of the selected trials are shown in **Table 1**.

Research Quality

We used the Cochrane Risk of Bias Tool to assess the quality of the above selected 21 studies. As shown in **Supplementary Figure 1**, 18 studies (85%) adopted a random sequence generation process using a computer random number generator or a random number table, 10 (47%) described the use of allocation or concealment methods, 5 (23%) described the blinding methods for researchers and participants, 16 (76%) described the blinding methods of outcomes assessment. In terms of incomplete outcome data, selective reporting and other bias, all included studies are assessed as low risk.

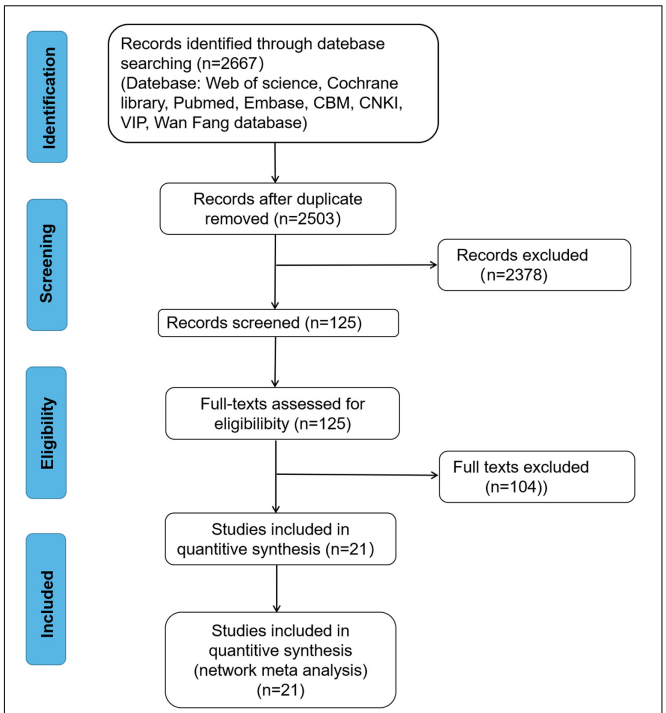


FIGURE 1 | Preferred reporting items for systematic reviews and meta-analysis (PRISMA) flow diagram.

Primary Outcome: Center for Epidemiologic Studies Depression Scale

In the network meta-analysis for the CES-D, 10 trials and 13 interventions were included, and the network plot is shown in **Figure 2A**. A forest plot showing the result of CG compared with other interventions is presented in **Figure 2B**. We found that most of the treatments show better efficacy on CES-D compared with CG alone. These interventions include electroacupuncture (MD −12.00, 95% CrI −15.00, −10.00), conventional acupuncture plus wheat-grain moxibustion (MD −9.70, 95% CrI −14.00, −5.30), the peripateticism pill plus group counseling with Chinese medicine (MD −9.00, 95% CrI −11.00, −6.70), cognitive behavioral treatment (MD −8.50, 95% CrI −13.00, −4.10), brain electrical biofeedback therapy instrument (MD −8.00, 95% CrI −10.00, −5.90), conventional acupuncture plus cognitive behavioral treatment (MD −7.60, 95% CrI −12.00, −3.00), conventional acupuncture (MD −7.60, 95% CrI −12.00, −3.50), group counseling with Chinese medicine (MD −5.00, 95% CrI −7.10, −3.00). SUCRA values is used to rank the curative effect of various treatments. As shown in **Figure 2C**, the results showed that electroacupuncture (2%) had the lowest SUCRA value across the various interventions, which means it had the highest probability of being the most effective treatment for CES-D. The following ranked treatments were conventional acupuncture plus wheat-grain moxibustion (16%), the peripateticism pill plus group counseling with Chinese medicine (24%), cognitive behavioral treatment (30%), brain electrical biofeedback therapy instrument (34%),

TABLE 1 Characteristic of included studies.

Study ID	Samples size	Scanning of SD	Treatment group interventions	Control group interventions	Course of treatment	Outcome
Tan, 2020	60	CES-D ≥ 16; 7 ≤ HAMD-17 < 17	CACUP + WGM	CACUP	4 weeks	CES-D
Xie, 2018	60	CES-D ≥ 16; 7 ≤ HAMD-17 < 17	EACUP	CG	6 weeks	CES-D
Li et al., 2017b	60	ZYYXH/T49-2008	PEPI + GCCM	GCCM	12 weeks	CES-D
Zhang, 2015	79	CES-D ≥ 16; 7 ≤ HAMD-17 < 17	CACUP/CBT/CACUP + CBT/CG		8 weeks/6 weeks	CES-D
Dong et al., 2015	60	CES-D > 16	BEBT	CG	3 weeks	CES-D
Tan et al., 2014	72	7 ≤ HAMD-17 < 17	GCCM	CG	8 weeks	CES-D
Ebert et al., 2018	204	CES-D ≥ 16	WCBT	CG	12 weeks	CES-D
Buntrock et al., 2015	406	CES-D ≥ 16	WCBT	WPE	3–6 weeks	CES-D
Joling et al., 2011	170	CES-D ≥ 16	BCBT	CG	12 weeks	CES-D
Kageyama et al., 2021	32	CES-D ≥ 16	VVSA	CG	8 weeks	CES-D, K-6
Jiang et al., 2020	142	CES-D ≥ 16; BDI-II ≥ 14	BLT/DLT/CG		8 weeks	BDI-II
Spek et al., 2007	301	CES-D ≥ 12	WCBT/GCBT/CG		10 weeks	BDI-II
Furukawa et al., 2012	118	BDI-II ≥ 10; K-6 ≥ 9	TCBT	CG	8 weeks	BDI-II, K-6, PHQ-9
Kasckow et al., 2014	23	CES-D ≥ 11	PST-PC	CG	6–8 weeks	BDI-II
Zhang et al., 2019	56	BDI > 14; SDS > 53	MBSR	CG	8 weeks	BDI-II
Takagaki et al., 2018	118	BDI-II ≥ 10	BAP	CG	5 weeks	BDI-II
Imamura et al., 2014	762	NM	WCBT	CG	6 weeks	BDI-II, K-6
Wong et al., 2018	231	5 < PHQ-9 < 9	BAM	CG	8 weeks	BDI-II
Xie, 2020	63	CES-D ≥ 16	Tai Chi	CG	12 weeks	PHQ-9
Morgan et al., 2012	1326	5 < PHQ-9 < 9	EBP	CG	6 weeks	PHQ-9
Gilbody et al., 2017	705	DSM-IV	COC	CG	8 weeks	PHQ-9

SD, subthreshold depression; CES-D, Center for Epidemiologic Studies Depression Scale; HAMD, Hamilton Rating Scale for Depression; BDI, Beck Depression Inventory Scale; K-6, Kessler Screening Scale for Psychological Distress; SDS, Self-rating Depression Scale; PHQ-9, the 9-item Patient Health Questionnaire; DSM-IV, Diagnostic and Statistical Manual of Mental Disorders (Fourth Edition); ZYYXH/T49-2008, Chinese society of traditional Chinese medicine issued the industry standard of depression; CACUP, conventional acupuncture; WGM, wheat-grain moxibustion; CG, control group; EACUP, electroacupuncture; PEPI, peripateticism pill; GCCM, group counseling with Chinese medicine; CBT, cognitive behavioral treatment; BEBT, brain electrical biofeedback therapy; WCBT, web-based cognitive behavioral treatment; BCBT, bibliotherapy-based cognitive behavioral treatment; VVSA, video viewing smartphone application; BLT, bright light therapy; DLT, dim light therapy; GCBT, group cognitive behavioral treatment; TCBT, telephone-based cognitive behavioral treatment; PST-PC, problem solving therapy for primary care; MBSR, mindfulness-based stress reduction; BAP, behavioral activation program; BAM, behavioral activation with mindfulness; EBP, email-based promotion; COC, collaborative care; WPE, web-based psycho education; NM, not mentioned.

conventional acupuncture plus cognitive behavioral treatment (40%), conventional acupuncture (41%), web-based cognitive behavioral treatment (52%), group counseling with Chinese medicine (62%), video viewing smartphone application (82%), bibliotherapy-based cognitive behavioral treatment (85%), and web-based psycho-education (90%).

Secondary Outcome: Beck Depression Inventory Scale

Eight trials and ten interventions were involved in measuring BDI outcome. The network geometry is presented in **Figure 3A**. From the forest plot of **Figure 3B**, we found that bright light therapy (MD −9.70, 95% CrI −13.00, −6.00), behavioral activation program (MD −5.70, 95% CrI −6.10, −5.40), dim light therapy (MD −6.30, 95% CrI −10.00, −2.20), telephone-based cognitive behavioral treatment (MD −4.70, 95% CrI −5.30, −4.10), group cognitive behavior treatment (MD −2.30, 95% CrI −4.60, 0.10), behavioral activation with mindfulness (MD −1.90, 95% CrI −4.40, 0.61), web-based cognitive behavioral treatment (MD −1.30, 95% CrI −2.40, −0.18), and mindfulness-based stress reduction (MD −1.20, 95% CrI −2.40, 0.03) had better efficacy compared with CG alone on BDI. As shown in **Figure 3C**, Bright light therapy (1%) showed the lowest SUCRA value in all treatments, which indicates that it had the highest probability of being the most effective treatment for BDI for SD. The following ranked treatments were behavioral activation program (18%), dim light therapy (19%), telephone-based cognitive behavioral treatment (32%), group cognitive behavior treatment (56%), behavioral activation with mindfulness (63%), web-based cognitive behavioral treatment (71%), problem solving therapy for primary care (72%), and mindfulness-based stress reduction (73%).

Secondary Outcome: Patient Health Questionnaire-9

To evaluate the PHQ-9 outcome, three studies and four interventions were included. The network plot is shown in **Figure 4A**. As shown in the forest plot of PHQ-9 (**Figure 4B**),

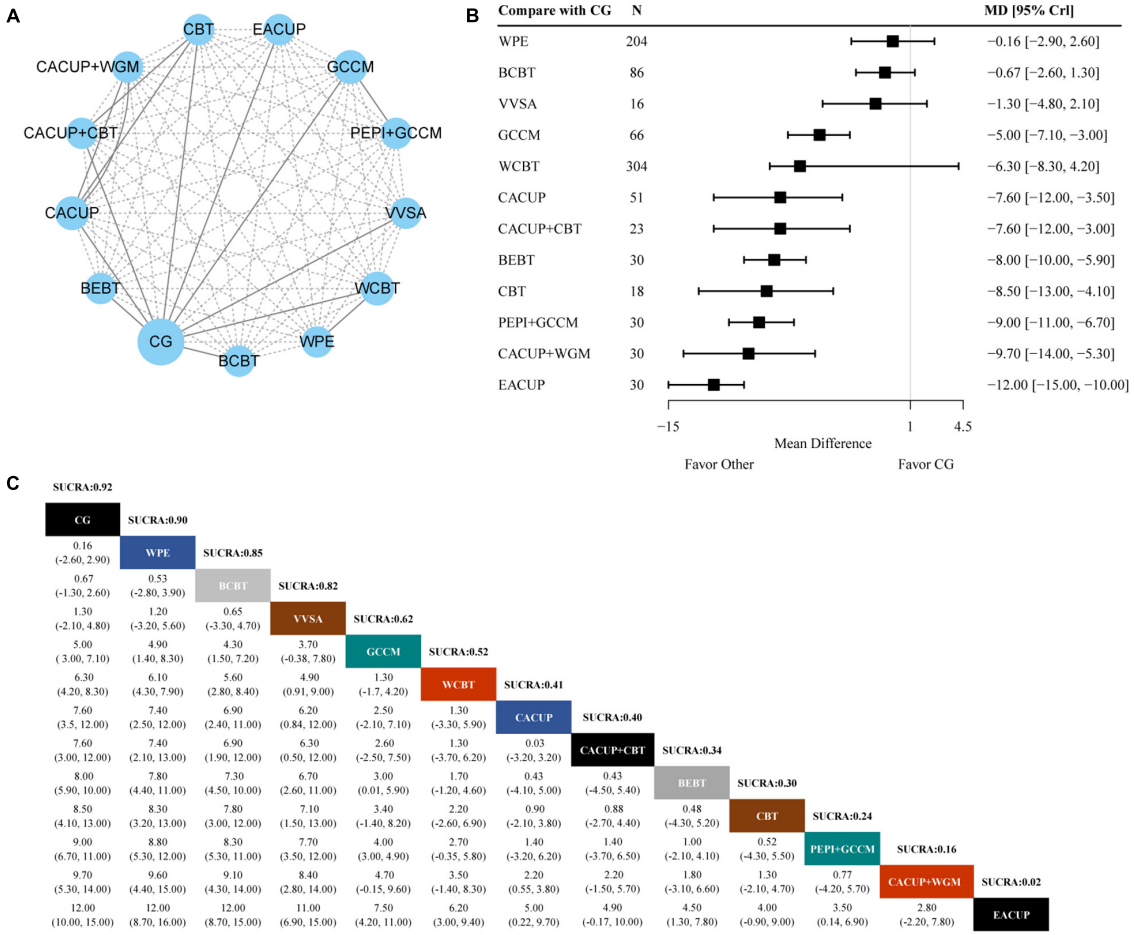


FIGURE 2 | Results of the network meta-analysis for CES-D. **(A)** Network geometry of eligible comparisons for CES-D. **(B)** Forest plot of the network meta-analysis compared with control group. **(C)** Ranking of each intervention based on the SUCRA values and the league table for the relative effects of all treatments. EACUP, electroacupuncture; CACUP, conventional acupuncture; WGM, wheat-grain moxibustion; GCCM, group counseling with Chinese medicine; CBT, cognitive behavioral treatment; BEBT, brain electrical biofeedback therapy; WCBT, web-based cognitive behavioral treatment; VVSA, video viewing smartphone application; BCBT, bibliotherapy-based cognitive behavioral treatment; WPE, web-based psycho education; CG, control group; SUCRA, surface under the cumulative ranking curve; MD, weighted mean difference; CrI, credible interval.

tai chi (MD −3.00, 95% CrI −4.00, −2.00), collaborative care (MD −1.30, 95% CrI −1.40, −1.20), and email-based promotion (MD −0.80 95% CrI −1.30, −0.27) showed better efficacy in PHQ-9 compared with CG alone. As shown in **Figure 4C**, tai chi (0%) had the lowest SUCRA value of the four interventions, which indicated that it had the highest probability of being the most effective treatment for PHQ-9 for SD. Other following effective treatments were collaborative care (34%) and email-based promotion (66%).

Secondary Outcome: Kessler Screening Scale for Psychological Distress

There were three trials and four interventions that measured a K-6 outcome. The network plot is presented in **Figure 5A**. As indicated in the forest plot for K-6 (**Figure 5B**), telephone-based cognitive behavioral treatment (MD −2.50 95% CrI −2.70, −2.30) had better efficacy compared with CG alone. As shown in **Figure 5C**, telephone-based cognitive behavioral treatment (0%) had the lowest SUCRA values among the four interventions, which indicated that it had the highest probability of being the most effective treatment for K-6. The outcome from other two treatments were web-based cognitive behavioral treatment (60%) and video viewing smartphone application (62%), respectively.

Model Convergence, Heterogeneity, and Publication Bias

The shrinking factors in the Brooks Gelman Rubin diagnostic plots for all outcomes were less than 1.05 (**Supplementary Figure 2**). The statistical heterogeneity for all outcomes were low (I^2 < 50%, ranging from 5% to 17%). According to results of Begg's and Egger's tests with a funnel plot, no publication bias was detected (p > 0.05) in our study (**Supplementary Figure 3** and **Supplementary Table 1**). There was no obvious inconsistency shown in this network meta-analysis.

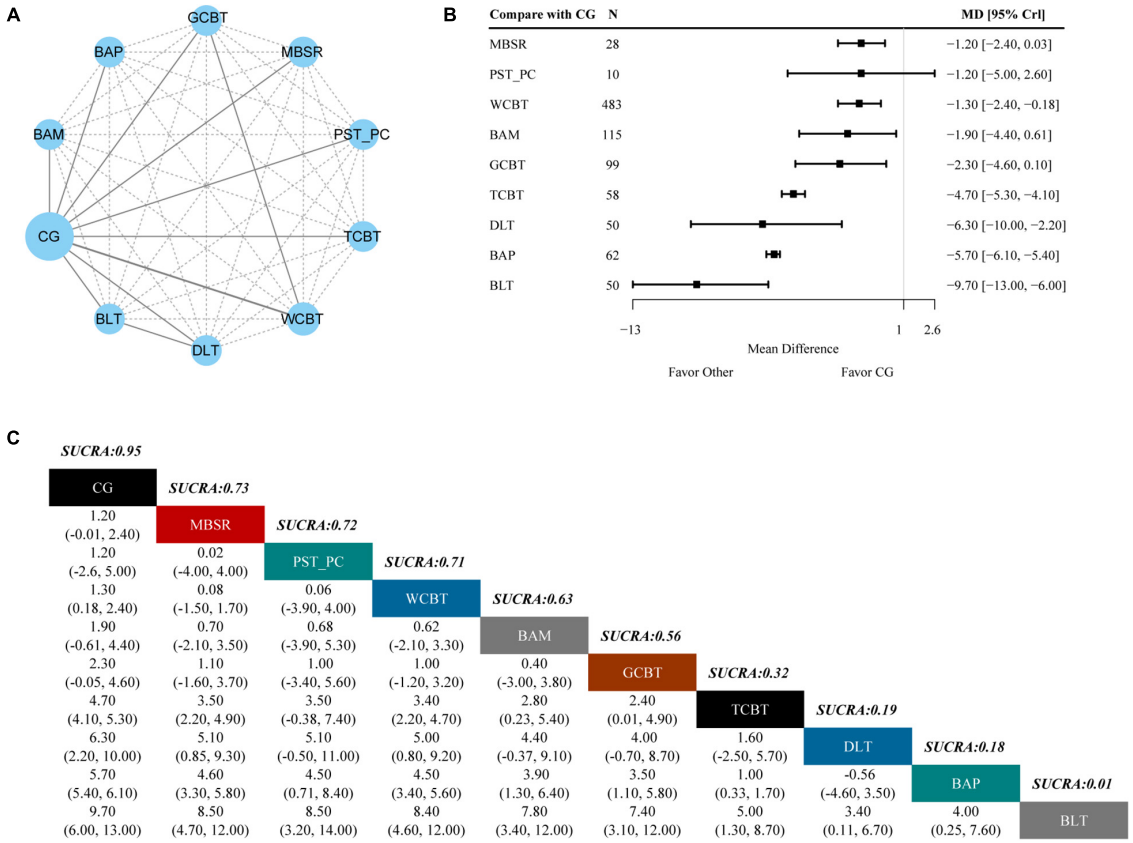


FIGURE 3 | Results of the network meta-analysis for BDI. **(A)** Network geometry of eligible comparisons for BDI. **(B)** Forest plot of the network meta-analysis compared with control group. **(C)** Ranking of each intervention based on the SUCRA values and the league table for the relative effects of all treatments. BLT, bright light therapy; DLT, dim light therapy; BAP, behavioral activation program; TCBT, telephone-based cognitive behavioral treatment; GCBT, group cognitive behavioral treatment; BAM, behavioral activation with mindfulness; WCBT, web-based cognitive behavioral treatment; PST-PC, problem solving therapy for primary care; MBSR, mindfulness-based stress reduction; CG, control group; SUCRA, surface under the cumulative ranking curve; MD, weighted mean difference; CrI, credible interval.

DISCUSSION

In this network meta-analysis, our results provided evidence that all the involved treatments for patients with SD showed advantages over waiting for treatment or no treatment. Furthermore, we found that electroacupuncture was the best therapy to improve outcome on the CES-D. Meanwhile, bright light therapy was the optimum treatment for the outcome on the BDI and tai chi was best effective in promoting outcome on the PHQ-9. However, telephone-based cognitive behavioral treatment was the best intervention for outcome on the K-6.

Electroacupuncture has been widely used to treat psychiatric conditions including depression (Woo et al., 2015), which may be associated with multiple mechanisms. First, relevant research found that the serotonin system plays an important role in the pathophysiology of depression (Mouri et al., 2016). Electroacupuncture was reported to restore hippocampal CA1 synaptic plasticity by regulating the level of serotonin system receptors, thereby improving depression-like behavior (Han et al., 2018). Second, it has been found that proinflammatory cytokines play an important role in neurogenesis and neuroprotection (Kim et al., 2016). Patients with depression have high levels of pro-inflammatory cytokines, acute phase proteins, chemokines, and cell adhesion molecules (Raison et al., 2006). Electroacupuncture reduced hippocampal neuroinflammation in depressed rats by reducing the expression of pyrin domain-containing protein 3 inflammatory components (ASC and caspase-1), activating microglia and ATP gated transmembrane protein (P2 × 7) receptor as well as reducing levels of interleukin-1 beta (IL-1β), IL-18, IL-6, and tumor necrosis factor alpha (Yue et al., 2018; Li et al., 2021). Furthermore, hypothalamic pituitary adrenal axis dysregulation, which is generally considered a diagnostic criterion for early stages of depression, is also implicated in the pathology of depression (Du and Pang, 2015). Studies have shown that electroacupuncture could exert anti-depressive activity by regulating the hypothalamic pituitary adrenal axis (Le et al., 2016).

Consistent with our results, light therapy has been widely used to treat seasonal depression (Avissar et al., 1999). Light can affect mood and learning through different retinal brain pathways (Fernandez et al., 2018). The antidepressant effects of light therapy have been found to be related to changes in neurotransmitters, including serotonin and catecholamine

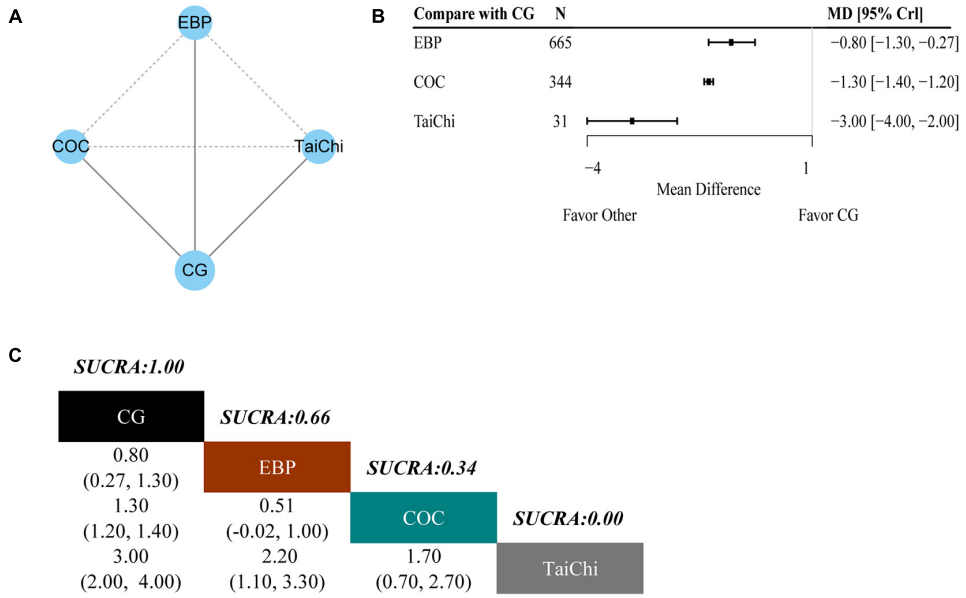


FIGURE 4 | Results of the network meta-analysis for PHQ-9. **(A)** Network geometry of eligible comparisons for PHQ-9. **(B)** Forest plot of the network meta-analysis compared with control group. **(C)** Ranking of each intervention based on the SUCRA values and the league table for the relative effects of all treatments. EBP, email-based promotion; COC, collaborative care; CG, control group; SUCRA, surface under the cumulative ranking curve; MD, weighted mean difference; CrI, credible interval.

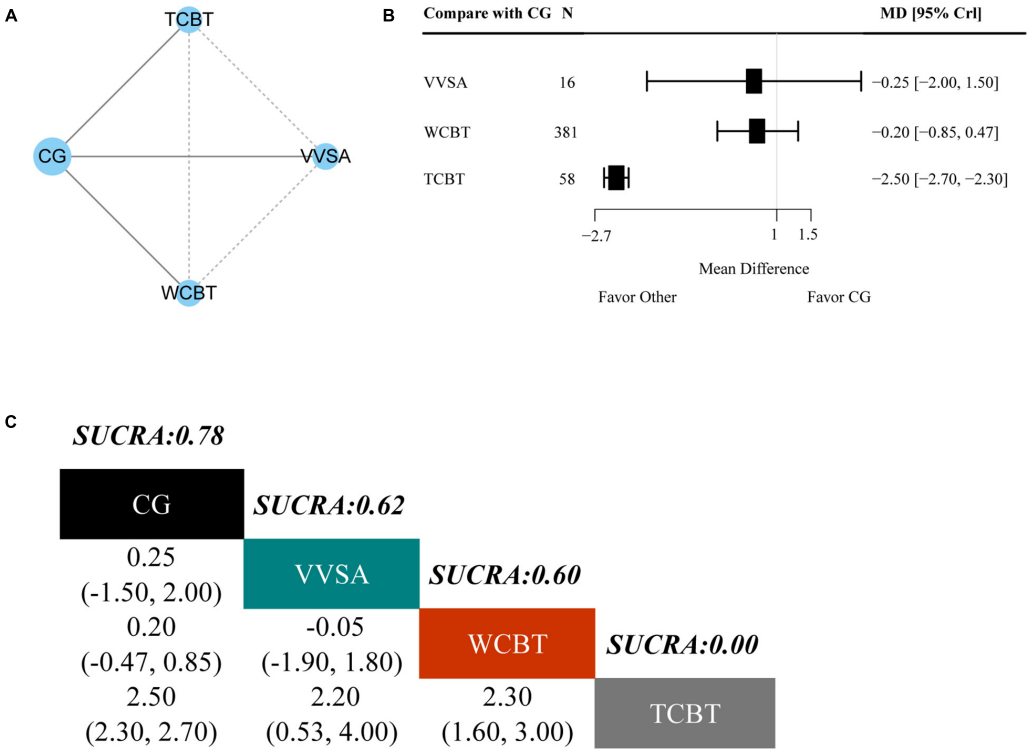


FIGURE 5 | Results of the network meta-analysis for K-6. **(A)** Network geometry of eligible comparisons for K-6. **(B)** Forest plot of the network meta-analysis compared with control group. **(C)** Ranking of each intervention based on the SUCRA values and the league table for the relative effects of all treatments. TCBT, telephone-based cognitive behavioral treatment; WCBT, web-based cognitive behavioral treatment; VVSA, video viewing smartphone application; CG, control group; SUCRA, surface under the cumulative ranking curve; MD, weighted mean difference; CrI, credible interval.

(Lam et al., 1996; Neumeister, 2003). Previous studies also found that light therapy could synchronize the biological clock with the circadian rhythm of the environment, which is considered to be closely related to depression (Lam and Levitan, 2000; Pail et al., 2011; Jagannath et al., 2013). Furthermore, light therapy has the advantages of low cost, high security, and direct availability (Bais et al., 2016). Together, all these studies provide further evidence that light therapy may be the optimal treatment for patients with SD.

As an adjuvant therapy for depression, physical exercise has attracted increasing attention (Legrand and Neff, 2016). One study found that aerobic exercise can increase the levels of brain-derived neurotrophic factor, which has been shown to decrease in individuals with severe depression (Bocchio-Chiavetto et al., 2010). Tai chi was reported to ameliorate the depressive symptoms of elderly Chinese patients (Cho, 2008). Meanwhile, other studies provide evidences that tai chi could regulate brain networks associated with depression and alleviate depressive symptoms by regulating cortisol levels and immune system (Walther et al., 2018; Kong et al., 2019). However, given that the sample sizes of clinical research studies were small, whether tai chi has a unique benefit for treatment of SD over other physical exercise requires further study.

Our results suggested that telephone-based cognitive behavioral treatment is the best treatment for improving K-6, which is used to screen for mental health problems and measure the severity of the impact of these problems (Mitchell and Beals, 2011). Telephone-based cognitive behavioral treatment is a novel method for the treatment of SD (Furukawa et al., 2012). It may overcome some of the obstacles preventing patients from receiving traditional psychotherapy services, including occupational or social constraints, residency in underserved areas, and the need to commute, and therefore may be a more appropriate choice in these situations (Bee et al., 2008). Taken together, our findings suggest that more attention should be paid to the application of telephone-based cognitive behavioral treatment.

There were some limitations in our study. First, many RCT studies did not include PHQ-9 and K-6, which limited the comprehensive evaluation of the efficacy on the two outcomes. Second, parts of the quality assessment risk were unsatisfactory because of the characteristics of waiting for treatment or no treatment, it hard to blind both therapist and participants. Finally, given that the number of patients included was relatively small, more multi-center and high-quality RCTs are needed in the future to validate our findings.

CONCLUSION

The results of this comprehensive network meta-analysis provides a complete evaluation of currently available therapies for patients with SD. Our results suggest that electroacupuncture or bright light therapy may be the preferred selection in the treatment of SD. This study provide new insights into clinical treatment selection and may help the development of guidelines for the management of SD.

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

YJC and XJ designed this network meta-analysis. XJ, YL, YWC, JY, and SH collected the data. XJ, YX, XW, YL, and YWC analyzed the data. XJ, LY, FW, and TW performed the analysis. XJ and YJC wrote the manuscript. All authors contributed to writing of this manuscript and approved the final version of the 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/fnbeh.2021.755547/full#supplementary-material

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Early Enhancement of Neuroplasticity Index, the Ratio of Serum Brain-Derived Neurotrophic Factor Level to HAMD-24 Score, in Predicting the Long-Term Antidepressant Efficacy

Yuxuan Zhang[1†], Bo Cui[2†], Tianyu Wang[3,4†], Yan Lu[5], Zhenlin Chen[5], Zhilu Zou[6], Jinlin Miao[5], Xiuli Zhao[5], Yonggui Yuan[3,4], Haosen Wang[5] and Gang Chen[2,7*]*

[1] Key Laboratory of Integrative Biomedicine for Brain Diseases, Center for Translational Systems Biology and Neuroscience, Nanjing University of Chinese Medicine, Nanjing, China, [2] Interdisciplinary Institute for Personalized Medicine in Brain Disorders, Jinan University, Guangzhou, China, [3] Department of Psychosomatics and Psychiatry, School of Medicine, Zhongda Hospital, Southeast University, Nanjing, China, [4] School of Medicine, Institute of Psychosomatics, Southeast University, Nanjing, China, [5] The Fourth People's Hospital of Taizhou, Taizhou, China, [6] Hubei University of Chinese Medicine, Wuhan, China, [7] Co-innovation Center of Neuroregeneration, Nantong University, Nantong, China

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Nuno Sousa,
University of Minho, Portugal

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Shaowu Cheng,
Hunan University of Chinese Medicine, China
Guo-qing Zheng,
Zhejiang Chinese Medical University, China

***Correspondence:**
Gang Chen
hdn_2001@yahoo.com
Haosen Wang
billet1116@163.com

[†] These authors have contributed equally to this work

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Background: Current mainstream treatment of major depressive disorder (MDD) has a disadvantage in delayed onset of efficacy, making detection of early signatures predicative of the long-term treatment efficacy urgent.

Methods: MDD patients were scored with HAMD-24 and serum brain-derived neurotrophic factor (BDNF) levels were measured at different times in two independent trials: a single-arm observation of Yueju pill, a clinically approved traditional multiherbal medicine, and a two-arm random placebo-controlled trial for Yueju vs escitalopram. The ratio of the BDNF level to HAMD-24 score, or neuroplasticity index (NI), and its derived parameters were used for correlation analysis and receiver operating characteristic (ROC) analysis.

Results: On both the early (4th) and final (28th) days, Yueju and escitalopram significantly reduced HAMD-24 scores, compared to baselines, but only Yueju increased BDNF at both times. For either Yueju or escitalopram treatment, NI, but not BDNF, at baseline was correlated to NIs at the early or final treatment day. NI at early time was significantly correlated to early NI enhancement from the baseline for both Yueju and escitalopram, and to final NI enhancement from the baseline for Yueju in both trials. ROC analysis supported the predictability of Yueju's final treatment efficacy from early NI enhancement.

Limitations: The small sample size and 28 days of treatment time may lead to the impossibility of ROC analysis of escitalopram.

Conclusion: Early NI enhancement is useful for prediction of long-term efficacy of Yueju and presumably some other antidepressants.

Clinical Trial Registration: [www.ClinicalTrials.gov], identifier [ChiCTR1900021114].

Keywords: Yueju pill, BDNF, antidepressant efficacy, prediction, neuroplasticity index

INTRODUCTION

The major depressive disorder (MDD), or depression, is characterized by the loss of pleasure or interest in everyday activities, as well as other characteristics, including changes in sleep and appetite, low motivation, and suicidal ideation or action (Organization American Psychiatric, 2013; Otte et al., 2016). Current conventional antidepressants, mainly selective serotonin reuptake inhibitors (SSRIs), only have moderate rates of response and remission, with some unwanted side effects (Kogoj, 2014). Usually, long-term treatment period is required before the antidepressant effects of SSRIs can be elicited symptomatically (Agius and Bonnici, 2017). As depression is a progressive disease, the long lag period of the efficacy may lead to the miss of the optimal window for the intervention (Lane, 1998). Therefore, detection of early markers that can predict the treatment outcome is important for the improvement of the treatment. In the past decades, great efforts have been made to find the potential early biomarkers, including neurotropic factors, neurotransmitters, cytokines, as well as other molecules in the blood (Nutt, 2008; Dowlati et al., 2010; Tavakolizadeh et al., 2018; Xu et al., 2019). However, up to date, none of the individual predictors is robust enough to guide first-line treatment options (Kessler, 2018).

Brain-derived neurotrophic factor (BDNF) is a neurotrophic factor critical for the viability of neurons in neural circuit (Monteggia et al., 2007). A number of studies have shown that BDNF is profoundly implicated in depression (Peng et al., 2018; Koo et al., 2019; Szuhany and Otto, 2020). Studies have shown that BDNF levels are lower in depression patients than healthy people (Karege et al., 2002). Decreasing BDNF expression leads to deficient neural plasticity in the brain of patients with MDD, which makes it a potential biomarker to monitor these diseases (Peng et al., 2018). BDNF in serum or plasma is increasingly used as a putative biomarker for depression, and some studies revealed that lower levels of BDNF in the blood of depressed patients are improved after long-term treatment of certain antidepressants (Shimizu et al., 2003; Gazal et al., 2012; Vanicek et al., 2019). However, a considerable number of studies demonstrated no effect or even opposite findings (Kheirouri et al., 2016; Ryan et al., 2018). Therefore, whether the changes of blood BDNF levels at the early time of antidepressants treatment alone predict the long-term treatment efficacy remains elusive (Kessler, 2018; Xu et al., 2019).

Traditional medicine has been used as an alternative monotherapy or an adjunct to conventional antidepressant therapy, whose efficacy and safety has been supported on the basis of increasing number of clinical trials (Zhang et al., 2007; Qin et al., 2011; Wang et al., 2012; Yang et al., 2020). Yueju pill is a multiherbal pill formulated 800 years ago to treat syndromes associated with mood disorders (Ren and Chen, 2017; Wang et al., 2019). It contains multiple antidepressant components and some of the bioactive compounds have been identified (Wei et al., 2008; Zhang et al., 2015; Wu et al., 2016). More recently, a number of preclinical studies using various animal models demonstrated that a single high dose of Yueju was capable to elicit antidepressant activity in a fast-onset and persistent manner (Xue et al., 2013), hippocampus is one of many regions that are involved in depression-related behavior (Ren et al., 2016; Xia et al., 2016). A pilot perspective random controlled clinic trial showed that the use of high dose of Yueju contributed to the early onset of the alleviation of depressive symptoms, starting at 4 days after treatment together with fluoxetine (Wu et al., 2015). Additionally, chronic treatment of conventional low dose of Yueju pill also elicited antidepressant activity, with overall superior efficacy to fluoxetine, in an animal model of depression (Zou et al., 2017). Both single high dose and repeated low dose of Yueju pill significantly induce hippocampal BDNF synthesis through transcriptional and translational mechanisms (Xue et al., 2013).

Here, in a clinical observation of the effect of low dose of Yueju pill treatment, we disclosed that the ratio of serum BDNF level to the scale of depression using the scores of HAMD-24, defined as neuroplasticity index (NI), may be useful for prediction of the antidepressant treatment outcome. We thus carried out a prospective random placebo-controlled clinical trial, in which the monotherapy of Yueju pill was compared with the mainstream antidepressant escitalopram. We assessed the correlation of baseline NI with the changes of NI at different treatment times. The results indicated significant correlations of NI or their changes at different times post treatment of Yueju as well as escitalopram. Additionally, the change of NI at the early time may be used for prediction of the long-term antidepressant efficacy.

MATERIALS AND METHODS

Experimental Design

This study consisted of two trials and was approved by the Institutional Review Board of The Fourth People's Hospital of Taizhou (**Figure 1**). The first trial was an open-labeled exploratory single-arm trial. Patients (HAMD-24 scores (20) were recruited (Addington et al., 1996) and treated with the conventional dose of Yueju pill. The second trial was a double-blinded, random and placebo-controlled parallel one. Patients were diagnosed with MDD, and randomly assigned to Yueju pill and escitalopram treatment groups. The procedures were conducted according to institutional guidelines, and all participants have provided written informed consent (Chinese Clinical Trial Registry: ChiCTR1900021114).

Trial 1: Exploratory Single-Arm Trial

The patients, who were consecutive recruited from August, 2016 to December, 2016, were diagnosed according to the DSM-IV of MDD (**Table 1**). The enrollment criteria were as follow: (1) 18–65 years old, (2) no psychotic characteristics but with diagnostic criteria for depression (DSM-IV), (3) no psychotropic medications previously, (4) HAMD-24 scores $\geq$20, (5) QIDS-SR16 scores $\geq$5, (6) never exposed to psychotropic drugs, (7) have not received antidepressant treatment within 1 week.

Treatments and Measurements

The medicinal plants used to prepare Yueju are *Cyperus rotundus* L. (Xiang Fu), *Ligusticum chuanxiong* Hort (Chuan Xiong),

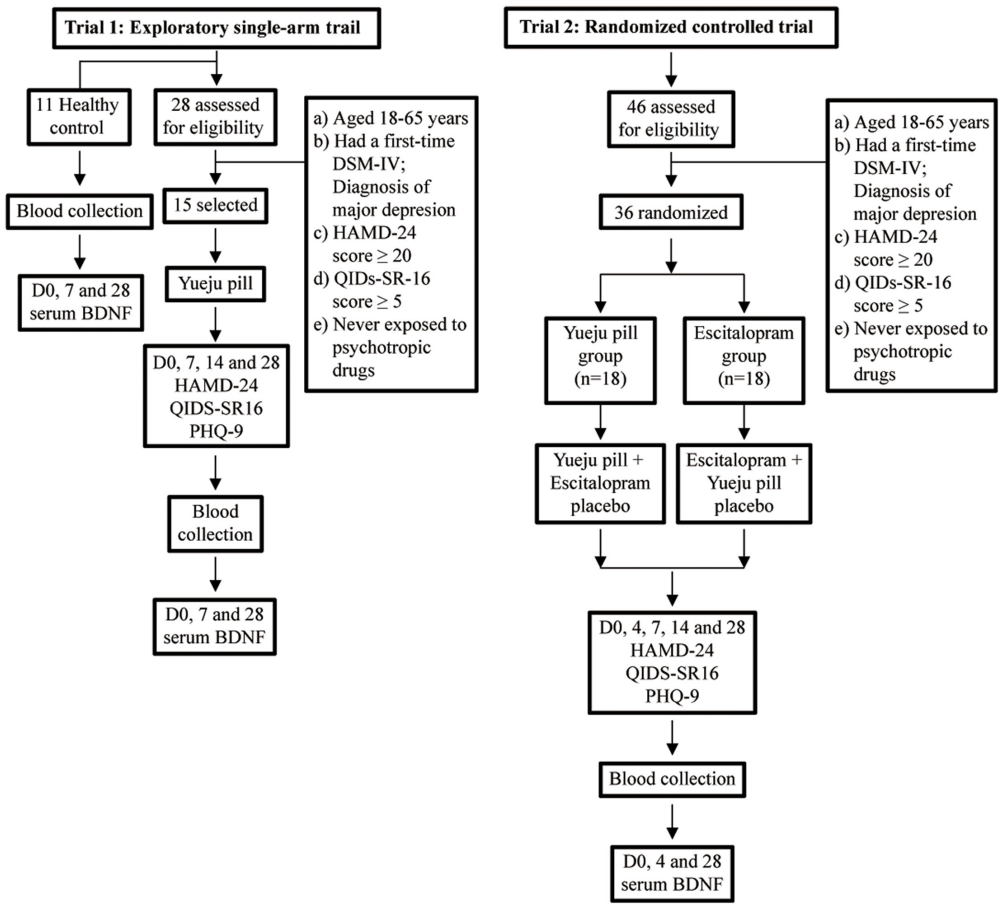

FIGURE 1 | Flowchart of enrollment, randomization, discontinuation, and completion of the treatment phases. HAMD-24, QIDS-SR16, and PHQ-9 scores and serum BDNF level were collected as indicated.

Gardenia jasminoides J. Ellis. (Zhi Zi), *Atractylodes lancea* (Thunb.) DC. (Cang Zhu), and *Massa Fermentata* (Shen Qu) (Ren and Chen, 2017). Patients who met the enrollment criteria were enrolled and treated with Yueju pills (12 g/day, Jiangsu 707 Natural Pharmaceutical Co., Ltd., approval number, Z32020738). On Day 7 (D7), D14, and D28, scales of depression were assessed with the HAMD-24, which was scored by a practicing physician, as well as QIDS-SR16 and PHQ-9, which were scored by the patients themselves. On the baseline day D0, as well as post treatment days of D7 and D28, peripheral blood was drawn for BDNF measurements. The peripheral blood samples were also collected from healthy counterpart controls.

TABLE 1 | Demographic data of the enrolled subjects with MDD or healthy controls.

Trials	Groups	Number	Male	Female	Age (years)
Trial 1 Open labeled	Yueju pill	15	1	14	42.93 ± 3.21
	Healthy control	11	6	5	41.7 ± 3.06
Trial 2 Double blinded	Yueju pill	18	5	13	45.17 ± 3.26
	Escitalopram	18	5	13	43.39 ± 3.51

Trial 2: Randomized Controlled Trial

Patients were consecutive recruited from March, 2017 to January, 2018, were diagnosed according to the DSM-IV of MDD (**Table 1**). The enrollment criteria were as follow: (1) 18–65 years old, (2) no psychotic characteristics but with diagnostic criteria for depression (DSM-IV), (3) no psychotropic medications previously, (4) HAMD-24 scores ≥20, (5) QIDS-SR16 scores ≥5, (6) never exposed to psychotropic drugs, (7) have not received antidepressant treatment within 1 week, (8) emmetropia and right hand. Patients who met the enrollment criteria were randomly assigned into two groups according to the enrollment order. The patients in the experimental group were treated with Yueju pills and the placebo for escitalopram. The patients in the escitalopram group were treated with escitalopram and herbal placebo. The placebo was prepared to be identical to the Yueju pill or escitalopram in shape, size, and color. The drugs and placebo are placed in the same packaging and the same box.

Treatments and Measurements

Yueju group: To accelerate the antidepressant response and then maintain the antidepressant activity, the patients took a high dose (23 g/time/day) of Yueju during the first week, followed by the

dose of 12 g/time/day of Yueju from the second to fourth week. *Escitalopram group*: Escitalopram was made by Yamagata Pharma Co., Ltd. (National Medicine Standard H20080599). Patients took the escitalopram or corresponding placebo 10 mg twice a day, whereas Yueju pills and the herbal placebo were taken once a day after dinner. HAMD-24, QIDS-SR16, and PHQ-9 scales were scored on D0, D4, D7, D14, and D28. Peripheral blood was drawn for BDNF level testing on D0, D4, and D28.

Blood Samples and Brain-Derived Neurotrophic Factor Measurements

Peripheral blood extractions in patients or healthy controls were performed between 9 and 10 am to minimize the effects of possible circadian rhythm alterations and the blood samples were collected into coagulation-promoting tubes. Patients were not exposed to drugs known to affect the coagulation system within 10 days prior to blood extraction. The blood samples were centrifuged (1,000 × *g* for 20 min) immediately and serum samples were collected and stored at −80°C until further analysis. BDNF level was measured using an anti-BDNF enzyme-linked immunosorbent assay (ELISA) kit (Boster Biological Technology Co., Ltd.) according to the manufacturer's instructions. Diluted serum (1:20) with sample buffer was used to conduct BDNF ELISA in duplicate. BDNF standard solution was diluted to concentrations from 0 to 4,000 pg/ml to create the standard curve. Four patients in each group of Trial 2 failed to take the blood for this test.

Statistics Analysis

All statistical analyses were conducted with SPSS 19.0 statistical software (Statistical Package for the Social Sciences, SPSS. Inc., Chicago, IL, United States), GraphPad Prism 6 (GraphPad Software, San Diego, CA, United States) and MedCalc statistical software (MedCalc Software Ltd., Ostend, Belgium). Analyses of variance with repeated measures as well as Pearson's correlations were used as appropriate. Pearson's correlations were performed with the raw values. For the receiver operating characteristic (ROC) analysis, HAMD-24 reduction rate (60% within 4 weeks) was defined to be quasi-effective. ROC curves were drawn to evaluate the prediction of the data collected in this study. Significance was evaluated at $p < 0.05$, two tailed. Data are reported as means ± SE.

RESULTS

Both Yueju Pill and Escitalopram Demonstrated Early Improvement and Continuous Antidepressant Effects

In Trial 1, 15 MDD patients were treated with low dose of Yueju pill for 4 weeks. Based on the analysis of HAMD-24 scores, there was a significant effect on time [$F(1.377,9.28) = 77.51$, $p < 0.001$, **Figure 2A**]. *Post hoc* tests indicated a trend of improvement of D7, and the significant improvement from D14 to D28 of treatment, compared to D0 (D7, $p = 0.116$; D14, $p < 0.001$, and D28, $p < 0.001$). The analysis of QIDS-SR16 [$F(1.534,21.48) = 6.83$, $p < 0.001$] showed the similar results, with a significant improvement from D14 to D28 (D7, $p = 0.152$, D14, $p = 0.002$, and D28, $p < 0.001$) (**Figure 2B**). For PHQ-9 [$F(1.699,23.78) = 26.63$, $p < 0.001$], the significant improvement was only on D28 (D7, $p = 0.653$, D14, $p = 0.211$, and D28, $p < 0.001$) (**Figure 2C**). The results from this observation demonstrated an antidepressant efficacy, with a plausible early symptomatic improvement.

In Trial 2, 36 patients were randomly assigned into placebo-controlled groups of escitalopram or Yueju (with high dose for the first week, followed with low dose for 3 weeks). For HAMD-24 scores, there was a significant effect for the interaction between drug and time [$F(4,68) = 2.854$, $p = 0.03$] and for time only [$F(4,68) = 148.8$, $p < 0.001$], but not for treatment only [$F(1,17) = 0.507$, $p = 0.486$]. Patients in Yueju group showed significant improvement at all times from D4 to D28, in comparison to D0 ($p < 0.001$ for D4, D7, D14, and D28, respectively). Similarly, escitalopram group showed significant improvement at all different time points (D4, $p = 0.013$; $p < 0.001$ for D7, D14, and D28, respectively). QIDS-SR16 scores started to improve from the fourth day and continued to the 28th day (**Figures 2D,E**). For PHQ-9 scores, Yueju group showed significant improvement at Day 7 (**Figure 2F**), whereas the escitalopram group started it at Day 14 (**Figure 2F**). These data indicate that escitalopram and initial high dose of Yueju elicited early antidepressant efficacy, and the significant improvement within the first 4 weeks were appreciable for both Yueju and escitalopram.

The Serum Brain-Derived Neurotrophic Factor Levels at the Early and Final Treatment Time Were Improved in Yueju but Not Escitalopram Group

In Trial 1, serum BDNF level before treatment of 15 selected patients is much lower than the healthy controls (**Figure 3A**). The BDNF levels showed a significant effect on time [$F(14,28) = 6.352$, $p < 0.001$]. *Post hoc* tests showed a significant improvement on day 7 and day 28 (D7, $p = 0.042$ and D28, $p = 0.023$), compared with D0, and BDNF levels for both D7 and D28 after Yueju treatment were comparable to healthy controls ($p > 0.05$) (**Figure 3A**).

In Trial 2, there was a significant interaction between treatment and time [$F(2,26) = 8.234$, $p = 0.002$], but not for time alone [$F(2,26) = 1.801$, $p = 0.185$] or drug alone [$F(1,13) = 0.729$, $p = 0.409$]. Only Yueju group showed significant changes overtime [$F(13,26) = 7.192$, $p = 0.0037$], and there was a significant improvement on D4, $p = 0.037$ and on D28, $p = 0.004$. In escitalopram group, the BDNF levels did not have a significant change overtime on time [$F(13,26) = 1.232$, $p = 0.3056$] (**Figure 3B**).

Correlation Analysis of Neuroplasticity Index and Neuroplasticity Index Enhancement

No significant correlation between serum BDNF levels with the degree of depression was found when the data from the two trials

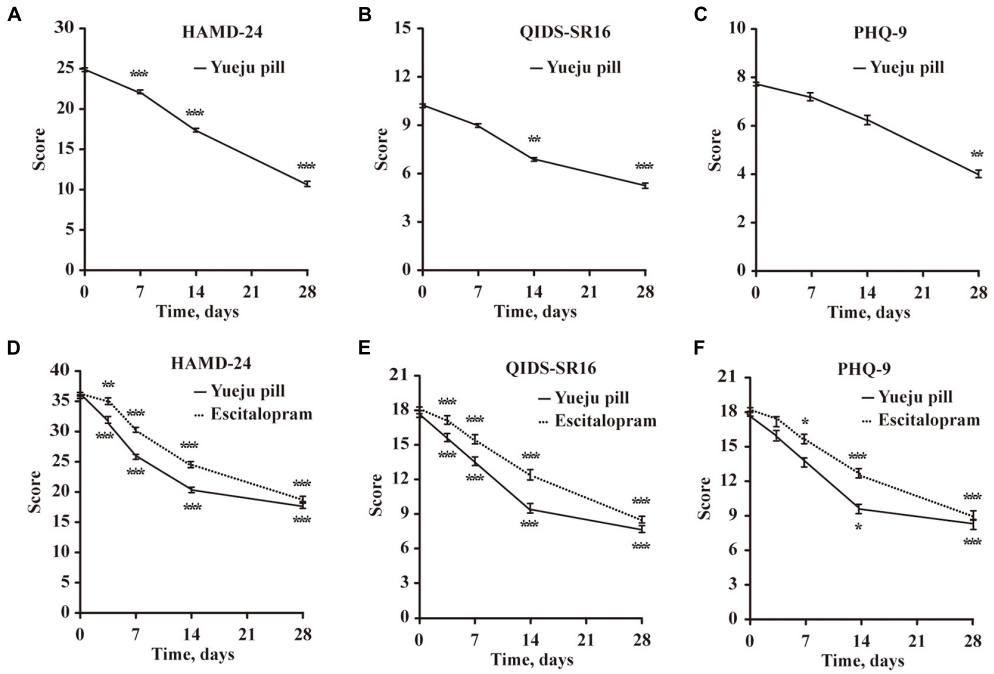

FIGURE 2 | Time course changes of depression scores measured with HAMD-24, QIDS-SR16, and PHQ-9 after treatment in Trial 1 and Trial 2. **(A–C)** Depression scores Trial 1 in a single-arm trial, with low dose of Yueju Pill treatment for 4 weeks (n = 15). **(D–F)** Depression scores in Trial 2 a two-arm randomized controlled trial, with Yueju Pill (n = 18) or escitalopram treatment (n = 18) for 4 weeks. Values are expressed as means and standard errors. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, compared with the baseline.

were analyzed separately. When the data in were collapsed, the baseline serum BDNF levels and HAMD-24 scores were found to be negatively correlated (R^2 = 0.21, p = 0.002). Additionally, analysis of collapsed data from two trials indicated serum BDNF levels and HAMD-24 scores at D28 post treatment were also negatively correlated (R^2 = 0.11, p = 0.03).

When the ratio of serum BDNF level and HAMD-24 score (BDNF/HAMD-24), or NI, was evaluated, the NI values on baseline D0 and on D7 post treatment of Yueju were significantly correlated in Trial 1 (R^2 = 0.37, p = 0.02, **Figure 4A**, left panel). NIs on D7 and D28 were also correlated (R^2 = 0.51, p = 0.0063, **Figure 4A**, right panel). Consistently, the correlation of NI at baseline D0 and NI on D4 was significant in Trial 2 for either Yueju (R^2 = 0.72, p = 0.0001, **Figure 4B**, left panel) or escitalopram (R^2 = 0.67, p = 0.0004, **Figure 4C**, left panel). Furthermore, there existed a significant correlation of NI on baseline D0 and on D28 for Yueju treatment (R^2 = 0.37, p = 0.02, **Figure 4B**, middle panel) or escitalopram treatment (R^2 = 0.38, p = 0.02, **Figure 4C**, middle panel) as well as correlations between NIs on D4 and on D28 for Yueju (R^2 = 0.42, p = 0.01, **Figure 4B**, right panel) or escitalopram treatment (R^2 = 0.34, p = 0.03, **Figure 4C**, right panel) in Trial 2.

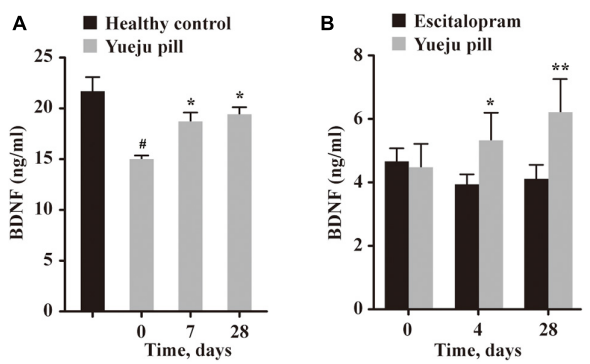

FIGURE 3 | Serum BDNF levels at different time after treatment. **(A)** BDNF levels in healthy control group and at different times in depressive patients treated with Yueju pill in Trial 1 (n = 14). **(B)** BDNF levels at different times in depressive patients treated with Yueju pill (n = 14) or escitalopram (n = 14) in Trial 2. $^{*}p < 0.05$, $^{**}p < 0.01$, compared with the baseline **(A,B)**; $^{\#}p < 0.05$, compared with healthy control **(A)**.

On the basis of NI, a derivative measurement of NI enhancement was further defined: NI enhancement referred to the difference in NI values following a given time period of treatment. There was a significant correlation between the early NI (D7) and the early NI enhancement from D0 to D7 (NI_{D7}–NI_{D0}) in Trial 1 (R^2 = 0.886, $p < 0.001$, **Figure 5A**, left panel), which was replicated in Trial 2 between the early NI (D4) and enhancement of NI from the D0 to D4 (NI_{D4}–NI_{D0}) for Yueju (R^2 = 0.876, $p < 0.001$, **Figure 5B**, middle panel). This correlation was also significant for escitalopram treatment (R^2 = 0.706, p = 0.0006, **Figure 5A**, right panel). Consistently, there was significant correlation between the final NI (D28) and final NI enhancement (NI_{D28}–NI_{D0}) for Yueju in Trial 1 (R^2 = 0.995, $p < 0.001$, **Figure 5B**, left panel) and Trial 2 (R^2 = 0.921,

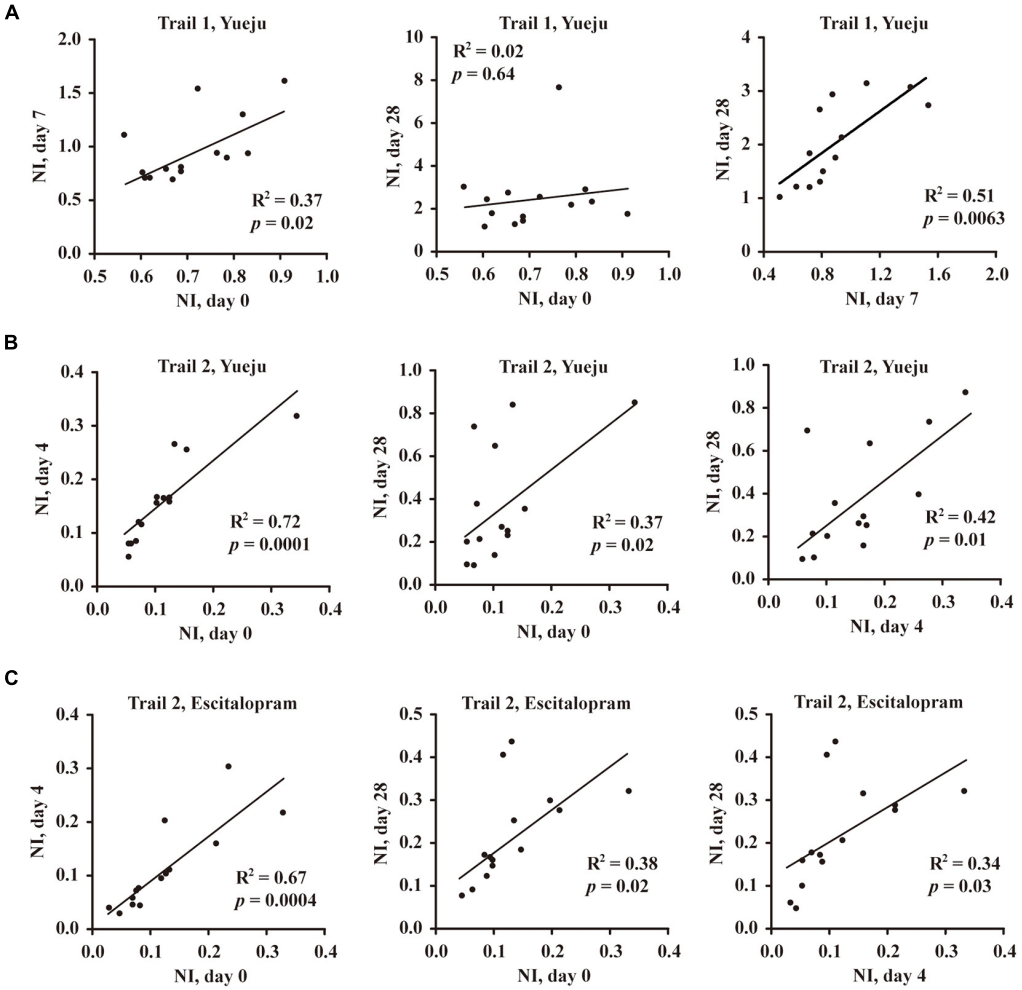

FIGURE 4 | Correlation analysis of neuroplasticity index (NI), the ratio of BDNF to HAMD-24 score in Trial 1 and Trial 2. **(A)** Correlation of NI between baseline (Day 0, D0), early treatment time (D7), and final treatment time (D28) after Yueju pill treatment in Trial 1. **(B)** Correlation of NI between baseline (D0), early treatment time (D4), and final treatment time (D28) after Yueju pill treatment in Trial 2. **(C)** Correlation of NI between baseline (D0), early treatment time (D4), and final treatment time (D28) after escitalopram treatment in Trial 2.

$p < 0.001$, **Figure 5B**, middle panel) and for escitalopram in Trial 2 ($R^2 = 0.599$, $p = 0.0012$, **Figure 5B**, right panel). Furthermore, there was a significant correlation between the early NI (D7) and the final NI enhancement from D0 to D28 (NI_{D28}–NI_{D0}) in Trial 1 ($R^2 = 0.424$, $p = 0.016$, **Figure 5C**, left panel), which was replicated in Trial 2 ($R^2 = 0.481$, $p = 0.008$, **Figure 5C**, middle panel). However, there was no such correlation for escitalopram.

Receiver Operating Characteristic Curve Analysis of Early Neuroplasticity Index Enhancement

Brain-derived neurotrophic factor levels were only used for ROC analysis of diagnosis of depression, as no correlation was found for treatment effect by it alone. The AUC (area under the ROC curve) for detection of depression using BDNF was 0.949, with a diagnostic sensitivity and specificity of 90.7 and 90.9% (**Figure 6A**). As the NI values showed good correlation between baseline, early time, final time or the enhancement from the baseline, we tested if they can be further used for ROC curve analysis to predict the long-term antidepressant response. Using 60% of reduction of HAMD-24 scores by 28 days of treatment as an efficacy index, we found that early NI enhancement showed considerable sensitivity and specificity for Yueju treatment prediction in Trial 1 and Trial 2 with all values of AUC (0.7 (**Figures 6B,C**). However, due to the insufficient number of subjects meeting the criteria of treatment efficacy, it was impossible to perform the ROC analysis using NI enhancement for escitalopram treatment.

DISCUSSION

In this study, we investigated whether the ratio of serum BDNF level and depression scale of HAMD-24, or NI, was useful for prediction of the long-term antidepressant outcome, using Yueju

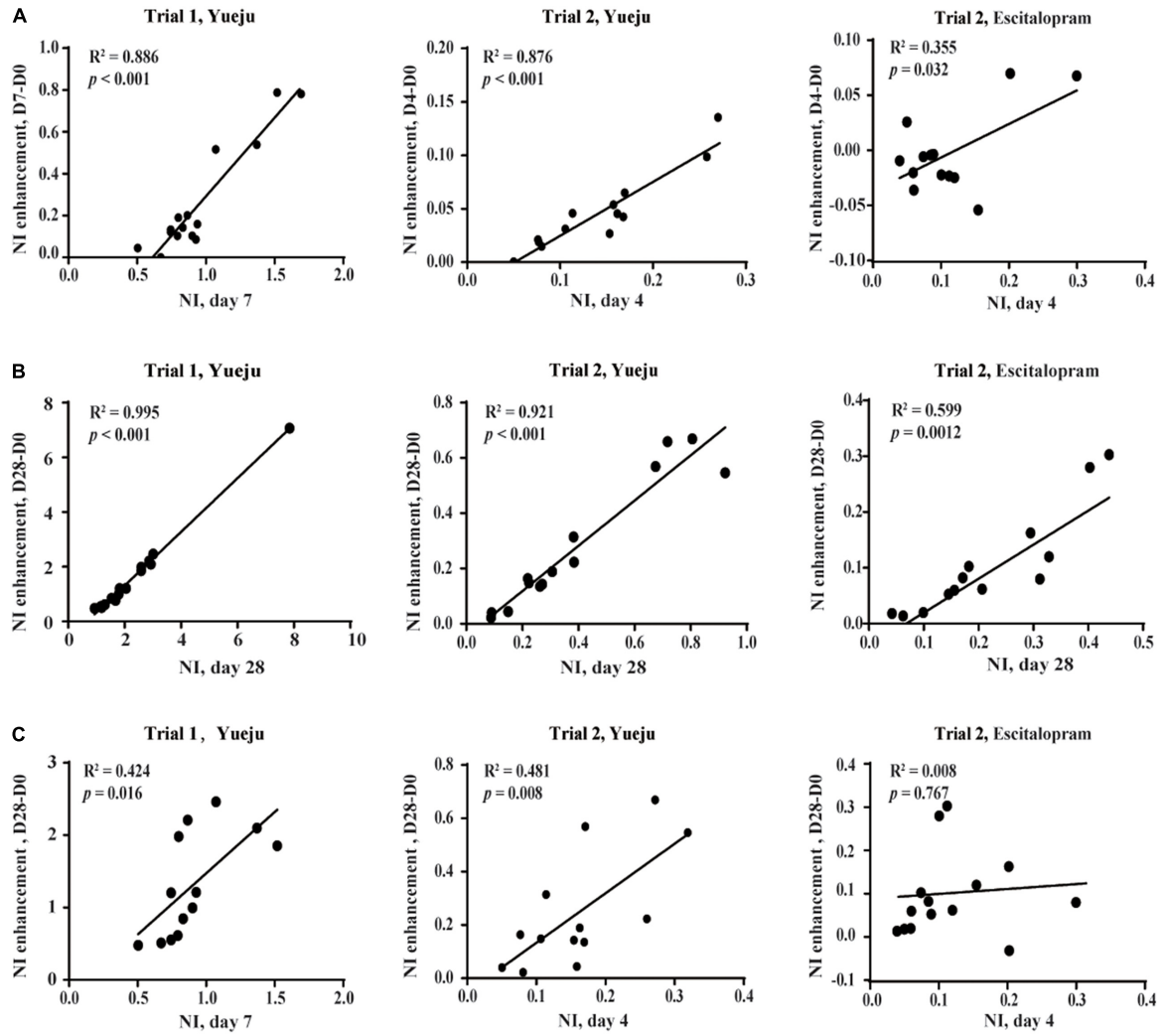

FIGURE 5 | Correlation analysis of NI enhancement, the change of NI within a given period of time. **(A)** The correlation between early NI and early NI enhancement. **(B)** The correlation between final NI and final NI enhancement. **(C)** The correlation between early NI and final NI enhancement. The early NI was on D7 of Yueju treatment in Trial 1 and on D4 of Yueju or escitalopram in Trial 2. The early NI enhancement was the difference of NI between D7 and D0 in Trial 1 and between D4 and D0 in Trial 2. The final NI was on D28 and the final NI enhancement was the difference of NI between D28 and D0 in both trials.

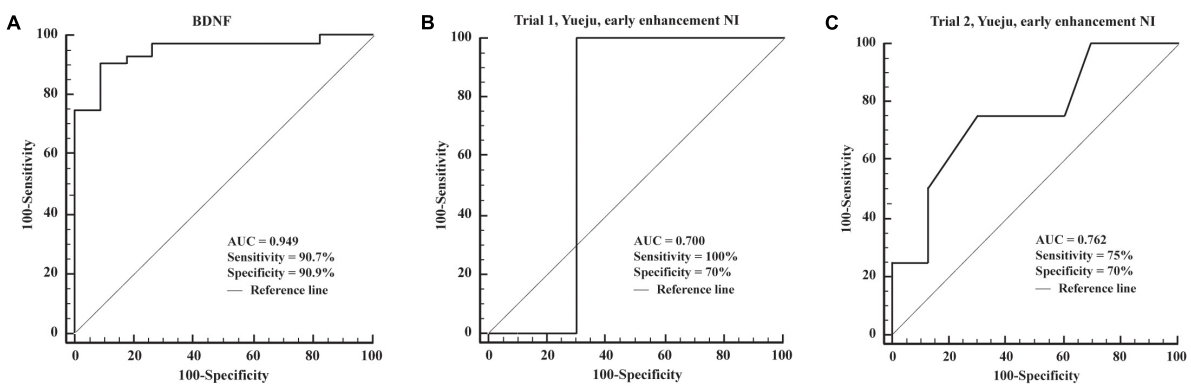

FIGURE 6 | Receiver operating characteristic curves of the detection power of different markers. **(A)** ROC curves of the detection of BDNF. **(B,C)** ROC curves of the detection of the biomarker of early NI enhancement in Trial 1 and Trial 2.

and/or escitalopram in two trials. In the preliminary open-labeled trial, we found both symptom alleviation and serum BDNF enhancement as early as 1 week post a conventional low dose of Yueju. We also revealed significant correlation between NIs at baseline and at the early treatment time. In the confirmatory random controlled double-blinded clinical trial, we found both escitalopram and Yueju pill resulted in early improvement of depression symptom, and comparable antidepressant outcome after 4 weeks of treatment. Unlike Yueju, escitalopram treatment failed to change BDNF levels overtime. However, baseline NI, early treatment NI and final treatment NI were all significantly correlated for both Yueju and escitalopram. Furthermore, the early NI was also correlated to the final enhancement of NI for Yueju in both trials. Finally, ROC analysis indicated the final antidepressant efficacy of Yueju treatment was predictable on the basis of early NI enhancement. The results suggest that the NI may represent an individual's trait that links BDNF with depression symptom, and early change of NI may predict long-term antidepressant efficacy.

Brain-derived neurotrophic factor is a neurotrophin that has been linked to the viability of neurons in brain circuits for depression regulation (Molendijk et al., 2011), and it has been argued that BDNF is a key player for neural plasticity underlying depression and antidepressant activity (Warner-Schmidt and Duman, 2006). Several lines of studies demonstrated the reduced levels of BDNF in MDD patients (Dreimüller et al., 2012). Consistent with this, the present study showed lower serum BDNF levels in MDD patients. The serum BDNF levels of depression patients also were negatively correlated with the degree of depression, when the data from the two trials were collapsed. Furthermore, we found MDD can be reliably predicted with high sensitivity and specificity on the basis of serum BDNF levels. Despite the utility of BDNF for prediction of MDD, our data indicated that it was not useful for prediction of antidepressant efficacy (Dreimüller et al., 2012; Wilkinson et al., 2018). Unlike the increase of BDNF level post treatment of Yueju in both trials, BDNF levels were not changed in the escitalopram treatment group even escitalopram induced significant symptom improvement, consistent with a finding that escitalopram did not affect BDNF levels at either early and late time points (Matrisciano et al., 2009).

The recovery of neuroplasticity is considered to be an important neural mechanism for antidepressant activity (Castrén and Antila, 2017; Koopman and El Aidy, 2017; Franklin et al., 2018; Fox and Lobo, 2019). NI is used to characterize the relative contribution of neuroplasticity indicated by the BDNF level to depression symptoms indicated by the score of HAMD-24. The differences of NI in individuals may reflect different levels of sensitivity of depression symptoms to BDNF levels. NIs at the baseline, the early time of treatment and the final time of treatment were all correlated. Importantly, this correlation was found for both Yueju and escitalopram treatment, even though escitalopram group did not show increased BDNF levels or different doses of Yueju was used overtime. These suggest that NI may represent a trait constantly determining the role of BDNF in an individual's depressive symptoms both at the disease baseline condition and following a certain antidepressant treatment. These intrinsic characteristics also led to the association between early or final treatment time NI with early or final NI enhancement, respectively. It is also noticeable that the measurement of HAMD-17 was not as sensitive as HAMD-24, which may indicate the importance of completeness of the depression symptom for NI. As HAMD-24 scaling and BDNF Elisa measurement are well developed techniques, NI can be reliably calculated and used in the clinical setting.

Partly due to the correlation of baseline NI with the NI at early or final treatment time, it was not surprising that early/final NI was significantly correlated with early/final NI enhancement for either Yueju or escitalopram treatment. This may contribute to the predictability with early NI enhancement to the final treatment efficacy of Yueju. The present study was unable to predict the treatment efficacy of escitalopram. This is technically ascribed to the insufficient number of subjects that met the criteria of treatment efficacy after 4 weeks of treatment of escitalopram. Other factors may also account for the difficulty: in contrast to the general increase of NI at both early and final treatment time of Yueju, NI was mostly reduced at the early treatment time, but augmented by the final treatment time of escitalopram. Consistently, we found the early treatment NI was correlated with the final NI enhancement for Yueju, but not for escitalopram. The definition of NI or related index may have some applications in testing other antidepressants. For example, ketamine is known for the neuroplasticity-dependent mechanism. For escitalopram or other 5-HT based antidepressants, we speculate to use the index like the ratio of the 5-HT improvement value to HAMD-24 score at somehow early stage, to predict the outcome from the contribution of early 5-HT response to behavioral improvement. Certainly, these warrant further investigations. These possibilities should be addressed in future studies by use of a variety of antidepressants for a longer treatment time with a larger number of MDD subjects.

In the present study, we also revealed that the clinical symptom improvement as early as 4 days after treatment of escitalopram. To the best of our knowledge, this finding was reported for the first time. It warrants further clinical and preclinical investigations as escitalopram has become one of the most frequently prescribed antidepressants for its relatively fast action among SSRIs (Pastoor and Gobburu, 2014). Consistent with the finding on combination of the high dose of Yueju pill with fluoxetine achieved early symptom improvement, the present study provided evidence directly showing this dose of Yueju pill by itself was capable to elicit early-onset antidepressant effects in patients (Wu et al., 2015). It appeared there was a larger attenuation of the depressive symptoms by Yueju, compared to escitalopram, although it did not reach the statistic significance. These are in agreement with the findings using different animal models that displayed an immediate and lasting antidepressant effect following a single high dose of Yueju pill (Tang et al., 2015). Additionally, the present study also showed the long-term antidepressant efficacy of conventional low dose of Yueju in both trials. As low dose of Yueju has been used safely over 800 years clinically, it may be useful for a

long-term treatment that many times is required for prevention of recurrence of the disorder.

LIMITATIONS AND CONCLUSION

In conclusion, we demonstrate that NI characterized the role of neuroplasticity in antidepressant treatment. The change of the NI after a short period time of treatment may indicate how the neural plasticity contributes to symptom improvement, which is helpful for the prediction of long-term antidepressant efficacy. Whether this measurement can be generalized to other antidepressant treatment remains to be determined. Nonetheless, the present study indicates the putative composite biomarker at the early time to predict the long-term antidepressant response, which offers novel insights to develop effective biomarker for antidepressant treatment efficacy to improve the treatment outcomes.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Institutional Review Board of The Fourth People's Hospital of Taizhou, Jiangsu Province. The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

YZ, BC, TW, YL, ZC, ZZ, JM, XZ, YY, HW, and GC performed the experiments and analyzed the data. YZ and GC conceived the experiments and contributed to the interpretation and to writing the manuscript. All authors revised the manuscript.

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Induction of Relaxation by Autonomous Sensory Meridian Response

*Noriko Sakurai[1], Ken Ohno[1], Satoshi Kasai[1], Kazuaki Nagasaka[2], Hideaki Onishi[2] and Naoki Kodama[1]**

[1] Department of Radiological Technology, Niigata University of Health and Welfare, Niigata, Japan, [2] Department of Physical Therapy, Niigata University of Health and Welfare, Niigata, Japan

Background: Autonomous sensory meridian response (ASMR) is used by young people to induce relaxation and sleep and to reduce stress and anxiety; it comprises somatosensation caused by audiovisual stimuli (triggers) that lead to positive emotions. Auditory stimuli play the most important role among the triggers involved in ASMR and have been reported to be more triggering than visual stimuli. On the other hand, classical music is also known to have a relaxing effect. This is the first study to clarify the difference in brain activation associated with relaxation effects between ASMR and classical music by limiting ASMR to auditory stimulation alone.

Methods: Thirty healthy subjects, all over 20 years of age, underwent fMRI while listening to ASMR and classical music. We compared the differences in brain activation associated with classical music and ASMR stimulation. After the experiment, the subjects were administered a questionnaire on somatosensation and moods. After the experiment, the participants were asked whether they experienced ASMR somatosensation or frisson. They were also asked to rate the intensity of two moods during stimulation: "comfortable mood," and "tingling mood".

Result: The results of the questionnaire showed that none of the participants experienced any ASMR somatosensation or frisson. Further, there was no significant difference in the ratings given to comfort mood, but there was a significant difference in those given to tingling mood. In terms of brain function, classical music and ASMR showed significant activation in common areas, while ASMR showed activation in more areas, with the medial prefrontal cortex being the main area of activation during ASMR.

Conclusion: Both classical music and the ASMR auditory stimulus produced a pleasant and relaxed state, and ASMR involved more complex brain functions than classical music, especially the activation of the medial prefrontal cortex. Although ASMR was limited to auditory stimulation, the effects were similar to those of listening to classical music, suggesting that ASMR stimulation can produce a pleasant state of relaxation even if it is limited to the auditory component, without the somatic sensation of tingling. ASMR stimulation is easy to use, and appropriate for wellness purposes and a wide range of people.

Keywords: ASMR, auditory perception, relaxation, social behavior, classical music, mPFC

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****Correspondence:***
Naoki Kodama
kodama@nuhw.ac.jp

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INTRODUCTION

Autonomous sensory meridian response (ASMR) videos have gained attention among young people in recent years. ASMR is a type of somatosensation or reaction caused by audiovisual stimuli (Barratt and Davis, 2015; Barratt et al., 2017; Fredborg et al., 2017; McErlean and Banissy, 2017). The triggered response usually extends to the spine, arms, and legs, with a pleasant ASMR somatosensation on the scalp. The purpose of ASMR is to promote sleep, and relaxation, relieve anxiety, and improve work efficiency (Barratt and Davis, 2015; Barratt et al., 2017). The content of ASMR videos varies widely, from gentle whispers in the ear to simulated actions, such as touching the hair or applying makeup, as well as the sounds of chewing, cutting, typing, and nature. Previous research on ASMR analyzed the triggers and identified four prominent categories: whispering, personal attention, vivid sounds, and slow movements; however, these are representative and include many triggers that do not belong to these categories (Barratt and Davis, 2015). Fredborg et al. (2017) then categorized them into five categories (watching, touching, repetitive sounds, simulations, and mouth sounds). The physiological response to ASMR somatosensation is emotionally positive and is accompanied by a sense of calm. The skin conductance response, a measure of autonomic nervous system arousal, is increased, and heart rate is decreased. These seemingly contradictory results reflect the high complexity of ASMR (Poerio et al., 2018).

Functional magnetic resonance imaging (fMRI) has been used to measure brain function. An fMRI study conducted by Lochte et al. (2018) investigated somatosensory brain activity during ASMR stimulation consisting of ASMR videos. ASMR somatosensation involves the activation of secondary somatosensory areas, such as the medial prefrontal cortex, accumbens, insula, inferior frontal gyrus, supplementary motor cortex, and dorsal anterior cingulate cortex (Lochte et al., 2018). Smith et al. also reported on the neural connectivity of the default mode network in subjects who experience the somatosensory component of ASMR and subjects who do not. The DMN of ASMR-sensitive individuals showed decreased connectivity in the precuneus and thalamus, and increased connectivity in the frontal gyrus and temporal gyrus. The study showed differences in brain activation between individuals who experience ASMR and those who do not (Smith et al., 2017). Lee et al. (2020) examined how functional connectivity changes during ASMR video viewing compared to resting state, and assessed its relevance to ASMR-induced emotional states. ASMR-induced changes in emotional state are negatively correlated with functional connectivity for visual information processing.

Personality trait analysis of subjects who have experienced ASMR somatosensation and those who have not has shown that those who experience this sensation are significantly more imaginative, excitable, curious, and open-minded. This is consistent with the personality analysis of those who experience musical frisson (McCrae, 2007; Kovacevichi and Huron, 2018), an emotional response to music and a feeling involving chills and goosebumps (Craig, 2005). The two phenomena, ASMR somatosensation sensation and musical frisson, are similar; however, they differ in that the somatosensation of frisson tends to spread rapidly throughout the body, whereas that of ASMR may last longer than a few minutes (del Campo and Kehle, 2016). In addition, frisson involves an exciting or emotionally stimulating experience; whereas, ASMR somatosensation is more often associated with relaxation and satisfaction (Barratt and Davis, 2015).

Some studies have reported on the wellness benefits of ASMR; ASMR is also already being used in wellness programs and is an efficient way of mind relaxation (Cash et al., 2018). Cash et al. suggest that expectations about the placebo effect of ASMR may lead to somatosensory responses and stress reduction effects. ASMR contributes to home-based stress management and pain management programs due to its ease of use (Cash et al., 2018). The sleep-inducing effects of ASMR have also been proposed as a way to improve sleep quality (Lee et al., 2019; Vardhan et al., 2020). While most studies have considered the somatosensory component of ASMR, this type of sleep induction is limited to auditory stimuli consisting of natural sounds that have no potential to induce ASMR. The combined auditory stimulation of binaural beats for sleeping is measured by electroencephalography, suggesting that this combined stimulation helps the transition to sleep (Lee et al., 2019). Paszkiel et al. (2020) investigated the effects of four different sound stimuli on stress levels as measured using four different methods (EEG, blood pressure, pulse, and questionnaire), and reported that both relaxing music and ASMR induce relaxation at a fast rate and reduce stress levels.

ASMR users do not necessarily experience ASMR somatosensation. However, even in the absence of ASMR somatosensation, mood is improved and chronic pain symptoms are greatly reduced (Barratt and Davis, 2015). Thus, even without ASMR somatosensation, ASMR is being incorporated into wellness programs for the purpose of stress reduction, depressed mood improvement, and pain relief. On the other hand, classical music also is known to have a relaxing effect (Menon and Levitin, 2005), but not all people experience or seek frisson.

This study focuses on the relaxation effects of ASMR and classical music to clarify the differences in brain activation between the two. Auditory stimulation plays the most important role among the triggers that constitute ASMR, and is reported to be more triggering than visual stimulation (Barratt et al., 2017). The present study is the first to clarify the differences in brain activation associated with the relaxation effects listening to classical music vs. the auditory component of ASMR.

MATERIALS AND METHODS

Participants

The subjects of this study were 36 healthy, non-psychiatrically impaired individuals over 20 years of age, who had no experience with ASMR somatosensation, were not enthusiasts, and did not watch ASMR videos for 1 week prior to the experiment. The

Abbreviations: ASMR, Autonomous sensory meridian response; MFG, middle frontal gyrus; MSFG, medial superior frontal gyrus.

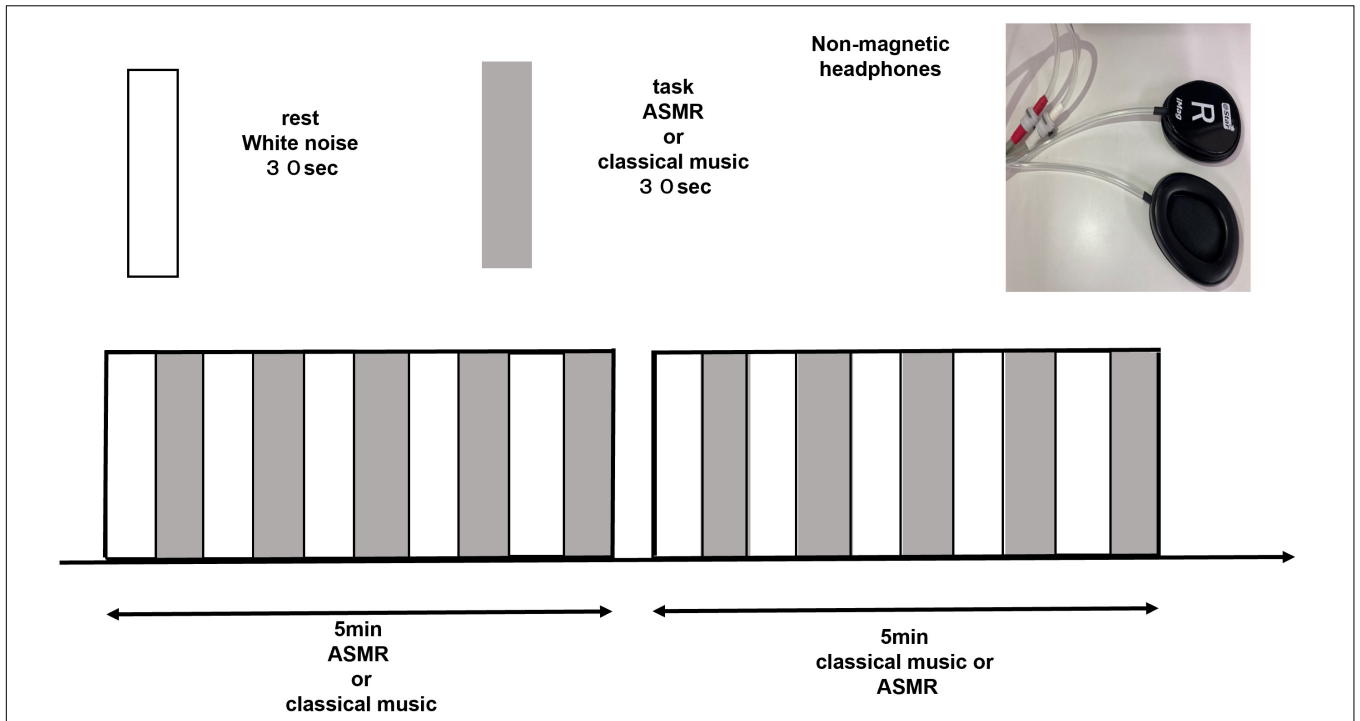

FIGURE 1 | Block design of 5-min with a 30-s rest and a 30-s task repetition (ASMR, classical music). Each subject listened to ASMR stimulation and classical music in random order. ASMR, autonomous sensory meridian response.

six participants whose temporal lobe activation could not be confirmed were excluded, as they were probably not listening to the task. Therefore, 30 subjects (18 men and 12 women; mean age, 20.3 years; $SD = 0.7$) were included in the analysis. This study was approved by the Research Ethics Committee of Niigata University of Health and Welfare (Approval No. 18218-190722). Written informed consent was obtained from all participants. Participant interview was performed to ensure the safety of MRI imaging.

Stimuli Task

We limited the triggers of ASMR to auditory stimuli in order to explore the differences in relaxation effects between ASMR and classical music in terms of brain function. Auditory stimuli are the most important trigger of ASMR and are reported to be more triggering than visual stimuli (Barratt et al., 2017). Lee et al. (2019) used nature sounds as triggers in their sleep-induction experiments, which are limited to auditory stimuli and have no potential for further inducing ASMR. As a sound stimulus task in this study, for ASMR, we selected a sound source with strong auditory effects. We excluded activities of the language cortex, such as whispering, and activities of the visual cortex, such as touching, and prepared 10 patterns of repetitive, crisp, and refreshing sounds. The participants listened to and selected beforehand from among the following sounds: ear scratching, eating a cucumber, typing, pouring soda, stepping in a puddle, grilling a steak, cutting a carrot, wind chimes, flowing water, and rain. Since ASMR is based on personal preference, the subjects listened to all 10 types of ASMR before the experiment and selected the stimulus that they liked the most and that they found most relaxing. Among classical music, Mozart's music has been reported to be comfortable and to have a great effect on relaxation, resonating with human biological rhythms, balancing the autonomic nervous system, and lowering blood pressure and heart rate (Trappe and Voit, 2016). Thus, we selected Eine kleine Nachtmusik, a piece of music by Mozart that was employed in a previous study (Menon and Levitin, 2005). The resting state task for comparison with the stimulus task was white noise (Cardona et al., 2020). We used white noise because the same auditory stimulus is a necessary condition for the resting task, and because it is a random signal with equal power at any frequency in a given bandwidth without emotional involvement. The block design consisted of a 30-s repetition of the resting task and a 30-s repetition of the stimulus task for a total of 5 min (**Figure 1**). In fMRI experiments of emotional changes in response to music, music is used as a task in 30-s increments (Pereira et al., 2011). Similarly, in the present study the design was a block of 30-s increments to obtain mood responses without aiming to induce the somatosensory component of ASMR. Subjects

TABLE 1 | Frequency of ASMR tingling sensation and musical frisson.

ASMR somatosensation	Classic musical frisson
Yes/No	Yes/No
0/30	0/30

No subject experienced tingling during ASMR stimulation, and all of them (30) reported not feeling it. No subject experienced musical frisson while listening to classical music, and all of them reported not experiencing it.

TABLE 2 | Moods while listening to ASMR and classical music.

Likert scale point		1	2	3	4	5	
Comfortable mood	Classic	3 (10.0%)	2 (6.7%)	5 (16.7%)	14 (46.7%)	6 (20.0%)	$P = 0.130$
	ASMR	2 (6.7%)	9 (30.0%)	6 (20.0%)	11 (36.7%)	2 (6.7%)	
Tingling mood	Classic	6 (20.0%)	14 (46.7%)	2 6.7%)	8 (26.7%)	0 (0.0%)	$P < 0.001$
	ASMR	3 (10.0%)	2 (6.7%)	3 (10.0%)	17 (56.7%)	5 (16.7%)	

In a comparison of ASMR vs. classical music, there was a significant difference in tingling mood, but there was no significant difference in comfortable mood.

were instructed to concentrate on the sound during the 5-min experiment and to keep their eyes open. Cushions were used in the gaps to prevent head movements.

Apparatus

Imaging was performed on a 3 Tesla MRI system (Canon Vantage Galan) with a 16-channel head coil. The subject laid in the MRI machine and listened to the block design stimuli of ASMR and classical music. The order of stimulus (ASMR or classical music) presentation was determined randomly.

MRI Acquisition

A separate high-resolution MRI image is required to obtain detailed anatomical information prior to fMRI imaging. For this purpose, a high-resolution magnetization-prepared rapid-gradient-echo sequence of T1-weighted imaging was used, with the following parameters: repetition time, 5.8 ms; echo time, 2.7 ms; inversion time, 900 ms; flip angle, 9°; number of matrices (matrix), 256 × 256; effective field of view, 23 × 23 mm; and slice thickness, 1.2 mm. The echo planar imaging sequence was used to capture the fMRI images. The images were repeatedly obtained, and used to compare the two stimuli. The fMRI imaging conditions were as follows: repetition time, 2,000 ms; echo time, 25 ms; flip angle, 85°; matrix, 64 × 64; effective field of view, 24 × 24 mm; and slice thickness, 3 mm to cover the whole brain.

Functional Magnetic Resonance Imaging Data Analyses

The fMRI data were preprocessed and analyzed using Statistical Parametric Mapping 12 (Wellcome Trust Center for Neuroimaging) in Matlab (Mathworks Inc.). Slice timing correction was used to correct the time difference, and realignment was then used to correct the displacement caused by motion. In addition, a coregister was used to compare the structural images with the fMRI images. The coregister was corrected for misalignment between structural and functional images and the data was preprocessed by normalizing each participant's brain to a template of the Montreal Neurological Institute coordinate system of a standard brain. Normalized images were smoothed using a Gaussian kernel of 8 mm. After the preprocessing, we employed a general linear model GLM to confirm brain activity changes associated with ASMR or classical music, using a block design. Contrasts images were created at first level (single-subject) for the following contrast: (1) ASMR = 1, rest = 0, (2) classic = 1, rest = 0, (3) ASMR = 1, classic = −1, respectively. The contrast of (3) was used to identify brain regions with significantly increased activity in the ASMR relative to the classic condition. For group analysis (second level), a one-sample *t*-test was performed using the above three contrasts. The initial threshold for the voxel size was set to uncorrected $p < 0.001$. Clusters were considered significant when falling below $p = 0.05$, cluster-corrected for family wise error. ASMR and classical music were analyzed, and classical music was subtracted from ASMR.

TABLE 3 | Significantly activated areas and their *Z*-values and coordinates while listening to classical music.

Area	Hemisphere	K_E	*Z*-value	x	y	z (mm)
Thalamus proper	Left	14,823	5.56	−2	−34	−4
Middle frontal gyrus	Right	479	4.79	50	12	46

Regions of significance after family wise error cluster levels ($p < 0.05$).

Questionnaire

After the experiment, the subjects were administered a questionnaire on somatosensation and mood. For somatosensation, the subjects were asked to answer "yes" or "no" to the question of whether they experienced ASMR somatosensation or frisson. They were then asked to indicate the intensity of the two moods for each stimulus: the two moods were "comfortable mood," and "tingling mood." A Likert-type scale of 1-5 was used: 1, completely disagree; 2, disagree; 3, undecided; 4, agree; and 5, highly agree. We explained to the subjects that "comfortable mood" refers to a state of relaxation and peace of mind, while "tingling mood" while "tingling" was considered a mood, even in the absence of somatic sensations, even if it does not cause somatic sensations. The chi-square test was performed for the statistical analysis using SPSS (IBM SPSS Statistics Base) 26.0, and the significance level was set at 5%.

RESULTS

The results of the questionnaire showed that none of the subjects experienced somatosensation during ASMR stimulation; furthermore, none of the subjects experienced musical frisson. The results are shown in **Table 1**. The results of the ratings of the two moods for each of the ASMR and classical stimuli are shown in **Table 2**. A 5-point Likert scale questionnaire was used, with 3 points being neutral; the further away the score was from the lower end, the more negative the response was (the mood was not experienced). Conversely, the further away the points

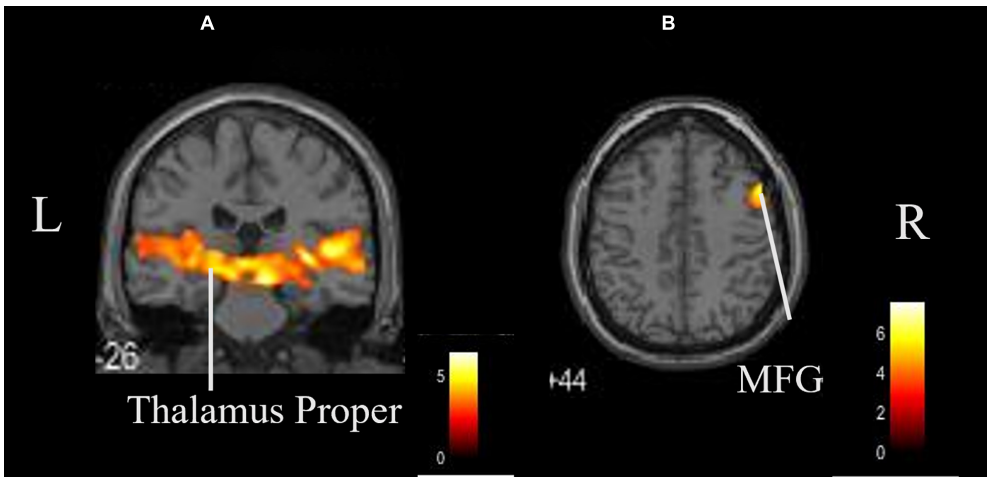

FIGURE 2 | Whole brain activation while listening to classical music compared with the resting state. Significant activation was observed in the right middle frontal gyrus and left thalamus proper. **(A)** Coronal view. **(B)** Axial view. L, left; R, right. MFG, middle frontal gyrus.

were from the higher end, the more positive the response was (the mood was experienced). As for the tingling mood, there was a significant difference between ASMR and classical music stimulation ($p < 0.001$). As for the comfortable mood, there was no significant difference between the ASMR and classical music stimulation ($p = 0.130$). We also found that there is no correlation between comfort and brain activity and between tingling and brain activity.

The coordinates of the areas that were significantly activated while listening to classical music are listed in **Table 3** and depicted in **Figure 2**. Significant activation was observed in the right middle frontal gyrus (MFG) and left thalamus proper while listening to classical music. The coordinates of the areas that were significantly activated during ASMR auditory stimulation are listed in **Table 4** and depicted in **Figure 3**. Significant activation was observed in the left thalamus proper, left anterior insula, right triangular part of the inferior frontal gyrus, right cerebellum exterior, left accumbens, right amygdala, left medial superior frontal gyrus (MSFG), and left planum polare. **Table 5** shows the coordinates of the areas that were significantly more activated by ASMR stimulation than by classical music. Images of the activated areas are shown in **Figure 4**. Significant activation was observed in the left calcarine cortex, right superior frontal gyrus medial segment, and right lingual gyrus during ASMR stimulation compared to classical music.

TABLE 4 | Significantly activated areas and their *Z*-values and coordinates during autonomous sensory meridian response (ASMR) listening.

Area	Hemisphere	K_E	*Z*-value	x	y	z (mm)
Thalamus proper	Left	2,543	5.01	−30	−26	−2
Insula	Left	648	4.93	−36	24	4
Triangular part of the inferior frontal gyrus	Right	1,142	4.78	44	32	6
Cerebellum exterior	Right	6,219	4.64	26	−64	−24
Accumbens area	Left	293	4.54	−20	2	−12
Amygdala	Right	272	4.52	22	0	−10
Superior frontal gyrus medial segment	Right	457	4.18	6	20	56
Planum polare	Left	262	3.64	−52	−22	4

Regions of significance after family wise error cluster levels ($p < 0.05$).

DISCUSSION

The present study is the first fMRI investigation focusing on the effects of ASMR sound without somatosensory perception on relaxation. We were able to compare the relaxation effects of ASMR with those of classical music by limiting the study to auditory stimuli.

Music is transmitted to the inner ear as air vibrations are captured by the eardrum. It is converted into nerve signals by sensory cells in the inner ear and enters the primary auditory cortex via the brainstem and thalamus. After perceptual processing in the auditory cortex, the information is sent to the association cortex, where it is linked with the parietal and frontal lobes for recognition. The results of this study showed that the thalamus proper and MFG were significantly activated when listening to classical music. The thalamus proper relays auditory sensory nerves to the cerebral cortex and basal ganglia. The same activation was observed in the thalamus while listening to ASMR stimuli. This activation included areas of brain function related to sleep. Sleep begins in the brainstem and enters the thalamus, where auditory sensory information is processed through sensory neural pathways (Tang et al., 2015). Ultimately, it is believed that auditory signals in the thalamus may influence the sleep regulatory system (Lee et al., 2019). The thalamus is involved in emotion and memory, and also acts on the autonomic nervous system. It has been suggested that both classical music and ASMR can induce relaxation and promote sleep.

In addition, more regions were activated during ASMR stimulation. Significant activation was also observed in the anterior insular cortex, nucleus accumbens, amygdala, MSFG,

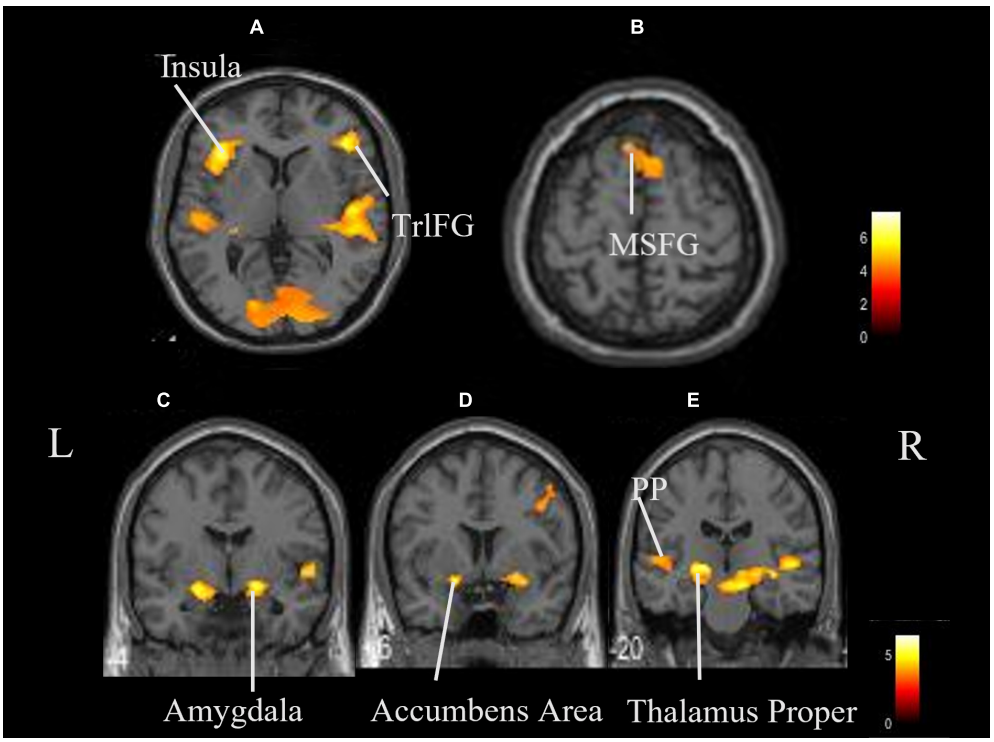

FIGURE 3 | Whole brain activation while listening to ASMR compared with the resting state. Significant activation was observed in the right triangular part of the left thalamus proper, left insula, right triangular part of the inferior frontal gyrus, right cerebellum exterior, left accumbens, right amygdala, left medial superior frontal gyrus, and left planum polare. **(A,B)** Axial view. **(C–E)** Coronal view. L, left; R, right. TrlFG, triangular part of the inferior frontal gyrus; MSFG, medial superior frontal gyrus; PP, planum polare.

triangular part of the inferior frontal gyrus, and planum polare. The planum polare is located in the temporal lobe, which is the first cortical region of the auditory signal processing system and constitutes the primary auditory cortex. In the basal ganglia and the surrounding limbic system, the amygdala and nucleus accumbens were activated by classical music in addition to the thalamus. The amygdala plays an important role in the control of emotional responses, primarily fear (Ohman, 2005). The nucleus accumbens is involved in reward, satisfaction, and emotion, and is largely responsible for the release of dopamine (Pereira et al., 2011). The insular cortex connects the primary and secondary somatosensory cortices and functions as a multisensory integration site that processes a variety of information (Nagai et al., 2007; Ibañez et al., 2010). In the frontal gyrus, activation was seen in the inferior frontal gyrus and MSFG, indicating that the prefrontal cortex was largely activated; the MSFG is located in the medial prefrontal cortex and has been suggested to contribute to the release of oxytocin (Sabihi et al., 2014).

In this study, we also identified the regions where ASMR stimulation induced a higher activation than classical music. These were the calcarine cortex, superior frontal gyrus medial segment, and lingual gyrus. Activation in the posterior part of the cerebrum is also related to auditory stimulation and is due to spatial hearing (Lewald et al., 2004). We think that ASMR has a more specific impact on the identification of location, direction, and distance of sound sources than classical music.

Particularly noteworthy is the activation of the superior frontal gyrus and the medial prefrontal cortex (mPFC), which is said to be the basis of social cognitive abilities (Feldman, 2012). The mPFC is thought to be activated because ASMR may contain many sound sources that are closely related to daily life and social activity. The mPFC region is also involved in the regulation of neurotransmitters such as dopamine, which projects to the prefrontal cortex through the dopamine pathway and has been shown to enhance stress resistance in response to short-term stress (Tanaka et al., 2012). It also involves the release of oxytocin, which is a stress-reducing, pro-social neuropeptide that is effective in modulating brain activity in depressed individuals (Pincus et al., 2010). These neurohormones are known to induce

TABLE 5 | Significantly activated regions and their *z*-values and coordinates in the difference between autonomous sensory meridian response (ASMR) stimulation and classical music.

Area	Hemisphere	K_E	*Z*-value	x	y	z (mm)
Calcarine cortex	Left	3,596	3.77	−18	−82	0
Superior frontal gyrus medial segment	Right	299	3.72	2	18	58
Lingual gyrus	Right	241	3.68	30	−46	−4

Regions of significance after family wise error cluster levels ($p < 0.05$).

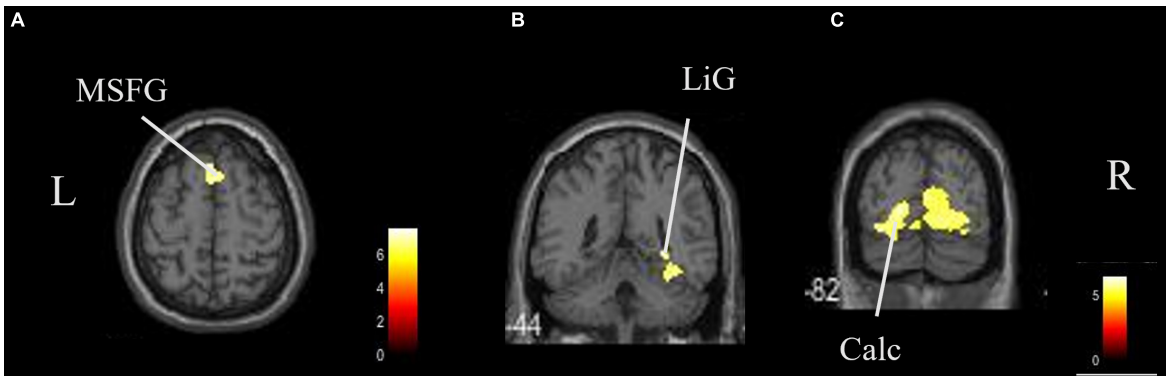

FIGURE 4 | Brain areas that were significantly more activated by ASMR stimulation than by classical music. Significantly more activation was observed in the right triangular part of the left calcarine cortex, right superior frontal gyrus medial segment, and right lingual gyrus in ASMR compared with classical music. **(A)** Axial view. **(B,C)** Coronal view. L, left; R, right. ASMR, autonomous sensory meridian response; LiG, lingual gyrus; MSFG, superior medial gyrus medial segment.

feelings of comfort, relaxation, and drowsiness (Lochte et al., 2018). ASMR stimulation is thought to be more effective in inducing relaxation and reducing stress than classical music because it activates brain regions associated with these functions.

Lochte et al. (2018) also investigated brain function during moments of relaxation without ASMR somatosensation while watching ASMR videos and found activation in the medial prefrontal cortex. In the present study, ASMR was limited to auditory stimulation, and the results are consistent with those of Lochte et al. (2018). The results of the present study proved using brain functional imaging that the auditory component of ASMR stimulation produced a comfortable and relaxed state, even though the tingling (somatic) sensation was not obtained.

To induce ASMR somatosensation, an individual normally needs a desirable, quiet environment (Barratt and Davis, 2015), which may not always be available. In addition, some persons are more likely than others to experience ASMR somatosensation due to personality traits (Kovacevichi and Huron, 2018). Based on the results of this study, we believe that ASMR stimulation can be used as a tool to easily obtain relaxation just by listening to it, without having to meet demanding requirements. The fact that ASMR is currently being used in wellness for stress reduction, depressive mood improvement, and pain relief further suggests the adequacy of an active use of ASMR. Future studies should examine how listening to ASMR sounds affects brain function in the elderly, and how it can counteract depression in the elderly. The biggest advantages of ASMR include its cost, ease of use, and that it can be completed by an individual at home. This opens up the possibility of proposing the use of ASMR for wellness purposes to an even wider range of persons in the future.

There are several limitations to this study; the results do not provide a quantitative demonstration of the relaxation effect of ASMR. Therefore, we believe that the results of this study can be further strengthened in the future by conducting experiments based on physiological indices and explore correlations. Then, in order to compare ASMR with baseline, we think it is necessary to try to examine a block design with longer rest and task times. Since there comparison group did not used in this study, future studies should compare between groups that are sensitive to ASMR and those that are not. In addition, a subclassification of the ASMR groups might also allow us to deepen our knowledge on this phenomenon. Further investigation of the neurotransmitters suggested in this study, such as dopamine and oxytocin, is needed, and the use of magnetic resonance spectroscopy to obtain information on the type, status, and quantity of neurotransmitters by placing ROIs at the activation sites may further clarify the involvement of specific metabolites.

CONCLUSION

ASMR felt as comfortable mood as classical music. In terms of brain function, ASMR stimulation significantly increased activation of mPFC compared to classical music. mPFC is involved in stress reduction and relaxation, suggesting that ASMR can induce relaxation even just by listening to it. Since ASMR stimulation is easy to use, it is expected to expand its use in the future.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Research Ethics Committee of Niigata University of Health and Welfare (Approval Nos. 18218-190722). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

NS and NK conceived the study and designed the experiments. NS, KO, and NK collected MR data. NS and KN interpreted the data. NS, KO, and KN performed the statistical analysis. SK, HO,

and NK helped draft the manuscript. All authors approved the final version of the manuscript.

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MINI REVIEW
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Novel Insights Into the Neurobiology of the Antidepressant Response From Ketamine Research: A Mini Review

*Michael Colla, Hanne Scheerer, Steffi Weidt, Erich Seifritz and Golo Kronenberg**

Department of Psychiatry, Psychotherapy and Psychosomatics, Hospital of Psychiatry, University of Zurich, Zurich, Switzerland

The serendipitous discovery of ketamine's antidepressant effects represents one of the major landmarks in neuropsychopharmacological research of the last 50 years. Ketamine provides an exciting challenge to traditional concepts of antidepressant drug therapy, producing rapid antidepressant effects seemingly without targeting monoaminergic pathways in the conventional way. In consequence, the advent of ketamine has spawned a plethora of neurobiological research into its putative mechanisms. Here, we provide a brief overview of current theories of antidepressant drug action including monoaminergic signaling, disinhibition of glutamatergic neurotransmission, neurotrophic and neuroplastic effects, and how these might relate to ketamine. Given that research into ketamine has not yet yielded new therapies beyond ketamine itself, current knowledge gaps and limitations of available studies are also discussed.

Keywords: depression, antidepressant, treatment-resistant depression, ketamine, BDNF, neurogenesis, monoamines, glutamate

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Reviewed by:
Juan Francisco Rodríguez-Landa, Universidad Veracruzana, Mexico
Darcy Litteljohn, Independent Researcher, Toronto, ON, Canada

***Correspondence:**
Golo Kronenberg
golo.kronenberg@pukzh.ch

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INTRODUCTION

Ketamine, synthesized in 1962 by the research team of Calvin Stevens, was the culmination of Parke-Davis's drive to find a short-acting intravenous anesthetic with favorable cardiovascular and respiratory characteristics (Mion, 2017). Ketamine replaced its congener phencyclidine (PCP), which, after a brief period of use as an anesthetic agent under the brand name Sernyl®, had to be abandoned owing to high rates of postoperative dysphoria and hallucinations (Meyer et al., 1959). The distinctive state produced by ketamine — characterized by analgesia, catalepsy, and amnesia, while maintaining respiratory reflexes and hemodynamic stability — was first described by Corssen and Domino (1965), who dubbed it "dissociative anesthesia." Its wide therapeutic index makes ketamine an excellent agent for use in emergency medical practice, battlefield pain management, and, more generally, in resource-stripped settings such as the developing world. Ketamine remains on the most recent WHO Model List of Essential Medicines as an injectable general anesthetic (World Health Organization [WHO], 2019).

Ketamine burst on the scene of antidepressant psychopharmacology in 2000 with the publication of its first double-blind placebo-controlled trial in major depression. This pilot investigation of seven patients found significant improvements in mood within 72 h of a single subanesthetic dose of intravenous racemic ketamine hydrochloride (Berman et al., 2000). A number of follow-up studies have confirmed the fast-onset antidepressant effects of ketamine infusions (Zarate et al., 2006a; aan het Rot et al., 2010; Murrough et al., 2013a,b). Moreover, adjunctive intravenous ketamine

has emerged as a powerful new treatment option for patients suffering from treatment-resistant depression (TRD; Diazgranados et al., 2010; Fava et al., 2020). In the interim, variant forms of ketamine therapy including treatment with the S-enantiomer (i.e., S-ketamine) and administration via the nasal (Popova et al., 2019) and oral route (Domany et al., 2019) have also been demonstrated to confer rapid antidepressant benefit. **Table 1** summarizes key studies of ketamine in depression.

From a neuroscience perspective, the uncanny rapidity of ketamine's antidepressant action (often within a few hours) sets it apart from conventional antidepressants, providing a new window on the neurobiology of the antidepressant response with exciting possibilities for translational and, maybe even more interesting, reverse translational research. The purpose of this mini-review is, therefore, to provide an overview of current thinking on ketamine's putative mechanisms of action within the context of antidepressant drug discovery and development.

MONOAMINE MECHANISMS

The short history of the development of antidepressant drugs is riddled with accidental yet transformative discoveries. At the risk of recounting well-known facts, here is a summary of the milestones: Iproniazid, initially developed and marketed by Hoffmann La-Roche as an antibiotic to treat tuberculosis (Marsilid®), was serendipitously identified as possessing antidepressant characteristics (Loomer et al., 1957; deVerteuil and Lehmann, 1958). A connection was quickly made with iproniazid's strong inhibitory effect on monoamine oxidase (MAO), paving the way for the targeted development of other, more refined, and ultimately safer MAO inhibitors, which are still widely prescribed today (e.g., Stefanis et al., 1982). The antidepressant activity of imipramine, the first tricyclic antidepressant, was recognized almost coevally with the discovery of iproniazid's antidepressant properties (Kuhn, 1957). Inhibition of the reuptake of biogenic amines was swiftly identified as the primary molecular mechanism of tricyclics (Axelrod et al., 1961; Carlsson et al., 1966, 1968; Fuxe and Ungerstedt, 1968). The observation that blood-pressure lowering drug reserpine may precipitate depression (Harris, 1957) provided further support for a link between brain levels of biogenic amines and mood states. Taken together, and in historical perspective, this "monoamine hypothesis" of depression has proven incredibly useful in the development of newer classes of antidepressants (such as selective serotonin reuptake inhibitors, noradrenaline reuptake inhibitors, dual reuptake inhibitors, etc.) that are usually superior to the older compounds with comparable efficacy yet fewer side effects and a greater therapeutic index. Nevertheless, the clinical limitations of monoamine-based agents, in particular relatively high rates of non-response and even resistance to treatment, have long led to calls to focus more research on alternative mechanisms (Berton and Nestler, 2006).

An obvious conceptual problem with the monoamine hypothesis lies in the fact that changes in neurotransmitter concentrations (along with the onset of typical side effects) occur within a few hours while conventional antidepressants typically require several days to weeks to take effect. Neurobiological research into the mechanisms underpinning the antidepressant response has therefore pivoted to longer-term adaptive changes downstream of the acute effects on biogenic amines.

Ketamine's principal pharmacological action is as an *N*-methyl-D-aspartate (NMDA) receptor antagonist. However, in a manner somewhat reminiscent of clozapine, ketamine is a "dirty" drug. Multiple off-target effects including on monoamine systems need to be considered. *In vitro*, ketamine displays affinity to dopamine D2 and serotonin 5-HT2 receptors in the same range as its affinity for the NMDA receptor (Kapur and Seeman, 2002). It has also been reported that ketamine inhibits monoamine transporters in cultured cells (Nishimura et al., 1998) and blocks the uptake of [3H]-dopamine into rat striatal synaptosomes (Keita et al., 1996).

Repeated ketamine injections increase the firing rate of norepinephrine neurons in the locus coeruleus and of dopaminergic neurons in the ventral tegmental area in rats (Iro et al., 2021). Microdialysis studies have demonstrated increased serotonin release by ketamine in the rodent prefrontal cortex (Ago et al., 2019; López-Gil et al., 2019). Several groups have found that serotonin depletion abrogates the antidepressive-like effects of ketamine in the forced swim test (Gigliucci et al., 2013; Fukumoto et al., 2015; du Jardin et al., 2016; Pham et al., 2017). Even so, measurable occupancy of the serotonin transporter *in vivo* was not detectable by positron emission tomography in twelve healthy human subjects after infusion of an antidepressant dose of ketamine (Spies et al., 2018).

While the available literature indicates that ketamine leads to increased dopamine levels in frontal cortex, striatum, and nucleus accumbens in rodents, the picture is less clear for the primate and human brain, given methodological issues and the scant available literature (Kokkinou et al., 2018). From a clinical perspective, the fact that haloperidol is able to ameliorate ketamine-induced psychosis argues for a role of dopaminergic pathways in ketamine's psychotropic effects (Giannini et al., 2000).

KETAMINE AND THE GLUTAMATERGIC SYSTEM

Racemic ketamine acts as a non-competitive NMDA receptor antagonist (**Figure 1A**). It is believed that the dissociative and psychotomimetic effects of PCP and ketamine relate directly to the affinity of these molecules to the NMDA receptor. Based on displacement binding studies with [3H]-MK801 as the marker ligand, S-ketamine exhibits an approximately three- to fourfold higher affinity to the NMDA receptor than R-ketamine (Moaddel et al., 2013). The pharmacokinetic profiles of racemic ketamine and its two enantiomers do not differ significantly in humans (White et al., 1985). Serum ketamine concentrations at the point of regaining consciousness and orientation during the course of experimental anesthesia of human volunteers indicate an S:R ketamine isomer potency ratio of 4:1. Similarly, S-ketamine has an approximately three- to fivefold greater ability to impair psychomotor function than R-ketamine (White et al., 1985). The

TABLE 1 | Overview of key studies of ketamine in depression.

Number of patients investigated	Study design	Route of administration	Patient characteristics	Results	References
9 (2 drop-outs)	Randomized, double-blind study of single dose of ketamine hydrochloride (0.5 mg/kg); two treatment days, at least 1 week apart	Intravenous	Recurrent unipolar depression and bipolar depression; unmedicated patients	Significant improvement within 72 h after ketamine (HDRS)	Berman et al., 2000
18 (1 drop-out)	Randomized, placebo-controlled, double-blind crossover study of single dose of ketamine hydrochloride (0.5 mg/kg)	Intravenous	Major depressive disorder, recurrent, without psychotic features; unmedicated patients	Significant improvement within 110 min after ketamine which remained significant throughout the following week (HDRS)	Zarate et al., 2006a
10	Repeated-dose open-label ketamine hydrochloride (0.5 mg/kg; six infusions over 12 days)	Intravenous	Medication free symptomatic patients suffering from treatment-resistant depression (patients excluded if they had lifetime history of psychotic symptoms or hypomania/mania)	The mean (SD) reduction in MADRS scores after sixth infusion was 85% (12%).	aan het Rot et al., 2010
73	Two-site, parallel-arm, randomized controlled trial of a single dose of ketamine hydrochloride (0.5 mg/kg) compared to active placebo (i.e., midazolam, 0.045 mg/kg) in a 2:1 ratio.	Intravenous	Treatment-resistant major depression (patients excluded if they had lifetime history of psychotic symptoms or bipolar disorder); unmedicated patients (with the exception of a stable dose of a non-benzodiazepine hypnotic).	Ketamine group showed greater improvement (MADRS score) than midazolam group 24 h after treatment	Murrough et al., 2013a
24	Series of up to six infusions of ketamine hydrochloride (0.5 mg/kg) administered open-label three times weekly over a 12-day period.	Intravenous	Treatment-resistant major depression (patients excluded if they had lifetime history of psychotic symptoms or bipolar disorder); patients free of antidepressant medication during infusion period	Large mean decrease in MADRS score at 2 h after first ketamine infusion which was largely sustained for the duration of the infusion period.	Murrough et al., 2013b
18	Randomized, placebo-controlled, double-blind, crossover, add-on study of ketamine hydrochloride (0.5 mg/kg) or placebo combined with lithium or valproate therapy on 2 test days 2 weeks apart	Intravenous	Treatment resistant bipolar I or II depression without psychotic features	Depressive symptoms significantly improved within 40 min in subjects receiving ketamine compared with placebo; improvement remained significant through day 3.	Diazgranados et al., 2010
99	Double-blind ketamine or placebo added to ongoing antidepressant therapy; patients randomly assigned to one of five study arms in a 1:1:1:1:1 fashion: single dose of ketamine 0.1 mg/kg ($n = 18$), 0.2 mg/kg ($n = 20$), 0.5 mg/kg ($n = 22$), 1.0 mg/kg ($n = 20$), and a single dose of midazolam 0.045 mg/kg ($n = 19$)	Intravenous	Treatment-resistant MDD (patients excluded if they had history of bipolar disorder, schizophrenia, or schizoaffective disorders, or any history of psychotic symptoms in current or previous depressive episodes)	Evidence for the efficacy of the 0.5 mg/kg and 1.0 mg/kg subanesthetic doses of IV ketamine, no clear or consistent evidence for clinically meaningful efficacy of lower doses	Fava et al., 2020
197 patients completed 28-day double-blind treatment phase.	Phase 3, double-blind, active-controlled, multicenter study of esketamine (56 and 84 mg versus placebo)	Intranasal	Treatment resistant moderate to severe MDD (key exclusion criteria: diagnosis of psychotic disorder, major depressive disorder with psychotic features, bipolar or related disorders, borderline, antisocial, histrionic, or narcissistic personality disorder)	Change in MADRS score with esketamine plus antidepressant significantly greater than with antidepressant plus placebo at day 28, clinically meaningful improvement observed in the esketamine plus antidepressant arm at earlier time points	Popova et al., 2019
41	Randomized, double-blind, placebo-controlled, proof-of-concept trial; participants received either 1 mg/kg oral ketamine or placebo thrice weekly for 21 days	Oral	Treatment-resistant MDD (key exclusion criteria: psychotic disorder or psychotic symptoms, bipolar disorder)	Reduction in MADRS score on day 21 significantly greater in the ketamine group than in the control group. Six participants in ketamine group (27.3%) achieved remission compared with none of the controls.	Domany et al., 2019

available literature, though scant, seems to suggests that, in humans, subanesthetic doses of R-ketamine lack the dissociative potential of racemic ketamine (Vollenweider et al., 1997; Leal et al., 2021).

It is tempting to speculate that the dissociative and the antidepressant effects of ketamine might be separable. In the context of double-blind placebo-controlled drug testing, this is a complex issue because questions around functional unblinding due to ketamine's dissociative effects and the potential use of active comparators have to be considered (Ballard and Zarate, 2020). At least so far, the bulk of the available clinical evidence seems to favor an association between racemic ketamine's hallucinogenic/dissociative and antidepressant effects (Mathai et al., 2020).

While clinical research into a possible role for R-ketamine in depression is still in its infancy, sufficient data has already accrued to recommend the use of S-ketamine. Intravenous S-ketamine has been shown to produce rapid onset of robust antidepressant effects in patients with TRD after a 40-min infusion (Singh et al., 2016). Further, there is meta-analytical evidence for the adjunctive intranasal use of S-ketamine in TRD and in depressed patients with acute suicidality (Papakostas et al., 2020). Moreover, a recent randomized double-blind head-to-head comparison of intravenous S-ketamine (0.25 mg/kg) and racemic ketamine (0.5 mg/kg) as adjunctive therapy in TRD confirmed non-inferiority of S-ketamine (Correia-Melo et al., 2020).

N-methyl-D-aspartate receptor blockade may augment glutamatergic outflow, e.g., in the prefrontal cortex. Indeed, one plausible mechanism of this seemingly paradoxical effect is that ketamine, when administered in a subanesthetic dose, blocks NMDA receptors on γ-aminobutyric acid interneurons, thereby increasing presynaptic release of glutamate (Moghaddam et al., 1997; Pothula et al., 2020). According to this "disinhibition hypothesis" (**Figure 1B**), downstream activation of α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA) receptors in mood-regulating synapses is believed to play a crucial role in mediating ketamine's rapid antidepressant response as evidenced by the fact that pre-treatment with NBQX, an AMPA receptor antagonist, attenuates the behavioral effects of ketamine in experimental mice and rats (Maeng et al., 2008; Koike and Chaki, 2014). It thus appears that, ultimately, ketamine produces increased glutamatergic throughput of AMPA receptors, as compared to NMDA receptors, triggering rapid downstream changes on the molecular, structural, and network levels (**Figure 1B**; Jourdi et al., 2009; Li et al., 2010; Autry et al., 2011).

While clinical research has, so far, focused primarily on the S-ketamine stereoisomer, it has been hypothesized, based on behavioral studies in experimental mice, that R-ketamine should show the greater antidepressant potency (Yang et al., 2015; Zanos et al., 2016). To our knowledge, there is currently only one small pilot trial that has investigated the effects of R-ketamine in major depression (Leal et al., 2021). That open-label study of seven patients reported a significant decrease in Montgomery-Åsberg Depression Rating Scale scores within 24 h of a single intravenous infusion of R-ketamine (0.5 mg/kg).

There is extensive metabolism of ketamine stereoisomers via cytochrome P450 enzymes producing a broad array of catabolites including norketamine, hydroxyketamines, dehydronorketamine, and the hydroxynorketamines (Kharasch and Labroo, 1992; Desta et al., 2012). In particular, potent antidepressant properties have been ascribed to the (2R,6R)-hydroxynorketamine [(2R,6R)-HNK] metabolite (**Figure 1A**), which is exclusively derived from R-ketamine (Zanos et al., 2016). Mechanistically, (2R,6R)-HNK acts through AMPA receptor-mediated mechanisms, with the AMPA receptor antagonist NBQX reversing its antidepressant-like effects (Zanos et al., 2016). Moreover, (2R,6R)-HNK recapitulates key downstream events observed in the rodent brain in response to ketamine such as increased neurotrophic signaling and rapid dendritic and synaptic plasticity (Autry et al., 2011; Zanos et al., 2016).

NEUROTROPHIC SIGNALING, NEUROPLASTICITY, AND STRESS

Profound structural changes such as neuronal atrophy, loss of synapses, and a decrease in hippocampal neurogenesis reflect the deleterious effects of stress, stress hormones, and major depression on the brain (Duman et al., 2016). Brain-derived neurotrophic factor (BDNF)/tropomyosin receptor kinase B (TrkB) signaling is of crucial importance to neuronal plasticity, morphogenesis, and survival (Huang and Reichardt, 2001). Numerous pre-clinical studies have connected stress and an excess of corticosteroids with reduced BDNF signaling in depression-related brain areas (Smith et al., 1995; Schaaf et al., 2000; Vollmayr et al., 2000; Oh et al., 2019). Conversely, bilateral infusion of BDNF into the hippocampal dentate gyrus has been shown to produce antidepressant-like effects in behavioral models of depression (Shirayama et al., 2002). Antidepressant interventions such as electroconvulsive therapy (Nibuya et al., 1995), physical activity (Sleiman et al., 2016), and conventional antidepressant pharmacotherapy (Conti et al., 2002) have all been linked with a rise in brain BDNF levels. Likewise, ketamine administration has been shown to raise BDNF mRNA and protein levels in hippocampus (Choi et al., 2017). BDNF signaling seems to be central to ketamine's distinct antidepressant activity because ketamine fails to produce rapid antidepressant-like effects in either BDNF or TrkB conditional knockout mice (Autry et al., 2011). Quite unexpectedly, some very recent research has demonstrated an exciting new mode of action of several antidepressants, including ketamine, beyond increasing BDNF concentrations, namely, to directly bind to TrkB (Casarotto et al., 2021). Antidepressant binding to TrkB could then facilitate BDNF action and the attendant cellular as well as structural plasticity (Casarotto et al., 2021). An important intracellular signaling pathway activated in response to ketamine is the mammalian target of rapamycin pathway. Activation of this pathway promotes rapid synaptic plasticicity with increased synaptic signaling proteins and increased number and function of synapses (Li et al., 2010). In this context, and given that the anti-dementia drug memantine, which shares with ketamine the property of non-competitive NMDA antagonism, is widely prescribed in Alzheimer's disease, it may be worthwhile

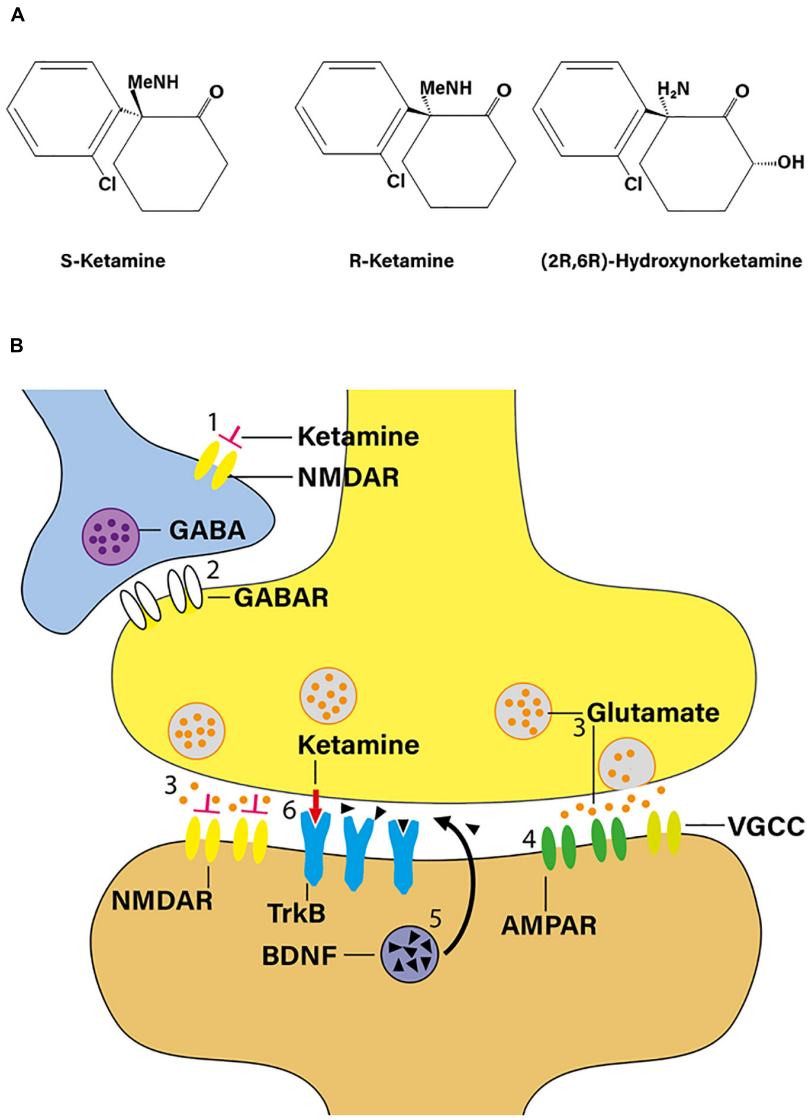

FIGURE 1 | Ketamine as a novel antidepressant. **(A)** Structural formula of S-ketamine, R-ketamine, and R-ketamine metabolite 2R,6R-hydroxynorketamine (adapted from Zanos et al., 2016). Me, methyl moiety. **(B)** According to the "disinhibition hypothesis" of ketamine action, NMDA receptor blockade by ketamine may increase glutamatergic outflow. When administered in a subanesthetic dose, ketamine blocks NMDA receptors on γ-aminobutyric acid (GABA) interneurons (1), thereby reducing GABA release (2) on principal neurons, and, in turn, increasing presynaptic release of glutamate (3). Preferential activation of postsynaptic α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid receptors (AMPAR) in mood-regulating synapses (4) is believed to play a critical role in mediating ketamine's rapid antidepressant response, triggering downstream changes such as inducing BDNF signaling (5). In addition, ketamine may also interact with the TrkB receptor directly (red arrow; 6). TrkB, tropomyosin receptor kinase B; VGCC, voltage-gated calcium channel (adapted from Shinohara et al., 2020).

to assess the effects of ketamine in patients with dementia (Smalheiser, 2019).

So far, few studies have investigated the effects of ketamine on hippocampal neurogenesis (Deyama and Duman, 2020). It has been reported that ketamine increases cell proliferation in the hippocampal dentate gyrus of rats showing a depressive-like phenotype (Michaëlsson et al., 2019). However, since neurogenesis is a multi-step process that unfolds over several weeks (Kempermann et al., 2004), it is unlikely that an overall increase in neurogenesis explains ketamine's rapid antidepressant effects. Still, increased recruitment of adult-born neurons into hippocampal circuitry (i.e., an acceleration in the final stages of neurogenesis) in response to ketamine is an obvious possibility, especially considering the importance of these immature cells for shaping memory processes (Anacker and Hen, 2017).

OPEN QUESTIONS AND OUTLOOK

An honest appraisal of where the field stands today must acknowledge the fact that, so far, "decades of 'murinization'" have contributed relatively little to antidepressant development

(Holsboer, 2014). From the sole perspective of drug discovery, the poor predictability of antidepressant efficacy based on behavioral assays in rodents is probably chief among today's challenges. On the other hand, it should be noted that the concept of NMDA antagonism in the treatment of depression was developed against a rich backdrop of experimental research (reviewed in Skolnick et al., 1996), demonstrating, among other things, that chronic administration of desipramine inhibits glutamatergic neurotransmission at NMDA receptors (Mjellem et al., 1993), and that both conventional antidepressants and electroconvulsive therapy alter the ligand-binding properties of the NMDA receptor complex (Paul et al., 1993, 1994). Given the short nature of this mini-review, the considerable body of preclinical evidence demonstrating ketamine's antidepressant activity in rodent models of depression has been largely passed over. For a detailed overview of this subject, the reader is referred to Polis et al. (2019) and Rincón-Cortés and Grace (2020).

How will the field evolve in the future? As a logical next step, the R-ketamine enantiomer is currently in the early stages of clinical development. Moreover, certain ketamine metabolites may hold promise as possessing equal antidepressant efficacy to the racemic parent molecule, possibly with fewer side effects, especially (2R,6R)-HNK. From a broader view, however, the prospect of discovering other molecules, not directly related to ketamine itself but tapping into the same neurobiological mechanisms, remains uncertain, at least for the time being. So far, the principle of NMDA antagonism has, unfortunately, not translated into tangible new drugs. Also, side-effects beyond psychotic symptoms have to be considered. Merck & Co's dizocilpine (commonly referred to as MK-801 in the lab), a strong NMDA receptor antagonist, was shown to produce acute pathomorphological lesions in specific populations of neurons when administered acutely to adult rats in comparatively low doses (Olney et al., 1989). MK-801 is no longer in active clinical development for this reason. Similar evidence of neurotoxicity (the eponymous "Olney's" lesions) has also been observed in experimental rodents after ketamine and PCP (reviewed in Ellison, 1995) and, more worryingly, in human ketamine addicts (Wang et al., 2013). Given these findings, it will be important, from a safety standpoint, to monitor the long-term effects of NMDA antagonist therapy (including with ketamine and S-ketamine) on brain structure and patients' cognitive trajectories.

Moving beyond neurotoxicity, which may represent a class effect, investigations of NMDA receptor antagonists other than ketamine in depression have, so far, failed to produce clinically relevant outcomes. Memantine proved ineffective as an antidepressant in two double-blind placebo-controlled trials (Zarate et al., 2006b; Smith et al., 2013). Similarly, rislenemdaz (also known as MK-0657), an NR2B subunit-specific NMDA receptor antagonist, failed to produce antidepressant effects in TRD, either when used as a monotherapy or in conjunction with other antidepressants (Ibrahim et al., 2012; Henter et al., 2021). Lanicemine, an NMDA blocker with low rates of associated psychotomimetic effects, does not come near to replicating ketamine's antidepressant effects (Zarate et al., 2013; Sanacora et al., 2017). More recently, three phase-III clinical trials of rapastinel, an NMDA receptor modulator with glycine-site partial agonist features, also failed to demonstrate antidepressant effects (Henter et al., 2021). This outcome is sobering, given that pre-clinical research had demonstrated antidepressant-like effects of rapastinel in mice and rats (Burgdorf et al., 2013; Yang et al., 2016).

The possibility of still other modes of action should also not be overlooked. It has long been known that ketamine possesses certain anti-inflammatory properties, which may especially benefit patients undergoing major surgery or septic patients requiring sedation (Kawasaki et al., 1999; Welters et al., 2011). Intriguingly, lipopolysaccharide-induced sickness behavior in mice can be blocked by ketamine (Walker et al., 2013). Moreover, the antidepressant effects of the two ketamine enantiomers in the chronic social defeat stress model of depression have been linked with restoration of gut microbiota in mice (Yang et al., 2017).

Opioid effects have also been implicated in ketamine's clinical profile. Both S- and R-ketamine bind to and activate mu and kappa opioid receptors (Bonaventura et al., 2021). Further, it has recently been reported that naltrexone blocks the antidepressant effects of ketamine in depressed patients (Williams et al., 2018).

In the aggregate, ketamine represents the first major breakthrough in antidepressant development in the last half-century. As described above, it engages novel mechanisms beyond monoaminergic neurotransmission, resulting in a much faster onset of action than conventional monoamine-based therapeutics. Although much remains to be elucidated, the advent of ketamine signals exciting new opportunities to extend and refine our knowledge of the neurobiological mechanisms underlying the antidepressant response. Given the accruing evidence of ketamine's therapeutic effects in TRD, it seems that the time has arrived to assign a central position to ketamine as an augmentation in the treatment algorithms for TRD patients.

AUTHOR CONTRIBUTIONS

GK drafted the manuscript with substantive input from all authors. All authors contributed to the article and approved the submitted version.

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Sex-Specific Behavioral Response to Early Adolescent Stress in the Genetically More Stress-Reactive Wistar Kyoto More Immobile, and Its Nearly Isogenic Wistar Kyoto Less Immobile Control Strain

*Sarah Kim†, Stephanie A. Gacek†, Madaline M. Mocchi and Eva E. Redei**

Department of Psychiatry and Behavioral Sciences, Feinberg School of Medicine, Northwestern University, Chicago, IL, United States

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Ravid Doron,
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Nafissa Ismail,
University of Ottawa, Canada

***Correspondence:**
Eva E. Redei
e-redei@northwestern.edu

†These authors share first authorship

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Genetic predisposition and environmental stress are known etiologies of stress-related psychiatric disorders. Environmental stress during adolescence is assumed to be particularly detrimental for adult affective behaviors. To investigate how genetic stress-reactivity differences modify the effects of stress during adolescence on adult affective behaviors we employed two inbred strains with differing stress reactivity. The Wistar Kyoto More Immobile (WMI) rat strain show increased stress-reactivity and despair-like behaviors as well as passive coping compared to the nearly isogenic control strain, the Wistar Kyoto Less Immobile (WLI). Males and females of these strains were exposed to contextual fear conditioning (CFC) during early adolescence (EA), between 32 and 34 postnatal days (PND), and were tested for the consequences of this mild EA stress in adulthood. Early adolescent stress significantly decreased anxiety-like behavior, measured in the open field test (OFT) and increased social interaction and recognition in adult males of both strains compared to controls. In contrast, no significant effects of EA stress were observed in adult females in these behaviors. Both males and females of the genetically less stress-reactive WLI strain showed significantly increased immobility in the forced swim test (FST) after EA stress compared to controls. In contrast, immobility was significantly attenuated by EA stress in adult WMI females compared to controls. Transcriptomic changes of the glucocorticoid receptor (*Nr3c1*, GR) and the brain-derived neurotrophic factor (*Bdnf*) illuminate primarily strain and stress-dependent changes, respectively, in the prefrontal cortex and hippocampus of adults. These results suggest that contrary to expectations, limited adolescent stress is beneficial to males thru decreasing anxiety and enhancing social behaviors, and to the stress more-reactive WMI females by way of decreasing passive coping.

Keywords: Wistar Kyoto More Immobile, passive coping, depression, social recognition, sex differences, brain-derived neurotrophic factor, glucocorticoid receptor

INTRODUCTION

Adolescence has gained increasing attention as a sensitive period of development, a period in which pubertal transitions may increase the vulnerability to stressors (McCormick et al., 2017). Adolescence is defined as a transition between childhood and adulthood, and puberty is thought to be an important hallmark of it. Adolescence is also of great clinical importance; it is the time in which many mental health problems such as mood disorders emerge. Further, the risk of adult drug abuse and addiction is greater in adults who were exposed to drugs in their adolescence, rather than those who started drug use in adulthood (Odgers et al., 2008). Animal studies has confirmed the effect of stress during adolescence to be deleterious for adult affective and cognitive functions as well as stress reactivity (Pohl et al., 2007; McCormick et al., 2008; Arnett et al., 2015). However, these effects of adolescent stress were found to be sex dependent (McCormick and Mathews, 2007; Weintraub et al., 2010). It is often thought that females are more vulnerable to the effects of adolescent stress, particularly in their depressive behavior (Mathews et al., 2008; Bourke and Neigh, 2011). In contrast, differences have been observed in anxiety-like behaviors of adult males after acute adolescent stress, with no or very limited effect in females (Lovelock and Deak, 2019).

In animal studies, stressors can be administered to rodents at postnatal day (PD) 22–32 during early adolescence (EA) or peripubertal period, later in adolescence up to PD 45, or post-pubertally (Lo Iacono and Carola, 2018). Stress experienced in the peripubertal phase is of clinical importance, as it is associated with the development of disorders such as depression, anxiety, and post-traumatic stress disorder in adulthood (Spear, 2000; Bale et al., 2010; McCrory et al., 2012; Turecki, 2012; Brown and Spencer, 2013). It is known that acute stress produces more prolonged activation of the hypothalamic-pituitary-adrenal (HPA) axis in peripubertal animals than in adults, thus, the consequence of acute stress during this developmental period could be long lasting (Lui et al., 2012). However, how genetic predisposition to increased stress-reactivity interacts with a mild developmental challenge is less explored. Specifically, animals of differing stress-reactivity that encounter a mild, acute stress during the prepubertal EA period may produce lesser or differential affective behaviors in adulthood.

Two inbred rat strains with differential stress-reactivity were employed in this study. These strains were generated by bidirectional selection from the Wistar Kyoto (WKY) parental strain using immobility behavior in the forced swim test (FST) as a functional selector (Will et al., 2003). The by now inbred Wistar Kyoto More Immobile (WMI) strain consistently show greater immobility compared to the inbred WKY Less Immobile (WLI) strain (Andrus et al., 2012; Mehta et al., 2013). Adult WMI males have decreased anxiety-like behavior in the open field test (OFT) compared to WLI males, while females of both strains have shown the same high levels of anxiety in adulthood (Mehta et al., 2013). WMI males also show greater behavioral stress reactivity than WLI males, indicated by their increased fear memory after stress (Lim et al., 2018).

Our hypothesis was that EA stress would exacerbate the behavioral deficits of WMI rats and generate a vulnerability in the WLI strain in adulthood. Additionally, we hypothesized that the genetic differences in stress-reactivity between the strains would interact with the sex differences observed in other rodent studies in adult behaviors after EA stress. To test these hypotheses, males and females of the more stress-reactive WMI, and their nearly isogenic less stress-reactive control WLIs were exposed to a stressor during early adolescence. This stressor was a mild foot-shock received during the contextual fear conditioning (CFC) test. In adulthood, we tested remote fear memory (RM) to determine the EA stress effects. After RM, we tested affective behaviors that are known to differ between the strains and sexes in adult WMI and WLIs. Previously, strain and sex differences have been seen in the OFT and in the FST, the latter being the functional selector for the original selective breeding (Will et al., 2003; Mehta et al., 2013). After adult stress, social interaction and recognition also differed within the strains by sex (Schaack et al., 2021).

Expression of the brain-derived neurotrophic factor (BDNF) has been shown to be region-specifically responsive to stress and thought to be a marker of neuronal plasticity (Bath et al., 2013). The BDNF stress-sensitivity hypothesis posits that disruption in the endogenous BDNF activity potentiates sensitivity to stress (Notaras and van den Buuse, 2020). Since the WMI strain show enhanced sensitivity to stress, *Bdnf* expression in relevant brain regions, specifically the hippocampus and the prefrontal cortex, has been studied. Although the nature of the BDNF-glucocorticoid connection is very complex (Choy et al., 2008; Numakawa et al., 2013), a brain region and development-dependent connection has been suggested between them (Choy et al., 2008; Daskalakis et al., 2015). Therefore, we examined the glucocorticoid receptor (*Nr3c1*) expression in the same brain regions.

The purpose of this study was to determine whether genetic hypersensitivity to stress would alter behaviors in adulthood after a limited mild stress during early adolescence. Additionally, sex differences in the effects of EA stress could also be determined, and thereby ascertain the significance of sex in the consequences of genetic—environmental interactions.

MATERIALS AND METHODS

Animals

Animal procedures were approved by the Institutional Animal Care and Use Committee of Northwestern University. The Wistar Kyoto (WKY) More Immobile (WMI) rat strain, a genetic rat model of enhanced stress-reactivity and depressive-like behavior, and the Wistar Kyoto Less Immobile (WLI) control strain are derived from the WKY parental strain. The inbred WMI and the nearly isogenic inbred WLI animals were of the 39–41st generation. Due to the low fecundity of these animals (Luo et al., 2020), animals from multiple generation were used to achieve the numbers needed for the study. The rats were maintained at Northwestern University Feinberg School of Medicine by the Center for Comparative Medicine. Animals were group housed

(2–3 per cage), maintained on a 12-h light and dark cycle (lights on at 0600h) in a temperature and humidity-controlled room with *ad libitum* access to food and water.

Adolescent male and female WMI and WLI rats were used (n = 7–8/sex/strain) in the stress group at postnatal day (PND) 32–34. Control WMI and WLI animals (n = 6–7/sex/strain) were not disturbed until adulthood. Adult behavioral testing started at PND 70–75.

Behavioral testing was carried out in the following time sequence: Adolescent FC at PND 32–34; Remote Memory in the CFC at PND 70–75; OFT at PND 77–82; Social interaction (SI) and recognition (SR) at 90–125; FST at PND 120–150, and animals were sacrificed at PND 180–210. The rest periods between the tests were selected to minimalize carryover effects from the previous behavioral tests.

All behavioral experiments and sacrificing the animals were conducted between 1,000 and 1,600 h. Between 1,000 and 1,600 h, there is no major change in the circadian rhythm-driven plasma CORT levels (Solberg et al., 2001; Sage et al., 2004). The behavioral experiments were either recorded and analyzed by the computerized behavioral system, or video recorded, and behaviors were analyzed by trained observers during repeated viewing.

Behavioral Measures

Contextual Fear Conditioning During Adolescence

Male and female, WLI and WMI adolescents (PND 32–34) were placed into an automated fear conditioning apparatus of Technical and Scientific Equipment (TSE, Bad Homburg, Germany) for 3 min of habituation, followed by one foot shock (0.8 mA, 1 s duration). The animals spent 1 min after the shock in the chamber. Twenty-four hours later, the rats were placed in the same apparatus for 3 min, without a shock, and their percent freezing, and total distance traveled were recorded automatically.

Remote Contextual Fear Memory in Adulthood

Remote fear memory of the animals was measured between PND 70–75. The animals were placed back into the automated fear-conditioning apparatus for 3 min, without a shock, and their percent freezing, and total distance traveled were recorded.

Open Field Test

Control and early adolescent stressed (EA-stressed) animals were tested between PND 77–82, a week after the remote memory test as described. Rats were placed in the center of an 82-cm diameter arena for 10 min. The time spent in the center and distance traveled by the animal were measured in the center 50-cm diameter area by TSE Videomot 2 version 5.75 software (TSE, Bad Homburg, Germany).

Social Interaction and Recognition

At PND 90–125, animals were singly housed for 48 h and tested for social interaction and recognition. The SI test employed in this study was selected in order to avoid provoking anxiety in the test animal. Anxiety-like behavior is known to differ between WLI and WMI males and females (Mehta et al., 2013). Therefore, in the present SI test, juveniles were chosen as stimulus animals, because their much smaller size does not threaten the test animals and generate anxiety (Engelmann et al., 1995). The wider than usual age range of the animals was due to the low fecundity of these strains (Luo et al., 2020), and therefore, the infrequent availability of the juvenile stimulus animals.

During the sample trial of the SI test (T1), one 25–28 days old juvenile rat was placed in the home cage of the test animal for 4 min. The juvenile was removed, and the test animal was given a 45-min rest period. This rest period was chosen as the maximum time for social recognition of familiar conspecifics in rats (Squires et al., 2006). After the 45-min rest, the original (familiar) juvenile and a novel juvenile were placed in the home cage with the adult for a 4-min test trial (T2). Juveniles were distinguished by different color non-toxic marks on their tails. Both trials were videotaped from the long side of the home cage, and behaviors were analyzed by trained observers using multiple stopwatches and repeated viewing.

The behavior of the test animals was scored for olfactory investigation (direct contact sniffing and following) in both the sample and test trials. Olfactory investigation was recorded separately for each juvenile and was defined as seconds spent sniffing within 1 cm of each juvenile. Social recognition was characterized by subtracting the time the test animal spent time investigating the familiar juvenile in the test trial from investigating the same juvenile in the sample trial (T2-T1). A larger number indicates that the test animal remembers the familiar juvenile.

Forced Swim Test

A minimum of 2 weeks after the social interaction, social recognition test, FST was carried out as described by Porsolt et al. (1977). The adult, 4–5 months old animals were placed into a glass cylinder (30 cm diameter, 45 cm deep) of 23°C tap water for 15 min. Twenty four hours later, rats were again placed into the cylinder of water for 5 min. Activity during the second swim test was video-recorded for subsequent scoring using a time-sampling technique previously described (Detke et al., 1995) in which behavior was scored as immobility, climbing, or swimming every 5 s. We have previously shown that immobility in the FST is not related to body weight (Solberg et al., 2001). Increased immobility indicates increased despair-like behavior, or according to another interpretation increased passive coping.

Brain Dissection and RNA Isolation

Animals were sacrificed at 6–7 months of age via swift decapitation. Whole brains were removed and stored in RNAlater (Invitrogen, Carlsbad, CA) at room temperature for 24 h and then at −80°C until dissection. Brains were thawed on ice and dissected. Prefrontal cortex and hippocampi were dissected on a brain matrix and immediately stored in RNAlater (Invitrogen, Carlsbad, CA) at −80°C. Paxinos coordinates were used: Prefrontal cortex (AP 5.20–1.70, ML 0–3.3, DV 28 9.0–4.4) and hippocampus (AP −2.12 to −6.0, ML 0–5.0, DV 5.4–7.6) (Wilcoxon et al., 2005).

The tissue was homogenized using TRI Reagent (Sigma-Aldrich, Saint Louis, MO) and a handheld tissue homogenizer (Kinetica Polytronic). Total RNA was isolated from brain samples

using the Direct-zol RNA MiniPrep Plus kit (Zymo Research, Irvine, CA) according to manufacturer's instructions. RNA quality was determined using a NanoDrop (Thermo Fisher Scientific). Accepted quality ranged from 1.8 to 2.2 for 260/280 and 260/230 ratios. Samples were stored at −80°C.

Reverse Transcription and Quantitative Polymerase Chain Reaction

Reverse transcription was carried out using the Super Script VILO Master Mix (Invitrogen). 1.0 μg of total RNA was used with this manufacturer's protocol. qPCR was performed with 5 ng cDNA, specific primer pairs for *Nr3c1* (forward: 5′AACAGACTTTCGGCTTCTGGAA 3′; reverse 5′ TGGAACGCTGGTCGACCTAT 3′), and for *Bdnf* (forward: 5′ ATTACCTGGATGCCGCAAAC 3′; reverse 5′ GGGACTTTCTCCAGGACTGT 3′) SYBR Green Master Mix (Applied Biosystems, Foster City, CA, United States), using the QuantStudio™ 6 Flex Real-Time PCR System (Applied Biosystems). Triplicate reactions were performed for each cDNA sample. Relative quantification levels of gene expression, or RQ values, were determined relative to *Gapdh* (forward: 5′ CAACTCCCTCAAGATTGTCAGCAA 3′; reverse: 5′ GGCATGGACTGTGGTCATGA 3′) and a general cDNA calibrator (from blood or hippocampus), using the $2^{-\Delta\Delta Ct}$ method, performed by the QuantStudio™ Software (Applied Biosystems).

Statistics

All data were represented as mean ± standard error of the mean. All measures were analyzed using a 3-way analysis of variance (ANOVA, stress x strain x sex) followed by false discovery rate (FDR) for multiple comparisons (GraphPad 9.0, San Diego, CA; Benjamini and Hochberg, 1995). Significance was established at $q \leq 0.05$, but occasionally when the comparison did not reach significance by correction for multiple comparison, individual $p \leq 0.05$ values were also indicated on the figures. ANOVA results are reported in the results, while *post hoc* significance is shown on the figures. Cohen's d effect sizes were also calculated, and the ranges are described in the figure legends where it could be compared with the q and p statistics.

RESULTS

Behavioral Consequences of Stress During Early Adolescence

During EA, there were no significant differences in fear memory due to strain or sex as measured by percent freezing (**Figure 1**). When adults were placed into the CFC without foot-shock to indicate remote memory (RM) after over a month, percent freezing was dramatically and significantly higher in adult WLI males after the EA stress of CFC compared to adolescent WLI males, adult WLI females and adult WMI males [sex × age, $F(1, 52) = 18.26$, $p < 0.01$; strain × age, $F(1, 52) = 9.35$, $p < 0.01$; strain × sex, $F(1, 52) = 5.28$, $p < 0.05$]. This elevated percent freezing of EA-stressed adult WLI males contributed to the significant main effect of sex and age [sex, $F(1, 52) = 11.99$, $p < 0.01$; age, $F(1, 52) = 4.66$, $p < 0.05$]. There was no effect of EA stress on adult females' RM.

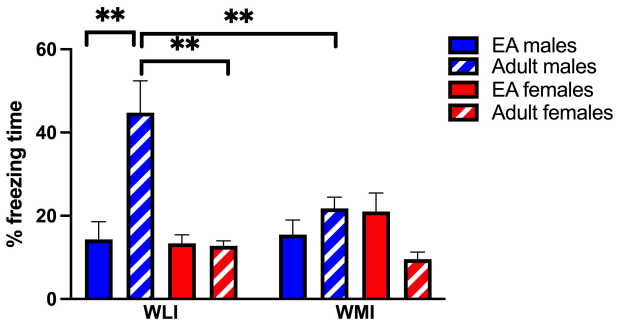

FIGURE 1 | Contextual fear memory of early adolescents and remote memory of adult WLI and WMI males and females expressed as percent freezing. Early adolescent WLI and WMI males and females were tested in the CFC on postnatal day (PND) 32–34. The same animals were placed back into the CFC apparatus as adults and their percent freezing determined without foot-shock for measuring their remote memory. There was no difference in fear memory in early adolescents, but when the animals were returned to the context, WLI males froze significantly more than adult WLI females and WMI males. Data are presented as mean ± standard error of mean (SEM). N = 5–8/sex/strain. *Post hoc* comparisons are indicated as $^{**}q < 0.01$ and determined by two stage linear step-up procedure of Benjamini, Krieger and Yekutieli (FDR) after three-way ANOVA. Cohen's d effect sizes were 1.77–2.60 in *post hoc* comparisons.

Interestingly, this elevated percent freezing of adult WLI male after EA in the CFC coincided with increased time spent in the anxiety-provoking center of the OFT, but this effect was also present in EA stressed adult WMI males compared to non-stressed controls [**Figure 2A**; stress × sex, $F(1, 46) = 8.06$, $p < 0.01$]. Significant main effects were also seen for sex and strain [sex, $F(1, 46) = 34.54$, $p < 0.01$; stress, $F(1, 46) = 10.23$, $p < 0.01$]. There was no effect of EA stress on females of either strain.

Distance covered in the center showed a similar pattern to time in center (**Figure 2B**). However, it also revealed that adult WMI males after EA stress explored the center significantly more than stressed adult WLI males. Furthermore, adult stressed WMI males traveled significantly more in the center than their female counterparts after stress [strain × stress × sex, $F(1, 49) = 4.7$, $p < 0.05$; stress × sex, $F(1, 49) = 15.0$, $p < 0.001$]; [strain × stress, $F(1, 49) = 8.6$, $p < 0.01$]; [sex, $F(1, 49) = 15.0$, $p < 0.01$]; [stress, $F(1, 49) = 24.0$, $p < 0.01$]; strain, [$F(1, 49) = 5.5$, $p < 0.05$]. There was no effect of EA stress on females of either strain.

Curiously, adult EA-stressed WLI males also showed significantly increased ambulation compared to controls, which suggest that their increased distance traveled in the center are not necessarily a reflection of decreased anxiety, but more of enhanced activity (**Figure 2C**). Stressed WLI males also showed significantly greater activity compared to EA-stressed adult WLI females [stress × sex, $F(1, 49) = 5.67$, $p < 0.05$]; [sex, $F(1, 49) = 11.55$, $p < 0.01$]. WMIs showed no differences in total distance regardless of adolescent stress status or sex. There was no effect of EA stress on females of either strain.

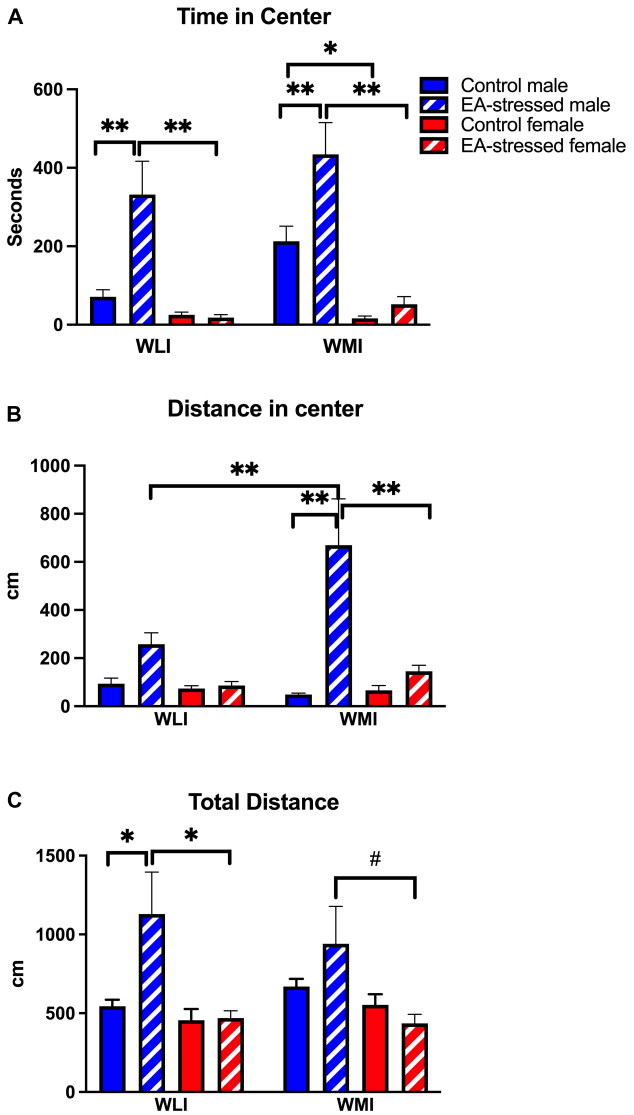

FIGURE 2 | Stress during early adolescence decreases anxiety-like behavior in males of both strains compared to controls in the OFT without any effects on females. **(A)** Male WLIs and WMIs spent significantly more time in the center after stress during early adolescence (EA) compared to control males and EA-stressed females. Cohen's d effect sizes ranged between 1.21 and 2.9. **(B)** Distance traveled by EA-stressed WMI males in the center of open field arena was significantly greater compared to control WMI males and EA-stressed WLI males and WMI females. Cohen's d effect sizes ranged between 2.93 and 4.56. **(C)** Total distance traveled during OFT differed only by EA-stressed WLI males from control WLI males and EA-stressed WLI females. Cohen's d effect sizes ranged between 1.04 and 1.40. Data are presented as mean ± SEM. N = 6–10/sex/strain/stress status. $^{*}q < 0.05$; $^{**}q < 0.01$ by FDR *post hoc* test after three-way ANOVA. $^{\#}p < 0.05$ represent independent p-values not corrected for multiple comparisons and shown for interpretation purposes.

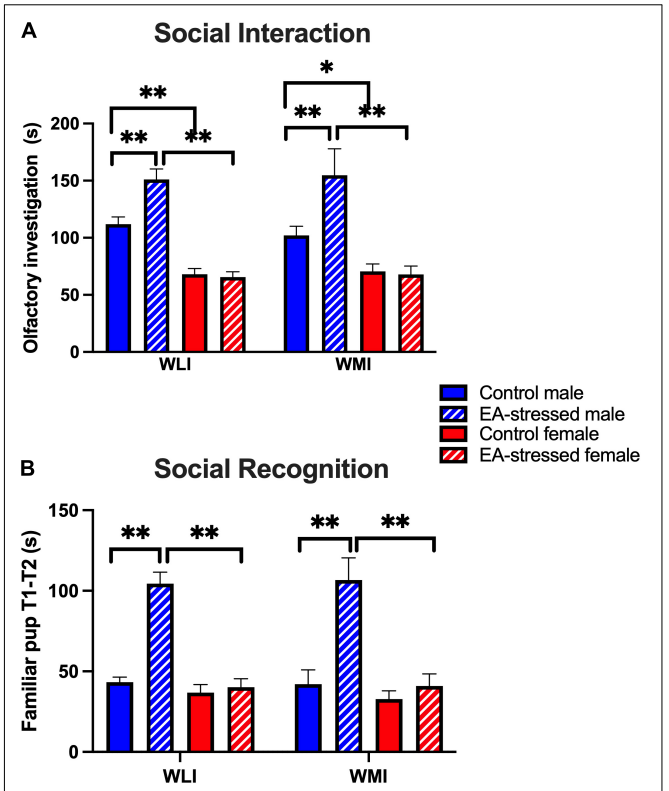

FIGURE 3 | Social interaction and recognition are increased in EA-stressed males of both strains compared to controls, no change in females. **(A)** Social interaction measured by total time spent (s) with olfactory investigation of the juvenile. Cohen's d effect sizes ranged between 3.04 and 11.7. **(B)** Social recognition or memory is characterized by the time difference in investigating the familiar pup in the first and second phases. Cohen's d effect sizes ranged between 2.43 and 3.98. Data are presented as mean ± SEM.
N = 4–10/sex/strain/stress status. $^{*}q < 0.05$; $^{**}q < 0.01$ by FDR *post hoc* test after three-way ANOVA.

In the social interaction test with non-threatening juveniles, EA stressed adult males interacted significantly more than their controls regardless of strain (**Figure 3A**). In addition, control adult males interacted significantly more than control females and EA stressed males interacted significantly more than EA stressed females [stress × sex, $F(1, 51) = 15.00$, $p < 0.01$]; [stress, $F(1, 51) = 12.00$, $p < 0.01$]; [sex, $F(1, 51) = 95.00$, $p < 0.01$]. There was no effect of EA stress on females of either strain.

Social recognition was measured by the difference in time the animal spent with investigating the same juvenile during the original interaction when the juvenile was alone and the second interaction when there was also a novel juvenile in the cage. Increased social recognition indicated that the animals spent less time investigating the familiar animal during the second interactive test stage. Stressed adult males recognized the familiar juvenile more than control males and more than EA stressed adult females, regardless of strain [**Figure 3B**; stress × sex, $F(1, 42) = 32.58$, $p < 0.01$]; [stress, $F(1, 42) = 27.54$, $p < 0.01$]; [sex, $F(1, 42) = 35.26$, $p < 0.01$]. There was no effect of EA stress on females of either strain.

The WLI and WMI inbred strains were selectively bred originally by their behavior in the FST. Thus, not surprisingly, EA stress had major, and sex-dependent effects on immobility in the FST, differently in WLIs and WMIs (**Figure 4**). Stressed adult

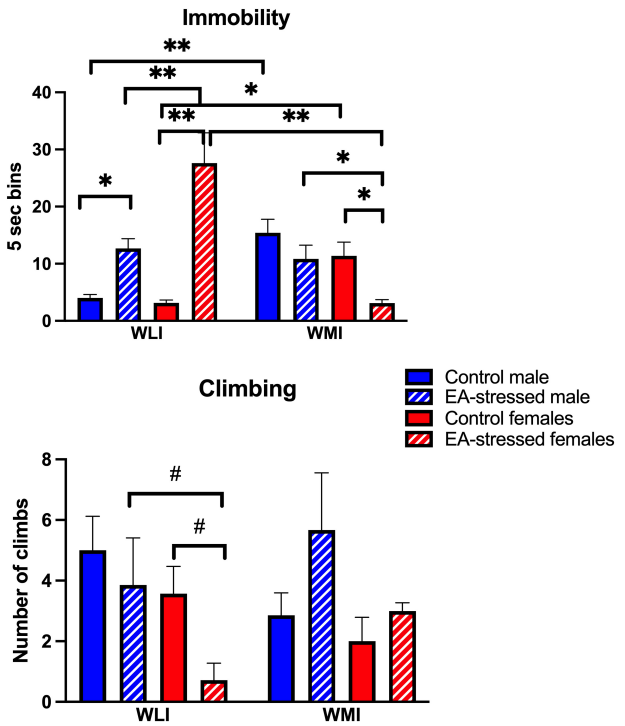

FIGURE 4 | Forced swim test demonstrating immobility behavior in WMI and WLI. Early adolescent stress increases immobility behavior in adult WLIs while it reduces immobility behavior in WMI females. Immobility behavior (floating) was measured every 5s (bins) on the second day of FST. Climbing behavior was observed against the wall of the glass chamber and showed greater variations. Cohen's d effect sizes for immobility ranged between 1.67 and 2.89. Cohen's d effect sizes for climbing were 1.02 and 1.44. Data are presented as mean ± SEM. N = 4–10/sex/strain/stress status. $^{*}q < 0.05$; $^{**}q < 0.01$ by FDR *post hoc* after three-way ANOVA. $^{\#}p < 0.05$ represent independent p-values not corrected for multiple comparisons and shown for interpretation purposes.

WLI males and females showed significantly greater immobility than control WLIs. In contrast, adult WMI females decreased their immobility after EA stress, and no significant effects of EA stress were seen in adult WMI males [strain × sex × stress, F (1,51) = 8.50, $p < 0.01$]; [strain × stress, $F(1, 51) = 48.00$, $p < 0.01$]; [strain × sex, $F(1, 51) = 15.00$, $p < 0.01$]; [stress, $F(1, 51) = 9.30$, $p < 0.01$].

Climbing was not always easy to observe, and it did not reflect an inverse relationship with immobility (**Figure 4**). The effect of EA stress tended to decrease climbing in adult WLI females compared to controls and stressed WLI males [strain × stress, $F(1, 47) = 7.10$, $p = 0.01$]; [sex, $F(1, 47) = 7.60$, $p < 0.01$].

Transcript Levels in the Prefrontal Cortex and the Hippocampus

Glucocorticoid receptor (*Nr3c1*) expression was significantly higher in the prefrontal cortex of control and EA-stressed adult WLI females than males, and significantly higher compared to control WMI females [**Figure 5A**; strain × sex × stress, $F(1, 40) = 4.26$, $p < 0.05$, sex × stress, $F(1, 40) = 24.10$, $p < 0.01$]; [strain × stress, $F(1, 40) = 19.31$, $p < 0.01$]; [strain × sex, $F(1, 40) = 14.14$, $p < 0.01$]. Analysis of hippocampal *Nr3c1* transcript levels resulted in a significant main effect of strain, although all *post hoc* tests showed only individual p-value significance [**Figure 5A**; strain, $F(1, 40) = 8.90$, $p < 0.01$]. Expression of *Nr3c1* in both control and EA-stressed adult WLI males were higher than those of WMI males, and control WLI females had greater expression than control WMI females.

Bdnf expression was higher in the prefrontal cortex of adult control WMIs compared to control WLIs, although EA stress eliminated this difference by reducing expression in stressed male WMIs [**Figure 5B**; strain, $F(1, 39) = 9.91$, $p < 0.01$]; [stress, $F(1, 39) = 7.30$, $p = 0.01$]. Interestingly, this latter difference was the opposite in the hippocampus of adult WMI males and females, who showed decreased *Bdnf* expression after EA stress [**Figure 5B**; stress, $F(1, 37) = 9.96$, $p < 0.01$]. Sex differences were seen between EA stressed adult WLI males and WLI females in hippocampal *Bdnf* expression [sex, $F(1, 37) = 10.46$, $p < 0.01$].

DISCUSSION

The goals of the study were to identify the role of genetic stress-reactivity in the long-lasting behavioral effects of stress during early adolescence. The adolescent stress was a contextual fear memory test, in which no strain and sex differences were found. The remote memory of this stress was affected in adult males, specifically in WLI males, but not in females. Similarly, adult females were not affected by EA stress in their anxiety and social behaviors, while this EA stress brought positive changes in these behaviors to adult males. However, the enhanced immobility of the less stress-reactive WLI males and females after EA stress in the FST suggest that similarly to other strains, EA stress enhances depression-like behavior or passive coping in adulthood. The opposite effect of EA stress on WMI females question whether this mild stress can generate a resilience in the stress-reactive strain, particularly in females that were not affected by EA stress in any of the other measures. The lack of effect of EA stress on *Nr3c1* expression in either brain region, but the WMI-specific changes in *Bdnf* transcript levels propose that glucocorticoid independent processes regulate these latter changes.

Adult-like CFC emerges by PD 24 (Casey et al., 2015), therefore, the lack of strain and sex differences in fear memory at EA suggests no differences in developmental trajectory between the strains. Still, despite this lack of differences in EA responses to CFC, major strain and sex differences were found in remote memory, particularly in WLI males. The increased percent freezing of adult EA-stressed WLI males is seemingly contradictory with their increased time spent in the center of the OFT. Would this mean a clear separation between fear and anxiety as increased percent freezing suggests increased fear, while increased time spent in the anxiety-provoking center of the OFT indicates decreased anxiety?

In neurobiological research, the distinction between anxiety and fear is ambiguous, and often one is used to define the other (Perusini and Fanselow, 2015). Fear is thought to be specific to responses directed to a present threat associated with specific

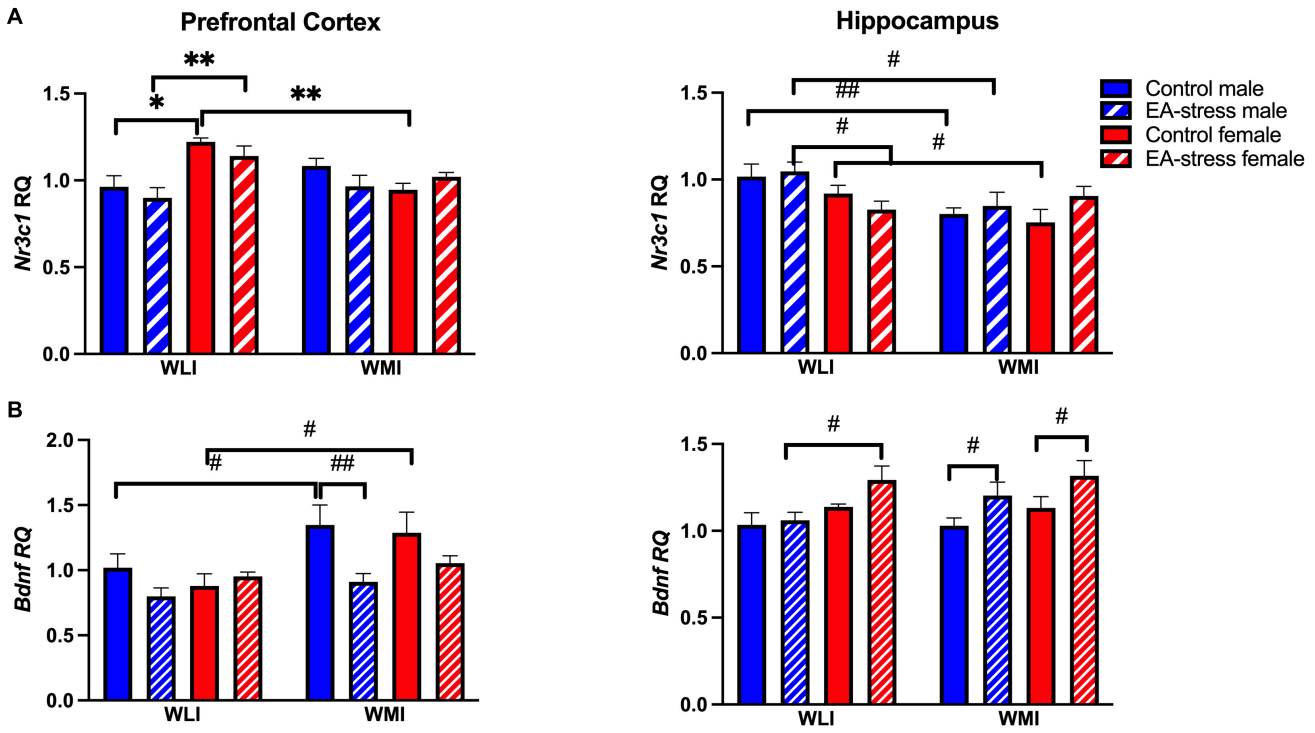

FIGURE 5 | Prefrontal cortex and hippocampal expression of *Nr3c1* **(A)** and *Bdnf* **(B)** in WLI and WMI. Transcript levels were measured by quantitative RT-qPCR and shown as Relative Quantification (RQ). Data are presented as mean ± SEM. N = 5–7/sex/strain/stress status. $^{*}q < 0.05$; $^{**}q < 0.01$ by FDR *post hoc* test after three-way ANOVA. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ represent independent p-values not corrected for multiple comparisons and shown for interpretation purposes.

cues or contexts, while anxiety is in preparation for threats that are future-oriented (Davis et al., 2010). The distinct definition of fear and anxiety are supported by opposing changes found after environmental challenges in these two facets of danger-induced behaviors (Heinz et al., 2021). However, human and animal studies support the opposing hypothesis showing correlations between these behaviors (Indovina et al., 2011; Ahn et al., 2013). Since anxiety measurements are based on the rodents' natural tendency to avoid open areas, it is thought to measure intrinsic anxiety-related characteristics of the animal. Since male WMIs already show less anxiety than WLIs (Mehta et al., 2013), the exaggerated effect of EA stress on decreasing anxiety of the adult WMI males could be due this innate difference. Indeed, the genetically high anxiety HR rats also show increased contextual fear memory compared to the low anxiety LR animals (Lehner et al., 2010), just like the EA-stressed WLI males.

The finding of decreased anxiety-like behaviors in males of both strains after EA stress is similar to some, but dissimilar to other findings in the literature. Acute lipopolysaccharide injection at PND 26 shown to be anxiolytic in male, but not in female, Wistar rats as measured by time spent in the open arm of the elevated plus maze (EPM) (Ariza Traslaviña et al., 2014). No other acute stressor applied at the same developmental period showed this response in the study. Sex specific effect is also reported for Wistar rats, when acute stress during adolescence increases anxiety behavior in male Sprague-Dawley rats, while females are not affected (Lovelock and Deak, 2019). Most studies report increased anxiety-like behaviors in adult male rodents after a single or repeated mild stress during the same developmental period (Tsoory et al., 2007; Mancini et al., 2021; Meyer et al., 2021). In contrast, a 3-day stress in the same EA period increases anxiety-like measures in the OFT and the EPM in both adult male and female rats (Jacobson-Pick and Richter-Levin, 2010).

The current findings of decreased anxiety-like behaviors in adult males after EA stress is novel and at variance with other studies. The cause of these differing results is likely related to the strains of the animals employed. The parental strain of the WLI and WMI, the WKY strain, show increased anxiety and depression-like behavior as measured by immobility in the FST. However, immobility in the FST is also considered to be more of a measure of passive coping with stress (de Kloet and Molendijk, 2016). The selective breeding was based on the immobility behavior, and anxiety behavior in the OFT did not segregate consistently between the strains. In the present study, males of both strains showed decreased adult anxiety-like behavior after the relatively mild acute stress during early adolescence, suggesting a resilience generated by the EA stress. In contrast, only adult WMI females had a positive effect of adolescent stress on immobility in the FST. Interestingly, in a recent study we found that stress during adolescence in another strain of rats with genetic predisposition to increased passive coping led to increased anxiety in males, but decreased immobility in the FST in females (Ji et al., 2021). Would females genetically predisposed

to increased passive coping be more resilient to the effects of adolescent stress than males of the same strain? Would the resilience generated in females after the double hit of adolescent stress and the stress of FST in adulthood be like the resilience generated by social defeat in adolescence to the single stress in adulthood (Mancini et al., 2021)? Future studies modifying the nature of the stressor in adolescence and the strain of animals used could in part answer this question.

The increased social interaction and recognition observed in adult male rats after EA stress may be related to the decreased anxiety-like behavior observed. This hypothesis could be confirmed if other genetically anxious or stress-prone strains show similar phenomena. The Roman Low Avoidance (RLA) strain has consistently shown high anxiety compared to the Roman High Avoidance (RHA) animals (Giorgi et al., 2019). However, after neonatal handling, social interaction increases in males of both strains in parallel with their decreased anxiety (Sampedro-Viana et al., 2021). The authors of that study argue that the effects of neonatal handling on social interaction seem to be independent from the reduction of anxiety. In a similar vein, we could argue that these phenotypes are also independent in our study as anxiety-like behavior as measured by distance traveled in the center of the OFT is less improved in EA-stressed WLI males compared to WMI males. Still social interaction is improved by EA stress to a similar degree in males of both strains.

Females of either strain were resistant to the behavioral changes caused by EA stress in agreement with many studies, using similar or dissimilar stressors and prepubertal or adolescent developmental time frame (Klinger et al., 2019). Other studies have found no effect in females (different strains, stressors and ages of exposure to stress) in the EPM and/or in the OFT (Barna et al., 2003; Slotten et al., 2006; Prusator and Greenwood-Van Meerveld, 2015; Guadagno et al., 2018). Although examined at earlier time points (i.e., PD3, PD9, or PD11) and different stressors, others have also found no impact of early life stress on female rats with respect to the EPM, NOR, and object recognition location test (Loi et al., 2017). A recent study conducted in female MAM rats also found no differences in anxiety-like behavior in the EPM (Perez et al., 2019).

The passive coping measure of immobility responded to EA stress in both adult males and females. EA stress increased immobility in the adult WLI males, similarly to our findings after chronic stress in adulthood (Mehta-Raghavan et al., 2016). However, it did have the opposite effect on adult females; increased immobility in WLIs, but decreased immobility in WMI females after EA stress. While one can argue that in the case of males, control WMI males show a ceiling effect in immobility, this is clearly not the case for females, as immobility of EA-stressed WLI females is higher than that of control WMI females. The exaggerated immobility of stressed WLI females is inverse of their decreased climbing behavior, confirming this finding. As the WLI and WMI inbred strains were originally selected for immobility behavior in the FST, these changes induced by EA stress suggest that in the absence of genetic predisposition, even a minor stress during early adolescence could have adverse long-lasting effects on the individual. However, the same minor stress could generate resilience in the genetically predisposed individual. A recent very illuminating review of resilience discuss novel interpretation of resilience, and pathways that can lead to it (Malhi et al., 2019).

Brain-derived neurotrophic factor is a growth factor acting through TrkB tyrosine kinase receptors to promote neuronal survival and differentiation. The evidence for a critical role of BDNF in resilience during development is largely based on animal models of chronic stress (Taliaz et al., 2011), which significantly implicate hippocampal BDNF mediation. BDNF genes are also involved in the development of neural circuits that control coping mechanisms (Cattaneo et al., 2015). BDNF is also involved in social behaviors as demonstrated by animal models of social behaviors (Branchi et al., 2013). It is also critical for experience-dependent synaptic plasticity and memory, including fear learning (Chao, 2003; Lee et al., 2003; Andero and Ressler, 2012; Casey et al., 2015). Loss of *Bdnf* expression in adult genetic knockout mice leads to impaired fear learning and increased anxiety-related behaviors (Chen et al., 2006). Furthermore, *Bdnf* expression in the hippocampus is linearly correlated with time spent in the center of the OFT (Bahi, 2017). Thus, we expected that at least some of the behavioral phenotypes show correlations with *Bdnf* expression. This was not the case. It is proposed that decreased *Bdnf* does not cause depressive behaviors but does hamper the effect of antidepressant drugs (Martinowich et al., 2007), therefore, the lack of correlation between *Bdnf* expression and the behavioral phenotypes may only reflect that. There was also no correlation found between the expression of the glucocorticoid receptor and that of *Bdnf* in the same brain regions. However, both *Nr3c1* and *Bdnf* expression were measured after the series of behavioral tests, which could clearly obscure any effects of EA stress on the expression of these genes.

This study is novel, as for the first time it demonstrates the complex effect of early adolescent stress on two nearly isogenic inbred strains exhibiting high, and low stress-reactivity. The sex-specific findings are also very illuminating, particularly the major sex differences in vulnerability to EA stress. One caveat of our study is the nature and the timing of the stress employed. Stronger and different, for example metabolic, stressors may lead to different outcomes. Additionally, despite our efforts to eliminate carryover effects of the different behavioral measurements, a cumulative stress affecting the adult animals could not be excluded. Future studies can investigate these questions and characterize whether the observed sex differences are related to differences in the early adolescent neurodevelopmental stages between males and females. Additionally, further exploration is needed to determine if the resilience caused by the EA stress can be generated at different time frames or by different stressors in both sexes.

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/s.

ETHICS STATEMENT

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

AUTHOR CONTRIBUTIONS

SK, MM, and SG: experimental work, analyzing, interpreting data, and editing the manuscript. ER: conceptualization, writing editing of manuscript, analyzing, and interpreting data. All authors contributed to the article and approved the submitted version.

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Novel Opipramol-Baclofen Combination Alleviates Depression and Craving and Facilitates Recovery From Substance Use Disorder—An Animal Model and a Human Study

*Tzofnat Bareli[1], Hadas Levi Ahdoot[1], Hila Ben Moshe[1], Royi Barnea[1], Gal Warhaftig[1], Iris Gispan[1], Rachel Maayan[2,3], Paola Rosca[4,5], Abraham Weizman[2,3,6] and Gal Yadid[1]**

[1] Faculty of Life Sciences, Leslie and Gonda (Goldschmied) Multidisciplinary Brain Research Center, Bar Ilan University, Ramat Gan, Israel, [2] The Laboratory of Molecular Psychiatry, Felsenstein Medical Research Center, Petah Tikva, Israel, [3] Faculty of Medicine, Tel Aviv University, Tel Aviv, Israel, [4] Department for the Treatment of Substance Abuse and Mental Health Services, Israeli Ministry of Health, Jerusalem, Israel, [5] Faculty of Medicine, The Hebrew University of Jerusalem, Jerusalem, Israel, [6] Research Unit, Geha Mental Health Center, Petah Tikva, Israel

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Matthew O. Parker,
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Spain
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University of Melbourne, Australia

***Correspondence:**
Gal Yadid
yadidg@gmail.com

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Substance use disorders (SUDs) are associated with depression and anxiety, with the latter being one of the major factors in substance-seeking and relapse. Due to dose-dependent sedative side effects there is limited efficacy of baclofen treatment for SUDs. Here we suggest the use of a novel combination of opipramol and baclofen (O/B) which is known to attenuate anxiety and depression, for the facilitation of recovery from SUDs. Since both opipramol and baclofen have a common downstream signal transduction, their individual doses could be reduced while still maintaining the benefits of the combination. We tested the O/B combination in both animals and patients. Rats treated with O/B showed significant attenuation in craving behavior and in relapse rate during withdrawal from cocaine. In a double-blind, placebo-controlled pilot study, conducted in a residential detoxification center, 14 males and 3 females, aged 28–60 years were assigned to a study (n = 6) and a placebo (n = 11) group (placebo group: 40 ± 10.5 years; O/B group 40 ± 10.8 years). The participants completed scales measuring depression, anxiety and craving symptoms and provided saliva samples for stress hormone examination [cortisol and dehydroepiandrosterone-sulfate (DHEA-S)]. Participants with polysubstance use disorder (PsUD) treated with O/B showed a reduction in cravings and depression and an increase in DHEA-S and in the DHEA-S/cortisol ratio. Our findings indicate a beneficial effect of O/B treatment. This study suggests a novel candidate for pharmacological treatment of patients with SUD and comorbid mood/anxiety disorders that may facilitate their rehabilitation.

Keywords: stress, substance-induced depressive disorder, addiction, treatment, therapeutic center, baclofen, opipramol

INTRODUCTION

Substance use disorder (SUD) affects every aspect of a person's life, as well as their social environment. It may destroy relationships, lead to unemployment, poor health, and mortality due to overdose. The Diagnostic and Statistical Manual of Mental Disorders (DSM-5) substituted in 2013 the previous dichotomous definition between substance abuse and dependence, with the term SUD relating to it as a continuum. Substance-induced depressive disorder (SIDD) refers to depression induced by substance use (Magidson et al., 2013; Conner et al., 2014; Tolliver and Anton, 2015; Tirado-Muñoz et al., 2018).

Opipramol is a well-tolerated anti-anxiety and antidepressant agent (Möller et al., 2001). Clinical trials with opipramol showed it to be effective in anxiety associated with somatic complaints (Möller et al., 2001). Opipramol is a σ-1 receptor agonist. This receptor is defined as a chaperone and can modulate the activity of several ion channels thus affecting neuronal excitability and synaptic activity (Su et al., 2016; Yang et al., 2019) and may be involved in cocaine addiction (Ferris et al., 1991; Katz et al., 2016). Recently, we showed significant reduction of cocaine-primed reinstatement in 75% of the opipramol-treated group as well as demonstrating the involvement of σ-1 receptor in a rat model of cocaine self-administration (SA) (Bareli et al., 2021). In addition, we found that those rats which responded to opipramol (Responders = Rs) exhibited significantly less Ras-related C3 botulinum toxin substrate 1 (Rac1) mRNA expression in the nucleus accumbens (NAc), compared with opipramol non-responder (NR) rats. Hence, we concluded that Rac1 differentiated Rs from NRs (Bareli et al., 2021). A study by Tsai et al. (2012) demonstrated that σ-1 receptor knock-down in the hippocampus is associated with a parallel downregulation of gamma-aminobutyric acid B receptor 1 ($GABA_B$,Gabbr1) and Rac1.

Baclofen is a potent, selective Gabbr1 agonist (Bowery et al., 1980, 1981; Davidoff, 1985) and is used in clinical practice as a muscle relaxant in patients suffering from multiple sclerosis and spinal cord diseases (Kuroiwa et al., 2009; Woolf and Baum, 2017; Lake and Shah, 2019; Chisari et al., 2020). Additionally, a variety of studies show that the $GABA_B$ receptor is involved in SUDs (Roberts and Andrews, 1997; Di Ciano and Everitt, 2003; Agabio et al., 2013; Phillips and Reed, 2014), including ethanol (EtOH) consumption. In mice, the $GABA_B$ receptor agonist baclofen decreases EtOH intake (Rabat et al., 2019) and attenuates social-interaction deficits when given before ethanol exposure or anxiety during abstinence (Knapp et al., 2007). However, side effects, such as severe sedation, dizziness, and confusion, were observed as a result of high-doses (de Beaurepaire et al., 2019). In a study on people with severe cocaine dependence, the benefit of baclofen as a sole treatment was inconclusive compared to placebo (Kahn et al., 2009).

Chronic anxiety enhances the susceptibility to SUDs (Sinha and Jastreboff, 2013). In a previous study the authors showed that relieving anxiety in rats with the neurosteroid dehydroepiandrosterone (DHEA), a σ-1 receptor agonist, is associated with a reduction in cocaine SA and cocaine-seeking during abstinence (Yadid et al., 2012). The DHEA sulfate ester (DHEA-S) and DHEA, are the most abundant steroid hormones in the body. In humans with SUD, the levels of DHEA and DHEA-S are altered (Buydens-Branchey et al., 2002). Notably, DHEA-S levels were significantly lower in patients with SUD who relapsed, compared to patients that did not relapse (Shoptaw et al., 2004).

The present study suggests attenuating anxiety and depression during withdrawal from SUD by using an opipramol and baclofen (O/B) combination. Baclofen at low doses is known to be well-tolerated and safe. The downstream signal transduction which is common to both opipramol and baclofen allows reduction of the baclofen dose, thus diminishing the sedative side effects. We postulated that the O/B combination mediated by σ-1 receptor/Gabbr1 interaction and possibly by other proteins like Rac1, could provide a new strategy for decreasing drug cravings. The current study evaluated the efficacy of O/B treatment for SUD as well as comorbid anxiety/depressive symptoms. In the preclinical phase the efficacy was examined in a rat model of cocaine use disorder. The second clinical phase consisted of a double-blind, placebo-controlled pilot study conducted in a detoxification residential center including patients with polysubstance use disorder (PsUD). The diagnosis of PsUD refers, in our study, to a situation in which an individual uses at least two different classes of substances repeatedly (excluding caffeine or nicotine) during a 12-months period, but no single substance predominates (Martinotti et al., 2009).

In the current study, the authors aim to show that the O/B combination can alleviate anxiety and depression associated with SUD thus facilitating rehabilitation.

STUDY 1—ANIMAL MODEL

Materials and Methods

Ethics

All experimental procedures were approved by the Animal Care and Use Committee of Bar Ilan University, Ramat Gan, and were performed in accordance with the National Institutes of Health guidelines for animal experiments.

Animals

Male Sprague-Dawley rats (250–280 g) were maintained on a reverse 12–12-h dark-light cycle with free access to food and water.

Intravenous Catheterization

Rats were anesthetized with ketamine hydrochloride (100 mg/kg, i.p.)/xylazine (10 mg/kg, i.p.) and then implanted with intravenous silastic catheters (Dow Corning, Midland, MI, United States) into the right jugular vein. The catheter was secured to the vein with silk sutures and passed subcutaneously to the top of the skull, where it exited into a connector (a modified 22-gauge cannula; Plastics One, Roanoke, VA, United States) mounted to the skull with MX-80 screws (Small Parts, Inc., Miami Lakes, FL, United States) and dental cement (Yates & Birds, Chicago, IL, United States).

Cocaine Self-Administration Training

Rats were trained to self-administer cocaine (between 10 and 16 days depending on the rate of addiction in every experiment) under a fixed ratio (FR)-1 schedule of reinforcement. Five days after catheterization, rats were transferred to operant conditioning chambers (Med-Associates, Inc., St. Albans, VT, United States) for 1 h daily during their dark cycle. The SA chambers (Med-Associates, Inc., St Albans, VT, United States) had active and inactive levers. An active lever press generated a cocaine infusion (0.5 mg/kg, 0.13 ml, 5 s/infusion; cocaine was obtained from the National Institutes of Drug Abuse, North Bethesda, MD, United States) through the i.v. catheter, which was connected to an infusion pump. Throughout cocaine infusion intervals, a light located above the active lever was lit for 20 s, 15 s beyond the cocaine infusion period, which lasted only 5 s. During the 15-s intervals, active lever presses were recorded; no additional cocaine reinforcement was provided. Presses on the inactive lever did not activate the infusion pump and light. The number of active lever responses, infusions, and inactive lever responses was recorded. Rats were returned to their home cages at the end of the daily session.

Extinction Training

Rats received daily i.p. injections of opipramol (12.5 mg/kg) or baclofen (3.2 mg/kg or 0.1 mg/kg), or O/B (Sigma-Aldrich, Darmstadt, Germany) or saline before each extinction test session. Opipramol was administered 15 min before entering the operant chamber and baclofen 30 min before entering it. The difference in administration time was due to differences in pharmacokinetic properties (Di Ciano and Everitt, 2003; Bareli et al., 2021). An opipramol dose of 12.5 mg/kg was chosen since it was the optimal dose still having an impact but not affecting the locomotion behavior (Bareli et al., 2021). After preliminary experiments with two doses of baclofen (3.2 or 0.1 mg/kg) the lower dose of 0.1 mg/kg was chosen since it was shown to be without sedative side effects (data presented in the first part of Results). Rats were then placed in the operant conditioning chambers for 1-h daily sessions, with no cocaine available and only the light cue turning on, upon active lever presses. Active and inactive lever presses were recorded.

To find the effective dose of baclofen, which is high enough to initiate a therapeutic effect and at the same time does not cause side effects, we examined the effect of two doses of baclofen. In the first one we used a dose of 3.2 mg/kg. This dose was chosen based on previous studies reporting that baclofen in doses 3–5 mg/kg effectively reduces cocaine craving in a rat model. Compared to control rats, the baclofen-treated rats did not move in the experimental cages, so this dose caused side effects. Administration of baclofen (0.1 mg/kg) throughout the extinction phase to rats trained for cocaine self-administration did not reduce active lever presses compared to controls. In Addition, providing a boost of baclofen (0.1 mg/kg) before the second reinstatement to rats that did not respond to opipramol resulted in no change in active lever presses compared to controls.

Cocaine-Primed Reinstatement

Twenty-four hours after the last opipramol or baclofen or O/B combination or saline treatment, rats received a single injection of cocaine (10 mg/kg; i.p.) and were placed in the operant chambers for 1 h, with no cocaine available. At the conclusion of the session, rats were immediately anesthetized and decapitated for NAc isolation and further molecular analysis (below).

Tissue Preparation

Rats were decapitated. Their brains were removed rapidly after reinstatement and placed in a rat brain mold (constructed at Bar Ilan University) on ice. Then, the tissue was dissected into serial 1 mm slices and placed on chilled microscope slides. Tissue punches of the NAc were procured rapidly according to the following coordinates: AP, +1.4 mm; ML, +1.2 mm to bregma (Paxinos and Watson, 2007), using a stainless-steel cannula with an inner diameter of 1.1 mm. The tissue samples were immediately frozen on dry ice and stored at −80°C until they were extracted for analysis.

RNA Extraction and Reverse Transcription

We performed analyses of gene expression for Gabbr1 of saline-treated rats (n = 8–9) and opipramol-treated rats (n = 12) and for Rac1 of O/B treated rats (n = 7), also. Total RNA was extracted using a Total RNA Purification Kit (Norgen Biotek Corp., Thorold, Canada). Purity, integrity, and concentration of the isolated RNA samples were determined by spectrophotometric absorbance at 260 nm. RNA samples were reverse transcribed to generate cDNA (qScript cDNA Synthesis; Quanta BioSciences Inc., Gaithersburg, MD, United States). These were later used as templates for quantitative real-time PCR analysis. Real-time PCR reactions were carried out on a Step One Plus Real-time PCR system (Thermo Fisher Scientific, Waltham, MA, United States) using fluorescent SYBR Green fast mix technology (qScript cDNA Synthesis; Quanta BioSciences Inc., Gaithersburg, MD, United States). Reaction protocols were as follows: 30 s at 95°C for enzyme activation followed by 45 cycles of 5 s at 95°C and 30 s at 60°C. Melting curve analysis examined the specificity of the amplification products. Primers [synthesized by Integrated DNA Technologies (IDT), Coralville, IA, United States] for tested genes were designed as follows: Gabbr1 (F:5′-ACGTGGCTTGGCATTTTCTATG-3′; R:5′-TTC AGTGGACACGCTCTTGG-3′), Rac1 (F: 5′-AAAACCAGTGA ATCTGGGCCT-3′; R: 5′-AACACGTCTGTTTGCGGGTA-3′) and beta-actin (F: 5′-GTAGCCATCCAGGCTGTGTT-3′; R: 5′-CCCTCATAGATGGGCACAGT-3′).

THE RATIONALE OF THE STUDY

The study is based on our previous publication (Tsai et al., 2012) in which we showed that treatment with opipramol in a cocaine self- administration (SA) rat model, caused reduction of cocaine-primed reinstatement in 75% of the

opipramol-treated group (i.e., Rs). Using a K-mean cluster analysis, the opipramol-treated group was shown in our previous study to constitute two separate groups: Rs and Non-Responders (NR) to opipramol treatment in a cocaine SA model. The separation between the two groups had a specific cutoff point.

In an attempt to augment the efficacy of opipramol we added baclofen (0.1 mg/kg) to opipramol and assessed the rate of rat-R to the combined treatment in comparison to opipramol alone.

The aim of the present study was to assess whether of addition of baclofen to opipramol increases the rate of treatment success achieved by opipramol alone. The focus of this study were rats not affected by opipramol alone [opip (NR)]. To this end, in the first stage we identified the opip (NR) rats as assessed by their craving state in the reinstatement test. Once identified, we gave these rats an acute baclofen injection and retested their craving in a second reinstatement.

In our previous study using the same model, we demonstrated that there is no order effect in the reinstatement tests (Bareli et al., 2021).

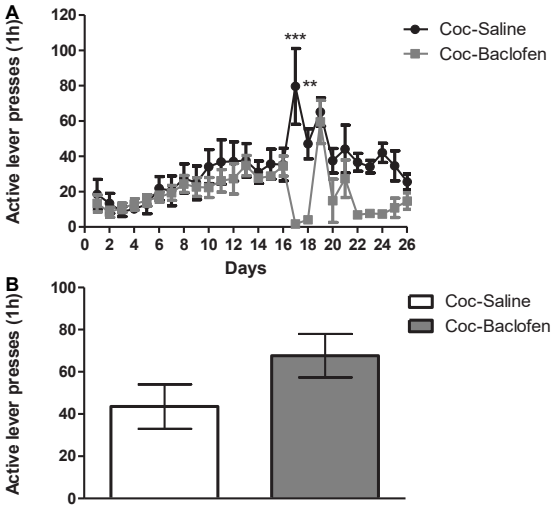

FIGURE 1 | Effects of a dose of 3.2 mg/kg of baclofen on extinction, withdrawal and reinstatement phases in a cocaine (Coc) self-administration (SA) rat model. **(A)** Effect of baclofen on cocaine-seeking during extinction and withdrawal phases. Rats (n = 6) were trained for cocaine (0.5 mg/kg) or saline SA on days 1–16. Coc-Saline, control, trained for cocaine SA and received saline at the withdrawal phase, and Coc-Baclofen, trained for cocaine SA and received baclofen (3.2 mg/kg) at the withdrawal phase. Baclofen treatment, given on days 17–18, significantly reduced cocaine-seeking behavior throughout extinction training, compared with saline-treated controls. A two-way ANOVA with repeated measures revealed a significant effect of treatment [$F(1, 250) = 13.93$, $p = 0.0039$], time [$F(25, 250) = 4.77$, $p < 0.0001$], and the interaction between them [$F(25, 250) = 3.11$, $p < 0.0001$] (Bonferroni, ***$p < 0.001$ day 17, **$p < 0.01$ day 18). A lack of baclofen administration on day 19 increased active lever presses immediately and resembled control. Baclofen administration on days 20–26 reduced active lever presses but not significantly. **(B)** Effect of Baclofen on cocaine-primed reinstatement. Rats received a priming injection of cocaine (10 mg/kg, i.p, day 27) and were placed in the SA chamber with no cocaine available in the relapse test. Baclofen treatment did not decrease active lever pressing compared to controls (Student's t-test, $p = 0.1323$). Values are expressed as mean ± SEM.

RESULTS

Baclofen Administered in High Doses of 3.2 mg/kg Induces Sedative Effects and Is Ineffective in Lowering Cocaine Cravings

A high-dose of baclofen (3.2 mg/kg) was administered in a classical cocaine SA rat model according to a FR-1 schedule. This dose was chosen based on previous studies reporting that baclofen in doses of 3–5 mg/kg effectively reduces cocaine cravings. The rats (n = 6) demonstrated stable cocaine SA behavior during 1-h/day training sessions over 16-days (**Figure 1A**). At stable maintenance, rats were divided into two groups: one received consecutive injections (i.p.) of baclofen (3.2 mg/kg per day, 30 min before the session) and the other at the same time point, of saline. The saline injected rats served as control group. Both groups went through extinction on days 17–26. The baclofen-treated rats stopped completely active lever presses at the beginning of the extinction phase. Compared to control rats, the baclofen-treated rats showed little movement in the experimental cages, probably due to the sedative effect of the high dose of baclofen. We tested the consequences of not administrating baclofen treatment on the following day (day 19). Active lever presses were dramatically increased to the same level as that of the saline-treated rats. As our aim was to examine whether chronic baclofen treatment attenuates cravings, we continued administration on the consecutive days (days 20–26). Baclofen injections were discontinued on day 26 (last extinction session), and 24 h later, both baclofen-treated and control rats received a priming injection of cocaine (10 mg/kg; i.p.) and were placed in the SA chambers for 1 h. We did not find any differences in active lever presses between baclofen-treated rats and controls (**Figure 1B**).

Baclofen Administered at Low Doses of 0.1 mg/kg, as Sole Treatment and in Combination With Opipramol, to Rats That Are NRs to Opipramol Alone, Did Not Reduce Cocaine Cravings

The reinstatement timeline is presented in **Figure 2A**. We previously reported that in the SA model of cocaine SA, two groups emerged with regard to chronic opipramol administration: Rs (75%) and NRs (25%) to opipramol (Bareli et al., 2021). Those groups were defined by K- mean cluster analysis as Rs being rats that showed a significantly lower number of active lever presses during reinstatement in contrast to NRs, which did not show such reduction. Among cocaine-trained rats, 75% showed a reduction in cocaine cravings (see **Figure 2C**), probably resulting from activation of the σ-1

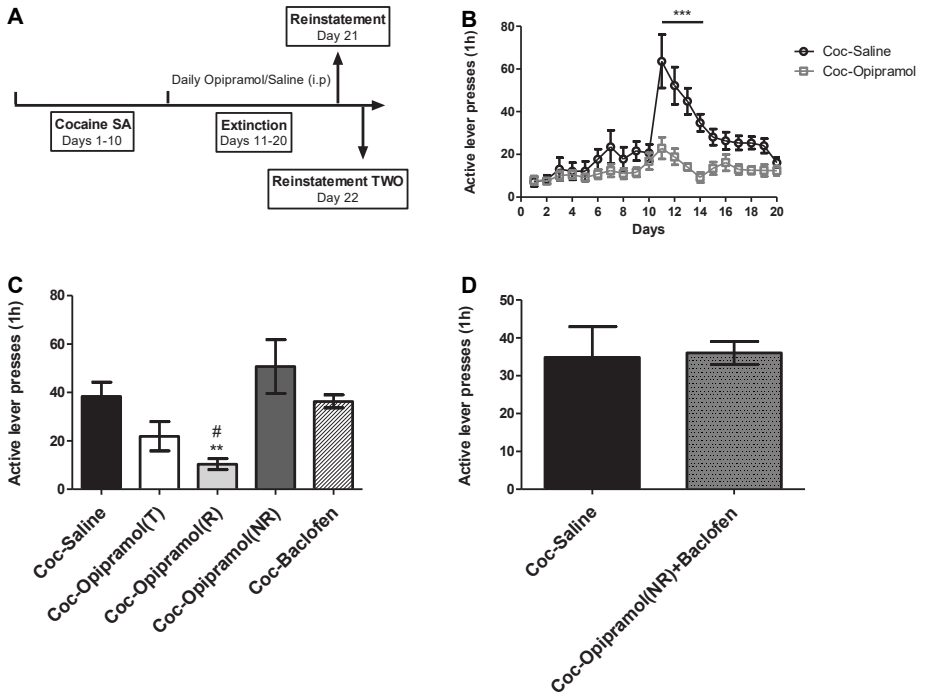

FIGURE 2 | Effect of opipramol (12.5 mg/kg) and low doses of baclofen (0.1 mg/kg) on extinction, withdrawal and reinstatement phases in a cocaine (Coc) self-administration (SA) rat model. **(A)** The fixed ratio 1 schedule (FR-1) of cocaine SA model that was used. Rats (n = 10–14) were trained (T) for cocaine (0.5 mg/kg) or saline SA on days 1–10. Next, we created two groups: Coc-Saline, control, trained for cocaine SA and received saline at the withdrawal phase, and Coc-Opipramol, trained for cocaine SA and received opipramol (12.5 mg/kg) throughout the withdrawal phase. **(B)** Effect of opipramol on cocaine-seeking during extinction and withdrawal phases. Opipramol treatment, given on days 11–20, significantly reduced cocaine-seeking behavior during extinction training, compared with saline-treated controls. A two-way ANOVA with repeated measures revealed a significant effect of treatment [$F(1, 418) = 12.64$, $p = 0.0018$], time [$F(19, 418) = 15.49$, $p < 0.0001$] and the interaction between them [$F(19, 418) = 6.65$, $p < 0.0001$] (Bonferroni, $^{***}p < 0.001$ days 11–14). **(C)** Effect of opipramol or baclofen on cocaine-primed reinstatement. Classification of treated rats according to "responders" or "non-responders" to chronic opipramol treatment revealed significantly decreased active lever presses for responder rats compared with non-responders or controls (Coc-Saline). A one-way ANOVA revealed a significant effect of treatment [$F(4, 34) = 5.35$, $p = 0.0019$] in the relapse test (Bonferroni, $^{**}p < 0.01$ opipramol responders (R) vs. non-responders (NR), $^{\#}p < 0.05$ opipramol responders vs. controls). Baclofen administration did not decrease active lever presses compared to controls. Coc-Opipramol (T) included the total rats that received opipramol, i.e., opipramol responders [Coc-Opipramol (R)] and non-responders [Coc-Opipramol (NR)]. **(D)** Examination of baclofen (0.1 mg/kg) one injection to opipramol non-responder rats, before a second cocaine-primed reinstatement. A Student's t-test did not reveal a significant treatment effect in the relapse test ($p = 0.9207$). Values are expressed as mean ± SEM.

receptor, which is present in the NAc as are Rac1 and Gabbr1. As the baclofen's target is the Gabbr1, we postulated that the O/B combination may increase the number Rs, compared to opipramol alone. As described earlier, rats that had been trained for cocaine SA were divided into three groups: one group received injections (i.p.) of opipramol (12.5 mg/kg per day, 15 min before the session), the second group received injections (i.p.) of baclofen (0.1 mg/kg per day, 30 min before the session) and the third group received saline (30 min before the session) as a control for the baclofen. Next all groups went through an extinction test session (on days 11–20). Active lever presses decreased significantly in the opipramol-treated rats, compared to the saline-treated group, throughout the extinction phase (**Figure 2B**). Administration, throughout the extinction phase, to rats trained for cocaine SA of baclofen (0.1 mg/kg; Coc-Baclofen) did not reduce active lever presses compared to controls (Coc-Saline; **Figure 2C**). In Addition, providing a boost of baclofen (0.1 mg/kg) before the second reinstatement to rats that were NRs to opipramol (Coc-Opipramol (NRs)) resulted in no change in active lever presses compared to controls (**Figure 2D**).

Chronic Treatment With a Combination of Opipramol and a Low Dose of Baclofen During the Extinction Phase Is More Effective in Reducing Cocaine Craving Than Opipramol Alone

The above-described results and the narrow therapeutic index of baclofen led us to examine whether chronic administration of a combination of opipramol and a low dose of baclofen (O/B) throughout the extinction phase would increase the number of rats responding to opipramol. The rats were put through the same cocaine SA paradigm as described above. Active lever presses decreased significantly in the O/B-treated rats compared to controls (**Figure 3A**). Furthermore, in the relapse test, the combination treatment reduced active lever presses and increased the rate of Rs compared to R-rate to opipramol alone (**Figure 3B**).

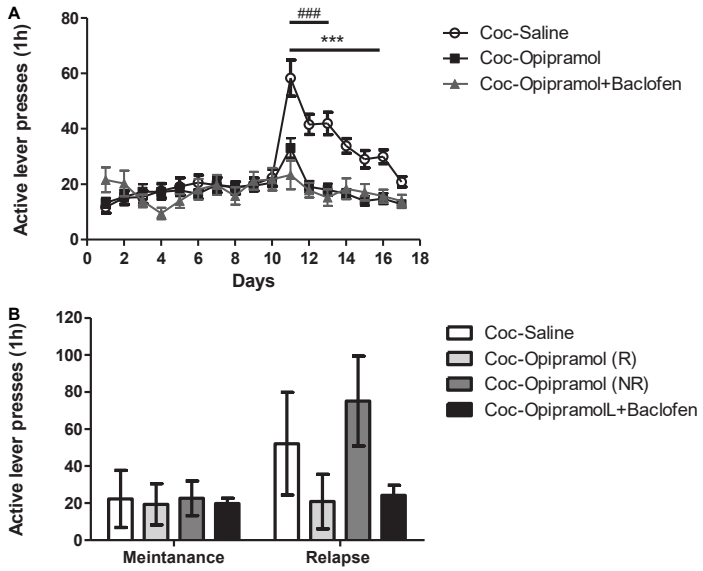

FIGURE 3 | Effect of the combination of opipramol and baclofen on extinction, withdrawal and reinstatement phases in a cocaine (Coc) self-administration rat (SA) model. **(A)** Effect of the selected treatment; opipramol (12.5 mg/kg) or opipramol (12.5 mg/kg) and baclofen (0.1 mg/kg) combination on cocaine-seeking during extinction and withdrawal phases. Opipramol alone and the combination treatments given on days 11–17 significantly reduced cocaine-seeking behavior during extinction training, compared with saline-treated controls. A two-way ANOVA with repeated measures revealed a significant effect of treatment [$F(2, 1712) = 7.42$, $p = 0.001$], time [$F(16, 1712) = 16.97$, $p < 0.0001$] and the interaction between them [$F(32, 1712) = 7.75$, $p < 0.0001$]. (Bonferroni, $^{***}p < 0.001$ days 11–16 opipramol treatment vs. controls, $^{###}p < 0.001$ days 11–13 O/B treatment vs. controls). **(B)** Effect of opipramol or the combination of O/B on cocaine-primed reinstatement. Active lever presses were decreased in rats that were responders (R) to opipramol [Coc-Opipramol (R)] and in rats that received O/B combination (Coc-Opipramol + Baclofen) compared with opipramol non-responder (NR) rats [Coc-Opipramol (NR)] and controls (Coc-Saline). A one-way ANOVA revealed a significant effect of treatment [$F(3, 89) = 28.97$, $p < 0.0001$] in the relapse test (Bonferroni, $^{***/###}p < 0.001$ opipramol responders and O/B vs. non-responders and controls, respectively). Values are expressed as mean ± SEM.

Combination of Opipramol and Baclofen Changes Expression Levels of Proteins in the Nucleus Accumbens

In a previous study we showed that opipramol alone acts on a complex containing σ-1r and Rac1 proteins (Bareli et al., 2021). The present study found that craving behavior was affected by administration of O/B to cocaine-trained rats.

Baclofen's main target is Gabbr1 thus we examined Gabbr1 involvement in the beneficial effect of the treatment. qPCR analysis revealed a significant decrease in the mRNA expression levels of Gabbr1 in the opipramol treated group compared to controls ($^{**}p < 0.01$, **Figure 4A**). High doses of baclofen (3.2 mg/kg) did not affect Rac1 mRNA expression levels (**Figure 4B**). Administering the combination of opipramol (12.5 mg/kg) with a low dose of baclofen (0.1 mg/kg) did not lead to a significant decrease in Rac1 mRNA expression levels (**Figure 4B**). According to the STRING analysis, σ-1r, Rac1, and Gabbr1 all the examined proteins interact (**Figure 4C**).

STUDY 2—HUMAN TRIAL

Materials and Methods

Clinical Trial Design

Residential detoxification centers provide a controlled environment that enables a balanced diet and a fairly stable daily routine, thus providing a controlled research setting. We designed a 1-month study during which the patients were administered either O/B treatment or placebo (**Figure 5**). Behavioral, clinical, and hormonal/biochemical parameters were monitored (**Figure 6**). All adverse effects, spontaneously reported by the study participants, were assessed for severity and association with study medication.

Ethics

The study was approved by the review board of the Israeli Ministry of Health (proposal no. 066-2015, Aug 2017). All participants signed a written informed consent form.

Participants

Thirty people with PsUD from a residential detoxification center (Dolphine, Ashdod, Israel) participated in the study. Inclusion criteria were a diagnosis of PsUD, age in the range of 18–60 years. Exclusion criteria consisted of the following: serious kidney, lung, liver, neurological, prostatic or cardiovascular disease, presence of suicidal thoughts or behaviors (as assessed in a clinical interview), acute or chronic psychosis, bipolar disorder, major depressive episodes, intellectual disability, organic brain syndrome, current treatment with monoamine oxidase (MAO) inhibitors or benzodiazepines, being pregnant or breast-feeding and finally, active HIV or hepatitis C. Formal cognitive assessments were not conducted in this study. However, all participants underwent a clinical interview at

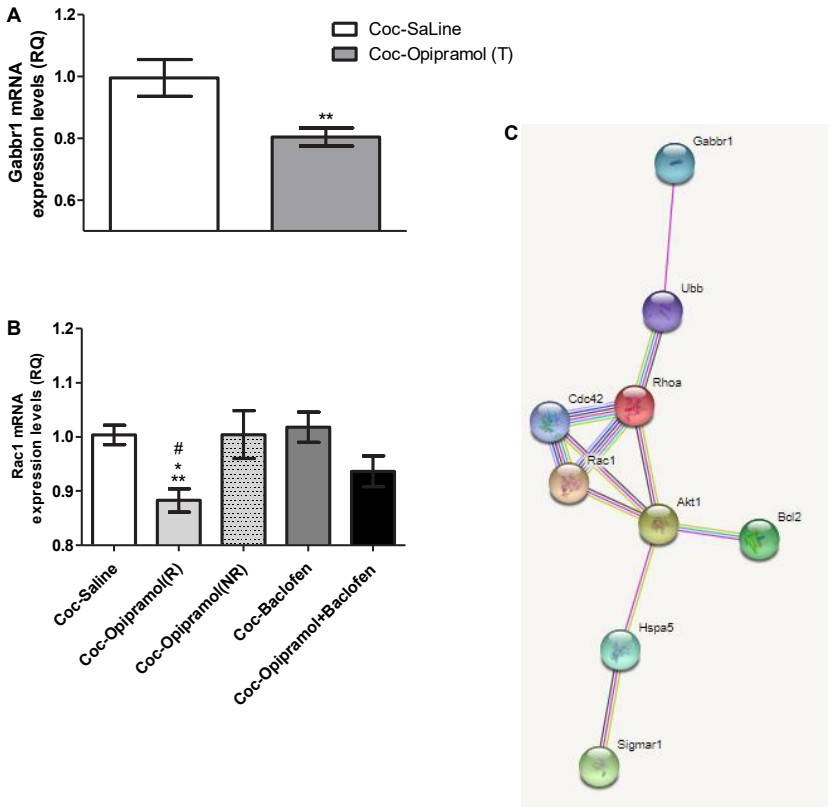

FIGURE 4 | Effects of the various drug-combinations on Gabbr1 and Rac1 mRNA expression levels in the nucleus accumbens (NAc). **(A)** A Student's *t*-test revealed a significant effect of opipramol treatment alone on the expression of Gabbr1 (**$p = 0.0063$). **(B)** Newman-Keuls *post hoc* test revealed a significant decrease in Rac1 mRNA expression levels in the opipramol responders (R) [Coc-Opipramol (R)] compared with the non-responder (NR) rats [Coc-Opipramol (NR)], as well as compared to high dose baclofen (3.2 mg/kg) alone treated rats (Coc-Baclofen), and compared to controls (Coc-Saine) (one-way ANOVA, *$p < 0.05$, $^{\#}p < 0.05$, and **$p < 0.01$, respectively). Values are expressed as mean ± SEM. **(C)** STRING software (version 11.0) revealed a complex of interacting proteins including Gabbr1, Rac1, and σ-1 receptor in a PPI enrichment analysis, $p = 0.0272$. The software indicates that the examined proteins are at least partially biologically connected.

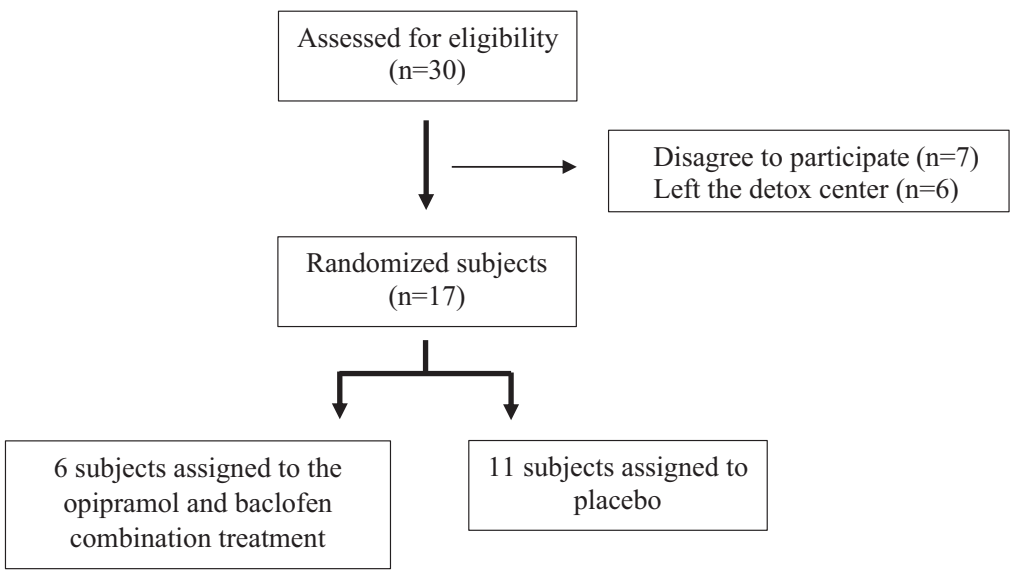

FIGURE 5 | Subject recruitment. Participants were randomly assigned to either the opipramol-baclofen (O/B) combination group or to the placebo group.

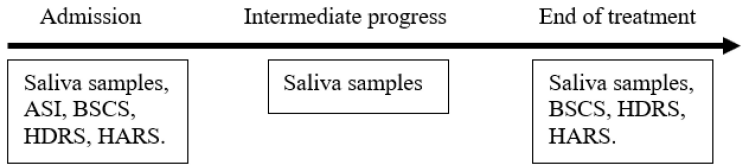

FIGURE 6 | Study timeline. The clinical scales and saliva samples were collected on days 2 and 7 of the trial and 1 day before the release from the detox center. ASI, Addiction Severity Index; BSCS, Brief Substance Craving Scale; HDRS, Hamilton Depression Rating Scale; HARS, Hamilton Anxiety Rating Scale.

baseline that included assessment of their basic cognitive skills. Those found to have gross cognitive impairment were also excluded from the study.

Protocol and Medication Procedure

Participants were randomly assigned to either the O/B combination group or to the placebo group. Each participant was assigned a random number generated by a random number generator, thus keeping the participants' identity unknown to the researchers (Matthews, 2003). O/B or placebo were prepared by Nextar Chempharma Solutions Ltd. (Rehovot, Israel) and were administered by a registered nurse. For the duration of 1 month, those assigned to the study group received 30 mg of baclofen and 50 mg of opipramol that were given daily by oral administration in 3 divided doses over the day. All patients were asked at baseline, to stop using drugs or medications that affect cravings (such as benzodiazepines, antidepressants, metadoxine, naltrexon, acamprosate, γ-hydroxybutyric acid, antihistamines) for the duration of the study. Each patient was monitored throughout the study period (**Figure 6**). Professional staff provided a standard program for the treatment of SUDs, including relapse prevention, 12 steps, motivational enhancement and daily group interventions. At specific time points, patients completed a battery of scales measuring cravings for addictive substances (Addiction Severity Index—ASI; Brief Substance Craving Scale—BSCS), anxiety [Hamilton Anxiety Rating Scale—HARS)] and depression [Hamilton Depression Rating Scale—HDRS)]. Saliva samples were also collected in order to evaluate DHEA-S and cortisol hormone levels (**Figure 6**). Throughout the detoxification period all participants received group psychotherapy along with the pharmacotherapy.

Assessments

Testing started in the first 2 days of a patient's admission to the detox center. Data recorded at baseline included standard demographics consisting of sex, age, marital status, immigration status, date of immigration, and education level. Additionally, each patient's history of psychiatric disorders was recorded, as well as the patient's SUD history. The following details were collected for each participant: (1) age at first substance use, (2) age at first binge, (3) duration of substance consumption, (4) number of prior detoxification programs (inpatient and outpatient), (5) average daily substance consumption in the last 6 months, (6) the number of days in the last month when substances were consumed.

Patients were administered the following measurement tools: (1) ASI, a semi-structured interview designed to address the following 7 potential problem areas in patients with SUD: medical status, source of income, drug use, alcohol use, legal status, family/social status, and psychiatric status. The ASI provides a general overview of problems related to substance use rather than focusing on any single problem area. (2) BSCS, a self-report instrument that assesses cravings for multiple substances with regard to: intensity and frequency of cravings, number of times per week, thoughts of cravings for a substance). (3) HDRS, a scale frequently used by clinicians to assess depressive symptoms. (4) HARS, a scale used by clinicians to assess anxiety symptoms. In our analysis we calculated three parameters related to SUD—depression, helplessness, and worthlessness feelings from Hamilton Depression Rating Scale (HDRS) (Yadid et al., 2012; Barnett et al., 2018).

TABLE 1 | Demographic and clinical characteristics of the study population.

	Placebo (n = 11)	O/B treatment (n = 6)
Age (years; mean ± SD)	40 ± 10.5	40.5 ± 10.8
Male: Female	5:1	9:2
Stimulants	6	7
Opioids	5	11
Depressants	3	6
Hallucinogens	4	6

O/B, Opipramol and baclofen.

As shown in **Figure 6** below, saliva samples were taken from the participants three times during the study: at the beginning of the study, in its middle and at the end. At the beginning and the end of the study along with the saliva samples that were taken for hormone analysis scales for clinical assessments were completed as well.

Analysis of Saliva Samples

Saliva samples were taken before breakfast, between 06:30 and 07:30 a.m., and were used to analyze levels of DHEA-S and cortisol. The latter were measured with DHEA-S/Cortisol Saliva ELISA kits (IBL International GmbH, Hamburg, Germany). The sensitivity of DHEA-S and cortisol kits are 0.05 ng/ml and 0.005 μg/dl, respectively, and both present low cross-reactivity to other hormones.

Data Analysis

All data are expressed as mean ± SD. One-way ANOVA or two-way ANOVA with repeated measures (days) followed by Bonferroni or Student-Newman-Keuls *post hoc* test were used as appropriate. A Student's *t*-test was used for comparisons between

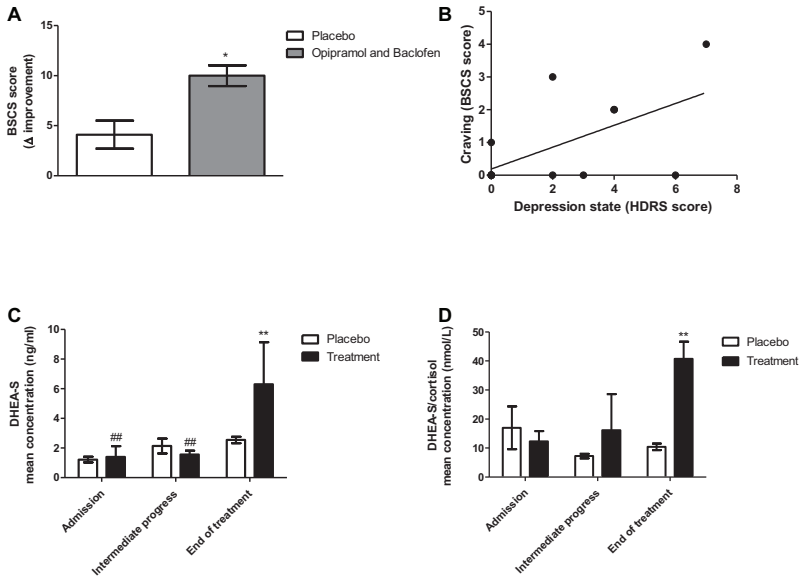

FIGURE 7 | Effects of administering a combination of opipramol and baclofen on rehabilitation factors of patients with polysubstance use disorder (PsUD). **(A)** The individual magnitude of craving change tested by the Brief Substance Craving Scale (BSCS) questionnaire (score before/after O/B treatment was significantly improved in comparison with placebo treatment (Student's *t*-test, $p < 0.031$). **(B)** A correlation between depression state and craving symptoms at the end of the trial (Pearson rank correlation, $^{*}p = 0.022$, $r = 0.36$). **(C)** Dehydroepiandrosterone- sulfate (DHEA-S) levels were increased at the end of the treatment compared with placebo (Bonferroni, $^{**}p < 0.01$) and over time (Bonferroni, $^{\#\#}p < 0.01$, admission vs. end of treatment, intermediate progress vs. end of treatment). A two-way ANOVA with repeated measures revealed a significant effect of time [$F(2, 20) = 12.85$, $p = 0.0003$] and interaction between treatment and time [$F(2, 20) = 6.22$, $p = 0.0079$], but did not reveal a significant effect of treatment [$F(1, 20) = 2.27$, $p = 0.1628$]. **(D)** DHEA-S and cortisol ratio were increased at the end of the treatment compared with placebo (Bonferroni, $^{**}p < 0.01$). A two-way ANOVA with repeated measures revealed a significant effect of treatment [$F(1, 16) = 9.2$, $p = 0.0162$ and interaction between treatment and time [$F(2, 16) = 3.67$, $p = 0.0488$], but did not reveal a significant effect of time [$F(2, 16) = 2.52$, $p = 0.1119$].

the two groups. All the data were analyzed using Prism 5 software (GraphPad, San Diego, CA, United States).

Results

Opipramol and Baclofen Combination Treatment Improves Depressive Symptoms, Craving and Stress Hormones in People With Polysubstance Use Disorder

Following the promising results from the rat model study, we performed a double-blind placebo-controlled pilot study to investigate whether the O/B combination can lower addiction and anxiety/depressive symptoms. The demographic and clinical characteristics of the study population are presented in **Table 1**.

The outcomes of the treatments are shown in **Figure 7**. Patients with PsUD receiving the treatment combination three times a day, demonstrated higher levels of improvement in cravings compared to placebo. Moreover, we observed a significant correlation between depression state and craving. Remarkably, at the end of the study DHEA-S levels were higher in the study group than in the placebo group. The ratio of DHEA-S/cortisol was also high throughout the trial.

Discussion

The current study shows that administration of the O/B combination in humans with PsUD, lowers cocaine cravings and possibly the risk of relapse and reduces the depressive symptoms and increases at the end-point, the level DHEA-S. In the rat model, the addition of baclofen to opipramol resulted in a significant reduction in the relapse to cocaine rate of rats that did not respond to opipramol.

On the molecular level, our findings suggest that the beneficial effect of O/B may be mediated by the interaction of proteins, including $GABA_B$ receptors, σ-1 receptor, and Rac1.

Our previous study Bareli et al. (2021) showed that Rac1 has a critical role in drug-seeking and cravings. We also showed that opipramol significantly decreased σ-1 receptor mRNA levels, indicating a correlation between Rac1 and σ-1 receptor when responding to opipramol. Both σ-1 and $GABA_B$ receptors are proteins that interact during changes in cellular function through crosstalk with other proteins, regulating signal transduction, receptor localization, and pharmacological profiles (Hayashi and Su, 2007; Nguyen et al., 2015; Terunuma, 2018). Changes in the $GABA_B$ receptor functions have been linked with an array of psychiatric disorders, including depression, anxiety, cognition, nociception, and SUD (Möhler, 2012; Felice et al., 2016; Terunuma, 2018). Gabbr1 associates with the σ-1 receptor, as demonstrated by increased expression of Gabbr1 in the CA1 region of the hippocampus in σ-1 receptor-knockout-mice (Vavers et al., 2021) and, as shown in the current study, changes in Gabbr1 and in Rac1 in the NAc of rats treated with opipramol.

Animal studies revealed that the efficacy of baclofen depends on the dose selected, both for cocaine and baclofen, and the setup, i.e., the requirement of a FR schedule rather than a progressive

ratio schedule. Campbell et al. (1999) showed that high-doses of baclofen (2.5, 5 mg/kg) decrease SA of low doses of cocaine and attenuate cravings (Campbell et al., 1999). Their addiction model, however, was not a longitudinal one, testing the effect on substance withdrawal. Another study, using 5 mg/kg of baclofen for 5 days under FR-5, significantly decreased cocaine SA (Shoaib et al., 1998). In contrast, Roberts et al. (1996) demonstrated that a dose of 1.5 mg/kg baclofen did not reduce cocaine SA at an FR-1 schedule but had a positive effect on a progressive ratio schedule that emphasizes the stressful/obsessive manner of substance consumption (Roberts et al., 1996). Eleven randomized clinical trials that tested baclofen efficacy in patients with withdrawal syndrome or SUDs reported inconsistent results. About half of those studies reported baclofen as being effective (Agabio et al., 2013), whereas others showed minor or no differences between baclofen and placebo (Ahmadi-Abhari et al., 2001; Heinzerling et al., 2006; Garbutt et al., 2010; Bschor et al., 2018).

In our study, we attempted to find an optimal dose of opipramol in the O/B combination, for the treatment of SUD. To the best of our knowledge, opipramol was never tested in pre-clinical trials in relation to SUD. We used a rat model in order to identify the dose that elicits a cocaine-craving inhibiting effect without affecting locomotion (Bareli et al., 2021).

In clinical trials, it is common to administer a target dose of 200 mg/kg opipramol a day for treating generalized anxiety and somatoform disorders (Volz et al., 2000; Möller et al., 2001). Most clinical trials use a maintenance dose of 30–300 mg baclofen a day (de Beaurepaire et al., 2019).

In our animal model for cocaine cravings, the dose of baclofen administered to the rats was reduced to a minimal one. We thus decided that in order to achieve a clinical effect with minimal side effects when combining opipramol and baclofen, which share a common molecular target, a dose of 150 mg/kg opipramol, and 90 mg/kg baclofen a day, may help inhibit cocaine cravings.

SUDs often co-occur with mood disorders. The reasons vary from genetic tendencies (Quello et al., 2005) to using substances as self-medication in an attempt to relieve depression (Markou et al., 1998). It is often difficult to differentiate between pure mood disorders and SUD associated with or mimicking the symptoms of mood disorders. In either case it is essential to manage the depression that is associated with SUD. In the past treatment was directed at either SUD or depression (Westermeyer, 2003), however, it was shown that both components have to be addressed simultaneously (Pettinati, 2004).

Treatments for SIDD include mood- stabilizers for withdrawal symptoms and antidepressants for mood symptoms (Nunes and Levin, 2004). In clinical trials, using antidepressants, especially selective serotonin reuptake inhibitors (SSRIs), resulted in unclear outcomes (Nunes and Levin, 2004; Pettinati et al., 2013; Agabio et al., 2018). A large multi-center randomized, single-blind placebo trial, conducted in patients with co-occurring major depressive disorder and alcohol dependence, showed that administration of the SSRI sertraline did not improve depression or drinking behavior, compared to placebo (Kranzler et al., 2006). However, the simultaneous administration of two medications, sertraline for depression and naltrexone for alcohol dependence, to patients with depression co-morbid with alcohol dependence showed an advantage in decreasing symptoms of both disorders (Pettinati et al., 2010). In another double-blind, placebo-controlled trial, patients were randomly assigned to four groups receiving 14 weeks of treatment with sertraline, naltrexone, or sertraline/naltrexone combination, vs. placebo. Patients treated with the combination exhibited higher rates of alcohol abstinence, their time to relapse to heavy drinking was longer and they tended to have fewer depression symptoms by the end of the treatment (Pettinati et al., 2010). This study supports our results with regard to the beneficial effects of combining two medications, like opipramol and baclofen to treat co-occurring mood disorders cocaine cravings.

The findings of this study indicate that patients receiving daily treatment of combined O/B exhibited improvement in craving symptoms compared to placebo. In addition, mood state was correlated with craving improvement (**Figure 7**), suggesting a role of mood alterations in the rehabilitation process. We also found at the end of the study, an increase in DHEA-S concentration levels and a significantly higher ratio of DHEA-S/cortisol in patients who received the O/B combination, compared to those who received placebo. This outcome is consistent with studies showing that the ratio between those hormones indicates the levels of anxiety (Ohana et al., 2016).

Limitations

While the results verify proof of concept to the behavioral effect in treatment with O/B, there are also limitations. The small sample size resulting from the difficulties recruiting patients and the level of attrition during the study are some of those limitations. Additionally, it is unclear if the short-term effects of our treatment are maintained long-term.

All the participants received group psychotherapy interventions along with pharmacotherapy, throughout the detoxification period. Thus, some of our outcomes may have been affected by the psychotherapy, however, both study subjects and controls received the same psychotherapy.

The O/B combination may be a promising approach for SUD, but this combination may produce more secondary effects than using each medication alone (Khezrian et al., 2020). Future studies should evaluate the beneficial effect of each one of these drugs alone in comparison to the combination, in a similar population.

CONCLUSION

Our preliminary clinical trial results may encourage researchers to conduct a long-term, double blind, placebo controlled, multi-center clinical trial to examine the effectiveness of the O/B combination as a novel anti-anxiety, anti-depression and anti-craving treatment on a large sample of patients with PsUD.

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/s.

ETHICS STATEMENT

The study was approved by the Israeli Ministry of Health (proposal no. 066-2015, Aug 2017). The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by Animal Care and Use Committee of Bar Ilan University, Ramat Gan, and were performed in accordance with the guidelines for animal experiments of the National Institutes of Health.

AUTHOR CONTRIBUTIONS

TB contributed to behavioral, molecular, and data analyses, wrote the first draft of the manuscript, conducted the clinical trial, and performed the questionnaires and hormones analysis. HA and RB assisted with the behavioral experiments. HB contributed to the initial behavioral experiments and the former concept of the manuscript. GW assisted with the molecular analysis. IG contributed to the surgical procedures and animal care and rehabilitation. RM drafted the manuscript. PR conceived of the clinical trial and provided the clinical setting for the trial. AW provided a significant revision of the article and prepared the final version of the manuscript. GY contributed to the research concept and design, interpretation of the results, and critical revision of the manuscript. All authors reviewed the content and approved the final version for publication.

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The Regulation of Glutamate Transporter 1 in the Rapid Antidepressant-Like Effect of Ketamine in Mice

Yaping Chen[1,2,3†], Mengxin Shen[1†], Xu Liu[4†], Jiangping Xu[2] and Chuang Wang[1*]*

[1] Zhejiang Provincial Key Laboratory of Pathophysiology, Ningbo University School of Medicine, Ningbo, China, [2] School of Pharmaceutical Sciences, Southern Medical University, Guangzhou, China, [3] College of Pharmacy, Fujian University of Traditional Chinese Medicine, Fuzhou, China, [4] Department of Pharmacy, General Hospital of Chinese People's Armed Police Forces, Beijing, China

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Alon Shamir,
Mazor Mental Health Center, Israel

Reviewed by:
Qi-Gang Zhou,
Nanjing Medical University, China
Kathryn J. Reissner,
University of North Carolina at Chapel Hill, United States

****Correspondence:***
Jiangping Xu
jpx@smu.edu.cn
orcid.org/0000-0002-0447-9229
Chuang Wang
wangchuang@nbu.edu.cn;
wanglovechuang@163.com
orcid.org/0000-0002-3816-230X

† These authors have contributed equally to this work

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Accumulating evidence suggests that glutamate clearance plays a critical role in the pathophysiology and treatment of depression. Preclinical and clinical studies have demonstrated that ketamine provides an immediate and sustained antidepressant effect. However, the precise mechanism of its action remains to be elucidated. Glutamate transporter 1 (GLT1) participates in glutamate clearance; therefore, we hypothesized that GLT1 may play an important role in the antidepressant effect of ketamine. In this study, we determined that GLT1 inhibition blocks the antidepressant-like properties of ketamine and alters the phosphorylation of the mammalian target of rapamycin (mTOR) in the prefrontal cortex (PFC). Our results show that pretreatment with dihydrokainic acid (DHK), a GLT1 inhibitor, alleviated the antidepressant-like effect of ketamine, and decreased the level of phosphorylated mTOR (pmTOR) in mice (which is normally upregulated by ketamine). In addition, inhibition of α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA) receptor and L-type voltage-dependent calcium channel (L-VDCC) significantly abolished the antidepressant-like effect of ketamine. Moreover, inhibition of L-VDCC significantly blocked the upregulation of GLT1 and BDNF in the PFC of mice. The inhibition of the AMPA receptor only significantly alleviated BDNF. Our results provide insight into the role of GLT1 as the critical presynaptic molecule participating in the pathophysiological mechanism of depression and contributing to the antidepressant-like effect of ketamine. In addition, our study confirms that both AMPA receptor and L-VDCC are crucial factors in the immediate antidepressant-like effect of ketamine.

Keywords: glutamate transporter 1, depression, ketamine, α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor, L-type voltage-dependent calcium channel

INTRODUCTION

Depression is one of the most prevalent psychiatric disorders, characterized by high incidence and treatment resistance. However, currently available antidepressants have several major drawbacks, such as low response rates and delayed therapeutic effects (Gaynes et al., 2009). The pathophysiology of depression as well as targets of pharmacological treatments have been defined

by the monoamine hypothesis of depression for the last decades. Antidepressants aiming at these previously defined targets normally require from 3 to 8 weeks to produce a therapeutic response; however, they affect neurotransmitters immediately (Machado-Vieira et al., 2008). A single sub-anesthetic dose of ketamine, a glutamate N-methyl-D-aspartate (NMDA) receptor antagonist, based on two meta-analyses of randomized placebo-controlled trials (Fond et al., 2014; McGirr et al., 2015), meets the needs of a rapid-acting antidepressant treatment. However, the mechanism underlying this immediate antidepressant effect in animal models remains largely unknown (Kavalali and Monteggia, 2015).

Ketamine has been described as a powerful antagonist of NMDA receptors, however, researchers have shown that the mechanisms underlying this response are likely to be more complex than a selective blockade of NMDA receptors (Naughton et al., 2014; Chaki and Fukumoto, 2015). Glutamatergic systems have been found to play an important role in the rapid antidepressant effect of ketamine (Krystal et al., 2013; Lener et al., 2017; Machado-Vieira et al., 2017). Glial cells regulate glutamatergic systems by clearing glutamate from extracellular space via excitatory amino acid transporter (EAAT). The glutamate is then recycled in the glutamate-glutamine cycle. Without the activity of glutamate transporters, glutamate builds up and kills cells in a process called excitotoxicity. It has been reported that β-lactam antibiotic ceftriaxone increases the uptake of glutamate by upregulating the expression of EAATs, and therefore exerts neuroprotective (Rothstein et al., 2005) and antidepressant effects (Mineur et al., 2007). There are five subtypes of EAATs in humans, as well as rodents. Subtypes EAAT1-2 are found in the membranes of glial cells (Lehre et al., 1995). EAAT2, also known as glutamate transporter 1 (GLT1), is responsible for over 90% of glutamate reuptake in the central nervous system (CNS) (Matsugami et al., 2006; Holmseth et al., 2009; Rao et al., 2015). GLT1 has been reported to play a critical role in the antidepressant effect of ketamine, and its mechanism of action may be associated with brain-derived neurotrophic factor (BDNF)/tropomyosin receptor kinase B (TrkB) signaling (Liu et al., 2016). However, the upstream regulatory mechanism by which GLT1 participates in the antidepressant effect of ketamine remains to be clarified.

In the present study, we examined the effects of GLT1 on the rapid antidepressant-like effect of ketamine in chronic unpredictable mild stress (CUMS) mice and explored the pathways that may participate in the regulation of GLT1 in the prefrontal cortex (PFC) of mice. First, we inhibited the activity of GLT1 with dihydrokainic acid (DHK) in CUMS mice. We then determined the level of mammalian target of rapamycin (mTOR), which is responsible for the rapid action of ketamine (Jernigan et al., 2011). Furthermore, we aimed to clarify the roles of the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR) and L-type voltage-dependent calcium channel (L-VDCC) in the regulation of GLT1. These two proteins participate in the upstream pathways of BDNF as well as TrkB, and have been reported to play an important role in the rapid antidepressant-like effects of ketamine (Duman et al., 2012). Additionally, BDNF and TrkB activation regulated by AMPAR and L-VDCC are required for the rapid antidepressant effects of ketamine (Autry et al., 2011), and BDNF is a potent endogenous activator of mTOR, which has also been suggested to underlie the antidepressant action of ketamine (Li et al., 2010).

MATERIALS AND METHODS

Animals and Drugs

The adult male C57BL/6J mice (6–8 weeks) used for the experiment were supplied by the Laboratory Animal Center of the Southern Medical University (Guangzhou, China). The animals were housed in an air-conditioned room at 22 ± 3°C and 60 ± 5% relative humidity under a 12 h light/12 h dark cycle (lights on at 7:00 a.m.) with *ad libitum* access to food and water. All experiments were carried out in accordance with the principles of the "NIH Guide for the Care and Use of Laboratory Animals" (NIH Publications No. 80–23, revised 1996). The procedures were approved by the Animal Care and Use Committee of the Southern Medical University. Ketamine hydrochloride, NBQX, and DHK used in this work were obtained from Sigma (St. Louis, MO or Shanghai, China). Verapamil was purchased from Aladdin Ltd., (Shanghai, China). All of them were dissolved in saline to the required concentration.

Experimental Design and Drug Treatment

The first aim of the present study was to explore the role of GLT1 in ketamine-induced rapid-acting antidepressant-like effects in CUMS mice. The selective inhibitor of GLT-1, DHK (1.0 μg), or vehicle was microinjected by intracerebroventricular (i.c.v.) injection in mice. Intraperitoneal injection of ketamine (10 mg/kg, i.p) was administered 30 min after the DHK treatment, and then the open field test (OFT) and forced swim test (FST) were conducted successively in the light phase between 8:00 a.m. and 4:00 p.m. in day 1 and 2 (as shown in **Figure 1A**).

In addition, to assess the roles of AMPAR and L-VDCC in the antidepressant-like actions of ketamine, the NBQX (0.5 μg, i.c.v) and Verapamil (5.0 nmol, i.c.v) were pretreatment 30 min before the ketamine administration, respectively, in mice. And then the OFT and FST were conducted successively in the same day (as shown in **Figure 1B**).

Surgery for Brain Cannula Implantation and Drug Injections

Intracerebroventricular cannulation implantation was performed as described (Li et al., 2017). The stereotaxic coordinates for the lateral ventricle were carried out in accordance with the Paxinos/Franklin mouse atlas (Paxinos and Franklin, 2001). All mice were anesthetized with 2% isoflurane and mounted on a stereotactic frame (R WD Kopf Instruments, Shenzhen, China) with a Kopf model mouse adaptor. Stainless steel guide cannula were implanted in the lateral ventricle at 0.70 mm posterior to the bregma, 1.30 mm lateral to the midline, and 2.00 mm below the skull surface. The guide cannula was anchored to the skull with dental cement and a stainless-steel stylet was inserted to maintain patency for microinjection. Animals were housed individually and allowed 7 days for recovery.

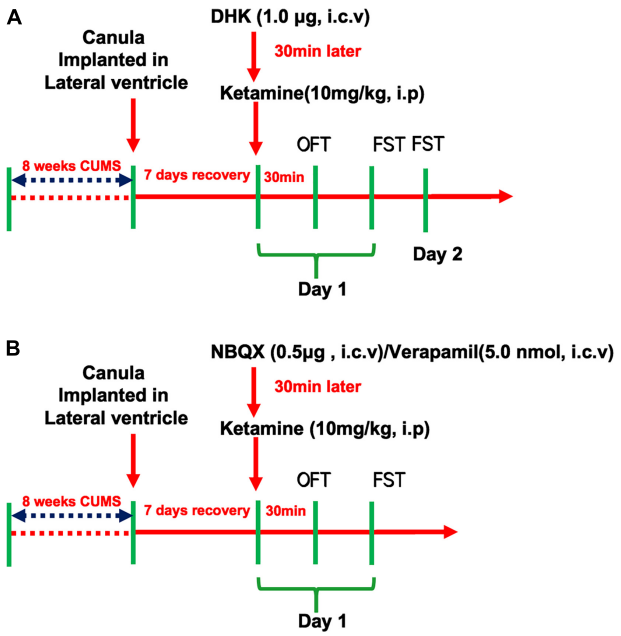

FIGURE 1 | Schedule of drug treatment and behavioral tasks orders. The experiment design of GLT1 inhibition in the rapid antidepressant-like effect of Ketamine **(A)** and the involvement of AMPAR/L-VDCC in the regulation of Ketamine on GLT1 **(B)**.

During the microinfusion, the mice were indisposed with a gaseous anesthetic and woke up 10∼20 min after infusion, and could be applied to behavior tests to explore the rapid antidepressant-like effects of ketamine in mice. The infusions were performed via a 10-μl Hamilton microsyringe connected to the microinfusion cannula via 0.26-mm ID polyethylene tubing. Microinfusions were carried out over 5 min with an infusion pump at 1 μL/min, and the cannulas were left in place for 3 additional minutes to avoid backflow. To verify the correct placement of the cannula after i.c.v drug delivery, mice were sacrificed after behavioral tests and cryostat sections of lateral ventricle cut through the cortex determine the cannula track. Only animals with the correct cannula placement were used for further analysis. Mice were sacrificed by dislocated spine method under anesthesia (ether), the tissues used for western blot analysis were collected 6 h after ketamine treatment.

Chronic Unpredictable Mild Stress Procedure

This animal model of stress consists of chronic exposure to variable unpredictable stressors, none of which is sufficient alone to induce long-lasting effects. Briefly, the CUMS procedure (Huang et al., 2017) involved 12 different stressors that were randomly arranged throughout the day and night over 56 consecutive days. The stressors were (1) 24 h of food deprivation, (2) 1 h of exposure to 4°C room (3) 24 h of exposure to a 45° cage tilt, (4) overnight illumination, (5) 24 h of exposure to a wet cage (100 ml of water per individual cage, which is enough to make the sawdust bedding wet), (6) 5 min of swimming in 6∼8°C water, (7) tail clamp for 5 min, (8) 24 h of water deprivation, (9) unpredictable shocks for 5 min (15 mA, one shock/5, 10 s duration), (10) Swimming for 15 min, (11) 4 h of restricted movement, and (12) 4 h of disrupting the cage. The behavioral tests were performed and scored by trained and experienced observers who were blinded to the animals' conditions.

Open Field Test

Briefly, the Open Field Test (OFT) was conducted according to the previous protocols that we recently reported (Li et al., 2017; Lv et al., 2018). The 50 × 50-cm arena with 39-cm high walls is made of a white Plexiglas box. Two black lines were drawn on the floor. Mice were placed into the center of the arena and allowed to explore the apparatus for 5 min. The number of the line crossings and rearings were considered parameters of locomotor activity and recorded over a 5-min period by a digital system.

Forced Swimming Test

The FST was conducted in a sound-attenuated room according to the previous studies with minor changes (Wu et al., 2016; Zanos et al., 2016; Lv et al., 2018). Briefly, mice were placed individually for 6 min into a clear plastic cylinder (diameter 10 cm, height 25 cm) containing 10 cm of fresh water, maintained at 23 ± 2°C. The immobility time was recorded over the following 4 min of the 6-min testing duration. The immobility time was defined as time when a mouse floated with only the bare minimum activity necessary to keep their heads above the water.

Western Blot Analysis

Frozen PFC tissues in each group (n = 3) of mice were homogenized in ice-cold radio-immunoprecipitation assay (RIPA) lysis buffer containing protease and phosphatase inhibitors cocktail (Pierce Biotechnology, Rockford, IL, United States). Lysates were centrifuged at 12,000 × g for 30 min at 4°C. The protein concentration of each sample lysate was determined with the BCA kit (Thermo Scientific, Rockford, IL). Each sample (25 μg total protein) was separated on 10% SDS polyacrylamide gel electrophoresis (PAGE) gels and transferred to PVDF membranes (0.22 μm; Millipore, CA). Membranes were then incubated with anti-mTOR (1:1,000, Cell Signaling), anti-phospho-mTOR (1:1,000, Cell Signaling), anti-GLT1 (1:1,000, Santa Cruze), BDNF (1:800, Abcam, United States) and anti-GAPDH (1:2,000, Millipore, CA) at 4°C overnight. The membranes were then incubated with Alexa Fluor800-conjugated antibody (1:10,000, Invitrogen, Eugene, OR) for 60 min. Target bands were captured with the fluorescence scanner (Odyssey Infrared Imaging System, LI-COR Biotechnology, Lincoln, NE) and quantified with Image J.

Statistical Analysis

Data are presented as mean ± standard error of the mean (SEM). Statistical analysis of the data was performed using one-way analysis of variance (ANOVA) followed by Tukey's *post-hoc* test as appropriate using GraphPad Prism software (Version 5.0, Prism software for PC, GraphPad). Values of $P < 0.05$ were considered statistically significant.

RESULTS

Role of Glutamate Transporter 1 in the Antidepressant-Like Effects of Ketamine in Chronic Unpredictable Mild Stress Mice

The CUMS exposure did not alter the locomotor activities of the mice in OFT [$F(4, 51) = 1.712$, $P = 0.1616$, **Figure 2A**]. While, the immobility time of FST was significantly increased by CUMS in day 1 [$F(4, 51) = 5.228$, $P < 0.01$, **Figure 2B**] and day 2 [$F(4, 51) = 5.447$, $P < 0.01$, **Figure 2C**]. A single administration of ketamine (10 mg/kg) significantly reversed these two alterations in CUMS procedure (both $P < 0.01$). However, the decrease of immobility time produced by ketamine was only significantly blocked by intracerebroventricular (i.c.v.) injection of DHK on day 1 ($P < 0.01$, **Figure 2B**), suggesting that GLT1 plays a critical role in the rapid-acting antidepressant-like effects of ketamine in mice. We did not find the antidepressant-like actions of ketamine was abolished by DHK 24 h after infusion in the FST (Day 2, **Figure 2C**). As shown in **Supplementary Figure 1**, the different doses of ketamine (3, 10, and 30 mg/kg, i.p) did not significantly change the locomotor activities. However, these Three doses of ketamine produced significant antidepressant-like actions in the FST and TST of mice (**Supplementary Figure 2**).

Ketamine Activated Mammalian Target of Rapamycin in the Prefrontal Cortex of Chronic Unpredictable Mild Stress Mice and Was Significantly Alleviated by Dihydrokainic Acid

To investigate whether the activation of mTOR produced by ketamine in an animal model of depression was abolished by DHK, the expressions of phosphorylation of mTOR (pmTOR) and total mTOR were determined in the PFC of mice (**Figure 3A**). As shown in **Figure 3B**, the CUMS exposure resulted in a significant decrease in protein levels of pmTOR when compared with the control group ($P < 0.05$). In comparison with the CUMS group, ketamine treatment significantly elevated pmTOR protein expression ($P < 0.01$). However, pretreatment with DHK, the up-regulation on the ratio of pmTOR in the PFC of mice produced by ketamine was significantly abolished ($P < 0.01$). **Figure 3C** showed the changes of mTOR, there was no significant change among all groups [$F(4, 10) = 0.1079$, $P = 0.9770$].

The Involvement of α-Amino-3-Hydroxy-5-Methyl-4-Isoxazolepropionic Acid Receptor and L-Type Voltage-Dependent Calcium Channel in the Rapid-Acting Antidepressant-Like Effects of Ketamine in the Prefrontal Cortex of Mice

Given the growing evidence that AMPAR and L-VDCC may involve in the rapid-acting antidepressants (Duman et al., 2012; Lepack et al., 2014; Zanos et al., 2016), we aimed to explore whether the GLT1 and BDNF were regulated by AMPAR and L-VDCC in the antidepressant-like actions of ketamine in mice. As shown in **Figures 4A,C**, the locomotor activities of the mice were not changed in the line crossings [NBQX, $F(4, 45) = 1.523$, $P = 0.2115$, **Figure 4A**; Verapamil, $F(4, 45) = 0.8936$, $P = 0.4757$, **Figure 4C**] of OFT by all treatments. However, both pretreatment with NBQX [$F(4, 45) = 22.56$, $P < 0.001$, **Figure 4B**] and verapamil [$F(4, 45) = 10.03$, $P < 0.001$, **Figure 4D**] can completely reversed the antidepressant-like effects of ketamine in the FST of mice. As shown in **Supplementary Figure 3**. Single treatment with NBQX (microinjection) and verapamil (i.p.) had no effects alone in the OFT and FST of mice.

The Different Role of α-Amino-3-Hydroxy-5-Methyl-4-Isoxazolepropionic Acid Receptor and L-Type Voltage-Dependent Calcium Channel in the Regulation on the Glutamate Transporter 1 by Ketamine in the Prefrontal Cortex of Mice

Growing evidence has shown that the AMPAR and L-VDCC are activated by rapid-acting antidepressants, resulting in the fast release of BDNF and activation of downstream pathways (Lepack et al., 2014; Yao et al., 2017; Yu et al., 2017, 2018; Ghosal et al., 2018). To evaluate the roles of AMPAR and L-VDCC in the regulation of GLT1 and BDNF by ketamine, we analyzed levels of the GLT1 and BDNF by western blot analysis in the PFC of mice (**Figure 5A**). After 56 days of CUMS exposure, the levels of GLT1 [$F(4, 10) = 17.01$, $P = 0.0002$, **Figure 5B**] and BDNF [$F(4, 10) = 13.63$, $P = 0.0005$, **Figure 5B**] were significantly decreased in the PFC of mice. However, a single treatment with ketamine rapidly and significantly reversed these molecular changes (GLT1, $P < 0.01$; BDNF, $P < 0.01$, **Figure 5B**). In contrast, the levels of BDNF (NBQX, $P < 0.05$; verapamil, $P < 0.001$) in the PFC of mice were significantly abolished by pretreatment with verapamil, respectively. Notably, the up-regulation on the GLT1 of ketamine was significantly alleviated by verapamil in the PFC of mice ($P < 0.05$). Interestingly, pretreatment with NBQX, the up-regulation on the GLT1 by ketamine was not significantly abolished compared with single treatment with ketamine in the PFC of mice.

DISCUSSION

A variety of studies in patients have shown that glutamatergic and rodent models dysregulation is involved in depression (Li et al., 2019). GLT1 is responsible for the majority (90%) of extracellular and synaptic glutamate clearance in the CNS. Previous studies have suggested that a decrease in the level of GLT1 in the brain is a possible cause of depression (Cui et al., 2014; Rappeneau et al., 2016). Specifically, infusion of DHK (GLT1 inhibitor) into the brain has been shown to alter the levels of amino acids (Fallgren and Paulsen, 1996) and induce both anxiety and depressive-like symptoms (John et al., 2015; Gasull-Camos et al., 2017a). In the above reports, DHK exhibited its effects within 5–15 min, and biological assays and behavior tests were completed in 24

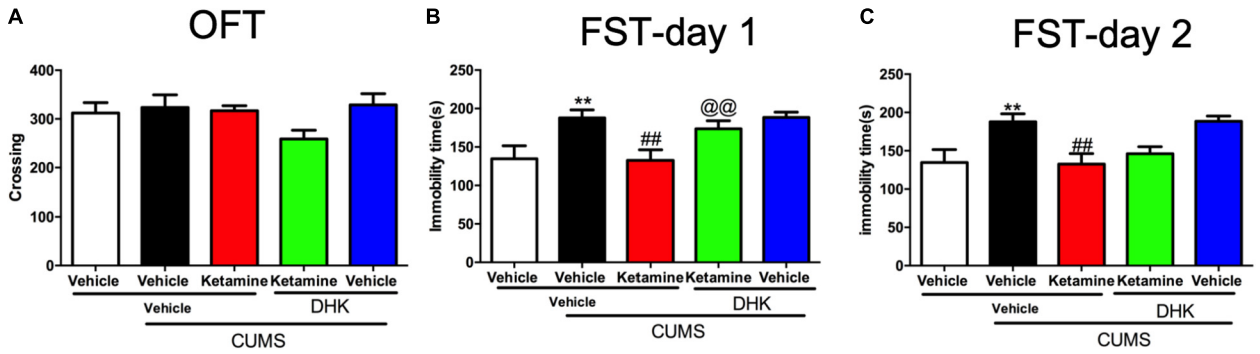

FIGURE 2 | The influence of DHK on the rapid antidepressant effect of Ketamine. The crossing of mice in OFT **(A)**; the immobility time of mice in day 1 **(B)**; the immobility time of mice in day 2 **(C)**. The data are expressed as means ± SEM, n = 10–12. $^{**}P < 0.01$ compared with vehicle + vehicle + non-stressed group; $^{\#\#}P < 0.01$ compared with vehicle + vehicle + CUMS group; $^{@@}P < 0.01$ compared with ketamine + vehicle + CUMS group.

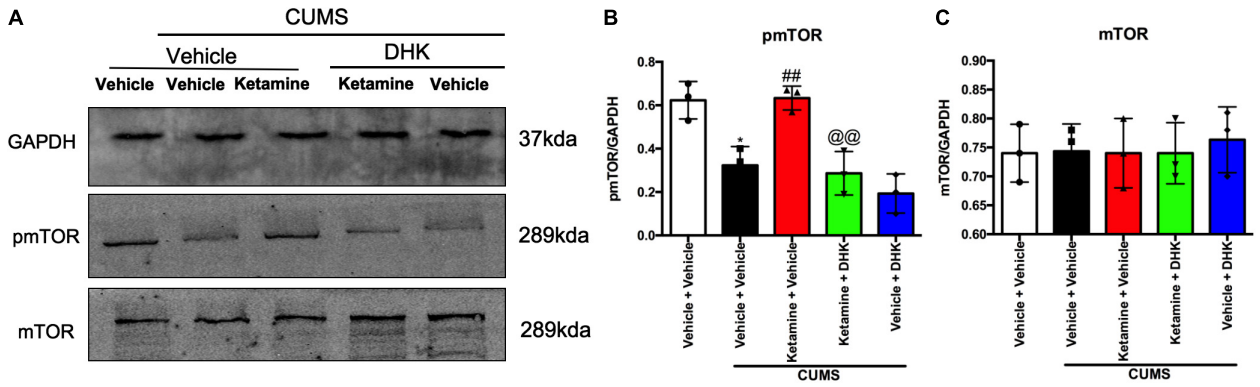

FIGURE 3 | The influence of DHK on the mTOR phosphorylation in the PFC of mice. mTOR and pmTOR protein expression were determined by Western blot. Representative western blot **(A)** and quantification **(B,C)** of fold changes in the protein levels of pmTOR and mTOR in the PFC of mice. The data are expressed as means ± SEM; n = 3; $^{*}P < 0.05$ compared with vehicle + vehicle + non-stressed group; $^{\#\#}P < 0.01$ compared with vehicle + vehicle + CUMS group; $^{@@}P < 0.01$ compared with ketamine + vehicle + CUMS group.

h, similar to the behavior tests in our study. GLT1 levels in the PFC corresponding to the effect after 48 h of DHK infusion were established by western blot analyses.

In our experiments, GLT1 expression levels in the PFC were significantly decreased following CUMS stimulation and returned to normal by ketamine treatment, which was consistent with the results of previous studies (Choudary et al., 2005; Liu et al., 2016). GLT1 inhibition is known to induce depression-like behaviors (Bechtholt-Gompf et al., 2010), whereas upregulation of GLT1 can have an antidepressant effect (Rothstein et al., 2005; Sanacora et al., 2007; Bechtholt-Gompf et al., 2010; Ding et al., 2017). Our current work confirmed that pretreatment with the GLT1 inhibitor DHK significantly alleviated the rapid antidepressant-like effect of ketamine infusion, which was consistent with the results of previous studies (Choudary et al., 2005; Liu et al., 2016). The decrease in astrocytic Glu uptake due to the decreased level of GLT1 might lead to a shortage of Gln in astrocytes, which is responsible for the release of Glu into the presynaptic neuron, which may support our findings. Our findings may be also confirmed by previous studies in which the infusion of DHK into the brain has been shown to change the levels of amino acids (Fallgren and Paulsen, 1996) and induce both anxiety and depressive-like symptoms (John et al., 2015; Gasull-Camos et al., 2017a).

Activation of the mTOR signaling pathway has been recently shown to be a critical factor in the antidepressant effect of ketamine (Hoeffer and Klann, 2010). The rapid induction of mTOR phosphorylation occurs within 30 min of ketamine administration. This, in turn, leads to the activation of mTOR-dependent protein synthesis (Duman et al., 2012). Here, we assessed changes in the level of the phosphorylated mTOR (pmTOR) after DHK treatment and found that the downregulation of GLT1 significantly inhibited the regulatory effect of ketamine on pmTOR. Therefore, it can be concluded that the regulatory action of ketamine on GLT1 influences mTOR activity. GLT1 as the major glial glutamate transporter is located in the membranes of pre-synaptic astrocytes and is responsible for more than 90% of glutamate uptake.

Glutamate is an important excitatory neurotransmitter in the CNS. This molecule as well as its cognate receptors have been described as new targets for rapid antidepressant action (Dutta et al., 2015; Machado-Vieira et al., 2017). Here, we speculated that

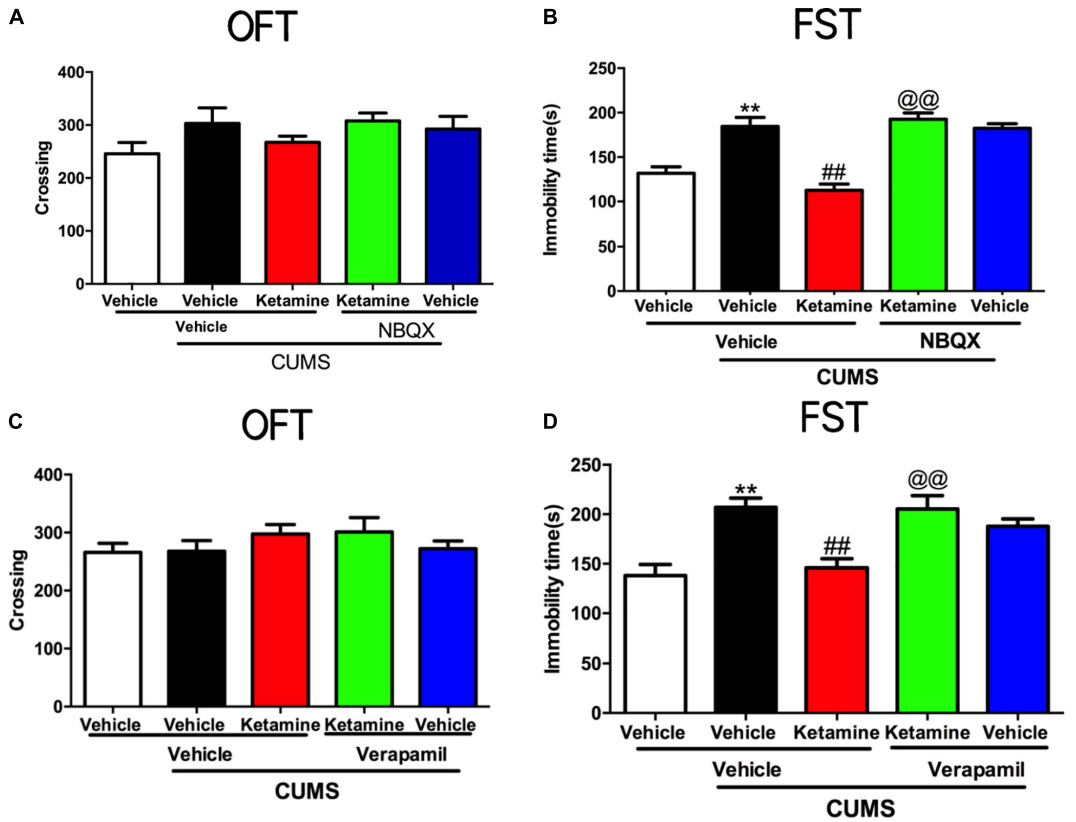

FIGURE 4 | The roles of AMPAR and L-VDCC played in the rapid antidepressant effect of Ketamine. The crossing of mice in an open field test in AMPAR blocked **(A)** and L-VDCC inhibition **(C)**; the immobility time of mice in forced swimming test in AMPAR blocked **(B)** and L-VDCC inhibition **(D)**. The data are expressed as means ± SEM, $n = 10$. $^{**}P < 0.01$ compared with vehicle + vehicle + non-stressed group; $^{\#\#}P < 0.01$ compared with vehicle + vehicle + CUMS group; $^{@@}P < 0.01$ compared with ketamine + vehicle + CUMS group.

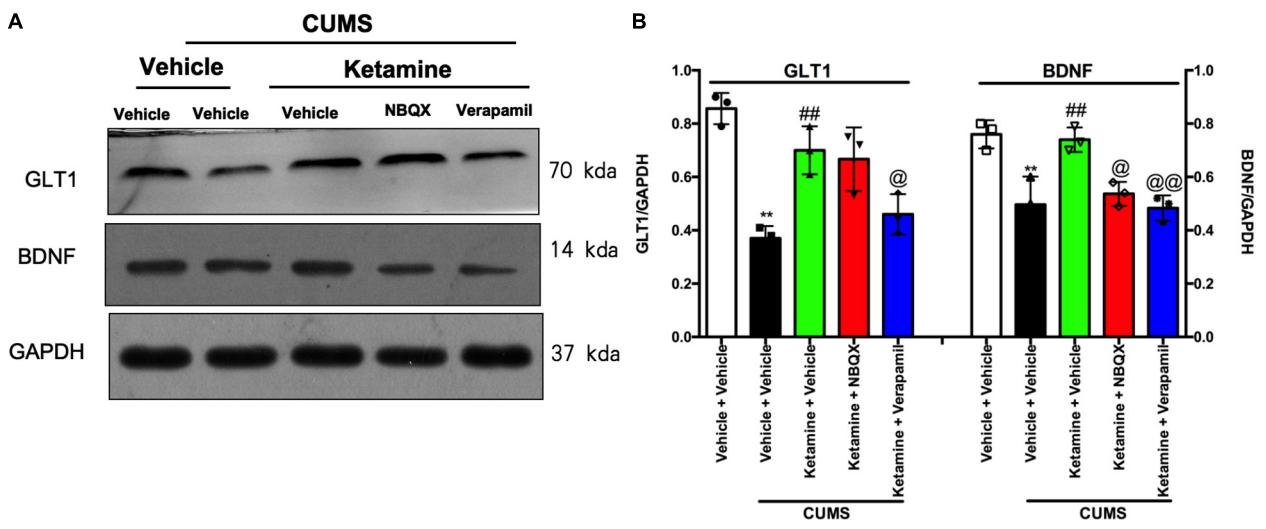

FIGURE 5 | The roles of AMPAR and L-VDCC in the regulation of Ketamine on GLT1 and BDNF in the PFC of mice. GLT1 and BDNF protein expression were determined by Western blot. Representative western blot (A) and quantification (B) of fold changes in the protein levels of GLT1, and BDNF in the PFC of mice. The data are expressed as means ± SEM; $n = 3$; $^{**}P < 0.01$ compared with vehicle + vehicle + non-stressed group; $^{\#\#}P < 0.01$ compared with vehicle + vehicle + CUMS group; $^{@}P < 0.05$, $^{@@}P < 0.01$ compared with ketamine + vehicle + CUMS group.

GLT1 indirectly influences the level of mTOR phosphorylation through the regulation of glutamate levels during the rapid antidepressant effect of ketamine.

AMPAR and L-VDCC have both been demonstrated to play important roles in the rapid antidepressant effect of ketamine as one of the critical downstream fast-acting factors. This finding has been confirmed by behavior tests in the current study. We also measured BDNF expression levels and found that ketamine regulated the levels of BDNF in CUMS mice. Both NBQX and verapamil pretreatment abolished the effects of ketamine on BDNF, consistent with previous reports (Jourdi et al., 2009; Lepack et al., 2014; Zhou et al., 2014). It is likely that L-VDCC may play a key role in the regulatory effect of ketamine on GLT1. However, the effect of L-VDCC inhibition on the level of GLT1 expression was different from that of AMPAR inhibition. AMPAR antagonist treatment increased GLT1 expression. We believe that this effect may be due to the participation of AMPA in the serotonergic activity in the PFC. It was reported that DHK and S-AMPA microinfusion in IL evoked similar antidepressant-like effects in the FST at 10 min post-administration (Gasull-Camos et al., 2018). Moreover, the GLT1 inhibition has been reported to induce a rapid increase in serotonergic activity in IL, which was blocked by NBQX (Gasull-Camos et al., 2017b). However, in our experiments we only detected the total GLT1 expressed in the PFC (not IL); therefore, further research is needed to clarify the changes in GLT1 expression in the IL after AMPA inhibition. The signaling pathway induced by the communication between glial and neuronal cells is often difficult to study. Data suggest that astrocytes signal to neurons through the Ca^{2+}-dependent release of glutamate (Haydon and Carmignoto, 2006; Fiacco et al., 2009). Additionally, our biochemical studies revealed that the calcium channel blocker verapamil significantly inhibited the effect of ketamine in CUMS mice.

This may be evidence of the influence of calcium channels on GLT1 expression. Growing evidence suggests that the antidepressant-like effects of ketamine and scopolamine in rodent models are caused by an influx of extracellular glutamate, elevated levels of BDNF, and activation of L-VDCC (Lepack et al., 2014; Wohleb et al., 2017; Ghosal et al., 2018; Yu et al., 2018). This may explain why the regulation of GLT1 by ketamine was blocked by t e calcium channel antagonist verapamil.

This study has some limitations. We found that GLT1 expression changed in both the hippocampus and PFC in mice under CUMS. As the PFC is an integral region in the top-down regulation of behavior and control of stress reactivity (Arnsten, 2015), we studied the biological changes in this area as a representation of the brain region. Accumulating evidence indicates that the hippocampus and nucleus accumbens (NAc) are also involved in depression-like phenotypes (Tsankova et al., 2006; Zhang et al., 2014; Yang et al., 2015). Thus, it is necessary to clarify the changes in these areas in future studies.

CONCLUSION

In conclusion, we showed that the antidepressant-like effect of ketamine on CUMS mice was prevented by GLT1 inhibition and that the regulation of mTOR phosphorylation in the PFC of mice affected the action of GLT1. Our results also indicated that L-VDCC in the PFC may influence the regulatory role of GLT1 in the therapeutic mechanism of ketamine.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

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

AUTHOR CONTRIBUTIONS

YC, MS, XL, JX, and CW performed the experiments and article preparation. CW wrote the first draft of the manuscript, which all other authors reviewed. All authors approved publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnbeh.2022.789524/full#supplementary-material

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DNA Methylation as a Therapeutic and Diagnostic Target in Major Depressive Disorder

*Elad Lax**

Department of Molecular Biology, Ariel University, Ariel, Israel

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Major Depressive Disorder (MDD) is a widespread debilitating neuropsychiatric disorder. While a broad range of drugs to treat MDD are available, a large portion of the patients fail to achieve a complete and sustained remission. It is estimated that only about half of the patients will be responsive to currently available antidepressant treatment (Rush et al., 2006), while others will be only partly responsive, and some will develop a treatment-resistant MDD (Akil et al., 2018).

The etiology of MDD is not clear and considering the large heterogeneity of symptoms and pathophysiologies it is likely to arise from a complex integration of genetic risk factors (Flint and Kendler, 2014; Geschwind and Flint, 2015) and environmental influences, mostly adverse life experiences (Gourion et al., 2008; LeMoult et al., 2020).

Adverse experiences such as early-life stress and poor maternal care are associated with increased risk for MDD in humans (Heim et al., 2010; Lippard and Nemeroff, 2020) and these findings were recapitulated in rodent models (Liu et al., 1997; Caldji et al., 1998) and non-human primates (Champoux et al., 2002; Barr et al., 2003). Maternal behavior and environmental stress alter the development of the hypothalamic–pituitary–adrenal (HPA) axis stress response leading to a stress susceptible phenotype associated with a greater risk for depression later in life (Liu et al., 1997; Anacker et al., 2014). Early-life stress effects can further interact with genetic factors that predispose individuals to depression (Heim and Binder, 2012).

The genome can integrate environmental signals through epigenetic mechanisms such as DNA methylation of CpG dinucleotides and histone modifications. Indeed, ample evidence has linked environmental stress to epigenetic alterations. Increased DNA methylation of the glucocorticoid receptor (GR) promoter was found in the hippocampus of rat pups with poor maternal care (Weaver et al., 2004) and in the post-mortem human brains of individuals who suffered childhood abuse (McGowan et al., 2009). Similar findings suggested conserved epigenetic signature of early life stress in rats and humans (Suderman et al., 2012). It was also demonstrated that peripheral tissues (including blood, saliva and buccal cells) can be used as surrogates for measuring epigenetic changes in the brain across many neuropsychiatric disorders (Fuchikami et al., 2011; Unternaehrer et al., 2012; Lax et al., 2018; McEwen et al., 2020). In addition, many CpGs show correlation of DNA methylation levels between blood and several brain regions, and hence can serve as disease biomarkers (Hannon et al., 2015; Edgar et al., 2017).

The observations that environmental factors, such as early-life stress, that make individuals prone to MDD, also modulate the epigenetic signals to ultimately reprogram brain gene-expression patterns encouraged studies that seek direct associations between MDD and DNA methylation. For example, a genome-wide DNA methylation study in post-mortem brain samples from MDD patients who died during a depressive episode and matched controls found more than a hundred differentially methylated regions between the groups (Nagy et al., 2015). Recently, a large-scale genome-wide study directly compared brain and blood DNA methylation patterns in MDD

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Edited by:
Ravid Doron,
Open University of Israel, Israel

Reviewed by:
Chong Chen,
Yamaguchi University Graduate School of Medicine, Japan

***Correspondence:**
Elad Lax
eladl@ariel.ac.il
orcid.org/0000-0002-0310-0520

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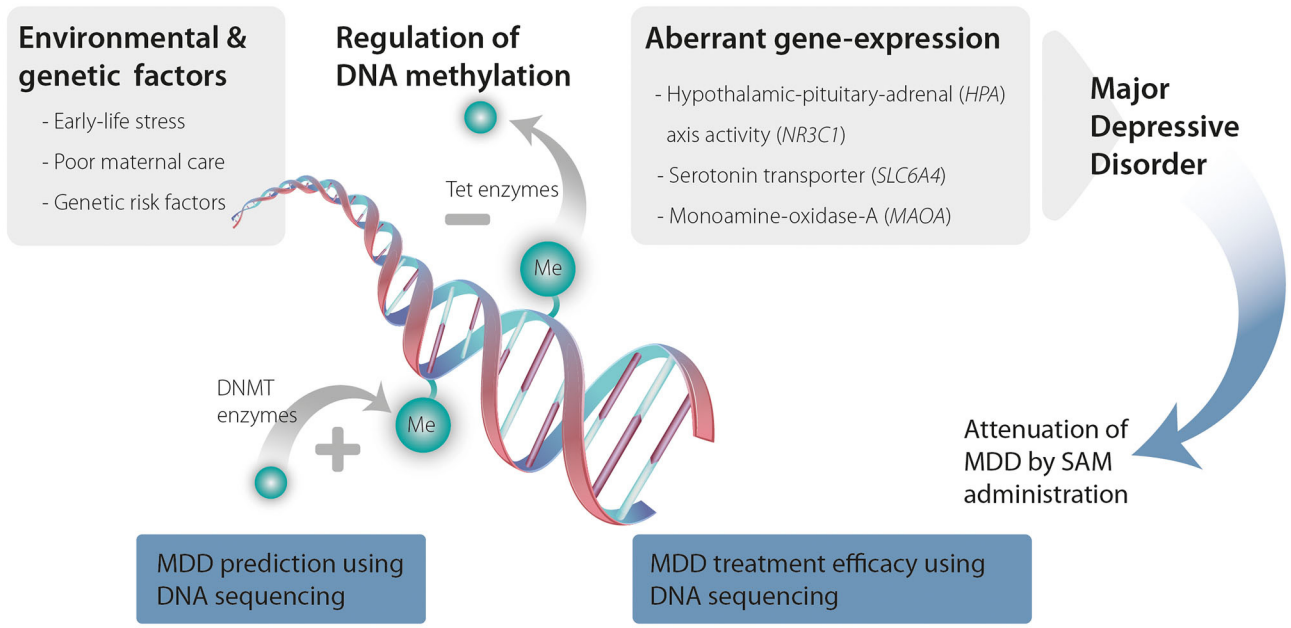

FIGURE 1 | Environmental and genetic risk factors promote changes in DNA methyltransferases (DNMTs) and ten-eleven translocation methylcytosine dioxygenases (TET) activity that increase DNA methylation and demethylation, respectively. Changes in DNA methylation lead to aberrant expression of genes that are associated with MDD ultimately causing MDD. SAM administration can attenuate these changes in DNA methylation. Further, genomic sequencing to detect changes in DNA methylation can serve as a predictive tool for MDD as well as a tool to predict treatment efficacy and responsiveness.

patients including replication cohorts and found differentially methylated sites in MDD patients (Aberg et al., 2020). Other researchers used a candidate gene approach and found changes in DNA methylation levels for the genes *MAOA* (encoding the monoamine-oxidase-A enzyme) and *NR3C1* (encoding the glucocorticoid-receptor) in individuals with MDD and childhood adversities (Melas et al., 2013).

Other studies aimed to assess DNA methylation levels of the promoter of *SLC6A4*, the gene that encodes the serotonin transporter, a major target of many antidepressant drugs. Kang et al. (2013) found an association between childhood adversity and worse clinical presentation of MDD and higher methylation levels of the *SLC6A4* promoter with no effect of antidepressant treatment on methylation levels of this region. Using the same approach, Okada et al. (2014) did not find a significant difference between DNA methylation levels of the *SLC6A4* promoter in healthy controls and MDD patients before antidepressant treatment. However, they found significantly increased methylation in some CpGs following a 6-week treatment. Similar findings were also found in additional studies (Vijayendran et al., 2012; Zhao et al., 2013; Domschke et al., 2014). Furthermore, several studies linked peripheral measures of *SLC6A4* promoter DNA methylation to brain connectivity in MDD (Chiarella et al., 2020), brain functions involved in emotional stimuli (Frodl et al., 2015), and hippocampal volume in MDD (Booij et al., 2015). Notably, heterogeneity in DNA methylation changes in MDD across experiments is to be expected due to factors such as genomic heterogeneity and the parameters of the sampled population. For example, a distinct DNA methylation signature was found for adult-onset and late-onset MDD (Yamagata et al., 2021). On the other hand, parameters such as ethnicity might have smaller effects. A meta-analysis of multiethnic epigenome-wide studies for depressive symptoms found DNA methylation signatures of depression which were robust across ethnicities (Story Jovanova et al., 2018).

Taken together, the findings that DNA methylation changes were observed in MDD led to efforts to pharmacologically manipulate DNA methylation levels as a potential antidepressant treatment. Administration of S-adenosyl methionine (SAM), a methyl donor that is used by DNA methyltransferases (DNMTs) to catalyze DNA methylation, can increase global DNA methylation. Therefore, many studies examined the effect of SAM administration as a monotherapy or an add-on to antidepressant treatment. The overall effects of SAM administration in MDD were analyzed in several thorough systemic reviews, which concluded that SAM shows promising results although additional larger randomized double-blind studies with long-term follow-up are required (Galizia et al., 2016; Sarris et al., 2016; Sharma et al., 2017; Cuomo et al., 2020). Animal models suggested some mechanistic insight into the beneficial effects of SAM. Saunderson et al. (2016) demonstrated that SAM administration attenuated stress-induced c-Fos and Egr-1 gene-promoter demethylation and protein expression in the dentate gyrus of stressed rats. Intracerebroventricular

infusion of methionine (SAM precursor) reversed stress response and DNA methylation levels of the GR promoter in rat offspring from poor maternal care dams (Weaver et al., 2005) and systemic methionine injections in the same animal model altered gene-expression of over 300 genes in the hippocampus (Weaver et al., 2006). Notably, *Dnmt3a* over-expression (which increases global methylation) specifically in the nucleus accumbens increased depressive-like behaviors, while DNMT inhibition with RG-108 decreased depressive-like behaviors in mice (LaPlant et al., 2010). Also, forebrain deletion of *Dnmt1*, but not *Dnmt3a*, showed anti-depressive effects (Morris et al., 2016).

In naïve newborn and adult rodents, systematic administration of the DNMT inhibitors 5-aza-2-deoxycytidine or 5-azacytidine reduced depressive-like behaviors through demethylation of the *Bdnf* gene promoter leading to increased brain Bdnf mRNA and protein levels (Sales et al., 2011; Li et al., 2017). While the findings of pharmacological inhibition of DNA methylation might be seen as contradictory to the beneficial effects of SAM observed in preclinical and clinical studies, it is important to note that different models, species, and administration routes were used, making it hard to directly compare these results. Also, while SAM treatment is a promising add-on MDD therapy, it increases DNA methylation globally and can potentially reprogram gene-expression beyond those that are causative for MDD. However, many human studies on the effects of SAM administration on MDD showed beneficial effects for this treatment with no major adverse side-effects reported. Currently, there are not pharmacological interventions that can manipulate DNA methylation levels of specific genomic loci. Novel technologies might allow this in the future, for example by targeting a catalytically inactive CRISPR/deadCAS9 protein fused to DNMT3a (dCAS9-DNMT3a) to loci of interest as was shown experimentally (Liu et al., 2016; Vojta et al., 2016; Xu and Heller, 2019).

Disentangling whether changes in DNA methylation are the cause or result of MMD is very difficult in human studies which can measure mostly associations. However, several studies found that DNA methylation can predict behavioral outcomes, including major depression, in humans (Ursini et al., 2011; Guintivano et al., 2014; Humphreys et al., 2019). In addition, studies in animal models for MDD showed that manipulating the methyl donor availability, DNMTs levels and DNMTs activity can induce MDD-like behavioral phenotypes (LaPlant et al., 2010; Sales et al., 2011; Morris et al., 2016; Li et al., 2017). Therefore, it is prudent to assume that alterations in DNA methylation, as a result to external stressful stimuli, are at least partly causative of MDD, although it is likely that some methylation alterations are secondary to MDD (and yet can serve as potential biomarkers).

The notion that manipulating DNA methylation has an impact on MDD encouraged studies measuring DNA methylation levels as potential biomarkers to predict MDD and its severity in vulnerable populations as well as treatment outcomes. For example, blood methylation levels were measured in several cohorts to successfully predict antenatal and postpartum depression (Guintivano et al., 2014; Payne et al., 2020). Some of these studies focused on one or a few candidate genes as potential MDD predictors, mostly the *BDNF* and *SLC6A4* genes (Booij et al., 2015; Kleimann et al., 2015), while others used genome-wide methods (Barbu et al., 2020).

A recent systematic review on DNA methylation in depression and the effects of MDD treatment on DNA methylation concluded that findings from studies that aimed to search for biomarkers for MDD treatment outcome are inconsistent; with some studies showing significant results while others had mixed findings. This is most likely due to larger heterogeneity compared to other studies, types and stages of treatment and small sample sizes in some of the studies. Overall, the most consistent effects were increased methylation of the *BDNF* and *SLC6A4* genes in MDD patients (Li et al., 2019).

Taken as a whole, a growing body of evidence support a role for DNA methylation in MDD (see summary in **Figure 1**). Drugs that modify DNA methylation are available and demonstrate significant effects across both preclinical and clinical studies. These drugs (mostly methionine and SAM) have the potential to be used as adjuvants increasing the efficacy of classic antidepressant treatments. Further, peripheral DNA methylation has the potential to become a non-invasive method for assessing MDD risk and treatment-efficacy estimation. Future large-scale research on MDD patients is needed for further study and validation to establish these approaches.

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The author confirms being the sole contributor of this work and has approved it for publication.

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