MECHANISMS UNDERLYING MOOD DISORDERS

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MECHANISMS UNDERLYING MOOD DISORDERS

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SYSTEMATIC REVIEW
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Efficacy and Safety of Botulinum Toxin vs. Placebo in Depression: A Systematic Review and Meta-Analysis of Randomized Controlled Trials

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Background: Major depressive disorder (MDD) is a serious mental disorder that represents a substantial public health problem. Several trials have been undertaken to investigate the role of botulinum toxin type A (BTX-A) in the treatment of MDD, but the conclusions were controversial. To examine the efficacy and safety of BTX-A vs. placebo on patients with a clinical diagnosis of MDD, we conducted this systematic review and meta-analysis.

Methods: A systematic search was conducted for all relevant randomized controlled trials (RCTs) in PubMed and Web of Science from inception to June 17, 2020. All published studies that investigated the efficacy and safety of BTX-A injections on patients with a clinical diagnosis of MDD were included. The overall effect size was summarized using a random-effects meta-analysis model. The primary outcomes of the present meta-analysis were the changes in depressive rating scale at week 6 after BTX-A injection compared with placebo. The safety of BTX-A injections also was assessed.

Results: Five RCTs with a total of 417 participants (189 patients in the BTX-A group, 228 patients in placebo group) were eligible in this meta-analysis. The results indicated an overall positive effect of BTX-A injections for reducing the depressive symptoms of patients with MDD (Hedges' g, −0.82; 95% CI, −1.38 to −0.27) with large effect size. Differences are likely explained by the dose of BTX-As and the gender of the participants. Our findings also highlighted that BTX-A injections were generally well-tolerated, with only mild and temporary adverse events reported.

Conclusions: The present meta-analysis provides evidence that BTX-A injections are associated with a statistically significant improvement in depressive symptoms. BTX-A injections are generally safe and may provide a new, alternative option for the treatment of depression.

Keywords: botulinum toxin type A, antidepressant, depression, systematic review, meta-analysis

INTRODUCTION

Major depressive disorder (MDD) is a common and severe mental disorder among the general population. It is related to psychosocial factors, heredity, and changes in the nervous system (1–4). According to the latest data provided by the Global Burden of Disease Study (GBD), ∼216 million people suffered from MDD worldwide in 2015 (5). The core symptoms of MDD include sadness, fatigue, and loss of interest or pleasure, which incur a tremendous burden on health and finances (6). Additionally, the high suicide rate associated with severe depression is considered a serious public health concern (7).

Botulinum toxin type A (BTX-A), also known as onabotulinumtoxinA or Botox®, is widely known for its cosmetic efficacy in treating glabellar frown lines (8). It was estimated that more than 1 million cases of BTX-A treatment were reported annually in the United States (9). Emerging evidence suggests that BTX-A injections may exert psychological effects (10, 11). In 2006, a case series first reported the role of BTX-A in the treatment of depression (12). Since this initial report, there has been a growing interest in studying the effect of BTX-A on depression. Wollmer et al. (13) subsequently conducted a randomized double-blind, placebo-controlled trial to explore the effect of BTX-A injections as an adjuvant therapy for MDD. The results showed that depressive symptoms were significantly improved in patients receiving BTX-A injections. The remission and response rates of MDD were also decreased in the BTX-A group compared with the placebo group. Several subsequent trials reported similar results (14, 15). However, a recent large study in 2019 showed that the effects of the high-dosage (50 U) BTX-A injections were similar to effects in the placebo group (16). Given the controversy among different studies, and the growing interest toward complementary and alternative medicine for depression, a systematic review and meta-analysis regarding the efficacy and safety of BTX-A on MDD is worth updating.

Hence, the objective of our study was to comprehensively compile results from the randomized controlled trials (RCTs) and to precisely investigate the efficacy and safety of BTX-A injections as an adjuvant treatment for MDD in comparison to placebo using a meta-analytic methodology. The evidence-based results will benefit further research on MDD.

MATERIALS AND METHODS

Search Strategy

This systematic review and meta-analysis was conducted following the guidelines of the Preferred Reporting Items of Systematic Reviews and Meta-Analyses (PRISMA) statement (17). We systematically searched PubMed and Web of Science to identify all potential literature concerning the role of BTX-A in depression, from inception to June 17, 2020. The following search strategy was adopted: ("*botulinum*" OR "*botox*" OR "*abobotulinumtoxin*" OR "*onabotulinum*" OR "*onabotulinumtoxin*" OR "*botulinumtoxin*" OR "*oculinum*" OR "*dysport*" OR "*botulinotherapy*") AND ("*antidepressant*" OR "*depression*" OR "*depressive*" OR "*depressed*" OR "*melancholia*" OR "*mood disorder**" OR "*affective disorder**" OR "*anxiety*"). The search results were restricted to articles published in English. Moreover, we manually checked the reference citations of all retrieved articles to identify additional publications.

Inclusion and Exclusion Criteria

Two independent investigators determined potentially relevant studies by screening the titles and abstracts, in duplicate. Next, the papers were assessed to identify eligible studies based on the predefined inclusion criteria. Any discrepancies noted were discussed and resolved with a third investigator.

According to the PICOS criteria, original articles that met the following explicit criteria were eligible: (1) Patients: individuals with the clinical diagnosis of MDD were recruited based on validated and effective diagnostic criteria [e.g., Diagnostic and Statistical Manual of Mental Disorders (DSM-V or DSM-IV)]. We included studies that reported MDD of any severity (mild, moderate, or severe). Studies that recruited patients with depressive symptoms different from MDD or individuals who did not meet the diagnostic thresholds of depression at baseline were excluded; (2) Intervention: BTX-A was administered as an effective intervention for MDD. No restrictions were placed on the form, dosage, or injection site; (3) Comparison: BTX-A injections vs. placebo injections; (4) Outcome: different rating scales of depression were applied to assess the change of depressive symptoms; (5) Study design: only randomized, placebo-controlled trials were included in the present analysis.

Data Extraction

We extracted effective data from all eligible studies using a standard data extraction checklist. Two independent investigators completed this process. Any discrepancies were discussed and resolved with a third investigator. We extracted the descriptive information, including the first author's name, publication year, country of the participants, interventional duration, study design, severity of depression, diagnosis criteria, dosage of BTX-A, primary outcome measures, and injection region, as well as number, mean age, and gender composition of the participants. Moreover, the pre- and post-treatment means and standard deviations (SDs) of depression scores or the pre- and post-treatment differences of means and SDs of depression scores from each included study were extracted. If a study provided valid data at multiple points after intervention, the time point of the primary outcome was utilized. If any of the eligible studies provided insufficient data, the corresponding authors would be contacted for further information.

Risk of Bias Assessment

Two investigators used the Cochrane risk of bias tool to assess the methodological quality of each selected study (18). Any discrepancies were discussed and resolved with a third investigator. We evaluated the risk of bias according to the seven following items: random sequence generation, allocation concealment, blinding of participants and personnel, blinding of outcome assessment, incomplete outcome data, selective outcome reporting, and other bias. The potential bias of each item was classified as high, low, or unclear risk. A study was considered

high risk of bias if any of the six items were classified as high risk (the item "other bias" was excluded). We assigned an overall low risk of bias if a study was considered low risk in all six items. Otherwise, the study was categorized as overall unclear risk of bias (19).

Statistical Analysis

To investigate differences in depressive symptoms between BTX-A injections vs. placebo, a meta-analysis method was used to pool extracted data from the included studies. Given the impact of a small sample size on the overall effect size, Hedges' g with corresponding 95% confidence interval (CI) were appropriate to analyze the continuous variables (mean and SDs). When pre-post changes of SDs in depression scores were not reported, an imputed correlation coefficient of 0.5 was used (20), according to the transformation formula in the Cochrane Handbook. When SDs from the original articles were not available, we calculated the estimates from the 95% CI (21). The effect sizes were interpreted under the guidelines (i.e., 0.2, small; 0.5, medium; 0.8 large) (22). We assessed the between-study heterogeneity of effect size using the inconsistency index (I^2) and Cochran Q-test (23). $I^2 > 50\%$ or $P < 0.05$ was considered statistically significant. The fixed-effects model was applied to calculate the pooled results when no statistically significant heterogeneity was presented; otherwise, a random-effects model was applied to provide more conservative estimates.

Subgroup analysis was conducted using the number of subjects, proportion of females, risk of bias, and measurement tool to investigate the sources of heterogeneity. We performed sensitivity analysis by successive exclusion of each study to test the reliability of the main outcomes. All the statistical analyses of this meta-analysis were performed with STATA software, version 15.1 (Stata Corp, College Station, TX, USA). $P < 0.05$ was considered statistically significant.

RESULTS

Search Results and Study Characteristics

The detailed literature screening process is depicted in **Figure 1**. Database searching yielded a total of 1,115 related studies, while five potentially eligible studies were obtained from reference citations of retrieved articles. After removing duplicates, 768 studies remained. We excluded 740 completely unrelated articles by evaluating titles and abstracts. For the remaining articles, we obtained the full-text articles for detailed assessment. Twenty-three articles were excluded; the reasons are presented in **Figure 1**. Finally, five RCTs met the inclusion criteria for this meta-analysis (13–16, 24).

Baseline characteristics of the included studies are listed in **Table 1**. We identified five eligible articles involving a total of 417 participants (189 patients in the BTX-A group, 228 patients in the placebo group), all of which were randomized, placebo-controlled trials published between 2012 and 2020. Notably, Brin et al. (16) carried out a two-dose parallel groups study of low dosage (30 U) and high dosage (50 U) BTX-A. There were 389 females and 28 males, with a mean (SD) sample size of 70 (44.0) and a mean (SD) age of 46.4 (4.1). The total follow-up period varied from 6 to 24 weeks after a single intervention at baseline. Several countries were involved in the analysis. Three studies were from America, one from Iran, and one from Switzerland and Germany.

Risk of Bias Assessment

A summarization regarding the risk of bias for the five included studies is presented in **Table 2**. All studies were blinded to participants, investigators, and outcome assessment (24). Only two articles were considered low risk of bias in terms of incomplete outcome data (13, 16), while others were considered high risk of bias because the data regarding the differences in pre- and post-treatment means and SDs were not given directly. For other bias, we only rated one study as high risk of bias (24), but the risk of bias was unclear for four other studies. Three articles were assigned an overall high risk of bias, but the rest were categorized as an overall low or unclear risk of bias.

Efficacy of BTX-A in MDD

Primary outcomes of all included studies were the changes in depressive rating scale at week 6 after BTX-A injections compared with placebo. The forest plot for the efficacy of BTX-A in MDD is shown in **Figure 2**. Compared with the placebo group, we found a statistically significant efficacy of BTX-A injections in MDD with a large pooled effect size (Hedge's g, −0.82; 95% CI, −1.38 to −0.27, for the random-effects model). Obvious heterogeneity was observed across the study data ($I^2 = 84.5\%$, P-heterogeneity < 0.001).

Subgroup analysis was performed to seek more information. The results stratified by potential modifying factors are shown in **Table 3**. There was no substantial difference in the overall results of stratified subgroups. After stratifying the number of subjects, the results showed that a relatively large sample size was a momentous source of heterogeneity ($I^2 = 86.1\%$, P-heterogeneity $= 0.222$), but not the small sample size. For studies with a high proportion of females, the pooled Hedge's g was −0.56 (95% CI, −1.15 to 0.03; $I^2 = 85.2$; P-heterogeneity < 0.001), but for studies with a low proportion of females, the pooled Hedges' g was −1.43 (95% CI: −2.02 to −0.84; $I^2 = 0.0\%$; P-heterogeneity $= 0.503$). Moreover, statistically significant heterogeneity was found in studies with low or unclear risk, as well as studies using the Montgomery–Åsberg Depression Rating Scale (MARDS) as the assessment for outcome.

We carried out sensitivity analysis to further explore the potential sources of heterogeneity. After excluding one study, (16) the heterogeneity decreased significantly ($I^2 = 0.0$; P-heterogeneity $= 0.621$), and a more significant effect of BTX-A was observed for the treatment of MDD (Hedges' g, −1.20; 95% CI: −1.54 to −0.86).

Safety Assessments

In short, the BTX-A injections were well tolerated, and no serious adverse events (AEs) were reported in any of the studies. Magid et al. (14) did not provide data on treatment-related AEs. In the RCT conducted by Zamanian et al. (24) none of the 28 patients with MDD experienced any AEs. The most common AEs, including headache, upper respiratory infection, eyelid

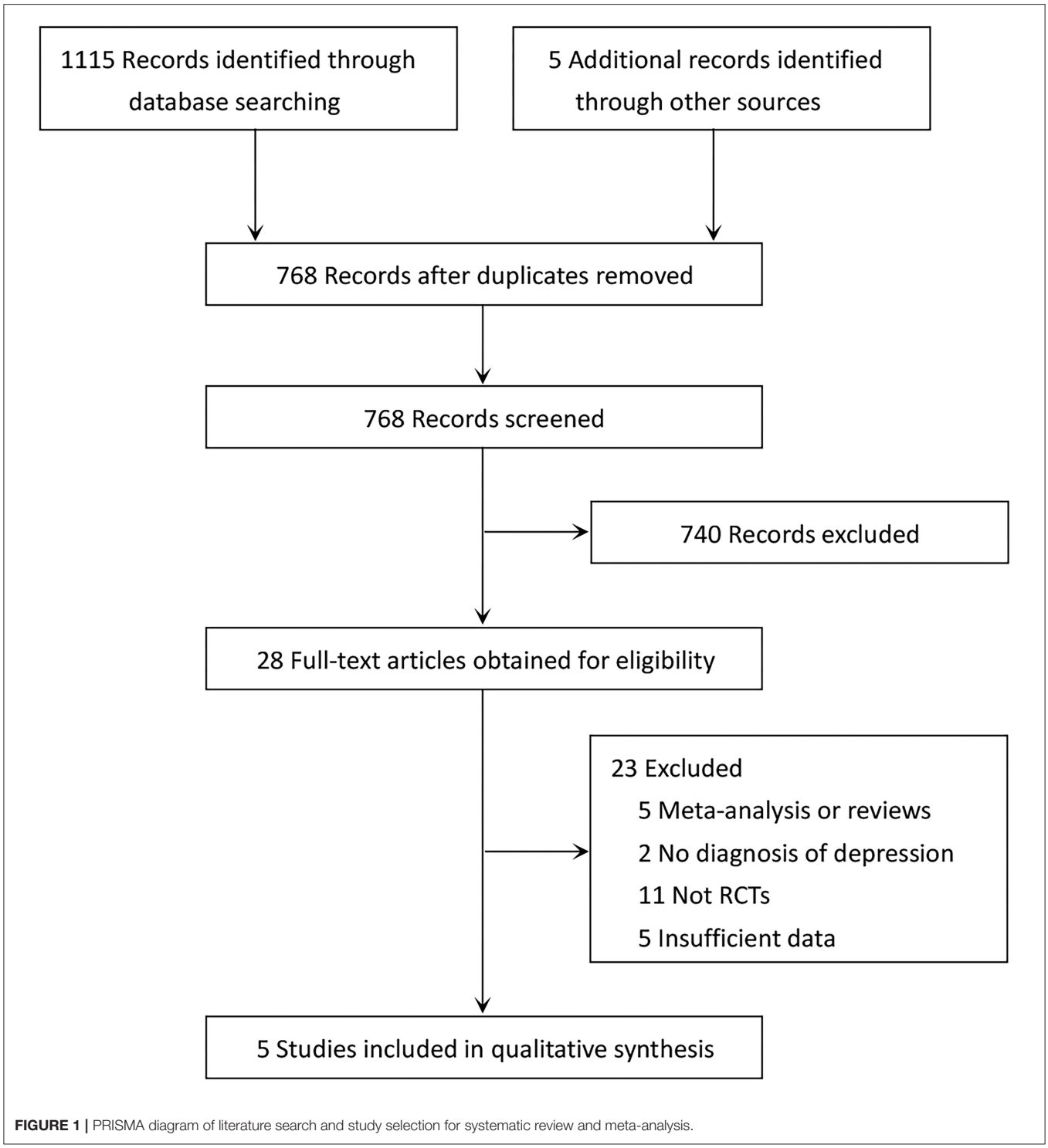

FIGURE 1 | PRISMA diagram of literature search and study selection for systematic review and meta-analysis.

ptosis, and injection pain, were noted in the remaining three studies. Brin et al. (16) reported that more than 10% of all patients experienced headaches, but the headaches seemed unrelated to treatment. Moreover, the incidence rates of eyelid ptosis and upper respiratory tract infection added up to 5% in the BTX-A group, which was significantly higher than the placebo group. Transient and mild headaches occurred in 3 of the 74 participants in the study conducted by Finzi and Rosenthal (15), and one patient in the placebo group also complained of nightmares and night terrors. In the study by Wollmer et al. (13), headaches occurred in 40.0 and 26.7% patients in the BTX-A and placebo groups, respectively (Fisher's exact, $P = 0.700$).

TABLE 1 | General characteristics of the five RCTs included in the meta-analysis.

Study ID	Country	Study Design	No. of Subjects (BTX-A, placebo)	Mean Age (range)	Gender (M/F)	Main Diagnosis (diagnostic tool)	InterventionDosage (M/F)	Duration of Active Treatment (Weeks)	Primary Outcome	Injection Region
(16)	USA	Double-blind RCT	Total dosage (30 U): 123 (65, 58) Total dosage (50 U): 132 (65, 67)	43.90 (18–65)	Only F	Moderate to severe MDD (DSM-IV)	30 U or 50 U	24	Change in MADRS score at week 6 after injection	Glabellar injections
(24)	Iran	RCT	28 (14,14)	39.43	14/14	MDD (DSM-V)	NR	6	Change in BDI score at week 6 after injection	NR
(14)	USA	Double-blind RCT	30 (11,19)	49.47 (24–65)	2/28	Mild to severe MDD (DSM-IV)	39 U/29 U	24	Change in HAM-D_{21} score at week 6 after injection	Glabellar injections
(15)	USA	Double-blind RCT	74 (33,41)	48.40 (18–65)	5/69	MDD (DSM-IV)	40 U/29 U	6	Change in MADRS score at week 6 after injection	Glabellar injections
(13)	Switzerland and Germany	Double-blind RCT	30 (15,15)	50.57 (25–65)	7/23	Mild to moderate MMD (DSM-IV)	39 U/29 U	16	Change in HAM-D_{17} score at week 6 after injection	Glabellar injections

RCT, randomized controlled trial; BTX-A, botulinum toxin type A; MDD, major depressive disorder; M, male; F, female; U, units; DSM, Diagnostic and Statistical Manual of Mental Disorders; MADRS, Montgomery–Åsberg Depression Rating Scale; NR, not reported; BDI, Beck Depression Inventory; HAM-D, Hamilton Depression Rating Scale.

TABLE 2 | Risk of bias assessment of five RCTs included in the meta-analysis.

Study ID	Random Sequence Generation (selection bias)	Allocation Concealment (selection bias)	Blinding of Participants and Personnel (performance bias)	Blinding of Outcome Assessment (detection bias)	Incomplete Outcome Data (attrition bias)	Selective Reporting (reporting bias)	Other Bias
(16)	Low	Low	Low	Low	Low	Low	Unclear
(15)	Low	Unclear	Low	Low	High	Low	Unclear
(14)	Low	Unclear	Low	Low	High	Low	Unclear
(13)	Low	Low	Low	Low	Low	Low	Unclear
(24)	Unclear	Unclear	High	High	High	Low	High

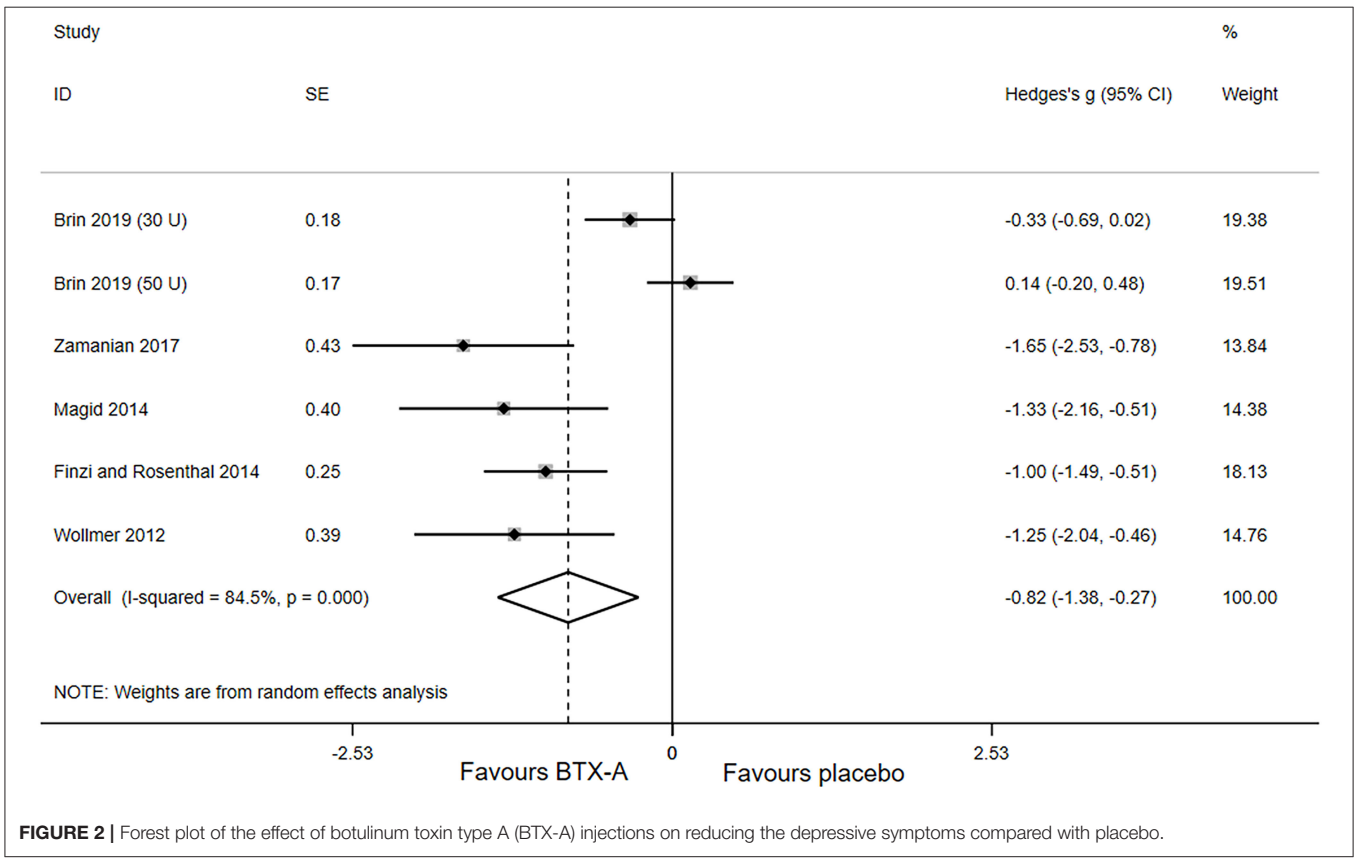

FIGURE 2 | Forest plot of the effect of botulinum toxin type A (BTX-A) injections on reducing the depressive symptoms compared with placebo.

DISCUSSION

This updated meta-analysis identified five independent studies and examined the efficacy and safety of BTX-A injections as an adjuvant treatment in MDD. The findings revealed that BTX-A injections were associated with a significant improvement in depressive symptoms when compared with placebo.

As observed in the sensitivity analysis, the study by Brin et al. (16) was the main source of heterogeneity, which had a significant impact on the summary results. That study used a two-dose parallel design (30 U BTX-A and 50 U BTX-A) and only recruited female patients with a clinical diagnosis of MDD. However, in three other studies [Zamanian et al. (24) did not report the dose of BTX-A injections], 39–40 U and 29 U BTX-A were injected into the glabellar muscles of male and female patients, respectively, and the proportion of male patients varied from 6.8 to 23.3%. Therefore, the dose of BTX-A injections and the gender of the participants may be the main reasons for the difference. In the research conducted by Brin et al. (16), the effect of high-dosage BTX-A injections (50 U) on MDD was similar to the placebo. One possible reason is that more placebo injections may lead to a greater placebo response. In addition, 50 U is higher than the dose commonly used for cosmetic purposes in women. It is possible that the women were actually over-treated and had a worse outcome due to poor cosmetic outcome or some other effect. Due to a limited number of studies, we failed to explore the

TABLE 3 | Subgroup analyses results for the efficacy of BTX-A vs. placebo in MDD.

Variable	Number of Studies	Hedges' g (95% CI)	*P*-value	I^2 (%)	*P*-value[a]	Model
All studies	5	−0.82 (−1.38 to −0.27)	0.004	84.5	<0.001	Random
Number of subjects						
>52	2	−0.38 (−0.98 to 0.23)	0.222	86.1	0.001	Random
≤52	3	−1.40 (−1.87 to −0.92)	<0.001	0.0	0.785	Fixed
Proportion of female						
>90%	3	−0.56 (−1.15 to 0.03)	0.063	85.2	<0.001	Random
≤90%	2	−1.43 (−2.02 to −0.84)	<0.001	0.0	0.503	Fixed
Risk of bias						
High risk	3	−1.19 (−1.57 to −0.81)	<0.001	0.0	0.415	Fixed
Low or unclear risk	2	−0.38 (−0.99 to 0.23)	0.117	82.0	0.004	Random
Measurement tool for outcome						
MADRS	2	−0.38 (−0.98 to 0.23)	0.222	86.1	0.001	Random
HAM-D	2	−1.29 (−1.86 to −0.72)	<0.001	0.0	0.885	Fixed
BDI	1	−1.65 (−2.53 to −0.78)	–	–	–	–

BTX-A, botulinum toxin type A; MDD, major depressive disorder; CI, confidence interval; MADRS, Montgomery–Åsberg Depression Rating Scale; HAM-D, Hamilton Depression Rating Scale; BDI, Beck Depression Inventory.

[a] *P-value for heterogeneity within each subgroup.*

association between different doses of BTX-A and MDD, as the linear or nonlinear relationship between the dose of BTX-A and MDD was still unknown. Further clinical trials are encouraged to explore the influence that BTX-A dose and gender composition may have in utilizing BTX-A as a treatment for depression.

The results of this meta-analysis showed that BTX-A has a unique advantage in the treatment of MDD. Although the risk of AEs from the BTX-A injections was increased compared to the placebo, the events were mild and brief. The safety of BTX-A was also proven during the treatment of other diseases, such as chronic migraine, primary hyperhidrosis, nocturnal molars, and dystonia (25–28). The long-term effect of a single dose may be conducive to improving compliance and cost-effectiveness. Moreover, the role of BTX-A injections in improving patients' quality of life, self-esteem, and satisfaction was gratifying (29, 30). Thus, BTX-A may provide a new option for the treatment of MDD in the future.

In the present meta-analysis, all trials recruited individuals with a clinical diagnosis of MDD. The effect of BTX-A on depressive symptoms secondary to other diseases or failing to meet the diagnostic criteria of MDD was still unknown. In two RCTs using BTX-A treatment for primary premature ejaculation and chronic tension-type headache, respectively, there was no significant difference in depressive scores between the trial and the control group (31, 32). However, due to the differences in study design and the lack of data, we failed to summarize the results of the two studies. It is worth noting that anxiety disorder is a common comorbidity of depression as nearly 85% of patients with depression are also affected by severe anxiety (33). To date, no RCT has been conducted that has studied the effect of BTX-A on anxiety with/without depression.

Several potential mechanisms have been proposed to explain the beneficial effect of BTX-A in depressive symptoms. The most common theory is the "facial feedback hypothesis" posited by Darwin in 1872, which states that facial expression can affect emotional states (34–36). In 1894, the psychologist James further elaborated this view. He proposed that emotions only change as the body changes, such as blood pressure, heart rate, and of course, expressive behavior (37). The evidence suggests that when corrugator muscles are activated in the forehead, this can lead to negative emotions (38). Furthermore, the study of Schwartz et al. (39) found that the facial muscles of patients with depression are relatively overactive compared to non-depressed individuals. BTX-A injection into the corrugator muscle might block normal sensory feedback from the nerves, especially the left amygdala to the brain (36). Excessive activation of the amygdala was associated with negative emotions (e.g., anger, anxiety, depression, and fear), but the BTX-A reduced the activation of the amygdala by blocking acetylcholine release to the synapses (40), which has a positive effect on mood. In addition, a recently published study has suggested that BTX-A may accomplish antidepressant effects after systemic distribution, although the content of circulating BTX-A is probably very low (41). This theory provides novel insights into the possible mechanism of BTX-A antidepressant effect.

There are some limitations that should be addressed. First, the limited number of studies included was insufficient to support the detection of publication bias. Second, it was difficult to reliably blind participants due to the potential cosmetic effects of BTX-A treatment; therefore, the antidepressant effects of BTX-A may be overestimated. The extent to which the cosmetic effect contributes to the observed improvement of depression symptoms remains unclear. However, a previous work showed that the antidepressant effect of BTX-A lasted for at least 24 weeks, which exceeds the duration of the cosmetic effect on glabellar lines (∼12–16 weeks) (14). Third, the diagnosis of MDD to date mostly relies on clinical review using depressive rating scale (e.g., DSM criteria), which may bias the diagnosis

results due to the subjectivity. Moreover, there is considerable heterogeneity in the symptoms and severity among different patients even if they were all diagnosed as depression. Fourth, given the statistically significant heterogeneity observed among studies, the use of the random-effects model allowed us to take into consideration the heterogeneity among studies. Finally, the patients included in the study were mainly female, which could be explained by the different interest in cosmetic treatment. Therefore, the results of this study may not be applicable to males. Despite the above limitations, the strengths should also be mentioned. First, considering the impact of the small sample size on the overall effect size, Hedge's g was adopted to analyze the continuous variables. Furthermore, the primary outcome of all the included studies was the changes in depressive rating scale at week 6 after injection, and the injection dose of BTX-A was essentially the same, which improved the comparability. In addition, the main sources of heterogeneity were revealed by sensitivity analysis.

CONCLUSION

The findings of this systematic review and meta-analysis confirmed that the glabellar injections of BTX-A were associated with a statistically significant improvement in depressive symptoms. BTX-A injections are generally safe, which may provide a new option for the treatment of MDD. However, further clinical trials are still needed to investigate the antidepressant effect of BTX-A and to explore the underlying mechanisms.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

YL, AS, and HQ planned and designed the study. YL and HQ conducted the database search and screened studies for inclusion. HQ extracted data. YL, FS, and HQ assessed risk of bias. HQ planned and performed the statistical analysis. HQ wrote the first draft of the manuscript. CL revised the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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REFERENCES

1. Sinyor M, Rezmovitz J, Zaretsky A. Screen all for depression. *BMJ.* (2016) 352:i1617. doi: 10.1136/bmj.i1617
2. Duchaine CS, Aube K, Gilbert-Ouimet M, Bruno Pena Gralle AP, Vezina M, Ndjaboue R, et al. Effect of psychosocial work factors on the risk of depression: a protocol of a systematic review and meta-analysis of prospective studies. *BMJ Open.* (2019) 9:e033093. doi: 10.1136/bmjopen-2019-033093
3. Kaufman J. Unraveling the genetics of major depression and stress-related psychiatric disorders: is it time for a paradigm shift? *Biol Psychiatry.* (2018) 84:82–84. doi: 10.1016/j.biopsych.2018.05.006
4. Konsman JP. Inflammation and depression: a nervous plea for psychiatry to not become immune to interpretation. *Pharmaceuticals (Basel).* (2019) 12:29. doi: 10.3390/ph12010029
5. Disease GBD, Injury I, Prevalence C. Global, regional, and national incidence, prevalence, and years lived with disability for 310 diseases and injuries, 1990-2015: a systematic analysis for the Global Burden of Disease Study 2015. *Lancet.* (2016) 388:1545–602. doi: 10.1016/S0140-6736(16)31678-6
6. American Psychiatric Association. *Diagnostic and Statistical Manual of Mental Disorders (DSM-5VR).* Arlington, VI: American Psychiatric Publisher (2013).
7. Kessler RC, Bromet EJ. The epidemiology of depression across cultures. *Annu Rev Public Health.* (2013) 34:119–38. doi: 10.1146/annurev-publhealth-031912-114409
8. Carruthers JD, Fagien S, Joseph JH, Humphrey SD, Biesman BS, Gallagher CJ, et al. DaxibotulinumtoxinA in the treatment of glabellar lines: results from each of two multicenter, randomized, double-blind, placebo-controlled, phase 3 studies (SAKURA 1 and SAKURA 2). *Plast Reconstr Surg.* (2020) 145:45–58. doi: 10.1097/PRS.0000000000006327
9. Mazidi M, Shivappa N, Wirth MD, Hebert JR, Vatanparast H, Kengne AP. The association between dietary inflammatory properties and bone mineral density and risk of fracture in US adults. *Eur J Clin Nutr.* (2017) 71:1273–7. doi: 10.1038/ejcn.2017.133
10. Heckmann M, Teichmann B, Schroder U, Sprengelmeyer R, Ceballos-Baumann AO. Pharmacologic denervation of frown muscles enhances baseline expression of happiness and decreases baseline expression of anger, sadness, and fear. *J Am Acad Dermatol.* (2003) 49:213–6. doi: 10.1067/S0190-9622(03)00909-5
11. Lewis MB, Bowler PJ. Botulinum toxin cosmetic therapy correlates with a more positive mood. *J Cosmet Dermatol.* (2009) 8:24–6. doi: 10.1111/j.1473-2165.2009.00419.x
12. Finzi E, Wasserman E. Treatment of depression with botulinum toxin A: a case series. *Dermatol Surg.* (2006) 32:645–9; discussion 649–50. doi: 10.1111/j.1524-4725.2006.32136.x
13. Wollmer MA, de Boer C, Kalak N, Beck J, Gotz T, Schmidt T, et al. Facing depression with botulinum toxin: a randomized controlled trial. *J Psychiatr Res.* (2012) 46:574–81. doi: 10.1016/j.jpsychires.2012.01.027
14. Magid M, Reichenberg JS, Poth PE, Robertson HT, LaViolette AK, Kruger TH, et al. Treatment of major depressive disorder using botulinum toxin A: a 24-week randomized, double-blind, placebo-controlled study. *J Clin Psychiatry.* (2014) 75:837–44. doi: 10.4088/JCP.13m08845
15. Finzi E, Rosenthal NE. Treatment of depression with onabotulinumtoxinA: a randomized, double-blind, placebo controlled trial. *J Psychiatr Res.* (2014) 52:1–6. doi: 10.1016/j.jpsychires.2013.11.006
16. Brin MF, Durgam S, Lum A, James L, Liu J, Thase ME, et al. OnabotulinumtoxinA for the treatment of major depressive disorder: a phase 2 randomized, double-blind, placebo-controlled trial in adult females. *Int Clin Psychopharmacol.* (2020) 35:19–28. doi: 10.1097/YIC.0000000000000290
17. Moher D, Liberati A, Tetzlaff J, Altman DG, Group P. Reprint–preferred reporting items for systematic reviews and meta-analyses: the PRISMA statement. *Phys Ther.* (2009) 89:873–80. doi: 10.1093/ptj/89.9.873
18. Cumpston M, Li T, Page MJ, Chandler J, Welch VA, Higgins JP, et al. Updated guidance for trusted systematic reviews: a new edition of the Cochrane Handbook for Systematic Reviews of Interventions. *Cochrane Database Syst Rev.* (2019) 10:ED000142. doi: 10.1002/14651858.ED000142
19. Fusar-Poli L, Vozza L, Gabbiadini A, Vanella A, Concas I, Tinacci S, et al. Curcumin for depression: a meta-analysis. *Crit Rev Food Sci Nutr.* (2020) 60:2643–53. doi: 10.1080/10408398.2019.1653260
20. Bruloy E, Sinna R, Grolleau JL, Bout-Roumazeilles A, Berard E, Chaput B. Botulinum toxin versus placebo: a meta-analysis of prophylactic

treatment for migraine. *Plast Reconstr Surg.* (2019) 143:239–50. doi: 10.1097/prs.0000000000005111
21. Parsaik AK, Mascarenhas SS, Hashmi A, Prokop LJ, John V, Okusaga O, et al. Role of botulinum toxin in depression. *J Psychiatr Pract.* (2016) 22:99–110. doi: 10.1097/PRA.0000000000000136
22. Higgins JPT, S. Green. *Cochrane Handbook for Systematic Reviews of Interventions Version 5.1. 0 (Updated March 2011)*. The Cochrane Collaboration (2011). Available online at: http://www.cochrane-handbook.org (accessed April 1, 2020).
23. Higgins JP, Thompson SG, Deeks JJ, Altman DG. Measuring inconsistency in meta-analyses. *BMJ.* (2003) 327:557–60. doi: 10.1136/bmj.327.7414.557
24. Zamanian A, Ghanbari Jolfaei A, Mehran G, Azizian Z. Efficacy of Botox versus placebo for treatment of patients with major depression. *Iran J Public Health.* (2017) 46:982–4. doi: 10.00000/28845410
25. Nawrocki S, Cha J. Botulinum toxin: pharmacology and injectable administration for the treatment of primary hyperhidrosis. *J Am Acad Dermatol*. (2020) 82:969–79. doi: 10.1016/j.jaad.2019.11.042
26. Herd CP, Tomlinson CL, Rick C, Scotton WJ, Edwards J, Ives NJ, et al. Cochrane systematic review and meta-analysis of botulinum toxin for the prevention of migraine. *BMJ Open.* (2019) 9:e027953. doi: 10.1136/bmjopen-2018-027953
27. Kwon KH, Shin KS, Yeon SH, Kwon DG. Application of botulinum toxin in maxillofacial field: part I. Bruxism and square jaw. *Maxillofac Plast Reconstr Surg.* (2019) 41:38. doi: 10.1186/s40902-019-0218-0
28. Drexel SC, Klietz M, Kollewe K, Paracka L, Kutschenko A, Kopp B, et al. Caregiver burden and health-related quality of life in idiopathic dystonia patients under botulinum toxin treatment: a cross-sectional study. *J Neural Transm (Vienna).* (2020) 127:61–70. doi: 10.1007/s00702-019-02109-6
29. Hexsel D, Brum C, Porto MD, Soirefmann M, Siega C, Schilling-Souza J, et al. Quality of life and satisfaction of patients after full-face injections of abobotulinum toxin type A: a randomized, phase IV clinical trial. *J Drugs Dermatol.* (2013) 12:1363–7. doi: 10.00000/30173431
30. Dayan SH, Arkins JP, Patel AB, Gal TJ. A double-blind, randomized, placebo-controlled health-outcomes survey of the effect of botulinum toxin type a injections on quality of life and self-esteem. *Dermatol Surg.* (2010) 36(Suppl 4):2088–97. doi: 10.1111/j.1524-4725.2010.01795.x
31. Schulte-Mattler WJ, Krack P, Bo NSG. Treatment of chronic tension-type headache with botulinum toxin A: a randomized, double-blind, placebo-controlled multicenter study. *Pain.* (2004) 109:110–4. doi: 10.1016/j.pain.2004.01.016
32. Li ZT, Li YF, Zhang Y, Luo Y, Zhu T, Li K, et al. [Injection of botulinum-A toxin into bulbospongiosus muscle for primary premature ejaculation: a preliminary clinical study]. *Zhonghua Nan Ke Xue.* (2018) 24:713–8. doi: 10.00000/24301237
33. Tiller JW. Depression and anxiety. *Med J Aust.* (2013) 199:S28–31. doi: 10.5694/mjao12.10628
34. Davis JI, Senghas A, Brandt F, Ochsner KN. The effects of BOTOX injections on emotional experience. *Emotion.* (2010) 10:433–40. doi: 10.1037/a0018690
35. Ekman P, Levenson RW, Friesen WV. Autonomic nervous system activity distinguishes among emotions. *Science.* (1983) 221:1208–10. doi: 10.1126/science.6612338
36. Shin LM, Liberzon I. The neurocircuitry of fear, stress, and anxiety disorders. *Neuropsychopharmacology.* (2010) 35:169–91. doi: 10.1038/npp.2009.83
37. Magid M, Keeling BH, Reichenberg JS. Neurotoxins: expanding uses of neuromodulators in medicine–major depressive disorder. *Plast Reconstr Surg.* (2015) 136:111S—9S. doi: 10.1097/PRS.0000000000001733
38. Ekman P. *Emotions Revealed: Recognizing Faces and Feelings to Improve Communication*. New York, NY: Owl Books (2007).
39. Schwartz GE, Fair PL, Salt P, Mandel MR, Klerman GL. Facial muscle patterning to affective imagery in depressed and nondepressed subjects. *Science.* (1976) 192:489–91. doi: 10.1126/science.1257786
40. Burgen AS, Dickens F, Zatman LJ. The action of botulinum toxin on the neuro-muscular junction. *J Physiol.* (1949) 109:10–24. doi: 10.1113/jphysiol.1949.sp004364
41. Makunts T, Wollmer MA, Abagyan R. Postmarketing safety surveillance data reveals antidepressant effects of botulinum toxin across various indications and injection sites. *Sci Rep.* (2020) 10:12851. doi: 10.1038/s41598-020-69773-7

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Alterations of Cognition and Cerebral Ventricle Volume in Manic and Euthymic Pediatric Bipolar Disorder

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Introduction: It remains unknown whether volumetric alterations of ventricles are similar or not in pediatric bipolar disorder (PBD) among different mood states. The present study aims to estimate ventricular volumetric alteration of PBD patients in manic and euthymic status, as well as the relationship between this alteration and cognitive changes.

Methods: T1 magnetic resonance images were obtained from 20 manic PBD patients, 21 euthymic PBD patients, and 19 healthy controls (HCs). Ventricular volumes were automatically obtained via FreeSurfer 6.0 software. Ventricular volumes and cognitive indices were compared among the three groups, and the relationship between ventricular volumes and cognitive/clinical indices was analyzed.

Results: In contrast to HCs, manic and euthymic PBD patients exhibited decreased cognitive scores of the Stroop color-word test and the digit span subtest. Manic PBD subjects presented enlarged volumes in the bilateral ventricles, third ventricle, and whole ventricles, and euthymic PBD participants displayed increased volumes in the third ventricle, fourth ventricle, and whole ventricles. No significant differences in cognitive performance and ventricular volumes were found between PBD groups. No significant correlation was discovered between ventricular volumes and cognitive/clinical indices in both manic and euthymic PBD patients.

Conclusions: No significant differences in cognitive performance and ventricle volume were observed between euthymic and manic PBD groups, which may imply that the alterations are not specific to mood state. It may indicate structural and functional damage of corresponding brain circuits in euthymic PBD patients similar with that of manic PBD, which may provide clues to the diagnosis and treatment of euthymic PBD.

Keywords: Pediatric bipolar disorder (PBD), mania, euthymia, ventricle volume, cognition

INTRODUCTION

Pediatric bipolar disorder (PBD) is a severe psychiatric illness that recurrently attacks patients, marked by manic or hypomanic depressed episodes, and separated by euthymic periods. Manic PBD can feature extreme joy or rage, full vigorousness and inattention, difficultly falling asleep and reduced sleep needs, and racing thoughts and increased speech, while depressed PBD is characterized by inattention and suicidal tendencies, increased sleep needs, and emotional instability, and euthymic PBD generally shows no clinical psychiatric manifestations. With heritability of about 59% (1), bipolar disorder (BD) is reported to relate with considerably high prevalence and psychiatric comorbidity rate (2), high risk of suicide (3), and impaired cognition (4).

It has been found that BD patients exhibit alterations of ventricular volume (5). Some studies reported enlarged lateral ventricular volume in adult BD patients (6–10), while other researchers reported no difference in lateral ventricular volume between adult BD patients and healthy controls (HCs) (11, 12). Most studies have focused on ventricles' alterations in one mood state of BD. In the manic state, the third and lateral ventricle volumes in the first episode of adult BD (13) and the cerebral ventricular size in young male BD patients were shown to be increased (14). In euthymic adult BD, the width of the third ventricle exhibited an increase (15). Nevertheless, different results have also been reported, such as finding no lateral ventricle volume alteration in euthymic and manic BD adults (16–18). One study compared the width of the third ventricle in depressed, manic, and euthymic adult BD type I (BD I), and the researchers found increased width of the third ventricle in BD patients. No width difference of the third ventricle was displayed among the three patient groups (19). Several studies focused on ventricles in PBD and found increased lateral ventricle volume (20), enlarged lateral ventricles, and ventricle asymmetry in manic PBD subjects (21). The ratio of ventricle cerebrospinal fluid to cerebral total tissue volume, showing no difference between PBD and HCs, has also been reported (22). The above studies mostly involved one mood state of BD and have only reported alteration of the lateral and third ventricles. However, the difference in ventricular volume among different mood states and the relationship between ventricular volume alteration and cognitive tests remains unknown in PBD.

In the current study, we aimed to investigate alterations of the ventricular volumes of PBD patients in manic and euthymic states. According to prior research, we hypothesized that the volumes of four ventricles would be larger in PBD patients than those of the HCs. The current study was designed to: (1) evaluate cognitive functions of the three groups; (2) compare volumes of the left lateral ventricle, right lateral ventricle, third ventricle, fourth ventricle, and whole ventricles (adding bilateral ventricles, third ventricle, and fourth ventricle) among the three groups; (3) evaluate left lateral ventricle-to-brain ratio (LLVBR), right lateral ventricle-to-brain ratio (RLVBR), third ventricle-to-brain ratio (TVBR), fourth ventricle-to-brain ratio (FVBR), and whole ventricle-to-brain ratio (WVBR) among the three groups; and (4) conduct a correlation analysis between ventricular volumes with cognitive and clinical variables.

MATERIALS AND METHODS

Participants

In the current study, 60 subjects were enrolled, including 20 manic PBD patients, 21euthymic PBD patients, and 19 age- and gender-matched HCs. All of the PBD patients were recruited from the clinical psychiatric department in the Second Xiangya Hospital of Central South University, while HCs were enlisted via advertisement. This study was conducted from January 2012 to July 2014.

The inclusion criteria of PBD patients were: (a) having met the Diagnostic and Statistical Manual for Mental Disorders, Fourth Edition (DSM-IV) criteria for BD with current manic and euthymic episodes; (b) manic PBD conformed to hypomania ($\geq$4 days) or mania ($\geq$7 days) and euthymic PBD patients were needed to have experienced a remission period for more than four consecutive weeks prior to the study; (c) 10–18 years old; and (d) right-handedness.

Exclusion criteria for all of the subjects were: (a) score of intelligence quotient (IQ) <80; (b) contraindications for MRI scanning, including the presence of a pacemaker, artificial metal heart valves, aneurysm clip, other foreign metal matter in the body, or claustrophobia; (c) history of alcohol or substance abuse in the 2 months prior to the study; (d) history of cranial trauma; and (e) other psychiatric illnesses, including schizophrenia, attention-deficit hyperactivity disorder, or anxiety disorder.

The study obtained approval from the Ethics Committee of the Second Xiangya Hospital of Central South University. All of the participants and at least one parent or legal guardian signed informed consent documents.

Clinical and Cognitive Evaluation

The children and at least one parent or legal guardian were assessed employing Affective Disorders and Schizophrenia for School-Age Children-Present and Lifetime Version (K-SADS-PL) (23) by clinical interviews. The diagnosis of PBD was carried out by two senior child psychiatrists who had extensive clinical experience of diagnosis in PBD. Demographic, clinical, and neurocognitive information was collected for all subjects. Clinical scales, including IQ, Young Mania Rating Scale (YMRS), Mood and Feelings Questionnaire (MFQ) scores, onset age, illness duration, and onset frequency, were recorded. YMRS (24) and MFQ (25) were used to evaluate the severity of manic and depressive symptoms, respectively.

Cognitive functions were measured by Stroop color-word test (SCWT), trail making test (TMT), and digit span subtest (DST). SCWT was utilized to examine processing speed, perceptual conversion, selective attention, and inhibition of habitual response patterns and sensibility for plasticity of mental control and response (26, 27). SCWT fell into three categories: word reading test (SCWT-A), color naming test (SCWT-B), and color word interference (SCWT-C). In SCWT-A, the subjects were asked to name the word printed on a white piece of paper (e.g., the word "red" printed in black) as quickly as possible.

In SCWT-B, words printed in three colors were presented (e.g., "red" printed in red), and the subjects were asked to name the color as quickly as possible. In SCWT-C, the subjects were asked to name the color of words whose meanings were different from the ink color (e.g., "blue" printed in red) as quickly as possible, while trying to ignore the meaning of the word. Participants were requested to finish every test within 45 s, and the number of correct answers was recorded as the SCWT score. TMT was used to assess the participants' abilities in mental processing speed, attention, cognitive order, spatial perception, eye and hand coordination, and flexibility in thinking. TMT was divided into TMT-A and TMT-B. In TMT-A, 25 consecutive numbers surrounded by circles were randomly shown on a paper, and subjects were asked to sequentially connect each circle in ascending order (e.g., 1-2-3…) as quickly as possible. In TMT-B, 25 consecutive numbers and 25 letters enclosed by circles were randomly displayed on a piece of paper, and participants were asked to alternately link every circle by numerical ascending order and alphabetical order (e.g., 1-a-2-b-3-c…). The score of TMT was the time taken to complete the task. DST is a part of the Wechsler scale of intelligence and it is utilized to test short-term memory attention, concentration, and memory. It includes forward DST (DST-F) and backward DST (DST-B). The subjects were required to repeat a series of random numbers from 1–9 in sequential order (DST-F) and in reverse order (DST-B) after hearing them. The DST score was recorded as the maximum number of digits that the subject was able to repeat correctly.

MRI Acquisition and Processing

MRI Scan

For the study, MRI scans were conducted using a Siemens 3.0T Trio scanner (Siemens, Munich, Germany). During the MRI scanning, foam pads were placed on two sides of the heads of the subjects to restrict head motion, and cotton earplugs were used to reduce noise and protect the hearing of the subjects. Every subject was requested to stay awake with eyes closed and not to think specific thoughts during the MRI scan. T1-weighted images were obtained by employing three-dimensional magnetization-prepared rapid acquisition gradient echo (3D MPRAGE) protocol. Acquisition parameters covering the whole brain included: repetition time (TR) = 2,300 ms, echo time (TE) = 2.98 ms, inversion time = 900 ms, thickness = 1 mm, gap = 0 mm, field of view (FOV) = 256 mm × 256 mm, matrix = 256 × 256, and flip angle = 9°.

Image Processing and Calculation of Volumes

T1-weighted images were processed by FreeSurfer 6.0 software (http://surfer.nmr.mgh.harvard.edu/). Ventricular volumes and total intracranial volume (TIV) were obtained by recon stream ("recon-all"). The procedure included steps such as: (a) Motion correction was performed to reduce the effect of head movement during scanning; (b) Skull stripping was conducted to remove the skull and extract the brain; (c) Talairach transformation was carried out for affine transformation from the original volume to the MNI305 atlas; (d) Intensity normalization was conducted to reduce the intensity difference in the same tissue due to a non-uniform magnetic field or other factors; (e) The brain was divided into gray matter, white matter, and cerebrospinal fluid (CSF); and (f) A transform was created in linear transform array format. In addition, the tessellation of the boundary was conducted between white and gray matter. To ensure proper segmentation of ventricles, a trained physician re-inspected all segmented ventricle borders and corrected them manually, if required. The volumes of five ventricles, namely, the bilateral ventricles, third ventricle, fourth ventricle, and whole ventricles, were used for analysis in the current study. Ventricle-to-brain ratio (VBR) was the value of dividing ventricle volume by TIV, and then multiplying by 100 (22). LLVBR, RLVBR, TVBR, FVBR, and WVBR were calculated for each subject.

Statistical Analysis

Statistical analysis was conducted using IBM SPSS (version 25.0, Armonk, NY, United States). The Pearson chi-square test was employed to evaluate categorical variables, including gender, psychotic symptoms, BD subtype, and familial BD history. Parametric tests were used when data satisfied both normal distribution and homogeneity of variance at the same time. When data did not satisfy normal distribution or homogeneity of variance, non-parametric tests were used. Shapiro-Wilk test and Levene's test were used to assess the normality of distribution and homogeneity of variance.

Age and gender were deemed as covariates in the comparison of cognitive variables and VBR. TIV, age, and gender were considered as covariates in the comparison of ventricular volumes. One-way ANCOVA or Kruskal-Wallis H test was applied for comparison of cognitive variables, ventricular volumes, and VBR among the three groups. False discovery rate (FDR) correction (http://www.sdmproject.com/utilities/?show=FDR) was used in the comparison of the main effects. Two-sample *t*-test or Kruskal–Wallis H test was employed for *post hoc* analysis and Bonferroni correction was used for correction of multiple comparisons. Because the sample size in each group was relatively small, statistical power (Cohen's *d*) was provided to show differences between the PBD patients and the HCs. Pearson correlation analysis was employed to assess correlations between ventricular volumes and cognitive and clinical indices, with age, gender, and TIV as covariates. Significant level for all of the analyses was set as $p < 0.05$. Normal distributed data were expressed as mean ± standard, and non-normal distributed data were reported as median and inter-quartile range [M(QU-QL)].

RESULTS

Demographic and Clinical Information

As shown in **Table 1**, there was no significant difference among the three groups in gender, age, education, IQ, or MFQ scores. YMRS scores showed a significant difference among the three groups. No significant difference was found between manic and euthymic patients in onset age, illness duration, onset frequency, psychotic symptoms, BD subtype, and familial BD history.

Cognitive Variables Analysis

As shown in **Table 2**, significant differences of cognitive variables among the three groups were found in SCWT-A, SCWT-B,

TABLE 1 | Demographic and clinical information of the participants.

Characteristics	Manic-PBD ($n = 20$)	Euthymic-PBD ($n = 21$)	Healthy controls ($n = 19$)	$H/U/T/\chi^2$	P
Gender (male/female)	7/13	11/10	7/12	1.54[#]	0.463
Age (years)[b]	14.50 (14.00–16.00)	15.00 (14.00–17.00)	15.00 (12.00–15.00)	3.82[&]	0.148
Education (years)[b]	8.00 (7.00–9.00)	8.00 (7.00–10.05)	8.00 (6.00–9.00)	1.97[&]	0.374
IQ[b]	97.00 (90.50–114.75)	111.00 (99.00–114.50)	106.00 (100.00–113.00)	3.58[&]	0.167
YMRS scores[b]	34.50 (29.25–40.00)	6.00 (4.00–7.00)	3.00 (2.00–5.00)	43.17[&]	***<0.001***
MFQ scores[b]	7.00 (5.25–8.75)	7.00 (2.50–10.00)	6.00 (3.00–9.00)	0.84[&]	0.657
Onset age (years)[a]	14.15 ± 1.87	13.57 ± 2.06	-	0.94[^]	0.354
Illness duration (months)[b]	12.00 (6.00–17.00)	13.00 (11.50–39.00)	–	139.50[~]	0.065
Onset frequency (times)[b]	3.00 (2.00–3.75)	3.00 (2.00–5.50)	–	190.50[~]	0.790
Psychotic symptoms (yes/no)	9/11	12/9	–	0.61[#]	0.437
BD-I/BD-II	16/4	13/8	–	1.62[#]	0.203
Familial BD history (yes/no)	7/13	6/15	–	0.20[#]	0.658
Medications					
Lithium	9 (45%)	8 (38%)	–	–	–
Valproate	10 (50%)	14 (67%)	–	–	–
Atypical antipsychotics	14 (70%)	16 (76%)	–	–	–
Antidepressants	3 (15%)	–	–	–	–

[a]*mean ± standard deviation;* [b]*Median (range).*
[#]*Pearson chi-square test;* [&]*Kruskal-Wallis H test;* [^]*Two-sample t-test;* ~*Mann–Whitney U test.*
IQ, intelligence quotient; YMRS, Young Manic Rating Scale; MFQ, Mood and Feelings Questionnaire; BD-I, bipolar disorder type I; BD-II, bipolar disorder type II.
Bold values indicated significant difference with $p < 0.05$.

SCWT-C, and DST-B. In comparison with the HCs group, the manic group and the euthymic group presented reductions of score in SCWT-A, SCWT-B, SCWT-C, and DST-B.

Ventricle Volume Analysis

As shown in **Table 3**, significant differences of ventricular volumes among the three groups were found in five ventricles, namely, the left lateral ventricle, right lateral ventricle, third ventricle, fourth ventricle, and whole ventricles. In comparison with the HCs group, the manic group presented enlarged volumes in the left lateral ventricle, right lateral ventricle, third ventricle, and whole ventricles, and the euthymic group displayed increased volumes in the third ventricle, fourth ventricle, and whole ventricles. No difference in ventricle volume was found between the manic and euthymic PBD patients.

Ventricle-To-Brain Ratio Analysis

As shown in **Table 4**, no significant difference of VBR among the three groups was found in five ratios: LLVBR, RLVBR, TVBR, FVBR, and WVBR.

Correlation Analysis

Pearson correlation analysis was performed between ventricular volumes with clinical and cognitive indices. No significant correlation ($p > 0.05$) was found between ventricular volumes or VBR with clinical and cognitive indices in either the manic PBD groups or euthymic PBD groups.

DISCUSSION

In the current study, cognitive function and brain ventricle volumes were evaluated among manic PBD patients, euthymic PBD patients, and healthy adolescents. When compared with HCs, the manic and euthymic PBD patients showed decreased scores of SCWT and DST-B. In terms of ventricle volume comparison, larger bilateral ventricle volumes in the manic PBD group, larger fourth ventricle volume in the euthymic PBD group, and enlarged third ventricle and whole ventricle volumes were found in both PBD groups.

In the current study, the manic as well as euthymic PBD patients exhibited significant impairment in cognitive tests compared with the HCs. The damage in cognition included poor performance in tests of processing speed, concentration and attention, working memory, and executive function. Specifically, the manic and euthymic PBD patients performed worse on SCWT and DST-B compared with the HCs. SCWT is a test of processing speed, cognitive flexibility, and response inhibition. Deficits of SCWT may imply that the manic and euthymic PBD subjects were damaged in brain areas such as the anterior cingulate cortex, dorsolateral prefrontal cortex, lingual gyrus, and extrastriate cortex, which are involved in executive control, speed of color processing, and word-form processing (28). Current results were in line with those of a previous study, that showed poorer performance of SCWT was discovered in PBD patients than in HCs (29). DST is a test of attention and concentration. A prior study found that DST-B of BD was obviously different than HCs, while no difference was displayed in DST-F between adult

TABLE 2 | Cognitive variables in Manic-PBD, Euthymic-PBD, and Healthy controls.

Cognitive scales	MPBD	EPBD	HCs	Main effect		post hoc								
						MPBD < HCs			EPBD < HCs			MPBD < EPBD		
				F/H	*P-value*	*T/H*	*P*-value	*d*	*T/H*	*P*-value	*d*	*T/H*	*P*-value	*d*
SCWT-A[e]	54.63 ± 15.32	53.38 ± 13.92	66.00 ± 12.26	11.25[b]	***<0.001***	3.72[c]	***0.001***	1.23	4.44[c]	***<0.001***	1.47	−0.63[c]	1.000 (MPBD > EPBD)	−0.19
SCWT-B[f]	74.00 (56.00–88.00)	76.00 (57.00–80.50)	89.00 (81.00–94.00)	21.04[a]	***<0.001***	20.95[a]	***0.001***	1.48	22.75[a]	***<0.001***	1.70	1.80[a]	1.000	0.06
SCWT-C[e]	29.74 ± 7.05	31.57 ± 8.98	40.74 ± 9.43	14.75[b]	***<0.001***	4.93[c]	***<0.001***	1.62	4.48[c]	***<0.001***	1.32	0.58[c]	1.000	0.20
TMT-A[e]	39.42 ± 11.90	38.76 ±12.79	29.74 ± 9.63	2.97[b]	0.083	-	-	-	-	-	-	-	-	-
TMT-B[f]	81.00 (70.00–98.00)	90.00 (67.50–119.00)	69.00 (63.00–93.00)	1.32[a]	0.518	-	-	-	-	-	-	-	-	-
DST-F[f]	8.00 (7.00–9.00)	8.00 (8.00–9.00)	9.00 (8.00–10.00)	3.88[a]	0.168	-	-	-	-	-	-	-	-	-
DST-B[e]	4.42 ± 1.17	4.67 ± 1.62	5.95 ± 1.68	7.79[b]	***0.002***	3.55[c]	***0.002***	1.21	3.31[c]	***0.005***	0.95	−0.33[c]	1.000	0.11

[a]Kruskal-Wallis H test; [b]One-Way ANCOVA; [c]Two-sample t-test; [d]adjusted Cohen's d.
[e]mean ± standard deviation; [f]Median (range).
MPBD, manic pediatric bipolar disorder; EPBD, euthymic pediatric bipolar disorder; HCs, healthy controls; SCWT, Stroop color-word test; TMT, trail making test; DST, digit span test.
FDR correction for main effect comparison; Bonferroni correction for post hoc analysis; Presented adjusted $p < 0.05$ was considered to indicate a significant difference.
Bold values indicated significant difference with $p < 0.05$.

TABLE 3 | Ventricular volumes in Manic-PBD, Euthymic-PBD, and Healthy controls.

Regions	MPBD	EPBD	HCs	Main effect		post hoc								
						MPBD > HCs			EPBD > HCs			MPBD < EPBD		
				F/H	*P-value*	*T/H*	*P*-value	*d*	*T/H*	*P*-value	*d*	*T/H*	*P*-value	*d*
Left lateral ventricle[f]	6.52 (5.37–9.97)	6.85 (5.75–9.91)	5.57 (4.17–6.60)	7.90[a]	***0.024***	14.09[a]	***0.035***	0.93	13.12[a]	0.053	0.88	0.97[a]	1.00	0.22
Right lateral ventricle[f]	5.61 (4.87–7.83)	5.73 (4.36–7.64)	4.06 (3.32–6.11)	8.60[a]	***0.023***	15.26[a]	***0.019***	0.91	12.92[a]	0.059	0.84	2.34[a]	1.00	0.22
Third ventricle[e]	1.22 ± 0.43	1.28 ± 0.30	0.94 ± 0.21	5.19[b]	***0.020***	2.79 [c]	***0.022***	0.90	2.83 [c]	***0.019***	1.08	0.01[c]	1.00	0.00
Fourth ventricle[f]	2.04 (1.60–2.34)	2.10 (1.77–2.72)	1.56 (1.39–1.83)	7.39[a]	***0.025***	12.52 [a]	0.076	0.81	13.71 [a]	***0.039***	0.99	1.19 [a]	1.00	0.12
Whole ventricles[f]	15.58 (13.15–21.63)	16.50 (12.94–21.83)	11.12 (10.11–15.57)	10.83[a]	***0.020***	16.57[a]	***0.009***	1.02	15.27[a]	***0.017***	1.08	1.30[a]	1.00	0.20

[a]Kruskal–Wallis H test; [b]One-Way ANCOVA; [c]Two-sample t-test; [d]adjusted Cohen's d.
[e]mean ± standard deviation; [f]Median (range); nit: cm^3.
MPBD, manic pediatric bipolar disorder; EPBD, euthymic pediatric bipolar disorder; HCs, healthy controls.
FDR correction for main effect comparison; Bonferroni correction for post hoc analysis; Presented adjusted $p < 0.05$ was considered to indicate a significant difference.
Bold values indicated significant difference with $p < 0.05$.

BD and HCs (30). Impairment of executive function was found in the manic, depressive, and euthymic BD I patients, including initial reaction, inhibition control, and strategic thinking (31). It had been reported that euthymic PBD patients exhibited impairment of cognitive functions, including verbal learning and working memory (32). That more severe damage of the DST-B in PBD patients than that of the HCs shown in the current study suggests a related dysfunction of executive control of phonological information in the manic and euthymic PBD patients. It is worth noting that manic and euthymic PBD patients exhibited similar cognitive damage, and no significant difference in SCWT and DST was found between the two groups. This may imply that although euthymic PBD patients expressed relatively stable mood states, they had cognitive impairment similar to that of manic patients, indicating impairment of brain structures and function in related cognitive circuits in euthymic PBD patients.

Bilateral ventricles' volumes were found to be increased in manic PBD compared to those of HCs in the study. No significant difference of lateral ventricles' volumes was found between euthymic patients and HCs, or between euthymic and manic patients. Similar results were reported in a study of adult BD, which showed the larger lateral ventricular volumes were related to the number of manic episodes in adult BD (10). It had been shown that patients with 22q11.2 deletion syndrome

TABLE 4 | Ventricle-to-brain ratio in Manic-PBD, Euthymic-PBD, and Healthy controls.

Regions	MPBD	EPBD	HCs	Main effect	
				F/H	*P-value*
LLVBR[d]	0.50 (0.36–0.66)	0.48 (0.39–0.66)	0.35 (0.28–0.47)	5.17[a]	0.076
RLVBR[c]	0.45 ± 0.17	0.42 ± 0.13	0.31 ± 0.10	4.04[b]	0.057
TVBR[d]	0.08 (0.06–0.10)	0.09 (0.08–0.10)	0.07 (0.05–0.08)	6.78[a]	0.057
FVBR[d]	0.14 (0.11–0.16)	0.14 (0.12–0.18)	0.11 (0.10–0.13)	5.30[a]	0.076
WVBR[d]	1.10 (0.90–1.47)	1.12 (0.93–1.45)	0.82 (0.68–1.05)	8.21[a]	0.057

[a] *Kruskal–Wallis H test;* [b] *One-Way ANCOVA.*

[c] *mean ±standard deviation;* [d] *Median (range).*

MPBD, manic pediatric bipolar disorder; EPBD, euthymic pediatric bipolar disorder; HCs, healthy controls; LLVBR, left lateral ventricle-to-brain ratio; RLVBR, right lateral ventricle-to-brain ratio; TVBR, third ventricle-to-brain ratio; FVBR, fourth ventricle-to-brain ratio; WVBR, whole ventricle-to-brain ratio.

FDR correction for main effect comparison; Presented adjusted $p < 0.05$ was considered to indicate a significant difference.

(22q11DS) exhibited enlarged lateral ventricle volumes compared with HCs (33). The 22q11DS, which affected multiple genes involved in neurodevelopment, was also reported to be related with early-onset BD (34). This may imply that the 22q11DS is a genetic risk factor for BD. In animal experiments with mice, ongoing dysregulation of neural cell adhesion molecules in neuropsychiatric disorders led to an increase in lateral ventricular volumes, and affected cognitive function via learning, synaptic plasticity, and long-term potentiation influences (35).

Concerning the third ventricle, we found that third ventricular volume in manic and euthymic PBD patients was larger than that of the HCs, and that third ventricle volume was not an obvious discrepancy between manic and euthymic patients. The result in PBD was not in line with that of previous studies in which adult BD patients were grouped into those with and without psychotic forms, and enlarged third ventricle volume was only discovered in psychotic BD patients (36). Increased third volume may be related to the surrounding anatomical boundary, such as decreased volumes of the thalamus and hypothalamus (5). In post-mortem BD research, total selective neurons robustly decreased by about 50% in the paraventricular nucleus of hypothalamus (37). In other ongoing research, we found a decrease of thalamic volume in the same PBD samples, matching the increased third ventricular volumes in the current study.

We found that the fourth ventricle of euthymic PBD was markedly greater than that of HCs. The shape and size of the fourth ventricle was believed to be influenced by the surrounding brainstem structure. The variation of CACNA1C gene was reported to increase the risk of mental diseases, particularly BD, and to affect brainstem volume (38). Few previous studies focused on the volume of the fourth ventricle in PBD. However, two studies reported larger volumes of the fourth ventricle in schizophrenia (39, 40). To some extent, previous findings elucidated that BD and schizophrenia partially shared heritability and showed some similar clinical features (1, 41, 42). In mental disorders, the region of the brainstem reticular activating system may play an essential role in supporting attention, but it was easily influenced by environmental and genetic influences, and these influences may be the etiology of attention impairment (43, 44). These research studies disclosed that impaired subcortical structures near the fourth ventricle likely indicated pathophysiology of sustained inattention in psychiatric disorders. Considering that euthymic PBD patients also show daily clinical presentation of inattention, the increased fourth ventricular volume exhibited in the current study may indirectly indicate a similar inattention mechanism in euthymic PBD with that of schizophrenia.

In the current study, increased whole ventricular volumes were found in manic and euthymic PBD patients compared to those of HCs. The differences in RLVBR, TVBR, and WVBR among the three groups closely reached statistical significance and the three ratios in manic and euthymic PBD patients were larger than those of the HCs, as shown in **Table 4**. Previous studies found increased LVBR in older BD patients (45) or else no alterations of LVBR and TVBR in adult BD patients (46). Nevertheless, two studies did not find the volumetric alteration of ventricles in adult BD (47, 48). The underlying mechanisms for BD seemed different in adults and children (49). Our results regarding whole ventricular volume and VBR provided more clues in the field of PBD.

Several limitations should be acknowledged in the current study. First, the sample size of our study was relatively small, which may cause an inability to observe some subtle changes in the ventricular volume or significant correlations between alterations of ventricular volume and cognitive and clinical indices. In our future work, much more data will be extracted to make the study more convincing. Second, medical treatment was a potential obstruction in interpreting the present results. Some studies, however, reported no significant effect of medication in behavioral (50) and neural differences between patients and HCs (51). Third, the ventricular volume corrected by the two methods in the present study, that is, the regression-based residuals in calculating brain volume (the residual method, **Table 3**) and the region-to-ICV ratio (the proportion method, **Table 4**), were different. After FDR correction, the significant differences in VBR among the three groups disappeared. This may be due to the influence

of random errors, which were combined in the numerator and denominator, and then the sources of the error were hidden in the final ratio values (52). In future work, some improvement may be needed to reduce the influence of the random errors.

In the current study, enlarged ventricle volumes were found in manic and euthymic PBD patients compared with those of HCs, which may provide some new clues to brain structural impairment of child and adolescent BD patients. No significant difference of cognitive performance and ventricle volume was observed between manic and euthymic PBD patients, which may imply that the alterations are not specific to mood state. However, when in remission, euthymic PBD patients behaved with similar abnormalities in cognition and ventricle volume as the manic patients, which may indicate damage of the brain structure and function of corresponding brain circuits in euthymic PBD patients.

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 Committee of the Second Xiangya Hospital of Central South University. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

QJ and GL: conception and study design. WG and LS: data collection or acquisition and clinical support. DC and LK: statistical analysis. YG and JQ: interpretation of results. LK and WC: drafting the manuscript or revising it critically for important intellectual content. All authors: approval of the final version to be published and agreement to be accountable for the integrity and accuracy of all aspects of the work. All authors contributed to the article and approved the submitted version.

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REFERENCES

1. Lichtenstein P, Yip BH, Bjork C, Pawitan Y, Cannon TD, Sullivan PF, et al. Common genetic determinants of schizophrenia and bipolar disorder in Swedish families: a population-based study. *Lancet.* (2009) 373:234–9. doi: 10.1016/S0140-6736(09)60072-6
2. Frias A, Palma C, Farriols N. Comorbidity in pediatric bipolar disorder: prevalence, clinical impact, etiology and treatment. *J Affect Disord.* (2015) 174:378–89. doi: 10.1016/j.jad.2014.12.008
3. Goldstein TR. Suicidality in pediatric bipolar disorder. *Child Adolesc Psychiatr Clin N Am.* (2009) 18:339–52, viii. doi: 10.1016/j.chc.2008.11.005
4. Martinez-Aran A, Vieta E, Reinares M, Colom F, Torrent C, Sanchez-Moreno J, et al. Cognitive function across manic or hypomanic, depressed, and euthymic states in bipolar disorder. *Am J Psychiatry.* (2004) 161:262–70. doi: 10.1176/appi.ajp.161.2.262
5. Beyer JL, Krishnan KR. Volumetric brain imaging findings in mood disorders. *Bipolar Disord.* (2002) 4:89–104. doi: 10.1034/j.1399-5618.2002.01157.x
6. Arnone D, Cavanagh J, Gerber D, Lawrie SM, Ebmeier KP, McIntosh AM. Magnetic resonance imaging studies in bipolar disorder and schizophrenia: meta-analysis. *Br J Psychiatry.* (2009) 195:194–201. doi: 10.1192/bjp.bp.108.059717
7. Kempton MJ, Geddes JR, Ettinger U, Williams SC, Grasby PM. Meta-analysis, database, and meta-regression of 98 structural imaging studies in bipolar disorder. *Arch Gen Psychiatry.* (2008) 65:1017–32. doi: 10.1001/archpsyc.65.9.1017
8. Nakamura M, Salisbury DF, Hirayasu Y, Bouix S, Pohl KM, Yoshida T, et al. Neocortical gray matter volume in first-episode schizophrenia and first-episode affective psychosis: a cross-sectional and longitudinal MRI study. *Biol Psychiatry.* (2007) 62:773–83. doi: 10.1016/j.biopsych.2007.03.030
9. McDonald C, Zanelli J, Rabe-Hesketh S, Ellison-Wright I, Sham P, Kalidindi S, et al. Meta-analysis of magnetic resonance imaging brain morphometry studies in bipolar disorder. *Biol Psychiatry.* (2004) 56:411–7. doi: 10.1016/j.biopsych.2004.06.021
10. Strakowski SM, DelBello MP, Zimmerman ME, Getz GE, Mills NP, Ret J, et al. Ventricular and periventricular structural volumes in first- versus multiple-episode bipolar disorder. *Am J Psychiatry.* (2002) 159:1841–7. doi: 10.1176/appi.ajp.159.11.1841
11. Brambilla P, Harenski K, Nicoletti M, Mallinger AG, Frank E, Kupfer DJ, et al. MRI study of posterior fossa structures and brain ventricles in bipolar patients. *J Psychiatr Res.* (2001) 35:313–22. doi: 10.1016/S0022-3956(01)00036-X
12. Lim KO, Rosenbloom MJ, Faustman WO, Sullivan EV, Pfefferbaum A. Cortical gray matter deficit in patients with bipolar disorder. *Schizophr Res.* (1999) 40:219–27. doi: 10.1016/S0920-9964(99)00063-8
13. Strakowski SM, Wilson DR, Tohen M, Woods BT, Douglass AW, Stoll AL. Structural brain abnormalities in first-episode mania. *Biol Psychiatry.* (1993) 33:602–9. doi: 10.1016/0006-3223(93)90098-X
14. Nasrallah HA, McCalley-Whitters M, Jacoby CG. Cerebral ventricular enlargement in young manic males. A controlled CT study. *J Affect Disord.* (1982) 4:15–9. doi: 10.1016/0165-0327(82)90014-3
15. Cousins DA, Moore PB, Watson S, Harrison L, Ferrier IN, Young AH, et al. Pituitary volume and third ventricle width in euthymic patients with bipolar disorder. *Psychoneuroendocrinology.* (2010) 35:1074–81. doi: 10.1016/j.psyneuen.2010.01.008
16. Lopez-Jaramillo C, Vargas C, Diaz-Zuluaga AM, Palacio JD, Castrillon G, Bearden C, et al. Increased hippocampal, thalamus and amygdala volume in long-term lithium-treated bipolar I disorder patients compared with unmedicated patients and healthy subjects. *Bipolar Disord.* (2017) 19:41–9. doi: 10.1111/bdi.12467
17. Arumugham SS, Torres IJ, Lang DJ, Su W, Lam RW, Honer WG, et al. Subcortical structural volumes in recently remitted first episode mania. *J Affect Disord.* (2017) 222:23–7. doi: 10.1016/j.jad.2017.06.051
18. McDonald WM, Krishnan KR, Doraiswamy PM, Blazer DG. Occurrence of subcortical hyperintensities in elderly subjects with mania. *Psychiatry Res.* (1991) 40:211–20. doi: 10.1016/0925-4927(91)90013-G
19. Krogias C, Hoffmann K, Eyding J, Scheele D, Norra C, Gold R, et al. Evaluation of basal ganglia, brainstem raphe and ventricles in bipolar

disorder by transcranial sonography. *Psychiatry Res.* (2011) 194:190–7. doi: 10.1016/j.pscychresns.2011.04.002
20. Friedman L, Findling RL, Kenny JT, Swales TP, Stuve TA, Jesberger JA, et al. An MRI study of adolescent patients with either schizophrenia or bipolar disorder as compared to healthy control subjects. *Biol Psychiatry.* (1999) 46:78–88. doi: 10.1016/S0006-3223(98)00351-5
21. Botteron KN, Vannier MW, Geller B, Todd RD, Lee BC. Preliminary study of magnetic resonance imaging characteristics in 8- to 16-year-olds with mania. *J Am Acad Child Adolesc Psychiatry.* (1995) 34:742–9. doi: 10.1097/00004583-199506000-00014
22. Chang K, Barnea-Goraly N, Karchemskiy A, Simeonova DI, Barnes P, Ketter T, et al. Cortical magnetic resonance imaging findings in familial pediatric bipolar disorder. *Biol Psychiatry.* (2005) 58:197–203. doi: 10.1016/j.biopsych.2005.03.039
23. Kaufman J, Birmaher B, Brent D, Rao U, Flynn C, Moreci P, et al. Schedule for Affective Disorders and Schizophrenia for School-Age Children-Present and Lifetime Version (K-SADS-PL): initial reliability and validity data. *J Am Acad Child Adolesc Psychiatry.* (1997) 36:980–8. doi: 10.1097/00004583-199707000-00021
24. Young RC, Biggs JT, Ziegler VE, Meyer DA. A rating scale for mania: reliability, validity and sensitivity. *Br J Psychiatry.* (1978) 133:429–35. doi: 10.1192/bjp.133.5.429
25. Wood A, Kroll L, Moore A, Harrington R. Properties of the mood and feelings questionnaire in adolescent psychiatric outpatients: a research note. *J Child Psychol Psychiatry.* (1995) 36:327–34. doi: 10.1111/j.1469-7610.1995.tb01828.x
26. Rivera D, Perrin PB, Stevens LF, Garza MT, Weil C, Saracho CP, et al. Stroop color-word interference test: normative data for the Latin American Spanish speaking adult population. *NeuroRehabilitation.* (2015) 37:591–624. doi: 10.3233/NRE-151281
27. Baune BT, Air T. Clinical, functional, and biological correlates of cognitive dimensions in major depressive disorder—rationale, design, and characteristics of the cognitive function and mood study (CoFaM-Study). *Front Psychiatry.* (2016) 7:150. doi: 10.3389/fpsyt.2016.00150
28. Roelofs A, Hagoort P. Control of language use: cognitive modeling of the hemodynamics of Stroop task performance. *Brain Res Cogn Brain Res.* (2002) 15:85–97. doi: 10.1016/S0926-6410(02)00218-5
29. Karakurt MN, Karabekirogllu MZ, Yuce M, Baykal S, Senses A. [Neuropsychological profiles of adolescents with bipolar disorder and adolescents with a high risk of bipolar disorder]. *Turk Psikiyatri Derg.* (2013) 24:221–30. doi: 10.5080/u7185
30. Soni A, Singh P, Shah R, Bagotia S. Impact of cognition and clinical factors on functional outcome in patients with bipolar disorder. *East Asian Arch Psychiatry.* (2017) 27:26–34.
31. Dixon T, Kravariti E, Frith C, Murray RM, McGuire PK. Effect of symptoms on executive function in bipolar illness. *Psychol Med.* (2004) 34:811–21. doi: 10.1017/S0033291703001570
32. Elias LR, Miskowiak KW, Vale AM, Kohler CA, Kjaerstad HL, Stubbs B, et al. Cognitive impairment in euthymic pediatric bipolar disorder: a systematic review and meta-analysis. *J Am Acad Child Adolesc Psychiatry.* (2017) 56:286–96. doi: 10.1016/j.jaac.2017.01.008
33. Ching CRK, Gutman BA, Sun D, Villalon Reina J, Ragothaman A, Isaev D, et al. Mapping subcortical brain alterations in 22q11.2 deletion syndrome: effects of deletion size and convergence with idiopathic neuropsychiatric illness. *Am J Psychiatry.* (2020) 77:589–600. doi: 10.1176/appi.ajp.2019.19060583
34. Aneja A, Fremont WP, Antshel KM, Faraone SV, AbdulSabur N, Higgins AM, et al. Manic symptoms and behavioral dysregulation in youth with velocardiofacial syndrome (22q11.2 deletion syndrome). *J Child Adolesc Psychopharmacol.* (2007) 17:105–14. doi: 10.1089/cap.2006.0023
35. Vawter MP. Dysregulation of the neural cell adhesion molecule and neuropsychiatric disorders. *Eur J Pharmacol.* (2000) 405:385–95. doi: 10.1016/S0014-2999(00)00568-9
36. Bhadoria R, Watson D, Danson P, Ferrier IN, McAllister VI, Moore PB. Enlargement of the third ventricle in affective disorders. *Indian J Psychiatry.* (2003) 45:147–50.
37. Manaye KF, Lei DL, Tizabi Y, Davila-Garcia MI, Mouton PR, Kelly PH. Selective neuron loss in the paraventricular nucleus of hypothalamus in patients suffering from major depression and bipolar disorder. *J Neuropathol Exp Neurol.* (2005) 64:224–9. doi: 10.1093/jnen/64.3.224
38. Franke B, Vasquez AA, Veltman JA, Brunner HG, Rijpkema M, Fernandez G. Genetic variation in CACNA1C, a gene associated with bipolar disorder, influences brainstem rather than gray matter volume in healthy individuals. *Biol Psychiatry.* (2010) 68:586–8. doi: 10.1016/j.biopsych.2010.05.037
39. Juuhl-Langseth M, Rimol LM, Rasmussen IA, Jr., Thormodsen R, Holmen A, Emblem KE, et al. Comprehensive segmentation of subcortical brain volumes in early onset schizophrenia reveals limited structural abnormalities. *Psychiatry Res.* (2012) 203:14–23. doi: 10.1016/j.pscychresns.2011.10.005
40. Andreone N, Tansella M, Cerini R, Rambaldelli G, Versace A, Marrella G, et al. Cerebral atrophy and white matter disruption in chronic schizophrenia. *Eur Arch Psychiatry Clin Neurosci.* (2007) 257:3–11. doi: 10.1007/s00406-006-0675-1
41. Brainstorm C, Anttila V, Bulik-Sullivan B, Finucane HK, Walters RK, Bras J, et al. Analysis of shared heritability in common disorders of the brain. *Science.* (2018) 360:8757. doi: 10.1126/science.aap8757
42. Cross-Disorder Group of the Psychiatric Genomics C, Lee SH, Ripke S, Neale BM, Faraone SV, Purcell SM, et al. Genetic relationship between five psychiatric disorders estimated from genome-wide SNPs. *Nat Genet.* (2013) 45:984–94. doi: 10.1038/ng.2711
43. Lindsley DB, Bowden JW, Magoun HW. Effect upon the EEG of acute injury to the brain stem activating system. *Electroencephalogr Clin Neurophysiol.* (1949) 1:475–86. doi: 10.1016/0013-4694(49)90221-7
44. Mirsky AF, Duncan CC. Pathophysiology of mental illness: a view from the fourth ventricle. *Int J Psychophysiol.* (2005) 58:162–78. doi: 10.1016/j.ijpsycho.2005.06.004
45. Young RC, Nambudiri DE, Jain H, de Asis JM, Alexopoulos GS. Brain computed tomography in geriatric manic disorder. *Biol Psychiatry.* (1999) 45:1063–5. doi: 10.1016/S0006-3223(98)00201-7
46. Dewan MJ, Haldipur CV, Lane EE, Ispahani A, Boucher MF, Major LF. Bipolar affective disorder. I. Comprehensive quantitative computed tomography. *Acta Psychiatr Scand.* (1988) 77:670–6. doi: 10.1111/j.1600-0447.1988.tb05186.x
47. Harvey I, Persaud R, Ron MA, Baker G, Murray RM. Volumetric MRI measurements in bipolars compared with schizophrenics and healthy controls. *Psychol Med.* (1994) 24:689–99. doi: 10.1017/S0033291700027847
48. Dutt A, McDonald C, Dempster E, Prata D, Shaikh M, Williams I, et al. The effect of COMT, BDNF, 5-HTT, NRG1 and DTNBP1 genes on hippocampal and lateral ventricular volume in psychosis. *Psychol Med.* (2009) 39:1783–97. doi: 10.1017/S0033291709990316
49. Terry J, Lopez-Larson M, Frazier JA. Magnetic resonance imaging studies in early onset bipolar disorder: an updated review. *Child Adolesc Psychiatr Clin N Am.* (2009) 18:421–39, ix-x. doi: 10.1016/j.chc.2008.12.004
50. Delvecchio G, Fossati P, Boyer P, Brambilla P, Falkai P, Gruber O, et al. Common and distinct neural correlates of emotional processing in bipolar disorder and major depressive disorder: a voxel-based meta-analysis of functional magnetic resonance imaging studies. *Eur Neuropsychopharmacol.* (2012) 22:100–13. doi: 10.1016/j.euroneuro.2011.07.003
51. Jiang X, Fu S, Yin Z, Kang J, Wang X, Zhou Y, et al. Common and distinct neural activities in frontoparietal network in first-episode bipolar disorder and major depressive disorder: preliminary findings from a follow-up resting state fMRI study. *J Affect Disord.* (2020) 260:653–9. doi: 10.1016/j.jad.2019.09.063
52. Sanfilipo MP, Benedict RH, Zivadinov R, Bakshi R. Correction for intracranial volume in analysis of whole brain atrophy in multiple sclerosis: the proportion vs. residual method. *Neuroimage.* (2004) 22:1732–43. doi: 10.1016/j.neuroimage.2004.03.037

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Nutrition as Metabolic Treatment for Anxiety

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Despite the overwhelming prevalence of anxiety disorders in modern society, medications and psychotherapy often fail to achieve complete symptom resolution. A complementary approach to medicating symptoms is to address the underlying metabolic pathologies associated with mental illnesses and anxiety. This may be achieved through nutritional interventions. In this perspectives piece, we highlight the roles of the microbiome and inflammation as influencers of anxiety. We further discuss the evidence base for six specific nutritional interventions: avoiding artificial sweeteners and gluten, including omega-3 fatty acids and turmeric in the diet, supplementation with vitamin D, and ketogenic diets. We attempt to integrate insights from the nutrition science-literature in order to highlight some practices that practitioners may consider when treating individual patients. Notably, this piece is not meant to serve as a comprehensive review of the literature, but rather argue our perspective that nutritional interventions should be more widely considered among clinical psychiatrists. Nutritional psychiatry is in its infancy and more research is needed in this burgeoning low-risk and potentially high-yield field.

Keywords: anxiety, inflammation, microbiome, nutrition, mental illness

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INTRODUCTION

Anxiety disorders are the most common type of psychiatric condition in the United States, with one-third of individuals suffering from some form of anxiety during their lifetime (1). Standard of care medications and psychotherapy are only successful in treating about half of patients, and only one-quarter experience complete symptomatic resolution (2).

While medications and behavioral therapies certainly have their place as part of a multifaceted approach to treat anxiety, the relatively high failure rate of such approaches is consistent with the broader failure of drug treatments for most neurological conditions. For example, antidepressants are efficacious in only about one-third of clinical cases (3) and there are no established disease-modifying medications for major neurodegenerative conditions like Parkinson's disease or Alzheimer's disease. With respect to the latter, the drug discovery failure rate for mere symptomatic management is 99.6% (4). It is therefore feasible, if not probable, that we are approaching neurological conditions with the wrong paradigm. As neurological conditions and mental illnesses (5) are characterized by a subset of fundamental metabolic disturbances [such as oxidative stress (6), insulin resistance (7), inflammation (8), and microbiome dysbiosis (9)] to which lifestyle factors are a contributor, it would makes sense that mental illnesses deserve complementary lifestyle approaches. In effect, lifestyle interventions for mental illness are a form of metabolic medicine complementary to metabolic disease (5). Nutrition is one such metabolic medicine.

Herein, we discuss the pathological correlates of anxiety disorders specifically, emphasizing the possible roles of microbiome dysbiosis and inflammation. We chose to structure this perspective piece as follows: First, we discuss microbiome dysbiosis and inflammation, pathologies that are particularly relevant to anxiety disorders in order to establish anxiety as a metabolic disease. Second, we discuss six nutritional strategies for which there is emerging evidence of their efficacy in anxiety. These are elimination of (i) artificial sweeteners and (ii) gluten, inclusion (iii) omega-3 fatty acids and (iv) turmeric (curcumin), maintaining adequate levels of (v) vitamin D, and (vi) and ketogenic diets. Within each section, we build up the evidence hierarchy from a mechanistic metabolic perspective, to animal models, to human studies. The purpose of this piece is not to delve into all the mechanisms of interventions (for which there is currently limited data), but demonstrate that anxiety is a metabolic disease and that nutritional therapy can be efficacious in its treatment.

MICROBIOME

The gut contains ∼40 trillion microorganisms and is the largest endocrine organ in the body. By communicating to the brain via the Vagus nerves, regulating hormones, and influencing inflammation, the gut can impact mental health (10). More specifically, the compositions of individuals' gut microbial ecosystems can regulate mental status and anxiety (11). It is, therefore, unsurprising that microbiome dysbiosis is associated with anxiety (9).

As a comprehensive description of the mechanisms by which the microbiome and gut-brain axis influence the neuroanatomy and neurochemistry of anxiety is beyond the scope of this piece, we will emphasize the role of the amygdala, short chain fatty acids (SCFAs), and gut peptides as examples [Please see the following references as starting points for further reading on the Vagus nerve (12) or microbiome influence of cytokine production (13)].

The amygdala is a structure in the brain largely responsible for the threat response that is hyperactive in anxiety disorders (14). Interestingly, germ-free mice exhibit larger and more active amygdalae (15, 16). Furthermore, fecal transplantation, or the introduction *Bifidobacterium infantis*, has been shown to correct excessive stress response in such germ-free mice (17), implicating the microbiome in amygdala dysfunction.

The amygdala has receptors for gut peptides, including neuropeptide Y (NPY), pancreatic polypeptide (PP), and glucagon-like peptide 1 (GLP-1) (11, 18). Addressing each of these, there is evidence that the NPY system affects anxiety (19, 20); the PP Y_4 receptor has been shown to modulate anxiety in rodents (21); and multiple GLP-1 receptor agonists have been used to address anxiety in animal models (22, 23). The release of each of these gut peptides is regulated by SCFAs produced by certain gut bacteria, which act through the G-protein coupled receptors, free fatty acid receptors 2 (FFAR2) and FFAR3 (11). Notably, populations of SCFA producing species tend to be reduced in individuals with anxiety (9).

In review, food influences the microbiome (24) and microbe-derived SCFAs bind to receptors on enteroendocrine cells to regulate the secretion of gut peptides, which themselves bind to receptors on the amygdala to influence the stress response and anxiety. This is just one cascade by which diet can influence the brain. SCFAs from gut microbes can also act through immune, inflammatory, and other endocrine mechanisms (25, 26), and lipopolysaccharide (LPS) from gram-negative bacteria can induce anxiety when leaked into circulation through a compromised gut barrier (27, 28). The mechanisms are many, but the point is simple: diet and nutrition influence anxiety by modulating the microbiome.

It is also to be emphasized that the microbiome-brain axis is a bidirectional relationship. Negative emotions can shift the microbial ecosystem by the release of stress hormones sympathetic neurotransmitters (29). Therefore, even if the current state of science does not enable precision medicine aimed at the microbiome, it is still important to consider the role that positive feedback loops between the gut and brain may be playing in anxiety disorders.

INFLAMMATION

Chronic inflammation is a feature of almost all neurological and neurodegenerative disorders, including anxiety (30). Individuals suffering from anxiety and anxiety-related disorders, like panic disorder (31), generalized anxiety disorder (32), and post-traumatic stress disorders (PTSD), exhibit elevated levels of inflammatory markers in their circulation and cerebral spinal fluid (33). These include C-reactive protein (CRP), IL-1β, IL-6, and TNFα (31, 34–38). These cytokines contribute to neurotransmitter imbalances in the brain (including, serotonin, dopamine, glutamate/GABA) and can pathologically increase amygdala responsivity (30).

Suggestions of causality exist in the literature and, because the existing literature is most highly focused on PTSD, we too will focus on PTSD as a case in point of potential causality. As examples, polymorphisms in *CRP* predict increased likelihood of being diagnosed with PTSD, and predict worse symptoms if diagnosed (36); a study on immune cells taken from patients with anxiety showed increased reactivity and secretion of the cytokines, IL-17 and TNFα (39); and, administration of LPS to 39 healthy subjects doubled amygdala activity, as measured by fMRI, in response to socially threatening images (40). Admittedly, the state of research on the mechanisms of inflammation-induced anxiety is in its infancy. Nevertheless, it is probable that

Abbreviations: AGEs, advanced glycation end products; ALA, alpha-linolenic acid; BDNF, brain-derived neurotrophic factor; CRP, C-reactive protein; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FFARs, free fatty acid receptors; GABA, gamma-aminobutyric acid; GLP-1, glucagon-like peptide 1; HDACs, histone deacetylases; IBDs, inflammatory bowel diseases; IBS, irritable bowel syndrome; IL, interleukin; LPS, lipopolysaccharide; MSFD2A, major facilitator superfamily domain-containing protein 2 A; NFκB, nuclear factor κ-light-chain enhancer of activated B cells; NLRP3, NOD-LRR-and pyrin domain-containing protein 3; NPY, neuropeptide Y; PP, pancreatic polypeptide; PTSD, post-traumatic stress disorder; SAD, Standard American Diet; SCFAs, short chain fatty acids; TNFα, tumor necrosis factor α.

inflammation contributes to anxiety in at least some, and possibly a majority, of patients.

The menu of "inflammatory foods" is extensive, but generally includes foods associated with the Standard American Diet (SAD). Holistically speaking, the two most metabolically challenging components of SAD are refined sugars and processed vegetable oils, both of which can contribute to inflammation through myriad mechanisms (41–43). To mention a few as illustrative points, refined sugars, and in particular high fructose corn syrup, contribute to *de novo* lipogenesis of pro-inflammatory visceral fat (43), and fructose now composes 10% of caloric intake in the United States (42). Sugar also attaches to molecules throughout the body to generate inflammatory advanced glycation end products (AGEs). It has even been demonstrated that sugar can increase the production of AGEs in the brain and that these AGEs increase neuroinflammation and contribute to metabolic diseases (41).

Processed vegetable oils, such as corn oil and soybean oil, that contain high levels of the omega-6 fatty acids, linoleic acid, are likewise inflammatory. Having been stripped of the antioxidants that protect omega-6 fats in whole foods, the linoleic acid in processed vegetable oils incorporates into cells and tissue throughout the body, gets oxidized, and can initiate a vicious cycle of oxidation, insulin resistance, and inflammation that perpetuates metabolic and inflammatory diseases from the gut to the brain (7, 44–46). Increased consumption of linoleic acid-containing vegetable oils has even been proposed as a driver of cardiovascular disease (47), an inflammatory disease and comorbidity of anxiety disorders (46, 48, 49). Elimination of refined sugars and processed vegetable oils from the diet, and their replacement with whole foods, is foundational for good physical, cognitive, and mental health. However, more specific dietary and nutritional interventions have been explored in the context of anxiety, and it is to these which we turn.

NUTRITIONAL STRATEGIES

Artificial Sweeteners

Administration of artificial sweeteners to animals has been shown to precipitate anxiety (50). The anxiolytic effects of sweeteners are likely mediated by their adverse impacts on the microbiome and inflammation. Negative effects of certain sweeteners on systemic metabolism have been shown to be causal in animal models and humans, although the precise pathways are unknown (51, 52). Other mechanisms exist as well. For example, aspartame given to rats increased the levels of stress hormones in the animals' amygdalae (53). Aspartame can also block the transport of dopamine and serotonin precursors into the brain and can increase the levels of excitatory neurotransmitters, shifting brain chemistry toward an anxiety prone state (54).

In humans, artificial sweeteners have been associated with neuropsychiatric problems, including anxiety (55). Further, it has been proposed that individuals suffering from mental disorders may be particularly susceptible to the adverse effects of artificial sweeteners. For example, a randomized, placebo-controlled, crossover study designed to assess the impact of aspartame on mood was prematurely terminated because of the severity of reactions in patients with a history of depression (56), which is highly comorbid with anxiety (57).

Unfortunately, the literature is currently limited to the investigation of only a narrow range of sweeteners (and predominantly the sweetener, aspartame, found under the trade names *Equal* and *NutraSweet*, and in popular low-fat snacks and drinks, like Diet Coke). Future human studies will hopefully reveal associations between specific sweeteners and specific neurological disorders so that nutritional psychiatrists can provide more specific recommendations.

For patients unwilling to give up sweeteners, stevia (a natural non-caloric, non-insulinogenic sweetener) and erythritol [a non-insulinogenic sugar alcohol that gets absorbed in the small intestine and is not fermented by gut bacteria (58)] may be reasonable alternatives to recommend to patients in a practical clinical setting because they are presumed to have minimal negative impact on insulin sensitivity and the microbiome and are, therefore, less likely to cause metabolic dysfunction. However, as absence of evidence does not equate to evidence of absence, the most conservative approach is still the complete elimination of sugar and sweeteners.

Gluten

Gluten can induce inflammation by causing "leaky gut." Gluten proteins increase zonulin expression, which increases gut permeability (59, 60). Thereafter, immune stimulating compounds, like LPS, leak from the gut into the bloodstream, leading to inflammation.

Zonulin protein is overexpressed in celiac disease, a condition that itself is associated with social phobias, panic disorder, and other forms of anxiety (61–63). Generalizing beyond celiac disease, zonulin has been linked as a biomarker of mental illnesses such as autism, attention deficient hyperactivity disorder, and schizophrenia (64). Even in anxiety patients with no reported history of gastrointestinal disturbances, zonulin and LPS are found at elevated levels in the blood relative to non-anxious control subjects (65). This is consistent with the hypothesis that gluten can cause "leaky gut" to precipitate inflammation and anxiety and suggests patients with anxiety may be particularly sensitive to gluten.

At this time, a gluten-free diet has been shown to decrease anxiety only in celiac patients (66). Nevertheless, we feel it is reasonable to include a gluten-free diet in the arsenal of metabolic treatments for anxiety, given the mechanistic link to "leaky gut" and associations between zonulin and mental illness and zonulin levels and anxiety.

Omega-3s

Omega-3 fatty acids, particularly the long-chain omega-3s, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), are potent anti-inflammatory signaling molecules that support the microbiome (67, 68) and are important in cognition and mental health (69, 70). Direct evidence that omega-3s themselves are healthful, in addition to their whole food sources, comes from the comparison of genetically engineered mice that can biosynthesize omega-3 and/or omega-6 fats. On identical diets, mice that biosynthesize omega-3s and have lower

omega-6/omega-3 ratios and exhibit healthier microbiomes, less inflammation, and less chronic disease (71). In preclinical studies on rats suffering from inflammation-induced anxiety, omega-3-rich diets have been shown to normalize dopamine levels (72) and reduce anxiety-like behaviors (73). And, in mice, omega-3s have been shown to improve serotonergic neurotransmission and increase levels of brain-derived neurotrophic factor (BDNF) (74). Thus, while the mechanisms by which omega-3s assist in addressing the metabolic foundations of anxiety are manifold, they likely include improving microbiome balance, decreasing inflammation, and balancing neurochemistry.

Turning to humans, Green et al. demonstrated that, in patients with social anxiety disorder, erythrocyte EPA and DHA levels are reduced 18–34%. Moreover, an inverse correlation exists between levels of these omega-3s and severity of anxiety (75). Similar observations have been made by others (76), and these associations are backed by interventional trials.

A randomized, double-blinded, placebo-controlled trial on 68 medical students showed that 12 weeks of omega-3 supplementation lowered anxiety by 20%. This study also revealed that lower omega-6/omega-3 ratios predicted lower levels of inflammatory markers and anxiety (77). Lastly, a meta-analysis of nineteen clinical trials, including 2,240 participants across eleven countries, concluded that omega-3 treatment is effective in reducing anxiety (78).

The aforementioned meta-analysis also highlights the fact that dose and omega-3s type are important to consider. Studies that used doses lower than 2 grams per day tended not to be effective in treating anxiety. Furthermore, subgroups analyses found that supplements with lower proportions of DHA were less effective in reducing anxiety, with supplements containing more than 60% EPA having no significant effect (78).

Practically speaking, on the topic of omega-3 types and sources, plant sources of omega-3 (such as flax seeds and chia seeds) contain primarily alpha linolenic acid (ALA), a shorter chain omega-3 that is converted in to the more bioactive EPA and DHA only at very low levels, on the order of 5% conversion (79–81). Fatty fish, such as mackerel, sardines, and Alaskan sockeye salmon are far richer in EPA and DHA. Salmon, in particular, includes the antioxidant, astaxanthin, which not only gives salmon their pink-red color but also protects omega-3s from oxidation and itself has neuroprotective properties (82). It is also worth mentioning that there is diversity among DHA forms. Specifically, lysophosphatidylcholine-conjugated DHA, found at its highest levels in fish roe and krill oil, has privileged transport to the brain via the major facilitatory superfamily domain-containing protein (MSFD2A) transporter, a transmembrane protein that exists within endothelial cells at the blood-brain barrier. Whereas, free DHA bound in the blood crosses into the brain via passive diffusion, the MSFD2A transporter actively shuttles lysophosphatidylcholine-conjugated DHA into the brain using energy derived from the sodium electrochemical gradient (83). This active transport mechanisms may be particularly beneficial in inflamed brains in which the blood-brain barrier is compromised. Therefore, when recommending omega-3 sources to patients, fish roe and krill oil may be the best options, followed by salmon and other fatty fish.

Thus, there is mechanistic rationale, animal and human data supporting the emphasis of dietary omega-3 for the treatment of anxiety.

Turmeric (Curcumin)

Turmeric is probably the most heavily studied spices for brain health. Its active component, curcumin, has been explored as a treatment for Alzheimer's disease, Parkinson's disease, depression, comorbidities of anxiety, and anxiety itself (84, 85). Curcumin's mechanisms of action are many and include improving the gut microbial ecosystem (86), decreasing inflammation by inhibiting NFκB and the NLRP3 inflammasome (87–90), altering dopamine, serotonin, and cortisol levels (91), and regulating microRNAs and histone deacetylases (HDACs) (92).

Preclinical trials of curcumin for anxiety in rodent models add to the promise of curcumin as an anti-anxietolytic. In rats treated with a food preservative to induce anxiety, curcumin treatment completed rescued anxiety-like behaviors (93). Similar findings have been reported in other animal models of anxiety (94, 95). In these and other studies, curcumin significantly reduced anxiety-like behaviors concomitant with complementary improvements in neurotransmitter and hormone levels (91, 94, 95).

Multiple randomized, double-blinded, placebo-controlled trials have shown that curcumin supplementation can reduce anxiety in human patients. In patients with diabetes, 8 weeks of curcumin supplementation decreased anxiety (96). A crossover trial on 30 obese individuals likewise found that curcumin supplementation for 30 days reduced anxiety scores (97). And, a meta-analysis of five studies reported an overall significant effect of curcumin on anxiety with a large effect size [Hedge's $g = -2.62$ (84)].

Admittedly, there are limitations to the curcumin literature. Some have challenged that the health benefits of turmeric and its active components are over sensationalized. Specifically, Nelson et al. performed a careful analysis of the medical chemistry of curcumin and make a compelling case that the positive results in model systems may be confounded by curcumin's chemical instability and potential for interfering with assay readouts. Furthermore, they point out that there is a great degree of variability among studies with respect to supplement purity and formulations, which confound the reproducibility of studies (98). For example, curcuminoids are fat-soluble and exhibit <1% bioavailability when administered alone or an aqueous solution (99). For this reason, curcuminoids should be consumed with fats, and lipid-based delivery systems have been and are being developed for the administration of curcumin, including liposomes and nanoparticles (100). Indeed, the two randomized controlled trials referenced in the previous paragraph that reported positive findings for curcumin on anxiety each employed techniques to increase the bioavailability of curcumin, including nano-curcumin (96) and co-administration of bioperine (97) (also known as piperine), which enhances curcumin absorption 20-fold (101). Thus, future research resources should be devoted to the study of these more bioavailable forms of curcumin, and also to the impact of

curcumin on the human microbiome, as this does not require systemic absorption.

Turmeric also relates to the previous section on omega-3s. Recall that humans are inefficient at converting ALA into EPA and DHA. Curcumin can increase ALA to DHA conversion by increasing levels of the DHA synthesis enzymes (102). This not only increases DHA in the brain but has direct functional implications on anxiety. Rodents treated with a combination of ALA and curcumin exhibit decreased anxiety (102).

Vitamin D

As most modern humans spend most of their time inside, fully clothed, or simply living at high latitudes, endogenous vitamin D production is often inadequate. It is also difficult to get enough vitamin D from the diet. Even using the most favorable numbers for vitamin D content of milk, one would need to consume five gallons of whole milk daily to meet the recommended 600 IU (103), ignoring that many practitioners believe higher doses may be optimal. On a population level, vitamin D insufficiency (<30 ng/mL) has been estimated at 77% in the United States (104), making low vitamin D levels a hormonal epidemic.

In the brain, vitamin D regulates calcium homeostasis and ion channels (105, 106), neurotransmitter levels, including dopamine and serotonin (107–110), and the secretion of nerve growth factor and BDNF (111, 112). The benefits of vitamin D are also likely mediated by its role in shaping the microbiome and reducing inflammation (113–117).

Lower levels of vitamin D are associated with multiple mental disorders, including schizophrenia (118), depression (119), and anxiety (120–122). One such association study found that vitamin D levels in patients with a wide range of anxiety disorders were <60% those of healthy controls (120).

In interventional studies, vitamin D supplementation to those with vitamin D deficiency has been effective in addressing anxiety. In a study of 30 anxiety patients, once weekly vitamin D supplementation at 50,000 IU for 3 months significantly improved symptoms (123). A similar study in 51 women with type II diabetes also showed that 50,000 IU vitamin D fortnightly decreased inflammation and reduced symptoms of anxiety over 4 months (124).

It may be that vitamin D supplementation for anxiety is only effective in those with vitamin D insufficiency, with one association study finding elevated anxiety only on those with extreme vitamin D deficiency (<10 ng/mL) (125). Nonetheless, vitamin D insufficiency persists amongst Americans, sufficient vitamin D intake is difficult to obtain through diet or sun exposure, when living at higher latitudes, and adequate vitamin D levels are important to overall health. Therefore, vitamin D supplementation should be considered for patients with anxiety as most will be vitamin D insufficient and the collateral effects on patient health are likely to be positive.

Ketogenic Diets

Ketogenic diets—high-fat, low-carbohydrate diets that induce the body to produce ketones, a fuel source for the brain—are gaining traction as a metabolic treatment for a wide range of chronic metabolic diseases (126–131). Ketogenic diets have been used for a century to treat drug-resistant pediatric epilepsy, are still widely used for epilepsy (132, 133), and are gaining in popularity for the treatment of neurodegenerative conditions, such as Parkinson's disease (131, 134, 135) and Alzheimer's disease (128, 130, 136). For reviews specifically on the topic of ketogenic diets for neurological diseases and mental illnesses, we recommend the following recent reviews, both published this year, which cover the literature supporting the therapeutic implementation of ketogenic diets for a wide range of conditions including attention deficit hyperactivity disorder (ADHD), bipolar disorder, schizophrenia, autism spectrum disorder, major depressive disorder, and binge eating disorder (5, 137).

Ketogenic diets help to address many of the biopathological foundations of chronic neurological diseases and mental illnesses, including glucose hypometabolism, neurotransmitter imbalances, oxidative stress, and inflammation (5). Ketones produced by the liver during carbohydrate restriction are not only a more efficient fuel substrate for the brain, but are also signaling molecules that bind their own G-protein coupled receptors, inhibit HDACs, directly modify histones, shift the gut microbiome and improve gut barrier function, and reduce oxidative stress and inflammation (134, 138–142).

Preclinical models offer promise. Rats orally administered exogenous ketone supplements to achieve ketone levels comparable to those achieved by patients on ketogenic diets (~0.8 mmol/L) exhibited significant decreases in anxiety (143).

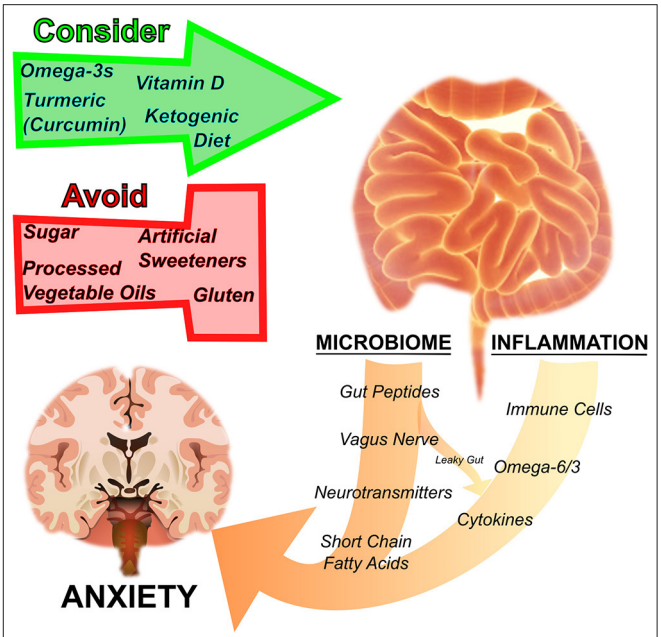

FIGURE 1 | Nutrition regulates anxiety disorders by influencing the microbiome and inflammation. The gut microbiome and inflammation are interrelated and influence anxiety. By acting on the gut microbial ecosystem, regulating inflammation, as well as through other pathways mentioned in the text, particular nutritional strategies have been suggested to either harm or help disorders of anxiety. Sugar, processed vegetable oils rich in inflammatory omega-6 fatty acids, artificial sweeteners, and gluten have a negative effect on anxiety, whereas omega-3 fatty acids, turmeric (curcumin), vitamin D, and ketogenic diets are thought to have a therapeutic effect.

Another rat study found that ketosis induced anti-anxiolytic changes in brain metabolism in association with a reduction of anxiety-like behaviors (144). There is also evidence that intermittent fasting, an intervention that induces ketosis, induces neurological adaptations overtime (including an upregulation of the mitochondrial sirtuin, SIRT3, and GABAergic activity) that and neuroprotective and reduce anxiety (145). No clinical trials assessing the efficacy of ketogenic diets for anxiety have yet been conducted. We include ketogenic diets as a potentially promising future option and to provide clinicians with a perspective on the emerging research on nutrition and mental health.

Other Strategies

Additional nutritional strategies hold potential for the treatment of anxiety disorders, including caffeine reduction (146, 147), prebiotics (148, 149), and probiotics (150) to support the microbiome, and supplementation with or magnesium (151, 152) or tryptophan (153) to potentially increase serotonin synthesis.

CONCLUSION

Herein, we provide biological rationales and translational evidence that nutritional strategies aimed at addressing disturbances in metabolism and brain function can protect against anxiety disorders (**Figure 1**). Anxiety and other mental illnesses are metabolic diseases as much as they are psychological. And, in our opinions, metabolic diseases deserve metabolic medicine. Nutrition is one form of metabolic medicine, and one which patients and clinicians interact with every day. It is important to leverage this metabolic tool to better offer persons suffering with anxiety a full spectrum of relief.

However, the clinical challenge of bioindividuality persists. Different patients are afflicted with different deficiencies and comorbidities. We each carry genetic polymorphisms and have distinct microbiomes. Therefore, future research should be focused on determining the mechanisms by which various interventions operate such that the medical community can turn nutritional psychiatry from a shotgun approach into precision personalized medicine.

In closing, we pose the question, "if patients needs to eat everyday anyway, why not turn a gustatory pleasure into an experimental one as well?"

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.

AUTHOR CONTRIBUTIONS

Both authors contributed to the work and approved it for publication.

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REFERENCES

1. Bandelow B, Michaelis S. Epidemiology of anxiety disorders in the 21st century. *Dialogues Clin Neurosci*. (2015) 17:327–35. doi: 10.31887/DCNS.2015.17.3/bbandelow
2. Roy-Byrne P. Treatment-refractory anxiety; definition, risk factors, and treatment challenges. *Dialogues Clin Neurosci*. (2015) 17:191–206. doi: 10.31887/DCNS.2015.17.2/proybyrne
3. Blackburn TP. Depressive disorders: treatment failures and poor prognosis over the last 50 years. *Pharmacol Res Perspect*. (2019) 7:e00472. doi: 10.1002/prp2.472
4. Cummings JL, Morstorf T, Zhong K. Alzheimer's disease drug-development pipeline: few candidates, frequent failures. *Alzheimers Res Ther*. (2014) 6:37. doi: 10.1186/alzrt269
5. Norwitz NG, Sethi SD, Palmer CM. Ketogenic diet as a metabolic treatment for mental illness. *Curr Opin Endocrinol Diabetes Obes*. (2020) 27:269–74. doi: 10.1097/MED.0000000000000564
6. Bouayed J, Rammal H, Soulimani R. Oxidative stress and anxiety: relationship and cellular pathways. *Oxid Med Cell Longev*. (2009) 2:63–7. doi: 10.4161/oxim.2.2.7944
7. Norwitz NG, Mota AS, Norwitz SG, Clarke K. Multi-loop model of Alzheimer disease: an integrated perspective on the Wnt/GSK3beta, alpha-synuclein, and type 3 diabetes hypotheses. *Front Aging Neurosci*. (2019) 11:184. doi: 10.3389/fnagi.2019.00184
8. Firth J, Veronese N, Cotter J, Shivappa N, Hebert JR, Ee C, et al. What is the role of dietary inflammation in severe mental illness? A review of observational and experimental findings. *Front Psychiatry*. (2019) 10:350. doi: 10.3389/fpsyt.2019.00350
9. Jiang HY, Zhang X, Yu ZH, Zhang Z, Deng M, Zhao JH, et al. Altered gut microbiota profile in patients with generalized anxiety disorder. *J Psychiatr Res*. (2018) 104:130–6. doi: 10.1016/j.jpsychires.2018.07.007
10. Cryan JF, O'Riordan KJ, Cowan CSM, Sandhu KV, Bastiaanssen TFS, Boehme M, et al. The Microbiota-gut-brain axis. *Physiol Rev*. (2019) 99:1877–2013. doi: 10.1152/physrev.00018.2018
11. Lach G, Schellekens H, Dinan TG, Cryan JF. Anxiety, depression, and the microbiome: a role for gut peptides. *Neurotherapeutics*. (2018) 15:36–59. doi: 10.1007/s13311-017-0585-0
12. Breit S, Kupferberg A, Rogler G, Hasler G. Vagus nerve as modulator of the brain-gut axis in psychiatric and inflammatory disorders. *Front Psychiatry*. (2018) 9:44. doi: 10.3389/fpsyt.2018.00044
13. Schirmer M, Smeekens SP, Vlamakis H, Jaeger M, Oosting M, Franzosa EA, et al. Linking the human gut microbiome to inflammatory cytokine production capacity. *Cell*. (2016) 167:1125–36 e1128. doi: 10.1016/j.cell.2016.10.020
14. Engel K, Bandelow B, Gruber O, Wedekind D. Neuroimaging in anxiety disorders. *J Neural Transm*. (2009) 116:703–16. doi: 10.1007/s00702-008-0077-9
15. Hoban AE, Stilling RM, Moloney G, Shanahan F, Dinan TG, Clarke G, et al. The microbiome regulates amygdala-dependent fear recall. *Mol Psychiatry*. (2018) 23:1134–44. doi: 10.1038/mp.2017.100

16. Luczynski P, Whelan SO, O'Sullivan C, Clarke G, Shanahan F, Dinan TG, et al. Adult microbiota-deficient mice have distinct dendritic morphological changes: differential effects in the amygdala and hippocampus. *Eur J Neurosci*. (2016) 44:2654–66. doi: 10.1111/ejn.13291
17. Sudo N, Chida Y, Aiba Y, Sonoda J, Oyama N, Yu XN, et al. Postnatal microbial colonization programs the hypothalamic-pituitary-adrenal system for stress response in mice. *J Physiol*. (2004) 558(Pt. 1):263–75. doi: 10.1113/jphysiol.2004.063388
18. Anesten F, Dalmau Gasull A, Richard JE, Farkas I, Mishra D, Taing L, et al. Interleukin-6 in the central amygdala is bioactive and co-localised with glucagon-like peptide-1 receptor. *J Neuroendocrinol*. (2019) 31:e12722. doi: 10.1111/jne.12722
19. Farzi A, Reichmann F, Holzer P. The homeostatic role of neuropeptide Y in immune function and its impact on mood and behaviour. *Acta Physiol*. (2015) 213:603–27. doi: 10.1111/apha.12445
20. Reichmann F, Holzer P. Neuropeptide Y: a stressful review. *Neuropeptides*. (2016) 55:99–109. doi: 10.1016/j.npep.2015.09.008
21. Painsipp E, Wultsch T, Edelsbrunner ME, Tasan RO, Singewald N, Herzog H, et al. Reduced anxiety-like and depression-related behavior in neuropeptide Y Y4 receptor knockout mice. *Genes Brain Behav*. (2008) 7:532–42. doi: 10.1111/j.1601-183X.2008.00389.x
22. Komsuoglu Celikyurt I, Mutlu O, Ulak G, Uyar E, Bektas E, Yildiz Akar F, et al. Exenatide treatment exerts anxiolytic- and antidepressant-like effects and reverses neuropathy in a mouse model of type-2 diabetes. *Med Sci Monit Basic Res*. (2014) 20:112–7. doi: 10.12659/MSMBR.891168
23. Sharma AN, Pise A, Sharma JN, Shukla P. Glucagon-like peptide-1 (GLP-1) receptor agonist prevents development of tolerance to anti-anxiety effect of ethanol and withdrawal-induced anxiety in rats. *Metab Brain Dis*. (2015) 30:719–30. doi: 10.1007/s11011-014-9627-z
24. Asnicar F, Berry SE, Valdes AM, Nguyen LH, Piccinno G, Drew DA, et al. Microbiome connections with host metabolism and habitual diet from 1,098 deeply phenotyped individuals. *Nat Med*. (2021). doi: 10.1038/s41591-020-01183-8. [Epub ahead of print].
25. Dalile B, Van Oudenhove L, Vervliet B, Verbeke K. The role of short-chain fatty acids in microbiota-gut-brain communication. *Nat Rev Gastroenterol Hepatol*. (2019) 16:461–78. doi: 10.1038/s41575-019-0157-3
26. Silva YP, Bernardi A, Frozza RL. The role of short-chain fatty acids from gut microbiota in gut-brain communication. *Front Endocrinol*. (2020) 11:25. doi: 10.3389/fendo.2020.00025
27. Sulakhiya K, Keshavlal GP, Bezbaruah BB, Dwivedi S, Gurjar SS, Munde N, et al. Lipopolysaccharide induced anxiety- and depressive-like behaviour in mice are prevented by chronic pre-treatment of esculetin. *Neurosci Lett*. (2016) 611:106–11. doi: 10.1016/j.neulet.2015.11.031
28. Yang TY, Jang EY, Ryu Y, Lee GW, Lee EB, Chang S, et al. Effect of acupuncture on Lipopolysaccharide-induced anxiety-like behavioral changes: involvement of serotonin system in dorsal Raphe nucleus. *BMC Complement Altern Med*. (2017) 17:528. doi: 10.1186/s12906-017-2039-y
29. Malan-Muller S, Valles-Colomer M, Raes J, Lowry CA, Seedat S, Hemmings SMJ. The gut microbiome and mental health: implications for anxiety- and trauma-related disorders. *OMICS*. (2018) 22:90–107. doi: 10.1089/omi.2017.0077
30. Felger JC. Imaging the role of inflammation in mood and anxiety-related disorders. *Curr Neuropharmacol*. (2018) 16:533–58. doi: 10.2174/1570159X15666171123201142
31. Hoge EA, Brandstetter K, Moshier S, Pollack MH, Wong KK, Simon NM. Broad spectrum of cytokine abnormalities in panic disorder and posttraumatic stress disorder. *Depress Anxiety*. (2009) 26:447–55. doi: 10.1002/da.20564
32. Bankier B, Barajas J, Martinez-Rumayor A, Januzzi JL. Association between C-reactive protein and generalized anxiety disorder in stable coronary heart disease patients. *Eur Heart J*. (2008) 29:2212–7. doi: 10.1093/eurheartj/ehn326
33. Michopoulos V, Powers A, Gillespie CF, Ressler KJ, Jovanovic T. Inflammation in fear- and anxiety-based disorders: PTSD, GAD, and beyond. *Neuropsychopharmacology*. (2017) 42:254–70. doi: 10.1038/npp.2016.146
34. Baker DG, Ekhator NN, Kasckow JW, Hill KK, Zoumakis E, Dashevsky BA, et al. Plasma and cerebrospinal fluid interleukin-6 concentrations in posttraumatic stress disorder. *Neuroimmunomodulation*. (2001) 9:209–17. doi: 10.1159/000049028
35. Bersani FS, Wolkowitz OM, Lindqvist D, Yehuda R, Flory J, Bierer LM, et al. Global arginine bioavailability, a marker of nitric oxide synthetic capacity, is decreased in PTSD and correlated with symptom severity and markers of inflammation. *Brain Behav Immun*. (2016) 52:153–60. doi: 10.1016/j.bbi.2015.10.015
36. Michopoulos V, Rothbaum AO, Jovanovic T, Almli LM, Bradley B, Rothbaum BO, et al. Association of CRP genetic variation and CRP level with elevated PTSD symptoms and physiological responses in a civilian population with high levels of trauma. *Am J Psychiatry*. (2015) 172:353–62. doi: 10.1176/appi.ajp.2014.14020263
37. Oganesyan LP, Mkrtchyan GM, Sukiasyan SH, Boyajyan AS. Classic and alternative complement cascades in post-traumatic stress disorder. *Bull Exp Biol Med*. (2009) 148:859–61. doi: 10.1007/s10517-010-0836-0
38. von Kanel R, Hepp U, Kraemer B, Traber R, Keel M, Mica L, et al. Evidence for low-grade systemic proinflammatory activity in patients with posttraumatic stress disorder. *J Psychiatr Res*. (2007) 41:744–52. doi: 10.1016/j.jpsychires.2006.06.009
39. Vieira MM, Ferreira TB, Pacheco PA, Barros PO, Almeida CR, Araujo-Lima CF, et al. Enhanced Th17 phenotype in individuals with generalized anxiety disorder. *J Neuroimmunol*. (2010) 229:212–8. doi: 10.1016/j.jneuroim.2010.07.018
40. Inagaki TK, Muscatell KA, Irwin MR, Cole SW, Eisenberger NI. Inflammation selectively enhances amygdala activity to socially threatening images. *Neuroimage*. (2012) 59:3222–6. doi: 10.1016/j.neuroimage.2011.10.090
41. Gao Y, Bielohuby M, Fleming T, Grabner GF, Foppen E, Bernhard W, et al. Dietary sugars, not lipids, drive hypothalamic inflammation. *Mol Metab*. (2017) 6:897–908. doi: 10.1016/j.molmet.2017.06.008
42. Jang C, Hui S, Lu W, Cowan AJ, Morscher RJ, Lee G, et al. The small intestine converts dietary fructose into glucose and organic acids. *Cell Metab*. (2018) 27:351–61 e353. doi: 10.1016/j.cmet.2017.12.016
43. Softic S, Cohen DE, Kahn CR. Role of dietary fructose and hepatic *de novo* lipogenesis in fatty liver disease. *Dig Dis Sci*. (2016) 61:1282–93. doi: 10.1007/s10620-016-4054-0
44. Borkman M, Storlien LH, Pan DA, Jenkins AB, Chisholm DJ, Campbell LV. The relation between insulin sensitivity and the fatty-acid composition of skeletal-muscle phospholipids. *N Engl J Med*. (1993) 328:238–44. doi: 10.1056/NEJM199301283280404
45. Guyenet SJ, Carlson SE. Increase in adipose tissue linoleic acid of US adults in the last half century. *Adv Nutr*. (2015) 6:660–4. doi: 10.3945/an.115.009944
46. Lopez-Candales A, Hernandez Burgos PM, Hernandez-Suarez DF, Harris D. Linking chronic inflammation with cardiovascular disease: from normal aging to the metabolic syndrome. *J Nat Sci*. (2017) 3:e341.
47. DiNicolantonio JJ, O'Keefe JH. Omega-6 vegetable oils as a driver of coronary heart disease: the oxidized linoleic acid hypothesis. *Open Heart*. (2018) 5:e000898. doi: 10.1136/openhrt-2018-000898
48. Easton K, Coventry P, Lovell K, Carter LA, Deaton C. Prevalence and measurement of anxiety in samples of patients with heart failure: meta-analysis. *J Cardiovasc Nurs*. (2016) 31:367–79. doi: 10.1097/JCN.0000000000000265
49. Grace SL, Abbey SE, Irvine J, Shnek ZM, Stewart DE. Prospective examination of anxiety persistence and its relationship to cardiac symptoms and recurrent cardiac events. *Psychother Psychosom*. (2004) 73:344–52. doi: 10.1159/000080387
50. Ashok I, Sheeladevi R, Wankhar D. Effect of long-term aspartame (artificial sweetener) on anxiety, locomotor activity and emotionality behavior in Wistar Albino rats. *Biomed Prev Nutr*. (2014) 4:39–43. doi: 10.1016/j.bionut.2013.04.002
51. Bian X, Chi L, Gao B, Tu P, Ru H, Lu K. Gut microbiome response to sucralose and its potential role in inducing liver inflammation in mice. *Front Physiol*. (2017) 8:487. doi: 10.3389/fphys.2017.00487
52. Suez J, Korem T, Zeevi D, Zilberman-Schapira G, Thaiss CA, Maza O, et al. Artificial sweeteners induce glucose intolerance by altering the gut microbiota. *Nature*. (2014) 514:181–6. doi: 10.1038/nature13793

53. Yokogoshi H, Wurtman RJ. Acute effects of oral or parenteral aspartame on catecholamine metabolism in various regions of rat brain. *J Nutr*. (1986) 116:356–64. doi: 10.1093/jn/116.3.356
54. Rycerz K, Jaworska-Adamu JE. Effects of aspartame metabolites on astrocytes and neurons. *Folia Neuropathol*. (2013) 51:10–7. doi: 10.5114/fn.2013.34191
55. Choudhary AK, Lee YY. Neurophysiological symptoms and aspartame: what is the connection? *Nutr Neurosci*. (2018) 21:306–16. doi: 10.1080/1028415X.2017.1288340
56. Walton RG, Hudak R, Green-Waite RJ. Adverse reactions to aspartame: double-blind challenge in patients from a vulnerable population. *Biol Psychiatry*. (1993) 34:13–7. doi: 10.1016/0006-3223(93)90251-8
57. Zhou Y, Cao Z, Yang M, Xi X, Guo Y, Fang M, et al. Comorbid generalized anxiety disorder and its association with quality of life in patients with major depressive disorder. *Sci Rep*. (2017) 7:40511. doi: 10.1038/srep40511
58. Arrigoni E, Brouns F, Amado R. Human gut microbiota does not ferment erythritol. *Br J Nutr*. (2005) 94:643–6. doi: 10.1079/BJN20051546
59. Clemente MG, De Virgiliis S, Kang JS, Macatagney R, Musu MP, Di Pierro MR, et al. Early effects of gliadin on enterocyte intracellular signalling involved in intestinal barrier function. *Gut*. (2003) 52:218–23. doi: 10.1136/gut.52.2.218
60. Fasano A. Zonulin, regulation of tight junctions, and autoimmune diseases. *Ann N Y Acad Sci*. (2012) 1258:25–33. doi: 10.1111/j.1749-6632.2012.06538.x
61. Addolorato G, Mirijello A, D'Angelo C, Leggio L, Ferrulli A, Vonghia L, et al. Social phobia in coeliac disease. *Scand J Gastroenterol*. (2008) 43:410–5. doi: 10.1080/00365520701768802
62. Carta MG, Hardoy MC, Boi MF, Mariotti S, Carpiniello B, Usai P. Association between panic disorder, major depressive disorder and celiac disease: a possible role of thyroid autoimmunity. *J Psychosom Res*. (2002) 53:789–93. doi: 10.1016/S0022-3999(02)00328-8
63. Jackson JR, Eaton WW, Cascella NG, Fasano A, Kelly DL. Neurologic and psychiatric manifestations of celiac disease and gluten sensitivity. *Psychiatr Q*. (2012) 83:91–102. doi: 10.1007/s11126-011-9186-y
64. Fasano A. All disease begins in the (leaky) gut: role of zonulin-mediated gut permeability in the pathogenesis of some chronic inflammatory diseases. *F1000Res*. (2020) 9:F1000 Faculty Rev-69. doi: 10.12688/f1000research.20510.1
65. Stevens BR, Goel R, Seungbum K, Richards EM, Holbert RC, Pepine CJ, et al. Increased human intestinal barrier permeability plasma biomarkers zonulin and FABP2 correlated with plasma LPS and altered gut microbiome in anxiety or depression. *Gut*. (2018) 67:1555–7. doi: 10.1136/gutjnl-2017-314759
66. Addolorato G, Capristo E, Ghittoni G, Valeri C, Masciana R, Ancona C, et al. Anxiety but not depression decreases in coeliac patients after one-year gluten-free diet: a longitudinal study. *Scand J Gastroenterol*. (2001) 36:502–6. doi: 10.1080/00365520119754
67. Menni C, Zierer J, Pallister T, Jackson MA, Long T, Mohney RP, et al. Omega-3 fatty acids correlate with gut microbiome diversity and production of N-carbamylglutamate in middle aged and elderly women. *Sci Rep*. (2017) 7:11079. doi: 10.1038/s41598-017-10382-2
68. Parolini C. Effects of fish n-3 PUFAs on intestinal microbiota and immune system. *Mar Drugs*. (2019) 17:374. doi: 10.3390/md17060374
69. Lauritzen L, Brambilla P, Mazzocchi A, Harslof LB, Ciappolino V, Agostoni C. DHA Effects in brain development and function. *Nutrients*. (2016) 8:6. doi: 10.3390/nu8010006
70. Weiser MJ, Butt CM, Mohajeri MH. Docosahexaenoic acid and cognition throughout the lifespan. *Nutrients*. (2016) 8:99. doi: 10.3390/nu8020099
71. Kaliannan K, Li XY, Wang B, Pan Q, Chen CY, Hao L, et al. Multi-omic analysis in transgenic mice implicates omega-6/omega-3 fatty acid imbalance as a risk factor for chronic disease. *Commun Biol*. (2019) 2:276. doi: 10.1038/s42003-019-0521-4
72. Song C, Li X, Kang Z, Kadotomi Y. Omega-3 fatty acid ethyl-eicosapentaenoate attenuates IL-1beta-induced changes in dopamine and metabolites in the shell of the nucleus accumbens: involved with PLA2 activity and corticosterone secretion. *Neuropsychopharmacology*. (2007) 32:736–44. doi: 10.1038/sj.npp.1301117
73. Song C, Li X, Leonard BE, Horrobin DF. Effects of dietary n-3 or n-6 fatty acids on interleukin-1beta-induced anxiety, stress, and inflammatory responses in rats. *J Lipid Res*. (2003) 44:1984–91. doi: 10.1194/jlr.M300217-JLR200
74. Zemdegs J, Rainer Q, Grossmann CP, Rousseau-Ralliard D, Grynberg A, Ribeiro E, et al. Anxiolytic- and antidepressant-like effects of fish oil-enriched diet in brain-derived neurotrophic factor deficient mice. *Front Neurosci*. (2018) 12:974. doi: 10.3389/fnins.2018.00974
75. Green P, Hermesh H, Monselise A, Marom S, Presburger G, Weizman A. Red cell membrane omega-3 fatty acids are decreased in nondepressed patients with social anxiety disorder. *Eur Neuropsychopharmacol*. (2006) 16:107–13. doi: 10.1016/j.euroneuro.2005.07.005
76. Liu JJ, Galfalvy HC, Cooper TB, Oquendo MA, Grunebaum MF, Mann JJ, et al. Omega-3 polyunsaturated fatty acid (PUFA) status in major depressive disorder with comorbid anxiety disorders. *J Clin Psychiatry*. (2013) 74:732–8. doi: 10.4088/JCP.12m07970
77. Kiecolt-Glaser JK, Belury MA, Andridge R, Malarkey WB, Glaser R. Omega-3 supplementation lowers inflammation and anxiety in medical students: a randomized controlled trial. *Brain Behav Immun*. (2011) 25:1725–34. doi: 10.1016/j.bbi.2011.07.229
78. Su KP, Tseng PT, Lin PY, Okubo R, Chen TY, Chen YW, et al. Association of use of omega-3 polyunsaturated fatty acids with changes in severity of anxiety symptoms: a systematic review and meta-analysis. *JAMA Netw Open*. (2018) 1:e182327. doi: 10.1001/jamanetworkopen.2018.2327
79. Anderson BM, Ma DW. Are all n-3 polyunsaturated fatty acids created equal? *Lipids Health Dis*. (2009) 8:33. doi: 10.1186/1476-511X-8-33
80. Brenna JT. Efficiency of conversion of alpha-linolenic acid to long chain n-3 fatty acids in man. *Curr Opin Clin Nutr Metab Care*. (2002) 5:127–32. doi: 10.1097/00075197-200203000-00002
81. Brenna JT, Salem N Jr., Sinclair AJ, Cunnane SC, International Society for the Study of Fatty, Acide and Lipids. α-Linolenic acid supplementation and conversion to n-3 long-chain polyunsaturated fatty acids in humans. *Prostaglandins Leukot Essent Fatty Acids*. (2009) 80:85–91. doi: 10.1016/j.plefa.2009.01.004
82. Barros MP, Poppe SC, Bondan EF. Neuroprotective properties of the marine carotenoid astaxanthin and omega-3 fatty acids, and perspectives for the natural combination of both in krill oil. *Nutrients*. (2014) 6:1293–317. doi: 10.3390/nu6031293
83. Patrick RP. Role of phosphatidylcholine-DHA in preventing APOE4-associated Alzheimer's disease. *FASEB J*. (2019) 33:1554–64. doi: 10.1096/fj.201801412R
84. Fusar-Poli L, Vozza L, Gabbiadini A, Vanella A, Concas I, Tinacci S, et al. Curcumin for depression: a meta-analysis. *Crit Rev Food Sci Nutr*. (2019) 60:2643–53. doi: 10.1080/10408398.2019.1653260
85. Zhou H, Beevers CS, Huang S. The targets of curcumin. *Curr Drug Targets*. (2011) 12:332–47. doi: 10.2174/138945011794815356
86. Di Meo F, Margarucci S, Galderisi U, Crispi S, Peluso G. Curcumin, gut microbiota, and neuroprotection. *Nutrients*. (2019) 11:2426. doi: 10.3390/nu11102426
87. Ambegaokar SS, Wu L, Alamshahi K, Lau J, Jazayeri L, Chan S, et al. Curcumin inhibits dose-dependently and time-dependently neuroglial cell proliferation and growth. *Neuro Endocrinol Lett*. (2003) 24:469–73.
88. Marquardt JU, Gomez-Quiroz L, Arreguin Camacho LO, Pinna F, Lee YH, Kitade M, et al. Curcumin effectively inhibits oncogenic NF-kappaB signaling and restrains stemness features in liver cancer. *J Hepatol*. (2015) 63:661–9. doi: 10.1016/j.jhep.2015.04.018
89. Shakibaei M, John T, Schulze-Tanzil G, Lehmann I, Mobasheri A. Suppression of NF-kappaB activation by curcumin leads to inhibition of expression of cyclo-oxygenase-2 and matrix metalloproteinase-9 in human articular chondrocytes: implications for the treatment of osteoarthritis. *Biochem Pharmacol*. (2007) 73:1434–45. doi: 10.1016/j.bcp.2007.01.005
90. Yin H, Guo Q, Li X, Tang T, Li C, Wang H, et al. Curcumin Suppresses IL-1beta secretion and prevents inflammation through inhibition of the NLRP3 inflammasome. *J Immunol*. (2018) 200:2835–46. doi: 10.4049/jimmunol.1701495
91. Xia X, Cheng G, Pan Y, Xia ZH, Kong LD. Behavioral, neurochemical and neuroendocrine effects of the ethanolic extract from *Curcuma longa* L. in the mouse forced swimming test. *J Ethnopharmacol*. (2007) 110:356–63. doi: 10.1016/j.jep.2006.09.042

92. Reuter S, Gupta SC, Park B, Goel A, Aggarwal BB. Epigenetic changes induced by curcumin and other natural compounds. *Genes Nutr*. (2011) 6:93–108. doi: 10.1007/s12263-011-0222-1
93. Noorafshan A, Vafabin M, Karbalay-Doust S, Asadi-Golshan R. Efficacy of curcumin in the modulation of anxiety provoked by sulfite, a food preservative, in rats. *Prev Nutr Food Sci*. (2017) 22:144–8. doi: 10.3746/pnf.2017.22.2.144
94. Benammi H, El Hiba O, Romane A, Gamrani H. A blunted anxiolytic like effect of curcumin against acute lead induced anxiety in rat: involvement of serotonin. *Acta Histochem*. (2014) 116:920–5. doi: 10.1016/j.acthis.2014.03.002
95. Lee B, Lee H. Systemic administration of curcumin affect anxiety-related behaviors in a rat model of posttraumatic stress disorder via activation of serotonergic systems. *Evid Based Complement Alternat Med*. (2018) 2018:9041309. doi: 10.1155/2018/9041309
96. Asadi S, Gholami MS, Siassi F, Qorbani M, Sotoudeh G. Beneficial effects of nano-curcumin supplement on depression and anxiety in diabetic patients with peripheral neuropathy: a randomized, double-blind, placebo-controlled clinical trial. *Phytother Res*. (2020) 34:896–903. doi: 10.1002/ptr.6571
97. Esmaily H, Sahebkar A, Iranshahi M, Ganjali S, Mohammadi A, Ferns G, et al. An investigation of the effects of curcumin on anxiety and depression in obese individuals: a randomized controlled trial. *Chin J Integr Med*. (2015) 21:332–8. doi: 10.1007/s11655-015-2160-z
98. Nelson KM, Dahlin JL, Bisson J, Graham J, Pauli GF, Walters MA. The essential medicinal chemistry of curcumin. *J Med Chem*. (2017) 60:1620–37. doi: 10.1021/acs.jmedchem.6b00975
99. Yang KY, Lin LC, Tseng TY, Wang SC, Tsai TH. Oral bioavailability of curcumin in rat and the herbal analysis from Curcuma longa by LC-MS/MS. *J Chromatogr B Analyt Technol Biomed Life Sci*. (2007) 853:183–9. doi: 10.1016/j.jchromb.2007.03.010
100. Sun M, Su X, Ding B, He X, Liu X, Yu A, et al. Advances in nanotechnology-based delivery systems for curcumin. *Nanomedicine*. (2012) 7:1085–100. doi: 10.2217/nnm.12.80
101. Shoba G, Joy D, Joseph T, Majeed M, Rajendran R, Srinivas PS. Influence of piperine on the pharmacokinetics of curcumin in animals and human volunteers. *Planta Med*. (1998) 64:353–6. doi: 10.1055/s-2006-957450
102. Wu A, Noble EE, Tyagi E, Ying Z, Zhuang Y, Gomez-Pinilla F. Curcumin boosts DHA in the brain: implications for the prevention of anxiety disorders. *Biochim Biophys Acta*. (2015) 1852:951–61. doi: 10.1016/j.bbadis.2014.12.005
103. Schmid A, Walther B. Natural vitamin D content in animal products. *Adv Nutr*. (2013) 4:453–62. doi: 10.3945/an.113.003780
104. Ginde AA, Liu MC, Camargo CA Jr. Demographic differences and trends of vitamin D insufficiency in the US population. 1988-2004. *Arch Intern Med*. (2009) 169:626–32. doi: 10.1001/archinternmed.2008.604
105. Annweiler C. Vitamin D in dementia prevention. *Ann N Y Acad Sci*. (2016) 1367:57–63. doi: 10.1111/nyas.13058
106. Brewer LD, Thibault V, Chen KC, Langub MC, Landfield PW, Porter NM. Vitamin D hormone confers neuroprotection in parallel with downregulation of L-type calcium channel expression in hippocampal neurons. *J Neurosci*. (2001) 21:98–108. doi: 10.1523/JNEUROSCI.21-01-00098.2001
107. Kalueff AV, Tuohimaa P. Neurosteroid hormone vitamin D and its utility in clinical nutrition. *Curr Opin Clin Nutr Metab Care*. (2007) 10:12–9. doi: 10.1097/MCO.0b013e328010ca18
108. Kesby JP, Turner KM, Alexander S, Eyles DW, McGrath JJ, Burne THJ. Developmental vitamin D deficiency alters multiple neurotransmitter systems in the neonatal rat brain. *Int J Dev Neurosci*. (2017) 62:1–7. doi: 10.1016/j.ijdevneu.2017.07.002
109. Lima LAR, Lopes MJP, Costa RO, Lima FAV, Neves KRT, Calou IBF, et al. Vitamin D protects dopaminergic neurons against neuroinflammation and oxidative stress in hemiparkinsonian rats. *J Neuroinflammation*. (2018) 15:249. doi: 10.1186/s12974-018-1266-6
110. Patrick RP, Ames BN. Vitamin D and the omega-3 fatty acids control serotonin synthesis and action, part 2: relevance for ADHD, bipolar disorder, schizophrenia, and impulsive behavior. *FASEB J*. (2015) 29:2207–22. doi: 10.1096/fj.14-268342
111. Gezen-Ak D, Dursun E, Yilmazer S. The effect of vitamin D treatment on Nerve Growth Factor (NGF) release from hippocampal neurons. *Noro Psikiyatr Ars*. (2014) 51:157–62. doi: 10.4274/npa.y7076
112. Khairy EY, Attia MM. Protective effects of vitamin D on neurophysiologic alterations in brain aging: role of brain-derived neurotrophic factor (BDNF). *Nutr Neurosci*. (2019) 16:1–10. doi: 10.1080/1028415X.2019.1665854
113. Aranow C. Vitamin D and the immune system. *J Investig Med*. (2011) 59:881–6. doi: 10.2310/JIM.0b013e31821b8755
114. Gominak SC. Vitamin D deficiency changes the intestinal microbiome reducing B vitamin production in the gut. The resulting lack of pantothenic acid adversely affects the immune system, producing a "pro-inflammatory" state associated with atherosclerosis and autoimmunity. *Med Hypotheses*. (2016) 94:103–7. doi: 10.1016/j.mehy.2016.07.007
115. Tabatabaeizadeh SA, Tafazoli N, Ferns GA, Avan A, Ghayour-Mobarhan M. Vitamin D, the gut microbiome and inflammatory bowel disease. *J Res Med Sci*. (2018) 23:75. doi: 10.4103/jrms.JRMS_606_17
116. Yamamoto EA, Jorgensen TN. Relationships between vitamin D, gut microbiome, and systemic autoimmunity. *Front Immunol*. (2019) 10:3141. doi: 10.3389/fimmu.2019.03141
117. Yin K, Agrawal DK. Vitamin D and inflammatory diseases. *J Inflamm Res*. (2014) 7:69–87. doi: 10.2147/JIR.S63898
118. Cieslak K, Feingold J, Antonius D, Walsh-Messinger J, Dracxler R, Rosedale M, et al. Low vitamin D levels predict clinical features of schizophrenia. *Schizophr Res*. (2014) 159:543–5. doi: 10.1016/j.schres.2014.08.031
119. Maddock J, Berry DJ, Geoffroy MC, Power C, Hypponen E. Vitamin D and common mental disorders in mid-life: cross-sectional and prospective findings. *Clin Nutr*. (2013) 32:758–64. doi: 10.1016/j.clnu.2013.01.006
120. Bicikova M, Duskova M, Vitku J, Kalvachova B, Ripova D, Mohr P, et al. Vitamin D in anxiety and affective disorders. *Physiol Res*. (2015) 64:S101–103. doi: 10.33549/physiolres.933082
121. Han B, Zhu FX, Yu HF, Liu S, Zhou JL. Low serum levels of vitamin D are associated with anxiety in children and adolescents with dialysis. *Sci Rep*. (2018) 8:5956. doi: 10.1038/s41598-018-24451-7
122. Wu C, Ren W, Cheng J, Zhu B, Jin Q, Wang L, et al. Association between serum levels of vitamin D and the risk of post-stroke anxiety. *Medicine*. (2016) 95:e3566. doi: 10.1097/MD.0000000000003566
123. Eid A, Khoja S, AlGhamdi S, Alsufiani H, Alzeben F, Alhejaili N, et al. Vitamin D supplementation ameliorates severity of generalized anxiety disorder (GAD). *Metab Brain Dis*. (2019) 34:1781–6. doi: 10.1007/s11011-019-00486-1
124. Fazelian S, Amani R, Paknahad Z, Kheiri S, Khajehali L. Effect of vitamin D supplement on mood status and inflammation in vitamin D deficient type 2 diabetic women with anxiety: a randomized clinical trial. *Int J Prev Med*. (2019) 10:17. doi: 10.4103/ijpvm.IJPVM_174_18
125. Armstrong DJ, Meenagh GK, Bickle I, Lee AS, Curran ES, Finch MB. Vitamin D deficiency is associated with anxiety and depression in fibromyalgia. *Clin Rheumatol*. (2007) 26:551–4. doi: 10.1007/s10067-006-0348-5
126. Athinarayanan SJ, Adams RN, Hallberg SJ, McKenzie AL, Bhanpuri NH, Campbell WW, et al. Long-term effects of a novel continuous remote care intervention including nutritional ketosis for the management of type 2 diabetes: a 2-year non-randomized clinical trial. *Front Endocrinol*. (2019) 10:348. doi: 10.3389/fendo.2019.00348
127. Cunnane SC, Courchesne-Loyer A, Vandenberghe C, St-Pierre V, Fortier M, Hennebelle M, et al. Can ketones help rescue brain fuel supply in later life? Implications for cognitive health during aging and the treatment of Alzheimer's disease. *Front Mol Neurosci*. (2016) 9:53. doi: 10.3389/fnmol.2016.00053
128. Fortier M, Castellano CA, Croteau E, Langlois F, Bocti C, St-Pierre V, et al. A ketogenic drink improves brain energy and some measures of cognition in mild cognitive impairment. *Alzheimers Dement*. (2019) 15:625–34. doi: 10.1016/j.jalz.2018.12.017
129. Hyde PN, Sapper TN, Crabtree CD, LaFountain RA, Bowling ML, Buga A, et al. Dietary carbohydrate restriction improves metabolic syndrome independent of weight loss. *JCI Insight*. (2019) 4:e128308. doi: 10.1172/jci.insight.128308

130. Newport MT, VanItallie TB, Kashiwaya Y, King MT, Veech RL. A new way to produce hyperketonemia: use of ketone ester in a case of Alzheimer's disease. *Alzheimers Dement*. (2015) 11:99–103. doi: 10.1016/j.jalz.2014.01.006
131. Phillips MCL, Murtagh DKJ, Gilbertson LJ, Asztely FJS, Lynch CDP. Low-fat versus ketogenic diet in Parkinson's disease: a pilot randomized controlled trial. *Mov Disord*. (2018) 33:1306–14. doi: 10.1002/mds.27390
132. Levy RG, Cooper PN, Giri P. Ketogenic diet and other dietary treatments for epilepsy. *Cochrane Database Syst Rev*. (2012). 2:CD001903. doi: 10.1002/14651858.CD001903.pub2
133. Liu H, Yang Y, Wang Y, Tang H, Zhang F, Zhang Y, et al. Ketogenic diet for treatment of intractable epilepsy in adults: a meta-analysis of observational studies. *Epilepsia Open*. (2018) 3:9–17. doi: 10.1002/epi4.12098
134. Norwitz NG, Hu MT, Clarke K. The mechanisms by which the ketone body D-beta-hydroxybutyrate may improve the multiple cellular pathologies of Parkinson's disease. *Front Nutr*. (2019) 6:63. doi: 10.3389/fnut.2019.00063
135. Vanitallie TB, Nonas C, Di Rocco A, Boyar K, Hyams K, Heymsfield SB. Treatment of Parkinson disease with diet-induced hyperketonemia: a feasibility study. *Neurology*. (2005) 64:728–30. doi: 10.1212/01.WNL.0000152046.11390.45
136. Rusek M, Pluta R, Ulamek-Koziol M, Czuczwar SJ. Ketogenic diet in Alzheimer's disease. *Int J Mol Sci*. (2019) 20:3892. doi: 10.3390/ijms20163892
137. Kraeuter A-K, Phillips R, Sarnyai Z. Ketogenic therapy in neurodegenerative and psychiatric disorders: from mice to men. *Prog Neuro Psychopharmacol Biol Psychiatry*. (2020) 101:109913. doi: 10.1016/j.pnpbp.2020.109913
138. Cheng CW, Biton M, Haber AL, Gunduz N, Eng G, Gaynor LT, et al. Ketone body signaling mediates intestinal stem cell homeostasis and adaptation to diet. *Cell*. (2019) 178:1115–31 e1115. doi: 10.1016/j.cell.2019.07.048
139. Forsythe CE, Phinney SD, Fernandez ML, Quann EE, Wood RJ, Bibus DM, et al. Comparison of low fat and low carbohydrate diets on circulating fatty acid composition and markers of inflammation. *Lipids*. (2008) 43:65–77. doi: 10.1007/s11745-007-3132-7
140. Lindefeldt M, Eng A, Darban H, Bjerkner A, Zetterstrom CK, Allander T, et al. The ketogenic diet influences taxonomic and functional composition of the gut microbiota in children with severe epilepsy. *NPJ Biofilms Microbiomes*. (2019) 5:5. doi: 10.1038/s41522-018-0073-2
141. Xie Z, Zhang D, Chung D, Tang Z, Huang H, Dai L, et al. Metabolic regulation of gene expression by histone lysine beta-hydroxybutyrylation. *Mol Cell*. (2016) 62:194–206. doi: 10.1016/j.molcel.2016.03.036
142. Youm YH, Nguyen KY, Grant RW, Goldberg EL, Bodogai M, Kim D, et al. The ketone metabolite beta-hydroxybutyrate blocks NLRP3 inflammasome-mediated inflammatory disease. *Nat Med*. (2015) 21:263–9. doi: 10.1038/nm.3804
143. Ari C, Kovacs Z, Juhasz G, Murdun C, Goldhagen CR, Koutnik AP, et al. Exogenous ketone supplements reduce anxiety-related behavior in Sprague-Dawley and Wistar Albino Glaxo/Rijswijk rats. *Front Mol Neurosci*. (2016) 9:137. doi: 10.3389/fnmol.2016.00137
144. Hollis F, Mitchell ES, Canto C, Wang D, Sandi C. Medium chain triglyceride diet reduces anxiety-like behaviors and enhances social competitiveness in rats. *Neuropharmacology*. (2018) 138:245–56. doi: 10.1016/j.neuropharm.2018.06.017
145. Liu Y, Cheng A, Li YJ, Yang Y, Kishimoto Y, Zhang S, et al. SIRT3 mediates hippocampal synaptic adaptations to intermittent fasting and ameliorates deficits in APP mutant mice. *Nat Commun*. (2019) 10:1886. doi: 10.1038/s41467-019-09897-1
146. Smith JE, Lawrence AD, Diukova A, Wise RG, Rogers PJ. Storm in a coffee cup: caffeine modifies brain activation to social signals of threat. *Soc Cogn Affect Neurosci*. (2012) 7:831–40. doi: 10.1093/scan/nsr058
147. Wikoff D, Welsh BT, Henderson R, Brorby GP, Britt J, Myers E, et al. Systematic review of the potential adverse effects of caffeine consumption in healthy adults, pregnant women, adolescents, and children. *Food Chem Toxicol*. (2017) 109(Pt. 1):585–648. doi: 10.1016/j.fct.2017.04.002
148. Foster JA, McVey Neufeld KA. Gut-brain axis: how the microbiome influences anxiety and depression. *Trends Neurosci*. (2013) 36:305–12. doi: 10.1016/j.tins.2013.01.005
149. Taylor AM, Holscher HD. A review of dietary and microbial connections to depression, anxiety, and stress. *Nutr Neurosci*. (2020) 23:237–50. doi: 10.1080/1028415X.2018.1493808
150. Selhub EM, Logan AC, Bested AC. Fermented foods, microbiota, and mental health: ancient practice meets nutritional psychiatry. *J Physiol Anthropol*. (2014) 33:2. doi: 10.1186/1880-6805-33-2
151. Boyle NB, Lawton C, Dye L. The effects of magnesium supplementation on subjective anxiety and stress-a systematic review. *Nutrients*. (2017) 9:429. doi: 10.3390/nu9050429
152. Grases G, Perez-Castello JA, Sanchis P, Casero A, Perello J, Isern B, et al. Anxiety and stress among science students. Study of calcium and magnesium alterations. *Magnes Res*. (2006) 19:102–6.
153. Lindseth G, Helland B, Caspers J. The effects of dietary tryptophan on affective disorders. *Arch Psychiatr Nurs*. (2015) 29:102–7. doi: 10.1016/j.apnu.2014.11.008

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Glymphatic Dysfunction: A Bridge Between Sleep Disturbance and Mood Disorders

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Mounting evidence demonstrates a close relationship between sleep disturbance and mood disorders, including major depression disorder (MDD) and bipolar disorder (BD). According to the classical two-process model of sleep regulation, circadian rhythms driven by the light–dark cycle, and sleep homeostasis modulated by the sleep–wake cycle are disrupted in mood disorders. However, the exact mechanism of interaction between sleep and mood disorders remains unclear. Recent discovery of the glymphatic system and its dynamic fluctuation with sleep provide a plausible explanation. The diurnal variation of the glymphatic circulation is dependent on the astrocytic activity and polarization of water channel protein aquaporin-4 (AQP4). Both animal and human studies have reported suppressed glymphatic transport, abnormal astrocytes, and depolarized AQP4 in mood disorders. In this study, the "glymphatic dysfunction" hypothesis which suggests that the dysfunctional glymphatic pathway serves as a bridge between sleep disturbance and mood disorders is proposed.

Keywords: glymphatic system, depression, sleep, bipolar disorder, astrocyte, aquaporin-4

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INTRODUCTION

Mood disorders are a group of complex debilitating psychiatric diseases identified by symptoms centered on markedly disrupted emotions, including major depressive disorder (MDD) and bipolar disorder (BD) (1). Due to their high prevalence, the risk for recurrence and suicide, they remain a serious health concern worldwide (2, 3). However, the exact neurobiological mechanisms underlying mood disorders remain unclear, resulting in unsatisfactory treatment (2, 3).

Sleep disturbance is a common concomitant and prodromal symptom of mood disorders (1, 4, 5). Specifically, both the two processes of sleep regulation—circadian oscillator and sleep pressure—are disrupted in mood disorders (4, 6). On one hand, circadian rhythms are approximately 24-h patterns in physiology and behavior, which are regulated by molecular clocks in the suprachiasmatic nuclei (SCN) of the hypothalamus (7). Mounting evidence suggests that there are abnormalities of the clock genes in mood disorders, such as single nucleotide polymorphisms (SNPs) (8–13), gene expression (14, 15), and gene–gene interactions (8). Excitingly, antidepressants including fluoxetine (16–18), ketamine (19, 20), and agomelatine (21) can reset the circadian clock along with the amelioration of mood symptoms. On the other hand, sleep pressure fluctuates with the sleep–wake cycle (6). Whereas, disturbance of the sleep–wake cycle has often been reported in mood disorders (22–24). Disturbed sleep architecture, especially decreased percentage of stage 3

non-rapid eye movement sleep (NREM III), represents decreased homeostatic drive for sleep (6). Actually, NREM III serves as a deep and recovery sleep, playing a vital role in the operation of the glymphatic system, and clearance of metabolic wastes (25, 26).

The glymphatic system is considered as an effective waste-removal system in the brain, which facilitates the exchange between the cerebrospinal fluid (CSF) and interstitial fluid (ISF), along with the potentially neurotoxic proteins such as amyloid-β (Aβ) (27), tau protein (28), and α-synuclein (29). Therefore, glymphatic impairment caused by sleep disturbance results in protein aggregation and increased risk for neurological diseases, such as Alzheimer's disease (AD) (30), Parkinson's disease (PD) (31), stroke (32, 33), and idiopathic normal cranial pressure hydrocephalus (iNPH) (34, 35). The water channel protein aquaporin-4 (AQP-4) is highly expressed on astrocytic endfeet and exerts significant influence in glymphatic transport (36). At present, accumulating evidence suggests the presence of abnormal astrocytes (37–43), depolarized AQP-4 (44–46), and dysfunctional glymphatic system (47, 48) in mood disorders. Therefore, we speculated that glymphatic dysfunction serves as an imperative intermediary factor between sleep disturbance and mood disorders.

In this study, we integrated available data from both animal and human studies regarding sleep in mood disorders and highlighted the core role of the glymphatic system. Furthermore, we discussed the glymphatic system dysfunction in mood disorders and identified the potential therapeutic opportunities for mood disorders based on sleep regulation and the glymphatic pathway.

SLEEP DISTURBANCE AND MOOD DISORDERS

The Model of Sleep Regulation

The classical two-process model of sleep regulation was first proposed by Borbély, and it consists of the process controlled by the circadian oscillator (Process C) and the homeostatic drive for the sleep–wake cycle (Process S). The two processes closely interact with each other but are also relatively independent (6) (**Figure 1**).

Circadian rhythms (Process C) are approximately 24-h rhythms in physiology and behavior, which are primarily driven by a hierarchy of cellular pacemakers located in the SCN (7). The most common measurements of the circadian rhythm are core body temperature and endogenous melatonin, other than the chronotype or morningness-eveningness (49). In fact, circadian rhythms are generated by a molecular clock in a network of positive and negative feedback loops. At the core of SCN timekeeping, the heterodimeric transcription factors CLOCK/BMAL1 translated from *CLOCK* and *Brain and muscle ARNT-like 1 (BMAL1)* genes, activate the *Period (PER1–3*) and *Cryptochrome* (*CRY1–2*) genes and initiate the circadian cycle. In turn, the dimer complex protein PER/CRY inhibit the activity of the CLOCK/BMAL1 proteins (50), exerting dominant effect in the negative feedback. As a critical complementary loop, the *BMAL1* transcription is activated by the retinoic acid-related orphan receptor (ROR) protein at night, and repressed by the nuclear receptors REV-ERB α/β (encoded by *NR1D1/2 genes*) at daytime (51), respectively. In addition, other clock genes also participate in the regulation of circadian rhythms. The *neuronal PAS domain protein 2 (NPAS2)* functions similarly to *CLOCK*, while *albumin gene D-site binding protein (DBP)* acts cooperatively with CLOCK/BMAL1 (52, 53). The *casein kinase I isoform δ/ε (CSNK1D/E)* regulates levels of PER by phosphorylation-mediated degradation, and thus inhibits the activity of CLOCK/BMAL1 (54). The *basic helix-loop-helix family 40/41* (*BHLHE40/41*, also known as *DEC1/2*) suppresses *PER* gene transcription via competing with *CLOCK-BMAL1* for *e-box* element binding (55). The *TIMELESS* gene is also conceived required for circadian rhythmicity, however, the exact role in human clockwork is still unclear (56). These circadian genes expression rise and fall in rhythm, contributing to the regulation of 24-h physical and behavioral cycles (15).

Process S, also referred to as the sleep pressure gradually accumulates during wakefulness and declines during sleep (6). Especially, as deep sleep (NREM III) dominates in the early phases of sleep and dwindles with decreasing sleep pressure in the late phases. Conversely, sleep deficit such as sleep deprivation results in a longer and deeper NREM III to achieve recovery (57), implying greater sleep pressure. Therefore, NREM III sleep is considered as a representation of sleep pressure (6). Sleep electroencephalogram (EEG) and actigraphy are effective assessments of sleep pressure to detect sleep architecture.

According to the two-process model, proper alignment of Process C and S is essential for recovery sleep. Otherwise, the daytime sleep fails to fulfill the homeostatic sleep drive, manifesting as lighter and lacking of recovery sleep (NREM III) (58). Moreover, the daytime sleep decreases sleep pressure, causing a negative influence on the more effective nighttime sleep.

Sleep Disturbance in Mood Disorders

Disturbed Circadian Rhythms in Mood Disorders

Disruptions of the circadian rhythms are common in people exposed to jet-lag, social jet-lag, shift-work, as well as light

Abbreviations: MDD, major depression disorder; BD, bipolar disorder; AQP4, aquaporin-4; SCN, suprachiasmatic nuclei; SNP, single nucleotide polymorphisms; NREM III, stage 3 non-rapid eye movement sleep; CSF, cerebrospinal fluid; ISF, interstitial fluid; Aβ, amyloid-β; AD, Alzheimer's disease; PD, Parkinson's disease; iNPH, idiopathic normal cranial pressure hydrocephalus; EEG, electroencephalogram; DLMO, dim light melatonin onset; MDR, multifactor-dimensionality reduction; REM, rapid eye movement sleep; MT, melatonin; SSRI, selective serotonin reuptake inhibitor; LHb, lateral habenula; ROS, reactive oxygen species; CNS, central nervous system; PET, positron emission tomography; RBD, REM sleep behavior disorder; DTI, diffusion tensor imaging; ALPS, analysis along the perivascular space; CUMS, chronic unpredictable mild stress; PUFA, polyunsaturated fatty acid; GFAP, glial fibrillary acidic protein; TMS, transcranial magnetic stimulation; BA, Brodmann area; ADC uh, the apparent diffusion coefficient from ultra-high b-values; eDWI, enhanced diffusion-weighted imaging; SCP, superior cerebellar peduncles; PVS, perivascular space; ALDH1L1, aldehyde dehydrogenase 1 family member L1; qPCR, quantitative polymerase chain reaction.

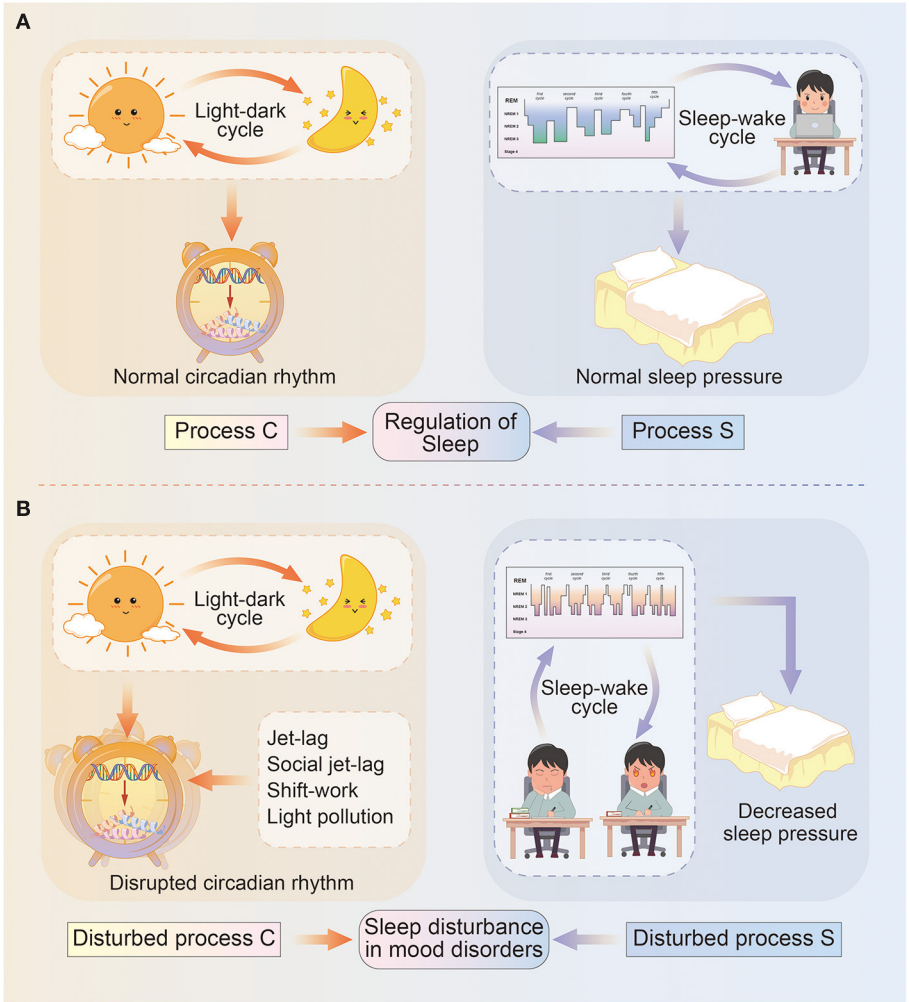

FIGURE 1 | Diagram illustrating the two-process model of sleep regulation. **(A)** In normal circumstances, sleep regulation depends on the interaction between process C and process S. Specifically, process C represents the circadian rhythm driven by light–dark cycles, and circadian genes deliver circadian information via transcriptional–translational feedback loops and control physical and behavioral states. Process S means sleep pressure influenced by sleep–wake cycles, and include sleep architecture and daytime wakefulness. **(B)** In mood disorders, circadian rhythms (process C) are misaligned with light–dark cycles due to events such as jet-lag, social jet-lag, shift-work, light pollution, and so on; while sleep pressure (process S) is remarkably decreased due to longer sleep onset latency, a higher percentage of REM sleep, daytime sleepiness, or reduced need for sleep.

pollution (light exposure at night) (59), and may lead to mood alterations (60, 61). Recently, a large population cross-sectional study (n = 91,105) using a wrist-worn accelerometer reported that lower relative amplitude of the circadian rhythm is associated with the lifetime prevalence of both MDD and BD (4). Individuals with circadian misalignment have higher depressive scores (62, 63). Moreover, a strong correlation between depressive symptoms and advances in dim light melatonin onset (DLMO) has been reported following an adjunctive multimodal chronobiological intervention organically combining psychoeducation, behavioral manipulation, and agomelatine intake (64). Bipolar disorder patients show delayed and decreased melatonin secretion during depressive and euthymic episodes (24, 65), with impaired psychosocial functioning and worse quality of life (24). In addition, manic and mixed episodes present with sustained phase advances, as well as a lower degree of rhythmicity corresponding to the severity of manic symptoms (66, 67). Apart from the daily (solar) cycle mentioned above, the lunar tidal cycles seem to entrain the mood cycles. In patients with rapid cycling BD, the periodicities in mood cycles have been observed to be synchronous with multiples of bi-weekly lunar tidal cycles (68).

The relationship between circadian rhythms and mood disorders is further supported by emerging genomic studies. In depressive cases, genetic association analyses have found SNPs in *PER2* (10870), *BMAL1* (rs2290035), *NPAS2* (S471L), *CRY2* (rs10838524), *BHLHB2* (rs6442925), *CLOCK* (rs12504300), *CSNK1E* (rs135745), and *TIMELESS* (rs4630333 and rs1082214) (8, 9, 13). Single nucleotide polymorphisms in *CSNK1E* (rs135745), *TIMELESS* rs4630333, *CRY2* (rs10838524), *PER3*

(rs707467 and rs10462020), *RORB* (rs1157358, rs7022435, rs3750420, and rs3903529), *REV-ERBA* (rs2314339) are strongly related to BD (8, 10–12, 69). In particular, *CLOCK* SNP rs1801260 contribute to the recurrence of mood episodes, while *CRY2* SNP rs10838524 is significantly associated to rapid cycling BD (10, 70). Moreover, the arrhythmic expression of circadian genes including *BMAL1*, *PER1–3, REV-ERBA*, *DBP,* and *BHLHE40/41*, has been observed in postmortem brain tissues of MDD patients (15). Reduced amplitude of rhythmic expression for BMAL1, REV-ERBα, and DBP has been reported in fibroblast cultures of 12 BD patients (14). Recently, Park et al. have explored gene–gene interactions of clock genes using the non-parametric model-free multifactor-dimensionality reduction (MDR) method, and revealed optimal SNP combination models for predicting mood disorders (8). Specifically, the four-locus model differs between MDD (*TIMELESS* rs4630333, *CSNK1E* rs135745, *BHLHB2* rs2137947, *CSNK1E* rs2075984) and BD (*TIMELESS* rs4630333, *CSNK1E* rs135745, *PER3* rs228669, *CLOCK* rs12649507), supporting the clinical observation of different circadian characteristics in two disorders.

The Unbalanced Homeostatic Drive of Sleep in Mood Disorders

The sleep–wake cycle is significantly affected by mood disorders. Firstly, a disturbed sleep–wake cycle is one of the most common diagnostic criteria for mood disorders. Individuals suffering from manic or hypomanic episodes often show a reduced demand for the sleep, while depressive patients experience insomnia or hypersomnia (1). Delayed sleep–wake phase and evening chronotype is common in patients with mood disorders (24, 71, 72), and strongly associated with the severity of mood symptoms (73). Sleep deficits predict a poor prognosis with a higher risk of suicide (74). Furthermore, both polysomnography and self-reported studies have revealed longer sleep onset latency, a higher percentage of rapid eye movement (REM) sleep, more fragmentation of the sleep/wake rhythm, and daytime dysfunction in patients with mood disorders during the remission state relative to healthy controls (22, 75, 76). More importantly, sleep disturbance often serves as a prodrome of manic or depressive episodes. Several retrospective studies have revealed that sleep disturbance is the most robust early symptom of manic episodes and the sixth most common prodromal symptom of manic episodes (5, 23). Recently, a 10-year prospective study among adolescents and young adults reported that the sleep problem is a risk factor for the development of BD (77). Sleep abnormalities have also been highly related to subsequent depression (23, 78, 79). Moreover, sleep deprivation is reported to trigger manic-like behavior in animal models (80). Thus, some researchers speculate that a disturbed sleep–wake cycle is probably a causal factor triggering mood episodes. However, because of ethical reasons, sleep generally cannot be manipulated in human research and this weakens the causal evidence between the sleep–wake rhythm and mood disorders.

Chronotherapeutic Treatments in Mood Disorders

In response to the vital roles that Process C and S play in the onset and course of mood disorders, chronotherapeutic interventions have been successfully used. Sleep deprivation combined with bright light therapy has been implicated in improving depressive symptoms (72, 81–83), while virtual darkness therapy via blue-light-blocking increases the regularity of sleep and a rapid decline in manic symptoms (84). These treatments exert great influence on mood recovery by resetting the circadian clock. Also, the hormone melatonin (MT) secreted by the pineal gland acts on the circadian clock via MT1 receptors (85, 86), while the MT agonist agomelatine shows important properties for phase shifts of the clock and anti-depressive effects (21). Additionally, agomelatine functions as an antagonist for 5-HT_{2c} receptors and modulates the master SCN clock via 5-HT innervations (87, 88). Similarly, other antidepressants can regulate the expression of the clock genes and thus affect the circadian rhythms (89). Fluoxetine, a selective serotonin reuptake inhibitor (SSRI) can shift electrical rhythms of the SCN and thus affect the behavior rhythm (16–18). Ketamine results in a rapid increase in glutamate level in the SCN and directly acts on NMDA receptors of the circadian clock in the epi-thalamic lateral habenula (LHb) (19, 20), suggesting that the rapid anti-depressive effects of ketamine might also be through the resetting of the circadian system (90). However, the mood stabilizer lithium is considered a clock-modifying drug in that it delays the sleep–wake cycle in healthy human and increase the length of the circadian period in non-human primates (91, 92). At the molecular level, lithium treatment can not only regulate the rhythm period via increasing *PER2* mRNA levels, but also significantly augment the oscillation amplitude PER2 and CRY1 protein rhythms via inhibiting the phosphorylation of glycogen synthase kinase 3β (GSK3B) (93, 94). Furthermore, the lithium efficacy is influenced by two *GSK3B* SNPs (rs334558 and rs3755557) (95). Considering all the above evidence, more pharmacological manipulations targeting the circadian rhythm and sleep drive are increasingly becoming plausible in the treatment of mood disorders.

Taken together, there seems to be a clear link between sleep disturbance and mood disorders, even though the underlying mechanisms remain unclear. The discovery of the glymphatic system provides researchers with insights into sleep-related diseases.

SLEEP AND THE GLYMPHATIC SYSTEM

Overview of the Glymphatic System

The lymphatic system accounts for the clearance of ISF and it is also critical to both hydrostatic and homeostatic maintenance (96). With regard to lymphatic system in central nervous system (CNS), it consists of two interacting system, the glymphatic (glia-lymphatic) system and the meningeal lymphatic vessels (97). The glymphatic system is responsible for exchanging between CSF and ISF, and clearing solutes and metabolites from the brain parenchyma through a unique system of perivascular tunnels. More specifically, CSF produced by the choroid plexus and capillary influx is pumped deep into the brain parenchyma via arterial pulsation (36, 98). In the perivascular space (PVS), CSF exchanges with ISF, accompanied by clearance of soluble metabolic waste like Aβ (36). Indeed, large and eccentric PVS provides considerably less hydraulic resistance

to CSF-ISF flow compared to concentric annular tunnel (99, 100). During the clearance of solutes, convection coexists with diffusion in the glymphatic system (101–103). It is argued that in the brain interstitium, small molecule transport is best explained by diffusion while convection becomes more predominant with increasing molecular size (104). However, the exact contributions of the two processes are highly dynamic and remain controversial, with one of the reasons being that the glymphatic influx and efflux are influenced by arousal state, pulse, respiration, body position, and more (98, 103, 105, 106). Moreover, CSF–ISF and solutes drain from the CNS via meningeal and cervical lymphatic vessels, as well as the cranial and spinal nerve roots (107, 108). Therefore, interference of the lymphatic system, such as ultraviolet photoablation of meningeal lymphatic vessels and ligation of cervical lymphatics, accounts for the stagnation of glymphatic flow and aggregation of metabolic wastes like Aβ (109, 110).

More importantly, the glymphatic system is supported by the water channel AQP4 which is primarily expressed by the astrocytic endfeet (36). Animals lacking AQP4 exhibit slower CSF influx and less interstitial solute clearance (70% reduction) (36, 111, 112). Deletion of the AQP4 in APP/PS1 transgenic mice results in increased interstitial Aβ plaque accumulation, cerebral amyloid angiopathy, as well as loss of synaptic protein and brain-derived neurotrophic factor in the hippocampus and cortex (113). However, it should be noted that the role of AQP4 in glymphatic clearance function are debated (103, 106). Smith et al. have found that *AQP4* gene deletion mice exhibited a similar Aβ distribution as wildtype mice, suggesting that *AQP4* gene deletion did not impair clearance of Aβ (114).

Sleep-Dependent Glymphatic Cycling

Emerging evidence reveals that the function of the glymphatic system fluctuates daily along with the sleep–wake cycle. A two-photon imaging study reported a 60% increase in the interstitial space and two-fold faster clearance of Aβ in natural sleep or anesthesia mice compared with awake mice (27). A coherent pattern of slow-wave activity and CSF influx has been observed during NREM sleep in humans, supporting the exciting possibility of sleep-regulated glymphatic function (25). However, recent evidence using contrast-enhanced MRI has revealed that the glymphatic system is controlled by the circadian rhythm rather than by the sleep–wake cycle (115, 116). The parenchymal redistribution of contrast agent is lowest during the light phase and highest during the dark phase in fully awake rats, regardless of normal or reversed light–dark cycles (115). The diurnal variation of glymphatic cycling persists even under constant light or anesthesia, suggesting the hypothesis that endogenous circadian oscillations determine glymphatic function (116). The discrepancy may be related to the extreme differences in the circadian rhythm between humans and rodents (117). Rodents are nocturnal animals with opposite circadian phase, and they are also poly-phasic sleepers with relatively low sleep drive (118). Presently, the exact contributions of the light–dark cycle, sleep–wake cycle, and other physiological rhythms remain unknown (116). Further studies are warranted to confirm the circadian control of the glymphatic system in humans.

Surprisingly, the deletion of AQP4 effectively eliminates the circadian rhythm in glymphatic fluid transport (116). A recent genomic study reports that AQP4-haplotype influences sleep homeostasis in NREM sleep and response to prolonged wakefulness (119), providing supporting evidence for the sleep-dependent glymphatic pathway. The high polarization of AQP4 in astrocytic endfeet is under the control of the circadian rhythm, and thus, modulates bulk fluid movement, CSF–ISF exchange, and solutes clearance (116). Conversely, there is also evidence that astrocytes repress SCN neurons and regulate circadian timekeeping via glutamate signaling (120). Thus, astrocytes and AQP4 present a checkpoint for the functional glymphatic system during deep sleep.

Considerable evidence suggests a causal relationship between sleep and regulation of the glymphatic flow, thus modulating protein clearance. Sleep disturbance (including shorter total sleep time, sleep fragmentation, and lack of NREM III) causes suppressed glymphatic function and a decline in the clearance of metabolic waste, hence contributing to the development and progression of various neurological diseases including AD (30), PD (31), stroke (32, 33), and iNPH (34, 35).

Taken together, the glymphatic function is considered as a brain fluid transport with astrocyte-regulated mechanisms, while glymphatic dysfunction is intimately associated with neurological diseases, especially neurodegenerative diseases with cognitive decline (30, 31).

GLYMPHATIC DYSFUNCTION IN MOOD DISORDERS

Abnormalities of Glymphatic Flow, Astrocytes, and AQP4 in Depression

Individuals suffering from depressive episodes always show diverse cognitive decline (1), including attention, memory, response inhibition, decision speed, and so on. Depression has been considered as a prodrome of dementia (121), with increased Aβ deposition reported in an (18) F-florbetapir positron emission tomography (PET) imaging study (122). These observations raise the exciting possibility that wide-spread disruption of the glymphatic system exists in depression. Recent animal studies using chronic unpredictable mild stress (CUMS) model have provided supporting evidence for the glymphatic dysfunction in depression (47, 48) (**Table 1**). In the CUMS model, animals were exposed to the various stressors randomly for several weeks and injected with fluorescence tracers from cisterna magna to estimate the glymphatic function (47, 48). The CSF tracer penetration in the brain of CUMS-treated mice was significantly decreased, and recovered to the control level after fluoxetine administration or polyunsaturated fatty acid (PUFA) supplementation (47, 48). In parallel with the impaired glymphatic circulation, the increased deposition of Aβ has been observed (47, 48). Amyloid-β accumulation along the blood vessels, in turn, could impair glymphatic function by reducing PVS and increasing hydraulic resistance, and thus result in a more severe parenchymal build-up of Aβ and neuronal death (134). Another plausible explanation of PVS closure induced

by CUMS is the alteration of arterial pulsation and compliance that triggered by neuroinflammation and restored by daily PUFA supplementation (48) (**Table 1**).

During the neuroinflammatory response, reactive astrocytosis, and AQP4 depolarization have been widely reported in depression (48). Abundant evidence indicated astrocytic abnormalities in patients with depression (**Table 2**). Golgi-staining of postmortem tissues from depressed suicide cases has revealed reactive astrocytosis within the cingulate cortex (37). Additionally, glial fibrillary acidic protein (GFAP), one of the astrocyte-specific biomarkers, is reduced in depression-associated brain regions including the prefrontal cortex, cingulate cortex (38, 39), hippocampus (40), amygdala (41), locus coeruleus (44), cerebellum (146), thalamus, and caudate nuclei (42). A lower density of S100β-immunopositive astrocytes has been reported in the bilateral hippocampus and locus coeruleus of depressive patients compared to that of healthy controls (44, 135). Downregulated expression of AQP4 has been found in postmortem locus coeruleus and hippocampus in MDD patients (44, 136). More importantly, the reduction in astrocyte density is passed on to offsprings of depressive females via an epigenetic mechanism (123) (**Table 1**). Nevertheless, there are several contradictory results (**Table 2**). The density of astrocytes has been observed unchanged in the cingulate cortex and hippocampus of MDD patients (142, 144). A postmortem study using quantitative polymerase chain reaction (qPCR) have observed upregulated expression of GFAP and aldehyde dehydrogenase 1 family member L1 (ALDH1L1) in the basal ganglia of MDD patients (145). Another postmortem study using microarray analysis and qPCR has found upregulated expression of AQP4 in the prefrontal cortex of MDD patients. Obviously, the variety of studied methods involving Glogi-staining, Nissl-staining, qPCR, western blotting, and immunohistochemistry, contributes to the discrepancies.

However, emerging animal studies provide powerful evidence implying the pathological alterations of astrocytes and AQP4 in depression. Decreased astrocytes and downregulated AQP4 expression have been reported in various animal models of depression (47, 48, 123, 124, 130) (**Table 1**), supporting dysfunctional glymphatic transport in depression. Effective antidepressant therapy, such as fluoxetine (47, 124, 125), escitalopram (48), mirtazapine (126), ketamine (127, 128), and repetitive high-frequency transcranial magnetic stimulation (TMS) (129) could benefit the functioning of both astrocytes and AQP4, and hence alleviate depressive-like behaviors. Additionally, the synergistic agents of antidepressant—lithium—can attenuate the reduction of AQP4 and disruption of the neurovascular unit in the hippocampus of CUMS rats (130), resulting in a functioning glymphatic system. These therapeutic effects can be suppressed by AQP4 knockout. More specifically, AQP4 deficiency abolishes fluoxetine treatment-induced hippocampal neurogenesis and behavioral improvement in depressive mice (133). Recent studies indicate that the therapeutic option for depression is via the restoration of astrocytes function, AQP4, and glymphatic system (131, 132), which provide further supporting evidence for the critical role of glymphatic flow in depression.

Abnormalities of Astrocytes and AQP4 in Bipolar Disorders

To date, the role of the glymphatic function in BD has not been widely studied. However, astrocytic dysfunction has undoubtedly been implicated in the development of BD (43). Different from MDD, pictures from human postmortem studies in BD appear to be highly heterogeneous (**Table 2**). The density of GFAP-positive astrocytes is reported to be significantly increased in Brodmann area (BA) 9 (137) and reduced in BA10 (138), BA24 (38), BA11, and BA 47 (139), while the level of S100β has been reported to be increased in BA40 and reduced in BA9 (140). Other studies on human postmortem tissues from BD exhibit an unchanged density of astrocytes in the frontal cortex (141), cingulate cortex (142), amygdala (41, 143), hippocampus (144), entorhinal cortex (143), basal ganglia (145), dorsal raphe nucleus, and cerebellum (146). The considerable discrepancy is on account of various confounding factors, including phenotype (depressive episode, manic episode, or remission state) (150), cause of death (depressive suicide or physical diseases) (141, 144), comorbidity (150, 151), the methodology used (137, 144), and the brain regions studied (139, 140). Therefore, additional studies regarding diverse phenotypes of BD are essential to investigate state-related abnormalities of astrocytes (152). In patients with bipolar depression, a reduction in S100β-immunopositive astrocytes has been observe, but with no change in GFAP-immunopositive astrocytes (135, 147). As for manic states, *in vivo* studies have revealed increased serum levels of S100β, suggesting astrocytic activation (148).

Upregulated expression of AQP4 in the prefrontal cortex has been revealed in BD (149). Evaluation of the qualitative alterations of astrocytes (especially AQP4 function) is far much valuable than quantitative alterations. The apparent diffusion coefficient from ultra-high b-values (ADC uh), a parameter of enhanced diffusion-weighted imaging (eDWI), can reflect the function of AQP4 (45). In individuals suffering from bipolar depression, increased ADC uh values in bilateral superior cerebellar peduncles (SCP) and cerebellar hemisphere is positively associated with depressive scores, implying that a positive correlation exists between the upregulated expression of AQP4 and severity of depression (46). A plausible explanation is that increased and depolarized AQP4 impair water homeostasis and glymphatic transport in BD (149). Lithium is a classical mood-stabilizer, and its effect of regulating AQP4 function is discussed above (130). Additionally, other mood-stabilizers such as valproic acid, topiramate, and lamotrigine have been shown to inhibit AQP4 (153), and hence regulate directed glymphatic flow.

Even though direct evidence for glymphatic impairment in mood disorders is lacking, astrocytes and AQP4 abnormalities provide support to the hypothesis that glymphatic dysfunction functions as a bridge between sleep disturbance and mood disorders. Additionally, treatments for mood improvement, including medicines, light therapy, sleep invention, and TMS can

TABLE 1 | Glymphatic flow, astrocytes, and AQP4 in animal studies.

References	Studied cohort	Method	Main findings
Xia et al. (47)	CUMS model mice	Injection of tracers, immunohistochemistry	Impaired glymphatic circulation and increased accumulation of Aβ42, which can be reversed by fluoxetine treatment. Downregulated AQP4 expression in cortex and hippocampus, which can be reversed by fluoxetine treatment.
Liu et al. (48)	CUMS model mice	Injection of tracers, immunohistochemistry	Impaired glymphatic circulation and cerebrovascular reactivity, which can be reversed by PUFA supplementation. Decreased Aβ40 clearance, which can be reversed by PUFA supplementation and escitalopram treatment. Decreased astrocytes and AQP4 expression, which can be reversed by PUFA supplementation and escitalopram treatment.
Gong et al. (123)	CMS model mice	Immunohistochemistry	Decreased hippocampal astrocyte is passed on to offsprings via an epigenetic mechanism.
Czéh et al. (124)	Chronic psychosocial stress mice	Immunohistochemistry	Fluoxetine treatment prevented the stress-induced numerical decrease of astrocytes.
Kinoshita et al. (125)	VNUT-knockout mice	Immunohistochemistry, qPCR	Fluoxetine increased ATP exocytosis and BDNF in astrocytes.
Hisaoka-Nakashima et al. (126)	Rat primary astrocytes, C6 astroglia cells	qPCR, ELISA, western blotting	Mirtazapine treatment increased mRNA expression of GDNF and BDNF in astrocytes.
Wang et al. (127)	Mice	Western blotting	Ketamine promotes the activation of astrocyte.
Lasič et al. (128)	Rat primary astrocytes	Structured illumination microscopy and image analysis	Ketamine induced cholesterol redistribution in the plasmalemma of astrocytes.
Xue et al. (129)	CUS model rats	Immunohistochemistry, qPCR	Repetitive TMS at 5 Hz increased the expression of DAGLα and CB1R in hippocampal astrocytes and neurons.
Taler et al. (130)	CUMS model rats	Immunohistochemistry, western blotting, ELISA	Lithium can attenuate the reduction of AQP4 and disruption of the neurovascular unit in hippocampus.
Wang et al. (131)	LPS-induced depression model mice	Immunohistochemistry, qPCR	Inhibition of activated astrocytes ameliorates LPS-induced depressive-like behavior.
Portal et al. (132)	Cx43 KD male mice	Immunohistochemistry, western blotting	Inactivation of astroglial connexin 43 potentiated the antidepressant-like effects of fluoxetine.
Kong et al. (133)	CMS model mice	Immunohistochemistry, western blotting	AQP4 knockout disrupted fluoxetine-induced enhancement of hippocampal neurogenesis, as well as behavioral improvement.

Aβ, amyloid-β; AQP4, aquaporin-4; ATP, adenosine triphosphate; BDNF, brain-derived neurotrophic factor; CB1R, cannabinoid type 1 receptor; CMS, chronic mild stress; CUMS, chronic unpredictable mild stress; CUS, chronic unpredictable stress; Cx43 KD, connexin 43 knock-down; DAGLα, diacylglycerol lipase alpha; ELISA, enzyme-linked immunosorbent assays; GDNF, glial cell line-derived neurotrophic factor; GFAP, glial fibrillary acidic protein; LPS, lipopolysaccharide; mRNA, messenger RNA; PUFA, polyunsaturated fatty acid; qPCR, quantitative polymerase chain reaction; TMS, transcranial magnetic stimulation; VNUT, vesicular nucleotide transporter.

regulate the function of astrocytes and AQP4. Therefore, AQP4-dependent glymphatic system may serve as a new therapeutic target in mood disorders.

CONCLUSION AND OUTLOOK

Mood symptoms often occur with the onset of sleep disturbance and ameliorate with improved sleep disturbance. Moreover, early-life sleep problems due to jet-lag, social jet-lag, shift-work, or light pollution can significantly increase the lifetime risk of mood disorders (60). In addition, sleep deprivation can directly trigger mania-like symptoms (80). Based on considerable evidence, a causal relationship between sleep disturbance and mood disorders is hypothesized (154). Therefore, how does disrupted sleep affect the development and phenotype of mood disorders? An intriguing possibility has emerged that glymphatic dysfunction serves as a bridge between sleep disturbance and mood disorders. Adequate sleep, especially deep sleep (NREM III), is a key factor in the functioning of the glymphatic system which accounts for the clearance of metabolic wastes. The effects of sleep on the glymphatic system are mainly dependent on the dynamic alterations of astrocytic function and AQP4 distribution (113, 119, 155). Significantly, suppressed glymphatic circulation, astrocytic abnormalities, and AQP4 depolarization are consistently

TABLE 2 | Astrocytes and AQP4 in patients with mood disorder.

References	Studied cohort	Tested sample	Method	Main findings
Torres-Platas et al. (37)	10 Depressed suicides, 10HC	Postmortem tissue	Golgi-staining	Reactive astrocytosis within the cingulate cortex of depressive patients.
Torres-Platas et al. (42)	22 Depressed suicides, 22HC	Postmortem tissue	Immunohistochemistry, qPCR	Downregulation of GFAP mRNA and protein in the mediodorsal thalamus and caudate nucleus of depressed suicides.
Webster et al. (38)	15MDD, 15BD, 15HC	Postmortem tissue	*In situ* hybridization	Decreased level of GFAP mRNA in the cingulate cortex of BD patients. Decreased level of GFAP mRNA in the cingulate cortex of MDD patients (not significantly).
Gittins et al. (39)	5MDD, 2BD, 9HC	Postmortem tissue	Immunohistochemistry	Decreased GFAP protein in the anterior cingulate cortex of patients with mood disorders.
Cobb et al. (40)	17MDD, 17HC	Postmortem tissue	Immunohistochemistry	Decreased GFAP-positive astrocytes in the left hippocampus of depressive patients.
Altshuler et al. (41)	11MDD, 10BD, 14HC	Postmortem tissue	Immunohistochemistry	Decreased GFAP-positive astrocytes in the amygdala of depressive patients. Unchanged GFAP-positive astrocytes in the amygdala of BD patients.
Bernard et al. (44)	12MDD, 6BD, 9HC	Postmortem tissue	*In situ* hybridization	Downregulated expression of GFAP, S100B and AQP4 in locus coeruleus of MDD patients.
Gos et al. (135)	9MDD, 6BD, 13HC	Postmortem tissue	Immunohistochemistry	Decreased S100β-immunopositive astrocytes in the bilateral hippocampus of depressive patients.
Medina et al. (136)	13MDD, 10HC	Postmortem tissue	Microarray analysis, qPCR	Downregulated AQP4 mRNA expression in hippocampus of MDD patients.
Feresten AH et al. (137)	34BD, 35HC	Postmortem tissue	Western blotting	Increased GFAP expression of in BA9 of BD patients. Unchanged levels of vimentin and ALDH1L1 in BA9 of BD patients.
Johnston-Wilson et al. (138)	19MDD, 23BD, 23HC	Postmortem tissue	Western blotting	Decreased GFAP-positive astrocytes in BA10 of BD patients.
Toro et al. (139)	15MDD, 15BD, 15HC	Postmortem tissue	Immunohistochemistry	Decreased GFAP-positive astrocytes in BA11/47 of BD patients.
Dean et al. (140)	8BD, 20HC	Postmortem tissue	Western blotting, qPCR	Increased S100β in BA40 of BD patients. Decreased S100β in BA9 of BD patients.
Hercher et al. (141)	20BD, 20HC	Postmortem tissue	Immunohistochemistry	Unchanged density of astrocytes in the frontal cortex of BD patients.
Williams et al. (142)	20MDD, 16BD, 20HC	Postmortem tissue	Immunohistochemistry	Unchanged density of astrocytes in the cingulate cortex of patients with mood disorder.
Pantazopoulos et al. (143)	11BD, 15HC	Postmortem tissue	Immunohistochemistry	Unchanged density of astrocytes in the amygdala and entorhinal cortex of BD patients.
Malchow et al. (144)	8MDD, 8BD, 10HC	Postmortem tissue	Nissl-staining	Unchanged density of astrocytes in the hippocampus of patients with mood disorder.
Barley et al. (145)	14MDD, 14BD, 15HC	Postmortem tissue	qPCR	Upregulated expression of GFAP and ALDH1L1 the basal ganglia of MDD patients. Upregulated expression of GFAP and ALDH1L1 the basal ganglia of BD patients (not significantly).
Fatemi et al. (146)	15MDD, 15BD, 15HC	Postmortem tissue	Western blotting	Decreased GFAP in the cerebellum of patients with mood disorders.
Steiner et al. (147)	9MDD, 5BD, 10HC	Postmortem tissue	Immunohistochemistry	No change in GFPA-immunopositive astrocytes of patients with mood disorder.
da Rosa et al. (148)	52 manic BD, 52HC	Serum	meta-analysis	Increased S100β levels in serum of patients with manic episodes.
Zhao et al. (46)	50BD II, 43HC	eDWI	ADCuh	Increased ADCuh values in bilateral SCP and cerebellar hemisphere, which positively associated with depressive scores.
Iwamoto et al. (149)	11MDD, 11BD, 15HC	Postmortem tissue	Microarray analysis, qPCR	Upregulated expression of AQP4 in the prefrontal cortex of patients with mood disorders.

AQP4, aquaporin-4; ADCuh, apparent diffusion coefficient from ultra-high b-values; ALDH1L1, aldehyde dehydrogenase 1L1; BA, Brodmann area; BD, bipolar disorder; eDWI, enhanced diffusion-weighted imaging; GFAP, glial fibrillary acidic protein; HC, health control; mRNA, messenger RNA; qPCR, quantitative polymerase chain reaction; SCP, superior cerebellar peduncles.

reported in mood disorders, providing support for the posited hypothesis.

However, several limitations exist in this study. First, much of the existing evidence on the glymphatic system has been conducted in rodents and only a few in humans. Although sleep is an evolutionarily conserved physiological behavior, the reversed circadian rhythms and polyphasic sleep which reduces sleep pressure in rodents make it less representative. Most of the current human studies use invasive methods such as intrathecal injection of contrast agents, while the ADCuh value obtained from the emerging eDWI fails to identify the distribution of AQP4. Therefore, non-invasive methods to explore the glymphatic system in humans are necessary for future studies. Secondly, there is a lack of evidence of known metabolic wastes that fail to be cleared by the glymphatic system and trigger or exacerbate mood symptoms, such as Aβ in AD and *α-synuclein* in PD. Exploring the excessive metabolic wastes in mood disorders is warranted, and can provide promising biomarkers for indicating the occurrence and severity of mood disorders.

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.

AUTHOR CONTRIBUTIONS

TY, YQ, and LY defined the research questions and aims of the study. TY and YQ carried out the literature search, selected and interpreted relevant articles, and wrote the first draft of the manuscript. XY made the original figure and tables. LY and XY critically appraised the texts, figure and tables, corrected them, and made suggestions for further improvement. All authors contributed to the article and approved the submitted version.

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REFERENCES

1. American Psychiatric Association. *Diagnostic and Statistical Manual of Mental Disorders (DSM).* 5th edition. Washington, DC: American Psychiatric Publishing (2013).
2. Grande I, Berk M, Birmaher B, Vieta E. Bipolar disorder. *Lancet (London, England).* (2016) 387:1561–72. doi: 10.1016/S0140-6736(15)00241-X
3. O'Leary OF, Dinan TG, Cryan JF. Faster, better, stronger: towards new antidepressant therapeutic strategies. *Eur J Pharmacol.* (2015) 753:32–50. doi: 10.1016/j.ejphar.2014.07.046
4. Lyall LM, Wyse CA, Graham N, Ferguson A, Lyall DM, Cullen B, et al. Association of disrupted circadian rhythmicity with mood disorders, subjective wellbeing, and cognitive function: a cross-sectional study of 91 105 participants from the UK Biobank. *Lancet Psychiatry.* (2018) 5:507–14. doi: 10.1016/S2215-0366(18)30139-1
5. Jackson A, Cavanagh J, Scott J. A systematic review of manic and depressive prodromes. *J Affect Disord.* (2003) 74:209–17. doi: 10.1016/S0165-0327(02)00266-5
6. Borbély AA, Daan S, Wirz-Justice A, Deboer T. The two-process model of sleep regulation: a reappraisal. *J Sleep Res.* (2016) 25:131–43. doi: 10.1111/jsr.12371
7. Yamazaki S, Numano R, Abe M, Hida A, Takahashi R, Ueda M, et al. Resetting central and peripheral circadian oscillators in transgenic rats. *Science.* (2000) 288:682–5. doi: 10.1126/science.288.5466.682
8. Park M, Kim SA, Yee J, Shin J, Lee KY, Joo EJ. Significant role of gene-gene interactions of clock genes in mood disorder. *J Affect Disord.* (2019) 257:510–7. doi: 10.1016/j.jad.2019.06.056
9. Lavebratt C, Sjöholm LK, Soronen P, Paunio T, Vawter MP, Bunney WE, et al. CRY2 is associated with depression. *PLoS ONE.* (2010) 5:e9407. doi: 10.1371/journal.pone.0009407
10. Sjöholm LK, Backlund L, Cheteh EH, Ek IR, Frisén L, Schalling M, et al. CRY2 is associated with rapid cycling in bipolar disorder patients. *PLoS ONE.* (2010) 5:e12632. doi: 10.1371/journal.pone.0012632
11. Brasil Rocha PM, Campos SB, Neves FS, da Silva Filho HC. Genetic association of the PERIOD3 (Per3) clock gene with bipolar disorder. *Psychiatry Investig.* (2017) 14:674–80. doi: 10.4306/pi.2017.14.5.674
12. McGrath CL, Glatt SJ, Sklar P, Le-Niculescu H, Kuczenski R, Doyle AE, et al. Evidence for genetic association of RORB with bipolar disorder. *BMC Psychiatry.* (2009) 9:70. doi: 10.1186/1471-244X-9-70
13. Partonen T, Treutlein J, Alpman A, Frank J, Johansson C, Depner M, et al. Three circadian clock genes Per2, Arntl, and Npas2 contribute to winter depression. *Ann Med.* (2007) 39:229–38. doi: 10.1080/07853890701278795
14. Yang S, Van Dongen HP, Wang K, Berrettini W, Bućan M. Assessment of circadian function in fibroblasts of patients with bipolar disorder. *Mol Psychiatry.* (2009) 14:143–55. doi: 10.1038/mp.2008.10
15. Li JZ, Bunney BG, Meng F, Hagenauer MH, Walsh DM, Vawter MP, et al. Circadian patterns of gene expression in the human brain and disruption in major depressive disorder. *Proc Natl Acad Sci USA.* (2013) 110:9950–5. doi: 10.1073/pnas.1305814110
16. Horikawa K, Yokota S, Fuji K, Akiyama M, Moriya T, Okamura H, et al. Nonphotic entrainment by 5-HT1A/7 receptor agonists accompanied by reduced Per1 and Per2 mRNA levels in the suprachiasmatic nuclei. *J Neurosci.* (2000) 20:5867–73. doi: 10.1523/JNEUROSCI.20-15-05867.2000
17. Horikawa K, Shibata S. Phase-resetting response to (+)8-OH-DPAT, a serotonin 1A/7 receptor agonist, in the mouse *in vivo*. *Neurosci Lett.* (2004) 368:130–4. doi: 10.1016/j.neulet.2004.06.072
18. Shibata S, Tsuneyoshi A, Hamada T, Tominaga K, Watanabe S. Phase-resetting effect of 8-OH-DPAT, a serotonin1A receptor agonist, on the circadian rhythm of firing rate in the rat suprachiasmatic nuclei *in vitro*. *Brain Res.* (1992) 582:353–6. doi: 10.1016/0006-8993(92)90156-4
19. Orozco-Solis R, Montellier E, Aguilar-Arnal L, Sato S, Vawter MP, Bunney BG, et al. A circadian genomic signature common to ketamine and sleep deprivation in the anterior cingulate cortex. *Biol Psychiatry.* (2017) 82:351–60. doi: 10.1016/j.biopsych.2017.02.1176
20. Cui Y, Hu S, Hu H. Lateral habenular burst firing as a target of the rapid antidepressant effects of ketamine. *Trends Neurosci.* (2019) 42:179–91. doi: 10.1016/j.tins.2018.12.002
21. de Bodinat C, Guardiola-Lemaitre B, Mocaër E, Renard P, Muñoz C, Millan MJ. Agomelatine, the first melatonergic antidepressant: discovery, characterization and development. *Nat Rev Drug Discov.* (2010) 9:628–42. doi: 10.1038/nrd3140
22. Jones SH, Hare DJ, Evershed K. Actigraphic assessment of circadian activity and sleep patterns in bipolar disorder. *Bipolar Disord.* (2005) 7:176–86. doi: 10.1111/j.1399-5618.2005.00187.x
23. Jaussent I, Bouyer J, Ancelin ML, Akbaraly T, Pérès K, Ritchie K, et al. Insomnia and daytime sleepiness are risk factors for depressive symptoms in the elderly. *Sleep.* (2011) 34:1103–10. doi: 10.5665/SLEEP.1170
24. Bradley AJ, Webb-Mitchell R, Hazu A, Slater N, Middleton B, Gallagher P, et al. Sleep and circadian rhythm disturbance in bipolar disorder. *Psychol Med.* (2017) 47:1678–89. doi: 10.1017/S0033291717000186

25. Fultz NE, Bonmassar G, Setsompop K, Stickgold RA, Rosen BR, Polimeni JR, et al. Coupled electrophysiological, hemodynamic, and cerebrospinal fluid oscillations in human sleep. *Science.* (2019) 366:628–31. doi: 10.1126/science.aax5440
26. Hablitz LM, Vinitsky HS, Sun Q, Stæger FF, Sigurdsson B, Mortensen KN, et al. Increased glymphatic influx is correlated with high EEG delta power and low heart rate in mice under anesthesia. *Sci Adv.* (2019) 5:eaav5447. doi: 10.1126/sciadv.aav5447
27. Xie L, Kang H, Xu Q, Chen MJ, Liao Y, Thiyagarajan M, et al. Sleep drives metabolite clearance from the adult brain. *Science.* (2013) 342:373–7. doi: 10.1126/science.1241224
28. Simon MJ, Wang MX, Murchison CF, Roese NE, Boespflug EL, Woltjer RL, et al. Transcriptional network analysis of human astrocytic endfoot genes reveals region-specific associations with dementia status and tau pathology. *Sci Rep.* (2018) 8:12389. doi: 10.1038/s41598-018-30779-x
29. van Dijk KD, Bidinosti M, Weiss A, Raijmakers P, Berendse HW, van de Berg WD. Reduced α-synuclein levels in cerebrospinal fluid in Parkinson's disease are unrelated to clinical and imaging measures of disease severity. *Eur J Neurol.* (2014) 21:388–94. doi: 10.1111/ene.12176
30. Roh JH, Huang Y, Bero AW, Kasten T, Stewart FR, Bateman RJ, et al. Disruption of the sleep-wake cycle and diurnal fluctuation of β-amyloid in mice with Alzheimer's disease pathology. *Sci Transl Med.* (2012) 4:150ra122. doi: 10.1126/scitranslmed.3004291
31. Sundaram S, Hughes RL, Peterson E, Müller-Oehring EM, Brontë-Stewart HM, Poston KL, et al. Establishing a framework for neuropathological correlates and glymphatic system functioning in Parkinson's disease. *Neurosci Biobehav Rev.* (2019) 103:305–15. doi: 10.1016/j.neubiorev.2019.05.016
32. Gaberel T, Gakuba C, Goulay R, Martinez De Lizarrondo S, Hanouz JL, Emery E, et al. Impaired glymphatic perfusion after strokes revealed by contrast-enhanced MRI: a new target for fibrinolysis? *Stroke.* (2014) 45:3092–6. doi: 10.1161/STROKEAHA.114.006617
33. Pu T, Zou W, Feng W, Zhang Y, Wang L, Wang H, et al. Persistent malfunction of glymphatic and meningeal lymphatic drainage in a mouse model of subarachnoid hemorrhage. *Exp Neurobiol.* (2019) 28:104–18. doi: 10.5607/en.2019.28.1.104
34. Ringstad G, Vatnehol SAS, Eide PK. Glymphatic MRI in idiopathic normal pressure hydrocephalus. *Brain.* (2017) 140:2691–705. doi: 10.1093/brain/awx191
35. Ringstad G, Valnes LM, Dale AM, Pripp AH, Vatnehol SS, Emblem KE, et al. Brain-wide glymphatic enhancement and clearance in humans assessed with MRI. *JCI Insight.* (2018) 3:e121537. doi: 10.1172/jci.insight.121537
36. Iliff JJ, Wang M, Liao Y, Plogg BA, Peng W, Gundersen GA, et al. A paravascular pathway facilitates CSF flow through the brain parenchyma and the clearance of interstitial solutes, including amyloid β. *Sci Transl Med.* (2012) 4:147ra111. doi: 10.1126/scitranslmed.3003748
37. Torres-Platas SG, Hercher C, Davoli MA, Maussion G, Labonté B, Turecki G, et al. Astrocytic hypertrophy in anterior cingulate white matter of depressed suicides. *Neuropsychopharmacology*. (2011) 36:2650–8. doi: 10.1038/npp.2011.154
38. Webster MJ, O'Grady J, Kleinman JE, Weickert CS. Glial fibrillary acidic protein mRNA levels in the cingulate cortex of individuals with depression, bipolar disorder and schizophrenia. *Neuroscience.* (2005) 133:453–61. doi: 10.1016/j.neuroscience.2005.02.037
39. Gittins RA, Harrison PJ. A morphometric study of glia and neurons in the anterior cingulate cortex in mood disorder. *J. Affect. Disord.* (2011) 133:328–32. doi: 10.1016/j.jad.2011.03.042
40. Cobb JA, O'Neill K, Milner J, Mahajan GJ, Lawrence TJ, May WL, et al. Density of GFAP-immunoreactive astrocytes is decreased in left hippocampi in major depressive disorder. *Neuroscience*. (2016) 316:209–20. doi: 10.1016/j.neuroscience.2015.12.044
41. Altshuler LL, Abulseoud OA, Foland-Ross L, Bartzokis G, Chang S, Mintz J, et al. Amygdala astrocyte reduction in subjects with major depressive disorder but not bipolar disorder. *Bipolar Disord.* (2010) 12:541–9. doi: 10.1111/j.1399-5618.2010.00838.x
42. Torres-Platas SG, Nagy C, Wakid M, Turecki G, Mechawar N. Glial fibrillary acidic protein is differentially expressed across cortical and subcortical regions in healthy brains and downregulated in the thalamus and caudate nucleus of depressed suicides. *Mol Psychiatry.* (2016) 21:509–15. doi: 10.1038/mp.2015.65
43. Peng L, Li B, Verkhratsky A. Targeting astrocytes in bipolar disorder. *Exp Rev. Neurotherap.* (2016) 16:649–57. doi: 10.1586/14737175.2016.1171144
44. Bernard R, Kerman IA, Thompson RC, Jones EG, Bunney WE, Barchas JD, et al. Altered expression of glutamate signaling, growth factor, and glia genes in the locus coeruleus of patients with major depression. *Mol Psychiatry.* (2011) 16:634–46. doi: 10.1038/mp.2010.44
45. Xueying L, Zhongping Z, Zhoushe Z, Li G, Yongjin T, Changzheng S, et al. Investigation of apparent diffusion coefficient from ultra-high b-values in Parkinson's disease. *Eur Radiol.* (2015) 25:2593–600. doi: 10.1007/s00330-015-3678-3
46. Zhao L, Luo Z, Qiu S, Jia Y, Zhong S, Chen G, et al. Abnormalities of aquaporin-4 in the cerebellum in bipolar II disorder: an ultra-high b-values diffusion weighted imaging study. *J Affect Disord.* (2020) 274:136–43. doi: 10.1016/j.jad.2020.05.035
47. Xia M, Yang L, Sun G, Qi S, Li B. Mechanism of depression as a risk factor in the development of Alzheimer's disease: the function of AQP4 and the glymphatic system. *Psychopharmacology (Berl).* (2017) 234:365–79. doi: 10.1007/s00213-016-4473-9
48. Liu X, Hao J, Yao E, Cao J, Zheng X, Yao D, et al. Polyunsaturated fatty acid supplement alleviates depression-incident cognitive dysfunction by protecting the cerebrovascular and glymphatic systems. *Brain Behav Immun.* (2020) 89:357–70. doi: 10.1016/j.bbi.2020.07.022
49. Bauducco S, Richardson C, Gradisar M. Chronotype, circadian rhythms and mood. *Curr Opin Psychol.* (2020) 34:77–83. doi: 10.1016/j.copsyc.2019.09.002
50. Takahashi JS. Molecular components of the circadian clock in mammals. *Diabetes Obes Metab.* (2015) 17(Suppl. 1):6–11. doi: 10.1111/dom.12514
51. Preitner N, Damiola F, Lopez-Molina L, Zakany J, Duboule D, Albrecht U, et al. The orphan nuclear receptor REV-ERBalpha controls circadian transcription within the positive limb of the mammalian circadian oscillator. *Cell.* (2002) 110:251–60. doi: 10.1016/S0092-8674(02)00825-5
52. DeBruyne JP, Weaver DR, Reppert SM. CLOCK and NPAS2 have overlapping roles in the suprachiasmatic circadian clock. *Nat Neurosci.* (2007) 10:543–5. doi: 10.1038/nn1884
53. Yamaguchi S, Mitsui S, Yan L, Yagita K, Miyake S, Okamura H. Role of DBP in the circadian oscillatory mechanism. *Mol Cell Biol.* (2000) 20:4773–81. doi: 10.1128/MCB.20.13.4773-4781.2000
54. Aryal RP, Kwak PB, Tamayo AG, Gebert M, Chiu PL, Walz T, et al. Macromolecular assemblies of the mammalian circadian clock. *Mol Cell.* (2017) 67:770.e6–82.e6. doi: 10.1016/j.molcel.2017.07.017
55. Honma S, Kawamoto T, Takagi Y, Fujimoto K, Sato F, Noshiro M, et al. Dec1 and Dec2 are regulators of the mammalian molecular clock. *Nature.* (2002) 419:841–4. doi: 10.1038/nature01123
56. Mazzoccoli G, Laukkanen MO, Vinciguerra M, Colangelo T, Colantuoni V. A timeless link between circadian patterns and disease. *Trends Mol Med.* (2016) 22:68–81. doi: 10.1016/j.molmed.2015.11.007
57. Rodriguez AV, Funk CM, Vyazovskiy VV, Nir Y, Tononi G, Cirelli C. Why does sleep slow-wave activity increase after extended wake? Assessing the effects of increased cortical firing during wake and sleep. *J. Neurosci*. (2016) 36:12436–47. doi: 10.1523/JNEUROSCI.1614-16.2016
58. Nedergaard M, Goldman SA. Glymphatic failure as a final common pathway to dementia. *Science.* (2020) 370:50–6. doi: 10.1126/science.abb8739
59. Foster RG, Peirson SN, Wulff K, Winnebeck E, Vetter C, Roenneberg T. Sleep and circadian rhythm disruption in social jetlag and mental illness. *Prog Mol Biol Transl Sci.* (2013) 119:325–46. doi: 10.1016/B978-0-12-396971-2.00011-7
60. Inder ML, Crowe MT, Porter R. Effect of transmeridian travel and jetlag on mood disorders: evidence and implications. *Aust N Z J Psychiatry.* (2016) 50:220–7. doi: 10.1177/0004867415598844
61. Kalmbach DA, Pillai V, Cheng P, Arnedt JT, Drake CL. Shift work disorder, depression, and anxiety in the transition to rotating shifts: the role of sleep reactivity. *Sleep Med.* (2015) 16:1532–8. doi: 10.1016/j.sleep.2015.09.007
62. Nguyen C, Murray G, Anderson S, Filipowicz A, Ingram KK. *In vivo* molecular chronotyping, circadian misalignment, and high rates of depression in young adults. *J Affect Disord.* (2019) 250:425–31. doi: 10.1016/j.jad.2019.03.050

63. Murray JM, Sletten TL, Magee M, Gordon C, Lovato N, Bartlett DJ, et al. Prevalence of circadian misalignment and its association with depressive symptoms in delayed sleep phase disorder. *Sleep.* (2017) 40:zsw002. doi: 10.1093/sleep/zsw002
64. Robillard R, Carpenter JS, Feilds KL, Hermens DF, White D, Naismith SL, et al. Parallel changes in mood and melatonin rhythm following an adjunctive multimodal chronobiological intervention with agomelatine in people with depression: a proof of concept open label study. *Front Psychiatry.* (2018) 9:624. doi: 10.3389/fpsyt.2018.00624
65. Robillard R, Naismith SL, Rogers NL, Scott EM, Ip TK, Hermens DF, et al. Sleep-wake cycle and melatonin rhythms in adolescents and young adults with mood disorders: comparison of unipolar and bipolar phenotypes. *Eur Psychiatry.* (2013) 28:412–6. doi: 10.1016/j.eurpsy.2013.04.001
66. Salvatore P, Ghidini S, Zita G, De Panfilis C, Lambertino S, Maggini C, et al. Circadian activity rhythm abnormalities in ill and recovered bipolar I disorder patients. *Bipolar Disord.* (2008) 10:256–65. doi: 10.1111/j.1399-5618.2007.00505.x
67. Gonzalez R, Tamminga CA, Tohen M, Suppes T. The relationship between affective state and the rhythmicity of activity in bipolar disorder. *J Clin Psychiatry.* (2014) 75:e317–22. doi: 10.4088/JCP.13m08506
68. Wehr TA. Bipolar mood cycles and lunar tidal cycles. *Mol Psychiatry.* (2018) 23:923–31. doi: 10.1038/mp.2016.263
69. Kripke DF, Nievergelt CM, Joo E, Shekhtman T, Kelsoe JR. Circadian polymorphisms associated with affective disorders. *J Circadian Rhythms.* (2009) 7:2. doi: 10.1186/1740-3391-7-2
70. Lee KY, Song JY, Kim SH, Kim SC, Joo EJ, Ahn YM, et al. Association between CLOCK 3111T/C and preferred circadian phase in Korean patients with bipolar disorder. *Prog Neuropsychopharmacol Biol Psychiatry.* (2010) 34:1196–201. doi: 10.1016/j.pnpbp.2010.06.010
71. Drennan MD, Klauber MR, Kripke DF, Goyette LM. The effects of depression and age on the Horne-Ostberg morningness-eveningness score. *J Affect Disord.* (1991) 23:93–8. doi: 10.1016/0165-0327(91)90096-B
72. Maruani J, Geoffroy PA. Bright light as a personalized precision treatment of mood disorders. *Front Psychiatry.* (2019) 10:85. doi: 10.3389/fpsyt.2019.00085
73. Park H, Lee HK, Lee K. Chronotype and suicide: the mediating effect of depressive symptoms. *Psychiatry Res.* (2018) 269:316–20. doi: 10.1016/j.psychres.2018.08.046
74. Stange JP, Kleiman EM, Sylvia LG, Magalhães PV, Berk M, Nierenberg AA, et al. Specific mood symptoms confer risk for subsequent suicidal ideation in bipolar disorder with and without suicide attempt history: multi-wave data from step-BD. *Depress Anxiety.* (2016) 33:464–72. doi: 10.1002/da.22464
75. Cretu JB, Culver JL, Goffin KC, Shah S, Ketter TA. Sleep, residual mood symptoms, and time to relapse in recovered patients with bipolar disorder. *J Affect Disord.* (2016) 190:162–6. doi: 10.1016/j.jad.2015.09.076
76. Wichniak A, Wierzbicka A, Jernajczyk W. Sleep as a biomarker for depression. *Int Rev Psychiatry.* (2013) 25:632–45. doi: 10.3109/09540261.2013.812067
77. Ritter PS, Höfler M, Wittchen HU, Lieb R, Bauer M, Pfennig A, et al. Disturbed sleep as risk factor for the subsequent onset of bipolar disorder–data from a 10-year prospective-longitudinal study among adolescents and young adults. *J Psychiatr Res.* (2015) 68:76–82. doi: 10.1016/j.jpsychires.2015.06.005
78. Chang PP, Ford DE, Mead LA, Cooper-Patrick L, Klag MJ. Insomnia in young men and subsequent depression. The Johns Hopkins Precursors Study. *Am J Epidemiol.* (1997) 146:105–14. doi: 10.1093/oxfordjournals.aje.a009241
79. Buysse DJ, Angst J, Gamma A, Ajdacic V, Eich D, Rössler W. Prevalence, course, and comorbidity of insomnia and depression in young adults. *Sleep.* (2008) 31:473–80. doi: 10.1093/sleep/31.4.473
80. Arent CO, Valvassori SS, Steckert AV, Resende WR, Dal-Pont GC, Lopes-Borges J, et al. The effects of n-acetylcysteine and/or deferoxamine on manic-like behavior and brain oxidative damage in mice submitted to the paradoxal sleep deprivation model of mania. *J Psychiatr Res.* (2015) 65:71–9. doi: 10.1016/j.jpsychires.2015.04.011
81. Lopez-Rodriguez F, Kim J, Poland RE. Total sleep deprivation decreases immobility in the forced-swim test. *Neuropsychopharmacology.* (2004) 29:1105–11. doi: 10.1038/sj.npp.1300406
82. Zhang B, Gao Y, Li Y, Yang J, Zhao H. Sleep deprivation influences circadian gene expression in the lateral habenula. *Behav Neurol.* (2016) 2016:7919534. doi: 10.1155/2016/7919534
83. Terman M, Terman JS. Light therapy for seasonal and nonseasonal depression: efficacy, protocol, safety, and side effects. *CNS Spectr.* (2005) 10:647–63;quiz 672. doi: 10.1017/S1092852900019611
84. Henriksen TE, Skrede S, Fasmer OB, Hamre B, Grønli J, Lund A. Blocking blue light during mania - markedly increased regularity of sleep and rapid improvement of symptoms: a case report. *Bipolar Disord.* (2014) 16:894–8. doi: 10.1111/bdi.12265
85. Yokota H, Vijayasarathi A, Cekic M, Hirata Y, Linetsky M, Ho M, et al. Diagnostic performance of glymphatic system evaluation using diffusion tensor imaging in idiopathic normal pressure hydrocephalus and mimickers. *Curr Gerontol Geriatr Res.* (2019) 2019:5675014. doi: 10.1155/2019/5675014
86. Scott FF, Belle MD, Delagrange P, Piggins HD. Electrophysiological effects of melatonin on mouse Per1 and non-Per1 suprachiasmatic nuclei neurones *in vitro*. *J Neuroendocrinol.* (2010) 22:1148–56. doi: 10.1111/j.1365-2826.2010.02063.x
87. Antle MC, Ogilvie MD, Pickard GE, Mistlberger RE. Response of the mouse circadian system to serotonin 1A/2/7 agonists *in vivo*: surprisingly little. *J Biol Rhythms.* (2003) 18:145–58. doi: 10.1177/0748730403251805
88. Dudley TE, DiNardo LA, Glass JD. Endogenous regulation of serotonin release in the hamster suprachiasmatic nucleus. *J Neurosci.* (1998) 18:5045–52. doi: 10.1523/JNEUROSCI.18-13-05045.1998
89. Spencer S, Falcon E, Kumar J, Krishnan V, Mukherjee S, Birnbaum SG, et al. Circadian genes Period 1 and Period 2 in the nucleus accumbens regulate anxiety-related behavior. *Eur J Neurosci.* (2013) 37:242–50. doi: 10.1111/ejn.12010
90. Duncan WC Jr., Slonena E, Hejazi NS, Brutsche N, Yu KC, Park L, et al. Motor-activity markers of circadian timekeeping are related to ketamine's rapid antidepressant properties. *Biol Psychiatry.* (2017) 82:361–9. doi: 10.1016/j.biopsych.2017.03.011
91. Kripke DF, Judd LL, Hubbard B, Janowsky DS, Huey LY. The effect of lithium carbonate on the circadian rhythm of sleep in normal human subjects. *Biol Psychiatry.* (1979) 14:545–8.
92. Welsh DK, Moore-Ede MC. Lithium lengthens circadian period in a diurnal primate, *Saimiri sciureus*. *Biol Psychiatry.* (1990) 28:117–26. doi: 10.1016/0006-3223(90)90629-G
93. Li J, Lu WQ, Beesley S, Loudon AS, Meng QJ. Lithium impacts on the amplitude and period of the molecular circadian clockwork. *PLoS ONE.* (2012) 7:e33292. doi: 10.1371/journal.pone.0033292
94. Schnell A, Sandrelli F, Ranc V, Ripperger JA, Brai E, Alberi L, et al. Mice lacking circadian clock components display different mood-related behaviors and do not respond uniformly to chronic lithium treatment. *Chronobiol Int.* (2015) 32:1075–89. doi: 10.3109/07420528.2015.1062024
95. Iwahashi K, Nishizawa D, Narita S, Numajiri M, Murayama O, Yoshihara E, et al. Haplotype analysis of GSK-3β gene polymorphisms in bipolar disorder lithium responders and nonresponders. *Clin Neuropharmacol.* (2014) 37:108–10. doi: 10.1097/WNF.0000000000000039
96. Aukland K, Reed RK. Interstitial-lymphatic mechanisms in the control of extracellular fluid volume. *Physiol Rev.* (1993) 73:1–78. doi: 10.1152/physrev.1993.73.1.1
97. Louveau A, Plog BA, Antila S, Alitalo K, Nedergaard M, Kipnis J. Understanding the functions and relationships of the glymphatic system and meningeal lymphatics. *J Clin Invest.* (2017) 127:3210–9. doi: 10.1172/JCI90603
98. Mestre H, Tithof J, Du T, Song W, Peng W, Sweeney AM, et al. Flow of cerebrospinal fluid is driven by arterial pulsations and is reduced in hypertension. *Nat Commun.* (2018) 9:4878. doi: 10.1038/s41467-018-07318-3
99. Rey J, Sarntinoranont M. Pulsatile flow drivers in brain parenchyma and perivascular spaces: a resistance network model study. *Fluids Barriers CNS.* (2018) 15:20. doi: 10.1186/s12987-018-0105-6
100. Tithof J, Kelley DH, Mestre H, Nedergaard M, Thomas JH. Hydraulic resistance of periarterial spaces in the brain. *Fluids Barriers CNS.* (2019) 16:19. doi: 10.1186/s12987-019-0140-y
101. Koundal S, Elkin R, Nadeem S, Xue Y, Constantinou S, Sanggaard S, et al. Optimal mass transport with Lagrangian workflow reveals advective and

diffusion driven solute transport in the glymphatic system. *Sci Rep.* (2020) 10:1990. doi: 10.1038/s41598-020-59045-9
102. Thomas JH. Fluid dynamics of cerebrospinal fluid flow in perivascular spaces. *J R Soc Interface.* (2019) 16:20190572. doi: 10.1098/rsif.2019.0572
103. Mestre H, Mori Y, Nedergaard M. The brain's glymphatic system: current controversies. *Trends Neurosci.* (2020) 43:458–66. doi: 10.1016/j.tins.2020.04.003
104. Ray L, Iliff JJ, Heys JJ. Analysis of convective and diffusive transport in the brain interstitium. *Fluids Barriers CNS.* (2019) 16:6. doi: 10.1186/s12987-019-0126-9
105. Kiviniemi V, Wang X, Korhonen V, Keinänen T, Tuovinen T, Autio J, et al. Ultra-fast magnetic resonance encephalography of physiological brain activity - glymphatic pulsation mechanisms? *J Cereb Blood Flow Metab.* (2016) 36:1033–45. doi: 10.1177/0271678X15622047
106. Christensen J, Yamakawa GR, Shultz SR, Mychasiuk R. Is the glymphatic system the missing link between sleep impairments and neurological disorders? Examining the implications and uncertainties. *Prog Neurobiol.* (2021) 198:101917. doi: 10.1016/j.pneurobio.2020.101917
107. Ma Q, Ries M, Decker Y, Müller A, Riner C, Bücker A, et al. Rapid lymphatic efflux limits cerebrospinal fluid flow to the brain. *Acta Neuropathol.* (2019) 137:151–65. doi: 10.1007/s00401-018-1916-x
108. Ahn JH, Cho H, Kim JH, Kim SH, Ham JS, Park I, et al. Meningeal lymphatic vessels at the skull base drain cerebrospinal fluid. *Nature.* (2019) 572:62–6. doi: 10.1038/s41586-019-1419-5
109. Wang L, Zhang Y, Zhao Y, Marshall C, Wu T, Xiao M. Deep cervical lymph node ligation aggravates AD-like pathology of APP/PS1 mice. *Brain Pathol.* (2019) 29:176–92. doi: 10.1111/bpa.12656
110. Da Mesquita S, Louveau A, Vaccari A, Smirnov I, Cornelison RC, Kingsmore KM, et al. Functional aspects of meningeal lymphatics in ageing and Alzheimer's disease. *Nature.* (2018) 560:185–91. doi: 10.1038/s41586-018-0368-8
111. Mestre H, Hablitz LM, Xavier AL, Feng W, Zou W, Pu T, et al. Aquaporin-4-dependent glymphatic solute transport in the rodent brain. *Elife.* (2018) 7:e40070. doi: 10.7554/eLife.40070
112. Tarasoff-Conway JM, Carare RO, Osorio RS, Glodzik L, Butler T, et al. Clearance systems in the brain-implications for Alzheimer disease. *Nat Rev Neurol.* (2015) 11:457–70. doi: 10.1038/nrneurol.2015.119
113. Xu Z, Xiao N, Chen Y, Huang H, Marshall C, Gao J, et al. Deletion of aquaporin-4 in APP/PS1 mice exacerbates brain Aβ accumulation and memory deficits. *Mol Neurodegener.* (2015) 10:58. doi: 10.1186/s13024-015-0056-1
114. Smith AJ, Yao X, Dix JA, Jin BJ, Verkman AS. Test of the 'glymphatic' hypothesis demonstrates diffusive and aquaporin-4-independent solute transport in rodent brain parenchyma. *Elife.* (2017) 6:e27679. doi: 10.7554/eLife.27679
115. Cai X, Qiao J, Kulkarni P, Harding IC, Ebong E, Ferris CF. Imaging the effect of the circadian light-dark cycle on the glymphatic system in awake rats. *Proc. Natl. Acad. Sci. USA.* (2020) 117:668–76. doi: 10.1073/pnas.1914017117
116. Hablitz LM, Plá V, Giannetto M, Vinitsky HS, Stæger FF, Metcalfe T, et al. Circadian control of brain glymphatic and lymphatic fluid flow. *Nat Commun.* (2020) 11:4411. doi: 10.1038/s41467-020-18115-2
117. Cederroth CR, Albrecht U, Bass J, Brown SA, Dyhrfjeld-Johnsen J, Gachon F, et al. Medicine in the fourth dimension. *Cell Metab.* (2019) 30:238–50. doi: 10.1016/j.cmet.2019.06.019
118. Simasko SM, Mukherjee S. Novel analysis of sleep patterns in rats separates periods of vigilance cycling from long-duration wake events. *Behav Brain Res.* (2009) 196:228–36. doi: 10.1016/j.bbr.2008.09.003
119. Ulv Larsen SM, Landolt HP, Berger W, Nedergaard M, Knudsen GM, Holst SC. Haplotype of the astrocytic water channel AQP4 is associated with slow wave energy regulation in human NREM sleep. *PLoS Biol.* (2020) 18:e3000623. doi: 10.1371/journal.pbio.3000623
120. Brancaccio M, Patton AP, Chesham JE, Maywood ES, Hastings MH. Astrocytes control circadian timekeeping in the suprachiasmatic nucleus via glutamatergic signaling. *Neuron.* (2017) 93:1420.e5–35.e5. doi: 10.1016/j.neuron.2017.02.030
121. Mirza SS, Wolters FJ, Swanson SA, Koudstaal PJ, Hofman A, Tiemeier H, et al. 10-year trajectories of depressive symptoms and risk of dementia: a population-based study. *Lancet Psychiatry.* (2016) 3:628–35. doi: 10.1016/S2215-0366(16)00097-3
122. Wu KY, Liu CY, Chen CS, Chen CH, Hsiao IT, Hsieh CJ, et al. Beta-amyloid deposition and cognitive function in patients with major depressive disorder with different subtypes of mild cognitive impairment: (18)F-florbetapir (AV-45/Amyvid) PET study. *Eur J Nucl Med Mol Imaging.* (2016) 43:1067–76. doi: 10.1007/s00259-015-3291-3
123. Gong Y, Sun X-L, Wu F-F, Su C-J, Ding J-H, Hu G. Female early adult depression results in detrimental impacts on the behavioral performance and brain development in offspring. *CNS Neurosci Ther.* (2012) 18:461–70. doi: 10.1111/j.1755-5949.2012.00324.x
124. Czéh B, Simon M, Schmelting B, Hiemke C, Fuchs E. Astroglial plasticity in the hippocampus is affected by chronic psychosocial stress and concomitant fluoxetine treatment. *Neuropsychopharmacology.* (2006) 31:1616–26. doi: 10.1038/sj.npp.1300982
125. Kinoshita M, Hirayama Y, Fujishita K, Shibata K, Shinozaki Y, Shigetomi E. Anti-depressant fluoxetine reveals its therapeutic effect via astrocytes. *EBioMedicine.* (2018) 32:72–83. doi: 10.1016/j.ebiom.2018.05.036
126. Hisaoka-Nakashima K, Taki S, Watanabe S, Nakamura Y, Nakata Y, Morioka N. Mirtazapine increases glial cell line-derived neurotrophic factor production through lysophosphatidic acid 1 receptor-mediated extracellular signal-regulated kinase signaling in astrocytes. *Eur J Pharmacol.* (2019) 860:172539. doi: 10.1016/j.ejphar.2019.172539
127. Wang Y, Xie L, Gao C, Zhai L, Zhang N, Guo L. Astrocytes activation contributes to the antidepressant-like effect of ketamine but not scopolamine. *Pharmacol Biochem Behav.* (2018) 170:1–8. doi: 10.1016/j.pbb.2018.05.001
128. Lasič E, Lisjak M, Horvat A, BožIć M, Šakanović A, Anderluh G, et al. Astrocyte specific remodeling of plasmalemmal cholesterol composition by ketamine indicates a new mechanism of antidepressant action. *Sci Rep.* (2019) 9:10957. doi: 10.1038/s41598-019-47459-z
129. Xue S-S, Xue F, Ma Q-R, Wang S-Q, Wang Y, Tan Q-R, et al. Repetitive high-frequency transcranial magnetic stimulation reverses depressive-like behaviors and protein expression at hippocampal synapses in chronic unpredictable stress-treated rats by enhancing endocannabinoid signaling. *Pharmacol Biochem Behav.* (2019) 184:172738. doi: 10.1016/j.pbb.2019.172738
130. Taler M, Aronovich R, Henry Hornfeld S, Dar S, Sasson E, Weizman A, et al. Regulatory effect of lithium on hippocampal blood-brain barrier integrity in a rat model of depressive-like behavior. *Bipolar Disord.* (2020) 23:55–65. doi: 10.1111/bdi.12962
131. Wang Y, Ni J, Zhai L, Gao C, Xie L, Zhao L, et al. Inhibition of activated astrocyte ameliorates lipopolysaccharide- induced depressive-like behaviors. *J Affect Disord.* (2019) 242:52–9. doi: 10.1016/j.jad.2018.08.015
132. Portal B, Delcourte S, Rovera R, Lejards C, Bullich S, Malnou CE, et al. Genetic and pharmacological inactivation of astroglial connexin 43 differentially influences the acute response of antidepressant and anxiolytic drugs. *Acta Physiol. (Oxf).* 2020:e13440. doi: 10.1111/apha.13440
133. Kong H, Sha LL, Fan Y, Xiao M, Ding JH, Wu J, Hu G. Requirement of AQP4 for antidepressive efficiency of fluoxetine: implication in adult hippocampal neurogenesis. *Neuropsychopharmacology.* (2009) 34:1263–76. doi: 10.1038/npp.2008.185
134. Hughes TM, Kuller LH, Barinas-Mitchell EJ, Mackey RH, McDade EM, Klunk WE, et al. Pulse wave velocity is associated with β-amyloid deposition in the brains of very elderly adults. *Neurology.* (2013) 81:1711–8. doi: 10.1212/01.wnl.0000435301.64776.37
135. Gos T, Schroeter ML, Lessel W, Bernstein H-G, Dobrowolny H, Schiltz K, et al. S100B-immunopositive astrocytes and oligodendrocytes in the hippocampus are differentially afflicted in unipolar and bipolar depression: a postmortem study. *J Psychiatr Res.* (2013) 47:1694–9. doi: 10.1016/j.jpsychires.2013.07.005
136. Medina A, Watson SJ, Bunney W Jr, Myers RM, Schatzberg A, Barchas J, et al. Evidence for alterations of the glial syncytial function in major depressive disorder. *J Psychiatr Res.* (2016) 72:15–21. doi: 10.1016/j.jpsychires.2015.10.010
137. Feresten AH, Barakauskas V, Ypsilanti A, Barr AM, Beasley CL. Increased expression of glial fibrillary acidic protein in prefrontal cortex in psychotic illness. *Schizophr Res.* (2013) 150:252–7. doi: 10.1016/j.schres.2013.07.024

138. Johnston-Wilson NL, Sims CD, Hofmann JP, Anderson L, Shore AD, Torrey EF, et al. Disease-specific alterations in frontal cortex brain proteins in schizophrenia, bipolar disorder, and major depressive disorder. The Stanley Neuropathology Consortium. *Mol Psychiatry.* (2000) 5:142–9. doi: 10.1038/sj.mp.4000696
139. Toro CT, Hallak JE, Dunham JS, Deakin JF. Glial fibrillary acidic protein and glutamine synthetase in subregions of prefrontal cortex in schizophrenia and mood disorder. *Neurosci Lett.* (2006) 404:276–81. doi: 10.1016/j.neulet.2006.05.067
140. Dean B, Gray L, Scarr E. Regionally specific changes in levels of cortical S100beta in bipolar 1 disorder but not schizophrenia. *Aust N Z J Psychiatry.* (2006) 40:217–24. doi: 10.1111/j.1440-1614.2006.01777.x
141. Hercher C, Chopra V, Beasley CL. Evidence for morphological alterations in prefrontal white matter glia in schizophrenia and bipolar disorder. *J Psychiatry Neurosci.* (2014) 39:376–85. doi: 10.1503/jpn.130277
142. Williams MR, Hampton T, Pearce RKB, Hirsch SR, Ansorge O, Thom M, et al. Astrocyte decrease in the subgenual cingulate and callosal genu in schizophrenia. *Eur Arch Psychiatry Clin Neurosci.* (2013) 263:41–52. doi: 10.1007/s00406-012-0328-5
143. Pantazopoulos H, Woo T-UW, Lim MP, Lange N, Berretta S. Extracellular matrix-glial abnormalities in the amygdala and entorhinal cortex of subjects diagnosed with schizophrenia. *Arch Gen Psychiatry.* (2010) 67:155–66. doi: 10.1001/archgenpsychiatry.2009.196
144. Malchow B, Strocka S, Frank F, Bernstein H-G, Steiner J, Schneider-Axmann T, et al. Stereological investigation of the posterior hippocampus in affective disorders. *J Neural Transm (Vienna).* (2015) 122:1019–33. doi: 10.1007/s00702-014-1316-x
145. Barley K, Dracheva S, Byne W. Subcortical oligodendrocyte- and astrocyte-associated gene expression in subjects with schizophrenia, major depression and bipolar disorder. *Schizophr Res.* (2009) 112:54–64. doi: 10.1016/j.schres.2009.04.019
146. Fatemi SH, Laurence JA, Araghi-Niknam M, Stary JM, Schulz SC, Lee S, et al. Glial fibrillary acidic protein is reduced in cerebellum of subjects with major depression, but not schizophrenia. *Schizophr Res.* (2004) 69:317–23. doi: 10.1016/j.schres.2003.08.014
147. Steiner J, Bielau H, Brisch R, Danos P, Ullrich O, Mawrin C, et al. Immunological aspects in the neurobiology of suicide: elevated microglial density in schizophrenia and depression is associated with suicide. *J Psychiatr Res.* (2008) 42:151–7. doi: 10.1016/j.jpsychires.2006.10.013
148. da Rosa MI, Simon C, Grande AJ, Barichello T, Oses JP, Quevedo J. Serum S100B in manic bipolar disorder patients: systematic review and meta-analysis. *J Affect Disord.* (2016) 206:210–5. doi: 10.1016/j.jad.2016.07.030
149. Iwamoto K, Kakiuchi C, Bundo M, Ikeda K, Kato T. Molecular characterization of bipolar disorder by comparing gene expression profiles of postmortem brains of major mental disorders. *Mol Psychiatry.* (2004) 9:406–6. doi: 10.1038/sj.mp.4001437
150. Woo T-UW, Walsh JP, Benes FM. Density of glutamic acid decarboxylase 67 messenger RNA-containing neurons that express the N-methyl-D-aspartate receptor subunit NR2A in the anterior cingulate cortex in schizophrenia and bipolar disorder. *Arch Gen Psychiatry.* (2004) 61:649–57. doi: 10.1001/archpsyc.61.7.649
151. Seredenina T, Sorce S, Herrmann FR, Ma Mulone XJ, Plastre O, Aguzzi A, et al. Decreased NOX2 expression in the brain of patients with bipolar disorder: association with valproic acid prescription and substance abuse. *Transl Psychiatry.* (2017) 7:e1206. doi: 10.1038/tp.2017.175
152. Harrison PJ, Geddes JR, Tunbridge EM. The emerging neurobiology of bipolar disorder. *Trends Neurosci.* (2018) 41:18–30. doi: 10.1016/j.tins.2017.10.006
153. Huber VJ, Tsujita M, Kwee IL, Nakada T. Inhibition of aquaporin 4 by antiepileptic drugs. *Bioorg Med Chem.* (2009) 17:418–24. doi: 10.1016/j.bmc.2007.12.038
154. Morton E, Murray G. An update on sleep in bipolar disorders: presentation, comorbidities, temporal relationships and treatment. *Curr Opin Psychol.* (2020) 34:1–6. doi: 10.1016/j.copsyc.2019.08.022
155. Zeppenfeld DM, Simon M, Haswell JD, D'Abreo D, Murchison C, Quinn JF, et al. Association of perivascular localization of aquaporin-4 with cognition and alzheimer disease in aging brains. *JAMA Neurol.* (2017) 74:91–9. doi: 10.1001/jamaneurol.2016.4370

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Repeated Dosing of Ketamine in the Forced Swim Test: Are Multiple Shots Better Than One?

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The anesthetic drug ketamine has been successfully repurposed as an antidepressant in human subjects. This represents a breakthrough for clinical psychopharmacology, because unlike monoaminergic antidepressants, ketamine has rapid onset, including in Major Depressive Disorder (MDD) that is resistant to conventional pharmacotherapy. This rapid therapeutic onset suggests a unique mechanism of action, which continues to be investigated in reverse translational studies in rodents. A large fraction of rodent and human studies of ketamine have focused on the effects of only a single administration of ketamine, which presents a problem because MDD is typically a persistent illness that may require ongoing treatment with this drug to prevent relapse. Here we review behavioral studies in rodents that used repeated dosing of ketamine in the forced swim test (FST), with an eye toward eventual mechanistic studies. A subset of these studies carried out additional experiments with only a single injection of ketamine for comparison, and several studies used chronic psychosocial stress, where stress is a known causative factor in some cases of MDD. We find that repeated ketamine can in some cases paradoxically produce *increases* in immobility in the FST, especially at high doses such as 50 or 100 mg/kg. Several studies however provide evidence that repeated dosing is more effective than a single dose at *decreasing* immobility, including behavioral effects that last longer. Collectively, this growing literature suggests that repeated dosing of ketamine has prominent depression-related effects in rodents, and further investigation may help optimize the use of this drug in humans experiencing MDD.

Keywords: chronic ketamine, forced swim test, major depression, literature search, sex differences, strain differences, sustained effects, subchronic

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INTRODUCTION

In the last two decades, the anesthetic drug *(R,S)*-ketamine (this racemic mixture is referred to as "ketamine" hereafter) has been successfully repurposed as an antidepressant in humans suffering from Major Depressive Disorder (MDD) (1–4). This has been a significant advance in clinical psychopharmacology because, unlike commonly used monoaminergic antidepressants such as selective serotonin reuptake inhibitors (SSRIs), ketamine has rapidly acting antidepressant properties, including in treatment resistant MDD (2, 4, 5). Monoaminergic antidepressants are also not therapeutically effective in all individuals, and can have significant side effects, creating demand for novel agents such as ketamine (6). The initial repurposing of ketamine was based on a foundation of rodent behavioral studies largely

conducted in the 1990's, using a variety of compounds that, like ketamine, block glutamatergic NMDA receptors (7–11), although the detailed molecular mechanisms of ketamine (a non-competitive NMDA receptor antagonist) began being elucidated later (8, 12). Thus, a combination of preclinical and clinical studies, a large number of which continue to be carried out, has established that use of ketamine is an important pharmacological option for clinicians in the treatment of MDD.

Rodent neuroscience models offer an excellent opportunity to elucidate the neural network mechanisms of new or repurposed medications such as ketamine. This in turn would enable us to understand limbic circuitry itself in new ways. Many studies have focused on this and an ongoing debate, particularly in the rodent preclinical literature, concerns the precise molecular and circuit-based mechanisms through which ketamine produces its favorable effects (13–16). This ongoing debate includes investigating the efficacy of the R enantiomer of ketamine in a rat learned helplessness model (15), and also potentially dose-dependent sex differences in response to ketamine (16). It is not clear at this time whether the molecular or circuit-based mechanisms of single dose ketamine differ just in magnitude or instead qualitatively from those of repeated administration, but qualitative differences are possible (17).

To date most rodent studies have used only single doses of ketamine, whereas in clinical settings repeated dosing is increasingly used to treat MDD (18–20). These rodent studies typically administer a single, systemic injection of ketamine, and then monitor behavior in the acute period afterward, or 24 h or more later (14). It is important to measure behavior beyond the acute time window, such as at the 24 h point, since ketamine has acute dissociative-like properties that can result in hyperlocomotion (21, 22) and be confounded with immobility-related behavior in the FST. For these reasons, when studying any drug in the FST it is important to also measure locomotor activity in an assay such as the open field test, to gauge whether changes observed in the FST are confounded with generalized hyperactivity (or hypoactivity). The topic of dosing frequency of ketamine is of particular importance, since in clinical settings multiple doses are often given over the course of weeks to months in individuals with MDD (18–20, 23) to prevent relapse (19, 20). While understanding the effects of repeated dosing of ketamine is important clinically, this topic is only beginning to be addressed preclinically in rodent settings where neural mechanisms can be deciphered. We can then ask, why does ketamine initially produce favorable effects? And why do these favorable effects then typically fade? These questions can only be answered if we have effective and reliable behavioral models in rodents to allow us to subsequently delve deeper into neural mechanisms.

In this brief review, we investigate the behavioral effects of repeated dosing of ketamine in rodents, focusing on the widely used behavioral assay of antidepressant compounds, the forced swim test (FST) (24, 25). We focus on the FST since at this time there are only a limited number of studies on the behavioral effects of repeated dose ketamine and those using the FST are the most numerous. That said, this appears to be a growing literature, which we summarize below. Importantly, some of these studies use chronic psychosocial stress, such as chronic unpredictable stress (CUS) [also known as unpredictable chronic mild stress (UCMS)], to determine how this affects treatment with ketamine. This is crucial since chronic stress often triggers MDD (26, 27) and stress models in rodents are one of our primary means for studying mood-related brain circuits (28). The single injection ketamine literature in the FST provides conflicting evidence as to whether prior chronic stress exposure modulates the behavioral response to this drug, with some studies showing stress-sensitivity (28, 29) and a number of others not (16, 30). This topic remains to be adequately addressed in future studies that use repeated ketamine administration.

We should point out that in recent years, the FST has been increasingly criticized as not being a direct measure of depression-related behavior (31). For example, it has been suggested that immobility in the FST represents a passive coping strategy that is a behavioral adaptation to an inescapable acute stressor, rather than modeling depression-like behavior (32–34), or represents an extinction-like response (35). In this scenario, the FST may be more of a screening test for putative antidepressant compounds in response to an acute stressor, although questions remain regarding the utility of the FST for predicting response to non-monoaminergic, fast-acting glutamatergic agents (33, 35). For all of these reasons, future rodent studies aimed at investigating the putative antidepressant properties of ketamine (or other drugs) should consider including a battery of other behavioral tests [sucrose preference, novelty suppressed feeding, splash; as well as open field (to test generalized hyperactivity)] in addition to the FST. Repeated ketamine administration is only beginning to be investigated in these other tests (36–38).

LITERATURE SEARCH DETAILS

As recently as July 6, 2020, we conducted a literature search of PubMed using the following terms: ketamine + repeated/twice/subchronic/chronic + "forced swim"/"forced swimming." We identified 24 relevant studies that used repeated administration of ketamine to mice or rats. These FST data are summarized in **Table 1**, and comprise immobility as a behavioral readout. In the studies described in **Table 1**, ketamine was injected from 2 to 30 times (and in one study was orally administered), over a period of up to 7 weeks, at a dose that varied from 0.1 to 100 mg/kg. The time delay between the final administration of ketamine and when the FST was conducted varied greatly, from 30 min to 2 months, which should be considered when interpreting acute versus sustained effects of this drug. A subset of these publications also used a *single* injection of ketamine for comparison with the multiple injections, and these data were also included in **Table 1**. During some of the experiments, chronic unpredictable stress (CUS) or unpredictable chronic mild stress (UCMS) was used, prior to the FST. The table consists of mice and rats of both sexes and various strains, ranging in age from adolescents to adults. The studies primarily used racemic (*R*,*S*)-ketamine, although one study (42) as noted in the table only used the (*S*)-ketamine enantiomer.

TABLE 1 | Summary of repeated dosing of (*R,S*)-ketamine in the rodent forced swim test (FST).

Publication	Species	Strain	Sex	Age at start	Dose (mg/kg)	# of repeats	Time delay	Stressor	Immobility
Thelen et al. (39)	Mice	C57	M	8–12 wk	3	Once daily for 21 days	24 h	Unstr	No Ch
		C57	M	8–12 wk	5	Once daily for 21 days	24 h	Unstr	No Ch
		C57	M	8–12 wk	10	Once daily for 21 days	24 h	Unstr	Decr
		C57	F	8–12 wk	3	Once daily for 21 days	24 h	Unstr	No Ch
		C57	F	8–12 wk	5	Once daily for 21 days	24 h	Unstr	No Ch
		C57	F	8–12 wk	10	Once daily for 21 days	24 h	Unstr	Incr
Clarke et al. (40)	Mice	CD-1	M	8–10 wk	10	Single injection	1 h	Unstr	Decr
		CD-1	M	8–10 wk	10	Single injection	2, 5, or 8 days	Unstr	No Ch
		CD-1	M	8–10 wk	10	3 repeats over 2 weeks	2 days	Unstr	Decr
		CD-1	M	8–10 wk	10	3 repeats over 2 weeks	8 days	Unstr	Decr
Krimmel et al. (41)	Mice	CD-1	M	7 wk	10	Single injection	1 h	Unstr	Decr
		CD-1	M	7 wk	10	Single injection	24 h	Unstr	Decr
		CD-1	F	7 wk	10	Single injection	1 h	Unstr	Decr
		CD-1	F	7 wk	10	Single injection	24 h	Unstr	No Ch
		CD-1	M	7 wk	10	Every 2nd week for 4 weeks	1 h	Unstr	Decr
		CD-1	M	7 wk	10	Every 2nd week for 4 weeks	24 h	Unstr	Decr
		CD-1	F	7 wk	10	Every 2nd week for 4 weeks	1 h	Unstr	Decr
		CD-1	F	7 wk	10	Every 2nd week for 4 weeks	24 h	Unstr	No Ch
		CD-1	M	7 wk	10	Every 2nd week for 3 weeks	1 h	Unstr	Decr
		CD-1	M	7 wk	10	Every 2nd week for 3 weeks	24 h	Unstr	Decr
		CD-1	F	7 wk	10	Every 2nd week for 3 weeks	1 h	Unstr	No Ch
		CD-1	F	7 wk	10	Every 2nd week for 3 weeks	24 h	Unstr	No Ch
Neves et al. (42)	Mice	CF-1	M	Adult	30 (*S*-ket)	Single injection	24 h	Unstr	No Ch
		CF-1	M	Adult	30 (*S*-ket)	Once daily for 14 days	2 days	Unstr	Decr
		CF-1	M	Adult	30 (*S*-ket)	Once daily for 14 days	4 days	Unstr	No Ch
		CF-1	M	Adult	30 (*S*-ket)	Once daily for 14 days	8 days	Unstr	No Ch
		CF-1	M	Adult	30 (*S*-ket)	Once daily for 14 days	15 days	Unstr	No Ch
		CF-1	M	Adult	30 (*S*-ket)	Once daily for 14 days	22 days	Unstr	No Ch
		CF-1	M	Adult	30 (*S*-ket)	Once daily for 5 days	24 h	Unstr	No Ch
Kara et al. (43)	Mice	ICR	Mix	10–12 wk	5	Single injection	30 min	Unstr	No Ch
		ICR	Mix	10–12 wk	10	Single injection	30 min	Unstr	Decr
		ICR	Mix	10–12 wk	5	Once daily for 3 weeks	30 min	Unstr	No Ch
		ICR	Mix	10–12 wk	10	Once daily for 3 weeks	30 min	Unstr	No Ch
Suárez-Santiago et al. (38)	Mice	NIH	M	2 months	10	Once daily for 5 days	20 min	Unstr	Incr
Chatterjee et al. (44)	Mice	Swiss	M	Not stated	100	Single injection	24 h	Unstr	No Ch
		Swiss	M	Not stated	100	Once daily for 10 days	30 min	Unstr	Incr
		Swiss	M	Not stated	100	Once daily for 10 days	5 days	Unstr	Incr
		Swiss	M	Not stated	100	Once daily for 10 days	10 days	Unstr	Incr
Popik et al. (45)	Mice	Swiss	M	Not stated	1.25	Single injection	30 min, 2 weeks	Unstr	No Ch
		Swiss	M	Not stated	2.5	Single injection	30 min, 2 weeks	Unstr	No Ch
		Swiss	M	Not stated	5	Single injection	30 min, 2 weeks	Unstr	No Ch

(Continued)

TABLE 1 | Continued

Publication	Species	Strain	Sex	Age at start	Dose (mg/kg)	# of repeats	Time delay	Stressor	Immobility
		Swiss	M	Not stated	10	Single injection	30 min, 2 weeks	Unstr	No Ch
		Swiss	M	Not stated	50	Single injection	30 min, 2 weeks	Unstr	Decr
Singh et al. (46)	Mice	Swiss	M	Adult	100	Once daily for 10 days	24 h	Unstr	Incr
Hou et al. (47)	Mice	Sw-K	M	Not stated	25	Single injection	24 h	Unstr	No Ch
		Sw-K	M	Not stated	50	Single injection	24 h	Unstr	No Ch
		Sw-K	M	Not Stated	100	Single injection	24 h	Unstr	No Ch
		Sw-K	M	Not stated	25	Once daily for 7 days	24 h	Unstr	No Ch
		Sw-K	M	Not stated	50	Once daily for 7 days	24 h	Unstr	No Ch
		Sw-K	M	Not stated	100	Once daily for 7 days	24 h	Unstr	Incr
Owolabi et al. (48)	Mice	Albino	Mix	Adult	3	Single injection	5 min	Unstr	Decr
		Albino	Mix	Adult	15	Single injection	5 min	Unstr	Decr
		Albino	Mix	Adult	15	Once daily for 21 days	24 h	Unstr	Incr
Zhang et al. (49)	Rat	S-D	M	7 wk	10	Once daily for 3 days	15–17 h	UCMS	Decr
		S-D	M	7 wk	10	Once daily for 7 days	15–17 h	UCMS	Decr
		S-D	M	7 wk	10	Once every 3 days for 21 days	15–17 h	UCMS	Decr
		S-D	M	7 wk	10	Once every 7 days for 21 days	15–17 h	UCMS	Decr
Jiang et al. (50)	Rat	S-D	M	35–49 days	10	Once daily for 14 days	1 week	CUS	No Ch
		S-D	M	35–49 days	10	Once daily for 14 days	7 weeks	CUS	Decr
Li et al. (51)	Rat	S-D	M	28 days old	20	Once daily for 21 days	24 h	Unstr	No Ch
		S-D	M	28 days old	30	Once daily for 21 days	24 h	Unstr	No Ch
Parise et al. (36)	Rat	S-D	M	35–49 days	20	Single injection	1 h	CUS	Decr
		S-D	M	75–89 days	20	Twice daily for 15 days	2 months	CUS	Decr
		S-D	M	35–49 days	20	Twice daily for 15 days	2 months	CUS	Decr
		S-D	M	35–49 days	5	Twice in a day	24 h	Unstr	No Ch
		S-D	M	35–49 days	10	Twice in a day	24 h	Unstr	Decr
		S-D	M	35–49 days	20	Twice in a day	24 h	Unstr	Decr
Getachew and Tizabi (52)	Rat	Wist	M	8–10 wk	2.5 + alc	Once daily for 7 days	18 h	Unstr	Decr
Garcia et al. (53)	Rat	Wist	M	60 days	5	Once daily for 14 days	1 h	Unstr	Decr
		Wist	M	60 days	10	Once daily for 14 days	1 h	Unstr	Decr
		Wist	M	60 days	15	Once daily for 14 days	1 h	Unstr	Decr
Chindo et al. (54)	Rat	Wist	Mix	Adult	30	Single injection	24 h	Unstr	No Ch
		Wist	Mix	Adult	1	Once daily for 10 days	24 h, then weekly	Unstr	No Ch
		Wist	Mix	Adult	10	Once daily for 10 days	24 h, then weekly	Unstr	No Ch
		Wist	Mix	Adult	30	Once daily for 10 days	24 h, then weekly	Unstr	Incr
		Wist	Mix	Adult	50	Once daily for 10 days	24 h, then weekly	Unstr	Incr
De Cartágenes et al. (55)	Rat	Wist	F	35 days	10	Once daily for 3 days	3 h	Unstr	Incr
Réus et al. (56)	Rat	Wist	M	3 months	30	Once daily for 14 days	1 h	Unstr	Decr
Ecevitoglu et al. (57)	Rat	Wist	M	Adult	Up to 0.2/day	16 days in drinking water	24 h then 48 h	Unstr	No Ch
		Wist	M	Adult	Up to 0.4/day	16 days in drinking water	24 h then 48 h	Unstr	Decr
Popik et al. (45)	Rat	Wist	M	Not Stated	160	Single injection	6 days, 7 days	Unstr	No Ch

(Continued)

TABLE 1 | Continued

Publication	Species	Strain	Sex	Age at start	Dose (mg/kg)	# of repeats	Time delay	Stressor	Immobility
		Wist	M	Not Stated	50	Once daily for 2 days	40 min	Unstr	Decr
		Wist	M	Not Stated	50	Twice daily for 2 weeks	40 min	Unstr	Decr
Aricioglu et al. (58)	Rat	Wist	M	8–10 wk	10	Single injection	12 h	UCMS	No Ch
		Wist	M	8–10 wk	10	Once daily for 3 weeks	12 h	UCMS	Decr
Tizabi et al. (59)	Rat	Wist	F	Adult	0.5	Single injection	30 min	Unstr	No Ch
		Wist	F	Adult	2.5	Single injection	30 min	Unstr	No Ch
		Wist	F	Adult	5	Single injection	30 min	Unstr	No Ch
		WKY	F	Adult	0.5	Single injection	30 min	Unstr	No Ch
		WKY	F	Adult	2.5	Single injection	30 min	Unstr	Decr
		WKY	F	Adult	5	Single injection	30 min	Unstr	Decr
		Wist	F	Adult	0.5	Once daily for 10 days	20–22 h	Unstr	No Ch
		Wist	F	Adult	2.5	Once daily for 10 days	20–22 h	Unstr	No Ch
		Wist	F	Adult	5	Once daily for 10 days	20–22 h	Unstr	No Ch
		WKY	F	Adult	0.5	Once daily for 10 days	20–22 h	Unstr	No Ch
		WKY	F	Adult	2.5	Once daily for 10 days	20–22 h	Unstr	Decr
		WKY	F	Adult	5	Once daily for 10 days	20–22 h	Unstr	Decr
		WKY	F	Adult	2.5	Once daily for 10 days	1 week	Unstr	Decr
		WKY	F	Adult	2.5	Once daily for 10 days	2 weeks	Unstr	No Ch
Akinfiresoye and Tizabi (60)	Rat	WKY	M	Adult	0.25	Once daily for 11 days	20 min	Unstr	No Ch
		WKY	M	Adult	0.5	Once daily for 11 days	20 min	Unstr	Decr

This table comprises the 24 mice and rat studies from our literature search that used repeated administration of ketamine in the FST. The "Immobility" column in table indicates whether ketamine decreased or increased this measure relative to vehicle-injected animals subjected to the same stress condition (i.e., stressed or unstressed), in a statistically significant manner where $p < 0.05$. The "Time Delay" column represents the amount of time between the last (or only) administration of ketamine and when the FST was carried out. All experiments that used a single injection are marked in yellow, and those that used chronic stress are marked in red. Experiments that showed a statistically significant decrease in immobility are marked in green, whereas those with a significant increase are marked in blue. C57, C57BL/6J; Sw-K, Swiss-Kunming; S-D, Sprague-Dawley; Wist, Wistar; M, male; F, female; Mix, males and females; wk, weeks; S-ket, (S)-ketamine; alc, alcohol; Unstr, unstressed; Decr, decreased; Incr, increased; No Ch, no change; UCMS, unpredictable chronic mild stress; CUS, chronic unpredictable stress.

SUMMARY OF LITERATURE

While the literature on repeated ketamine administration in the FST is somewhat limited at this time, we summarize these findings in **Table 1**. This table may already yield several principles or themes. One principle is that when the effects of ketamine are acutely measured in the FST (within an hour of final administration), whether after only a single injection or multiple injections, this drug tends to decrease immobility [for example: (41, 53)]. However, as noted above, this time window comprises the acutely intoxicating or dissociative-like effects of ketamine, and one remarkable aspect of this drug is its capacity to affect brain and behavior for hours or days *after* it has been largely systemically eliminated *in vivo* (in about 4 h). Therefore, we focus on the time period in the FST beginning 24 h or later after the last drug administration (14). While a number of the studies listed in **Table 1** investigated acute effects of ketamine, many examined longer time delays after drug injection.

Inspection of **Table 1** also reveals that not many studies of repeated ketamine have used chronic stress, even though as described earlier chronic stress or trauma is an etiological factor in human MDD (26, 27). While we identified only four studies (all carried out in rats) that used chronic stress in **Table 1**, in each of the experiments carried out there was a decrease in immobility, after either a single injection or multiple injections, that was statistically significant in most cases and lasted up to 2 months for repeated injections (36, 49, 50, 58). These daily unpredictable stress procedures varied in length from 15 (36) to 42 days (50, 58), and used either one (58) or two (36, 49, 50) stressors per day, such as cage tilting or placing wet bedding in the cage. These behavioral findings after chronic stress was administered are consistent with experiments from our laboratory that used C57BL/6J mice, and found that chronic stress was either necessary for (28) or amplified (29) the increases in swimming or decreases in immobility produced by a single injection of ketamine. Future studies should further investigate how repeated ketamine modulates the behavioral and neural effects of chronic stress.

In comparing mice with rats in **Table 1**: while studies often used different parameters such as varied dosing and none of the mouse studies used chronic stress, we can conclude that repeated ketamine is capable of producing either decreases or increases in immobility in the FST in both species, under various experimental conditions, with higher doses such 50 or 100 mg/kg favoring immobility. Regarding different strains of mice or rats: the three studies that used CD-1 and CF-1 mice only found

decreases in immobility (40–42), whereas studies of other albino mouse strains and C57BL/6J mice showed either increases or decreases in immobility depending on the study parameters and strain used (39, 44–48). Likewise, Sprague-Dawley or WKY rats were only observed to have decreases in immobility (36, 49–51, 59, 60). One of these studies directly compared female Wistar and WKY rats, and found that the latter strain was more responsive to the immobility decreasing effects of this drug (59). As mentioned above, Neves et al. (42) was the only study in **Table 1** that used the enantiomer, (*S*)-ketamine. It is noteworthy that this study showed an advantage of repeated over single dose ketamine at 30 mg/kg administered daily for 2 weeks, although the favorable effect of repeated drug did not persist beyond 2 days (42).

These preliminary observations on species and strain should be tested in further studies in a controlled manner. It is not clear whether these differences in the FST reflect differential response to ketamine or qualitatively different engagement by the strains with the FST itself. For example, in one strain, higher mobility may indicate more motivation or exploratory drive, but in another it may indicate depressive-like behavior that resembles psychomotor agitation—-which are on opposing ends of the typical spectrum of neuropsychiatric health states. Some of our own recent data suggest that male C57BL/6J mice, for example, differ in FST behavior from other common inbred and outbred strains, perhaps showing greater sensitivity to the immobility enhancing effects of one injection of 10 mg/kg ketamine in unstressed animals (14). We have also recently demonstrated that female C57BL/6J mice can be more sensitive than males to the favorable effects of a single dose of 30 mg/kg ketamine in the FST, an effect that is amplified by prior exposure to chronic stress (29). Finally, there is growing evidence that, in addition to factors such as animal strain, sex, and age, the sex of the experimenter may play a prominent role in the outcomes of behavioral experiments such as the FST (61, 62). These factors need to be considered in future studies that investigate repeated dosing of ketamine and other drugs.

Most of the experiments in **Table 1** used male rather than female mice and rats. Inspection of the table reveals that both male and female mice and rats are capable of exhibiting either increases or decreases in immobility in response to repeated ketamine under different experimental conditions. For example, in females repeated doses as low as 10 mg/kg can produce *increases* in immobility (39, 55). There appears to be only a limited literature directly comparing males and females after repeated dosing of ketamine in the FST, although studies such as Thelen et al. (39) and Krimmel et al. (41) suggest that female mice may be less sensitive than males to the immobility decreasing effects of repeated ketamine. Several studies in the single injection ketamine literature suggest that female mice or rats show decreased immobility after lower doses (such as 10 mg/kg or less), but the duration of action may be longer in males, although our recent study may be an exception to this latter point (29, 63–65).

Is there an optimal dose for repeated ketamine that emerges from these studies? High doses, such as 50 or 100 mg/kg, can be associated with *increases* in immobility in **Table 1** both acutely and up to 10 days later (44, 46, 54), although 50 mg/kg is still a subanesthetic dose. While we have previously suggested (14) that the field has converged on using 10 mg/kg as a standard dose in single injection rodent FST experiments (16), our own data have suggested that 30 mg/kg is more effective at reducing immobility in male C57BL/6J mice (28). A repeated dose as low as 10 mg/kg in **Table 1** has actually been associated with increases in immobility in mice (38, 39, 55). The field has not converged on a single repeated dose found to be generally efficacious in all labs and contradictory results are frequent. This will encumber efforts to consistently study these phenomena. The variety of results may stem from strain differences (including between inbred versions of the same strain from different vendors or laboratories), handling differences (both by laboratory staff and animal housing staff), environmental differences, or differences in the testing and quantification itself. These problems are not new to behavioral pharmacology, but ketamine research has not avoided them.

Lastly and perhaps most importantly, are multiple administrations of ketamine more effective in the FST than a single administration of this drug, based on the limited data set described in **Table 1**? As stated above, chronic treatment with ketamine can increase immobility in some studies, especially at moderate to high doses (30–100 mg/kg). Whether repeated use of lower doses is more effective at reducing immobility than a single administration of that same dose or a different one, seems to be unclear at this time. Some studies that used both single and repeated injections show an advantage for chronic dosing over a single dose (40, 42, 58), whereas others show the opposite pattern (47, 54) or no clear advantage for either dosing regimen (41). The Chindo et al. (54) and Hou et al. (47) studies found immobility increasing effects of repeated ketamine at relatively high doses of this drug (30–100 mg/kg), which may suggest that repeated dosing can be more favorable than a single dose if a sufficiently low dose (such as 10 mg/kg or lower) is used repeatedly, or perhaps if there is a longer interval between administrations. Beyond there being some degree of difference in dose used in these above five studies, it is difficult to draw further conclusions from them on the efficacy of repeated dosing due to different experimental parameters used, such as (*S*)-ketamine (42) or chronic stress (58). In general, the scarcity of studies reviewed here that have used repeated ketamine in the FST precludes definitive conclusions on the potential interactions between variables such as strain, sex, stress, and dose. To better compare a single dose with repeated dosing, future studies should keep all other experimental variables the same, including the time lag between the last (or only) dose of ketamine and the start of the FST or other behavioral tests [sucrose preference, novelty suppressed feeding, splash; as well as open field (to test generalized hyperactivity)]. It should be noted that in a recent study using rats selectively bred for depressive-like behavior, repeated ketamine (10 mg/kg/day for 7 consecutive days) did not produce favorable brain network topological effects relative to vehicle or a single ketamine injection, calling into question the general approach of using repeated ketamine in this animal model (17).

A related topic is whether the immobility decreasing effects of repeated ketamine last longer than a single dose. For example, studies such as Clarke et al. (40) and Neves et al. (42), which directly compare in mice a single injection vs. multiple injections at the same dose, suggest that the immobility decreasing effects of repeated ketamine may last longer than those of one injection. Another point is that repeated dosing regimens are capable of producing a decrease in immobility when ketamine is given at different fixed intervals, including less frequently than once a day (40, 41, 49), exactly once a day (39, 42, 49, 50, 52, 53, 58–60), or twice a day (36). In Sprague-Dawley rats weekly administration of ketamine, for 5 weeks at 20 or 50 mg/kg, has previously been shown to induce locomotor sensitization (66). All of the experiments in **Table 1** that repeatedly administered ketamine used a fixed interval. It would also be informative to study this drug when given at a variable interval. One possibility is that a variable interval may be less likely to produce mood cycling or therapeutic habituation, since drug administration would not occur at a fixed, repeating interval.

Future reverse translational studies of ketamine in rodents may benefit from investigating dosing regimens, such as three times a week for 2 weeks and related protocols, that have been used effectively in human MDD studies (1, 19, 67, 68). Another consideration in future rodent studies, as well as clinical trials, would be to test whether monoaminergic antidepressants can amplify or further sustain the antidepressant properties of ketamine (69), including the combination of esketamine with SSRIs or SNRIs in rodents or treatment resistant human subjects (70). Likewise, if treatment with a monoaminergic antidepressant is initiated but only results in a weak response for a given individual, perhaps ketamine can then be added to amplify the response. It has already been demonstrated in MDD that repeated intranasal esketamine plus a conventional antidepressant can be more effective than an antidepressant alone (71). Several studies in the rodent literature also suggest that SSRIs modulate the behavioral effects of ketamine. For example, chronically administered citalopram can hasten and sustain the anti-immobility effect of repeated ketamine (10 mg/kg once every 7 days for 3 weeks) (49), although another study that gave daily ketamine for 2 weeks at a higher dose (30 mg/kg/day) found no effect of a single dose of fluoxetine (72). A study that reported an *increase* in immobility with 30 mg/kg/day ketamine for 5 days showed that a single dose of paroxetine could attenuate this effect (54). One or two injections of sertraline can also counteract hyperlocomotion induced by 10 consecutive days of 15 mg/kg ketamine (73). Combining repeated ketamine with conventional monoaminergic antidepressants is a promising line of inquiry that should be investigated further.

Another approach has been suggested for prolonging the antidepressant properties of a single administration of ketamine: using (*R*)-ketamine instead of the racemic mixture of this drug or (*S*)-ketamine. A number of rodent studies that used a variety of depression-related tests including the FST, have suggested that a single administration of (*R*)-ketamine has greater therapeutic potency and more sustained effects than (*S*)-ketamine (74–76). These studies also suggest that (*R*)-ketamine has fewer side effects, such as dissociative-like properties and hyperlocomotion, than (*S*)-ketamine. A body of repeated dosing studies in rodents also suggests that (*R*)-ketamine has more favorable effects on: bone mineral density in ovariectomized mice (77), MPTP-induced dopaminergic neurotoxicity (78), and phencyclidine-induced cognitive deficits (79).

CONCLUSIONS

Based on our literature search, we find that administering ketamine multiple times has prominent effects in the rodent FST. Perhaps most importantly, several studies that directly compared single with multiple injections of ketamine found evidence for greater efficacy at decreasing immobility, that is possibly of longer duration, with repeated drug administration, especially when lower doses (10 mg/kg or lower) are used. Further, repeated injections of this drug are capable of producing both increases in immobility (especially at higher doses such as 30–100 mg/kg) and decreases, both acutely and in a more sustained fashion. Within the 1st h after administration, ketamine more reliably reduces immobility, but this may be confounded with dissociative-related hyperactivity. In the few studies that used chronic psychosocial stress, either single or repeated ketamine tended to reduce immobility. Both mice and rats (including males and females) are capable of exhibiting either increases or decreases in immobility after repeated ketamine (depending on experimental parameters such as dose or administration interval), and there is some evidence that this could differ by strain. Two studies suggest that female mice are less sensitive than males to the favorable effects of this drug in the FST, which is an important topic with respect to higher rates of MDD in women. Future studies should further investigate the optimal dosing interval (and dose) of repeated ketamine treatment in the rodent FST and related tests. The potential sex differences in response to ketamine have been particularly understudied at this time, and we are also only beginning to appreciate whether certain strains such as C57BL/6J mice are better than others for modeling depression-related behavior and its response to ketamine (14). These future studies should be conducted with an eye toward maximizing the sustained antidepressant effects of this unique pharmacological agent in the treatment of MDD in human subjects.

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RW, PF, and BW conceived of this review, wrote, and edited the manuscript. RW performed the literature search and constructed the table, with assistance from PF. All authors contributed to the article and approved the submitted version.

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REFERENCES

1. Aan Het Rot M. Safety and efficacy of repeated-dose intravenous ketamine for treatment-resistant depression. *Biol Psychiatry.* (2010) 67:139–45. doi: 10.1016/j.biopsych.2009.08.038
2. Berman RM, Cappiello A, Anand A, Oren DA, Heninger GR, Charney DS, et al. Antidepressant effects of ketamine in depressed patients. *Biol Psychiatry.* (2000) 47:351–54. doi: 10.1016/S0006-3223(99)00230-9
3. Krystal JH, Abdallah CG, Sanacora G, Charney DS, Duman RS. Ketamine: a paradigm shift for depression research and treatment. *Neuron.* (2019) 101:774–8. doi: 10.1016/j.neuron.2019.02.005
4. Zarate CA, Singh JB, Carlson PJ, Brutsche NE, Ameli R, Luckenbaugh DA, et al. A randomized trial of an N-methyl-D-aspartate antagonist in treatment-resistant major depression. *Arch Gen Psychiatry.* (2006) 63:856–64. doi: 10.1001/archpsyc.63.8.856
5. Duman RS. Ketamine and rapid-acting antidepressants: a new era in the battle against depression and suicide. *F1000Research.* (2018) 7:F1000 Faculty Rev−659. doi: 10.12688/f1000research.14344.1
6. Ruhe HG, Huyser J, Swinkels JA, Schene AH. Switching antidepressants after a first selective serotonin reuptake inhibitor in major depressive disorder: a systematic review. *J Clin Psychiatry.* (2006) 67:1836–55. doi: 10.4088/JCP.v67n1203
7. Layer RT, Popik P, Olds T, Skolnick P. Antidepressant-like actions of the polyamine site NMDA antagonist, eliprodil (SL-82.0715). *Pharmacol Biochem Behav.* (1995) 52:621–7. doi: 10.1016/0091-3057(95)00155-P
8. Maeng S, Zarate CA, Du J, Schloesser RJ, McCammon J, Chen G, et al. Cellular mechanisms underlying the antidepressant effects of ketamine: role of α-Amino-3-Hydroxy-5-Methylisoxazole-4-Propionic acid receptors. *Biol Psychiatry.* (2008) 63:349–52. doi: 10.1016/j.biopsych.2007.05.028
9. Meloni D, Carla Gambarana I, Graziella Montis MDE, Dal Pra P, Taddei I, Tagliamonte A, et al. Dizocilpine antagonizes the effect of chronic imipramine on learned helplessness in rats. *Pharmacol Biochem Behav.* (1993) 46:423–6. doi: 10.1016/0091-3057(93)90374-3
10. Papp M, Moryl E. Antidepressant-like effects of 1-aminocyclopropanecarboxylic acid and D-cycloserine in an animal model of depression. *Eur J Pharmacol.* (1996) 316:145–51. doi: 10.1016/S0014-2999(96)00675-9
11. Trullas R, Skolnick P. Functional antagonists at the NMDA receptor complex exhibit antidepressant actions. *Eur J Pharmacol.* (1990) 185:1–10. doi: 10.1016/0014-2999(90)90204-J
12. Li N, Lee B, Liu RJ, Banasr M, Dwyer JM, Iwata M, et al. mTOR-dependent synapse formation underlies the rapid antidepressant effects of NMDA antagonists. *Science.* (2010) 329:959–64. doi: 10.1126/science.1190287
13. Autry AE, Adachi M, Nosyreva E, Na ES, Los MF, Cheng PF, et al. NMDA receptor blockade at rest triggers rapid behavioural antidepressant responses. *Nature.* (2011) 475:91–5. doi: 10.1038/nature10130
14. Polis AJ, Fitzgerald PJ, Hale PJ, Watson BO. Rodent ketamine depression-related research: Finding patterns in a literature of variability. *Behav Brain Res.* (2019) 376:112153. doi: 10.1016/j.bbr.2019.112153
15. Shirayama Y, Hashimoto K. Lack of antidepressant effects of (2R,6R)-hydroxynorketamine in a rat learned helplessness model: comparison with (R)-ketamine. *Int J Neuropsychopharmacol.* (2018) 21:84–8. doi: 10.1093/ijnp/pyx108
16. Zanos P, Moaddel R, Morris PJ, Georgiou P, Fischell J, Elmer GI, et al. NMDAR inhibition-independent antidepressant actions of ketamine metabolites. *Nature.* (2016) 533:481–6. doi: 10.1038/nature17998
17. Gass N, Becker R, Reinwald J, Cosa-Linan A, Sack M, Weber-Fahr W, et al. The influence of ketamine's repeated treatment on brain topology does not suggest an antidepressant efficacy. *Transl Psychiatry.* (2020) 10:56. doi: 10.1038/s41398-020-0727-8
18. Daly EJ, Singh JB, Fedgchin M, Cooper K, Lim P, Shelton RC, et al. Efficacy and safety of intranasal esketamine adjunctive to oral antidepressant therapy in treatment-resistant depression: a randomized clinical trial. *JAMA Psychiatry.* (2018) 75:139–48. doi: 10.1001/jamapsychiatry.2017.3739
19. Murrough JW, Perez AM, Pillemer S, Stern J, Parides MK, Aan Het Rot M, et al. Rapid and longer-term antidepressant effects of repeated ketamine infusions in treatment-resistant major depression. *Biol Psychiatry.* (2013) 74:250–6. doi: 10.1016/j.biopsych.2012.06.022
20. Wilkinson ST, Katz RB, Toprak M, Webler R, Ostroff RB, Sanacora G. Acute and longer-term outcomes using ketamine as a clinical treatment at the yale psychiatric hospital. *J Clin Psychiatry.* (2018) 79:1–16. doi: 10.4088/JCP.17m11731
21. McDougall SA, Park GI, Ramirez GI, Gomez V, Adame BC, Crawford CA. Sex-dependent changes in ketamine-induced locomotor activity and ketamine pharmacokinetics in preweanling, adolescent, adult rats. *Eur Neuropsychopharmacol.* (2019) 29:740–55. doi: 10.1016/j.euroneuro.2019.03.013
22. Wilson C, Cone K, Kercher M, Hibbitts J, Fischer J, Van Lake A, et al. Naloxone increases ketamine-induced hyperactivity in the open field in female rats. *Pharmacol Biochem Behav.* (2005) 81:530–4. doi: 10.1016/j.pbb.2005.03.018
23. Singh JB, Fedgchin M, Daly E, Xi L, Melman C, De Bruecker G, et al. Intravenous esketamine in adult treatment-resistant depression: a double-blind, double-randomization, placebo-controlled study. *Biol Psychiatry.* (2016) 80:424–31. doi: 10.1016/j.biopsych.2015.10.018
24. Porsolt RD, Bertin A, Jalfre M. Behavioral despair in mice: a primary screening test for antidepressants. *Arch Int Pharmacodyn Ther.* (1977) 229:327–36.
25. Slattery DA, Cryan JF. Using the rat forced swim test to assess antidepressant-like activity in rodents. *Nat Protocols.* (2012) 7:1009–14. doi: 10.1038/nprot.2012.044
26. Bonde JP, Utzon-Frank N, Bertelsen M, Borritz M, Eller NH, Nordentoft M, et al. Risk of depressive disorder following disasters and military deployment: systematic review with meta-analysis. *Br J Psychiatry.* (2016) 208:330–6. doi: 10.1192/bjp.bp.114.157859
27. Hosang GM, Shiles C, Tansey KE, McGuffin P, Uher R. Interaction between stress and the BDNF Val66Met polymorphism in depression: a systematic review and meta-analysis. *BMC Med.* (2014) 12:7. doi: 10.1186/1741-7015-12-7
28. Fitzgerald PJ, Yen JY, Watson BO. Stress-sensitive antidepressant-like effects of ketamine in the mouse forced swim test. *PLoS ONE.* (2019) 14:e0215554. doi: 10.1371/journal.pone.0215554
29. Fitzgerald PJ, Kounelis-Wuillaume SK, Gheidi A, Morrow JD, Spencer-Segal JL, Watson BO. Sex- and stress-dependent effects of a single injection of ketamine on open field and forced swim behavior. *Stress.* (2021) 1:1–9. doi: 10.1080/10253890.2021.1871600
30. Pham TH, Defaix C, Xu X, Deng SX, Fabresse N, Alvarez JC, et al. Common neurotransmission recruited in (R,S)-ketamine and (2R,6R)-hydroxynorketamine–induced sustained antidepressant-like effects. *Biol Psychiatry.* (2018) 84:e3–6. doi: 10.1016/j.biopsych.2017.10.020
31. Reardon S. Depression researchers rethink popular mouse swim tests. *Nature.* (2019) 571:456–7. doi: 10.1038/d41586-019-02133-2
32. Commons KG, Cholanians AB, Babb JA, Ehlinger DG. The rodent forced swim test measures stress-coping strategy, not depression-like behavior. *ACS Chem Neurosci.* (2017) 8:955–60. doi: 10.1021/acschemneuro.7b00042
33. Molendijk ML, de Kloet ER. Immobility in the forced swim test is adaptive and does not reflect depression. *Psychoneuroendocrinology.* (2015) 62:389–91. doi: 10.1016/j.psyneuen.2015.08.028
34. Molendijk ML, de Kloet ER. Coping with the forced swim stressor: current state-of-the-art. *Behav Brain Res.* (2019) 364:1–10. doi: 10.1016/j.bbr.2019.02.005
35. Campus P, Colelli V, Orsini C, Sarra D, Cabib S. Evidence for the involvement of extinction-associated inhibitory learning in the forced swimming test. *Behav Brain Res.* (2015) 278:348–55. doi: 10.1016/j.bbr.2014.10.009
36. Parise EM, Alcantara LF, Warren BL, Wright KN, Hadad R, Sial OK, et al. Repeated ketamine exposure induces an enduring resilient phenotype in adolescent and adult rats. *Biol Psychiatry.* (2013) 74:750–9. doi: 10.1016/j.biopsych.2013.04.027
37. Schumacher A, Sivanandan B, Tolledo EC, Woldegabriel J, Ito R. Different dosing regimens of repeated ketamine administration have opposite effects on novelty processing in rats. *Prog Neuro-Psychopharmacol Biol Psychiatry.* (2016) 69:1–10. doi: 10.1016/j.pnpbp.2016.03.007
38. Suárez-Santiago JE, Briones-Aranda A, Espinosa-Raya J, Picazo O. Agonist E-6837 and antagonist SB-271046 of 5-HT 6 receptors both reverse the depressive-like effect induced in mice by subchronic ketamine administration. *Behav Pharmacol.* (2017) 28:582–5. doi: 10.1097/FBP.0000000000000327

39. Thelen C, Sens J, Mauch J, Pandit R, Pitychoutis PM. Repeated ketamine treatment induces sex-specific behavioral and neurochemical effects in mice. *Behav Brain Res.* (2016) 312:305–12. doi: 10.1016/j.bbr.2016.06.041
40. Clarke M, Razmjou S, Prowse N, Dwyer Z, Litteljohn D, Pentz R, et al. Ketamine modulates hippocampal neurogenesis and pro-inflammatory cytokines but not stressor induced neurochemical changes. *Neuropharmacology.* (2017) 112:210–20. doi: 10.1016/j.neuropharm.2016.04.021
41. Krimmel SR, Zanos P, Georgiou P, Colloca L, Gould TD. Classical conditioning of antidepressant placebo effects in mice. *Psychopharmacology.* (2020) 237:93–102. doi: 10.1007/s00213-019-05347-4
42. Neves G, Borsoi M, Antonio CB, Pranke MA, Betti AH, Rates SMK. Is forced swimming immobility a good endpoint for modeling negative symptoms of schizophrenia? - study of sub-anesthetic ketamine repeated administration effects. *An Acad Bras Cienc.* (2017) 89:1655–69. doi: 10.1590/0001-3765201720160844
43. Kara NZ, Agam G, Anderson GW, Zitron N, Einat H. Lack of effect of chronic ketamine administration on depression-like behavior and frontal cortex autophagy in female and male ICR mice. *Behav Brain Res.* (2017) 317:576–80. doi: 10.1016/j.bbr.2016.09.056
44. Chatterjee M, Jaiswal M, Palit G. Comparative evaluation of forced swim test and tail suspension test as models of negative symptom of schizophrenia in rodents. *ISRN Psychiatry.* (2012) 2012:1–5. doi: 10.5402/2012/595141
45. Popik P, Kos T, Sowa-Kućma M, Nowak G. Lack of persistent effects of ketamine in rodent models of depression. *Psychopharmacology.* (2008) 198:421–30. doi: 10.1007/s00213-008-1158-z
46. Singh S, Choudhury A, Gusain P, Parvez S, Palit G, Shukla S, et al. Oral acetate supplementation attenuates N-methyl D-aspartate receptor hypofunction-induced behavioral phenotypes accompanied by restoration of acetyl-histone homeostasis. *Psychopharmacology.* (2016) 233:1257–68. doi: 10.1007/s00213-016-4213-1
47. Hou Y, Zhang H, Xie G, Cao X, Zhao YN, Liu Y, et al. Neuronal injury, but not microglia activation, is associated with ketamine-induced experimental schizophrenic model in mice. *Prog Neuro-Psychopharmacol Biol Psychiatry.* (2013) 45:107–16. doi: 10.1016/j.pnpbp.2013.04.006
48. Owolabi RA, Akanmu MA, Adeyemi OI. Effects of ketamine and N-methyl-d-aspartate on fluoxetine-induced antidepressant-related behavior using the forced swimming test. *Neurosci Lett.* (2014) 566:172–6. doi: 10.1016/j.neulet.2014.01.015
49. Zhang GF, Liu WX, Qiu LL, Guo J, Wang XM, Sun HL, et al. Repeated ketamine administration redeems the time lag for citalopram's antidepressant-like effects. *Eur Psychiatry.* (2015) 30:504–10. doi: 10.1016/j.eurpsy.2014.11.007
50. Jiang Y, Wang Y, Sun X, Lian B, Sun H, Wang G, et al. Short- and long-term antidepressant effects of ketamine in a rat chronic unpredictable stress model. *Brain Behav.* (2017) 7:1–11. doi: 10.1002/brb3.749
51. Li Q, Wu HR, Fan SJ, Liu DX, Jiang H, Zhang Q, et al. The effects of sub-anesthetic ketamine plus ethanol on behaviors and apoptosis in the prefrontal cortex and hippocampus of adolescent rats. *Pharmacol Biochem Behav.* (2019) 184:172742. doi: 10.1016/j.pbb.2019.172742
52. Getachew B, Tizabi Y. Both ketamine and NBQX attenuate alcohol-withdrawal induced depression in male rats. *J Drug Alcohol Res.* (2019) 8:236069. doi: 10.4303/jdar/236069
53. Garcia LS, Comim CM, Valvassori SS, Réus GZ, Andreazza AC, Stertz L, et al. Chronic administration of ketamine elicits antidepressant-like effects in rats without affecting hippocampal brain-derived neurotrophic factor protein levels. *Basic Clin Pharmacol Toxicol.* (2008) 103:502–6. doi: 10.1111/j.1742-7843.2008.00210.x
54. Chindo BA, Adzu B, Yahaya TA, Gamaniel KS. Ketamine-enhanced immobility in forced swim test: a possible animal model for the negative symptoms of schizophrenia. *Prog Neuro-Psychopharmacol Biol Psychiatry.* (2012) 38:310–6. doi: 10.1016/j.pnpbp.2012.04.018
55. De Carvalho Cartágenes S, Fernandes LMP, Carvalheiro TCVS, De Sousa TM, Gomes ARQ, Monteiro MC, et al. "Special K" drug on adolescent rats: Oxidative damage and neurobehavioral impairments. *Oxid Med Cell Longevity.* (2019) 2019:5452727. doi: 10.1155/2019/5452727
56. Réus GZ, Abelaira HM, dos, Santos MAB, Carlessi AS, Tomaz DB, et al. Ketamine and imipramine in the nucleus accumbens regulate histone deacetylation induced by maternal deprivation and are critical for associated behaviors. *Behav Brain Res.* (2013) 256:451–6. doi: 10.1016/j.bbr.2013.08.041
57. Ecevitoglu A, Canbeyli R, Unal G. Oral ketamine alleviates behavioral despair without cognitive impairment in Wistar rats. *Behav Brain Res.* (2019) 372:112058. doi: 10.1016/j.bbr.2019.112058
58. Aricioglu F, Yalcinkaya C, Ozkartal CS, Tuzun E, Sirvanci S, Kucukali CI, et al. NLRP1-mediated antidepressant effect of ketamine in chronic unpredictable mild stress model in rats. *Psychiatry Investig.* (2020) 17:283–91. doi: 10.30773/pi.2019.0189
59. Tizabi Y, Bhatti BH, Manaye KF, Das JR, Akinfiresoye L. Antidepressant-like effects of low ketamine dose is associated with increased hippocampal AMPA/NMDA receptor density ratio in female Wistar-Kyoto rats. *Neuroscience.* (2012) 213:72–80. doi: 10.1016/j.neuroscience.2012.03.052
60. Akinfiresoye L, Tizabi Y. Antidepressant effects of AMPA and ketamine combination: role of hippocampal BDNF. synapsin, and mTOR. *Psychopharmacology.* (2013) 230:291–8. doi: 10.1007/s00213-013-3153-2
61. Chapman CD, Benedict C, Schiöth HB. Experimenter gender and replicability in science. *Sci Adv.* (2018) 4:e1701427. doi: 10.1126/sciadv.1701427
62. Georgiou P, Zanos P, Jenne C, Highland J, Gerhard D, Duman R, et al. Human experimenter sex modulates mouse behavioral responses to stress and to the antidepressant ketamine. *Biol Psychiatry.* (2018) 83:S277. doi: 10.1016/j.biopsych.2018.02.715
63. Carrier N, Kabbaj M. Sex differences in the antidepressant-like effects of ketamine. *Neuropharmacology.* (2013) 70:27–34. doi: 10.1016/j.neuropharm.2012.12.009
64. Franceschelli A, Sens J, Herchick S, Thelen C, Pitychoutis PM. Sex differences in the rapid and the sustained antidepressant-like effects of ketamine in stress-naïve and "depressed" mice exposed to chronic mild stress. *Neuroscience.* (2015) 290:49–60. doi: 10.1016/j.neuroscience.2015.01.008
65. Sarkar A, Kabbaj M. Sex differences in effects of ketamine on behavior, spine density, and synaptic proteins in socially isolated rats. *Biol Psychiatry.* (2016) 80:448–56. doi: 10.1016/j.biopsych.2015.12.025
66. Trujillo KA, Zamora JJ, Warmoth KP. Increased response to ketamine following treatment at long intervals: implications for intermittent use. *Biol Psychiatry.* (2008) 63:178–83. doi: 10.1016/j.biopsych.2007.02.014
67. Ionescu DF. Repeat-dose ketamine augmentation for treatment-resistant depression with chronic suicidal ideation: a randomized, double blind, placebo controlled trial. *J Affect Disord.* (2019) 243:516–24. doi: 10.1016/j.jad.2018.09.037
68. Phillips JL, Norris S, Talbot J, Birmingham M, Hatchard T, Ortiz A, et al. Single, repeated, and maintenance ketamine infusions for treatment-resistant depression: a randomized controlled trial. *Am J Psychiatry.* (2019) 176:401–9. doi: 10.1176/appi.ajp.2018.18070834
69. Melo A, Kokras N, Dalla C, Ferreira C, Ventura-Silva AP, Sousa N, et al. The positive effect on ketamine as a priming adjuvant in antidepressant treatment. *Transl Psychiatry.* (2015) 5:1–10. doi: 10.1038/tp.2015.66
70. Fedgchin M, Trivedi M, Daly EJ, Melkote R, Lane R, Lim P, et al. Efficacy and safety of fixed-dose esketamine nasal spray combined with a new oral antidepressant in treatment-resistant depression: results of a randomized, double-blind, active-controlled study (TRANSFORM-1). *Int J Neuropsychopharmacol.* (2019) 22:616–30. doi: 10.1093/ijnp/pyz039
71. Popova V, Daly EJ, Trivedi M, Cooper K, Lane R, Lim P, et al. Efficacy and safety of flexibly dosed esketamine nasal spray combined with a newly initiated oral antidepressant in treatment-resistant depression: a randomized double-blind active-controlled study. *Am J Psychiatry.* (2019) 176:428–38. doi: 10.1176/appi.ajp.2019.19020172
72. Borsoi M, Nunes LED, Barbosa AR, Lima MS, Medeiros I, Pranke MA, et al. Intermittent repeated stress but not ketamine changes mice response to antidepressants. *Neurosci Lett.* (2021) 741:135452. doi: 10.1016/j.neulet.2020.135452
73. Onaolapo OJ, Paul TB, Onaolapo AY. Comparative effects of sertraline, haloperidol or olanzapine treatments on ketamine-induced changes in mouse behaviours. *Metab Brain Dis.* (2017) 32:1475–89. doi: 10.1007/s11011-017-0031-3

74. Fukumoto K, Toki H, Iijima M, Hashihayata T, Yamaguchi JI, Hashimoto K, et al. Antidepressant potential of (R)-ketamine in rodent models: comparison with (S)-ketamine. *J Pharmacol Exp Therap*. (2017) 361:9–16. doi: 10.1124/jpet.116.239228
75. Yang C, Shirayama Y, Zhang JC, Ren Q, Yao W, Ma M, et al. R-ketamine: a rapid-onset and sustained antidepressant without psychotomimetic side effects. *Transl Psychiatry*. (2015) 5:e632. doi: 10.1038/tp.2015.136
76. Zhang JC, Li SX, Hashimoto K. R (-)-ketamine shows greater potency and longer lasting antidepressant effects than S (+)-ketamine. *Pharmacol Biochem Behav*. (2014) 116:137–41. doi: 10.1016/j.pbb.2013.11.033
77. Fujita Y, Hashimoto K. Decreased bone mineral density in ovariectomized mice is ameliorated after subsequent repeated intermittent administration of (R)-ketamine, but not (S)-ketamine. *Neuropsychopharmacol Rep*. (2020) 40:401–6. doi: 10.1002/npr2.12132
78. Fujita A, Fujita Y, Pu Y, Chang L, Hashimoto K. MPTP-induced dopaminergic neurotoxicity in mouse brain is attenuated after subsequent intranasal administration of (R)-ketamine: a role of TrkB signaling. *Psychopharmacology*. (2020) 237:83–92. doi: 10.1007/s00213-019-05346-5
79. Tan Y, Fujita Y, Qu Y, Chang L, Pu Y, Wang S, et al. Phencyclidine-induced cognitive deficits in mice are ameliorated by subsequent repeated intermittent administration of (R)-ketamine, but not (S)-ketamine: role of BDNF-TrkB signaling. *Pharmacol Biochem Behav*. (2020) 188:172839. doi: 10.1016/j.pbb.2019.172839

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M2-AChR Mediates Rapid Antidepressant Effects of Scopolamine Through Activating the mTORC1-BDNF Signaling Pathway in the Medial Prefrontal Cortex

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Background: Scopolamine, a non-selective muscarinic acetylcholine receptor (M1∼5-AChR) antagonist, has rapid and robust antidepressant effects in humans and other species. However, which of the five M-AChRs mediates these therapeutic effects has not been fully identified. Several studies implicate M2-AChR as a potential antidepressant target of scopolamine. This study aimed to explore the role of M2-AChR in scopolamine's antidepressant-like effects and determine the underlying mechanisms.

Methods: We used the classic novelty suppressed feeding test (NSFT), open field test (OFT) and forced swim test (FST) to observe antidepressant-related behaviors of normal rats, medial prefrontal cortex (mPFC) neuron silenced rats and M2-AChR knockdown rats treated with scopolamine. In a further experiment, the M2 cholinergic receptor antagonist methoctramine (MCT) was injected intracerebroventricularly into normal rats. Levels of mTORC1 and brain-derived neurotrophic factor (BDNF) in the mPFC of animals were analyzed by Western blotting.

Results: Consistent with previous studies, mPFC was required for the antidepressant-like effects of scopolamine, and intracerebroventricular injection of MCT into rats could produce similar antidepressant-like effects. Use of AAV-shRNA to knock down M2-AChR in the mPFC resulted in the antidepressant-like effects of scopolamine being blunted. Furthermore, Western blotting demonstrated increased expression of mTORC1 signaling and BDNF in MCT-treated rats.

Conclusion: Our results indicate that M2-AChR in the mPFC mediates the antidepressant-like effects of scopolamine by increasing the expression of BDNF and activating the mTORC1 signaling pathway.

Keywords: scopolamine, muscarinic acetylcholine receptor-2, medial prefrontal cortex, mammalian target of rapamycin complex 1, brain-derived neurotrophic factor

INTRODUCTION

Major depressive disorder (MDD) is characterized by anhedonia, loss of motivation and depressive mood. The prevalence of MDD in China is about 3.4%, causing an enormous economic burden on society (1). Drug therapy is the main treatment for MDD. However, the commonly used antidepressant drugs, including selective serotonin reuptake inhibitors (SSRIs) and selective serotonin and norepinephrine reuptake inhibitors (SNRIs), usually take 4–6 weeks to become effective, reducing the medication compliance of patients and having detrimental effects on long-term prognosis (2). Therefore, in recent years there has been an urgent need to research and develop novel, faster-acting antidepressants.

Scopolamine is a non-selective muscarinic acetylcholine receptor (M-AChR) antagonist and has affinity for five M-AChR subtypes, being most selective for M1- and M2-AChR. As an M-AChR antagonist, scopolamine has mainly been used in the treatment of motion sickness, Parkinson's disease, and pregnancy-related vomiting (3). In recent years, however, evidence from clinical trials indicated that scopolamine has a rapid and robust antidepressant effect in both bipolar and unipolar depression. Essentially, a single intravenous injection of low dose (4.0 μg/kg, i.v.) scopolamine can significantly improve depressive symptoms within 3 days and this effect can last for approximately 2 weeks, without serious adverse events. The available clinical evidence shows that it is unlikely to be addictive (4, 5).

It is known that scopolamine exerts rapid antidepressant effects by promoting the release of brain-derived neurotrophic factor (BDNF) and glutamate, activating the mammalian target of rapamycin complex 1 (mTORC1) and enhancing synaptogenesis in the medial prefrontal cortex (mPFC) (6–8). These effects are reported to be mediated by blockade of M1-AChR (8, 9). However, M2-AChR may also be involved in the antidepressant effects of scopolamine (10, 11). Animal studies have shown that scopolamine has no antidepressant-like effects in M1- or M2-AChR knockout mice, but the effects are retained in M3-, M4- or M5-AChR knockout mice (10). Selective M2-AChR antagonists can mimic the antidepressant-like effects of scopolamine (10, 11). Additionally, human studies support the association between M2-AChR and MDD. For example, a polymorphism of the M2-AChR encoding gene, CHRM2, is significantly correlated with development of MDD (12, 13) and MDD patients show reduced binding activity of M2-AChR, but not M1-, M3- and M4-AChR, in the dorsolateral prefrontal cortex (14, 15). Accordingly, we speculate that M2-AChR may be an important antidepressant target of scopolamine. The present study was therefore undertaken to confirm its key role and investigate the underlying mechanisms of M2-AChR in scopolamine's antidepressant-like effects.

MATERIALS AND METHODS

Animals

SPF Sprague Dawley (SD) male rats (total 160), aged 7 weeks and weighing 180–200 g, were pair-housed and maintained in standard conditions with a 12-h light/dark cycle and access to food and water *ad libitum*. They were purchased from Beijing Vitalriver Experimental Animal Center, laboratory animal license number: SCXK (Beijing) 2016-0011. Animal procedures were under the authority of the Animal Ethics Committee of Capital Medical University (ethical permission number: AEEI-2018-017).

Drugs and Treatments

Scopolamine (25 μg/kg, Tocris, UK), dissolved in 0.9% saline, was injected intraperitoneally (i.p.). Muscimol (1.25 μg/2 μl, Abcam, USA) dissolved in 0.9% saline was bilaterally injected into the mPFC 1 h prior to scopolamine. M2-AChR antagonist methoctramine (MCT, GlpBio, USA), dissolved in 0.9% saline (0.5 μg/2 μl, 1 μg/2 μl or 2 μg/2 μl), was injected intracerebroventricularly (i.c.v.) (8). M3-AChR antagonist 4-diphenylacetoxy-N-methylpiperidine methiodide (4-DAMP, 100 pmol, Cayman, USA) was dissolved in 0.9% saline (1 μg/2 μl) and injected i.c.v (8). Rapamycin (Solarbio, China), dissolved in dimethyl sulfoxide (0.2 nmol/2 μl), was delivered i.c.v. 30 min prior to MCT injection. Groups of control animals for the above experiments received equal volumes of vehicle alone (0.9% saline or dimethyl sulfoxide). The experimental procedures are illustrated in **Figure 1**.

Behavioral Tests

Twenty-four hours after administration of scopolamine, MCT or vehicle, rats were sequentially tested for depression-associated behaviors using the open field test (OFT) followed by forced swim test (FST). Animals were then put back into the cage and 48 h later novelty suppressed feeding tests (NSFT) were conducted.

OFT

This test is used to evaluate autonomous behavior and tension levels of rats in a novel environment (16). The rats were individually placed in a coverless plastic square box (100 × 100 × 40 cm). A digital camera covering the entire field was placed above the box. At the beginning of the test, animals were placed in the center of the area and allowed to explore freely for 5 min. The total distance covered was recorded and analyzed by video tracking software (Supermaze, Shanghai XinRuan Information Technology Co., Ltd, China) to estimate effects of drug treatment on locomotor activity.

FST

This test is widely used in antidepressant drug research (17). Animals behave with desperation in this test, which is sensitive to most antidepressants. The rats were placed individually into a plexiglass cylinder (barrel height 50 cm, diameter 30 cm, purchased from Wuhan ProBeCare Scientific Inc., China) filled with room temperature water (25 ± 1°C) to a depth of 37 cm, to ensure that animals could not touch the bottom of the container with their hind paws or tails. The time to immobility (only the head of the rat above the water, body floating in the water, limbs slightly moving but not struggling) was recorded within 5 min. The rats were placed in the same environment for 15 min for pre-swimming 24 h before the formal test. EthoVision XT software

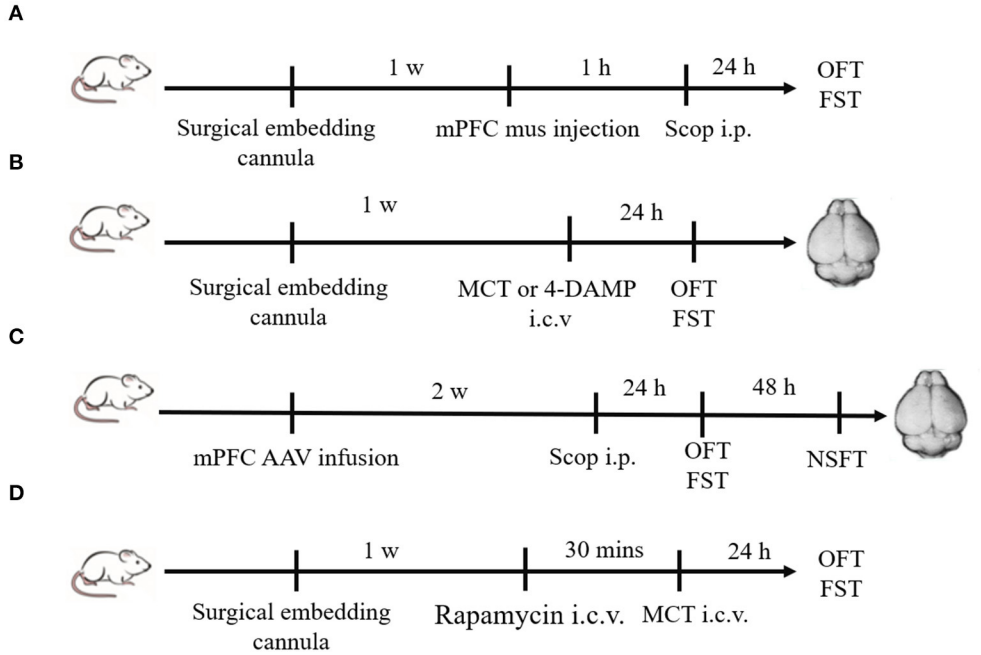

FIGURE 1 | Schematic diagram of experimental procedures. **(A)** Plastic guide cannulas were pre-implanted bilaterally into the mPFC. One week after recovery, muscimol (1.25 μg/2μl) was bilaterally injected through the cannulas 1 h prior to scopolamine administration (i.p.), and behavior tests were conducted 24 h after scopolamine administration. **(B)** Drugs (MCT or 4-DAMP) were bilaterally injected into the lateral ventricle through pre-implanted cannulas, and behavior tests were conducted 24 h later. Then animals were sacrificed, and brains were rapidly removed. **(C)** Two weeks after mPFC AAV infusion, scopolamine was injected (i.p.). Twenty-four hours later, OFT and FST were sequentially conducted. After another 48 h, NSFT were conducted. Then animals were sacrificed, and brains were rapidly removed. **(D)** Rapamycin (0.2 nmol/2 μl) were bilaterally injected into the lateral ventricle through pre-implanted cannulas 30 min prior to MCT administration (i.c.v.). OFT and FST were sequentially conducted 24 h after MCT administration.

(version 10, Noldus Information Technology Co., Netherland) was used to analyze the immobility time.

NSFT

This test is used to evaluate the anxiety and depression of animals in novel environments (18). Rats were food-deprived for 24 h and placed into a new dimly lit environment (100 × 100 × 40 cm) with food in the center. A digital camera covering the entire field was placed above the box and the latency for feeding (reflecting anxiety-like behavior) in 5 min was recorded. After that, animals were then returned to the home cage immediately and food consumption within 30 min was recorded to eliminate the influence of appetite.

Adeno-Associated Virus Construction

AAV-CHRM2-shRNA (pAAV-U6-shRNA (Chrm2)-CMV-EGFP-pA) (2.41E + 12vg/ml) purchased from Wuhan BrainVTA Scientific Inc. (Wuhan, China) was injected bilaterally into the mPFC (1 μl in each side). Its empty vector (pAAV-U6-BBSI-shRNA-CMV-EGFP-pA, 2.97E + 12vg/ml) was used as a negative control. The sequence for CHRM2-shRNA was GCCACCTTCAGACTGTCAACA.

Stereotactic Injection

Rats were anesthetized by 3% sodium pentobarbital solution (30 mg/kg, i.p.) and fixed in the stereotaxic apparatus. For intracerebral injection of drugs, plastic guide cannulas were pre-implanted bilaterally into the infralimbic cortex (IL) of mPFC (AP = 2.8 mm, ML = ±0.6 mm, DV = −3.8 mm) and bilateral lateral ventricle (AP = 0.9 mm, L = ± 1.5 mm, DV = −3.5 mm) (19). One week after recovery, drugs were slowly injected into the target regions at a speed of 0.2 μl/min via a syringe pump (LongerPump Co. LTD, China). Viruses (1 μl on each side) were directly injected bilaterally into the mPFC through a Hamilton syringe using a syringe pump without pre-implantation of guide cannulas. The animals were kept for 2 weeks to allow for virus infection.

Western Blotting

Animals were sacrificed and brains were rapidly removed and frozen in liquid nitrogen. mPFC was dissected out bilaterally using a brain mold on ice. Homogenates of the dissected tissue were lysed using lysis buffer (Beyotime Biotechnology, China) containing protease inhibitors (Sigma, USA) and phosphorylase inhibitors (Sigma, USA). Electrophoresis was performed on 4–15% Mini-PROTEAN TGX precast gels (Bio-Rad, USA). Primary antibodies used included anti-BDNF (1:500, Abcam, Cat GR3227037-2, USA), -M2-AChR (1:500, Abcam, Cat GR50911-14, USA), -mTORC1 (1:500, Cell Signaling Technology, Cat 2983S, USA), -phospho-mTORC (1:500, Cell Signaling Technology, Cat 5536S, USA) and -β-Actin (1:1,000, Santa Cruz, Cat SC47778,

USA). Secondary antibodies used included HRP anti-rabbit antibody (1:5,000, Beyotime, China) and HRP anti-mouse antibody (1:5,000, Beyotime, China). Bands were detected using a chemiluminescence imaging system (Bio-Rad, USA). Image J (NIH, USA) software was used to analyze band densitometry.

Statistical Analysis

Data are presented as the mean ± standard deviation of the mean (SD). Statistical analysis was conducted using two-tailed Student's *t*-tests (parametric test) and Mann-Whitney *U*-test (non-parametric test) for two-group comparisons. One-way ANOVA or two-way ANOVA was used for three or four-group comparisons, followed by Tukey's multiple comparisons. GraphPad Prism software (version 8.0, GraphPad, USA) was used and differences were considered significant at $p < 0.05$.

RESULTS

The mPFC Is Required for Antidepressant-Like Effects of Scopolamine

Previous studies have demonstrated that scopolamine at a dose of 25 μ/kg (i. p.) has rapid and robust antidepressant-like effects on rats, (10, 20–22), and mPFC is a target region of these effects. We replicated these through using a gamma-amino-butyric acid type A (GABA-A) receptor agonist (muscimol) to silence neurons in the mPFC. At the same time, saline and scopolamine were used as two control groups. As shown in **Figure 2A** ($F = 6.624$, $p = 0.0053$), compared with the saline control group, immobility time in the FST was significantly reduced by scopolamine ($p = 0.0049$), and muscimol significantly increased the immobility time of scopolamine treated rats in the FST ($p = 0.0412$). But there was no significant difference in the locomotor activity in the OFT among these three groups ($p > 0.05$, **Figure 2B**).

M2-AChR Mediates the Antidepressant-Like Effects of Scopolamine

MCT, a selective M2 cholinergic receptor antagonist, was used to further explore M2-AChR participation in antidepressant-like effects of scopolamine. Consistent with our previous study (23), we found that intracerebroventricular injection of 1 μg MCT significantly reduced immobility time in the FST ($p = 0.0081$, $t = 3.906$, **Figure 3A**), while 4-DAMP (100 pmol, i.c.v.), a selective M3-AChR antagonist had no such effect ($p > 0.05$, **Figure 3C**). In addition, neither significantly affected locomotor activity in the OFT ($p > 0.05$, **Figures 3B,D**).

To further investigate the role of M2-AChR in the antidepressant effects of scopolamine, we injected an AAV vector expressing CHRM2-shRNA (AAV-CHRM2-shRNA) into the mPFC bilaterally, which produced an ∼35% reduction in M2-AChR protein levels in the mPFC ($p = 0.0253$, $t = 3.484$, **Figures 4A,B**). Compared with empty vector + saline group, M2-AChR knockdown (KD) induced a significant decrease of immobility time in the FST ($F = 5.261$, $p = 0.0317$, **Figure 4C**). However, scopolamine could no longer reduce the immobility time in M2-AChR KD rats (**Figure 4C**) and M2-AChR KD rats showed a similar effect of scopolamine ($p = 0.0351$, **Figure 4C**). Similar results were obtained in the NSFT, scopolamine significantly reduced the latency to feeding in the empty vector group ($F = 7.077$, $p = 0.0309$, **Figure 4D**), while M2-AChR KD blocked this effect (**Figure 4D**). Home cage food consumptions among the four groups were not statistically different. There was no statistical difference in total distance in the OFT among these groups ($p > 0.05$, **Figure 4E**). Taken together, these findings indicate that M2-AChR is required for the antidepressant-like effects of scopolamine.

mTORC1 Signaling Is Essential for the Antidepressant Effects of MCT

It has been demonstrated that scopolamine exerts rapid antidepressant-like effects through activation of the mTORC1 signaling pathway. Here, we observed similar effects of MCT

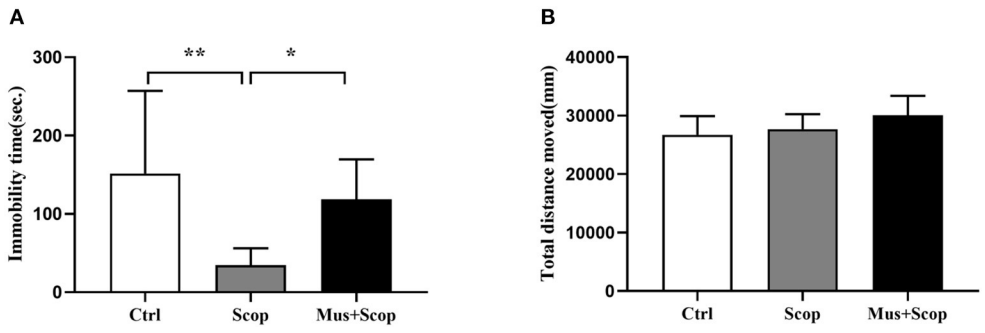

FIGURE 2 | Results for effects of scopolamine on animal behaviors. **(A)** compared with saline group, scopolamine significantly reduces immobility time in the FST in rats, but after silencing of neurons in the mPFC by muscimol, the immobility time of scopolamine is significantly increased compared with scopolamine only. **(B)** total distance among the three groups shows no significant difference. $n = 10$/group; Crtl: control; Scop: scopolamine; Mus: muscimol; *: $p < 0.05$; **: $p < 0.01$.

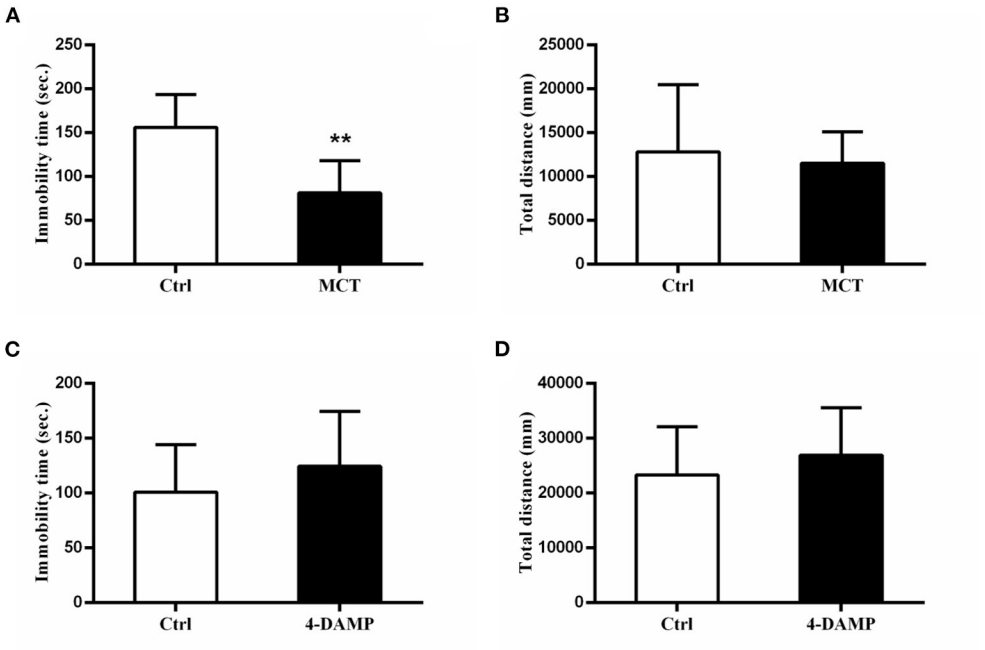

FIGURE 3 | Effects of MCT and 4-DAMP on animal behavior. **(A)** using 1 μg/μl MCT to block M2-AChR in the mPFC significantly reduces the immobility time in the FST. **(B)** total distance between the two groups is not significantly different. **(C)** using 4-DAMP to block M3-AChR cannot significantly reduce the immobility time in the FST. **(D)** total distance between the two groups is not significantly different. $n = 7$–9/group; Crtl: control; **: $p < 0.01$.

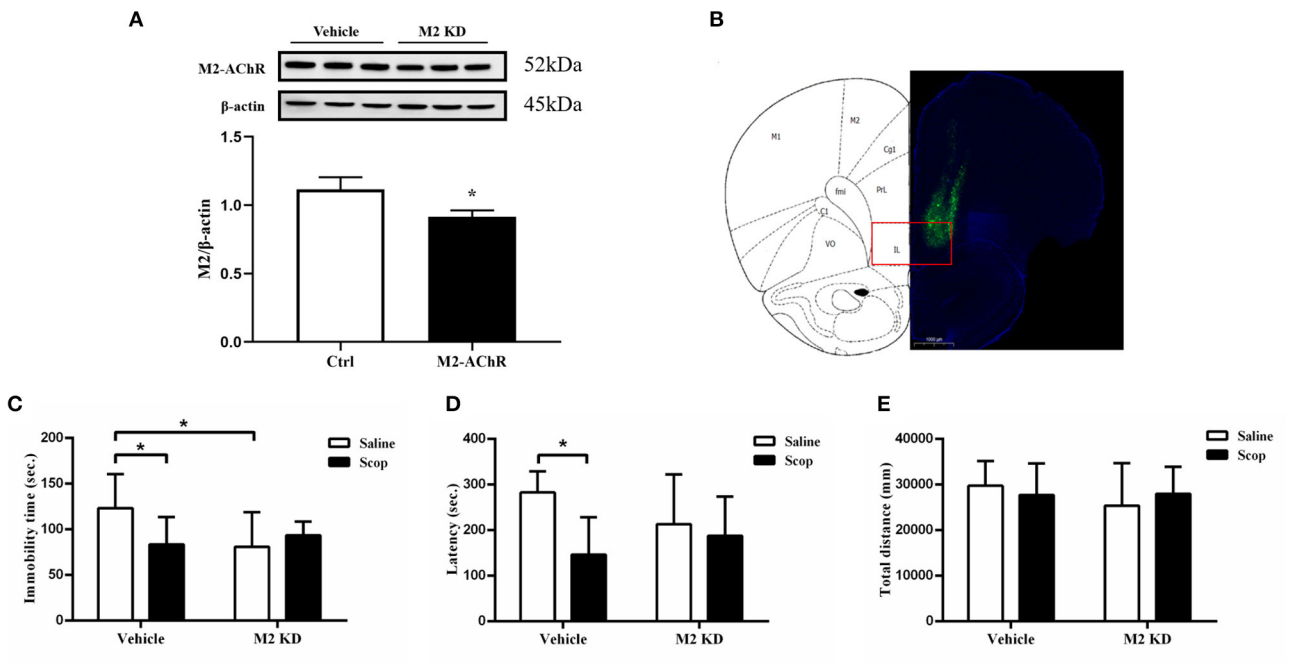

FIGURE 4 | (A) Expression of M2-AChR in the M2-KD group was significantly reduced. **(B)** location of frozen section needle path, showing that AAV-CHRM2-shRNA (pAAV-U6-shRNA (Chrm2)-CMV-EGFP-pA) was injected into the mPFC. **(C)** immobility time of the M2-KD-saline group and Ctrl-scop group is significantly reduced. **(D)** latency of the Ctrl + saline group in NSFT is significantly reduced. **(E)** total distance between these groups shows no significant difference. $n = 7$–9/group; Crtl: control; Scop: scopolamine; *: $p < 0.05$.

on mTORC1 signaling in the mPFC ($p = 0.0023$, $t = 5.053$, **Figures 5A,B**). In addition, MCT significantly increased the expression of BDNF in the mPFC ($p = 0.0182$, $t = 3.218$, **Figures 5A,C**). MCT induced a significant decrease of immobility time in the FST ($F = 5.371$, $p = 0.0131$; $p = 0.0447$, **Figure 5D**). However, after pretreatment with the mTORC1 inhibitor rapamycin (0.2 nmol/2 μl, i.c.v,), the antidepressant-like effect of MCT in the FST was completely inhibited ($p = 0.0163$, **Figure 5D**). These results suggest that the BDNF/mTORC1 signaling pathway is critical to the antidepressant-like effects of MCT.

DISCUSSION

Both human and animal studies have demonstrated that scopolamine has rapid and robust antidepressant activities. However, as a non-selective M-AChR antagonist, scopolamine acts at all five M-AChRs (M1-M5) with equal potency, and it has not been fully determined which one of them mediates these therapeutic effects. In the present study, we found that M2-AChR in the mPFC is required for the antidepressant-like effects of scopolamine, and enhancement of the BDNF/mTORC1 signaling pathway may be the downstream mechanism antagonizing M2-AChR.

Dysfunction of the mPFC has been linked to the cognitive and emotional deficits in depression (24). Herein, our results reveal that silencing neurons in the mPFC could abolish the antidepressant-like effects of scopolamine (**Figure 2**), identifying the mPFC as a critical brain region for the behavioral effects of scopolamine. This is consistent with the finding by Voleti et al. that scopolamine administration rapidly increased neuronal activity in the mPFC (20). Also, ketamine, as a non-competitive glutamate N-methyl-D-aspartate (NMDA) receptor antagonist, has been shown to rapidly relieve depression symptoms through enhancement of α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor (AMPA) receptor signaling in the mPFC (25). Moreover, both scopolamine and ketamine have been demonstrated to rapidly stimulate glutamate/GABA-glutamine cycling in the mPFC, which is critical in initiating rapid-acting antidepressant activity (25, 26). In addition to the mPFC, the hippocampus may also be involved in the antidepressant like effects of scopolamine. Several rodent studies have shown that scopolamine induces PKA-dependent AMPA receptor potentiation and increased expression of BDNF and the neuropeptide VGF in the hippocampus (11, 22). Other brain regions, such as the lateral habenula, nucleus accumbens and others, are also reactive to scopolamine administration (20). Therefore, it is likely that scopolamine acts on emotion-related circuits composed by multiple brain regions to exert its antidepressant effects, and this complexity necessitates further study.

In relation to the specific antidepressant target of scopolamine, most of the previous studies focus on the

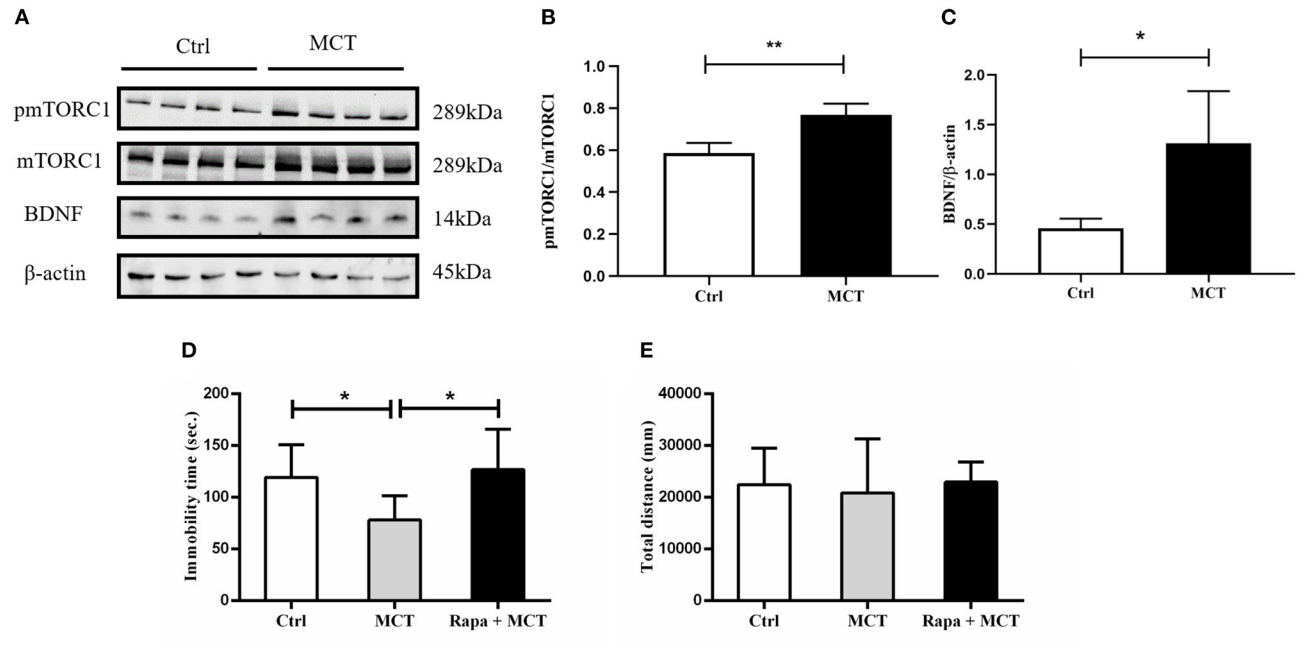

FIGURE 5 | Western blot results from the mPFC and the effect of rapamycin on animal behavior. **(A)** Western blot images of the control group and MCT group; **(B)** MCT significantly increases the ratio of p-mTORC1/mTORC1; **(C)** MCT significantly increases the expression of BDNF; **(D)** compared to the control group, MCT significantly reduces the immobility time in the FST; **(E)** total distance in the OFT is not significantly different from controls, the MCT group and rapamycin + MCT group. $n = 4$/group; Crtl: control; Scop: scopolamine; Rapa: rapamycin; *: $p < 0.05$; ** $p < 0.01$.

M1-AChR. In the brain, M1-AChR is mainly located in the hippocampus, striatum medium spiny neurons, cortical pyramidal neurons, amygdala, and thalamus postsynaptic. It is expressed in glutamatergic pyramidal neurons and GABA interneurons with increased excitability and its physiological functions include learning, memory, inflammatory cytokine production, etc. (27, 28). A recent study showed that M1-AChR in mPFC somatostatin-GABA interneurons is antagonized by scopolamine, leading to disinhibition of pyramidal glutamate neurons, increased glutamate release and enhanced numbers and function of synapses. A study of the M1- and M3-AChR antagonist penehyclidine hydrochloride (PHC) also gave similar results: PHC could improve depressive-like behaviors in depression model mice and increased BDNF expression could be observed in the hippocampus (29). By contrast, specifically knocking down the M1-AChR of SST-GABA interneurons weakened the rapid antidepressant-like effect of scopolamine (9). These results show that M1-AChR is a main target of scopolamine's rapid antidepressant-like effects. In our study, we found that intracerebroventricular injection of MCT produced similar antidepressant-like effects to scopolamine in the FST (**Figure 3**) and knocking down M2-AChR in the mPFC blocked scopolamine's antidepressant-like effects in the FST (**Figure 4**). This is consistent with the results of a previous study that M2-AChR knockout (KO) mice had a blunted response to scopolamine in the FST and the antagonist SCH226206, which has selectivity for M2-AChR over M1-AChR, was effective in the FST but the effect was negated in M2-AChR KO mice (10). M2-AChR is mainly located in the basal forebrain, thalamus, brainstem, heart and exocrine glands (27), and scopolamine therapy may have some dry mouth and cardiovascular-related adverse effects. However, no trials have been halted due to serious adverse events (21, 30).

M1-AChR studies have found that scopolamine can stimulate glutamate transmission or glutamate burst, resulting in a long-term potentiation–like synaptogenic effect (8). Reports of the downstream mechanisms of M2-AChR antidepressant-like effects, however, are rare. Here, we found that MCT activated mTORC1 signaling and increased BDNF expression in the mPFC and inhibiting mTORC1 with rapamycin could block antidepressant-like effects of MCT (**Figure 5**). The mTORC1 pathway is implicated in activity-dependent synaptic plasticity, especially in neuronal dendrites and spines (31). Dong et al. reported that scopolamine blocks M2-AChR, activating the PKA signaling pathway, which results in mTORC1 pathway activation and synaptic plasticity enhancement (11). The rapid antidepressant effect of ketamine also relies on the rapid activation of mTORC1 signaling, resulting in activation of synapse-associated proteins, such as extracellular signal–regulated kinase (ERK), protein kinase B (PKB/Akt) and phosphorylated 70S6 kinase (p70S6K), increasing in spine number in the PFC and release of BDNF in the amygdala (32, 33). Increased expression of mTORC1 related proteins including phosphorylated ERK 1/2, phosphorylated Akt, p70S6K and others, is required for synaptic and antidepressant effects (6). Animal studies have similar conclusions: both the phosphorylation of mTORC1 and its downstream protein p70S6K are significantly reduced in depressive model animals (34). Protein kinase is well-known to be involved in glutamic synaptic enhancement and synaptic plasticity and patients with depression have altered AMPA receptor expression, which is closely related to the pathophysiology of depression (35). Nonetheless our study has the limitation that its related mechanisms were not explored. As mentioned above, scopolamine could stimulate BDNF and VGF release in the hippocampus and PFC and after using verapamil to block L-type voltage-dependent calcium channels (L-VDCC), antidepressant-like behavior and upregulation of BDNF and VGF were blunted (22). BDNF is an important neurotransmitter involved in regulation of emotions. BDNF expression in depressive animal models is decreased (36) and animal work has also shown that acute administration of ketamine improves BDNF and mTOR levels in the hippocampus, so the mTORC1-BDNF signaling pathway is probably the target of the rapid antidepressant effect (37). Together, these data indicate that regulating BDNF expression and mTORC1 signaling in the PFC and hippocampus may be involved in the rapid antidepressant effects of scopolamine. Further research is underway and answering these remaining questions will have a profound impact on the development of novel rapid-acting antidepressants.

As mentioned, there are limitations in the current study. First, we did not directly examine whether scopolamine activates mTORC1 and increases BDNF expression via M2-AChRs in the mPFC. Secondly, our findings were not validated in animal models of depression (e.g., the chronic unpredictable stress model). Lastly, the current study used only male rats, so our findings may not be generalizable to female rats.

CONCLUSION

Our current data from rats suggest that the antidepressant-like behaviors of scopolamine may depend on blockade of M2-AChR and activation of the mTORC1-BDNF signaling pathway in the mPFC. In addition, our findings indicate that intracerebroventricular injection of a defined dose of the M2-AChR antagonist MCT has similar behavioral effects and responses at a molecular level.

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 Ethics Committee of Capital Medical University.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for 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/fpsyt.2021.601985/full#supplementary-material

REFERENCES

1. Huang Y, Wang Y, Wang H, Liu Z, Yu X, Yan J, et al. Prevalence of mental disorders in China: a cross-sectional epidemiological study. *Lancet Psychiatry.* (2019) 6:211–24. doi: 10.1016/S2215-0366(18)30511-X
2. Papakostas GI. Limitations of contemporary antidepressants: tolerability. *J Clin Psychiatry.* (2007) 68(Suppl 10):11–7. doi: 10.1016/j.foodres.2003.08.001
3. Renner UD, Oertel R, Kirch W. Pharmacokinetics and pharmacodynamics in clinical use of scopolamine. *Ther Drug Monit.* (2005) 27:655–65. doi: 10.1097/01.ftd.0000168293.48226.57
4. Drevets WC, Furey ML. Replication of scopolamine's antidepressant efficacy in major depressive disorder: a randomized, placebo-controlled clinical trial. *Biol Psychiatry.* (2010) 67:432–8. doi: 10.1016/j.biopsych.2009.11.021
5. Furey ML, Drevets WC. Antidepressant efficacy of the antimuscarinic drug scopolamine: a randomized, placebo-controlled clinical trial. *Arch Gen Psychiatry.* (2006) 63:1121–9. doi: 10.1001/archpsyc.63.10.1121
6. Ghosal S, Bang E, Yue W, Hare BD, Lepack AE, Girgenti MJ, et al. Activity-dependent brain-derived neurotrophic factor release is required for the rapid antidepressant actions of scopolamine. *Biological Psychiatry.* (2018) 83:29–37. doi: 10.1016/j.biopsych.2017.06.017
7. Dulawa SC, Janowsky DS. Cholinergic regulation of mood: from basic and clinical studies to emerging therapeutics. *Mol Psychiatry.* (2019) 24:694–709. doi: 10.1038/s41380-018-0219-x
8. Navarria A, Wohleb ES, Voleti B, Ota KT, Dutheil S, Lepack AE, et al. Rapid antidepressant actions of scopolamine: role of medial prefrontal cortex and M1-subtype muscarinic acetylcholine receptors. *Neurobiol Dis.* (2015) 82:254–61. doi: 10.1016/j.nbd.2015.06.012
9. Wohleb ES, Wu M, Gerhard DM, Taylor SR, Picciotto MR, Alreja M, et al. GABA interneurons mediate the rapid antidepressant-like effects of scopolamine. *J Clin Invest.* (2016) 126:2482–94. doi: 10.1172/JCI85033
10. Witkin JM, Overshiner C, Li X, Catlow JT, Wishart GN, Schober DA, et al. M1 and m2 muscarinic receptor subtypes regulate antidepressant-like effects of the rapidly acting antidepressant scopolamine. *J Pharmacol Exp Ther.* (2014) 351:448–56. doi: 10.1124/jpet.114.216804
11. Dong J, Zhou Q, Wei Z, Yan S, Sun F, Cai X. Protein kinase A mediates scopolamine-induced mTOR activation and an antidepressant response. *J Affect Disord.* (2018) 227:633–42. doi: 10.1016/j.jad.2017.11.041
12. Wang JC, Hinrichs AL, Stock H, Budde J, Allen R, Bertelsen S, et al. Evidence of common and specific genetic effects: association of the muscarinic acetylcholine receptor M2 (CHRM2) gene with alcohol dependence and major depressive syndrome. *Human Mol Genet.* (2004) 13:1903–11. doi: 10.1093/hmg/ddh194
13. Comings DE, Wu S, Rostamkhani M, McGue M, Iacono WG, MacMurray JP. Association of the muscarinic cholinergic 2 receptor (CHRM2) gene with major depression in women. *Am J Med Genet.* (2002) 114:527–9. doi: 10.1002/ajmg.10406
14. Gibbons AS, Scarr E, McLean C, Sundram S, Dean B. Decreased muscarinic receptor binding in the frontal cortex of bipolar disorder and major depressive disorder subjects. *J Affect Disord.* (2009) 116:184–91. doi: 10.1016/j.jad.2008.11.015
15. Gibbons AS, Jeon WJ, Scarr E, Dean B. Changes in muscarinic M2 receptor levels in the cortex of subjects with bipolar disorder and major depressive disorder and in rats after treatment with mood stabilisers and antidepressants. *Int J Neuropsychopharmacol.* (2016) 19:pyv118. doi: 10.1093/ijnp/pyv118
16. Walsh RN, Cummins RAJPB. The open-field test: a critical review. (1976) 83:482.
17. Lucki, Pharmacology IJB. The forced swimming test as a model for core and component behavioral effects of antidepressant drugs. *Behav Pharmacol.* (1997) 8:523–32. doi: 10.1097/00008877-199711000-00010
18. Camargo A, Pazini FL, Rosa JM, Wolin IAV, Research ALSRJJoP. Augmentation effect of ketamine by guanosine in the novelty-suppressed feeding test is dependent on mTOR signaling pathway. *J Psychiatr Res.* (2019) 115:103–112. doi: 10.1016/j.jpsychires.2019.05.017
19. Paxinos G, Watson C. *The Rat Brain in Stereotaxic Coordinates.* (1986).
20. Voleti B, Navarria A, Liu RJ, Banasr M, Li N, Terwilliger R, et al. Scopolamine rapidly increases mammalian target of rapamycin complex 1 signaling, synaptogenesis, and antidepressant behavioral responses. *Biol Psychiatry.* (2013) 74:742–9. doi: 10.1016/j.biopsych.2013.04.025
21. Zhou J, Yang J, Zhu X, Zghoul T, Wang GJTAiP. The effects of intramuscular administration of scopolamine augmentation in moderate to severe major depressive disorder: a randomized, double-blind, placebocontrolled trial. *Ther Adv Psychopharmacol.* (2020) 10:1–11. doi: 10.1177/2045125320938556
22. Yu H, Li M, Shen X, Lv D, Sun X, Wang J, et al. The requirement of L-type voltage-dependent calcium channel (L-VDCC) in the rapid-acting antidepressant-like effects of scopolamine in mice. *Int J Neuropsychopharmacol.* (2018) 21:175–86. doi: 10.1093/ijnp/pyx080
23. Shi DD, Yang J, Zhang JN, Wang G. Effects of scopolamine on forced swimming behavior in rats and its possible action target. *J Cap Med Univ.* (2018) 39:630–5. doi: 10.3969/j.issn.1006-7795.2018.05.002
24. Seo JS, Wei J, Qin L, Kim Y, Yan Z, Greengard P. Cellular and molecular basis for stress-induced depression. *Mol Psychiatry.* (2017) 22:1440–7. doi: 10.1038/mp.2016.118
25. Krystal JH, Sanacora G, Duman RS. Rapid-acting glutamatergic antidepressants: the path to ketamine and beyond. *Biol Psychiatry.* (2013) 73:1133–41. doi: 10.1016/j.biopsych.2013.03.026
26. Chowdhury GM, Zhang J, Thomas M, Banasr M, Ma X, Pittman B, et al. Transiently increased glutamate cycling in rat PFC is associated with rapid onset of antidepressant-like effects. *Mol Psychiatry.* (2017) 22:120–6. doi: 10.1038/mp.2016.34
27. Hasselmann H. Scopolamine and depression: a role for muscarinic antagonism? *CNS & Neurological Disorders - Drug Targets.* (2014) 13:673–83. doi: 10.2174/18715273136661406181057l0
28. Brown DA. Muscarinic acetylcholine receptors (mAChRs) in the nervous system: some functions and mechanisms. *J Mol Neurosci.* (2010) 41:340–6. doi: 10.1007/s12031-010-9377-2
29. Sun X, Sun C, Zhai L, Dong W. A selective M1 and M3 receptor antagonist, penehyclidine hydrochloride, exerts antidepressant-like effect in mice. *Neurochem Res.* (2019) 44:2723–32. doi: 10.1007/s11064-019-02891-5
30. Drevets WC, Zarate CA, Jr., Furey ML. Antidepressant effects of the muscarinic cholinergic receptor antagonist scopolamine: a review. *Biol Psychiatry.* (2013) 73:1156–63. doi: 10.1016/j.biopsych.2012.09.031
31. Hoeffer CA, Klann E. mTOR signaling: at the crossroads of plasticity, memory and disease. *Trends Neurosci.* (2010) 33:67–75. doi: 10.1016/j.tins.2009.11.003
32. Li N, Lee B, Liu RJ, Banasr M, Dwyer JM, Iwata M, et al. mTOR-dependent synapse formation underlies the rapid antidepressant effects of NMDA antagonists. *Science.* (2010) 329:959–64. doi: 10.1126/science.1190287

33. Lepack AE, Bang E, Lee B, Dwyer JM, Duman RS. Fast-acting antidepressants rapidly stimulate ERK signaling and BDNF release in primary neuronal cultures. *Neuropharmacology*. (2016) 111:242–52. doi: 10.1016/j.neuropharm.2016.09.011
34. Chandran A, Iyo AH, Jernigan CS, Legutko B, Austin MC, Karolewicz B. Reduced phosphorylation of the mTOR signaling pathway components in the amygdala of rats exposed to chronic stress. *Prog Neuropsychopharmacol Biol Psychiatry*. (2013) 40:240–5. doi: 10.1016/j.pnpbp.2012.08.001
35. Freudenberg F, Celikel T, Reif A. The role of alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors in depression: central mediators of pathophysiology and antidepressant activity? *Neuroscience & Biobehavioral Reviews*. (2015) 52:193–206. doi: 10.1016/j.neubiorev.2015.03.005
36. Angelucci F, Brenè S, Mathé AA. BDNF in schizophrenia, depression and corresponding animal models. *Mol Psychiatry*. (2005) 10:345–52. doi: 10.1038/sj.mp.4001637
37. Yang C, Hu YM, Zhou ZQ, Zhang GF, Yang JJ. Acute administration of ketamine in rats increases hippocampal BDNF and mTOR levels during forced swimming test. *Ups J Med Sci*. (2013) 118:3–8. doi: 10.3109/03009734.2012.724118

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Gut Microbiome Composition Associated With Major Depressive Disorder and Sleep Quality

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The microbiota–gut–brain axis plays a critical role in the pathogenesis of major depressive disorder (MDD) and related subclinical symptoms. However, studies on the gut microbiota in MDD are inconsistent, and data on MDD's effects on sleep are lacking. This study aimed to analyze the gut microbiota composition and sleep quality of patients with MDD. We performed 16S rRNA sequencing of stool samples from 36 patients with MDD and 45 healthy controls (HC). Sleep quality was assessed using the Pittsburgh Sleep Quality Index, depressive severity with the Hamilton Depression Scale, and insomnia severity using the Insomnia Severity Index. Forty-eight microbiota targets showed significant differences between MDD and HC. In MDD, six microbiota targets were associated with the severity of depression, 11 with sleep quality, and 3 with sleep severity. At the genus level, *Dorea* was simultaneously related to depression and sleep quality, while *Intestinibacter* was more closely related to sleep problems. *Coprococcus* and *Intestinibacter* were associated with sleep quality independent of the severity of depression. In conclusion, the present findings enable a better understanding of the relationship between gut microbiota and MDD-related symptoms. Gut microbiota alterations may become potential biomarkers and/or treatment targets for sleep quality in MDD.

Keywords: gut microbiome, major depressive disorder, sleep quality, 16S rRNA sequencing, insomnia

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INTRODUCTION

Major depressive disorder (MDD) is a common psychiatric illness influencing ∼300 million people (1), with lifetime prevalence rates of about 10.8% as reported in a survey of 30 countries (2) and exerting a huge clinical and social burden.

Several hypotheses, including the monoamine hypothesis, the subclinical inflammation hypothesis (3, 4), and hypothalamic–pituitary axis (HPA) dysregulation, have been proposed to explain its underlying etiology (5). Another promising hypothesis is gut–brain axis (GBA) dysfunction (6). The gut microbiota affects both the gastrointestinal system and central nervous system (CNS) function. Bacteria can produce neurotransmitters such as GABA, dopamine, and serotonin which affect emotional and sleep states (7). Because of their neuroactive properties and their effects on other gut–brain signaling pathways, including immune and endocrine systems, the main metabolites produced by intestinal dietary fiber bacterial fermentation, short-chain fatty acids (SCFAs), are speculated to be directly or indirectly involved in communication along the brain–gut axis (8, 9).

Several studies have attempted to prove the association between the gut microbiota and depression, but with inconsistent results. For instance, Jiang et al. found that the gut microbiome diversity in MDD was higher than that in HC, and the proportion of *Bacteroides*, *Proteus*, and *Actinomycetes* was significantly higher than in HC (10). Chung et al. found no significant difference in bacterial abundance and diversity between the two groups, but found increasing *Actinobacteria* and *Firmicutes* and decreasing *Bacteroidetes* and *Proteobacteria* in MDD. Furthermore, they found that the severity of depression correlated with bacterial composition (11). Aside from anhedonia and the enduring depressed mood, sleep disturbance is a common issue for MDD patients, present during the whole course of the disease even as a residual symptom. More than 90% of patients with depression have sleep disorders, and a small number of patients complain of drowsiness (12). Previous hypothesis considered that sleep and depression were a one-way causal relationship in which depression leads to sleep disorders, but new evidence seems to claim a bidirectional relationship between them. However, at present, no universally acknowledged theory explains the pathophysiologic mechanism between depression and sleep. In the past decade, known theories including the S-deficiency hypothesis (13), HPA dysfunction hypothesis (14), circadian rhythm (15, 16), rapid eye movement (REM) phase advance hypothesis (17), and neuroimmune mechanisms (18) can only partly explain the cause of insomnia in depression. Therefore, a novel mechanism involving the gut–brain axis has been proposed (12, 13, 19).

There is considerable evidence indicating that gut microbiome can regulate sleep and mental states (20). Previous studies have shown a positive association between sleep quality, the F/B ratio, a greater relative abundance of *Blautia* and *Ruminococcus* (*Firmicutes*), and lower proportions of *Prevotella*, *Bacteroidetes*, and *TM7-3a* (21, 22). Despite the lack of research on this topic in the context of depression, three cross-sectional studies analyzing sleep and intestinal microbiota in bipolar disorder and irritable bowel syndrome found negative correlations between *Faecalibacterium*, *Lactobacillus*, and sleep quality (23, 24) among bipolar disorder patients, while baseline intestinal and gut microbiota diversity had a negative correlation with the Hamilton Depression Rating Scale (HAM-D) score in irritable bowel syndrome (25). Therefore, to fill the evidence gap in MDD, the current study focused on (1) characterizing the gut microbiota distributions of participants with MDD and (2) determining whether gut bacteria differentially correlate with sleep quality.

METHODS AND MATERIALS

Participants

We recruited 36 patients with MDD and 45 healthy controls from inpatients at Peking University Huilongguan Clinical Medical School, Beijing Huilongguan Hospital between January 2020 and October 2020. The MDD group inclusion criteria were as follows: (1) 18–55 years of age, (2) diagnosed with MDD and a depressive episode of at least moderate severity according to ICD-10 criteria (F32.1, F32.2, F33.1, F33.2) by two trained psychiatrists (26), (3) total scores of the 17-item version HAM-D >17 (26), and (4) drug naive or without treatment for ≥ 1 week and without long-acting antipsychotics >6 months before the study. Healthy controls were recruited from nearby communities and were screened out with any history of psychiatric disorders or psychosis among their first-degree relatives. The candidates of HC were hospital staff, care workers, and patients' accompanying family members and friends with no consanguinity. For all participants in our study, physical examination results, laboratory test results (blood and urine analyses), imaging results, and past history were collected before admission to exclude those with history of persistent infection, allergy, or inflammatory diseases whether systematic or local inflammation. Exclusion criteria for both patients and control groups were as follows: (1) a prior medical history of central nervous system disease, severe head injury, substance abuse or dependence, intellectual disability, and other severe medical records; (2) recent use of antibiotics or probiotic synbiotics within 30 days of study participation; (3) history of gastrointestinal surgery or severe congenital abnormalities; (4) night shift or rotating schedule within the past 3 months; (5) history of electroconvulsive therapy within the previous 6 months; (6) pregnancy; and (7) comorbidities associated with other sleep disorders (e.g., sleep apnea).

All candidates were subjected to similar living conditions during the entire hospitalization period and received the same hospital diet and followed a similar daily routine.

This study was approved by the Institutional Review Board of Beijing Huilongguan Hospital (Beijing Huilongguan Ethics Committee # 2019-43), and all participants provided written informed consent.

Data Collection

All participants were interviewed on the day of admission. Patients' clinical symptoms were assessed by trained psychiatrists or psychologists using the 17-item HAM-D. The Pittsburgh Sleep Quality Index (PSQI) was assessed based on self-reported (subjective) sleep quality, including sleep duration, onset latency, sleep efficiency, sleep quality, sleep disturbance, sedative-hypnotic drugs, daily function, and total score over the past month (27). We used the Insomnia Severity Index (ISI) to evaluate the subjective perception of insomnia severity (28). As a seven-item and five-point Likert self-report questionnaire, the global score can range from 0 to 28 and is classified as follows: not clinically significant insomnia (0–7), subthreshold insomnia (8–14), moderate insomnia (15–21), and severe insomnia (22–28).

Fecal samples (≥ 1 g) collected within 2 days after admission or after the elution period were placed in a sample tube containing 2 ml of RNA stabilization solution (TinyGen, Bio-Tech, Shanghai, China) and stored at -80°C until DNA extraction.

Details on the methods for library preparation and 16S gene amplicon sequencing are provided in the **Supplementary Methods**. After sequence processing, according to different similarity levels, all sequences were divided into operational taxonomic units (OTUs), which were usually based on biological information statistical analysis at 97% similarity level.

Determination of Bacterial Counts

DNA amplification was performed in the V4–V5 regions of the 16S rRNA gene and barcode sequences were added. Unique fusion primers were designed based on the general primers (515F 5′-GTGCCAGCMGCCGCGGTAA-3′, 926R 5′-CCGTCAATTCMTTTGAGTTT-3′). Sequencing was performed with Illumina 5′ (Illumina, San Diego, CA, USA) following the manufacturer's instructions (29). One unit Phusion DNA Polymerase (New England Biolabs, USA) was used to complete the initial PCR reactions. A DNA gel extraction kit (Axygen, USA) was used to purify the barcodes. PCR products and the FTC-3000^{TM} real-time PCR (Funglyn, Shanghai) were used for quantification. Thermal cycling included an initial denaturation at 94°C for 2 min, followed by 25 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 30 s, elongation at 72°C for 30 s, and a final extension at 72°C for 5 min. The PCR products from different samples were mixed at equal ratios. Eight PCR cycles were used to incorporate two unique barcodes to either of the ends of the amplicons. Finally, we used a DNA gel extraction kit (Axygen, USA) to purify the library and a 2 × 250-bp paired-end sequencing on the NovaSeq platform using NovaSeq 6000 SP 500 Cycle Reagent Kit (Illumina, USA) at TinyGen Bio-Tech (Shanghai) Co., Ltd.

Raw pyrosequencing reads were run through Trimmomatic (version 0.35) (30), and in order to remove low-quality base pairs, we used the parameters—SLIDINGWINDOW: 50:20, MINLEN: 50—while the FLASH program (version 1.2.11) was used to process default parameters. Low-quality contigs were removed based on the screen.seqs command using the following filtering parameters: maxambig = 0, minlength = 200, maxlength = 485, maxhomop = 8. A combination of software mothur (31) (version 1.33.3), UPARSE (usearch version v8.1.1756, http://drive5.com/uparse/) (32), and R (version 3.6.0) was used as quality filter. After sequence processing, according to different similarity levels, all sequences were divided into OTUs. Since OTUs are usually based on biological information statistical analysis at 97% similarity level, singleton OTUs were deleted using the UPARSE pipeline (http://drive5.com/uparse/).

Bioinformatics and Statistical Analyses

For alpha diversity (Shannon, Simpson, and evenness indices), rarefaction curves were calculated using mothur and plotted by R. Phylogenetic beta diversity measures, weighted and unweighted UniFrac distance matrix were calculated using mothur and visualized with principal coordinate analysis (PCoA). Bray–Curtis and Jaccard metrics were calculated using the vegan package in R and visualized by R as UniFrac analysis. To compare within- and between-group similarity, analysis of similarity (ANOSIM) was performed with "vegan" package of R, based on (un) Weighted.unifrac distance. Canoco 5 RDA software was used to analyze the correlation between clinical indices and intestinal community variation.

Linear discriminant analysis effect size (LEfSe) analysis was used to identify taxa significantly enriched in the MDD and HC groups. The linear discriminant analysis (LDA) score was computed for taxa differentially abundant between the two groups. A taxon at $p < 0.05$ (Kruskal–Wallis test) and log10[LDA] $\geq$2.0 (or $\leq$-2.0) were considered significant.

Demographic and clinical variables were compared between MDD and HC using the chi-square test for categorical variables, the independent-samples *t*-test for normal continuous variables, and the Mann–Whitney *U*-test for non-normal continuous variables. Taxa that have abundance >0.01% were reserved for analysis. The MDD group and HC were compared at the levels of phylum, class, order, family, genus, and species by the Wilcoxon signed-rank test. We performed partial correlations (adjusted for age, sex, and BMI) between bacterial counts and the total HAM-D-17 score. Moreover, partial correlation analysis (adjusted for age, sex, and BMI) was used to examine the correlations between bacterial counts and other variables such as total PSQI and ISI scores. Linear regression analyses were used to assess the most influential taxa on sleep quality in MDD at the genus and species levels after correcting for age, gender, BMI, and HAM-D scores. Differences were considered statistically significant when the two-tailed $p < 0.05$. False discovery rate (FDR, Benjamini–Hochberg) was used for perform multiple testing. $p < 0.05$ was considered significant. Analysis was performed using the Statistical Package for the Social Sciences version 25.0 (IBM Corp, Chicago, Illinois, USA).

RESULTS

Demographic and Clinical Characteristics

Stool samples were collected from 36 patients with MDD and 45 healthy controls. Demographic and clinical characteristics were displayed in **Table 1**. There were no significant demographic differences between MDD and HC. However, the differences in HAM-D, ISI, and PSQI between the MDD and HC groups were significant. The mean age of onset was 31.39 ± 13.53 years, and illness duration was 5.36 ± 6.24 years. By thorough clinical tests, including blood and imaging examinations, participants had no local or systemic inflammation. We also collected the data of high-sensitivity C-reactive protein (hs-CRP) to exclude inflammation. They were all below the clinical threshold, and there was no significant difference between the two groups (**Table 1**).

Gut Microbiota Diversity Index Between MDD and HC

Chao, ACE, Shannon, and Simpson indices were used to compare the alpha diversity between the MDD and HC groups. The Chao and ACE diversity indices reflect microbial species richness, while Shannon and Simpson diversity indices reflect community richness and evenness. No difference in alpha diversity between patients with MDD and HC was observed ($p > 0.05$). Differences in community structure dispersion between the two groups were measured using Bray–Curtis analysis (**Figure 1A**), and community membership dispersion was assessed by Jaccard dissimilarity (**Figure 1B**). We used Canoco 5 RDA software to analyze the correlation between clinical indices and intestinal community variation. The relationship between the distribution of microflora and clinical indices is shown in **Figure 2**. PSQI, HAM-D, and ISI played important

TABLE 1 | Demographic and clinical characteristics in MDD patients and HC.

Parameter	MDD ($N = 36$)	HC ($N = 45$)	$\chi^2/t/Z$	*p*-value
Age, years	36.81 ± 13.52	39.29 ± 11.44	−1.297	0.195
Gender, female/male	15/21	26/19	2.077	0.150
BMI, kg/m^2	24.47 ± 4.16	23.94 ± 3.05	−0.656	0.513
Smoking, yes/no	6/30	5/40	0.526	0.468
Age of onset, years	31.39 ± 13.53	NA		
Illness duration, years	14.06 ± 3.04	NA		
HAM-D	5.36 ± 6.24	0.51 ± 1.01	−22.362	<0.001***
PSQI	23.06 ± 5.98	1.93 ± 1.29	−10.407	<0.001***
ISI	13.19 ± 7.79	0.40 ± 1.01	−9.755	<0.001***
hs-CRP	1.39 ± 1.34	1.03 ± 0.60	−0.57	0.954

*Data are presented as numbers or mean ± SD. MDD, major depressive disorder; HC, healthy controls (***$p < 0.001$). t stands for t score of independent-samples t-test. χ^2 stands for χ^2 score of the chi-square test. Z stands for Z score of the Mann–Whitney U-test.*

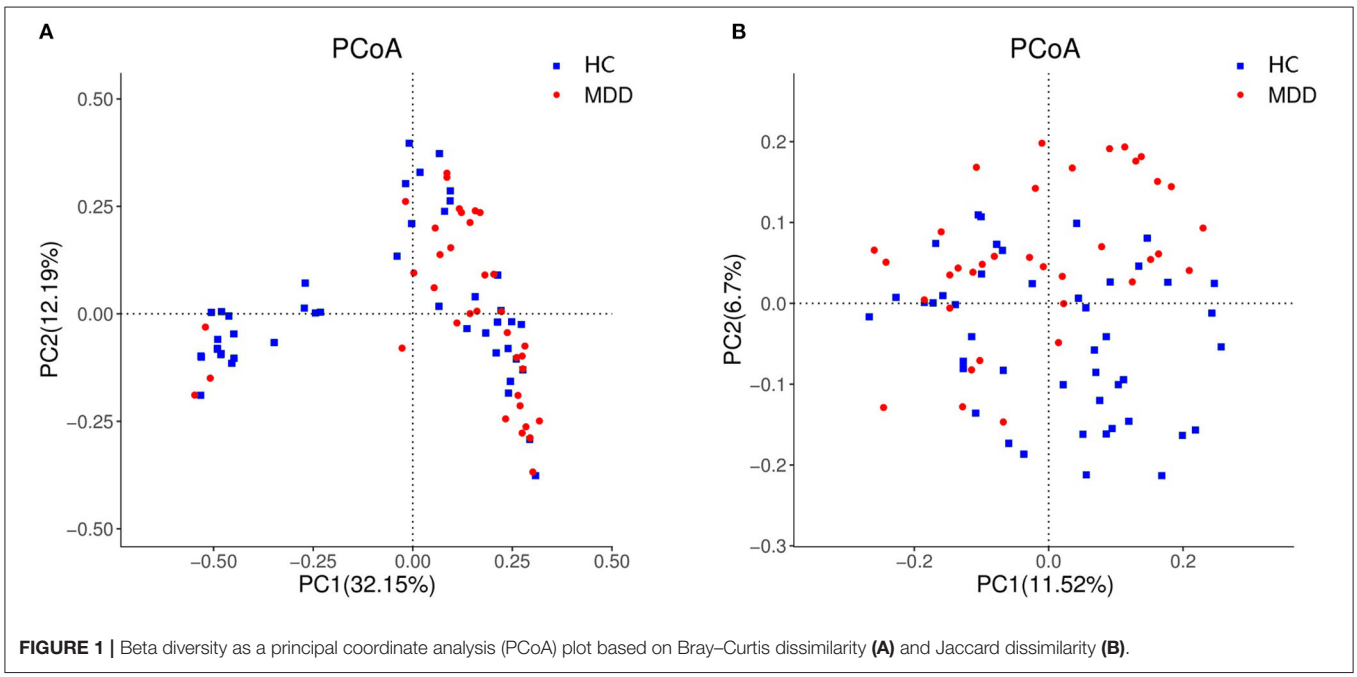

FIGURE 1 | Beta diversity as a principal coordinate analysis (PCoA) plot based on Bray–Curtis dissimilarity **(A)** and Jaccard dissimilarity **(B)**.

roles on the distribution of microflora in MDD patients, while BMI and age played important roles on HC. Among them, HAM-D has the most significant effect on community variation ($p < 0.05$) (**Supplementary Table 5**). Furthermore, unweighted and weighted ANOSIMs revealed significant differences between the two groups. Beta diversity was measured based on the unweighted ($r = 0.067$, $p = 0.007$) and the weighted ($r = 0.075$, $p = 0.007$) UniFrac distance matrix of the differences between groups, as shown in **Figure 3**, suggesting dissimilar microbiota composition.

Composition of Microbial Communities Between MDD and HC

We obtained 3,565,920 quality-filtered read pairs from 81 study participants (36 MDD patients and 45 HC), with an average of 44,024 read pairs per sample. Gut bacterial communities at the phylum, family, and genus levels detected in MDD and HC subjects are shown in **Figure 4**. In order to explore the differences among groups, all OTUs with ≥0.01% fractional representation in either of the groups were considered. As expected, *Bacteroidetes* and *Firmicutes* accounted for about 93% of all bacteria and were the two most common dominant taxa among the two groups. At the phylum and class levels, there were no significant differences between groups ($p > 0.05$).

A significant difference (Wilcoxon signed-rank test, $p < 0.05$) between the two groups affected two orders: *Pasteurellales* and *Actinomycetae*. At the family level, eight families showed significant differences between the two groups: the top four being *Bacteroidaceae*, *Prevotellaceae*, *Porphyromonadaceae*, and *Rikenellaceae*. At the genus level, 19 genera showed

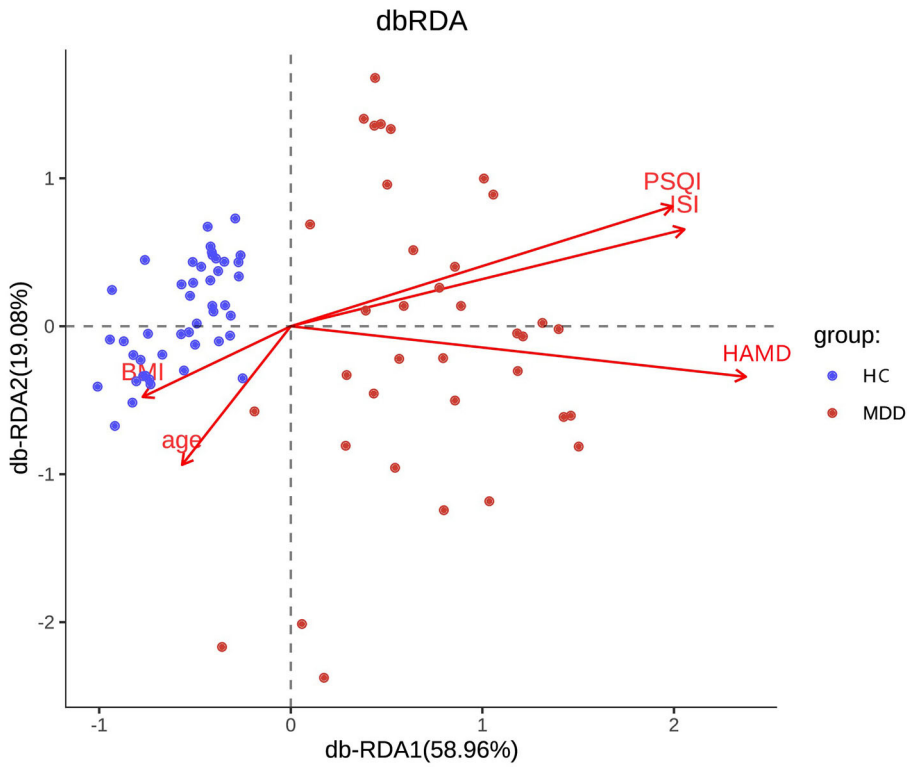

FIGURE 2 | RDA two-way sequence diagram of intestinal bacterial community and its main environmental factors driven by variation.

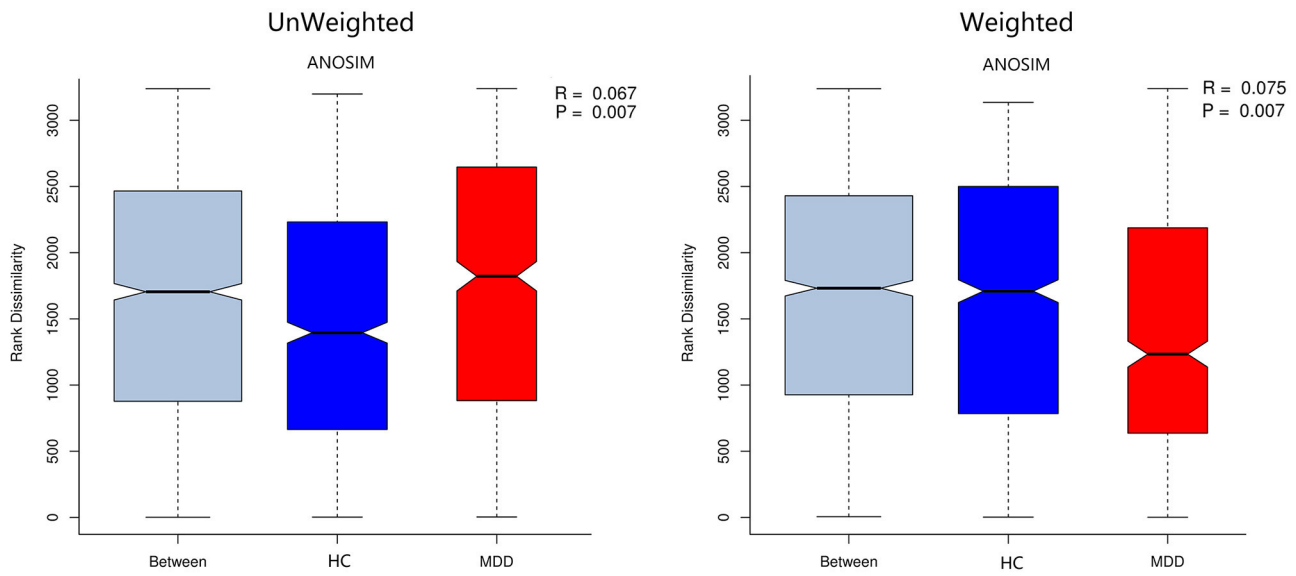

FIGURE 3 | Unweighted and weighted analyses of similarities (ANOSIMs) based on the distance matrix of UniFrac differences of the fecal microbial communities in the MDD group and HC. $r > 0$ means the difference between groups is greater than that within groups.

significant differences between the two groups; the top four were *Bacteroides*, *Prevotella*, *Parabacteroides*, and *Escherichia*. At the species level, 18 species showed significant differences between the two groups; the top four were *uncultured_bacterium*, *uncultured_organism*, *Bacteroides_thetaiotaomicron*, and *Bacteroides_uniformis*. After the strict FDR correction, at the

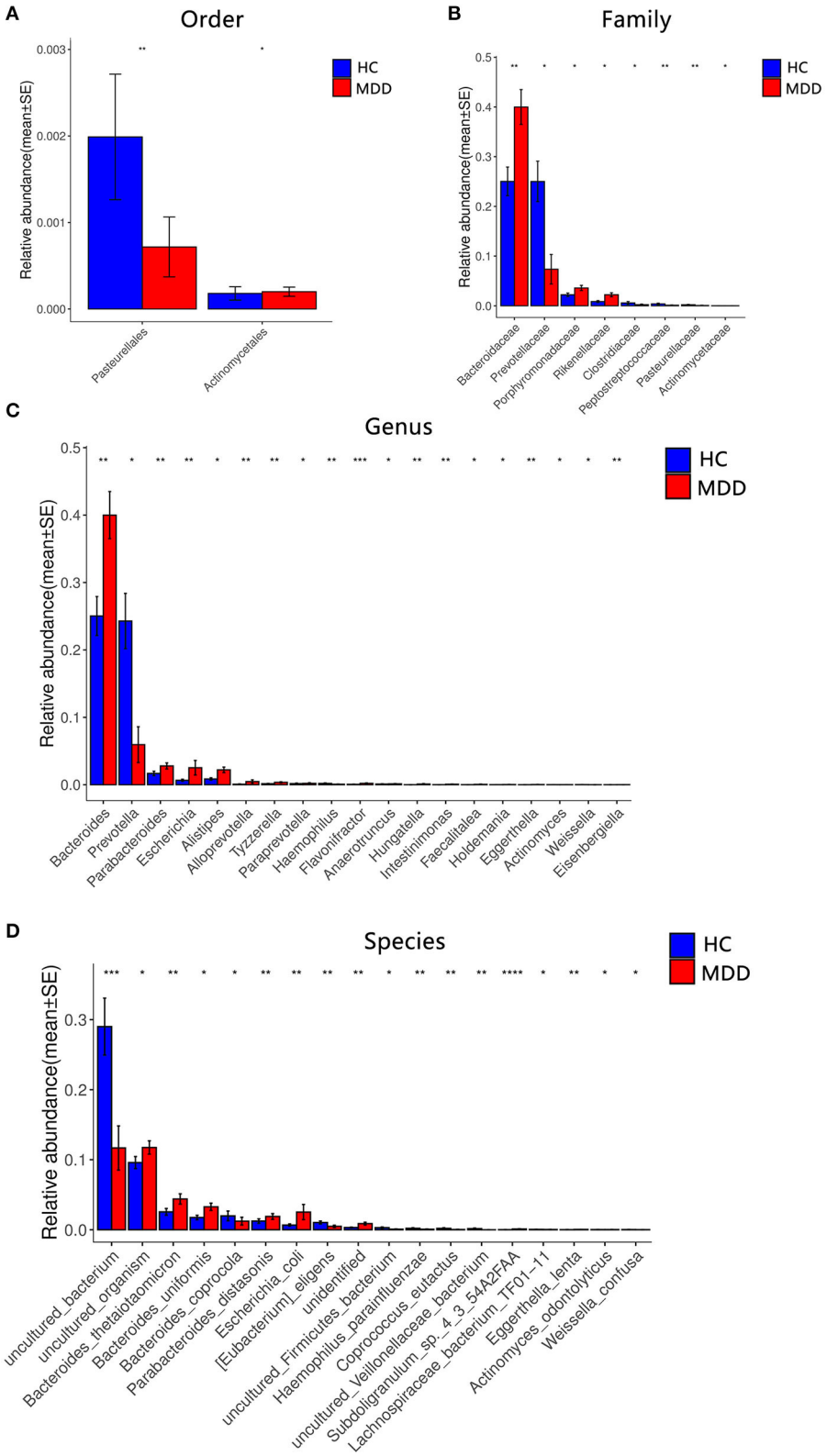

FIGURE 4 | Comparison of the microbial abundance among the MDD group and HC. Dominant bacteria with relative abundances >0.01%. After exclusion, the Wilcoxon signed-rank test was applied to identify the differentially abundant orders **(A)**, families **(B)**, genera **(C)**, and species **(D)**. Among these, the highest means of the phylogenetic abundance in the enriched cohort were drawn as bar plots (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$).

genus level, 10 genera showed significant differences between the two groups; the top four were *Flavonifractora*, *Alloprevotella*, *Parabacteroides*, and *Hungatella*. At the species level, four species showed significant differences between the two groups; they were *Subdoligranulum_sp._4_3_54A2FAA*, *uncultured_bacterium*, *Eggerthella_lenta*, and *uncultured_Veillonellaceae_bacterium*. No significant differences were identified at the family and order levels. The abundance of these microbiota targets is presented in **Figure 4**, **Table 2**, and **Supplementary Table 1**.

Based on the LDA score, we found that *Bacteroidaceae*, *Bacteroides*, and *uncultured_Mesorhizobium_sp.* were associated with healthy controls and *uncultured_bacterium*, *Prevotellaceae*, and *Prevotella* with the MDD group (**Figure 5**).

Correlations of Microbiota Abundance With Severity of Depression and Sleep Quality in MDD

After controlling for potential confounders (age, sex, BMI), the results of partial correlation analysis indicated no correlation between alpha diversity and ISI and HAM-D scores, while a moderate correlation was observed between Chao and PSQI scores ($r = -0.345$, $p = 0.049$). At the genus level, *Dorea* ($r = 0.355$, $p = 0.042$), *Butyricicoccus* ($r = 0.364$, $p = 0.037$), and *Peptococcus* ($r = 0.403$, $p = 0.020$) showed moderate correlations with the HAM-D score, while *Blautia* ($r = 0.406$, $p = 0.019$), *Coprococcus* ($r = 0.508$, $p = 0.003$), *Dorea* ($r = 0.399$, $p = 0.022$), and *Intestinibacter* ($r = -0.559$, $p = 0.001$) showed moderate to strong correlations with the PSQI score, and *Intestinibacter* ($r = -0.489$, $p = 0.004$) a moderate one with the ISI score. In addition, the species *Bacteroides_stercoris* ($r = -0.368$, $p = 0.035$), *Bacteroides_uniformis* ($r = 0.424$, $p = 0.014$), and *Parasutterella_secunda* ($r = 0.358$, $p = 0.041$) showed moderate correlations with HAM-D score; *Blautia_obeum* ($r = -0.392$, $p = 0.024$), *Streptococcus_salivarius_subsp._salivarius* ($r = -0.352$, $p = 0.045$), *Coprococcus_comes* ($r = 0.408$, $p = 0.018$), *Blautia_sp.* ($r = -0.411$, $p = 0.018$), *butyrate_producing_bacterium_L250* ($r = -0.357$, $p = 0.041$), *Dorea_formicigenerans* ($r = -0.419$, $p = 0.015$), and *uncultured_Coprococcus_sp.* ($r = -0.345$, $p = 0.049$) show moderate correlation with PSQI score; and *uncultured_Clostridiales_bacterium* ($r = 0.357$, $p = 0.042$) and *Blautia_obeum* ($r = -0.350$, $p = 0.046$) show moderate correlation with ISI. Correlations with HAM-D, PSQI, and ISI scores are shown in **Table 3** and **Supplementary Table 2**. Notably, *Dorea* commonly correlated with HAM-D and PSQI scores in patients with MDD. After FDR correction, at the genera level, *Intestinibacter* with PSQI was still significant. At the species level, no correlation was shown.

In HC, after controlling for potential confounders (age, sex, BMI), the results of partial correlation analysis are shown in **Supplementary Table 2**. At the genus level, *Haemophilus* ($r = 0.517$, $p < 0.001$) showed strong correlations with the HAM-D score, *Acidaminococcus* ($r = -0.345$, $p = 0.025$) showed moderate correlations with the PSQI score, and *Haemophilus* ($r = 0.753$, $p < 0.001$) had a strong correlation with the ISI score. In addition, the species *Haemophilus_parainfluenzae* ($r = 0.517$, $p < 0.001$) showed strong correlations with HAM-D score. *Haemophilus_parainfluenzae* ($r = 0.752$, $p < 0.001$) and *Clostridium_paraputrificum* ($r = 0.670$, $p < 0.001$) showed strong correlations with ISI. After FDR correction, at the genera level, the correlations between *Haemophilus* and HAM-D and ISI were still significant. At the species level, *Haemophilus_parainfluenzae* with HAM-D, *Haemophilus_parainfluenzae*, and *Clostridium_paraputrificum* with ISI were still significant.

When adding severity of depression to potential confounding factors, the most relevant taxa with sleep quality in MDD at the genus levels were *Coprococcus* ($\beta = -0.322$, $p = 0.021$) and *Intestinibacter* ($\beta = -0.455$, $p = 0.006$), and the results are shown in **Table 4**.

Differences of MDD Patients With or Without Sleep Disorder

We divided the MDD patients into two groups according PSQI scores (>5). **Figures 6A,B** show the relative abundance of all the patients at the genus level and species level, respectively. A significant difference was shown between the two groups (**Supplementary Table 3**). At the genus level, *Streptococcus*, *Dorea*, *Barnesiella*, and *Intestinibacter* decreased in MDD patients with sleep disorder, while *Coprococcus* increased. At the species level, *Blautia_obeum*, *Streptococcus_salivarius_subsp._salivarius*, *Dorea_formicigenerans*, *uncultured_Coprococcus_sp.*, and *Ruminococcus_lactaris* decreased in MDD patients with sleep disorder, and *Clostridium_sp.* increased. After FDR correction, no significant difference was found between the two groups.

DISCUSSION

Our study demonstrated significant gut flora differences between MDD and HC and found partially similar results to previous studies, as well as differences. Notably, we explore the composition of the microbiota of patients with MDD in relation to sleep quality and the severity of insomnia. We considered medication factors by limiting drug natural washout period, and we enrolled patients without any treatment >1 week and without long-acting antipsychotics >6 months.

In our study, some of the microbiota targets at the order, family, genus, and species levels indicated significant differences between MDD and HC. At the family level, a higher abundance of *Actinomycineae* and *Porphyromonadaceae* and a lower abundance of *Prevotellaceae* were observed in MDD (11, 33). At the genus level, higher *Bacteroides*, *Parabacteroides*, and *Alistipes* and lower *Prevotella* and *Eggerthella* were observed in MDD (11, 34–36). These results are consistent with previous reports. Transplanting the fecal flora from depressive patients to mice showed similar behaviors and microflora phenotype in those mice as the donor patients, indicating that the gut microbiome could cause depressive symptoms by affecting the metabolism (37). Growing research supports the effect of microbiota on brain networks and the regulation of negative affect. *Alistipes* can produce indole to influence tryptophan metabolism, which is vital for emotion regulation (38). However,

TABLE 2 | Taxa abundances in MDD and HC.

Parameter	MDD (*N* = 36)	HC (*N* = 45)	*p*-value	*p* adj.
Order				
Pasteurellales	0.07%	0.20%	0.005**	0.082
Actinomycetales	0.02%	0.02%	0.015*	0.131
Family				
Bacteroidaceae	40.00%	25.03%	0.003**	0.099
Prevotellaceae	7.36%	25.02%	0.028*	0.119
Porphyromonadaceae	3.59%	2.22%	0.017*	0.086
Rikenellaceae	2.22%	0.86%	0.046*	0.172
Clostridiaceae	0.22%	0.57%	0.015*	0.089
Peptostreptococcaceae	0.11%	0.39%	0.009**	0.086
Pasteurellaceae	0.07%	0.20%	0.005**	0.068
Actinomycetaceae	0.02%	0.02%	0.015*	0.109
Genus				
Bacteroides	40.00%	25.03%	0.003**	0.045*
Prevotella	5.95%	24.29%	0.015*	0.079
Parabacteroides	2.79%	1.68%	0.005**	0.043*
Escherichia	2.53%	0.67%	0.009**	0.054
Alistipes	2.22%	0.86%	0.044*	0.155
Alloprevotella	0.47%	0.08%	0.002**	0.042*
Tyzzerella	0.35%	0.16%	0.007**	0.049*
Paraprevotella	0.20%	0.17%	0.028*	0.110
Haemophilus	0.07%	0.20%	0.006**	0.046
Flavonifractor	0.21%	0.07%	<0.001**	0.010*
Anaerotruncus	0.14%	0.11%	0.033*	0.123
Hungatella	0.10%	0.01%	0.005**	0.044*
Intestinimonas	0.08%	0.01%	0.003**	0.046*
Faecalitalea	0.06%	0.02%	0.027*	0.113
Holdemania	0.04%	0.01%	0.017*	0.082
Eggerthella	0.04%	0.01%	0.002**	0.059
Actinomyces	0.02%	0.02%	0.015*	0.081
Weissella	0.00%	0.02%	0.024*	0.109
Eisenbergiella	0.01%	0.01%	0.004**	0.048*
Species				
uncultured_bacterium	11.68%	29.01%	<0.001***	0.027*
uncultured_organism	11.75%	9.59%	0.049*	0.207
Bacteroides_thetaiotaomicron	4.39%	2.56%	0.006**	0.055
Bacteroides_uniformis	3.28%	1.74%	0.026*	0.134
Bacteroides_coprocola	1.23%	1.98%	0.028*	0.128
Parabacteroides_distasonis	1.89%	1.25%	0.010*	0.060
Escherichia_coli	2.53%	0.67%	0.009**	0.060
Unidentified	0.88%	0.32%	0.008**	0.061
uncultured_Firmicutes_bacterium	0.09%	0.30%	0.026*	0.128
Haemophilus_parainfluenzae	0.07%	0.20%	0.006**	0.060
Coprococcus_eutactus	0.03%	0.19%	0.005**	0.054
uncultured_Veillonellaceae_bacterium	0.00%	0.16%	0.002**	0.040*
Subdoligranulum_sp._4_3_54A2FAA	0.10%	0.01%	<0.001***	<0.001***
Lachnospiraceae_bacterium_TF01–11	0.03%	0.05%	0.040*	0.017*
Eggerthella_lenta	0.04%	0.01%	0.002**	0.039*
Actinomyces_odontolyticus	0.02%	0.02%	0.016*	0.092
Weissella_confusa	0.00%	0.02%	0.024*	0.134
[Eubacterium]_eligens	0.48%	1.04%	0.005**	0.057

*Data are presented as mean relative abundance of gut microbiome in MDD groups and HC (*p < 0.05, **p < 0.01, ***p < 0.001). p adj. stands for p value after FDR correction.*

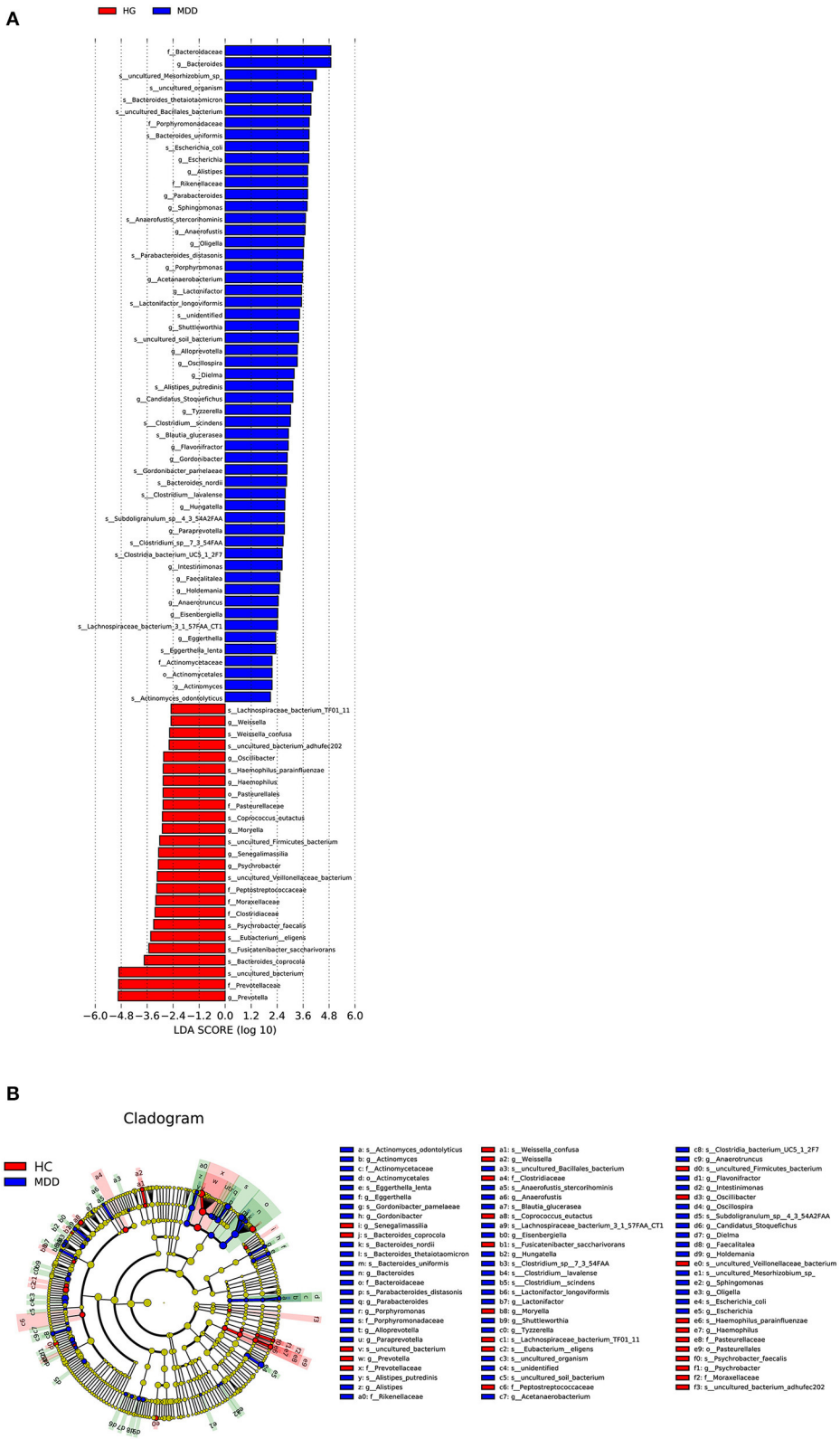

FIGURE 5 | (A) Taxonomic biomarkers found by LEfSe in HC (blue) and MDD (red). Only taxa with $p < 0.05$ and LDA score (log10) are shown. **(B)** Cladogram indicating the phylogenetic relatedness of the discriminant taxa.

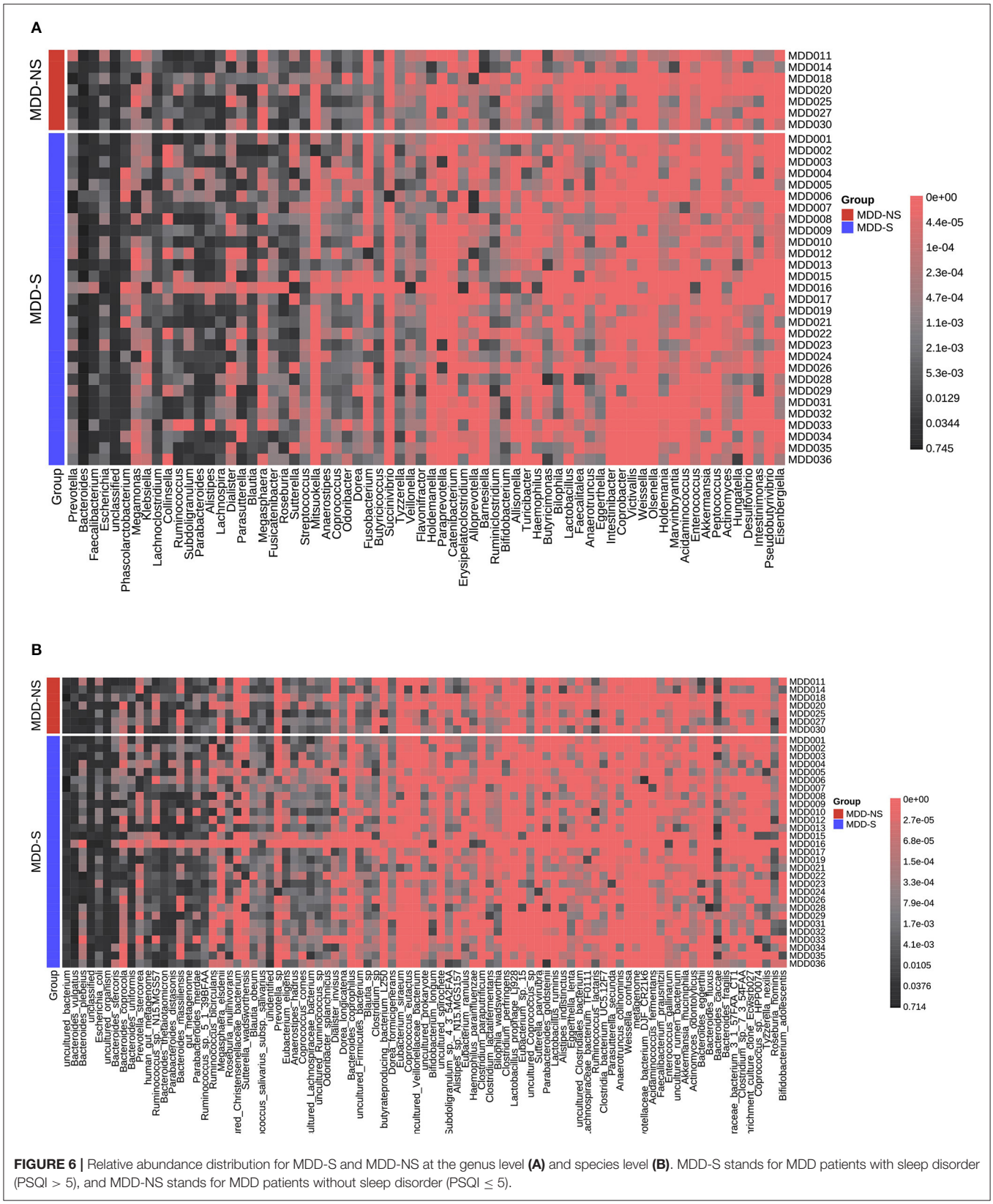

FIGURE 6 | Relative abundance distribution for MDD-S and MDD-NS at the genus level **(A)** and species level **(B)**. MDD-S stands for MDD patients with sleep disorder (PSQI > 5), and MDD-NS stands for MDD patients without sleep disorder (PSQI ≤ 5).

TABLE 3 | Partial correlations between taxa and HAM-D, PSQI, and ISI scores in MDD.

Taxa	HAM-D MDD			PSQI MDD			ISI MDD		
	r	*p*-value	*p* adj.	*r*	*p*-value	*p* adj.	*r*	*p*-value	*p* adj.
Genus									
Blautia	0.039	0.830	0.99	−0.406	0.019*	0.431	−0.226	0.207	0.999
Coprococcus	−0.236	0.186	0.99	−0.508	0.003**	0.101	−0.242	0.174	0.999
Dorea	−0.355	0.042*	0.99	−0.399	0.022*	0.374	−0.247	0.166	0.999
Butyricicoccus	0.364	0.037*	0.99	−0.069	0.705	0.888	0.065	0.720	0.999
Intestinibacter	−0.215	0.229	0.99	−0.559	0.001**	0.048*	−0.489	0.004**	0.272
Peptococcus	0.403	0.020*	0.99	−0.195	0.277	0.897	0.038	0.835	0.999
Species									
Bacteroides_stercoris	−0.368	0.035*	0.999	−0.007	0.97	0.994	0.072	0.689	0.833
Bacteroides_uniformis	0.424	0.014*	0.999	−0.025	0.89	0.994	0.231	0.196	0.838
uncultured_Clostridiales_bacterium	−0.117	0.516	0.999	0.124	0.491	0.815	0.357	0.042*	0.84
Blautia_obeum	−0.072	0.692	0.999	−0.392	0.024*	0.528	−0.35	0.046*	0.855
Streptococcus_salivarius_subsp._salivarius	−0.192	0.284	0.999	−0.352	0.045*	0.566	−0.164	0.362	0.857
Coprococcus_comes	−0.178	0.323	0.999	−0.408	0.018*	0.792	−0.061	0.736	0.863
Blautia_sp.	0.061	0.735	0.999	−0.411	0.018*	0.528	−0.098	0.588	0.864
butyrate-producing_bacterium_L2-50	−0.249	0.162	0.999	−0.357	0.041*	0.601	−0.204	0.255	0.878
Dorea_formicigenerans	−0.285	0.108	0.950	−0.419	0.015*	0.994	−0.197	0.273	0.881
uncultured_Coprococcus_sp.	0.049	0.787	0.999	−0.345	0.049*	0.539	−0.192	0.284	0.881
Parasutterella_secunda	0.358	0.041*	0.999	−0.052	0.775	0.922	0.229	0.200	0.882

*Partial correlation analysis between taxa and HAM-D, PSQI, and ISI in MDD or controls, after controlling for age, sex, and BMI. HAM-D, Hamilton Depression Rating Scale; PSQI, Pittsburgh Sleep Quality Index; ISI, Insomnia Severity Index (*p < 0.05, **p < 0.01). p adj. stands for p-value after FDR correction.*

TABLE 4 | Linear regression analysis results for PSQI at the genus level.

	Unstandardized		Standardized	*t*	*p*-value
	Beta	SE	Beta		
HAM−D score	0.160	0.126	0.199	1.269	0.214
Gender	−1.381	1.296	−0.143	−1.066	0.295
Age	0.095	0.052	0.266	1.832	0.077
BMI	−0.135	0.169	−0.117	−0.799	0.431
Intestinibacter	−10505.272	3521.040	−0.455	−2.984	0.006**
Coprococcus	−281.100	115.270	−0.322	−2.439	0.021*

*Linear regression analyses were used to assess the most influential taxa on sleep quality in MDD at the genus and species levels after correcting for age, gender, BMI, and HAM-D scores. PSQI, Pittsburgh Sleep Quality Index (*p < 0.05, **p < 0.01).*

the lower abundance of *Peptostreptococcaceaea* and *Rikenellaceae* in MDD was not found in our study. *Rikenellaceae* is a butyrate producer that can attenuate inflammation levels in order to improve emotion (39). *Peptostreptococcaceaea* belongs to *Firmicutes* which can affect glucose metabolism to mediate inflammation levels (40). A neuroimaging study found that patients with MDD had higher concentrations of *Bacteroides* and lower *Prevotella* and showed differences of brain structure and function associated with emotion (41). Age, BMI, gender, nations, diet, region, diagnostic criteria, mediation, lifestyles, sample size, and subclinical symptoms often disturb the reproducibility and accuracy of results (11, 42). In our study, age, BMI, and gender were similar between the two groups, and a mediation factor was also considered. Interestingly, our study found that *Dorea*, *Butyricicoccus*, and *Peptococcus* were associated with HAM-D scores measuring the severity of depression, which has never been reported before (9, 26, 43, 44).

Another contribution of our study was to further clarify the correlation of gut microbiota and concomitant sleep symptoms. We clustered the gut bacterial distributions based on existing similarity. PSQI and ISI represent different aspects of sleep, unlike ISI, sleep latency, sleep duration, and sleep efficiency components based on free-text numerical responses, while ISI concentrated on perceived feeling (45). At the genus level, *Blautia*, *Coprococcus*, *Dorea*, and *Intestinibacter* were negatively correlated with PSQI, with *Intestinibacter* being simultaneously

negatively correlated with PSQI and ISI. Moreover, in our study, after controlling for the HAM-D score, *Coprococcus* and *Intestinibacter* were associated with sleep quality, independent of the severity of depression. However, contrary to our findings, a recent study showed that higher sleep quality was associated with a high proportion of bacteria from the *Verrucomicrobia* and *Lentisphaerae* phyla (46). Recent reviews have summarized the potential mechanism of sleep and MBGA: immunoregulatory pathway (cytokines), neuroendocrine pathway (HPA axis, CNS, neurotransmitters), vagus nerve pathway, and gut microbial metabolite pathway (SCFAs) (20, 47). Butyrate producers (*Blautia*, *Coprococcus*) may influence sleep quality because butyrate may potentially serve as a sleep-inducing signal molecule to enhance sleep (48), and the results were consistent with those of previous studies (49). Depressive patients were observed to exhibit similar phenomena, indicating that the two gut bacteria were more relevant to sleep rather than depressive symptoms. Depression and sleep are both affected by circadian activity, and no single hypothesis can explain the complex mechanisms of the comorbidity of depression and insomnia (20). *Dorea* was observed to decrease with HAM-D and PSQI. Thus, altered gut microbiota composition may correlate not only with an increased MDD severity but also with lower sleep quality. Indeed, Huang et al. found that *Dorea* decreased in depressive patients, although no previous study has reported its link with sleep (50). Since *Dorea* is known for fermenting polysaccharides into SCFAs (51) and SCFAs, including butyrate and acetate, play important roles in clock gene expression, which is closely related to circadian rhythm and sleep quality, it is possible that *Dorea* mediates sleep deficits in MDD (52). However, it is not clear why *Intestinibacter* is associated with sleep, whether *via* immune inflammatory mechanisms or carbohydrate metabolism (53–55).

In our study, the correlation of gut microbiome and clinical symptoms in patients and in cases and controls was different. Previous studies reported the correlation between gut microbiome and sleep quality in animals or in healthy controls. *Blautia* and *Ruminococcus* were reported to have a negative correlation with PSQI score, and *Prevotella* was positively correlated with PSQI score in young healthy individuals (22). A recent study reported that probiotics could improve sleep quality and has a role in anti-inflammatory mechanism (47). Our study identified that *Acidaminococcus* was associated with better sleep quality, and it also identified the heterogeneity of microbiome in MDD and HC. Different gut flora were associated with different metabolites, metabolic pathways, and inflammatory pathways. Sleep and circadian rhythms could influence microbiome composition by inflammation and breakdown of the epithelial barrier (56). The inflammatory reaction mechanism is induced by the microbiome and then triggers the CNS and aggravates insomnia and depression (57).

As for alpha diversity, our results are consistent with the latest meta-analysis, which found no difference in alpha diversity between patients with MDD and HC (58). History studies showed ambiguous results of beta diversity, including increasing and no difference due to individual heterogeneity and using different measuring instruments and analysis software (35, 36, 59). Our results reported different microbiota compositions between the two groups, similar to Chung et al. (11). In the future, more extensive sampling is needed to verify the results. Most scholars believe that the higher the microbiome diversity, the better the health (60). Zhang et al. reported no overt changes in microbiome richness or composition after sleep restriction (21). However, another study indicated that alpha diversity was positively associated with sleep efficiency and total sleep time and negatively associated with sleep fragmentation (49). The marginal significance in community richness observed in our study may be limited by the small sample size, while the short-term loss of sleep may not affect the gut microbiome diversity (61). It is worth noting that RDA analysis pointed out that sleep quality, severity of insomnia, and depression were main environmental factors, which drove the variation of microbiome in MDD patients not in HC. Therefore, in MDD patients, clinical symptoms were significantly related to the changes of microflora.

Considering our results, some limitations were noted. First, part of our results did not pass strict FDR correction (p adj. value should be <0.05), and we believe the negative results failing multiple testing would give a systematic view regarding our study. Also, the small sample size with limited power makes it impossible to identify more related factors. Further studies are needed to enlarge the sample size. Second, a cross-sectional study cannot prove causality; therefore, a longitudinal study is warranted. Although we controlled medication and diet after admission to minimize confounding, long-term dietary and medication effects should be considered. Our healthy controls consisted of hospital workers ($N = 14$) and patients' families or friends with no consanguinity ($N = 31$). They were all from Beijing, with similar geographical location, and in relatively consistent dietary habits. As a previous study pointed out that there were differences in the microbiome of hospital workers and non-hospital workers (62), we further compared the difference between healthy hospital workers (HCW) and non-hospital workers (non-HCW). There was no significant difference between them in our study (**Supplementary Table 4**). Recruiting patients focusing on the same area, applying the dietary questionnaire, and collecting the patients' medication history will help increase the reliability of results. Thirdly, we did not measure the stool moisture in our research, because the stools were immediately placed in a sample tube containing 2 ml of RNA stabilization solution. Further study should consider the impact of stool consistency. Additionally, as a common sequencing method, we used 16S RNA sequences, despite new methods such as shotgun metagenomics often reflecting more reliability and repeatability, but at a higher cost. Lastly, functional microbiota analysis, immunological status, gut barrier integrity, and metabolomics should also be integrated in future studies.

CONCLUSIONS

The microbiomes of patients with major depressive disorder in China were found to be significantly different from those of healthy controls. In summary, 48 microbiota targets were associated with MDD, 6 with severity of depression, 11 with sleep quality, and 3 with insomnia severity. At the genus level,

Dorea was simultaneously related to depression and sleep quality, and *Intestinibacter* was more closely associated with sleep-related problems. The most interesting finding was that the presence of *Coprococcus* and *Intestinibacter* was associated with sleep quality independent of the severity of depression. We identified several specific taxa related to sleep health, which suggests that the microbiome may be related to both sleep and severity of illness at the same time. Overall, our findings are consistent with previous microbiome studies on depression and constitute a preliminary exploration of the role of dysbacteriosis in sleep disorders in MDD patients.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: https://www.ncbi.nlm.nih.gov/, PRJNA687871.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Institutional review board of Beijing Huilongguan Hospital (# 2019–43). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

QZ conducted data collection and analysis and drafted and revised the manuscript. FY and ZW designed the experiments. YY, WZ, and TM collected the data. HA proofread the manuscript. All authors contributed to the article 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/fpsyt.2021.645045/full#supplementary-material

REFERENCES

1. Gotlib IH, Hammen CL. *Handbook of Depression*, 3rd Edition. New York, NY: Guilford Publications (2015).
2. Lim G Y, Tam WW, Lu Y, Ho C S, Zhang M W, Ho R C. Prevalence of depression in the community from 30 countries between 1994 and 2014. *Sci Rep.* (2018) 8:2861. doi: 10.1038/s41598-018-21243-x
3. Breit S, Kupferberg A, Rogler G, Hasler G. Vagus nerve as modulator of the brain-gut axis in psychiatric and inflammatory disorders. *Front Psychiatry.* (2018) 9:44. doi: 10.3389/fpsyt.2018.00044
4. Feng Q, Chen WD, Wang YD. Gut microbiota: an integral moderator in health and disease. *Front Microbiol.* (2018) 9:151. doi: 10.3389/fmicb.2018.00151
5. Asarnow LD. Depression and sleep: what has the treatment research revealed and could the HPA axis be a potential mechanism? *Curr Opin Psychol.* (2020) 34:112–6. doi: 10.1016/j.copsyc.2019.12.002
6. Liang S, Wu X, Hu X, Wang T, Jin F. Recognizing depression from the Microbiota-Gut-Brain axis. *Int J Mol Sci.* (2018) 19:1592. doi: 10.3390/ijms19061592
7. Averina OV, Danilenko VN. [Human intestinal microbiota: role in development and functioning of the nervous system]. *Mikrobiologiia.* (2017) 86:5–24. doi: 10.1134/S0026261717010040
8. Stilling RM, van de Wouw M, Clarke G, Stanton C, Dinan TG, Cryan JF. The neuropharmacology of butyrate: the bread and butter of the microbiota-gut-brain axis? *Neurochem Int.* (2016) 99:110–32. doi: 10.1016/j.neuint.2016.06.011
9. Cheung SG, Goldenthal AR, Uhlemann AC, Mann JJ, Miller JM, Sublette ME. Systematic review of gut microbiota and major depression. *Front Psychiatry.* (2019) 10:34. doi: 10.3389/fpsyt.2019.00034
10. Jiang HY, Zhang X, Yu ZH, Zhang Z, Deng M, Zhao JH, et al. Altered gut microbiota profile in patients with generalized anxiety disorder. *J Psychiatr Res.* (2018) 104:130–6. doi: 10.1016/j.jpsychires.2018.07.007
11. Chung YE, Chen HC, Chou HL, Chen IM, Lee MS, Chuang LC, et al. Exploration of microbiota targets for major depressive disorder and mood related traits. *J Psychiatr Res.* (2019) 111:74–82. doi: 10.1016/j.jpsychires.2019.01.016
12. Tsuno N, Besset A, Ritchie K. Sleep and depression. *J Clin Psychiatry.* (2005) 66:1254–69. doi: 10.4088/JCP.v66n1008
13. Borbély AA. The S-deficiency hypothesis of depression and the two-process model of sleep regulation. *Pharmacopsychiatry.* (1987) 20:23–9. doi: 10.1055/s-2007-1017069
14. Antonijevic I. HPA axis and sleep: identifying subtypes of major depression. *Stress.* (2008) 11:15–27. doi: 10.1080/10253890701378967
15. Ivanov FI. [Biological rhythms in psychopathology (biological patterns in psychiatry)]. *Zh Nevropatol Psikhiatr Im S S Korsakova.* (1981) 81:589–97.
16. Salvatore P, Indic P, Murray G, Baldessarini RJ. Biological rhythms and mood disorders. *Dialog Clin Neurosci.* (2012) 14:369–79. doi: 10.31887/DCNS.2012.14.4/psalvatore
17. Armitage R. Sleep and circadian rhythms in mood disorders. *Acta Psychiatr Scand Suppl.* (2007) 2007:104–15. doi: 10.1111/j.1600-0447.2007.00968.x
18. Imeri L, Opp MR. How (and why) the immune system makes us sleep. *Nat Rev Neurosci.* (2009) 10:199–210. doi: 10.1038/nrn2576
19. Biological rhythms in psychiatry: order and disorder. Society of Biological Psychiatry. *Forty-Fifth Annual Convention & Scientific Program*. New York, NY: Program and abstracts (1990).
20. Li Y, Hao Y, Fan F, Zhang B. The role of microbiome in insomnia, circadian disturbance and depression. *Front Psychiatry.* (2018) 9:669. doi: 10.3389/fpsyt.2018.00669
21. Zhang SL, Bai L, Goel N, Bailey A, Jang CJ, Bushman FD, et al. Human and rat gut microbiome composition is maintained following sleep restriction. *Proc Natl Acad Sci USA.* (2017) 114:E1564–71. doi: 10.1073/pnas.1620673114
22. Grosicki GJ, Riemann BL, Flatt AA, Valentino T, Lustgarten MS. Self-reported sleep quality is associated with gut microbiome composition in young, healthy individuals: a pilot study. *Sleep Med.* (2020) 73:76–81. doi: 10.1016/j.sleep.2020.04.013
23. Evans SJ, Bassis CM, Hein R, Assari S, Flowers SA, Kelly MB, et al. The gut microbiome composition associates with bipolar disorder and illness severity. *J Psychiatr Res.* (2017) 87:23–9. doi: 10.1016/j.jpsychires.2016.12.007
24. Aizawa E, Tsuji H, Asahara T, Takahashi T, Teraishi T, Yoshida S, et al. Bifidobacterium and *Lactobacillus* counts in the gut microbiota of patients with bipolar disorder and healthy controls. *Front Psychiatry.* (2018) 9:730. doi: 10.3389/fpsyt.2018.00730

25. Kurokawa S, Kishimoto T, Mizuno S, Masaoka T, Naganuma M, Liang KC, et al. The effect of fecal microbiota transplantation on psychiatric symptoms among patients with irritable bowel syndrome, functional diarrhea and functional constipation: An open-label observational study. *J Affect Disord.* (2018) 235:506–12. doi: 10.1016/j.jad.2018.04.038
26. Liśkiewicz P, Kaczmarczyk M, Misiak B, Wroński M, Baba-Kubiś A, Skonieczna-Zydecka K, et al. Analysis of gut microbiota and intestinal integrity markers of inpatients with major depressive disorder. *Prog Neuropsychopharmacol Biol Psychiatry.* (2020) 106:110076. doi: 10.1016/j.pnpbp.2020.110076
27. Buysse DJ, Reynolds CF, Monk TH, Berman SR, Kupfer DJ. The Pittsburgh sleep quality index: a new instrument for psychiatric practice and research. *Psychiatry Res.* (1989) 28:193–213. doi: 10.1016/0165-1781(89)90047-4
28. Bastien CH, Vallieres A, Morin CM. Validation of the Insomnia Severity Index as an outcome measure for insomnia research. *Sleep Med.* (2001) 2:297–307. doi: 10.1016/S1389-9457(00)00065-4
29. Caporaso JG, Lauber CL, Walters WA, Berg-Lyons D, Huntley J, Fierer N, et al. Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. *Isme J.* (2012) 6:1621–4. doi: 10.1038/ismej.2012.8
30. Bolger AM, Lohse M, Usadel B. Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics.* (2014) 30:2114–20. doi: 10.1093/bioinformatics/btu170
31. Schloss PD, Westcott SL, Ryabin T, Hall JR, Hartmann M, Hollister EB, et al. Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Appl Environ Microbiol.* (2009) 75:7537–41. doi: 10.1128/AEM.01541-09
32. Edgar RC. UPARSE: highly accurate OTU sequences from microbial amplicon reads. *Nat Methods.* (2013) 10:996–8. doi: 10.1038/nmeth.2604
33. Chen Z, Li J, Gui S, Zhou C, Chen J, Yang C, et al. Comparative metaproteomics analysis shows altered fecal microbiota signatures in patients with major depressive disorder. *Neuroreport.* (2018) 29:417–25. doi: 10.1097/WNR.0000000000000985
34. Naseribafrouei A, Hestad K, Avershina E, Sekelja M, Linløkken A, Wilson R, et al. Correlation between the human fecal microbiota and depression. *Neurogastroenterol Motil.* (2014) 26:1155–62. doi: 10.1111/nmo.12378
35. Jiang H, Ling Z, Zhang Y, Mao H, Ma Z, Yin Y, et al. Altered fecal microbiota composition in patients with major depressive disorder. *Brain Behav Immun.* (2015) 48:186–94. doi: 10.1016/j.bbi.2015.03.016
36. Lai WT, Deng WF, Xu SX, Zhao J, Xu D, Liu YH, et al. Shotgun metagenomics reveals both taxonomic and tryptophan pathway differences of gut microbiota in major depressive disorder patients. *Psychol Med.* (2019) 51:90–101. doi: 10.1017/S0033291719003027
37. Zheng P, Zeng B, Zhou C, Liu M, Fang Z, Xu X, et al. Gut microbiome remodeling induces depressive-like behaviors through a pathway mediated by the host's metabolism. *Mol Psychiatry.* (2016) 21:786–96. doi: 10.1038/mp.2016.44
38. Song Y, Könönen E, Rautio M, Liu C, Bryk A, Eerola E, et al. *Alistipes onderdonkii* sp. nov. and *Alistipes shahii* sp. nov., of human origin. *Int J Syst Evol Microbiol.* (2006) 56:1985–90. doi: 10.1099/ijs.0.64318-0
39. Ye J, Lv L, Wu W, Li Y, Shi D, Fang D, et al. Butyrate protects mice against methionine-choline-deficient diet-induced non-alcoholic steatohepatitis by improving gut barrier function, attenuating inflammation and reducing endotoxin levels. *Front Microbiol.* (2018) 9:1967. doi: 10.3389/fmicb.2018.01967
40. Doumatey AP, Adeyemo A, Zhou J, Lei L, Adebamowo SN, Adebamowo C, et al. Gut microbiome profiles are associated with Type 2 diabetes in Urban Africans. *Front Cell Infect Microbiol.* (2020) 10:63. doi: 10.3389/fcimb.2020.00063
41. Tillisch K, Mayer EA, Gupta A, Gill Z, Brazeilles R, Le Nevé B, et al. Brain structure and response to emotional stimuli as related to gut microbial profiles in healthy women. *Psychosom Med.* (2017) 79:905–13. doi: 10.1097/PSY.0000000000000493
42. Łoniewski I, Misera A, Skonieczna-Zydecka K, Kaczmarczyk M, Kazmierczak-Siedlecka K, Misiak B, et al. Major depressive disorder and gut microbiota - association not causation. A scoping review. *Prog Neuropsychopharmacol Biol Psychiatry.* (2020) 106:110111. doi: 10.1016/j.pnpbp.2020.110111
43. Madan A, Thompson D, Fowler JC, Ajami NJ, Salas R, Frueh BC, et al. The gut microbiota is associated with psychiatric symptom severity and treatment outcome among individuals with serious mental illness. *J Affect Disord.* (2020) 264:98–106. doi: 10.1016/j.jad.2019.12.020
44. Chen JJ, Zheng P, Liu YY, Zhong XG, Wang HY, Guo YJ, et al. Sex differences in gut microbiota in patients with major depressive disorder. *Neuropsychiatr Dis Treat.* (2018) 14:647–55. doi: 10.2147/NDT.S159322
45. Omachi TA. Measures of sleep in rheumatologic diseases: Epworth Sleepiness Scale (ESS), Functional Outcome of Sleep Questionnaire (FOSQ), Insomnia Severity Index (ISI), and Pittsburgh Sleep Quality Index (PSQI). *Arthritis Care Res.* (2011) 63(Suppl. 11):S287–96. doi: 10.1002/acr.20544
46. Anderson JR, Carroll I, Azcarate-Peril MA, Rochette AD, Heinberg LJ, Peat C, et al. A preliminary examination of gut microbiota, sleep, and cognitive flexibility in healthy older adults. *Sleep Med.* (2017) 38:104–7. doi: 10.1016/j.sleep.2017.07.018
47. Matenchuk BA, Mandhane PJ, Kozyrskyj AL. Sleep, circadian rhythm, and gut microbiota. *Sleep Med Rev.* (2020) 53:101340. doi: 10.1016/j.smrv.2020.101340
48. Szentirmai É, Millican NS, Massie AR, Kapás L. Butyrate, a metabolite of intestinal bacteria, enhances sleep. *Sci Rep.* (2019) 9:7035. doi: 10.1038/s41598-019-43502-1
49. Smith RP, Easson C, Lyle SM, Kapoor R, Donnelly CP, Davidson EJ, et al. Gut microbiome diversity is associated with sleep physiology in humans. *PLoS ONE.* (2019) 14:e0222394. doi: 10.1371/journal.pone.0222394
50. Huang Y, Shi X, Li Z, Shen Y, Shi X, Wang L, et al. Possible association of firmicutes in the gut microbiota of patients with major depressive disorder. *Neuropsychiatr Dis Treat.* (2018) 14:3329–37. doi: 10.2147/NDT.S188340
51. Leone V, Gibbons SM, Martinez K, Hutchison AL, Huang EY, Cham CM, et al. Effects of diurnal variation of gut microbes and high-fat feeding on host circadian clock function and metabolism. *Cell Host Microbe.* (2015) 17:681–9. doi: 10.1016/j.chom.2015.03.006
52. Jiao N, Baker SS, Nugent CA, Tsompana M, Cai L, Wang Y, et al. Gut microbiome may contribute to insulin resistance and systemic inflammation in obese rodents: a meta-analysis. *Physiol Genomics.* (2018) 50:244–54. doi: 10.1152/physiolgenomics.00114.2017
53. Poggiogalle E, Jamshed H, Peterson CM. Circadian regulation of glucose, lipid, and energy metabolism in humans. *Metabolism.* (2018) 84:11–27. doi: 10.1016/j.metabol.2017.11.017
54. Rosario D, Benfeitas R, Bidkhori G, Zhang C, Uhlen M, Shoaie S, et al. Understanding the representative gut microbiota dysbiosis in metformin-treated Type 2 diabetes patients using genome-scale metabolic modeling. *Front Physiol.* (2018) 9:775. doi: 10.3389/fphys.2018.00775
55. Forbes JD, Chen CY, Knox NC, Marrie RA, El-Gabalawy H, de Kievit T, et al. A comparative study of the gut microbiota in immune-mediated inflammatory diseases-does a common dysbiosis exist? *Microbiome.* (2018) 6:221. doi: 10.1186/s40168-018-0603-4
56. Bravo JA, Forsythe P, Chew MV, Escaravage E, Savignac HM, Dinan TG, et al. Ingestion of *Lactobacillus* strain regulates emotional behavior and central GABA receptor expression in a mouse via the vagus nerve. *Proc Natl Acad Sci USA.* (2011) 108:16050–5. doi: 10.1073/pnas.1102999108
57. Hsiao EY, McBride SW, Hsien S, Sharon G, Hyde ER, McCue T, et al. Microbiota modulate behavioral and physiological abnormalities associated with neurodevelopmental disorders. *Cell.* (2013) 155:1451–63. doi: 10.1016/j.cell.2013.11.024
58. Sanada K, Nakajima S, Kurokawa S, Barceló-Soler A, Ikuse D, Hirata A, et al. Gut microbiota and major depressive disorder: a systematic review and meta-analysis. *J Affect Disord.* (2020) 266:1–13. doi: 10.1016/j.jad.2020.01.102
59. Liu RT, Rowan-Nash AD, Sheehan AE, Walsh RFL, Sanzari CM, Korry BJ, et al. Reductions in anti-inflammatory gut bacteria are associated with depression in a sample of young adults. *Brain Behav Immun.* (2020) 88:308–24. doi: 10.1016/j.bbi.2020.03.026

60. Matsuoka K, Kanai T. The gut microbiota and inflammatory bowel disease. *Semin Immunopathol.* (2015) 37:47–55. doi: 10.1007/s00281-014-0454-4
61. Benedict C, Vogel H, Jonas W, Woting A, Blaut M, Schürmann A, et al. Gut microbiota and glucometabolic alterations in response to recurrent partial sleep deprivation in normal-weight young individuals. *Mol Metab.* (2016) 5:1175–86. doi: 10.1016/j.molmet.2016.10.003
62. Zheng N, Li S, Dong B, Sun W, Li H, Zhang Y, et al. Structural changes in the gut microbiome of short-term and long-term medical workers compared to normal controls. *bioRxiv [Preprint].* (2019). doi: 10.1101/772590

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Sinisan Protects Primary Hippocampal Neurons Against Corticosterone by Inhibiting Autophagy via the PI3K/Akt/mTOR Pathway

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Objective: Corticosterone causes significant neurotoxicity in primary hippocampal neurons which is associated with depression. Dysfunctional autophagy is implicated in cognitive impairment and depressive-like behavior. The traditional Chinese medicine Sinisan (SNS) is highly effective in clinical treatment of depression. However, the molecular mechanisms underlying therapeutic effects of SNS are unknown.

Purpose: The aim of this study was to elucidate the protective effect of SNS and the underlying mechanisms against corticosterone-induced neuronal damage.

Study Design: The effects of serum derived from rats containing SNS (or untreated controls) on the expression of autophagy-related molecules in primary rat hippocampal neurons exposed to different concentrations of corticosterone for different intervals were explored.

Methods: CCK-8 assay, LDH assay were used to analyze cell viability and LDH activity. Western blot, qRT-PCR, and immunofluorescence assays were used to determine protein and mRNA expression levels of molecules such as LC3, p62, Beclin1, ULK1, PI3K, p-PI3K, Akt p-Akt, mTOR, p-mTOR, p70S6, p-p70S6, 4ebp1 and p-4ebp1.

Results: Corticosterone induced a dose- and time-dependent reduction in cellular viability. Moreover, corticosterone (100–400 μM) treatment for 24 h increased LC3-II/LC3-I protein ratio, increased Beclin1 and ULK1 protein expression levels, and decreased p62, PI3K, p-PI3K, p-Akt, p-mTOR, p-p70S6, and p-4ebp1 protein expression levels. Notably, SNS-containing serum reversed corticosterone-induced reduction of neuronal viability, and increased p62, PI3K, p-Akt, p-mTOR, p-p70S6, and p-4ebp1 protein and mRNA expression levels. In addition, SNS-containing serum decreased LC3-II/LC3-I protein ratio, and downregulated Beclin1, and ULK1 protein and mRNA expression in primary hippocampal neurons.

Conclusion: SNS protects primary hippocampal neurons against corticosterone-induced neurotoxicity by preventing excessive autophagy through activation of PI3K/AKT/mTOR pathway.

Keywords: sinisan, primary hippocampal neurons, corticosterone, autophagy, depression

INTRODUCTION

Depression is a condition characterized by low mood, loss of interest, low self- esteem, and suicidal impulse. Depression is one of the most common mental conditions worldwide (1, 2). Incidence of depression worldwide has increased by more than 18% between 2005 and 2015, and currently over 300 million people are living with depression. However, the cause of increase in prevalence of depression are unknown. Excessive stress plays an important role in development of depression (3, 4). Hyperactivity of the hypothalamic Pituitary Adrenal (HPA) axis is implicated in depression. Hippocampus is the major target of corticosterone which is the final product of HPA axis, and is correlated with pathogenesis and progression of depression (5). Therefore, we hypothesized that drugs used for inhibition of corticosterone-induced neurotoxicity in the hippocampus can be applied for clinical treatment of depression. Use of antidepressants is associated with various side effects such as cardiac toxicity, sexual dysfunction, obesity, and insomnia. Moreover, the rate of successful treatment of depression is approximately 70% (6, 7). Therefore, studies should explore better tolerated and more effective drugs for treatment of depression.

Traditional Chinese medicine (TCM) has been used successfully for treatment of depression. Sinisan decoction (SNS) reported by Zhongjing Zhang in "Treatise on Febrile Diseases" in 200–201 AD contains Bupleuri Radix (BR), Paeoniae Radix Alba (PRA), Aurantii Fructus Immaturus (AFI), and Glycyrrhizae Radix et Rhizoma Praeparata Cum Melle (GRM) (8). SNS is regarded as an effective and essential anti-depression therapy in TCM (9, 10). SNS treatment improves weight loss, activity in open field test (OFT), and sucrose preference test (SPT) performance in mouse models with depressive-like behavior (11). However, the mechanisms underlying the anti-depressive effects of SNS have not been explored.

Macro-autophagy (hereafter referred to as autophagy) is implicated in protein degradation in cells. In autophagy process, target proteins are packaged in a double-membrane vesicle (autophagosome), which fuses with lysosomes for degradation of its contents. Autophagy is a cell survival mechanism involved in removal of damaged cytosolic organelles and misfolded proteins to maintain homeostasis. Dysfunctional autophagy results in a variety of pathological processes. Prenatal stress induces cognitive impairment and depressive-like behavior through autophagy (12). Previous study reported that inhibition of autophagy prevents depressive-like behavior induced by ecstasy in rats (13).

PI3K/Akt/mTOR pathway is implicated in modulation of hippocampal activities that result in long-term depression (14). Eight-week chronic unpredictable mild stress (CUMS) exposure downregulates mTOR and the upstream and downstream signaling proteins (15). Therefore, PI3K/Akt/mTOR pathway is associated with depression (16).

In this study, primary hippocampal neurons were used as an *in vitro* system to explore the effects of SNS treatment on corticosterone-induced neuronal damage and elucidate the underlying mechanisms.

MATERIALS AND METHODS

Animals

Adult male (8 weeks old) and newborn Sprague-Dawley (SD) rats were obtained from Guangzhou University of Chinese Medicine. Adult animals were housed under normal environmental conditions (12 h light/dark cycle; lights on at 8:00 a.m., 20 ± 2°C). Rats were housed in plastic cages at a density of 5 per cage with free access to food and tap water. Animals were allowed to acclimatize for a week prior to experimental sessions. All animal experiments were approved by the Animal Care and Committee of Guangzhou University of Chinese Medicine. Experimental protocols were designed to minimize animal suffering.

Primary Hippocampal Neuron Culture and Treatment

Primary hippocampal neurons were obtained from newborn SD rats (∼1 day old). Animals were euthanized and immediately hippocampus from each animal was separated and dissected into 0.5-mm pieces in DMEM-F12 (Gibco, America). After harvesting of hippocampus, the meninges were gently removed under sterile conditions. The samples were treated with 0.125% trypsin (Gibco, USA) at 37°C for 30 min with continuous shaking. Samples were further suspended in DMEM-F12 containing 10% FBS (Gibco, America). Disassociated neurons were centrifuged at 152 g for 5 min and loaded at a density of 1.5×10^5-1×10^6 cells/well on poly-d-lysine-coated plates (sigma, America) for cell viability, immunofluorescence, western blot, and qRT-PCR analysis. Cells were initially maintained in DMEM-F12 containing 10% FBS. At 4 h post-plating, the media was replaced with neurobasal media (Gibco, USA) containing 2% B27 (Gibco, USA) and 1% L-glutamine (Gibco, USA). Cells were cultured at 37°C in a humidified 5% CO2 incubator, and half of the medium was changed every 2 days.

Primary hippocampal neurons were cultured for 7 days, then different concentrations and time of corticosterone were incubated to determine the appropriate damaging concentration of corticosterone. Neurons were then grouped including untreated (control group), corticosterone group, corticosterone + normal serum group, and corticosterone + SNS-containing serum group to explore the protective effect of SNS on

corticosterone-induced neurons. Cells in the corticosterone + normal serum group and corticosterone + SNS-containing serum group were incubated in serum with corticosterone for 24 h. Normal serum and SNS-containing-serum were administered for 0.5 h prior to treatment with corticosterone.

Preparation of Chinese Herbal Solutions

SNS concentrate particles were provided by the Department of Guangdong Yi Fang Pharmaceutical Company (Guangdong, China). Concentrate particles comprised 12 g Bupleuri Radix (BR), 12 g Paeoniae Radix Alba (PRA), 12 g Aurantii Fructus Immaturus (AFI), and 12 g Glycyrrhizae Radix et Rhizoma Praeparata Cum Melle (GRM). Concentrate particles were dissolved, sealed, and stored at 4°C. The concentration of crude concentrations of SNS solutions was 0.49 g/mL.

Drug-Containing Serum Preparation

Twenty SD rats were randomly divided into normal serum and SNS groups ($n = 10$ per group). SNS (2 mL, once a day) was administered to rats in the SNS group by gavage methods for seven days. Equal volumes of physiological saline solution were administered to rats in the normal serum group for seven successive days. Rats were anesthetized using sodium pentobarbital (40 mg/kg) 2 h after the last drug administration. Blood was collected through the abdominal aorta, centrifuged at 1,452 g for 10 min, and allowed to stand at 4°C for 4 h. Supernatant serums of the same group were pooled, filtered using a 0.22 μmol/L filter, inactivated at 56°C for 30 min, split into several samples, and stored at −20 °C.

Small Interfering RNA (si-RNA)

Target sequences were 5′-GCAAGACACC ATGAACCAT-3′ for mTOR α1 sense strand, 5′-CCAAAGCACTACACTACA A-3′ for mTOR α2, and 5′-GCTAGAAGCCTTTGTCTAT-3′ for mTOR α3. The target sequence for the mTOR α3 sense strand was selected. A nonspecific siRNA sequence was used as negative control. Transfection reagents (Ribobio, China) were prepared using riboFECTTM CP Transfection Kit (Ribobio, China) following the manufacturer's instructions. Neurons were transfected with 50 nM siRNA-mTOR (Ribobio, China). After transfection for 24 h, mTOR protein expression levels were determined using western blot.

CCK-8 Assay

Cell Counting Kit-8 (CCK-8) assay was used to determine the viability of primary hippocampal neurons following the protocol of the kit. Neurons were washed 3 times with PBS (Gibco, America). After washing, 100 μL neurobasal medium and 10 μL CCK-8 solution (DOJINDO, Japan) were added to each well. The plates were then incubated for 1.5 h at 37°C under dark conditions. A microplate reader (Bio-Rad, America) was used to determine the optical density (OD) for each well at 570 nm.

LDH Assay

Neuronal injury was evaluated by determination of LDH activity 24 h after administration of corticosterone and drug-containing serum, using colorimetric assay (Solarbio, china). Absorbance was determined at a wavelength of 450 nm to measure LDH activity and determine the number of damaged cells following the manufacturer's instructions. Data were normalized based on LDH activity of the control culture media (100%).

Immunofluorescence

Cells were loaded in 12 well-plate, washed 3 times and neurons fixed with 4% paraformaldehyde (Meilun, China) for 15 min at room temperature. Samples were then washed 3 times, blocked and permeabilized with 5% normal goat serum in PBS (PBS containing 0.2% BSA (Roche, America) and 0.2% Triton X-100 (Solarbio, China) for 30 min (PBS/BSA/Triton). Primary antibodies against LC3 (CST, USA) were diluted to 1:200 in PBS/BSA/Triton and added to samples and were incubated overnight at 4°C. Samples were then washed 3 times for 10 min and incubated with Alexa-Fluor 488-labeled secondary antibodies (CST, USA) diluted to 1:500 in PBS/BSA/Triton for 1 h in the dark. After washing the samples 3 times for 10 min, nuclei were counterstained with DAPI stain diluted to 1:1,000 PBS (Beyotime, China) for 5 min. Coverslips were washed 3 times for 10 min and mounted on glass slides with Polyvinylpyrrolidone (Beyotime, China). Immunofluorescence was determined using laser scanning confocal microscope (ZEISS, Germany).

Western Blot

After removal of culture medium, neurons were washed three times with PBS, and immediately lysed using 1 ml of RIPA (Sigma, America) containing protease and phosphatase inhibitor cocktails (Roche, America) to obtain total proteins. BCA kit (Beyotime, China) was used to quantify proteins in the samples. For immunoblotting, equal amounts of proteins were separated by 8–16% SurePageTM Gels (GenScript, USA), transferred to polyvinylidene difluoride membrane (Roche, America) and then blocked for 1 h using 5% BSA diluted in TBS-T. Membranes containing proteins were incubated with LC3, Beclin1, p62, ULK1, PI3K, p-PI3K, Akt, p-Akt, mTOR, p-mTOR, p70S6, p-p70S6, 4ebp1, p-4ebp1, β-actin (1:2,000; all from CST, America) primary antibodies overnight. Membranes were then incubated with the corresponding secondary HRP-coupled anti-rabbit antibody (1:5,000; CST, America) for 2 h. ECL detection system (Tanon, China) was used to visualize bands. For quantitative analysis, the bands were analyzed using ImageJ software.

qRT-PCR

Trizol (Invitrogen, America) was used to extract total cellular RNA following the manufacturer's instructions. Quality and quantity of RNA were determined using Nanodrop One (ThermoFisher, America) at 260 nm and 260/280 nm, respectively. One μg of total RNA was reverse transcribed to obtain cDNA using RevertAid First Strand cDNA Synthesis Kit (Invitrogen, USA) in the T100TM Thermal Cycler (Bio-rad, America). Quantitative gene expression was performed following the FastStart Universal SYBR@ Green Master (Invitrogen, America) protocol using a CFX96TM Real-Time PCR System (Bio-rad, America). Primer sequences used (Takara, Japan) for qRT-PCR are presented in **Table 1**. qRT-PCR data was analyzed using the $2^{-\Delta\Delta Ct}$ method with threshold cycle values.

TABLE 1 | Sequences of qRT-PCR primers used.

Gene	Primer (5′-3′)	
β-actin	Forward:GGAGATTACTGCCCTGGCTCCTA	Reverse:GACTCATCGTACTCCTGCTTGCTG
mTOR	Forward:GCTTATCAAGCAAGCGACATCTCA	Reverse:TCCACTGGAAGCACAGACCAAG
LC3	Forward:AGCTCTGAAGGCAACAGCAACA	Reverse:GCTCCATGCAGGTAGCAGGAA
p62	Forward:AAGCTGCCCTGTACCCACATC	Reverse:ACCCATGGACAGCATCTGAGAG
P70S6	Forward:AGGATGCAGGCTCTGAGGA	Reverse:ACCAAGTACCCGAAGTAGCTCAA
Beclin1	Forward:GAAACTGGACACGAGCTTCAAGA	Reverse:ACCATCCTGGCGAGTTTCAATA
4ebp1	Forward:TCACTAGCCCTACCAGCGATGAG	Reverse:CCAGAAGCATCACTGCGTCCTAT
ULK1	Forward:CCACTGCGTGGCTCACCTAA	Reverse:TAGCCAACAGGGTCAGCAAACTC
AKT	Forwar:ATGGACTTCCGGTCAGGTTC	Reverse:GCCCTTGCCCAGTAGCTTCA

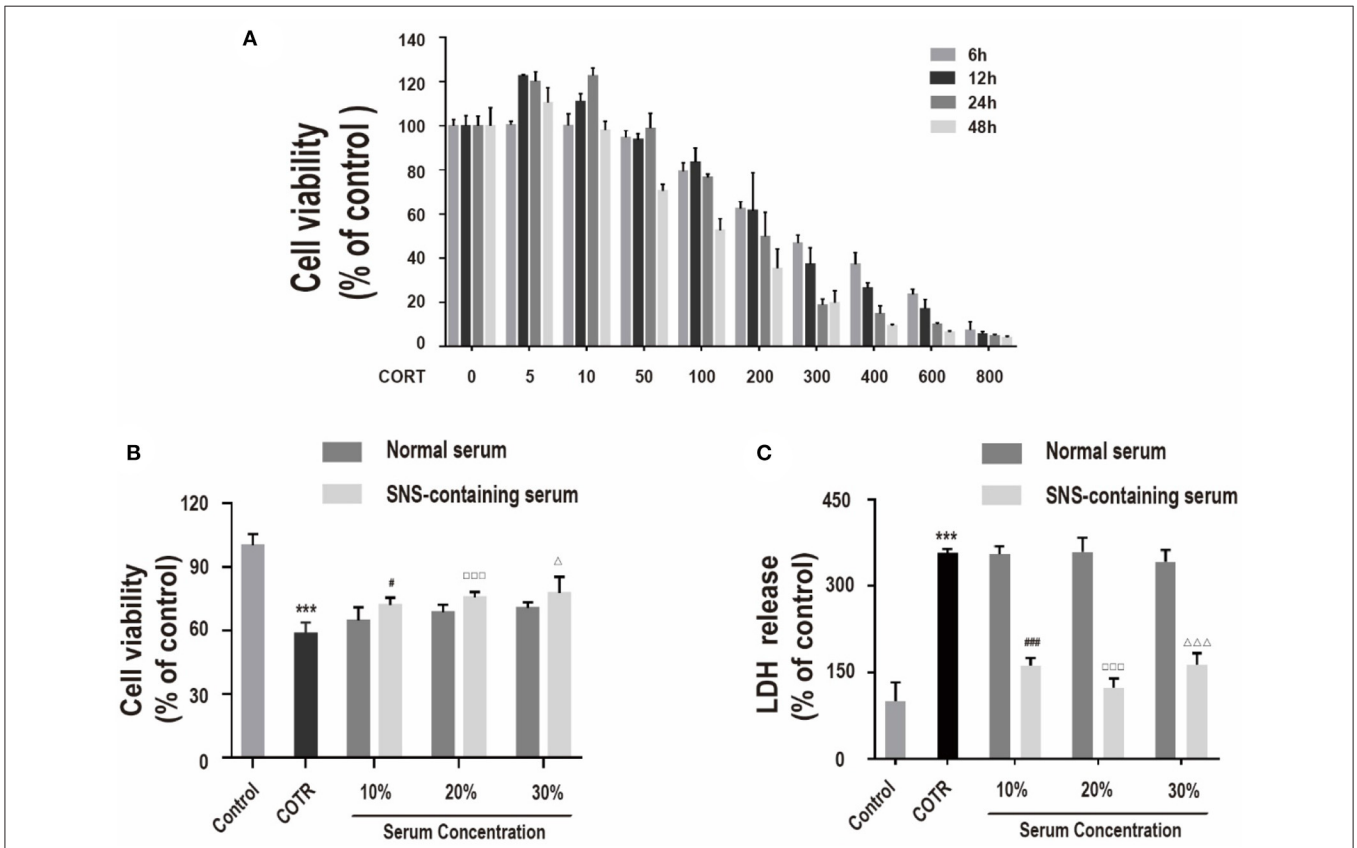

FIGURE 1 | (A) Viability of cells incubated with different concentrations of corticosterone (0, 5, 10, 50, 100, 200, 300, 400, 600, 800 μM) for different intervals (6, 12, 24, 48 h), determined by CCK-8 assay. ($n = 4$). **(B)** Viability of cells co-incubated with 200 μM corticosterone and 10%, 20%, or 30% SNS-containing serum for 24 h. Each column represents mean ± SD ($n = 6$). **(C)** LDH assay. ($n = 3$) $^{***}P < 0.01$, vs. control group; $^{\#}P < 0.05$, $^{\#\#\#}P < 0.01$ vs. 10% normal serum group; $^{\square\square\square}P < 0.01$ vs. 20% normal serum group; $^{\triangle}P < 0.05$, $^{\triangle\triangle\triangle}P < 0.01$ vs. 30% normal serum group.

Statistical Analysis

Experimental data were presented as mean ± S.E.M. of at least three independent experiments. Statistical analysis was performed using SPSS 23.0 software (SPSS, Chicago, IL, USA). Multiple group comparisons were performed using one-way analysis of variance (ANOVA) followed by Dunnett's test for inter-group differences. $P < 0.05$ was considered statistically significant.

RESULTS

SNS Protects Primary Hippocampal Neurons Against Corticosterone Induced Neurotoxicity

Effects of different concentrations of corticosterone (0, 5, 10, 50, 100, 200, 300, 400, 600, and 800 μM) were determined at different intervals (6, 12, 24, and 48 h) using CCK-8 assay to determine the optimal concentration and treatment time

of corticosterone *in vitro* injury model. Treatment of primary hippocampal neurons with corticosterone at a low dose (0–50 μM) did not significantly decease cell viability compared with the control group (**Figure 1A**). Cell death was induced by high corticosterone doses (100–800 μM) in a concentration- and time-dependent manner. Reduction of cell viability to ∼50% was achieved through treatment with 200 μM corticosterone for 24 h. Therefore, 200 μM corticosterone was chosen as the optimal concentration for subsequent experiments.

CCK-8 assay and LDH assay were used to measure cell viability and LDH release to determine the effect of SNS on corticosterone-induced damage in neurons. Primary hippocampal neurons were incubated with 10, 20, and 30% SNS-containing serum and 200 μM corticosterone for 24 h. Administration with 10, 20, and 30% SNS-containing serum significantly reversed corticosterone-induced decrease incell viability and increase in LDH release (**Figures 1B,C**). Notably, 20% SNS-containing serum had the highest neuroprotective effect, therefore it was used in subsequent experiments.

Corticosterone Increases Autophagy and Inactivates the PI3K/Akt/mTOR Pathway in Primary Hippocampal Neurons

To explore the role of autophagy on corticosterone-induced damage in primary hippocampal neurons, expression levels of specific intracellular autophagy-related proteins such as LC3-II/LC3-I, Beclin1, p62, ULK1, were determined. Prior to protein level analysis, different corticosterone concentrations (10, 50, 100, 200, and 400 μM) were administered for 24 h. Corticosterone administration significantly increased LC3-II/LC3-I ratio Beclin1 and ULK1 protein expression levels, and decreased p62 protein expression level in a concentration-dependent manner (**Figures 2A–E**). To explore the possible molecular mechanism of corticosterone in autophagy, PI3K, p-PI3K, Akt, p-Akt, mTOR, p-mTOR, p70S6, p-p70S6, 4ebp1 and p-4ebp1 protein expression levels were determined. Notably, corticosterone decreased p- PI3K/PI3K, p-Akt/Akt, p-mTOR/mTOR, p-p70S6/p70S6, and p-4ebp1/4ebp1 protein expression levels in a concentration- dependent manner (**Figures 2F–K**).

SNS-Containing Serum Inhibits Autophagy in Corticosterone-Treated Primary Hippocampal Neurons

To explore the effect of SNS on autophagy in corticosterone-induced injury, LC3-II/LC3-I protein ratio, Beclin1, p62 and ULK1 protein expression levels in hippocampal neurons treated with different concentrations of SNS-containing serum were determined. Administration of 10, 20, and 30% SNS-containing serum reduced LC3-II/LC3-I protein ratio, decreased Beclin1 and ULK1 protein expression levels, and increased protein expression level of p62 (**Figures 3A–E**). SNS-containing serum significantly decreased mRNA levels of LC3 and Beclin1, increased mRNA level of p62 (**Figures 3F–H**). In addition, SNS-containing serum down-regulated LC3 protein expression level (**Figure 3I**).

Further, the relationship between protective effect of SNS on corticosterone-treated neurons and downregulation of autophagy-related proteins was determined. Corticosterone-induced neurons exposed to SNS-containing serum were treated with 3-methyladenine (3-MA), an autophagy inhibitor. Analysis showed significant decrease in LC3-II/LC3-I protein ratio, and decrease in Beclin1, and ULK1 protein expression levels, increase in p62 protein expression level in the 3-MA-treated group compared with the levels in the group treated with 20% normal serum (**Figures 4A–E**). Furthermore, administration of 3-MA had no significant effect on neuronal viability, whereas a combination of 3-MA and 20% SNS-containing serum increased neuronal viability (**Figure 4F**).

SNS-Containing Serum Activates PI3K/Akt/mTOR Pathway in Corticosterone-Induced Injury of Primary Hippocampal Neurons

PI3K/Akt/mTOR pathway plays an essential role in neuroprotection (17). Therefore, we hypothesized that activation of the PI3K/Akt/mTOR pathway may be involved in exerting neuroprotective effects of SNS against corticosterone. Protein expression levels of PI3K, p-Akt, p-mTOR, p-p70S6, and p-4ebp1 in corticosterone-induced primary hippocampal neurons treated with SNS-containing serum were determined. Analysis showed that SNS-containing serum significantly increased PI3K, p-AKT, p-mTOR, p-p70S6, and p-4ebp1 protein and mRNA expression levels (**Figures 5A–J**).

Notably, the mTOR inhibitor, rapamycin and siRNA-mTOR significantly decreased p-mTOR, p-p70S6, and p-4ebp1 protein levels (**Figures 6A–H**), implying that the PI3K/Akt/mTOR pathway was induced by SNS-containing serum. Further, to explore the role of PI3K/Akt/mTOR pathway on SNS-mediated neuroprotection of corticosterone-induced neurons, cells were treated with rapamycin. Administration of 20% SNS-containing serum increased neuronal cell viability, whereas neuronal viability decreased in rapamycin +20% SNS-containing serum group compared with 20% SNS-containing serum group (**Figure 6I**), (–).

DISCUSSION

In this study, pharmacological and genetic tools were used to explore the mechanisms of corticosterone-induced injury and the protective effects of SNS in primary hippocampal neurons. The findings of this study show that SNS protects neurons against corticosterone-induced injury by inhibiting autophagy through induction of PI3K/AKT/mTOR pathway. These findings provides a basis for development of a therapeutic agent for prevention or treatment of corticosterone-induced neuronal injury.

Exogenous administration of high doses of corticosterone causes depression and neuronal damage in rodents, thus affecting cognitive functions. Corticosterone is used to establish models of *in vitro* and *in vivo* depression (18, 19). In this study, 100–800 μM corticosterone caused neuronal damage *in vitro*, which is consistent with findings from previous studies that

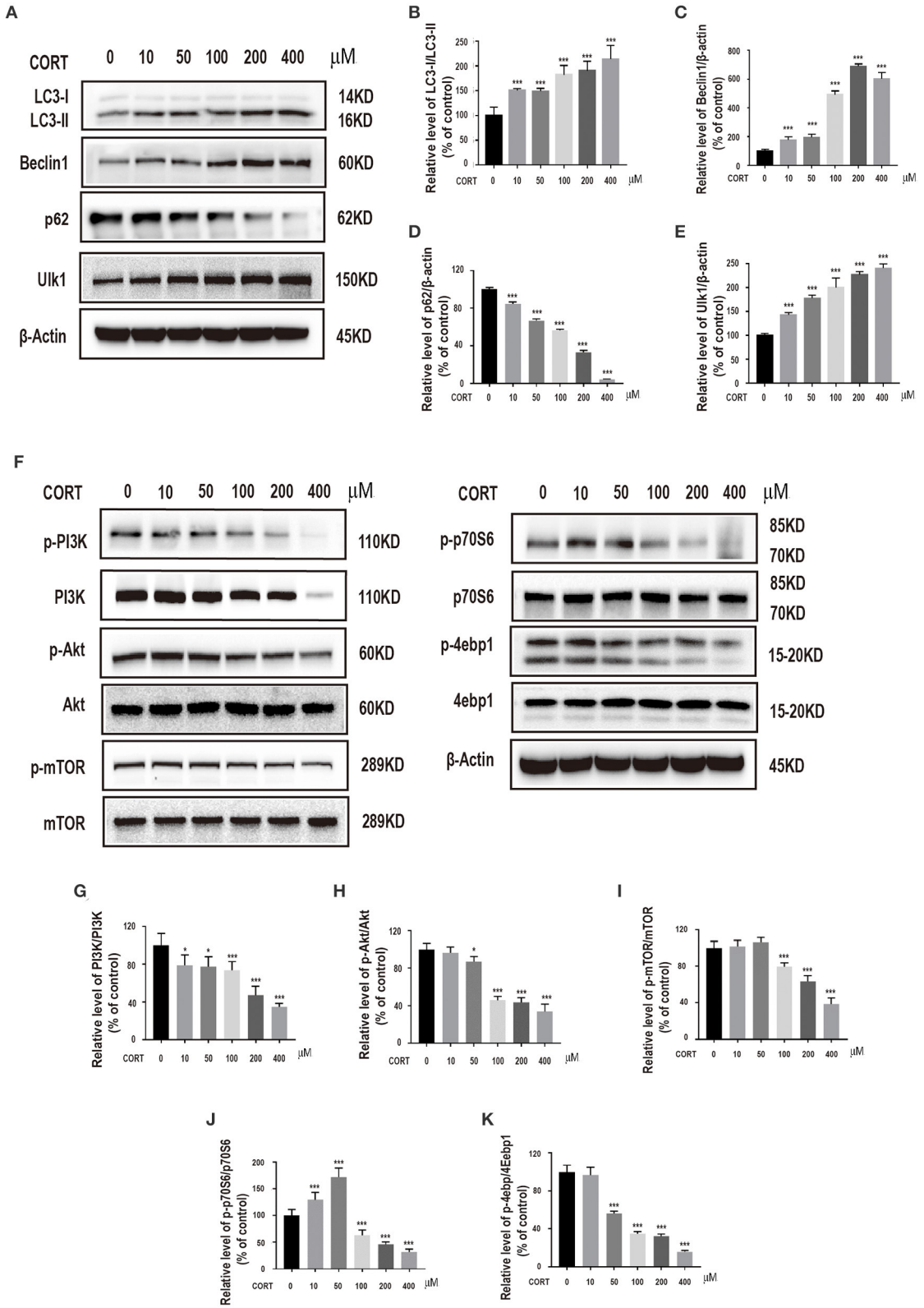

FIGURE 2 | Incubation of cells with different concentrations of corticosterone (10, 50, 100, 200, 400 μM) for 24 h. **(A–E)** Western blot and quantitative analyses of LC3-II/LC3-I protein ratio, and Beclin1, p62 and ULK1 protein expression levels. **(F–K)** Western blot and quantitative analyses of PI3K, p-PI3K, Akt, p-Akt, mTOR, p-mTOR, p70S6, p-p70S6, 4ebp1 and p-4ebp1 protein expression. Each column represents mean ± SD ($n = 3$). *$P < 0.05$, ***$P < 0.01$ vs. control group.

exposure to high doses of corticosterone is required to cause significant neurotoxicity in primary hippocampal neurons (20). On the contrary, Liu et al. reported that 1 μM corticosterone is sufficient to cause effects in neuronal survival (21). This difference in susceptibility might be because, unlike hippocampal neurons, astrocytes are more prone to injury resulting from corticosterone-induced apoptosis, have lower levels of reactive oxygen species and are more resistant to cytotoxic effects of corticosterone (22).

TCM is used for treatment of depression in China for centuries and is currently widely used in Western countries. In addition, drug-containing serum allows study of therapeutic effects of TCM. Normal serum subgroup and SNS-containing serum subgroup were included for comparison to rule out the effect of serum on experimental results (23). SNS is a traditional Chinese herbal formula used for treatment of depression. Li Y et al. reported that SNS is more effective in reducing depression-associated symptoms compared with use of fluoxetine (24). The main active chemical components in SNS include paeoniflorin and saikosaponin. Pretreatment with paeoniflorin prevents cell death in glutamate-induced PC12 cells (25). In addition, saikosaponin alleviates corticosterone-induced neurotoxicity in

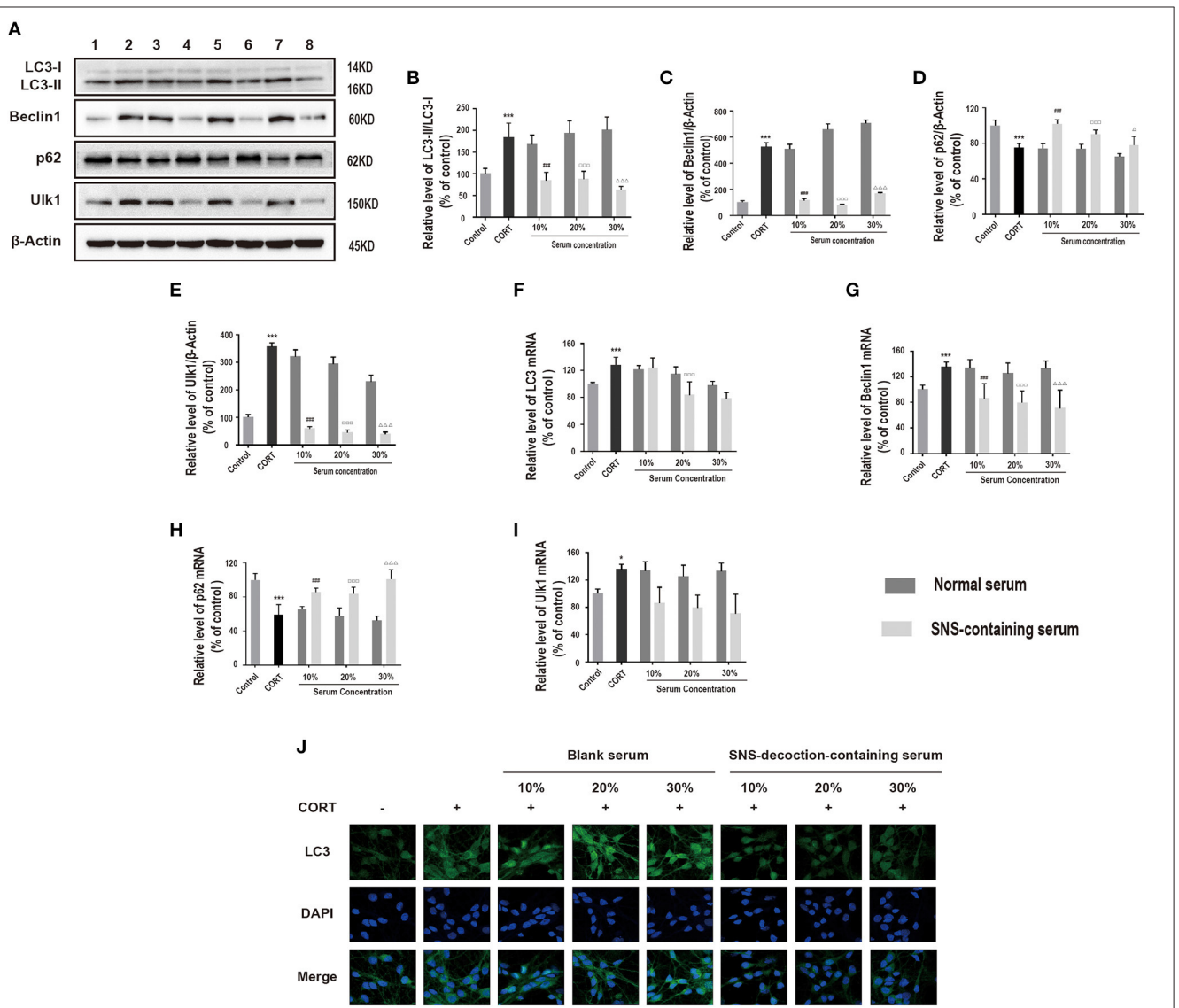

FIGURE 3 | Cells were incubated with 200 μM corticosterone and SNS-containing serum for 24 h. **(A–E)** Western blot and quantitative analyses of LC3-II/LC3-I protein ratio, and Beclin1, p62 and ULK1 protein expression levels. ($n = 3$). **(F–I)** qRT-PCR analysis of LC3, Beclin1, p62 and ULK1 expression. ($n = 3$). **(J)** Immunofluorescence analysis of LC3 expression. $^{*}P < 0.05$, $^{***}P < 0.01$ vs. control group; $^{\#\#\#}P < 0.01$ vs. 10% normal serum group; $^{\triangle}P < 0.05$, $^{\triangle\triangle\triangle}P < 0.01$ vs. 20% normal serum group; $^{\triangle\triangle\triangle}P < 0.01$ vs. 30% normal serum group, ($n = 3$). 1:control group; 2: corticosterone group; 3: 10% normal serum + corticosterone group; 4: 10% SNS-containing serum + corticosterone group; 5: 20% normal serum + corticosterone group; 6: 20% SNS-containing serum + corticosterone group; 7: 30% normal serum + corticosterone group; 8:30% SNS-containing serum + corticosterone group.

PC12 cells in a dose dependent manner. Notably, 10, 20, and 30% SNS-containing serum increased survival rate and inhibited LDH release in corticosterone-induced neurons. This finding shows that SNS protects primary hippocampal neurons against neurotoxicity induced by corticosterone. Therefore, SNS exerts antidepressant-like effects *in vitro*.

Previous studies reported that depression is associated with autophagy. Several autophagy related genes (Atgs) play key roles in the process of autophagy. Beclin1 is implicated in autophagosome formation, and studies reported that the level of autophagy is up-regulated by over-expression of Beclin1. LC3-I is lipidated to form LC3-II on autophagosomal membranes. LC3-II plays a key role in formation of autophagosomal membranes, therefore, LC3-II /LC3-I is an effective indicator of the level of autophagy activity. p62 is an autophagic adapter that mediates selective recognition and degradation of specific autophagy substrates, and is widely used as a marker to monitor autophagic flux (26–28). In addition, ULK1-FIP200-Atg13 complex formation is a characteristic of the initial stage of autophagy. Previous studies reported that paeoniflorin effectively protects PC12 cells from autophagic pathways (29), and saikosaponin D and A prevent autophagy after EV-A71 infection (30). These findings indicate that the protective function of SNS in depression may be correlated with autophagy.

In this study, administration of corticosterone reduced LC3-II/LC3-I protein ratio, decreased the levels of Beclin1 and ULK1 protein, increased p62 protein expression level in a dose-dependent manner. This finding implies that corticosterone treatment induces autophagy in primary hippocampal neurons. Similar findings were reported previously that corticosterone exposure causes a dose-dependent increase in LC3-II expression in PC12 cells (31). However, a recent study reported a dose-dependent decrease in autophagy in prefrontal cortex and hippocampus of CUMS mice (32). The differences in results can be attributed to differences in cell types and stimuli used. SNS-containing serum, 3-MA, and a combination of SNS-containing serum and 3-MA significantly decreased LC3-II/LC3-I protein ratio, decreased Beclin1, and ULK1 and increased p62 protein expression levels. Notably, treatment with SNS serum-3-MA combination showed the highest effects. These findings indicate that SNS inhibits excessive autophagy in corticosterone-injured neurons.

PI3K/AKT/mTOR pathway regulates protein synthesis, cell cycle, and cell metabolism by phosphorylating downstream proteins thus modulating cell growth, proliferation, apoptosis, and autophagy (33). mRNA expression of AKT1 and mTOR are downregulated in bipolar depression, and may induce autophagy (34). Reduced mTOR signaling is reported in major depressive disorder, compared with healthy controls (35). mTOR pathway is a major modulator of autophagy. PI3K and AKT are upstream targets of mTOR, which are activated by receptors of neurotrophins and growth factors. Activation of mTOR induces activation of two major downstream substrates, p70S6 and p-4ebp1, resulting in induction of

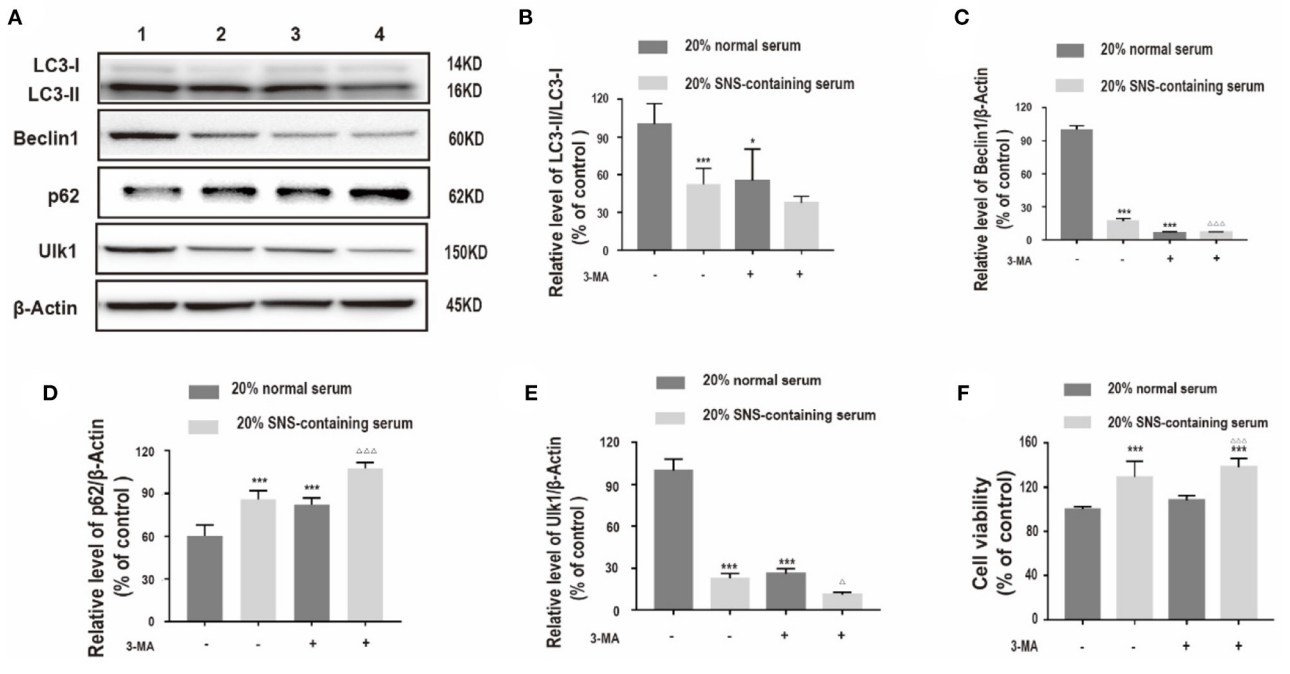

FIGURE 4 | (A–E) Western blot and quantitative analyses of LC3II/LC3I ratio, and Beclin1, p62 and ULK1 protein expression levels. 1: 20% normal serum + corticosterone group; 2: 20% SNS-containing serum + corticosterone group; 3: 20% normal serum + corticosterone + 3-MA group; 4: 20% SNS-containing serum + 3-MA + corticosterone group. ($n = 3$). Each column represents mean ± SD. $^{*}P < 0.05$, $^{***}P < 0.01$ vs. 20% normal serum + corticosterone group; $^{\Delta}P < 0.05$, $^{\Delta\Delta\Delta}P < 0.01$ vs. 20% SNS-containing serum group. **(F)** cells were incubated with 200 μM corticosterone, 1mM 3-MA, and 20% serum for 24 h. CCK-8 assay was used to determine cell viability. $^{***}P < 0.01$ vs. 20% normal serum group, $^{\Delta\Delta\Delta}P < 0.01$ vs. 20% normal serum + 3-MA group ($n = 4$).

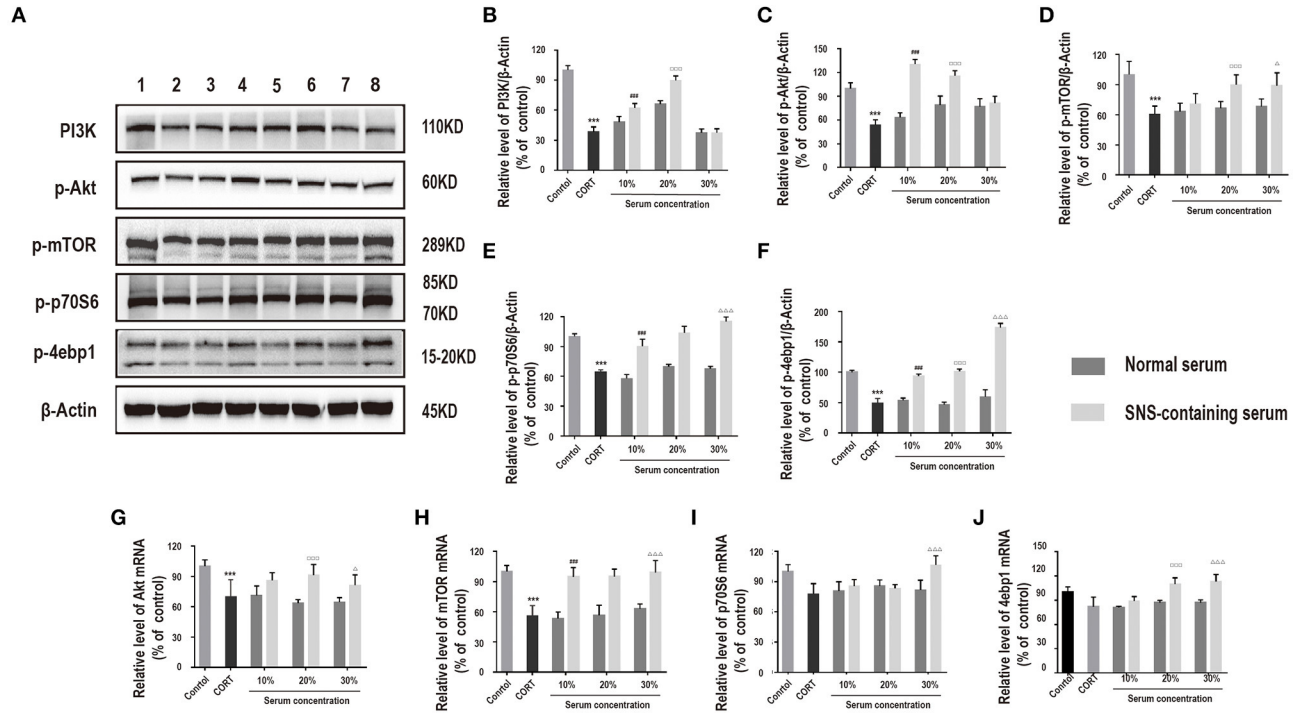

FIGURE 5 | Cells were incubated with 200 μM corticosterone and SNS-containing serum for 24 h. **(A–F)** Western blot and quantitative analyses of PI3K, p-Akt, p-mTOR, p-p70S6, and p-4ebp1 protein, ($n = 3$). **(G–J)** qRT-PCR analysis of Akt, mTOR, p70S6, and 4ebp1 expression levels. $^{***}P < 0.01$ vs. control group; $^{\#\#\#}P < 0.01$ vs. 10% normal serum group; $^{\square\square\square}P < 0.01$ vs. 20% normal serum group; $^{\Delta}P < 0.05$, $^{\Delta\Delta\Delta}P < 0.01$ vs. 30% normal serum group ($n = 3$).

protein translation (36, 37). Cui L et al. reported that saikosaponin thus inhibits autophagy in pancreatic fibrosis through regulation of PI3K/Akt/mTOR pathway (38). Therefore, we hypothesized that PI3K/Akt/mTOR pathway is a major target of SNS in prevention of excessive autophagy in corticosterone-induced neurons.

In this study, 100–400 μM corticosterone decreased expression of PI3K, p-Akt, p-mTOR, p-p70S6, and p-4ebp1, indicating that PI3K/Akt/mTOR pathway is activated by treatment with corticosterone. The findings of this study show that corticosterone activates neuronal autophagy and inhibits PI3K/AKT/mTOR pathway. Treatment with SNS-containing serum increased PI3K, p-AKT, p-mTOR, p-p70S6, and p-4ebp1 protein and mRNA expression levels in corticosterone-induced neurons. Notably, rapamycin and siRNA-mTOR significantly reduced protein levels of these autophagy-related factors. This finding implies that SNS activates the PI3K/Akt/mTOR pathway. Further, Administration of 20% SNS-containing serum increased neuronal cell viability, whereas neuronal viability decreased in rapamycin +20% SNS-containing serum group compared with 20% SNS-containing serum group,indicating that SNS protects neurons against damage through modulation of the PI3K/Akt/mTOR pathway. 3-MA is a selective inhibitor of PI3K, however, it had no significant effects on increase in neuronal viability, whereas a combination of 3-MA and 20% SNS-containing serum increased neuronal viability. This observation may be because other pathways, such as AMPK/mTOR signaling pathway in addition to PI3K/Akt/mTOR pathway may be involved in autophagy activated by corticosterone (39) which are then modulated by SNS.

Autophagy is a complex process and SNS comprises various active components, therefore, further studies should explore the effects and mechanisms of SNS-containing serum. In addition, qualitative and quantitative profiling of SNS should be performed using high performance liquid chromatography (HPLC). Furthermore, a limitation of this study is that anti-depression mechanism of SNS was only explored through *in vitro* experiment. However, a previous study by our group using CUMS model reported that SNS activates PI3K/ Akt /mTOR pathway and reduces autophagy in hippocampus of rats which is consistent with the findings of the current study (40).

CONCLUSIONS

In summary, the findings of this study show that 200 μM corticosterone decreases survival rate, activates autophagy, and inhibits PI3K/Akt/mTOR pathway in corticosterone-induced neuronal injury. SNS-containing serum inhibits autophagy through the PI3K/Akt/mTOR pathway. These findings provide information on the mechanism of SNS activity on depression, and provide a theoretical basis for future application of SNS in clinical treatment of depression. Further studies should be

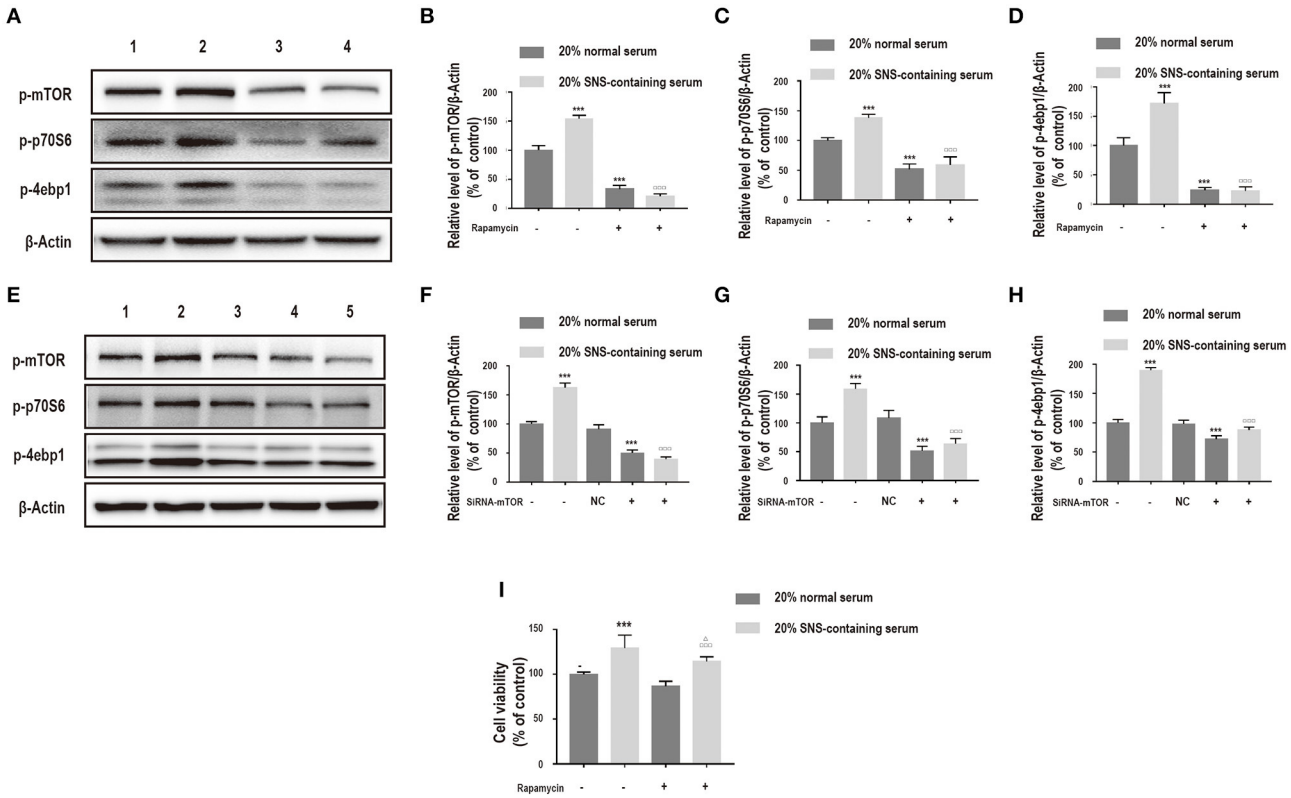

FIGURE 6 | (A–D) Cells were incubated with 200 μM corticosterone, 1 μM rapamycin, and 20% serum for 24 h. Western blot and quantitative analyses of p-mTOR, p-p70S6, and p-4ebp1 expression. 1: 20% normal serum + corticosterone group; 2: 20% SNS-containing serum + corticosterone group; 3: 20% normal serum + corticosterone + rapamycin group; 4: 20% SNS-containing + corticosterone + rapamycin group. $^{***}P < 0.01$ vs. 20% normal serum + corticosterone group; $^{□□□}P < 0.01$ vs. 20% SNS-containing serum group ($n = 3$). **(E–H)** siRNA-mTOR transfected cells were incubated with 200 μM corticosterone and 20% SNS-containing serum for 24 h. Western blot and quantitative analyses of p-mTOR, p-p70S6, and p-4ebp1 protein expression levels. 1: 20% normal serum + corticosterone group; 2: 20% SNS-containing serum + corticosterone group; 3: 20% normal serum + corticosterone + siRNA-NC group; 4: 20% normal serum + corticosterone + siRNA-mTOR group; 5: 20% + SNS-containing serum + siRNA-mTOR group ($n = 3$). **(I)** CCK-8 assay for determination of cell viability. $^{***}P < 0.01$ vs. 20% normal serum group + corticosterone group; $^{□□□}P < 0.01$ vs. 20% normal serum+ corticosterone +rapamycin;$^{\Delta}P < 0.05$ vs. 20% SNS-containing serum group+ corticosterone group ($n = 4$).

conducted to optimize the clinical use of SNS in management of neurodegenerative complications.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The animal study was reviewed and approved by the Animal Care and Committee of Guangzhou University of Chinese Medicine.

AUTHOR CONTRIBUTIONS

MZ performed the experiments and wrote the manuscript. HA and SH conceptualized and designed the experiments. YZ organized generation, collection, assembly, and interpretation of data. HN supplemented experimental data. HS, JS, XY, WC, WZ, XZ, and CH performed the experiments. All authors contributed to the article and approved the submitted version.

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REFERENCES

1. Hankin BL. Adolescent depression: description, causes, and interventions. *Epilepsy Behav.* (2006) 8:102–14. doi: 10.1016/j.yebeh.2005.10.012

2. Perahia DG, Quail D, Desaiah D, Montejo AL, Schatzberg AF. Switching to duloxetine in selective serotonin reuptake inhibitor non- and partial-responders: effects on painful physical symptoms of depression. *J Psychiatr Res.* (2009) 43:512–8. doi: 10.1016/j.jpsychires.2008.07.001

3. Wang J. Work stress as a risk factor for major depressive episode(s). *Psychol Med.* (2005) 35:865–71. doi: 10.1017/S0033291704003241
4. Risch N, Herrell R, Lehner T, Liang KY, Eaves L, Hoh J, et al. Interaction between the serotonin transporter gene (5-HTTLPR), stressful life events, and risk of depression: a meta-analysis. *JAMA.* (2009) 301:2462–71. doi: 10.1001/jama.2009.878
5. Saveanu RV, Nemeroff CB. Etiology of depression: genetic and environmental factors. *Psychiatr Clin North Am.* (2012) 35:51–71. doi: 10.1016/j.psc.2011.12.001
6. Bostwick JM. A generalist's guide to treating patients with depression with an emphasis on using side effects to tailor antidepressant therapy. *Mayo Clin Proc.* (2010) 85:538–50. doi: 10.4065/mcp.2009.0565
7. Murrough JW. Ketamine as a novel antidepressant: from synapse to behavior. *Clin Pharmacol Ther.* (2012) 91:303–9. doi: 10.1038/clpt.2011.244
8. Zhou J, Cai H, Tu S, Duan Y, Pei K, Xu Y, et al. Identification and analysis of compound profiles of sinisan based on 'individual herb, herb-pair, herbal formula' before and after processing using UHPLC-Q-TOF/MS coupled with multiple statistical strategy. *Molecules.* (2018) 23:3128. doi: 10.3390/molecules23123128
9. Feng DD, Tang T, Lin XP, Yang ZY, Yang S, Xia ZA, et al. Nine traditional Chinese herbal formulas for the treatment of depression: an ethnopharmacology, phytochemistry, and pharmacology review. *Neuropsychiatr Dis Treat.* (2016) 12:2387–402. doi: 10.2147/NDT.S114560
10. Shen X, Zhao Z, Luo X, Wang H, Hu B, Guo Z. Systems pharmacology based study of the molecular mechanism of SiNiSan formula for application in nervous and mental diseases. *Evid Based Complement Alternat Med.* (2016) 2016:9146378. doi: 10.1155/2016/9146378
11. Shen C, Cao K, Cui S, Cui Y, Mo H, Wen W, et al. SiNiSan ameliorates depression-like behavior in rats by enhancing synaptic plasticity via the CaSR-PKC-ERK signaling pathway. *Biomed Pharmacother.* (2020) 124:109787. doi: 10.1016/j.biopha.2019.109787
12. Zhang H, Shang Y, Xiao X, Yu M, Zhang T. Prenatal stress-induced impairments of cognitive flexibility and bidirectional synaptic plasticity are possibly associated with autophagy in adolescent male-offspring. *Exp Neurol.* (2017) 298:68–78. doi: 10.1016/j.expneurol.2017.09.001
13. Shih JH, Chiu CH, Ma KH, Huang YS, Shiue CY, Yeh TY, et al. Autophagy inhibition plays a protective role against 3, 4-methylenedioxymethamphetamine (MDMA)-induced loss of serotonin transporters and depressive-like behaviors in rats. *Pharmacol Res.* (2019) 142:283–93. doi: 10.1016/j.phrs.2019.02.026
14. Hou L, Klann E. Activation of the phosphoinositide 3-kinase-Akt-mammalian target of rapamycin signaling pathway is required for metabotropic glutamate receptor-dependent long-term depression. *J Neurosci.* (2004) 24:6352–61. doi: 10.1523/JNEUROSCI.0995-04.2004
15. Chandran A, Iyo AH, Jernigan CS, Legutko B, Austin MC, Karolewicz B. Reduced phosphorylation of the mTOR signaling pathway components in the amygdala of rats exposed to chronic stress. *Prog Neuropsychopharmacol Biol Psychiatry.* (2013) 40:240–5. doi: 10.1016/j.pnpbp.2012.08.001
16. Tao W, Dong Y, Su Q, Wang H, Chen Y, Xue W, et al. Liquiritigenin reverses depression-like behavior in unpredictable chronic mild stress-induced mice by regulating PI3K/Akt/mTOR mediated BDNF/TrkB pathway. *Behav Brain Res.* (2016) 308:177–86. doi: 10.1016/j.bbr.2016.04.039
17. Walker CL, Wu X, Liu NK, Xu XM. Bisperoxovanadium mediates neuronal protection through inhibition of PTEN and activation of PI3K/AKT-mTOR signaling after traumatic spinal injuries. *J Neurotrauma.* (2019) 36:2676–87. doi: 10.1089/neu.2018.6294
18. Sawamoto A, Okuyama S, Yamamoto K, Amakura Y, Yoshimura M, Nakajima M, et al. 3,5,6,7,8,3',4'-Heptamethoxyflavone, a citrus flavonoid, ameliorates corticosterone-induced depression-like behavior and restores brain-derived neurotrophic factor expression, neurogenesis, and neuroplasticity in the hippocampus. *Molecules.* (2016) 21:541. doi: 10.3390/molecules21040541
19. Lew SY, Lim SH, Lim LW, Wong KH. Neuroprotective effects of Hericium erinaceus (Bull: Fr) Pers. against high-dose corticosterone-induced oxidative stress in PC-12 cells. *BMC Complement Med Ther.* (2020) 20:340. doi: 10.1186/s12906-020-03132-x
20. Zhao X, Li R, Jin H, Jin H, Wang Y, Zhang W, et al. Epigallocatechin-3-gallate confers protection against corticosterone-induced neuron injuries via restoring extracellular signal-regulated kinase 1/2 and phosphatidylinositol-3 kinase/protein kinase B signaling pathways. *PLoS ONE.* (2018) 13:e0192083. doi: 10.1371/journal.pone.0192083
21. Liu B, Zhang H, Xu C, Yang G, Tao J, Huang J, et al. Neuroprotective effects of icariin on corticosterone-induced apoptosis in primary cultured rat hippocampal neurons. *Brain Res.* (2011) 1375:59–67. doi: 10.1016/j.brainres.2010.12.053
22. Yu S, Yang S, Holsboer F, Sousa N, Almeida OF. Glucocorticoid regulation of astrocytic fate and function. *PLoS ONE.* (2011) 6:e22419. doi: 10.1371/journal.pone.0022419
23. Li Z, Wang H, Wang Q, Sun J. Buyang huanwu decoction vigorously rescues PC12 cells against 6-OHDA-induced neurotoxicity via Akt/GSK3beta pathway based on serum pharmacology methodology. *Rejuvenation Res.* (2016) 19:467–77. doi: 10.1089/rej.2015.1798
24. Li Y, Sun Y, Ma X, Xue X, Zhang W, Wu Z, et al. Effects of Sini San used alone and in combination with fluoxetine on central and peripheral 5-HT levels in a rat model of depression. *J Tradit Chin Med.* (2013) 33:674–81. doi: 10.1016/S0254-6272(14)60041-8
25. Zhang Y, Qiao L, Xu W, Wang X, Li H, Xu W, et al. Paeoniflorin attenuates cerebral ischemia-induced injury by regulating Ca(2+)/CaMKII/CREB signaling pathway. *Molecules.* (2017) 22:359. doi: 10.3390/molecules22030359
26. Pankiv S, Clausen TH, Lamark T, Brech A, Bruun JA, Outzen H, et al. p62/SQSTM1 binds directly to Atg8/LC3 to facilitate degradation of ubiquitinated protein aggregates by autophagy. *J Biol Chem.* (2007) 282:24131–45. doi: 10.1074/jbc.M702824200
27. Clausen TH, Lamark T, Isakson P, Finley K, Larsen KB, Brech A, et al. p62/SQSTM1 and ALFY interact to facilitate the formation of p62 bodies/ALIS and their degradation by autophagy. *Autophagy.* (2010) 6:330–44. doi: 10.4161/auto.6.3.11226
28. Mizushima N, Yoshimori T, Levine B. Methods in mammalian autophagy research. *Cell.* (2010). 140:313–26. doi: 10.1016/j.cell.2010.01.028
29. Cao BY, Yang YP, Luo WF, Mao CJ, Han R, Sun X, et al. Paeoniflorin, a potent natural compound, protects PC12 cells from MPP+ and acidic damage via autophagic pathway. *J Ethnopharmacol.* (2010) 131:122–9. doi: 10.1016/j.jep.2010.06.009
30. Li C, Huang L, Sun W, Chen Y, He ML, Yue J, et al. Saikosaponin D suppresses enterovirus A71 infection by inhibiting autophagy. *Signal Transduct Target Ther.* (2019) 4:4. doi: 10.1038/s41392-019-0037-x
31. Ma RD, Zhou GJ, Qu M, Yi JH, Tang YL, Yang XY, et al. Corticosterone induces neurotoxicity in PC12 cells via disrupting autophagy flux mediated by AMPK/mTOR signaling. *CNS Neurosci Ther.* (2020) 26:167–76. doi: 10.1111/cns.13212
32. Geng J, Liu J, Yuan X, Liu W, Guo W. Andrographolide triggers autophagy-mediated inflammation inhibition and attenuates chronic unpredictable mild stress (CUMS)-induced depressive-like behavior in mice. *Toxicol Appl Pharmacol.* (2019) 379:114688. doi: 10.1016/j.taap.2019.114688
33. Shaw RJ, Cantley LC. Ras, PI(3)K and mTOR signalling controls tumour cell growth. *Nature.* (2006). 441:424–30. doi: 10.1038/nature04869
34. Machado-Vieira R, Zanetti MV, Teixeira AL, Uno M, Valiengo LL, Soeiro-de-Souza MG, et al. Decreased AKT1/mTOR pathway mRNA expression in short-term bipolar disorder. *Eur Neuropsychopharmacol.* (2015) 25:468–73. doi: 10.1016/j.euroneuro.2015.02.002

35. Jernigan CS, Goswami DB, Austin MC, Iyo AH, Chandran A, Stockmeier CA, et al. The mTOR signaling pathway in the prefrontal cortex is compromised in major depressive disorder. *Prog Neuro Psychoph.* (2011) 35:1774–9. doi: 10.1016/j.pnpbp.2011.05.010
36. Gingras AC, Raught B, Gygi SP, Niedzwiecka A, Miron M, Burley SK, et al. Hierarchical phosphorylation of the translation inhibitor 4E-BP1. *Genes Dev.* (2001) 15:2852–64. doi: 10.1101/gad.912401
37. Klann E, Dever TE. Biochemical mechanisms for translational regulation in synaptic plasticity. *Nat Rev Neurosci.* (2004) 5:931–42. doi: 10.1038/nrn1557
38. Cui LH, Li CX, Zhuo YZ, Yang L, Cui NQ, Zhang SK. Saikosaponin d ameliorates pancreatic fibrosis by inhibiting autophagy of pancreatic stellate cells via PI3K/Akt/mTOR pathway. *Chem Biol Interact.* (2019) 300:18–26. doi: 10.1016/j.cbi.2019.01.005
39. Xu B, Lang L, Li S, Yuan J, Wang J, Yang H, et al. Corticosterone excess-mediated mitochondrial damage induces hippocampal neuronal autophagy in mice following cold exposure. *Animals.* (2019) 9:682. doi: 10.3390/ani9090682
40. 倪慧 (2019). "调肝治法介导mTORCl细胞自噬信号通路发挥抗抑郁作用的机理研究," in.: 广州中医药大学).

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Unchanged Cognitive Performance and Concurrent Prefrontal Blood Oxygenation After Accelerated Intermittent Theta-Burst Stimulation in Depression: A Sham-Controlled Study

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Aim: Intermittent theta-burst stimulation (iTBS) delivered over the dorsomedial prefrontal cortex (DMPFC) has shown promise as a treatment for anhedonia and amotivation in patients with depression. Here, we investigated whether this protocol modulates cognitive performance and concurrent prefrontal blood oxygenation. We also examined whether depressed patients exhibit cognitive dysfunction and prefrontal hypoactivity at baseline compared to healthy controls.

Methods: This sham-controlled study comprises 52 patients randomized to either active or sham accelerated iTBS over the DMPFC (applied twice daily) for 10 consecutive treatment days, and 55 healthy controls. Cognitive performance was assessed at baseline and once again 4 weeks later using a cognitive test battery targeting attention, inhibitory control, and numerical, verbal, and visual working memory. Concurrent prefrontal oxygenated hemoglobin (oxy-Hb) was captured with functional near-infrared spectroscopy.

Results: Active iTBS over DMPFC did not affect cognitive performance or concurrent oxy-Hb change compared to sham iTBS in patients with depression. Compared to controls, patients at baseline showed impaired performance in the Trail Making Test, the Rey Auditory Verbal Learning Test, the Animal Naming Test, and the Digit Symbol Substitution Test, however no difference in prefrontal oxy-Hb was observed.

Conclusion: Patients with treatment-resistant depression displayed cognitive deficits, however without prefrontal hypoactivity, compared to healthy controls at baseline. iTBS treatment did not alter cognitive performance, nor concurrent prefrontal blood oxygenation, in patients. Taken together, iTBS can likely be considered a cognitively safe treatment option in this sample of patients.

Keywords: cognition, fNIRS, iTBS, repetitive transcranial magnetic stimulation, rTMS

INTRODUCTION

Depression, the mental health condition with the highest global disease burden (1), is characterized by persistent low mood, loss of interest, and low energy (2). Depression is also accompanied by impaired cognitive functions, in domains such as processing speed, working memory, and episodic memory (3). It is crucial to understand the neurobiological underpinnings of cognitive symptoms in depression, such as prefrontal hypoactivity (4), to develop appropriate treatment options.

For treatment-resistant depression, there are some treatment alternatives, with electroconvulsive therapy (ECT) being the most effective. ECT is a neuromodulatory therapy with global impact on the brain with high remission rate but also cognitive side effects (5). Repetitive transcranial magnetic stimulation (rTMS) over the dorsolateral prefrontal cortex (DLPFC) is an established add-on treatment alternative, with a more focal brain impact (6). The DLPFC is a node in the central executive network (7) and given that rTMS modulates local excitability over the DLPFC this in turn has effect on network signaling (8). There were early concerns that rTMS over the DLPFC might have cognitive side effects as well, however this could not be confirmed (9). While rTMS has been shown to potentially even cause cognitive enhancement in healthy subjects (10), a recent meta-analysis on depression and DLPFC-rTMS protocols found only modest task-specific cognitive improvement after treatment, and this only for the Trail Making Test (11). Using the shorter intermittent theta-burst stimulation (iTBS) (12) over the DLPFC in treatment-resistant depression, a sham-controlled study suggests cognitive improvement also in the Wisconsin Card Sorting Test (13).

While rTMS is effective in treatment-resistant depression, some of the symptoms, such as anhedonia and amotivation, have been less successful to treat with conventional rTMS protocols targeting the DLPFC (14). Anhedonia and amotivation are symptoms that cross over diagnostic boundaries and might have a common origin from an emotional and cognitive network that may be reached by instead targeting the dorsomedial prefrontal cortex (DMPFC) (15). Based on its suggested involvement in a depression network, the DMPFC has been proposed as an alternative rTMS target (16). An initial open-label study has indeed shown that the DMPFC is well-suited for rTMS treatment of depression (17). This target might be of interest also in the context of cognition, as DMPFC and DLPFC are involved in the same cognitive control network, with the DMPFC monitoring cognitive performance and the DLPFC adjusting behavior (18).

In a recent randomized clinical trial with a transdiagnostic approach, accelerated iTBS delivered twice daily over the DMPFC in patients with uni- or bipolar depression or schizophrenia conducted by our group (19), we found a treatment effect only in patients with depression and specifically for negative symptoms such as anhedonia and amotivation, but not for depressive symptoms overall. While we found that active and sham iTBS were similar in their reports of subjective memory deficits, fatigue, and headache, we do not yet know about the effects of this specific protocol on actual cognitive performance. The cognitive effects of iTBS over DMPFC in depression have been assessed in an open-label case series which suggests cognitive safety with task-specific improvement for the Trail Making Test and the Stroop Test (20), but no sham-controlled studies have been published yet.

Functional near-infrared spectroscopy (fNIRS) is a non-invasive technique that can be used to measure changes in cortical oxygenated hemoglobin levels (oxy-Hb) (21). Due to its relative insensitivity to body motion, fNIRS is well-suited to be applied during cognitive tests. This technique has indeed been used to capture changes in oxy-Hb during cognitive performance in patients with depression, such as prefrontal hypoactivity (22, 23). A sham-controlled trial of iTBS for panic disorder investigated changes in the prefrontal fNIRS signal during a verbal fluency task (24) and the Emotional Stroop Test (25), but to our knowledge there are yet no studies of depression in this regard.

In the present study, we explored whether accelerated iTBS delivered over the DMPFC in depression affected cognitive performance in a neurocognitive test battery, as well as the concurrent blood oxygenation response by applying prefrontal fNIRS during cognitive testing. The fNIRS signal was recorded from sites roughly corresponding to the left and right DLPFC, thus probing for indirect effects on the cognitive control network. We also examined whether patients at baseline exhibited cognitive dysfunction and prefrontal hypoactivity as measured with oxy-Hb response, during cognitive testing, compared to a group of healthy controls.

MATERIALS AND METHODS

Participants

This study comprised 52 patients from two randomized controlled trials with shared methodology at the Brain Stimulation Unit, Uppsala University Hospital, Sweden. Parts of this data has been reported elsewhere (19). The current patient sample included all 40 patients from our previous study (19) plus additional 12 patients with depression from an add-on study. Patients were recruited via the psychiatric clinic at Uppsala University Hospital, Sweden and were required to have a current depressive episode in either a uni- or bipolar disorder and unchanged psychotropic medication 1 month before treatment start. Patients' current medication was kept constant throughout the study and the study protocol did not allow benzodiazepine use. Data from the patient group were also compared with a cohort of 55 healthy controls recruited via advertisement over the internet. An overall inclusion criterion was age 18–59. Exclusion criterion for the control group was any ongoing, or history of, psychiatric disorder, and for all participants epilepsy, intracranial metallic implants, pacemaker or implantable cardioverter defibrillator, pregnancy, or an active substance use disorder. All participants underwent a Mini International Neuropsychiatric Interview (M.I.N.I.) (26). Comorbid clinical diagnoses of attention deficit hyperactivity disorder (ADHD) or attention deficit disorder (ADD) were collected from the medical records. Written informed consent was obtained from all participants. The study was approved by the Ethical Review Board, Uppsala University and performed in accordance with the principles of The Declaration of Helsinki.

Procedures

Patients were randomized to either active or sham iTBS treatment. The study was double-blinded, with patients and the symptom assessors being blinded to the treatment allocation. To ensure blinding, the magnetic stimulator operating nurse received a randomization code for each patient, prepared by an independent research organization. The code was entered in the stimulator research software upon each treatment session, which then directed the operator which side of the TMS coil to be angled toward the patient. At baseline, sociodemographic and clinical data were collected. Clinical data include the Clinical Assessment Interview for Negative Symptoms (CAINS) (27), a clinician-rated 13-item interview on a five-point scale, ranging from zero to four, assessing feelings of pleasure, motivation, and emotional expression. Further, the degree of treatment resistance was assessed with the Maudsley Staging Method for treatment resistant depression (MSM) (28), with a maximum score of 15, assessing duration, symptom severity, and failed treatment attempts of the depressive episode, as well as add-on psychotropic medications and electroconvulsive treatment. Patients' overall psychiatric symptoms were assessed using the clinical-rated Brief Psychiatric Rating Scale (BPRS), a seven-point scale, ranging from one to seven, with 24 items (29). Patients rated their overall health on the EQ-5D VAS, a visual analog scale ranging from 0 to 100 (30), and their depressive symptoms on the Montgomery-Åsberg Depression Rating Scale (MADRS-S) (31), a seven -point scale, ranging from zero to six with nine items. Controls did not receive any iTBS and only underwent baseline assessments. Both the cognitive test battery and the fNIRS acquisition took place at baseline 1 day before treatment start, and once again 4 weeks later. This time interval was chosen to test for potential delayed treatment effects and to synchronize with a neuroimaging study (32).

Repetitive Transcranial Magnetic Stimulation

The rTMS procedure has been described in detail elsewhere (19, 33). In short, the iTBS treatment was performed using the Cool D-B80 A/P butterfly coil (MagVenture, Denmark), designed for sham-controlled stimulation with one of the two identically looking coil sides being internally shielded to prevent the magnetic field from spreading. Treatment was given twice daily on weekdays with a 15 min break between the treatment sessions (34), aiming at 10 treatment days resulting in 20 iTBS sessions at target intensity. Target intensity was defined as 90% of resting foot motor threshold (35). As rTMS of non-motor areas does not systematically change the motor cortical excitability (36), the determination of the motor threshold was performed only at baseline. Treatment was delivered over the DMPFC following MRI-guided neuronavigation (TMS Navigator, Localite, Bonn, Germany), aiming at the dorsal anterior cingulate cortex (ACC) defined as $x = 0$, $y = 30$, $z = 30$ in the Montreal Neurological Institute coordinates (37) (**Supplementary Figure 1**). As shown in Hayward et al. (38), the ACC may be reached via rTMS over the medial PFC. Each iTBS session comprised 40 trains of stimulation (2 s on, 8 s off), each train consisting of 10 bursts at 5 Hz and each burst consisting of three biphasic pulses delivered at 50 Hz. For all patients, transcutaneous electrical nerve stimulation (TENS) electrodes were applied medially on the forehead directly beneath the TMS coil. However, only in the sham iTBS allocation, patients received a mild TENS with a maximum current of 4 mA, proportional to stimulation intensity and synchronous with the described rTMS pulses to mimic the sensation of active stimulation. The sham side of the coil was shielded to prevent magnetic stimulation to reach the cortex. No TENS current was applied in the active iTBS allocation.

Cognitive Tests

The test battery consisted of the Trail Making Test A and B (39), Rey Auditory Verbal Learning Test (RAVLT) (40), Animal Naming Test (41), Digit Symbol Coding Test (42), Sternberg Memory Test (43), Emotional Stroop Test (44), and Corsi Block Tapping Test (45). Except for RAVLT and Animal Naming Test, all tests were administered using the software Inquisit 4 (2015) with computerized adaptations of the tests from the online Millisecond Test Library[1]. **Table 1** lists the cognitive tests in the order they were conducted, and details on cognitive domain, duration, outcome, maximum score, and direction of results for each test. All participants undertook the cognitive tests in the same order, at all time points, reflecting increasing difficulty.

Trail Making Test A and B

The TMT consists of two parts requiring to connect a set of sequential targets by mouse press as fast as possible, with TMT A consisting of a set of numbers to connect, and TMT B of switching between connecting numbers and letters. The TMT assesses executive functioning, attention and processing speed and was operationalized as TMT B test completion speed in seconds in the present study.

Rey Auditory Verbal Learning Test

The RAVLT consists of four parts: in the first three trials, the same set of 15 words is read out by the test instructor and the participant is instructed to immediately verbally recall as many words as possible. The fourth part is a delayed free verbal recall of the word set 30 min later. The RAVLT assesses verbal learning and memory and was operationalized as sum of the first three parts. Two different sets of words were used at baseline and 4 weeks later.

Animal Naming Test

The participant is instructed to verbally name as many animals as possible within a 1 min time frame. The test assesses verbal fluency and was operationalized as number of animals named.

Digit Symbol Coding Test

The participant is instructed to code via key press as many symbols as possible to their corresponding digit with a digit-symbol pair key within a 2 min time frame. The tests assesses attention and processing speed and was operationalized as number of correctly coded digits.

[1]https://www.millisecond.com/download/library/

TABLE 1 | Cognition domain, time or trial number, outcome measure, maximum score and result direction for each cognitive test used in the cognitive test battery.

Test	Domain	Time/ Trials	Outcome	Maximum score	Direction
Trail Making Test A and B	Attention, processing speed	2 trails	Test completion speed of Trail B (in s)	N/A	Negative
RAVLT	Verbal memory and learning	15 items	Remembered words (sum of parts I-III)	45	Positive
Animal Naming Test	Verbal fluency	60 s	Number of words	N/A	Positive
Digit Symbol Coding Test	Attention, processing speed	120 s	Total score	144	Positive
Sternberg Memory Test	Numerical working memory	21 trials	Proportion of correct trials	100	Positive
Emotional Stroop Test	Inhibitory control	125 trials	RT to negative words minus RT to neutral words (in ms)	N/A	Negative
Corsi Block Tapping Test	Visuospatial working memory	16 trials	Correct trials * blockspan level	144	Positive

RAVLT, Rey Auditory Verbal Learning Test; RT, Reaction time; N/A, not applicable. A "positive" direction means that higher values indicate higher performance; a "negative" direction means that higher values indicate lower performance.

Sternberg Memory Test

Participants are presented with sequences of two to seven digits and subsequently being prompted with a digit, and thus to state by key press whether the digit was in the prior sequence or not. The test assesses numerical working memory and was operationalized as percentage of correct responses.

Emotional Stroop Test

Participants are presented with words from five different categories (neutral, negative, positive, aggressive, and color), written in four different colors. Patients are asked to indicate the word color by key press, regardless of the word meaning. The tests assesses inhibitory control and was operationalized as reaction time to negative words subtracted from the reaction time to neutral words ("interference effect").

Corsi Block Tapping Test

Participants are presented with a screen of nine boxes, lighting up sequentially in a predefined order, and ranging in sequence length from two to nine boxes. The participant is instructed to click on the boxes in the correct order. The test assesses visuospatial working memory and was operationalized as the total correct score.

fNIRS Data Acquisition

The fNIRS signal was obtained from a two-channel NIRS system (NIRO-200 NX, Hamamatsu Photonics, Hamamatsu, Japan). The probe holders were positioned over the left and right forehead, aiming at a site roughly corresponding to the DLPFC, with the detection optode lateral to the emission optode (**Supplementary Figure 2**). This optode placement allowed for probing effects in the cognitive control network (18), while indirectly even probing for DMPFC-iTBS effects via the targeted corticolimbic network (46). Distance between detection and emission optode was 3.5 cm. Light attenuation changes at three wavelengths, i.e., 735, 810, and 850 nm, were measured using the modified Beer-Lambert law. Based on an estimated differential path length factor of 5.93 according to the NIRS device manual, concentration changes of oxygenated hemoglobin (oxy-Hb), deoxygenated hemoglobin (deoxy-Hb), and total hemoglobin were calculated. In accordance with previous fNIRS studies, oxy-Hb was chosen as main outcome measure, but deoxy-Hb was also reported to complement the hemodynamic picture (47). Sampling frequency was 5 Hz.

Data were collected simultaneously to the cognitive test battery. Signal acquisition started after the patient was seated comfortably in the chair and the fNIRS probe holders were attached. First, an initial 5-min resting-state measurement was conducted with the participant being instructed to sit calmly and rest. Subsequently, the test battery started and was conducted in the above named order, without breaks or resting periods in between cognitive tests. The fNIRS signal acquisition was continuous over the whole test battery duration. Event markers, indicated by button press on the NIRS device by the operating staff, marked the beginning and the end of each cognition test. Due to technical restraints in the experimental software used for the cognition tests, it was not possible to send specific event trigger (i.e., stimulus presentation or data entry by the participant) to the fNIRS device.

fNIRS Data Analysis

The fNIRS data were analyzed using MATLAB R2020b. For data preprocessing, a low-pass filter with a cutoff frequency of 0.1 Hz was applied to remove potential noise stemming from respiration or heartbeats (48). The fNIRS signal was recorded over a long time period, thus potential changes between tasks being rather slow. To avoid filtering out differences in mean oxy-Hb or deoxy-Hb levels between tasks, no high-pass filter or detrending was applied.

For participant-level analysis, baseline correction was performed by subtracting the mean oxy-Hb or deoxy-Hb signal of the last 60 s within the resting-state measurement, i.e., when the signal had reached equilibrium based on visual inspection of the grouped data, from the filtered fNIRS signal. The fNIRS data were segmented into each cognitive test and contained the initial 60 s of each test performance. With the test length depending on test performance in most tests, we assumed that participants were still somewhat more comparable in their task processing in the beginning rather than in the end of each cognitive test. Given that the Animal Naming Test had a set length of 60 s and thus was the shortest test in the test battery, this length

TABLE 2 | Baseline demographic and clinical characteristics [Mean (SD)] of controls and patients, and the patients further divided into sham and active iTBS treatment allocation, and the respective statistical tests.

	Controls ($n = 55$)	Patients ($n = 51$)	*p*	Sham ($n = 26$)	Active ($n = 25$)	*p*
Age, years	30.20 (10.55)	29.53 (9.31)	0.874	29.04 (8.73)	30.04 (9.84)	0.821
Sex, *n* male:female	18:37	23:28	0.191	12:14	11:14	0.877
CAINS	–	29.18 (7.63)		30.92 (7.21)	27.36 (7.63)	0.105
EQ-5D VAS	–	34.08 (15.49)		34.38 (14.44)	33.76 (16.50)	0.962
MADRS-S	–	29.81 (7.57)		30.42 (6.96)	29.20 (8.10)	0.604
BPRS subscale	–	20.92 (4.54)		21.19 (4.19)	20.64 (5.04)	0.925
MSM	–	10.06 (1.85)		10.44 (2.00)	9.68 (1.59)	0.181
Education, *n*			0.050			0.127
9th year completed[†]	2	9		6	3	
12th year completed	32	28		16	12	
Higher education	21	14		4	10	
Primary diagnosis, *n*	–					0.382
Depressive episode		28		13	15	
Recurrent depression		18		9	9	
Bipolar depression		5		4	1	
Comorbidities, *n*	–					
Anxiety disorder		21		12	9	0.461
ADHD/ADD		11		8	3	0.173
Medication, *n*	–					
Antidepressants		41		19	22	0.291
Antidopaminergic drugs		10		6	4	0.726
Mood stabilizers		14		8	6	0.588
Stimulants		8		7	1	0.050
No medication		2		0	2	0.235

[†] *contains data by one patient (in the sham iTBS allocation) who had <9 years of education.*

CAINS, Clinical Assessment Interview for Negative Symptoms; EQ-5D VAS, self-rated health status; MADRS-S, Montgomery Åsberg Depression Rating Scale, self-rating version; BPRS, Brief Psychiatric rating scale, subscale mood disturbance including the following items: "depression," "anxiety," "suicidality," "guilt," "suspiciousness," and "self-neglect;" MSM, Maudsley Staging Method for Treatment Resistant Depression.

was chosen for all fNIRS data segments. Means were calculated for each cognitive test segment, for both left and right oxy-Hb and deoxy-Hb.

For group-level analysis, the resulting participant-level values were entered into the statistical analysis.

Statistical Analysis

Statistical analysis was performed using MATLAB R2020b. Demographic, clinical, and cognitive performance characteristics were assessed by means of Mann-Whitney *U*-tests (age, CAINS, EQ-5D VAS, MSM, MADRS-S, BPRS) or χ^2 tests (sex, education, primary diagnosis, comorbidities, and medication), comparing the two iTBS treatment allocations. To analyze cognitive performance, mean oxy-Hb, and mean deoxy-Hb, linear mixed-effects (LME) models were conducted with the fixed effect factors treatment allocation (active/sham), time (baseline/4 weeks after iTBS), treatment allocation*time interaction, and random intercept per subject. To analyze differences between the control group and the patient group (i.e., active and sham iTBS together) at baseline, Mann-Whitney *U*-tests were conducted for cognitive performance and LME models were conducted for mean oxy-Hb, with the fixed effect factors group (controls/patients) and random intercept per subject. Pearson correlation analyses were calculated for (I) patients' anhedonia symptoms and cognitive performance at baseline, (II) patients' negative symptoms and prefrontal oxy-Hb at baseline, (III) patients' cognitive performance and concurrent oxy-Hb at baseline, and (IV) the controls' cognitive performance and concurrent oxy-Hb. The alpha level was set to 0.05 for all statistical tests.

RESULTS

Demographic and Clinical Characteristics

After study inclusion, one patient allocated to active iTBS terminated study participation, resulting in 51 patients (23 male, 28 female, mean age = 30) and 55 controls (18 male, 37 female, mean age = 30). Twenty-five patients were randomized to active iTBS, and 26 patients to sham iTBS. Controls had a slightly higher education level but did not differ from patients with respect to age or sex ratio (**Table 2**). Among patients, there were no baseline differences between the two treatment allocations in either demographic or clinical variables (**Table 2**).

TABLE 3 | Cognitive performance [Mean (SD)] for controls and patients (at baseline), and the patients further divided into sham and active iTBS treatment allocation (at baseline and 4 weeks later), and the respective statistical tests.

	Baseline					4 weeks later		
	Controls ($n = 55$)	Patients ($n = 51$)	Mann-Whitney U-test†	Sham ($n = 26$)	Active ($n = 25$)	Sham ($n = 26$)	Active ($n = 25$)	Linear mixed-effect model‡
Trail Making Test	62.56 (26.52)	73.91 (30.73)	$p = 0.026$	74.89 (30.22)	72.89 (31.22)	65.15 (25.39)	72.79 (54.37)	n.s.
Rey Auditory Verbal Learning Test	30.96 (4.98)	27.47 (6.10)	$p < 0.001$	26.92 (5.95)	28.04 (6.19)	27.00 (6.89)	28.04 (5.70)	n.s.
Animal Naming Test	27.78 (6.51)	24.06 (6.12)	$p = 0.004$	23.04 (5.91)	25.12 (6.17)	24.88 (5.96)	26.12 (7.31)	n.s.
Digit Symbol Coding Test	57.24 (15.88)	49.69 (18.22)	$p = 0.016$	50.88 (16.44)	48.44 (19.83)	56.64 (20.22)	56.80 (14.94)	n.s.
Sternberg Memory Test	91.15 (11.39)	92.59 (10.19)	n.s.	91.24 (13.00)	93.99 (5.67)	93.37 (9.87)	95.76 (4.53)	n.s.
Emotional Stroop Test	−4.11 (79.98)	7.15 (113.92)	n.s.	−23.02 (116.80)	38.53 (104.00)	−13.69 (104.56)	−26.03 (143.46)	n.s.
Corsi Block Tapping Test	64.07 (22.61)	61.45 (21.80)	n.s.	60.50 (22.29)	62.44 (21.23)	64.31 (22.77)	64.96 (28.51)	n.s.

*For detailed test information see **Table 1**.*

*†Mann-Whitney U-Test for baseline comparison of controls (n = 55) and patients (n = 51), ‡Linear Mixed-Effect (LME) models with the fixed effect factors treatment allocation (active/sham) and time (baseline/4 weeks later iTBS). Reported are the p-values for each group*time interaction.*

Baseline Cognitive Performance (Patients vs. Healthy Controls)

At baseline, when comparing the patient group ($n = 51$) with the healthy control group, Mann-Whitney U-tests showed a difference in cognitive performance between groups, with lower performance scores for the patients in the Trail Making Test ($p = 0.026$), the RAVLT ($p < 0.001$), the Animal Naming Test ($p = 0.004$), and the Digit Symbol Substitution Test ($p = 0.016$) (**Table 3**). There were no performance differences between patients and controls in the Sternberg Memory Test ($p = 0.463$), the Emotional Stroop Test ($p = 0.557$), and the Corsi Block Tapping Test ($p = 0.571$).

Cognitive Performance Following DMPFC-iTBS (Active vs. Sham iTBS)

Cognitive performance for each cognitive test at baseline and 4 weeks later is shown in **Table 3**. LME models for each cognition test showed no treatment allocation*time interaction, reflecting no differences in trajectories between active and sham iTBS (**Table 3**).

Baseline Prefrontal Blood Oxygenation (Patients vs. Healthy Controls)

At baseline, when comparing the whole patient group ($n = 51$) with the healthy control group ($n = 55$), LME models for each cognitive test showed no main effect of group, reflecting no mean oxy-Hb change differences between patients and controls (**Figure 1**, **Supplementary Figures 3**, **4**). Similarly, LME models showed no mean deoxy-Hb differences between the groups (**Supplementary Figure 5**).

Prefrontal Blood Oxygenation Following DMPFC-iTBS (Active vs. Sham iTBS)

For concurrent mean oxy-Hb levels, LME models showed no main effect of treatment allocation or time, and no treatment allocation*time interaction for either cognitive test (**Supplementary Table 1**), reflecting no differences in trajectories from baseline to 4 weeks later between active and sham iTBS (**Figure 2**, **Supplementary Figures 6**, **7**). Similarly, LME models showed no mean deoxy-Hb differences over the treatment course between active and sham iTBS (**Supplementary Figure 8**).

Correlations

Pearson correlation analyses between baseline patients' negative symptoms and cognitive performance showed negative correlations for the RAVLT ($r = -0.29$, $p = 0.037$) and the Animal Naming Test ($r = -0.28$, $p = 0.049$), and trend-level correlations for the Trail-Making-Test ($r = 0.27$, $p = 0.055$), reflecting worse cognitive performance with more negative symptoms (**Supplementary Table 2**). However, no correlation was found between negative symptoms and cognitive performance assessed 4 weeks later (data not shown). For baseline prefrontal oxy-Hb during cognitive performance, there was a negative correlation with patients' negative symptoms at oxy-Hb during the Trail-Making-Test ($r = -0.29$, $p = 0.042$), reflecting lower cortical activation with more negative symptoms (**Supplementary Table 3**). However, such correlation was no longer observed after the iTBS treatment (data not shown). No correlations were found between baseline cognitive performance and concurrent oxy-Hb for either patients (**Supplementary Table 4**) or healthy controls (**Supplementary Table 5**).

DISCUSSION

To the best of our knowledge, this is the first sham-controlled study to investigate how iTBS over the DMPFC affects cognitive performance, and concurrent prefrontal blood oxygenation changes. While patients at baseline did show impaired cognitive performance in several cognition tests, we did not see the predicted concurrent prefrontal hypoactivity reflected in the fNIRS signal. Further, we did not detect any differences between active and sham iTBS regarding cognitive performance or

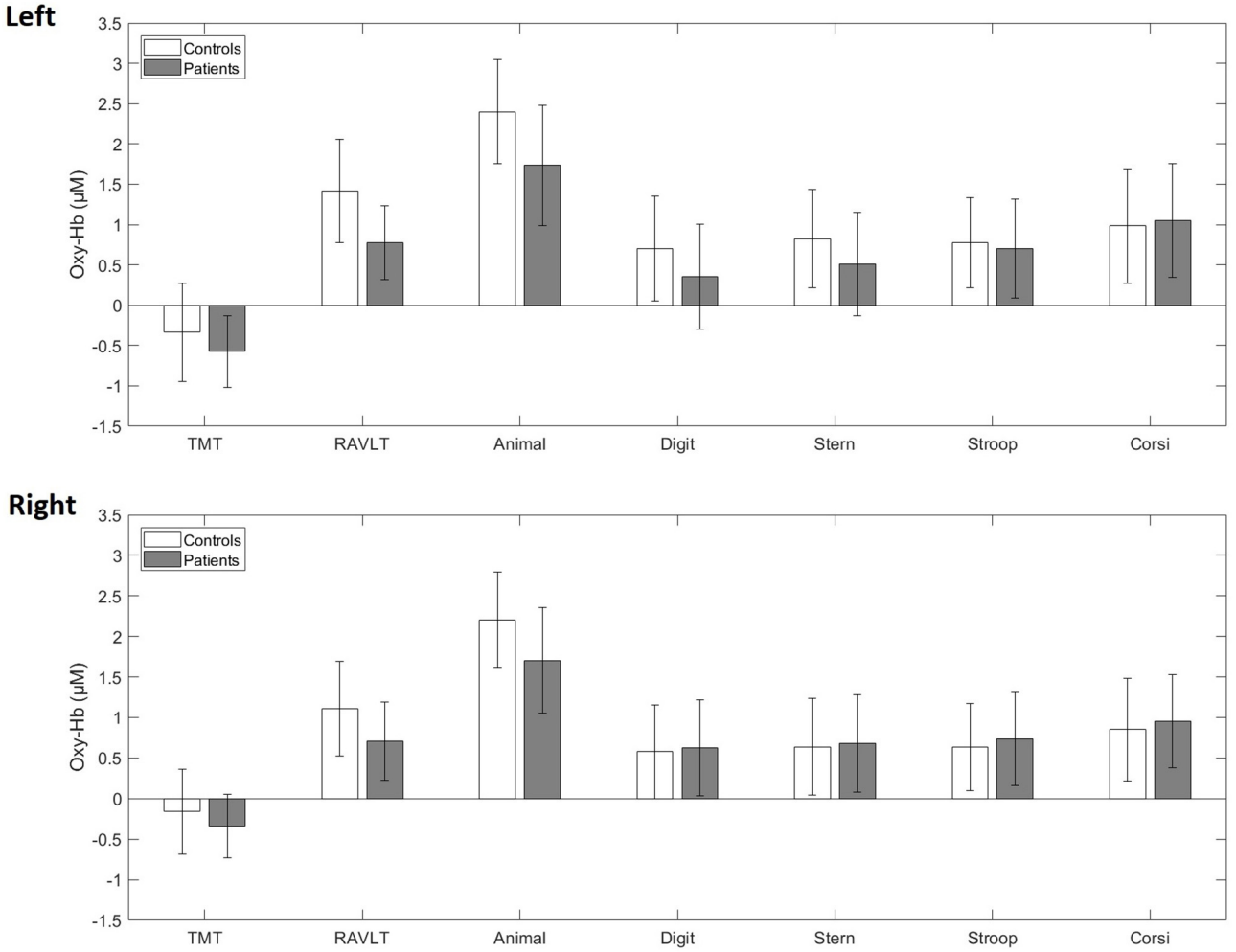

FIGURE 1 | Averaged blood oxygenation (oxy-Hb) and confidence intervals in patients (gray) and controls (white) during the cognitive test battery at baseline in the left and right fNIRS channel. TMT, Trail-Making-Test; RAVLT, Rey Auditory Verbal Learning Test; Animal, Animal Naming Test; Digit, Digit Symbol Coding Test; Stern, Sternberg Memory Test; Stroop, Emotional Stroop Test; Corsi, Corsi Block Tapping Test.

blood oxygenation changes over time. Negative symptoms correlated with cognitive performance to some extent, however there were no correlations between cognitive performance and blood oxygenation.

Our cognition results showed that active iTBS over DMPFC did not affect cognitive performance. The Trail Making Test performance has been reported to be positively affected by left DLPFC-rTMS (11) as well as DMPFC-iTBS (14). Improvement has also been observed in the Wisconsin Card sorting after iTBS to DLPFC. However, our results are in line with several studies showing no difference in cognitive performance following high frequency rTMS over left DLPFC, for example in the Digit Symbol Substitution Test (49), the Stroop Color Test (50), the Stroop Color-Word Interference Test (51), and the digit span (52). Further, a recent meta-analysis of the mostly small sample size studies, revealed no overall DLPFC-rTMS effects on attention, executive functioning, processing speed, verbal fluency, verbal learning, and social cognition were reported (53). DMPFC and DLPFC are engaged in the same cognitive control network (18), and it is possible that this network is less sensitive to manipulation by rTMS/iTBS than hitherto suggested.

Our fNIRS results showed that active iTBS over the DMPFC did not modulate blood oxygenation during cognitive testing. As the DLPFC is suggested as the main hub in executive functioning (54), we chose this region for recording of the blood oxygenation signal. With the DMPFC also being part in the cognitive control network (18), this allowed us to probe for indirect cognitive network effects. In a fNIRS study with iTBS over left DLPFC in panic disorder patients, active iTBS did not modulate prefrontal activity during a verbal fluency task (24). This is in line with our observations during the Animal Naming Test in the present study.

We did not observe a prefrontal hypoactivity when comparing the fNIRS signal of our depressed patients to the controls.

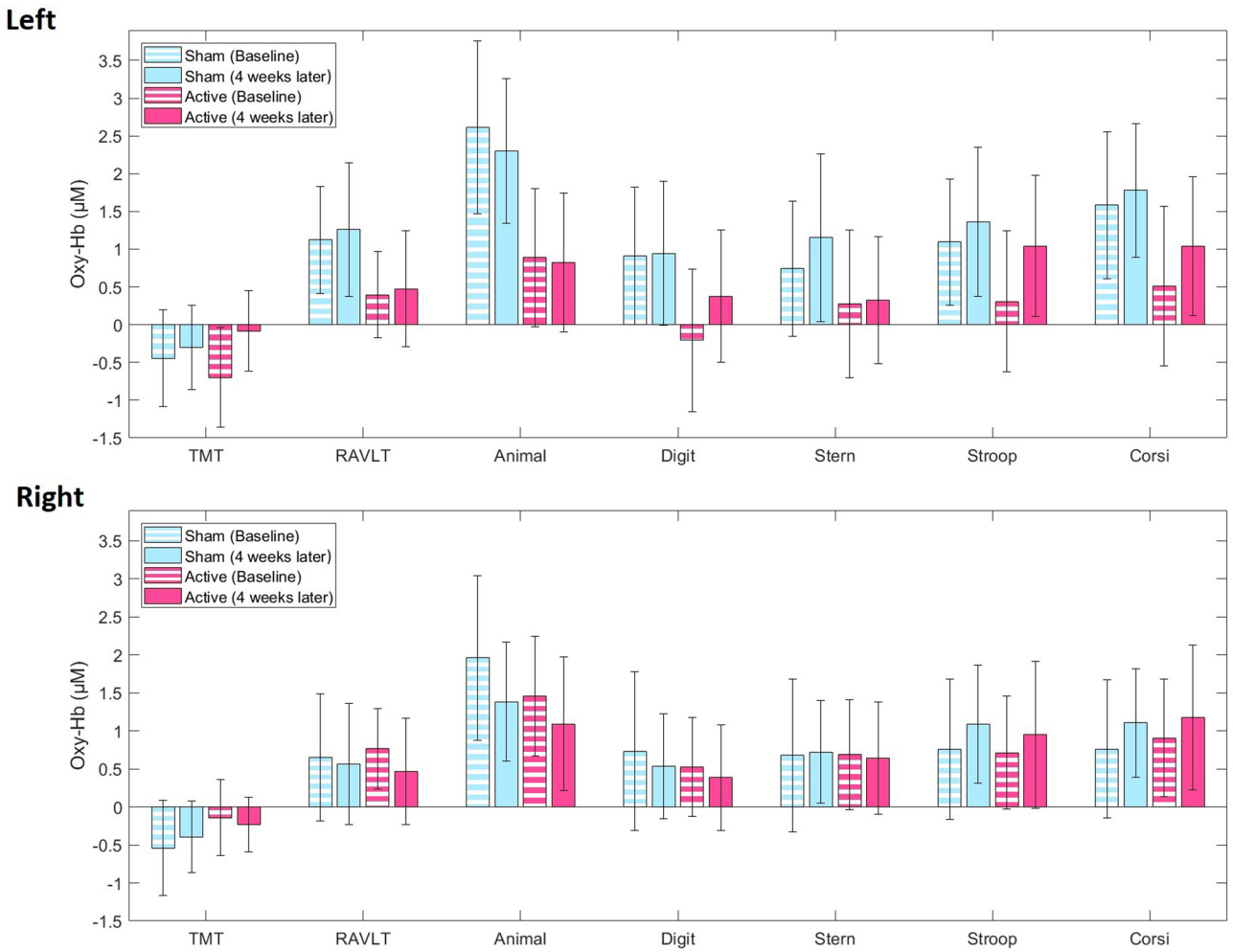

FIGURE 2 | Averaged blood oxygenation (oxy-Hb) and confidence intervals in the active (pink) and sham (blue) iTBS group during the cognitive test battery at baseline (stripes) and 4 weeks later (full color) in the left and right fNIRS channel. TMT, Trail-Making-Test; RAVLT, Rey Auditory Verbal Learning Test; Animal, Animal Naming Test; Digit, Digit Symbol Coding Test; Stern, Sternberg Memory Test; Stroop, Emotional Stroop Test; Corsi, Corsi Block Tapping Test.

This is somewhat surprising, as previous fMRI studies showed hypoactivity of prefrontal regions during various cognitive tasks, including a verbal fluency task (55) and a working memory task (56). Also with fNIRS, such hypofrontality has been reported in depression (57), and even with a similar two-channel device as the one used in our study (58). However, descriptively, our patients did exhibit lower prefrontal blood oxygenation throughout the cognitive testing. This trend not reaching significance however might partly be due to that in our study, we measured fNIRS continuously over a relatively long timespan, without applying an event-related paradigm as the majority of studies investigating working memory (57). Similarly, this might at least partly explain the lack of association between the fNIRS signal and cognitive performance.

Though patients did not show a cognitive improvement following the active compared to sham iTBS treatment course, it is important to stress that neither did active iTBS over the DMPFC cause worsened cognitive performance, making it likely to be a cognitively safe treatment option in this patient sample. rTMS targeting the DLPFC has not been reported to have cognitive side-effects such as temporary memory loss (59) which is the case for electroconvulsive therapy (5), and our study extends this observation to be also valid for iTBS over DMPFC.

This study is subject to some limitations. First, the observation that accelerated protocols yield a more rapid treatment response has been questioned by a recent study (60) and it is possible that the targeted treatment duration of 10 days thus was too short to yield a substantial treatment effect on cognitive functioning. In this context, the 15 min intersession interval used in our study might have been too short to increase cortical excitability (61). While longer intervals of 60 min are more reliably linked to excitatory effects (62), there are also reports of 15 min intervals yielding increased excitability (34). As of now, the optimal intersession interval length between

two daily sessions to yield maximum excitatory effects is yet to be determined. Second, we cannot fully exclude that the sham iTBS might have been partly active, as has been suggested by a recent rTMS study with the same coil (63). Third, the fNIRS signal may be sensitive also to signal changes from the skin blood flow, thus picking up psychophysiological arousal due to the cognitive performance situation (64). Fourth, as the patients had psychotropic medication this may have affected the NIRS-signal (65), making comparisons with healthy controls less reliable. However, as the patients' medications were unchanged 1 month before and during the blinded randomized phase of the trial, it is unlikely that the medication introduced any systematic bias in the comparison between sham and active iTBS. Finally, it should be noted that our two-channel fNIRS device only obtained signals from a limited brain region.

This sham-controlled study did not detect any changes in the prefrontal blood oxygenation during cognitive performance, and no changes in cognitive performance following a full treatment course of active iTBS over the DMPFC in depression. Together with our previous findings (19), showing a treatment effect for negative symptoms, such as anhedonia, while not causing more side effects, such as self-reported memory deficits or fatigue, than sham iTBS, we conclude that the treatment is likely to be considered cognitively safe in this sample of patients with depression.

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 Central Ethical Review Board, Uppsala University. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

JP, MG, WW, CW, and RB: conception and design of the study. WS, JP, MG, and RB: acquisition and analysis of data. WS, JP, MG, WW, CW, and RB: drafting the manuscript or figures. All authors contributed to the article 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/fpsyt.2021.659571/full#supplementary-material

Supplementary Figure 1 | Coil placement over the dorsomedial prefrontal cortex (DMPFC). For detailed coil information see Methods.

Supplementary Figure 2 | Position of the fNIRS probe holders, aiming at a site roughly corresponding to the dorsolateral prefrontal cortex (DLPFC), with the detection optode lateral to the emission optode.

Supplementary Figure 3 | Averaged blood oxygenation (oxy-Hb) and confidence intervals in patients (blue) and controls (yellow) during the cognitive test battery at baseline in the left fNIRS channel. Displayed are the initial 60 s of each cognitive test. Gaps between tests stem from time spent for test instruction. TMT, Trail-Making-Test; RAVLT, Rey Auditory Verbal Learning Test; Animal, Animal Naming Test; Digit, Digit Symbol Coding Test; Sternberg, Sternberg Memory Test; Stroop, Emotional Stroop Test; Corsi, Corsi Block Tapping Test.

Supplementary Figure 4 | Averaged blood oxygenation (oxy-Hb) and confidence intervals in patients (blue) and controls (yellow) during the cognitive test battery at baseline in the right fNIRS channel. Displayed are the initial 60 s of each cognitive test. Gaps between tests stem from time spent for test instruction. TMT, Trail-Making-Test; RAVLT, Rey Auditory Verbal Learning Test; Animal, Animal Naming Test; Digit, Digit Symbol Coding Test; Sternberg, Sternberg Memory Test; Stroop, Emotional Stroop Test; Corsi, Corsi Block Tapping Test.

Supplementary Figure 5 | Averaged blood oxygenation (deoxy-Hb) and confidence intervals in patients (gray) and controls (white) during the cognitive test battery at baseline in the left and right fNIRS channel. TMT, Trail-Making-Test; RAVLT, Rey Auditory Verbal Learning Test; Animal, Animal Naming Test; Digit, Digit Symbol Coding Test; Stern, Sternberg Memory Test; Stroop, Emotional Stroop Test; Corsi, Corsi Block Tapping Test.

Supplementary Figure 6 | Averaged blood oxygenation (oxy-Hb) and confidence intervals in the active (red) and sham (blue) iTBS group during the cognitive test battery at baseline (lighter color) and 4 weeks later (darker color) in the left fNIRS channel. Displayed are the initial 60 s of each cognitive test. Gaps between tests stem from time spent for test instruction. TMT, Trail-Making-Test; RAVLT, Rey Auditory Verbal Learning Test; Animal, Animal Naming Test; Digit, Digit Symbol Coding Test; Sternberg, Sternberg Memory Test; Stroop, Emotional Stroop Test; Corsi, Corsi Block Tapping Test.

Supplementary Figure 7 | Averaged blood oxygenation (oxy-Hb) and confidence intervals in the active (red) and sham (blue) iTBS group during the cognitive test battery at baseline (lighter color) and 4 weeks later (darker color) in the right fNIRS channel. Displayed are the initial 60 s of each cognitive test. Gaps between tests stem from time spent for test instruction. TMT, Trail-Making-Test; RAVLT, Rey Auditory Verbal Learning Test; Animal, Animal Naming Test; Digit, Digit Symbol Coding Test; Sternberg, Sternberg Memory Test; Stroop, Emotional Stroop Test; Corsi, Corsi Block Tapping Test.

Supplementary Figure 8 | Averaged blood oxygenation (deoxy-Hb) and confidence intervals in the active (pink) and sham (blue) iTBS group during the cognitive test battery and baseline (stripes) and 4 weeks later (full color) in the left and right fNIRS channel. TMT, Trail-Making-Test; RAVLT, Rey Auditory Verbal Learning Test; Animal, Animal Naming Test; Digit, Digit Symbol Coding Test; Stern,

Sternberg Memory Test; Stroop, Emotional Stroop Test; Corsi, Corsi Block Tapping Test.

Supplementary Table 1 | Depicted are the results of linear mixed-effects models for concurrent oxy-Hb assessed during a cognitive test battery, i.e., the T- and *p*-values of the treatment allocation (active, sham) x time (baseline, 4 weeks later) interactions. RAVLT, Rey Auditory Verbal Learning Test. fNIRS, functional near-infrared spectroscopy.

Supplementary Table 2 | Baseline correlations between patients' negative symptoms as assessed by the Clinical Assessment Interview for Negative Symptoms (CAINS) and cognitive performance at baseline. RAVLT, Rey Auditory Verbal Learning Test. Significant correlations are marked with an asterisk.

Supplementary Table 3 | Baseline correlation between patients' negative symptoms as assessed by the Clinical Assessment Interview for Negative Symptoms (CAINS) and prefrontal oxy-Hb during cognitive performance at baseline. RAVLT, Rey Auditory Verbal Learning Test. Significant correlations are marked with an asterisk.

Supplementary Table 4 | Baseline correlation between patients' cognitive performance and concurrent prefrontal oxy-Hb at baseline. RAVLT, Rey Auditory Verbal Learning Test.

Supplementary Table 5 | Correlations between controls' cognitive performance and concurrent prefrontal oxy-Hb. RAVLT, Rey Auditory Verbal Learning Test.

REFERENCES

1. James SL, Abate D, Abate KH, Abay SM, Abbafati C, Abbasi N, et al. Global, regional, and national incidence, prevalence, and years lived with disability for 354 Diseases and Injuries for 195 countries and territories, 1990-2017: a systematic analysis for the Global Burden of Disease Study 2017. *Lancet.* (2018) 392:1789–858. doi: 10.1016/S0140-6736(18)32279-7
2. *ICD-10: International Statistical Classification of Diseases and Related Health Problems: Tenth Revision.* 2nd ed. Geneva: World Health Organisation (2004).
3. Millan MJ, Agid Y, Brüne M, Bullmore ET, Carter CS, Clayton NS, et al. Cognitive dysfunction in psychiatric disorders: characteristics, causes and the quest for improved therapy. *Nat Rev Drug Discov.* (2012) 11:141–68. doi: 10.1038/nrd3628
4. Beck AT. The evolution of the cognitive model of depression and its neurobiological correlates. *Am J Psychiatry.* (2008) 165:969–77. doi: 10.1176/appi.ajp.2008.08050721
5. Semkovska M, McLoughlin DM. Objective cognitive performance associated with electroconvulsive therapy for depression: a systematic review and meta-analysis. *Biol Psychiatry.* (2010) 68:568–77. doi: 10.1016/j.biopsych.2010.06.009
6. Lefaucheur JP, Aleman A, Baeken C, Benninger DH, Brunelin J, Di Lazzaro V, et al. Evidence-based guidelines on the therapeutic use of repetitive transcranial magnetic stimulation (rTMS): an update (2014–2018). *Clin Neurophysiol.* (2020) 131:474–528. doi: 10.1016/j.clinph.2020.02.003
7. Miller EK, Cohen JD. An integrative theory of prefrontal cortex function. *Annu Rev Neurosci.* (2001) 24:167–202. doi: 10.1146/annurev.neuro.24.1.167
8. Hoogendam JM, Ramakers GMJ, Di Lazzaro V. Physiology of repetitive transcranial magnetic stimulation of the human brain. *Brain Stimul.* (2010) 3:95–118. doi: 10.1016/j.brs.2009.10.005
9. Schulze-Rauschenbach SC, Harms U, Schlaepfer TE, Maier W, Falkai P, Wagner M. Distinctive neurocognitive effects of repetitive transcranial magnetic stimulation and electroconvulsive therapy in major depression. *Br J Psychiatry.* (2005) 186:410–6. doi: 10.1192/bjp.186.5.410
10. Luber B, Lisanby SH. Enhancement of human cognitive performance using transcranial magnetic stimulation (TMS). *Neuroimage.* (2012) 23:1–7. doi: 10.1016/j.neuroimage.2013.06.007
11. Martin DM, McClintock SM, Forster JJ, Lo TY, Loo CK. Cognitive enhancing effects of rTMS administered to the prefrontal cortex in patients with depression: a systematic review and meta-analysis of individual task effects. *Depress Anxiety.* (2017) 34:1029–39. doi: 10.1002/da.22658
12. Blumberger DM, Vila-rodriguez F, Thorpe KE, Feffer K, Noda Y, Giacobbe P, et al. Effectiveness of theta burst versus high-frequency repetitive transcranial magnetic stimulation in patients with depression (THREE-D): a randomised non-inferiority trial. *Lancet.* (2018) 391:1683–92. doi: 10.1016/S0140-6736(18)30295-2
13. Cheng CM, Juan CH, Chen MH, Chang CF, Lu HJ, Su TP, et al. Different forms of prefrontal theta burst stimulation for executive function of medication- resistant depression: evidence from a randomized sham-controlled study. *Prog Neuro-Psychopharmacol Biol Psychiatry.* (2016) 66:35–40. doi: 10.1016/j.pnpbp.2015.11.009
14. Krepel N, Rush AJ, Iseger TA, Sack AT, Arns M. Can psychological features predict antidepressant response to rTMS? A Discovery-Replication approach. *Psychol Med.* (2019) 50:264–72. doi: 10.1017/S0033291718004191
15. Price JL, Drevets WC. Neural circuits underlying the pathophysiology of mood disorders. *Trends Cogn Sci.* (2012) 16:61–71. doi: 10.1016/j.tics.2011.12.011
16. Downar J, Daskalakis ZJ. New targets for rTMS in depression: a review of convergent evidence. *Brain Stimul.* (2013) 6:231–40. doi: 10.1016/j.brs.2012.08.006
17. Bakker N, Shahab S, Giacobbe P, Blumberger DM, Daskalakis ZJ, Kennedy SH, et al. RTMS of the dorsomedial prefrontal cortex for major depression: safety, tolerability, effectiveness, and outcome predictors for 10 Hz versus intermittent theta-burst stimulation. *Brain Stimul.* (2015) 8:208–15. doi: 10.1016/j.brs.2014.11.002
18. Taren AA, Venkatraman V, Huettel SA. A parallel functional topography between medial and lateral prefrontal cortex: evidence and implications for cognitive control. *J Neurosci.* (2011) 31:5026–31. doi: 10.1523/JNEUROSCI.5762-10.2011
19. Bodén R, Bengtsson J, Thörnblom E, Struckmann W, Persson J. Dorsomedial prefrontal theta burst stimulation to treat anhedonia, avolition, and blunted affect in schizophrenia or depression – a randomized controlled trial. *J Affect Disord.* (2021) 290:308–15. doi: 10.1016/j.jad.2021.04.053
20. Schulze L, Wheeler S, McAndrews MP, Solomon CJE, Giacobbe P, Downar J. Cognitive safety of dorsomedial prefrontal repetitive transcranial magnetic stimulation in major depression. *Eur Neuropsychopharmacol.* (2016) 26:1213–26. doi: 10.1016/j.euroneuro.2016.04.004
21. Lu C, Zhang Y, Biswal BB, Zang Y, Peng D, Zhu C. Use of fNIRS to assess resting state functional connectivity. *J Neurosci Methods.* (2010) 186:242–9. doi: 10.1016/j.jneumeth.2009.11.010
22. Dieler AC, Tupak SV, Fallgatter AJ. Functional near-infrared spectroscopy for the assessment of speech related tasks. *Brain Lang.* (2012) 121:90–109. doi: 10.1016/j.bandl.2011.03.005
23. Matsuo K, Onodera Y, Hamamoto T, Muraki K, Kato N, Kato T. Hypofrontality and microvascular dysregulation in remitted late-onset depression assessed by functional near-infrared spectroscopy. *Neuroimage.* (2005) 26:234–42. doi: 10.1016/j.neuroimage.2005.01.024
24. Deppermann S, Vennewald N, Diemer J, Sickinger S, Haeussinger FB, Notzon S, et al. Does rTMS alter neurocognitive functioning in patients with panic disorder/agoraphobia? An fNIRS-based investigation of prefrontal activation during a cognitive task and its modulation via sham-controlled rTMS. *Biomed Res Int.* (2014) 2014:542526. doi: 10.1155/2014/542526
25. Deppermann S, Vennewald N, Diemer J, Sickinger S, Haeussinger FB, Dresler T, et al. Neurobiological and clinical effects of fNIRS-controlled rTMS in patients with panic disorder/agoraphobia during cognitive-behavioural therapy. *NeuroImage Clin.* (2017) 16:668–77. doi: 10.1016/j.nicl.2017.09.013
26. Sheehan DV, Lecrubier Y, Sheehan KH, Amorim P, Janavs J, Weiller E, et al. The Mini-International Neuropsychiatric Interview (M.I.N.I): the development and validation of a structured diagnostic psychiatric interview for DSM-IV and ICD-10. *J Clin Psychiatry.* (1998) 59:22–33. doi: 10.1037/t18597-000
27. Kring AM, Gur RE, Blanchard JJ, Horan WP, Reise SP. The clinical assessment interview for negative symptoms (CAINS): final development and validation. *Am J Psychiatry.* (2013) 170:165–72. doi: 10.1176/appi.ajp.2012.12010109
28. Fekadu A, Wooderson S, Donaldson C, Markopoulou K, Masterson B, Poon L, et al. A multidimensional tool to quantify treatment resistance in

depression: the maudsley staging method. *J Clin Psychiatry.* (2009) 70:177–84. doi: 10.4088/JCP.08m04309
29. Zanello A, Berthoud L, Ventura J, Merlo MCG. The Brief Psychiatric Rating Scale (version 4.0) factorial structure and its sensitivity in the treatment of outpatients with unipolar depression. *Psychiatry Res.* (2013) 210:626–33. doi: 10.1016/j.psychres.2013.07.001
30. Herdman M, Gudex C, Lloyd A, Janssen M, Kind P, Parkin D, et al. Development and preliminary testing of the new five-level version of EQ-5D (EQ-5D-5L). *Qual Life Res.* (2011) 20:1727–36. doi: 10.1007/s11136-011-9903-x
31. Svanborg P, Åsberg M. A new self-rating scale for depression and anxiety states based on the Comprehensive Psychopathological Rating Scale. *Acta Psychiatr Scand.* (1994) 89:21–8. doi: 10.1111/j.1600-0447.1994.tb01480.x
32. Persson J, Struckmann W, Gingnell M, Fällmar D, Bodén R. Intermittent theta burst stimulation over the dorsomedial prefrontal cortex modulates resting-state connectivity in depressive patients: a sham-controlled study. *Behav Brain Res.* (2020) 394:112834. doi: 10.1016/j.bbr.2020.112834
33. Struckmann W, Persson J, Weigl W, Gingnell M, Bodén R. Modulation of the prefrontal blood oxygenation response to intermittent theta-burst stimulation in depression: a sham-controlled study with functional near-infrared spectroscopy. *World J Biol Psychiatry.* (2020) 0:1–10. doi: 10.1080/15622975.2020.1785007
34. Tse NY, Goldsworthy MR, Ridding MC, Coxon JP, Fitzgerald PB, Fornito A, et al. The effect of stimulation interval on plasticity following repeated blocks of intermittent theta burst stimulation. *Sci Rep.* (2018) 8:1–10. doi: 10.1038/s41598-018-26791-w
35. Salomons TV, Dunlop K, Kennedy SH, Flint A, Geraci J, Giacobbe P, et al. Resting-state cortico-thalamic-striatal connectivity predicts response to dorsomedial prefrontal rTMS in major depressive disorder. *Neuropsychopharmacology.* (2013) 39:488–98. doi: 10.1038/npp.2013.222
36. Nordmann G, Azorina V, Langguth B, Schecklmann M. A systematic review of non-motor rTMS induced motor cortex plasticity. *Front Hum Neurosci.* (2015) 9:1–11. doi: 10.3389/fnhum.2015.00416
37. Mir-Moghtadaei A, Giacobbe P, Daskalakis ZJ, Blumberger DM, Downar J. Validation of a 25% nasion–inion heuristic for locating the dorsomedial prefrontal cortex for repetitive transcranial magnetic stimulation. *Brain Stimul.* (2016) 9:793–5. doi: 10.1016/j.brs.2016.05.010
38. Hayward G, Mehta MA, Harmer C, Spinks TJ, Grasby PM, Goodwin GM. Exploring the physiological effects of double-cone coil TMS over the medial frontal cortex on the anterior cingulate cortex: An H215O PET study. *Eur J Neurosci.* (2007) 25:2224–33. doi: 10.1111/j.1460-9568.2007.05430.x
39. Reitan RM. The relation of the Trail Making Test to organic brain damage. *J Consult Psychol.* (1955) 19:393–4. doi: 10.1037/h0044509
40. Badcock JC, Dragović M, Dawson L, Jones R. Normative data for rey's auditory verbal learning test in individuals with schizophrenia. *Arch Clin Neuropsychol.* (2011) 26:205–13. doi: 10.1093/arclin/acr005
41. Tombaugh TN, Kozak J, Rees L. Normative data stratified by age and education for two measures of verbal fluency: FAS and animal naming. *Arch Clin Neuropsychol.* (1999) 14:167–77. doi: 10.1016/S0887-6177(97)00095-4
42. Joy S, Kaplan E, Fein D. Speed and memory in the WAIS-III Digit Symbol - Coding subtest across the adult lifespan. *Arch Clin Neuropsychol.* (2004) 19:759–67. doi: 10.1016/j.acn.2003.09.009
43. Sternberg S. High-speed scanning in human memory. *Science.* (1966) 153:652–4. doi: 10.1126/science.153.3736.652
44. Williams MJ, Mathews A, MacLeod C. The emotional Stroop task and psychopathology. *Psychol Bull.* (1996) 120:3–24. doi: 10.1037/0033-2909.120.1.3
45. Kessels RPC, Zandvoort MJE Van, Postma A, Kappelle LJ, Haan EHF De. The Corsi block-tapping task: standardization and normative data. *Appl Neuropsychol.* (2010) 4282:37–41. doi: 10.1207/S15324826AN0704_8
46. Tik M, Hoffmann A, Sladky R, Tomova L, Hummer A, Navarro de Lara L, et al. Towards understanding rTMS mechanism of action: Stimulation of the DLPFC causes network-specific increase in functional connectivity. *Neuroimage.* (2017) 162:289–96. doi: 10.1016/j.neuroimage.2017.09.022
47. Curtin A, Tong S, Sun J, Wang J, Onaral B, Ayaz H. A systematic review of integrated functional near-infrared spectroscopy (fNIRS) and transcranial magnetic stimulation (TMS) studies. *Front Neurosci.* (2019) 13:84. doi: 10.3389/fnins.2019.00084
48. Hirosawa R, Narumoto J, Sakai Y, Nishida S, Ishida T, Nakamae T, et al. Reduced dorsolateral prefrontal cortical hemodynamic response in adult obsessive-compulsive disorder as measured by near-infrared spectroscopy during the verbal fluency task. *Neuropsychiatr Dis Treat.* (2013) 9:955–62. doi: 10.2147/NDT.S45402
49. Myczkowski ML, Fernandes A, Moreno M, Valiengo L, Lafer B, Moreno RA, et al. Cognitive outcomes of TMS treatment in bipolar depression: safety data from a randomized controlled trial. *J Affect Disord.* (2018) 235:20–6. doi: 10.1016/j.jad.2018.04.022
50. Nadeau SE, Bowers D, Jones TL, Wu SS, Triggs WJ, Heilman KM. Cognitive effects of treatment of depression with repetitive transcranial magnetic stimulation. *Cogn Behav Neurol.* (2014) 27:77–87. doi: 10.1097/WNN.0000000000000031
51. Wajdik C, Claypoole KH, Fawaz W, Holtzheimer III PE, Neumaier J, Dunner DL, et al. No change in neuropsychological functioning after receiving repetitive transcranial magnetic stimulation (TMS) treatment for major depression. *J ECT.* (2014) 30:320–4. doi: 10.1097/YCT.0000000000000096
52. Mogg A, Pluck G, Eranti SV, Landau S, Purvis R, Brown RG, et al. A randomized controlled trial with 4-month follow-up of adjunctive repetitive transcranial magnetic stimulation of the left prefrontal cortex for depression. *Psychol Med.* (2008) 38:323–33. doi: 10.1017/S0033291707001663
53. Begemann MJ, Brand BA, Curcic-Blake B, Aleman A, Sommer IE. Efficacy of non-invasive brain stimulation on cognitive functioning in brain disorders : a meta-analysis. *Psychol Med.* (2020) 50:2465–86. doi: 10.1017/S0033291720003670
54. Yuan P, Raz N. Prefrontal cortex and executive functions in healthy adults: a meta-analysis of structural neuroimaging studies. *Neurosci Biobehav Rev.* (2014) 42:180–92. doi: 10.1016/j.neubiorev.2014.02.005
55. Takamura M, Okamoto Y, Okada G, Toki S, Yamamoto T, Yamamoto O, et al. Disrupted brain activation and deactivation pattern during semantic verbal fluency task in patients with major depression. *Neuropsychobiology.* (2017) 74:69–77. doi: 10.1159/000453399
56. Korgaonkar MS, Grieve SM, Etkin A, Koslow SH, Williams LM. Using standardized fMRI protocols to identify patterns of prefrontal circuit dysregulation that are common and specific to cognitive and emotional tasks in major depressive disorder: First wave results from the iSPOT-D study. *Neuropsychopharmacology.* (2013) 38:863–71. doi: 10.1038/npp.2012.252
57. Ho CSH, Lim LJH, Lim AQ, Chan NHC, Tan RS, Lee SH, et al. Diagnostic and predictive applications of functional near-infrared spectroscopy for major depressive disorder: a systematic review. *Front Psychiatry.* (2020) 11:378. doi: 10.3389/fpsyt.2020.00378
58. Herrmann MJ, Ehlis AC, Fallgatter AJ. Bilaterally reduced frontal activation during a verbal fluency task in depressed patients as measured by near-infrared spectroscopy. *J Neuropsychiatry Clin Neurosci.* (2004) 16:170–5. doi: 10.1176/jnp.16.2.170
59. Rossi S, Hallett M, Rossini PM, Pascual-Leone A, Avanzini G, Bestmann S, et al. Safety, ethical considerations, and application guidelines for the use of transcranial magnetic stimulation in clinical practice and research. *Clin Neurophysiol.* (2009) 120:2008–39. doi: 10.1016/j.clinph.2009.08.016
60. Kaster TS, Chen L, Daskalakis ZJ, Hoy KE, Blumberger DM, Fitzgerald PB. Depressive symptom trajectories associated with standard and accelerated rTMS. *Brain Stimul.* (2020) 13:850–7. doi: 10.1016/j.brs.2020.02.021
61. Chung SW, Hill AT, Rogasch NC, Hoy KE, Fitzgerald PB. Use of theta-burst stimulation in changing excitability of motor cortex: a systematic review and meta-analysis. *Neurosci Biobehav Rev.* (2016) 63:43–64. doi: 10.1016/j.neubiorev.2016.01.008
62. Thomson AC, Sack AT. How to design optimal accelerated rTMS protocols capable of promoting therapeutically beneficial metaplasticity. *Front Neurol.* (2020) 11:1–6. doi: 10.3389/fneur.2020.599918
63. Dunlop K, Sheen J, Schulze L, Fettes P, Mansouri F, Feffer K, et al. Dorsomedial prefrontal cortex repetitive transcranial magnetic stimulation for treatment-refractory major depressive disorder: a three-arm, blinded, randomized controlled trial. *Brain Stimul.* (2020) 13:337–40. doi: 10.1016/j.brs.2019.10.020
64. Takahashi T, Takikawa Y, Kawagoe R, Shibuya S, Iwano T, Kitazawa S. Influence of skin blood flow on near-infrared spectroscopy signals measured on the forehead during a verbal fluency task. *Neuroimage.* (2011) 57:991–1002. doi: 10.1016/j.neuroimage.2011.05.012

65. Takamiya A, Hirano J, Ebuchi Y, Ogino S, Shimegi K, Emura H, et al. High-dose antidepressants affect near-infrared spectroscopy signals: a retrospective study. *NeuroImage Clin.* (2017) 14:648–55. doi: 10.1016/j.nicl.2017.02.008

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Every Night and Every Morn: Effect of Variation in *CLOCK* Gene on Depression Depends on Exposure to Early and Recent Stress

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The role of circadian dysregulation is increasingly acknowledged in the background of depressive symptoms, and is also a promising treatment target. Similarly, stress shows a complex relationship with the circadian system. The *CLOCK* gene, encoding a key element in circadian regulation has been implicated in previous candidate variant studies in depression with contradictory findings, and only a few such studies considered the interacting effects of stress. We investigated the effect of *CLOCK* variation with a linkage-disequilibrium-based clumping method, in interaction with childhood adversities and recent negative life events, on two phenotypes of depression, lifetime depression and current depressive symptoms in a general population sample.

Methods: Participants in NewMood study completed questionnaires assessing childhood adversities and recent negative life events, the Brief Symptom Inventory to assess current depressive symptoms, provided data on lifetime depression, and were genotyped for 1054 SNPs in the *CLOCK* gene, 370 of which survived quality control and were entered into linear and logistic regression models with current depressive symptoms and lifetime depression as the outcome variable, and childhood adversities or recent life events as interaction variables followed by a linkage disequilibrium-based clumping process to identify clumps of SNPs with a significant main or interaction effect.

Results: No significant clumps with a main effect were found. In interaction with recent life events a significant clump containing 94 SNPs with top SNP rs6825994 for dominant and rs6850524 for additive models on current depression was identified, while in interaction with childhood adversities on current depressive symptoms, two clumps, both containing 9 SNPs were found with top SNPs rs6828454 and rs711533.

Conclusion: Our findings suggest that *CLOCK* contributes to depressive symptoms, but via mediating the effects of early adversities and recent stressors. Given the increasing

burden on circadian rhythmicity in the modern lifestyle and our expanding insight into the contribution of circadian disruption in depression especially as a possible mediator of stress, our results may pave the way for identifying those who would be at an increased risk for depressogenic effects of circadian dysregulation in association with stress as well as new molecular targets for intervention in stress-related psychopathologies in mood disorders.

Keywords: clock gene, depression, stress, childhood adversities, negative life events, gene-environment interactions, circadian rhythm

INTRODUCTION

In order to successfully adapt to environmental changes including day-night cycles signaling rhythmic alterations in the availability of resources and presence of dangers, regulation of rhythmic metabolic, cognitive, and behavioral functions via optimization of physiological and biological processes to 24-h cycles is necessary (1). Circadian disruption may compromise survival and lead to the emergence of several somatic and mental disorders including depression (2, 3). Self-sustained biological rhythms are controlled by the circadian system composed of tissues expressing endogenous 24-h timekeeping activity (4). The nucleus suprachiasmaticus acts as central pacemaker, the activity of which is based on a transcriptional/posttranslational feedback loop with rhythmic expression of circadian clock genes (5), including the *CLOCK* gene (circadian locomotor output cycles kaput gene) which possesses a transcriptional activator role in the circadian clock mechanism.

Genetic mutations in clock genes may lead to disruptions in the period, phase and amplitude of circadian rhythms (6). In animal models, manipulation and mutation of clock genes causes alterations in behavioral and affective phenotypes, suggesting a direct connection between clock genes and brain functions relevant to psychiatric illness (7). Depression has been linked to circadian abnormalities for decades as suggested by somatic rhythms showing disruption in depressed patients, as well as a typical circadian rhythmicity of various symptoms (8). Prominent circadian disturbances in mood disorders include sleep problems, morning worsening and evening improvement of symptoms, changes in appetite, social interactions, as well as alteration in the circadian rhythmicity of blood pressure, body temperature or hormone levels (9–11). These have implicated a rhythm disruption of central origin involving the core molecular machinery underlying circadian rhythm generation, thus suggesting that disruption of circadian regulation is a factor possibly underlying the development and maintenance of the disorder (12–14).

There is a strong genetic background of mood disorders (15, 16) and some studies point to an association between *CLOCK* gene variation and bipolar disorder (17, 18) but weak association with major depressive disorder (MDD) (12, 17, 19). However, in case of depression, the heterogeneity of the disorder and the underlying neurobiological etiological processes raised the possibility that inaccurate or misinterpreted phenotypes (19) and lack of differentiation between depression subtypes may have obscured existing associations. Furthermore, the circadian clock and stress response systems are closely related (7), and stress/increased vulnerability to stress are risk factors for multiple psychiatric disorders. Modulation of stress response is a common mechanism by which circadian clock genes affect such illnesses including depression (7). It has been suggested that effects of SNPs in candidate gene studies and GWAS-s may be masked by lack of consideration of the interacting effects of various types of environmental events in spite of our increasing understanding that the majority of genes and variants contributing to the emergence of depression act via modulating sensitivity toward stress (16, 20, 21).

In previous studies, only a few candidate polymorphisms in the *CLOCK* gene, and most frequently rs1801260 (also known as 3111T/C) were investigated in association with depression with inconsistent findings. GWAS-s have not confirmed the role of this variant or the *CLOCK* gene, although suggested a role for circadian system genes (19, 22, 23). However, GWAS-s may overlook existing association due to strict *p*-value criteria to compensate for multiple testing with even true positive SNPs not achieving genome-wide significance (19, 24). One way of reducing multiple testing burden yet overcoming the hit-and-miss approach of candidate variant studies is employing a gene-wide approach focusing on variations along the *CLOCK* gene.

The high prevalence of depression coupled with the remarkable lack of efficacy of currently available antidepressive medications leaving ∼30–35% of patients treatment resistant (25) on the one hand reflects the lack of in-depth understanding of the etiological processes in the background of depression, and on the other hand highlights the need for understanding novel processes and identifying novel molecular targets for intervention. Furthermore, given the well-known heterogeneity of depressive disorders not only on the clinical-symptomatic level, but also in the neurobiological and genetic background of such divergent clinical manifestations, understanding genetic background of various processes, such as circadian disruption, playing a role in the development of different depressive symptoms and syndromes may also help subtypization and, in the end, precision and personalized treatments of depression.

The aim of the present study was to investigate the association between variation in the *CLOCK* gene, lifetime depression, and current depressive symptoms in interaction with childhood adversities and recent negative life events in a general European population.

MATERIALS AND METHODS

Study Sample

The present study was part of the NewMood study funded by the European Union (New Molecules in Mood Disorders, Sixth Framework Program of the EU, LSHM-CT-2004-503474). Seven hundred sixty-seven non-related participants (238 males, 529 females) of self-reported European white ethnic origin aged between 18 and 60 years were recruited from the general population through advertisements, a website and general practices; provided self-reported data on sociodemographic factors including age and gender, as well as lifetime and current depression, and early childhood adversities and recent negative life events occurring in the past year; and provided genetic data by a saliva sampling kit.

NewMood aimed to recruit participants with a diverse exposure to different types on environmental influences and adversities and with diverse socioeconomical background to allow for generalisability of results to real-life settings. Furthermore, as NewMood focused on a general population with a continuum approach to affective symptoms and disorders, our sample included previously depressed, currently depressed and never depressed participants as well. A detailed description of the study population is provided in previously published reports (26–28). The study was carried out in accordance with the Declaration of Helsinki, and it was approved by the Scientific and Research Ethics Committee of the Medical Research Council, Budapest, Hungary. All participants provided written informed consent prior to participating in the study.

Phenotypes

The present study focused on measuring two aspects of depression and two types of stressors. Lifetime depression (DEP) was ascertained based on self-report using a background questionnaire. This measure to capture the lifetime presence of major depression has been validated previously with face-to-face structured diagnostic interviews (SCID-I) within a subpopulation of our sample, yielding a 91.7% sensitivity and 89.8% specificity (28).

Current level of depression (BSI-Depression) was measured using the Brief Symptom Inventory (29), a questionnaire measuring psychopathological symptoms in several scales with items scored between 0 and 4 depending on the distress caused. For the present study, only the depression subscale score was used to reflect the actual levels of depression, calculated as the sum of depression and additional item scores divided by the number of completed items. Use of BSI-Depression to capture actual depressive symptom severity has been validated in a previous study in a subsample of our population using the Montgomery-Asberg Depression Rating Scale (MADRS) administered by trained interviewers (28).

The two types of stressors measured in our study included early childhood adversities (CHA) as distal and etiological stressors and recent negative life events (RLE) occurring in the past year as proximal, trigger stressors. The early childhood adversity measure was derived from the Childhood Trauma Questionnaire (CTQ) (30), and included four items referring to emotional and physical abuse and emotional and physical neglect, and two items about the loss of parents. This short childhood adversity measure has previously been validated with the 28-item CTQ within a subpopulation of our sample, yielding a high correlation between the original and derived measures (28). The sum of item scores was used in the analyses. Recent stressful life events (RLE) occurring in the past year related to financial difficulties, illnesses/injuries, personal problems, and intimate relationship or social network difficulties were measured using the List of Threatening Experiences (31, 32). The number of recent negative life events (RLEs) was used in the statistical analyses.

Genotyping and Imputation

Participants provided buccal mucosa cells collected by a cytology brush (Cytobrush plus C0012, Durbin PLC). Genomic DNA was extracted according to the protocol of Freeman et al. (33). Genotyping was performed by Illumina's CoreExom PsychChip. All laboratory work was performed under the ISO 9001:2000 quality management requirements and was blinded with regard to phenotype. Variants were positioned on the genome based on GRCh37/hg19. SHAPEIT was used to determine haplotype information, then missing genotypes were imputed using IMPUTE2 on the *CLOCK* gene with boundaries extended by 10 kilobase pairs on both sides. Imputation and subsequent filtering were carried out in line with multiple quality control steps (34), except that missingness rate (MR), Hardy-Weinberg equilibrium (HWE) and minor allele frequency (MAF) steps were limited to SNPs within the *CLOCK* gene. Variants with an imputation score certainty <0.7 or info <0.5 were excluded.

Statistical Analyses

Plink v1.90 was used to calculate MR (<0.05), HWE ($>1 \times 10^{-5}$) and MAF (>0.01) as part of quality control steps prior to the analyses; for clumping; and for building linear and logistic regression models to test for main and interaction effects of genetic variation in the *CLOCK* gene. Analyses were supported by scripts individually written in R 3.0.2 (35). R was also used to illustrate the effects of significant findings (version 4.0.3 with the ggplot2 package). Descriptive statistics were run using IBM SPSS Statistics 25.

Genotyping provided a dataset incorporating 1054 SNPs in the region of *CLOCK* gene (with boundaries extended by 10 kb) available in the NewMood database. Three hundred and seventy SNPs survived quality control steps were analyzed with linear (for current depression with BSI-dep as the outcome variable) and logistic (for lifetime depression with DEP as the outcome variable) regression models to test for main effects of *CLOCK* variation on lifetime and current depression. To test for gene-environment correlation (rGE) effects, the main effects of *CLOCK* variants surviving quality control on childhood adversities (CHA) and recent life events (RLE) were also analyzed in linear regression models. After tests for main effects of genetic variants on the outcome variables, gene $\times$ environment interaction models with early childhood adversities (CHA) and recent negative life events (RLE) were also run. Regression data on all *CLOCK* SNPs surviving the quality control in all

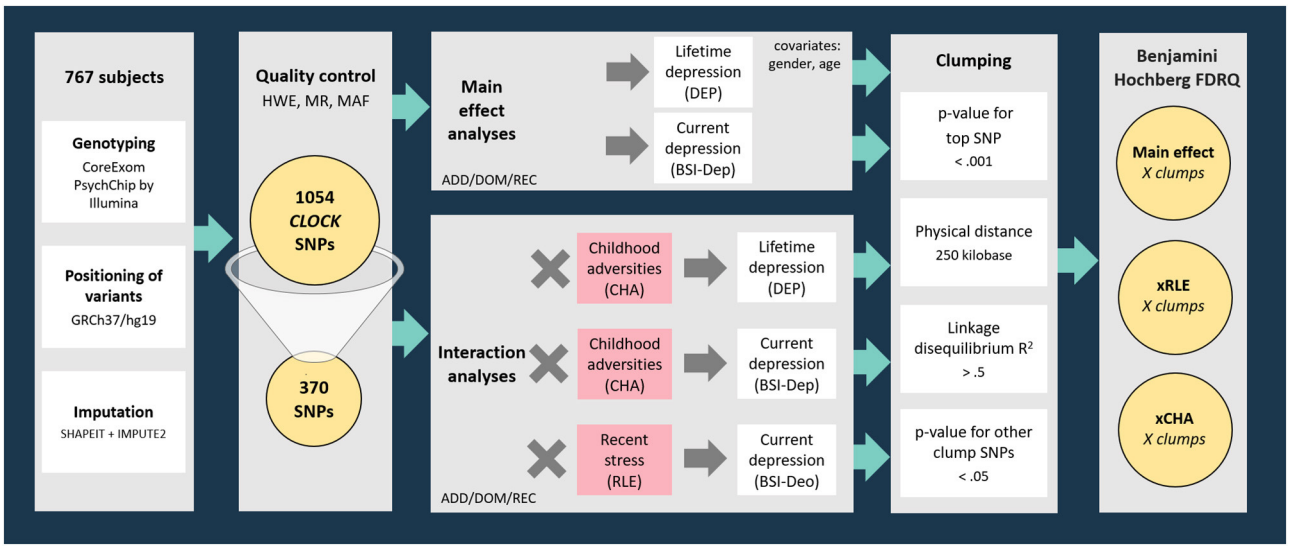

FIGURE 1 | Methods of investigating the effects of variation in *CLOCK* in interaction with childhood adversities and recent life events on lifetime and current depression: study population, quality control steps, and statistical analyses. RLE, recent life events; CHA, Childhood adversities; BSI-Dep, Brief Symptom Inventory depression score; ADD, additive model; DOM, dominant model; REC, recessive model; HWE, Hardy-Weinberg Equilibrium; MR, missingness rate; MAF, minor allele frequency; DEP, lifetime depression; FDRQ, False Discovery Rate Q value.

models are shown in **Supplementary Tables 1–4**). Regression models were in the next step followed by a clumping procedure both for main effect and for GxE interaction effects based on linkage disequilibrium (LD) estimates between the SNPs using the CLUMP function in Plink. The four parameters used for clumping included: (1) maximum *p*-value of the clump's top SNP was set at 0.001; (2) physical distance with top SNP was 250 kilobase; (3) minimum linkage disequilibrium R^2 with top SNP was 0.5; and (4) maximum *p*-value for the clump's other SNPs was 0.05. Results of top SNPs, that is, the most significant SNP representing correlated SNPs in individual clumps are reported (**Figure 1**).

All analyses, including main effect and interaction effect models, were run according to additive, dominant, and recessive models. Age and gender were covariates in all Plink regression models. When testing an SNP × CHA/RLE interaction effect, main effects of both the SNP and CHA/RLE were also included as covariates in the model. Nominal significance threshold was $p < 0.05$. To correct for multiple comparisons in analyses for each of the above outcome variables, Benjamini-Hochberg False Discovery Rate (FDR) Q-values were calculated; results with a $Q \leq 0.05$ were considered significant.

The data presented in this study are openly available in FigShare at https://doi.org/10.6084/m9.figshare.14258567.v1 (36).

RESULTS

Descriptive Statistics

Descriptive statistics of our study sample are provided in **Table 1**.

Main Effects of Variation in *CLOCK* on Current Depressive Symptoms and Lifetime Depression

Linear and logistic regression models on BSI-Dep (for current depression) and DEP (for lifetime depression), respectively, identified a few SNPs with a nominally significant main effect, but significant clumps could not be formed, furthermore, all *p*-values exceeded the maximum threshold for top SNP ($p = 0.001$) [**Supplementary Tables 1**, **2** for lifetime depression (DEP) and current depressive symptoms (BSI-Dep, respectively), but significant clumps for main genetic effects could not be identified]. Nevertheless, top SNPs of significant clumps emerging in the interaction models were tested in main effect models for lifetime and current depression as well, the results of which are shown in **Table 2**.

Gene-Environment Correlation (rGE): Main Effects of Variation in *CLOCK* on Recent Life Events (RLE) and Childhood Adversities (CHA)

Main effect linear regression models on recent life events (RLE) and childhood adversities (CHA) did not identify any nominally significant SNPs (**Supplementary Tables 5**, **6** for RLE and CHA, respectively) suggesting no gene-environment correlation (rGE) effects in case of either early or recent adversities. Linear regression results for potential rGE effects on RLE or CHA in case of top SNPs of significant clumps emerging in the interaction models are also shown in **Table 3**.

TABLE 1 | Descriptive statistics of the study sample.

Gender	n	%		
Male	238	21.76%		
Female	529	48.36%		
	Minimum	*Maximum*	*Mean*	*SEM*
Age	18	60	30.724	0.375
Clinical data	*n*	%		
Subjects reporting lifetime depression	166	21.64%		
Subjects reporting previous suicide attempts or self-harm	35	4.56%		
	Minimum	*Maximum*	*Mean*	*SEM*
BSI-depression score	0	4	0.546	0.024
Environmental influences	*Minimum*	*Maximum*	*Mean*	*SEM*
Childhood adversity	0	15	2.761	0.104
Recent negative life events	0	8	1.091	0.042
Sociodemographic descriptors	*n*	%		
Employment status				
Working full-time	405	52.80%		
Working part-time	27	3.52%		
Student	295	38.46%		
Retired	13	1.69%		
Housewife/househusband	12	1.56%		
Unemployed	15	1.95%		
Marital status				
Single	360	46.94%		
Married	251	32.72%		
Cohabiting	98	12.78%		
Divorced	35	4.56%		
Separated	11	1.43%		
Widowed	7	0.91%		
Data missing	5	0.65%		
Financial situation				
Living comfortably	495	64.54%		
Just getting by	259	33.76%		
Not able to get along	5	0.65%		
Data missing	8	0.91%		

BSI-depression score, current depressive symptom scores as measured by Brief Symptom Inventory; SEM, standard error of the mean.

Gene x Environment Effects of Variation in *CLOCK* on Current Depressive Symptoms: Interaction With Recent Life Events (RLE)

Our analyses for interaction with recent stressful life events (RLE) on current depressive symptoms (BSI-Dep) yielded one significant clump containing 94 SNPs (**Supplementary Table 3**) and with top SNPs rs6825994 for dominant and rs6850524 for additive models, both surviving correction for multiple testing (**Table 4**).

In case of rs6825994, we found a nominally significant interaction effect with recent negative life events (RLE) on BSI-depression in dominant ($p = 0.0004$) model as the lead SNP, and its effect in the additive model was also nominally significant ($p = 0.0017$), both of which remained significant after correction for multiple testing ($FDRQ_{add} = 0.0149$ and $FDRQ_{dom} = 0.0232$, respectively) (**Table 4**). Subjects carrying minor A allele of rs6825994 had significantly higher current depression scores when exposed to recent stressful life events suggesting the minor allele to be a risk allele (**Figure 2**).

In case of rs6850524 we found nominally significant interaction effects with recent life events (RLE) on BSI-depression as top SNP in the additive model ($p = 0.0004$) which survived correction for multiple testing ($FDRQ_{add} = 0.0127$). This SNP, although not as a top SNP, was also nominally significant in interaction with recent life events on BSI-depression in dominant ($p = 0.0028$) and recessive ($p = 0.0066$) models which all remained significant after correction for multiple testing ($FDRQ_{dom} = 0.0187$, $FDRQ_{rec} = 0.0398$) (**Table 4**). In these models, presence of the minor C allele was associated with a lower BSI-depression score if the subject was exposed to recent negative life events indicating a risk effect (**Figure 3**).

Gene × Environment Effects of Variation in *CLOCK* on Current Depressive Symptoms and Lifetime Depression: Interaction With Childhood Adversities (CHA)

In case of gene x environment interaction models with childhood adversity (CHA) on current depression (BSI-Dep), we identified two clumps with top SNPs rs6828454 and rs711533 (**Table 4**), both of them containing 9 SNPs (**Supplementary Table 4**).

TABLE 2 | Main effects of *CLOCK* variants emerging as top SNPs in the interaction models on lifetime depression and on current depression (BSI-dep) severity.

		Additive					Dominant					Recessive				
	Model	**β**	**95% C.I.**		***p*-value**	**FDRQ**	**β**	**95% C.I.**		***p*-value**	**FDRQ**	**β**	**95% C.I.**		***p*-value**	**FDRQ**
rs6828454	Lifetime depression	1.122	0.883	1.426	0.3482	0.497	1.034	0.710	1.506	0.8605	0.906	1.342	0.898	2.005	0.1514	0.293
	BSI depression	0.015	−0.050	0.079	0.6571	0.773	0.031	−0.07	0.133	0.5456	0.668	0.006	−0.106	0.118	0.9189	0.951
rs711533	Lifetime depression	1.524	1.114	2.084	*0.0084*	**0.042**	1.497	1.045	2.144	*0.0279*	0.084	2.945	1.152	7.530	*0.0241*	0.080
	BSI depression	0.070	−0.019	0.159	0.1233	0.264	0.074	−0.024	0.174	0.1463	0.293	0.129	−0.171	0.431	0.3988	0.532
rs6825994	Lifetime depression	1.465	1.149	1.868	*0.0021*	**0.015**	1.648	1.145	2.371	*0.0071*	**0.039**	1.730	1.102	2.715	*0.0171*	0.064
	BSI depression	0.058	−0.009	0.124	0.0913	0.211	0.086	−0.010	0.181	0.0757	0.182	0.059	−0.074	0.192	0.3822	0.533
rs6850524	Lifetime depression	1.351	1.061	1.721	*0.0149*	0.059	1.535	1.048	2.248	*0.0279*	0.088	1.470	0.963	2.242	0.0741	0.185
	BSI depression	0.063	−0.003	0.129	0.0608	0.159	0.106	0.007	0.205	0.0358	0.102	0.054	−0.067	0.175	0.3832	0.523

BSI-current depressive symptom scores as measured by Brief Symptom Inventory. DEP-self-reported lifetime depression. ADD, DOM, REC, additive, dominant and recessive heritability models. FDR, false discovery rate; Italic type shows nominally significant p-values, bold type denotes significant Q-values surviving correction for multiple testing (P < 0.05, FDR Q < 0.05).

TABLE 3 | Main effects of *CLOCK* variants emerging as top SNPs in the interaction models on recent stressful life events and childhood adversities (rGE models).

		Additive				Dominant				Recessive			
	Model	**β**	**95% C.I.**		***p*-value**	**β**	**95% C.I.**		***p*-value**	**β**	**95% C.I.**		***p*-value**
rs6828454	RLE	0.020	−0.094	0.133	0.7341	0.061	−0.117	0.239	0.5019	−0.015	−0.212	0.181	0.8788
	CHA	−0.146	−0.423	0.131	0.3012	−0.269	−0.704	0.166	0.2251	−0.111	−0.592	0.369	0.6504
rs711533	RLE	0.037	−0.119	0.193	0.6441	0.0505	−0.125	0.226	0.5722	−0.037	−0.566	0.492	0.8910
	CHA	−0.224	−0.604	0.157	0.2494	−0.263	−0.691	0.164	0.2279	−0.177	−1.470	1.115	0.7879
rs6825994	RLE	0.005	−0.113	0.123	0.9337	0.073	−0.095	0.241	0.3967	−0.122	0.120	−0.356	0.113
	CHA	0.011	−0.276	0.298	0.9417	0.113	−0.295	0.522	0.5874	−0.122	−0.356	0.113	0.3085
rs6850524	RLE	0.046	−0.071	0.164	0.4404	0.110	−0.067	0.287	0.223	−0.008	0.110	−0.223	0.207
	CHA	−0.014	−0.302	0.275	0.9245	0.039	−0.395	0.473	0.8597	−0.008	−0.223	0.207	0.9439

RLE, recent life stress; CHA, Childhood Adversity. ADD, DOM, REC, additive, dominant, and recessive heritability models.

TABLE 4 | Interactions of *CLOCK* top SNPs rs6828454, rs711533, rs6850524, and rs6825994 with recent negative life events (RLE) and childhood adversity (CHA) on current depression (BSI-Dep) and lifetime depression (DEP) (GxE models).

		Additive				Dominant				Recessive			
	Model	**β**	**95% C.I.**		***p***	**FDRQ**	**β**	**95% C.I.**	***p*-value**	**FDRQ**	**β**	**95% C.I.**	***p***	**FDRQ**
rs6828454	xRLE on BSI	0.017	−0.036	0.071	0.5240	0.655	0.046	−0.044	0.136	0.476	0.003	−0.083	0.089	0.9469	0.947
	xCHA on BSI	0.034	0.013	0.055	*0.0015*	**0.0145**	0.032	−0.000	0.065	0.1390	0.066	0.028	0.104	*0.0006*	**0.0118**
	xCHA on DEP	0.992	0.920	1.070	0.8424	0.903	0.974	0.865	1.096	0.6606	0.762	1.016	0.887	1.164	0.8143	0.905
rs711533	xRLE on BSI	0.088	0.018	0.158	0.0141	0.060	0.095	0.016	0.174	0.0188	0.066	0.139	−0.085	0.364	0.2244	0.396
	xCHA on BSI	0.053	0.022	0.084	*0.0008*	**0.0092**	0.058	0.024	0.092	*0.0008*	**0.0114**	0.066	−0.054	0.186	0.2815	0.4692
	xCHA on DEP	1.045	0.934	1.168	0.4458	0.581	1.070	0.944	1.213	0.2878	0.467	0.892	0.605	1.315	0.5621	0.675
rs6850524	xRLE on BSI	0.098	0.044	0.152	*0.0004*	**0.013**	0.127	0.044	0.210	*0.0028*	**0.019**	0.132	0.037	0.228	*0.0066*	**0.040**
	xCHA on BSI	0.014	−0.008	0.036	0.2160	0.3927	0.017	−0.016	0.050	0.3099	0.4768	0.020	−0.020	0.061	0.3192	0.4671
	xCHA on DEP	1.057	0.973	1.148	0.1866	0.350	0.986	0.872	1.114	0.8173	0.892	1.230	1.047	1.445	0.0117	*0.054*
rs6825994	xRLE on BSI	0.091	0.034	0.147	*0.0017*	**0.015**	0.141	0.064	0.219	*0.0004*	**0.023**	0.061	−0.055	0.176	0.3046	0.481
	xCHA on BSI	0.013	−0.010	0.036	0.2622	0.4495	0.024	−0.008	0.056	0.1408	0.2913	0.002	−0.043	0.047	0.9237	0.9394
	xCHA on DEP	1.028	0.946	1.117	0.5139	0.656	0.982	0.873	1.104	0.7614	0.862	1.145	0.967	1.357	0.1171	0.260

BSI–current depressive symptom scores as measured by Brief Symptom Inventory; DEP–self–reported lifetime depression. ADD, DOM, REC, additive, dominant and recessive heritability models; CHA, Childhood Adversity; RLE, recent life stress; FDR, false discovery rate. Italic type shows nominally significant p-values, bold type denotes significant Q-values surviving correction for multiple testing (P < 0.05, FDR Q < 0.05). In case of all top SNPs statistical parameters are shown for all analyses, not only for those models where they emerged as top SNPs, thus gray shading indicates results for the models where the given SNP was top SNP.

In case of rs6828454 we found a significant interaction effect with childhood adversities (CHA) on current depression (BSI-Dep) in the recessive model as top SNP ($p = 0.0006$, FDRQ $= 0.0118$). Minor C allele carriers reported significantly higher current depression levels when exposed to moderate or severe childhood adversity indicating the minor allele to be a risk allele (**Figure 4**).

In case of rs711533 we found significant interaction effects with CHA on current depression (BSI-Dep) as top SNP in both additive ($p = 0.0008$, FDRQ $= 0.0092$) and dominant ($p = 0.0008$, FDRQ $= 0.0114$) models remaining significant after correcting for multiple testing (**Table 4**). Subjects carrying the minor C allele scored significantly higher on BSI-depression scale when exposed to childhood maltreatment reflecting a risk effect for the minor allele (**Figure 5**).

Interaction with childhood adversity did not have a significant effect on lifetime depression in case of any SNPs, therefore significant clumps could not be calculated (**Table 4**).

In silico Characterization and Functional Prediction of Identified Top SNPs rs6828454, rs711533, rs6825994, and rs6850524 as Well as SNPs in the Clumps Showing a Significant Effect on Depression

Genomic location of significant SNPs and top SNPs identified in the clumping procedure are shown in **Figure 6**. To detect the functional effect of the top significant SNPs, we utilized FuncPred tool (https://snpinfo.niehs.nih.gov). Two SNPs (rs28448438, rs28463765) are located in the transcriptional-factor-binding site of *CLOCK* gene, furthermore, rs726967 is located in the microRNA-binding site. Among the significant polymorphisms, several SNP showed high regulatory potential and conservation score.

In the literature exploratory analyzes on SNPedia (https://www.snpedia.com/), ClinVar (https://figshare.com/articles/dataset/CLOCK_stress_depression/14258567), GWAS Catalog (https://www.ebi.ac.uk/gwas/), we found some relevant sources related to the following SNPs: rs6853192, rs11726609, rs11133399, rs4865010, rs1000254, rs2412646, rs3736544, rs6811520, rs11931061, rs3817444, rs6832769, rs726967, and rs11735267. Results are shown in **Supplementary Table 7** and discussed in the Discussion part.

DISCUSSION

In our study investigating the effect of variation in the *CLOCK* gene with a linkage disequilibrium-based clumping method, we found no clumps of SNPs with a significant main effect either on lifetime depression or current depressive symptoms. However, we have identified significant clumps of SNPs in interaction with both early childhood adversities and recent negative life events on current depressive symptoms, but not in case of lifetime depression. While our results confirm the role of *CLOCK* variation in emergence of depressive symptoms, they also indicate that this effect is observable only in case of exposure to stress. Notably, we found that clumps of SNPs in

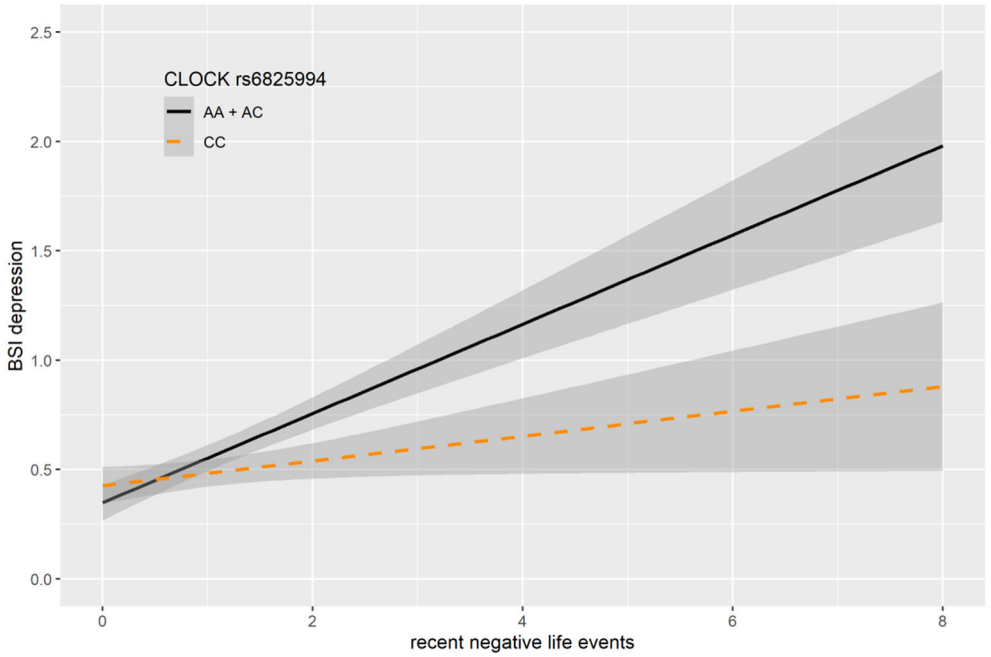

FIGURE 2 | Linear regression indicated a significant interaction between top SNPs rs6825994 of a clump containing 94 SNPs in the *CLOCK* gene and exposure to recent life events (RLE) on current depressive symptoms (BSI-Dep) in the dominant model, with the minor A allele as a risk allele. Linear regression indicated a significant interaction between *CLOCK* rs6825994 genotype and recent stressful life events (RLE) on current depression scores according to the dominant (p = 0.0004, FDR Q = 0.023, as top SNP) and additive (p = 0.0017, FDR Q = 0.015) models. Presence of the minor A allele was associated with higher depression scores in subjects exposed to more severe recent life events conveying a risk effect. On the vertical axis weighted depression (BSI-Dep) scores are shown. The horizontal axis shows recent life events (RLE) occurring within the past year as measured by the List of Threatening Experiences (31). BSI-Brief symptom inventory. Gray shading denotes 95%CI.

the *CLOCK* gene interacted with both distal childhood traumas and adversities, which contribute to the emergence of a diathesis promoting susceptibility for affective disorders, and recent, proximal stressors, which have a role in triggering onset of the actual symptoms or illness episodes based on a diatheses. Finally, we detected no gene-environment correlation effects suggesting that *CLOCK* variation does not influence risk of exposure to early or recent adversities.

The Involvement of Circadian Disruption in Depression

Circadian rhythms regulate a multitude of physiological processes coordinating them both with each other and with the external environment, integrating outer sensory information, and environmental cues with internal physiological and psychological states, thus also influencing human cognition, affect, and behavior, which suggests that dysregulated or disturbed circadian rhythms are involved in the etiopathology of mental and mood disorders as well (6, 19). The presence of circadian abnormalities in depression have been evidenced for a long time with alterations in depressed patients in the circadian rhythmicity of somatic functions including body temperature, blood pressure, or urine metabolite excretion; altered hormone rhythms including prolactin, cortisol, GH, thyrotropin, and melatonin; and in the diurnal fluctuation of symptoms of depression including alteration in sleep-wake cycles, timing and structure of sleep, appetite, or social rhythms (8–14, 37). While in clinical studies risk and severity of depression correlated with the degree of circadian rhythm misalignment (19, 38), there is no definitive evidence whether circadian dysregulation precedes and plays a causative role in, or follows and results from, or merely coincides with depression, however, the complex relationship between the circadian system and mood disorders appears to be bidirectional (7, 39). Nevertheless, conditions leading to circadian rhythm disruption such as shift work may precipitate mood symptoms in those susceptible (40, 41), and in post-mortem brain studies in depressed patients severe disruption and desynchronization of daily rhythmic gene expression patterns were reported (42). One possible linking factor between the circadian system and mood disorders is that neural systems playing a key role in affective illness including the HPA-axis, limbic regions, and monoamine neurotransmitter household are under circadian regulation, and alterations in the biological clock could lead to neurobiological changes in neurotransmitter systems triggering depressive states (12, 14, 42–44). While variation in clock genes encoding elements of circadian rhythm mechanisms is associated with smaller alterations of circadian behaviors in healthy subjects, it appears to have a more pronounced impact on psychopathological features in mood disorder patients affecting key clinical and course features such as timing of disease onset and recurrence and treatment response (45–47).

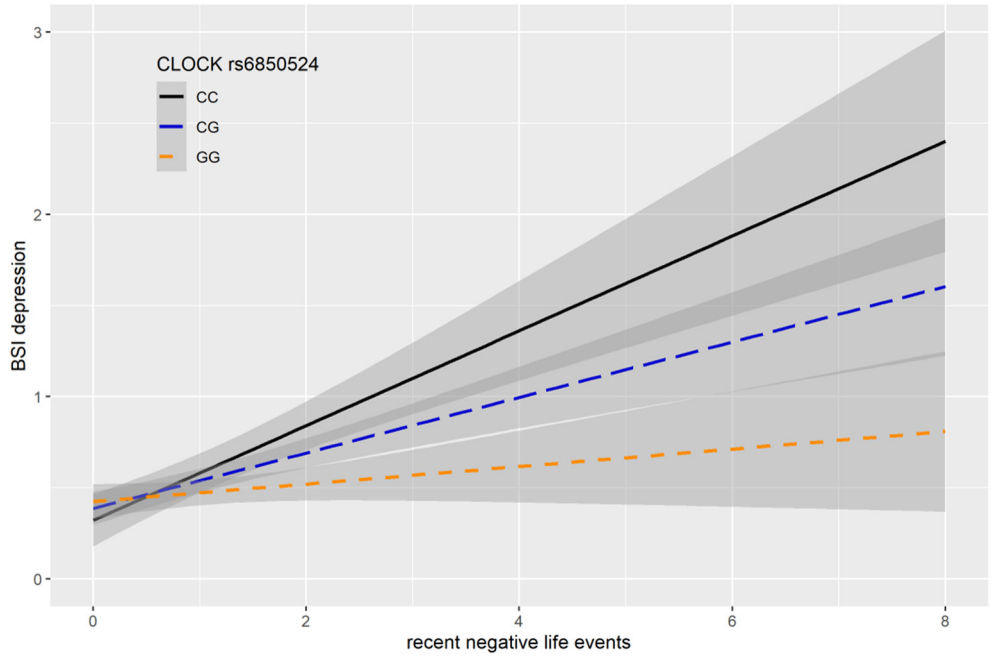

FIGURE 3 | Linear regression indicated a significant interaction between top SNP rs6850524 of a clump containing 94 SNPs in the *CLOCK* gene and exposure to recent life events (RLE) on current depression symptoms (BSI-dep) in the additive model, with the minor C allele as a risk allele. Linear regression indicated a significant interaction between *CLOCK* rs6850524 genotype and recent stressful life events (RLE) on current depression scores according to the additive model as top SNP (p = 0.0004, FDR Q = 0.013). Presence of the minor C allele was associated with higher depression scores in subjects exposed to more severe recent life events conveying a risk effect. On the vertical axis weighted depression (BSI-Dep) scores are shown. The horizontal axis shows recent life events (RLE) occurring within the past year as measured by the List of Threatening Experiences (31). BSI-Brief symptom inventory. Gray shading denotes 95%CI.

Variation in the *CLOCK* Gene Does Not Directly Impact Lifetime Depression or Current Depressive Symptoms

Our findings indicate that variation in the *CLOCK* gene exerts no significant main effect either on lifetime depression, or current depressive symptoms. The *CLOCK* gene, located at chr4q12, is one of the chief genes in the endogenous master clock system playing a key role in the formation of circadian rhythms. Genomic variation in *CLOCK* gene in common polymorphisms with a MAF of ≥1% in 1000 Genomes Project (48) comprises 406 SNPs for African, 271 for European, 278 for Asian, and 277 for American continental populations (49). The *CLOCK* gene, as part of CLOCK(NPAS2)/ARNTL complex regulates rhythmic transcription of clock-controlled genes (CCG) in several tissues, including at least 15% of mammalian transcripts many of which gene expression patterns are disrupted in MDD (11, 19, 50, 51). The *CLOCK* machinery could provide mechanisms for control of circadian gene expression and responsivity to stimuli on cellular levels influencing activity of brain structures which control emotions and behavior (47). Furthermore, the function of *CLOCK* protein as a transcription factor and histone acetyltransferase also implies that genetic and epigenetic variations could contribute to physiological changes possibly leading to altered susceptibility of psychiatric disorders including depression (49). *CLOCK* in the mammalian brain is expressed in several brain structures beyond the master clock, the nucleus suprachiasmaticus, including the cortex (52), and molecular and behavioral studies support that *CLOCK* gene plays an important role in neuronal function involved in the regulation of several pathways implicated in psychiatric disorders and its genetic manipulation leads to marked changes in neurotransmitter activity and behavior. For example, *CLOCK* regulates expression of neurogenic transcription factors influencing the differentiation of adult neural stem cells in mice (53), controls transcription of tyrosine hydroxylase and cholecystokinin and other regulators of monoaminerg transmission (54), and disruption of *CLOCK* gene function leads to alterations in glutamatergic and GABAergic signaling (49, 55). Animal experiments on *CLOCK* gene in psychiatric condition-related behaviors have shown biological plausibility and promising findings (3), while in humans *CLOCK* variations have been implicated in susceptibility to phenotypes of common psychiatric disorders including autism spectrum disorders, schizophrenia, attention deficit/hyperactivity disorder, substance use disorder, major depressive disorder, bipolar disorder, and anxiety (49).

Several studies suggested an association between *CLOCK* and mood disorders, however, results are inconsistent in case of unipolar depression (56, 57). Previous studies employing a candidate variant approach focused only on a few variants and mainly on rs1801260, speculated to affect mRNA, which has been found to be associated with evening preference and a

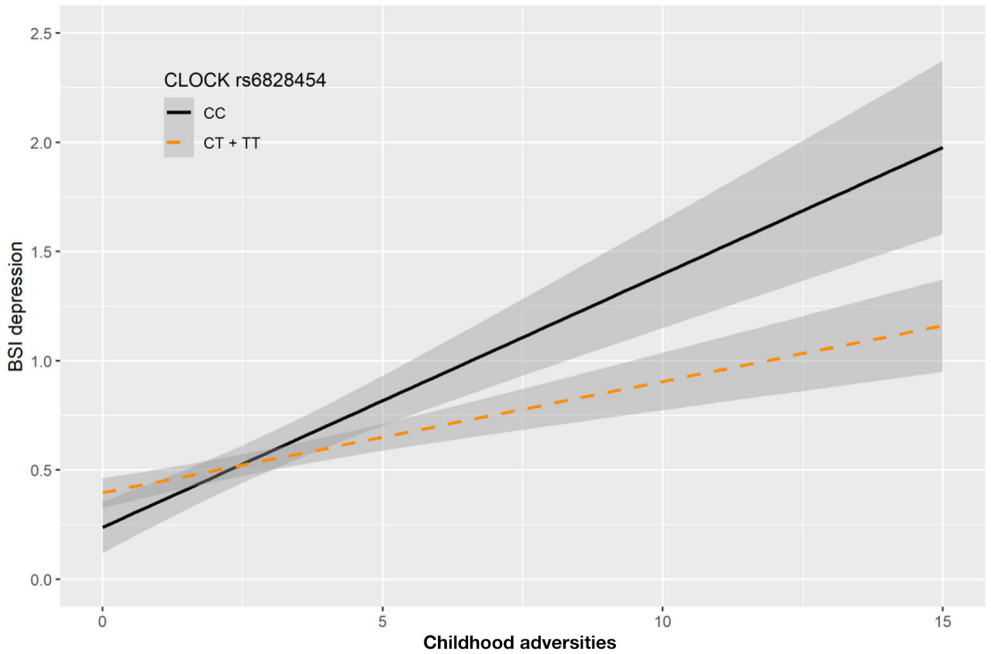

FIGURE 4 | Linear regression indicated a significant interaction between top SNP rs6828454 of a clump containing 9 SNPs in the *CLOCK* gene and exposure to childhood adversities on current depression symptoms (BSI-dep) in the recessive model, with the minor C allele being a risk allele. Linear regression indicated a significant interaction between *CLOCK* rs6828454 genotype and childhood adverse life events (CHA) on current depression scores according to the recessive model as top SNP ($p = 0.0006$, FDR $Q = 0.0118$). Homozygous presence of the minor C allele was associated with higher depression scores in subjects exposed to more severe childhood adverse events conveying a risk effect. On the vertical axis weighted depression (BSI-Dep) scores are shown. The horizontal axis shows childhood adverse life events (CHA) as measured by an instrument derived from the CTQ (30). BSI-Brief symptom inventory. Gray shading denotes 95%CI.

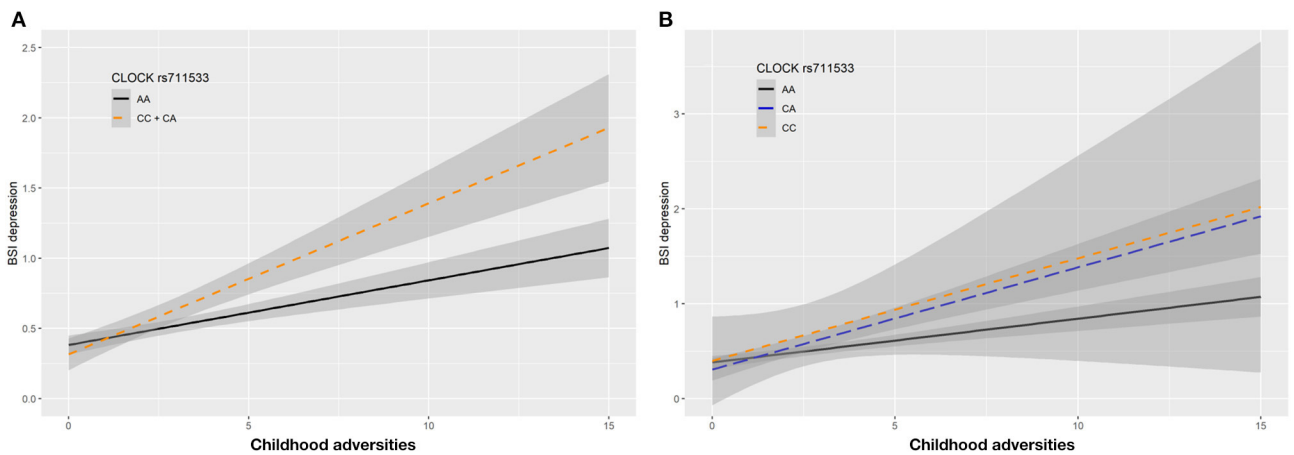

FIGURE 5 | Linear regression indicated a significant interaction between top SNP rs711533 of a clump containing 9 SNPs in the *CLOCK* gene and exposure to childhood adversity (CHA) on current depression symptoms (BSI-Dep) in the dominant **(A)** and additive **(B)** models, with the minor C allele being a risk allele. Linear regression indicated a significant interaction between *CLOCK* rs711533 genotype and childhood adverse life events (CHA) on current depression scores according to the dominant and additive models as top SNP ($p = 0.0006$, FDR $Q = 0.0118$). Presence of the minor C allele was associated with higher depression scores in subjects exposed to more severe childhood adverse events conveying a risk effect. On the vertical axis weighted depression (BSI-Dep) scores are shown. The horizontal axis shows childhood adverse life events (CHA) as measured by an instrument derived from the CTQ (30). BSI-Brief symptom inventory. Gray shading denotes 95%CI.

substantial, 10–44-min delay in preferred timing for activity and sleep in healthy C carriers (58). In some studies rs1801260 has been shown to be associated with clinical features mostly in case of bipolar disorder, especially with sleep problems including increased occurrence of lifetime and episode-related insomnia (59), higher recurrence of initial, middle and late insomnia in

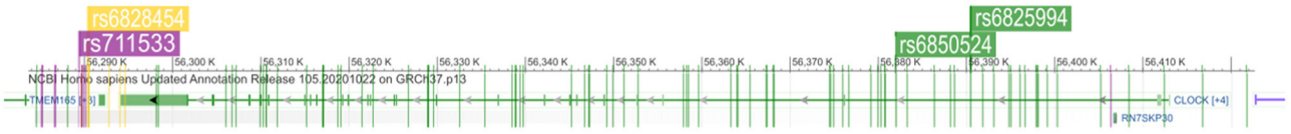

FIGURE 6 | Genomic location of significant SNPs identified in the clumping procedure, in the analysis of variation in *CLOCK* in interaction with recent life events and early childhood adversities on current depressive symptoms. In interaction with recent life events (RLE), our analyses identified one clump containing 94 SNPs with top SNPs rs6825994 and rs6850524 (for dominant and additive models, respectively) on current depressive symptoms (BSI-Dep), marked in green. In interaction with childhood adversities (CHA), two clumps, each with 9 SNPs and top SNPs rs6828454 and rs711533 were identified on current depression symptoms which are shown in yellow and purple, respectively. UCSC Genome Browser on Human Feb. 2009 (GRCh37/hg19) was used to visualize the location of polymorphisms on *CLOCK* gene.

both MDD and BD patients (60), number and recurrence of manic episodes (17), and appetite disturbances in women (49), but a meta-analysis could not confirm an association between rs1801260 and either unipolar or bipolar mood disorder (61). These findings suggest that this variant is not associated with mood symptoms itself but only with certain, mainly sleep-related symptoms accompanying mood disorders. Other, less numerous studies focused on other *CLOCK* variants, with a nominally significant relationship between rs11932595 and SSRI efficacy, rs534654 and weight loss and rs12504300 and rs3825148 and SSRI side effects including nausea, constipation and vomiting in a Han Chinese population (11).

In light of the above contradictory findings and the general lack of robust and replicable positive associations between candidate *CLOCK* SNPs and depression, our present findings, investigating variation along the *CLOCK* gene corroborate previous reports suggesting that *CLOCK* does not have a significant direct main effect on lifetime depression or current depressive symptoms (61, 62).

CLOCK Variation Mediates the Effect of Childhood Adversities and Recent Negative Life Events on Current Depressive Symptoms

More importantly, in spite of a lack of a main effect of *CLOCK* variation on depressive phenotypes, we did detect a robust effect of several clumps of SNPs on current depressive symptoms in interaction with both distal, early childhood, and proximal, recent stressors, which is in line with our paradigm postulating that the majority of genes impact depression by increasing susceptibility toward the negative effects of stress rather than exerting a direct effect (16, 21).

According to the social Zeitgeber theory, mood disorders are triggered by life stressors disrupting normal routines and circadian rhythms thus altering mood and biological rhythms (63). Several depression-relevant brain functions are controlled simultaneously by the circadian clock and stress response systems which are themselves closely connected (7). The relationship between stress and the circadian rhythm regulation is bidirectional, with both circadian rhythms impacting stress response and the effects of stress impacting regulation of circadian rhythms. Diurnal cycles of glucocorticoids, regulating various stress response-related physiological and behavioral responses, are one of the most prominent endocrine manifestations of circadian rhythms and themselves play a role in synchronizing peripheral and circadian oscillators with promoters of several clock genes containing glucocorticoid responsive elements (GREs) (7, 64, 65). Furthermore, many stress-inducible genes are also under control of the circadian clock (7). The circadian clock and stress response systems show an extensive overlap and regulate multiple systems which control cognition, affect, reward processing, and other systems and functions implicated in depression (7), suggesting that modulation of glucocorticoid-mediated stress response may constitute a common mechanism by which circadian clock affects mood disorders (19). Animal studies have shown that chronic mild stress led to anhedonic behavior associated with disturbed diurnal oscillation in circadian gene expression and higher *Clock* expression in the basolateral amygdala (66) and reduced *CLOCK* protein levels in the prefrontal cortex (67). As variants of *CLOCK* gene show differential transcriptional response to glucocorticoids (19), it is possible that variation in the *CLOCK* gene may mediate the effects of stress on the development of depressive symptoms.

Although only a few human studies took stressors into consideration when investigating the effects of *CLOCK* variants, some both in healthy and depressed subjects reported that, similar to our findings, the impact of the investigated variants was detectable only in those exposed to some type of stress. In healthy subjects, presence of the C allele of rs1801260 has been associated with a greater disruption of sleep patterns following stressful life events, and a few studies in mood disorder patients found that this variant was associated with sleep change only in case of prior stressful experiences (10) concluding that environmental stress may increase vulnerability to circadian rhythm disruption (10) and suggesting an interaction between *CLOCK* variants in shaping individual risk for deleterious effects of environmental stress including depression (47). In another study in bipolar disorder patients, a significant interaction between *CLOCK* variants and early stress exposure on hopelessness and suicide in BP patients was reported (47). In a non-clinical Chinese population, G allele of rs11932595 was associated with altered sleep duration only in those exposed to high job stress, but showed no effect in case of low stress, while AA carriers showed less daytime dysfunction under low stress and more in high stress

conditions compared to G carriers suggesting not only a gene-environment interaction effect, but also the role of the *CLOCK* as both a vulnerability and resilience gene with both positive and negative effects depending on stress (68).

Our present findings showing that *CLOCK* variation increases depressive symptoms only in those exposed to either early childhood adversities or recent stressful life events extend research suggesting that *CLOCK* gene is involved in the development of depressive symptoms by increasing vulnerability toward the disruptive effects of stress. More importantly, we found that both distal, early stressors, with a diathesis-forming etiological role, and recent negative life events, proximal stressors with a triggering role, interacted with *CLOCK* variation on severity of actual depressive symptoms. The exact mechanisms and phenotypes through which *CLOCK* in interaction with stress influences risk or severity of depression needs further study, but it has been suggested that *CLOCK* could hypothetically directly influence neural activity in brain structures playing a key role in generation and control of emotions and affect thus biasing depressive cognition in depression and resilience to detrimental effects of exposure to early stress (10, 69) and the interplay between *CLOCK* variation could influence the effects of stress on the central nervous system determining both vulnerability to or expression of phenotypes related to depression (7).

In silico Characterization and Functional Prediction of Top SNPs and SNPs in the Significant Clumps

In the final step of our analysis we carried out *in silico* characterization and functional prediction for the SNPs in the three clumps significantly impacting current depressive symptoms in interaction with either childhood adversities or current stressors. The search yielded several relevant findings (**Supplementary Table 7**). Notably, two SNPs, rs28448438 and rs28463765 are located in the transcriptional binding site of the *CLOCK* gene, while a third SNP, rs726967 is located in the microRNA-binding site possibly influencing transcriptional activity.

Of the four top SNPs, rs6850524, which significantly interacted with recent life events on current depressive symptoms has previously been associated with depression-relevant phenotypes including susceptibility to bipolar disorder (70), sleep quality (71), and risk of sleep disorders (72), which indicates its involvement in affective disorders and their symptomatology. This variant has also been found to be associated with somatic conditions including risk of obesity (73, 74), and non-alcoholic fatty liver disease (75) which may be clinically relevant considering the frequent comorbidity of the above illnesses with depression (76–78).

Among SNPs in the clumps significantly interacting with recent life events symptoms, rs6832769 has been reported to be related to emotional prosociality and agreeableness (79, 80), with response and remission with fluvoxamine in MDD but was not found to be associated with affective disorders (81–84), whereas rs2412646 has been associated with depression-comorbid alcohol use disorders and susceptibility to restless leg syndrome in schizophrenic patients (85–87). Several SNPs in the clumps significantly interacting with recent life events including rs11931061, rs3817444, and rs726967 have been reported to be associated with ADHD (88) or sleep problems, including rs11735267 showing a nominal association with late insomnia (70), and rs6853192 with longer sleep duration in general community sample of African Americans (89). In addition, several SNPs in the clumps interacting with recent life events were associated with risk of somatic conditions, including rs10002541 with abdominal obesity and diabetes (74), rs11726609 with BMI in African Americans (89), rs11133399 with gastric cancer overall survival and recurrence free survival and also a primary risk factor contributing to prognosis (90), rs4865010 with essential hypertension and coronary artery disease (91) and in men with hypertonia with testosterone levels corresponding to an androgenic deficient state itself a risk factor for cardiovascular complications (92), and rs6811520 with myocardial infarction (93), multiple sclerosis (94), male infertility (95).

In case of SNPs in clumps significantly interacting with childhood adversities on current depression, rs3749473 was associated with cognitive aging (96), rs6858749 with habitual sleep duration in interaction with protein intake (97, 98), and rs534654 with ADHD (88), disrupted sleep-wake cycles in BD (70), and development of depression (11).

Possible Clinical Implications of *CLOCK* Variation in Interaction With Early and Recent Stress in Depression

The association of stress and circadian vulnerability especially in case of mood disorders has significant clinical relevance in case of stress chronotherapy. As circadian disruptions and frequent stress exposure—especially in interaction—amplify the risks of development of a wide range of health-related disorders including mood disorders, simultaneous reduction of circadian and classical stressors including strategies stabilizing endogenous rhythms to counteract circadian perturbations would be beneficial. While the majority of such practices are hardly feasible in the modern lifestyle, there are several effective therapies for depression which act via modulating circadian parameters, including bright light, sleep deprivation, and phase resetting paradigms (12, 19, 99, 100) which improve symptoms within hours (101, 102). Scheduled meals or activity, or specific interventions such as social rhythm therapy to enhance circadian alignment of peripheral and central clocks also offer means of reducing circadian stress to prevent or improve mood disorder symptoms (103). Our study identifying an interaction between *CLOCK* variation and both childhood and recent stressors in the development of depressive symptoms could in the future help predict those at a higher risk for development of depression in case of circadian disruption, and also those who would benefit from chronotherapies for depression.

Furthermore, antidepressants including SSRIs, SNRIs and agomelatine also directly impact circadian rhythms as part

of their effects improving depressive symptoms (6, 104–106). For example, fluoxetine normalizes disrupted light-induced entrainment, fragmented ultradian rhythms and altered hippocampal *CLOCK* expression in animal models of depression (107), while agomelatine, a melatonergic antidepressant is hypothesized to resynchronize disrupted circadian rhythms (108, 109). It has also been suggested that *CLOCK* expression may predict efficacy of antidepressants exerting their effects via influencing circadian mechanisms (68). Thus, understanding the role of *CLOCK* variation in depression may help identify new targets for pharmacological treatment possibly via modulating the impact of stress on brain function (7) as well as prediction of efficacy especially in subtypes of depression aiding precision therapy.

Limitations

When interpreting the impact of our findings, several limitations of our study must also be taken into account. First, both early childhood adversities and recent life events occurring in the past year were assessed retrospectively, and based on self-report of the subjects but not ascertained by other informants, and are thus subject to recall and reporting biases. Second, both lifetime depression and current depression severity was similarly based on a self-reported measure. Third, our population sample was relatively small, consisting approximately two thirds of female subjects, and limited to European white participants. Fourth, scoring childhood adversity and counting the number of recent negative life events does not take into consideration the differing severity and subjective impact of individual life events. Nevertheless, our study also has several strengths, including considering several hundred variants along the *CLOCK* gene with a clumping method rather than individual, hypothesis-based candidate SNPs, employing a dimensional approach capture current depression symptom severity, and using a GxE paradigm with two etiologically different types of stressors.

CONCLUSION

In conclusion, the results of our study investigating variation along the *CLOCK* gene with a linkage disequilibrium-based clumping of SNPs support the role of *CLOCK* in the background of depressive symptoms but only in association with recent stress and early adversities, underlining not only the depressogenic importance of circadian disruption but also that it acts in interaction with both early, etiological, and recent, trigger-like stressors. Thus, our findings strengthen the rationale of looking at the circadian system in search of new molecular targets for pharmacological interventions, and by specifically reporting that effects in clock variation are only observable in interaction with stress also helps to explain previous lack of consistent findings.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: https://figshare.com/articles/dataset/CLOCK_stress_depression/14258567.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Scientific and Research Ethics Committee of the Medical Research Council, Budapest, Hungary. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

XG, DG, DB, GJ, and GB: conceptualization. DB, NE, SS, and PP: methodology. XG, NE, DB, DT, and ZG: data collection. DG, ZK, SS, PP, DB, NE, DT, and ZG: data analysis. XG, DG, SS, ZK, ZG, DT, and DB: writing—original draft preparation. XG, DG, NE, ZG, DT, DB, ZK, SS, PP, GJ, and GB: writing—review and editing. All authors contributed to the article 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/fpsyt.2021.687487/full#supplementary-material

Supplementary Table 1 | Main effect of *CLOCK* SNPs in the NewMood database surviving quality control on lifetime depression (DEP). Results of logistic

regression models and quality control steps are shown. While there were nominally significant SNPs, significant clumps could not be identified.

Supplementary Table 2 | Main effect of *CLOCK* SNPs in the NewMood database surviving quality control on current depressive symptoms (BSI-DEP). Results of linear regression models and quality control steps are shown. While there were nominally significant SNPs, significant clumps could not be identified.

Supplementary Table 3 | *CLOCK* SNPs in the NewMood database surviving quality control, in interaction with recent life events (RLE) on current depressive symptoms (BSI-DEP). Results of linear regression models and quality control steps are shown. SNPs in significant clumps are marked in red, top SNP of the clump is marked in bold red. Results for additive, dominant, and recessive models are shown.

Supplementary Table 4 | *CLOCK* SNPs in the NewMood database surviving quality control, in interaction with childhood adversities (CHA) on current depressive symptoms (BSI-DEP). Results of linear regression models and quality control steps are shown. SNPs in significant clumps are marked in red, top SNP of the clump is marked in bold red. Results for additive, dominant, and recessive models are shown.

Supplementary Table 5 | Main effect of *CLOCK* SNPs in the NewMood database surviving quality control on recent negative life events (RLE). Results of linear regression models are shown. There were no nominally significant SNPs, significant clumps could not be identified.

Supplementary Table 6 | Main effect of *CLOCK* SNPs in the NewMood database surviving quality control on childhood adversities (CHA). Results of linear regression models are shown. There were no nominally significant SNPs, significant clumps could not be identified.

Supplementary Table 7 | *In silico* characterization and functional prediction of SNPs in the clumps significantly interacting with recent life events (RLE) and childhood adversities (CHA). Top SNP of each clump is marked in bold red.

REFERENCES

1. Roenneberg T, Wirz-Justice A, Merrow M. Life between clocks: daily temporal patterns of human chronotypes. *J Biol Rhythms.* (2003) 18:80–90. doi: 10.1177/0748730402239679
2. Takahashi JS, Hong HK, Ko CH, Mcdearmon EL. The genetics of mammalian circadian order and disorder: implications for physiology and disease. *Nat Rev Genet.* (2008) 9:764–75. doi: 10.1038/nrg2430
3. Mendoza J, Vanotti G. Circadian neurogenetics of mood disorders. *Cell Tissue Res.* (2019) 377:81–94. doi: 10.1007/s00441-019-03033-7
4. Dibner C, Schibler U, Albrecht U. The mammalian circadian timing system: organization and coordination of central and peripheral clocks. *Annu Rev Physiol.* (2010) 72:517–49. doi: 10.1146/annurev-physiol-021909-135821
5. Hansen KF, Sakamoto K, Obrietan K. MicroRNAs: a potential interface between the circadian clock and human health. *Genome Med.* (2011) 3:10. doi: 10.1186/gm224
6. Pitsillou E, Liang J, Hung A, Karagiannis TC. The circadian machinery links metabolic disorders and depression: a review of pathways, proteins and potential pharmacological interventions. *Life Sci.* (2021) 265:118809. doi: 10.1016/j.lfs.2020.118809
7. Landgraf D, Mccarthy MJ, Welsh DK. Circadian clock and stress interactions in the molecular biology of psychiatric disorders. *Curr Psychiatry Rep.* (2014) 16:483. doi: 10.1007/s11920-014-0483-7
8. Van Cauter E, Turek FW. Depression: a disorder of timekeeping? *Perspect Biol Med.* (1986) 29:510–519. doi: 10.1353/pbm.1986.0033
9. Reinink E, Bouhuys AL, Gordijn MC, Van Den Hoofdakker RH. Prediction of the antidepressant response to total sleep deprivation of depressed patients: longitudinal versus single day assessment of diurnal mood variation. *Biol Psychiatry.* (1993) 34:471–81. doi: 10.1016/0006-3223(93)90238-9
10. Antypa N, Mandelli L, Nearchou FA, Vaiopoulos C, Stefanis CN, Serretti A, et al. The 3111T/C polymorphism interacts with stressful life events to influence patterns of sleep in females. *Chronobiol Int.* (2012) 29:891–7. doi: 10.3109/07420528.2012.699380
11. Ma HY, Liu ZF, Xu YF, Hu XD, Sun N, Li XR, et al. The association study of CLOCK gene polymorphisms with antidepressant effect in Chinese with major depressive disorder. *Per Med.* (2019) 16:115–22. doi: 10.2217/pme-2018-0123
12. Mcclung CA. Circadian genes, rhythms and the biology of mood disorders. *Pharmacol Ther.* (2007) 114:222–32. doi: 10.1016/j.pharmthera.2007.02.003
13. Vadnie CA, Mcclung CA. Circadian rhythm disturbances in mood disorders: insights into the role of the suprachiasmatic nucleus. *Neural Plast.* (2017) 2017:1504507. doi: 10.1155/2017/1504507
14. Walker WH III, Walton JC, Devries AC, Nelson RJ. Circadian rhythm disruption and mental health. *Transl Psychiatry.* (2020) 10:28. doi: 10.1038/s41398-020-0694-0
15. Sullivan PF, Neale MC, Kendler KS. Genetic epidemiology of major depression: review and meta-analysis. *Am J Psychiatry.* (2000) 157:1552–62. doi: 10.1176/appi.ajp.157.10.1552
16. Gonda X, Petschner P, Eszlari N, Baksa D, Edes A, Antal P, et al. Genetic variants in major depressive disorder: from pathophysiology to therapy. *Pharmacol Therap.* (2019) 194:22–43. doi: 10.1016/j.pharmthera.2018.09.002
17. Benedetti F, Serretti A, Colombo C, Barbini B, Lorenzi C, Campori E, et al. Influence of CLOCK gene polymorphism on circadian mood fluctuation and illness recurrence in bipolar depression. *Am J Med Genet B Neuropsychiatr Genet.* (2003) 123B:23–6. doi: 10.1002/ajmg.b.20038
18. Mccarthy MJ, Nievergelt CM, Kelsoe JR, Welsh DK. A survey of genomic studies supports association of circadian clock genes with bipolar disorder spectrum illnesses and lithium response. *PLoS ONE.* (2012) 7:e32091. doi: 10.1371/journal.pone.0032091
19. Shi SQ, White MJ, Borsetti HM, Pendergast JS, Hida A, Ciarleglio CM, et al. Molecular analyses of circadian gene variants reveal sex-dependent links between depression and clocks. *Transl Psychiatry.* (2016) 6:e748. doi: 10.1038/tp.2016.9
20. Bagdy G, Juhasz G, Gonda X. A new clinical evidence-based gene-environment interaction model of depression. *Neuropsychopharmacol Hung.* (2012) 14:213–20. doi: 10.5706/nph201212001
21. Gonda X, Hullam G, Antal P, Eszlari N, Petschner P, Hokfelt TG, et al. Significance of risk polymorphisms for depression depends on stress exposure. *Sci Rep.* (2018) 8:3946. doi: 10.1038/s41598-018-22221-z
22. Garriock HA, Kraft JB, Shyn SI, Peters EJ, Yokoyama JS, Jenkins GD, et al. A genomewide association study of citalopram response in major depressive disorder. *Biol Psychiatry.* (2010) 67:133–8. doi: 10.1016/j.biopsych.2009.08.029
23. Terracciano A, Tanaka T, Sutin AR, Sanna S, Deiana B, Lai S, et al. Genome-wide association scan of trait depression. *Biol Psychiatry.* (2010) 68:811–7. doi: 10.1016/j.biopsych.2010.06.030
24. International Schizophrenia C, Purcell SM, Wray NR, Stone JL, Visscher PM, O'donovan MC, et al. Common polygenic variation contributes to risk of schizophrenia and bipolar disorder. *Nature.* (2009) 460:748–52. doi: 10.1038/nature08185
25. Trivedi MH, Rush AJ, Wisniewski SR, Nierenberg AA, Warden D, Ritz L, et al. Evaluation of outcomes with citalopram for depression using measurement-based care in STAR*D: implications for clinical practice. *Am J Psychiatry.* (2006) 163:28–40. doi: 10.1176/appi.ajp.163.1.28
26. Lazary J, Lazary A, Gonda X, Benko A, Molnar E, Juhasz G, et al. New evidence for the association of the serotonin transporter gene (SLC6A4) haplotypes, threatening life events, depressive phenotype. *Biol Psychiatry.* (2008) 64:498–504. doi: 10.1016/j.biopsych.2008.03.030
27. Juhasz G, Chase D, Pegg E, Downey D, Toth ZG, Stones K, et al. CNR1 gene is associated with high neuroticism and low agreeableness and interacts with recent negative life events to predict current depressive symptoms. *Neuropsychopharmacology.* (2009) 34:2019–27. doi: 10.1038/npp.2009.19

28. Juhasz G, Dunham JS, Mckie S, Thomas E, Downey D, Chase D, et al. The CREB1-BDNF-NTRK2 pathway in depression: multiple gene-cognition-environment interactions. *Biol Psychiatry.* (2011) 69:762–71. doi: 10.1016/j.biopsych.2010.11.019
29. Derogatis LR. *BSI: Brief Symptom Inventory: Administration, Scoring, and Procedures Manual Minneapolis: National Computer Systems*, Minneapolis, MN, (1993).
30. Bernstein DP, Fink L, Handelsman L, Foote J, Lovejoy M, Wenzel K, et al. Initial reliability and validity of a new retrospective measure of child abuse and neglect. *Am J Psychiatry.* (1994) 151:1132–6. doi: 10.1176/ajp.151.8.1132
31. Brugha T, Bebbington P, Tennant C, Hurry J. The list of threatening experiences: a subset of 12 life event categories with considerable long-term contextual threat. *Psychol Med.* (1985) 15:189–94. doi: 10.1017/S003329170002105X
32. Brugha TS, Cragg D. The list of threatening experiences: the reliability and validity of a brief life events questionnaire. *Acta Psychiatr Scand.* (1990) 82:77–81. doi: 10.1111/j.1600-0447.1990.tb01360.x
33. Freeman B, Smith N, Curtis C, Huckett L, Mill J, Craig IW. DNA from buccal swabs recruited by mail: evaluation of storage effects on long-term stability and suitability for multiplex polymerase chain reaction genotyping. *Behav Genet.* (2003) 33:67–72. doi: 10.1023/A:1021055617738
34. Eszlari N, Millinghoffer A, Petschner P, Gonda X, Baksa D, Pulay AJ, et al. Genome-wide association analysis reveals KCTD12 and miR-383-binding genes in the background of rumination. *Transl Psychiatry.* (2019) 9:119. doi: 10.1038/s41398-019-0454-1
35. R Core Team (2013). *R: A Language and Environment for Statistical Computing*. Vienna: R Foundation for Statistical Computing. Available online at: http://www.R-project.org/
36. Gonda X, Gyorik D, Eszlari N, Gal Z, Torok D, Baksa D, et al. *"CLOCK, Stress, Depression"*. (FigShare) (2021).
37. Rusting CL, Larsen RJ. Diurnal patterns of unpleasant mood: associations with neuroticism, depression, and anxiety. *J Pers.* (1998) 66:85–103. doi: 10.1111/1467-6494.00004
38. Emens J, Lewy A, Kinzie JM, Arntz D, Rough J. Circadian misalignment in major depressive disorder. *Psychiatry Res.* (2009) 168:259–61. doi: 10.1016/j.psychres.2009.04.009
39. Lamont EW, Legault-Coutu D, Cermakian N, Boivin DB. The role of circadian clock genes in mental disorders. *Dialogues Clin Neurosci.* (2007) 9:333–42. doi: 10.31887/DCNS.2007.9.3/elamont
40. Bedrosian TA, Nelson RJ. Timing of light exposure affects mood and brain circuits. *Transl Psychiatry.* (2017) 7:e1017. doi: 10.1038/tp.2016.262
41. Lee A, Myung SK, Cho JJ, Jung YJ, Yoon JL, Kim MY. Night shift work and risk of depression: meta-analysis of observational studies. *J Korean Med Sci.* (2017) 32:1091–96. doi: 10.3346/jkms.2017.32.7.1091
42. Li JZ, Bunney BG, Meng F, Hagenauer MH, Walsh DM, Vawter MP, et al. Circadian patterns of gene expression in the human brain and disruption in major depressive disorder. *Proc Natl Acad Sci USA.* (2013) 110:9950–5. doi: 10.1073/pnas.1305814110
43. Oster H, Damerow S, Kiessling S, Jakubcakova V, Abraham D, Tian J, et al. The circadian rhythm of glucocorticoids is regulated by a gating mechanism residing in the adrenal cortical clock. *Cell Metab.* (2006) 4:163–73. doi: 10.1016/j.cmet.2006.07.002
44. Harbour VL, Robinson B, Amir S. Variations in daily expression of the circadian clock protein, PER2, in the rat limbic forebrain during stable entrainment to a long light cycle. *J Mol Neurosci.* (2011) 45:154–61. doi: 10.1007/s12031-010-9469-z
45. Mccarthy MJ, Welsh DK. Cellular circadian clocks in mood disorders. *J Biol Rhythms.* (2012) 27:339–52. doi: 10.1177/0748730412456367
46. Benedetti F, Terman M. Much ado about.a moody clock. *Biol Psychiatry.* (2013) 74:236–7. doi: 10.1016/j.biopsych.2013.05.037
47. Benedetti F, Riccaboni R, Dallaspezia S, Locatelli C, Smeraldi E, Colombo C. Effects of CLOCK gene variants and early stress on hopelessness and suicide in bipolar depression. *Chronobiol Int.* (2015) 32:1156–61. doi: 10.3109/07420528.2015.1060603
48. Sudmant PH, Rausch T, Gardner EJ, Handsaker RE, Abyzov A, Huddleston J, et al. An integrated map of structural variation in 2,504 human genomes. *Nature.* (2015) 526:75–81. doi: 10.1038/nature15394
49. Schuch JB, Genro JP, Bastos CR, Ghisleni G, Tovo-Rodrigues L. The role of CLOCK gene in psychiatric disorders: evidence from human and animal research. *Am J Med Genet B Neuropsychiatr Genet.* (2018) 177:181–98. doi: 10.1002/ajmg.b.32599
50. Doi M, Hirayama J, Sassone-Corsi P. Circadian regulator CLOCK is a histone acetyltransferase. *Cell.* (2006) 125:497–508. doi: 10.1016/j.cell.2006.03.033
51. Hirayama J, Sahar S, Grimaldi B, Tamaru T, Takamatsu K, Nakahata Y, et al. CLOCK-mediated acetylation of BMAL1 controls circadian function. *Nature.* (2007) 450:1086–90. doi: 10.1038/nature06394
52. Abe H, Honma S, Namihira M, Masubuchi S, Ikeda M, Ebihara S, et al. Clock gene expressions in the suprachiasmatic nucleus and other areas of the brain during rhythm splitting in CS mice. *Brain Res Mol Brain Res.* (2001) 87:92–9. doi: 10.1016/S0169-328X(00)00295-3
53. Kimiwada T, Sakurai M, Ohashi H, Aoki S, Tominaga T, Wada K. Clock genes regulate neurogenic transcription factors, including NeuroD1, and the neuronal differentiation of adult neural stem/progenitor cells. *Neurochem Int.* (2009) 54:277–85. doi: 10.1016/j.neuint.2008.12.005
54. Sidor MM, Spencer SM, Dzirasa K, Parekh PK, Tye KM, Warden MR, et al. Daytime spikes in dopaminergic activity drive rapid mood-cycling in mice. *Mol Psychiatry.* (2015) 20:1479–80. doi: 10.1038/mp.2015.8
55. Mcclung CA, Sidiropoulou K, Vitaterna M, Takahashi JS, White FJ, Cooper DC, et al. Regulation of dopaminergic transmission and cocaine reward by the Clock gene. *Proc Natl Acad Sci USA.* (2005) 102:9377–81. doi: 10.1073/pnas.0503584102
56. Kishi T, Kitajima T, Ikeda M, Yamanouchi Y, Kinoshita Y, Kawashima K, et al. Association study of clock gene (CLOCK) and schizophrenia and mood disorders in the Japanese population. *Eur Arch Psychiatry Clin Neurosci.* (2009) 259:293–7. doi: 10.1007/s00406-009-0869-4
57. Dmitrzak-Weglarz MP, Pawlak JM, Maciukiewicz M, Moczko J, Wilkosc M, Leszczynska-Rodziewicz A, et al. Clock gene variants differentiate mood disorders. *Mol Biol Rep.* (2015) 42:277–88. doi: 10.1007/s11033-014-3770-9
58. Katzenberg D, Young T, Finn L, Lin L, King DP, Takahashi JS, et al. A CLOCK polymorphism associated with human diurnal preference. *Sleep.* (1998) 21:569–76. doi: 10.1093/sleep/21.6.569
59. Benedetti F, Dallaspezia S, Fulgosi MC, Lorenzi C, Serretti A, Barbini B, et al. Actimetric evidence that CLOCK 3111 T/C SNP influences sleep and activity patterns in patients affected by bipolar depression. *Am J Med Genet B Neuropsychiatr Genet.* (2007) 144B:631–5. doi: 10.1002/ajmg.b.30475
60. Serretti A, Benedetti F, Mandelli L, Lorenzi C, Pirovano A, Colombo C, et al. Genetic dissection of psychopathological symptoms: insomnia in mood disorders and CLOCK gene polymorphism. *Am J Med Genet B Neuropsychiatr Genet.* (2003) 121B:35–8. doi: 10.1002/ajmg.b.20053
61. Kishi T, Yoshimura R, Fukuo Y, Kitajima T, Okochi T, Matsunaga S, et al. The CLOCK gene and mood disorders: a case-control study and meta-analysis. *Chronobiol Int.* (2011) 28:825–33. doi: 10.3109/07420528.2011.609951
62. Calati R, Gaspar-Barba E, Yukler A, Serretti A. T3111C clock single nucleotide polymorphism and mood disorders: a meta-analysis. *Chronobiol Int.* (2010) 27:706–21. doi: 10.3109/07420521003681480
63. Grandin LD, Alloy LB, Abramson LY. The social zeitgeber theory, circadian rhythms, and mood disorders: review and evaluation. *Clin Psychol Rev.* (2006) 26:679–94. doi: 10.1016/j.cpr.2006.07.001
64. Nader N, Chrousos GP, Kino T. Interactions of the circadian CLOCK system and the HPA axis. *Trends Endocrinol Metab.* (2010) 21:277–86. doi: 10.1016/j.tem.2009.12.011
65. Kalsbeek A, Van Der Spek R, Lei J, Endert E, Buijs RM, Fliers E. Circadian rhythms in the hypothalamo-pituitary-adrenal (HPA) axis. *Mol Cell Endocrinol.* (2012) 349:20–9. doi: 10.1016/j.mce.2011.06.042
66. Savalli G, Diao W, Schulz S, Todtova K, Pollak DD. Diurnal oscillation of amygdala clock gene expression and loss of synchrony in a mouse model of depression. *Int J Neuropsychopharmacol.* (2014) 18:pyu095. doi: 10.1093/ijnp/pyu095
67. Calabrese F, Savino E, Papp M, Molteni R, Riva MA. Chronic mild stress-induced alterations of clock gene expression in rat prefrontal cortex: modulatory effects of prolonged lurasidone treatment. *Pharmacol Res.* (2016) 104:140–50. doi: 10.1016/j.phrs.2015.12.023
68. Li Y, Cao Z, Wu S, Wang C, He S, Dong Y, et al. Association of job stress, CLOCK gene polymorphism and their interaction with poor sleep quality. *J Sleep Res.* (2020) 30:e13133. doi: 10.1111/jsr.13133

69. Benedetti F, Radaelli D, Bernasconi A, Dallaspezia S, Falini A, Scotti G, et al. Clock genes beyond the clock: CLOCK genotype biases neural correlates of moral valence decision in depressed patients. *Genes Brain Behav.* (2008) 7:20–5. doi: 10.1111/j.1601-183X.2007.00312.x
70. Shi J, Wittke-Thompson JK, Badner JA, Hattori E, Potash JB, Willour VL, et al. Clock genes may influence bipolar disorder susceptibility and dysfunctional circadian rhythm. *Am J Med Genet B Neuropsychiatr Genet.* (2008) 147B:1047–55. doi: 10.1002/ajmg.b.30714
71. Shi L, Liu Y, Jiang T, Yan P, Cao F, Chen Y, et al. Relationship between mental health, the CLOCK gene, and sleep quality in surgical nurses: a cross-sectional study. *Biomed Res Int.* (2020) 2020:4795763. doi: 10.1155/2020/4795763
72. Ning L, Shi L, Tao N, Li R, Jiang T, Liu J. Effects of occupational stress and circadian CLOCK gene polymorphism on sleep quality of oil workers in Xinjiang, China. *Med Sci Monit.* (2020) 26:e924202. doi: 10.12659/MSM.924202
73. Sookoian S, Gemma C, Gianotti TF, Burgueno A, Castano G, Pirola CJ. Genetic variants of Clock transcription factor are associated with individual susceptibility to obesity. *Am J Clin Nutr.* (2008) 87:1606–15. doi: 10.1093/ajcn/87.6.1606
74. Ye D, Cai S, Jiang X, Ding Y, Chen K, Fan C, et al. Associations of polymorphisms in circadian genes with abdominal obesity in Chinese adult population. *Obes Res Clin Pract.* (2016) 10(Suppl. 1):S133–41. doi: 10.1016/j.orcp.2016.02.002
75. Sookoian S, Castano G, Gemma C, Gianotti TF, Pirola CJ. Common genetic variations in CLOCK transcription factor are associated with nonalcoholic fatty liver disease. *World J Gastroenterol.* (2007) 13:4242–8. doi: 10.3748/wjg.v13.i31.4242
76. Marx P, Antal P, Bolgar B, Bagdy G, Deakin B, Juhasz G. Comorbidities in the diseasome are more apparent than real: What Bayesian filtering reveals about the comorbidities of depression. *PLoS Comput Biol.* (2017) 13:e1005487. doi: 10.1371/journal.pcbi.1005487
77. Hullam G, Antal P, Petschner P, Gonda X, Bagdy G, Deakin B, et al. The UKB envirome of depression: from interactions to synergistic effects. *Sci Rep.* (2019) 9:9723. doi: 10.1038/s41598-019-46001-5
78. Soto-Angona O, Anmella G, Valdes-Florido MJ, De Uribe-Viloria N, Carvalho AF, Penninx B, et al. Non-alcoholic fatty liver disease (NAFLD) as a neglected metabolic companion of psychiatric disorders: common pathways and future approaches. *BMC Med.* (2020) 18:261. doi: 10.1186/s12916-020-01713-8
79. Terracciano A, Sanna S, Uda M, Deiana B, Usala G, Busonero F, et al. Genome-wide association scan for five major dimensions of personality. *Mol Psychiatry.* (2010) 15:647–56. doi: 10.1038/mp.2008.113
80. Ci H, Wu N, Su Y. Clock gene modulates roles of OXTR and AVPR1b genes in prosociality. *PLoS ONE.* (2014) 9:e109086. doi: 10.1371/journal.pone.0109086
81. Kishi T, Kitajima T, Ikeda M, Yamanouchi Y, Kinoshita Y, Kawashima K, et al. CLOCK may predict the response to fluvoxamine treatment in Japanese major depressive disorder patients. *Neuromolecular Med.* (2009) 11:53–57. doi: 10.1007/s12017-009-8060-7
82. Kripke DF, Nievergelt CM, Joo E, Shekhtman T, Kelsoe JR. Circadian polymorphisms associated with affective disorders. *J Circadian Rhythms.* (2009) 7:2. doi: 10.1186/1740-3391-7-2
83. Mccarthy MJ, Nievergelt CM, Shekhtman T, Kripke DF, Welsh DK, Kelsoe JR. Functional genetic variation in the Rev-Erbalpha pathway and lithium response in the treatment of bipolar disorder. *Genes Brain Behav.* (2011) 10:852–61. doi: 10.1111/j.1601-183X.2011.00725.x
84. Porcelli S, Drago A, Fabbri C, Gibiino S, Calati R, Serretti A. Pharmacogenetics of antidepressant response. *J Psychiatry Neurosci.* (2011) 36:87–113. doi: 10.1503/jpn.100059
85. Sjoholm LK, Kovanen L, Saarikoski ST, Schalling M, Lavebratt C, Partonen T. CLOCK is suggested to associate with comorbid alcohol use and depressive disorders. *J Circadian Rhythms.* (2010) 8:1. doi: 10.1186/1740-3391-8-1
86. Jung JS, Lee HJ, Cho CH, Kang SG, Yoon HK, Park YM, et al. Association between restless legs syndrome and CLOCK and NPAS2 gene polymorphisms in schizophrenia. *Chronobiol Int.* (2014) 31:838–44. doi: 10.3109/07420528.2014.914034
87. Partonen T. Clock genes in human alcohol abuse and comorbid conditions. *Alcohol.* (2015) 49:359–65. doi: 10.1016/j.alcohol.2014.08.013
88. Carpena MX, Hutz MH, Salatino-Oliveira A, Polanczyk GV, Zeni C, Schmitz M, et al. CLOCK polymorphisms in Attention-Deficit/Hyperactivity Disorder (ADHD): further evidence linking sleep and circadian disturbances and ADHD. *Genes.* (2019) 10:88. doi: 10.3390/genes10020088
89. Riestra P, Gebreab SY, Xu R, Khan RJ, Gaye A, Correa A, et al. Circadian CLOCK gene polymorphisms in relation to sleep patterns and obesity in African Americans: findings from the Jackson heart study. *BMC Genet.* (2017) 18:58. doi: 10.1186/s12863-017-0522-6
90. Chen Y, Wang D, Song Y, Zhang X, Jiao Z, Dong J, et al. Functional polymorphisms in circadian positive feedback loop genes predict postsurgical prognosis of gastric cancer. *Cancer Med.* (2019) 8:1919–29. doi: 10.1002/cam4.2050
91. Kolomeychuk S, Kurbatova I, Topchieva LV, Korneva VA, Poltorak AN, Chambers TC, et al. Association between CLOCK genetic variants and individual susceptibility to essential hypertension and coronary artery disease in Russian population. *Exp Clin Cardiol.* (2014) 20:1–17.
92. Kurbatova I, Topchieva LV, Korneva VA. The level of circulating testosterone and the blood-plasma lipid profile in men with essential Hypertension. carriers of GG genotype in relation to the−257T>G polymorphic marker of the clock gene. *Proc Karelian Res Centre Russian Acad Sci.* (2016) 6:39–48. doi: 10.17076/eb291
93. Skrlec I, Milic J, Steiner R. The impact of the circadian genes CLOCK and ARNTL on myocardial infarction. *J Clin Med.* (2020) 9:484. doi: 10.3390/jcm9020484
94. Lavtar P, Rudolf G, Maver A, Hodzic A, Starcevic Cizmarevic N, Zivkovic M, et al. Association of circadian rhythm genes ARNTL/BMAL1 and CLOCK with multiple sclerosis. *PLoS ONE.* (2018) 13:e0190601. doi: 10.1371/journal.pone.0190601
95. Hodzic A, Ristanovic M, Zorn B, Tulic C, Maver A, Novakovic I, et al. Genetic variation in circadian rhythm genes CLOCK and ARNTL as risk factor for male infertility. *PLoS ONE.* (2013) 8:e59220. doi: 10.1371/journal.pone.0059220
96. Lin CH, Lin E, Lane HY. Genetic biomarkers on age-related cognitive decline. *Front Psychiatry.* (2017) 8:247. doi: 10.3389/fpsyt.2017.00247
97. Dashti HS, Follis JL, Smith CE, Tanaka T, Cade BE, Gottlieb DJ, et al. Habitual sleep duration is associated with BMI and macronutrient intake and may be modified by CLOCK genetic variants. *Am J Clin Nutr.* (2015) 101:135–43. doi: 10.3945/ajcn.114.095026
98. Sridhar GR, Sanjana NS. Sleep, circadian dysrhythmia, obesity and diabetes. *World J Diabetes.* (2016) 7:515–22. doi: 10.4239/wjd.v7.i19.515
99. Wehr TA, Wirz-Justice A, Goodwin FK, Duncan W, Gillin JC. Phase advance of the circadian sleep-wake cycle as an antidepressant. *Science.* (1979) 206:710–3. doi: 10.1126/science.227056
100. Lewy AJ, Sack RL, Miller LS, Hoban TM. Antidepressant and circadian phase-shifting effects of light. *Science.* (1987) 235:352–4. doi: 10.1126/science.3798117
101. Fritzsche M, Heller R, Hill H, Kick H. Sleep deprivation as a predictor of response to light therapy in major depression. *J Affect Disord.* (2001) 62:207–15. doi: 10.1016/S0165-0327(00)00154-3
102. Dallaspezia S, Benedetti F. Chronobiological therapy for mood disorders. *Expert Rev Neurother.* (2011) 11:961–70. doi: 10.1586/ern.11.61
103. Koch CE, Leinweber B, Drengberg BC, Blaum C, Oster H. Interaction between circadian rhythms and stress. *Neurobiol Stress.* (2017) 6:57–67. doi: 10.1016/j.ynstr.2016.09.001
104. Abe M, Herzog ED, Block GD. Lithium lengthens the circadian period of individual suprachiasmatic nucleus neurons. *Neuroreport.* (2000) 11:3261–3264. doi: 10.1097/00001756-200009280-00042
105. Sprouse J, Braselton J, Reynolds L. Fluoxetine modulates the circadian biological clock via phase advances of suprachiasmatic nucleus neuronal firing. *Biol Psychiatry.* (2006) 60:896–9. doi: 10.1016/j.biopsych.2006.03.003
106. Germain A, Kupfer DJ. Circadian rhythm disturbances in depression. *Hum Psychopharmacol.* (2008) 23:571–85. doi: 10.1002/hup.964

107. Schaufler J, Ronovsky M, Savalli G, Cabatic M, Sartori SB, Singewald N, et al. Fluoxetine normalizes disrupted light-induced entrainment, fragmented ultradian rhythms and altered hippocampal clock gene expression in an animal model of high trait anxiety- and depression-related behavior. *Ann Med.* (2016) 48:17–27. doi: 10.3109/07853890.2015.1122216
108. Kasper S, Hajak G, Wulff K, Hoogendijk WJ, Montejo AL, Smeraldi E, et al. Efficacy of the novel antidepressant agomelatine on the circadian rest-activity cycle and depressive and anxiety symptoms in patients with major depressive disorder: a randomized, double-blind comparison with sertraline. *J Clin Psychiatry.* (2010) 71:109–20. doi: 10.4088/JCP.09m05347blu
109. Zajecka J, Schatzberg A, Stahl S, Shah A, Caputo A, Post A. Efficacy and safety of agomelatine in the treatment of major depressive disorder: a multicenter, randomized, double-blind, placebo-controlled trial. *J Clin Psychopharmacol.* (2010) 30:135–44. doi: 10.1097/JCP.0b013e3181d420a7

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REVIEW
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A Hypothesis of Gender Differences in Self-Reporting Symptom of Depression: Implications to Solve Under-Diagnosis and Under-Treatment of Depression in Males

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The phenomenon of female preponderance in depression has been well-reported, which has been challenged by higher rates of suicide and addictive behaviors in males, and a longer life-span in females. We thus propose an alternative hypothesis "Gender differences in self-reporting symptom of depression," suggesting mild-moderate depression tends to be reported more often by females, and severe depression and suicide tend to be reported more often by males. Potential mechanisms that account for this difference may include three aspects: covariation between estrogen levels and the incidence peak of female depression, gender differences in coping style (e.g., comparative emotional inexpressiveness and non-help-seeking in males), and gender differences in symptom phenotypes (e.g., atypical symptoms in male depression). Our newly presented hypothesis implied the overlooked under-diagnosis and under-treatment of depression in males. For effective diagnoses and timely treatment of male depression, it is critical to incorporate symptoms of depression in males into the relevant diagnostic criteria, encourage males to express negative emotions, and increase awareness of suicidal behavior in males.

Keywords: gender difference, male depression, suicide, self-reporting symptom, coping style, symptom phenotype

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INTRODUCTION

In the past several decades, gender differences in depression have been extensively discussed. A few studies have found the gender difference in depression to be small or absent (1), and no gender difference has been indicated in psychotic or melancholic depression (2). However, most studies have confirmed that depression is twice as common in women than in men (3, 4), which has been reported across different cultures (5). Depression is disproportionately reported by women (almost twice as often as by men) during reproductive age (3, 6–8). For example, the worldwide annual prevalence of depression in 2010 for females and males was 5.5 and 3.2%, respectively (i.e., 1.72 vs. 1) (9, 10). In Canada, the prevalence was 5.0% in women and 2.9% in men in 2002 (i.e., 1.72

vs. 1), and it increased to 5.8% in women and 3.6% in men in 2012 (i.e., 1.61 vs. 1) (11). In the USA, women had an ∼2-fold higher risk of depression than men, with 21.3% of women and 12.9% of men experiencing major depressive episodes during their lifetimes (12). In a cross-sectional study of Pakistan, the majority (78.9%) of people diagnosed with major depression were women (13). Consistently, a review of studies between 1994 and 2014 with community participants from 30 countries showed that the point prevalence of depression in the community was significantly higher in females (14.4%) compared with males (11.5%) (i.e., 1.25 vs. 1) (14).

Even in specific populations, a female preponderance of depression has been confirmed. Among students of pedagogy, 9.42% of females reported depression, compared with 1.23% males (i.e., 7.66 vs. 1) (15). In Polish adolescents, being female was reportedly a major risk factor for depression (16). In a study of individuals with diabetes, the prevalence of comorbid depression was significantly higher in women (28%) than in men (18%) (i.e., 1.56 vs. 1) (17), which was further confirmed by a later review (18). In a similar cross-sectional study conducted in a gastroenterology clinic, compared with males, females reported more symptoms of depression (44 vs. 32%) (i.e., 1.38 vs. 1) (19). In brief, women have reported depression and been diagnosed with depression substantially more often than men (5, 20, 21).

CHALLENGES FOR THE FEMALE PREPONDERANCE HYPOTHESIS

Although women evidently tend to report depression more than men (3, 4), several observations raise questions as to the root cause of this, e.g., the higher suicide rate in males (22), longer life-span in females (23), and greater rates of alcoholism and other addictive behaviors in males (24).

With regard to suicide rates which correlates with age, gender, and socioeconomic status (25), although females reported significantly higher rates of suicidal ideation (OR 1.32) (26); however, the ratio of male to female completed suicides was 1.97:1 (27). It has also been reported that compared with women, men were three times more likely to die from suicide (28). Moreover, in one study, compared with patients who attempted suicide unsuccessfully, those who successfully committed suicide were more likely to be male (29). The finding inspired the argument that higher suicide rates in males may result from the more lethal methods used by males to attempt suicide (30). However, in this study (29), attempted suicide was defined as "acts of self-inflicted injury or self-poisoning with overdose drugs," which should be more accuracy with the term of "self-harm behavior." Notably, in a recent review, the ratio of male to female non-fatal attempted suicides was 1.21:1 (27). Indeed, being male is reported significantly associated with all types of suicidal behavior (31). The gender ratio in suicide behavior persisted even in the elder sample (2.74:1) aged above 60 (32). Importantly, higher suicide rates are associated with more severe depression (33, 34). Without regard for age and socioeconomic status, if the female preponderance hypothesis is true, these aforementioned observations suggest that women suffer from depression more but suicide less, whereas men suffer from depression less but suicide more. This can-not be simply explained by weaker suicide intention in female depression due to their social responsibility, and is conflicted with the fact that over 90% of people who die by suicide had a psychiatric disease, mainly depression (35). It may be that males with mild depression are less likely to express it and seek help, which results in more suicide causing by depression in males (36).

Another challenge for the female preponderance hypothesis is the longer life-span of females. Historically, women live longer than men in almost every country in the world (23). The life expectancy at birth is 79.3 years for females (86.3 in Japan) and 71.9 years for males (78.3 in Japan) (37). A recent review concluded that in developed countries women live ∼4–7 years longer than men (38). Despite the complex interaction of environmental, historical, and genetic factors on age-related diseases and longevity between genders (37), however, if the female preponderance hypothesis is true, these results suggest that females suffer from depression more and live longer. This challenges our understanding of depression because that depression is detrimental to physical health (39, 40), and is associated with a higher morbidity rate (41). It has also recently been reported that even in depressed subpopulations, mortality was higher in men than in women (42). Obviously, high morbidity in males can-not attribute to pure genetic factors or life-style, since that particular life-style (e.g., addiction and aggression) is highly correlated with depression in males (43, 44). Thus, the truth might be that females tend to express more depressive symptoms (45, 46) as a way of help-seeking, even they perceive similar symptoms as male (47).

A third challenge for the female preponderance hypothesis is the greater rate of addictive behaviors in males such as alcoholism and substance use disorder. Some studies indicated that gender difference is not observed as to the intensity of internet dependence (48). However, a gender difference in the risk for developing an addictive behavior was confirmed, with a significantly higher risk in males for several addiction tendencies (49), including alcohol dependence/abuse (48.1 vs. 24.5%) (24), substance use disorder (50) and tobacco dependence (51). Notably, the presence of either depression or alcoholism doubled the risks for another (OR range from 2.00 to 2.09) (52). Nearly one-third of patients with the major depressive disorder also suffered from substance use disorders, which yielded a higher risk of suicide (53). Thus, if the female preponderance hypothesis is true, these results suggest that males suffer from depression less, but report alcoholism, substance use disorder or other addictive behaviors more. This prompts the question as to what causes men to be more prone to addictive behavior. In a previous study, the incidence of depression was equal in males and females after controlling for alcoholism (54). Therefore, addictive behaviors in males may result in under-diagnosis of male depression, because that addictive behavior is not a typical symptom of depression and it can mask traditional symptoms of depression (55).

The three phenomena listed above strongly challenge the female preponderance hypothesis of depression, and suggest a need for a more comprehensive hypothesis. Herein we proposed an alternative hypothesis—gender differences in self-reporting

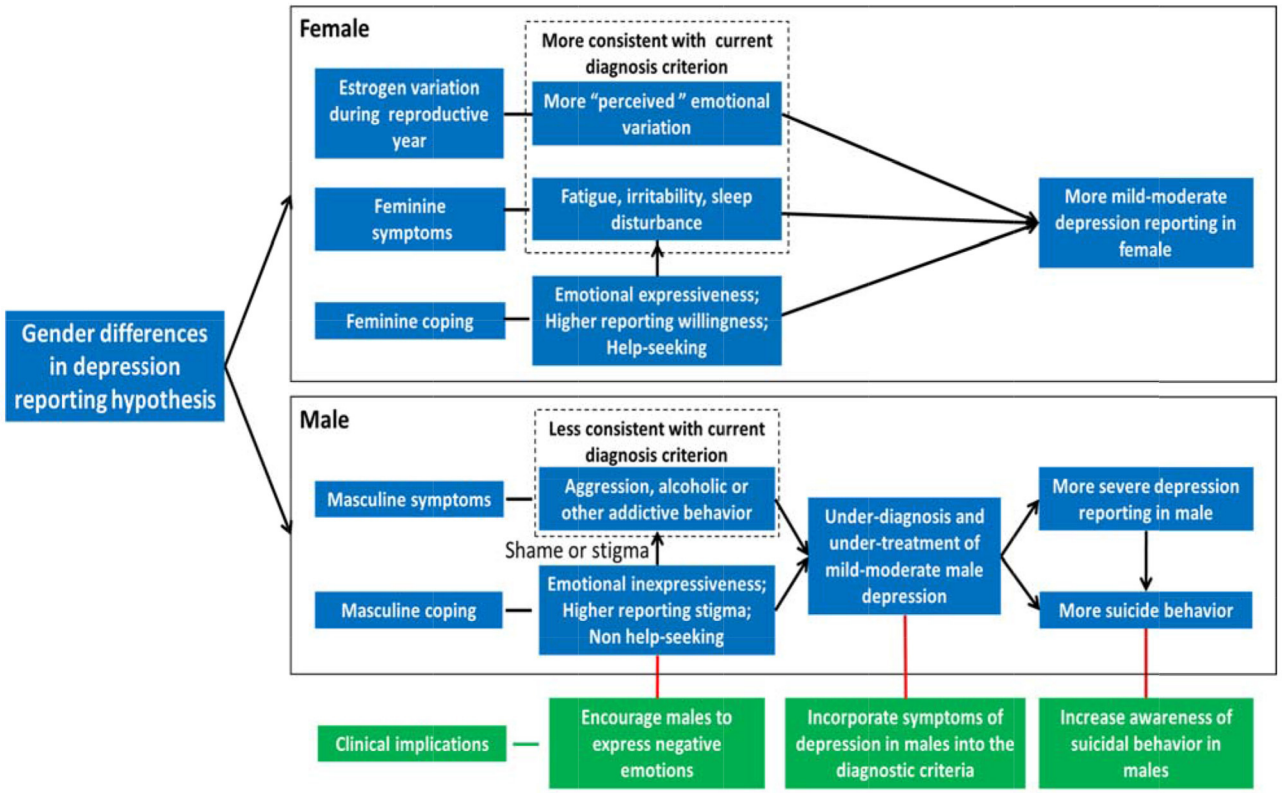

FIGURE 1 | Gender differences in self-reporting symptom of depression hypothesis. For females, estrogen variation during the reproductive year in female leads to more "perceived" emotional variation in the woman. Meanwhile, feminine coping styles (e.g., help-seeking and emotional expressiveness) and social expectations for females lead to traditional female depressive symptoms (e.g., fatigue, irritability, and sleep disturbance), which are more consistent with the current diagnosis criterion of depression. As a result, more mild-moderate depression is diagnosed in females. For males, masculine coping style (e.g., non-help-seeking and emotional inexpressiveness) and social expectation for males lead to more shame or stigma to express depression, results in traditional male depressive symptoms (e.g., aggression, alcohol, or substance abuse), which are less consistent with current diagnosis criterion of depression. As a result, mild-moderate male depression is under-diagnosed and under-treated, which leads to more severe depression and higher suicide reporting in males, and needs immediate attention. To diagnose and treat male depression early and effectively, it is critical to incorporate symptoms of depression in males into the relevant diagnostic criteria, encourage males to express negative emotions, and increase awareness of suicidal behavior in males.

symptom of depression, in an effort to better reconcile the numerous different observations and forms of evidence together.

GENDER DIFFERENCES IN SELF-REPORTING SYMPTOM OF DEPRESSION HYPOTHESIS

The basic tenets of the gender differences in depression severity reporting hypothesis (**Figure 1**) are that women are more likely to report mild-moderate symptoms of depression (56), and men intend to report more severe depression (56, 57) and higher suicide (28, 31).

More Mild-Moderate Symptom Reporting in Females

Females evidently exhibited a greater tendency to recognize subtle emotional changes than males, alternatively, they may have actually "perceived" more emotional symptoms (45). Similarly, female patients with depression reported more emotional experiences, particularly negative emotional experiences, than male patients (58). Therefore, females constantly reported more mild-moderate depression across all age bands (59). As a result, more mild-moderate depression was reported and diagnosed in females, whereas mild-moderate male depression was under-reported and under-diagnosed (56). Consistently, a gender difference was only significant when including minor depression based on of the general Danish population (60), suggesting that the female preponderance is more pronounced in less severe depression status. Study also suggested that amongst patients with severe depression, the gender ratio of patients was no longer significant (i.e., the female preponderance in depression was decreased along with the severity of depression) (59). Thus, in a recent report, it was recommended that optimal cut-off points for depression should be much higher for females (19/20, sensitivity 74.5% and specificity 73.8%) than for males (13/14, sensitivity 72.2% and specificity 64.1%) (61). Hence, with equal depression cut-off scores for both males and females, more

women would report symptoms and reach the diagnostic criteria for depression, and male depression would be less likely to be detected and diagnosed.

More Severe Depression and Higher Suicide Reporting in Males

When untreated, 53% mild to moderate depression will remit within 12 months (62). Meanwhile, untreated mild-moderate depression leads to a high immediate and subsequent suicide risk (63), which is also the largest risk factor for suicide (64). Compared with women, men are less likely to sought treatment due to the shame of seeking help for hegemonic masculinity (65). Under-diagnosis and under-treatment of male depression lead to prolonged depression and higher a suicide rate in men (65, 66). In one recent study, experiencing social pressure not to express negative feelings predicted increases in symptoms of depression, which may be particularly true in males due to their hegemonic masculinity (67). In another study, when required to be dependent on others, men exhibited more severe depression than women (57), in which 58% of depression occurred in men. Indeed, severe depression was not significantly differed between genders (59), indicating a higher male/female reporting ratio of severe depression compared with the gender ratio of reporting mild-moderate depression.

Higher suicide rates are associated with more severe depression (33, 34). Despite the report of gender paradox in suicidal behavior (26, 68, 69): Over representation of females in non-fatal suicidal behavior and a preponderance of males in committed suicide. However, females are only over-represented in suicidal ideation (26, 69) or self-harm behavior (70). In fact, the ratios of male to female attempted suicides and completed suicides were 1.21:1 and 1.97:1, respectively (27). The gender ratio of attempted suicide was even higher in adolescent (2.07:1) (71), while the suicidal commitment risk continued to elders aged 60 (2.74:1) (32). Depressed men often experience a loss of control, a hidden self, and substance use or abuse, which may cause them to commit suicide as a definitive means of eliminating their sense of a loss of control (72). In a previous study, suicide rates were significantly negatively correlated with rates of treatment for depression (73), i.e., higher suicide rates were associated with lower treatment rates. The above-described results suggest that men report more severe depression and higher suicide rates than women (56, 65, 66, 74), which may result from lower rates of diagnosis and treatment of male depression (36).

POTENTIAL MECHANISMS

Biological Dimension: Covariation Between Estrogen Levels and Incidence Peak of Female Depression

Rates of depression in males and females vary during their life-spans. Before puberty, girls and boys have similar rates of depression or a slight higher in boys (3). The prevalence of depression tends to be doubled in girls (3, 6, 75, 76) from the age of 12 years, and this trend persists until the age of 45 or 54 years (26, 77), then declines after menopause (3, 8, 78). Although one study indicated that the gender difference in depression persisted in elders (79), most studies reported that at ages > 65 years, both men and women exhibit declines in depression rate, which becomes similar between them again (11, 76, 80, 81). These results indicate that the "female preponderance in depression" could be relevant to the sex hormonal level (e.g., estrogen).

Longitudinal studies indicate that as soon as estrogen levels rise (first menstruation of girls) (82), the rate of major depression in girls increases in tandemly (83). Consistently, the peak incidence of depression during childbearing years is reportedly associated with cyclic estrogen changes, with a higher prevalence of depression in females at the premenstrual stage, during pregnancy, and at postpartum and peri-menopausal stages (84). About 48% of females who suffer from premenstrual syndrome (85) reported a depressed mood and fatigue in the week before the onset of menstruation (86). Consistently, during the menopausal transition, when sex hormones strongly fluctuated, "depressed mood" and "sleeping problems" were common complaints (87). In summary, the incidence of female depression evidently fluctuates with estrogen levels, leading to more "perceived" fluctuation in mood and more reporting of depression in female.

Psychological Dimension: Gender Differences in Coping Styles

Another explanation for the female preponderance of depression is that women show a more feminine coping style, and they are more willing to express affective symptoms and seek medical help (88). As previously introduced, women evidently report more mild depression whereas men report more severe depression (56). In one study, females reported more symptoms of depression than males (44 vs. 32%), and were more likely to subsequently seek help at private clinics (23 vs. 14%) or from a Quran therapist (11 vs. 5%) (19). Even in a 70-year-old population, femininity was associated with higher levels of depression (89).

In contrast, the masculine coping style was being emotionally unexpressive (90) and reluctant to seek help (91). Indeed, males reported less depression, even they experienced more intense emotions (92), indicating that they need greater symptom severity to ask for help. The results also suggested that males intend to "omit" symptoms, while females "notice" symptoms. Consequently, females may start to report depressed moods with mild or moderate severity, while males might only begin to report depression with much severer severity (56). Similarly, it has been reported that men were more likely to forget episodes that had generally not reached "case" criteria, whereas women were more likely to remember them (46). Due to the shame of seeking help or showing weakness, males tend to hide symptoms of depression from people and try hard to appear cheerful and exhibit happiness in the presence of others (93). Even after stratification by clinically significant impairment and paid employment status, men reported fewer symptoms of depression than women, and as a result men reached the diagnostic threshold less often (47). Instead, they tended to mask symptoms of depression, leading providers to under-diagnose and under-treat men for depression

(94–96). Even though males seek help, they intend to report fewer symptoms and low severity to maintain masculine status (97, 98). Thus, gender differences in coping style may have resulted in a "masculine" form of depression in the general population that is under-diagnosed and under-treated (99).

Social Dimension: Gender Differences in Phenotypic Symptoms of Depression

Hegemonic masculinity indicates how a gender role is enacted with depression expression, in which social expectation for males was proactive, aggression, and violent, while female stereotype was affective, passive, and selfless (90). Thus, although no evidence for a gender-related somatic factor was reported in one study (100), and the lower rate of poor appetite (OR 0.69) in females was indicated in another study (26). Most studies indicated a female predominance not in "pure depression" but in a specific phenotype in women, i.e., "somatic depression" (appetite, sleep, and fatigue) (43, 44, 101), which is generally consistent with current diagnostic criteria for depression. The female rates were consistently higher across all age bands only in DSM-IV mood disorder, major depressive disorder and non-melancholic mood disorder (59). In contrast, men exhibit more atypical signs of depression such as aggression and antisocial behaviors (102). In a study of 18,807 Korean, female depression was significantly associated with fatigue, hypersomnia, and psychomotor retardation (103). In another study, women commonly reported concurrent symptoms consistent with anxiety disorders, somatoform disorder, and bulimia, whereas drug and alcohol abuse was more common in men (104). Relatively, comorbid anxiety was reportedly more prevalent in women, whereas comorbid alcohol abuse was a major concern in men (20). In conclusion, women evidently exhibit more symptoms of fatigue, irritability, and sleep disturbance (somatic depression) which are consistent with current diagnostic criteria for depression, whereas men exhibit more atypical symptoms of aggression or substance addiction which are less consistent with current diagnostic criteria for depression (44, 105, 106). Thus, it is reasonable to assume that this results in comparatively less diagnosis and treatment of male depression.

The social role of male, especially hegemonic masculinity guaranteed that male depression might manifest as substance abuse, aggressive, and/or violent practices (107). Due to the fact that for males showing weakness is contrary to social expectations (being strong, independent, and exhibiting self-control), depressive symptoms (e.g., tiredness and weakness) bring men more shame or stigma to admit or seek help (108). Additional evidence is concordant with a "hegemonic" view—particularly concerning independence—that men "should" be reluctant to seek help; in fact they tend to repress symptoms of depression and/or hide them from others (109). Research suggests that men who are depressed may experience a trajectory of emotional distress that results in avoidant, numbing, and escapist behavior that can lead to substance use, aggression, violence, and suicide (110). Constantly, males exhibit significantly higher rates of substance use and physical violence (111, 112). Gender roles, in particular hegemonic masculinity, may primarily influence the expression of depression rather than the actual experience of depression *per se*, and this may in turn contribute to under-diagnosis and under-treatment of male depression.

CLINICAL IMPLICATIONS OF THE GENDER DIFFERENCES IN SELF-REPORTING SYMPTOM OF DEPRESSION HYPOTHESIS

The principal value of the above newly proposed hypothesis is to emphasize that male depression is under-diagnosed and under-treated, which requires immediate attention and action. The critical implication of this theory is summarized in the following three points.

Incorporating Symptoms of Depression in Males Into Current Diagnostic Criteria

Differential symptoms of depression in males and females (28) and low diagnosis and treatment rates of male depression (29, 54) suggest a greater need to improve the current screening for depression. Evidence suggests that standard assessments of depression omit several key components of male depression, mainly substance use and violence (107). Instead, a combination of the Patient Health Questionnaire and the Gotland Male Depression Scale (to be introduced[1]) may facilitate a more sensitive and accurate identification of male depression (116). It has been suggested that using gender-sensitive assessment strategies would assure that more men would be identified and treated for depression (117). In a study that utilized these alternative male-oriented diagnostic tools, the prevalence of depression was higher in men than in women (112). Notably, the results of another study suggest that male-type depressive symptoms may also be highly prevalent in females (118). Collective reports to date indicate a need to incorporate typical male symptoms of depression into the current diagnostic criteria for depression, i.e., substance abuse, aggression or violence, stress perception, and emotional suppression, which are suggested by mature questionnaire (112, 119) and hegemonic masculinity (107, 108), and need further validation.

Encouraging Males to Express Negative Emotions and Seek Help

Empirical evidence indicates that low treatment rates in men cannot be explained by better health, but are instead attributable

[1]Both Gotland Male Depression Scale (113, 114) and the Masculine Depression Scale (115) are scales available for evaluating the presence of alternative "male-type" depression symptoms in the clinic. Based on the above two scales, the Male Symptoms Scale (MSS) and the Gender Inclusive Depression Scale (GIDS) (112) have also been published on the given topic. The MSS was developed to assess eight constructs that have been proposed in the literature as externalizing symptoms of depression in men: irritability, anger attacks/aggression, sleep disturbance, alcohol/other drug abuse, risk-taking behavior, hyperactivity, stress, and loss of interest in pleasurable activities (112). The GIDS consisted of 15 symptoms, including the eight constructs contained in the Male Symptoms Scale as well as seven traditional symptoms of depression: sad/depressed mood, loss of vitality, tiredness, ambivalence, anxiety/uneasiness, and "complaintiveness" (feeling pathetic) (112).

to a discrepancy between perceptions of need and help-seeking behavior in men. It has been well-documented that males use drugs and alcohol to mask their depression (117, 120, 121). In fact, the common notion that people who seek psychological help because of mental disorders are weak or incapable (122) brings strong stigma to people who seek psychological help, especially for males (108), which prevents them from expressing symptom and seeking help. To fight stigma and prejudice against mental disorders, international campaigns were carried out previously, with a 5.6% increase in people who access mental health services reporting no experienced discrimination, and a fall in average levels of reported discrimination to 28.4% from 41.6% in response to the campaigns (123). These campaigns included an international campaign which is initiated by the World Psychiatric Association (124), "Time to Change" which is launched in 2007 by charities Mind and Rethink Mental Illness (https://www.time-to-change.org.uk/sites/default/files/Stigma%20Shout.pdf), "Heads Together" which is a campaign set up by the Duke and Duchess of Cambridge and Prince Harry in 2016 (https://www.headstogether.org.uk/about-heads-together/), as well as "See Me" which is a similar campaign run in Scotland (https://www.seemescotland.org/). Efforts at the social level deserve continuing to achieve a profound influence and effect.

The social expectation for males (being strong, independent, and exhibiting self-control) holding in different cultures, is contributed to form the traditional masculinity (125), which inhibits the emotional expressiveness and help-seeking behaviors in males (74). Thus, to better diagnose and treat male depression, reversing general expectations shared by the ordinary population toward males might encourage males to seek psychological help, e.g., to allow males to be weak or ill sometimes, and need help occasionally. Moreover, primary healthcare workers, as well as family members should encourage males to open up emotionally and communicate personal feelings of distress (126, 127). Using social media to encourage men with symptoms of depression to seek help should focus on their general trust in doctors, accepting lack of control, and reducing feelings of weakness associated with asking for help (128). Furthermore, as part of recovery from depression, men could reconstruct a valued sense of themselves and their own masculinity, and incorporate values associated with hegemonic masculinity into narratives (re-establishing control, and responsibility to others), which may be useful in reducing depression as well as suicide (129).

Awareness of Suicidal Behavior in Males With Depression

Studies suggest that undiagnosed and untreated depression in men may be one reason why many more men than women commit suicide (117), since that untreated or inadequately treated depression is the largest risk factor for suicide (63) and 90% of people who die from suicide have a previous psychiatric diagnosis mainly depression (35). Accordingly, increasing the rates of diagnosis and treatment of male depression may be critical to reducing the rate of male suicide. Concerning gender differences in suicidal behaviors, the ingestion of drugs was common for women; and hanging and use of sharp objects for men (130). Moreover, men with depression are less likely to mention suicide before committing suicide (29), rendering male suicide less preventable. Thus, to prevent male suicide more effectively, better recognition of subtle indicators of suicidal thoughts or intentions in males with depression is required.

FUTURE STUDIES

The current study is only a theoretical proposal on the "gender differences in self-reporting symptom of depression." The direct empirical evidence to supporting the above theory is lacking. To test it, more community-based investigations worldwide covering both genders and all age-bands are warranted, since that data from hospitals or private clinics might be biased. The survey tools should be more integrated considering symptoms of male depression. Notably, different degrees of depression (mild, moderate, and severe) and suicide (suicidal ideation, attempted suicide, and committed suicide) need to be clearly classified in the community sample. In addition, study has highlighted the importance of the emotional brain (prefrontal cortex, parietal lobe, central gyrus, and midbrain) in causing depression for females, while the socio cognitive brain (orbitofrontal, posterior, and cingulate cortices; insula) for males (131). These results implied that the medicine or brain stimulation treatment for depression should be adopted differently for female and male patients with depression [also see the findings of "microglia-neuro inflammation-BDNF" interconnection (132)].

CONCLUSION

In sum, due to the above-described strong challenges to the female preponderance of depression hypothesis, herein we propose an alternative hypothesis of the gender differences in self-reporting symptom of depression. The main tenets of this alternative hypothesis are that females are more likely to report mild-moderate symptoms of depression, while more severe depression and higher suicide reporting are evident in males. Potential mechanisms behind these observations include covariation between estrogen levels and the incidence peak of female depression, gender differences in coping style, and gender differences in symptom phenotypes. One of the primary aims of developing this hypothesis presented herein is to emphasize that male depression is under-diagnosed and under-treated. To diagnose and treat male depression timely and effectively, it is critical to incorporate male symptoms of depression into the relevant diagnostic criteria, encourage males to express negative emotions, and increase awareness of suicidal behavior in male patients.

AUTHOR CONTRIBUTIONS

QD raised the topic and opinion and further revised the manuscript. PS explored the literature and wrote the draft.

All authors contributed to the article and approved the submitted version.

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REFERENCES

1. Parker G, Brotchie H. Gender differences in depression. *Int Rev Psychiatry.* (2010) 22:429–36. doi: 10.3109/09540261.2010.492391
2. Kessing LV. Gender differences in patients presenting with a single depressive episode according to ICD-10. *Soc Psychiatry Psych Epidemiol.* (2005) 40:197–201. doi: 10.1007/s00127-005-0885-2
3. Cyranowski JM, Frank E, Young E, Shear MK. Adolescent onset of the gender difference in lifetime rates of major depression: a theoretical model. *Arch Gen Psychiatry.* (2000) 57:21–7. doi: 10.1001/archpsyc.57.1.21
4. Ford DE, Erlinger TP. Depression and C-reactive protein in US adults: data from the Third National Health and Nutrition Examination Survey. *Arch Intern Med.* (2004) 164:1010–4. doi: 10.1001/archinte.164.9.1010
5. Bromet E, Andrade LH, Hwang I, Sampson NA, Alonso J, de Girolamo G, et al. Cross-national epidemiology of DSM-IV major depressive episode. *BMC Med.* (2011) 9:90. doi: 10.1186/1741-7015-9-90
6. Bijl RV, De Graaf R, Ravelli A, Smit F, Vollebergh WA. Gender and age-specific first incidence of DSM-III-R psychiatric disorders in the general population Results from the Netherlands Mental Health Survey and Incidence Study (NEMESIS). *Soc Psychiatry Psychiatr Epidemiol.* (2002) 37:372–9. doi: 10.1007/s00127-002-0566-3
7. Kessler RC. Epidemiology of women and depression. *J Affect Disord.* (2003) 74:5–13. doi: 10.1016/S0165-0327(02)00426-3
8. Leach LS, Christensen H, Mackinnon AJ, Windsor TD, Butterworth P. Gender differences in depression and anxiety across the adult lifespan: the role of psychosocial mediators. *Soc Psychiatry Psychiatr Epidemiol.* (2008) 43:983–98. doi: 10.1007/s00127-008-0388-z
9. Whiteford HA, Degenhardt L, Rehm J, Baxter AJ, Ferrari AJ, Erskine HE, et al. Global burden of disease attributable to mental and substance use disorders: findings from the Global Burden of Disease Study 2010. *Lancet.* (2013) 382:1575–86. doi: 10.1016/S0140-6736(13)61611-6
10. Baxter AJ, Scott KM, Ferrari AJ, Norman RE, Vos T, Whiteford HA. Challenging the myth of an "epidemic" of common mental disorders: trends in the global prevalence of anxiety and depression between 1990 and 2010. *Depress Anxiety.* (2014) 31:506–16. doi: 10.1002/da.22230
11. Patten SB, Wang JL, Williams JV, Currie S, Beck CA, Maxwell CJ, et al. Descriptive epidemiology of major depression in Canada. *Can J Psychiatry.* (2006) 51:84–90. doi: 10.1177/070674370605100204
12. Kessler RC, McGonagle KA, Swartz M, Blazer DG, Nelson CB. Sex and depression in the National Comorbidity Survey I: lifetime prevalence, chronicity and recurrence. *J Affect Disord.* (1993) 29:85–96. doi: 10.1016/0165-0327(93)90026-G
13. Kausar N, Akram B, Khan SD. Major depression in Jalal Pur Jattan, district Gujrat, Pakistan: prevalence and gender differences. *J Pakistan Med Assoc.* (2015) 65:292–5.
14. Lim GY, Tam WW, Lu Y, Ho CS, Zhang MW, Ho RC. Prevalence of depression in the community from 30 countries between 1994 and 2014. *Sci Rep.* (2018) 8:2861. doi: 10.1038/s41598-018-21243-x
15. Pietras T, Witusik A, Panek M, Zielińska-Wyderkiewicz E, Kuna PP. Górski, Intensity of depression in pedagogy students (Article in Polish). *Polski Merkuriusz Lekarski.* (2012) 32:163–6.
16. Kalinowska S, Nitsch K, Duda P, Trześniowska-Drukała B, Samochowiec J. Depression in children and adolescents -symptoms, etiology, therapy (Article in Polish). *Ann Acad Med Stetin.* (2013) 59:32–6.
17. Anderson RJ, Freedland KE, Clouse RE, Lustman PJ. The prevalence of comorbid depression in adults with diabetes: a meta-analysis. *Diabetes Care.* (2001) 24:1069–78. doi: 10.2337/diacare.24.6.1069
18. Lloyd CE, Brown FJ. Depression and diabetes. *Curr Womens Health Rep.* (2002) 2:188–93. doi: 10.1007/s11892-002-0112-4
19. Alosaimi FD, Al-Sultan OA, Alghamdi QA, Almohaimeed IK, Alqannas SI. Gender-specific differences in depression and anxiety symptoms and help-seeking behavior among gastroenterology patients in Riyadh, Saudi Arabia. *Neurosciences.* (2014) 19:203–9.
20. Karger A. Gender differences in depression (Article in German). *Bundesgesundheitsblatt Gesundheitsforschung Gesundheitsschutz.* (2014) 57:1092–8. doi: 10.1007/s00103-014-2019-z
21. Altemus M, Sarvaiya N, Neill Epperson C. Sex differences in anxiety and depression clinical perspectives. *Front Neuroendocrinol.* (2014) 35:320–30. doi: 10.1016/j.yfrne.2014.05.004
22. Mackenzie S, Wiegel JR, Mundt M, Brown D, Saewyc E, Heiligenstein E, et al. Depression and suicide ideation among students accessing campus health care. *Am J Orthopsychiatry.* (2011) 81:101–7. doi: 10.1111/j.1939-0025.2010.01077.x
23. Austad SN. Why women live longer than men: sex differences in longevity. *Gend Med.* (2006) 3:79–92. doi: 10.1016/S1550-8579(06)80198-1
24. Schuch JJ, Roest AM, Nolen WA, Penninx BW, de Jonge P. Gender differences in major depressive disorder: results from the Netherlands study ofdepression and anxiety. *J Affect Disord.* (2014) 156:156–63. doi: 10.1016/j.jad.2013.12.011
25. Bachmann S. Epidemiology of suicide and the psychiatric perspective. *Int J Environ Res Public Health.* (2018) 15:1425. doi: 10.3390/ijerph15071425
26. Adewuya AO, Coker OA, Atilola O, Ola BA, Zachariah MP, Adewumi T, et al. Gender difference in the point prevalence, symptoms, comorbidity, and correlates of depression: findings from the Lagos State Mental Health Survey (LSMHS), Nigeria. *Arch Womens Ment Health.* (2018) 21:591–9. doi: 10.1007/s00737-018-0839-9
27. Moore F, Taylor S, Beaumont J, Gibson R, Starkey C. The gender suicide paradox under gender role reversal during industrialisation. *PLoS ONE.* (2018) 13:e0202487. doi: 10.1371/journal.pone.0202487
28. Flaskerud JH. Depression in men: issues for practice and research. *Issues Ment Health Nurs.* (2014) 35:635–9. doi: 10.3109/01612840.2014.903015
29. Younes N, Melchior M, Turbelin C, Blanchon T, Hanslik T, Chee CC. Attempted and completed suicide in primary care: not what we expected. *J Affect Disord.* (2015) 170:150–4. doi: 10.1016/j.jad.2014.08.037
30. Dabbagh N. Behind the statistics: the ethnography of suicide in Palestine. *Cult Med Psychiatry.* (2012) 36:286–305. doi: 10.1007/s11013-012-9251-5
31. Al-Habeeb AA, Sherra KS, Al-Sharqi AM, Qureshi NA. Assessment of suicidal and self-injurious behaviours among patients with depression. *Eastern Mediterranean Health J.* (2013) 19:248–54. doi: 10.26719/2013.19.3.248
32. Crestani C, Masotti V, Corradi N, Schirripa ML, Cecchi R. Suicide in the elderly: a 37-years retrospective study. *Acta Biomed.* (2019) 90:68–76. doi: 10.23750/abm.v90i1.6312
33. Hawton K, Casanas C, Comabella I, Haw C, Saunders K. Risk factors for suicide in individuals with depression: a systematic review. *J Affect Disord.* (2013) 147:17–28. doi: 10.1016/j.jad.2013.01.004
34. Sanchez-Gistau V, Baeza I, Arango C, González-Pinto A, de la Serna E, Parellada M, et al. Predictors of suicide attempt in early-onset, first-episode psychoses: a longitudinal 24-month follow-up

study. *J Clin Psychiatry.* (2013) 74:59–66. doi: 10.4088/JCP.12m07632
35. Barrigon ML, Cegla-Schvartzman F. Sex, gender, and suicidal behavior. *Curr Top Behav Neurosci.* (2020) 46:89–115. doi: 10.1007/7854_2020_165
36. Rutz W, Wålinder J, Von Knorring L, Rihmer Z, Pihlgren H. Prevention of depression and suicide by education and medication: impact on male suicidality. An update from the Gotland study. *Int J Psychiatry Clin Pract.* (1997) 1:39–46. doi: 10.3109/13651509709069204
37. Candore G, Balistreri CR, Listì F, Grimaldi MP, Vasto S, Colonna-Romano G, et al. Immunogenetics, gender, and longevity. *Ann N Y Acad Sci.* (2006) 1089:516–37. doi: 10.1196/annals.1386.051
38. Ginter E, Simko V. Women live longer than men. *Bratislavské Lekárske Listy.* (2013) 114:45–9. doi: 10.4149/BLL_2013_011
39. Blumenfield M, Suojanen JK, Weiss C. Public awareness about the connection between depression and physical health: specifically heart disease. *Psychiatr Q.* (2012) 83:259–69. doi: 10.1007/s11126-011-9199-6
40. Whooley MA, de Jonge P, Vittinghoff E, Otte C, Moos R, Carney RM, et al. Depressive symptoms, health behaviors, and risk of cardiovascular events in patients with coronary heart disease. *J Am Med Assoc.* (2008) 300:2379–88. doi: 10.1001/jama.2008.711
41. Zhang Y, Chen Y, Ma L. Depression and cardiovascular disease in elderly: current understanding. *J Clin Neurosci.* (2018) 47:1–5. doi: 10.1016/j.jocn.2017.09.022
42. Cuijpers P, Vogelzangs N, Twisk J, Kleiboer A, Li J, Penninx BW. Is excess mortality higher in depressed men than in depressed women? A meta-analytic comparison. *J Affect Disord.* (2014) 161:47–54. doi: 10.1016/j.jad.2014.03.003
43. Halbreich U, Kahn LS. Atypical depression, somatic depression and anxious depression in women: are they gender-preferred phenotypes. *J Affect Disord.* (2007) 102:245–58. doi: 10.1016/j.jad.2006.09.023
44. Silverstein B, Edwards T, Gamma A, Ajdacic-Gross V, Rossler W, Angst J. The role played by depression associated with somatic symptomatology in accounting for thegender difference in the prevalence of depression. *Soc Psychiatry Psychiatr Epidemiol.* (2013) 48:257–63. doi: 10.1007/s00127-012-0540-7
45. Dunn VJ, Abbott RA, Croudace TJ, Wilkinson P, Jones PB, Herbert J, et al. Profiles of family-focused adverse experiences through childhood and early adolescence: the ROOTS project a community investigation of adolescent mental health. *BMC Psychiatry.* (2011) 11:109. doi: 10.1186/1471-244X-11-109
46. Wilhelm K, Parker G. Sex differences in lifetime depression rates: fact or artefact. *Psychol Med.* (1994) 24:97–111. doi: 10.1017/S0033291700026878
47. Angst J, Gamma A, Gastpar M, Lépine JP, Mendlewicz J, Tylee A, et al. Gender differences in depression. Epidemiological findings from the European DEPRES I and IIstudies. *Eur Archiv Psychiatry Clin Neurosci.* (2002) 252:201–9. doi: 10.1007/s00406-002-0381-6
48. Soulioti E, Stavropoulos V, Christidi S, Papastefanou Y, Roussos P. The relationship of internet addiction with anxiety and depressive symptomatology [in Modern Greek]. *Psychiatriki.* (2018) 29:160–71. doi: 10.22365/jpsych.2018.292.160
49. Munno D, Saroldi M, Bechon E, Sterpone SC, Zullo G. Addictive behaviors and personality traits in adolescents. *CNS Spectr.* (2016) 21:207–13. doi: 10.1017/S1092852915000474
50. McHugh RK, Votaw VR, Sugarman DE, Greenfield SF. Sex and gender differences in substance use disorders. *Clin Psychol Rev.* (2018) 66:12–23. doi: 10.1016/j.cpr.2017.10.012
51. Stepankova L, Kralikova E, Zvolska K, Kmetova A, Blaha M, Bortlicek Z, et al. Tobacco treatment outcomes in patients with and without a history of depression, Czech Republic: 2005–2010. *Prev Chronic Dis.* (2013) 10:E158. doi: 10.5888/pcd10.130051
52. Boden JM, Fergusson DM. Alcohol and depression. *Addiction.* (2011) 106:906–14. doi: 10.1111/j.1360-0443.2010.03351.x
53. Davis L, Uezato A, Newell JM, Frazier E. Major depression and comorbid substance use disorders. *Curr Opin Psychiatry.* (2008) 21:14–8. doi: 10.1097/YCO.0b013e3282f32408
54. Egeland JA, Hostetter AM, Eshleman SK. Amish Study, III: the impact of cultural factors on diagnosis of bipolar illness. *Am J Psychiatry.* (1983) 140:67–71. doi: 10.1176/ajp.140.1.67
55. Uher R, Payne JL, Pavlova B, Perlis RH. Major depressive disorder in DSM-5: implications for clinical practice and research of changes from DSM-IV. *Depress Anxiety.* (2014) 31:459–71. doi: 10.1002/da.22217
56. Hajduk A, Korzonek M, Przybycień K, Ertmański A, Stolarek J. Study of depressiveness with Beck Depression Inventory in patients with cardiac arrhythmias (Article in Polish). *Ann Acad Med Stetin.* (2011) 57:45–8.
57. Mbelesso P, Senekian VP, Yangatimbi E, Tabo A, Zaoro-Kponsere AJ, Kette GC, et al. Depression post stroke in Africa: myth or reality (Article in French). *Bulletin de la Société de pathologie exotique.* (2014) 107:350–5. doi: 10.1007/s13149-014-0399-9
58. Li M, Lu S, Wang G, Zhong N. The effects of gender differences in patients with depression on their emotional working memory and emotional experience. *Behav Neurol.* (2015) 2015:807343. doi: 10.1155/2015/807343
59. Bogren M, Brådvik L, Holmstrand C, Nöbbelin L, Mattisson C. Gender differences in subtypes of depression by first incidence and age of onset: a follow-up of the Lundby population. *Eur Arch Psychiatry Clin Neurosc.* (2018) 268:179–89. doi: 10.1007/s00406-017-0778-x
60. Olsen LR, Mortensen EL, Bech P. Prevalence of major depression and stress indicators in the Danish general population. *Acta Psychiatr Scand.* (2004) 109:96–103. doi: 10.1046/j.0001-690X.2003.00231.x
61. Araya R, Montero-Marin J, Barroilhet S, Fritsch R, Gaete J, Montgomery A. Detecting depression among adolescents in Santiago, Chile: sex differences. *BMC Psychiatry.* (2013) 13:122. doi: 10.1186/1471-244X-13-122
62. Whiteford HA, Harris MG, McKeon G, Baxter A, Pennell C, Barendregt JJ, et al. Estimating remission from untreated major depression: a systematic review and meta-analysis. *Psychol Med.* (2013) 43:1569–85. doi: 10.1017/S0033291712001717
63. Bitter I, Filipovits D, Czobor P. Adverse reactions to duloxetine in depression. *Expert Opin Drug Saf.* (2011) 2011:839–50. doi: 10.1517/14740338.2011.582037
64. Weissmann MM, Wolk S, Goldstein RB. Depressed adolescents grown up. *J Am Med Assn.* (1999) 281:1707–13. doi: 10.1001/jama.281.18.1707
65. Uhlenhuth EH, Paykel ES. Symptom configuration and life events. *Arch Gen Psychiatry.* (1973) 28:744–8. doi: 10.1001/archpsyc.1973.01750350112021
66. Rutz W, von Knorring L, Pihlgren H, Rihmer Z, Wålinder J. Prevention of male suicides: lessons from Gotland study. *Lancet.* (1995) 345:524. doi: 10.1016/S0140-6736(95)90622-3
67. Dejonckheere E, Bastian B, Fried EI, Murphy SC, Kuppens P. Perceiving social pressure not to feel negative predicts depressive symptoms in daily life. *Depress Anxiety.* (2017) 34:836–44. doi: 10.1002/da.22653
68. Schrijvers DL, Bollen J, Sabbe BG. The gender paradox in suicidal behavior and its impact on the suicidal process. *J Affect Disord.* (2012) 138:19–26. doi: 10.1016/j.jad.2011.03.050
69. Lewinsohn PM, Rohde P, Seeley JR, Baldwin CL. Gender differences in suicide attempts from adolescence to young adulthood. *J Am Acad Child Adolesc Psychiatry.* (2001) 40:427–34. doi: 10.1097/00004583-200104000-00011
70. Lungu O, Potvin S, Tikàsz A, Mendrek A. Sex differences in effective fronto-limbic connectivity during negative emotion processing. *Psychoneuroendocrinology.* (2015) 62:180–8. doi: 10.1016/j.psyneuen.2015.08.012
71. Lee S, Dwyer J, Paul E, Clarke D, Treleaven S, Roseby R. Differences by age and sex in adolescent suicide. *Aust N Z J Public Health.* (2019) 43:248–53. doi: 10.1111/1753-6405.12877
72. Heifner C. The male experience of depression. *Perspect Psychiatr Care.* (1997) 33:10–8. doi: 10.1111/j.1744-6163.1997.tb00536.x
73. Rihmer Z, Barsi J, Veg K, Katona CL. Suicide rates in Hungary correlate negatively with reported rates of depression. *J Affect Disord.* (1990) 20:87–91. doi: 10.1016/0165-0327(90)90121-N
74. Moller-Leimkuhler AM. Barriers to help-seeking by men: a review of sociocultural and clinical literature with particular reference to depression. *J Affect Disord.* (2002) 71:1–9. doi: 10.1016/S0165-0327(01)00379-2
75. Salk RH, Hyde JS, Abramson LY. Gender differences in depression in representative national samples: meta-analyses of diagnoses and symptoms. *Psychol Bull.* (2017) 143:783–822. doi: 10.1037/bul0000102
76. Faravelli C, Alessandra Scarpato M, Castellini G, Lo Sauro C. Gender differences in depression and anxiety: the role of age. *Psychiatry Res.* (2013) 210:1301–3. doi: 10.1016/j.psychres.2013.09.027

77. Cairney J, Wade TJ. The influence of age on gender differences in depression: further population-based evidence on the relationship between menopause and the sex difference in depression. *Soc Psychiatry Psychiatr Epidemiol.* (2002) 37:401–8. doi: 10.1007/s00127-002-0569-0
78. Bebbington PE, Dunn G, Jenkins R, Lewis G, Brugha T, Farrell M, et al. The influence of age and sex on the prevalence of depressive conditions: report from the National Survey of Psychiatric Morbidity. *Psychol Med.* (1998) 28:9–19. doi: 10.1017/S0033291797006077
79. Kuehner C. Gender differences in unipolar depression: an update of epidemiological findings and possible explanations. *Acta Psychiat Scand.* (2003) 108:163–74. doi: 10.1034/j.1600-0447.2003.00204.x
80. Bebbington P, Dunn G, Jenkins R, Lewis G, Brugha T, Farrell M, et al. The influence of age and sex on the prevalence of depressive conditions: report from the National Survey of Psychiatric Morbidity. *Int Rev Psychiatry.* (2003) 15:74–83. doi: 10.1080/0954026021000045976
81. Forlani C, Morri M, Ferrari B, Dalmonte E, Menchetti M, De Ronchi D, et al. Prevalence and gender differences in late-life depression: a population-based study. *Am J Geriatr Psychiatry.* (2014) 22:370–80. doi: 10.1016/j.jagp.2012.08.015
82. Johnson EO, Roth T, Schultz L, Breslau N. Epidemiology of DSM-IV insomnia in adolescence: lifetime prevalence, chronicity, and an emergent gender difference. *Pediatrics.* (2006) 117:2. doi: 10.1542/peds.2004-2629
83. Angold A, Costello E, Worthman C. Puberty and depression: the roles of age, pubertal status and pubertal timing. *Psychol Med.* (1998) 28:51–61. doi: 10.1017/S003329179700593X
84. Sagud M, Hotujac LJ, Mihaljević-Peles A, Jakovljević M. Gender differences in depression. *Coll Antropol.* (2002) 26:149–57.
85. Direkvand-Moghadam A, Sayehmiri K, Delpisheh A, Satar K. Epidemiology of premenstrual syndrome, a systematic review and meta-analysis study. *J Clin Diagn Res.* (2014) 8:106–9. doi: 10.7860/JCDR/2014/8024.4021
86. Baker FC, Driver HS. Circadian rhythms, sleep, and the menstrual cycle. *Sleep Med.* (2007) 8:613–22. doi: 10.1016/j.sleep.2006.09.011
87. Soares CN. Can depression be a menopause-associated risk. *BMC Med.* (2010) 8:79. doi: 10.1186/1741-7015-8-79
88. Weissman MM, Klerman GL. Sex differences and the epidemiology of depression. *Archiv Gen Psychiatry.* (1977) 34:98–111. doi: 10.1001/archpsyc.1977.01770130100011
89. Rydberg Sterner T, Gudmundsson P, Falk H, Seidu N, Ahlner F, Wetterberg H, et al. Depression in relation to sex and gender expression among Swedish septuagenarians-results from the H70 study. *PLoS ONE.* (2020) 15:e0238701. doi: 10.1371/journal.pone.0238701
90. Möller-Leimkühler AM. The gender gap in suicide and premature death or: why are men so vulnerable. *Eur Arch Psychiatry Clin Neurosci.* (2003) 253:1–8. doi: 10.1007/s00406-003-0397-6
91. Sen B. Adolescent propensity for depressed mood and help seeking: race and gender differences. *J Ment Health Policy Econ.* (2004) 7:133–45.
92. Deng Y, Chang L, Yang M, Huo M, Zhou R. Gender differences in emotional response: inconsistency between experience and expressivity. *PLoS ONE.* (2016) 11:e0158666. doi: 10.1371/journal.pone.0158666
93. Pederson EL, Vogel DL. Male gender role conflict and willingness to seek counseling: testing a mediation model on college-aged men. *J Couns Psychol.* (2007) 54:373–84. doi: 10.1037/0022-0167.54.4.373
94. Aday LA. *At Risk in America the Health and Health Care Needs of Vulnerable Populations in the United States*. San Francisco: Jossey-Bass (2001).
95. Ramirez JL, Badger TA. Men navigating inward and outward through depression. *Arch Psychiatr Nurs.* (2014) 28:21–8. doi: 10.1016/j.apnu.2013.10.001
96. Association AP. *Diagnostic and Statistical Manual of Mental Disorders*. 5th ed. Arlington: American Psychiatric Publishing (2013).
97. Cleary A. Suicidal action, emotional expression, and the performance of masculinities. *Soc Sci Med.* (2012) 74:498–505. doi: 10.1016/j.socscimed.2011.08.002
98. Johnson JL, Oliffe JL, Kelly MT, Galdas P, Ogrodniczuk JS. Men's discourses of help-seeking in the context of depression. *Sociol Health Illn.* (2012) 34:345–61. doi: 10.1111/j.1467-9566.2011.01372.x
99. Kilmartin C. Depression in man: communication, diagnosis and therapy. *J Men's Health Gender.* (2005) 2:95–9. doi: 10.1016/j.jmhg.2004.10.010
100. Thompson AH, Bland RC. Gender similarities in somatic depression and in DSM depression secondary symptom profiles within the context of severity and bereavement. *J Affect Disord.* (2018) 227:770–6. doi: 10.1016/j.jad.2017.11.052
101. Silverstein B. Gender difference in the prevalence of clinical depression: the role played by depression associated with somatic symptoms. *Am J Psychiatry.* (1999) 156:480–2.
102. Moller-Leimkuhler A, Bottlender R, Staub A, Rutz W. Barriers to help seek by is there evidence for a male depressive syndrome in inpatients with major depression. *J Affect Disord.* (2003) 80:87–93. doi: 10.1016/S0165-0327(03)00051-X
103. Kim JH, Cho MJ, Hong JP, Bae JN, Cho SJ, Hahm BJ, et al. Gender differences in depressive symptom profile: results from nationwide general population surveys in Korea. *J Korean Med Sci.* (2015) 30:1659–66. doi: 10.3346/jkms.2015.30.11.1659
104. Marcus SM, Young EA, Kerber KB, Kornstein S, Farabaugh AH, Mitchell J, et al. Gender differences in depression: findings from the STAR*D study. *J Affect Disord.* (2005) 87:141–50. doi: 10.1016/j.jad.2004.09.008
105. Romm KL, Rossberg JI, Berg AO, Barrett EA, Faerden A, Agartz I, et al. Depression and depressive symptoms in first episode psychosis. *J Nerv Mental Dis.* (2010) 198:67–71. doi: 10.1097/NMD.0b013e3181c81fc0
106. Scott C, McKinlay A, McLellan T, Britt E, Grace R, MacFarlane M, et al. comparison of adult outcomes for males compared to females following pediatric traumatic brain injury. *Neuropsychology.* (2015) 29:501–8. doi: 10.1037/neu0000074
107. Branney P, White A. Big boys don't cry: depression and men. *Adv Psychiatr Treat.* (2008) 14:256–62. doi: 10.1192/apt.bp.106.003467
108. Lasalvia A, Zoppei S, Van Bortel T, Bonetto C, Cristofalo D, Wahlbeck K, et al. Global pattern of experienced and anticipated discrimination reported by people with major depressive disorder: a cross-sectional survey. *Lancet.* (2013) 381:55–62. doi: 10.1016/S0140-6736(12)61379-8
109. O'Brien R, Hunt K, Hart G. 'It's caveman stuff, but that is to a certain extent how guys still operate': men's accounts ofmasculinity and help seeking. *Soc Sci Med.* (2005) 61:503–16. doi: 10.1016/j.socscimed.2004.12.008
110. Brownhill S, Wilhelm K, Barclay L, Schmied V. "Big build": hidden depression in men. *Aust N Z J Psychiatry.* (2005) 39:921–31. doi: 10.1080/j.1440-1614.2005.01665.x
111. Harrison CM, Britt H, Harrison CM, Britt H. The rates and management of psychological problems in Australian general practice. *Austral N Zeal J Psychiatry.* (2004) 38:781–8. doi: 10.1080/j.1440-1614.2004.01462.x
112. Martin LA, Neighbors HW, Griffith DM. The experience of symptoms of depression in men vs. women: analysis of the National Comorbidity Survey Replication. *J Am Med Assoc Psychiatry.* (2013) 70:1100–6. doi: 10.1001/jamapsychiatry.2013.1985
113. Zierau F, Bille A, Rutz W, Bech P. The Gotland Male Depression Scale: a validity study in patients with alcohol use disorder. *Nordic J Psychiatry.* (2002) 56:265–71. doi: 10.1080/08039480260242750
114. Chu CL, Chen C-Y, Huang T-H, Chen Y, Jiang J-R, Hsieh M-L. Reliability, validation and clinical utilization of a Chinese version of Gotland Male Depression Scale in a men's health jointed clinic. *J Men's Health.* (2009) 6:262. doi: 10.1016/j.jomh.2009.08.136
115. Tarsuslu B, Durat G. Psychometric properties of the Masculine Depression Scale in Turkish male University students. *Perspect Psychiatr Care.* (2021) 57:287–94. doi: 10.1111/ppc.12561
116. Sharpley CF, Bitsika V, Christie DR. Diagnosing "male" depression in men diagnosed with prostate cancer: the next step in effective translational psycho-oncology interventions. *Psychooncology.* (2014) 23:1042–8. doi: 10.1002/pon.3530
117. Cochran SV, Rabinowitz FE. Gender-sensitive recommendations for assessment and treatment of depression in men. *Prof Psychol Res Pract.* (2003) 34:132–40. doi: 10.1037/0735-7028.34.2.132
118. Möller-Leimkühler AM, Yücel M. Male depression in females. *J Affect Disord.* (2010) 121:22–9. doi: 10.1016/j.jad.2009.05.007
119. Möller-Leimkühler AM, Mühleck J. Development and preliminary validation of a gender-sensitive depression screening (GSDS) [German]. *Psychiatr Prax.* (2020) 47:79–86. doi: 10.1055/a-1067-0241
120. Epperly TD, Moore KE. Health issues in men: part II common psychosocial disorders. *Am Fam Physician.* (2000) 6:117–1124.

121. Zamarripa MX, Wampold BE, Gregory E. Male gender role conflict, depression and anxiety: clarification and generalizability to women. *J Couns Psychol.* (2003) 50:333–8. doi: 10.1037/0022-0167.50.3.333
122. Tzouvara V, Papadopoulos C. Public stigma towards mental illness in the Greek culture. *J Psychiatr Ment Health Nurs.* (2014) 21:931–8. doi: 10.1111/jpm.12146
123. Corker E, Hamilton S, Robinson E, Cotney J, Pinfold V, Rose D, et al. Viewpoint survey of mental health service users' experiences of discrimination in England 2008-2014. *Acta Psychiatr Scand.* (2016) 134:6–13. doi: 10.1111/acps.12610
124. Sartorius N. Fighting schizophrenia and its stigma: a new World Psychiatric Association educational programme. *Br J Psychiatry.* (1997) 170:297. doi: 10.1192/bjp.170.4.297
125. Pleck JH. The gender role strain paradigm: an update. In: Levant R, Pollack W, editors, *A New Psychology of Men.* New York, NY: Basic Books (1995). p. 11–32.
126. Danielsson UE, Bengs C, Samuelsson E, Johansson EE. "My greatest dream is to be normal": the impact of gender on the depression narratives ofyoung Swedish men and women. *Qual Health Res.* (2011) 21:612–24. doi: 10.1177/1049732310391272
127. Banks I. New models for providing men with health care. *J Men's Health Gender.* (2004) 1:155–8. doi: 10.1016/j.jmhg.2004.07.023
128. Bass SB, Muñiz J, Gordon TF, Maurer L, Patterson F. Understanding help-seeking intentions in male military cadets: an application of perceptualmapping. *BMC Public Health.* (2016) 16:413. doi: 10.1186/s12889-016-3092-z
129. Emslie C, Ridge D, Ziebland S, Hunt K. Men's accounts of depression: reconstructing or resisting hegemonic masculinity. *Soc Sci Med.* (2006) 62:2246–57. doi: 10.1016/j.socscimed.2005.10.017
130. Alves Vde M, Silva AM, Magalhães AP, Andrade TG, Faro AC, Nardi AE. Suicide attempts in a emergency hospital. *Arq Neuropsiquiatr.* (2014) 72:123–8. doi: 10.1590/0004-282X20130212
131. Sundström Poromaa I, Comasco E, Georgakis MK, Skalkidou A. Sex differences in depression during pregnancy and the postpartum period. *J Neurosci Res.* (2017) 95:719–30. doi: 10.1002/jnr.23859
132. Liu LL, Li JM, Su WJ, Wang B, Jiang CL. Sex differences in depressive-like behaviour may relate to imbalance of microglia activation in the hippocampus. *Brain Behav Immun.* (2019) 81:188–97. doi: 10.1016/j.bbi.2019.06.012

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The Association Between Concentrations of Arginine, Ornithine, Citrulline and Major Depressive Disorder: A Meta-Analysis

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Alterations in the peripheral (e.g., serum, plasma, platelet) concentrations of arginine and its related catabolic products (i.e., ornithine, citrulline) in the urea and nitric oxide cycles have been reported to be associated with major depressive disorder (MDD). The meta-analysis herein aimed to explore the association between the concentration of peripheral arginine, its catabolic products and MDD, as well as to discuss the possible role of arginine catabolism in the onset and progression of MDD. PubMed, EMBASE, PsycINFO and Web of Science were searched from inception to June 2020. The protocol for the meta-analysis herein has been registered at the Open Science Framework [https://doi.org/10.17605/osf.io/7fn59]. In total, 745 (47.5%) subjects with MDD and 823 (52.5%) healthy controls (HCs) from 13 articles with 16 studies were included. Fifteen of the included studies assessed concentrations of peripheral arginine, eight assessed concentrations of ornithine, and six assessed concentrations of citrulline. Results indicated that: (1) the concentrations of arginine, ornithine, and citrulline were not significantly different between individuals with MDD and HCs when serum, plasma and platelet are analyzed together, (2) in the subgroups of serum samples, the concentrations of arginine were lower in individuals with MDD than HCs, and (3) concurrent administration of psychotropic medications may be a confounding variable affecting the concentrations of arginine, ornithine, and citrulline. Our findings herein do not support the hypothesis that arginine catabolism between individuals with MDD and HCs are significantly different. The medication status and sample types should be considered as a key future research avenue for assessing arginine catabolism in MDD.

Keywords: arginine, depression, nitric oxide, metabolism, catabolism, bipolar disorder, cognition

INTRODUCTION

Major depressive disorder (MDD) is one of the most common mental disorders affecting more than 350 million people worldwide (1, 2). Furthermore, MDD is one of the leading causes of global burden of disease. Major depressive disorder affects approximately 16% of the world's population (3), continues to be a major cause of disability worldwide, and is the number one cause of suicide (4). Excessive inflammation and neurodegenerative changes have been reported in MDD, both of which have been associated with cognitive impairment (5). However, the underlying pathology of MDD remains poorly understood, in part due to the heterogeneity of genetic and environmental factors related to proposed mechanisms of action (6). Moreover, the pathogenesis of acute depression may be different from recurrent or chronic depression, which is characterized by long-term decline in social or occupational function and cognitive ability (7).

Intensified research efforts are devoted to predicting onset of MDD and treatment response through exploring possible biomarkers/biosignatures including but not limited to metabolic pathways (8–10). Extensive literature indicates that amino acids may be candidate biomarkers for a variety of diseases, including metabolic syndrome, cancer, and mental disorders (11–16). With increasing research focusing on the dysfunction of oxidative and nitrosative stress in MDD, arginine catabolism regulation has received increasing attention (17–19).

Arginine is a semi-essential amino acid and a substrate of important metabolic pathways in the physiological processes of the central nervous system and immune defense [e.g., urea and nitric oxide (NO) cycles] (20, 21). Arginine is transformed into NO and citrulline by endothelial nitric oxide synthase (eNOS), inducible nitric oxide synthase (iNOS) and neuronal nitric oxide synthase (22, 23). The alteration of arginine may lead to abnormalities of NO metabolism and the urea cycle metabolic pathway (24, 25).

The dysregulation of the L-arginine-NO metabolic pathway has been linked to the pathogenesis of severe depression (26, 27). For example, a study reported a positive correlation between increased plasma NO concentrations and suicide attempts in individuals with mild depression (28). A separate study reported that the inhibition of NOS induced antidepressant effects in rats (29). Hitherto, reducing or blocking the synthesis of NO (i.e., blocking NOS) in the brain may be protective against depression (i.e., antidepressant effects) (29, 63).

Available clinical evidence has demonstrated that the NO signaling pathway is associated with schizophrenia, anxiety disorders, and affective disorders (30). Notably, the NO system has previously been reported to be a potential target of antidepressant and anti-anxiety drugs in acute therapy and prevention (31).

Previous cross-sectional studies among patients with MDD and experimental studies based on animal models of depression have reported the altered arginine levels in blood related to the catabolite and NO imbalance in pathophysiology of MDD (18, 32). The dysfunction of NO signaling pathway has been suggested as a nexus between MDD and commonly encountered comorbidities via platelet activation, endothelial dysfunction (i.e., low circulating endothelial NO concentrations and impaired vasodilation), and elevated concentrations of proinflammatory circulating cytokines (33, 34).

Arginine, citrulline, and ornithine are key amino acids of the urea cycle (35). Arginine is the substrate for arginase, the enzyme that produces urea while converting arginine to ornithine (36). Previous studies have shown that arginase activity is elevated in people with depression (37). Moreover, L-arginine is reported to be a risk factor for the development of mild depression (38). It has been separately reported that patients with depression have lower circulating L-arginine concentrations (33). Arginine has also been reported to affect concentrations of aminobutyric acid and glutamate in the prefrontal cortex of the brain, which are important to cellular bioenergetics and oxidative stress (39). In addition, two clinical trials have shown that ketamine and esketamine, glutamatergic N-methyl-D aspartate receptor (NMDAR) antagonists with established antidepressant effects, contributed to the changes in arginine in the urea cycle (40, 41). Taken together, extensive literature has supported the notion that arginine may be implicated in mechanisms that are relevant to MDD.

Ornithine is a metabolite of arginine (42). Citrulline is derived not only from the production of NO but also from the action of the enzyme ornithine carbamoyltransferase (43). Previous findings regarding the function of arginine catabolism underlying MDD are inconsistent. L-Arginine competes with asymmetric dimethylarginine for NOS (44) and increases endothelial NO production and reverses the endothelial dysfunction associated with vascular risk factors (64). L-citrulline can be converted to L-arginine via the citrulline-NO cycles as well as the urea cycle. The possibility that decreased L-cirtulline contributes to decreased L-arginine concentrations in physically healthy patients with MDD cannot be excluded (45). It has been previously demonstrated that L-arginine may have the potential to treat MDD (46). Taken together, the foregoing studies provide the rational to assess the potential association between L-arginine concentrations and MDD.

To our knowledge, there has been no previous meta-analysis evaluating the association between arginine catabolism and MDD. Herein, the current meta-analysis aims to compare peripheral arginine and its related catabolic products (i.e., ornithine and citrulline) between patients with MDD and healthy controls (HCs) (i.e., healthy volunteers who do not have current or previous history of mental illness).

METHODS

Literature Search

We performed this study according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) (47), and a systematic retrieval of literature from inception to June 2020. The literature search was conducted using the following online databases: PubMed, EMBASE, PsycINFO and Web of Science. The keywords of the search strategy were "major depressive disorder (MDD)", "depression", "mood disorder",

"arginine", "ornithine", "citrulline", "L-arginine", "amino acid", "argininosuccinate". The flow diagram outlining the study selection process is shown in **Figure 1**. The protocol of current meta-analysis has been registered at the Open Science Framework [https://doi.org/10.17605/osf.io/7fn59 (48)].

Selection Criteria

The inclusion of studies were based on the following criteria: (1) adults ($\geq$ 18 years old) with Diagnostic and Statistical Manual of Mental Disorders (DSM) diagnosed MDD (i.e., DSM-III-R, DSM-IV, DSM-IV-TR and DSM-V); (2) healthy volunteers who are not diagnosed with psychiatric illness and do not have a history of mental illness were used as the HC group; (3) measures of the concentrations of arginine, citrulline, or ornithine (one of which was sufficient) assessed in all subjects; and (4) the study type was either a case-control study or cohort study.

Studies were excluded based on the following criteria: (1) non-original research, articles or conference abstracts; (2) case reports, case studies, case series studies, clinical trials and other articles that did not meet the required research type; (3) non-human studies, the research objective did not include patients with MDD, or the case group contains other mental disorders (e.g., schizophrenia, bipolar disorder); (4) comparison of patients before and after treatment; people with other diseases as control group; (5) concentrations of arginine, citrulline, or ornithine were not available; (6) no full-text or studies were repetitive publications from the same datasets by the same or different authors.

Data Extraction and Quality Assessment

Two investigators (CB and LL) independently screened and reviewed articles, **Supplementary Materials**, and extracted relevant information. Several articles may not be captured by our search because their keywords are not exactly matched our search strategies. All reference lists of the retrieved articles were reviewed to identify potential studies for inclusion. The additional articles were manually retrieved from the official website of the journals from the reference lists. The study used standardized tables to extract information for each eligible article. The following information was extracted from each study: first author, publication year, study design, country, geographic location, age, sex (i.e., female, male), body mass index (BMI), type of blood sample specimen required for test (i.e., plasma, serum, platelet), sample detection method [i.e., High Performance Liquid Chromatography (HPLC), Liquid Chromatography Mass Spectrometry (LC-MS), or other], sample size, subjects' mean arginine or ornithine or citrulline concentrations, and standard deviation (SD).

Statistical Analysis

All data analysis was performed using Stata (version 15.0, Stata Corp LP, College Station, TX, USA). Forest plots was used to estimate the association between arginine, its related catabolic products, and MDD, which was evaluated by standardized mean difference (SMD) with a 95% confidence interval (CI). We assigned weights (%) based on the inverse of the variance. The greater weights represent the greater impact on the combined results. The heterogeneity of all studies was assessed by chi-square statistics and the I-Squared (I^2) test. If $P < 0.10$ or $I^2 > 50\%$, we considered that the heterogeneity had statistical differences and a random effects model would be used. Otherwise, the fixed effect meta-analysis would be applied (9).

Subgroup analysis was performed to explore the potential impact of the inclusion characteristics of the studies on the pooled effect size. The effect sizes of arginine, ornithine and citrulline concentrations were calculated for each subgroup. The subgroups were created based on medication status (prescribed medication vs. medication-free), sample types (plasma, serum, or platelet), published year (before vs. after 2010), regional distribution (Asia, Europe, America, or Oceania) of arginine and detection method (Amino acid Analyzer, HPLC, LC-MS). To create subgroups based on living standards of past decade or earlier, studies were divided into two groups according to their publication date (i.e., before 2010 vs. after 2010) (49, 50). Meta-regression was used to investigate the source of heterogeneity, and the effects of both continuous and categorical factors on the study were assessed simultaneously. Sensitivity analysis was used to investigate whether any single study would have an effect on the heterogeneity of total measurements in each meta-analysis. The funnel plot with Begg's test and Egger's test were used to test publication bias.

The Newcastle-Ottawa Scale was used to detect the risk of bias in observational studies. According to the total scores of the Newcastle-Ottawa Scale, the observational studies were divided into three categories: extremely high risk of bias (0–3 points), high risk of bias (4–6 points) and low risk of bias (7–9 points) (51). The bias risk assessment of the included articles is shown in **Supplementary Table 1**.

RESULTS

Basic Characteristics of Included Studies

A total of 8,227 articles were identified from the preliminary search. After screening titles and abstracts, excluding review articles and duplicated articles, and studies that did not meet the inclusion criteria, 29 articles were selected for full-text review. After evaluation, we identified 9 articles that met the inclusion criteria and were selected for analysis. Four articles were retrieved by manual search. The current meta-analysis includes 13 articles with 16 studies (18, 22, 33, 41, 52–60) (**Figure 1**). Of the thirteen articles included, ten articles reported the results of a single study each, and 3 articles each of which reported results from two studies.

The basic characteristics of included studies are illustrated in **Table 1**. The current meta-analysis included 745 (47.5%) individuals with MDD and 823 (52.5%) HCs. 15 of the studies assessed concentrations of arginine, 8 assessed ornithine concentrations, and 6 assessed citrulline concentrations. For the geographic location, 5 studies were conducted in Asia, 6 studies were conducted in Europe, 4 studies were conducted in the United States, and 1 study was conducted in Oceania. Most studies used the detection methods of HPLC ($n = 10$), followed by LC-MS ($n = 4$), and Amino Acid Analyses ($n = 2$). Only one study conducted semi-structured interviews, and the

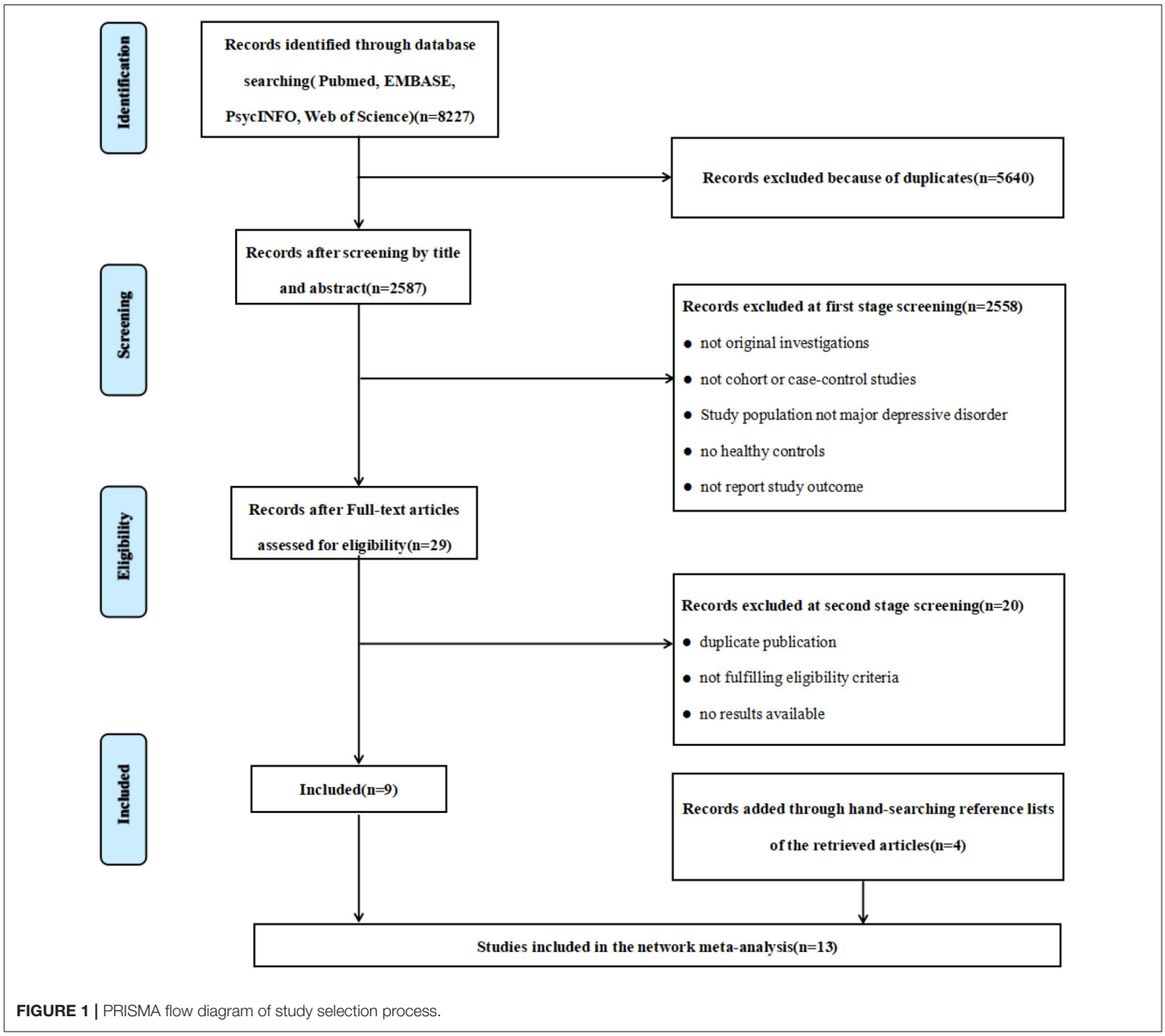

FIGURE 1 | PRISMA flow diagram of study selection process.

remaining studies conducted structured interviews for diagnosis. Participants in 9 studies had received antidepressant medications, and participants in 7 studies reported to be medication-free. The sample types for 11 studies was plasma, 3 studies were serum, and two were platelets. According to the results of the Newcastle-Ottawa Scale, there were 2 articles defined as "high bias risk" with scores of 6, while the others were all defined as "low bias risk" with scores higher than 7.

Homogeneity Analysis and Effect Estimation

Due to the high heterogeneity of arginine ($I^2 = 80.6\%$, $P < 0.001$), ornithine ($I^2 = 87.3\%$, $P < 0.001$) and citrulline ($I^2 = 72.1\%$, $P = 0.003$) reported in the included studies, random effect models were selected for the meta-analysis. No statistical differences in the concentrations of arginine (SMD = 0.02; 95%CI: −0.24, 0.29; $P = 0.86$), ornithine (SMD = −0.01; 95%CI: −0.38, 0.36; $P = 0.96$) and citrulline (SMD = −0.12; 95%CI: −0.43, 0.19; $P = 0.46$) were found between the subjects with MDD and HCs when serum, plasma and platelet are analyzed together. The forest plots are shown in **Figure 2**. Separate meta-analyses of arginine, ornithine and citrulline for serum, plasma and platelet are shown in **Supplementary Figures 5–7**.

Subgroup Analysis and Meta-Regression Results

Subgroup analysis were conducted to explore potential subgroup effects. For the subgroup analysis of arginine concentrations, the distribution trend of whether samples were prescribed medications (medication-free: SMD = −0.25; 95%CI: −0.83,

TABLE 1 | Characteristics of the included studies in the meta-analysis.

References	Country	Case		Control		Sample type	Detection method	semi-structured interview	Diagnosed criteria	Disease state and illness duration	Arginine	Ornithine	Citrulline	Quality Score*
		Sex (M/F)	Age (years)	Sex (M/F)	Age (years)									
Abou-Saleh MT et al.Arab (52)		33/30	36.92 ± 11.33	32/38	35.53 ± 10.12	Plasma	Amino Acid Analyser	No	DSM-III-R	All patients were receiving antidepressant medication	Female (MDD:89.15 ± 30.49 μm/L; HCs:65.40 ± 24.13 μm/L)	Male (MDD:69.23 ± 22.6 μm/L HCs:84.69 ± 25.02 μm/L); Female (MDD:65.45 ± 24.03 μm/L HCs:94.89 ± 40.11 μm/L)		6
Maes et al., (53)	Belgium	17/18	49.65 ± 14.78	10/5	47.5 ± 15.0	Serum	HPLC	Yes	DSM-III-R	8 Non-TRD and 27 TRD	Non-TRD:132 ± 27 μmol/l; TRD:121 ± 20 μmol/ml; HCs:137 ± 28 μmol/l			7
Mauri et al., (54)[a]	Italy	15/14	47.41 ± 10.85	12/16	42.46 ± 14.19	Plasma	HPLC	No	DSM-IV	outpatients, without melancholia, drug-free for at least 4 weeks	MDD:83.49 ± 100.59 nmol/ml HCs:87.58 ± 30.92 nmol/ml			7
Mauri et al., (55)[b]	Italy	NA	47.41 ± 10.85	NA	42.46 ± 14.19	Platelet	HPLC	No	DSM-IV	outpatients, without melancholia, drug-free for at least 4 weeks	MDD:0.86 ± 1.99 $\mu mol/10^{10}$ HCs:1.53 ± 2.94 $\mu mol/10^{10}$			7
Mitani et al., (56)	Japan	11/12	32.78 ± 12.41	17/14	41.46 ± 19.26	Plasma	HPLC	No	DSM-IV	Six depressed patients were drug-free. Seventeen of 23 depressed patients were on antidepressant medication at the time of examination with dosages ranging from 0 to 225 mg imipramine equivalents	Depression:116.8 (54.8) nnol/ml; HCs: 93.2 (42.8) nnol/ml;			6
Mauri et al., (55)[a]	Italy	5/11	50.18 ± 11.55	9/2	39.90 ± 13.39	Plasma	HPLC	No	DSM-IV	outpatients, all affected by recurrent unipolar depression	MDD: 119.24 ± 129.21 nnmol/ml; HCs: 76.97 ± 32.64 nnmol/ml;			7
Mauri et al., (55)[b]	Italy	NA	50.18 ± 11.55	NA	39.90 ± 13.39	Platelet	HPLC	No	DSM-IV	outpatients, all affected by recurrent unipolar depression	MDD:0.93 ± 1.89 $\mu mol/10^{10}$; HCs:1.78 ± 2.38 $\mu mol/10^{10}$			7
Pinto et al., (33)	Brazil	NA	34 ± 4	NA	34 ± 3	Plasma	HPLC	No	DSM-IV	current unipolar major depressive episode or with a diagnosis of comorbid anxiety disorder but no other Axis I disorder	HCs:130 ± 8 μM/ml MDD:104 ± 4 μM/ml	HCs:62 ± 12 μM/ml MDD:97 ± 2 μM/ml		7
Woo et al., (57)	Korea	16/52	65	4/18	68	Plasma	LC-MS/MS	No	DSM-IV	single episode or recurrent	HCs:58.7(39.1–75.2) μmol/l MDD: 69.7(49.0–94.1) μmol/l	HCs:109(88.2–132) μmol/l; MDD: 98.1 (70.5–129) μmol/l	HCs:30.3 (25.7–36.6) μmol/l; MDD: 34.0(25.2–45.6) μmol/l	7

(Continued)

TABLE 1 | Continued

References	Country	Case		Control		Sample type	Detection method	semi-structured interview	Diagnosed criteria	Disease state and illness duration	Arginine	Ornithine	Citrulline	Quality Score*
		Sex (M/F)	Age (years)	Sex (M/F)	Age (years)									
Baranyi et al., (22)	Austria	48/23	49.65 ± 9.84	31/17	46.23 ± 27.51	Plasma	HPLC	No	DSM-IV	all in-patients with major depression	HCs: 97.440(86.117-104.697) μmol/L; MDD: 101.500(86.190-112.940) μmol/L	HCs: 94.050(72.550-103.775) μmol/L; MDD: 82.64(70.390-101.290) μmol/L	HCs: 30.648 ± 7.63μmol/L; MDD: 29.887 ± 6.23μmol/L	8
Hess et al., (58)	Canada	20/15	27.06 ± 9.43	20/16	25.97 ± 8.47	Serum	Amino Acid Analyser	No	DSM-IV-TR	current unipolar major depressive episode or with comorbid anxiety disorders	MDD:73.54 ± 21.53 μmol/L HCs: 84.89 ± 25.16 μmol/L		MDD:31.58 ± 6.05 μmol/L; HCs 35.19 ± 6.85 μmol/L	7
Ali-Sisto et al., (18)	Finland	43/56	39.41 ±11.94	124/129	55.28 ± 10.08	Serum	LC-MS/MS	No	DSM-IV	outpatients, 84 of the patients used antidepressant medication and 48 used antipsychotic medication	MDD:99.15 (83.1–115.92) μmol/L; HCs:116.9 (94.87–142.18) μmol/L	MDD:86.43 (69.46–115.93) μmol/L; HCs:86.97 (65.14–107.94) μmol/L	MDD:29.01 (22.01–33.69) μmol/L; HCs:29.44 (23.31–36.61) μmol/L	8
Ogawa et al., (59)[a]	Japan	85/79	41.77 ± 11.89	100/117	41.2 ± 13.9	Plasma	HPLC	No	DSM-IV	currently depressed [dMDD] and remitted [rMDD])	dMDD:77.91 ± 21.89μM; HCs:79.20 ± 25.70μM	dMDD:74.86 ± 27.37μM; HCs: 87.09 ± 28.58μM		9
Ogawa et al., (59)[b]	Japan	39/26	43.98 ± 13.10	20/45	43.4 ± 13.4	Plasma	LC-MS	No	DSM-IV	currently depressed [dMDD] and remitted [rMDD])	rMDD:83.73 ± 24.08μM; HCs:90.56 ± 20.67μM			9
Moaddel et al., (41)	America	NA	NA	NA	NA	Plasma	LC-MS/MS	No	DSM-IV	recurrent MDD without psychotic features			HCs: 23.80 ± 7.22μM; MDD:19.25 ± 5.17μM	7
Ozden et al., (60)	America	28/49	41.01 ± 12.22	7/20	38.44 ± 12.23	Plasma	HPLC	No	DSM-IV	first or recurrent episode	MDD:6.64 (7.24) μM; HCs:1.42 (1.32) μM	MDD:7.83 (4.33) μM; HCs:4.51 (1.88) μM	MDD:5.25 (1.85) μM; HCs: 4.42 (1.03) μM	8

*DSM, Diagnostic and Statistical Manual of Mental Disorders; MDD, major depressive disorder; HC, healthy control; HPLC, High Performance Liquid Chromatography; LC-MS, Liquid Chromatography Mass Spectrometry; TRD, treatment-resistant depression; NA, Not Available. Mauri et al., (54)[a]: sample type was plasma; Mauri et al., (54)[b]: sample type was platelet; Mauri et al., (55)[a]: sample type was plasma; Mauri et al., (55)[b]: sample type was platelet; Ogawa et al., (59)[a]: dMDD, depressed (non-remitted) patients with major depressive disorder; Ogawa et al., (59)[b]: rMDD, remitted patients; *The quality score was evaluated by the Cochrane's Newcastle–Ottawa scale.*

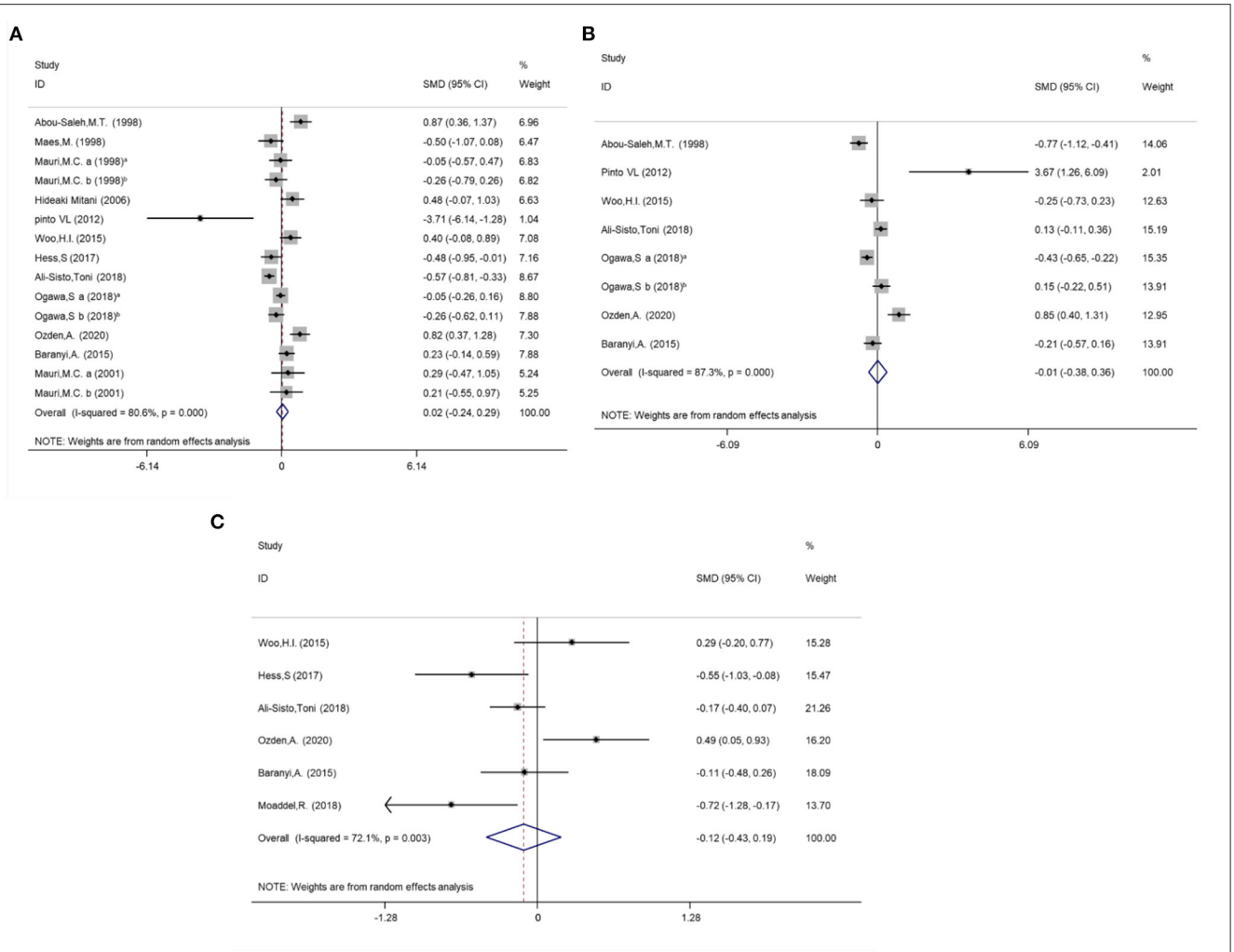

FIGURE 2 | Meta-analysis for the difference of the Arginine, Ornithine and Citrulline concentrations between MDD individuals and controls by random effect analysis. **(A)** Arginine; **(B)** Ornithine. **(C)** Citrulline. Mauri et al. (54) a: sample type was plasma; Mauri et al., (54) b: sample type was platelet; Mauri et al., (55) a: sample type was plasma; Mauri et al., (55) b: sample type was platelet; Ogawa et al., (59) a: dMDD, depressed (non-remitted) individuals with major depressive disorder; Ogawa et al., (59) b: rMDD, remitted individuals.

0.33; $P = 0.39$; $I^2 = 82.9\%$, $P < 0.001$; prescribed medications: SMD = 0.13; 95%CI: −0.17, 0.44; $P = 0.39$; $I^2 = 81.4\%$, $P < 0.001$) and different sample types (plasma: SMD = 0.23; 95%CI: −0.07, 0.53; $P = 0.14$; $I^2 = 76.1\%$, $P < 0.001$; serum: SMD = −0.54; $P < 0.001$; 95%CI: −0.74, −0.35; $I^2 = 0.0\%$, $P = 0.93$; platelet: SMD = −0.11; 95%CI: −0.54, 0.32; $P = 0.62$; $I^2 = 1.8\%$, $P = 0.313$) were also analyzed (**Figure 3**). The subgroup analysis results of medication status and sample types for ornithine and citrulline concentrations were shown in **Supplementary Figure 1**. We also conducted the subgroup analyses with year of publication, regional distribution, and detection methods (**Supplementary Figure 2**).

Meta-regression was performed on five aspects (i.e., published year, medication use status, geographic location, sample types, and detection methods) to investigate the sources of heterogeneity. None of the variables above could explain the heterogeneity of meta-analysis (all $P > 0.05$).

Sensitivity Analysis and Publication Bias

The results of our sensitivity analysis indicated that there was no significant change by omitting a single study, indicating that the models were relatively robust (**Supplementary Figure 3**). The Egger's test and the Begg's test were used to evaluate the publication bias in the study (**Supplementary Figure 4**). The publication bias test was only performed for arginine studies since these were the only studies with a sample size greater than 10. The funnel plots of the included meta-analysis indicated that there was no publication bias of the included studies assessing the concentrations of arginine (Egger's intercept = 0.82; 95%CI = (−2.21, 3.85), $P = 0.57$ and Begg's test Z = 0.10, $P = 0.92$).

DISCUSSION

The main findings of the meta-analysis herein are as follows: (1) the peripheral concentrations of arginine, ornithine, and

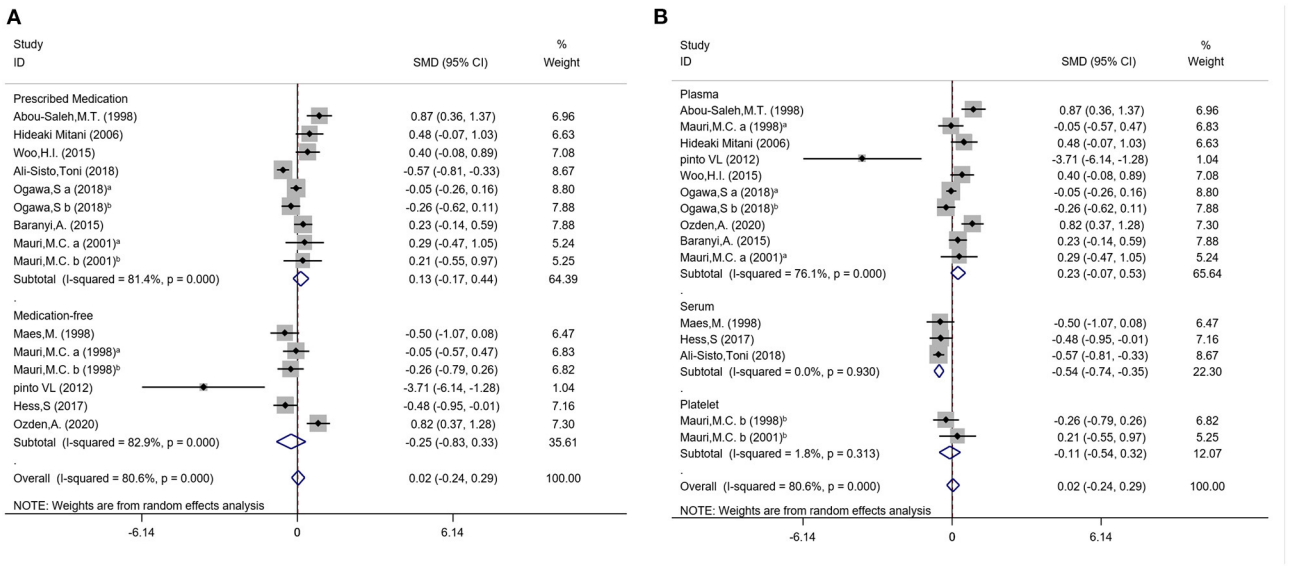

FIGURE 3 | Subgroup differences of the arginine concentrations in subjects with MDD and controls. **(A)** Medication status of arginine; **(B)** Sample types of arginine.

citrulline were not significantly different between patients with MDD and HCs; (2) the peripheral concentrations of arginine were lower in individuals with MDD than the HCs in the subgroups of serum samples; and (3) concurrent medication may contribute to altered peripheral concentrations of arginine, ornithine, and citrulline.

Through subgroup analysis, we found that the status of medication and sample types may contribute to the reduced heterogeneity of the sample. Although no statistically significant results were observed in the subgroup analysis of the medication status, the various trends of prescribed medication and medication-free were observed in arginine concentrations between individuals with MDD and HCs (55). Arginine concentrations reported in individuals with MDD with prescribed medication were generally higher than that of the HC population, while the arginine concentrations in individuals with MDD who were not taking medication were lower in comparison to HCs (58). Ali-Sisto et al. did not report a significant difference of arginine concentrations between responders and non-responders of antidepressants at baseline (18).

The subgroup analysis of sample types indicated that the arginine and citrulline concentrations in the serum samples were significantly lower in individuals with MDD when compared with HCs. The samples from plasma and serum represent circulating concentrations of amino acids. A previous study also illustrated that serum L-arginine concentrations do reflect intracellular L-arginine (61). Pinto et al. reported that reduced plasma L-arginine concentrations were associated with reduced L-arginine flow in platelets in a small sample comparing individuals with MDD and HCs (33).

Both the medication status and fasting status were potential confounders for the amino acid concentrations in blood samples. Psychotropic medications may have direct/indirect effects on NO metabolite concentrations in individuals with MDD. For example, medication may alter the protein-binding of amino acids, possibly leading to dysregulation in renal clearance of these metabolites. Additionally, antidepressants may cause the inhibition of related enzymes (18).

Moreover, no previous research has indicated a significant difference in amino acid concentrations between serum and EDTA-K2 anticoagulated plasma samples in individuals with MDD. A recent methodological study reported that the concentrations of amino acids were higher when compared to heparin plasma, EDTA plasma, and fluoride plasma (62). From the current meta-analysis, we cannot determine the reasons behind the observed L-arginine differences in serum vs. plasma samples between cases and controls. The current findings provide instruction from a methodological perspective on the importance of evaluating serum vs. plasma highlighting the importance of evaluating these variables separately.

To our knowledge, this is the first meta-analysis to explore the associations between arginine and related catabolites and MDD. We cannot conclude that arginine catabolism or the bioavailability of arginine has the potential to decrease or increase in individuals with MDD. Herein, our findings provide a meaningful direction for researchers engaged in the study of the metabolic mechanism of MDD. Our current findings require replication, preferrably with a large, well-characterized sample, sufficient to conduct disparate covariate analysis including buy not limited to subgrouping on the basis of medication status.

Limitations

The findings in our study should be interpreted with caution due to the following methodological aspects affecting the outcomes of this review. Firstly, 16 studies from 13 articles were included in the current study, thus the sample sizes of overall and subgroup analysis were relatively small, and the results of subgroup analysis. Secondly, there was

high heterogeneity in data comparison and data pooling. Through subgroup analysis and meta-regression analysis, the heterogeneity cannot be fully explained. Thirdly, studies that included populations categorized as "prescribed medication" may be limited in their results as some subjects may not have followed the medication regimen according to study protocol and/or included subjects that did not take any of the prescribed medication.

CONCLUSION

Taken together, using meta-analytic techniques we were unable to identify compelling evidence that arginine and/or its catabolic products exhibit significant alteration in adults with MDD vs. HCs. Subgroup analysis indicated that the concentrations of arginine were lower in individuals with MDD than HCs in the subgroups of serum samples. Our findings do not exclude the possibility of a type II error due to confounding factors.

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.

AUTHOR CONTRIBUTIONS

MF, XG, and BC conceived and designed the study. BC and LLi collected the data. LLi performed the statistical analysis. MF, RM, PD, KT, ZR, and LLui contributed to the discussion. All authors revised the paper and approved the final version of this article.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpsyt.2021.686973/full#supplementary-material

REFERENCES

1. Otte C, Gold S, Penninx B, Pariante C, Etkin A, Fava M, et al. Major depressive disorder. *Nat Rev Dis Primers.* (2016) 2:16065. doi: 10.1038/nrdp.2016.65
2. James SL, Abate D, Abate KH, Abay SM, Abbafati C, Abbasi N, et al. Global, regional, and national incidence, prevalence, and years lived with disability for 354 diseases and injuries for 195 countries and territories, 1990and#x2013;2017: a systematic analysis for the Global Burden of Disease Study 2017. *Lancet.* (2018) 392:1789–858. doi: 10.1016/S0140-6736(17)32154-2
3. Kessler RC, Berglund P, Demler O, Jin R, Koretz D, Merikangas KR, et al. The epidemiology of major depressive disorder: results from the National Comorbidity Survey Replication (NCS-R). *JAMA.* (2003) 289:3095–105. doi: 10.1001/jama.289.23.3095
4. Olin B, Jayewardene AK, Bunker M, Moreno F. Mortality and suicide risk in treatment-resistant depression: an observational study of the long-term impact of intervention. *PLoS ONE.* (2012) 7:e48002. doi: 10.1371/journal.pone.0048002
5. Krishnadas R, Cavanagh J. Depression: an inflammatory illness? *J Neurol Neurosurg Psychiatry.* (2012) 83:495–502. doi: 10.1136/jnnp-2011-301779
6. McIntosh A, Hall L, Zeng Y, Adams M, Gibson J, Wigmore E, et al. Genetic and environmental risk for chronic pain and the contribution of risk variants for major depressive disorder: a family-based mixed-model analysis. *PLoS Med.* (2016) 13:e1002090. doi: 10.1371/journal.pmed.1002090
7. Belmaker RH, Agam G. Major depressive disorder. *N Engl J Med.* (2008) 358:55–68. doi: 10.1056/NEJMra073096
8. Block A, Schipf S, Van der Auwera S, Hannemann A, Nauck M, John U, et al. Sex- and age-specific associations between major depressive disorder and metabolic syndrome in two general population samples in Germany. *Nord J Psychiatry.* (2016) 70:611–20. doi: 10.1080/08039488.2016.1191535
9. Cao B, Chen Y, Brietzke E, Cha D, Shaukat A, Pan Z, et al. Leptin and adiponectin levels in major depressive disorder: a systematic review and meta-analysis. *J Affect Disord.* (2018) 238:101–10. doi: 10.1016/j.jad.2018.05.008
10. Nasca C, Bigio B, Lee F, Young S, Kautz M, Albright A, et al. Acetyl-l-carnitine deficiency in patients with major depressive disorder. *Proc Natl Acad Sci U S A.* (2018) 115:8627–32. doi: 10.1073/pnas.1801609115
11. Altamura C, Maes M, Dai J, Meltzer HY. Plasma concentrations of excitatory amino acids, serine, glycine, taurine and histidine in major depression. *Eur Neuropsychopharmacol.* (1995) 5:71–75. doi: 10.1016/0924-977X(95)00033-L
12. Do K, Lauer C, Schreiber W, Zollinger M, Gutteck-Amsler U, Cuénod M, et al. gamma-Glutamylglutamine and taurine concentrations are decreased in the cerebrospinal fluid of drug-naive patients with schizophrenic disorders. *J Neurochem.* (1995) 65:2652–62. doi: 10.1046/j.1471-4159.1995.65062652.x
13. Fonteh AN, Harrington RJ, Tsai A, Liao P, Harrington MG. Free amino acid and dipeptide changes in the body fluids from Alzheimer's disease subjects. *Amino Acids.* (2007) 32:213–24. doi: 10.1007/s00726-006-0409-8
14. Cheng S, Rhee E, Larson M, Lewis G, McCabe E, Shen D, et al. Metabolite profiling identifies pathways associated with metabolic risk in humans. *Circulation.* (2012) 125:2222–31. doi: 10.1161/CIRCULATIONAHA.111.067827
15. Leichtle A, Nuoffer J, Ceglarek U, Kase J, Conrad T, Witzigmann H, et al. Serum amino acid profiles and their alterations in colorectal cancer. *Metabolomics.* (2012) 8:643–53. doi: 10.1007/s11306-011-0357-5
16. Xu H, Fang L, Hu Z, Chen Y, Chen J, Li F, et al. Potential clinical utility of plasma amino acid profiling in the detection of major depressive disorder. *Psychiatry Res.* (2012) 200:1054–7. doi: 10.1016/j.psychres.2012.05.027
17. Maurya P, Noto C, Rizzo L, Rios A, Nunes S, Barbosa D, et al. The role of oxidative and nitrosative stress in accelerated aging and major depressive disorder. *Progress Neuro-Psychopharmacol Biol Psychiatr.* (2016) 65:134–44. doi: 10.1016/j.pnpbp.2015.08.016
18. Ali-Sisto T, Tolmunen T, Viinamaki H, Mantyselka P, Valkonen-Korhonen M, Koivumaa-Honkanen H, et al. Global arginine bioavailability ratio is decreased in patients with major depressive disorder. *J Affect Disord.* (2018) 229:145–51. doi: 10.1016/j.jad.2017.12.030
19. Maes M, Landucci Bonifacio K, Morelli N, Vargas H, Barbosa D, Carvalho A, et al. Major differences in neurooxidative and neuronitrosative stress pathways between major depressive disorder and types I and II bipolar disorder. *Mol Neurobiol.* (2019) 56:141–56. doi: 10.1007/s12035-018-1051-7

20. Mariotti F, Petzke K, Bonnet D, Szezepanski I, Bos C, Huneau J, et al. Kinetics of the utilization of dietary arginine for nitric oxide and urea synthesis: insight into the arginine-nitric oxide metabolic system in humans. *Am J Clin Nutr.* (2013) 97:972–9. doi: 10.3945/ajcn.112.048025
21. Clark TC, Tinsley J, Sigholt T, Macqueen DJ, Martin SA. Arginine, ornithine and citrulline supplementation in rainbow trout: Free amino acid dynamics and gene expression responses to bacterial infection. *Fish Shellfish Immunol.* (2020) 98:374–90. doi: 10.1016/j.fsi.2020.01.026
22. Baranyi A, Amouzadeh-Ghadikolai O, Rothenhäusler H, Theokas S, Robier C, Baranyi M, et al. Nitric oxide-related biological pathways in patients with major depression. *PLoS ONE.* (2015) 10:e0143397. doi: 10.1371/journal.pone.0143397
23. Narvik J, Vanaveski T, Innos J, Philips M-A, Ottas A, Haring L, et al. Metabolic profile associated with distinct behavioral coping strategies of 129Sv and Bl6 mice in repeated motility test. *Sci Rep.* (2018) 8:1–11. doi: 10.1038/s41598-018-21752-9
24. Guelzim N, Mariotti F, Martin P, Lasserre F, Pineau T, Hermier DJA, et al. A role for PPARα in the regulation of arginine metabolism and nitric oxide synthesis. *Amino Acids.* (2011) 41:969–79. doi: 10.1007/s00726-010-0797-7
25. Zhang R, Zhang T, Ali A, Al Washih M, Pickard B, Watson DJC, et al. Metabolomic profiling of post-mortem brain reveals changes in amino acid and glucose metabolism in mental illness compared with controls. *Comput Struct Biotechnol J.* (2016) 14:106–16. doi: 10.1016/j.csbj.2016.02.003
26. Papageorgiou C, Grapsa E, Christodoulou NG, Zerefos N, Stamatelopoulos S, Christodoulou gnjp, et al. Association of serum nitric oxide levels with depressive symptoms: a study with end-stage renal failure patients. *Psychother Psychosom.* (2001) 70:216–20. doi: 10.1159/000056256
27. Suzuki E, Yagi G, Nakaki T, Kanba S, Asai MJJAD. Elevated plasma nitrate levels in depressive states. *J Affect Disord.* (2001) 63:221–4. doi: 10.1016/S0165-0327(00)00164-6
28. Kim YK, Paik JW, Lee SW, Yoon D, Han C, Lee BHJP, et al. Increased plasma nitric oxide level associated with suicide attempt in depressive patients. *Progress Neuro-Psychopharmacol Biol Psychiatr.* (2006) 30:1091–6. doi: 10.1016/j.pnpbp.2006.04.008
29. Joca SRL, Guimarães FS. Inhibition of neuronal nitric oxide synthase in the rat hippocampus induces antidepressant-like effects. *Psychopharmacology.* (2006) 185:298–305. doi: 10.1007/s00213-006-0326-2
30. Harvey BH and Experimental. Affective disorders and nitric oxide: a role in pathways to relapse and refractoriness? *Human Psychopharmacol Clin Exper.* (2015) 11:309–19. doi: 10.1002/(SICI)1099-1077(199607)11:4<309::AID-HUP775>3.0.CO;2-B
31. Wegener G, Volke V, Harvey BH. S.08.04 The nitric oxide pathway in anxiety and stress-related disorders. *Eur Neuropsychopharmacol.* (2007) 23:S2–3. doi: 10.1016/j.eurpsy.2008.01.010
32. Jesse CR, Bortolatto CF, Savegnago L, Rocha JB, Nogueira CW. Involvement of L-arginine-nitric oxide-cyclic guanosine monophosphate pathway in the antidepressant-like effect of tramadol in the rat forced swimming test. *Progress Neuro-Psychopharmacol Biol Psychiatr.* (2008) 32:1838–43. doi: 10.1016/j.pnpbp.2008.08.010
33. Pinto VL, de Souza PF, Brunini TM, Oliveira MB, Moss MB, Siqueira MA, et al. Low plasma levels of L-arginine, impaired intraplatelet nitric oxide and platelet hyperaggregability: implications for cardiovascular disease in depressive patients. *J Affect Disord.* (2012) 140:187–92. doi: 10.1016/j.jad.2012.02.008
34. Lundberg JO, Gladwin MT, Weitzberg E. Strategies to increase nitric oxide signalling in cardiovascular disease. Nature reviews. *Drug Disc.* (2015) 14:623–41. doi: 10.1038/nrd4623
35. Fromm HJ, Hargrove M. *Essentials of Biochemistry*. Springer Science & Business Media. (2012). doi: 10.1007/978-3-642-19624-9
36. Bersani FS, Wolkowitz OM, Lindqvist D, Yehuda R, Flory J, Bierer LM, et al. Global arginine bioavailability, a marker of nitric oxide synthetic capacity, is decreased in PTSD and correlated with symptom severity and markers of inflammation. *Brain Behav Immun.* (2016) 52:153–60. doi: 10.1016/j.bbi.2015.10.015
37. Elgün S, Kumbasar H. Increased serum arginase activity in depressed patients. *Progress Neuropsychopharmacol Biol Psychiatr.* (2000) 24:227–32. doi: 10.1016/S0278-5846(99)00100-1
38. Mayoral-Mariles A, Cruz-Revilla C, Vega-Manriquez X, Aguirre-Hernandez R, Severiano-Perez P, Aburto-Arciniega E, et al. Plasma amino acid levels discriminate between control subjects and mildly depressed elderly women. *Arch Med Res.* (2012) 43:375–82. doi: 10.1016/j.arcmed.2012.07.006
39. Liu P, Jing Y, Zhang HJN. Age-related changes in arginine and its metabolites in memory-associated brain structures. *Neuroscience.* (2009) 164:611–28. doi: 10.1016/j.neuroscience.2009.08.029
40. Rotroff D, Corum D, Motsinger-Reif A, Fiehn O, Bottrel N, Drevets W, et al. Metabolomic signatures of drug response phenotypes for ketamine and esketamine in subjects with refractory major depressive disorder: new mechanistic insights for rapid acting antidepressants. *Transl Psychiatry.* (2016) 6:e894. doi: 10.1038/tp.2016.145
41. Moaddel R, Shardell M, Khadeer M, Lovett J, Kadriu B, Ravichandran S, et al. Plasma metabolomic profiling of a ketamine and placebo crossover trial of major depressive disorder and healthy control subjects. *Psychopharmacology (Berl).* (2018) 235:3017–30. doi: 10.1007/s00213-018-4992-7
42. Kurata K, Shigemi K, Tomonaga S, Aoki M, Furuse MJN. L-Ornithine attenuates corticotropin-releasing factor-induced stress responses acting at GABA A receptors in neonatal chicks. *Neuroscience.* (2010) 172:226–31. doi: 10.1016/j.neuroscience.2010.10.076
43. Hoekstra R, Fekkes D, Pepplinkhuizen L, Loonen AJ, Tuinier S, Verhoeven WM. Nitric oxide and neopterin in bipolar affective disorder. *Neuropsychobiology.* (2006) 54:75–81. doi: 10.1159/000096042
44. Cooke RBSB-BASPTJCOTTBJ. Asymmetric dimethylarginine (ADMA): a novel risk factor for endothelial dysfunction: its role in hypercholesterolemia. *Circulation.* (1998) 98:1842–7. doi: 10.1161/01.CIR.98.18.1842
45. Erez A, Nagamani SCS, Shchelochkov OA, Premkumar MH, Campeau PM, Chen Y, et al. Requirement of argininosuccinate lyase for systemic nitric oxide production. *Nat Med.* (2011) 17:1619–26. doi: 10.1038/nm.2544
46. Selley ML. Increased (E)-4-hydroxy-2-nonenal and asymmetric dimethylarginine concentrations and decreased nitric oxide concentrations in the plasma of patients with major depression. *J Affect Disord.* (2004) 80:249–56. doi: 10.1016/S0165-0327(03)00135-6
47. Moher D, Liberati A, Tetzlaff J, Altman DG, Prisma Group. Preferred reporting items for systematic reviews and meta-analyses: the PRISMA statement. *PLoS Med.* (2009) 6:e1000097. doi: 10.1371/journal.pmed.1000097
48. Cao B, Deng R, Wang D, Li L, Ren Z, Xu L, et al. Association between arginine catabolism and major depressive disorder: a protocol for the systematic review and meta-analysis of metabolic pathway. *Medicine.* (2020) 99:e21068. doi: 10.1097/MD.0000000000021068
49. Shariati A, Dadashi M, Moghadam MT, van Belkum A, Yaslianifard S, Darban-Sarokhalil D. Global prevalence and distribution of vancomycin resistant, vancomycin intermediate and heterogeneously vancomycin intermediate Staphylococcus aureus clinical isolates: a systematic review and meta-analysis. *Sci Rep.* (2020) 10:12689. doi: 10.1038/s41598-020-69058-z
50. Yi ZH, Luther Y, Xiong GH, Ni YL, Yun F, Chen J, et al. Association between diabetes mellitus and lung cancer: Meta-analysis. *Eur J Clin Invest.* (2020) 50:e13332. doi: 10.1111/eci.13332
51. Lo CK, Mertz D, Loeb M. Newcastle-Ottawa Scale: comparing reviewers' to authors' assessments. *BMC Med Res Methodol.* (2014) 14:45. doi: 10.1186/1471-2288-14-45
52. Abou-Saleh MT, Karim L, Krymsky M. The biology of depression in Arab culture. *Nord J Psychiatry.* (1998) 52:177–82. doi: 10.1080/08039489850139067
53. Maes M, Verkerk R, Vandoolaeghe E, Lin A, Scharpe S. Serum levels of excitatory amino acids, serine, glycine, histidine, threonine, taurine, alanine and arginine in treatment-resistant depression: modulation by treatment with antidepressants and prediction of clinical responsivity. *Acta Psychiatr Scand.* (1998) 97:302–8. doi: 10.1111/j.1600-0447.1998.tb10004.x
54. Mauri MC, Ferrara A, Boscati L, Bravin S, Zamberlan F, Alecci M, et al. Plasma and platelet amino acid concentrations in patients affected by major depression and under fluvoxamine treatment. *Neuropsychobiology.* (1998) 37:124–9. doi: 10.1159/000026491
55. Mauri MC, Boscati L, Volonteri LS, Scalvini ME, Steinhilber CP, Laini V, et al. Predictive value of amino acids in the treatment of major depression with fluvoxamine. *Neuropsychobiology.* (2001) 44:134–8. doi: 10.1159/000054933

56. Mitani H, Shirayama Y, Yamada T, Maeda K, Ashby CRJr, Kawahara R. Correlation between plasma levels of glutamate, alanine and serine with severity of depression. *Prog Neuropsychopharmacol Biol Psychiatry.* (2006) 30:1155–8. doi: 10.1016/j.pnpbp.2006.03.036
57. Woo HI, Chun MR, Yang JS, Lim SW, Kim MJ, Kim SW, et al. Plasma amino acid profiling in major depressive disorder treated with selective serotonin reuptake inhibitors. *CNS Neurosci Ther.* (2015) 21:417–24. doi: 10.1111/cns.12372
58. Hess S, Baker G, Gyenes G, Tsuyuki R, Newman S, Le Melledo JM. Decreased serum L-arginine and L-citrulline levels in major depression. *Psychopharmacology.* (2017) 234:3241–7. doi: 10.1007/s00213-017-4712-8
59. Ogawa S, Koga N, Hattori K, Matsuo J, Ota M, Hori H, et al. Plasma amino acid profile in major depressive disorder: Analyses in two independent case-control sample sets. *J Psychiatr Res.* (2018) 96:23–32. doi: 10.1016/j.jpsychires.2017.09.014
60. Ozden A, Angelos H, Feyza A, Elizabeth W, John P. Altered plasma levels of arginine metabolites in depression. *J Psychiatr Res.* (2020) 120:21–8. doi: 10.1016/j.jpsychires.2019.10.004
61. Loscalzo J. An experiment of nature: genetic L-arginine deficiency and NO insufficiency. *J. Clin Invest.* (2001) 108:663–4. doi: 10.1172/JCI13848
62. Sotelo-Orozco J, Chen SY, Hertz-Picciotto I, Slupsky CM. A comparison of serum and plasma blood collection tubes for the integration of epidemiological and metabolomics data. *Front Mol Biosci.* (2021) 8:682134. doi: 10.3389/fmolb.2021.682134
63. Dhir A, Kulkarni SK. Involvement of nitric oxide (NO) signaling pathway in the antidepressant action of bupropion, a dopamine reuptake inhibitor. *Eur J Pharmacol.* (2007) 568:177–85. doi: 10.1016/j.ejphar.2007.04.028
64. Wu G, Meininger CJ. Arginine nutrition and cardiovascular function. *J Nutr.* (2000) 130:2626–9. doi: 10.1093/jn/130.11.2626

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