Methylation levels in the GRIN2B promoter region are associated with bipolar disorder and its anxiety and insomnia symptoms

HAO YU, YAO WU and SHAOHONG ZOU

Department of Clinical Psychology, People's Hospital of Xinjiang Uygur Autonomous Region, Urumqi, Xinjiang 830001, P.R. China

Received January 30, 2025; Accepted April 30, 2025

DOI: 10.3892/etm.2025.12894

Abstract. This study investigated the potential role of promoter-region DNA methylation in *GRIN2B* in bipolar disorder (BD) and its association with anxiety and insomnia symptoms among patients with BD. The DNA methylation levels in the *GRIN2B* promoter region were quantified using MassARRAY technology in peripheral blood samples from 31 patients with BD and 32 healthy controls. To assess associations, binary logistic regression and partial correlation analyses were performed to examine potential links between *GRIN2B* methylation, BD diagnosis and symptom severity. The exploratory analysis revealed six differentially methylated CpG sites between groups, four of which exhibited hypermethylation in patients with BD compared with the controls. Significant positive correlations were observed between Hamilton Anxiety Rating Scale scores and CpG9 methylation (r=0.408, P=0.038), as well as between Pittsburgh Sleep Quality Index scores and CpG8 methylation (r=0.419, P=0.033). These findings provide preliminary evidence supporting an association between *GRIN2B* methylation levels and BD, as well as related clinical symptoms. However, given the small sample size and the exploratory nature of this study, these results should be interpreted with caution. Further replication in larger, longitudinal cohorts is warranted to confirm these observations and evaluate their clinical implications.

Introduction

Bipolar disorder (BD) is associated with severe mental disability and an increased risk of suicide, significantly contributing to the societal burden (1,2). Insomnia and anxiety, two common concomitant symptoms of BD, often persist throughout the course of the illness. Approximately 45% of individuals with BD will experience co-morbid anxiety disorders over their lifetime (3). This comorbidity further exacerbates the risk of substance dependence and suicide (4), worsening social functioning and quality of life in individuals with BD (5). Crucially, anxiety symptoms have become a key target for early intervention in individuals at risk of BD (6), anxiety in BD is associated with an increased risk of subjective sleep disturbances (7). Sleep, as a key regulator of both metabolic homeostasis and oxidative stress, plays a particularly significant role; when disrupted, it can contribute to the onset and progression of BD. Given their role as cross-diagnostic precursors, insomnia and anxiety represent high-priority therapeutic targets for preventing BD episodes (8,9).

The N-methyl-D-aspartate receptor subunit 2B (*GRIN2B*) gene, which is located at 12p13.1, spans 419 kilobases and consists of 15 exons. N-methyl-D-aspartate receptors (NMDARs), a class of ionotropic glutamate receptors, are involved in regulating neuronal activity, synaptic plasticity and excitatory transmission, with substantial implications for the pathophysiology and treatment of affective disorders (10). The *GRIN2B* gene encodes the NMDAR subunit 2B (NR2B), which is crucial for determining both the structure and functional dynamics of NMDARs; overexpression of NR2B leads to long-lasting enhancement and increased synaptic efficacy (11). Light, which is the primary driver of sleep rhythm regulation (12), triggers numerous intracellular cascades through NMDARs in the suprachiasmatic nucleus (SCN), ultimately affecting the expression of clock genes (13) studies have demonstrated that circadian rhythm disturbances in mice correlate with reduced expression of the NR2B subunit of NMDARs in the SCN (14,15). Additionally, rats subjected to maternal separation showed a significant increase in anxiety, accompanied by upregulated expression of the *GRIN2B* gene (16), while ethanol-exposed mice displayed anxiety-like behaviors with elevated levels of *GRIN2B* mRNA expression in the cerebellum (17).

DNA methylation, one of the most stable epigenetic mechanisms, is increasingly recognized as a potential biomarker for numerous neuropsychiatric disorders (18) and various malignancies (19). It plays a significant role in the pathophysiology of BD (20) and may serve as a biomarker for variability in BD treatment response (21). Numerous studies have identified significant DNA methylation changes in patients with BD, particularly in regions such as the prefrontal cortex and

Correspondence to: Professor Shaohong Zou, Department of Clinical Psychology, People's Hospital of Xinjiang Uygur Autonomous Region, 91 Tianchi Road, Jiefang South Road, Tianshan, Urumqi, Xinjiang 830001, P.R. China
E-mail: zoushaohong@126.com

Key words: GRIN2B, DNA methylation, bipolar disorder, anxiety, insomnia

peripheral leukocytes (22-24), with potential links to suicidal behavior (25), cognitive impairment (26) and substance dependence (27). Emerging evidence from cancer epigenetics demonstrates that methylation patterns can effectively stratify disease subtypes and predict clinical outcomes (28,29), suggesting similar precision medicine applications could be explored in BD. However, few studies have specifically examined the associations of DNA methylation with anxiety and insomnia in BD, despite established methodological frameworks for epigenetic analysis in other neuropsychiatric conditions, such as depression (30) and schizophrenia (31).

Based on these findings, *GRIN2B* epigenetics appears to play a significant role in the development of BD and related anxiety and insomnia symptoms. However, the role of *GRIN2B* in BD remains controversial; a study detected no disease-associated variants in glutamatergic genes (including *GRIN2B*) through targeted sequencing in patients with BD (32), whereas another study reported associations between *GRIN2B* genotypes and both psychotic symptoms and disease relapse in BD (33). Research on *GRIN2B* gene inheritance as it relates to anxiety and insomnia has been largely restricted to animal models (34,35). Therefore, to further clarify the relationship between *GRIN2B* gene inheritance (particularly DNA methylation) and BD, as well as its potential role in anxiety and insomnia among patients with BD, DNA methylation levels were measured in the *GRIN2B* gene's promoter region in peripheral blood leukocytes from patients with BD and healthy controls via the MassARRAY method and the possibility of an association was analyzed.

Patients and methods

Participants and procedure. A total of 31 patients diagnosed with BD (in the depressive phase) were recruited from the inpatient units of the Clinical Psychology Department at the People's Hospital of Xinjiang Uygur Autonomous Region (Urumqi, China) between April and December 2023, while 32 healthy controls (HCs) were concurrently enrolled from hospital staff and students at the affiliated Medical University during the same study period. All participants were aged 18-55 years. The upper age limit was set to 55 years to minimize age-related confounding factors. This threshold aligns with evidence that epigenetic drift accelerates after age 55 (36), age-associated methylation changes increasingly interact with inflammatory/metabolic pathways beyond age 50 (37) and neurodegenerative comorbidities in older populations may introduce spurious associations (38). BD diagnosis was confirmed using the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition, Text Revision (DSM-IV-TR) criteria (39), based on structured clinical interviews conducted by two senior psychiatrists. Eligible patients met the following criteria: i) No medication, psychotherapy or physical therapy for ≥6 months before recruitment; ii) Hamilton Depression Rating Scale (HAMD)-24 [specifying the 24-item Hamilton Depression Rating Scale (HAMD-24); total score range: 0-76] to distinguish clinical severity thresholds from the 17-item version (HAMD-17), total score >20 (40); and iii) Young Mania Rating Scale (YMRS) total score <7 (41). The exclusion criteria (applied to all participants) were as follows: i) Comorbid psychiatric disorders (DSM-IV-TR axis I/II) (42); ii) history of organic brain disease or severe systemic illness; iii) pregnancy or lactation (women); and iv) substance abuse/dependence (drugs, alcohol or psychoactive substances). This study was reviewed and approved by the Ethics Committee of the People's Hospital of Xinjiang Uygur Autonomous Region (Urumqi, China; approval no. KY2023020968). All subjects volunteered to participate in the study and provided written informed consent prior to the study.

Data collection. Demographic data, including age, gender, education level and history of smoking and alcohol use, were collected from all participants. Clinical data, such as age at onset, illness duration, subtype, HAMD-24 score, depression severity, YMRS score, 14-item Hamilton Anxiety Scale (HAMA-14) (43) score and Pittsburgh Sleep Quality Index (PSQI) (44) score, were obtained from patients only.

DNA extraction and bisulfite conversion. Peripheral fasting venous blood samples (~5 ml) were collected from patients with BD using EDTA anticoagulant tubes (BD Biosciences) within 24 h of hospital admission, prior to the initiation of any new pharmacotherapy. Healthy control samples were obtained the following morning after enrollment. Genomic DNA was subsequently extracted using the solution-based Genenode DNA extraction kit (Wuhan Genenode Biotech) according to the manufacturer's protocol and stored at -80˚C. Quality control assessments included spectrophotometric analysis for determining DNA concentration and agarose gel electrophoresis to verify DNA integrity. Following extraction, the isolated DNA underwent modification and purification using the EZ DNA Methylation-Gold™ Kit (Zymo Research Corp.) according to the manufacturer's protocol. During this process, cytosine residues that were non-methylated were deaminated to uracil, while methylated cytosine residues remained stable.

DNA methylation assay

Primer design. The CpG island prediction website was utilized to predict potential CpG islands in the *GRIN2B* promoter region gene sequences, identifying two CpG islands (Fig. 1A). The Agena EpiDesigner program (http://www.epidesigner.com) was used to design primers for the target *GRIN2B* sequences (Fig. 1B), and a fragment with a high level of CpG methylation (#14 in Fig. 2A) was selected as the primer sequence (Fig. 2A and B). Details of the primer sequences are provided in Table I.

Methylation assay of GRIN2B. The bisulfite-converted *GRIN2B* DNA was amplified by PCR using the primers mentioned above. Optimal amplification conditions were achieved through digestion with shrimp alkaline phosphatase, transcriptional cleavage and resin-based purification. The purified PCR product was then transferred to a 384-well SpectroCHIP® bioarray (Axygen; Corning, Inc.) for precise spot sampling using the Agena Nanodispenser RS1000 spotter (Agena Bioscience). The spotted bioarray was analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry with the MassARRAY Analyzer 4.0 (Axygen; Corning, Inc.) to generate mass spectra. Methylation levels were then quantified from these spectra using Epityper 1.2 software (Sequenom).

A

Observed vs Expected

Obs/Exp

Bose number

Percentage

Percentage

Bose number

Putative islands

Threshold

Bose number

B

ctgatttatggaaaatacagcaagggtcttcaatttCGaactacagctcCG[1]ccttggagatgctttcCG[2]cagccCGCGggcCG[3]ccc
aCG[4]gggggcactgtctagctcccctCG[5]cccctcagcCGctctcCGtCGgtgctgttcccCGctgCGgcCGggaggaggCG[6]
ccaCGgactCG[7]ggctaagcttcttccccttcCGtccccttccatccaccttaccaccccacttccccagcacatgaCG[8]tgggaagct
gcctcCG[9]ccagagtcttagcaagaggagtgagggggtgcaggggcttgggggtggggtggggtggggggtgtggagaaagaggaca
CGCGCG[10]ctcacaccCG[11]cccctgggagcagaagcagtatcttccccagcttCGtgggtttctcctcccccCGgagtcagggtgt
gCG[12]aggtagggggtgtgtgtgtgtctgtgtgtgtgtgCGCGgcaCGtttttgagtgtgagacccaaatccaga

Figure 1. (A) CpG islands predicted by EMBOSS. CpG island locations: Island 1 (1,157-1,475, 319 bp); Island 2 (1,670-1,908, 239 bp); Core criteria: Obs/Exp, >0.6; GC%, >50; length, >200 bp. (B) The target GRIN2B sequences: The black boxes highlight the contiguous CpG sites analyzed, with the reported values representing the average methylation level across these sites. Undetectable CpG sites are indicated in gray, while the 12 successfully interrogated CpG sites are marked in yellow. Obs, observed; Exp, expected.

Statistical analysis. All analyses were performed using SPSS 27.0 (IBM Corp.) and OriginPro 8.5.0 (OriginLab Corp.). Continuous variables were assessed for normality using Shapiro-Wilk tests. Normally distributed data were expressed as the mean ± standard deviation and compared using independent-samples t-tests. Non-normally distributed data were reported as the median (interquartile range) and analyzed with Mann-Whitney U-tests. Categorical variables were presented as frequencies (percentages) and evaluated by χ^2 tests. All group comparisons were adjusted using the Bonferroni method to control for Type I error inflation. Binary logistic regression models adjusted for age, sex and education duration were constructed to examine the association between *GRIN2B* promoter methylation (independent variable) and bipolar disorder diagnosis (dependent variable). Partial correlation analyses (based on the Pearson correlation coefficient) controlling for age, sex, education level and HAMD scores were conducted to assess relationships between *GRIN2B* methylation levels and clinical symptom severity, specifically insomnia severity (PSQI) and anxiety severity (HAMA-14). A two-sided α-level of 0.05 was set as the statistical significance threshold for all analyses. All tests were performed with 95% confidence intervals (95% CI).

Results

Sample description. In Table II, the demographic and clinical profiles of patients with BD and healthy controls

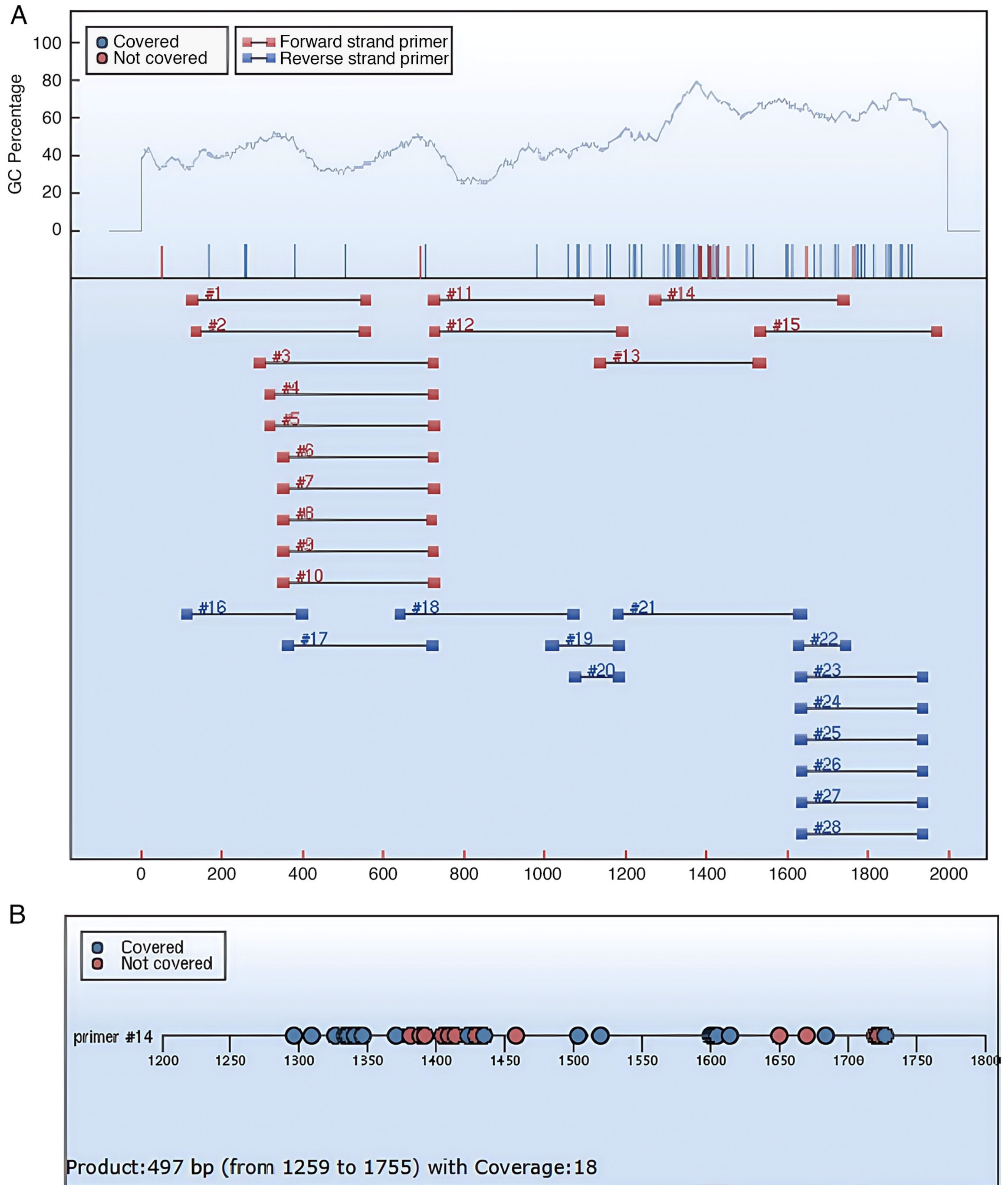

Figure 2. Target gene fragments. (A) Candidate Programs for Targeted Regions at a Glance. (B) Program 14 information: The sequences shown in the figure are forward sequences. Dots indicate CpG sites, with blue-marked CpG sites representing those that can be detected. Red-labeled CpG sites, however, cannot be detected due to issues with the sequence.

were presented. The BD group included 31 participants [8 male (25.8%), 23 female (74.2%)] with a median age of 23 years [interquartile range (IQR), 20-39], while the HC group comprised 32 individuals [11 male (34.4%), 21 female (65.6%)] with a median age of 26.5 years (IQR, 22.0-37.5). No statistically significant differences were observed between the groups in terms of age, years of education, gender or smoking and drinking history (P>0.05).

GRIN2B DNAm and BD

Altered GRIN2B DNAm patterns in patients with BD. In the present study, the DNA methylation levels of 12 CpG sites in

Table I. Primer information.

Primer	5' end primer sequence	3' end primer sequence
Start, bp	1,259	1,755
Size, bp	28	27
Tm, ˚C	58.14	57.06
GC%	25	22
Cs	4	5
Original sequence	CTGATTTATGGAAAATACAGCAAGGGTC	TCTGGATTTGGGTCTCACACTCAAAAA
Methylation sequence	TTGATTTATGGAAAATATAGTAAGGGTT	TCTAAATTTAAATCTCACACTCAAAAA
Insert sequence	aggaagagag	cagtaatacgactcactatagggagaaggct

Product size, 497; no. of CpGs, 30; coverage, 18. Tm, melting temperature.

Table II. Demographic and clinical information of BD and HC groups.

Item	BD (n=31)	HC (n=32)	χ^2/t/Z	P-value
Age, years	23.0 (20.0-39.0)	26.5 (22.0-37.5)	-1.343	0.179
Education, years	16.0 (15.0-16.0)	16.0 (16.0-16.0)	-0.748	0.455
Sex, male/female	(8,23)	(11,21)	0.207	0.649
Smoking, yes/no	(6,25)	(8,24)	0.290	0.590
Drinking, yes/no	(6,25)	(7,25)	0.061	0.805
Age of onset, years	20 (16.58-31.00)	-	-	-
Disease duration, years	4 (1.42-8.00)	-	-	-
HAMD-24	32 (23-36)	-	-	-
YMRS	2 (2-3)	-	-	-
HAMA-14	19 (15-29)	-	-	-
PSQI	14.32±4.05	-	-	-
Subtype (BDI/BDII)	(13,18)	-	-	-
Depression severity (moderate/severe)	(18,13)	-	-	-

Age, education, age of onset, disease duration, HAMD-24, YMRS, HAMA-14 and PSQI are expressed as the median (interquartile range). HAMD-24 (total score range: 0-76 to distinguish clinical severity thresholds from the HAMD-17) severity criteria: 20-34, moderate (n=18); ≥35, severe (n=13). BD, bipolar disorder; HC, healthy controls; HAMD, Hamilton Depression Rating Scale; YMRS, Young Mania Rating Scale; HAMA, Hamilton anxiety scale; PSQI, Pittsburgh Sleep Quality Index scale.

the *GRIN2B* promoter region were compared between patients with BD and HCs. An independent-samples t-test showed that 6 out of the 12 CpG sites (50%) had statistically significant differences in DNA methylation levels between the groups (Fig. 3). Specifically, CpG3, CpG5, CpG10 and CpG12 showed hypermethylation, while CpG1 and CpG7 showed hypomethylation in patients with BD compared to HCs. Among these, CpG3 (BD: 52.52±10.00%; HC: 32.60±25.70%) and CpG1 (BD: 36.03±13.94%; HC: 19.15±12.33%) exhibited the most significant differences in methylation levels between the two groups (P<0.001).

Association of BD with GRIN2B methylation. The predictive role of GRIN2B DNA methylation in the development of BD was analyzed using a binary logistic regression model with stepwise forward selection. Age, gender and years of education were included as covariates and the final model chosen through stepwise selection yielded a chi-square test value of χ^2=44.49 (P<0.001), indicating model significance. The Hosmer-Lemeshow test result was χ^2=3.896 (P=0.866), suggesting a good fit. The model identified four variables associated with the likelihood of BD development, specifically the hypermethylation of CpG3, CpG5, CpG10 and CpG12. Among these, CpG3 [P=0.002, odds ratio (OR)=1.079, 95% CI: 1.029-1.132] and CpG10 (P=0.002, OR=1.113, 95% CI: 1.042-1.190) demonstrated a significant predictive role (Table III).

Association of GRIN2B DNA methylation with anxiety and insomnia in BD. Partial correlation analysis (Table IV) revealed that the HAMA-14 score was positively associated with methylation levels of CpG9 (r=0.408, P=0.038) after controlling for age, gender and years of education (r=0.419, P=0.033). Similarly, the PSQI score showed a positive correlation with methylation levels of CpG8 at the *GRIN2B* locus.

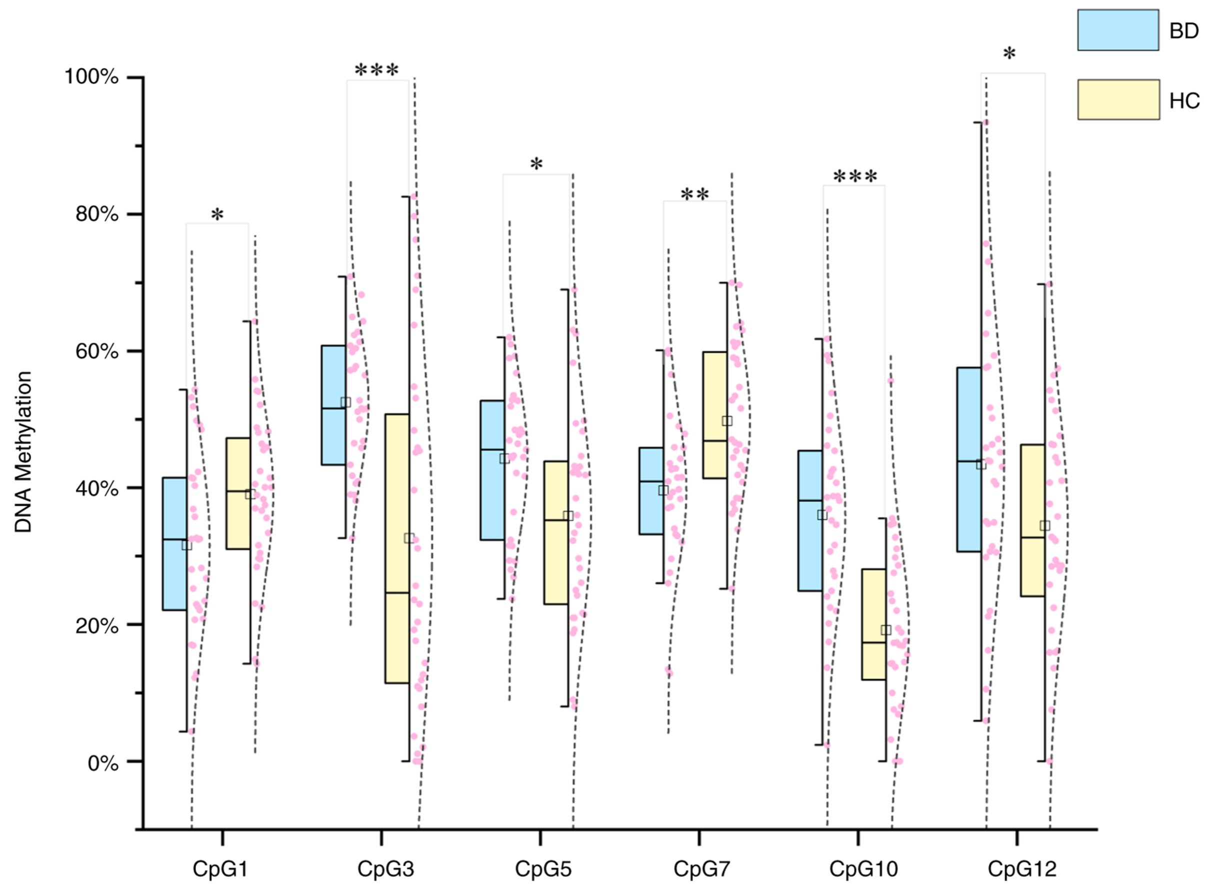

Figure 3. Comparative analysis of *GRIN2B* methylation profiles in BD and HC cohorts. Boxplots display the median (central line), mean (black square), interquartile range (boxes), theoretical normal distribution (black dashed curve), individual measurements (pink dots) and error bars represent the standard deviation. Significance gradient (group comparisons by independent samples t-test): *P<0.05, **P<0.01, ***P<0.001 (two-tailed). BD, bipolar disorder; HC, healthy controls.

No significant correlations were observed between other CpG sites and either the HAMA or PSQI scores (P>0.05).

Discussion

To our knowledge, this study provides the first evidence of differential DNA methylation patterns in the *GRIN2B* promoter region among patients with BD. The present findings not only demonstrate the association between *GRIN2B* methylation status and BD diagnosis but also reveal clinically meaningful correlations with comorbid anxiety symptoms and sleep disturbances in this population.

The present analysis revealed differential DNA methylation patterns at specific CpG sites within the *GRIN2B* promoter region between patients with BD and HCs. Among the 12 analyzed CpG loci, six exhibited methylation alterations: CpG3, CpG5, CpG10 and CpG12 showed hypermethylation, whereas CpG1 and CpG7 displayed hypomethylation. Notably, after adjusting for covariates through logistic regression, only the hypermethylation patterns at CpG3, CpG5, CpG10 and CpG12 maintained statistical significance, suggesting that site-specific epigenetic modifications may be particularly relevant to BD pathophysiology. The *GRIN2B* gene encodes a critical subunit of the NMDAR complex and DNA methylation-dependent transcriptional modulation may alter NMDAR subunit composition, potentially disrupting synaptic plasticity in prefrontal-temporal-limbic circuits. The functional impact of BD-associated *GRIN2B* polymorphisms identified in German-Jewish (45) and Chinese Han populations (46) could be modulated by the local methylation status. Notably, the clinical relevance of these mechanisms is underscored by prior findings that the *GRIN2B* genotype predicts psychotic symptom severity and relapse frequency in patients with BD (33), suggesting methylation-mediated expression changes may similarly impact the disease course. Intriguingly, the therapeutic efficacy of quetiapine in BD may involve *GRIN2B*-related pathways, as molecular docking studies suggest its interaction with neuroactive ligand-receptor systems regulated by *GRIN2B* (47). Hypermethylation-induced *GRIN2B* downregulation could reduce NMDAR density in key brain regions (e.g., prefrontal cortex and hippocampus) (48) creating a neurobiological state amenable to mood stabilizer modulation. While the present findings align with gene-environment interaction models proposing methylation as a dynamic interface between genetic predisposition and environmental stressors, three critical considerations emerge: First, the observed methylation differences may represent either compensatory adaptations or pathogenic processes. Second, glutamatergic dysfunction (49) could serve as both precursor and consequence of neurodevelopmental alterations in BD; and third, the functional consequences of site-specific methylation require validation through allele-specific expression analyses.

Table III. Binary logistic regression analysis of the effect of *GRIN2B* DNA methylation on bipolar disorder.

CpG methylation site	B	SE	Wald	P-value	OR	95%CI
CpG3	0.076	0.024	9.759	0.002	1.079	1.029-1.132
CpG5	0.082	0.034	5.798	0.016	1.085	1.015-1.160
CpG10	0.107	0.034	10.058	0.002	1.113	1.042-1.190
CpG12	0.045	0.022	4.153	0.042	1.046	1.002-1.093

SE, standard error; OR, odds ratio.

Table IV. Correlation analysis of *GRIN2B* DNA methylation with anxiety and insomnia in bipolar disorder.

Methylation % at CpG site	HAMA score		PSQI score	
	r	P-value	r	P-value
CpG1	-0.178	0.383	-0.172	0.402
CpG2	0.098	0.635	-0.033	0.874
CpG3	-0.036	0.862	-0.061	0.767
CpG4	0.107	0.602	0.194	0.342
CpG5	-0.043	0.833	-0.286	0.157
CpG6	0.169	0.408	-0.029	0.887
CpG7	-0.240	0.237	-0.198	0.332
CpG8	0.159	0.439	0.419	0.033
CpG9	0.408	0.038	0.312	0.121
CpG10	0.144	0.484	0.251	0.215
CpG11	-0.081	0.696	0.057	0.783
CpG12	-0.313	0.119	-0.021	0.921

Control variables: Age, gender, education level and HAMD. HAMD, Hamilton Depression Rating Scale; HAMA, Hamilton Anxiety Scale; PSQI, Pittsburgh Sleep Quality Index Scale.

In the present study, it was also found that DNA methylation levels of *GRIN2B* were associated with anxiety and insomnia symptoms in BD, independent of age, gender, education or depression severity. This epigenetic alteration may contribute to BD-related psychopathology by suppressing *GRIN2B* transcription, thereby reducing the availability of functional NR2B subunits-a critical component of NMDARs implicated in synaptic plasticity and glutamatergic signaling (50), diminished NR2B activity could impair glutamate-mediated neurotransmission in limbic circuits (e.g., prefrontal cortex-amygdala connectivity and hippocampal networks), which are essential for emotion regulation and stress adaptation (34,51-53).

The association between *GRIN2B* methylation levels and anxiety symptoms may stem from dysregulated NMDAR-dependent excitatory-inhibitory balance. Specifically, hippocampal NMDARs are critical to behavioral inhibitory systems (40), and NR2B subunit deficiency-induced functional impairment could exacerbate anxiety-related neural hyperactivity. For instance, preclinical studies demonstrate that environmental stressors [e.g., benzo (a) pyrene exposure or high-fat diets] induce anxiety-like behaviors in tandem with elevated *GRIN2B* methylation in brain regions governing fear responses (34,54) epigenetic silencing likely attenuates NMDAR-mediated synaptic potentiation in the ventral hippocampus and anterior cingulate cortex-key regions where NR2B modulates anxiety-like phenotypes (52,53,55), ketamine's rapid anxiolytic effects-mediated by NMDAR antagonism (56)-further support the hypothesis that *GRIN2B* methylation-driven NMDAR hypofunction could perpetuate anxiety states in BD.

Insomnia is a hallmark symptom of anti-NMDAR encephalitis, with clinical evidence underscoring the critical role of NMDAR dysfunction in sleep architecture disruption (57), aberrant *GRIN2B* methylation may perturb sleep-wake regulation through two interconnected pathways: i) Diminished NR2B expression in the hypothalamic SCN, where NR2B-containing NMDARs are essential for circadian rhythm entrainment (15,58); and ii) impaired Non-Rapid Eye Movement (NREM) sleep homeostasis caused by reduced NMDAR-mediated excitability in the lateral preoptic hypothalamus, a key region for sleep initiation and maintenance (59). The findings of the present study align with chronic sleep deprivation studies in young mice demonstrating reduced NR2B levels (35), suggesting that *GRIN2B* aberrant methylation may exacerbate insomnia by impairing NMDAR-dependent synaptic adaptation to sleep pressure. Additionally, selective NR2B antagonism disrupts cortical gamma oscillations during REM sleep (60), indicating that *GRIN2B* epigenetic silencing could destabilize sleep architecture through dysregulated glutamatergic neurotransmitter interactions.

While the present study yielded promising results, several critical limitations must be acknowledged. First, the conclusions may be constrained by the relatively small sample size, which reduces statistical power to detect robust epigenetic associations. Given the established clinical and biological heterogeneity of BD, the limited sample of the present study may not adequately represent the full spectrum of disease subtypes. These factors, combined with the cross-sectional design, preclude causal inferences; it cannot be determined whether the observed *GRIN2B* methylation alterations are causes or consequences of BD pathophysiology. Second, the reliance on peripheral blood methylation profiles raises questions about their biological relevance to brain processes. While blood-based biomarkers offer clinical practicality, it remains elusive whether these patterns mirror those in the central nervous system. Future validation should incorporate postmortem brain tissue analyses or emerging cerebrospinal

fluid-based epigenetic profiling techniques. Third, the present study relied on retrospective data collection and self-reported medical histories, which may have resulted in the under-reporting of comorbidities. This could lead to unintentional inclusion of patients with undiagnosed conditions that met exclusion criteria, potentially confounding the observed methylation patterns. Fourth, while cohort homogeneity was improved by exclusively recruiting patients with BD in the depressive phase, this design limits the generalizability of findings to other disease phases (e.g., manic or euthymic states). Epigenetic markers may exhibit phase-dependent fluctuations, implying the results could reflect state-specific alterations rather than trait characteristics of BD. Fifth, the lack of integrated single-nucleotide polymorphism analyses and gene expression data limits our ability to elucidate gene-environment interactions underlying the observed methylation changes. Most importantly, these preliminary findings require replication in larger, independent cohorts with longitudinal designs to establish clinical generalizability. Future investigations should prioritize multicenter collaborations, incorporate pharmaco-epigenetic analyses and employ multi-omics approaches to comprehensively characterize *GRIN2B* methylation dynamics in BD progression, and explicitly address phase-specific biomarker variations through cross-state comparisons.

In conclusion, the present study demonstrated for the first time that DNA hypermethylation in the *GRIN2B* promoter region is associated with BD and may play a role in mediating the development of anxiety and insomnia symptoms in patients with BD. These findings underscore the importance of gene-environment interactions in BD and advance a better understanding of its complex etiology. Early intervention strategies targeting these mechanisms could improve timely diagnosis, symptom management and long-term outcomes in BD. Further studies are warranted to validate *GRIN2B* methylation as both a diagnostic biomarker and therapeutic target for BD.

Acknowledgements

Not applicable.

Funding

This study was supported by grants from the Natural Science Foundation of Xinjiang Uygur Autonomous Region (grant no. 2022D01C606) and the Tianshan Innovation Team Plan of Xinjiang Uygur Autonomous Region (grant no. 2022D14011).

Availability of data and materials

The datasets generated and/or analyzed during the current study are not publicly available due to ethical issues involving the participants' data and privacy but may be requested from the corresponding author.

Authors' contributions

SZ and HY conducted and designed the study. HY and YW collected the data. HY analyzed the data and drafted the manuscript. SZ revised the manuscript. SZ and YW have independently checked and verified the authenticity of the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

This study was approved by the Human Research and Ethics Committee of Xinjiang Uygur Autonomous Region People's Hospital (Urumqi, China; approval no. KY2023020968). This study adhered to the guidance listed in the latest version of the Declaration of Helsinki. All subjects volunteered to participate in the study provided written informed consent prior to the study. The participants were also informed that they could withdraw from the study at any time without any reason or consequence.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. GBD 2019 Mental Disorders Collaborators: Global, regional, and national burden of 12 mental disorders in 204 countries and territories, 1990-2019: A systematic analysis for the global burden of disease study 2019. Lancet Psychiatry 9: 137-150 2022.
2. Grande I, Berk M, Birmaher B and Vieta E: Bipolar disorder. Lancet 387: 1561-1572, 2016.
3. Pavlova B, Perlis RH, Alda M and Uher R: Lifetime prevalence of anxiety disorders in people with bipolar disorder: A systematic review and meta-analysis. Lancet Psychiatry 2: 710-717, 2015.
4. Lopes FL, Zhu K, Purves KL, Song C, Ahn K, Hou L, Akula N, Kassem L, Bergen SE, Landen M, *et al*: Polygenic risk for anxiety influences anxiety comorbidity and suicidal behavior in bipolar disorder. Transl Psychiatry 10: 298, 2020.
5. Gamage N, Senanayake S, Kumbukage M, Mendis J and Jayasekara A: The prevalence of anxiety and its association with the quality of life and illness severity among bipolar affective disorder patients in a developing country. Asian J Psychiatr 52: 102044, 2020.
6. Buckley V, Young AH and Smith P: Child and adolescent anxiety as a risk factor for bipolar disorder: A systematic review of longitudinal studies. Bipolar Disord 25: 278-288, 2023.
7. Oakes DJ, Pearce HA, Roberts C, Gehrman PG, Lewis C, Jones I and Lewis KJS: Associations between comorbid anxiety and sleep disturbance in people with bipolar disorder: Findings from actigraphy and subjective sleep measures. J Affect Disord 309: 165-171, 2022.
8. Choi J, Kang J, Kim T and Nehs CJ: Sleep, mood disorders, and the ketogenic diet: Potential therapeutic targets for bipolar disorder and schizophrenia. Front Psychiatry 15: 1358578, 2024.
9. Uher R, Pavlova B, Najafi S, Adepalli N, Ross B, Howes VE, Freeman K, Parker R, Propper L and Palaniyappan L: Antecedents of major depressive, bipolar, and psychotic disorders: A systematic review and meta-analysis of prospective studies. Neurosci Biobehav Rev 160: 105625, 2024.
10. Ghasemi M, Phillips C, Trillo L, De Miguel Z, Das D and Salehi A: The role of NMDA receptors in the pathophysiology and treatment of mood disorders. Neurosci Biobehav Rev 47: 336-358, 2014.
11. Tang YP, Shimizu E, Dube GR, Rampon C, Kerchner GA, Zhuo M, Liu G and Tsien JZ: Genetic enhancement of learning and memory in mice. Nature 401: 63-69, 1999.
12. Carrier J, Semba K, Deurveilher S, Drogos L, Cyr-Cronier J, Lord C and Sekerovick Z: Sex differences in age-related changes in the sleep-wake cycle. Front Neuroendocrinol 47: 66-85, 2017.

13. Nakamura TJ, Nakamura W, Yamazaki S, Kudo T, Cutler T, Colwell CS and Block GD: Age-related decline in circadian output. J Neurosci 31: 10201-10205, 2011.
14. Biello SM, Bonsall DR, Atkinson LA, Molyneux PC, Harrington ME and Lall GS: Alterations in glutamatergic signaling contribute to the decline of circadian photoentrainment in aged mice. Neurobiol Aging 66: 75-84, 2018.
15. Steponenaite A, Biello SM and Lall GS: Aging clocks: Disrupted circadian rhythms. Aging (Albany NY) 10: 3065-3066, 2018.
16. Cevik OS, Cevik K, Temel GO and Sahin L: Maternal separation increased memory function and anxiety without effects of environmental enrichment in male rats. Behav Brain Res 441: 114280, 2023.
17. Healey K, Waters RC, Knight SG, Wandling GM, Hall NI, Jones BN, Shobande MJ, Melton JG, Pandey SC, Swartzwelder HS, *et al*: Adolescent intermittent ethanol exposure alters adult exploratory and affective behaviors, and cerebellar Grin2b expression in C57BL/6J mice. Drug Alcohol Depend 253: 111026, 2023.
18. Shirvani-Farsani Z, Maloum Z, Bagheri-Hosseinabadi Z, Vilor-Tejedor N and Sadeghi I: DNA methylation signature as a biomarker of major neuropsychiatric disorders. J Psychiatr Res 141: 34-49, 2021.
19. Papanicolau-Sengos A and Aldape K: DNA methylation profiling: An emerging paradigm for cancer diagnosis. Annu Rev Pathol 17: 295-321, 2022.
20. Fries GR, Li Q, McAlpin B, Rein T, Walss-Bass C, Soares JC and Quevedo J: The role of DNA methylation in the pathophysiology and treatment of bipolar disorder. Neurosci Biobehav Rev 68: 474-488, 2016.
21. Goud AC, Etain B, Bellivier F and Marie-Claire C: DNA methylation as a biomarker of treatment response variability in serious mental illnesses: A systematic review focused on bipolar disorder, schizophrenia, and major depressive disorder. Int J Mol Sci 19: 3026, 2018.
22. Bundo M, Ueda J, Nakachi Y, Kasai K, Kato T and Iwamoto K: Decreased DNA methylation at promoters and gene-specific neuronal hypermethylation in the prefrontal cortex of patients with bipolar disorder. Mol Psychiatry 26: 3407-3418, 2021.
23. Ceylan D, Scola G, Tunca Z, Isaacs-Trepanier C, Can G, Andreazza AC, Young LT and Özerdem A: DNA redox modulations and global DNA methylation in bipolar disorder: Effects of sex, smoking and illness state. Psychiatry Res 261: 589-596, 2018.
24. Carrard A, Salzmann A, Malafosse A and Karege F: Increased DNA methylation status of the serotonin receptor 5HTR1A gene promoter in schizophrenia and bipolar disorder. J Affect Disord 132: 450-453, 2011.
25. Jeremian R, Chen YA, De Luca V, Vincent JB, Kennedy JL, Zai CC and Strauss J: Investigation of correlations between DNA methylation, suicidal behavior and aging. Bipolar Disord 19: 32-40, 2017.
26. Lima C, Suchting R, Scaini G, Cuellar VA, Favero-Campbell AD, Walss-Bass C, Soares JC, Quevedo J and Fries GR: Epigenetic GrimAge acceleration and cognitive impairment in bipolar disorder. Eur Neuropsychopharmacol 62: 10-21, 2022.
27. Shi X, Li M, Yao J, Li MD and Yang Z: Alcohol drinking, DNA methylation and psychiatric disorders: A multi-omics Mendelian randomization study to investigate causal pathways. Addiction 119: 1226-1237, 2024.
28. Pierconti F, Rossi ED, Cenci T, Carlino A, Fiorentino V, Totaro A, Sacco E, Palermo G, Iacovelli R, Larocca LM, *et al*: DNA methylation analysis in urinary samples: A useful method to predict the risk of neoplastic recurrence in patients with urothelial carcinoma of the bladder in the high-risk group. Cancer Cytopathol 131: 158-164 2023.
29. Wei W, Fan P, Zhang Z, Wu D, Liu J, Wang L, Duan X, Zhang X and Ding D: A urine-based liquid biopsy for detection of upper tract urothelial carcinoma: A self-matched study. BMC Cancer 24: 1180, 2024.
30. Zhu JH, Bo HH, Liu BP and Jia CX: The associations between DNA methylation and depression: A systematic review and meta-analysis. J Affect Disord 327: 439-450, 2023.
31. Srivastava A, Dada O, Qian J, Al-Chalabi N, Fatemi AB, Gerretsen P, Graff A and De Luca V: Epigenetics of schizophrenia. Psychiatry Res 305: 114218, 2021.
32. Gaynor SC, Breen ME, Monson ET, de Klerk K, Parsons M, DeLuca AP, Scheetz TE, Zandi PP, Potash JB and Willour VL: A targeted sequencing study of glutamatergic candidate genes in suicide attempters with bipolar disorder. Am J Med Genet B Neuropsychiatr Genet 171: 1080-1087, 2016.
33. Dalvie S, Horn N, Nossek C, van der Merwe L, Stein DJ and Ramesar R: Psychosis and relapse in bipolar disorder are related to GRM3, DAOA, and GRIN2B genotype. Afr J Psychiatry (Johannesbg) 13: 297-301, 2010.
34. Zhang W, Tian F, Zheng J, Li S and Qiang M: Chronic administration of Benzo(a)pyrene induces memory impairment and anxiety-like behavior and increases of NR2B DNA methylation. PLoS One 11: e0149574, 2016.
35. Li Y, Xiao X, Wang L, Wang Q, Liang R, Zheng C, Yang J and Ming D: Comparison effects of chronic sleep deprivation on juvenile and young adult mice. J Sleep Res 31: e13399, 2022.
36. Horvath S: DNA methylation age of human tissues and cell types. Genome Biol 14: R115, 2013.
37. Johansson A, Enroth S and Gyllensten U: Continuous aging of the human DNA methylome throughout the human lifespan. PLoS One 8: e67378, 2013.
38. Hannum G, Guinney J, Zhao L, Zhang L, Hughes G, Sadda S, Klotzle B, Bibikova M, Fan JB, Gao Y, *et al*: Genome-wide methylation profiles reveal quantitative views of human aging rates. Mol Cell 49: 359-367, 2013.
39. First M, Spitze R, Gibbon M and Williams J: Structured clinical interview for DSM-IV-TR axis I disorders, research version, patient edition. (SCID-I/P). New York: Biometrics Research, New York State Psychiatric Institute. 2002.
40. HAMILTON M: A rating scale for depression. J Neurol Neurosurg Psychiatry 23: 56-62, 1960.
41. Young RC, Biggs JT, Ziegler VE and Meyer DA: A rating scale for mania: Reliability, validity and sensitivity. Brit J Psychiatry 133: 429-435, 1978.
42. APA. Diagnostic and Statistical Manual of Mental Disorders, Text Revision (DSM-IV-TR), 4th Edn. Washington, DC: American psychiatric association; 1994.
43. HAMILTON M: The assessment of anxiety states by rating. Br J Med Psychol 32: 50-55, 1959.
44. Buysse DJ, Reynolds CR III, Monk TH, Berman SR and Kupfer DJ: The pittsburgh sleep quality index: A new instrument for psychiatric practice and research. Psychiatry Res 28: 193-213, 1989.
45. Avramopoulos D, Lasseter VK, Fallin MD, Wolyniec PS, McGrath JA, Nestadt G, Valle D and Pulver AE: Stage II follow-up on a linkage scan for bipolar disorder in the Ashkenazim provides suggestive evidence for chromosome 12p and the GRIN2B gene. Genet Med 9: 745-751, 2007.
46. Zhao Q, Che R, Zhang Z, Wang P, Li J, Li Y, Huang K, Tang W, Feng G, Lindpaintner K, *et al*: Positive association between GRIN2B gene and bipolar disorder in the Chinese Han Population. Psychiatry Res 185: 290-292, 2011.
47. Li C, Tian H, Li R, Jia F, Wang L, Ma X, Yang L, Zhang Q, Zhang Y, Yao K and Zhuo C: Molecular mechanisms of quetiapine bidirectional regulation of bipolar depression and mania based on network pharmacology and molecular docking: Evidence from computational biology. J Affect Disord 355: 528-539, 2024.
48. Weiss F, Caruso V, De Rosa U, Beatino MF, Barbuti M, Nicoletti F and Perugi G: The role of NMDA receptors in bipolar disorder: A systematic review. Bipolar Disord 25: 624-636, 2023.
49. Beneyto M and Meador-Woodruff JH: Lamina-specific abnormalities of NMDA receptor-associated postsynaptic protein transcripts in the prefrontal cortex in schizophrenia and bipolar disorder. Neuropsychopharmacology 33: 2175-2186, 2008.
50. Riaza BC, Perez-Rodriguez MM, Vaquero-Lorenzo C and Baca-Garcia E: New perspectives in glutamate and anxiety. Pharmacol Biochem Behav 100: 752-774, 2012.
51. Bannerman DM, Sprengel R, Sanderson DJ, McHugh SB, Rawlins JN, Monyer H and Seeburg PH: Hippocampal synaptic plasticity, spatial memory and anxiety. Nat Rev Neurosci 15: 181-192, 2014.
52. Guo H, Hu WC, Xian H, Shi YX, Liu YY, Ma SB, Pan KQ, Wu SX, Xu LY, Luo C and Xie RG: CCL2 potentiates inflammation pain and related anxiety-like behavior through NMDA signaling in anterior cingulate cortex. Mol Neurobiol 61: 4976-4991, 2024.
53. Ren D, Li JN, Qiu XT, Wan FP, Wu ZY, Fan BY, Zhang MM, Chen T, Li H, Bai Y and Li YQ: Anterior cingulate cortex mediates hyperalgesia and anxiety induced by chronic pancreatitis in rats. Neurosci Bull 38: 342-358, 2022.
54. Yan Z, Jiao F, Yan X and Ou H: Maternal chronic folate supplementation ameliorates behavior disorders induced by prenatal high-fat diet through methylation alteration of BDNF and Grin2b in offspring hippocampus. Mol Nutr Food Res 61: doi.org/10.1002/mnfr.201700461, 2017.

55. Barkus C, McHugh SB, Sprengel R, Seeburg PH, Rawlins JN and Bannerman DM: Hippocampal NMDA receptors and anxiety: At the interface between cognition and emotion. Eur J Pharmacol 626: 49-56, 2010.
56. Tully JL, Dahlen AD, Haggarty CJ, Schioth HB and Brooks S: Ketamine treatment for refractory anxiety: A systematic review. Br J Clin Pharmacol 88: 4412-4426, 2022.
57. Arino H, Munoz-Lopetegi A, Martinez-Hernandez E, Armangue T, Rosa-Justicia M, Escudero D, Matos N, Graus F, Sugranyes G, Castro-Fornieles J, *et al*: Sleep disorders in anti-NMDAR encephalitis. Neurology 95: e671-e684, 2020.
58. Burgdorf JS, Vitaterna MH, Olker CJ, Song EJ, Christian EP, Sorensen L, Turek FW, Madsen TM, Khan MA, Kroes RA and Moskal JR: NMDAR activation regulates the daily rhythms of sleep and mood. Sleep 42: zsz135, 2019.
59. Miracca G, Anuncibay-Soto B, Tossell K, Yustos R, Vyssotski AL, Franks NP and Wisden W: NMDA receptors in the lateral preoptic hypothalamus are essential for sustaining NREM and REM sleep. J Neurosci 42: 5389-5409, 2022.
60. Kocsis B: State-dependent increase of cortical gamma activity during REM sleep after selective blockade of NR2B subunit containing NMDA receptors. Sleep 35: 1011-1016, 2012.

Copyright © 2025 Yu et al. This work is licensed under a Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International (CC BY-NC-ND 4.0) License.