SLEEP AND MOOD DISORDERS

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SLEEP AND MOOD DISORDERS

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EDITORIAL
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Editorial: Sleep and Mood Disorders

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Editorial on the Research Topic

Sleep And Mood Disorders

Sleep occupies almost one third of our life and it is necessary for survival of all species, including man. There is little doubt that sufficient, restorative sleep plays a critical role in maintaining physical and mental health. Evaluation of a good sleep always includes its quality, timing, quantity and rhythm. Sleep disturbances, including hypersomnia, insomnia, or irregular sleep patterns, result in various cognitive impairments and mood disorders (1, 2). Prolonged sleep deprivation or chronic sleep abnormalities are risk factors for the major depressive disorder (MDD) (3) and bipolar disorder (BD) (4), whereas disturbed sleep appears as a key symptom of mental diturbances (5, 6). Mechanisms connecting sleep deprivation and mood disorders remain, however, unclear.

The current Research Topic represents a collection of papers investigating the relationship between sleep abnormalities and mood disorders, as well as studies analyzing potential mechanisms connecting these pathologies. As described in the review by Steardo et al., the worldwide prevalence of sleep disorders is about 50% with even higher occurrence in psychiatric population. Sleep abnormalities are frequently associated with BD and are often a good predictor of a mood disorders. In this review, the alterations in the structure or duration of sleep are considered in all stages of BD. In particular the role of neuroglia in BD and the contributions of the different types of glial cells to BD and sleep abnormalities are discussed in depth. Specially, astrocytes are suggested to have an important role to the pathophysiology of BD through the loss of glial support and neuroprotection in disease-specific regions (7, 8). Furthermore, neuroglia cells are also reported to be a key target for the drugs used for the treatment of BD.

In the article by Li Y. et al., the relationship between stressful life events and sleep quality is discussed. The effects of stress on the sleep quality was investigated on more than 1,000 college students from a single province in China high correlation between scores of stressful life events and sleep quality has been reported. These results demonstrate that stressful life events can disturb the sleep quality of college students directly and indirectly, through rumination. The latter is defined as a response style that an individual tends to repeatedly think about the problematic situations being focused on negative emotions (9). Increasing psychological resilience could decrease both the direct and indirect effects stressful life events.

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The influence of sleep problems in childhood and the relevant risk for mood disorders were further researched are in the focus of original paper by Wescott et al. This study is focused on the sleep parameters in offspring of parents with MDD or BD during both weeknights and weekends. Children of parents with MDD had longer sleep periods and total sleep time during weeknights than healthy controls. Thus, hypersomnia may be an early indicator of increased risk for depression in children and could be a relevant target for sleep-oriented interventions for individuals with high familial risk.

Effects of combined treatment with alprazolam and bailemian on the sleep quality of the patients after stroke were analyzed by Wang et al. The post-stroke insomnia is a very common symptom, albeit the treatment is often ignored (10). Alprazolam was discovered to improve the sleep efficiency and decrease the arousal times, rapid eye movement (REM) sleep and sleep latency, whereas bailemian significantly improved the sleep efficiency, total sleep time and the duration of N3. Moreover, the combination of these two drugs has synergistic qualities.

Besides the above three clinical reports, a novel approach for continuous recording the sleep–wake cycle in rodents is reported by Cui et al. Such a continuous sleep recording is required to reveal the sleep–wake, cycle which can reflect the brain maturational stages in postnatal rodent life (11). This paper reports a new method of continuous sleep recording involving two types of EEG electrodes, a milk-feeding system and temperature-controlled incubator. This technique allows characterization of sleep–wake cycle in the first postnatal month. This approach is suitable for recording continuous polysomnographic in infant rats and uncovers the ontogenetic features of sleep–wake cycle.

The sleep deprivation (SD) is known to aggravate various pathological processes including neuroinflammation. SD can induce the activation of nucleotide-binding domain and leucine-rich repeat protein-3 (NLRP3) inflammasome (12). The original article by Li X. et al., demonstrates that the activation of NLRP3 inflammasome is involved in the depressive-like behaviors induced by SD. Activation of NLRP3 inflammasome requires brain-derived neurotrophic factor (BDNF), which is decreased by SD. This study also shows that leptin augmented the anti-depressive effects of fluoxetine via increasing the expression of 5-HT_{2B} receptors in astrocytes. The decrease in BDNF by the activated NLRP3 inflammasome in astrocytes is the main pathological event of the depressive-like behaviors induced by SD, while the combination of fluoxetine and leptin can improve the therapeutic outcome for the depression triggered by SD.

The article by Lima et al. analyzed the consequences of the exposure of low- and high-exploratory rats during peri-adolescence; the authors evaluated and evaluated hippocampal immune-inflammatory/oxidative impairments as consequence of environmental stress (ES). The authors also analyzed the beneficial role of mood stabilizing drugs, lithium and valproate in prevention of ES-induced memory alterations. It appears that low- and high-exploratory rats exposed to ES present inflammatory phenotype, distinct anxiety-related behaviors and similar memory deficits, which all can be partially normalized by mood-stabilizing drugs.

The last two articles report potential pharmacological mechanisms of novel antidepressants, ketamine and vortioxetine. Ketamine, which is an ionotropic glutamatergic NMDA receptor antagonist, has been widely used as an anesthetic; recently however the rapid anti-depressant effect of ketamine treatment has been discovered. Rapid improvement of anhedonia and the depressive-like or anxious-like behaviors induced by the chronic unpredictable mild stress is described in the article of Wen et al. In the report by Cao et al., 100 patients with MDD were treated with vortioxetine, which is a multimodal antidepressant with multiple effects on neurotransmitter systems. It appears that vortioxetine significantly reduces anhedonia, which improves quality of life.

Taken together, this Research Topic covers several aspects of sleep disturbances and mood disorders and updates readers on the latest research in this field. These articles will provide researchers in many fields insights into which tools are available and thereby to further explore the relationship between sleep and mood disorders with the ultimate goal of developing effective therapeutic strategies.

AUTHOR CONTRIBUTIONS

Each of the authors have contributed intellectual content to the actual writing of the editorial.

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ORIGINAL RESEARCH
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Leptin Increases Expression of 5-HT$_{2B}$ Receptors in Astrocytes Thus Enhancing Action of Fluoxetine on the Depressive Behavior Induced by Sleep Deprivation

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The long-lasting loss of sleep is a generally acknowledged risk factor for the occurrence of major depressive disorder (MDD), whereas sleep abnormalities being a key clinic symptom of the MDD. In our previous work, we demonstrated that the sleep deprivation (SD) stimulates activation of nucleotide-binding domain and leucine-rich repeat protein-3 (NLRP3) inflammasomes as well as the release of IL-1β and IL-18 from astrocytes. However, the underlying mechanism connecting SD and MDD still requires further study. Apart of the secretion of the pro-inflammatory cytokines, SD affects production of brain-derived neurotrophic factor (BDNF) while release of BDNF from astrocytes appears a key contributor to mood disorders. If and how the activation of NLRP3 inflammasome following SD affects the level of BDNF remains unknown. Antidepressant fluoxetine acts through astroglial 5-hydroxytryptamine receptor 2B (5-HT$_{2B}$); these receptors are also related to the sleep-wake cycle. Contribution of leptin to MDD has been discovered recently, although the mechanistic links between leptin and the depressive-like behaviors has not been revealed. In this study, we discovered: (i) that activation of NLRP3 inflammasome was involved in the depressive-like behaviors induced by SD; (ii) decrease in BDNF following SD required the activation of NLRP3 inflammasomes; (iii) leptin augmented the anti-depressive action of fluoxetine through an increase in expression of astrocytic 5-HT$_{2B}$ receptors. We suggest that decrease in BDNF by the activated NLRP3 inflammasomes in astrocytes is the key pathological event of the depressive-like behaviors induced by SD, while the combined treatment with fluoxetine and leptin improves therapeutic outcome for the depression induced by SD.

Keywords: sleep deprivation, 5-HT$_{2B}$ receptors, astrocytes, fluoxetine, leptin, NLRP3 inflammasomes, BDNF

INTRODUCTION

Sleep occupies almost one third of our life and it is necessary for human survival. Sleep loss results in various pathological manifestations, such as cognitive impairments (1), mood disorders (2), the increased circulating levels of inflammatory cytokines (3) and the dysfunction of the metabolite clearance from brain (4). Prolonged sleep deprivation or chronic sleep abnormalities are risk factors for the major depressive disorders (MDD) (5), whereas sleep disorders appear as a key symptom of MDD (6). However, the underlying mechanisms connecting sleep deprivation and MDD remain unclear.

Sleep deprivation (SD) can aggravate various pathological processes including neuroinflammation. Previously we have demonstrated that 6 h daily SD for 3 weeks triggers the activation of nucleotide-binding domain and leucine-rich repeat protein-3 (NLRP3) inflammasomes, and increases astroglial secretion of IL-1β and IL-18 (7). In addition, SD increases serum levels of brain-derived neurotrophic factor (BDNF) (8), which is related to sleep, cognition and learning (9–11), The BDNF released from astrocytes is also known to be associated with mood disorders (10). Both neuroinflamamtory (12) and the neurotrophin (10) hypothesis of MDD have been considered recently, albeit the links between inflammasome activation or BDNF secretion to the depressive symptoms induced by SD are unknown.

There is mounting evidence suggesting astrocytic abnormality in the major depression (13, 14), we previously reports the changes in the expression of 5-hydroxytryptamine 2B (5-HT_{2B}) receptors in astrocytes following chronic mild stress (CMS) (15). The antidepressant effects of fluoxetine, a serotonin specific reuptake inhibitor (SSRIs), depend on astroglial 5-HT_{2B} receptors (16). We found that fluoxetine suppresses the activation of NLRP3 inflammasomes triggered by SD in astrocytes (7). The 5-HT_{2B} receptors are associated with regulation of sleep-wake cycle, as selective blockade of 5-HT_{2B} receptors increases the phase of wakefulness (14), reflecting the role of serotonin (5-HT) as the neurotransmitter contributing to physiological and pathological regulation of sleep-wake cycle (17). However, the role of 5-HT_{2B} receptors in the emergence of depressive-like behaviors induced by SD unknown. Recently, the therapeutic potential of leptin in the context of MDD has been discovered (18), although leptin efficacy in treating depressive-like behaviors following by SD has not been studied. The serum level of leptin is increased in the depressed women with normal weight with sleep disturbances, however the level of leptin is not changed in the overweight women (19).

In this work, we studied the mechanisms of leptin-induced facilitation of effects of fluoxetine on the depressive-like behaviors induced by SD and linked this effect to the increased expression of 5-HT_{2B} receptors in astrocytes.

METHODS AND MATERIALS

Animals

Male C57BL/6 mice and FVB/N-Tg(GFAPGFP)14Mes/J (GFAP-GFP; #003257, RRID:IMSR_JAX:003257) mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). As described previously (7), the mice were around 3 months old (∼25 g) and were kept in standard housing conditions with food and water freely available. All operations were carried out in accordance with the USA National Institutes of Health's Guide for the Care and Use of Laboratory Animals (NIH Publications No. 8023) and its 1978 revision, and all experimental protocols were approved by the Institutional Animal Care and Use Committee of China Medical University.

Materials

Most chemicals, including fluoxetine (fluoxetine hydrochloride; F132), SB204741 [*N*-(1-Methyl-1H-5-indolyl)-*N*′-(3-methyl-5-isothiazolyl)urea; S0693], U0126 [1,4-Diamino-2,3-dicyano-1,4-bis(o-aminophenylmercapto)butadiene monoethanolate; U120], LY294002 [2-(4-Morpholinyl)-8-phenyl-1(4H)-benzopyran-4-one hydrochloride; L9908], and a primary antibody raised against β-actin (A5441) were purchased from Sigma (MO, USA). Other primary antibodies were acquired from Millipore (MA, USA). Horseradish peroxidase-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology (CA, USA). MCC950 (CP-456773; HY-12815) was purchased from MedChem Express (Shanghai, China). WP1066 [(E)-3(6-bromopyridin-2-yl)-2-cyano-N-(S0-1phenylethyl)acrylamide] was from EMD Chemicals (San Diego, CA, USA). Recombinant murine leptin (obesity protein; 450-31) was obtained from Preprotech Inc. (Jiangsu, China). BDNF enzyme-linked immunosorbent assay (ELISA) kit was from R&D Systems (MN, USA).

Sleep Deprivation (SD) and Drug Treatment

FVB/N-Tg(GFAPGFP)14Mes/J mice were used for SD models. SD was induced by "gentle handling" according to standard protocols (20), including the introduction of new objects into the cage or gentle touching with a brush to keep the mice awake. As described previously (7), SD was induced for 6 h, from 7 a.m. to 1 p.m. During the treatment of SD, the mice were offered food and water *ad libitum*. Animals in the sham group were kept undisturbed in a separate room with the same light/dark cycle. The mice were treated with sham or SD stimulation for 3 weeks. During the third week, MCC950 (50 mg/kg for intraperitoneal injection), SB204741 (0.5 μmM in 2 μL for intracerebroventricular (ICV) infusion), U0126 (10 μM in 2 μL for ICV infusion), LY294002 (10 μM in 2 μL for ICV infusion), or vehicle (artificial cerebrospinal fluid, ACSF) was injected each day, then fluoxetine (10 mg/kg/day), leptin (4 mg/kg/day) or PBS (phosphate buffered saline) used as control group was injected intraperitoneally.

Dissociation and Fluorescence-Activated Cell Sorting (FACS)

FVB/N-Tg(GFAPGFP)14Mes/J mice were used for collecting astrocytes. A purified astrocyte suspension isolated from the cortex and hippocampus was prepared as described previously (7). Briefly, tissue from two transgenic mice were pooled for one sample. Wavelengths of GFP excitation and emission were 488 and 530/30 nm, respectively. The purity of the sorted astrocytes

has been confirmed in our previous study (21) by checking mRNA expression of cell markers of astrocytes, neurons and oligodendrocytes.

Primary Culture of Astrocytes

Astrocytes were cultured according to our previous description (22, 23). In brief, primary cultures of mouse astrocytes were prepared from the cerebral hemispheres of newborn C57BL/6 mice. Cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 7.5 mM glucose. For the entire third week, 0.25 mM dibutyryl cyclic AMP (dBcAMP) was included in the medium. All dishes of primary cultured astrocytes were randomly separated into different experimental groups.

RNA Interference

As described previously (24), the cultured astrocytes were incubated in DMEM without serum for half day before transfection. A transfection solution containing 2 μl

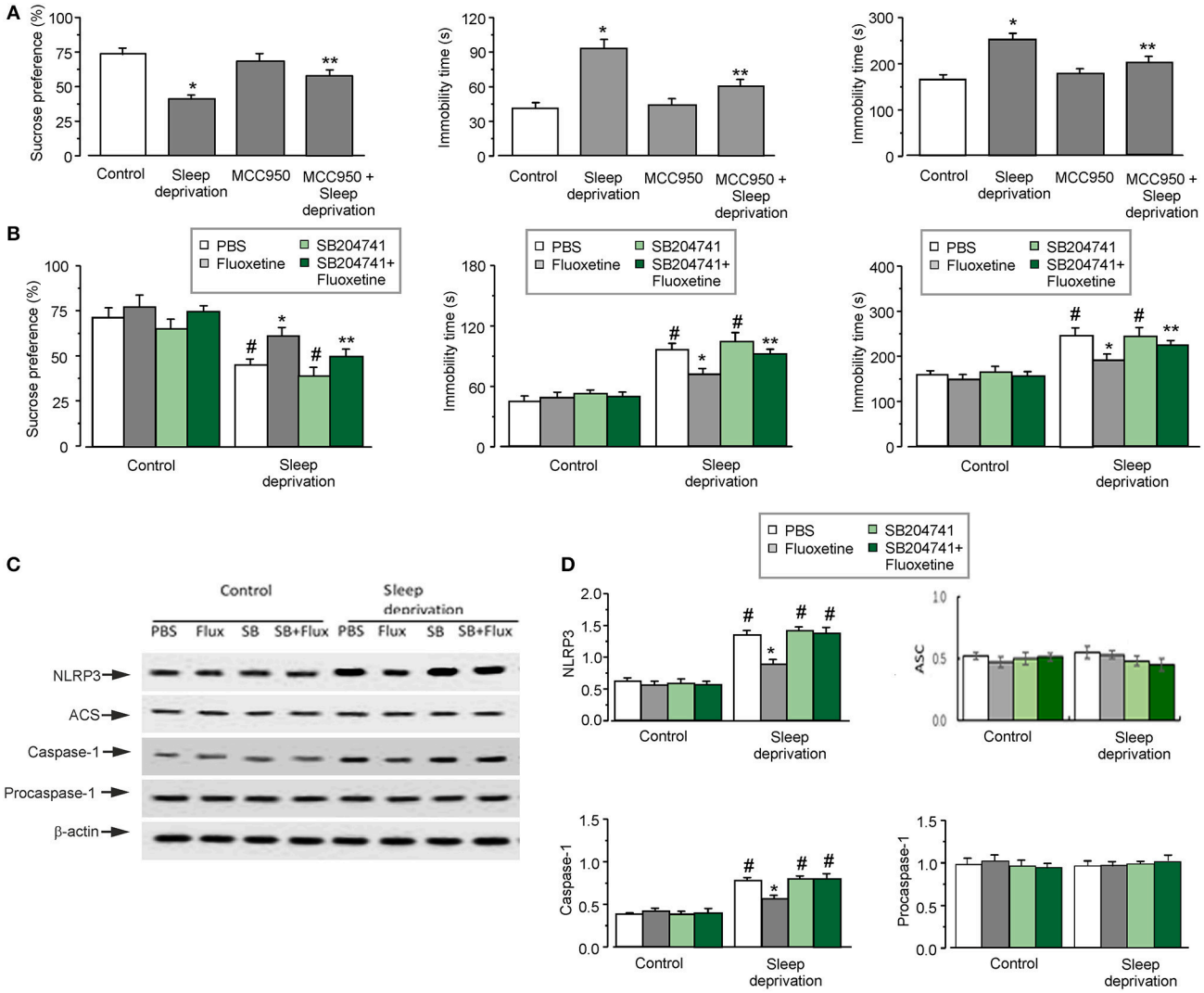

FIGURE 1 | The depressive-like behaviors and the activation of NLRP3 inflammasomes induced by SD. **(A–D)** The GFAP-GFP transgenic mice were treated with sham (Control) or exposed to SD for 3 weeks, in the final week the mice were injected with or without MCC950 (a NLRP3 inhibitor), fluoxetine, 5-HT$_{2B}$ receptors antagonist (SB204741; SB) or SB plus fluoxetine. The percentage of sucrose preference was measured (the left panels of **A** and **B**), the time of immobility were recorded in force-swimming test (the middle panels of **A** and **B**) and tail suspension test (the right panels of **A** and **B**), the values are expressed as mean ± SEM, $n = 12$. $^{*}p < 0.05$, statistically significant difference compared with any other group **(A)**; $^{**}p < 0.05$, statistically significant difference compared with control and SD groups **(A)**; $^{*}p < 0.05$, statistically significant difference compared with any other group **(B)**; $^{**}p < 0.05$, statistically significant difference compared with PBS and SD groups treated with sham (Control) or SD **(B)**; $^{\#}p < 0.05$, statistically significant difference compared with any other group except for each other **(B)**. The astrocytes sorted from GFAP-GFP mice were collected, representative blots for the expression of NLRP3, ASC, caspase-1, procaspase-1 was shown in C1. Average protein levels were quantified as the ratio between the protein and β-actin, respectively, $n = 6$ **(D)**. $^{*}p < 0.05$, statistically significant difference compared with any other group **(D)**; $^{\#}p < 0.05$, statistically significant difference compared with any other group except for each other **(D)**.

oligofectamine (Promega, Madison, WI, USA), 40 μl Opti-MEMI, and 2.5 μl siRNA (666 ng) was added to the culture in every well for 8 h. In the siRNA-negative control cultures, transfection solution without siRNA was added. Thereafter, DMEM with three times serum was added to the cultures. The siRNA duplex chains of leptin receptor (LepR), 5-HT$_{2B}$ receptor (5-HT$_{2B}$R) and *c-fos* were purchased from Santa Cruz Biotechnology (CA, USA).

Behavior Tests

Twenty-four mice were randomly separated into different groups, and every mouse was scheduled for tests, and the test sequences were randomly assigned to avoid interference from different tests.

Tail Suspension Test

The tail suspension test is a behavioral despair-based test. As described previously (25), every mouse was suspended by its tail around 2 cm from the tip. Behavior was recorded for 6 min. The duration of immobility was calculated by an observer blinded to the treatment groups.

Forced Swimming Test

The forced swimming test is also a behavior despair-based test. Briefly, each mouse was trained to swim 15 min on the first day and was placed into a glass cylinder that contained 30 cm deep water (25 ± 1°C) for 6 min on the second day. The time of immobility was calculated during the last 4 min period which followed 2 min of habituation (25).

Sucrose Preference Test

The sucrose preference test is a reward-based test and a measure of anhedonia, as previously described by our group (15, 26). In brief, after 12 h of food and water deprivation, the mice were provided with two pre-weighed bottles, including one bottle that contained 2.5% sucrose solution and a second bottle filled with water, for 2 h. The percentage preference was calculated according to the following formula: % preference = [sucrose intake/(sucrose + water intake)] × 100%.

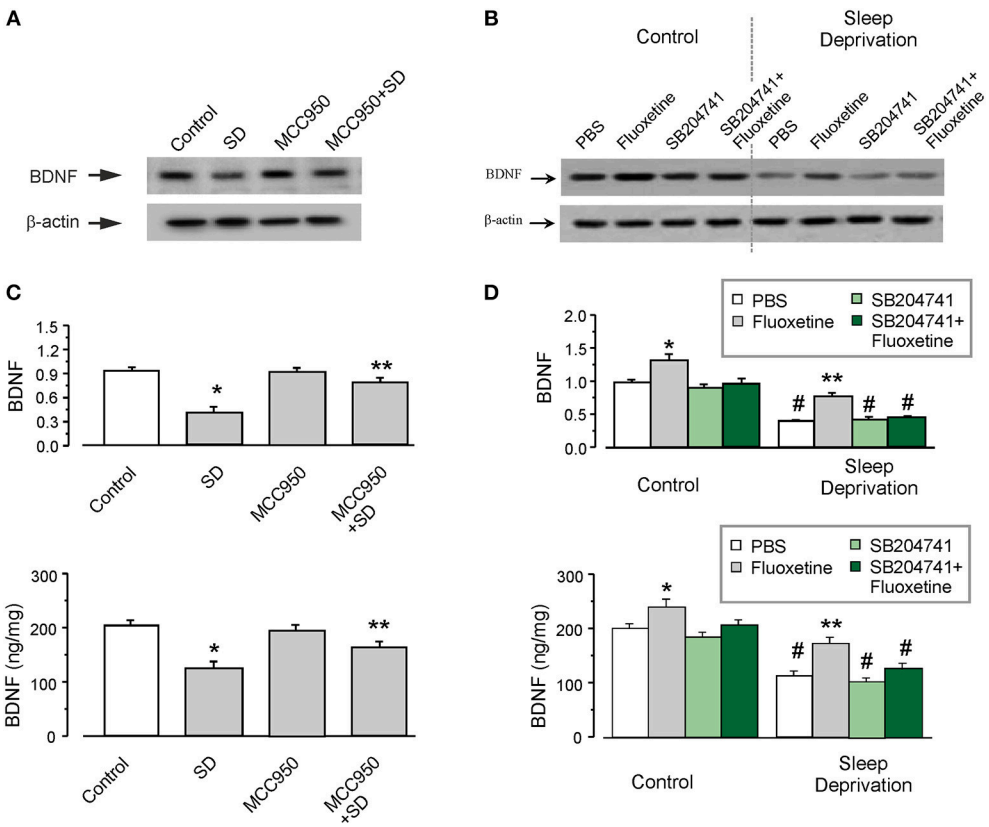

FIGURE 2 | The protein level of BDNF decreased by SD. **(A–D)** The GFAP-GFP transgenic mice were treated with sham (Control) or exposed to SD for 3 weeks, in the final week the mice were intraperitoneally injected with or without MCC950, fluoxetine, 5-HT$_{2B}$ receptors antagonist SB204741 (SB) or SB plus fluoxetine. The astrocytes sorted from GFAP-GFP mice were collected, representative blots for the expression of BDNF was shown in **(A,C)**. Average BDNF level was quantified as the ratio between the BDNF and β-actin, $n = 6$ (the upper panels of **B** and **D**). Meanwhile, the BDNF level was also measured via ELISA, $n = 6$ (the lower panels of **B** and **D**). $^{*}p < 0.05$, statistically significant difference compared with any other group **(B)**; $^{**}p < 0.05$, statistically significant difference compared with control and SD groups **(B)**; $^{*}p < 0.05$, statistically significant difference compared with any other group **(D)**; $^{**}p < 0.05$, statistically significant difference compared with fluoxetine group treated with sham (Control) and the other three groups treated with SD **(D)**; $^{\#}p < 0.05$, statistically significant difference compared with any other group except for each other **(D)**.

Western Blotting

Western blotting was performed as previously described (23, 25). The protein content was determined by using BSA as the standard. Samples containing 50 μg protein were added into 10% polyacrylamide slab gels. After protein transfer to nitrocellulose membranes, the membranes were blocked by 5% skimmed milk powder in TBS-T for 2 h and then incubated with the primary antibody for 2 h at room temperature. After washing of the membrane, the specific binding was detected by goat-anti-rabbit or goat-anti-mouse horseradish peroxidase-conjugated secondary antibodies. Staining was visualized with ECL detection reagents, and images were acquired with an electrophoresis gel imaging analysis system. Band density was measured in Windows AlphaEase FC 32-bit software.

Enzyme-Linked Immunosorbent Assay (ELISA)

According to our previously described (25, 27), BDNF level in the sorted astrocytes from GFAP-GFP transgenic mice or in the cultured astrocytes was measured via a total BDNF immunoassay kit methods. In brief, the measurements were performed according to the manufacturer's protocol. Samples were collected in pyrogen/endotoxin-free tubes. The lower limit of detection was <10 pg/ml.

Quantitative PCR (qPCR)

Total RNA was reverse transcribed, and PCR amplification was performed with a Robocycler thermocycler, as our previously described (28). In brief, the relative quantities of the transcripts were assessed using 5-fold serial dilutions of RT product (200 ng). The RNA quantities were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) before calculation of the relative expression of 5-HT$_{2B}$R, c-fos, and LepR. Values were first calculated as the ratio of the relative expression of 5-HT$_{2B}$R and GAPDH, then the values were normalized by control group.

Statistical Analysis

Differences between multiple groups with one or two variables were evaluated by one-way or two-way analysis of variance (ANOVA) followed by Fisher's least significant difference (LSD) or a Tukey-Kramer *post-hoc* multiple comparison test for unequal replications using GraphPad Prism 5 software (GraphPad Software Inc., La Jolla, CA). Sample size was not predetermined by formal power calculation, and no samples or data were excluded from the analysis. All statistical data in the

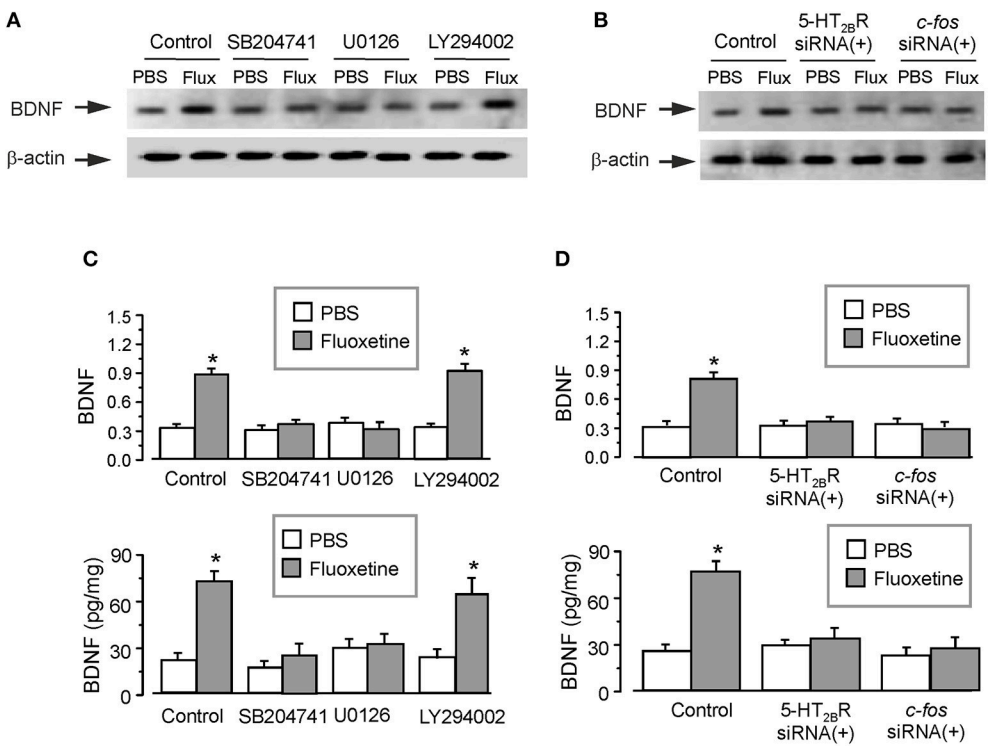

FIGURE 3 | The regulation of fluoxetine on the level of BDNF in primary culture astrocytes. In the primary cultured astrocytes, after the pretreatment with or without 200 nM SB204741 (SB), 10 μM U0126 or 10 μM LY294002 **(A,C)**, or after RNA interfering the expression of 5-HT$_{2B}$ receptors or cfos with siRNA duplex **(B,D)**, then the astrocytes were treated with PBS or fluoxetine for 1 week. Representative blots for the expression of BDNF was shown in **(A,B)**. Average BDNF level was quantified as the ratio between the BDNF and β-actin, $n = 6$ (the upper panels of **C** and **D**). Meanwhile, the average BDNF level was also measured via ELISA, $n = 6$ (the lower panels of **C** and **D**). *$p < 0.05$, statistically significant difference compared with any other group except for each other **(B)**; *$p < 0.05$, statistically significant difference compared with any other group **(D)**.

text are expressed as the mean ± SEM; the level of significance was set at $p < 0.05$.

RESULTS

SD-Induced Depressive-Like Behaviors Depends On the Activation of NLRP3 Inflammasome

Three weeks of SD triggered depressive-like behaviors, as shown in **Figures 1A,B**. Exposure to SD decreased the percentage of sucrose preference by 44 ± 3.5% ($n = 12$) of control group (the left panel of **Figure 1A**). Meanwhile, 3-weeks of SD significantly elevated the immobility time by 126 ± 7.3 and 53 ± 9.5% ($n = 12$) of control group, in the forced swimming and tail suspension tests, respectively (the middle and right panels of **Figure 1A**). The depressive-like behaviors induced by SD required the activation of NLRP3 inflammasomes. Treatment with MCC950 (an inhibitor of NLRP3 inflammasomes) alleviated the depressive-like behaviors induced by SD: the percentage of sucrose preference was significantly increased by about 40 ± 4.2% ($n = 12$) compared with SD group (the left panel of **Figure 1A**), and the immobility time was reduced to 64 ± 6.3 or 80 ± 10.7% ($n = 12$) of SD group in the forced swimming or tail suspension test, respectively (the middle and right panels of **Figure 1A**).

Fluoxetine Alleviation of SD-Induced Depressive-Like Behaviors and SD-Activated NLRP3 Inflammasomes Are Mediated by 5-HT$_{2B}$ Receptor

Antidepressant fluoxetine effectively remedied the depressive-like behaviors induced by SD, as shown in **Figure 1B**. This effect of fluoxetine on depressive-like behaviors required the involvement of 5-HT$_{2B}$ receptor. Treatment with SB204741 (an antagonist of 5-HT$_{2B}$ receptor) inhibited effects of fluoxetine in behavioral tests: the pre-treatment with SB204741 decreased sucrose preference to the 73 ± 3.3% ($n = 12$) of the fluoxetine group (the left panel of **Figure 1B**), significantly prolonged the immobility time by 75 ± 6.1% ($n = 12$) in forced swimming test (the middle panel of **Figure 1B**), and by 35 ± 8.3% ($n = 12$) in tail suspension test compared with fluoxetine treated-SD group (the right panel of **Figure 1B**). However, sole treatment with SB204741 did not affect results of behaviors tests compared with neither control nor SD groups.

As have been shown previously, fluoxetine inhibits the activation of NLRP3 inflammasomes in astrocytes, which was induced by 3 weeks of SD of 3 weeks (7). Here, we further corroborated that inhibition of fluoxetine effect on NLRP3 inflammasome was mediated by 5-HT$_{2B}$ receptor. In astrocytes isolated from transgenic mice fluoxetine reduced the protein level of NLRP3 and caspase-1 to 35 ± 4.3 and 72 ± 2.9% ($n = 6$) of

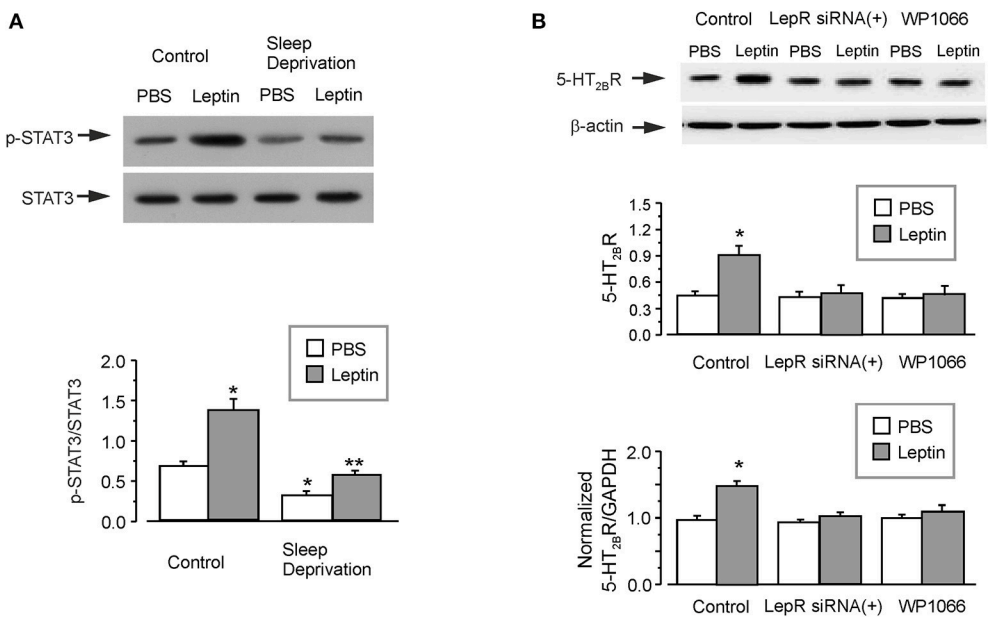

FIGURE 4 | The regulation of leptin on the expression of 5-HT$_{2B}$ receptors. The GFAP-GFP transgenic mice were treated with sham (Control) or exposed to SD for 3 weeks, in the final week the mice were injected with or without leptin **(A)**. Representative blots for the expression of p-STAT3 was shown in A1. The astrocytes sorted from GFAP-GFP mice were collected by FACS. The average phosphorylation level of STAT3 was quantified as the ratio between the p-STAT3 and STAT3, $n = 6$ **(A)**. $^*p < 0.05$, statistically significant difference compared with any other group **(A)**; $^{**}p < 0.05$, statistically significant difference compared with leptin group treated with sham (Control) and PBS group treated with SD **(A)**. After RNA interfering the expression of leptin receptors with siRNA duplex or 3 μM WP1066, an inhibitor of STAT3 **(B)**, then the primary cultured astrocytes were treated with PBS or leptin for 1 week. Representative blots for the expression of 5-HT$_{2B}$ receptors was shown in B1. The average expression of 5-HT$_{2B}$ receptors was quantified as the ratio between the 5-HT$_{2B}$ receptors and β-actin, $n = 6$ (the upper panel of **B**). The mRNA expression of 5-HT$_{2B}$ receptors was shown in **(C)**, the relative expression ratios of 5-HT$_{2B}$R/GAPDH were normalized by the control group. Data represent mean ± SEM, $n = 6$. $^*p < 0.05$, statistically significant difference compared with any other group **(A,B)**.

SD group, respectively (**Figure 1D**). However, the pretreatment with SB204741 eliminated effects of fluoxetine on the expression of NLRP3 and caspase-1 in SD models (**Figure 1D**). Meanwhile, SD with or without fluoxetine had no effects on the protein level of ASC or procaspase-1 (**Figure 1D**).

SD Decreases Astrocytic Level of BDNF via Activating NLRP3 Inflammasomes

The level of BDNF was significantly decreased in the astrocytes isolated from SD-treated transgenic mice, as shown in **Figures 2A,B**. Exposure to SD suppressed the protein expression of BDNF to 43 ± 3.3% ($n = 6$) of control group measured by western blotting (the upper panel of **Figure 2B**), the level of BDNF measured by ELISA was decreased by 39 ± 4.3% ($n = 6$), as compared with control group (the lower panel of **Figure 2B**). Furthermore, MCC950 (the inhibitor of NLRP3 inflammasomes) partly reversed this suppressive effect of SD on the level of BDNF (**Figures 2A,B**). In the isolated astrocytes, the protein expression of BDNF was elevated by 90 ± 2.7% ($n = 6$; the upper panel of **Figure 2B**), whereas the release of BDNF was increased by 30 ± 3.9% ($n = 6$; the lower panel of **Figure 2B**), as compared with SD group.

The Level of BDNF Elevated by Fluoxetine Requires 5-HT$_{2B}$ Receptor

As shown in **Figure 2C**, fluoxetine increased the expression of BDNF by 92 ± 4.2 or 36 ± 5.7% ($n = 6$), with or without SD treatment, respectively. Pretreatment with SB204741 (an antagonist of 5-HT$_{2B}$ receptors), completely abolished the effect of fluoxetine on BDNF. Similarly, as compared with PBS group exposed to SD in animals, fluoxetine increased the level of BDNF by 50 ± 7.5% ($n = 6$), but after the pretreatment with SB204741,

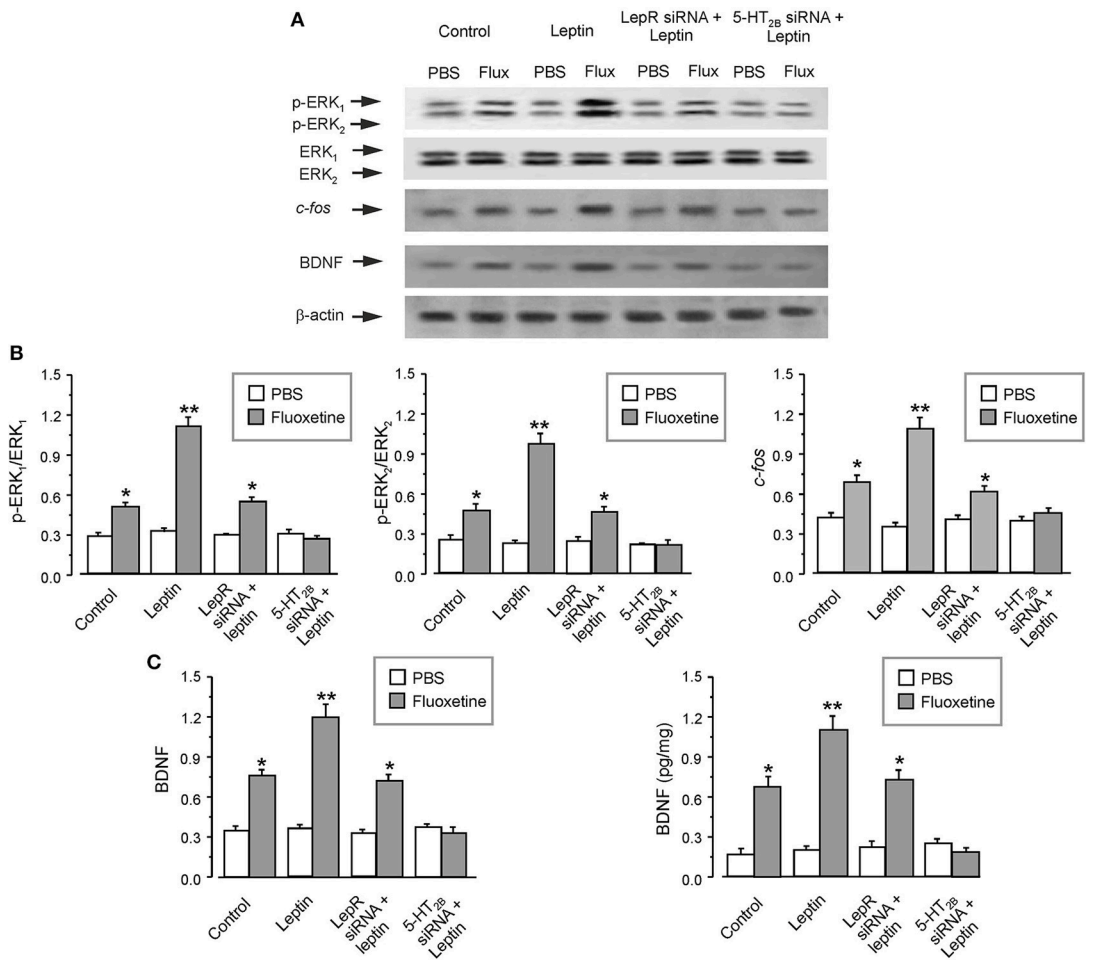

FIGURE 5 | The regulation of leptin on the effects of fluoxetine in astrocytes. After RNA interfering the expression of leptin receptors or cfos with siRNA duplex, the primary cultured astrocytes were pre-treated with PBS (Control) or leptin, then the cells were treated with PBS or fluoxetine for 30 min to measure the phosphorylation of ERK$_{1/2}$ or for 1 week to check the expression of the other proteins **(A–C)**. Representative blots for the level of p-ERK$_{1/2}$, cfos and BDNF were shown in **(A)**. The average phosphorylation of ERK1 and ERK2 was quantified as the ratio between the p-ERK$_1$ and ERK$_1$ or p-ERK$_2$ and ERK$_2$, $n = 6$ (the left and middle panels of **B**), the average expression of cfos and BDNF were normalized by β-actin, $n = 6$ (the right panel of **B** and the left panel of **C**). The average BDNF level measured via ELISA was shown in **(B)**, $n = 6$. *$p < 0.05$, statistically significant difference compared with any other group except for each other **(B,C)**; **$p < 0.05$, statistically significant difference compared with any other group **(B,C)**.

the elevated BDNF by fluoxetine was decreased by 10 ± 7.2% (n = 6; **Figure 2D**).

The Signaling Cascade Contributing to Fluoxetine-Depended Decrease of Astrocytic BDNF

Administration of fluoxetine to the primary cultured astrocytes increased the expression of BDNF by 180 ± 5.1% (n = 6; **Figure 3A**). Similarly to *in vivo* experiments, SB204741 completely abolished the fluoxetine induced increase in BDNF expression (by 60 ± 4.2% (n = 6) of fluoxetine group). Pretreatment with U0126 (a selective inhibitor of MEK) completely abolished the fluoxetine-induced increase in expression of BDNF by 66 ± 4.7% of fluoxetine group (n = 6; **Figure 3A**). However, after the pretreatment with LY294002 [a selective inhibitor of phosphatidylinositol-3-kinase (PI3K)], fluoxetine still significantly increased the expression of BDNF by 176 ± 5.9% (n = 6) of control (PBS) group (**Figure 3A**). As shown in the lower panel of **Figure 3C**, the level of BDNF measured by ELISA had a similar tendency as the expression of BDNF measured by western blotting. SB204741 and U0126 dramatically abolished the fluoxetine induced increase in the level of BDNF by 65 ± 5.2 and 57 ± 4.7% (n = 6) of fluoxetine group, respectively. After pretreatment with LY294002, fluoxetine still increased the level of BDNF by 171 ± 6.2% (n = 6) of PBS group (the lower panel of **Figure 3C**).

Meanwhile, after treatment with interfering RNA of 5-HT$_{2B}$ receptor, the protein level of BDNF increased by fluoxetine was suppressed to 111 ± 4.5 or 117 ± 6.9% (n = 6) of PBS group, as shown in **Figures 3B,D**. We also found that transcription factor *c-fos* was required for the fluoxetine induction of the expression of BDNF. Pretreatment with the siRNA duplex chains of *c-fos* eliminated differences between PBS group and fluoxetine group (**Figures 3B,D**). The relative expression of 5-HT$_{2B}$R and c-fos were shown in **Supplementary Figures 1A,B**.

Leptin Increases the Expression of 5-HT$_{2B}$ Receptor on Astrocytes

The administration of leptin significantly induced the phosphorylation of STAT3 by about two times in the isolated astrocytes from GFAP-GFP mice (**Figure 4A**). However, in the SD-treated group, the ratio of p-STAT3/STAT3 was decreased to 46 ± 3.2% (n = 6) of control group (**Figure 4A**). Leptin re-elevated the phosphorylation of STAT3 decreased by SD to 177 ± 3.5% (n = 6) of SD-treated PBS group, but it was still only 80 ± 3.3% (n = 6) of PBS group without SD treatment (**Figure 4A**).

In the primary cultured astrocytes, treatment with leptin significantly increased the protein and mRNA expression of 5-HT$_{2B}$ receptors by 100 ± 5.5 and 52 ± 4.7% (n = 6), respectively (**Figure 4B**). Treatment with RNA interfering with the expression of leptin receptors with siRNA duplex chains, completely the abolished effect of leptin on the expression of 5-HT$_{2B}$ receptors. Pretreatment with WP1066 (an inhibitor of JAK2/STAT3) similarly decreases the protein and mRNA expression of 5-HT$_{2B}$ receptors to the level of PBS group (**Figure 4B**). The relative expression of LepR was shown in **Supplementary Figure 1C**.

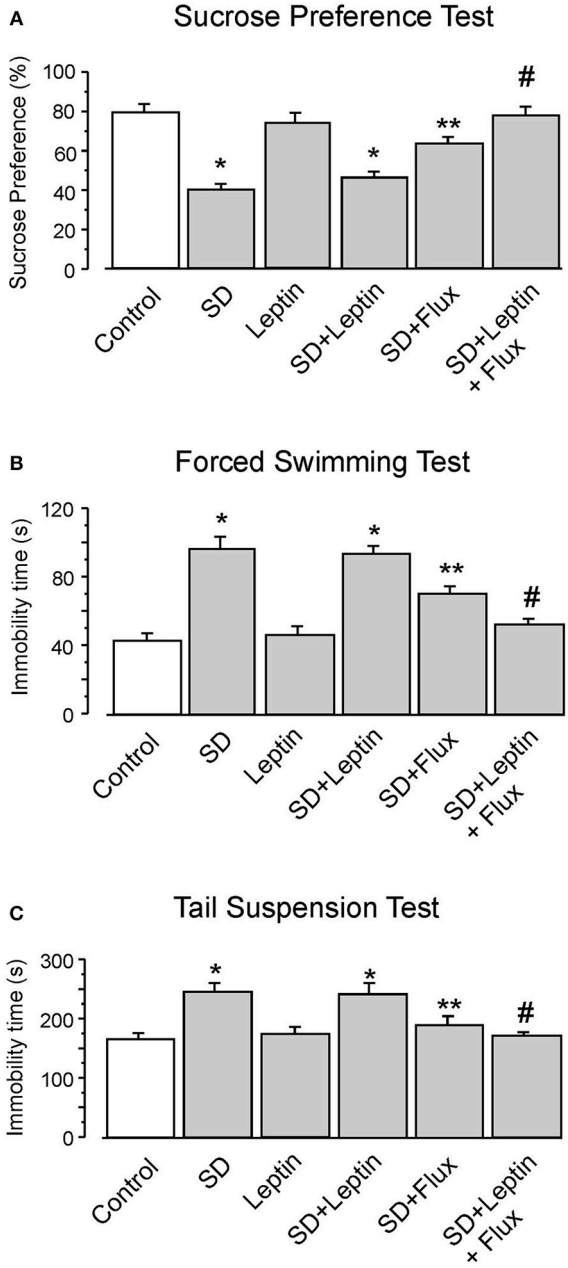

FIGURE 6 | The GFAP-GFP transgenic mice were treated with sham (Control) or exposed to SD for 3 weeks, in the final week the mice were intraperitoneally injected with PBS, leptin, fluoxetine or leptin plus fluoxetine. The percentage of sucrose preference was measured **(A)**, the time of immobility were recorded in force-swimming test **(B)** and tail suspension test **(C)**, the values are expressed as mean ± SEM, n = 12. *$p < 0.05$, statistically significant difference compared with any other group except for each other **(A–C)**; **$p < 0.05$, statistically significant difference compared with any other group **(A–C)**; $^{\#}p < 0.05$, statistically significant difference compared with SD, SD plus leptin and SD plus fluoxetine groups **(A–C)**.

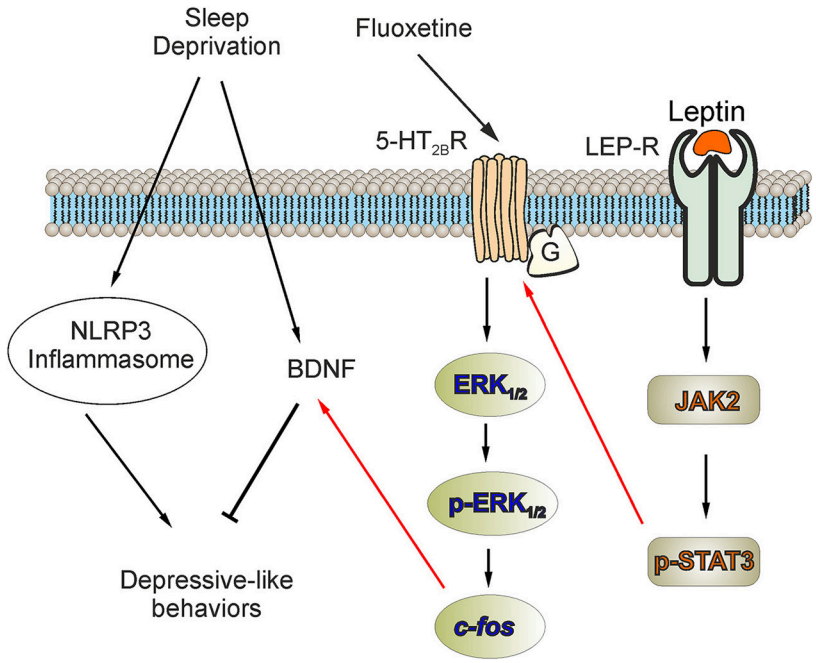

FIGURE 7 | The facilitation of leptin on the improvement of fluoxetine for the depressive-like behaviors induced by SD. The activation of NLRP3 inflammasomes or the elimination of BDNF plays an important role in the depressive-like behaviors induced by SD. In our previous research, we reported that antidepressant fluoxetine could decrease the activation of NLRP3 inflammasomes triggered by SD. In this study, we discover that the activation of NLRP3 inflammasomes is involved in the elimination of BDNF in SD-treated mice. Meanwhile, fluoxetine increases the expression of BDNF via 5-HT$_{2B}$ receptors. In primary cultured astrocytes, the expression of BDNF elevated by fluoxetine also requires the phosphorylation of ERK$_{1/2}$ and the up-regulation of *c-fos*. Moreover, the pretreatment with leptin significantly increase the expression of 5-HT$_{2B}$ receptors via LepR/JAK2/STAT3 pathway in astrocytes, so the improvement of fluoxetine for the level of BDNF and the depressive-like behaviors are obviously enhanced.

Leptin Enhances the Function of Fluoxetine via the Increased 5-HT$_{2B}$ Receptor

After pretreatment of the primary cultured astrocytes with leptin, fluoxetine significantly increased the phosphorylation of ERK$_{1/2}$ by 120 ± 7.2 and 106 ± 7.7% ($n = 6$), and enhanced the protein expression of *c-fos* or BDNF by 57 ± 6.5 or 57 ± 7.5% ($n = 6$), as compared with fluoxetine group (**Figures 5A–C**). The effect of leptin was mediated through leptin receptors, because after RNA interfering the expression of leptin receptors, leptin lost its ability to augment effects of fluoxetine on expression of p-ERK$_{1/2}$, *c-fos* and BDNF (**Figures 5A–C**). At the same time after suppressing the expression of 5-HT$_{2B}$ receptors, the effects of fluoxetine and leptin on the level of p-ERK$_{1/2}$, *c-fos*, and BDNF were all abolished (**Figures 5A–C**).

Leptin Augments Effects of Fluoxetine on the Depressive-Like Behaviors Induced by SD

In experiments *in vivo*, leptin augments effects of fluoxetine on the depressive-like behaviors. In sucrose preference test, SD decreased the sucrose uptake ratio by 49 ± 2.9% ($n = 12$), the administration of leptin alone had no effect. However, while fluoxetine increased this ratio by 59 ± 3.1% ($n = 12$) of SD group, treatment with leptin and fluoxetine combined increased the sucrose preference by 92 ± 5.7% ($n = 12$) of SD group, and by 21 ± 3.3% ($n = 12$) of SD plus fluoxetine group (**Figure 6A**). Similarly, in forced swimming test, leptin did not change the immobility time increased by SD, whereas treatment with leptin and fluoxetine decreased the immobility time to 54 ± 3.7% ($n = 12$) of SD group, and to 73 ± 3.1% ($n = 12$) of SD plus fluoxetine group (**Figure 6B**). Similar effect of leptin in tail suspension test is shown in **Figure 6C**, the immobility time of leptin plus fluoxetine in SD-treated group was decreased by 31 ± 2.9% ($n = 12$) in SD group, and by 12 ± 2.2% ($n = 12$) in SD plus fluoxetine group.

DISCUSSION

In this study, we discovered that activation of NLRP3 inflammasomes contributes to the development of the depressive-like behaviors induced by SD; the NLRP3 inflammasome also was associated to the decreased level of BDNF. The antidepressant fluoxetine triggers (through activation of 5-HT$_{2B}$ receptors) the phosphorylation of ERK$_{1/2}$ and increases the downstream expression of *c-fos*, which in turn increases the expression of BDNF in astrocytes. At the same time, leptin increases the expression of 5-HT$_{2B}$ receptors via LepR/JAK2/STAT3 pathway in astrocytes, and fluoxetine could be more effective to elevate the level of BDNF and improve the depressive-like behaviors via the increased 5-HT$_{2B}$ receptors induced by leptin (**Figure 7**).

The pathological mechanism of MDD is related to the activation of NLRP3 inflammasomes and the suppression

of BDNF secretion (10, 12). For the first time, we show that SD decreases the level of BDNF and triggers the depressive-like behaviors via activating NLRP3 inflammations in astrocytes. Previously, we have discovered that SD activates NLRP3 inflammasomes and augments production of IL1β/18 in astrocytes, while fluoxetine obliterates neuroinflammation and neuronal apoptosis induced by SD (7). Sleep disturbance is often associated with stress-related mental disorders (29), but the potential mechanism linking these two diseases remains unknown. In the long term, sleep disturbance and MDD are both related to the decreased BDNF, whereas a part of SD-treatment induce a fast increase in BDNF serum level within hours, which has antidepressive effects (29), In this study, we demonstrate, that activated NLRP3 inflammasomes contribute to the SD-induced decrease in astrocytic BDNF levels. Although we did not measure the effect of astrocytic NLRP3 on integral or neuronal BDNF, the suppression of NLRP3 significantly decreased CUMS-induced inflammatory cytokines (caspase-1 and IL-1β) and apoptosis in hippocampus (30). And CUMS-induced the down-regulated BDNF and the activation of NLRP3 inflammasome in hippocampus or cortex could be both reversed in neuroligin3 (NLGN3) knockout mice (31).

Leptin is a 16 kDa hormone associated with regulation of energy balance and appetite (32); with growing evidence indicating its role in the MDD; it remains however a degree of controversy. Low CSF levels of leptin were detected in female suicide attempters with MDD (33), however, the serum leptin levels were not significantly changed in women with postpartum depression (34). The administration of leptin can produce a dose-dependent alleviation of depressive behaviors triggered by chronic stress and measured by forced swimming test or tail suspension test (35). But, the serum level of leptin in SD participants is 9.43 ± 8.87 ng/ml, which has no significant difference from the serum level of leptin in non-sleep-deprived persons (36). According to our results, the sole treatment with leptin does not improve the depressive like behaviors induced by SD in forced swimming or tail suspension test. In leptin receptors (LepR) knock-out mice, the anti-depressive-like behavioral effects of fluoxetine were unaffected (37). Our present study demonstrates that leptin enhances the antidepressive effects of fluoxetine through an increase of the expression of 5-HT_{2B} receptors in astrocytes.

Targeting astroglial 5-HT_{2B} receptors is an important part of therapy of depression (14). The therapeutic potential of fluoxetine and other serotonin-specific re-uptake inhibitors (SSRIs) is mediated, in part, through direct stimulation of astroglial 5-HT_{2B} receptors (16). In this work, we only checked the effects of fluoxetine on the increased expression of 5-HT_{2B} receptors induced by leptin, but the other SSRIs may play more antidepressive roles via the increased 5-HT_{2B} receptors. We demonstrated previously that acute treatment with fluoxetine induces the transactivation of epidermal growth factor (EGF) receptors (EGFR) and the phosphorylation of $ERK_{1/2}$ by stimulating 5-HT_{2B} receptors in astrocytes (22). Furthermore, chronic treatment of astrocytes with fluoxetine increases the expression of *c-fos* through the 5-HT_{2B} receptors-EGFR-$ERK_{1/2}$ pathway; this subsequently regulates expression of proteins, such as for example $cPLA_{2a}$ and caveolin-1 (38, 39). Here we show that the decrease of astrocytic BDNF following SD is reversed by fluoxetine acting through *c-fos* induced by 5-HT_{2B} receptors-mediated $ERK_{1/2}$ phosphorylation. At the same time leptin enhances the anti-depressive effects of fluoxetine through increasing the expression of 5-HT_{2B} in astrocytes under SD-condition.

In the clinic, sleep disorders and MDD are common pathologies; patients with sleep disturbances have a higher risk of the occurrence of MDD. To summarize our study, we found that chronic SD triggers the depressive-like behaviors by stimulating the activation of NLRP3 inflammasomes and by decreasing the expression of BDNF in astrocytes. We further found that fluoxetine alleviates the depressive-like behaviors and rescues decreased BDNF release induced by SD by stimulating 5-HT_{2B} receptors. Leptin enhances fluoxetine effects by increasing the expression of astroglial 5-HT_{2B} receptors. Thus, the decrease in astrocytic BDNF release induced by the activated NLRP3 inflammasomes is the key pathological mechanism of the depressive-like behaviors induced by SD, while the co-treatment with fluoxetine and leptin can provide an effective therapy for the depressive-like behaviors induced by SD by regulating expression of astroglial 5-HT_{2B} receptors.

AUTHOR CONTRIBUTIONS

MX and BL designed the experiments. XL and SSL built the animal models and analyzed the data. XL operated cell culture. ZL and SL performed other experiments. AV and BL wrote the paper.

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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.2018.00734/full#supplementary-material

Supplementary Figure 1 | The mRNA expressions after RNA interfering. The expression of 5-HT_{2B} receptors (5-HT_{2B}R), c-fos and leptin receptors (LepR) with negative control or siRNA duplex, the relative expression ratios of 5-HT_{2B}R/GAPDH, c-fos/GAPDH and LepR/GAPDH were normalized by the control group and shown in **(A–C)**. Data represent mean ± SEM, $n = 6$. *$p < 0.05$, statistically significant difference compared with any other group.

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The Efficacy of Vortioxetine on Anhedonia in Patients With Major Depressive Disorder

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Background: Anhedonia is a common, persistent, and disabling phenomenon in treated adults with Major Depressive Disorder (MDD). Hitherto, relatively few antidepressant agents have been evaluated with respect to their effect on anhedonia in MDD.

Methods: This is a *post-hoc* analysis of a primary study that sought to evaluate the sensitivity to change of the THINC-integrated tool (THINC-it) in MDD (ClinicalTrials.gov Identifier: NCT03053362). Adults meeting DSM-5 criteria for MDD with at least moderate depressive symptom severity [i.e., Montgomery Åsberg Depression Rating Scale (MADRS) total score ≥20] were eligible. Subjects were recruited between October 2017 and August 2018 in Toronto, Ontario at the Brain and Cognition Discovery Foundation. All subjects received open-label vortioxetine (10–20 mg/day, flexibly-dosed) for 8 weeks. Herein, the primary outcome of interest was the change from baseline to endpoint in the Snaith-Hamilton Pleasure Scale (SHAPS) total score, as well as the MADRS anhedonia factor. The mediational effects of improvements in anhedonia on general function and quality of life, as measured by the Sheehan Disability Scale (SDS) and the 5-Item World Health Organization Well-Being Index (WHO-5), were secondarily assessed.

Results: A total of 100 subjects with MDD were enrolled in the primary study and began treatment with vortioxetine. Vortioxetine significantly improved anhedonia as evidenced by significant baseline to endpoint improvements in SHAPS and MADRS anhedonia factor scores ($p < 0.0001$). Improvements in the SHAPS and the MADRS anhedonia factor correlated with improvements in general function (i.e., SDS) and quality of life (i.e., WHO-5) ($p < 0.0001$). Notably, improvements in anhedonia were found to mediate the association between improvements in overall depressive symptom severity (i.e., MADRS total score) and social functioning (i.e., social life component of the SDS) ($p = 0.026$).

Conclusion: The unmet need in depression is to improve patient functioning and other patient-reported outcomes (e.g., quality of life). Antidepressant interventions capable of attenuating anhedonia as well as cognitive dysfunction in MDD may help in this regard, as improvement in these domains have been associated with improvement in psychosocial function and quality of life.

Keywords: major depressive disorder, anhedonia, function, vortioxetine, quality of life, antidepressants

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INTRODUCTION

Major depressive disorder (MDD) is a leading cause of disability worldwide and is associated with significant economic burden (1). Approximately 50% of the illness burden and costs attributable to MDD is due to impairments in function (e.g., impaired workplace function, short-/long-term disability) (2, 3). Replicated evidence indicate that disturbances in motivation (and cognition) are persisting deficits in MDD and mediate poor functional outcomes in MDD.

Anhedonia is defined as an impaired capacity to experience or anticipate pleasure (4). According to the Diagnostic and Statistical Manual of Mental Disorders, fifth edition (DSM-5), anhedonia and depressed mood are among the key diagnostic features that characterize a major depressive episode (MDE) as part of MDD (5). Notably, anhedonia has been associated with disturbances of central dopaminergic, mesolimbic, and mesocortical reward circuit pathways, which involve brain regions such as the ventral tegmental area (VTA), ventral striatum, and pre-frontal cortex (6).

Anhedonia is a common symptom of MDD, and is reported in ∼75% of patients (7). Importantly, anhedonia and impaired reward learning have been associated with poorer disease prognosis and suboptimal treatment response (8). In addition to being a common symptom of MDD, it is often a persisting dimension amongst individuals with MDD receiving disparate treatments (9). The hazards posed by anhedonia, as well as the suboptimal anti-anhedonia effects of many available antidepressants, provides the impetus for specifically evaluating the efficacy of newer treatments on this dimension. Preliminary evidence suggests, for example, that agomelatine and ketamine may exert clinically relevant effects on measures of anhedonia (10, 11).

Vortioxetine is a multimodal antidepressant with multiple effector neurotransmitter systems, including serotonin (5-HT), norepinephrine (NE), dopamine, amino acids, histamine (HA), and cholinergic systems (12–15). Results from meta- and network analyses indicate that vortioxetine is generally well-tolerated and efficacious at reducing MDD illness severity (16–18). Moreover, in 2018, the product insert for vortioxetine in the USA was updated to include mention of vortioxetine's independent pro-cognitive effects in MDD.

The pharmacodynamic profile of vortioxetine, as well as the pro-cognitive effects of this agent (13), provide the basis for hypothesizing that vortioxetine may be able to attenuate measures of anhedonia in adults with MDD. In addition, no previous study has evaluated the anti-anhedonia effects of vortioxetine, and since anhedonia (like cognition) has been shown to be an important mediator of overall clinical improvement in MDD. Herein, we sought to determine whether vortioxetine improved measures of anhedonia and to what extent improvements in anhedonia correlate with overall function and quality of life.

METHODS

Study Population

This is a *post-hoc* analysis of a primary study that sought to evaluate the sensitivity to change of the THINC-integrated tool (THINC-it) in MDD (ClinicalTrials.gov Identifier: NCT03053362). Adults meeting DSM-5 criteria for MDD with at least moderate depressive symptom severity [i.e., Montgomery Åsberg Depression Rating Scale (MADRS) total score ≥20] were eligible. Subjects were recruited between October 2017 and August 2018 in Toronto, Ontario at the Brain and Cognition Discovery Foundation. All subjects received open-label vortioxetine (10–20 mg/day, flexibly-dosed) for 8 weeks. Ninety-five female and male patients with DSM-5-defined MDD between the ages of 18 and 65 were included in the analysis. Approval from a local Institutional Review Board was obtained prior to initiating the study and all eligible participants provided written informed consent.

Eligibility Criteria

Patients who met the following eligibility criteria were included into the study: (1) provided written informed consent, (2) male or female between 18 and 65 years of age, (3) current diagnosis of a major depressive episode (MDE) as part of MDD as per DSM-5 criteria, (4) current MDE was confirmed by the Mini International Neuropsychiatric Interview (M.I.N.I 5.0.), (5) outpatient of a psychiatric setting, (6) MADRS score ≥20 at screening and baseline, (7) history of at least one prior MDE formally diagnosed by a healthcare provider or validated by previous treatment (e.g., guideline-informed pharmacotherapy and/or manual-based psychotherapy).

The exclusion criteria were: (1) current alcohol and/or substance use disorder as confirmed by the M.I.N.I 5.0, (2) presence of a comorbid psychiatric disorder that was a focus of clinical concern (3) medications approved and/or employed off-label for cognitive dysfunction (e.g., psychostimulants), (4) medications for a general medical disorder that, in the opinion of the investigator, could affect cognitive function, (5) use of benzodiazepines within 12 h of cognitive assessments, (6) consumption of alcohol within 8 h of cognitive assessments, (7) inconsistent use or abuse of marijuana, (8) physical, cognitive, or language impairments sufficient to adversely affect data derived from cognitive assessments, (9) diagnosed reading disability or dyslexia, (10) clinically significant learning disorder by history, (11) electroconvulsive therapy (ECT) in the last 6 months, (12) history of moderate or severe head trauma (e.g., loss of consciousness for >1 h), other neurological disorders, or unstable systemic medical diseases that, in the opinion of the investigator, are likely to affect the central nervous system, (13) pregnant and/or breastfeeding, (14) received investigational agents as part of a separate study within 30 days of the screening visit, (15) actively suicidal or evaluated as being at high suicide risk as per clinical judgment using the Columbia-Suicide Severity Rating Scale, (16) currently receiving treatment with Monoamine Oxidase Inhibitors (MAOIs), antibiotics such as linezolid, or intravenous methylene blue, (17) previous

hypersensitivity reaction to vortioxetine or any components of the formulation.

Study Procedure

Patients taking other medications for depression were tapered off these drugs as instructed by the treating clinician (the tapering period was based on the time it takes for vortioxetine to reach therapeutically significant plasma levels in the bloodstream). Subjects with MDD were dosed with vortioxetine (10–20 mg, flexibly dosed) daily for 8 weeks. All enrolled participants will receive vortioxetine 10 mg/day for the first 2 weeks. They also had the option of moving up to 20 mg; however, dose adjustment was based on tolerability and clinical response as assessed by the treating clinician. Subjects underwent five visits (screening, week 0: baseline, week 2, week 4, and week 8: endpoint). The MADRS total score was evaluated at all five visits, and the SHAPS and SDS scores were evaluated at three different time points (i.e., week 0, week 2, and week 8).

Outcome Measures

The primary outcome of the analysis was change in anhedonia, as measured by the baseline to endpoint change in Snaith–Hamilton Pleasure Scale (SHAPS) and the MADRS anhedonia factor [i.e., which was based on items 1 (apparent sadness), 2 (reported sadness), 6 (concentration difficulties), 7 (lassitude), 8 (inability to feel)]. Relevant secondary measures were functional impairment, as measured by the Sheehan Disability Scale (SDS), which is a brief self-report measure that evaluates three functional domains (i.e., work/school, social life, and family life or home responsibilities); and quality of life, as measured by the 5-Item World Health Organization Well-Being Index (WHO-5), which is a five-item self-report measure assessing subjective psychological well-being. For the logistic regression analyses, clinical response for anhedonia was defined as having a SHAPS score improvement of $\geq$50% from baseline, and remission of anhedonia was defined as having a follow-up SHAPS score of $\leq$3 (19).

Statistical Analysis

Continuous variables were summarized as means and standard deviations (SDs) or medians and interquartile ranges (IQRs). Categorical variables were summarized as frequencies and proportions. The repeated measures mixed model analysis was used to evaluate changes in the SHAPS and MADRS scores. A multivariate logistic regression model was used to analyze differences in demographic and clinical variables (i.e., sex; age; total years of education; current use of alcohol, nicotine, marijuana; age of MDD onset, length of current MDE; family history of mental illness) that might affect anhedonia in patients with MDD. Odds ratios (ORs) and their 95% confidence intervals (CIs) were estimated using maximum likelihood methods. Correlations among different scale scores (i.e., MADRS, SHAPS, SDS, WHO-5) were calculated using the Pearson correlation coefficient. Mediational analysis was used to estimate indirect effects. Herein, a mediation analysis was used to determine the extent to which the association between changes in depressive symptom severity (ΔMADRS), changes in functional impairment (ΔSDS), and changes in well-being (ΔWHO-5) were mediated by improvements in anhedonia (ΔSHAPS) in patients treated with vortioxetine over 8 weeks. Significance was set to $p < 0.05$, two-sided. All statistical analyses were conducted using SPSS software, version 22.0.

RESULTS

Characteristics of Patients With MDD at Baseline

One hundred and forty-four subjects with MDD provided written, informed consent, and underwent screening. Of 95 eligible subjects from the primary study, 92 (96.8%) and 79 (83.2%) completed the week 2 visit and week 8 visit, respectively. The mean age of subjects at baseline was 38.9

TABLE 1 | Characteristics of patients with MDD at baseline.

Variables	Baseline clinical characteristics of patients ($n = 95$)
CATEGORICAL VARIABLES	
Sex (female/male); *n* (%)	62/33 (65.3/34.7)
Current alcohol use (at least weekly) (yes/no); *n* (%)	32/63 (33.7/66.3)
Current Nicotine use (yes/no); *n* (%)	22/73 (31.6.2/68.4)
Current Marijuana use (yes/no); *n* (%)	29/66 (30.5/69.5)
Family history of mental illness (yes/no); *n* (%)	61/34 (64.2/35.8)
Presence of psychiatric comorbidity (yes/no); *n* (%)	49/45 (51.6/48.4)
Presence of general comorbidity (yes/no); *n* (%)	59/36 (62.1/37.9)
CONTINUOUS VARIABLES	
Age in years; mean (SD)	38.9 (12.9)
BMI (kg/m^2); mean (SD)	28.5 (6.5)
Total years of education; mean (SD)	15.7 (3.1)
Age of MDD onset in years; median (IQR)	16.0 (13.0, 25.0)
Number of lifetime episodes; median (IQR)	5.0 (3.0, 20.0)
Duration of illness in years; mean (SD)	15.0 (8.0, 26.0)
Length of Current MDE in months; median (IQR)	8.0 (5.0, 24.0)
Number of current psychiatric medications; median (IQR)	1.0 (0.0, 2.0)
Number of past psychiatric medications; median (IQR)	1.0 (0.0, 2.0)
MADRS total score; mean (SD)	32.3 (7.3)
SDS total score; mean (SD)	20.9 (6.0)
SDS work; mean (SD)	6.9 (2.9)
SDS social; mean (SD)	7.2 (2.3)
SDS family life; mean (SD)	7.1 (2.2)
WHO-5; mean (SD)	14.8 (11.3)

MDE, major depressive episode; MADRS, Montgomery Åsberg Depression Rating Scale; SHAPS, Snaith-Hamilton Pleasure Scale; SDS, Sheehan Disability Scale; WHO-5, The World Health Organization- Five Well-Being Index.

years (SD = 12.9), and 62 (65.3%) subjects were female. The median age of MDD onset was 16.0 years (IQR = 13.0–25.0), and the median length of current MDE duration was 8 months (IQR = 5.0–25.0). Sixty-one subjects (64.8%) reported a positive family history of mental illness. With respect to clinical characteristics, the mean (SD) MADRS and SDS of subjects at baseline were 32.3 (7.3) and 20.9 (6.0), respectively. The characteristics of MDD patients at baseline are described in **Table 1**.

Efficacy of Vortioxetine on Anhedonia Outcomes

Treatment with vortioxetine significantly improved measures of anhedonia between baseline and endpoint (i.e., week 8) (ΔSHAPS = −2.9, 95% CI: −3.7, −2.2, $z = -7.88$, $p < 0.0001$; ΔMADRS anhedonia factors= −7.1, 95% CI: −8.2, −5.9, $z = -11.90$, $p < 0.0001$). Response and remission rates at endpoint were 56.9 and 51.72%, respectively (**Figure 1**). Significant correlations were found between the SHAPS and MADRS anhedonia factor at baseline ($r = 0.474$, $p < 0.0001$), week 2 ($r = 0.669$, $p < 0.0001$), and week 8 ($r = 0.474$, $p < 0.0001$).

Repeated measures mixed model analyses were used to assess for changes in anhedonia after 2 and 8 weeks of treatment with vortioxetine when compared to baseline (**Table 2**). Among the subjects who completed the 2 weeks follow-up, 71.7% (66/92) had SHAPS >3 at baseline (i.e., the presence of clinically significant anhedonia). Between baseline and week 2, these subjects exhibited significant improvements in anhedonia, as indicated by both the change in SHAPS score (ΔSHAPS = −1.0, 95% CI: −1.7, −0.3, $z = -2.88$, $p = 0.004$) and the change in MADRS anhedonia factor (ΔMADRS anhedonia factor= −3.8, 95% CI: −4.9, −2.7, $z = -7.22$, $p < 0.0001$). Anhedonia response and remission rates for the 66 MDD patients with anhedonia at week 2 were 28.79 and 27.27%, respectively (**Figure 1**). Among the subjects who completed the 8 weeks follow-up, 73.4% (58/79) had SHAPS >3 at baseline. Furthermore, among the subjects withdrew, 70.6% (11/16) had SHAPS >3 at baseline. No significant difference was observed between the patients completed the follow-up and withdrew. Both the changes of SHAPS and MADRS anhedonia factor scores have significant improvement between baseline and endpoint (ΔSHAPS= −2.9, 95% CI: −3.7, −2.2, $z = -7.88$, $p < 0.0001$; ΔMADRS anhedonia factors= −7.1, 95% CI: −8.2, −5.9, $z = -11.90$, $p < 0.0001$). The anhedonia response and remission rate were reached 56.9 and 51.72%, respectively (**Figure 1**). In addition, the changes of SHAPS and MADRS anhedonia factor scores with treatment of vortioxetine in the subgroup of patients with anhedonia (i.e., SHAPS score >3 at baseline) were shown in **Supplemental Table 1**. The changes in the subgroup was similar as overall patient both in weeks 2 and 8.

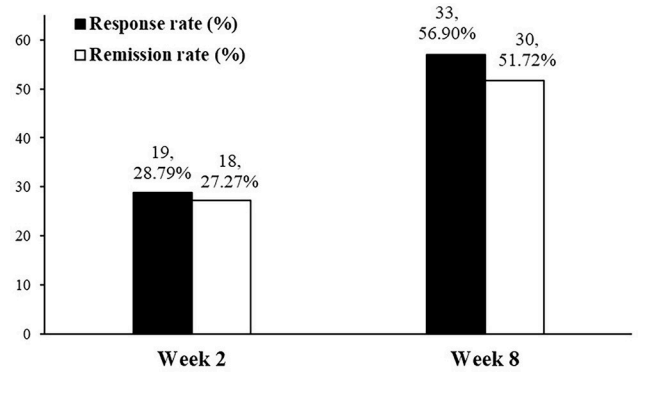

FIGURE 1 | Response and remission rate of anhedonia in patients with MDD using SHAPS at weeks 2 and 8.

The logistic regression analysis indicated that none of the analyzed demographic and clinical variables significantly influenced SHAPS-defined anhedonia at baseline. However, the results demonstrated that gender was predictive of remission at endpoint (i.e., females AOR = 0.139, 95% CI: 0.024, 0.802, $p = 0.027$). Moreover, current marijuana use (AOR = 6.056, 95% CI: 1.168, 31.397, $p = 0.032$), older age of MDD onset (AOR = 1.086, 95% CI: 1.009, 1.169, $p = 0.028$), and having a family history of mental illness (AOR = 5.476, 95%CI: 1.156, 25.947, $p = 0.032$) increased the probability of non-remission at endpoint (**Table 3**).

The Correlation Between Anhedonia and Functional Impairment

Correlations between changes in depressive symptom severity, anhedonia, functional impairment, and quality of life were assessed. All measures were found to be significantly different between baseline and endpoint (all $p < 0.05$). This is shown in **Table 4**. Mediational analysis was used to evaluate he indirect effect of improvements in anhedonia (ΔSHAPS) on the association between changes in depressive symptom severity (ΔMADRS) and changes in function (ΔSDS) [as well as well-being (ΔWHO-5)] (**Figures 2**, **3**). The results showed

TABLE 2 | The changes of SHAPS and MADRS anhedonia factor scores with treatment of vortioxetine.

Variables	Baseline ($n = 95$)	Week 2 ($n = 92$)				Week 8 ($n = 79$)			
	M ± SD	M ± SD	Δbaseline (95% CI)	z	p	M ± SD	Δbaseline (95% CI)	z	p
SHAPS score	6.2 ± 3.8	5.2 ± 4.5	−1.0 (−1.7, −0.3)	−2.88	0.004*	3.3 ± 3.8	−2.9 (−3.7, −2.2)	−7.88	<0.0001*
MADRS anhedonia factor	18.3 ± 3.7	14.5 ± 5.1	−3.8 (−4.9, −2.7)	−7.22	<0.0001*	11.4 ± 6.1	−7.1 (−8.2, −5.9)	−11.90	<0.0001*

*Indicates significant differences from baseline.

TABLE 3 | Logistic regression of the factors affecting anhedonia in patients with MDD.

Factors	AOR	95%CI	*p-values*
TOTAL PATIENTS (*N* = 95): WITHOUT ANHEDONIA VS. WITH ANHEDONIA AT BASELINE			
Age (years)	1.013	0.971, 1.056	0.550
Sex (male/female)	1.165	0.443, 3.059	0.757
Total years of education (years)	1.027	0.875, 1.205	0.745
Current alcohol use (at least weekly) (yes/no)	0.504	0.196, 1.299	0.156
Current Nicotine use (yes/no)	2.141	0.588, 7.802	0.248
Current Marijuana use (yes/no)	0.648	0.237, 1.774	0.399
Age of MDD onset (years)	0.986	0.941, 1.032	0.539
Length of Current MDE (months)	1.008	0.993, 1.024	0.297
Family history of mental illness (yes/no)	0.762	0.276, 2.104	0.601
PATIENTS WITH ANHEDONIA* (*N* = 58): REMITTER VS. NON-REMITTER AT ENDPOINT			
Age (years)	1.022	0.968, 1.080	0.428
Sex (male/female)	**0.139**	**0.024, 0.802**	**0.027**
Total years of education(years)	1.115	0.875, 1.421	0.378
Current alcohol use (at least weekly) (yes/no)	0.250	0.053, 1.182	0.080
Current Nicotine use (yes/no)	1.004	0.203, 4.961	0.996
Current Marijuana use (yes/no)	**6.056**	**1.168, 31.397**	**0.032**
Age of MDD onset (years)	**1.086**	**1.009, 1.169**	**0.028**
Length of Current MDE (months)	1.007	0.989, 1.025	0.468
Family history of mental illness (yes/no)	**5.476**	**1.156, 25.947**	**0.032**

**Patients who had anhedonia at baseline and did not drop out at endpoint. Bold values denote statistical significance at the $p < 0.05$ level.*

that anhedonia improvement was a strong mediator of the association between improvement in depressive symptoms and improvement in social functioning [i.e., improvement in the social life component of the SDS (ΔSDS-S)] with $p = 0.026$, and explained 39.9% of the total variance. Anhedonia improvement was not found to mediate the association between improvement in depressive symptoms and improvement in other SDS domains or WHO-5.

DISCUSSION

Anhedonia is subserved by a dysregulation of central nervous system reward circuits and structures (20), and is a composite symptom with two primary dimensions (i.e., motivational/appetitive and consummatory dimensions) (21, 22). The present analysis indicated that both outcome measures (i.e., SHAPS and MADRS anhedonia component) were highly correlated (with each other) and could reliably assess anhedonia symptoms. We evaluated anhedonia as a continuous and categorical measure. Definitions for response and remission with the SHAPS have been reported elsewhere (19). Herein, we observed that 70.5% of our sample met SHAPS-defined criteria for clinically significant anhedonia symptoms at baseline, underscoring the high prevalence of this disturbance. After 8 weeks of flexibly dosed vortioxetine, it was observed that 56.9% of participants met SHAPS-defined response criteria.

Previous studies have reported anti-anhedonia effects in adults with MDD treated with select antidepressants including, but not limited to agomelatine, bupropion, venlafaxine, fluoxetine, amitifadine, levomilnacipran, escitalopram, and ketamine (22–26). Moreover, some antidepressants (e.g., SSRIs) have been shown to help treat emotional blunting in MDD, which phenotypically overlaps with anhedonia in some subjects (27). The results of the present study indicate that vortioxetine may also be an effective treatment of anhedonia in MDD. Herein, we observed a significant benefit of vortioxetine on anhedonia, as measured by the SHAPS and MADRS anhedonia factor. We observed that the improvement in anhedonia with vortioxetine treatment was significantly correlated with improvement in function and quality of life. Moreover, we observed a significant mediational effect of improvement in anhedonia on social functioning, which was independent of the effect of vortioxetine treatment on total depression symptom severity.

We also identified four variables that may affect remission of anhedonia in patients treated with vortioxetine (i.e., sex, marijuana use, age of MDD onset, and family history of mental illness). We found that female subjects with MDD treated with vortioxetine were more likely to achieve remission status (i.e., SHAPS total score <3). In other words, the anti-anhedonia effects with vortioxetine may be more pronounced in women with MDD. Although sex differences have been reported in MDD with respect to phenomenology, comorbidity, illness trajectory, and response to treatment, it is not known whether sex differences exist with respect to the likelihood of exhibiting an anti-anhedonia effect with antidepressant treatment. It is also noteworthy that subjects in our study who reported regular current use of marijuana were less likely to exhibit an anti-anhedonia effect with treatment. The foregoing observation requires replication and comports with separate lines of evidence indicating that recreational marijuana utilization exerts both anhedonia-promoting and anti-cognitive effects in users (28, 29). Furthermore, we further observed that individuals with MDD reporting with a family history positive for psychiatric illness were less likely to exhibit an improvement in anhedonia. It could be conjectured that the increased loading of psychopathology in families represents a more complex, less treatment responsive phenotype and/or ongoing environmental stressors as a general non-response predictor (30).

Improvements in overall function and quality of life have been prioritized as a primary therapeutic objective with respect to treatment of patients with MDD (31). Relatively few antidepressants have been evaluated with respect to their primary effect on psychosocial function, workplace performance, and return work (32). Several lines of research indicate that

TABLE 4 | The correlations of the endpoint changes between functional impairment, well-being and anhedonia from baseline.

Correlations	SDS total score		SDS work		SDS social life		SDS family life		WHO-5	
	r	*p*	*r*	*p*	*r*	*p*	*r*	*p*	*r*	*p*
MADRS total score	0.527	<0.001	0.422	<0.001	0.46	<0.001	0.486	<0.001	−0.604	<0.001
SHAPS score	0.392	<0.001	0.309	0.006	0.403	<0.001	0.364	0.001	−0.336	0.002
MADRS anhedonia factor score	0.511	<0.001	0.423	<0.001	0.41	<0.001	0.507	<0.001	−0.570	<0.001

MADRS, Montgomery Åsberg Depression Rating Scale; SHAPS, Snaith-Hamilton Pleasure Scale; SDS, Sheehan Disability Scale; WHO-5, The World Health Organization- Five Well-Being Index.

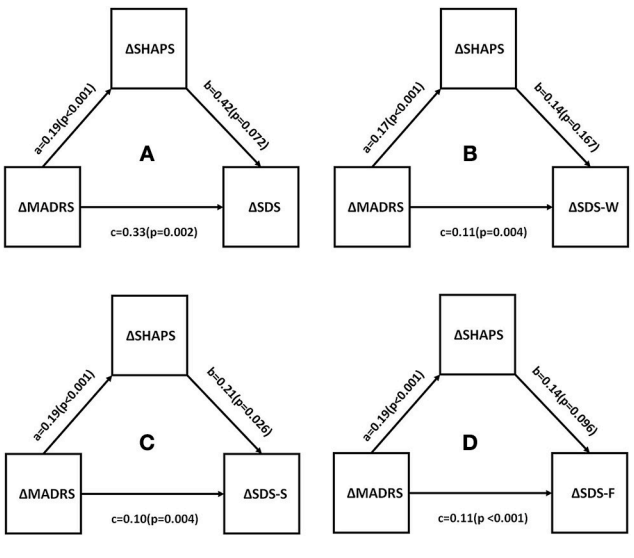

FIGURE 2 | Mediation analysis to estimate indirect effects of anhedonia improvement (ΔSHAPS) in the improvement of depressive symptoms (ΔMADRS) and function (ΔSDS). a, b, and c are path coefficients. **(A)** SDS total score, **(B)** SDS- work/school; **(C)** SDS -social life; **(D)** SDS-family life or home responsibilities. MADRS, Montgomery Åsberg Depression Rating Scale; SHAPS, Snaith-Hamilton Pleasure Scale; SDS, Sheehan Disability Scale.

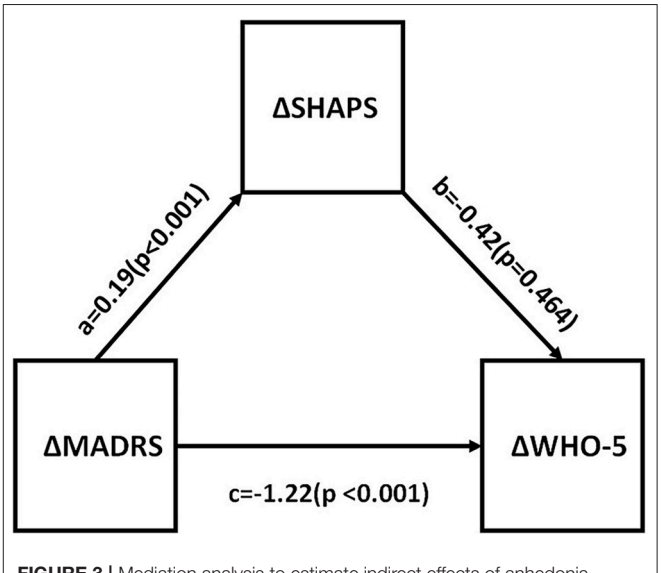

FIGURE 3 | Mediation analysis to estimate indirect effects of anhedonia improvement (ΔSHAPS) in the improvement of depressive symptoms (ΔMADRS) and well-being (ΔWHO-5). a, b, and c are path coefficients. MADRS, Montgomery Åsberg Depression Rating Scale; SHAPS, Snaith-Hamilton Pleasure Scale; WHO-5, The World Health Organization- Five Well-Being Index.

vortioxetine is capable of improving psychosocial function, as assessed by self-report and/or performance based measures (33–35). The results from the present study replicate previous studies showing that vortioxetine improves both psychosocial function and quality of life. We extend further existing knowledge by observing that improvements in function and quality of life were mediated by improvement in measures of anhedonia. Final, the foregoing observations with vortioxetine comports with previous research findings demonstrating that improvement in anhedonia mediates the association between improvements in depressive symptoms and improvements psychosocial functioning (19).

There are methodological limitations that affect inferences and interpretations of our data analyses. First, our analyses were done *post-hoc*. Second, evaluating the effect of vortioxetine on anhedonia (and the mediational effect of anhedonia on function and quality of life) was not the primary aim of the study. Third, our study was open-label and not placebo-controlled, increasing the likelihood of expectancy affecting our outcomes of interest. In addition, we did not exclude the patients received medications could effect anhedonia at screening or measure the anhedonia status during the tapering off period. Last, our analysis did not include a more rigorous performance-based measure of reward/motivation (e.g., EEFRT) (36). Notwithstanding, our analyses were meant to be more hypothesis-generating and to provide pilot data supporting a larger rigorous study evaluating determinants of improved functional outcomes with vortioxetine.

In summary, vortioxetine improved measures of anhedonia, which significantly correlated with improvements in function. Moreover, the effect of improvement in anhedonia on patient-reported outcomes (i.e., social functioning) was independent of the overall improvement in depressive symptoms. Our results, if replicated, would indicate that measures of reward/motivation, along with cognitive disturbance are

critical determinants of health outcomes in patients with MDD.

ETHICS STATEMENT

The study has been reviewed and approved by an independent Institutional Review Board that is accredited by the Association for the Accreditation of Human Research Protection.

AUTHOR CONTRIBUTIONS

RSM was responsible for designing the study. BC was responsible for analyzing data and writing the original manuscript. CP, MS, HZ, MI, and RBM collected the research data. RSM, CP, MS, MI, YL, and LP revised the manuscript. All authors have read and approved the final version of this article.

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Sleep in Offspring of Parents With Mood Disorders

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Background: Sleep problems in childhood are an early predictor of mood disorders among individuals at high familial risk. However, the majority of the research has focused on sleep disturbances in already diagnosed individuals and has largely neglected investigating potential differences between weeknight and weekend sleep in high-risk offspring. This study examined sleep parameters in offspring of parents with major depressive disorder or bipolar disorder during both weeknights and weekends.

Methods: We used actigraphy, sleep diaries, and questionnaires to measure several sleep characteristics in 73 offspring aged 4–19 years: 23 offspring of a parent with major depressive disorder, 22 offspring of a parent with bipolar disorder, and 28 control offspring.

Results: Offspring of parents with major depressive disorder slept, on average, 26 min more than control offspring on weeknights (95% confidence interval, 3 to 49 min, p = 0.027). Offspring of parents with bipolar disorder slept, on average, 27 min more on weekends than on weeknights compared to controls, resulting in a significant family history × weekend interaction (95% confidence interval, 7 to 47 min, p = 0.008).

Conclusions: Sleep patterns in children and adolescents were related to the psychiatric diagnosis of their parent(s). Future follow-up of these results may clarify the relations between early sleep differences and the risk of developing mood disorders in individuals at high familial risk.

Keywords: sleep, severe mental illness, major depressive disorder, bipolar disorder, actigraphy, high-risk offspring, cohort study

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INTRODUCTION

Sleep disturbances are core symptoms of mood disorders including major depressive disorder and bipolar disorder (1). Additionally, sleep problems have been associated with more severe symptoms, greater functional impairment, and increased risk for relapse among individuals with mood disorders (2). Over 40% of children and youth experience sleep disturbances during development (3). Early sleep disturbances have been shown to predict depressive symptoms (4, 5), manic episodes (6, 7), and psychotic-like experiences (8). However, there have been some inconsistencies in the reported relationship between sleep and psychopathology (9). The persistence of sleep problems may represent a key factor in the true relationship between sleep disturbance in childhood and later mental illness. Many sleep problems resolve during development, but those that persist predict symptoms of depression, anxiety, and externalizing behaviors into adulthood (10).

These findings highlight the importance of treating sleep disturbances as a contributing factor to, and not just a symptom of, mood disorders, and emphasize the need for longitudinal investigations of these relationships.

Many previous studies examining the relationship between sleep disturbances and mood disorders focused on individuals who had already been diagnosed (11). There has been less research examining sleep disturbances among children and youth at high familial risk of mood disorders. This gap in the literature is surprising given that offspring of parents with a mood disorder have a 1 in 3 chance of developing a mental illness themselves (12).

Mood disorders typically have an onset in the second or third decade of life; however, they are often preceded by earlier manifestations, which have been referred to as antecedents (13), defined as conditions that precede the onset of mood disorders by at least several years (14). Longitudinal high-risk studies suggest that sleep disturbances likely represent an antecedent to mood disorders (14–16). However, there is limited information on early sleep disturbances among offspring of parents with mood disorders. Offspring of mothers with postpartum depression are more likely than control offspring to have sleep problems (17) and to develop depression themselves (18). Consistent with these findings, there is evidence that sleep disturbances are prevalent among offspring of a parent with bipolar disorder (7, 19, 20).

Previous research on sleep in high-risk offspring has relied largely on self-reported measures of sleep (7, 14) despite the known benefits of combining self-report and objective measures (21). Actigraphy is an objective, indirect measure of sleep that uses accelerometer-based devices to estimate sleep parameters (22) and provides an ecologically valid representation of typical sleep patterns in a participant's natural environment (23). Studies that have used objective sleep measures have typically not reported sleep data from weeknight and weekends separately, despite recommendations to do so (24, 25). Discrepancies in sleep patterns between weeknights and weekends are prevalent during adolescence. These discrepancies are linked to emotional disturbances and have been shown to be independently associated with self-harm among adolescents (26). Additionally, sleep duration on free days (e.g., weekends or vacation) has been shown to be more heritable than sleep duration on sleep-constrained days (e.g., weeknights preceding school days) (27), and variability in weeknight and weekend sleep is a common sleep pattern observed in individuals with bipolar disorder (14). This reinforces the importance of evaluating these sleep parameters on weeknights and weekends separately among offspring at high familial risk of mood disorders.

The current study used actigraphy, sleep diaries, and self-report sleep questionnaires to examine sleep among offspring of parents with mood disorders during both weeknights and weekends. Sleep period length, total sleep time (TST), sleep onset latency (SOL), and wake after sleep onset (WASO) were compared between offspring at high familial risk of mood disorders and offspring of control parents. We hypothesized that children and youth at familial risk of developing a mood disorder would have more disturbed sleep than offspring of controls, as indicated by longer SOLs, increased WASO, and larger weeknight–weekend discrepancies in TST.

MATERIALS AND METHODS

Sample Description

We measured sleep in children and youth aged 4–19 years who participate in the Families Overcoming Risks and Building Opportunities for Well-being (FORBOW) cohort, which includes offspring of parents with severe mental illness and compares them to controls with no known familial risk (28).

Offspring of parents with mood disorders were recruited from February 2015 to June 2018 through their parents' contact with mental health services in Nova Scotia, Canada. Offspring of control parents matched for age and socioeconomic status were recruited through local school boards. In this study, we included offspring with a parent diagnosed with major depressive disorder or bipolar disorder, as well as offspring of control parents. Participants and at least one parent were required to have the capacity to read, write, and speak English. We excluded youth with severe intellectual disability or autism spectrum disorders. No exclusions were made based on sex, ethnicity, living arrangements, medication, or other psychopathology.

Parent Assessment

Diagnoses of mental disorders according to the *Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (DSM-IV)* and *DSM-5* were established using the *Schedule for Affective Disorders and Schizophrenia* (SADS for DSM-IV) and the *Structured Clinical Interview for DSM-5 Disorders* (SCID-5) (29). Diagnoses were confirmed in consensus meetings with a psychiatrist blind to offspring psychopathology. Offspring were placed in family history groups based on their biological parent's diagnosis. In cases where offspring had two parents with different diagnoses, they were placed in the family history group based on the DSM-5 diagnostic hierarchy. Thus, offspring with *at least* one parent with bipolar disorder were placed in the bipolar disorder family history group, because a bipolar disorder diagnosis is considered definite. This group included a subset of offspring who also had a parent with a diagnosis of major depressive disorder, which are referred to here as dual-risk offspring (see the section Results).

Actigraphy and Sleep Diary

Participants wore Micro Motionlogger actigraphs (Ambulatory Monitoring Inc., Ardsley NY) on their nondominant wrist every day for a 2-week period, beginning on a Sunday. They were instructed to remove the actigraph during any water-related activities and while participating in sports. Communication check-ins were made approximately once per week to ensure that there were no difficulties with the protocol or with the actigraphs. Each night was analyzed independently. Additionally, weekdays and weeknights were analyzed separately. Weeknights were defined as Sunday

through Thursday nights, and weekends were defined as Friday and Saturday nights.

Data were sampled in 60-s epochs, collected in Zero-Crossing Mode, and analyzed with Action W2.7 using the Cole–Kripke scoring algorithm (30). The Cole–Kripke algorithm has been validated in adult and child populations and has been found to be more accurate in estimating TST than the alternative Sadeh algorithm (24, 31). From the actigraphy data, we extracted information about the sleep period, TST, SOL, and WASO separately to determine the impact of familial history on each characteristic of sleep. Sleep period was defined as the duration between sleep onset (time fell asleep) and sleep end (morning wake time). TST was the number of actual sleep minutes during the sleep period (i.e., excluding intervening wake episodes).

In accordance with best practice parameters for actigraphy (32, 33), sleep diaries were used to clarify any ambiguous actigraphy data and provided a timeline for when and how long the participants wore the actigraphs. Participants filled out a sleep diary every day during the 2-week period as recommended by Buysse et al. (21). Participants older than 15 completed a self-report sleep diary, and parents completed a parent report sleep diary for participants younger than 15. Information collected in the sleep diaries included nightly bedtimes, time required to fall asleep, morning wake times, and daytime naps.

Self-Reported Measures of Sleep

Children's Sleep Habits Questionnaire

This 35-item parent report measure assesses sleep problems in children aged 4–14 years (34). This questionnaire contains eight subscale scores including bedtime resistance, sleep onset delay, sleep duration, sleep anxiety, night waking, parasomnias, sleep disordered breathing, and daytime sleepiness. The Children's Sleep Habits Questionnaire (CSHQ) has been validated in clinical and community populations and has been shown to have good internal consistency and test–retest reliability. Scoring of some items was reversed in order to make a higher score consistently indicative of more disturbed sleep (34).

School Sleep Habits Survey

This self-report measures sleep/wake habits, sleep patterns during school nights compared to weekends, and daytime functioning of adolescents 15–17 years old (35). The School Sleep Habits Survey (SSHS) consists of 63 questions and has been used in other studies looking at high-risk offspring (7). The SSHS contains three subscales: 1) a depressed mood scale, 2) a sleepiness scale, and 3) a sleep/wake problem behavior scale. The depressed mood scale (36) comprises six items assessing participants' negative emotions in the 2 weeks prior to completing the questionnaire. This scale has high internal reliability and high test–retest reliability for adolescents (35). The sleepiness scale queries the frequency that participants struggled to stay awake in 10 different situations during the 2 weeks prior to completing the questionnaire. The sleep/wake problem scale comprises 10 items examining the frequency of erratic sleep/wake behaviors in the 2 weeks prior to completing the questionnaire (35).

Puberty Measurements

During adolescence, there are noticeable changes in sleep–wake behaviors due to a variety of psychosocial and biological factors (37, 38), which are strongly influenced by the stages of puberty (39). To accurately compare sleep parameters between family history groups, we controlled for puberty onset in our analyses. Pubertal onset was determined by the "Growing and Changing Questionnaire" (GCQ) administered annually in the FORBOW cohort at age 8 and older. The GCQ was based on two measures of pubertal status: Pearson's Puberty Development Scale (PDS) (40) and the Sexual Maturation Scale (SMS) (41, 42). The PDS components of the GCQ asked about body hair, skin changes, growth of facial hair for males, and onset/age of menstruation for females. The SMS section comprises five drawings displaying progressive stages of pubertal development of secondary sexual characteristics ranging from pre-pubertal to post-pubertal. Both the PDS and SMS sections were scored on a 1–5 scale according to Carskadon and Acebo (40) and then averaged to create a composite puberty score. A dichotomous puberty onset score was created to indicate whether each participant was pre- or post-pubertal at the time of sleep assessment. Composite puberty scores ≤2 were scored as pre-pubertal and scores ≥3 were scored as post-pubertal.

Statistical Analysis

The actigraphy measures of sleep parameters (sleep period, TST, SOL, and WASO) were calculated using Action W2.7 software. Sleep parameters were analyzed separately for weeknights and weekend nights as recommended by Galland et al. (25).

All statistical procedures were conducted using STATA software package, version 15.1 (43). We fitted mixed-effects generalized linear models to test the effect of family history (no diagnosis, major depressive disorder, or bipolar disorder) on sleep period, TST, SOL, and WASO. We included age, sex, pubertal onset, and current diagnosis of Attention Deficit Hyperactivity Disorder (ADHD) as fixed covariates in each model. We accounted for the non-independence of observations from related individuals and from repeated measures from the same individual by including family and individual identifiers as random effects in the models. We conducted sensitivity analyses controlling for stimulant use, cannabis use, and a current diagnosis of anxiety. We also conducted sensitivity analyses removing offspring with current diagnoses of bipolar disorder (n = 1) or major depressive disorder (n = 2). We assessed the subscales for the CSHQ and the SSHS for internal consistency using Cronbach's α-coefficients. We used mixed-effects generalized linear models with the same fixed covariates and random effects described above for comparisons between family history groups.

RESULTS

Sample Description

A total of 86 participants completed the study. Actigraphy data from 13 participants were not included in the analysis due to loss of the actigraph before retrieving the data (n = 3), long periods of time without wearing the actigraph (n = 2), or corrupted

TABLE 1 | Demographic and clinical information for the sample.

	Control offspring (*n* = 28)	Offspring of MDD (*n* = 23)	Offspring of BD (*n* = 22)	Total (*n* = 73)
Age, mean (SD)	11.01 (2.97)	11.44 (3.44)	12.36 (3.32) *11.99 (3.86)*[1]	11.55 (3.23)
Female, *n* (%)	8 (29%)	11 (48%)	8 (36%) *4 (44%)*[1]	27 (37%)

[1] *Demographic and clinical information for dual-risk offspring of one parent with bipolar disorder and one parent with major depressive disorder (n = 9).*

actigraphy data (n = 8). Of the remaining 73 participants (actigraphy completion rate 86.2%), 23 were offspring of a parent with major depressive disorder (9 of whom had both parents with major depressive disorder), 22 were offspring of a parent with bipolar disorder (9 of whom had one parent with bipolar disorder and one parent with major depressive disorder), and 28 were offspring of controls (**Table 1**). There were 45 pre-pubertal and 28 post-pubertal participants. There were 20 offspring with a current diagnosis: 9 with ADHD, 8 with anxiety, 2 with major depressive disorder, and 1 with bipolar disorder.

Questionnaire Scores

Among the participants who completed all questionnaires (total completion rate, 75.3%), offspring of a parent with bipolar disorder scored significantly higher on the sleep/wake problems scale of the SSHS compared to controls (β = 8.79, 95% CI: 0.83, 16.75; p = 0.030). There were no significant differences among offspring from the three family history groups for the depressed mood subscale or the sleepiness subscale calculated from the SSHS (see **Table 2**). There were no group differences for the eight subscales of the CSHQ or the total CSHQ score (see **Table 2**).

Sleep Diary Results

As recommended by Meltzer et al (24), we calculated the average self- or parent-reported bedtime and waketime for each family history group (see **Table 3**). We also calculated the average sleep period as the time between bedtime and wake time. There were no significant differences between family history groups in bedtimes, waketimes, or sleep periods on either weeknights or weekends.

Weeknight Actigraphy Results

In contrast to the sleep diary results, we found that the offspring of a parent with major depressive disorder had a significantly longer sleep period (β = 25 min, 95% CI: 4.54, 46.43; p = 0.017) and TST than control offspring on weeknights (β = 26 min, 95% CI: 3.01, 49.46; p = 0.027; see **Table 3**). We looked separately at the results from offspring with only one or with two parents with major depressive disorder. Those with two parents with a diagnosis of major depressive disorder (n = 9) showed a pattern similar to that of the entire group with a longer sleep period than controls that did not reach threshold for statistical significance (β = 23 min, 95% CI: −5.08, 51.53; p = 0.108) and TST (β = 26 min, 95% CI: −5.15, 56.54; p = 0.103). Offspring of only one parent with major depressive disorder (n = 14) showed a significantly longer sleep period (β = 26.95 min, 95% CI: 2.52, 51.36; p = 0.031) and a similar, but not statistically significant, increase in TST (β = 26.61 min, 95% CI: −0.72, 53.94; p = 0.056).

Offspring of a parent with bipolar disorder did not differ significantly from control offspring in their sleep period (β = −3 min, 95% CI: −23.99, 17.95; p = 0.777) or TST (β = −0.79 min, 95% CI: −23.86, 22.28; p = 0.946) on weeknights. Average differences between TST calculated from actigraphy and "predicted" TST on weeknights for each family history group are shown in **Figure 1**. Predicted TST was calculated using a linear prediction from a model based on age, sex, and pubertal status for controls during weeknights.

TABLE 2 | Mean and standard deviations of parent-reported Children's Sleep Habits Questionnaire (CSHQ) total score and subscales by family history group for offspring aged 4–14. Mean and standard deviations of self-reported School Sleep Habits Survey (SSHS) subscales by family history group for offspring aged 15–17.

	Offspring of controls	Offspring of MDD	Offspring of BD
Children's Sleep Habits Questionnaire[1]			
Participants (*n*)	17	14	9
	Mean (SD)	Mean (SD)	Mean (SD)
Subscale Item[2]			
Bedtime resistance	8.50 (2.89)	6.64 (0.84)	7.89 (2.15)
Sleep onset delay	1.56 (0.71)	1.36 (0.63)	1.78 (0.67)
Sleep duration	4.47 (1.74)	3.29 (0.47)	4.00 (1.58)
Sleep anxiety	5.50 (2.20)	4.79 (1.48)	6.00 (2.24)
Night wakings	3.50 (0.92)	3.21 (0.58)	3.56 (0.76)
Parasomnias	7.28 (1.23)	6.36 (0.63)	7.89 (0.92)
Sleep disordered breathing	1.12 (0.33)	1.43 (0.76)	1.11 (0.33)
Daytime sleepiness	8.50 (2.92)	9.93 (2.56)	9.78 (3.93)
Total score	40.44 (9.79)	37.00 (2.63)	42.00 (7.38)
School Sleep Habits Survey[3]			
Participants (*n*)	4	3	5[4]
	Mean (SD)	Mean (SD)	Mean (SD)
Subscale item			
Depressed mood scale	6.90 (1.80)	7.00 (0.00)	11.00 (3.74)
Sleepiness scale	11.00 (2.00)	12.00 (3.46)	12.80 (2.68)
Sleep/wake problems behavior scale	19.29 (3.15)	23.57 (5.96)	**28.96***(6.77)

[1] *Parents of 48 offspring between ages 4 and 14 completed the CSHQ (completion rate, 70.6%).*
[2] *The names of the items in this table are those used in the CSHQ.*
[3] *Twelve offspring between ages 15 and 17 completed the SSHS (completion rate, 92.3%).*
[4] *Two offspring in the bipolar family history group were dual-risk offspring who also had a parent with major depressive disorder.*
*$p < 0.05$.

Weekend Actigraphy Results

Offspring of a parent with bipolar disorder showed longer, but not statistically significantly different, sleep periods than controls (β = 23 min, 95% CI: −1.10, 49.04; p = 0.061) and TST (β = 20 min, 95% CI: −8.59, 48.79; p = 0.170; see **Table 3**). The "dual-risk" subgroup of this group (offspring of one parent with bipolar disorder and one with major depressive disorder) showed a significantly longer sleep period compared to controls

TABLE 3 | Sleep parameters derived from 14 days of actigraphy data by family history group.

	Control offspring (*n* = 28)		Offspring of MDD (*n* = 23)		Offspring of BD only (*n* = 13)		Offspring of BD and MDD (*n* = 9)		All Offspring of BD (*n* = 22)	
	Mean	SD (min)	Mean	SD (min)	Mean	SD (min)	Mean	SD (min)	Mean	SD (min)
Weekdays										
Sleep onset (HH : MM)	22:06	117.61	22:05	108.63	22:17	118.08	22:56	155.55	22:33	82.95
Sleep end (HH : MM)	7:21	60.03	7:33	78.45	07:22	94.02	8:05	131.04	7:40	69.69
Variables of Interest										
Sleep period (min)	555.36	64.81	**572.08***	68.00	559.73	66.14	534.41	84.50	538.14	74.96
TST (min)	488.87	64.26	**510.53***	61.34	496.03	61.51	473.17	91.61	477.83	72.01
SOL (min)	11.17	12.25	8.35	6.88	9.72	11.53	10.00	11.10	8.8	14.39
WASO (min)	46.88	34.89	42.74	27.04	44.85	31.13	37.02	25.49	40.27	29.55
Weekends										
Sleep onset (HH : MM)	22:34	138.68	22:39	116.15	22:44	120.35	23:01	141.21	22:54	83.95
Sleep end (HH : MM)	7:50	123.26	7:59	97.23	8:09	158.54	8:54	129.43	8:28	68.52
Variables of Interest										
Sleep period (min)	557.05	80.88	561.25	90.59	557.85	80.61	**594.03***	66.19	**577.89***	67.19
TST (min)	484.18	83.58	492.73	93.63	484.65	83.31	519.43	51.56	502.46	64.95
SOL (min)	9.86	10.7	7.67	6.77	9.67	11.04	10.57	8.45	11.67	15.24
WASO (min)	49.42	35.58	44.88	25.77	47.86	31.59	45.20	29.30	47.24	33.13

*$p < 0.05$.

on weekend nights (β = 35 min, 95% CI: 2.55, 68.71; $p = 0.035$). Offspring of a parent with major depressive disorder did not differ significantly from controls in their sleep period (β = 5 min, 95% CI: −18.94, 28.95; $p = 0.682$) or TST (β = 8 min, 95% CI: −19.93, 35.74; $p = 0.578$) on weekends.

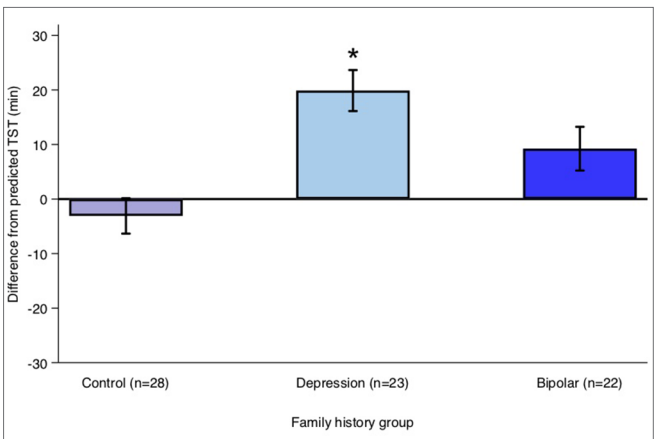

FIGURE 1 | Average difference between actual total sleep time (TST) and predicted TST on weeknights for each family history group. Predicted TST was calculated using a linear prediction from a model predicting TST using age, sex, and pubertal onset for controls during weeknights. Error bars represent the standard error of the mean. *Note:* *$p < 0.05$.

Family History by Weekend Interaction Actigraphy Results

Offspring of a parent with bipolar disorder showed a significant family history by weekend interaction for TST (β = 27 min, 95% CI: 7.16, 47.49; $p = 0.008$) compared to controls (see **Figure 2**). When this family history group was separated into its two subgroups, dual-risk offspring ($n = 9$; offspring of one parent with bipolar disorder and one with major depressive disorder) showed a family history by weekend interaction for TST (β = 49 min, 95% CI: 22.33, 75.21; $p < 0.001$), but offspring with one parent with bipolar disorder and a healthy co-parent ($n = 13$) did not (β = 11 min, 95% CI: −12.12, 35.31; $p = 0.338$).

The family history by weekend interaction was also significant for sleep period in offspring of a parent with bipolar disorder (β = 36 min, 95% CI: 15.76, 57.07; $p = 0.001$). Dual-risk offspring showed a family history by weekend interaction for sleep period (β = 56 min, 95% CI: 28.73, 82.69; $p < 0.001$) compared to controls, but offspring with one parent with bipolar disorder and a healthy co-parent showed only a weaker tendency toward the same effect (β = 22 min, 95% CI: −1.96, 46.49; $p = 0.072$). Offspring of a parent with major depressive disorder did not show a family history by weekend interaction for either TST (β = −13 min, 95% CI: −32.80, 6.45; $p = 0.188$) or sleep period (β = −11 min, 95% CI: −31.83, 8.38; $p = 0.253$) compared to control offspring.

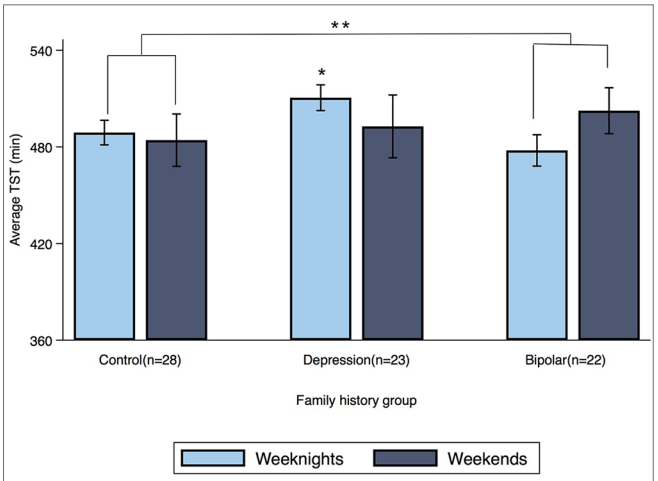

FIGURE 2 | Average TST for weeknights and weekends by family history group. Error bars represent the standard error of the mean. *Note:* $^{*}p < 0.05$, $^{**}p < 0.01$.

Sensitivity Analyses

The significant effects remained unchanged in sensitivity analyses that excluded participants who used stimulant medication or cannabis as determined by self-report measures in the FORBOW study. The significant effects also remained when removing offspring with a current diagnosis of bipolar disorder, major depressive disorder, or anxiety. When offspring with anxiety were removed from the sample, offspring of a parent with bipolar disorder had a significantly longer sleep period on weekends (β = 37 min, 95% CI: 11.71, 62.70; p = 0.004). There were no other significant differences in actigraphy results between the groups.

DISCUSSION

Sleep disturbances, including hypersomnia, insomnia, and irregular sleep patterns, are associated with increased risk of mood disorders (7, 44, 45), as are sleep features such as higher Rapid Eye Movement sleep density in high-risk individuals (46). Using actigraphy, we found differences in sleep patterns and durations between offspring of parents with mood disorders and control offspring. We found longer sleep periods and TST on weeknights among offspring of a parent with major depressive disorder and weeknight–weekend differences from controls in sleep duration among offspring of a parent with bipolar disorder and a parent with major depressive disorder. It is noteworthy that these effects seen during 2 weeks of actigraphic recording were not matched by any significant group differences in sleep diaries, nor according to the CSHQ. However, we did find significantly more sleep/wake problems in offspring of a parent with bipolar disorder compared to controls as reported on the SSHS.

The increased sleep duration on weekends compared to weeknights among offspring of parents with bipolar disorder is consistent with earlier results based on interviews, which found that at-risk offspring had high rates of sleep disorders (14). Among those at risk for bipolar disorder, a small pilot study suggested that an intervention aimed at stabilizing sleep–wake patterns over the week may be beneficial in reducing sleep disturbances (47).

When we analyzed data separately for the dual-risk offspring (one parent with bipolar disorder and one with major depressive disorder) and the offspring of only one parent with bipolar disorder, we found that the dual-risk offspring were driving these weeknight–weekend differences in TST. It is unclear whether this pattern is specific to this diagnostic configuration or reflects additional familial loading of risk resulting from having two parents with any mood disorder diagnosis. However, our results comparing offspring with one or two parents with major depressive disorder do not support a generalized risk of having both parents with any mood disorder diagnosis. Rather, they highlight a potential specific risk associated with having one parent with bipolar disorder and one parent with major depressive disorder. In this context, the fact that major depressive disorder is sometimes re-diagnosed as bipolar disorder allows for the possibility that some of these families will include two parents with bipolar disorder. Future follow-ups may permit assessment of this possibility. This result suggests that while altered weeknight–weekend sleep patterns may be an early indicator of increased risk for development of bipolar disorder, those who have two parents with this specific diagnostic configuration may be at greater risk.

Offspring of parents with major depression had longer sleep periods and TST during weeknights than controls. Most treatments for sleep disturbances associated with major depressive disorder to date have focused on treating insomnia (2). Our results suggest that hypersomnia may be an early indicator of increased risk for depression in some children and could be a relevant target for future sleep-based interventions for individuals at high familial risk.

Strengths and Limitations

Our study benefited from the inclusion of offspring at high familial risk for multiple forms of mood disorders across a broad age range and the use of an objective measure of sleep quality in a relatively large sample of high-risk youth. Actigraphic recording for 2 weeks allowed us to assess weeknight–weekend differences, which proved to be informative. Questionnaire data, which are known to detect sleep abnormalities in clinical populations, did not reproduce the results found with actigraphy in this high-risk population, with the exception of identifying increased sleep problems in offspring of a parent with bipolar disorder. These findings suggest caution in interpreting results of questionnaires and short-term actigraphy data in at-risk children.

Several factors in this study limit the strength of our conclusions. Because of the number of participants in each group, we could not compare results among groups at different ages across the study sample. We did, however, control for both age and pubertal status in our statistical analyses. Because of power limits, we also reported

our results as nominal significance levels. It should be noted that there were only nine participants in the dual-risk group; thus, the results for this subgroup should be considered preliminary. Overall, our results suggest that familial risk has an effect on offspring's sleep patterns; however, it is not possible to distinguish whether parentally contributed genes, the impact of parental illness on the family/offspring, or an interaction of the two contributes to these differences, especially in dual-risk offspring.

The FORBOW study is designed as a cohort with ongoing recruitment of children and adolescents. We anticipate adding additional participants to each of the groups, with a special focus on increasing the number of dual-risk offspring. The cohort will be followed for at least a 5-year period, which will allow for determination of the clinical outcomes for these children and adolescents at high risk and for the evaluation of the predictive power of early sleep–wake characteristics in the development of mood disorders. The results should also facilitate development of early sleep interventions tailored to the characteristics of different at-risk groups and the evaluation of their effects on risk of illness.

ETHICS STATEMENT

The protocol was approved by the Research Ethics Board of the Nova Scotia Health Authority, in conformity with the Canadian Tri-Council Policy Statement 2: Ethical Conduct of Research involving Humans (2014). We obtained informed consent from participants who had the capacity to provide it. For participants who did not have the capacity to make an informed decision, a parent or guardian provided written informed consent and the participant provided assent.

AUTHOR CONTRIBUTIONS

All authors listed have contributed sufficiently to the project to be included as authors, and all those who are qualified to be authors are listed in the author byline. DW contributed substantially to the conception and design, acquisition of data, and analysis and interpretation of data. DW also drafted the article and gave final approval of the version to be published. JM contributed to the conception and design and to the acquisition of data, revised the manuscript critically for important intellectual content, and gave final approval of the version to be published. AZ contributed to the analysis and interpretation of data, revised the manuscript critically for important intellectual content, and gave final approval of the version to be published. JC contributed to the acquisition of data and gave final approval of the version to be published. RU contributed substantially to the conception and design, acquisition of data, and analysis and interpretation of data; revised the manuscript critically for important intellectual content; and gave final approval of the version to be published. BR contributed substantially to the conception and design and to the analysis and interpretation of data, revised the manuscript critically for important intellectual content, and gave final approval of the version to be published.

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Regulation of Tau Protein on the Antidepressant Effects of Ketamine in the Chronic Unpredictable Mild Stress Model

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Tau protein is known to play an important role in maintaining microtubule assembly and stabilization, and maintaining the normal morphology of neurons, but several studies have found that chronic stress leads to Tau hyperphosphorylation. A large number of clinical trials have found that ketamine, which is an N-methyl-D-aspartate receptor antagonist, produces a rapid, long-lasting, and potent antidepressant effect in patients suffering from major depression. This rapid antidepressant effect of ketamine, which involves many mechanisms, has attracted wide attention. However, the relationship between ketamine's antidepressant effects and Tau protein has rarely been examined. We used C57BL/6 and Tau KO mice exposed to 42 days of chronic unpredictable mild stress (the CUMS model) to investigate the effect of ketamine on behavioral changes and synaptic functioning of the hippocampus. The results showed that a single treatment of ketamine rapidly relieved the CUMS-induced anhedonia, depression-like, and anxious behaviors of the C57BL/6 mice. The abnormal behaviors were accompanied by increased levels of specific alterations of hyperphosphorylated Tau protein in cytoplasm and synapse in the hippocampus of the C57BL/6 mice, but ketamine reduced the aggregation of hyperphosphorylated Tau protein only in the synapse. We also found that CUMS exposure reduced the levels of GluA1 and PSD95 in the hippocampus of the C57BL/6 mice and that these deficits were reversed by ketamine. However, the Tau KO mice did not develop any stress-induced depressive behaviors or deficits of hippocampal function. The antidepressant effect of ketamine may decrease the levels of hyperphosphorylated Tau protein in synapse of C57BL/6 mice.

Keywords: depression, hippocampus, Tau protein phosphorylation, synapse, ketamine

INTRODUCTION

Depressive disorders were reported to be the leading cause of the global disease burden in 2014, with prevalence rates throughout the world ranging from 3% to 17% (1). Studies indicate that early-life depression is associated with a 2–4-fold increased risk of developing Alzheimer's disease (AD) (2, 3), the main feature of which is the aggregation of neurofibrillary tangles (NFTs) after Tau hyperphosphorylation,

which is one of the main pathological characteristics of AD (4). The Tau protein, one of the microtubule-associated proteins (MAPs), plays an important role in maintaining neuron morphology and promoting axonal development in the cytoskeleton system. Tau proteins are the transport channels of axons and dendrites (5), and phosphorylated Tau regulates this function during normal neuron maturation. However, Tau can evoke neurotoxicity when it is abnormally hyperphosphorylated (6). Hyperphosphorylation of Tau protein affects intracellular material transportation and eventually leads to hippocampus neuron atrophy, which is also a key factor in the pathogenesis of AD (7).

Recent studies on C57BL/6 mice have shown that chronic unpredictable mild stress (CUMS) can induce hyperphosphorylation of Tau protein at the pSer202, pThr231, pSer262, and pSer396/404 sites of hippocampal neurons (8). Yang et al. (9), who produced hyperphosphorylation of Tau at sites Ser356 and Thr231 in the hippocampus and prefrontal cortex of C57BL/6 mice by CUMS, found that fluoxetine reduced the levels of hyperphosphorylated Tau protein of whole protein after 3 weeks of treatment. Although fluoxetine is widely used, it often takes weeks to reach efficacy. Ketamine, an ionotropic glutamatergic NMDA receptor antagonist, has been used by millions of people as an anesthetic over the past 40 years (10). In recent years, clinical studies have found that ketamine produced an antidepressant effect within 2–4 h that lasts from several days to 2 weeks. It has also been found to reduce the suffering of patients with major depressive disorder (11–13), but its mechanism of action is not clear. Therefore, the rapid and effective antidepressant effects of ketamine have become a "hot spot" in the field.

Studies have found that the expression of the synaptic proteins GluR1 and PSD95 on the hippocampal neuron membrane decreased after rats showed depression-like behavior (14). GluR1, a major subunit of AMPA, plays a vital role in maintaining synaptic plasticity and the processing of long-term depression (LTD) and long-term potentiation (LTP) (15). AMPA receptors are transported to the postsynaptic membrane and then anchored on the PSD95 scaffold protein of the postsynaptic membrane to perform the function of synaptic transmission (16). PSD95 is the most important and abundant scaffold protein on the postsynaptic membrane, which mainly exists in mature excitatory glutamate synapses (17). PSD95 is involved in the formation and reconstruction of synapses, and decreases in its expression indicate the loss of synapses (18). The antidepressant effect of ketamine is related to the rapid synthesis of brain-derived neurotrophic factors (BDNFs) (19), m-TOR (rapamycin target protein) (20), and eukaryotic elongation factor 2 (eEF2) (21). Therefore, it is crucial to explore the role of Tau protein in the pathogenesis of depression and whether Tau protein is involved in the ketamine antidepressant process.

Abbreviations: AD, Alzheimer's disease; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; ANOVA, one-way analysis of variance; BDNF, brain-derived neurotrophic factor; CUMS, chronic unpredictable mild stress; eEF2, eukaryotic elongation factor 2; EPM, elevated plus-maze; FST, forced-swim test; GluA1, glutamate receptor 1; KO, knockout; LTD, long-term depression; LTP, long-term potentiation; m-TOR, rapamycin target protein; NFT, neurofibrillary tangles; NMDA, N-methyl-D-aspartate; OFT, open-field test; PSD95, postsynaptic density protein 95; PVDF, polyvinylidence difluoride; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SPT, sucrose-preference test; TBST, Tris-buffered saline–Tween.

MATERIALS AND METHODS

Animals

The study's subjects were 63 adult male C57BL/6 mice and 63 adult male Tau knockout (KO) mice. The C57BL/6 mice were 2 months old (20–23 g) from the Laboratory Animal Center of China Medical University, and the 63 Tau KO mice [B6.129S4-Mapttm/(EGFP)Kit] were 2 months old (20–23 g) from the Jackson Laboratory, bred at the China Medical University. The mice were housed individually in ventilated cages on a 12-h light/dark cycle with free access to water and food. Each strain of mice was randomly divided into three groups (n = 21 per group): a control (CON) group, and two experimental groups—a CUMS group and a CUMS plus ketamine treatment (CUMS+KET) group. The experimental animals were exposed to a series of mild unpredictable stressors for 42 days, as explained below. CUMS+KET and CUMS groups were given an intraperitoneal injection of ketamine hydrochloride 10 mg/kg (14, 22) and the identical dose of normal saline at 8:00 am on the 43rd day, respectively. The sucrose-preference test (SPT) and the open-field test (OFT) were conducted on the 44th day, the elevated plus-maze (EPM) test was conducted on the 45th day, and the forced-swim test (FST) was conducted on the 46th day. All animal procedures were conducted in accordance with the Guidelines for the Care and Use of Laboratory Animals of China Medical University. The protocols were approved by the Institutional Animal Care and Use Committee of China Medical University.

Chronic Mild Unpredictable Stress

The mice were exposed to a variable sequence of unpredictable and mild stressors (23–28) for 6 weeks. These stressors included 45° cage tilting for 4 h, damp sawdust (200 ml water, 200 g sawdust in a cage) for 4 h, 24 h of food or water deprivation, restriction in a 115 mm × 29 mm cylindrical plastic restrainer for 4 h, alterations of the light–dark cycle, 120-db noise overnight, and strobe lights overnight. The animals were exposed to two stressors everyday; stressors were never presented simultaneously.

Ketamine Administration

Ketamine hydrochloride (Fujian Gutian Pharmaceutical Co., Ltd., Gutian, Fujian, China) was dissolved in physiological saline immediately before use. An intraperitoneal injection of ketamine (10 mg/kg) or the identical dosage of saline was administered to the mice on the 43rd day.

Behavioral Tests

Sucrose-Preference Test

We used the SPT to assess anhedonia, which is the core symptom of depression. The mice were given two bottles containing 2% sugar water (Tianjin Guangfu Technology Development Co. Ltd). on the first day, and a bottle of water and a bottle of 2% sucrose

on the second day. On the third day, all the mice were deprived of water for 24 h, and on the fourth day, they were given a bottle of 2% sucrose solution and a bottle of water for 1 h at 8:00–9:00 am. The procedure was originally described by Willner et al. (29). Sucrose and water consumption were determined by measuring the change in the volume of the fluid consumed; the sucrose-preference percentage = sugar water consumption/(sugar water consumption + water consumption) × 100%.

Open-Field Test

The OFT used a procedure described in a previous report (30). Each mouse was tested separately in an open field to assess their locomotor activity and "anxious" behaviors. The open field consisted of a plastic structure of 40 cm × 40 cm × 30 cm, with a white floor. We defined the area adjacent to the surrounding wall as the peripheral area and the rest of the field as the central area (23 cm × 23 cm). The observation of the animals and the data analysis were performed using the SMART™ tracking software system (San Diego Instruments, San Diego, CA). The total distance traversed in the open field and time spent in the central area were observed for 10 min.

Elevated Plus-Maze

The EPM used a procedure described in a previous report (31) to evaluate the "anxiety" of the mice. Each mouse was placed in the EPM, which consisted of two open arms (30 cm × 8 cm) and two closed arms (30 cm × 8 cm × 16 cm) that were elevated 50 cm above the floor in a dimly illuminated room. The open arms and closed arms were perpendicular to each other, and cameras were installed directly above the maze. Each mouse was placed on the central platform of the maze, with its head aligned to one of the open arms. The mouse freely explored the maze for 5 min. The time spent in the open arms was recorded by the SMART™ tracking analysis system.

Forced-Swim Test

The FST was used to evaluate the "depression-like" behavior of the mice (32, 33). Each mouse was placed into a transparent cylinder (10 cm in diameter, depth of 22 cm), filled with water (23°C to 25°C). The mice could not touch the bottom for support. The test was conducted for 6 min: the first 2 min was an adaption phase, after which the immobility of the mouse in the water was recorded for 4 min (immobility refers to the absence of active struggle, with the mouse's body floating in the water).

Western Blot

The mice were anesthetized with excessive sevoflurane and decapitated at the end of the last test. The brain was quickly separated from the skull, and the hippocampus was dissected from both hemispheres, while the brain was on an ice plate. The hippocampus samples were kept at −80°C until use.

The analysis of the synaptosomal fraction was performed using the Syn-PER Reagent (Thermo Scientific) with a protease/phosphatase (50×) inhibitor (P1046, Beyotime, Shanghai, China). Briefly, samples were centrifuged at 1,200×*g* for 10 min, and the remaining supernatant was centrifuged at 15,000×*g* for 20 min to obtain synaptosome pellets and supernatant cytoplasm. In order to verify whether Tau KO mice express Tau protein, we extracted the whole protein of C57BL/6 mice and Tau KO mice, and samples were dissolved with RIPA (P0013B, Beyotime, Shanghai, China) and then centrifuged at 1,200×*g* for 5 min. Then, protein concentrations were determined using a bicinchoninic acid (BCA) Protein Assay Kit (P0012, Beyotime, Shanghai, China). Equal amounts of protein (10 μl) for each Western-blot sample were loaded into 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to polyvinylidence difluoride (PVDF) membranes (Millipore). After being blocked with 8% nonfat milk in Tris-buffered saline–Tween-20 (TBST-20) at room temperature for 2 h, the membranes were incubated with primary antibodies [total Tau, 1:2,000, BD Biosciences; phospho-Tau pSer199, 1:2,000, Abcam; phospho-Tau AT8 (pSer202+Thr205), 1:2,000, Invitrogen; phospho-Tau pSer396, 1:1,000, Life Technologies; phospho-Tau pSer404, 1:2,000, Abcam; GluR1, 1:2,000, Millipore; PSD95, 1:2,000, Abcam; β-actin, 1:5,000, ZSGB-BIO] overnight at 4°C. The blots were washed three times in TBST the next day and incubated with appropriate horseradish peroxidase-conjugated secondary antibodies at room temperature for 1.5 h. The blots were visualized with a luminal reagent, and immunoreactive bands were imaged by Electrophoresis Gel Imaging Analysis System (Tanon 5500, Shanghai, China). Quantification of proteins was performed using National Institutes of Health (NIH) ImageJ software and then normalized to the respective actin band.

Statistical Analysis

One-way analysis of variance (ANOVA) and Tukey *post hoc* tests were used to compare differences between the groups. T test was used to compare C57BL/6 mice and Tau KO mice of their respective CON groups. The statistical analyses were conducted with GraphPad Prism 6.0 software; *p*-values less than 0.05 were considered statistically significant.

RESULTS

In this study, C57BL/6 and Tau KO mice were used. For Tau KO mice, Western blot results of anti-Tau protein specific antibodies showed that Tau KO mice had no positive bands at 50–55 kDa (Tau protein position), while C57BL/6 mice normally expressed Tau protein (**Figure 1**).

The C57BL/6 mice exhibited depressive behaviors, including anxiety, anhedonia, and depression-like after CUMS, and ketamine treatment alleviated their depressive behavior. Tau KO mice did not exhibit depressive behavior after CUMS. After treatment with ketamine at the same dose, there was no significant change in the behavioral results between CUMS+KET and CON and CUMS groups.

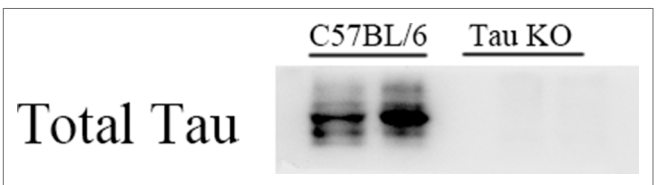

FIGURE 1 | Tau knockout (KO) mice did not express Tau protein.

Sucrose-Preference Test

The CUMS procedures simulated anhedonia in the C57BL/6 mice, and ketamine reduced this behavior. **Figure 2A** shows the sucrose preference of the three groups of C57BL/6 mice. Six weeks of chronic stress produced a significant decrease in sucrose consumption in the CUMS group compared to the CON group of C57BL/6 mice, and ketamine reversed this decrease [**Figure 2A**: $F_{(2,60)} = 7.222$, $p < 0.05$]. However, there was no significant difference in sucrose preference between the CUMS and CON groups of Tau KO mice, and the CUMS+KET group did not differ significantly for the CON and CUMS groups of Tau KO mice (**Figure 3A**: $F = 0.8971$, $p > 0.05$).

Open-Field Test

There was no significant difference in the total distance traversed by the CON, CUMS, and CUMS+KET groups of C57BL/6 mice [**Figure 2B**: $F_{(2,60)} = 0.2402$, $p > 0.05$] or by the CON, CUMS, and CUMS+KET groups of Tau KO mice [**Figure 3B**: $F_{(2,60)} = 0.3049$, $p > 0.05$]. However, the CUMS group of C57BL/6 mice spent significantly more time in the central area than did the CON group. Treatment with ketamine decreased time in the center area, indicating that the anxiety behavior of the C57BL/6 mice was alleviated by ketamine treatment [**Figure 2C**: $F_{(2,60)} = 5.838$, $p < 0.05$]. There was no significant difference in spent time in central area among the three groups of Tau KO mice [**Figure 3C**: $F_{(2,60)} = 0.1705$, $p > 0.05$].

Elevated Plus-Maze

CUMS significantly reduced the duration of time spent in the open arms of the maze by C57BL/6 mice, and antidepressant treatment with ketamine reversed this measure of anxious behavior [**Figure 2D**: $F_{(2,60)} = 5.936$, $p < 0.05$]. There was no significant difference in time spent in the open arms between the three groups of Tau KO mice after stress exposure or ketamine treatment [**Figure 3D**: $F_{(2,60)} = 0.4545$, $p > 0.05$].

Forced-Swim Test

Figure 2E shows that the time the CUMS group was immobile during the FST was significantly longer than that of the CON group, indicating greater depression-like after CUMS exposure among the C57BL/6 mice; however, ketamine treatment significantly reduced the duration of immobility [$F_{(2,60)} = 8.656$, $p < 0.05$]. The duration of immobility in the FST did not differ significantly among the three groups of Tau KO mice [**Figure 3E**: $F_{(2,60)} = 0.4808$, $p > 0.05$].

C57BL/6 mice showed depressive behavior after CUMS, whereas Tau KO mice did not show depressive behavior after CUMS. We used *t*-tests to compare the C57BL/6 mice and Tau KO mice of their respective CON groups. The results showed that there was no statistical difference in the behavioral manifestations of C57BL/6 and Tau KO mice not exposed to CUMS: sucrose preference, $t = 0.82$, $p > 0.05$; total distance of open field test, $t = 0.62$, $p > 0.05$; time spent in the center of the open field, $t = 0.33$, $p > 0.05$; EPM, $t = 0.99$, $p > 0.05$; FST, $t = 0.75$, $p > 0.05$.

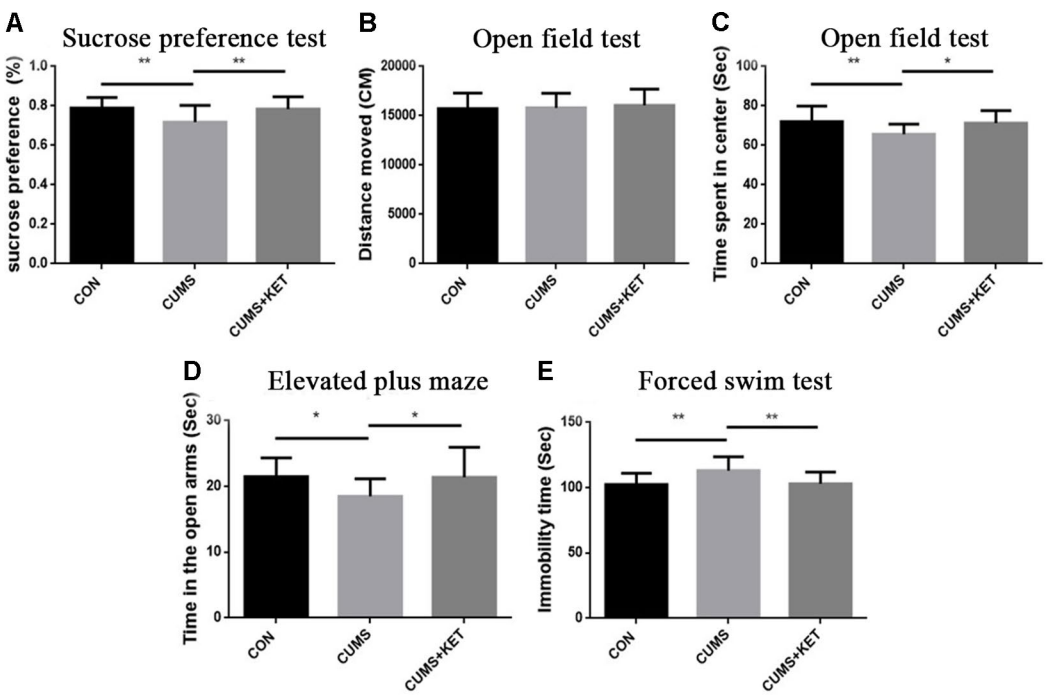

FIGURE 2 | Effects of chronic unpredictable mild stress (CUMS) on the anhedonia, anxiety, and depression-like behaviors of C57BL/6 mice, and ketamine reversal of these behaviors. **(A)** Preference for the sucrose solution on the sucrose-preference test. **(B)** Total distance traversed in the open-field test. **(C)** Time spent in the center area of the open-field test. **(D)** Time in the open arms of the elevated plus-maze. **(E)** Duration of immobility in the forced-swim test. Data are expressed as mean ± SD; n = 21 per group. One-way ANOVA was used to compare differences among the CON, CUMS, and CUMS+KET groups (*$p < 0.05$, **$p < 0.01$).

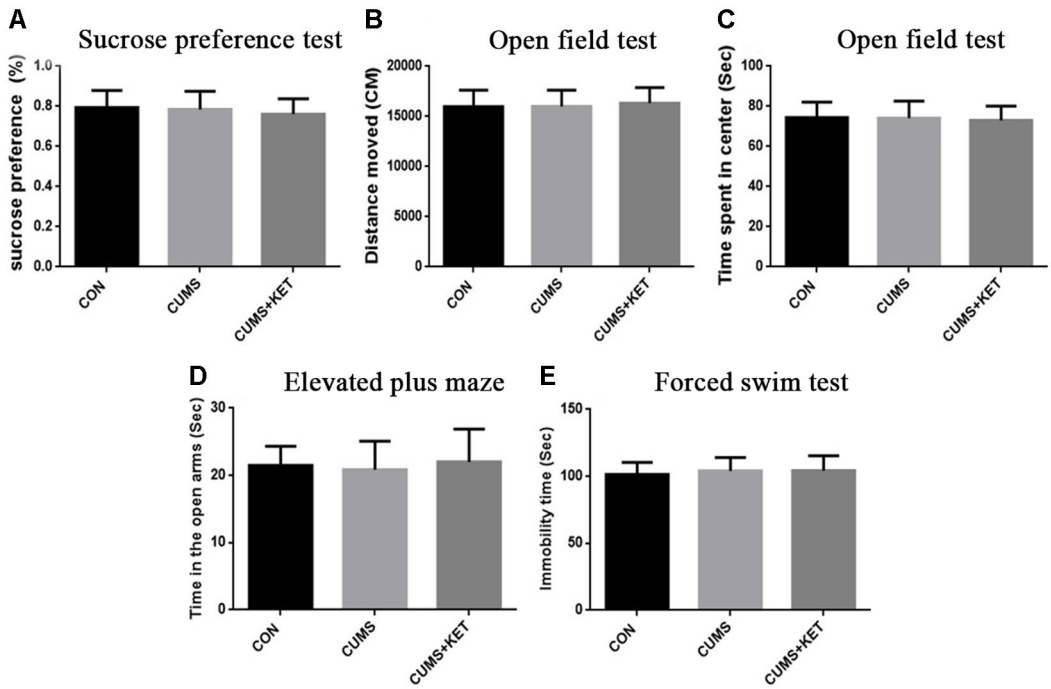

FIGURE 3 | Effects of CUMS on the anhedonia, anxiety, and depression-like behaviors for Tau KO mice. **(A)** Preference for the sucrose solution on the sucrose-preference test. **(B)** Total distance traversed in the open-field test. **(C)** Time spent in the center area of the open-field test. **(D)** Time in the open arms of the elevated plus-maze. **(E)** Duration of immobility in the forced-swim test. Data are expressed as mean ± SD; n = 21 per group. One-way ANOVA was used to compare difference among the CON, CUMS, and CUMS+KET groups.

CUMS-induced total Tau and phosphorylation of Tau at pSer396 and pSer404 increased in the cytoplasm and synapses, while the expression level of phosphorylated Tau at pSer199 and AT8 sites remained unchanged. Ketamine decreased hyperphosphorylated Tau protein in synapses at the pSer396 and pSer404 sites.

We observed the effect of CUMS on Tau and its phosphorylation state in the cytoplasm and synapses of the hippocampus of C57BL/6 mice in **Figure 4A** and **Figure 5A**. CUMS induced a significant increase in cytoplasm levels of total Tau, accompanied by increased levels at pSer396 and pSer404, as seen in **Figure 4F** [$F_{(2,27)} = 5.304$, $p < 0.05$], **Figure 4B** [$F_{(2,27)} = 6.370$, $p < 0.05$], and **Figure 4C** [$F_{(2,27)} = 8.550$, $p < 0.05$]. However, there was no change after CUMS at pSer199 [**Figure 4D**: $F_{(2,27)} = 0.3675$, $p > 0.05$] or AT8 [**Figure 4E**: $F_{(2,27)} = 0.7435$, $p > 0.05$]. We found that CUMS increased total Tau levels [**Figure 5F**: $F_{(2,27)} = 8.088$, $p < 0.05$], as well as the levels of hyperphosphorylation at pSer396 [**Figure 5B**: $F_{(2,27)} = 8.963$, $p < 0.05$] and pSer404 [**Figure 5C**: $F_{(2,27)} = 7.374$, $p < 0.05$] in the synapses of the hippocampus of C57BL/6 mice, but not the pSer199 [**Figure 5D**: $F_{(2,27)} = 0.6130$, $p > 0.05$] and AT8 sites [**Figure 5E**: $F_{(2,27)} = 1.100$, $p > 0.05$]. Total and hyperphosphorylated Tau protein did not change in the cytoplasm after ketamine treatment. However, at the synapse, ketamine decreased the levels of hyperphosphorylation at the pSer396 (**Figure 5B**: $p < 0.05$) and pSer404 sites (**Figure 5C**: $p < 0.05$). Total Tau did not change at the synapse after ketamine treatment (**Figure 5F**).

Ketamine Reversed the CUMS-Induced Decrease in GluA1 and PSD95 in the Hippocampus Synapses of C57BL/6 Mice

CUMS exposure in our experiment decreased the levels of GluA1 and PSD95 in the hippocampus synapses of C57BL/6 mice (**Figure 6A**). A single injection of ketamine treatment reversed the deficits of these synaptic proteins [**Figure 6B**: $F_{(2,27)} = 7.554$, $p < 0.05$; **Figure 6C**: $F_{(2,27)} = 4.495$, $p < 0.05$].

CUMS in our experiment did not change the levels of GluA1 and PSD95, and ketamine treatment had no effects on the Tau KO mice (**Figure 6D**) [**Figure 6E**: $F_{(2,27)} = 0.7851$, $p > 0.05$, **Figure 6F**: $F_{(2,27)} = 0.8668$, $p > 0.05$].

DISCUSSION

A large number of animal experiments are needed to study the etiology of depression and test antidepressants in addition to the study of clinical patients with depression. Therefore, it is particularly valuable to establish effective animal models. Common animal models of depression include the CUMS model (25), social defeat model (34), chronic restraint stress model (35), maternal separation model (36), and the repeated corticosterone injection model (37). We chose to use the CUMS model because CUMS is the best model to mimic the long-term, low-intensity stress of human daily life.

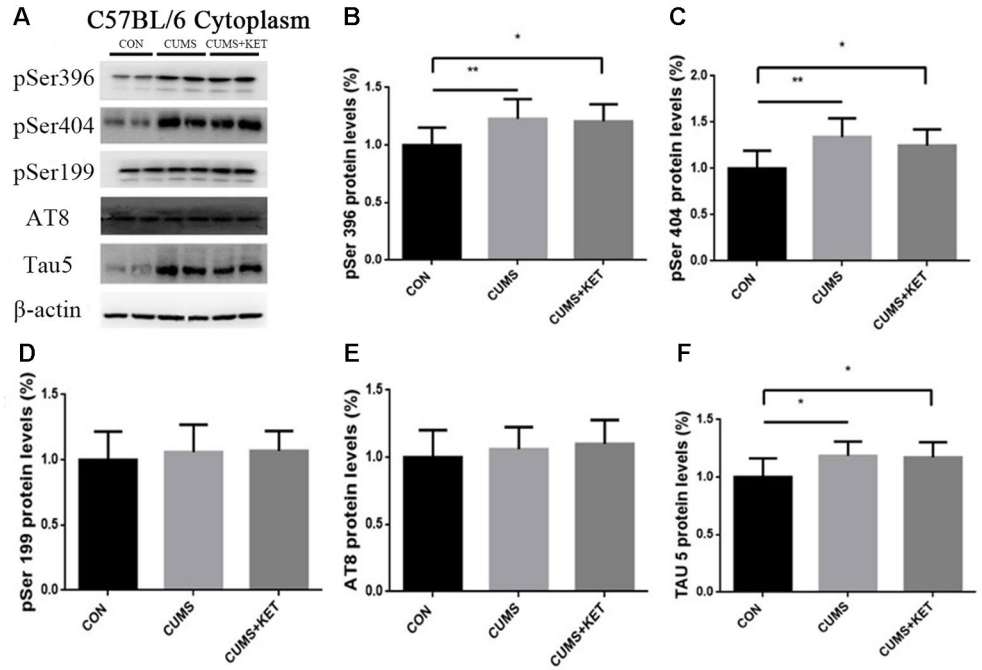

FIGURE 4 | The effects of CUMS and ketamine on Tau protein in the cytoplasm of the hippocampus of C57BL/6 mice. **(A)** Total Tau and hyperphosphorylated Tau protein in the cytoplasm of the hippocampus. **(B, C,** and **F)** After CUMS, the levels of pSer396, pSer404, and total Tau protein increased; ketamine did not change cytoplasm levels. **(D** and **E)** Levels of pSer199 and AT8 of Tau protein did not change after CUMS or ketamine treatment. Data are expressed as mean ± SD; n = 10 per group. One-way ANOVA was used to compare differences among the CON, CUMS, and CUMS+KET groups.

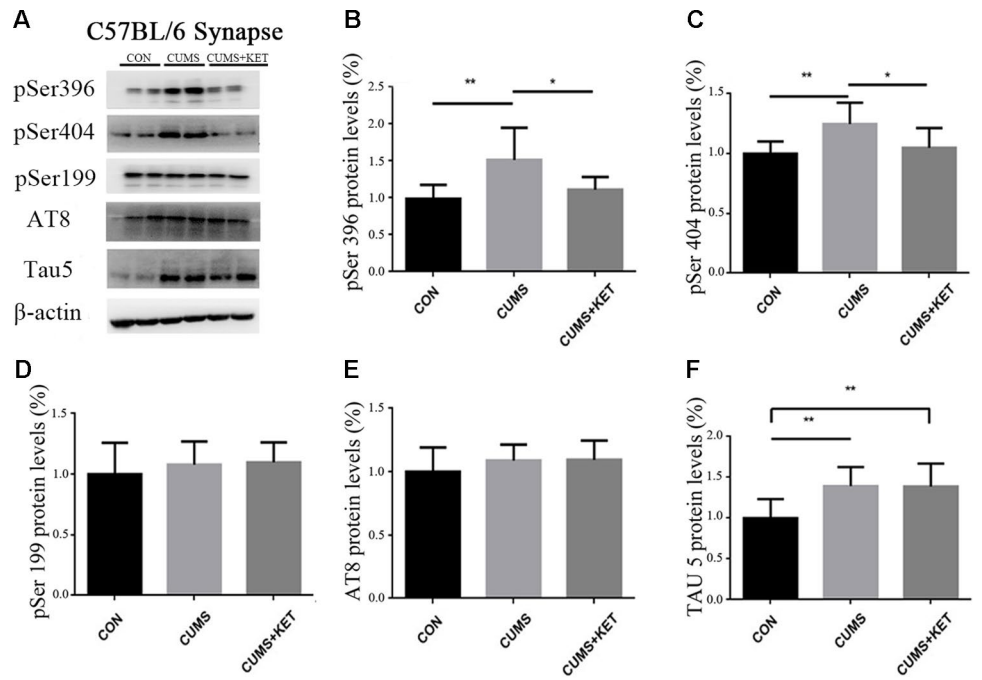

FIGURE 5 | The effects of CUMS and ketamine on Tau protein in the synapses of the hippocampus of C57BL/6 mice. **(A)** Total Tau and hyperphosphorylated Tau protein in the synapses of the hippocampus. **(B, C,** and **F)** After CUMS, the levels of pSer396, pSer404, and total Tau protein increased; ketamine decreased the levels of hyperphosphorylation at pSer396 and pSer404 sites in the synapses, but not total Tau. **(D** and **E)** Levels of pSer199 and AT8 did not change after CUMS and ketamine treatment did not alter the levels in the synapses. Data are expressed as mean ± SD; n = 10 per group. One-way ANOVA was used to compare differences among the groups (*$p < 0.05$, **$p < 0.01$).

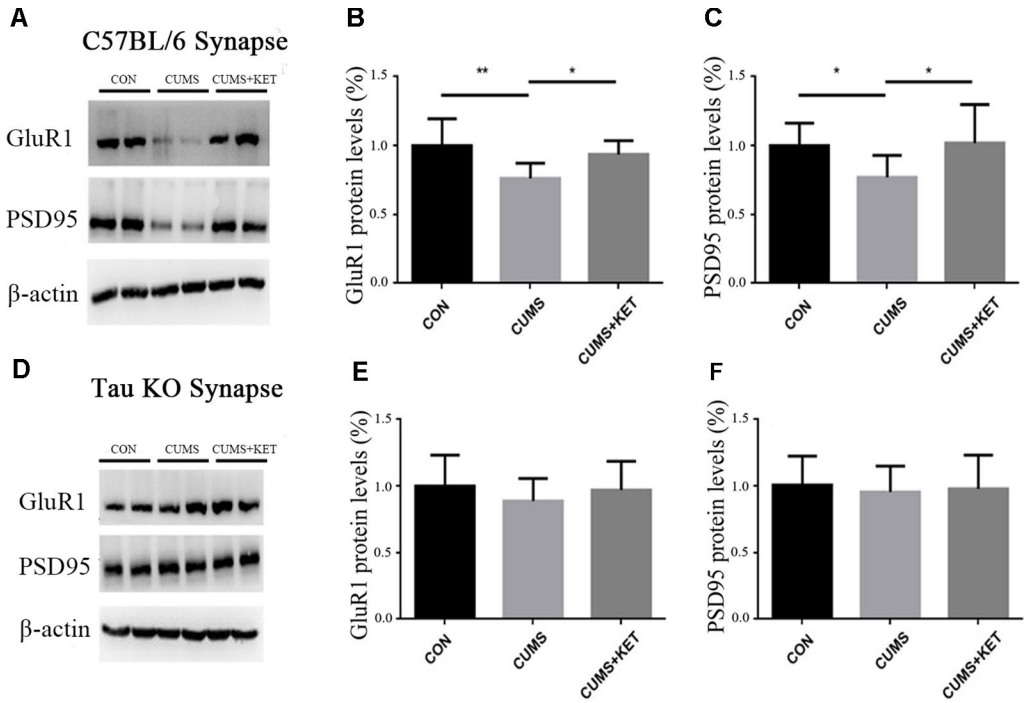

FIGURE 6 | CUMS decreased the levels GluA1 and PSD95 of hippocampus synapses in C57BL/6 mice, with a rapid reversal by ketamine. CUMS did not decrease the levels of GluA1 and PSD95 of hippocampus synapses of Tau KO mice, and ketamine had no effect on the Tau KO mice. **(A)** Representative Western blot images of the GluA1 and PSD95 of C57BL/6 mice are shown. **(B** and **C)** CUMS decreased the expression of GluA1 and PSD95, and this deficit was reversed by a single dose of ketamine. **(D)** Representative Western blot images of GluA1 and PSD95 of Tau KO mice are shown. **(E** and **F)** CUMS and ketamine did not change the levels of GluA1 and PSD95 of Tau KO mice. Results were quantified and expressed as the mean ± SD percent of the control; n = 10 (*$p < 0.05$, **$p < 0.01$).

We used CUMS to establish a depression model in C57BL/6 mice followed by ketamine treatment and conducted behavioral tests on three groups of mice. There was no significant group difference in the total distance traversed in the OFT, indicating that the locomotor activity of mice in each group was not affected. The CUMS group, which was exposed to stress, spent increased time in the center area of the OFT and decreased time in the open arms of the EPM, indicating the C57BL/6 mice were anxious, and their decrease in sucrose preference indicated that these mice exhibited anhedonia. The prolonged duration of immobility time in the FST indicated that the mice experienced desperation. The CUMS group showed the core symptoms of depression—anhedonia and depression-like behaviors, indicating that our depression model was successfully established. Compared with the CUMS group, the CUMS+KET group spent decreased time in the center of the open field, increased time in the open arms of the EPM, increased sucrose preference in the SPT, and significantly less immobility in the FST, indicating that ketamine effectively alleviated the depressive symptoms induced by CUMS.

We used Tau KO mice of the same age to investigate the role of Tau protein in the pathogenesis of depression after undergoing exposure to CUMS exposure and ketamine treatment. Their distance traversed and time in the center area of the OFT, sucrose preference in the SPT, time in the open arms of the EPM, and duration of immobility in the FST did not differ statistically among the three groups (CON, CUMS, and CUMS+KET), showing that the Tau KO mice did not have anhedonia, anxiety, or depression-like behaviors after CUMS, and ketamine did not affect their behaviors. These results suggest that Tau protein plays an important role in the pathogenesis of depression. That is, after Tau protein was knocked out, the mice "escaped" the harmful effects caused by CUMS.

Tau is regulated by its different isomer forms and phosphorylation states. Hyperphosphorylation of Tau protein is generally a key step leading to its migration from axons to dendrites (38). Hyperphosphorylation of Tau protein aggregates in the synapse, leading to synapse loss and impaired neuron function (39, 40). The synapse is the connection between neurons and the key sites of information transmission. Therefore, the hyperphosphorylated Tau protein aggregates in synapses, which is significant for neuron damage. In current studies on depression, hyperphosphorylation forms of Tau protein in the hippocampus are mostly detected by whole protein components (9, 41), but not specifically at the synapse. Therefore, we isolated the cytoplasm and synapses from hippocampal tissue to observe the aggregation of hyperphosphorylated Tau protein in the cytoplasm and synapses, to investigate whether ketamine can affect hyperphosphorylated Tau protein in synapses. We found that C57BL/6 mice showed depressive behavior after CUMS exposure, while the levels of total Tau and

hyperphosphorylated Tau at pSer396 and pSer404 increased in the cytoplasm and synapse of hippocampal neurons. After ketamine treatment, hyperphosphorylated Tau decreased at pSer396 and pSer404 sites in the synapses of hippocampal neurons in C57BL/6 mice. These results suggest that ketamine may play an antidepressant role by reducing hyperphosphorylated Tau in the synapse.

Regan found that when Tau protein was hyperphosphorylated at Ser396 in hippocampal slices of Wistar rats, it could induce LTD (42). The pathogenesis of depression is similar to LTD, resulting in decreased synaptic efficiency, synaptic contraction, and loss of function (43). The 3xTg-AD transgenic mouse model is a good model to simulate AD. Studies have found that Tau protein in the hippocampal total protein of 3xTg-AD transgenic mice is over-phosphorylated at Ser396 and Ser262, which leads to decreased expression of synaptic GluR1 and PSD95 (44). We found the expression of GluR1 and PSD95 in hippocampal synapses of C57BL/6 mice was decreased after CUMS, whereas the expression of GluR1 and PSD95 in Tau KO mice was not affected by CUMS. We hypothesized that CUMS decreased the expression levels of GluR1 and PSD95 protein in the hippocampal synapses of C57BL/6 mice, which may be caused by hyperphosphorylated Tau protein expression in hippocampal synapses. Ketamine treatment reduced the expression of hyperphosphorylated Tau protein in the hippocampal synapses of C57BL/6 mice and increased the expression of GluR1 and PSD95. Tau protein may play an important role in both depression disorder induced by CUMS and hippocampal synaptic protein defects, as well as processes in which ketamine plays an antidepressant role.

Tau protein hyperphosphorylation involves many sites; in this experiment, the levels of Tau protein and the phosphorylated Tau protein at sites pSer396 and pSer404 increased in the hippocampal neuronal cytoplasm and synapses of C57BL/6 mice after CUMS. The levels of phosphorylated Tau protein at sites pSer199 and AT8 did not change in the cytoplasm or synapses. We speculate that CUMS may induce Tau protein hyperphosphorylation with site specificity. Studies have reported that repeated corticosterone injection in male Wistar rats results in no significant changes in Tau hyperphosphorylation at pSer199 and AT8 sites in hippocampal neurons, but increased expression at pSer396 (45). Tau hyperphosphorylation at pSer404 was induced in primary cell cultures of cortical neurons in mice by adding a beta peptide into the culture medium; the Tau hyperphosphorylation was not found at Thr205 (46). Research has shown if Tau protein is hyperphosphorylated at pSer396 and pSer404, Tau hyperphosphorylation is induced at Ser199 when plasmids are transferred into human embryonic kidney cells (47). The hyperphosphorylation of Tau protein at pSer396 in AD patients contributes to its dissociation from microtubules and affects the stability of microtubules (48). Hyperphosphorylation of Tau protein at pSer396 and pSer404 leads to a reduction in its solubility and the formation of NFTs (49).

Tau protein hyperphosphorylation at Ser396/404 and Ser422 in AD patients can also enhance the mutual polymerization of Tau protein to form NFT, while hyperphosphorylation at Ser262 can weaken its polymerization ability (50–52). We found that the phosphorylation sites of Tau protein are different in different diseases and animal studies. Therefore, we speculated that phosphorylation sites of pSer396 and pSer404 may be more sensitive to CUMS in the pathogenesis of depression. In our next experiment, we will closely study different phosphorylation sites of Tau protein that may be related to the development of depression. We will try to observe whether the Tau hyperphosphorylation sites in different models are consistent with the Tau hyperphosphorylation sites induced by CUMS and detect the sites of Tau hyperphosphorylation in cerebrospinal fluid of patients who have clinical depression with different degrees of severity. In addition, the specific mechanism by which phosphorylated Tau protein aggregates in hippocampal synapses to cause a decrease in GluR1 should be studied further.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the "Institutional Animal Care and Use Committee of China Medical University." The protocol was approved by the "Institutional Animal Care and Use Committee of China Medical University."

AUTHOR CONTRIBUTIONS

XWu and GW conceived and designed the experiments. YLi, RD, XR, WR, HYa, and YT performed the experiments. HYu, XZ, JY and XWa helped to analyze and interpret the data. GW drafted the manuscript. XWu, EX, YLu, and GZ provided critical revisions. All the authors reviewed and approved the final manuscript.

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Relationship Between Stressful Life Events and Sleep Quality: Rumination as a Mediator and Resilience as a Moderator

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Purpose: The aim of this study was to investigate the relationship between stressful life events and sleep quality and to probe the role of rumination and resilience in the relationship.

Method: The Adolescent Self-Rating Life Events Checklist, Ruminative Responses Scale, Connor–Davidson Resilience Scale, and Pittsburgh Sleep Quality Index were used among 1,065 college students. Statistical Product and Service Solutions (SPSS) 20.0 and the SPSS macro Process, which were specifically developed for assessing complex models including both mediators and moderators, were used to analyze the data.

Results: High scores of stressful life events predicted worse sleep quality. Rumination partially mediated the relations between stressful life events and sleep quality. Resilience moderated the direct and indirect paths leading from stressful life events to sleep quality.

Conclusions: The results demonstrate that stressful life events can directly affect the sleep quality of college students and indirectly through rumination. Additionally, increasing psychological resilience could decrease both the direct effect and the indirect effect of stressful life events affecting sleep quality. The results of this study may contribute to a better understanding of the effects, as well as the paths and conditions, of stressful life events on sleep quality in college students. Moreover, these findings can provide constructive suggestions for improving college students' sleep quality.

Keywords: stressful life events, sleep quality, psychological rumination, psychological resilience, college student

INTRODUCTION

There is little doubt that sufficient, restorative sleep plays a critical role in maintaining one's physical and mental health. Poor sleep is considered as a predictive sign and symptom for many diseases and is associated with substantial decrements in life quality (1–4). College students are in a special period of life development, and good sleep is significantly important for them. However, it is reported that 12.9% to 52.8% of college students in China have sleep problems (5, 6). Troubled sleep has caused decrements in academic performance and increased risk-taking behaviors, and it has also increased

the risk for subsequent declines in social, psychological, physical, and mental health. Therefore, it is important to identify and characterize the factors that modulate sleep quality and quantity.

A substantial body of literature has established the links between stressful events, negative consequences (7–10), and subsequent poor sleep quality (11–13). Stressful events refer to the things that compel people to make changes in their ongoing life patterns (14). Previous reports suggested that two important factors—rumination and resilience—are involved in psychological and physiological changes after stressful events. For example, Laura Blackburn's study found that combat exposure affects soldiers' posttraumatic stress disorder (PTSD) severity through intrusive and deliberate rumination, and resilience moderated the effect (15). Wu and his colleague examined the role of resilience and rumination in traumatic outcome and found that earthquake exposure-induced PTSD symptoms (including sleep disturbance) were modulated by brooding rumination and depression-related rumination (16). However, the roles of rumination and resilience have not been examined in the context of daily stressful events. Hence, we probe into the effects of stressful life events on sleep quality of college students in China and also the modulating roles of rumination and resilience in the process. We will introduce a new model about the mechanism of poor sleep quality in the context of daily stressful life events by examining factors that might predict poor sleep quality as well as those that might moderate the relationship.

Rumination

According to the appraisal theory of emotion, emotions result from people's interpretations and explanations of their circumstances. Richard Lazarus suggested that there are two major types of appraisals during stressful events (17): the first appraisal being automatic, unconscious, and fast activating; and the second appraisal being conscious and concerned with coping. Then there will be a reappraisal, which might be rumination. Rumination can be emotion-focused or problem-focused (18, 19) and can be defined as a response style whereby an individual tends to repeatedly think about the problematic situations or events, and focus on negative emotions and symptoms the adversity evoked (20–22). Previous studies indicated that rumination could induce many physiological changes, such as troubled sleep.

Harvey's cognitive model of insomnia provides a framework to explain how rumination is linked to sleep disorders, especially at problematic situations (23). It is suggested that individuals with a ruminative response style tend to negatively tone cognitive activity to trigger both autonomic arousal and emotional distress. It is proposed that this anxious state focuses selective attention toward internal and external sleep-related threat cues. The unfortunate consequence is that the excessive and escalating anxiety may culminate in a real deficit in sleep.

Some of the literature provides preliminary support for the model that rumination is associated with sleep problems. For example, Yang's meta-analysis found that rumination is associated with deficits in core executive functions (EFs) (24), which have been suggested to be closely related to sleep problems among young adults with histories of suicide attempts (25). A high level of rumination is also related with worse sleep quality in teenage participants (26, 27). Studies also indicate that rumination may serve as a potential mediator in the relationship between stressful life events and later sleep-related difficulties (28, 29). For example, Zoccola and his colleagues first used portable sleep monitors to measure objective sleep-onset latency, and they found that poststressor rumination could positively predict longer sleep-onset latency (30). Taken together, these results demonstrate correlative relationships between stressful life events and rumination, and between rumination and later sleep-related difficulties. However, no clear pathway leading from rumination to poor sleep quality, especially in problematic situations, has been found.

Accordingly, we expect that daily stressful life events will predict relative increases in college students' rumination. Furthermore, we expect that rumination will predict relative decreases in college students' sleep quality. Thus, our hypothesis is: Daily stressful life events will positively predict poor sleep quality, through rumination (Hypothesis 1).

Resilience

Resilience is a kind of ability to manage stressful events, and appears to have promise in buffering against adversity (31). Resilience can also be seen as an effective operation of the self-adjustment system (32). Why do some resilient individuals recover relatively quickly after a stressful event, returning to baseline functioning, whereas others are still struggling and having sleep difficulties? According to the meta-model, psychological resilience-related variables influence risk factors at multiple stages, from an individual's appraisal of risk, his/her metacognitions in response to felt emotions, to his/her selection of coping strategies. It has been found to serve a protective role against physical and mental health problems. Furthermore, as stated in Kumpfer's theory of resilience framework (33), the positive resiliency process could foster resilient reintegration, and individuals achieve a higher level of resilience after such a process. As a result, constant amplification of the protective effect promotes the virtuous circle of resilience.

Empirical research has provided strong support for the above theories. For example, several studies have suggested that psychological resilience can alleviate the adverse effects of traumatic childhood experiences on depression and reduce the risk of posttraumatic stress disorder (PTSD) while promoting posttraumatic growth (PTG) (16, 34, 35). A recent study by Houpy also found that resilience training is helpful for medical students to cope with occupational stress during the clinical year (36). Pietrzak's team further found that people with high levels of resilience tend to have more psychological resources, which can be used to overcome adversity and adapt to changes in daily life (37). According to the protective mechanism of psychosocial resilience proposed by Rutter (38), resilience may play a protective role in reducing negative chain reactions, etc.

Therefore, we expect that resilience might moderate the adverse effects in daily stressful life events. Hence, our second hypothesis is: The effects whereby daily stressful life events positively affect poor sleep quality through rumination are moderated by resilience.

More specifically, the impacts of daily stressful life events and rumination on sleep quality are expected to be decreased in the context of higher levels of resilience and to be increased in the context of lower levels of resilience (Hypothesis 2).

In this study, we sought to build on and extend prior studies on the relationship between stressful events and college students' well-being in two ways: first, examining direct and indirect pathways, in order to provide an understanding of how stressful events affect sleep quality in college students' daily life; second, investigating whether these pathways differ between individuals with different levels of resilience. Future prevention and intervention programs can be used to subsequently target their efforts at those students at risk. Our conceptual model in this study is as in **Figure 1**.

METHOD

Participants

A total of 1,065 college students from Jiangsu Province in China participated in this study (mean age = 20.21 years; SD = 1.35 years). These included 308 (28.92%) freshmen, 349 (32.76%) sophomores, and 408 (38.31%) juniors and seniors, consisting of 529 boys (49.67%) and 536 girls (50.33%) from different majors, including science and engineering (41.97%), medicine (38.22%), and liberal arts (19.81%). The exclusion criteria for subjects are average Symptom Check List 90 (SCL-90) scores >1.66, which means poor mental health, and students who failed the validity check items in the survey were excluded.

Measures

Stressful Life Events

The intensity and frequency of participants' stressful life events were evaluated with the Adolescent Self-Rating Life Events Checklist (ASLEC) (39), which includes 27 items grouped under six factors: interpersonal, learning stress, punishment, loss, health adaptation, and "other." Each item is rated on a 6-point Likert-type scale. Participants were told to recall whether such events had occurred during the preceding 12 months of their lives. If they answered "no," the score was 0; when answering "yes," they were required to assess the impact of the stressful life event from 1 (not at all) to 5 (very much). ASLEC has been widely used in previous studies, has also been demonstrated to have good reliability and validity, and can be used to evaluate stressful events among teens, especially students in middle and high school and students in university. In this study, the Cronbach's alpha of the ASLEC was 0.88 for the whole scale.

Rumination

Rumination was measured by the Ruminative Responses Scale (RRS), which is a subscale of the Response Styles Questionnaire (RSQ) (40). The RRS includes 22 items that describe the response to depression or other symptoms that arise from stressful events: Each item is rated on a 4-point Likert-type scale (from 1 = "almost never" to 4 = "almost always"). The RRS is composed of three dimensions, which are brooding, reflection, and depression-related. The Chinese version of RRS has been widely used and has reported good reliability and validity among Chinese college students (41). In this study, the Cronbach's alpha of the RRS was 0.81 for the whole scale.

Resilience

Resilience was measured by the Connor and Davidson Resilience Scale (CD-RISC) (42), which measures personal qualities that enable people to thrive after exposure to stress and trauma. The CD-RISC involves 25 items and five dimensions. Each item is rated on a 5-point Likert-type scale (from 0 = "not true at all" to 4 = "true nearly all the time"), with higher scores indicating a stronger degree of resilience. The instrument's reliability and validity is further evidenced by widespread use of the CD-RISC in research on Chinese students (43). In this study, the Cronbach's alpha of the CD-RISC was 0.79.

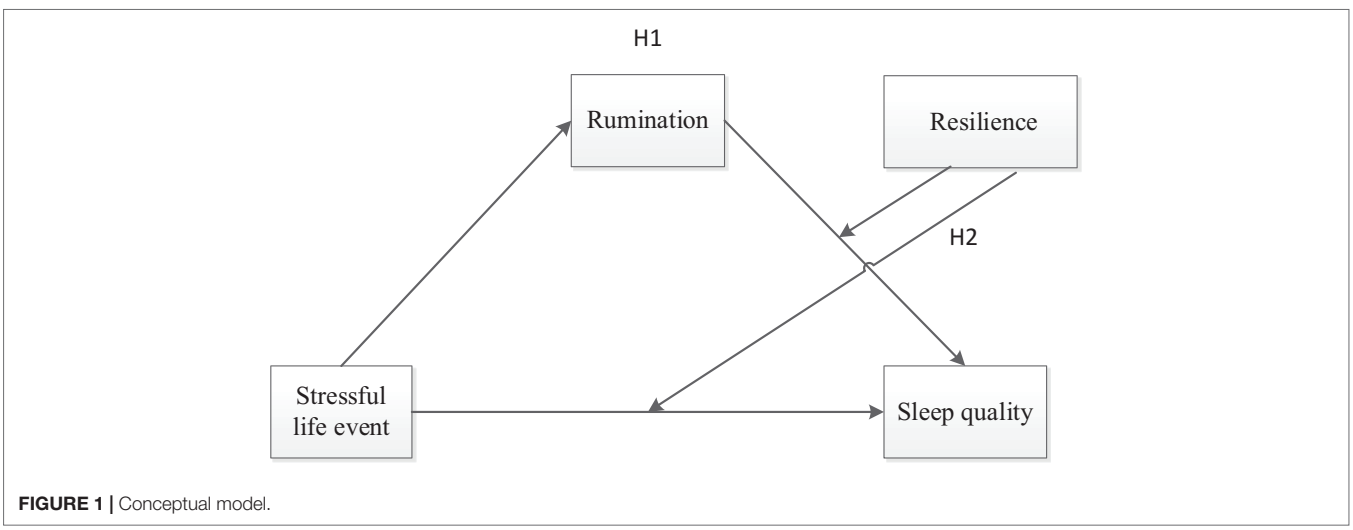

FIGURE 1 | Conceptual model.

Sleep Quality

Sleep quality and disturbance was measured by the Pittsburgh Sleep Quality Index (PSQI) (44), which includes 19 items and 7 factors, including sleep duration, sleep disturbance, sleep latency, daytime dysfunction due to sleepiness, sleep efficiency, overall sleep quality, and sleep medication use. Each of the factors yields a score ranging from 0 to 3. These 7 factors' scores are summed to yield a total score ranging from 0 to 21. Higher score indicates worse sleep quality. The PSQI has been widely used in Chinese college students' samples and shows great reliability and validity (45). In this study, the Cronbach's alpha of the PSQI was 0.74.

Procedures

The paper-and-pencil questionnaires were administrated to classes of 30–60 students during several data collection events within 1 week. We avoided the beginning of the semester, midterms, and finals, which are the periods that include more stressful events and may induce poorer sleep quality. Participants were guaranteed anonymity of their responses and confidentiality of the data. Completing the entire packet of instruments typically required 25–30 min. After data collection, SPSS 20.0 and the SPSS macro PROCESS (46), which were specifically developed for assessing complex models including mediators and moderators, were used to analyze the data. The study protocol conformed to the ethical guidelines of Jiangsu University and was approved by the institutional ethics committee, and written informed consent was obtained from each participant.

RESULTS

Preliminary Analyses

In order to evaluate the common method variance in this study, we ran the Harman's single-factor test. The results showed that no single factor can explain the majority of variance (the maximum component explained only 29.46% of total variance), which means that there was no common method bias in this study.

The means, standard deviations, and correlations for the measured variables are shown in **Table 1**. Data were first checked for normality, with a critical assumption underlying the asymptotically distribution-free procedure being used in the analysis. All results in this study indicated univariate normality for all measured variables.

The average score on the PSQI was 6.36 ± 3.07. Of the 1,065 participants, 358 (33.62%) subjects (177 males, 181 females) met the criteria of sleep disturbance in China (PSQI scores ≥8) (47). The independent sample *t*-test of PSQI scores showed no statistically significant difference according to gender ($t = 0.19$, $P = 0.85$), and the one-way ANOVA showed no statistically significant differences in PSQI scores in terms of major ($F = 1.15$, $P = 0.31$).

Correlation analysis showed that stressful life events were positively correlated with PSQI scores and rumination. Rumination had a positive correlation with the PSQI scores. There was a negative correlation between resilience and PSQI scores (with higher PSQI scores indicating worse sleep quality).

TABLE 1 | Means, standard deviations, and correlations among variables.

	Mean	SD	1	2	3
1. Sleep Quality	6.36	3.07	–		
2. Rumination	48.75	11.73	0.19**	–	
3. Resilience	58.90	16.81	−0.46**	−0.03	–
4. Stressful Life Events	45.09	12.87	0.18**	0.57**	−0.12**

*n = 1065. **P < 0.01.*

Mediation Analyses

We examined the indirect effects of rumination on the associations between stressful life events and sleep quality using the PROCESS procedure in SPSS 20.0 (46). The results are shown in **Table 2**, which indicated that stressful life events can positively predict sleep quality; when treating stressful life events and rumination as predictors, their effects on sleep quality were both significant. We created 1,000 bootstrap samples to further test the indirect effects of rumination. The results showed that the indirect effect was statistically significant (indirect effect = 0.08, 95% CI = 0.03–0.12), and the ratio of indirect to total effect of stressful life events on sleep quality was 41.10%. These results indicated that rumination serves a partial mediating function in the relation between stressful life events and sleep quality.

Moderation Analysis

A PROCESS analysis was used to test whether resilience moderates the links leading from stressful life events to sleep quality through rumination (**Table 3**), and the results showed that the standardized regression coefficient (β) of "resilience × rumination" remained significant; when resilience and rumination were controlled for, the same was true of "resilience × stressful life events." This implies that the effect of stressful life events and rumination on

TABLE 2 | Multiple regression analyses of the indirect effect of rumination.

Regression		Significance of regression coefficient			
Dependent variable	**Independent variable**	***β***	**LLCI**	**ULCI**	***t***
Sleep quality	Stressful Life events	0.18	0.12	0.24	6.01**
	Gender	0.03	−0.09	0.16	0.54
	Major	−0.05	−0.11	0.02	−1.47
	Grade	−0.02	−0.08	0.04	−0.63
Rumination	Stressful Life events	0.57	0.52	0.62	22.39**
	Gender	−0.08	−0.18	0.02	−1.53
	Major	0.03	−0.03	0.09	0.95
	Grade	−0.03	−0.08	0.02	−1.23
Sleep quality	Rumination	0.13	0.06	0.20	3.62**
	Stressful Life events	0.11	0.04	0.18	2.93**
	Gender	0.04	−0.08	0.17	0.72
	Major	−0.05	−0.12	0.01	−1.58
	Grade	−0.02	−0.08	0.05	−0.50

*n = 1,065. **P < 0.01. Continuous variables were normalized. LLCI, lower level for confidence interval; ULCI, upper level for confidence interval.*

TABLE 3 | Multiple regression analyses of the moderate effect of resilience.

Regression		Fit index		Significance of regression coefficient			
Dependent variable	**Independent variable**	R^2	F	β	LLCI	ULCI	*t*
Sleep quality	Resilience	0.34	92.81**	–0.39	–0.44	–0.34	–15.40**
	Rumination			0.19	0.14	0.24	7.49**
	Resilience × Rumination			–0.27	–0.32	–0.23	–12.77**
	Gender			0.01	–0.09	0.11	0.25
	Major			–0.01	–0.07	0.04	–0.49
	Grade			–0.01	–0.06	0.04	–0.38
Sleep quality	Resilience	0.29	70.49**	–0.39	–0.44	–0.34	–14.50**
	Stressful Life Events			0.12	0.07	0.17	4.49**
	Resilience × Stressful Life Events			–0.22	–0.26	–0.17	–9.19**
	Gender			0.03	–0.08	0.13	0.49
	Major			–0.01	–0.07	0.05	–0.38
	Grade			–0.02	–0.07	0.04	–0.59

*n = 1,065. **P < 0.01. Continuous variables were centered at their means.*

TABLE 4 | The direct and indirect effects at different levels of resilience.

Resilience	Effect		*LLCI*	*ULCI*
M–SD	Direct effect	0.03	0.01	0.04
	Indirect effect	0.05	0.04	0.07
M	Direct effect	0.01	–0.01	0.02
	Indirect effect	0.02	0.01	0.03
M+SD	Direct effect	–0.01	–0.02	0.02
	Indirect effect	–0.01	–0.02	0.01

LLCI, lower for confidence interval; ULCI, upper level for confidence level.

sleep quality decreased as the level of resilience increased. In other words, the direct and indirect effects of stressful life events on sleep quality are moderated by resilience.

The nature of the moderation was further explored using a simple slope analysis (48) and conditioned at one SD above and below the mean; the results are shown in **Table 4** and **Figure 2**. Compared with individuals who have higher levels of psychological resilience, individuals with lower levels of psychological resilience tend to show a stronger positive relationship between stressful life events/rumination and PSQI score.

We tested the statistical insignificance of the moderating effect of psychological resilience on the association between stressful life events/rumination and sleep quality using the Johnson–Neyman (J-N) technique provided by the PROCESS procedure (46). The results are shown in **Figure 3**. Following the J-N technique, the 95% confidence interval of the conditional effect containing 0 indicates that the association was lost. As shown in **Figure 3**, with increasing resilience, the conditional effect of stressful life events on sleep quality declined dramatically and became statistically insignificant for students scoring above 63.81, as did the conditional effect of rumination when scores were higher than 67.12.

DISCUSSION

This study aimed to explore the relationship between stressful life events and poor sleep quality, and also the mediating and moderating factors between the bivariate links. We proposed that daily stressful life events positively predict poor sleep quality through rumination, and resilience moderates the links. Data from 1,065 college students in the Jiangsu Province of China supported our model. The results have several theoretical and practical implications, which we consider in turn.

First, this study sheds light on the nature of the relationship between daily stressful life events and sleep quality. Our study shows that college students who have experienced more stressful life events have poorer sleep quality, which is consistent with previous reports (12, 13, 49, 50). Psychophysiological research has provided a possible explanation that stress caused by stressful life events may lead to greater activation of the locus coeruleus norepinephrine (LC-NE) system and the hypothalamic–pituitary–adrenocortical (HPA) axis, which can increase excitability and aggravate difficulty in falling asleep (51). It also offers experimental data for our hypothesis about the neuromodulator basis of core affect. We propose that there are four basic emotions—fear, anger, happiness, and sadness—that are associated with three core affects: reward (happiness), punishment (sadness), and stress (fear and anger) (52). These three core affects are analogous to the three primary colors (red, yellow, and blue) and are subsided by three monoamines [DA (Dopamine) NE (Norepinephrine) 5-HT (5-hydroxytryptamine)]. This new model is called the "*three primary color model of basic emotions.*" (17) The activation of NE release by stressful events can antagonize the activity of 5-HT, which is the major substance associated with sleep.

Moreover, our study illuminates rumination as a mechanism that accounts for the relationship between stressful life events and poor sleep quality. According to Morin's hyperarousal theory of insomnia (53), cognitive arousal may play an important role in sleep turbulence (54). Individuals with higher levels of rumination are more likely to have negative reappraisals of the past, which can increase cognitive arousal and consequently lower sleep quality. The result also offers some proof for our "emotional flow" theory. In this theory, we proposed that when something unexpected occurs, we first feel scared (fear) and then try to control the fearful situation (anger) (52), which could lead to a "fight" response. Afterwards, we might feel happy when

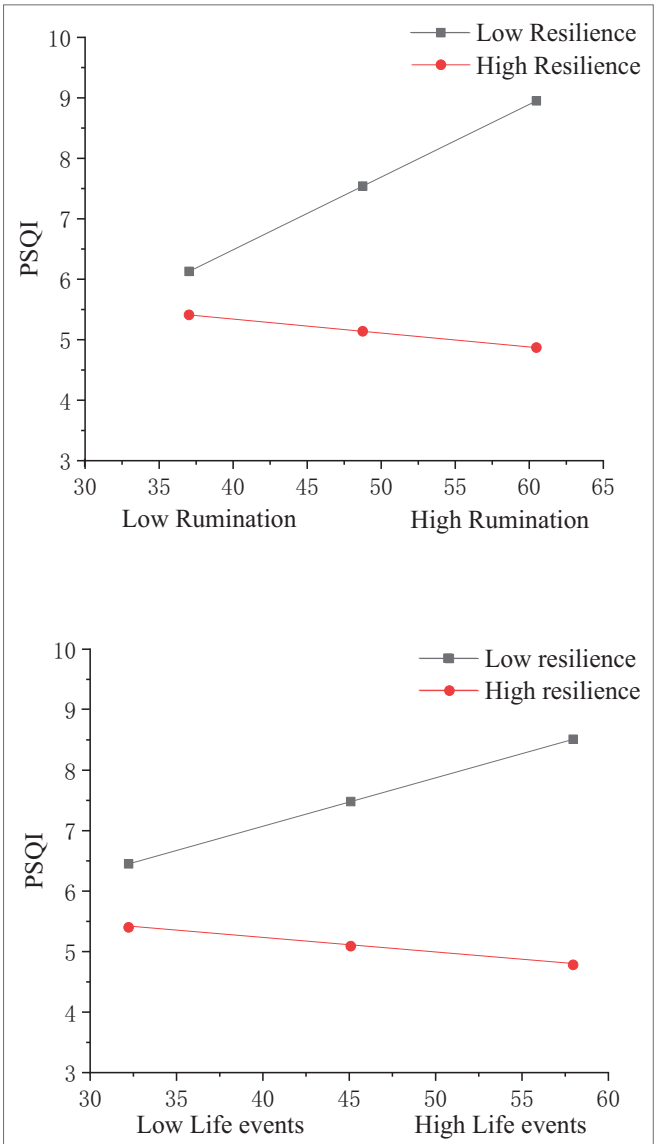

FIGURE 2 | Simple slope analyses of the moderating effect of psychological resilience.

successfully coping with the stressful event, or we may feel sad if we failed to cope (55). This kind of emotional flow constitutes emotions in sequence: fear–anger–happiness/sadness. According to Lazarus, fear and anger caused by stressful events are related to appraisal, while happiness or sadness is related to reappraisal, which might be manifested as rumination and affects other outcome variables, such as troubled sleep.

Finally, the data also show that the effects that daily stressful life events positively affect poor sleep quality through rumination are moderated by resilience. More specifically, the impact leading from daily stressful life events and rumination to sleep quality is expected to be decreased in the context of a higher level of resilience and be increased in the context of a lower level of resilience. Furthermore, according to the result of the J-N technique, resilience plays a significant moderating role in the majority of college students. The negative impact of stressful life events upon sleep quality declines with the increase of psychological resilience, and the effect may even disappear in highly resilient individuals (resilience scores greater than 63.81 and 67.12, respectively).

These new findings could reveal how stressful life events influence sleep quality and provide enlightenment for controlling sleep-related risk factors. Given the important influence of stressful life events on sleep quality, parents and teachers should pay attention to students' stress levels. Methods of reducing stress can help create a supportive environment and improve sleep quality. In addition, our study suggested that rumination worsens sleep quality in those suffering from stressful life events. Hence, interventions like mindfulness training and positive reevaluation training would be beneficial for students with sleep problems. Finally, considering that resilience acts as a buffer between stressful events and sleep quality as well as the relationship between rumination and sleep problems, it is feasible to solve sleeping problems by improving psychological resilience level.

LIMITATION AND FUTURE RESEARCH DIRECTIONS

Although our data provide new evidence pertaining to the mediating effect of rumination and moderating effect of resilience on the relationship between stressful life events and sleep quality, our results should be assessed with care to include the background of the limitations inherent in our study. First, we collected data at one point in time, which limits the conclusions that can be made regarding the causal order of relationships. Hence, we would plan a second wave of data collection next year to substantiate the causality of our hypotheses. Also, experimental or longitudinal research designs from other researchers are welcome. Second, we relied on individuals' self-reports for all variables in our model, which raises the concern of possible common method bias. However, our statistical analyses revealed that common method bias did not cause a major concern in our study. Therefore, we encourage future researchers to collect data from multiple sources to investigate our findings further. Moreover, sleep quality can be affected by many other factors, such as anxiety, depression, adult attachment, etc.; all these factors could serve as potential mediators in the stressful events–sleep relationship. Thus, multiple mediator models are suggested for future studies.

In conclusion, our study has contributed to debates about the role of stressful events in influencing sleep disturbances through the development and testing of a moderated mediation model. We introduced rumination and resilience as two key variables in this causal chain. We found that rumination partially mediated the relationship between daily stressful events and poor sleep quality in college students. We further found that resilience moderated the links. The findings are consistent with Lazarus's appraisal theory of emotion and our own emotional flow theory. From our study, we can make a conclusion that college students' sleep quality can be predicted by stress from day-to-day working

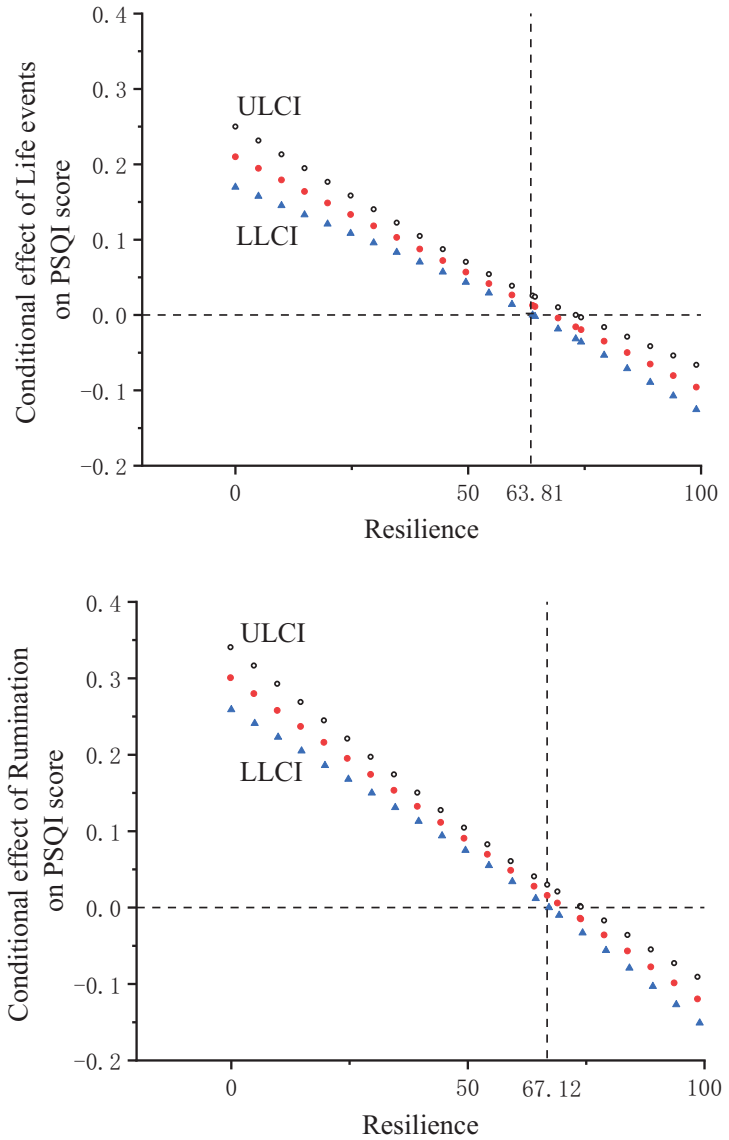

FIGURE 3 | Visual representation of the conditional effect of stressful life events/rumination on sleep quality as moderated by resilience (LLCI, lower level for confidence interval; ULCI, upper level for confidence interval).

lives directly, and sleep quality can also be predicted indirectly through ruminative response style. Increasing psychological resilience could decrease both the direct effect and the indirect effect that stressful life events affect sleep quality. Our findings suggest that resilience and rumination play very important roles not only in traumatic events but also in daily stressful events context. Our data reveal how stressful life events influence sleep quality and provide enlightenment for controlling sleep-related risk factors.

ETHICS STATEMENT

The study protocol conformed to the ethical guidelines of Jiangsu University and was approved by the institutional ethics committee. Informed consent was obtained from each participant.

AUTHOR CONTRIBUTIONS

SG, FW, GF, and JH designed the experiment. YL, ZW, SD, and HL collected the questionnaire. HZ, XM, and XX did the data analysis. YL, SG, and FW wrote the paper.

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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.2019.00348/full#supplementary-material

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Combination of Alprazolam and Bailemian Capsule Improves the Sleep Quality in Patients With Post-Stroke Insomnia: A Retrospective Study

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Insomnia is often ignored in the diagnosis and treatment of patients of stroke. The present study aimed to evaluate the efficacy of alprazolam (ALP) combined with Bailemian capsule (BC, a traditional Chinese patent medicine) in the treatment of post-stroke insomnia (PSI). A total of 231 stroke patients involved in this retrospective study were treated with ALP, BC, or ALP + BC for 3 weeks. The quality of sleep was evaluated by the Pittsburgh Sleep Quality Index (PSQI) and polysomnography (PSG), while self-care ability was monitored by the modified Rankin Scale (mRS) before and after treatment. Compared with the baseline, the self-care ability of patients in each group was significantly improved after treatment ($P < 0.01$). The PSQI data showed a significant improvement in all patients in all of the subjective PSQI items and global score ($P < 0.05$). Notably, ALP + BC administration had a significantly greater effect on sleep latency, quality, disturbance, and efficiency, as well as daytime dysfunction and global PSQI than the use of ALP or BC alone ($P < 0.05$). The PSG data showed that ALP significantly improved the sleep efficiency and decreased the arousal times, rapid eye movement (REM) sleep, and sleep latency ($P < 0.05$), while BC significantly improved the sleep efficiency, total sleep time, and the duration of N3 ($P < 0.05$). Strikingly, ALP + BC achieved the effect of both ALP and BC ($P < 0.05$). Importantly, the effect of the combination of ALP and BC was greater than the use of ALP or BC alone, which was consistent with the result of PSQI. In conclusion, the sleep quality and self-care ability of patients with PSI were improved by ALP and BC, thereby supporting the potential advantages of ALP combined with BC in the treatment of patients with PSI.

Keywords: stroke, insomnia, drug therapy, Bailemian capsule, alprazolam

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INTRODUCTION

Stroke is the second most common cause of deaths worldwide (1) as well as the leading cause of long-term disability (2, 3). Post-stroke insomnia (PSI) is a common symptom but often underestimated and is even ignored in the diagnosis and treatment (4). Up to 70% of the patients with acute stroke have sleep disorders including excessive daytime sleepiness, insomnia, hypersomnia, and fatigue (5). PSI affects the

functional recovery of the nervous system, aggravates the existing diseases such as hypertension and diabetes, and deteriorates the quality of life (6). Accumulating evidence demonstrated that poor quality of sleep could be detrimental to the immune system (7), delay the recovery (8), increase pain sensitivity (9), lead to depression and anxiety (10), and affect the functional well-being (11). Although the consequences of PSI and the potential clinical impact are severe, the condition is not well treated.

Reportedly, alprazolam (ALP) was the most commonly used Western drug in China due to its effectiveness on generalized anxiety, panic attacks with or without agoraphobia, and depression (12). ALP is a derivative of benzodiazepine, and the mechanism underlying the activity of the drug and the side effects have been described previously (13). Notably, ALP presents excessive side effects when more than 0.5 mg was used each time (14).

Drugs for stroke from traditional Chinese medicine have been developed (15, 16). According to clinical and basic research in traditional Chinese medicine, these drugs were beneficial in the prevention and treatment of stroke. BC is a traditional Chinese patent medicine. It can improve sleep quality and alleviate insomnia by elevating the level of brain contents 5-HT and GABA (17, 18). Previous studies have shown that sleep disorders were closely related to the decreased content of 5-HT and gamma-aminobutyric acid (GABA) in the central nervous system (19). The data were monitored, which showed that this drug might cause nausea, abdominal pain, rash, and itching. Despite mild adverse reactions, BC can alleviate PSI and anxiety, resulting in the improvement of life quality (20).

In this retrospective study, we evaluated the effect of BC, ALP, and BC combined with ALP on sleep quality and stroke outcome (self-care ability) in PSI patients.

PATIENTS AND METHODS

Participants

In the present retrospective study, we analyzed 231 patients (78 females and 153 males) who were hospitalized for stroke at the Department of Neurology in the General Hospital of Western Theater Command from January 1, 2014, to September 1, 2015. All the patients underwent identical treatment for stroke according to the guidelines of acute ischemic stroke (21). The degrees of insomnia and neurological impairment were evaluated after admission. This study has been approved by the Ethics Committee of The General Hospital of Western Theater Command. Also, we obtained the informed consent of patients and their families.

The patient selection process was applied according to the following criteria:

Inclusion criteria: 1) The diagnosis of stroke is based on clinical presentation, computerized tomography, and/or magnetic resonance imaging scan of the brain when the stroke occurred within 7 days prior to the admission. 2) Patients had varying degrees of insomnia [*Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition* (*DSM-IV*) criteria and the three insomnia-related items of the Hamilton depression scale] and received ALP, BC, or both during hospitalization. 3) Insomnia was present after stroke. 4) The score was at least 26 on the Mini-Mental State Exam. 5) The questionnaire was completed independently. And 6) antipsychotic medications, if administered, had been discontinued for more than 7 days.

Exclusion criteria: 1) Patients had a history of sleep disorders (based on the sleep disorders questionnaire designed in Hong Kong) (22). 2) Patients also presented with cardiovascular, liver, kidney, or any severe life-threatening diseases. 3) Patients had active epilepsy and unable to complete the entire treatment process. 4) Patients had language barrier and could not cooperate with the researchers. 5) Patients had typical mental diseases, such as anxiety and depression. 6) Patients had family history of mental disorders. 7) Patients had other severe diseases and could not complete the treatment and investigation. 8) Patients had restless legs syndrome, obstructive sleep apnea–hypopnea syndrome, central sleep apnea syndromes, and rapid eye movement (REM) sleep behavior disorder.

STUDY DESIGN

The patients were divided into three groups according to the treatment. The patients received ALP (12) ($n = 71$, 0.4 mg/day, taken 30 min before sleep at night; Qilu Pharmaceutical Group, Jinan, Shandong, China, national drug approval number: H37021277), BC (20) ($n = 87$, four capsules each time after breakfast and dinner; Yangtze River Pharmaceutical Co., Ltd, Taizhou, Jiangsu, China, national drug approval number: Z20020131), or ALP + BC ($n = 73$) for 3 weeks. Pittsburgh Sleep Quality Index (PSQI) and modified Rankin Scale (mRS) were applied to evaluate the sleep quality and the self-care ability of patients before and after treatment. Furthermore, we also objectively assessed the data of polysomnography (PSG) before and after treatment in order to evaluate the curative effect impersonally ($n = 5$ in each group).

ASSESSMENT CRITERIA AND QUESTIONNAIRES

Sleep quality: PSQI is a self-reported questionnaire tool for subjectively measuring the quality of sleep for adults in the last month. It includes seven sleep-related items, such as latency, quality, duration, disturbances, efficiency, the use of sleep medications, and daytime dysfunction. Each item is assigned a score of 0–3 points so that the total score of PSQI is 0–21. A total score of 7 means good sleep, 7–11 points indicate mild, 12–16 points moderate, and 17–21 points severe sleep disorder (23).

PSG: PSG was performed as reported previously (24). Two nights of PSG recording were recommended; however, the first night was regarded as the "adaptation" night and removed from the analysis as it was not representative of the usual sleeping patterns (25). Briefly, PSG was performed with an eight-channel Grass electroencephalograph in one night. The recordings included brain electrical activity, eye movements, chin muscle activity, nasal and oral airflow, thoracic and abdominal respiratory movements, heart rate, and leg movements. Also, the following parameters were recorded: total recording time, total sleeping time, sleep efficiency, sleep latency, paradoxical latency, duration of non-rapid eye movement sleep (NREM) and its

three stages (N1, N2, and N3), duration of rapid eye movement sleep (REM), wake time after sleep onset, and arousal time (26).

Clinical outcomes: The prognosis of stroke was assessed using mRS, a commonly used scale for measuring the self-care ability.

Adverse reactions: During the treatment, adverse effects such as headache, aggravated insomnia, blood pressure increase, hyperethism, nausea and vomiting, dizziness, palpitation, frequent urination, somnolence, and numbness were evaluated. A total of four cases presented nausea and upper abdominal discomfort in the BC group, while no adverse reactions were detected in the other two groups. Moreover, the symptoms were relieved spontaneously without any additional treatment.

STATISTICAL ANALYSIS

Data analysis was carried out using SPSS 21.0 software (IBM Corp, Armonk, NY, USA). Measurement data were expressed as means ± standard deviations (SDs). One-way ANOVA, Student's *t*-test, χ^2 test, or Mann–Whitney *U* test was utilized for group comparison. When ANOVA revealed significant differences, least significant difference (LSD) or Dunnett's T3 *post hoc* tests were used to identify significant differences among three groups. $P < 0.05$ was considered as a statistical significance.

RESULTS

Comparison of Demographic and Clinical Characteristics Between the Three Groups

No significant difference was observed in the baseline characteristics of age, sex, blood glucose, triglyceride (TG), cholesterol (TC), High-density lipoprotein cholesterol (HDL-C), Low-density lipoprotein cholesterol (LDL-C), serum uric acid, and homocysteine between the three groups ($P > 0.05$, **Table 1**).

Comparison of Sleep Quality by PSQI Before and After ALP, BC, and ALP + BC Treatment

The data did not show any significant difference in the sleep quality ($F_{(2,228)} = 1.055$, $P = 0.35$), sleep latency ($F_{(2,228)} = 0.205$, $P = 0.815$), sleep duration ($F_{(2,228)} = 0.169$, $P = 0.845$), sleep efficiency ($F_{(2,228)} = 0.074$, $P = 0.929$), sleep disturbances ($F_{(2,228)} = 0.046$, $P = 0.955$), daytime dysfunction ($F_{(2,228)} = 0.127$, $P = 0.881$), sleep medication ($F_{(2,228)} = 0.197$, $P = 0.822$), and global score ($F_{(2,228)} = 0.143$, $P = 0.867$) (**Figure 1**) at baseline. After 3 weeks of treatment, all the three treatment groups significantly improved in all of the subjective PSQI items and global score (**Figure 1**, $P < 0.05$). Notably, ALP + BC administration exerted a significantly greater effect on all the PSQI items and the global PSQI score than did the other groups, except for the sleep duration, which was greatly affected by ALP + BC or BC as compared with ALP (**Figure 1C**, $P < 0.05$).

Comparison of Sleep Quality by PSG Before and After ALP, BC, and ALP + BC Treatment

Five patients in each group were selected for PSG testing. The data did not reveal any significant difference in the arousal time, sleep efficiency, sleep latency, total sleep time, wake after sleep onset, REM sleep, and NREM sleep at baseline ($P > 0.05$). After 3 weeks of treatment, ALP improved the sleep efficiency and decreased the arousal times, sleep latency, and REM sleep (**Figure 2A**, **B**, **C** & **F**), while BC significantly improved the sleep efficiency, total sleep time, and the duration of N3 (**Figure 2B**, **D** & **I**); ALP + BC achieved the effect of both ALP and BC (**Figure 2A**, **B**, **C**, **D, E** & **I**; $P < 0.05$). There was a significant difference in sleep latency ($F_{(2,12)} = 28.407$, $P < 0.001$) and total sleep time ($F_{(2,12)} = 5.701$, $P = 0.018$) among the three groups after treatment. *Post hoc* analysis indicated that ALP significantly decreased sleep latency compared to BC (**Figure 2C**, $P = 0.028$), while BC significantly increased total sleep time compared to ALP (**Figure 2D**, $P = 0.026$). Importantly, the effect of the combination of ALP and BC was greater than that of ALP or BC alone, which was consistent with the result of PSQI.

Comparison of Self-Care Ability Before and After ALP, BC, and ALP + BC Treatment

Previous studies showed that PSI could affect the recovery of neurological function in stroke patients (6). Therefore, we explored the effect of insomnia improvement on stroke outcome. After treatment, the stroke outcome was improved in all of the three groups, in which the mRS score decreased ($P < 0.05$). The *post hoc* study demonstrated that this effect of ALP + BC was greater than that of ALP or BC alone (**Figure 3**, $P < 0.05$).

TABLE 1 | Demographic and clinical characteristics of the patients. (Data are expressed as *n* (%) or mean ± SD.)

		ALP + BC	ALP	BC	F	P
Sex	Male	48 (65.75)	49 (69.01)	56 (32.15)	0.174	0.677
	Female	25 (34.25)	22 (30.99)	31 (41.65)		
Age, y		58 ± 10.05	56.13 ± 14.28	57.95 ± 11.98	0.565	0.569
Serum glucose, mmol/L		5.8 ± 2.11	5.47 ± 1.28	5.48 ± 1.66	0.909	0.404
TG, mmol/L		1.72 ± 0.98	1.93 ± 1.41	1.86 ± 1.57	0.467	0.627
TC, mmol/L		4.53 ± 1.23	4.42 ± 1.25	4.58 ± 1.43	0.320	0.726
HDL-C, mmol/L		1.1 ± 0.22	1.14 ± 0.22	1.47 ± 2.16	1.849	0.160
LDL-C, mmol/L		2.84 ± 0.97	2.89 ± 0.88	2.75 ± 0.92	0.450	0.638
Serum uric acid, μmol/L		305.17 ± 86.15	315.3 ± 80.42	306.98 ± 86.05	0.298	0.742
Homocysteine, μmol/L		15.03 ± 6.7	14.24 ± 5.99	13.84 ± 6.18	0.720	0.488

ALP, alprazolam; BC, Bailemian capsule; TG, triglyceride; TC, cholesterol; HDL-C; LDL-C.

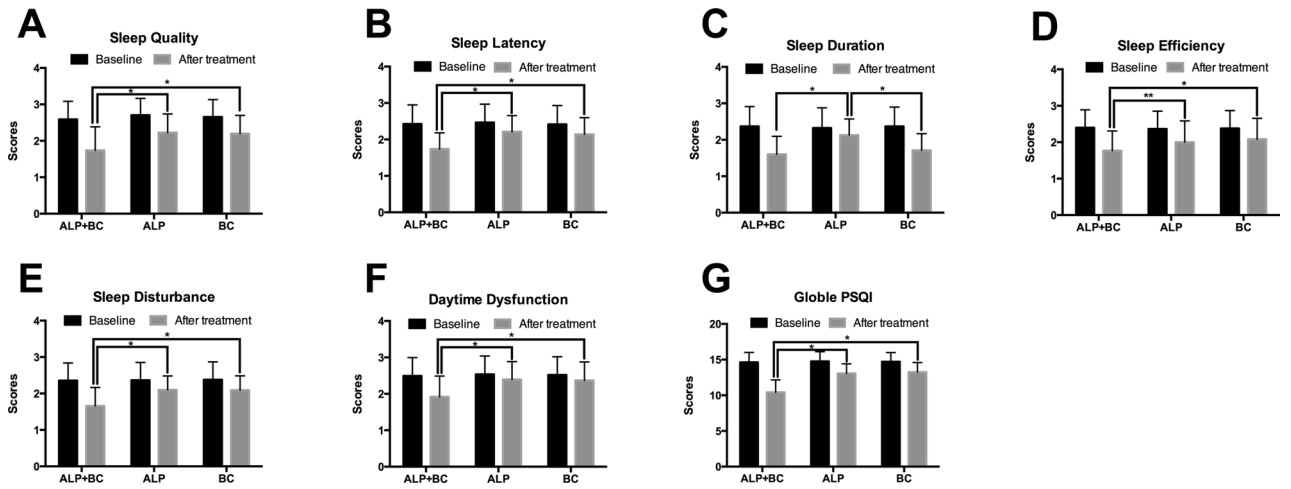

FIGURE 1 | Effects of alprazolam and Bailemian on PSQI components. **(A)** Sleep quality, **(B)** sleep latency, **(C)** sleep duration, **(D)** sleep efficiency, **(E)** sleep disturbances, **(F)** daytime dysfunction, and **(G)** global score were measured according to the PSQI. *$P < 0.01$, **$P < 0.05$. PSQI, Pittsburgh Sleep Quality Index; ALP, alprazolam; BC, Bailemian capsule.

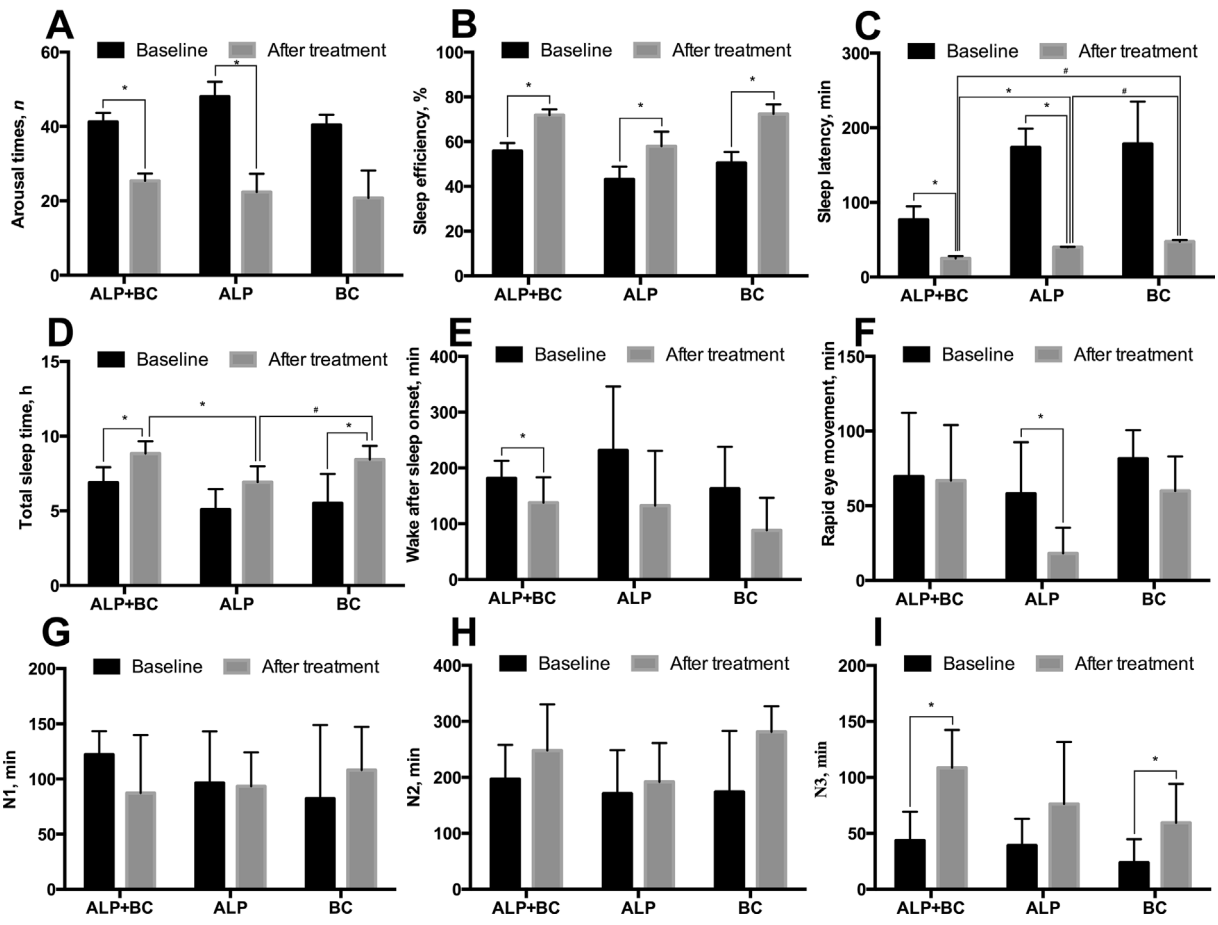

FIGURE 2 | Effects of alprazolam and Bailemian on the quality of sleep in patients tested by polysomnography (PSG). **(A)** Arousal times (total number across the night), **(B)** sleep efficiency, **(C)** sleep latency, **(D)** total sleep time, **(E)** wake after sleep onset, **(F)** duration of rapid eye movement, and duration of non-rapid eye movement sleep and its three stages **(G)** N1, **(H)** N2, and **(I)** N3 were measured and compared. *$P < 0.05$, #$P < 0.01$. ALP, alprazolam; BC, Bailemian capsule.

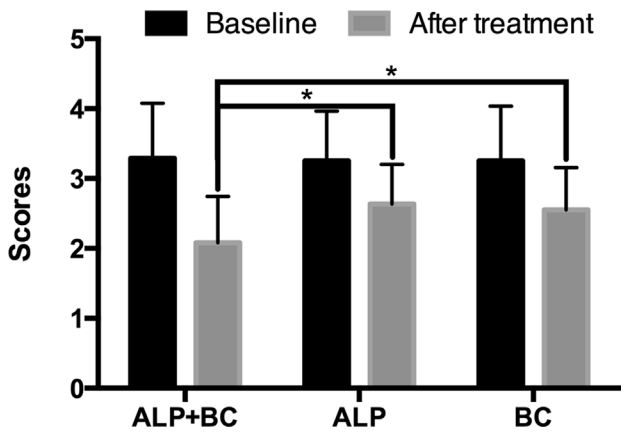

FIGURE 3 | Effects of alprazolam and Bailemian on stroke outcome assessed by the modified Rankin Scale (mRS). *$P < 0.001$.

DISCUSSION

The present study examined the therapeutic effect of ALP, BC, and the combined effect of ALP and BC on improving the sleep quality and self-care ability in patients with PSI. The results revealed that the oral administration of ALP and BC alone significantly but differentially improves the subjective perception of sleep quality by PSQI and objective sleep quality by PSG. However, the combined effect of ALP and BC is better than that of ALP or BC alone.

Next, compared with the baseline, ALP + BC administration had a significantly greater effect on sleep latency, quality, disturbance, and efficiency than did either ALP or BC alone, which was consistent with objective PSG and subjective PSQI. In addition, in comparison with the baseline after the use of BC or ALP, PSQI data showed that all items of PSQI were improved. Surprisingly, after 3 weeks of treatment, among the 15 patients ($n = 5$ in each group), the PSG data showed that ALP improved the sleep efficiency and decreased the arousal times, sleep latency, and REM sleep, while BC improved the sleep efficiency, total sleep time, and duration of N3 significantly. This discrepancy between subjective and objective data might be attributed to the smaller sample size of PSG patients than PSQI patients as well as the minor differences in the areas of sleep improvement targeted by ALP and BC therapies.

With the improvement in insomnia, patients' self-care ability has also been enhanced effectively. A previous study demonstrated that sleep disorders could impact the daytime functioning and quality of life (11), which was confirmed by improved daytime dysfunction of PSQI after treatment. Furthermore, the self-care ability can be enhanced by improving the nervous system and immune system and alleviating the basic diseases such as hypertension and diabetes (6, 7). Thus, the mechanism underlying the improvement of insomnia symptoms and self-care ability after stroke needs to be explored further along with the start time and course of treatment for PSI.

BC is a traditional Chinese patent medicine, composed of 15 Chinese herbal extractions. *Acanthopanax senticosus*, one of the main components of BC, plays an anxiolytic role *via* the regulation of autonomic function and increases the signal of the hippocampus *via* brain-derived neurotrophic factor (BDNF) (27). Semen Ziziphi spinosae (*Suanzaoren* in Chinese) and Radix et Rhizoma Salviae miltiorrhizae (*Danshen* in Chinese), the other two major components of BC, are well-known conventional herbal drugs in traditional Chinese medicine and have been used widely for the treatment of insomnia (28). The main compounds of Semen Ziziphi spinosae include saponins, swertisin, and fatty oils (29). The oral administration of spinosin and swertisin prolongs the sleeping time *via* a serotonergic mechanism (30). Moreover, the pharmacological activities of Radix et Rhizoma Salviae miltiorrhizae exert anti-inflammatory and cardioprotective effects, rendering it preferable for the treatment of insomnia coupled with inflammation or cardiovascular diseases (28). Polygalae Radix, also a main component of BC, is primarily used for treating insomnia and depression (31). Recent pharmacological studies in animals have demonstrated that the constituents of Polygalae Radix can improve cognition and potentially exert antipsychotic, antioxidant, and anti-inflammatory effects (31). Taken together, BC might improve the total sleep time of PSI through multiple targets and mechanisms.

In consideration of the mild and slow synergistic effects of the various herbal components, which are not conducive to inducing sleep directly, low-dose ALP plays a complementary but vital role and effectively reduces the latency of sleep. Also, only fewer side effects are observed in the low-dose ALP.

Nevertheless, the present study has several limitations: 1) The size of the sample is small, and the variability of the individual result is high. 2) It is a retrospective study, lacking long-term follow-up data for some patients. Thus, further randomized controlled trials are essential to clarify the efficiency and mechanism of BC on PSI. 3) A comprehensive evaluation of the dose and treatment duration of BC is absent in this study, and thus, future randomized controlled trials are needed to address these issues. 4) Although the evidence of using ALP in PSI is insufficient, it is still widely used in clinics due to its cost-efficiency. In recent years, with the improving economy, the use of eszopiclone has been increasing gradually. Thus, choosing eszopiclone as control and combination drug would seem to be a viable alternative.

ETHICS STATEMENT

This study was approved by the ethics committee of The General Hospital of Western Theater Command, and received informed consent of patients and their families and signed informed consent.

AUTHOR CONTRIBUTIONS

QW designed the study and made critical revision of the article; JW and ZW collected data and drafted the article; XW analyzed and interpreted the data; GD, BZ, and YL collected data.

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REVIEW
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Sleep Disturbance in Bipolar Disorder: Neuroglia and Circadian Rhythms

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The worldwide prevalence of sleep disorders is approximately 50%, with an even higher occurrence in a psychiatric population. Bipolar disorder (BD) is a severe mental illness characterized by shifts in mood and activity. The BD syndrome also involves heterogeneous symptomatology, including cognitive dysfunctions and impairments of the autonomic nervous system. Sleep abnormalities are frequently associated with BD and are often a good predictor of a mood swing. Preservation of stable sleep–wake cycles is therefore a key to the maintenance of stability in BD, indicating the crucial role of circadian rhythms in this syndrome. The symptom most widespread in BD is insomnia, followed by excessive daytime sleepiness, nightmares, difficulty falling asleep or maintaining sleep, poor sleep quality, sleep talking, sleep walking, and obstructive sleep apnea. Alterations in the structure or duration of sleep are reported in all phases of BD. Understanding the role of neuroglia in BD and in various aspects of sleep is in nascent state. Contributions of the different types of glial cells to BD and sleep abnormalities are discussed in this paper.

Keywords: astroglia, microglia, oligodendroglia, bipolar disorder, depressive behavior

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INTRODUCTION

Bipolar disorder (BD) is a recurrent disorder that affects in excess of 1% of the world population and usually has its onset in young age. The resulting cognitive deficits, the high risk of suicide, and the occurrence of severe psychiatric and medical comorbidities all make BD one of the major causes of mortality and disability worldwide (1). The concept of BD was introduced at the end of the 19th century by Emil Kraepelin (2) who referred to this disorder as "manic depressive insanity." About 70 years later, the term "bipolar" was proposed to indicate the condition in which both depression and mania, the opposite poles of mood, alternate in the course of the illness (3). In modern psychiatry, BD is conceptualized as a cyclical mood disorder involving episodes of mania, hypomania, and alternating or intertwining episodes of depression. The last edition of the *Diagnostic and Statistical Manual of Mental Disorders* (DSM-5) categorizes clinical features of BD according to severity (4). Classical BD type I is identified by the occurrence of major depression and full-blown manic episodes, whereas in BD type II, depression is more prominent, with interspersed episodes of less severe manic symptoms, classified as hypomanic episodes. However, despite this general description, the clinical presentation of BD is polymorphic with regard to symptomatology, progression, efficacy of therapies, and functional outcome. Consequently, the DSM-5 introduces additional specifics for diagnosis, such as BD "with mixed features," or "with rapid cycling," or "with melancholic features,"

or "with mood congruent or incongruent psychotic features," to mention only a few. Far from being a discrete diagnostic entity, there is increasing recognition of a spectrum of BDs that ranges from marked and severe mood disturbance into milder mood variations (5). In this context, "cyclothymia" is the term assigned to recurrent hypomanic episodes and subclinical episodes of depression. It represents a subsyndromal condition, although mood disturbance is a continuing problem and interferes with everyday functioning (5). Moreover, unlike previous versions that included BD along with all other mood disorders, DSM-5 now assigns a separate chapter to BD and places it between depressive disorders and the spectrum of schizophrenia and other psychotic disorders. The rationale for this new diagnostic taxonomy stems from the assumption that BD could be considered as a bridge that, in terms of genetics, familiarity, and clinical picture, holds together the other two pathologies, sharing some clinical aspects of both.

Despite numerous studies performed in recent decades, little is known about the etiopathogenetic mechanisms responsible for the BD. The most recent research is focusing on the possible biologic mechanisms underlying the disorder, including genetic components, neurochemical abnormalities, and morphostructural brain differences, along with psychosocial factors, such as life experience and social environment context (6). Hitherto, there is no sufficient explanation to account for the pathobiology of such a multiform condition while the disease heterogeneity prompts us to contemplate multifactorial genesis. Indeed, no single paradigm can explain the occurrence and the variability in course and severity of manic-depressive disorder. Because the key phenotype of BD is a biphasic dysregulation in mood, behavior and sleep remain of great interest and could help expand the understanding of pathogenic mechanisms.

Sleep has a critical significance in the regulation of mood, and sleep disturbances can be seen in BD primarily or because of BD itself (7). These alterations have been linked to a lower quality of life, suicide attempts, poorer clinical and cognitive functioning, and higher relapse rates of mood episodes (8).

SLEEP DISORDERS AND BIPOLAR DISORDER: EPIDEMIOLOGY

The "sleep disorders" are defined as every significant alteration of quality of sleep, timing, and quantity, with different adverse impacts on function and quality of life (9). Sleep disturbances are very common in the general population (10). The prevalence of symptoms of sleep disorders range between 41% and 52% worldwide, with the most widespread symptoms being insomnia, followed by excessive daytime sleepiness, nightmares, difficulty falling asleep or maintaining sleep, poor sleep quality, sleep talking, sleep walking, and obstructive sleep apnea (11).

Sleep disorders also have a high prevalence in the psychiatric population. Furthermore, sleep disturbances exert a negative impact on the course and treatment of every psychiatric illness, and aberrant sleep represents a core symptom of BD. For example, 23% to 78% of patients with BD have reported symptoms of hypersomnia (10). The circadian rhythm hypothesis of BD postulates that variability of the circadian rhythms represents a critical step in BD evolution, whereas disturbances in circadian rhythms are considered a core element for the onset and progress of BD (12, 13). It is universally acknowledged that the increased risk of suicidal ideation and manic switch is linked to insomnia (14, 15).

Sleep disturbances are frequent in BD patients in different phases of illness, including the euthymic state (16) and remission (17). These sleep aberrations are represented not only by insomnia but also by sleep–wake rhythm disorders, especially delayed sleep–wake phase disorders (18–20) albeit the disturbance pattern can change with the specific mood phase. During the manic state, most patients (66–99%) experience a reduced need for sleep (21–23) and longer sleep onset latency (7), and *vice versa* sleep deprivation is well known as a trigger factor for manic episodes (24). Likewise, in the depressive state, insomnia (40–100%) and hypersomnia (23–78%) are commonly observed (25–27). A prevalence of 32.4% of circadian rhythm sleep–wake disorders (CRSWD) was found in a sample of 127 patients affected by BD type I or II, whereas younger onset age of BD and family history of suicide were associated with CRSWD in BD patients (28). Meta-analyses of trials conducted on remitted BD patients demonstrated prolonged total sleep time, increased awakenings after sleep onset, greater variability of sleep–wake variables, and reduced sleep efficiency (16, 29).

Overall, all kinds of sleep disorders and parasomnias are very common especially in youth patients with BD (30). Thus, compared to the general population, youth with BD exhibit lower sleep efficiency, longer slow wave sleep, and reduced REM sleep, features that could affect the genesis and prognosis of the disorder (7, 31). Sleep disturbances may also be used as predictors of the onset of BD in a subset of high-risk young subjects (32).

CIRCADIAN RHYTHMS AND BIPOLAR DISORDER

Several types of rhythms rule the human body. Based on the approximate duration, these rhythms can be classified as circadian (about of 24 h), infradian (of longer duration), and ultradian (of shorter length). Temporal organization of physiological, cellular, organ, biochemical, and behavioral processes is controlled by circadian clocks (33).

Endogenously generated circadian rhythms are tuned by and adapted to the environment so that the body is able to synchronize the internal time with the geophysical time. The clock system captures exogenous time signals, called "zeitgebers," which include the day/night (or light/dark) cycle, temperature, and food intake (33). Environmental information is processed by a central clock, which is located in the anterior region of the hypothalamus, in the suprachiasmatic nuclei (SCN) (34). The central clock receives light and dark information from the visual input through the retino-hypothalamic tract; increased levels of light elevate alertness whereas decreased levels of light reduce sleep latency (35, 36). The processed information is transmitted to the peripheral clocks and to other clocks in the brain (located in other hypothalamic nuclei, thalamus, amygdala) to synchronize all

individual endogenous rhythms (33, 37). The stable relationship between internal rhythms and the external environment is ensured by exposure to a normal light–dark schedule (**Figure 1**) (36). Lack of coordination between the endogenous circadian system and the sleep/wake cycle is a critical factor in the clinical status of illness associated to the disruption of the circadian timing of sleep and the alteration levels of alertness, vigilance, and performance (36, 38). In pathological conditions, the SCN and peripheral clocks lose their normal phase relationship, and thus, a state of internal desynchronization develops that, if sustained, may predispose individuals to a disease (36). The SCN received multiple feedbacks from the periphery that include information regarding metabolic status and the levels of activity (39).

Various pathological conditions are associated with sleep and circadian disturbances, including allergies, hypothyroidism and hyperthyroidism, coronary artery disease, congestive heart failure, diabetes, arthritis, asthma, gastroesophageal reflux disease, and chronic pain (40). Disorderly circadian system contributes to the etiology and progression of major psychiatric disorders (38, 41, 42). About three-quarters of individuals with delayed sleep phase syndrome have a past or current history of depression, whereas depression severity correlates with circadian misalignment (43). Patients with different psychiatric conditions, such as anxiety disorders and schizophrenia, often show circadian deregulation contributing to major functional impairments (44).

Sleep disturbances are common in BD with a great variability in sleep duration (45). The decreased need for sleep predicts the onset of a manic or hypomanic episode the following day (46), whereas sleep extension occurs frequently in the depressive episode (13, 16, 47, 48). The disruption in sleep–awake cycle tends to precipitate or exacerbate mood episodes (49), and they are risk factors for the recurrence of a mood episode (**Figure 2**) (50). Sleep deprivation was also found to induce manic episodes in animal BD models (51, 52). Loss of sleep confers a poor prognosis, increasing the risk of suicide in patients with a suicide attempt history (53). Even in euthymia, sleep alterations occur in BD patients (16). Given all this evidence and based on the rhythmic nature of BD, it has been suggested that the endogenous circadian system may play a role in BD etiology, clinical manifestations, and outcome (42, 54).

The activity of a group of clock genes governs the generation of circadian rhythms. There are molecular positive and negative transcriptional/translational feedback loops that drive the expression of different genes to stabilize 24-h periodicity (55). Several of these clock genes have been linked directly to the abnormal sleep/circadian phenotypes (36). Mutations of any of these circadian genes can potentially have an impact on the circadian clock and thus subtly or dramatically alter sleep, mood, or behavior in ways that contribute to physical and mental illness, and indeed many circadian genes have been associated with BD (56). The strongest evidence for genetic abnormalities is associated with polymorphisms of clock genes and an increased

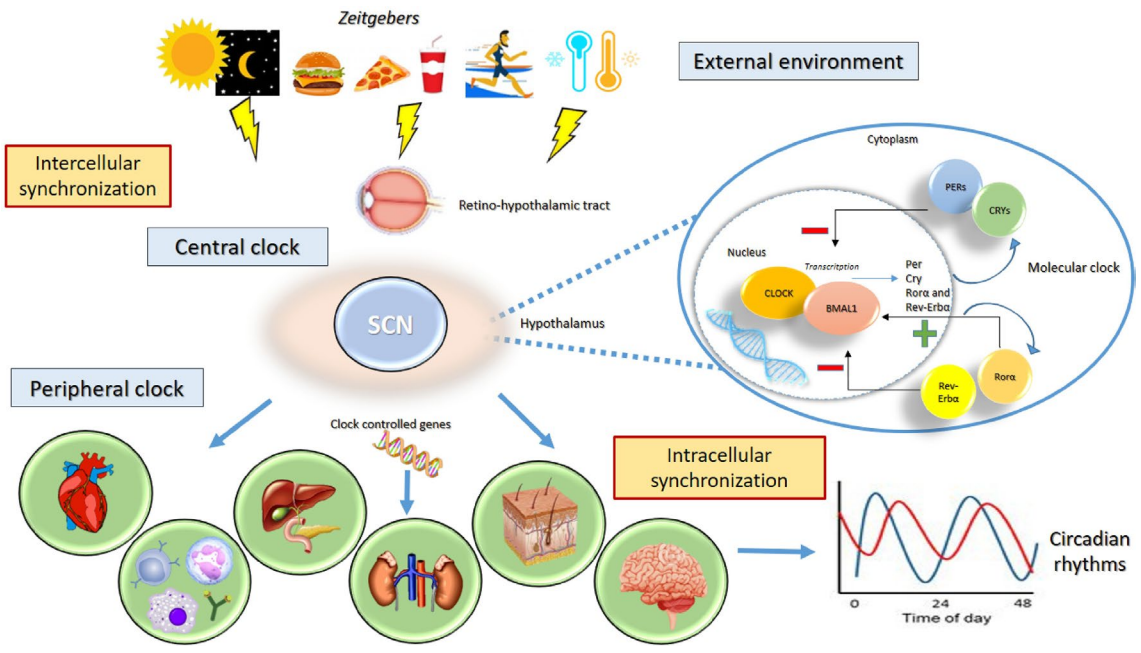

FIGURE 1 | Circadian rhythms. The clock system captures exogenous "zeitgebers" (light/dark cycle, temperature, exercise, food intake) and triggers the central clock in the suprachiasmatic nucleus (SCN) of the hypothalamus through the retino-hypothalamic tract. The activity the group of clock genes governs the generation of circadian rhythms. The genes CLOCK and ARNTL encode the transcription factors CLOCK and ARNTL, which together activate the transcription of Per, Cry, RORα, and REV-ERBα genes. The proteins PER1, PER2, PER3, CRY 1, and CRY 2 combine to inhibit their own transcription, whereas RORα and REV-ERBα act on ARNTL to activate and inhibit transcription, respectively. The processed information is transmitted to the peripheral clocks and to other clocks in the brain to stabilize 24-h periodicity. The stable relationship between internal rhythms and the external environment is needed to ensure the synchronization of individual endogenous rhythms.

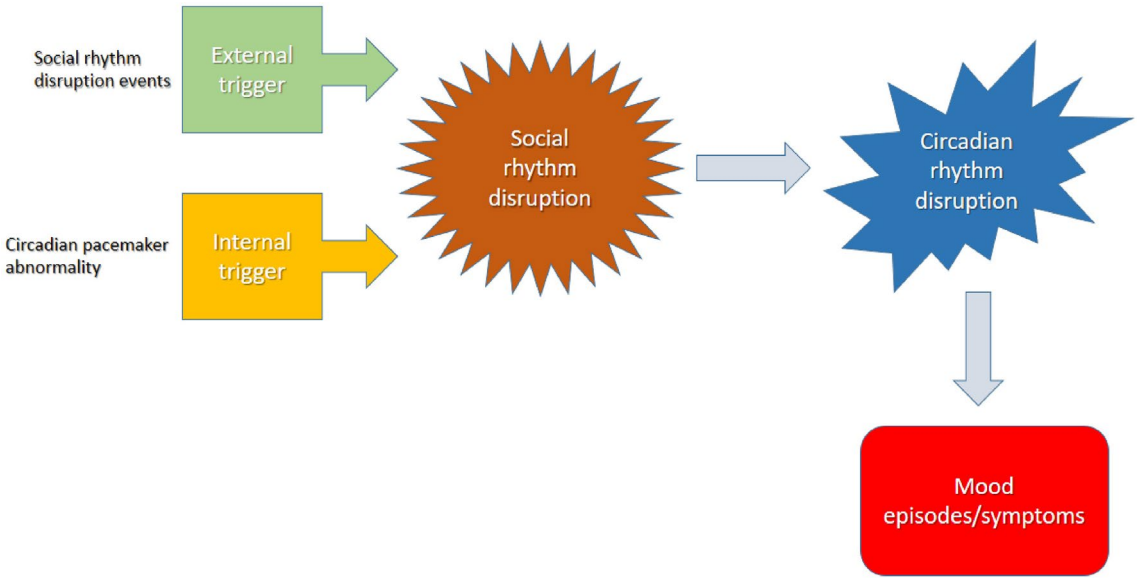

FIGURE 2 | The disruption in sleep–awake cycle ends to precipitate or exacerbate mood episodes.

susceptibility to BD (57). In humans, genetic association studies of patients with BD have suggested that polymorphisms in the period gene (PER) is linked to specific phenotypes, such as a good lithium responder (36).

Particularly, a variant of PER3 gene has been linked to different chronotypes. The PER3 gene contains a variable number tandem repeat polymorphism, in which a 54-nucleotide coding-region segment is repeated four ($PER^{4/4}$) or five ($PER^{5/5}$) times. The long allele variant of $PER^{5/5}$ has been linked to extreme morning chronotypes, whereas the shorter allele $PER^{4/4}$ is associated with extreme eveningness and delayed sleep phase syndrome (58). Incidentally, the early onset in BD is associated with the longer allele ($PER^{5/5}$), whereas the later onset is associated with the shorter allele ($PER^{4/4}$) (36) (**Table 1**).

TABLE 1 | Main polymorphism of clock genes and their association in bipolar disorder.

Clock gene	Polymorphism	Association found in bipolar disorder	References
PER3	rs57875989	Extreme morning and early onset Extreme eveningness and later onset	Dijk and Archer (58)
PER 2	rs934945	Diurnal preference for eveningness	Song et al. (59)
CLOCK	rs10462028	Association with BD aetiology	Nievergelt et al. (60) Dmitrzak-Weglarz et al. (61) Soria et al. (62)
	rs11932595	Sleep disorders	Maciukiewicz et al. (63)
	rs1801260	Early stress, history of suicide attempt and persistence of the idea of suicide	Benedetti et al. (64) Bollettini et al. (65)
	rs11932595	More depressive episode and appetite disorder	Maciukiewicz et al. (63)
	rs1801260	Influence on sleep pattern, daytime preference, age at onset, and response to treatment	Bollettini et al. (65)
ARNTL (BMAL1)	rs2279287	Seasonal pattern	Geoffroy et al. (66)
	rs1481892	Higher susceptibility to the disease	Rajendran and Janakarajan (67)
	rs1982350		
TIMELESS	rs2291738	Suicide attempts	Pawlak et al. (68)
	rs10876890	Insomnia	
PPIEL*	Lower methylation level	Altered dopaminergic transmission or neuroendocrine system functions	Kuratomi et al. (69)
NR1D1 promoter	rs2071427	Good response to treatment	McCarthy et al. (70)
CRY1	rs8192440	Good response to treatment	McCarthy et al. (70)
GSK-3β	rs6438552	Robust and additive response to treatment if associated with NR1D1 (rs2071427)	Oliveira et al. (71)

*ARNTL (BMAL1), Aryl hydrocarbon receptor nuclear translocator like protein-1; CRY 1-2, Cryptochrome circadian regulator; GSK-3β, Glycogen synthase kinase-3; NR1D1, Nuclear receptor subfamily 1 group D member 1; PER3, Period circadian regulator-3; PPIEL, E-like peptidilprolil isomerase; TIMELESS, Timeless Circadian Clock. *pseudogene.*

One of the consequences of sleep/circadian disruption is an abnormality in the stress axis, with particular emphasis on atypical neurotransmitter release. The hypercortisolemia can arise from a breakdown in glucocorticoid receptor-mediated negative feedback mechanisms in the hypothalamic–pituitary–adrenal (HPA) axis (36). Circadian disturbances, such as a phase advance of the diurnal rhythm of plasma melatonin (72) and plasma cortisol (73), have been observed in BD, although these were not universally confirmed (74). In relation to oxidative stress, circadian rhythm disturbance was associated with increased lipid peroxidation in BD (75). Studying alteration of the wake–sleep rhythm may provide yet unknown insights into the pathophysiology of BD.

NEUROGLIA IN BIPOLAR DISORDER AND SLEEP DISORDERS

Neuroglia: An Overview

Neuroglia represent the homeostatic and defensive arm of the nervous system; neuroglial cells of the central nervous system (CNS) are classified into astrocytes, microglia, and oligodendrocytes and their precursors, also known as NG2 glia (76). The functions of neuroglia are diverse; these nonexcitable cells are indispensable companions of neurons, supporting them in physiology and protecting them against pathological lesions. Astrocytes are the main homeostatic cells of the CNS, which control the homeostasis of the nerve tissue at all level of organization from molecular to organ (77, 78). Astroglial perisynaptic processes cover synaptic contacts and form synaptic cradle, which through various mechanisms control synaptogenesis, synaptic maturation, synaptic maintenance, and synaptic extinction (79). Microglial cells invade the neural tube early in development and are fundamental for early shaping of neuronal connections by synaptic stripping (80). Finally, oligodendrocytes support and protect axons and provide for gray and white matter myelination, which supports brain connectome (81). The fundamental role of neuroglia in neuropathology has been considered by many prominent neuroanatomists (including Santiago Ramon y Cajal, Alois Alzheimer, Nicolas Achucarro, and Franz Nissl, to name a few) a century ago. The recent decade has witnessed the revival of interest to pathological potential of neuroglia, challenging universally accepted neurono-centric neuropathological doctrine (82–86).

Pathological Classifications of Neuroglia

Conceptually, neuroglial cells contribute to all neurological diseases either as primary elements driving pathology or by responding to lesions through an evolutionary conserved defensive program of reactive gliosis. Neuroglial changes in pathological conditions are context- and disease-specific, are complex, and evolve through the stages of neuropathology. Astrogliopathology in particular is subclassified (86) into i) reactive astrogliosis, which represents a graded response to various types of lesions.Reactive astrogliosis is fundamentally neuroprotective and produces a wide spectrum of reactive phenotypes that are disease- and disease stage-specific (84, 86–90); ii) pathological remodeling of astrocytes—when astrocytes acquire new properties driving neuropathology, Alexander disease (91) being a signal example; and iii) astroglial atrophy and loss of function. Similarly, microglial cells in pathology assume a multitude of phenotypes with various degrees of activation with both neuroprotective and neurotoxic functions. In chronic pathologies, microglial cells often undergo degeneration that limits their defensive capabilities (92, 93) or pathological remodeling (94). Pathological classification of oligodendrocytes is yet to be produced.

Neuroglial Abnormalities in Psychiatric Disorders

Neuroglial abnormalities are widely manifested in all major psychiatric diseases; and they are particularly prominent in bipolar disease and in major depression (95–98). In contrast to many other neuropathologies, there are no signs of astroglial reactivity in BD (as well as in other major psychiatric diseases); instead, astrocytes demonstrate prominent atrophy and asthenia, which most likely is associated with loss of homeostatic and supportive functions that in turn underlie failures in information processing and neurotransmission. Already in early stereological studies using Nissl staining (that revealed a total glial population), a prominent decrease in the overall number of neuroglial cells has been described in human postmortem samples from both major depressive disorder and BD (99). Subsequent morphometric studies have confirmed a significant reduction in glial numbers (up to 20–40%) in relevant brain regions (including the prefrontal cortex, orbitofrontal cortex, subgenual cortex, anterior cingulate cortex, and amygdala) in BD and major depression (95, 100–104). The expression of glial fibrillary acidic protein (GFAP), the marker of astroglial reactivity, which reveals the cytoskeleton of astrocytes, is generally suppressed in brain samples from young or adult subjects with depression and BD (105–107). In older subjects, GFAP expression was sometimes increased, which reflects general age-dependent changes or neuroinflammatory changes (105). Very significant (up to 95%) decrease in GFAP expression and GFAP-positive astroglial profiles have been recently detected in the white matter of the ventral prefrontal cortex of subjects with major depression (108). Impairment of astroglial networks and aberrant signaling in astroglial syncytia were evidenced by a significant decrease in the expression of major astroglial connexins XC30 and Cx 43 in the prefrontal cortex of depression-associated suicide victims (109). Major depression (but not BD) was found to be associated with a significant decrease in the density of astrocytes expressing glutamine syntethase and with downregulation of astroglial expression of glutamate transporter GLT-1, suggesting thus aberrant operation of glutamine–glutamate shuttle (110, 111). Likewise, the population of S100B-positive astrocytes was decreased in hippocampi of patients with BD and major depression (112).

Similar reduction in glial numbers and GFAP expression and astroglial morphological profiles have been detected in animal models of depressive behavior. These models are often based on exposure of animals to various types of chronic stress that

instigate depressive-like behavior manifested by anhedonia or aberrant social communications. The density of GFAP-positive astrocytes and morphological astroglial profiles were reduced after the stress of separating juveniles from their family (113), chronic social defeat (114), or chronic mild stress (115), which induces prominent morphological atrophy of astroglial cells (116). Astroglial atrophy in chronic stress animal models may be associated with aberrant glycogen processing and decreased glycogen content (117). Significant astroglial atrophy was also observed in the repeated corticosterone injection-induced mouse depression model (118). Likewise, the density of astrocytes was significantly reduced in the prefrontal cortex, anterior cingulate cortex, amygdala, and hippocampus of Wistar–Kyoto strain of rats susceptible to depressive-like behavior (119). Chronic stress-induced astroglial asthenia and loss of function, as well as depressive behavior, were reversed by treating animals with riluzole, the drug that limits glutamate excitotoxicity (115). Selective ablation of astrocytes after injection of L-α aminoadipic acid into either rodent prefrontal cortex or prelimbic cortex triggered depressive-like behavior (120, 121); injection of neuronal toxin ibotenate had no such an effect (120). Emergence of depressive phenotype is associated with astroglia-specific decrease of expression of several genes associated with signaling systems, including serotonin 5-HT_{2B} receptors, cytosolic phospholipase 2α, ionotropic kainate receptor GluK2, and adenosine deaminase acting on RNA 2 (ADAR2); treatment with fluoxetine restored altered expression (122, 123). The chronic stress-induced depressive phenotypes were also linked to a downregulation of astroglial expression of multiple endocrine neoplasia type 1 gene encoding protein menin; the efficiency in menin was associated with increased activation of NF-κB activation and elevated production of IL-1β (124). Depression after traumatic brain injury was associated with a decrease in astroglial expression of glutamate transporters (125), this being another example of astroglial asthenia with loss of function. All in all, these data underlie the hypothesis of the role of astroglial asthenia in the pathophysiology of mood disorders, including BD (97, 126, 127).

Astrocytes are recognized as therapeutic targets for the treatment of psychiatric disorders and, in particular, depression and BD (128–130). Treatment of animals subjected to psychosocial stress prevented the loss of astrocytes (114), whereas riluzole (the drug that limits glutamate excitotoxicity) similarly prevented loss of astrocytes in animals subjected to mild chronic stress (115). Even electroconvulsive therapy (ECT) has been shown to increase the expression of GFAP in the piriform cortex, amygdala, and hippocampus (131). Recent findings identified astrocytes as primary targets for transcranial direct current stimulation used for the management of depression (132). Moreover, it has been documented that two classical mood stabilizers used as first-line therapy for BD, lithium (Li^+) and valproic acid (VPA), have a neuroprotective role reducing neuroinflammation through modulating the activation of astrocytes (133). Chronic treatments of astrocytes *in vitro* with Li^+, VPA, and another classic antidepressant, carbamazepine (CBZ), suppress glutamate release, thus contributing to alleviation of excitotoxicity (134). Long-lasting exposure of astrocytes to antidepressant fluoxetine, a selective serotonin reuptake inhibitor, increased cytosolic pH from 7.18 to 7.58 by stimulating sodium-proton transporter 1, thus affecting brain pH homeostasis (135). Fluoxetine, as well as Li^+, VPA, and CBZ, also affects astroglial glycogen content in a concentration-dependent manner, increasing glycogen at low concentrations and decreasing at high concentrations—this action being mediated by caveoline-1 (Cav-1) - phosphatase and tensin homologue (PTEN) - phosphoinositide 3-kinase (PI3K) - glycogen synthase kinase 3 (GSK-3β) cascade (**Figure 3**) (136, 137). These multiple actions of fluoxetine on astrocytes are mediated through direct activation of serotonin 5-HT_{2B} receptors and transactivation of epidermal growth factor receptor (EGFR) (138, 139). Chronic treatment with antidepressants, as well as stimulation of adrenoceptors, was also reported to stimulate astroglial secretion of brain-derived neurotrophic factor (BDNF), which may boost synaptic transmission and provide neuroprotection (140, 141).

Analysis of lipopolysaccharide (LPS)-induced inflammation in rat primary mixed (80% astrocytes and 15% microglia) glial cultures found that Li^+ decreases the secretion of TNF-α, IL1-β, prostaglandin E2, and nitric oxide (142). Pretreatment of LPS-stimulated microglial cells with Li^+ significantly inhibited LPS-induced microglial activation and proinflammatory cytokine production (143). Similarly, VPA modulates microglial response to inflammatory insults mediated by LPS and may affect the synaptic excitatory inhibitory balance through its effect on astrocytes in rats (144, 145).

ASTROCYTES AND SLEEP REGULATION

The role of astroglia in the regulation of sleep has been suggested more than a century ago by Santiago Ramon y Cajal, who suggested that astroglial processes, by entering the synaptic cleft, may slow down communication in neuronal networks, thus instigating sleep (146); a very similar mechanism was also considered by Carl-Ludwig Schleich (147) as a basis for general anesthesia.

Astrocytes of the suprachiasmatic nucleus do contain clock genes and do produce circadian rhythms of GFAP expression; astrocytes, in addition, may contribute to timekeeping through regulating glutamate levels (148). Nonetheless, it seems that the major role of astrocytes is the regulation of sleep homeostasis. The latter refers to a regulation mechanism that increases urge to sleep proportionally to the time spent awake (149). Sleep homeostasis is regulated by accumulation of adenosine in the brain during wakefulness (150), and the data accumulated demonstrated that the main source for adenosine in the physiological conditions is associated with astrocytes (151). Another important role of astroglia in sleep is associated with cleansing the brain parenchyma (152). It is, therefore, plausible to speculate that astroglial asthenia observed in mood disorders and in BD impairs astroglial sleep-regulating capabilities.

SLEEP, ASTROGLIA, AND BIPOLAR DISORDER

As has been mentioned above, sleep plays a key role in the clinical manifestations of BD. Alterations in the structure or

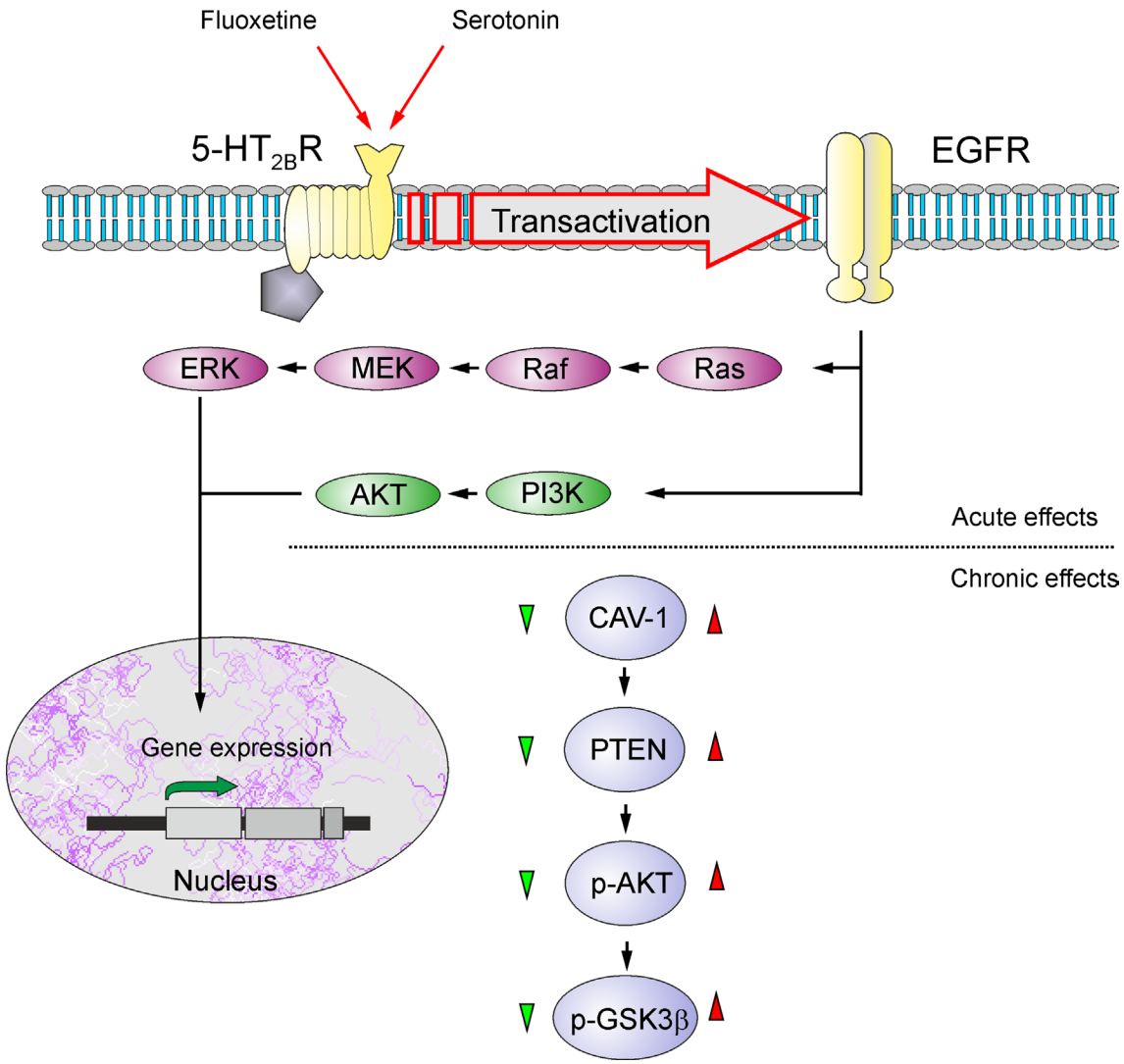

FIGURE 3 | Schematic illustration of biphasic concentration-dependent regulation of Cav-1 gene expression and GSK-3β activity by fluoxetine in astrocytes. Acute treatment with fluoxetine stimulates 5-HT_{2B} receptors, induces transactivation of EGF receptors and activates its MAPK/ERK and PI3K/AKT signal pathways that, in turn, regulate gene expression in astrocytes. Chronic treatment with fluoxetine at low concentrations (green triangle) decreases Cav-1 gene expression, which decreases membrane content of PTEN, induces dephosphorylation and inhibition of PI3K and elevates GSK-3β phosphorylation thus suppressing its activity. At higher concentrations (red triangle) fluoxetine increases Cav-1 gene expression that acts on PTEN/PI3K/AKT/GSK-3β in an inverse fashion. Reproduced from Ref. (139).

duration of sleep are reported in all phases of the disorder—in the manic, depressive, and euthymic phases (65). During manic or hypomanic episodes, there is a reduced need for sleep, whereas during depressive episodes, there may be difficulty in achieving adequate quality or amount of sleep or, alternatively, patients experience hypersomnia (12, 153). Sleep abnormalities are strongly associated with immune dysfunction. Aberrant sleep is associated with increased levels of proinflammatory cytokines with a bidirectional causal association identified (154, 155). As such, interest has grown in immune dysfunction as a potential link that underwent two-way interaction between sleep dysfunction and BD (156, 157). Both postmortem and *in vivo* studies showed that microglial activation is involved in the neurobiology of BD (158, 159). These findings agree with the presence of peripheral inflammatory markers and the blood–brain barrier disruption revealed by meta-analyses. If as it seems it is true that modifications of inflammatory markers and microglial function may play an important role in progression of BD, several drugs used in the treatment of this disorder could have effects on glial cells, and future studies may use these cells as targets for the development of new treatments in this way (160, 161).

CONCLUSION

Sleep disturbances are common in patients with BD; these sleep alterations are present even during euthymia, as insomnia, increased sleep latency, and variability in sleep hours. Recent

research has sought to identify the biological markers that underlie sleep disorders in patients with BD. The focus of the latest studies has highlighted the role for neuroglial cells. Astrocytes, the primary homeostatic cells of the CNS, undergo atrophy, asthenia, and loss in BD-specific brain regions, and deficiency in glial support and neuroprotection may have a key role to the pathophysiology of BD (84, 160), even though the precise mechanisms need to be further explored and clarified. Several drugs used for the treatment of BD have specific effects on glial cells indicating neuroglia as a target for the development of new treatments. Further research should concentrate on investigations of glial cells *in vivo* and in "humanized" preparations to increase our understanding of the role of glia in sleep regulation in people with BD. Additional systematic studies are also needed to highlight the importance of sleep disorders in patients with BD to offer a tailor-made treatment for these patients.

AUTHOR CONTRIBUTIONS

LS, AV conceived the manuscript. LS, AV, RF, EC wrote the manuscript. LS, AV, CS-G, PDF edited the text and supervised the paper. The manuscript was critically revised and finally approved by AV, LS, CS-G and PDF. LS and AV coordinated the work.

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High Exploratory Phenotype Rats Exposed to Environmental Stressors Present Memory Deficits Accompanied by Immune-Inflammatory/Oxidative Alterations: Relevance to the Relationship Between Temperament and Mood Disorders

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Low-exploratory (LE) and high-exploratory (HE) rodents mimic human depressive and hyperthymic temperaments, respectively. Mood disorders (MD) may be developed by the exposure of these temperaments to environmental stress (ES). Psychiatric symptoms severity in MD patients is related to the magnitude of memory impairment. Thus, we aimed at studying the consequences of the exposure of LE and HE male Wistar rats, during periadolescence, to a combination of ES, namely, paradoxical sleep deprivation (PSD) and unpredictable stress (US), on anxiety-related behavior in the plus maze test, working (WM) and declarative memory (DM) performance. We also evaluated hippocampal immune-inflammatory/oxidative, as consequences of ES, and prevention of ES-induced alterations by the mood-stabilizing drugs, lithium and valproate. Medium exploratory (ME) control rats were used for comparisons with HE- and LE-control rats. We observed that HE-controls presented increased anxiolytic behavior that was significantly increased by ES exposure, whereas LE-controls presented increased anxiety-like behavior relative to ME-controls. Lithium and valproate prevented anxiolytic alterations in HE+ES rats. HE+ES- and LE+ES-rats presented WM and DM deficits. Valproate and lithium prevented WM deficits in LE-PSD+US rats. Lithium prevented DM impairment in HE+ES-rats. Hippocampal levels of reduced glutathione (GSH) increased four-fold in HE+ES-rats,

being prevented by valproate and lithium. All groups of LE+ES-rats presented increased levels of GSH in relation to controls. Increments in lipid peroxidation in LE+ES- and HE+ES-rats were prevented by valproate in HE+ES-rats and by both drugs in LE+ES-rats. Nitrite levels were increased in HE+ES- and LE+ES-rats (five-fold increase), which was prevented by both drugs in LE+ES-rats. HE+ES-rats presented a two-fold increase in the inducible nitric oxide synthase (iNOS) expression that was prevented by lithium. HE+ES-rats showed increased hippocampal and plasma levels of interleukin (IL)-1β and IL-4. Indoleamine 2, 3-dioxygenase 1 (IDO1) was increased in HE+ES- and LE+ES-rats, while tryptophan 2,3-dioxygenase (TDO2) was increased only in HE+ES-rats. Altogether, our results showed that LE- and HE-rats exposed to ES present distinct anxiety-related behavior and similar memory deficits. Furthermore, HE+ES-rats presented more brain and plasma inflammatory alterations that were partially prevented by the mood-stabilizing drugs. These alterations in HE+ES-rats may possibly be related to the development of mood symptoms.

Keywords: temperament, animal model, bipolar disorder, paradoxical sleep deprivation, unpredictable stress, neuroinflammation, memory impairment

INTRODUCTION

The relationship between personality and mood disorders has been studied for decades. Indeed, Emil Kraepelin described four basic affective dispositions, namely, depressive, manic (hyperthymic), cyclothymic, and irritable, and proposed that imbalances between these affective temperaments could be the cause of mental disorders (1). Several decades later, the personality model proposed by Cloninger (2) and its derived instruments have been widely applied to mood disorders (3, 4). According to Cloninger's model, temperament is the emotional core of personality and represents the basic pattern of response to emotional stimuli that is heritable and moderately stable through life. Based on Cloninger classification, temperament is divided into four dimensions, namely, novelty seeking (NS), harm avoidance (HA), reward dependence (RD), and persistence (P) (5). NS and HA are dimensions evolutively conserved in humans and mammals and greatly influence exploratory behavior. This behavior consists of a complex act that allows the collection of information about the environment and increases the chances to find food, mating partner, shelter, and, lately, of survival (6, 7).

In preclinical research, the selection based on exploratory behavior has been applied as a useful approach to study temperament (8). This selection allows the separation of rodents in two extreme subgroups—high and low exploratory (named here HE and LE, respectively). This trait is stable over time and keeps important associations with other anxious and depression-like behaviors as well as response to psychoactive drugs (8–10). In humans, several studies reported that patients diagnosed with mood disorders (both unipolar depression and bipolar disorder) present high HA, while bipolar patients differentially express high NS and RD domains (11, 12). In this context, the selection of extreme temperamental features (based on exploratory behavior) has emerged as a valuable tool to study the biological basis of temperament and to understand the involvement of personality traits in adaptive responses to stress and stress-related disorders.

Despite the well-documented consequences of paradoxical sleep deprivation (PSD) in animal models, the effects of combining environmental contingencies in a model induced by sleep deprivation associated with stress in memory and learning have been neglected. Sleep deprivation associated with unpredictable stressors occurs more commonly in the modern society, thus representing a better translational model.

Previous reports show that sleep deprivation induces increases in brain oxidative stress. For instance, authors reported that 72 h of rapid eye movement (REM) sleep deprivation increases lipid oxidation in both hippocampal and cortex areas (13). In another study, 21 days of intermittent REM sleep was shown to increase lipid oxidation and decrease superoxide dismutase (SOD) and glutathione peroxidase (GPx) activity in rat hippocampus (14). In contrast, Ramanathan et al. (15) showed that sleep deprivation increases antioxidants in the hippocampus, cerebellum, and neocortex.

Additionally, it is well known that allosteric loading conditions to the central nervous system (CNS), such as sleeping deprivation and unpredictable stress, may lead to activation of microglial activity and thereby aid release of a variety of proinflammatory and neurotoxic factors, including cytokines, such as tumor necrosis factor (TNF-α), interleukin (IL) -1β, IL-6, and free radicals, such as nitric oxide (NO) and superoxide, resulting in a decline in cognitive function (16–20).

These proinflammatory cytokines lead to an increased NO production *via* direct activation of microglial inducible nitric oxide synthase (iNOS) and indirect activation of neuronal NOS (nNOS) through N-methyl-D-aspartate (NMDA) receptor complex activation (NMDA2R subunit) (21, 22). Furthermore, an increased release of proinflammatory cytokines, like IL-1β, has been shown to bring about an increase in quinolinic acid to

kynurenic acid ratio, as well as an inhibition of excitatory amino acid (glutamate) removal *via* focal loss of astroglial EAAT1/2 (excitatory amino acid transporter), leading to a net NMDA agonism (21, 23).

Lithium (Li) and valproic acid (VPA) are classical mood-stabilizing agents that are effective as maintenance therapy in mood disorders. Consistent evidence has shown that these drugs present neuroprotective action against several neuropathology models, which seems mainly due to their well-demonstrated antioxidant, anti-inflammatory, and neurotrophic properties (24–26). Regarding stress models, Li and VPA individually rescued the effects of chronic unpredictable stress on behavior and brain oxidative homeostasis (27, 28). Also, Li and VPA prevented the oxidative damage, mitochondrial dysfunction, and hypothalamic–pituitary–adrenal (HPA) hyperactivity induced by sleep deprivation (29, 30). Additionally, both these mood stabilizers protected against sleep deprivation-induced mania-like phenotype (29), while Li rescued some sleep deprivation-associated cognitive deficits (31). However, little is known about the effects of these mood-stabilizing drugs on behavioral and neurochemical consequences of the combination of sleep deprivation and unpredictable stress as well as the influence of temperament in this effect.

In the present study, we have established a rat model mimicking repetitive and intermittent sleep deprivation combined with unpredictable stress in humans. We evaluated anxiety-related behavior and cognitive behavioral parameters and provided neuropathological evidences for cognitive dysfunction in this model. Also, we tested the effects of the mood stabilizers drugs, Li and VPA, in the prevention of these abnormalities.

MATERIALS AND METHODS

Animals

The experiments were performed with 80 male *Wistar* periadolescent rats (weight: 50–60 g) obtained from the Animal House of Universidade Federal do Ceara, Brazil. Animals were housed at a maximum of five per cage in standard polycarbonate cages (42 × 20.5 × 20 cm) and standard environmental conditions (22 ± 1°C; humidity 60 ± 5%; 12-h light/dark cycle with lights on at 7:00 am) with access to food (Laboratory RodentDiet—LabDiet®) and water *ad libitum*. All behavioral procedures were conducted between ZT2 (8 am) and ZT5 (11 am) by raters blinded to the experimental groups. The methods were carried out in accordance with the NIH Guide for the Care and Use of Laboratory Animals (32) and with the approval of the local ethical committee of Universidade Federal do Ceara. All efforts were made to minimize animal suffering and to reduce the number of animals used.

Behavioral Analyses of High and Low Exploratory Rats

A psychogenic selection was employed to study the bases of temperament in rodents, dividing them into extremes of baseline exploratory activity based on the screening for locomotor response to novelty, as previously described (9, 33). The study started with 80 periadolescent animals on the 30th postnatal day (PN30). These animals were selected according to their performance in the open field apparatus for 10 min. Specifically, periadolescent rats were classified into three groups based on their vertical exploratory activity values (33) into high exploratory (HE) (75th percentile), medium exploratory (ME) (50th percentile), and low exploratory (LE) (25th percentile) (**Supplementary Material—Table S1**). Animals were tested again in the same apparatus in adult life (PN60), and those who maintained the same exploratory profile were randomly divided in groups for behavioral and neurochemical evaluations.

Drugs

Valproic acid (VPA, Tocris, USA) and lithium carbonate (Li; Sigma-Aldrich Corp., St Louis, USA) were used. The drugs were prepared freshly within 1–2 h of dosing. All other chemicals used were of analytical grade.

Experimental Protocol

The experiments were divided into three protocols as depicted in **Figure 1**. In protocol 1, the animals were selected by exploratory profile, namely, HE, ME, and LE, as previously described. In protocol 2, the animals grouped as HE and LE were divided into four experimental groups (n = 8/group), being exposed or not to paradoxical sleep deprivation (PSD) and unpredictable stressors (US): group 1—HE-control (not-stressed), group 2—HE-PSD+US, group 3—LE-control (not-stressed), group 4—LE-PSD+US. In protocol 3, in order to evaluate the effects of Li and VPA in the prevention of behavioral and neurochemical alterations induced by the exposure of HE- and LE-rats to PSD+US, four further groups were conducted, named group 5—HE-VPA+PSD+US, group 6—HE-Li+PSD+US, group 7—LE-VPA+PSD+US, and group 8—LE-Li+PSD+US.

In the present study, since our interest was in studying behavioral and biochemical consequences of the exposure of animals from extremes of temperament to environmental stressors, ME-control rats were used only for comparisons with HE- and LE-control rats. For this reason, we did not conduct a group of ME-rats exposed to PSD+US. For the environmental stress (ES) induction, the animals were subjected to a 10-day protocol with a five alternative day exposure to PSD combined with 5 days of US (as represented in **Figure 1**). Some groups exposed to PSD+US underwent a prevention treatment with the intraperitoneal administration of the mood-stabilizing drugs Li (47.5 mg/kg) or VPA (200 mg/kg). The administration of Li or VPA took place 30 min before the second section of PSD (PN42) and continued until the last day of stress exposure (PN49). The doses of VPA (35) and Li (36, 37) were based on previous studies.

The method of PSD used here was an adaptation of the multiple platform method developed for rats (38). Groups of five to six animals were placed in water tanks (41 cm × 34 cm × 16.5 cm) containing 12 platforms (3 cm in diameter) each, surrounded by water up to 1 cm beneath the surface, for 24 h beginning on PN40 and implemented on alternate days. The present study adopted 24 h of PSD since previous studies showed that it was able to induce hyperlocomotion (taken as a mania-like behavior) (39).

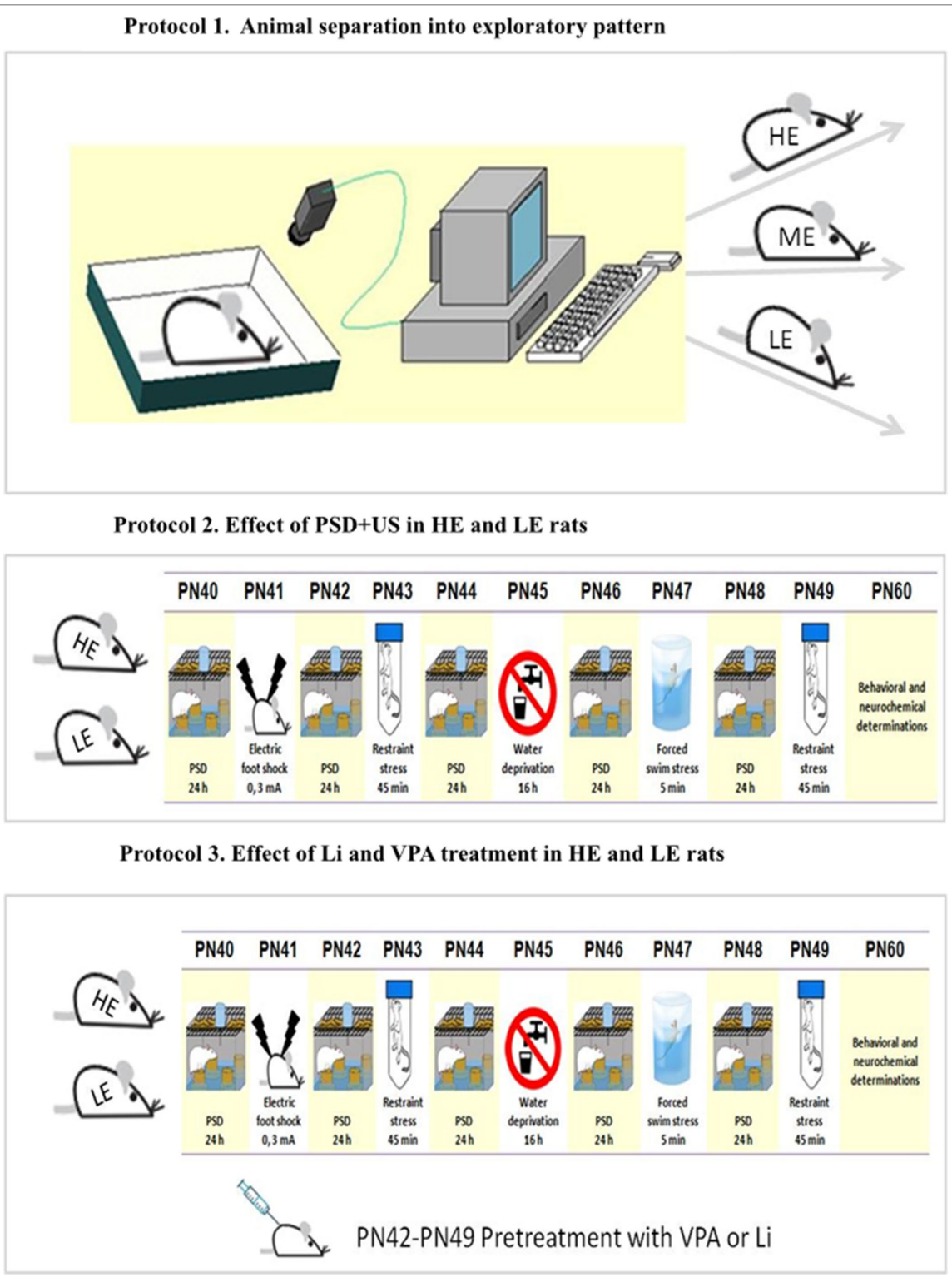

FIGURE 1 | Schematic representation of the experimental design. Protocol 1: On PN (postnatal day) 30, rats were separated according to exploratory activity in HE (high exploratory), ME (medium exploratory), and LE (low exploratory). Protocol 2: From PN40–49, the animals were exposed to daily alternated sections of paradoxal sleep deprivation (PSD) and unpredictable stress (US), day 40—PSD; day 41—foot shock; day 42—PSD; day 43—restraint stress; day 44—PSD; day 45—water deprivation; day 46—PSD; day 47—forced swim stress; day 48—PSD; and day 49—restraint stress or left undisturbed (controls). From PN42–49, some animals were administered valproate (VPA) or lithium (Li), as preventive strategies. The behavioral and neurochemical analysis were performed in brains from animals euthanized on PN60.

In this method, the animals could move inside the tank, jumping from one platform to the other. Control animals were maintained in their home cages in the same room. Food and water were made available through a grid placed on top of the water tank.

On alternate days, beginning on PN41, the animals were submitted to the following sessions of US (40), as detailed in the **Supplementary Material**: day 41—foot shock, day 43—restraint stress, day 45—water deprivation, day 47—forced swim stress, and day 49—restraint stress. From PN50–59, the animals were left undisturbed, and on PN60, the behavioral tests were conducted.

Behavioral Tests

Plus Maze Test

This test was classically designed to evaluate anxiety-like behavior (41). The apparatus has a total dimension of 1,000 (W) × 1,000 (D) × 1,000 (H) mm with two perpendicular open arms and two perpendicular closed arms, 100 (W) × 450 (D) mm arm's length, with a 650 (H) mm elevation from the floor. The open and closed arms are connected by a central platform. The platform and the lateral walls of the closed arms are made of transparent acrylic. The floor is made of black acrylic. After the respective treatment, each animal was placed at the center of the elevated plus maze with its nose in the direction of one of the closed arms and was observed for 5 min according to the following parameters: number of entries into open and closed arms and the amount of time spent by in open and closed arms of the maze. These data were used to calculate: % of open entries (open entries/total entries × 100), % of time in open arms (time spent in open arms/ total time × 100) and % time in the closed arms (time spent in closed arms/total time × 100).

Y-Maze Test

This test was used to evaluate working memory (42). Each rat could freely move through the maze during 8 min. The series of arm entries was recorded visually. Alternations were defined as entries in all three arms on consecutive occasions. The percentage of alternation was calculated as total of alternations/(total arm entries – 2) (43).

Novel Object Recognition Test (NOR)

This test is widely used to assess the ability to memorize and recognize new and already known objects, being related in rodents to declarative memory (44). On the first day, before any procedure (habituation), animals were placed in the center of the open field apparatus and left for 5 min with no object. After 24 h (training session), animals were placed back in the box with two identical objects (A1 and A2, double Lego® toys) placed in the middle of the box and were left for 5 min for the exploration of the new environment. The time spent exploring each object was recorded for further analysis through the recognition rate. On the same day, 1.5 h after the training session, rats were tested for short-term memory. In this procedure, animals were placed back into the box, with two similar objects (A1 and B1) in color and size, but of different shapes. Again, the animals were left to explore the objects for 5 min and the exploration time of each object was registered. To analyze the results, we used the recognition index, which is calculated as follows: T(B1) – T (A1)/TE, where T(B1) is the time spent by the animal in exploring the new object (B1), T(A1) is the time spent exploring the familiar object (A1), and TE is the total operating time (the sum of animal exploration time in the old and new objects) (45). The test was used to evaluate short-term memory.

Neurochemical and Plasma Determinations

Immediately after the memory tests, the rats were euthanized and the hippocampus was dissected and homogenized for neurochemical determinations.

Determination of Reduced Glutathione (GSH) Levels

The levels of GSH were evaluated to estimate endogenous defenses against oxidative stress. The method was based on Ellman's reagent (DTNB) reaction with free thiol groups (46). The reaction was read in the absorbance of 412 nm, and the product was expressed as ng of GSH/mg wet tissue.

Measurement of Lipid Peroxidation

Lipid peroxide formation was analyzed by measuring the thiobarbituric acid reacting substances (TBARS) in brain homogenates (47), as an index of reactive oxygen species (ROS) production. Lipid peroxidation was assessed by the absorbance at 532 nm and expressed as μmol of malonaldehyde (MDA)/mg of tissue.

Nitrite Levels

This method is based on the use of the Griess reagent, which detects the presence of nitrite in the sample through a diazotization reaction by formation of a chromophore pink color. The reagent is prepared using equal parts of 5% phosphoric acid, 0.1% N-1-naphthalenediamine (NEED), 1% sulfanilamide in 5% phosphoric acid, and distilled water. The assay was performed by the addition of 100 μl of the supernatant of the homogenate (diluted 10% in potassium phosphate buffer) to 100 μl of Griess reagent. The standard curve was obtained through serial dilutions (100, 50, 25, 12.5, 6.25, 3.12, 1.56 μM) of nitrite. The entire assay was performed in a 96-well plate, and absorbance readings were taken in the 560-nm range. Nitrite product is expressed as μmol of nitrite/mg of tissue (48).

Immunoassay for Interleukin (IL)-1β, IL-4, and IL-6

Brain tissues were homogenized in 8 volumes of PBS buffer with protease inhibitor (EMD Biosciences) and centrifuged (10,000 rpm, 5 min) for the collection of the supernatant. The concentration of cytokines (IL-1β, IL-4, and IL-6) in 50 ml samples was determined by Enzyme-Linked Immunosorbent Assay (ELISA) immunoenzymatic assays (R&D Systems, Minneapolis, MN, USA), according to the manufacturer's protocol, and expressed in pg/g tissue in hippocampal samples and in pg/ml in plasma samples.

Determination of Uric Acid

In the case of uric acid, the determination was based on the Cobas method. In this enzymatic colorimetric assay, uricase cleaves uric acid to form allantoin and hydrogen peroxide. In the presence of peroxidase, 4-aminophenazone is oxidized by

hydrogen peroxide to a quinone-diimine dye. The red color intensity of the quinone-diimine formed is directly proportional to the uric acid concentration and is determined by measuring the increase in absorbance. The results of plasma levels of uric levels are expressed in mg/dl.

RNA Isolation for Gene Expression Analyses

Total RNA was isolated from hippocampal samples using the SV Total RNA Isolation System from Promega (Madison, WI, USA). RNA was quantified by NanoDrop (Thermo Fisher Scientific), and RNA quality was determined by examining the 260/280 ratio > 1.8. A total of 1 µg RNA was then reverse transcribed using a high-capacity cDNA reverse transcription Kit (Applied Biosystems), according to the manufacturer's protocol. Messenger RNA (mRNA) expression was analyzed by quantitative PCR (qPCR) according to the manufacturer's instructions (Applied Biosystems, USA). The sequences of primers and ID numbers are listed in **Table 1**. Target gene expression was calculated relative to a stably expressed reference glyceraldehyde 3-phosphate dehydrogenase (*Gapdh* gene). A total of 50 ng cDNA was added to a mix containing the gene expression assay [1 µl indoleamine 2, dioxygenase 1 (*Ido1*), tryptophan 2,3-dioxigenase (*Tdo2*), inducible nitric oxide synthase (*Inos*), or *Gapdh*, Power SYBR Green PCR master mix (10 µl), and RNA-free water (5 µl) to a final volume of 20 µl]. After the reaction components were mixed by inverting the tube several times, the tube was briefly centrifuged. Then, 20 µl of PCR reaction mix was transferred to each well of a 96-well plate, which was loaded into the instrument, sealed, and centrifuged. The Light cycler 96 (Roche) thermo cycler parameters were 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 60 s. All fold changes were calculated by the $\Delta\Delta C_t$ method (49).

Data Analysis

The division of the animals, according to the exploratory activity, into HE, ME, and LE groups was based on frequency distribution. For the comparisons between HE-, ME-, and LE-control animals (results of Protocol 1), we used one-way ANOVA followed by Dunnett's multiple comparisons test, considering ME values as control column. Data obtained in Protocol 2 were analyzed by two-way ANOVA followed by Tukey *post hoc* test considering as factors "exploratory activity" (HE and LE) and "stress exposure" (no-stress and PSD+US). The effects of Li or VPA treatments in HE or LE rats (protocol 3) were analyzed by one-way ANOVA with Tukey as *post hoc* test. Data are presented as means ± standard error (S.E.M.). GraphPad Prism version 6.0 was used for data analyses and construction of graphs. The alpha level was set at 0.05.

RESULTS

HE- and LE-Rats Present Distinct Anxiety-Related Behavior That is Prevented by the Administration of the Mood-Stabilizing Drugs VPA and Li

In the analysis of the % of open arms entries in the plus maze test (**Figure 2A**), we observed that HE-control rats presented increased levels of this parameter relative to ME-control rats {$P < 0.0001$; one-way ANOVA [$F(2, 26) = 25.89$, $P < 0.0001$]}. The exposure of HE-rats to PSD+US induced a 1.5-fold increase in the % of open entries in relation to HE-controls {$P < 0.0001$; two-way ANOVA "exploratory activity" vs. "stress exposure" interaction [$F(1, 31) = 9.412$, $P = 0.0044$]}. The administration of VPA or Li significantly prevented PSD+US-induced increase in the % of open entries in HE-rats {HE-VPA+PSD+US vs. HE-PSD+US, $P = 0.0075$; HE-Li+PSD+US vs. HE-PSD+US, $P = 0.0075$; one-way ANOVA [$F(2, 29) = 8.077$, $P = 0.0027$]}.

In the analysis of the % of time in open arms (**Figure 2B**), HE-control rats presented a significant increase in this parameter in relation to ME-control rats {$P < 0.0001$; one-way ANOVA [$F(2, 26) = 18.12$, $P < 0.0001$]}. In the analysis of the interaction between "exploratory activity" and "stress exposure," we observed that HE-control rats presented a significant increase in the % of time in open arms in relation to LE-control rats ($P = 0.0038$) and also that the exposure of HE-rats to PSD+US caused a significant 1.4-fold increase in the % of time spent in the open arms relative to HE-control rats {HE-PSD+US vs. HE-control, $P < 0.0001$; two-way ANOVA interaction: [$F(1, 31) = 12.59$, $P = 0.0016$]}. The administration of VPA or Li to HE-PSD+US rats prevented from the alteration induced by PSD+US {$P < 0.0001$; one-way ANOVA [$F(3, 29) = 52.17$, $P < 0.0001$]}. No significant changes were observed by the exposure of LE-rats to PSD+US in relation to LE-control. Despite this, the administration of VPA or Li decreased the % of time in the open arms when compared to LE-control and to LE-PSD+US {$P < 0.0001$; one-way ANOVA [$F(3, 29) = 23.65$, $P < 0.0001$]}.

In the evaluation of the % of time spent in the closed arms (**Figure 2C**), we observed that HE-control rats presented

TABLE 1 | PCR amplification primers.

Gene name	GenBank accession no.	Forward primer 5′-3′	Reverse primer 5′-3′	Fragment size
iNOS	NM_012611.3	AGGCCACCTCGGATATCTCT	TGGGTCCTCTGGTCAAACTC	81
IDO1	NM_02397.1	CATCAAGACCCGAAAGCACT	GGTGTCTGGATCCACGAAGT	108
TOD2	NM_022403.2	GGGGGATCCTCAGGCTATTA	GGGAACCAGGTACGATGAGA	96

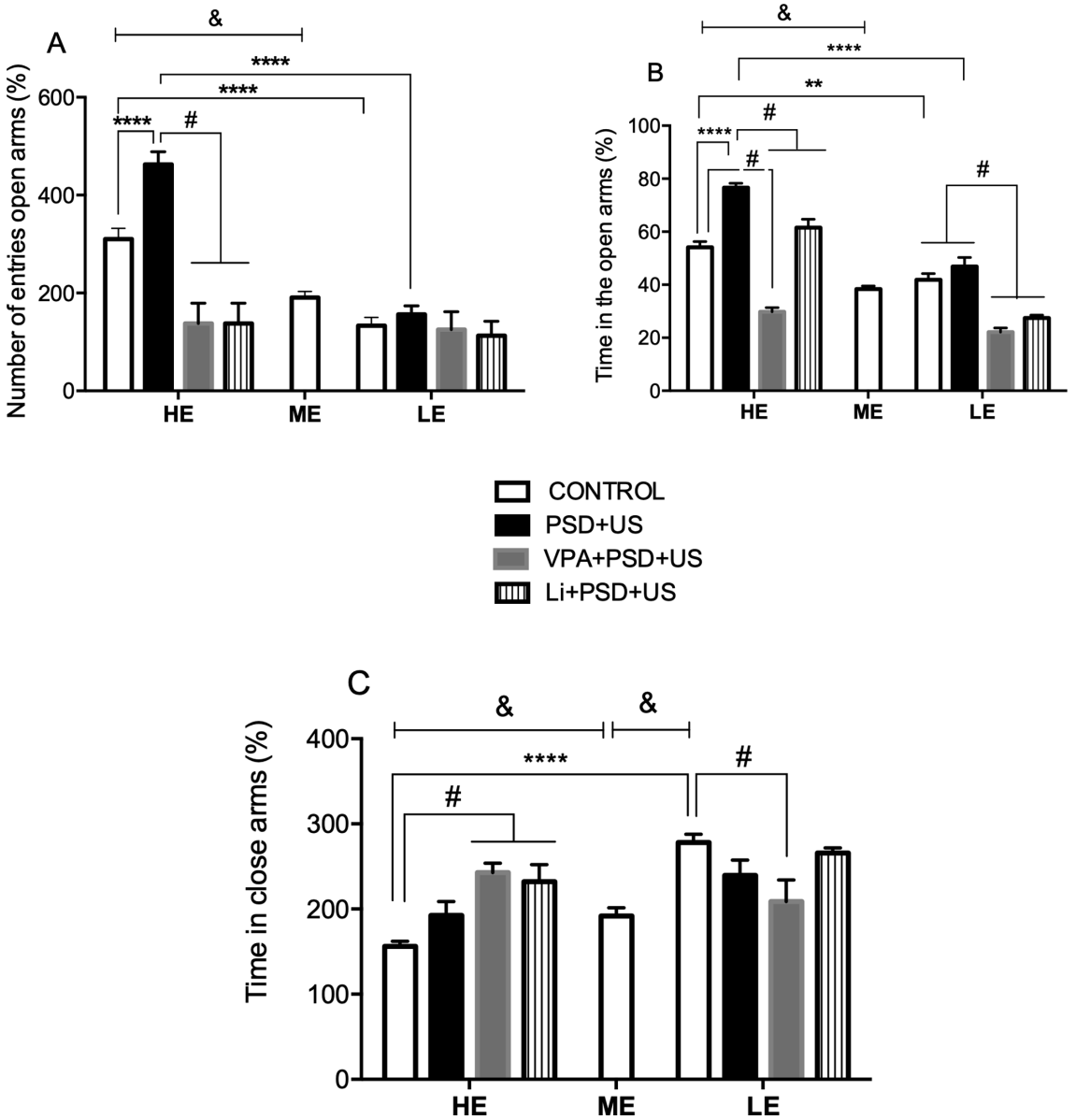

FIGURE 2 | Effects of the exposure of HE- and LE-rats to PSD+US in the plus maze test for the evaluation of anxiety-related behavior. The parameters measured were as follows: % of the number of entries in open arms **(A)**, % of the time in open arms **(B)**, and % of time in closed arms **(C)**. Animals were separated by exploratory pattern in HE (high exploratory), ME (medium exploratory), and LE (low exploratory). HE and LE rats, respectively, mimic the human temperaments hyperthymic and depressive. During periadolescence, PNs 40–49, HE- and LE-rats were exposed to daily alternated sections of paradoxal sleep deprivation (PSD) and unpredictable stress (US) or left undisturbed (controls). The preventive strategies, VPA or Li, were administered during PNs 42–49. Plus maze test was performed on PN60. Bars represent means ± SEM of eight animals/group. $^{\&}P < 0.05$ for comparisons between HE-, ME-, and LE- control rats according to one-way ANOVA followed by Dunnett *post hoc* test; $^{**}P < 0.01$, $^{****}P < 0.0001$ for two-way ANOVA comparisons with Tukey as *post hoc* test using "exploratory activity" and "stress exposure" as factors; $^{\#}P < 0.05$ for comparisons between groups pretreated with Li- and VPA- in relation to PSD+US and controls according to one-way ANOVA followed by Tukey *post hoc* test. HE, high exploratory; ME, medium exploratory; LE, low exploratory; PN, postnatal day; PSD, paradoxal sleep deprivation; US, unpredictable stress; VPA, valproate; Li, lithium.

a significant decrease in this parameter when compared to ME-control ($P < 0.05$), whereas LE-control presented increased levels relative to ME-rats ($P < 0.0001$). We also observed that HE-control presented lower % of time in closed arms in relation to LE-control {$P < 0.0001$; two-way ANOVA interaction [F (1, 31) = 8.788, $P = 0.0059$]}. The pretreatment with Li ($P = 0.0027$) or VPA ($P = 0.0006$) significantly increased the % of time in the closed arms of HE-PSD+US rats in relation to HE-control rats [one-way ANOVA F (3, 29) = 8.352, $P = 0.0004$], whereas LE-PSD+US rats pretreated with VPA presented decreased % time in closed arms when compared to LE-control {$P = 0.0214$; one-way ANOVA [F (3, 29) = 3.587, $P = 0.0265$]}.

The Exposure to PSD+US Impairs Memory Performance in HE- and LE-Rats That Is Distinctly Prevented by the Mood-Stabilizing Drugs

In the analysis of working memory (**Figure 3A**), we observed that both HE- ($P < 0.0001$) and LE-control ($P = 0.0070$) presented increased % of incorrect alternations when compared to ME-control {one-way ANOVA [$F(2, 22) = 26.82$, $P < 0.0001$]}. Two-way ANOVA indicated a significant "exploratory activity" vs. "stress exposure" interaction [$F(1, 28) = 4.472$, $P = 0.0435$]. *Post hoc* analysis showed that the percentage of incorrect alternations in HE-PSD+US and LE-PSD+US was significantly increased in relation to controls matched by exploratory activity ($P < 0.0001$). Additionally, HE-control rats presented increased % of incorrect alternations in relation to LE-control ($P = 0.0104$). Pretreatment with Li or VPA did not prevent working memory deficits in HE-rats, while prevented working memory deficits in LE-rats {LE-VPA+PSD+US vs. LE-PSD+US, $P = 0.0129$; LE-Li+PSD+US vs. LE-PSD+US, $P = 0.0016$; one-way ANOVA [$F(3, 27) = 12.02$, $P < 0.0001$]}.

As depicted in **Figure 3B**, we observed that both HE- and LE-control presented significant lower discrimination index relative to ME-control {$P < 0.0001$; one-way ANOVA [$F(2, 17) = 52.27$, $P < 0.0001$])}. Both HE-PSD+US ($P < 0.0001$) and LE-PSD+US ($P = 0.0009$) presented marked decreases in discrimination indexes when compared to controls matched by exploratory activity {two-way ANOVA interaction [$F(1, 20) = 5.236$, $P = 0.0331$]}. Pretreatment of HE-PSD+US rats with Li (HE-Li+PSD+US vs. HE-control, $P = 0.0076$) or VPA (HE-VPA+PSD+US vs. HE-control, $P < 0.0001$) maintained the significant decrease in discrimination ratio in relation to HE-control rats, while pretreatment of HE-PSD+US rats with Li increased the discrimination index in relation to HE-PSD+US group ($P = 0.0019$). Again, LE-VPA+PSD+US group presented decreased discrimination index when compared to LE-control ($P = 0.0021$) being this result akin to LE-PSD+US levels, whereas LE-Li+PSD+US group presented significant increased levels of discrimination index relative to LE-PSD+US group ($P = 0.0216$).

The Exposure of HE- and LE-Rats to PSD+US Increases Hippocampal Levels of GSH, Lipid Peroxidation, and Nitrite, Which Are Distinctly Altered by the Administration of Mood-Stabilizing Drugs

In the evaluation of hippocampal levels of GSH (**Figure 4A**), we observed that HE-control presented increased levels of this antioxidant defense relative to ME-control ($P = 0.0008$). On the other hand, decreased levels of GSH were observed in LE-control when compared to ME-control {$P < 0.0001$; one-way ANOVA [$F(2, 19) = 79.41$, $P < 0.0001$]}. There was a significant interaction between "exploratory activity" and "stress exposure" in GSH levels {two-way ANOVA [$F(1, 22) = 71.41$, $P < 0.0001$]}. In this regard, HE-control presented significant increased hippocampal levels of GSH when compared to LE-control ($P = 0.0003$). We detected a 3- and 2.3-fold increase in GSH levels, respectively, in HE-PSD+US and LE-PSD+US rats in relation to their controls ($P < 0.0001$). Furthermore, the exposure of HE-rats to PSD+US caused a marked increase in GSH levels that was significant in relation to LE-PSD+US ($P < 0.0001$). The administration of VPA or Li to HE-PSD+US rats prevented the alterations in GSH levels ($P < 0.0001$). On the contrary, the administration of these drugs to LE-PSD+US induced a further increase in the levels of GSH relative to LE-controls ($P < 0.05$).

Regarding lipid peroxidation (**Figure 4B**), we detected decreased levels of MDA in the hippocampus of ME-control in relation to LE- ($P = 0.0004$) and HE-control {$P < 0.0001$; one-way ANOVA [$F(2, 18) = 31.45$, $P < 0.0001$]}. HE-control presented increased hippocampal MDA levels when compared to LE-control ($P = 0.0358$). Both HE-PSD+US ($P = 0.0008$) and LE-PSD+US ($P < 0.0001$) presented increased levels of lipid peroxidation in relation to their respective controls {two-way ANOVA, significant main effect of "exploratory activity" [$F(1, 23) = 9.819$, $P = 0.0047$] and "stress exposure" [$F(1, 23) = 54.86$, $P < 0.0001$]}. VPA pretreatment significantly prevented PSD+US alterations in HE-rats, while both VPA and Li prevented the alterations induced by PSD+US in LE-rats, and also decreased the levels of MDA in relation to LE-control ($P < 0.05$).

Nitrite levels (**Figure 4C**) were decreased in LE-control relative to ME ones {$P = 0.0085$; one-way ANOVA [$F(2, 18) = 5.985$, $P = 0.0102$]}. Both HE- and LE-rats when exposed to PSD+US presented increased hippocampal levels of nitrite when compared to their respective controls (HE-PSD+US vs. HE-control, $P = 0.0002$; LE-PSD+US vs. LE-control, $P < 0.0001$). Furthermore, the levels of nitrite in LE-PSD+US rats were significantly increased when compared to HE-PSD+US {$P = 0.0030$; two-way ANOVA interaction [$F(1, 23) = 20.70$, $P = 0.0001$]}. The administration of both mood-stabilizing drugs prevented nitrite increase in LE-PSD+US rats (LE-VPA+PSD+US vs. LE-PSD+US, $P < 0.01$; LE-Li+PSD+US vs. LE-PSD+US, $P < 0.0001$), while Li increased the levels of nitrite in HE-PSD+US rats (HE-Li+PSD+US vs. HE-PSD+US, $P < 0.01$).

Exposure to PSD+US Increases Cytokine Levels in the Hippocampus and Plasma as Well as Uric Acid in the Plasma of HE-Rats

We found a significant increase in the hippocampal levels of IL-1β (**Figure 5A**) in both HE- and LE-rats exposed to PSD+US in relation to their respective control {HE-PSD+US vs. HE-control, $P < 0.0001$; LE-PSD+US vs. LE-control, $P = 0.0002$; two-way ANOVA significant main effect of "stress exposure" [$F(1, 33) = 69.63$, $P < 0.0001$]. The administration of the mood-stabilizing drugs did not prevent the alterations in this parameter. Regarding hippocampal IL-4 (**Figure 5B**), we found increased levels of this cytokine in HE-PSD+US rats relative to HE-control ($P = 0.0012$), being this increase also significant in relation to LE-PSD+US {$P = 0.0103$; two-way ANOVA interaction [$F(1, 25) = 4.713$, $P = 0.0396$]}. Again, the mood-stabilizing drugs could not prevent IL-4 changes in HE-PSD+US rats. Hippocampal IL-6 levels (**Figure 5C**) were increased in HE-control in relation to ME-control {$P = 0.0002$, one-way ANOVA [$F(2, 23) = 12.81$, $P = 0.0002$]}. We observed that IL-6 levels were higher in HE-control

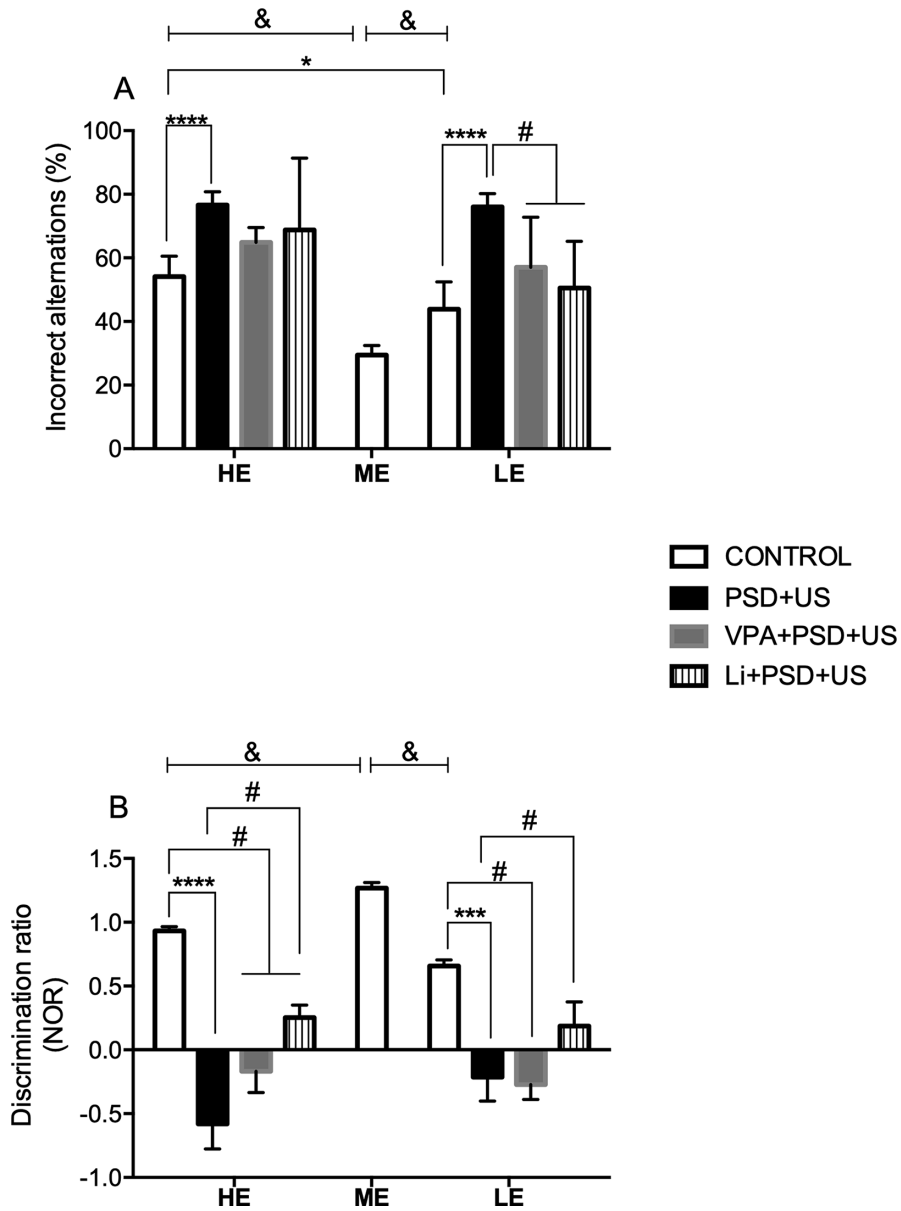

FIGURE 3 | Effects of the exposure of HE and LE rats to PSD+US in memory alterations evaluated by the % of incorrect alternations in the Y maze task **(A)** or discrimination ratio in the novel object recognition test (NOR) **(B)**. Animals were separated by exploratory pattern in HE (high exploratory), ME (medium exploratory), and LE (low exploratory). HE and LE rats, respectively, mimic the human temperaments hyperthymic and depressive. During periadolescence, PNs 40–49, HE- and LE-rats were exposed to daily alternated sections of paradoxal sleep deprivation (PSD) and unpredictable stress (US) or left undisturbed (controls). The preventive strategies, VPA or Li, were administered during PNs 42–49. Y maze task and novel object recognition test (NOR) were performed on PN60 for the evaluation of declarative memory and working memory, respectively. Bars represent means ± SEM of eight animals/group. $^{\&}P < 0.05$ for comparisons between HE-, ME-, and LE- control rats according to one-way ANOVA followed by Dunnett *post hoc* test; $^{*}P < 0.05$, $^{***}P < 0.001$, $^{****}P < 0.0001$ for two-way ANOVA comparisons with Tukey as *post hoc* test using "exploratory activity" and "stress exposure" as factors; $^{\#}P < 0.05$ for comparisons between groups pretreated with Li- and VPA- in relation to PSD+US and controls according to one-way ANOVA followed by Tukey *post hoc* test. HE, high exploratory; ME, medium exploratory; LE, low exploratory; PN, postnatal day; PSD, paradoxal sleep deprivation; US, unpredictable stress; VPA, valproate; Li, lithium.

relative to LE-control ($P = 0.0119$) and in HE-PSD+US in relation to LE-PSD+US {$P = 0.0207$; two-way ANOVA significant main effect of "exploratory activity" [F (1, 30) = 20.57, $P < 0.0001$]}.

Plasma levels of IL-1β (**Figure 5D**) were increased in LE-control when compared to ME-control {$P = 0.0010$; one-way ANOVA [F (2, 15) = 13.96, $P = 0.0017$]}. We observed a marked increase in IL-1β levels in HE-PSD+US animals in relation to HE-control {$P = 0.0001$; two-way interaction [F (1, 13) = 8.077, $P = 0.0139$]}. Pretreatment with VPA prevented the alterations in IL-1β induced by the exposure of HE-rats to PSD+US ($P < 0.05$). On the other hand, the administration of the mood-stabilizing drugs decreased the levels of this cytokine in LE-rats

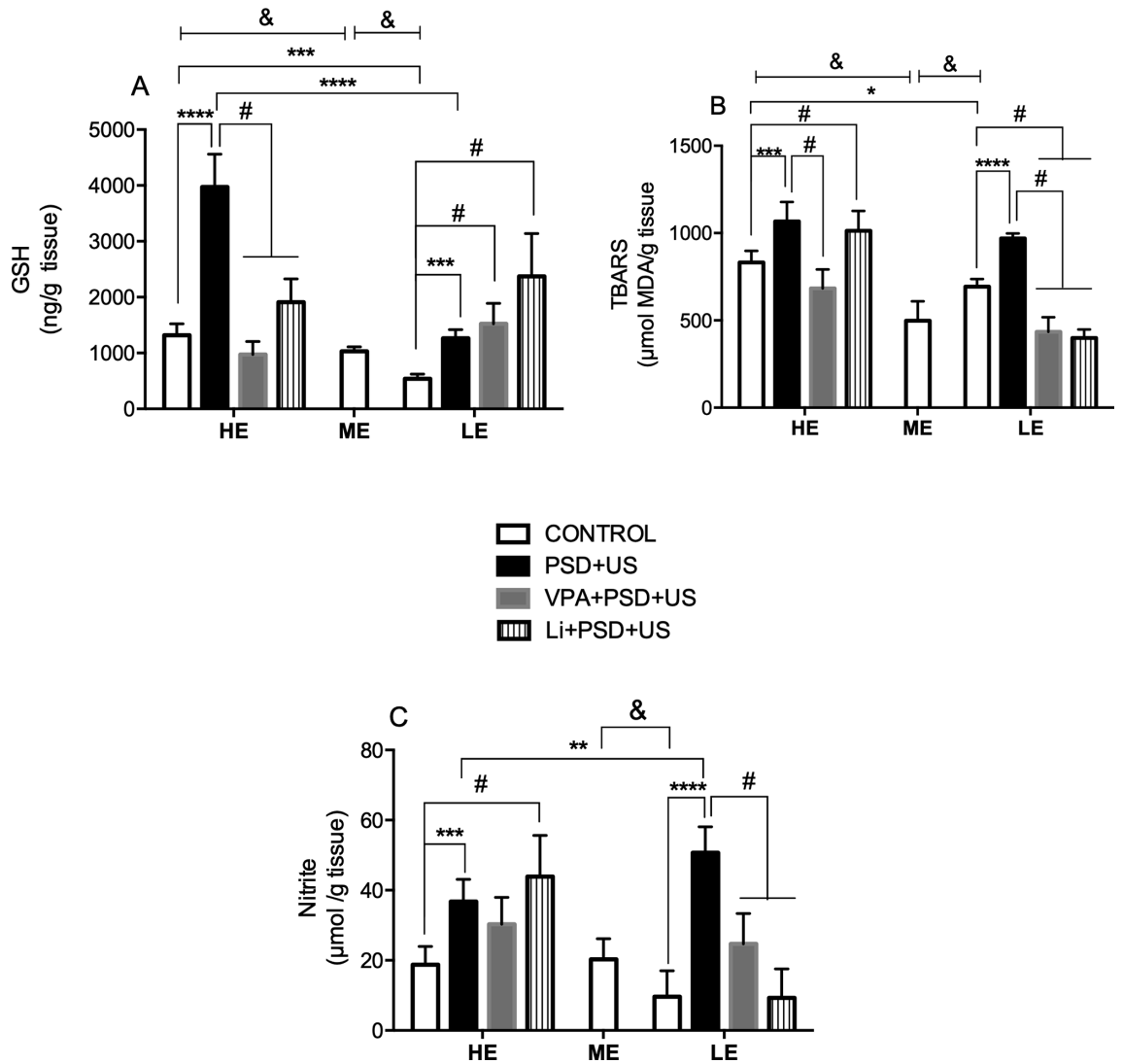

FIGURE 4 | Effects of the exposure of HE and LE rats to PSD+US in oxidative parameters, namely, GSH **(A)**, thiobarbituric acid reacting substances (TBARS) (for lipid peroxidation) **(B)**, and nitrite **(C)**. Animals were separated by exploratory pattern in HE (high exploratory), ME (medium exploratory), and LE (low exploratory). HE- and LE-rats, respectively, mimic the human temperaments hyperthymic and depressive. During periadolescence, PNs 40–49, HE- and LE-rats were exposed to daily alternated sections of paradoxal sleep deprivation (PSD) and unpredictable stress (US) or left undisturbed (controls). The preventive strategies, VPA or Li, were administered during PNs 42–49. The hippocampi were dissected on PN60. Bars represent means ± SEM of six to eight animals/group. $^{\&}P < 0.05$ for comparisons between HE-, ME-, and LE-control rats according to one-way ANOVA followed by Dunnett *post hoc* test; $^{*}P < 0.05$, $^{***}P < 0.001$, $^{****}P < 0.0001$ for two-way ANOVA comparisons with Tukey as *post hoc* test using "exploratory activity" and "stress exposure" as factors; $^{\#}P < 0.05$ for comparisons between groups pretreated with Li- and VPA- in relation to PSD+US and controls according to one-way ANOVA followed by Tukey *post hoc* test. HE, high exploratory; ME, medium exploratory; LE, low exploratory; PN, postnatal day; PSD, paradoxal sleep deprivation; US, unpredictable stress; VPA, valproate; Li, lithium.

($P < 0.01$). IL-4 levels (**Figure 5E**) were significantly increased in HE-rats exposed to PSD+US in relation to HE-control ($P < 0.0001$). This increase observed in HE-PSD+US rats was also significant in comparison with LE-PSD+US rats {$P < 0.0001$; two-way ANOVA interaction [$F(1, 27) = 9.736$, $P = 0.0043$]}. The administration of both mood-stabilizing drugs decreased IL-4 plasma levels in relation to HE-PSD+US animals ($P < 0.001$). In the analysis of plasma IL-6 levels (**Figure 5F**), we observed that HE-PSD+US rats presented increased levels of this cytokine in relation to LE-PSD+US group {$P = 0.0081$; two-way ANOVA significant main effect of "exploratory activity" [$F(1, 22) = 13.92$, $P = 0.0012$]}. We also observed a decrease in IL-6 levels by the administration of VPA and Li to HE-PSD+US rats ($P < 0.05$).

Uric acid plasma levels (**Figure 5G**) were two-fold higher in HE-PSD+US rats in relation to HE-control ($P = 0.0004$), being this increase also significant in relation to LE-PSD+US {$P < 0.0001$; two-way ANOVA interaction [$F(1, 22) = 22.13$, $P = 0.0001$]}. Pretreatment with VPA ($P = 0.0045$) and Li

($P = 0.0002$) prevented the increase in uric acid levels in HE-rats. No significant alterations were observed in LE-rats.

PSD+US Alters the mRNA Expression of the Enzymes iNOS, IDO1, and TDO2 in the Hippocampus of HE- and LE-Rats

Since we observed that the exploratory activity of rats influences brain and plasma cytokines' levels, we decided to evaluate the expression of the enzymes involved in tryptophan metabolism, regulated by pro-inflammatory environment (50), and by glucocorticoids, namely, IDO1 and TDO2, respectively. We also evaluated the expression of iNOS, because it is increased in an inflammatory environment, and also based on the five-fold increase in nitrite levels detected in the hippocampus of LE-PSD+US rats.

The exposure to environmental stressors significantly increased iNOS mRNA levels in HE-rats when compared to their respective control (HE-PSD+US vs. HE-control, $P = 0.0078$). Pretreatment with Li prevented iNOS increased expression in HE-PSD+US and also reduced its expression to minimum levels ($P < 0.01$). A similar reduction in iNOS expression was observed in the group LE-Li+PSD+US when compared to LE-control ($P < 0.0001$) (**Figure 6A**).

We detected a significant two-fold increase in IDO mRNA levels in both HE-PSD+US ($P = 0.02$) and LE-PSD+US ($P = 0.0379$) when compared to their respective controls. Pretreatment with VPA or Li significantly prevented the alterations in HE-rats ($P < 0.001$) (**Figure 6B**). In relation to TDO2 (**Figure 6C**), HE-PSD+US rats presented an almost three-fold increase in the expression of this enzyme when compared to HE-control, while both VPA and Li prevented this alteration ($P < 0.001$). On the other hand, the expression of TDO2 was higher in LE-controls in relation to HE groups ($P < 0.01$). VPA pretreatment of LE-rats decreased TDO2 expression when compared to PSD+US group ($P < 0.01$).

DISCUSSION

Variations in temperament are core traits that can be passed to descendants and lead to a greater risk of developing bipolar disorder (51). In line with this evidence, affective temperaments may help predict vulnerability to mood disorders, while exposure to stress triggers mood episodes (52). In the present study, we observed that the exposure of HE-rats (a pattern of exploratory activity in rats that mimics hyperthymic temperament) and LE-rats (a pattern of exploratory activity in rats that mimics depressive temperament) (8) to a combination of two distinct types of stressors, namely, PSD and US, causes distinct alterations in anxiety-related behavior, but similar working memory and declarative memory impairments accompanied by inflammatory and oxidative changes that varies according to the animals' exploratory profile. In other words, HE-rats exposed to PSD+US presented with more hippocampal pro-inflammatory alterations in relation to LE-rats, as evidenced by increased expression of iNOS and higher plasma levels of IL-1β and uric acid. Furthermore, HE-PSD+US rats presented a marked increase in plasma IL-4 levels. The effects of mood stabilizers appeared to be related to temperament. Indeed, both VPA and Li prevented LE-PSD+US rats from working memory impairment, while the alterations in declarative memory were partially prevented only by Li in both LE- and HE-PSD+US rats. Regarding hippocampal oxidative parameters, both VPA and Li prevented GSH alterations induced by PSD+US in HE-rats, while alterations in lipid peroxidation and nitrite levels were prevented by both drugs only in LE-rats. Plasma alterations of IL-4 and uric acid observed in HE-PSD+US rats were prevented by both drugs, while hippocampal IDO expression induced by PDS+US was prevented by both drugs in animals of both temperaments.

Evaluation of Anxiety-Related Behavior in HE and LE Rats Exposed to Environmental Stressors

In the present study, we observed increased percentage of open arms entries in the plus maze in HE-rats when compared to ME- and LE-rats, an indicative of anxiolytic behavior. Furthermore, when exposed to PSD+US, these rats presented a marked increase in this parameter that was prevented by both mood-stabilizing drugs, VPA and Li. Notably, this anxiolytic pattern observed in HE-rats may be related in patients to an increased risk-taking behavior that is observed in mood disorders, such as bipolar disorder and replicated in animal models of this disorder (53). On the contrary, LE-rats presented increased tendency to explore closed arms, which was not influenced by the exposure to environmental stressors.

In the last years, some attempts have been made to develop models of mood disorders based on the exposure of animals to environmental stressors. In this regard, the exposure of adult rodents (not selected by exploratory pattern) to chronic US is related to the development of depressive-like symptoms (54, 55), while the exposure to PSD is related to the occurrence of mania-like symptoms (39, 56). Based on this evidence, previous studies have been conducted with the exposure of adult rodents to US or PSD, separately. A recent study of our research group showed that the exposure of adult rats to a combination of PSD and hot air blast (HAB) (57), this last being a stressful stimulus related to the development of depressive-symptoms, can induce behavioral and memory alterations that resemble bipolar disorder endophenotypes (58).

In the present study, we exposed periadolescent animals to a combination of stressors. Adolescence is a developmental period characterized by significant neuronal and adrenocortical maturation (59–61). A previous study evaluating the consequences of one-night sleep deprivation on adolescent neurobehavioral performance found that one night of total sleep deprivation had significant deleterious effects upon neurobehavioral performance and subjective sleepiness, leading to impairment in daytime functioning (62). Indeed, sleep loss is known to trigger mania or depression (63). In adolescents, the acute glucocorticoid response, both in human and in rodents, requires a longer period to return to baseline levels compared to adults and prepubertal youth.

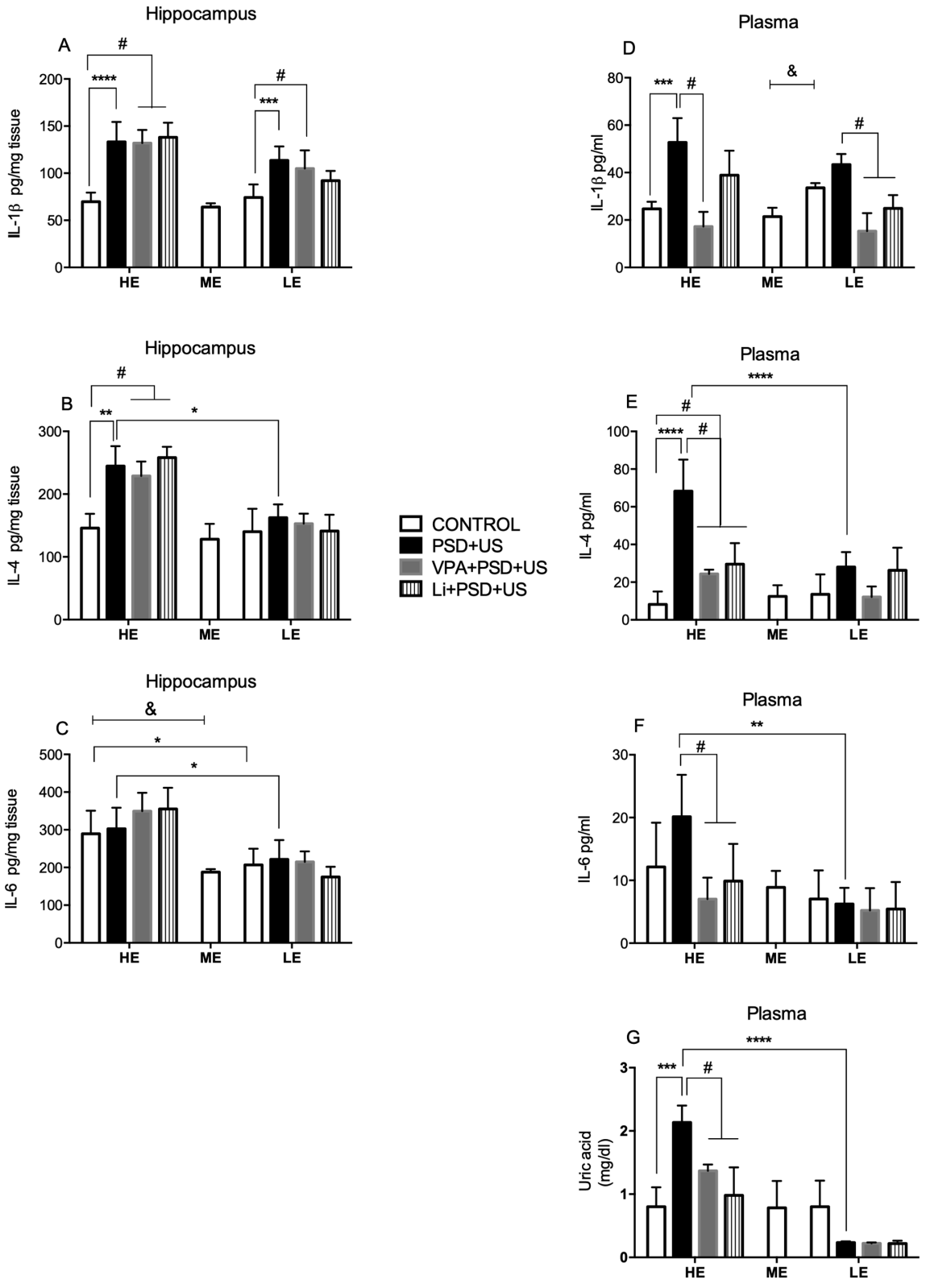

FIGURE 5 | Continued

FIGURE 5 | Effects of the exposure of HE and LE rats to PSD+US in hippocampal IL-1β **(A)**, IL-4 **(B)**, IL-6 **(C)** and plasma IL-1β **(D)**, IL-4 **(E)**, IL-6 **(F)**, uric acid **(G)** inflammatory parameters. Animals were separated by exploratory pattern in HE (high exploratory), ME (medium exploratory), and LE (low exploratory). HE- and LE-rats, respectively, mimic the human temperaments hyperthymic and depressive. During periadolescence, PNs 40–49, HE- and LE-rats were exposed to daily alternated sections of paradoxal sleep deprivation (PSD) and unpredictable stress (US) or left undisturbed (controls). The preventive strategies, VPA or Li, were administered during PNs 42–49. The hippocampi and blood samples were collected on PN60. Bars represent means ± SEM of six to eight animals/group. $^{\&}P < 0.05$ for comparisons between HE-, ME-, and LE-control rats according to one-way ANOVA followed by Dunnett *post hoc* test; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$ for two-way ANOVA comparisons with Tukey as *post hoc* test using "exploratory activity" and "stress exposure" as factors; $^{\#}P < 0.05$ for comparisons between groups pretreated with Li- and VPA- in relation to PSD+US and controls according to one-way ANOVA followed by Tukey *post hoc* test. HE, high exploratory; ME, medium exploratory; LE, low exploratory; PN, postnatal day; PSD, paradoxal sleep deprivation; US, unpredictable stress; VPA, valproate; Li, lithium.

Hence, adolescence is a developmental period for programming of adult adrenocortical responses (59, 63–65).

Contributions of Temperament Combined With Periadolescence Exposure to Environmental Stressors to the Development of Memory Impairment and Hippocampal Oxidative/Inflammatory Alterations

Our study showed that the exposure of periadolescent rats from extremes of temperaments (HE- and LE-rats) to PSD+US (for 10 days) impaired working and declarative memory. We also observed that the increase in the percentage of incorrect alternations and lower discrimination ratio observed in HE- and LE-rats was significant in relation to ME-controls. Taken together, our data suggest that the temperament itself induces working and declarative memory dysfunctions that are exacerbated by repeated and intermittent exposure to PSD+US. Indeed, extensive studies have shown an association between sleep deprivation (66–68) or US (69–72) and cognitive processes in humans and animals (73–77). In line with our findings, various studies have shown sleep deprivation-induced learning and memory impairments in different tests, such as avoidance tasks (78–80), Morris water maze (81, 82), radial maze (73), and the plus-maze discriminative avoidance tasks (83–85). Thus, both acute and chronic forms of sleep deprivation seem to interfere with cognitive functions. In addition, numerous studies have demonstrated that US in laboratory animals produces memory deficits in several behavioral models, such as the Morris water maze (86, 87), Y-maze (86), and elevated plus maze (86, 88).

We also observed that LE-controls presented a lower percentage of incorrect alternations in the Y-maze task when compared to HE-controls. This greater incidence of errors in the Y-maze observed in HE-controls may be related to the higher impulsivity of these rats (89), and also confirmed in our results, since high impulsives are more likely to be involved in risky behavior than low impulsive subjects (90).

Adult patients with obstructive sleep apnea have been shown to present decreased levels of antioxidants and lower performance on neuropsychological tasks (91). A recent meta-analysis revising studies on sleep deprivation and oxidative stress in animals revealed that in rats, 11 out of 13 reports showed oxidative alterations in at least one brain region. In this meta-analysis, one study showed decreased lipid peroxidation, whereas 10 studies showed an increase in oxidative stress (92).

Some molecular mechanisms may underlie the memory impairments induced by sleep deprivation and US. Several studies showed that the cognitive decline triggered by these environmental contingencies could be attributed to a hippocampal oxidative imbalance or a decrease in synaptic plasticity *via* the reduction of brain-derived neurotrophic factor (BDNF) levels (93) after sleep deprivation (94–97) or US (98–100). Thus, as an attempt to explore the mechanisms involved in the memory impairments observed by the exposure to both stressors (PSD+US), in the present study, we measured oxidative/immune-inflammatory parameters in order to better understand the impact of hippocampal changes triggered by the exposure of periadolescent animals with extreme temperaments to these two environmental stressors.

In our results, we observed that temperament alters oxidative homeostasis. Indeed, HE- and LE-controls when compared to ME-controls presented increased levels of hippocampal lipid peroxidation, while LE-controls showed decreased nitrite and GSH levels. On the other hand, both HE- and LE-rats presented increased lipid peroxidation and nitrite levels when exposed to PSD+US. The levels of GSH, the main antioxidant defense (37), were increased in both HE- and LE-PSD+US US groups, but in HE-rats, PSD+US-induced increase was greater when compared to LE-rats. A previous study searching for the time course of memory impairments and oxidative changes in animals exposed to carbon monoxide showed that in the initial period, these animals presented increments in lipid peroxidation and also increased levels of GSH during the course of memory impairment (93). We hypothesize that this increase in hippocampal GSH levels may be a compensatory mechanism to prevent excessive brain damage. This hypothesis is built on the fact that HE-rats present increased baseline brain levels of dopamine (101), which is at least partially responsible for the increased locomotor activity of these animals, whereas dopamine metabolism generates reactive oxygen species that may lead to brain damage (102). In line with this increase in dopamine metabolism in HE-rats, we observed here that HE-control presents increased levels of GSH when compared to ME- (1.3-fold) and LE-controls (2.4-fold) as well as presented higher levels of lipid peroxidation (1.7-fold in relation to ME and 1.4-fold in relation to LE).

Since the neurobiology of mood disorders involves both oxidative and inflammatory alterations (103, 104), we decided to study hippocampal and plasma levels of inflammatory markers. We observed that the hippocampal levels of IL-1β were increased in both HE- and LE-rats exposed to PSD+US, but this hippocampal change did not completely correlate with the alterations observed

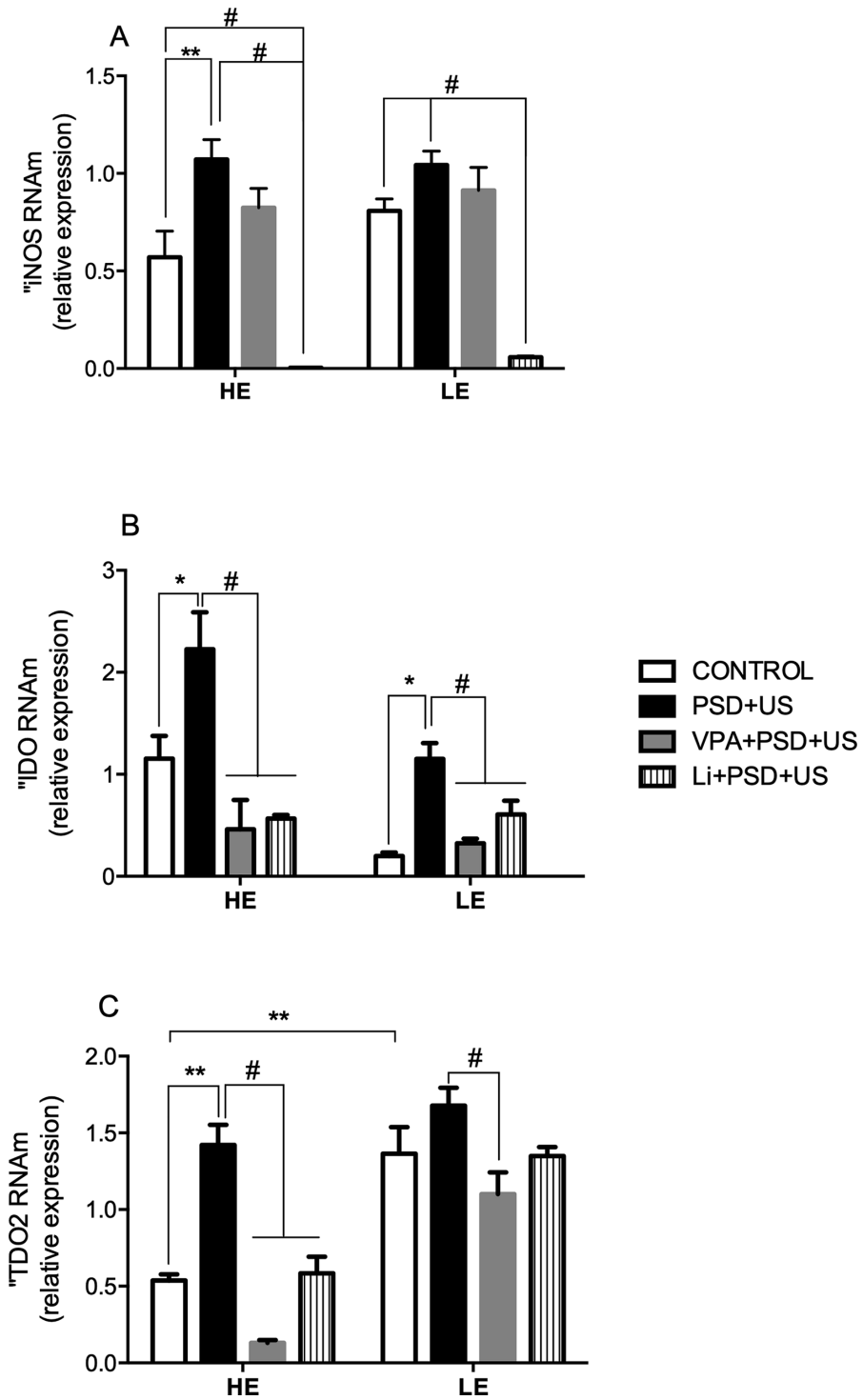

FIGURE 6 | Effects of the exposure of HE and LE rats to PSD+US in iNOS **(A)**, IDO **(B)**, and TDO **(C)** mRNA relative expression. Animals were separated by exploratory pattern in HE (high exploratory), ME (medium exploratory), and LE (low exploratory). HE- and LE-rats, respectively, mimic the human temperaments hyperthymic and depressive. During periadolescence, PNs 40–49, HE- and LE-rats were exposed to daily alternated sections of paradoxal sleep deprivation (PSD) and unpredictable stress (US) or left undisturbed (controls). The preventive strategies, VPA or Li, were administered during PNs 42–49. The hippocampi were dissected on PN60. Bars represent means ± SEM of five animals/group. $^{*}P < 0.05$, $^{**}P < 0.01$ for two-way ANOVA comparisons with Tukey as *post hoc* test using "exploratory activity" and "stress exposure" as factors; $^{\#}P < 0.05$ for comparisons between groups pretreated with Li- and VPA- in relation to PSD+US and controls according to one-way ANOVA followed by Tukey *post hoc* test. HE, high exploratory; ME, medium exploratory; LE, low exploratory; PN, postnatal day; PSD, paradoxal sleep deprivation; US, unpredictable stress; VPA, valproate; Li, lithium.

in the plasma levels of this cytokine. Indeed, since the increase in IL-1β levels was greater in the hippocampus of HE-PSD+US rats compared HE-control (almost two-fold change), and LE-PSD+US presented a 1.5-fold increase in IL-1β compared to LE-control, we believe that the increase in plasma levels of HE-rats and not LE-rats may reflect the difference in the magnitude of the brain IL-1β changes observed between HE- and LE-rats.

The extremes of temperament also influenced IL-4 brain and plasma levels. We observed that only HE-PSD+US rats presented increased hippocampal (1.6-fold increase) and plasma levels of IL-4 (8.5-fold increase). IL-4 is a cytokine related to memory function (105). While normal levels of IL-4 may have neuroprotective effects, high levels of this cytokine are observed in extreme situations, such as traumatic brain injury (106). Hence, IL-4 also presents pro-inflammatory activity (105). IL-4 is a T helper type (Th) 2 cytokine. Recently, Th2 response has been implicated in memory impairments based on the positive correlations between aging and memory deficits with eotaxin-1/CCL11 levels. To date, eotaxin-1/CCL11 is considered an endogenous cognitive deteriorating chemokine whose levels are increased in neurodegenerative disorders and schizophrenia (107). IL-4 induces the release of eotaxin-1/CCL11 from eosinophils and can cross the unaltered blood-brain barrier (108). Eotaxin-1/CCL11 may shift the immune response toward Th2 and promote microglia migration and activation with subsequent production of reactive oxygen species, potentiating glutamate-induced neuronal death (109).

Reinforcing the increase in plasma pro-inflammatory alterations in HE-PSD+US rats, we observed a marked increase in uric acid. Indeed, a previous study searching for deleterious effect of uric acid in memory revealed that a diet based on high-uric acid triggers the expression of proinflammatory cytokines, activates the Toll-like receptor 4 (TLR4)/nuclear factor (NF)-κB pathway, and increases gliosis in the hippocampus of *Wistar* rats (110). Furthermore, high uric acid levels have been associated with hyperthymic and irritable temperament scores, whereas low uric acid levels associate to depressive temperament scores. This finding has led to the assumption that uric acid levels may be a biological marker for the differentiation of unipolar and bipolar disorder (111).

Still in line with the immune-inflammatory changes, we observed that while both HE- and LE-rats exposed to PSD+US presented increased hippocampal expression of the enzyme IDO, only HE-PSD+US rats presented increased hippocampal expression of iNOS and TDO2. This result is in line with the hippocampal levels of cytokines (112), since we observed increased levels of IL-1β in both HE- and LE-PSD+US rats and increased IL-4 only in HE-PSD+US rats. On the other hand, TDO2 activity is associated with Th2 response. Similarly, NO modulates Th1/Th2 balance (113). Since increased expression of iNOS leads to an overproduction of NO, this mechanism of increased Th2 response may also be influenced by iNOS. As previously discussed, only HE-PSD+US rats presented increased hippocampal and plasma levels of IL-4. Hence, the results obtained in the present study suggest an association between memory impairment observed in HE-PSD+US rats and a Th2 mechanism. This needs to be further explored in future studies.

Effect of Mood-Stabilizing Drugs in the Prevention of Memory Alterations and Oxidative/Immune-Inflammatory Mechanisms in HE- and LE-Rats Exposed to PSD+US

The results of the present study showed that Li treatment partially prevented PSD+US-induced impairment in declarative memory in HE- and LE-rats, while both VPA and Li prevented working memory deficits only in LE-PSD+US group. We also measured the levels of cytokines and ROS to investigate possible mechanisms in VPA and Li's preventive effects on hippocampus-dependent learning and memory impairment in HE and LE rats. As a result, we found that VPA decreased plasmatic levels of IL-1β and IL-6 in HE rats. There was also a decrease of IL-1β levels in LE animals by both Li and VPA. Moreover, VPA was able to reduce the levels of lipid peroxidation in HE and LE rats. Interestingly, plasma levels of the cytokines IL-1β and IL-4 as well as of uric acid were predictive of the effects of the mood-stabilizing drugs only in HE-rats.

Taken together, these results show the role of VPA and Li in neuronal functions, neuroprotection, learning, and memory.

The present study has some limitations since we did not test the effects of each stressor separately. The reason for this decision was to follow the principle of the 3Rs, since there is a great amount of literature on US and PSD single exposure. Another limitation was that the protocol was conducted only in males and not in female rats. We also did not evaluate microglia alterations, nor the levels of eotaxin-1/CCL11 and corticosterone, which seems to be important parameter for determination in future studies.

In conclusion, our findings contribute to a limited body of literature investigating the cognitive consequences of sleep deprivation alternated with unpredictable stress in rats separated by temperament, as well as its response to mood stabilizers. Our results are congruent with the current knowledge that exposure to environmental stressors and temperamental susceptibility triggers mood disorder, and extend current literature, suggesting that a Th1/Th2 imbalance may contribute to neuroinflammatory and oxidative changes in HE and LE rats. In this context, the study of affective temperament, such as its genetic bases, phenotypic alterations, and influence in the treatment of mood disorders, becomes an instrument for personalized psychiatry.

DATA AVAILABILITY

All datasets generated for this study are included in the manuscript and the supplementary files.

ETHICS STATEMENT

The methods were carried out in accordance with the NIH Guide for the Care and Use of Laboratory Animals (12) and with the approval of the local ethical committee of Universidade Federal do Ceara. All efforts were made to

minimize animal suffering and to reduce the number of animals used.

AUTHOR CONTRIBUTIONS

CL, FS, AC, AQ, and AO treated the animals and performed the behavioral evaluations and neurochemical analyses. CL also helped in the study design. CL, FS, SV, and DFL performed the statistical analyses. GF, JQ, and MF contributed to the study design and to the first draft. DM designed the study, constructed the graphics, and wrote the final 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.2019.00547/full#supplementary-material

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A Novel Continuously Recording Approach for Unraveling Ontogenetic Development of Sleep-Wake Cycle in Rats

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Sleep-wake development in postnatal rodent life could reflect the brain maturational stages. As the altricial rodents, rats are born in a very undeveloped state. Continuous sleep recording is necessary to study the sleep-wake cycle profiles. However, it is difficult to realize in infant rats since they rely on periodic feeding before weaning and constant warming and appropriate EEG electrodes. We developed a new approach including two types of EEG electrodes and milk-feeding system and temperature-controlled incubator to make continuously polysomnographic (PSG) recording possible. The results showed that there was no evident difference in weight gaining and behaviors between pups fed through the milk-feeding system and warmed with temperature-controlled incubator and those kept with their dam. Evolutional profiles of EEG and electromyogram (EMG) activities across sleep-wake states were achieved perfectly during dark and light period from postnatal day (P) 11 to P75 rats. The ontogenetic features of sleep-wake states displayed that the proportion of rapid eye movement (REM) was 57.0 ± 2.4% and 59.7 ± 1.7% and non-REM (NREM) sleep was 5.2 ± 0.8% and 4.9 ± 0.5% respectively, in dark and light phase at P11, and then REM sleep progressively decreased and NREM sleep increased with age. At P75, REM sleep in dark and light phase respectively, reduced to 6.3 ± 0.6% and 6.9 ± 0.5%, while NREM correspondingly increased to 37.5 ± 2.1% and 58.4 ± 1.7%. Wakefulness from P11 to P75 in dark phase increased from 37.8 ± 2.2% to 56.2 ± 2.6%, but the change in light phase was not obvious. P20 pups began to sleep more in light phase than in dark phase. The episode number of vigilance states progressively decreased with age, while the mean duration of that significantly increased. EEG power spectra in 0.5–4 Hz increased with age accompanied with prolonged duration of cortical slow wave activity. Results also indicated that the dramatic changes of sleep-wake cycle mainly occurred in the first month after birth. The novel approaches used in our study are reliable and valid for continuous PSG recording for infant rats and unravel the ontogenetic features of sleep-wake cycle.

Keywords: polysomnographic recording, sleep-wake states, ontogeny of sleep, infant rats, milk-feeding, temperature-controlled

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INTRODUCTION

The ontogenetic hypothesis of sleep, proposed 50 years ago, postulates that early developmental sleep is essential for maturation of fundamental brain function (1). Daily sleep amounts are highest early in development across multiple species (2–5) that lead to promote normal brain development that give rise to adult critical behaviors for learning, memory consolidation, emotional processing and species propagation (4, 6, 7). Human studies have showed that impaired sleep during early periods of development can have severe and longlasting consequences such as cognitive, attentional, and psychosocial problems (8–10).

In all species studied so far, measures of sleep changes throughout development are fundamental ways for unraveling which regions of brain are most susceptible to sleep perturbations early in life. Sleep electroencephalogram (EEG) in humans and cortical EEG recordings in animals provide unique *in vivo* opportunities to observe regional changes in brain activity over the course of cortical maturation. In the human being, the distinct electrical patterns associated with the different sleep states begin to emerge approximately at 28 weeks' gestation age. By 30 weeks' gestation, the EEG patterns of rapid eye movement (REM) and non-REM (NREM) sleep (also named as active sleep (AS) and quiet sleep (QS) in the infant, respectively) appear but are not continuous (11). The ontogenetic changes of sleep in animal life are similar to those in humans (4, 12). The rat is an altricial born in a far less mature condition than humans and its cortical maturity during the first postnatal week corresponds to that of the young premature human brain (13–15). Thus, rats are good models to study the development of the sleep-wake cycle and its EEG rhythms because more immature stages of these processes can be studied in postnatal life when they are more experimentally accessible (16).

In recent studies, the sleep-wake states in rat pups are identified by combining visual observation with measurements of muscle activity and EEG recordings (2, 17–20). However, most of these studies observed and recorded intermittently, which means they cannot get 24 h continuous recordings, and cannot elaborated the dramatic changes and circadian development during the whole day in early life. The limitations of the early methods for intermittent recordings are largely due to periodic feeding before weaning and keeping warm during long-term sleep recording in pups after maternal separation. The sensitivity of pups to limosis and improper temperature around recording environment easily produces significant changes in sleep-wake pattern (21, 22). Furthermore, an inadequacy of the conventional screws as EEG electrodes for polysomnographic (PSG) recording are implanted into the neonatal rat skull and wired with a relatively large head plug cemented onto the small skull surface that is soft, fragile, and rapid growing. Therefore, it is necessary to make light and suitable EEG electrodes which could effectively achieve EEG signals from young pups.

We have recently developed the novel approaches including a milk-feeding system for the pre-weaned pups, a temperature-controlled incubator, and two types of EEG electrodes and well-matched plug according to the pup age for continuously (24 h/day) PSG recording that overcomes the above limitations of conventional methods. Moreover, we carried out a periodic stimulation on the anogenital region and grooming for the pre-weaned pups in order to induce micturition and defecation (a process usually performed by the lactating dam), and to minimize the stress response due to maternal deprivation (2, 23, 24). The present study describes the new PSG recording methods in detail and offers ontogenetic features of sleep-wake cycle in rats from postnatal day (P) 11 to P75.

MATERIALS AND METHODS

Animal Preparation

Adult male and female Sprague-Dawley rats (6–8 weeks old, weighing = 250 ± 35 g) were purchased from the Experimental Animal Center of Lanzhou University (Lanzhou, PR China). A male with two female rats were housed in a plastic cage (485 mm L × 350 mm W × 225 mm H) for mating and kept in an automatically controlled room in a 12:12-h light/dark cycle (lights on 8:00–20:00 h, illumination intensity = 100 lx) at an ambient temperature (23 ± 1°C) and 50% relative humidity with food and water available *ad libitum*. The mating procedure was repeated on successive days until copulation and confirmed on the basis of vaginal plug formation (monitored every morning). The pregnant rats were individually housed in cages and checked twice daily until birth. The day of birth was defined as P0, and 10 pups of a litter were generally kept in their dam and their behaviors were monitored by an infrared video camera. A total of 19 pregnant rats were used in this study, and 89 of offspring were successfully used to record sleep-wake states. All animals were cared for, and experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (2011 revision). The experimental protocol was approved by the Ethics Committee of Lanzhou University (permit number: SCXK Gan 2018–0002, Lanzhou, PR China). All possible efforts were made to reduce the number of animals used and discomfort to the animals.

EEG and EMG Electrodes and Their Implantation

In order to effectively acquire EEG and electromyogram (EMG) signals and to reduce weight of electrodes borne by pups, two types of EEG electrodes were used respectively, for ≤P16 and ≥P17 pups. For ≤P16 pups, 4 gold-plated pins (top of pin = 0.5 × 0.5 mm) served as EEG electrodes were assembled within a 6-pin pedestal (weighing = 0.3 g). As shown in **Figure 1A**, the interval between left and right pins and between anterior and posterior pins respectively, is 2.54 and 5.08 mm. For ≥P17 pups, 4 gold-plated screws (diameter = 1 mm) served as EEG electrodes were connected with insulted silver-plated copper wires and soldered into a 6-pin pedestal socket (**Figure 1E**). One end of a pair of 25 mm silver wires (diameter = 0.5 mm) insulated with fluorinated ethylene propylene was exposed to 5 mm and then looped and filled with soldering tin served as EMG electrodes, and the other end was soldered into the pedestal socket (**Figure 1A**). Finally, the exposed welding points were covered and insulated by hot melt adhesive for preventing

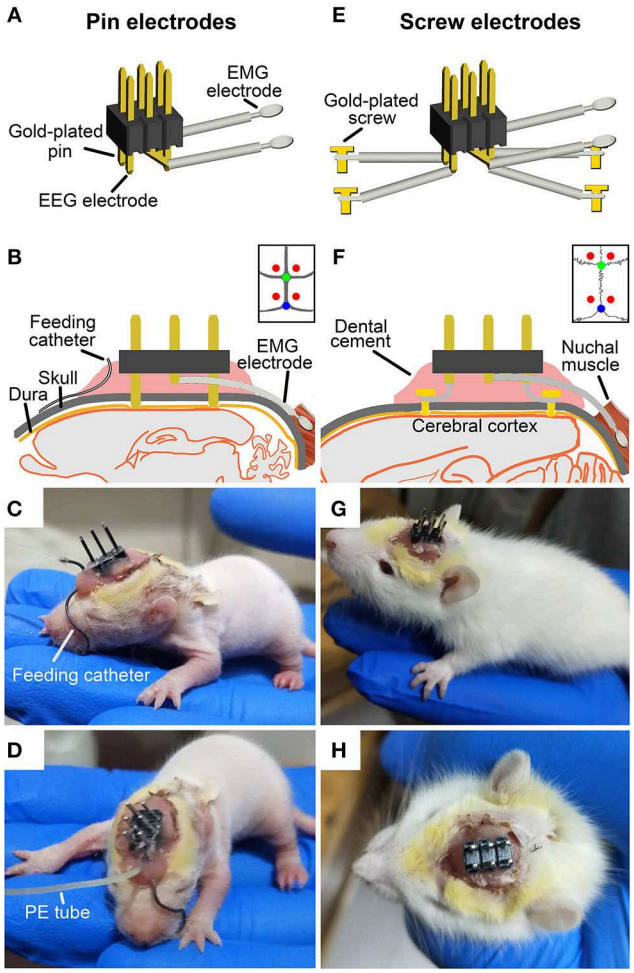

FIGURE 1 | Schematic showing the implantation of two types of EEG and EMG electrodes with or without a milk-feeding catheter respectively, in ≤P16 **(A–D)** and ≥P17 pups **(E–H)**. For ≤P16 pups, 4 gold-plated pins **(A)** as EEG electrodes were directly implanted through skull to contract with the dura (**B**, implanted sites are shown by red points in insert), and 2 silver loops filled tin as EMG electrodes were inserted into nuchal muscles. A metal milk-feeding catheter **(C)** connected with PE tube **(D)** was amenably placed along left angulus oris into side of oral cavity. For ≥P17 pups, 4 gold-plated screws as EEG electrodes **(E)** were screwed through skull into the dura **(F**, screwed sites are shown by red points in insert) and a pair of silver loops as EMG electrodes was inserted into nuchal muscles. **(G,H)** Respectively, show side and top view of the plug fixed chronically to skull with dental cement. Green and blue points in insert of **(B,F)** respectively, show bregma and lambda.

possible surgery-induced conduct electricity from neighboring wires. The electrical continuity between the electrodes and the outputs of pedestal socket was checked with a digital multimeter before the implantation of EEG and EMG electrodes.

Under isoflurane anesthesia (1%, flow rate of 0.4 L/min; R510-22, RWD Life science Co. Ltd, Shenzhen, PR China), pups were prepared for aseptic surgery, and secured in a SR-6R stereotaxic frame (Narishige, Tokyo, Japan) on a homeothermic heating mat (37°C, ThermoStar, 69020, Life science Co. Ltd., Shenzhen, PR China). Four pins EEG electrodes were directly inserted onto the dura mater through the two pairs of skull holes that are corresponded to the interval between the 4 pins, and were located, respectively, in the frontal (1.27 mm lateral and 1 mm anterior to the bregma) and parietal (1.27 mm lateral to the midline and 4.08 mm posterior to the bregma) cortices (**Figure 1B**). Four gold-plated screws EEG electrodes were screwed through the similar coordinates on the skull onto the dura mater (**Figure 1F**). The EMG electrodes were bilaterally placed into the nuchal muscles. The pedestal socket was chronically fixed to skull with dental cement (**Figures 1C,G**).

Feeding Catheter and Its Installation

Based on our explored experiments and previous report (25), the ≤P16 pups need to be lactated for getting adequate nutrition. A feeding catheter was made of a stainless-steel tube (internal diameter = 0.5 mm, length = 31 mm), one end of which was obtuse for inserting oral cavity, and the other end of which was connected with a pliable PE tube for pumping milk.

Under anesthesia, the oral end of feeding catheter was simultaneously inserted along left angulus oris into ipsilateral oral cavity near buccal mucosa for 2 mm after EEG and EMG electrodes implantation. The rest of feeding catheter was curved along the curvature of skull, and the end connected with PE tube was reached and fixed with dental cement to the anterior of pedestal socket (**Figures 1C,D**).

Care for Young Pups After Surgery

After surgery, the pups were placed singly in a temperature-controlled incubator and fed by milk-feeding system within a sound-attenuated, ventilated and electrically isolated sleep-recording chamber, and allowed to recover for 36 h (**Figures 2A,B**). The incubator (300 mm L × 300 mm W × 400 mm H) is made of acrylics, and its base part was immersed in temperature-controlled water bath. The pups were placed on a diaper covered the bottom of the incubator during the recovery and sleep-wake recording. The thermal environment was controlled with a temperature sensor that automatically switched on and off the calorifier (**Figures 2A,C**), and it was set by the age of P9-P20 (**Table 1**) based on our explorative studies and previous reports (24, 26, 27).

The milk-feeding system was consisted of a computer-assisted infusion pump (KDS210, KD Scientific, MA, USA) and a conterminous PE tube connected to feeding catheter. The feeding pump was programmed to infuse formula milk (20%, Nestle, Lactogen-1, Harbin, PR China) for 1 min per hour, and its delivering rate was set to be almost suckled by pups at each corresponding age (**Table 1**). The total volume of milk infused at each age was calculated to result in a weight gain similar to that occurs in pups lactated by mater over the same period of time (24) (**Table 1**). In addition to milk, rat chow soaked with milk was added to pups at P16. Based on our probe trials and previous studies, pups could be weaned at P16 without any major effects on their development (25). When pups were older than P17, standard rat chow, formula milk in bottle for P17-P20 and water for P21-P75 were available *ad libitum*. The pre-weaned pups were also ministered to stimulate anogenital region and groom body with moistened cotton swabs twice a

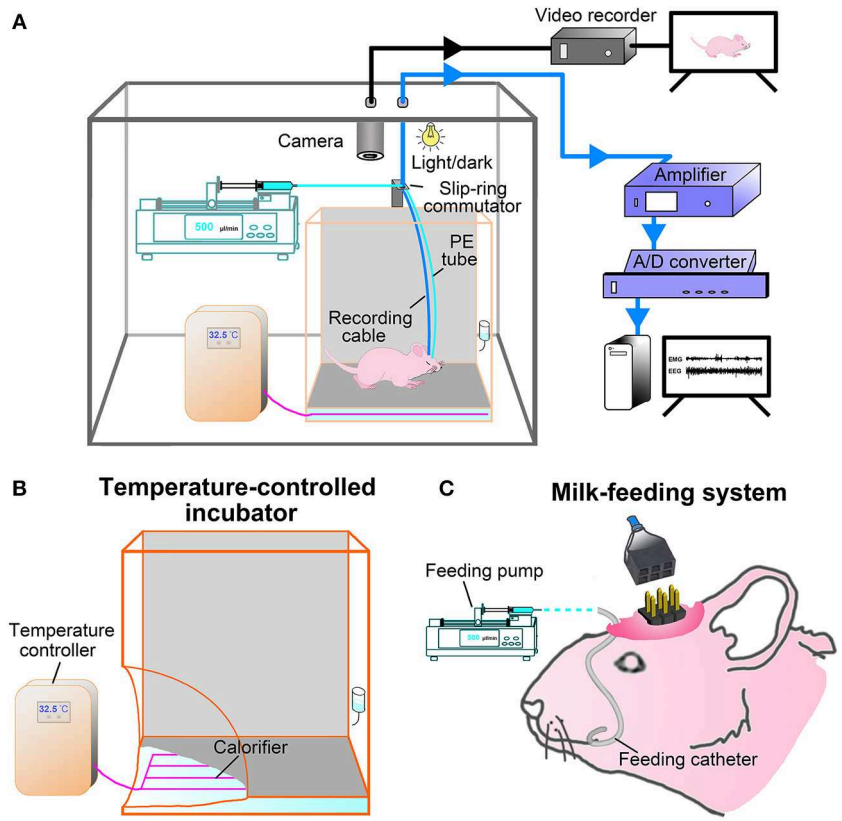

FIGURE 2 | Experimental setup. **(A)** Constructing illustration shows sleep-wake states and behavior recording and analyzing setup for freely moving infant rats. Pups were kept in a temperature-controlled incubator in which the ambient temperature was set according to its age and controlled by temperature sensor that automatically switched on and off the calorifier **(B)**, and their behaviors on a 12:12-hlight/dark cycle in the sleep recording chamber was monitored with an infrared camera and stored with video recorder (black line). The ≤P16 pups were fed once per hour by a milk-feeding system which was consisted of a programmable infusion pump and a conterminous PE tube connected to feeding catheter **(C)**. The ≥P17 pups freely accessed to rat chow and milk or water. EEG and EMG signals were transmitted through a flexible cable connected with slip ring to an amplifier, digitized with an A/D convertor, and stored and analyzed with Spike 2 scripts (CED, UK) installed in PC (blue line).

day, so as to induce micturition and defecation and reduce the stress of maternal deprivation. All pups from the same litters with or without the experimental process were weighed everyday, and their movement, hair growth, and eye-opening time were observed to evaluate the adequate nutritional status.

Sleep-Wake States Recording and Analysis

During recovery and sleep-wake recording, pups were kept in an automatically controlled sleep-recording chamber on a 12:12-h light/dark cycle (lights on 8:00–20:00 h, illumination intensity = 100 lx), and their wake- and sleep-related behaviors were simultaneously monitored and recorded by an infrared video camera above incubator (**Figure 2A**). After recovery, a 24-h sleep-wake cycle was recorded following pups acclimation to the recording cable connected to a slip-ring commutator for 12 h. In addition, the P21-P75 rats were kept in a barrel (300 mm L × 300 mm W × 400 mm H) within the sleep-recording chamber with an ambient temperature (23 ± 1°C) for recovery after surgery and sleep recording.

EEG and EMG signals were amplified (2000×) and filtered (0.5–30 Hz for EEG and 30-300 Hz for EMG; Model 3500, A-M Systems, WA, USA) and digitized with a resolution of 256 and 128 Hz, respectively, using CED 1401 MK II (Cambridge Electronic Design Limited, Cambridge, UK) (**Figure 2A**).

The sleep-waking states were defined by the EEG and EMG signal recordings and behaviors monitored with video camera. Using a Spike 2 (CED, Cambridge, UK) script and with the assistance of spectral analysis by the fast Fourier transform (FFT), PSG records were visually scored by 10-s epochs for wake, NREM and REM sleep according to previously described criteria validated for infant and adult rats (2, 20, 28–32). Briefly, wake was identified by the presence of low-voltage fast-EEG and sustaining high-EMG activities coupled with limbs crawling, moving or standing posture with eyes open. NREM sleep marked by continuous high-voltage slow-EEG and low-EMG activities coupled with limbs curling or uncurling immobility with eyes closed. REM sleep characterized by an appearance of theta waves, in addition to low-voltage fast EEG activity, and an occurrence of irregular burst in persistence of low-EMG activity associated with body twitches (phasic and rapid movements of limbs and tail) during pup's immobility with eyes closed.

TABLE 1 | The parameters of formula milk infusion and ambient temperature set at each age.

Postnatal days	Formula milk infusion		Ambient temperature (°C)
	Rate (mL/min)	Volume (mL/day)	
9	0.250	6.0	36
10	0.300	7.2	36
11	0.350	8.4	35
12	0.375	9.0	34
13	0.400	9.6	33
14	0.500	12.0	32
15	0.500	12.0	31
16	0.625	15.0	30
17	–	–	29
18	–	–	27
19	–	–	25
20	–	–	23

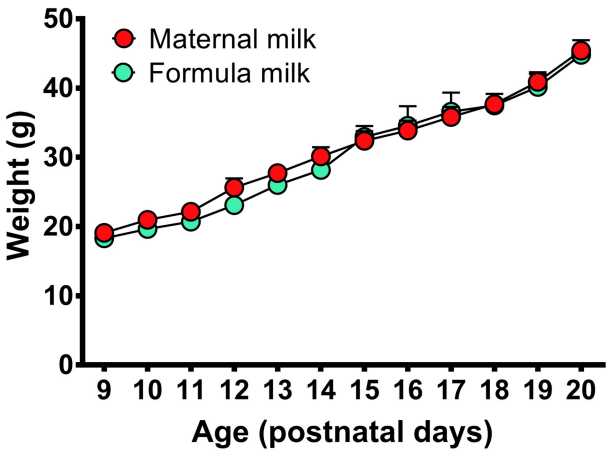

FIGURE 3 | Weight gains in the P9-P20 pups fed respectively, by formula milk and maternal milk. The weight of pups fed with formula milk were not significantly different from that fed with their dam, though the weight gains in the pups fed with formula milk had a slight reduction at P10-P14. Values are means ± SEM (n = 5–6 in each age). Data are analyzed by Student's t-test.

The changes of cortical EEG power spectra across the sleep-wake states in each postnatal day were computed for consecutive 10-s epochs in the frequency range 0.5 to 30 Hz by FFT using Spike 2 software with a frequency resolution of 0.5 Hz. A window weighting function (Hanning) was applied before FFT performance (33). In addition, the delta (0.5–4 Hz) power in NREM spectrum was analyzed to display the developmental biomarker in EEG power densities. The percentage of delta power densities relative to NREM spectrum that was performed with FFT in all 10-s epochs of NREM without obvious artifact was compared across the considered key postnatal days (P11, P15, P20, P30, and P75).

Statistics

All data are expressed as means ± SEM. IBM SPSS Statistics for Windows (version 21.0, NY, USA) was employed for the data analysis. The Student's t-test was used to compare weight gain at each age between pups fed by formula milk and by their mater. One-way analysis of variance (ANOVA) followed by *post hoc* Fisher's least significant difference (LSD) was used to analyze the developmental changes in delta power in NREM spectrum, and in the amount, episode number and mean duration of vigilance states and state transitions. Statistical significance was considered as $P < 0.05$.

RESULTS

Effectiveness of Temperature-Controlled Incubator and Milk-Feeding System on Growth of Young Pups

In this study, a milk-feeding system was used to feed formula milk for P9-P16 pups, and a temperature-controlled incubator was used to keep proper temperature of P9-P20 pups during recovery after surgery and PSG recording according to each age schedule (**Table 1**). In comparision with the pups fed by their dam, the pups fed with formula milk and warmed with temperature-controlled incubator showed no obvious differences in weight gain at each age (**Figure 3**) as well as hair growth.

Behavior Aspects

The behaviors in the rats implanted with EEG and EMG electrodes, and with or without a feeding catheter were monitored through video camera during recovery and PSG recording. The postoperative pups moved freely and easily, presumably because the implant apparatus and pedestal socket linking cable to the commutator were light, soft and pliable. The P9-P11 pups remained eyes closed, and presented distinct behavior patterns. Either they apeared to be awake, with crawling and wiggling movements and tonic extension of neck, or motionless with a muscular hypotonia interrupted by frenquent twitches of the entire body. The twitches were much more intense from P9 to P12. Pup's eyes generally opened during P12-P15, and body twitches were gradually less. The immobile posture with curling or uncurling limbs and without muscular twitches, as presumed NREM sleep, was increased with age. Behavior aspects of sleep and wakefulness at P20 became more adult-like.

Characteristics of EEG and EMG Activities Across Sleep-Wake States

Implantation of the gold-plated pins or screws as EEG electrodes into frontal and parietal dura respectively, for ≤P16 (n = 31) and ≥P17 (n = 58) rats, and of the silver loops as EMG electrodes into nuchal muscle successfully captured cortical EEG and muscular EMG signals. As summarized in **Figure 4**, the representative EEG and EMG patterns of each state were clearly distinguished in P11-P75 rats. Overall, P11-P13 pups spent their most time in REM sleep characterized by an appearance of theta waves, in addition to low-voltage fast EEG activity, and an occurrence of

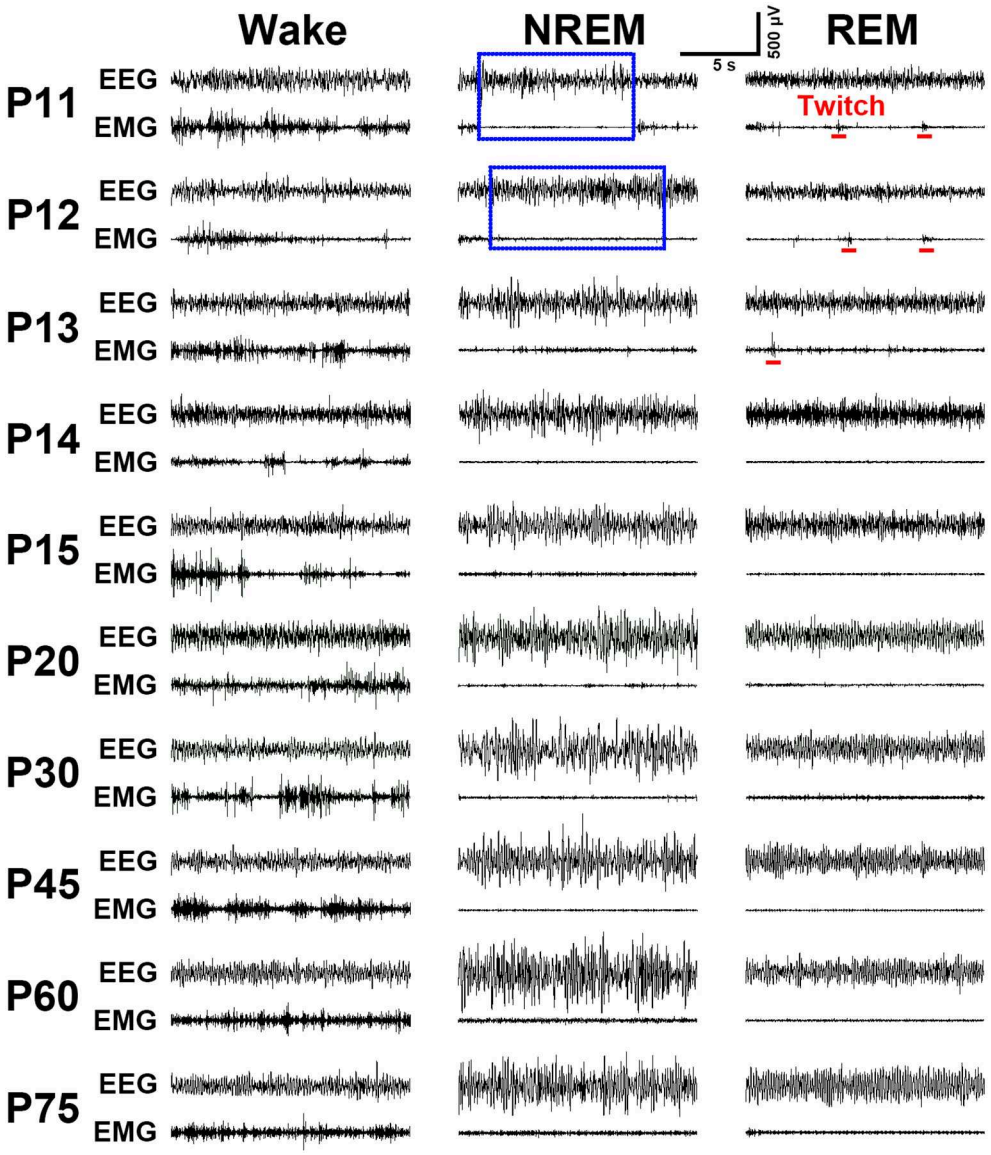

FIGURE 4 | Evolution of EEG and EMG activities in wake, NREM and REM sleep from P11 to P75 rats. Note the EMG bursts during REM sleep denoting the presence of muscular twitches (red bar) in P11-P13 pups. Calibrations: 500 μV, 5 s.

nuchal EMG bursts accompanying muscle twitches appearing on a background of very low EMG activity. P14-P20 pups progressively reduced the total time of REM sleep, meanwhile, the occurrence of EMG bursts associated with muscle twitches gradually declined and eventually disappeared. NREM sleep characterized by high-voltage slow-EEG and low-EMG activities in P11-P12 pups were very short and rare. However, its episode number and duration gradually increased during P13-P15. The EEG activity during NREM at P30 was similar to adult (P75). Wakefulness during P11-P75 was characterized by low-voltage fast-EEG activity and sustaining high-EMG activity, though EEG and EMG activities between P11 and P13 showed fragmentation and variation.

Development of Sleep-Wake Cycle

The developmental profile of EEG power spectra, EEG, EMG and sleep-wake states during dark (21:00–23:00 h) and light period (9:00–11:00 h) in P11-P75 rats were shown in **Figure 5A**. On the 11th day, the amount of REM sleep was very great (daily average 58.5 ± 1.4%) and its episode duration was short, and NREM occurrence was rare (daily average 5.1 ± 0.4%). The main transition between REM sleep and wakefulness was very quick so that the EEG power spectra displayed low density and scattering across the frequency of 0–30 Hz. P11-P15 pups still spent more time in REM than NREM sleep, though NREM sleep progressively increased and REM sleep decreased. The episode mean duration in each state progressively prolonged during this

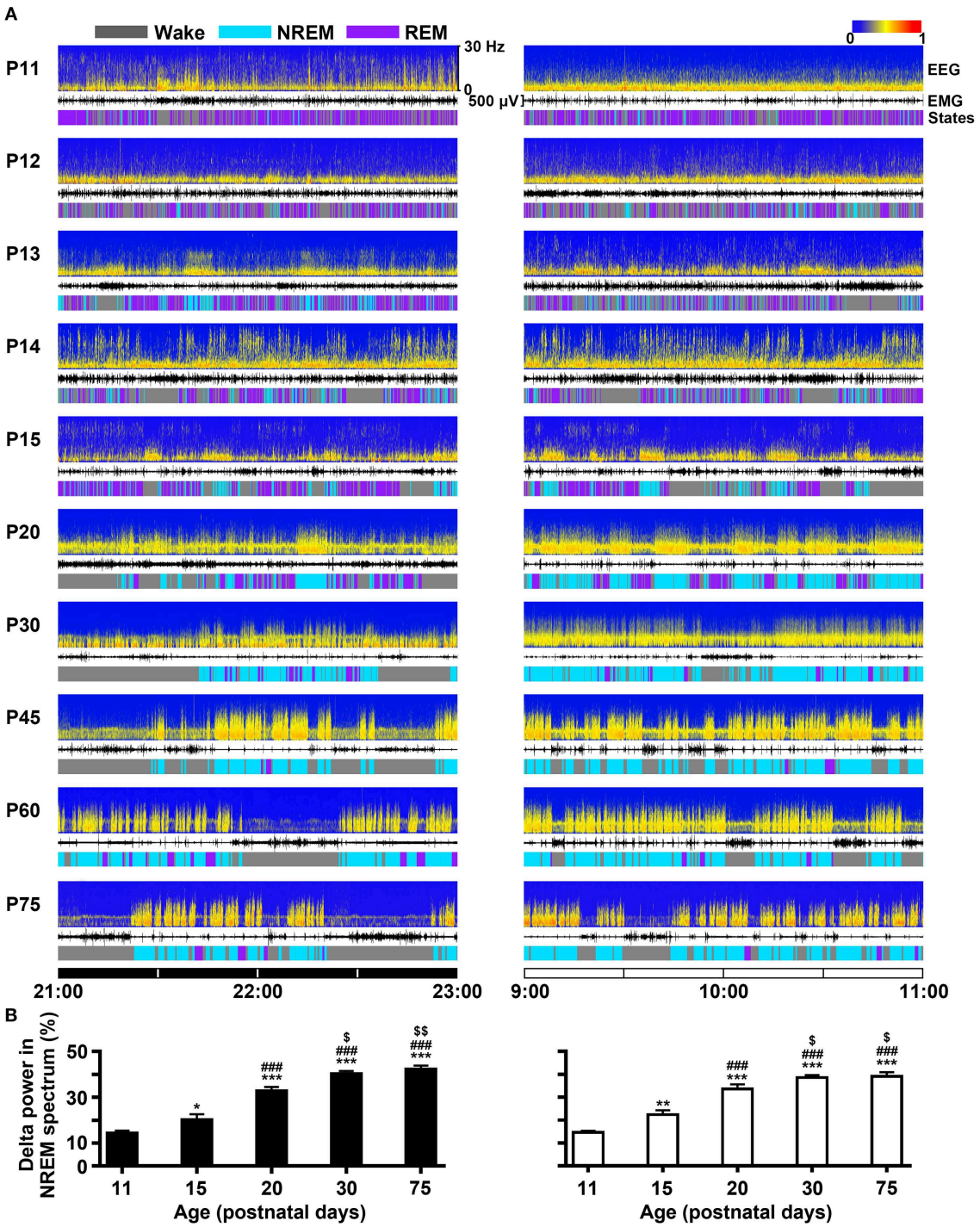

FIGURE 5 | Evolutional profiles of sleep-wake states in dark and light phase from infant to adult rats. **(A)** Representative EEG power spectra (frequency 0–30 Hz), EMG activity (500 μV) and sleep-wake states show the changes during 21:00–23:00 h of dark phase and 9:00–11:00 h of light phase in rats from P11 to P75. **(B)** The analysis of EEG power spectra displayed that the percentage of delta (0.5-4 Hz) power in NREM spectrum at the key postnatal days (P11, P15, P20, P30, and P75) was significantly increased in both dark (21:00–23:00 h) and light (9:00–11:00 h) phases from P11 to P30. Values are means ± SEM (n = 5-9 in each age), $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with P11; $^{\#\#\#}P < 0.001$ compared with P15; $^{\$}P < 0.05$, $^{\$\$}P < 0.01$ compared with P20. Statistics are analyzed by one-way ANOVA and followed by Fisher's LSD test.

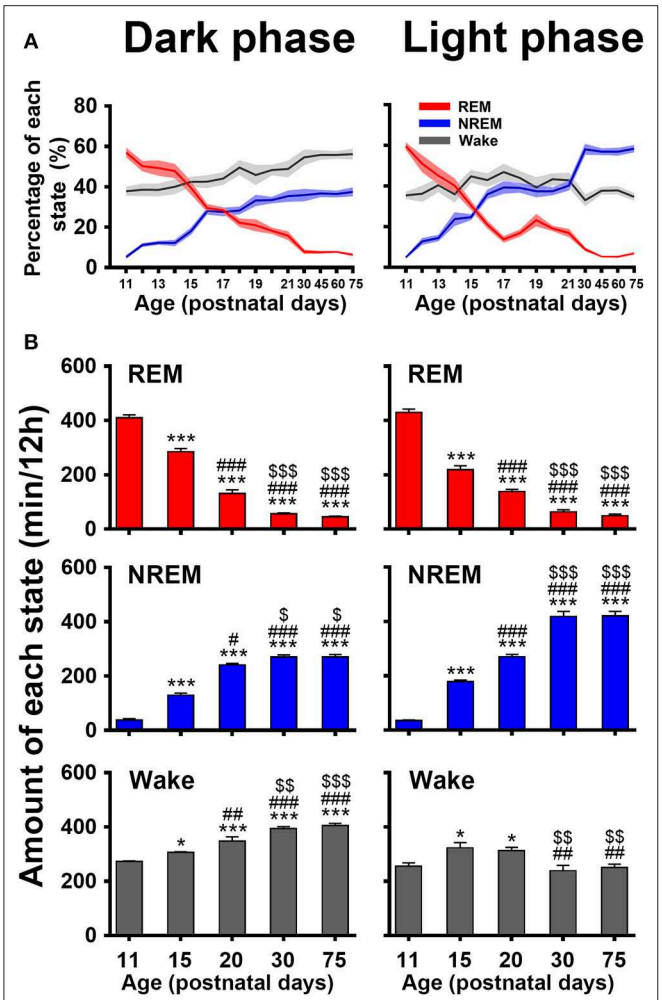

FIGURE 6 | Developmental changes of percentage **(A)** and amount **(B)** of REM, and NREM sleep and wakefulness in dark and light phase from infant to adult rats. The amount analysis at the key postnatal days displayed that REM sleep dramatically decreased in both dark and light phases from P11 to P30, and NREM sleep and wakefulness meanwhile increased, but the amount of each state in respective dark and light phase from P30 to P75 was not obviously different. Notably, the increased NREM sleep in light phase was more than that in dark phase from P20 to P30, while wakefulness significantly increased in dark phase and decreased in light phase. Values are means ± SEM (n = 5–9 in each age), $^{*}P < 0.05$, $^{***}P < 0.001$ compared with P11; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ compared with P15; $^{\$}P < 0.05$, $^{\$\$}P < 0.01$, $^{\$\$\$}P < 0.001$ compared with P20. Statistics are analyzed by one-way ANOVA and followed by Fisher's LSD test.

period. And interestingly, the immediate transition from wake to REM sleep occurred frequently. The amount of wakefulness and sleep between dark and light phase during the age from P11 to P19 was no obvious difference. However, the pups at P20 began to sleep more during light period than dark period. The diurnal rhythm of sleep-wake cycle at P30 was quite similar to that at P75. The EEG delta (0.5–4 Hz) power in NREM spectrum was markedly increased with age (**Figure 5B**). The stable amount, architecture and EEG power spectra across sleep-wake cycle during light and dark period were found in P30-P75 rats.

The evolutional process of wake, NREM and REM sleep in dark and light phase from P11 to P75 was illustrated in **Figure 6**. The proportion of REM and NREM sleep had a dramatic decrease and increase, respectively (**Figure 6A**). The REM sleep was reduced respectively, from 57.0 ± 2.4 to 6.3 ± 0.6% in dark phase and from 59.7 ± 1.7 to 6.9 ± 0.5% in light phase. The NREM sleep in dark and light phase increased respectively, from 5.2 ± 0.8% to 37.5 ± 2.1% and from 4.9 ± 0.5 to 58.4 ± 1.7%. Wakefulness in dark phase from P11 to P75 increased from 37.8 ± 2.2 to 56.2 ± 2.6%. Attractively, the increased NREM sleep from P20 to P30 in light phase was more than that in the dark. Furthermore, an amount analysis of each state in dark and light phase at the considered key postnatal days (P11, P15, P20, P30, and P75; **Figure 6B**) during developmental process demonstrated that REM sleep from P11 to P30 dramatically decreased in both dark (410.2 ± 10.7 min vs. 56.2 ± 3.4 min, $P <$ 0.001) and light (429.5 ± 12.4 min vs. 63.8 ± 7.1 min, $P < 0.001$) phases, while NREM sleep simultaneously increased in dark (37.6 ± 5.5 min vs. 270.5 ± 7.6 min, $P < 0.001$) and light phase (35.5 ± 1.9 min vs. 418.2 ± 18.7 min, $P < 0.001$). Notably, the increased NREM sleep from P20 to P30 in light phase was more than in dark phase (418.2 ± 18.7 min vs. 270.5 ± 7.6 min, $P = 0.001$). In the meantime, wakefulness significantly increased in dark phase (347.8 ± 15.8 min vs. 393.3 ± 7.7 min, $P < 0.01$) and decreased in light phase (312.4 ± 12.3 min vs. 238.0 ± 20.4 min, $P < 0.01$). However, the amount of each state in dark and light phase from P30 to P75 was not significantly different ($P > 0.05$).

An architectural profile of sleep-wake development showed that the episode number of each state progressively decreased in both dark and light phases with age, while the mean episode duration significantly increased (upper panels in **Figures 7A,B**). A further statistical analysis at the key postnatal days (lower panels in **Figures 7A,B**) demonstrated that REM and wake episode number from P11 to P75 in dark phase (REM, 665.3 ± 26.6 vs. 52.6 ± 1.7 and wake, 752.0 ± 23.4 vs. 99.2 ± 2.1) and light phase (REM, 677.0 ± 18.8 vs. 52.1 ± 12.3 and wake, 733.0 ± 22.6 vs. 124.8 ± 5.8) were significantly reduced ($P <$ 0.001), while their mean duration remarkably increased (REM, 0.62 ± 0.02 min vs. 0.88 ± 0.08 min, $P < 0.05$ and wake, 0.36 ± 0.01 min vs. 4.09 ± 0.11 min, $P < 0.001$ in dark phase; REM, 0.64 ± 0.04 min vs. 1.13 ± 0.13 min, $P < 0.05$ and wake, 0.35 ± 0.01 min vs. 2.04 ± 0.14 min, $P < 0.001$ in light phase). Notably, NREM episode number from P11 to P15 increased respectively, in dark (200.0 ± 34.6 vs. 336.0 ± 16.6, $P < 0.01$) and light phase (225.9 ± 12.3 vs. 345.6 ± 21.1, $P < 0.01$) and then decreased from P20 to P75 (251.8 ± 19.4 vs. 109.0 ± 4.2, $P < 0.001$ in dark phase and 254.1 ± 5.5 vs. 135.9 ± 9.4, $P < 0.001$ in light phase). Drastically, its episode duration from P20 to P75 was increased in both dark (0.98 ± 0.08 min vs. 2.50 ± 0.09 min, $P < 0.001$) and light phase (1.06 ± 0.04 min vs. 3.19 ± 0.19 min, $P < 0.001$). The analysis of state transitions (**Figure 7C**) demonstrated that the number of transitions of wake-REM (46.6 ± 2.8 in dark phase and 43.4 ± 1.5 in light phase) and REM-wake (54.4 ± 1.6 in dark phase and 55.3 ± 1.1 in light phase) were great at P11, and then sharply reduced and declined to a low level at P20 (wake-REM, 1.5 ± 0.2 in dark phase, $P < 0.001$ and 2.0 ± 0.6 in light phase, $P < 0.001$; REM-wake, 12.5 ± 0.8 in dark phase, $P < 0.001$ and 11.9 ± 0.6 in light phase, $P < 0.001$). And eventually the transitions of wake-REM was absent in >P30 rats. The transitions of wake-NREM and NREM-REM were increased

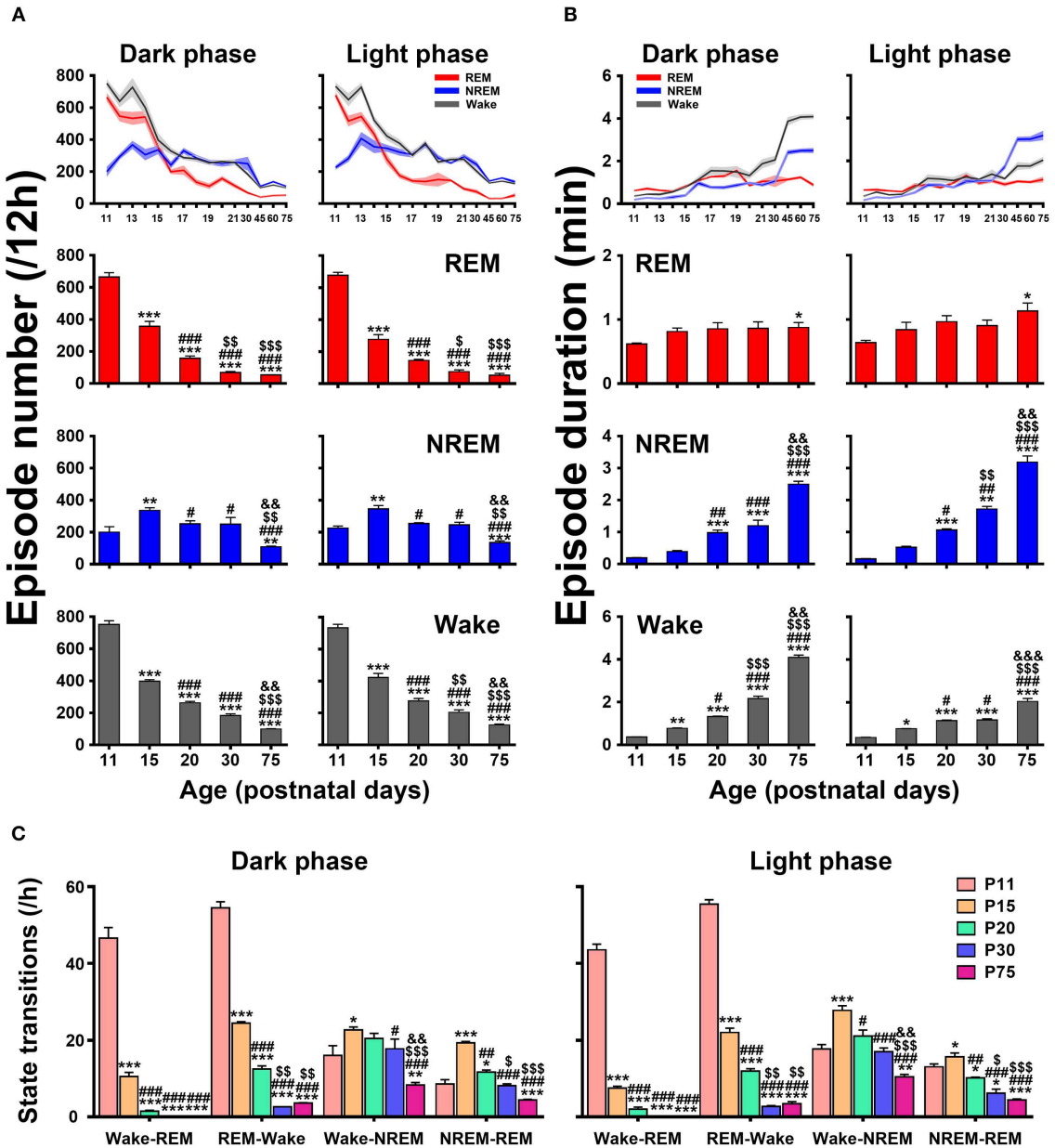

FIGURE 7 | Developmental changes of episode number **(A)**, mean duration **(B)**, and state transitions **(C)** of REM, and NREM sleep and wakefulness in dark and light phase from infant to adult rats. The architectural analysis of three vigilance states at the key postnatal days showed that the episode number gradually declined in both dark and light phases with age and the mean duration of episode meanwhile increased, though NREM episode number temporarily increased at P15. The number of state transitions progressively decreased. Note that the transitions between wake and REM sleep sharply decreased from P11 to P20, and the transitions from wake to REM sleep were eventually absent in >P30 rats. Values are means ± SEM (n = 5–9 in each age), $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with P11; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ compared with P15; $^{\$}P < 0.05$, $^{\$\$}P < 0.01$, $^{\$\$\$}P < 0.001$ compared with P20; $^{\&\&}P < 0.01$, $^{\&\&\&}P < 0.001$ compared with P30. Statistics are analyzed by one-way ANOVA and followed by Fisher's LSD test.

in both dark and light phases at P15 compared to P11 and then gradually decreased.

DISCUSSION

In our study, the new approaches including two types of EEG electrodes, a temperature-controlled incubator and a milk-feeding system in freely moving infant rats were to successfully developed to perform continuous and distinguished EEG and EMG recordings, And it also enable us to distinctly unravel the ontogenetic features and architectures and cortical EEG power spectra across sleep-wake development. Our study demonstrated in P11 to P75 rats that they first (at P11) spent their most time in REM sleep in dark (57.0 ± 2.4%) and light phase (59.7 ± 1.7%) and less time in NREM sleep (5.2 ± 0.8 and 4.9 ± 0.5% respectively, in dark and light phase). And then

REM sleep was sharply decreased with age and NREM sleep simultaneously increased. The dramatic evolution of reduction in REM sleep and of increase in NREM sleep mainly occurred between P11 and P30. Eventually, at P75 REM sleep reduced to 6.3 ± 0.6% in dark phase and 6.9 ± 0.5% in light phase, while NREM sleep increased up 37.5 ± 2.1% in dark phase and 58.4 ± 1.7% in light phase, respectively. EEG power spectra in 0.5–4 Hz increased with age accompanied with prolonged duration of cortical slow wave activity. Amount of sleep between light and dark phase were not obviously different until P20. The circadian rhythm of sleep-wake cycle in infants began to be comparable to adult rats from P30. The episode number of each state was gradually decreased with age in both dark and light phases, while the mean duration was significantly increased.

The feeding problem in the pup prior to weaning limits early sleep recording to only one or several hours a day. In the previous studies, formula milk was administered orally by syringe before sleep recording (2, 3, 17, 18, 20). The milk-feeding system used in our study was set up with a computer-assisted infusion pump and a conterminous PE tube connected to feeding catheter inserted into one side of oral cavity, and delivered milk once an hour (**Figure 2**). The programmable infusion pump could mimic the maternally periodic feeding throughout 24 h. And it allowed the pups to suckle the proper volume of milk at each corresponding age (**Table 1**) and eliminate the defect of manually unmanageable feeding at night (34). The temperature problem in maternally separated pups is another limitation for continuous PSG recording. The young pups are of poor adaptability to ambient temperature changes because their thermoregulatory system was immaturity (35, 36). When the ambient temperature decreases, the pups will experience a significant increase in wakefulness and decrease in sleep (22, 37). A temperature-controlled incubator was used in our study to keep the proper warmth of P9-P20 pups during recovery and PSG recording through setting temperature controller in line with the requirement of pup's age (**Table 1**) (20, 24, 27). Additionally, a potential probability of the changes of sleep-wake pattern caused by maternal separation before weaning (21) was also considered in our study. To induce micturition and defecation and reduce the stress induced by maternal deprivation (2, 23, 24), the pre-weaned pups were ministered to periodically groom and stimulate on the anogenital region. All in all, these approaches mentioned above employed in the present study largely ensured the growth, movement, eye-open time, gross appearance and weight gain (**Figure 3**) in maternally separated pups similar to those in control pups kept with their dam that sustained a continuously PSG recordings.

More importantly, the present study aims at an effective acquisition of cortical EEG and muscular EMG signals of the rat from neonatal to adult. This would contribute to the analysis of states and architectures during sleep-wake development. Gold-plated pins and screws served as EEG electrodes respectively, for P9-P16 and P17-P75 rats (**Figure 1**) accurately recorded a distinguishable EEG pattern of each state (**Figure 4**). The EMG activities of nuchal muscles in sleep-wake states at each age were stably recorded through sliver EMG electrodes (**Figure 4**). The innovation of 4-pins EEG electrodes assembled within a 6-pin pedestal used in P9-P16 pups was considered to be light and perfectly fit the small, soft and fragile skull surface. In addition, the infrared video camera was used during recovery after surgery and PSG recording through which to monitor the behavioral alterations in state development. The occurrences of myoclonic twitches, the discrete and spontaneous limb movements exclusively during REM sleep in early development that contribute to distinguish sleep-wake states in young pups (38, 39) were also observed in this study. Thus, the evolutional characteristics of both EEG and EMG activities and cortical EEG power spectra, and behavioral states were identified perfectly in ontogeny of sleep-wake states in dark and light phase (**Figure 5**).

Using the novel methods, the development information of sleep-wake states in P11-P75 rats was acquired in the present study. The developmental profiles showed that the amount of REM was 410.2 ± 10.7 min in dark phase and 429.5 ± 12.4 min in light phase at P11. For rats at P30, the REM sleep was dramatically declined to 56.2 ± 3.4 min ($P < 0.001$) in dark phase and 63.8 ± 7.1 min ($P < 0.001$) in light phase, respectively. Conversely, NREM sleep between P11 and P30 increased from 37.6 ± 5.5 min to 270.5 ± 7.6 min ($P < 0.001$) in dark phase and from 35.5 ± 1.9 min to 418.2 ± 18.7 min ($P < 0.001$) in light phase. Wakefulness between P11 and P30 significantly increased in both dark and light phases, except it decreased from P20 to P30 in light phase. The amount of each state in respective dark and light phase from P30 to P75 was not obviously different (**Figure 6**). The evidences indicate that the dramatic changes of sleep-wake cycle mainly occur in the first month of postnatal rats. Additionally, the pups at P20 began to sleep more during light period than dark period. And the diurnal rhythm of sleep-wake cycle built up completely at P30. The high amount and intensity of REM sleep in early life of mammals has been observed in previous studies (1, 3, 27, 29) that is proposed to serve as an indicator for the degree of brain maturation and the promoter of further brain development (40–42). Higher levels of REM sleep are needed by altricial mammals such as neonatal rats, which are born with relatively shorter gestational time and thus require relatively more brain development to reach adulthood (43). The decrease of REM sleep with age indicates the maturation of inhibitory mechanism of REM sleep generator center (41, 42). NREM sleep is less in early life. Actually, it does not show up until the brain has developed a certain maturity (3, 44). The dramatic increase of NREM sleep with age is considered to parallel cortical development including synaptic connections, energy use and metabolic homeostasis (45), though its mechanism involved in the brain maturation remains to elucidate. The diurnal rhythm of sleep-wake cycle emerged at the third postnatal week, suggesting that rats are born with an immature circadian system, which achieves its completion during postnatal development. Despite the presence of endogenous rhythms of the suprachiasmatic nucleus in the mammalian fetus, newborn animals do not display circadian organization (27, 45).

The study also demonstrated the evolutional features of sleep-wake architectures. The episode number of three vigilance states was progressively decreased with age, while the mean episode duration of that was significantly increased (**Figures 7A,B**).

Notably, the mean duration of NREM episode from P20 to P75 was increased in light phase more than in the dark. The number of state transitions was great in neonatal rats and then decreased with age (**Figure 7C**). The transitions between wake and REM sleep predominated at P11, and then sharply declined and eventually absented in > P30 rats. The characteristics of state transitions in infant rats are similar to that have been observed in early life in humans (1). The transitions between NREM and REM sleep, and wake and NREM sleep were greater at P15 and then decreased with age, suggesting that NREM sleep tardily forms than REM sleep. These results indicate that rats, similar to humans, have a dramatic change in sleep architecture in the first month (27, 45). The consolidation of sleep and waking episodes throughout development is gradual evolution through increasing the episode duration and decreasing the episode number or quick transitions among three vigilance states (27).

In conclusion, using special EEG and EMG electrodes in maternally separated infant rats, and supported by the milk-feeding system and temperature-controlled incubator, we successfully record the continuous and distinguished EEG and EMG signals. And this allows us to analyze the ontogenetic profiles, architecture and EEG power spectra of sleep-wake states. REM sleep predominates in early developmental sleep and subsequently reduces with age. NREM sleep is of very little amount in neonatal rats, and then augments accompanied with EEG delta activity with age. And finally it predominates in lately developmental sleep. In addition, episode number and duration of each state decreases and increases respectively, with age. The above dramatic changes occur mainly in the first month after birth, suggesting that REM and NREM sleep are required in developmental node from immature to mature brain, and play different role in different stages. Thus, the innovative approaches settle a fundamental method for unraveling the developmental relationship between sleep and brain in future.

DATA AVAILABILITY

All datasets generated for this study are included in the manuscript and/or the supplementary files.

ETHICS STATEMENT

This study was carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (2011 revision). The experimental protocol was approved by the Ethics Committee of Lanzhou University (permit number: SCXK Gan 2018-0002, Lanzhou, PR China).

AUTHOR CONTRIBUTIONS

Y-PH, G-FC, MH, and Y-FS designed the study. G-FC, MH, H-LC, Y-NC, and J-XG conducted the experiments. C-YC and F-QD collected and analyzed the data. J-FX conducted statistical analysis. Y-PH, G-FC, and Y-FS wrote the paper. All authors approved the final version and evaluated the accuracy and integrity of the work.

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