SEX HORMONE AND THE NEUROBIOLOGY OF AFFECTIVE DISORDERS

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SEX HORMONE AND THE NEUROBIOLOGY OF AFFECTIVE DISORDERS

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Effectiveness of Electroacupuncture and Electroconvulsive Therapy as Additional Treatment in Hospitalized Patients With Schizophrenia: A Retrospective Controlled Study

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Electroacupuncture (EA) and electroconvulsive therapy (ECT) are often used in the management of schizophrenia. This study sought to determine whether additional EA and ECT could augment antipsychotic response and reduce related side effects. In this retrospective controlled study, 287 hospitalized schizophrenic patients who received antipsychotics (controls, n = 50) alone or combined with EA (n = 101), ECT (n = 55) or both (EA + ECT, n = 81) were identified. EA and ECT were conducted for 5 and 3 sessions per week, respectively, with a maximum of 12 sessions for ECT during hospitalization. The Scale for the Assessment of Positive Symptoms (SAPS) and Scale for the Assessment of Negative Symptoms (SANS) were used to assess the severity of psychotic symptoms. Clinical response on SAPS and SANS, weight gain, and adverse events were compared. Survival analysis revealed that the ECT and EA + ECT groups had markedly greater clinical response rate than controls on SAPS [72.7 and 90.1% vs. 64.0%; relative risk (RR), 1.974 and 2.628, respectively, $P \leq 0.004$] and on SANS (67.3 and 70.4% vs. 42.0%; RR, 1.951 and 2.009, respectively, $P \leq 0.015$). A significantly greater response rate on SANS than controls was also observed in the EA group (64.4% vs. 42.0%; RR = 1.938, $P = 0.008$). EA-containing regimens remarkably reduced weight gain and incidences of headache, insomnia, dry mouth, and electrocardiographic abnormalities. These results suggest that EA and ECT can serve as additional treatment for enhancing antipsychotic response and reduce the side effects in hospitalized patients with schizophrenia.

Clinical Trial Registration: http://www.chictr.org.cn/showprojen.aspx?proj=38901, identifier ChiCTR1900023563.

Keywords: electroconvulsive therapy (ECT), acupuncture, schizophrenia, positive symptoms, negative symptoms, weight gain

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INTRODUCTION

Schizophrenia is a severe and highly disabling mental disorder that affects about 0.3% of the world population (Charlson et al., 2018). With antipsychotic drugs as the first-line treatment of schizophrenia, a large portion of the patients could not achieve satisfactory outcomes and develop relapse or become a chronic condition (Lally and MacCabe, 2015). Long-term antipsychotic

treatment also often lead to weight gain and other side effects, which possibly explains low medication adherence and poor outcomes (Zhao et al., 2016). Different antipsychotic drugs have been demonstrated to be differentially associated with weight gain (Leucht et al., 2013). These have led to an increasing desire of seeking non-pharmacological alternatives that could enhance efficacy and reduce related side effects.

Since electroconvulsive therapy (ECT) was introduced in 1950s, it has been widely used in the treatment of psychotic disorders (Ali et al., 2019; Grover et al., 2019). It is well documented that ECT is remarkably effective in rapidly relieving positive symptoms (Ali et al., 2019; Grover et al., 2019). Over the past two decades, ECT also has been increasingly utilized in the clinical practice of psychiatry in China, where ECT often serves as adjunctive therapy with antipsychotic drugs (Tang et al., 2012). One recent meta-analysis suggests that additional ECT has a positive effect on mid-term clinical response for patients with treatment-resistant schizophrenia (Sinclair et al., 2019). However, ECT is frequently associated with adverse effects, such as headache and transient global amnesia (Andrade et al., 2016).

As a highly recognized alternative therapy, acupuncture has been widely used in the clinical practice of psychiatry in China and other East Asian countries (Pilkington, 2013). Numerous studies have suggested that acupuncture has multiple benefits in treating psychiatric symptoms, particularly insomnia, anxiety, and depression (Pilkington, 2013). Add-on acupuncture has beneficial effects in improving the positive, negative, cognitive symptoms, and the accompanying sleep disorders in patients with schizophrenia (Shen et al., 2014; Bosch et al., 2015; van den Noort et al., 2018). Acupuncture is also effective in controlling weight gain (Zhang R. Q. et al., 2017; Kim et al., 2018) and reducing antipsychotic-associated side effects (Shen et al., 2014).

We hypothesized that ECT and acupuncture could augment antipsychotic efficacy and reduce the side effects in patients with schizophrenia. To test this hypothesis, a retrospective controlled study was conducted to determine whether additional electroacupuncture (EA) and ECT, either used in separate or in combination, could produce better outcomes than antipsychotics alone in improving psychotic symptoms and reducing side effects in patients with schizophrenia.

MATERIALS AND METHODS

Setting and Subjects

This retrospective study was conducted in the Xi'an Mental Health Center which is a public psychiatric hospital in Xi'an, Shaanxi, China, and has been registered in Chinese Clinical Trial Register (ChiCTR1900023563)[1]. The Xi'an Mental Health Center provides comprehensive services for patients with various psychiatric disorders. In November 2014, the Center has established a fully accessible and searchable electronic medical record system. This study then targeted and screened patients who were hospitalized between November 30, 2014 and December 1, 2018 in the Department of Early Intervention-I of the Center.

Patients were included in this study if they: (1) were either gender aged 18–75 years; (2) were hospitalized and diagnosed with chronic schizophrenia according to the International Classification of Diseases (10th version) (ICD-10) (World Health Organization [WHO], 2014); (3) had antipsychotic treatment comprising olanzapine or risperidone combined with or without EA or/and ECT during their stay in the hospital; and (4) had complete medical record and full clinical assessment.

Patients were excluded from the study if they had: (1) other nerve and brain stimulation therapies besides EA and ECT during hospitalization; (2) acupuncture treatment for 1 week or longer before admission to hospital; (3) a history of brain tumors or intracranial space-occupying lesions; (4) a history of substance abuse over the last one year; or (5) investigational treatments over the last year.

Antipsychotic Treatment

A large proportion of patients had received treatment with olanzapine- and risperidone-containing regimens during their stay in the hospital. These two drugs are commonly prescribed antipsychotics which have been recommended as first-line agents for the management of schizophrenia in China (Li et al., 2015). Olanzapine and risperidone doses generally commenced at 5 and 2 mg/day, and gradually increased to an optimal dose within 1 week, but limited to 20 and 8 mg/day, respectively. Other antipsychotic drugs, such as ziprasidone, aripiprazole, haloperidol, and mood stabilizers, anxiolytics, and hypnotics were also often prescribed as additional treatment at the psychiatrist's discretion, depending on patient's condition and response. For those who had significant agitation, hostility, and aggressive behavior, haloperidol injection was immediately given in combination with ECT. Previous studies have confirmed that haloperidol injection had particular effects in managing hostile and aggressive acutely schizophrenic patients and agitation (Tuason, 1986; Bieniek et al., 1998).

ECT Intervention

While whether patients had ECT treatment was at the discretion of a psychiatrist, depending on the severity of positive symptoms and patients' condition, ECT was immediately administered in combination with haloperidol if patients had significant agitation, hostility, or aggressive behavior at their admission to hospital. ECT has been used for acute management of schizophrenia with high aggression risk at time of admission in China (Zhang Q. E. et al., 2016). ECT was conducted for 3 sessions per week, but the total number should not exceed 12 sessions during hospitalization.

Patients were asked to have at least 8 h of fasting prior to ECT. For the ECT procedure, firstly patients received one dose of 0.5 mg atropine to reduce salivation and respiratory tract secretions, with one dose of 1–2 mg/kg propofol for anesthesia, followed by one dose of muscle relaxant succinylcholine (1–2 mg/kg). Subsequently ECT was performed on a spECTrum 5000Q machine (MECTA Corporation, United States) with the

[1]http://www.chictr.org.cn/showprojen.aspx?proj=38901

placement of stimulus electrodes on the right temple and the vertex of the scalp as previously reported (Nobler and Sackeim, 2008). The initial stimulus intensity (percentage of energy) was set at 5% equivalent to 25 mV with a fixed constant current of 0.9 A, and then gradually increased until a seizure discharge that lasted for a minimum of 20 s, as indicated in electroencephalogram (EEG) was achieved.

Electroacupuncture Treatment

Electroacupuncture has become a standard therapy provided to hospitalized patients at the Center. The treatment was carried out by acupuncturists at bedside for 5 sessions per week (once per day in weekdays). EA protocol for schizophrenia has been well established at the Center. Briefly, the following acupoints were used: Shen-Ting (GV24), Bai-Hui (GV20), Si-Shen-Cong (EX-HN1), Feng-Chi (GB20), Shen-Men (HT7), and Nei-Guan (P6). Disposable acupuncture needles (0.30 mm in diameter, 25–40 mm in length) were used for EA. The needles were penetrated 0–30 mm in depth at acupoints in a perpendicular or oblique direction. Manual manipulation was then carried out until the patients felt needling sensation (De-Qi). Electrical stimulation was then applied with connections between Shen-Ting (GV24) and Bai-Hui (GV20) and between left and right Feng-Chi (GB20). The output peak current and voltage of the machine (G6805-A electrical stimulator) were 6 V and 48 mA, respectively. Stimulus parameters were constant wave at frequency of 100 Hz and phase duration of 100 μs for 20 min. The intensity of stimulation was gradually increased to a level at which patients obtained considerable electric stimulus sensation.

Clinical Assessments

The Scale for the Assessment of Positive Symptoms (SAPS, Chinese version) (Andreasen, 1984) and the Scale for the Assessment of Negative Symptoms (SANS, Chinese version) (Andreasen, 1989) are routine clinical instruments used for the assessment of the severity of schizophrenia at the Center. The assessment was done once weekly during patients' hospitalization by designated psychiatrists who had received a series of training workshops to ensure assessment consistency and reliability. The Center held training workshops biannually. The primary outcome was the clinical response, which is defined as an at least 30% reduction from baseline in score on SAPS and SANS on a weekly basis. The clinical response rate was compared over time in the four groups using survival analysis (see below). Body weight gain was recorded on a weekly basis. Other side effects were recorded using the Treatment Emergent Symptom Scale (TESS) (Guy, 1976).

In China, the determination of discharge of patients from hospital was not only upon clinical remission of positive symptoms as defined previously (Lambert et al., 2010), but also upon administrative policies and patients' individual socioeconomic situation. Hospital stay days could not accurately reflect clinical remission and were therefore not included as a clinical outcome, but served as a covariate for outcome analysis (see below).

Data Analysis

Our previous study has shown that additional ECT could produce an approximately 25% greater clinical response rate than antipsychotics alone in acute treatment of psychotic episode (Zhang et al., 2012a). In this study, a sample size of 287 with an average 72 per group could yield at least an 80% power to detect a 25% difference in the clinical response rate at a significant level of 0.05 between any two groups.

Categorical baseline parameters, antipsychotic regimens, and incidence of adverse events were analyzed using Chi-square (χ^2) test. Continuous baseline data was analyzed using one-way analysis of variance (ANOVA), followed by Student–Newman–Keuls method to further detect between-group differences. Net weight gain was analyzed using analysis of covariance (ANCOVA) with age, duration of the illness, number of previous psychotic episodes, number of previous hospitalizations, and current hospital stay days as covariates. Whether patients were treated with a particular antipsychotic drug with which proportion of patients had significant differences among the four groups also served a covariate in the analysis of weight gain.

Despite the fact that hospital stay days largely varied, the majority of patients had achieved clinically meaningful improvement, stable partial, or even full remission within 14 weeks. Therefore, Week 14 was chosen as the endpoint for survival analysis. Cox regression proportional hazards model was used for survival analysis to examine differences in time to clinical response at 14 weeks among the four groups with adjustment for age, duration of the illness, number of previous psychotic episodes, number of previous hospitalizations, and current hospital stay days. Statistical significance was defined as a two-sided P-value of <0.05. The analyses were performed with SPSS version 19 software (Chicago, IL, United States).

RESULTS

Baseline Characteristics of Patients

Of 2,468 patients who were hospitalized in the Department of Early Intervention-I of the Center between November 30, 2014 and December 1, 2018, 287 met the inclusion criteria and were included in the study. While all had antipsychotic treatment, 50 were treated with pharmacotherapy alone; 101 and 50 received additional EA and ECT, respectively; other 81 had a combination of EA and ECT (**Figure 1**).

Baseline characteristics are summarized in **Table 1**. Significant differences among the four groups were observed on age, duration of the illness, number of previous psychotic episodes, number of previous hospitalization, and current hospital stay days. These variables served as covariates in survival analysis and weight gain analysis. Other baseline variables were not different among the four groups. The severity of psychotic symptoms measured with baseline SAPS and SANS scores and other baseline variables were not significantly different.

Psychotropic Medication Modes

The five most commonly prescribed antipsychotic drugs were olanzapine (75.6%, 217/287), risperidone (30.0%, 86/287),

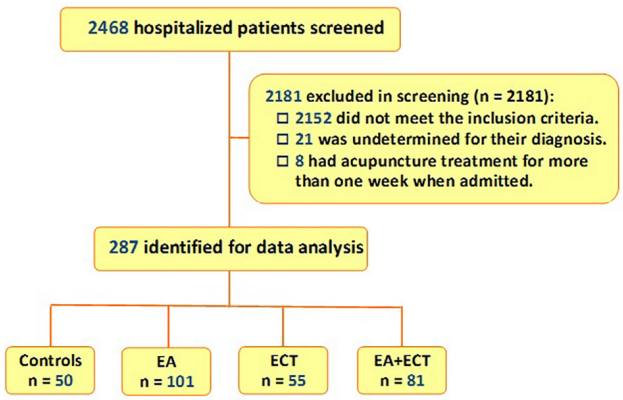

FIGURE 1 | Flowchart of selection of study subjects from schizophrenic patients who were hospitalized in Department of Early Intervention-I of the Xi'an Mental Health Center at Xi'an, China, between November 30, 2014 and December 1, 2018. EA, electroacupuncture; ECT, electroconvulsive therapy.

haloperidol (20.6%, 59/287), ziprasidone (18.1%, 52/287), and aripiprazole (5.6%, 16/287) (**Table 2**). The EA + ECT group had a markedly higher proportion of patients who were treated with haloperidol than the other three groups ($P = 0.003$) and most of them received haloperidol injection. Whether patients received haloperidol treatment then served an additional covariate in the analysis of weight gain. There were 56.1% (161/287) patients receiving antipsychotic combination regimens. No significant differences were detected on a proportion of patients who received mono- and combination therapy with antipsychotics among the four groups (**Table 2**).

Clinical Response Rate

Survival analysis revealed significant differences among the four groups in clinical response rate on SAPS ($P = 0.000$) and SANS ($P = 0.037$) over 14 weeks (**Figure 2**).

For SAPS, additional ECT and EA + ECT produced a significantly greater response rate (72.7 and 90.1%) compared to the control group (64.0%) with relative risk (RR) of 1.974 ($P = 0.004$) and 2.628 ($P = 0.000$), respectively. The response rate of both ECT and EA + ECT regimens did not differ with a RR of 0.751 ($P = 0.149$), but was markedly higher than that of additional EA (64.4%) with RR of 2.120 and 2.823 ($P = 0.000$), respectively. The response rate of additional EA and controls was not different with a RR of 0.971 ($P = 0.742$).

For SANS, all the three additional treatment groups (EA, ECT, and EA + ECT) displayed a markedly greater response rate than the control group (64.4, 67.3, and 70.4% vs. 42.0%) with RR of 1.938 ($P = 0.008$), 1.951 ($P = 0.015$), and 2.009 ($P = 0.006$), respectively. The response rate of the three additional regimens was not different.

TABLE 1 | Baseline characteristics[a].

Variables	Control ($n = 50$)	EA ($n = 101$)	ECT ($n = 55$)	EA + ECT ($n = 81$)	*P*-value
Age, year[b]	39.7 ± 13.7	40.2 ± 12.7	33.3 ± 9.6	36.3 ± 11.3	0.003
Gender, *n* (%)[c]					0.123
Male	22 (44.0)	51 (50.5)	17 (30.9)	38 (46.9)	
Female	28 (56.0)	50 (49.5)	38 (69.1)	43 (53.1)	
Residential areas, *n* (%)[c]					0.317
Urban and town	26 (52.0)	57 (56.4)	23 (41.8)	38 (46.9)	
Rural	24 (48.0)	44 (43.6)	32 (58.2)	43 (53.1)	
Educational degree, *n* (%)[c]					0.183
Primary and illiteracy	10 (20.0)	13 (12.9)	10 (18.2)	12 (14.8)	
Secondary	30 (60.0)	68 (67.3)	25 (45.5)	47 (58.0)	
College and above	10 (20.0)	20 (19.8)	20 (36.4)	22 (27.2)	
Employment status, *n* (%)[c]					0.251
On work	19 (38.0)	34 (33.7)	29 (52.7)	35 (43.2)	
Unemployed/retired	27 (54.0)	61 (60.4)	21 (38.2)	42 (51.9)	
Students	4 (8.0)	6 (5.9)	5 (9.1)	4 (4.9)	
Marital status, *n* (%)[c]					0.070
Single/divorce/widow	29 (58.0)	43 (42.6)	26 (47.3)	49 (60.5)	
Married	21 (42.0)	58 (57.4)	29 (52.7)	32 (39.5)	
Family history with mental disease, *n* (%)[c]	13 (26.0)	31 (30.7)	19 (34.5)	16 (19.8)	0.223
Duration of the illness, year[b]	11.5 ± 10.4	13.9 ± 10.6	7.3 ± 7.2	8.0 ± 8.2	< 0.001
no. of previous psychotic episodes[b]	3.4 ± 4.1	4.3 ± 4.5	2.9 ± 1.7	2.5 ± 1.9	0.002
no. of hospitalization[b]	3.7 ± 4.2	4.6 ± 4.4	2.9 ± 2.1	2.8 ± 2.3	0.005
Current hospital stay days[b]	41.9 ± 21.8	56.1 ± 27.1	38.1 ± 13.1	53.6 ± 19.7	< 0.001

[a] *EA, electroacupuncture; ECT, electroconvulsive therapy.* [b] *Continuous data are expressed mean ± SD and were examined using one-way analysis of variance (ANOVA).* [c] *Categorical data were examined using Chi-square (χ^2) test.*

TABLE 2 | Antipsychotic regimens used in patients with schizophrenia[a,b].

Antipsychotics	Control (n = 50)	EA (n = 101)	ECT (n = 55)	EA + ECT (n = 81)	P-value
5 most commonly used antipsychotics					
Olanzapine	36 (72.0)	73 (72.3)	43 (78.2)	65 (80.2)	0.551
Risperidone	17 (34.0)	30 (29.7)	14 (25.5)	25 (30.9)	0.812
Ziprasidone	10 (20.0)	14 (13.9)	16 (29.1)	12 (14.8)	0.093
Aripiprazole	4 (8.0)	6 (5.9)	3 (5.5)	3 (3.7)	0.772
Haloperidol [c]	6 (12.0)	16 (15.8)	9 (16.4)	28 (34.6)	0.003
Regimens					0.745
Monotherapy	24 (48.0)	44 (43.6)	26 (47.3)	32 (39.5)	
Combination therapy	26 (52.0)	57 (56.4)	29 (52.7)	49 (60.5)	

[a]EA, electroacupuncture; ECT, electroconvulsive therapy. [b]Data were examined using Chi-square (χ^2) test. [c]Most patients received haloperidol injection for their significant agitation, hostility, or aggressive behavior.

TABLE 3 | The incidence of adverse events[a,b].

	Control (n = 50)	EA (n = 101)	ECT (n = 55)	EA + ECT (n = 81)	P-value
Any	8 (16.0)	18 (17.8)	8 (14.5)	12 (14.8)	0.936
Headache	4 (8.0)	2 (2.0)	8 (14.5)	1 (1.2)	0.002
Decreased activity	8 (16.0)	5 (5.0)	4 (7.3)	12 (14.8)	0.062
Tremor	1 (2.0)	6 (5.9)	1 (1.8)	1 (1.2)	0.250
Akathisia	1 (2.0)	4 (4.0)	1 (1.8)	1 (1.2)	0.658
Heart pound	2 (4.0)	2 (2.0)	0	2 (2.5)	0.546
Muscle rigidity	3 (6.0)	1 (1.0)	2 (3.6)	1 (1.2)	0.223
Hypersalivation	1 (2.0)	2 (2.0)	0	1 (1.2)	0.758
Constipation	1 (2.0)	2 (2.0)	1 (1.8)	2 (2.5)	0.994
ECG abnormalities	19 (38.0)	14 (13.9)	15 (27.3)	10 (12.3)	< 0.001
Insomnia	8 (16.0)	4 (4.0)	7 (12.7)	3 (3.7)	0.014
Dry mouth	6 (12.0)	1 (1.0)	4 (7.3)	2 (2.5)	0.011

[a]EA, electroacupuncture; ECT, electroconvulsive therapy. [b]Data were examined using Chi-square (χ^2) test.

Net Weight Gain

Net weight gain was compared among the four groups with adjustment for age, duration of illness, number of previous psychotic episodes and hospitalization, current hospital stay days, and whether patients received haloperidol (**Figure 3**). ANCOVA revealed a significant difference (F = 9.383, P < 0.001). An average weight gain (1.06 kg) of the EA group was markedly lower than that of the control group (2.16 kg, P < 0.001) and the EA + ECT group (1.65 kg, P = 0.012). Net weight gain of the control group (2.16 kg) was significantly greater than that of the ECT group (1.33 kg, P = 0.002) and the EA + ECT group (1.65 kg, P = 0.016).

Adverse Events

Table 3 summarizes side effects observed, with no significant differences in the overall incidence of adverse events among the four groups; but the ECT group had a much higher rate of headache than the other three groups (P = 0.002). The control and ECT groups exhibited higher incidence of ECG abnormalities (P < 0.001), insomnia (P = 0.014), and dry mouth (P = 0.011) compared to the two EA-containing groups.

DISCUSSION

The central theme of this retrospective controlled study was to systematically evaluate the effectiveness and safety profile of EA and ECT and a combination of both as additional treatment in hospitalized patients with schizophrenia. All subjects included in this study were experiencing a severe psychotic episode, with multiple previous episodes and an average illness duration of 7–14 years, suggesting that most patients' condition was lingering and recurrent. The majority of patients were treated with olanzapine and risperidone in either monotherapy or combination regimens, as these two drugs are the most commonly prescribed antipsychotic agents in acute and long-term treatment of schizophrenia in China (Li et al., 2015). We noted that the EA + ECT group had a much higher proportion of patients who received additional haloperidol than the other three groups, with approximately 67% vs. 12–16%. This was because haloperidol injection was often combined with ECT for acute management of agitation, hostility, and aggressive behavior. ECT combined with haloperidol has been shown to induce a transient redistribution of haloperidol (Aoba et al., 1983). It appears that ECT may have effects on pharmacokinetic profile of haloperidol.

We found that the patients who received additional ECT-containing regimens had an approximately twofold to threefold greater odds of achieving clinical response on both positive and negative symptom domains compared to controls over the course of hospitalization; the greater improvement of most patients treated with ECT-containing regimens was observed as early as 1–4 weeks. These results clearly indicate that additional ECT can augment and accelerate antipsychotic response. This finding is highly consistent with a meta-analysis conclusion that a combination of ECT with antipsychotics could produce rapid global improvement and reduction of symptoms (Tharyan and Adams, 2005). Nevertheless, unlike previous studies in which ECT was generally used as a last option to treat refractory schizophrenia (Grover et al., 2018), in the present study, ECT was introduced immediately to antipsychotic regimens when patients were hospitalized. Our previous study also has confirmed that additional ECT augmented antipsychotic efficacy in adolescents with first-episode psychosis, without causing apparent side effects (Zhang et al., 2012a).

One meta-analysis has suggested that acupuncture may have some antipsychotic effects measured with global and mental state (Shen et al., 2014). This study further revealed that, like ECT, EA also had the augmenting effects in reducing negative symptoms, with an approximately twofold greater odds of achieving clinical response on SAPS compared to antipsychotic regimens alone, although it did not produce additional improvement on positive symptoms. Individuals with schizophrenia often experienced persistent negative symptoms, such as blunted mood, poverty of speech, apathy, anhedonia, and cognitive impairment (Veerman et al., 2017). Antipsychotic drugs are less effective and even ineffective in treating primary

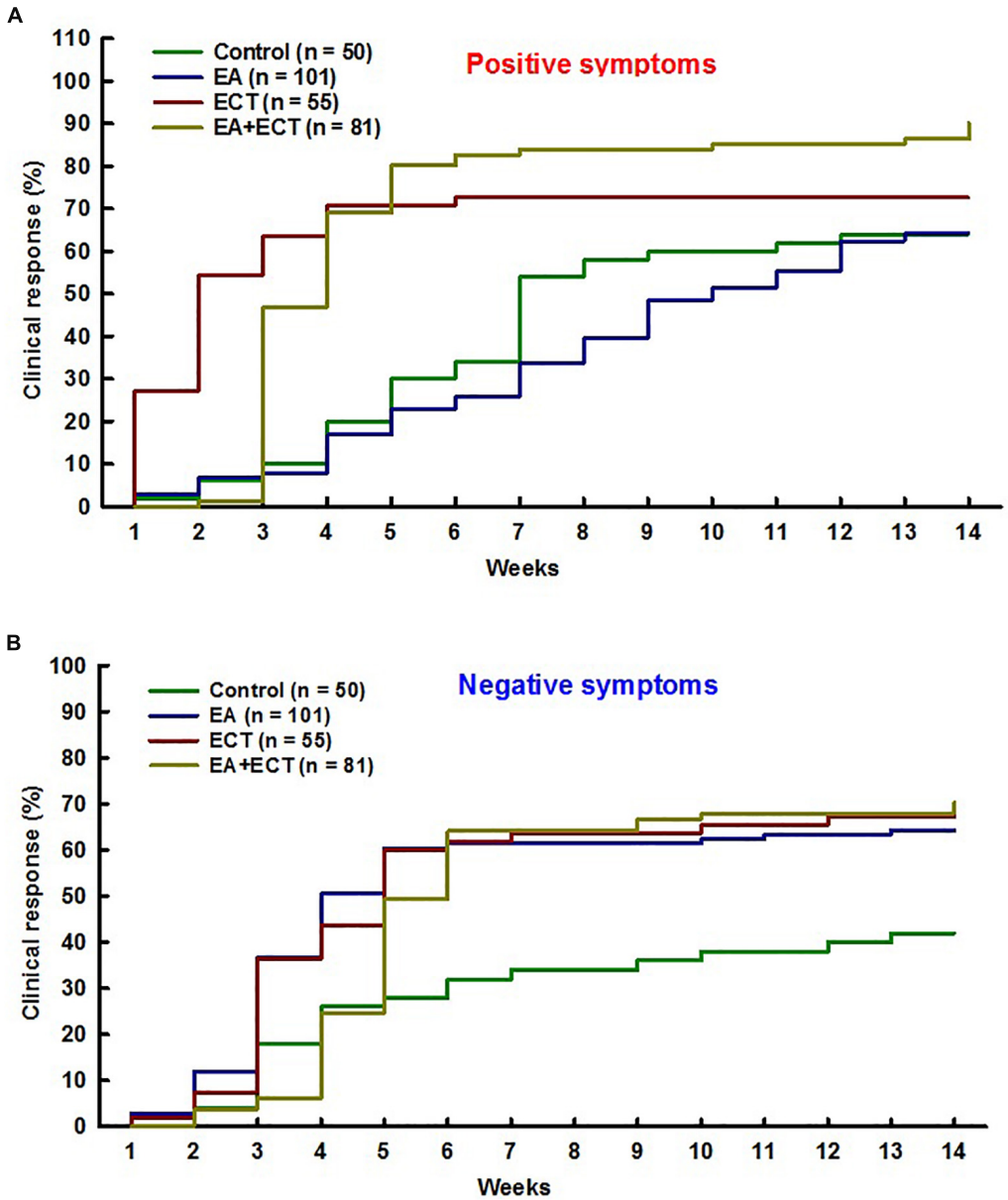

FIGURE 2 | Cox regression proportional hazards model with relative risk (RR) was used for survival analysis on odds of achieving clinical response on positive symptoms **(A)** and negative symptoms **(B)** over 14 weeks of hospitalization of schizophrenic patients treated with additional electroacupuncture (EA), electroconvulsive therapy (ECT), or a combination of both (EA + ECT). The analysis was adjusted for age, duration of the illness, number of previous psychotic episodes, number of previous hospitalization, and current hospital stay days. For positive symptoms, EA vs. control: RR = 0.931, $P = 0.742$; ECT vs. control: RR = 1.974, $P = 0.004$; EA + ECT vs. control: RR = 2.628, $P = 0.000$; ECT vs. EA: RR = 2.120, $P = 0.000$; EA + ECT vs. EA: RR = 2.823, $P = 0.000$; EA + ECT vs. ECT: RR = 1.331, $P = 0.149$. For negative symptoms, EA vs. control: RR = 1.938, $P = 0.008$; ECT vs. control: RR = 1.951, $P = 0.015$; EA + ECT vs. control: RR = 2.009, $P = 0.006$; ECT vs. EA: RR = 1.007, $P = 0.975$; EA + ECT vs. EA: RR = 1.037, $P = 0.844$; EA + ECT vs. ECT: RR = 1.030, $P = 0.890$.

negative symptoms (Veerman et al., 2017). Nevertheless, it has been found that acupuncture could improve working memory of patients with chronic schizophrenia (Bosch et al., 2016; van den Noort et al., 2018). A large body of evidence confirms the effectiveness of EA in alleviating various depressive disorders (Zhang et al., 2010) and mild cognitive impairment (Deng and Wang, 2016). EA also has therapeutic effects against anhedonic behavior in animal models (Yu et al., 2007). These studies, together with this study, suggest that EA may have particular effects in reducing negative symptoms and comorbid psychiatric symptoms of schizophrenia. On the other hand, the EA + ECT group had approximately 17–26% higher response rate than the other two groups on positive symptoms, although some differences did not reach statistical significance level. It seems that EA combined with ECT could produce additive and even synergistic augmenting effects on positive symptoms. However,

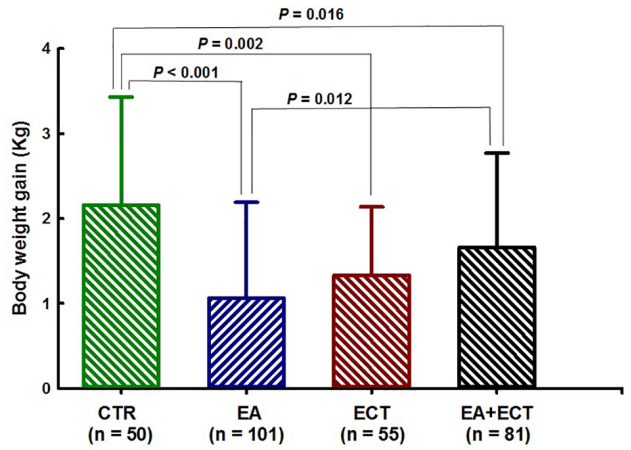

FIGURE 3 | Effects of additional electroacupuncture (EA), electroconvulsive therapy (ECT), or a combination of both (EA + ECT) on net weight gain of hospitalized patients with schizophrenia. Data are expressed mean ± SD and were analyzed using analysis of covariance (ANCOVA) with age, duration of the illness, number of previous psychotic episodes, number of previous hospitalization, current hospital stay days, and whether patients received haloperidol treatment as covariates, followed by Student–Newman–Keuls method to further detect between-group differences.

unlike positive symptoms, the clinical response rate of the EA + ECT group was similar to that of the EA and ECT groups on negative symptoms (between 64 and 70%). Additive effects of the two interventions seem least on negative symptoms, probably due to the fact that there exists a ceiling effect or the current length of treatment was not sufficient to produce additive effects on negative symptoms.

The possible mechanisms of acupuncture's efficacy on schizophrenia may be related to its broad neuromodulation (Zhang et al., 2012b). Acupuncture could robustly modulate multiple catecholaminergic and neuropeptidergic neuronal systems, including dopamine, serotonin, noradrenaline, and endogenous opiate neuropeptides of the brain (Ulett et al., 1998). It also broadly affects brain regions associated with the pathogenesis of psychotic disorders (Dhond et al., 2007).

Weight gain is a prevalent and serious side effect of antipsychotic treatment (Zhang J. P. et al., 2016). In this study, the control group treated only with antipsychotic drugs gained approximately 2.2 kg of body weight during hospitalization. The addition of EA, however, reduced weight gain by approximately 0.3–1.1 kg as compared to the other three groups. The ECT and EA + ECT groups also gained less weight than the control group. These results demonstrated the effectiveness of both EA and ECT in controlling antipsychotic-related weight gain. Similar results also have been observed in acupuncture treatment of olanzapine-induced obesity (Zhang L. et al., 2017) and simple obesity (Zhang R. Q. et al., 2017; Kim et al., 2018). One case report also has shown that ECT reduced weight gain in a patient with comorbid severe obesity, binge-eating disorder, and bipolar disorders (Rapinesi et al., 2013). It is noted that, as mentioned above, the EA + ECT group had a much higher proportion of patients who received haloperidol treatment than the other three groups (34.6% vs. 12.0–16.4%). As a typical antipsychotic, haloperidol-induced weight gain seems less responsive to acupuncture treatment, probably due to its higher metabolic liability compared to second-generation antipsychotics (Solmi et al., 2017). This could explain the smaller amount of weight loss in the EA + ECT group than the EA group.

Post-ECT headache is a commonly occurring side effect (Haghighi et al., 2016). ECG abnormalities, insomnia, and dry mouth are often associated with antipsychotic treatment (Orsolini et al., 2016). In this study, we observed that the EA and EA + ECT groups had much lower incidences of headache, ECG abnormalities, insomnia, and dry mouth compared to the control and ECT groups. Previous studies also have shown acupuncture was effective in alleviating sleep disturbance, dry mouth, and tachycardia in patients with schizophrenia (Shen et al., 2014; Bosch et al., 2016; van den Noort et al., 2018). These results indicate that acupuncture has apparent benefits in treating ECT- and antipsychotic-related side effects. This also could partly explain the particular effects of EA on negative symptoms observed in this study.

In summary, additional ECT can augment antipsychotic effects against both positive and negative symptoms. Additional EA may have particular effects in reducing negative symptoms and antipsychotic- and ECT-related side effects, including antipsychotic-induced weight gain and ECT-related headache. We suggest that EA and ECT can serve as additional treatment for enhancing antipsychotic response and reduce side effects in hospitalized patients with schizophrenia.

LIMITATIONS

Multiple limitations of this study should be noted. Firstly, as a retrospective controlled study, blinding was not established in psychiatrists and patients, their expectation on treatment outcomes might potentially interfered with data analysis. In addition, doses and treatment length of the targeted antipsychotics could not be well controlled, and there was a lack of the analysis of interrater reliability in this retrospective studies. Secondly, significant variations exist in multiple baseline variables, including age, duration of the illness, number of previous psychotic episodes, number of previous hospitalization, current hospital stay days, and proportion of patients who received haloperidol treatment. These variations suggest that older patients with longer hospital stays and more persistent and recurrent schizophrenia were more likely to receive acupuncture treatment. On the other hand, the subjects identified in this study represent a subpopulation of Chinese patients who may have distinctive perceptions of EA and ECT. Whether similar treatment outcomes of EA and ECT could be achieved in other subpopulations with schizophrenia needs further investigation. Thirdly, we did not develop sham EA and sham ECT procedure for controlled group.

As EA and ECT to some extent may cause fear or other adverse experience, potential bias caused by the procedure-related negative psychological effects should be considered, although we expect that such negative effects would not have significant interference with treatment outcomes. Finally, the choice of acupuncture points used in this study was mainly based on traditional Chinese medicine (TCM) doctrine and empirical evidence. Acupuncture regimens used for the treatment of schizophrenia also varied from one to another (Shen et al., 2014). Various acupuncture regimens for schizophrenia should be optimized and standardized in the future.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Medical Ethical Committee of the Xi'an Mental Health Center. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

JJ and Z-JZ were involved in the conception and design of the study, data analysis, and preparation of the manuscript. JJ, JS, and F-HL collected the data. HW helped for the statistical analysis. X-JY helped for registration and data collection. Q-JW, HZ, H-NW, and Q-RT provided the critical comments.

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Traumatic Stress Produces Delayed Alterations of Synaptic Plasticity in Basolateral Amygdala

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Acute traumatic event exposure is a direct cause of post-traumatic stress disorder (PTSD). Amygdala is suggested to be associated with the development of PTSD. In our previous findings, different activation patterns of GABAergic neurons and glutamatergic neurons in early or late stages after stress were found. However, the neural plastic mechanism underlying the role of basolateral amygdala (BLA) in post-traumatic stress disorder remains unclear. Therefore, this study mainly aimed at investigating time-dependent morphologic and electrophysiological changes in BLA during the development of PTSD. We used single prolonged stress (SPS) procedure to establish PTSD model of rats. The rats showed no alterations in anxiety behavior as well as in dendritic spine density or synaptic transmission in BLA 1 day after SPS. However, 10 days after SPS, rats showed enhancement of anxiety behavior, and spine density and frequency of miniature excitatory and inhibitory postsynaptic currents in BLA. Our results suggested that after traumatic stress, BLA displayed delayed increase in both spinogenesis and synaptic transmission, which seemed to facilitate the development of PTSD.

Keywords: single prolonged stress, post-traumatic stress disorder, dendritic spines, synaptic plasticity, basolateral amygdala

INTRODUCTION

As an intricate anxiety disorder, post-traumatic stress disorder (PTSD) generally occurs after traumatic stress exposure (Galea et al., 2007; Keyes et al., 2013; Scott et al., 2013; Olaya et al., 2015). PTSD has a high prevalence rate worldwide (Seal et al., 2009) and imposes a heavy burden to families and the society (Cohen et al., 2010). But the biological basis underlying PTSD was unclear. Single prolonged stress (SPS) model, an appropriate PTSD model of animal, has been established to explore the neurobiological mechanisms of PTSD considering the limitations of human studies (Souza et al., 2017; Fang et al., 2018). Rats exhibited abnormal behavior as well as hypothalamic-pituitary-adrenal (HPA)-axis dysfunction following SPS (Ding et al., 2010), which is a putative neuroendocrinological hallmark of PTSD (Mellman et al., 2009; Hughes and Shin, 2011;

Pratchett et al., 2011; Bailey et al., 2013). SPS paradigm is composed of the following procedures (Bradley et al., 2005): restraint, forced swim in water at 20–24°C, ether exposure, and stay at homecage undisturbedly for 7 days which is essential for the development of key symptoms of PTSD (Liberzon et al., 1997; Knox et al., 2012b). This model can mimic the symptoms of PTSD in humans, with behavioral changes including increased anxiety (Han et al., 2014; Fang et al., 2018), impaired social interaction and spatial memory (Wen et al., 2016), and disrupted extinction of fear memory (Iwamoto et al., 2007; Fang et al., 2018).

The amygdala, which is involved in the regulation of fear and memory (Dias et al., 2014) and emotion (Saghir et al., 2018; Abuhasan and Siddiqui, 2019), is located at the limbic system of the brain and consists of several subregions, such as corticomedial nucleus, basolateral nucleus (BLA), central nucleus. Pyramidal neurons account for about 85% of all neurons in the adult BLA, and the rest are mainly interneurons (Berdel et al., 1997; Duvarci and Pare, 2014). It has been suggested that the dysfunction of amygdala is associated with the pathogenesis of mental disorders, such as depression, anxiety, and autism (Rainnie et al., 2004; Shekhar et al., 2005; Truitt et al., 2007; Koob and Volkow, 2010). The clinical study on PTSD has revealed that the response of amygdala in patients to emotional stimuli was exaggerated (Rauch et al., 2000). Furthermore, amygdala's response to fear stimuli could be used to evaluate the treatment effect (Bryant et al., 2008). A series of molecular substrates in amygdala have been implicated in the PTSD-associated behaviors, such as glucocorticoid receptor (Kohda et al., 2007; Cohen et al., 2012), betaarrestin-2 (Ding et al., 2017), β-adrenoreceptor (Ronzoni et al., 2016), and mTOR signaling pathway (Oh et al., 2018). We also recently found different activating patterns of glutamatergic and GABAergic neurons in amygdala, specifically delayed enhancement of glutamatergic pyramidal neuron activation in BLA (Fang et al., 2018). However, fewer studies have showed how the synaptic plasticity in BLA changes in the animal model of PTSD (Cohen et al., 2014).

Dendrites and dendritic spines form the structural basis of synaptic plasticity (Spruston, 2008; Papoutsi et al., 2014). Neural circuits are shaped with dendritic morphology, and generation and storage of memory involves adjustment of structures of spines and dendrites in the brain (Papoutsi et al., 2014). Dendrites in amygdala are especially sensitive to stress. Significant changes have been exhibited in spine density of pyramidal neurons as well as dendritic morphology in amygdala in rats that underwent chronic or acute stress (Vyas et al., 2002, 2006; Mitra et al., 2005; Leuner and Shors, 2013; Padival et al., 2013; Suvrathan et al., 2014; Yasmin et al., 2016). Dendritic spines are usually classified according to shapes (stubby, thin, mushroom) (Wang et al., 2017), which are distinct in their functions (Noguchi et al., 2005; Bourne and Harris, 2007; Gipson and Olive, 2017). Studies have shown that the number of postsynaptic glutamatergic receptors decides the spine morphology to a great extent (Rochefort and Konnerth, 2012). Taken together, in order to identify changes in synaptic plasticity in BLA after traumatic stress, we used Golgi–Cox method to determine if SPS causes alterations of spine morphology and density, and recorded mEPSCs and mIPSCs to explore whether SPS leads to activity alterations in excitatory synapses and inhibitory synapses.

MATERIALS AND METHODS

Subjects

Sprague-Dawley rats (3-month old, male), with weight of 220–260 g, were gained from the Laboratory Animal Center, Peking University Health Science Center. The rats were kept in groups of five at temperature of 23 ± 2°C and humidity of 50 ± 5% with free access to water and food under a 12 h:12 h light:dark cycle. We performed all the behavioral experiments under the dark phase. Animal care and experimental procedures were conducted according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All experiments were permitted by Biomedical Ethics Committee of Peking University.

Single Prolonged Stress

Single prolonged stress is a commonly recognized PTSD model (Ding et al., 2010), which results in potent responses to stress via psychological (restraint), physiological (forced swimming), and pharmacological (exposure to ether) pathways. The SPS procedure was conducted based on previous study (Fang et al., 2018), including 2-h restraint, 20-min forced swimming, recovery in homecage for 15 min, and exposure to diethyl ether until a brief loss of consciousness. On the same day of SPS treatment, the control animals were handled. All animals were undisturbed in their homecages for 10 days before sensitization test (Liberzon et al., 1997).

Open Field Test

The apparatus of the open field test (OFT) had a square arena at 75 × 75 × 40 cm and was divided into 25 even squares with size of 15 × 15 cm (Xue et al., 2015; Fang et al., 2018). The apparatus was illuminated at 10 lux. Towels soaked with 75% ethanol were used to clear up the apparatus and wipe off odor of previous rat after each 5-min run. A rat was put in the center of the apparatus, and its movement was recorded by a digital video camera mounted on the roof and connected to a computer. Using an EthoVision System XT 10.1 (Noldus Information Technology, Netherlands), the time spent in the central part of the apparatus was analyzed.

Elevated Plus Maze

The elevated plus maze (EPM) test was conducted as previously described (Xue et al., 2015; Fang et al., 2018). Two open arms (50 × 10 cm), and two closed arms (50 × 10 × 40 cm), as well as a middle compartment (10 × 10 cm) constituted the shape of a plus, which were placed 70 cm above the ground. Each rat explored the apparatus *ad libitum* for 5 min after being placed in the middle compartment with head facing an open arm. Towels soaked with 75% ethanol were used to clear up the apparatus and wipe off odor of the previous rat after each run. Movement of rats was recorded using a video camera mounted on the roof and connected to a computer. The test was performed with illuminance level of 3 lx in the closed arms and 8 lx in the open arms (Suo et al., 2013). Using an EthoVision System XT

10.1 (Noldus Information Technology, Netherlands), the number of entries into the open arms and time spent (sec) in the open arms were analyzed.

Slice Preparation

The brains were rapidly removed after rats were anesthetized and then decapitated. The brains were immediately placed into cutting solution (in mM) at 0–4°C: 87 NaCl, 3.0 KCl, 1.5 $CaCl_2$, 1.3 $MgCl_2$, 1.0 NaH_2PO_4, 26 $NaHCO_3$, 20 D-glucose, and 75 sucrose, saturated with 95% O_2 and 5% CO_2 to obtain 250 μm-thick coronal sections with a vibratome (Leica VT1000 S). Transverse slices containing the BLA were cut and transferred into a holding chamber containing ACSF (in mM): 124 NaCl, 3.0 KCl, 1.5 $CaCl_2$, 1.3 $MgCl_2$, 1.0 NaH_2PO_4, 26 $NaHCO_3$, and 20 D-glucose, saturated with 95% O_2 and 5% CO_2 at 33°C for 30 min and then at room temperature for at least 30 min until being used for recordings.

Whole-Cell Patch-Clamp Recording

Neurons with obvious primary dendrites and spines were selected, which is the morphological characteristics of BLA principal neurons (McDonald, 1982; Padival et al., 2013). Whole-cell patch clamp pipettes were composed of borosilicate glass capillaries (1.5 mm outer diameter; World Precision Instruments, Sarasota, FL, United States). The resistances of electrodes were from 2 to 3.5 MΩ. Voltages were corrected for a liquid junction potential of 13–14 mV, calculated using pClamp 10.3. Recordings were performed at 32–33°C, with stable perfusion of ACSF (2 ml/min). Electrodes were filled with (in mM): 110 Cs methylsulfate, 0.3 Tris-GTP, 15 CsCl, 2 $MgCl_2$, 0.5 EGTA, 10 HEPES, 4 ATP-Mg, 4 QX-314 and 5 Na_2-phosphocreatine (pH 7.15–7.25 with CsOH, 270–280 mOsm with sucrose). To record miniature synaptic events (mEPSCs and mIPSCs), we bathed the slices in normal ACSF containing 1.0 μM TTX. After allowing for 5 min of stabilization after break in, mEPSCs and mIPSCs were recorded at a holding potential of −70 mV for 2 min and 0 mV for 2 min, respectively (Lippi et al., 2016; Nagode et al., 2017). The postsynaptic currents recorded at −70 mV were blocked after the addition of 20 μM CNQX and 50 μM AP5, whereas those recorded at 0 mV were blocked by 50 μM picrotoxin (**Supplementary Figure S1**). Series resistance was constantly monitored. Cell input resistance (R_{in}) was calculated by determining the current response from a holding potential of −70 mV to the steps of −5 mV hyperpolarization (Mizunuma et al., 2014; Nagode et al., 2017). Data were excluded when the series resistance reached above 16 MΩ or the change of series resistance reached more than 20%. In this study, the rise time represents 10–90% rise time, and the decay kinetics were measured as 90–37% decay time. The total number of events that occurred during 2-min recording epochs was analyzed. The number of mEPSCs used in the analysis for each cell ranged from 109 to 942, and the number of mIPSCs ranged from 55 to 899. Previous study showed that the mEPSC rise time was variable depending on the different electrotonic distances from the somatic recording site to the synaptic region where each mEPSC occurs, and events originating from the soma or dendrites presented as fast and slow rising events respectively (Han et al., 2013). In the present study, we did not detect the specific populations of mEPSCs via the rising time, and no criteria relating to rise time were used to further filter detected events. Thus, the mEPSCs and mIPSCs may be comprised of both proximal (i.e., somatic) and distal (dendritic) events. In this study, mEPSCs and mIPSCs were analyzed by the two exponential equations model fitting in Decay fit of MiniAnalysis software. This method was fit to an ensemble average generated for each cell. The equation is as follows: y = A1*e^(-x/t1) + A2*e^(-x/t2). Signals were amplified with a MultiClamp 700B amplifier (Molecular Devices, Union City, CA, United States), filtered at 2 kHz, and digitized at 10 kHz. Data were analyzed with the pCLAMP 10.3 data acquisition program (Molecular Devices). Miniature events were detected offline using MiniAnalysis (Synaptosoft), with the amplitude threshold set to 5 pA and an area threshold of 10.

Golgi-Cox Staining

Rats were anesthetized and transcardially perfused with 0.9% normal saline solution. Brains were dissected, and were immersed with a Golgi-Cox solution for 2 weeks based on previous studies (Yang et al., 2015; Han et al., 2016; Wang et al., 2017), and then in 30% sucrose solution for 2–5 days in darkness at room temperature. Coronal sections (200 μm) were prepared using a vibratome (Microm HM 650V, Thermo Scientific, Walldorf, Germany) according to previous studies (Yang et al., 2015; Wang et al., 2017). Slides were kept in the darkness during staining and afterward.

Neurons with obvious primary dendrites and spines were selected, which is the morphological characteristics of BLA principal neurons (McDonald, 1982; Padival et al., 2013). We excluded aspiny neurons showing small somata with few dendrites or large somata with bipolar primary dendrites. A recent study states that the distance from the soma affects the role of inhibitory shaft and spine synapses, and strengthens the role of axon initial segment (Boivin and Nedivi, 2018). Thus, in our study, dendritic segments with 50–150 μm distance from the soma (Christoffel et al., 2011), and 40–70 μm in length, were randomly chosen from pyramidal neurons in the BLA and were counted starting from the origin of a branch. Second-order apical dendrites were analyzed in our study. In order to meet the requirements of spinal analysis, dendritic segments must have the following qualifications: segments must be fully filled (excluding all endings); segments must have a distance of no less than 50 μm from the soma; segments did not show overlap with other branches, which may blur the visualization of spines (Christoffel et al., 2011). A 3D image was reconstructed with NIH ImageJ software[1]. The number of dendritic protrusions were calculated based on the morphology: thin spines have thin head and long neck; mushroom spines come with large head and short neck; stubby spines also have large head but no apparent neck (Montalbano et al., 2013; Geoffroy et al., 2019). For morphological quantification, one dendrite per neuron and 5–8 neurons per rat were analyzed in five rats in each group. The experimenter was blind to the grouping. All images were

[1]http://rsbweb.nih.gov/ij/

captured using Olympus BX53 microscope with a 100× oil-immersion objective. The average number of spines per 10 μm of dendrite was calculated.

Statistical Analysis

Waveform parameters (frequency, amplitude, rise-time 10–90%, half-width, decay time 90–37% and area) (Hendrich et al., 2012) were measured in the study. The results were showed as mean ± SEM. Normal distribution was validated with Shapiro–Wilks test, and homogeneity of variance was validated with Levene's test. Unpaired Student's *t*-test was used for comparisons between two groups. Analysis of variance (ANOVA) was used for data analysis with suitable between- and within-subject factors. When comparing three or more groups, one- or two-way ANOVA was adopted with *post hoc* analysis (one-way, Tukey; two-way, Sidak's multiple comparisons) for comparison of three of more groups. Cumulative probability was compared using Kolmogorov–Smirnov (KS) statistics (P_{KS}). Since large samples were analyzed, the significance level was mostly taken at $P_{KS} < 0.001$ (Simkus and Stricker, 2002; Miura et al., 2012; Calfa et al., 2015).

RESULTS

Previous investigations have revealed that significant alterations in anxiety-like behaviors occurred only in rats 10 days rather than 1 day after SPS (Fang et al., 2018). Experiment 1 aimed at demonstrating changes in anxiety-like behavior of the rats 1 and 10 days after SPS. Experimental procedure was displayed in **Figure 1A**. Normal healthy rats were kept separately 4–5 days before the tests to adapt to the feeding environment. Then rats in the experimental group underwent SPS procedure (be restrained for 2 h, forced swimming for 20 min, rest for 15 min and anesthetized with ether until being unconscious), followed by being kept in single cage with undisturbed feeding environment. Anxiety-like behaviors of rats were tested with the EPM and OFT on the first as well as the tenth day after SPS. The experiment mainly consisted of 4 groups: SPS(1d), NO SPS(1d), SPS(10d), NO SPS(10d) ($n = 8$ per group). The results of EPM and OFT were analyzed with two-way ANOVA, and we used SPS (SPS, No SPS) and Post-SPS Day (1 day, 10 day) as the between-subject factors. The analysis of time spent in the open arm in EPM showed significant effects of Post-SPS Day ($F_{1,28} = 7.92$, $p < 0.01$) and SPS × Post-SPS Day interaction ($F_{1,28} = 4.76$, $p < 0.05$). It was revealed that time spent in the open arm in SPS(10d) group was significantly less compared with NO SPS(10d) group via *post hoc* analysis ($p < 0.01$, **Figure 1B**). The analysis of entries into the open arms in EPM displayed significant effects of Post-SPS Day ($F_{1,28} = 7.07$, $p < 0.05$) and SPS × Post-SPS Day interaction ($F_{1,28} = 11.27$, $p < 0.01$). It was revealed that reducing entries were observed in SPS(10d) group in contrast to NO SPS(10d) group via *post hoc* analysis ($p < 0.01$, **Figure 1C**). The result of time spent in the center area in OFT illustrated significant effects of SPS × Post-SPS Day interaction ($F_{1,28} = 5.45$, $p < 0.05$). It was showed that time spent in the central area in SPS(10d) group was obviously less in contrast to the NO SPS(10d) group via *post hoc* analysis ($p < 0.01$, **Figure 1D**). For total locomotor distance, no obvious difference was shown in experimental conditions ($p > 0.05$, **Figure 1E**). In summary, these results showed that rats displayed delayed onset of anxiety-like behaviors after SPS, which were in line with previous findings (Knox et al., 2012a; Fang et al., 2018).

Next, we studied the synaptic mechanisms underlying deferred development of anxiety-like behaviors after SPS. Our previous study showed that activity of BLA glutamatergic neurons and BLA GABAergic neurons was increased day 10 after SPS in contrast to the control group (Fang et al., 2018). BLA is mainly composed of glutamatergic pyramidal neurons (∼85%) (Duvarci and Pare, 2014). Dendrites in the amygdala are especially sensitive to stress exposure (Chattarji et al., 2015). Thus, we analyzed the time-dependent changes in density of dendritic spines in BLA in the SPS model (**Figures 2A,B**). The experiment consisted of four groups ($n = 5$ per group). Results of the spine density were analyzed with two-way ANOVA, and we used SPS (SPS, No SPS) and Post-SPS Day (1 day, 10 days) as the between-subject factors. The analysis showed noticeable effects of SPS ($F_{1,16} = 6.74$, $p < 0.05$) and SPS × Post-SPS Day interaction ($F_{1,16} = 5.73$, $p < 0.05$) on the total spine density. The results showed that the density of spines increased obviously in SPS(10d) group in contrast to NO SPS(10d) via *post hoc* analysis ($p < 0.01$, **Figure 2C**). Dendritic spines are often categorized by morphology and the shape of these spines have correlation with their functions (Moench and Wellman, 2015). Thus, we analyzed the spine densities of different subtypes (Wang et al., 2017) in BLA after SPS. Analysis with two-way ANOVA revealed noticeable effects of SPS ($F_{1,16} = 29.60$, $p < 0.01$), Post-SPS Day ($F_{1,16} = 17.22$, $p < 0.01$) and SPS × Post-SPS Day interaction ($F_{1,16} = 7.76$, $p < 0.05$) on the density of mushroom spines, but the analysis on the density of thin spines revealed no significant effects ($p > 0.05$). *Post hoc* analysis showed that the density of mushroom spines increased remarkably in the SPS(10d) group ($p < 0.05$, **Figure 2E**), while no significant differences were found in the SPS(1d) group. The density of thin spines showed no significant difference in the SPS(1d) group or SPS(10d) group ($p > 0.05$, **Figure 2D**). Density of stubby spines, which were reckoned to be immature structures and had a certain relationship with the stress-induced increase of glutamatergic synapses, was increased both of day 1 and day 10 after SPS in contrast to corresponding control groups (both $p < 0.05$, **Figure 2F**) (Christoffel et al., 2011). To sum up, total and mushroom spine density were markedly increased in SPS(10d) group in our research, which accompanied an increase in anxiety-like behavior in SPS(10d) group.

Formation and elimination of dendritic spines may contribute to synaptic connectivity and function, especially mushroom spines positively correlating with synapse strength and age (Holtmaat and Svoboda, 2009; Moench and Wellman, 2015). Therefore, following the same SPS procedure, we recorded mIPSCs and mEPSCs of the same cell at different voltages in the SPS(1d) and SPS(10d) group, respectively to determine spontaneous quantal synaptic input onto BLA pyramidal neurons (**Figures 3**, **4**). For the input resistance, there was no

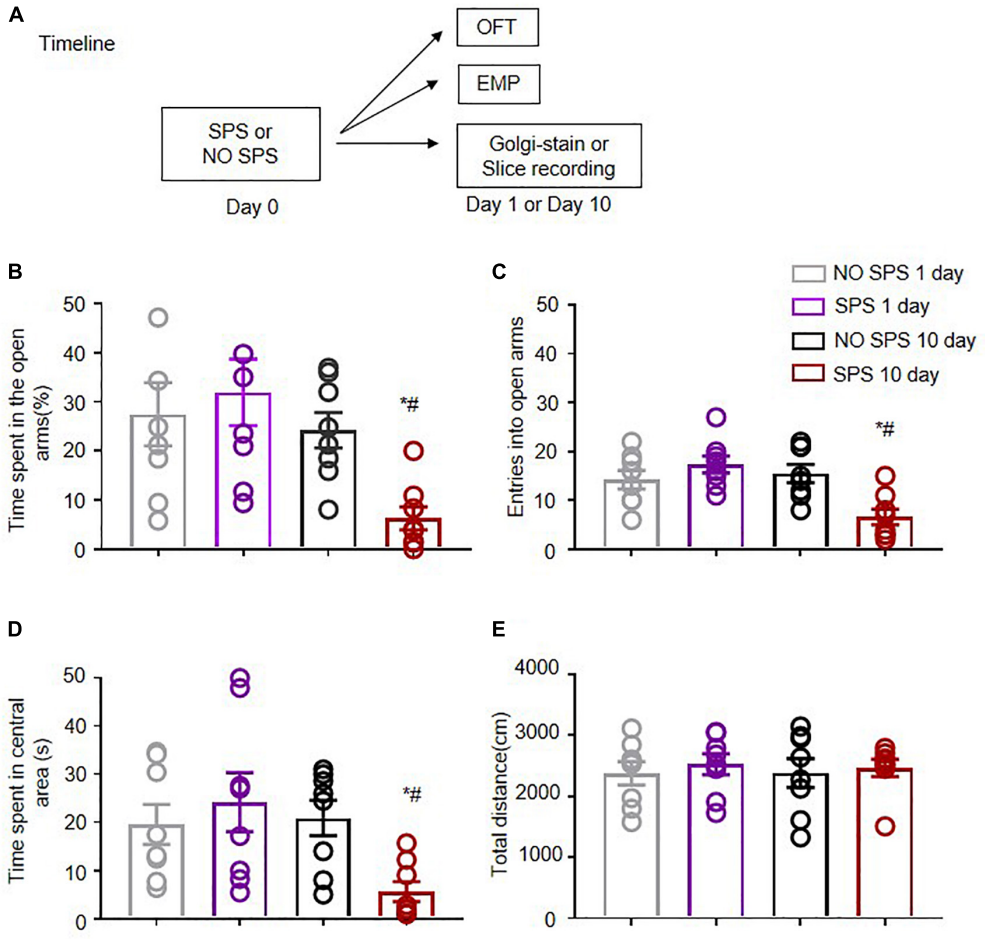

FIGURE 1 | Effect of single prolonged stress (SPS) on anxiety-like behaviors on the first and tenth day after stress. **(A)** Experimental procedures. **(B,C)** Time spent in open arms **(B)** and the entries into open arms **(C)** in different experimental conditions in EPM. **(D,E)** Time spent in the central area **(D)** and total distance (cm) **(E)** in different experimental conditions in OFT. $n = 8$ per experimental condition. #Different from SPS(1d) group, *Different from NO SPS group at each post-stress day, * #$p < 0.05$, two-way ANOVA. Data are shown as means ± SEM.

significant difference among experimental conditions ($p > 0.05$, **Supplementary Figure S2A**). Data of the frequency and amplitude of mEPSCs and mIPSCs were analyzed with one-way ANOVA, and we used SPS as the between-subjects factor ($n = 13$–18 cells per group). The mEPSCs frequency recorded in BLA pyramidal neurons of the SPS(10d) group was remarkably increased compared with the NO SPS group ($F_{1,29} = 20.93$, $p < 0.01$, **Figure 3J**), while no obvious difference was found in the day 1 group after SPS ($p > 0.05$, **Figure 3D**). For mEPSCs inter-event intervals of BLA pyramidal neurons, the cumulative probability distribution of SPS(10d) group was shifted left compared with NO SPS group ($P_{KS} < 0.001$, **Figure 3I**), which indicated that mEPSCs frequency in SPS(10d) group was increased. The cumulative probability distribution was mildly shifted toward the left ($P_{KS} = 0.0015$, **Figure 3C**), which may be due to a slight increase in mEPSCs frequency in SPS(1d) group. The mEPSCs amplitudes recorded in BLA pyramidal neurons of the SPS(1d) group as well as SPS(10d) group showed no significant difference with NO SPS groups (both $p > 0.05$, **Figures 3F,L**). The cumulative probability distribution of amplitudes of mEPSCs in SPS(1d) and SPS(10d) group BLA pyramidal neurons were not shifted compared to NO SPS pyramidal neurons (both $p > 0.05$, **Figures 3E,K**). Finally, we compared the amplitude and frequency of mEPSCs in BLA pyramidal neurons of the SPS(1d) group and SPS(10d) group. The excitatory synaptic frequency of pyramidal neurons of the SPS(10d) group was increased compared with that of the SPS(1d) group ($F_{1,30} = 9.18$, $p < 0.01$, **Table 1**), and no difference was observed for the amplitude ($p > 0.05$, **Table 1**). The results showed that the amplitude of mEPSCs of BLA pyramidal neurons after SPS was not obviously affected, but frequency of mEPSCs in BLA pyramidal neurons increased significantly day 10 after SPS. Finally, as larger spines often predict larger mEPSC amplitude (Segal, 2010; Ueno et al., 2014), we analyzed the mEPSCs after classifying spikes into different subgroups by different amplitude values (Lu et al., 2007; Biggs et al., 2010).

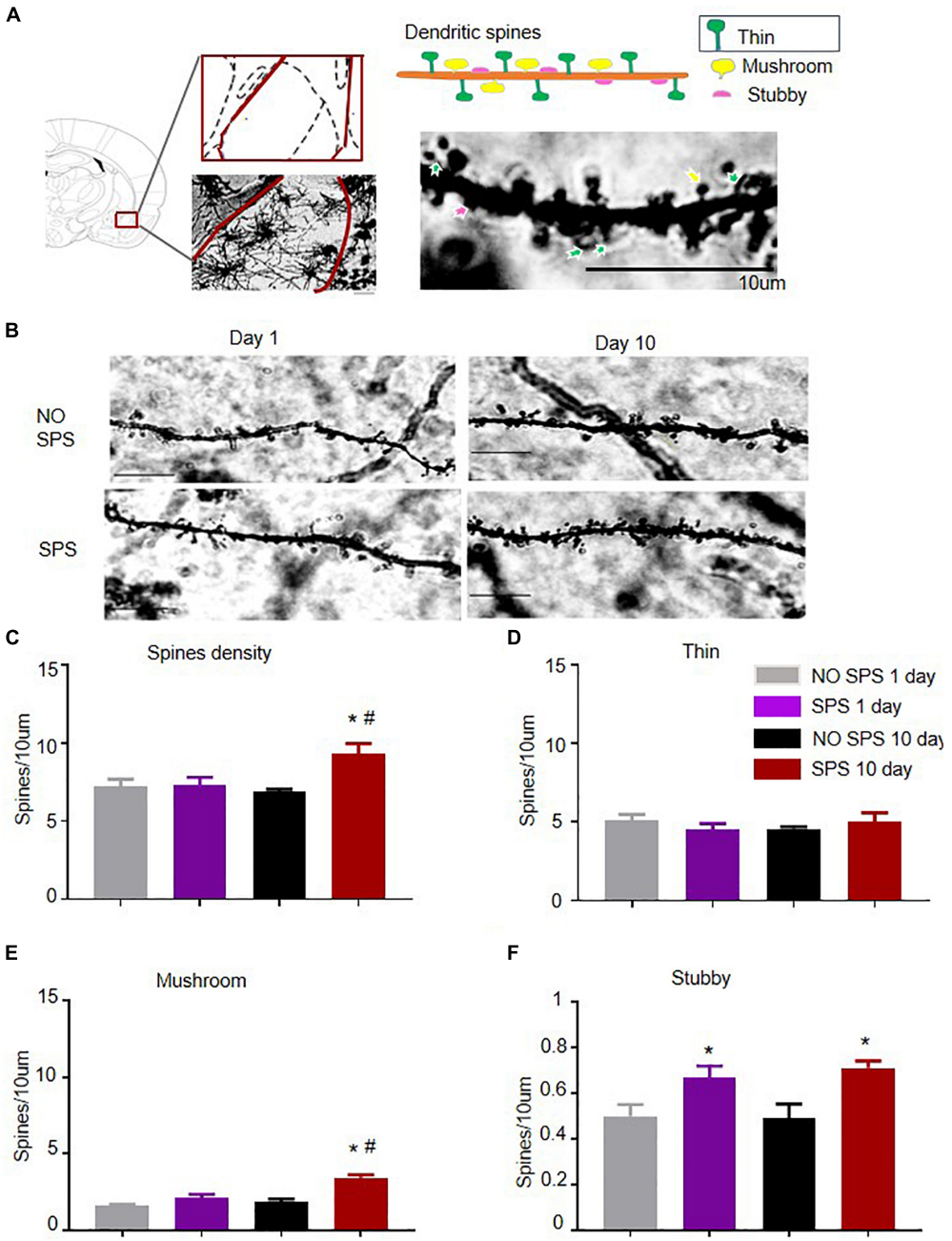

FIGURE 2 | Effect of SPS paradigms on spine density of BLA pyramidal neurons. **(A)** Low-power image of dendritic spines of BLA from control rats. Scale bar = 10 μm. Dendritic spines were classified based on morphology: thin dendritic spines have thin head and long neck (indicated by green arrows), mushroom dendritic spines come with large head and short neck (indicated by yellow arrows) and stubby dendritic spines have large head but no apparent neck (indicated by red arrows). Scale bar = 10 μm. **(B)** High-power image of representative dendrite segments (scale bar = 10 μm). **(C)** Spine density in BLA pyramidal dendrite segments in different experimental conditions (animals, rats = 5; segments, n = 5–8, total dendritic length = 40–70 μm). **(D–F)** Average density in mushroom **(D)**, thin **(E)**, and stubby **(F)** spines in BLA pyramidal dendrite segments sampled from four groups: NO SPS(1d)/SPS(1d)/NO SPS(10d)/SPS(10d). #Different from SPS(1d) group, #*Different from NO SPS group at each post-SPS day, #*$p < 0.05$, two-way ANOVA. Data are shown as means ± SEM.

One-way ANOVA analyzed the large amplitude events (>30 pA) of mEPSCs in BLA pyramidal neurons from four groups, the results showed that the large amplitude events (>30 pA) of mEPSCs in BLA pyramidal neurons of the SPS(10d) group was increased compared with control groups ($F_{3,45} = 4.16$, $p < 0.05$, **Supplementary Figure S2B**). In summary, the results showed that the excitatory synaptic transmission of BLA pyramidal neurons increased day 10 after SPS.

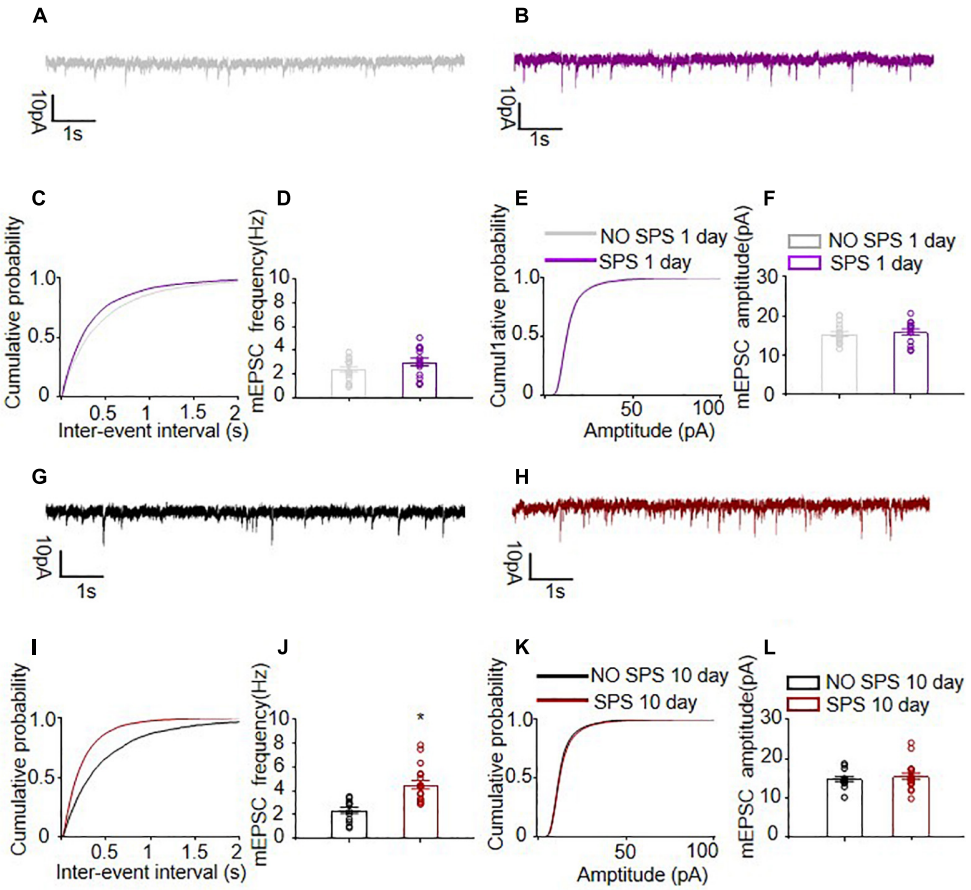

FIGURE 3 | Spontaneous excitatory quantal synaptic transmission onto BLA pyramidal neurons. **(A,B,G,H)** Representative examples of mEPSCs of different experimental conditions. **(C,I)** The cumulative probability distribution of mEPSCs inter-event intervals of BLA pyramidal neurons from SPS group and NO SPS group on the first and tenth day after SPS respectively. **(D,J)** The mEPSCs frequency of BLA pyramidal neurons from SPS group and NO SPS group on the first and tenth day after SPS respectively. **(E,K)** The cumulative probability distribution of mEPSCs amplitudes of BLA pyramidal neurons from SPS group and NO SPS group on the first and tenth day after SPS respectively. **(F,L)** The mEPSCs amplitudes of BLA pyramidal neurons from SPS group and NO SPS group on the first and tenth day after SPS respectively. *Different from NO SPS group at each post-SPS day, $*p < 0.05$, one-way ANOVA. Data are shown as means ± SEM.

We further recorded the mIPSCs of the same pyramidal neurons paired with mEPSCs after the SPS procedure (Calfa et al., 2015) with 1 μM TTX (**Figure 4**) at 0 mV (n = 13–18 cells per group). The mIPSCs frequency recorded in BLA pyramidal neurons 1 day after SPS exhibited no changes compared with controls ($p > 0.05$, **Figure 4D**), while the SPS(10d) group showed significantly higher mIPSCs frequency than control groups ($F_{1,29} = 11.60$, $p < 0.01$, **Figure 3J**). For mIPSCs inter-event intervals of BLA pyramidal neurons, the cumulative probability distribution of the SPS(10d) group was shifted left compared with that of NO SPS pyramidal neurons ($P_{KS} < 0.001$, **Figure 4I**), while no difference was found between the SPS(1d) group and NO SPS group. On the other hand, the mIPSCs amplitude recorded in BLA pyramidal neurons of either SPS(1d) or SPS(10d) group showed no difference compared with NO SPS groups (both $p > 0.05$, **Figures 4F,L**). In BLA pyramidal neurons, the curves of the cumulative probability distribution of mIPSCs amplitude from SPS(1d) group and SPS(10d) group were not shifted compared with control pyramidal neurons and they almost coincided (both $p > 0.05$, **Figures 4E,K**). Then, we compared the amplitude and frequency of mIPSCs in BLA pyramidal neurons of SPS(1d) group and SPS(10d) group, and the analysis showed that the inhibitory synaptic frequency of BLA pyramidal neurons of the SPS(10d) group was obviously higher compared to that of the SPS(1d) group ($F_{1,30} = 3.51$, $p > 0.05$, **Table 1**), and no changes were found in amplitude ($p > 0.05$, **Table 1**). In summary, the results showed that the inhibitory synaptic transmission of BLA pyramidal neurons increased day 10 after SPS. The results showed that BLA pyramidal neurons received enhanced inhibitory neuronal projections day 10 after SPS, and the frequency of mEPSCs and mIPSCs were increased.

DISCUSSION

Patients with PTSD typically have symptoms such as avoidance, interference and awakening, emotional and cognitive changes (Pitman et al., 2012). Extensive reports have used SPS procedure

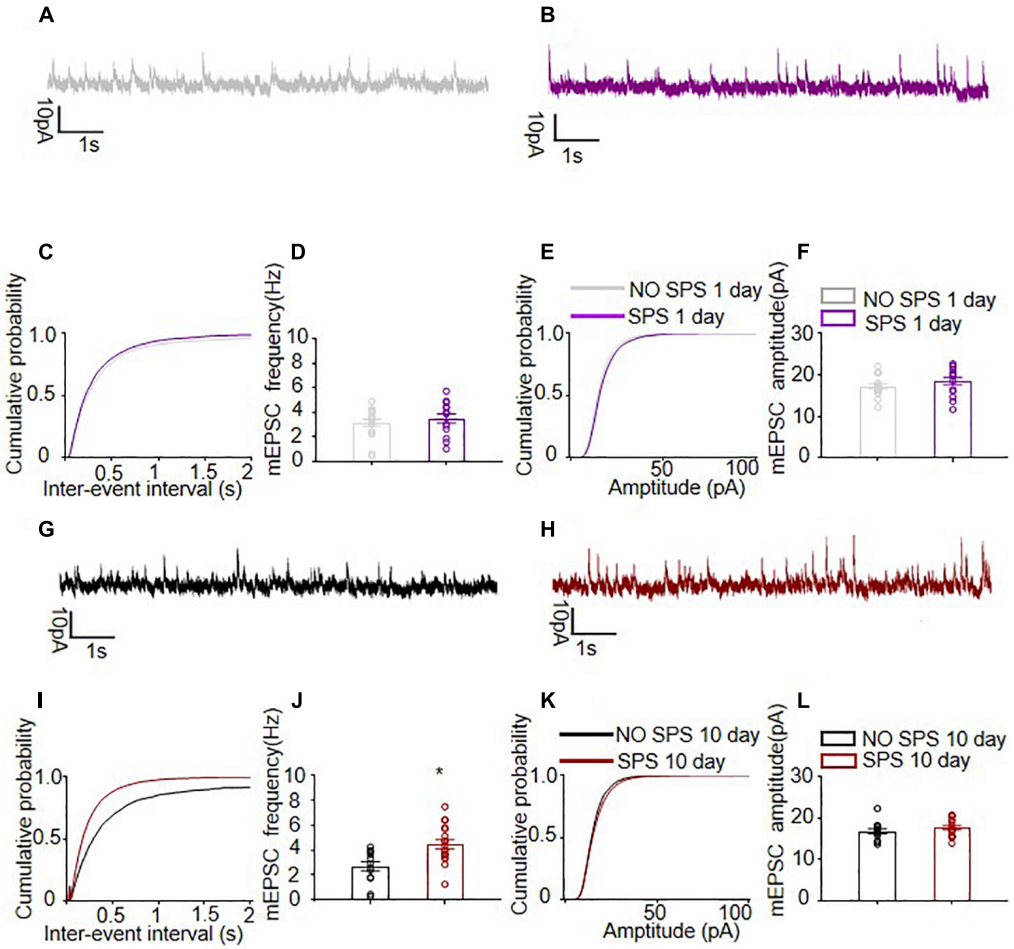

FIGURE 4 | Spontaneous inhibitory quantal synaptic transmission onto BLA pyramidal neurons. **(A,B,G,H)** Representative examples of mIPSCs of different experimental conditions. **(C,I)** The cumulative probability distribution of mIPSCs inter-event intervals of BLA pyramidal neurons from SPS group and NO SPS group on the first and tenth day after SPS respectively. **(D,J)** The mIPSCs frequency of BLA pyramidal neurons from rats on SPS group and NO SPS group. **(E,K)** The cumulative probability distribution of mIPSCs amplitudes of BLA pyramidal neurons from SPS group and NO SPS group on the first and tenth day after SPS respectively. **(F,L)** The mIPSCs amplitudes of BLA pyramidal neurons from rats on SPS group and NO SPS group on the first and tenth day after SPS respectively. *Different from NO SPS group at each post-SPS day, $*p < 0.05$, one-way ANOVA. Data are shown as means ± SEM.

to study the animal PTSD (Iwamoto et al., 2007; Wen et al., 2016; Fang et al., 2018). Research has revealed that SPS leads to diminished fear extinction (Knox et al., 2012a; Fang et al., 2018), enhanced stress-induced nociceptive sensitivity and increased anxiety-like behavior (Zhang et al., 2012), and SSRI may reverse the symptoms (Takahashi et al., 2006; Lin et al., 2016). We assessed PTSD-induced anxiety-like behavior through OFT and EPM. Our behavioral experiments revealed no notable alterations in the anxiety-like behavior of rats on the first day after SPS, but a significant increase consistent with previous findings on the 10th day after SPS (Fang et al., 2018). Furthermore, we found delayed changes in synaptic plasticity in BLA pyramidal neurons after SPS. Specifically, on day 10 after exposure to SPS, result indicated an increase in density of dendritic spine, and enhancement both in glutamatergic and GABAergic synaptic transmissions. In conclusion, SPS produced delayed increase in spinogenesis and synaptic transmission in BLA which is accompanied with enhanced anxiety-like behaviors.

The structural plasticity of dendritic spines is critical for diverse types of synaptic plasticity (Yang et al., 2009; Yin et al., 2009; Oe et al., 2013), including structural remodeling in response to stress (Chattarji et al., 2015; Duman and Duman, 2015; Qiao et al., 2016). The structural basis of synaptic connectivity in BLA is differentially modified by various forms of stress (Chattarji et al., 2015). Acute restraint stress induces an enhancement in dendritic spine density in the BLA pyramidal neurons several days after stress (Mitra et al., 2005; Maroun et al., 2013; Suvrathan et al., 2014; Yasmin et al., 2016). Chronic restraint stress induces dendritic hypertrophy in BLA pyramidal neurons, increased size of dendritic spine heads (Mitra et al., 2005; Vyas et al., 2006; Maroun et al., 2013; Zhang et al., 2019) and enhanced neuronal excitability (Rosenkranz et al., 2010). Consistently, our results

TABLE 1 | Summary of electrophysiological data.

Group		Frequency (Hz)	Amplitude (pA)	Risetime 10–90% (ms)	Half-width (ms)	Decay 90–37%(ms)
		Mean ± sem	Mean ± sem	Mean ± sem	Mean ± sem	Mean ± sem
NO SPS (1d)	mEPSCs	2.41 ± 0.22, *n* = 18	15.41 ± 0.59, *n* = 18	1.09 ± 0.05, *n* = 18	6.48 ± 0.15, *n* = 18	5.79 ± 0.26, *n* = 18
	mIPSCs	3.17 ± 0.28, *n* = 18	17.29 ± 0.57, *n* = 18	1.42 ± 0.19, *n* = 18	14.32 ± 0.43, *n* = 18	13.83 ± 0.52, *n* = 18
SPS (1d)	mEPSCs	3.03 ± 0.36, *n* = 14	15.87 ± 0.78, *n* = 14	1.00 ± 0.05, *n* = 14	5.37 ± 0.37, *n* = 14	4.83 ± 0.24, *n* = 14
	mIPSCs	3.52 ± 0.37, *n* = 14	18.53 ± 0.93, *n* = 14	1.34 ± 0.21, *n* = 14	13 ± 0.59, *n* = 14	12.8 ± 0.65, *n* = 14
NO SPS (10d)	mEPSCs	2.38 ± 0.25, *n* = 13	14.88 ± 0.64, *n* = 13	1.08 ± 0.06, *n* = 13	5.85 ± 0.38, *n* = 13	5.18 ± 0.33, *n* = 13
	mIPSCs	2.71 ± 0.37, *n* = 13	16.84 ± 0.62, *n* = 13	1.13 ± 0.21, *n* = 13	12.8 ± 0.51, *n* = 13	14.08 ± 0.59, *n* = 13
SPS (10d)	mEPSCs	4.53 ± 0.35*#, *n* = 18	15.56 ± 0.82, *n* = 18	1.10 ± 0.07, *n* = 18	5.91 ± 0.33, *n* = 18	5.07 ± 0.27, *n* = 18
	mIPSCs	4.48 ± 0.35*, *n* = 18	17.68 ± 0.51, *n* = 18	1.26 ± 0.15, *n* = 18	12.7 ± 0.54, *n* = 18	12.31 ± 0.47, *n* = 18

**Different from NO SPS group at each post-SPS day, #Different from SPS(1d) group, $p < 0.05$, one-way ANOVA. Data are shown as means ± SEM.*

showed that dendritic spine density in BLA pyramidal neurons of SPS(10d) group was increased. However, a recent study showed that acute elevated platform stress increased mushroom spine density and produced dendritic retraction in BLA pyramidal neurons 2 days later (Maroun et al., 2013). We presumed that the discrepancies between the findings on effects of stress on dendritic morphology of amygdala may be due to different types and procedures of stress. The present results showed that mushroom spines but not thin spines displayed delayed increase after SPS. Generally, thin spines have higher plasticity and lability compared with mushroom spines (Moench and Wellman, 2015). Thus, our results suggest that the mature and stable type of spines are gradually increased after traumatic stress which may be the structural substrates of delayed onset of anxiety-like behaviors. While the mechanisms underlying the delayed alteration of dendritic spines remain unclear, it is worthy to note the implications of NMDA and AMPA receptors in regulating structural plasticity (Krugers et al., 2010; Duman, 2014; Yasmin et al., 2016). NMDA receptors are considered to be implicated in the initial formation of spines by calcium influx and continuous downstream effects (Maletic-Savatic et al., 1999), and AMPA receptors are implicated in the strengthening of existing spines (Maletic-Savatic et al., 1999). In the amygdala, 10 days of chronic immobilization stress could enhance NMDAR-mediated synaptic responses (Suvrathan et al., 2014), and the NMDAR antagonist infused into the BLA during the acute stress prevented the enhanced effects on mEPSCs frequency and spine density 10 days later (Yasmin et al., 2016). It has been demonstrated that the ratio of GluA1-AMPAR-labeled spines to labeled dendritic shafts in the BLA was found to increase 6 and 14 days but not 1 day after stress, which accompanies enhanced frequency of mEPSCs in stressed animals without changes in mEPSCs amplitude (Hubert et al., 2014). Thus, we speculated that AMPA receptors are associated with the expression and maintenance of stress-induced structural plasticity, while NMDA receptors are important for the initiation of stress-induced structural plasticity. Interestingly, we found the stubby spines were increased on both day 1 and 10 after traumatic stress. A stubby spine with a large head and no neck is considered as a type of immature spines (Ebrahimi and Okabe, 2014; Berry and Nedivi, 2017). Although stubby structures are rarely studied and understood, it is reported that they predominate early in postnatal development (Boyer et al., 1998) and to proliferate in nucleus accumbens after social stress (Christoffel et al., 2011). Considering the roles of the geometry of the spine neck in synaptic plasticity, stubby spines may elicit strong signal diffusing through the surrounding dendrite (Hayashi and Majewska, 2005; Ebrahimi and Okabe, 2014), which may be involved in anxiety-like behaviors. The precise roles of stubby spines in the amygdala structural plasticity and maladaptive response to stress need to be further investigated.

Spines are important targets for excitatory synaptic transmission (Harris and Kater, 1994; Qiao et al., 2016) and are positively associated with synaptic transmission (Hayashi and Majewska, 2005; Alvarez and Sabatini, 2007; Ebrahimi and Okabe, 2014). In our current study, the analysis of the mEPSCs frequency showed a delayed enhancement in BLA after SPS, which is consistent with the findings that an increased number of excitatory pyramidal neurons were activated on the 10th day after SPS (Fang et al., 2018). Consistently, Yasmin and colleagues found that increase in mEPSCs frequency induced by stress is associated with an enhancement of the number of dendritic spines (Yasmin et al., 2016). Escalation in the frequency of mEPSCs is considered to be due to an increase in the number of glutamatergic synapses and a presynaptic suppression of glutamate release probability (Malgaroli and Tsien, 1992; Sastry and Bhagavatula, 1996). Considering the significant increase in the number of dendritic spines in BLA in SPS(10d) group, the observed enhancement of mEPSCs frequency in our study may be induced, at least in part, by the increase in the number of functional excitatory synapses. Under some circumstances, some other studies have reported that an increase in the number of dendrites spines accompanies an enhancement of frequency of mEPSCs (Wissman et al., 2011; Montalbano et al., 2013; Bochner et al., 2014; Yasmin et al., 2016; Schilling et al., 2017; Sun et al., 2018). However, the increased number of spines, especially large spines, would be predictive of an increase in the expression of postsynaptic excitatory receptors and subsequently larger mEPSCs amplitude (Lee et al., 2015; Udagawa et al., 2015; Awad et al., 2016; Deng et al., 2019). Consistently, we showed that the amplitude (>30 pA) of mEPSCs in BLA pyramidal neurons of SPS(10d) group was increased compared with No SPS groups, which fits with

the observed increasing density of mushroom spines on the 10th day after SPS.

Interestingly, we found an enhancement in the frequency of inhibitory synaptic transmission 10 days after stress. Combined with our previous findings that more inhibitory neurons are activated on the 10th day after SPS (Fang et al., 2018), we considered that also gradually activated inhibitory neurons which would be due to either an increase in the number of GABAergic synapses or an increase in the release probability. More data, such as spontaneous IPSC are required to confirm these explanations in the future. It is essential to explore the effects of inhibitory transmission on stress-induced BLA dysfunction and delayed appearance of PTSD-like behaviors. It has been shown that function of adult BLA is regulated by a reciprocal interaction between GABAergic interneurons and pyramidal neurons (Ehrlich et al., 2009, 2012; Ryan et al., 2012), so the delayed increase in inhibitory transmission may be attributed to a homeostatic mechanism which avoids excessive activation of the pyramidal neurons in BLA. The current finding was in line with previous results that chronic activity blockade leads to homeostatic plasticity that both mEPSCs and mIPSCs frequency were elevated (Echegoyen et al., 2007). We found that the frequency of IPSCs and EPSCs increase by similar amounts after stress, and the balance between inhibition and excitation seems to be unaltered. We presumed that other cellular and synaptic mechanisms may also contribute to the PTSD-like behaviors in rats, such as the alterations in a specific type of GABAergic neurons in BLA after traumatic stress or time-dependent distributions of inhibitory synapse on pyramidal neurons after stress. Furthermore, it is unclear if the excitability of pyramidal cells or activity-dependent network plasticity would be significantly altered. Further experiments investigating the effects of stress upon intrinsic excitability, spontaneous EPSCs/IPSCs and evoked EPSCs/IPSCs would be informative in this regard. Lastly, it should be noted, with various corticolimbic targets, that BLA pyramidal neurons are functionally heterogeneous and thus stress may differentially impact specific output circuits. Indeed, dendrites were hypertrophied caused by chronic restraint stress in BLA pyramidal neurons, and the size of dendritic spine heads was increased only in BLA pyramidal neurons targeting the nucleus accumbens (NAc) or the ventral hippocampus (vHPC) (Zhang et al., 2019). In addition, the excitatory glutamatergic transmission targeting the vHPC or the NAc in BLA PNs was selectively increased (Zhang et al., 2019). Therefore, which BLA projects exhibit changes of excitation-inhibition balance after SPS needs to be further investigated.

The underlying molecular mechanism of delayed increase in spine density and neural transmissions is still unknown, and previous evidence suggests that it may be related to dysregulation of the HPA axis, with significant lower concentrations of plasma and urinary cortisol (Yehuda et al., 1993). Previous studies speculated that hypercortisol and glucocorticoid negative feedback is specifically increased by PTSD (Zoladz and Diamond, 2013). Consistently, it has been shown that the delayed spinogenesis in the BLA can be impeded by prior exposure to glucocorticoids after acute stress, which could be blocked by bilateral adrenalectomy (Rao et al., 2012). Furthermore, some studies have revealed that SPS increases the expression level of glucocorticoid receptors (Ganon-Elazar and Akirav, 2013), and NMDA receptor subunit mRNAs (Yamamoto et al., 2008). However, another study showed that the expression level of CaMKII and MR/GR in BLA had not been obviously affected by SPS, and the improvement of NPY functions could regulate the alterations in the morphology of the BLA pyramidal neurons induced by SPS (Cui et al., 2008). Thus, more research is required to discover the molecular mechanisms of the increase in spinogenesis and synaptic transmission after SPS.

The results of present study revealed that rats showed increase in both spinogenesis and synaptic transmission in the BLA only on day 10 rather than day 1 after SPS, which means after traumatic stress, BLA displayed delayed changes in neuronal plasticity. The present findings revealed that BLA may be associated with the pathogenesis of PTSD, which is of great importance for future clinical research and targeted treatment.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

ETHICS STATEMENT

All experiments were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and Biomedical Ethics Committee of Peking University for animal use and protection. The protocol was approved by the Biomedical Ethics Committee of Peking University for animal use and protection.

AUTHOR CONTRIBUTIONS

H-HZ, S-QM, J-LY, and Y-XX designed the experiments. H-HZ, S-QM, X-YG, and Y-YC performed the experiments. H-HZ and Y-XX analyzed and interpreted the data. J-LZ, WZ, S-QM, and J-LY commented on the manuscript. H-HZ, J-LZ, Y-XX, and LL wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpsyg.2019.02394/full#supplementary-material

FIGURE S1 | (A) Experimental timeline. **(B)** In defect of antagonists of glutamatergic or GABAergic receptors, mEPSCs and mIPSCs were recorded at clamped voltages of −70 and 0 mV. After adding 20 μM CNQX and 50 μM AP5, the postsynaptic currents recorded at −70 mV were blocked, while those recorded at 0 mV were suppressed by 50 μM picrotoxin.

FIGURE S2 | (A) The input resistance of BLA pyramidal neurons among the different experimental conditions. **(B)** Large amplitude events (>30 pA) of mEPSCs in BLA pyramidal neurons from four groups: NO SPS(1d)/SPS(1d)/NO SPS(10d)/SPS(10d). *Different from NO SPS groups. One-way ANOVA, $^*p < 0.05$.

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Lowered Plasma Steady-State Levels of Progesterone Combined With Declining Progesterone Levels During the Luteal Phase Predict Peri-Menstrual Syndrome and Its Major Subdomains

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Background: It is unknown whether lowered steady state levels of sex hormones coupled with changes in those hormones during the menstrual cycle are associated with premenstrual syndrome (PMS).

Objective: To examine associations between levels of progesterone and oestradiol during the menstrual cycle and PMS considering different diagnostic criteria for PMS.

Methods: Forty-one women aged 18–45 years with a regular menstrual cycle completed the Daily Record of Severity of Problems (DRSP) for all 28 consecutive days of the menstrual cycle. Blood was sampled at days 7, 14, 21, and 28 to assay oestradiol and progesterone.

Results: We developed a new diagnosis of peri-menstrual syndrome, which is characterized by increased DRSP severity in pre and post-menstrual periods and increased scores on the major DRSP dimensions, i.e., depression, physio-somatic symptoms, breast tenderness and appetite, and anxiety. This new diagnosis performed better than classical diagnoses of PMS, including that of the American College of Obstetricians and Gynecologists (ACOG). Lowered steady state levels of progesterone, when averaged over the menstrual cycle, together with declining progesterone levels during the luteal phase predict severity of peri-menstrual symptoms. Steady state levels of oestradiol and declining oestradiol levels during the cycle are also related to DRSP severity although most of these effects appeared to be mediated by progesterone.

Conclusion: A significant increase in menstrual-cycle related symptoms can best be conceptualized as "peri-menstrual syndrome" and may result from insufficient progesterone production (relative corpus luteum insufficiency), which, in part may result from lowered oestradiol production indicating suboptimal pre-ovulatory follicular development.

Keywords: premenstrual syndrome, depression, anxiety, physio-somatic, fatigue, progesterone

INTRODUCTION

Premenstrual syndrome (PMS) comprises affective, behavioral, and physical symptoms appearing during the luteal phase of the menstrual cycle and ameliorating after the onset of menses (Deuster et al., 1999; Dickerson et al., 2003). Symptoms of PMS include fatigue, depression, cramps, bloating, anxiety and breast tenderness. A recent meta-analysis shows that the prevalence of PMS is 47.8% (95% CI: 32.6–62.9), with a lower prevalence in France, i.e., 12% (95% CI: 11–13), and a higher prevalence in Iran, namely 98% (95% CI: 97–100). This burdersome condition is commonly observed in adolescent girls and young women with prevalence rates between 58.1 to 92.3% among university students (Acikgoz et al., 2017; Hussein Shehadeh and Hamdan-Mansour, 2018). PMS is associated with substantial functional impairment comparable to that observed in dysthymia (Kues et al., 2016) and may lead to impaired work productivity (Chawla et al., 2002; Halbreich et al., 2003) and interfere with marital relationships (Frank et al., 1993), family/homemaking functions (Kuczmierczyk et al., 1992), hobbies and social activities (Heinemann et al., 2010), thereby decreasing health-related quality of life (Farrokh-Eslamlou et al., 2015). Furthermore, PMS is also an important predictor of perinatal depression (Studd and Nappi, 2012; Buttner et al., 2013; Roomruangwong et al., 2016; Stoner et al., 2017).

The normal menstrual cycle results from an integrated action of the hypothalamic-pituitary-ovary axis and the uterine endometrium. The hypothalamus releases gonadotropin-releasing hormone (GnRH) every 1–1.5 h during the follicular phase and every 2–4 h during the luteal phase. GnRH activates the pituitary gland thereby increasing levels of luteinizing hormone (LH) and follicle stimulating hormone (FSH). LH stimulates theca cells of the ovarian follicles to produce androstenedione, whereas FSH stimulates the synthesis of aromatase, which catalyzes the conversion of androstenedione into oestradiol (Barbieri, 2014). During the follicular phase, oestradiol promotes the proliferation of the uterine endometrium, while during mid-cycle, higher concentrations of oestradiol cause a positive feedback to the hypothalamus, resulting in an increased in GnRH secretion and a LH surge, which initiates "ovulation." After ovulation, the follicle is transformed into a corpus luteum, a yellow mass of cells which secretes progesterone thereby preparing the endometrium for implantation (so-called a "secretory endometrium") in case fertilization occurs (Barbieri, 2014).

The cyclical nature of PMS and the absence of symptoms among women who underwent bilateral oophorectomy and women during their anovulatory cycles (Rapkin and Winer, 2009; Rapkin and Akopians, 2012) suggest that PMS is associated with reproductive hormones, including progesterone and oestradiol (Seeman, 1996; Case and Reid, 1998; Tan, 2001; Doyle et al., 2007). There is some evidence that progesterone plays a relevant role in the pathophysiology of PMS as symptoms usually appear during the luteal phase when progesterone is produced and released from the corpus luteum, subsequently decreasing in the late luteal phase. For example, a study among 122 healthy, reproductive age women showed that increased progesterone levels in the luteal phase were accompanied by lowered levels of aggression, irritability and fatigue, and additionally that peak progesterone levels in the luteal phase are inversely associated with the same symptoms (Ziomkiewicz et al., 2012). Nevertheless, studies in large study samples failed to demonstrate any efficacy of progesterone in the treatment of PMS (Freeman et al., 1990; Ford et al., 2012). In addition, evidence indicates that PMS-like symptoms may be introduced or re-introduced during cyclical and continuous progesterone treatment (Baker and O'Brien, 2012). Fewer studies, however, have examined possible associations between oestradiol and PMS, although there is some evidence that combined estrogen-progesterone contraception may have some benefits in the treatment of PMS (Freeman et al., 2012; Lopez et al., 2012; Takeda et al., 2015). Such benefits have not been universally demonstrated across studies (Bakhshani et al., 2013). Fluctuations in oestradiol and progesterone levels during the cycle could be more closely associated with the onset of PMS symptoms than their steady-state levels (Schmidt et al., 2017). Nevertheless, it is still unknown whether PMS, severity of PMS and its relevant symptom factors (e.g., depression versus somatic) are associated with steady state levels coupled with changes in sex hormones during the menstrual cycle.

Hence, the aim of this study was to examine associations between steady state levels of progesterone and oestradiol and changes in both hormones during the menstrual cycle and the presence and severity of PMS. We *a priori* hypothesized that lowered steady levels of progesterone and oestradiol coupled with declining levels of these hormones during the menstrual cycle could be associated with the emergence of PMS symptoms.

MATERIALS AND METHODS

Participants

We recruited 41 participants by word of mouth, 21 women without subjective complaints of PMS and 20 women with subjective complaints of PMS. Participants were staff members or

friends and relatives of staff members and women accompanying a patient to the hospital. Inclusion criteria were: 1) women aged 18–45 years; 2) a regular menstrual cycle with cycle length 27–30 days during past year; 3) being able to read and write in Thai; 4) willing to have four blood samples drawn at day 7 (T1), day 14 (T2), day 21 (T3) and day 28 (T4) of the menstrual cycle; and 5) complete the DRPS daily for all consecutive days of the menstrual cycle. Exclusion criteria in both groups were: (1) those with a history of psychiatric illness, including schizophrenia, major depression, bipolar disorder, and obsessive compulsive disorder; (2) those with a history of major medical illness, including diabetes type 1, autoimmune or immune-inflammatory disorders such as inflammatory bowel disease, rheumatoid arthritis, psoriasis and multiple sclerosis; (3) those who are currently pregnant or lactating or using hormonal contraceptive agents; and (4) those who are using any psychotropic medications. The body mass index (BMI) was computed as weight (in kg) divided by height 2 (in meter). The study was approved by the Ethics Committee of the Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand (IRB No.611/60, COA No. 1111/2017). Written informed consent was obtained from all participants prior to the study.

Measures

Clinical Assessments

All participants were requested to complete questionnaires comprising personal information including menstrual history, age, education, height, weight, a history of alcohol or substance use, and diets. The latter lifestyle factor was examined in order to exclude subjects with unusual diets, which could affect the immune system (e.g., anti-inflammatory diets). Participants in both groups were evaluated by the same experienced psychiatrist (CR) before recruitment into the study for their potential diagnosis of PMS and psychiatric and medical exclusion criteria. Severity of PMS was scored using the Daily Record of Severity of Problems (DRSP), which was scored daily during the menstrual cycle by all participants. This scale consists of 21 items + 3 functional impairment items commonly used in the evaluation of PMS (Endicott et al., 2006). The DRSP is a self-report test that scores the "presence" and "severity" of premenstrual symptoms and that can be used to make the DSM-IV diagnosis of premenstrual dysphoric disorder (PMDD) (Biggs and Demuth, 2011).

Table 1 shows two different diagnoses of PMS used in the present study. Firstly, we used the American College of Obstetricians and Gynecologists (ACOG) diagnostic criteria for PMS (American College of Obstetricians and Gynecologists, 2014). The ACOG criteria include one or more affective and physical symptoms during 5 days prior to menses in three menstrual cycles and these symptoms must "be relieved within 4 days after onset of menses without recurrence until at least day 13 of the cycle" (American College of Obstetricians and Gynecologists, 2014). Moreover, the subject must experience identifiable dysfunctions in social, academic, or work performance. The diagnosis of PMS was also made when the total DRSP score $\geq$ 70 on day −5 to −1 of menses and when there was a difference of at least 30% between premenstrual (day −5 to −1) and postmenstrual (day 6–10) scores (Endicott et al., 2006; Biggs and Demuth, 2011; Qiao et al., 2012).

The DRSP and plasma hormone levels were measured at four different time points, namely day 7 (T1), day 14 (T2), day 21 (T3) and day 28 (T4) during the menstrual cycle in order to analyze changes in DRSP and sex hormone levels during the menstrual cycle. A normal menstrual cycle generally ranges between 26–35 days with a mean duration of 28 days. T1 DRSP values represent measurements of the mid-follicular phase when estrogen levels are rising. T2 represents mid-cycle values when there is a decline in estrogen levels and ovulation occurs. T3 values represent the mid-luteal phase when progesterone levels reach their peak values. T4 represents the end of the cycle when all hormones levels decline to their nadir (Owen, 1975; Mihm et al., 2011; Messinis et al., 2014).

Assays

Fasting blood was sampled at 8.00 a.m. to assay plasma oestradiol and progesterone using an immunoassay for the quantitative determination of oestradiol and progesterone using Cobas® 601. The methods to measure both sex hormones are described at great length in our previous published work (Roomruangwong et al., 2019a,b). The intra-assay CV value is 1.2% for oestradiol and 2.3% for progesterone.

Statistical Analysis

Analysis of contingency tables (χ^2 test) was used to check associations between categorical variables, while analysis of variance (ANOVAs) was used to check differences in continuous variables between diagnostic groups. Generalized estimating equation (GEE) analysis, repeated measures, was used to check the effects of time, diagnosis and the interaction time X diagnosis on the sex hormone levels, while adjusting for age, age menarche, length cycle and duration of menses. GEE analyses, repeated measurements, were also used to examine the associations between the DRSP values over time (T1, T2, T3, and T4) and steady state hormone levels (an average value of the sex hormones over the cycle) and changes in hormonal levels from T1 to T4. In addition, we used a distributed lag model to predict the DRPS values over time (dependent variable) by the current and lagged (1 week) values of the sex hormones. We also introduce the Δ hormone values in the analysis, i.e., current value – lagged value 1 week earlier (Δprog_lag, thus denoting the changes in progesterone levels 1 week before blood sampling). Moreover, we also used Generalized Linear Model (GLM) analysis, repeated measurements, and computed effect sizes for time, time X diagnosis and diagnosis. Binary logistic regression analysis was used to delineate the most important predictors of the diagnosis (dependent variables) using the sex hormone levels as explanatory variables. Factor analysis was used to examine the factor structure of the DRSP data. The factorability of the factor analysis was assessed using the KMO index, while we also computed Bartlett's test of sphericity. The number of factors was based on the number of factors with eigenvalues > 1. We performed equamax

TABLE 1 | Definition of four different diagnoses used in the current study to diagnose "premenstrual" syndrome.

Diagnostic Label	Abbreviation	Definition
Premenstrual syndrome (American college of obstetricians and gynecologists)	ACOG	Subjects report 1 or more of the following affective and somatic symptoms at day −5 before menses in each of 3 prior menstrual cycles Affective: Depression, Angry outbursts, Irritability, Anxiety, Confusion, Social withdrawal Somatic: Breast tenderness, Abdominal bloating, Headache, Swelling of extremities Symptoms relieved within 4 days after menses onset without recurrence until at least cycle day 13. Symptoms present in absence of any pharmacologic therapy, hormone ingestion, or drug or alcohol use. Symptoms occur reproducibly during two cycles of prospective recording Subjects suffer from identifiable dysfunction in social or economic performance
Premenstrual syndrome	PMS	PMS: subjects who scored ≥70 on the total Daily Record of Severity of Problems (DRSP) score during day 24–28 of menstrual cycle, and in addition there is a difference of at least 30% in DRSP scores between pre (late luteal phase day 24–28) and post (mid follicular day 6–10) menstrual phases
Peri-menstrual syndrome	PeriMS	Sum DRSP day 1 + day 2 + day 24 to 28 ≥ 307 (0.666 percentile value)
Menstrual cycle associated symptoms	MCAS	Sum of all DRSP scores from day 1 to day 28 ≥ 1,050 (0.666 percentile value)

rotation of the relevant factors in order to interpret the factors and loadings ≥ 0.5 were considered to be significant. Tests were 2-tailed and a p-value of 0.05 was considered for statistical significance. All statistical analyses were performed using IBM SPSS windows version 25. The number of subjects was computed *a priori* (G*Power 3.1.7): using a power of 0.8, effect size $f = 0.22$, two study groups and four measurements with a $r = 0.4$ correlation in a repeated measurement design (within–between interaction) showed that the total sample size should be 36. Therefore, we included 41 subjects divided into two groups.

RESULTS

Different PMS Diagnoses

Table 1 lists the four PMS diagnoses used in the present study, two of these PMS diagnoses were already described in the section "Materials and Methods." Based on the inspection of the daily values of the patients we decided to construct two new diagnoses, namely a first reflecting increased DRSP ratings in the peri-menstrual period (named: "PeriMS") and a second showing increased ratings all over the menstrual cycle (named "menstrual cycle associated symptoms" or MCAS). The PeriMS index was computed as sum of all daily DRSP values at days 1, 2, 24, 25, 26, 27, and 28 ≥ 307, that is the 0.666 percentile of the distribution of the DRSP sums. The MCAS was computed as sum of all DRSP scores from day 1 to day 28 ≥ 1050, that is the 0.666 percentile of the DRSP sum distribution.

Comparisons of the Effects of the Four Diagnoses on the DRSP Time Series

In order to evaluate the validity of the four diagnoses used in the current study, we have used GEE analysis with the time series of the total DRSP scores as dependent variables. Important predictors were the time effects (effects of time all over 28 days) and especially the diagnosis (four different diagnosis) X time interaction, namely the differences in the time series in participants with and without one of the four diagnoses. A greater impact of time X diagnosis is an important feature of a valid diagnosis, while also greater inter-group differences are important especially for the MCAS diagnosis. In addition, we have computed partial eta squared values using GLM repeated measurements analyses for time, time X diagnosis and group effects.

Table 2 shows the results of those GEE analyses. The ACOG diagnosis yielded significant time (partial eta squared $\eta^2 = 0.256$) and less significant time X diagnosis ($\eta^2 = 0.094$) effects, while the inter-group differences were not significant ($\eta^2 = 0.052$). The PMS diagnosis yielded a significant time effect ($\eta^2 = 0.282$), a significant time X diagnosis effects ($\eta^2 = 0.112$) and showed a lower impact of inter-group differences ($\eta^2 = 0.150$). The diagnosis PeriMS yielded significant time ($\eta^2 = 0.297$) and time x diagnosis ($\eta^2 = 0.132$) effects, while there were also highly significant inter-group differences ($\eta^2 = 0.689$). The diagnosis MCAS yielded significant time ($\eta^2 = 0.312$) and time x diagnosis ($\eta^2 = 0.103$) effects, while the inter-group differences were highly important ($\eta^2 = 0.825$).

Prediction of the Diagnostic Categories Using Hormonal Levels

Table 3 shows the results of binary regression analysis with the diagnosis as dependent variable and hormonal levels as explanatory variables. We also entered age, duration of menses, age menarche and length of the cycle, but these variables did not reach significance and therefore we omitted these extraneous variables from the final models shown in **Table 3**. We entered separately the four oestradiol values and the four progesterone data at T1, T2, T3, and T4 and based on these findings we

TABLE 2 | Results of GEE analysis with the daily total scores on the Daily Record of Severity of Problems (DRSP) as dependent variables and different PMS group, time and time X group interaction as independent variables.

Group variables	Time			Group			Time X diagnosis			Daily mean (SE)	
	$X2$	df	P	X^2	df	p	X^2	df	p	No	Yes
ACOG	362.77	27	<0.001	2.58	1	0.108	187.64	27	<0.001	31.37 (1.77)	34.87 (1.45)
PMS	605.89	27	<0.001	5.33	1	0.021	103.95	27	<0.001	30.60 (1.37)	36.32 (1.64)
PeriMS	822.05	27	<0.001	81.05	1	< 0.001	288.63	27	<0.001	27.72 (0.44)	40.00 (1.30)
MCAS	732.76	27	<0.001	180.56	1	< 0.001	285.67	27	<0.001	28.58 (0.54)	42.71 (0.95)

ACOG = Premenstrual syndrome diagnosis according to American College of Obstetricians and Gynecologists (ACOG) criteria. PMS = Subjects who scored ≥70 of total DRSP score during day 24–28 of menstrual cycle and there is a difference of at least 30% in DRSP scores between pre (late luteal phase day 24–28) and post (mid follicular day 6–10) menstrual phases. PeriMS = peri-menstrual syndrome. MCAS = menstrual cycle-associated symptoms.

TABLE 3 | Results of binary logistic regression analysis with different diagnoses as dependent variables.

Dichotomies	Exploratory variables	X^2	Nagelkerke	Wald	df	p	OR	95% CI
ACOG	–	–	–	–	–	–	–	–
PMS	Prog T1−(T2 + T3 + T4)	9.30	0.277	6.08	1	0.002	3.44	1.29–9.16
PeriMS	Prog T2 + T3 + T4	8.36	0.252	5.69	1	0.004	0.32	0.13–0.82
PeriMS	Oest T2 + T3 + T4	5.66	0.176	4.78	1	0.017	0.68	0.49–0.96
MCAS	Prog T2 + T3 + T4	9.44	0.289	6.55	1	0.002	0.31	0.13–0.76
MCAS	Oest T1 + T2 + T3 + T4	5.57	0.179	4.49	1	0.018	0.70	0.50–0.97

ACOG = Premenstrual syndrome diagnosis according to American College of Obstetricians and Gynecologists (ACOG) criteria. PMS = Subjects who scored ≥70 of total DRSP score during day 24–28 of menstrual cycle and there is a difference of at least 30% in DRSP scores between pre (late luteal phase day 24–28) and post (mid follicular day 6–10) menstrual phases. PeriMS = peri-menstrual syndrome. MCAS = menstrual cycle-associated symptoms. OR: Odd's ratio, 95% CI: 95% confidence intervals. Prog: progesterone; Oest: oestradiol. T1−(T2 + T3 + T4): computed as a z unit weighted composite score of zT1 − z(zT2 + zT3 + zT4). T1 + T2 + T3 + T4: sum of the z scores of the four time points. T2 + T3 + T4: sum of the z scores of the three time points in the luteal phase.

constructed new z unit weighted composite scores, namely (a) sum of z scores of T2 progesterone (z T2 progesterone) + z T3 progesterone + z T4 progesterone (Prog T2 + T3 + T4); and (b) z T1 progesterone – z(Prog T2 + T3 + T4) Prog T1 – (T2 + T3 + T4). For oestradiol we found that two composite scores were useful, namely (a) sum of all z scores of the four oestradiol measurements (Oest T1 + T2 + T3 + T4) and (b) sum of the *z* scores at T2, T3, and T4 (Oest T2 + T3 + T4).

Table 3 shows that the diagnosis according to ACOG criteria was not significantly predicted by any of the hormone levels or composite scores. PMS was significantly associated with the Prog T1 – (T2 + T3 + T4) score, but not with oestradiol levels. PeriMS was significantly predicted by Prog T2 + T3 + T4 and by Oest T2 + T3 + T4. The impact of the progesteron *z* score (Nagelkerke = 0.252) was greater than that of the oestradiol score (Nagelkerke = 0.176), and there were no cumulative effects of progesterone and oestradiol predicting PeriMS. We also found that MCAS was significantly predicted by Prog T2 + T3 + T4 or Oest T1 + T2 + T3 + T4.

Features of PeriPMS

Table 4 shows the features of PeriMS versus no PeriMS. Thus, there were no significant differences in age, family income, age at menarche, length of the cycle, duration of menses, education, gave birth yes or no, and BMI between both study groups (results of ANOVAs or X^2 tests). GEE analysis showed that the total DRSP score (sum of all 28 days) was significantly higher in subject with PeriMS than in those without (effect size: 0.689). The increases in the DRSP score in the pre- and post-menstrual weeks were significantly higher in women with than without PeriMS. Moreover, the impact of PeriMS on the DRSP ratings in the premenstrual week (η^2 = 0.441) were more important than in the postmenstrual week (η^2 = 0.217). The mean DRSP values averaged over T1, T2, T3, and T4 was significantly greater in women with PeriMS than in those without. This table shows also the measurements of oestradiol and progesterone at the four time points. We found that the levels of oestradiol at T2 and T3 and progesterone at T3 were significantly lower in women with PeriMS than in those without PeriMS.

Results of Principal Component Analysis

Table 5 shows the results of PCA performed on the items of the DRPS in order to detect meaningful latent constructs that consequently could be used as severity indices of the underlying constructs. The analysis was performed on the 40 participants including the four time points (thus 160 cases). One item (worthlessness) did not load significantly on the PCs and showed less variation and therefore this item was removed from the final analysis. The factorability of the analysis was adequate (KMO = 0.888) and Bartlett's test of sphericity was adequate (X^2 = 3545.91, df = 253, $p < 0.001$). There were four factors with eigenvalues > 1 and explaining 73.11% of the variance. **Table 2** shows the equamax rotated PCs: the first rotated PC explained 20.14% of the variance and loaded highly on

TABLE 4 | Measurement of Daily Record of Severity of Problems (DRSP) total score and subscales and demographic data in subjects with and without peri-menstrual syndrome (PeriMS).

Variables	No PeriMS	PeriMS	F/X^2	df	*p*	Partial eta squared
Age (years)	31.0 (6.6)	31.5 (7.7)	0.05	1/39	0.831	–
Family income (baht)	105.250 (104.245)	64.263 (42.911)	2.53	1/39	0.120	–
Age menarche (years)	12.7 (1.2)	12.9 (1.3)	0.30	1/39	0.588	–
Length cycle (days)	27.7 (2.4)	27.4 (5.7)	0.09	1/39	0.763	–
Duration menses (days)	4.4 (1.3)	5.0 (1.5)	2.17	1/39	0.149	–
Education (years)	16.0 (0.8)	15.9 (1.4)	0.05	1/39	0.818	–
Body mass index (kg/m2)	21.7 (3.5)	22.9 (3.8)	1.08	1/39	0.305	–
Children No/Yes	19/3	13/6	$\Psi = 0.216$	–	0.166	–
DRSP (28 days)	774.8 (58.0)	1119.6 (162.6)	86.53	1/39	<0.001	0.689
DRSP premenstrual week	132.6 (18.2)	252.2 (99.3)	30.82	1/39	<0.001	0.441
DRSP postmenstrual week	133.1 (19.9)	175.7 (56.9)	10.83	1/39	<0.001	0.217
DRSP (T1,T2,T3,T4)	27.0 (1.3)	38.3 (1.9)	35.56	1/157	<0.001	0.185
Oestradiol T1 (pmole/L)	266.1 (195.5)	315.3 (356.7)	0.019	1/38	0.892	–
Oestradial T2 (pmole/L)	723.9 (528.2)	476.5 (399.2)	4.50	1/38	0.040	–
Oestradiol T3 (pmole/L)	669.3 (260.1)	518.9 (292.7)	4.62	1/38	0.038	–
Oestradiol T4 (pmole/L)	308.9 (183.9)	252.1 (143.0)	0.85	1/38	0.361	–
Progesterone T1 (nmole/L)	0.51 (0.28)	0.56 (0.39)	1.45	1/38	0.236	–
Progesterone T2 (nmole/L)	4.33 (7.82)	2.68 (3.39)	0.26	1/38	0.614	
Progesterone T3 (nmole/L)	39.72 (23.43)	29.05 (30.16)	6.63	1/38	0.014	
Progesterone T4 (nmole/L)	13.71 (13.49)	8.95 (12.32)	2.54	1/38	0.120	
DRSP depression score	9.7 (0.5)	14.3 (0.6)	34.37	1/157	<0.001	0.180
DRSP physio-somatic score	6.8 (0.4)	10.1 (0.4)	36.77	1/157	<0.001	0.190
DSRP eating-breast score	5.0 (0.3)	6.6 (0.3)	13.42	1/157	<0.001	0.079
DRSP anxiety score	5.6 (0.3)	7.9 (0.3)	27.81	1/157	<0.001	0.150

All results are shown as mean (SD); All hormonal data were processed in Ln transformation.

depression, mood swings, sensitive to rejection, angry-irritable, more conflicts, less interest, out of control, and interference with hobbies and relationships. Therefore, we named this PC the "depressive dimension." The second rotated PC explained 18.02% of the variance and loaded highly on concentration disturbances, lethargy, sleepiness, headache, muscle/joint pain and lowered productivity, and therefore we named this PC the "physio-somatic dimension." The third rotated PC explained 17.83% of the variance and loaded highly on appetite and craving and breast tenderness and swelling, and therefore was named the "eating & breast PC." The fourth rotated PC explained 17.11% of the variance and scored highly on hopelessness, anxious, lethargy, insomnia, being overwhelmed, and muscle-joint pain and was therefore named the "anxiety PC." Consequently, we have computed the scores of the four different dimensions by adding up the symptoms belonging to the PCs and as such these sums reflect severity of the four underlying constructs of the DRSP.

Measurements and Predictions of the Four DRSP Dimensions

Table 4 shows the measurements of these four dimensions in subjects with and without PeriMS. Thus, PeriMS was characterized by significantly higher scores of the four dimensions, with a strong impact on the physio-somatic ($\eta^2 = 0.190$), depressive ($\eta^2 = 0.180$) and anxiety ($\eta^2 = 0.150$) dimensions and a lower effect size on the eating & breast dimension ($\eta^2 = 0.079$).

Table 6 shows the results of GEE analyses, which examined the effects of oestradiol and progesterone time series as well as the hormone composite scores on the time series of the DSRP and its four dimensions. These analyses were performed in all 40 patients considering the four repeated measurements of the DPRS and hormones (denoted as T1→T4) or the composite scores of three or four time points (thus one fixed variable per subject). We found that the total DRSP score was significantly predicted by Prog (T1→T4) (inversely), but not oestradiol (T1→T4) or other variables. The sum DRPS (T1→T4) was also significantly predicted by the cumulative effects of changes over time in oestradiol (T1→T4) (inversely) and Prog T1−(T2 + T3 + T4) (positively). The same combination of variables also predicted the severity of the depressive and physio-somatic dimensions. The sum of the DRSP items and the depression and physio-somatic symptoms was also significantly predicted by the lagged progesterone (but not oestradiol) values. The total DRSP and depression scores were significantly predicted by progesterone T2 + T3 + T4 coupled with and Δprog_lag. The four repeated measurements of the anxiety and the eating-breast dimension were best predicted by Prog T2 + T3 + T4 (inversely) and Prog_lag, although the latter was also predicted by Oest T1 + T2 + T3 + T4 coupled with Pro_lag. The changes over time in progesterone (T1→T4) were

TABLE 5 | Results of factor analysis (equamax rotation) performed on the items of the Daily Record of Severity of Problems (DRSP) rating scale during 28 days of the menstrual cycle.

	Component			
	1	2	3	4
Depression	**0.563**	0.294	0.203	0.488
Hopelessness	0.333	−0.032	0.220	**0.746**
Anxious	0.469	0.234	0.267	**0.578**
Mood swings	**0.717**	0.278	0.158	0.371
Sensitive to rejection	**0.524**	0.291	0.374	0.485
Angry-irritability	**0.719**	0.336	0.222	0.240
More conflicts	**0.785**	0.236	0.223	0.159
Less interest	**0.577**	0.391	0.401	0.182
Concentration	0.307	**0.799**	0.008	0.173
Lethargy	0.122	**0.601**	0.209	**0.629**
Appetite	0.117	0.466	**0.619**	0.316
Craving	0.132	0.313	**0.716**	0.317
Sleepiness	0.215	**0.593**	0.363	0.313
Insomnia	0.066	0.072	0.154	**0.831**
Overwhelmed	0.478	0.364	0.262	**0.522**
Out of control	**0.613**	0.185	0.488	0.290
Breast tenderness	0.252	0.094	**0.772**	0.187
Breast swelling	0.206	0.156	**0.838**	0.183
Headache	0.240	**0.663**	0.349	0.060
Muscle/joint pain	0.096	**0.514**	0.207	**0.630**
Productivity	0.405	**0.705**	0.415	0.082
Hobbies	**0.538**	0.496	0.490	0.199
Relationships	**0.557**	0.448	0.489	0.177

Extraction Method: Principal Component Analysis; Rotation Method: Equamax with Kaiser Normalization. Significant loadings are shown in bold (≥0.5). depression DRSP = Felt depressed, sad, "down," or "blue" (item 1a). hopelessness DRSP = Felt hopeless (item 1b). Anxious = Felt anxious, tense, "keyed up" or "on edge" (item 2). Swing = Had mood swings (e.g., suddenly felt sad or tearful) (item 3a). Sensitive = Was more sensitive to rejection or my feelings were easily hurt (item 3b). Angry = Felt angry, irritable (item 4a). Conflict = Had conflicts or problems with people (item 4b). less interest = Had less interest in usual activities (e.g., work, school, friends, hobbies) (item 5). concentration = Had difficulty concentrating (item 6). Lethargy = Felt lethargic, tired, fatigued, or had a lack of energy (item 7). Appetite = Had increased appetite or overate (item 8a). Craving = Had cravings for specific foods (item 8b). Sleep = Slept more, took naps, found it hard to get up when intended (item 9a). Insomnia = Had trouble getting to sleep or staying asleep (item 9b). Overwhelm = Felt overwhelmed or that I could not cope (item 10a). out of control = Felt out of control (item 10b). tenderness = Had breast tenderness (item 11a). Swelling = Had breast swelling, felt "bloated", or had weight gain (item 11b). Headache = Had headache (item 11c). muscle/joint pain = Had joint or muscle pain (item 11d). productivity = At work, at school, at home, or in daily routine, at least one of the problems noted above caused reduction of productivity or inefficiency. Hobbies = At least one of the problems noted above interfered with hobbies or social activities (e.g., avoid or do less). Relationships = At least one of the problems noted above interfered with relationships with others.

predicted by oestradiol T1 + T2 + T3 + T4, Δoestradiol_lag and a time factor.

DISCUSSION

The first major finding of the current study is that lowered steady state levels of sex hormones, mainly progesterone and to a lesser degree also oestradiol, when averaged over the menstrual cycle, predicted the presence of PMS as well as its severity. Previous studies that assessed associations between progesterone levels and the onset of PMS have yielded controversial results. For example, in women with PMDD peripheral levels of progesterone or its active metabolite allopregnanolone during the luteal phase were found to be either decreased (Wang et al., 1996; Rapkin et al., 1997; Ziomkiewicz et al., 2012) or increased (Backström et al., 1983; Hammarbäck et al., 1989; Girdler et al., 2001) across different studies, whilst some other studies found no significant changes in these hormones (Rubinow et al., 1988; Hsiao et al., 2004).

Discrepancies in some of the above-mentioned case-control studies and the current study may be explained by our findings that the severity of PMS symptoms (which as a scale variable is a more sensitive score of PMS symptoms than a categorical PMS diagnosis) is predicted by steady state levels of progesterone combined with changes over time in progesterone levels in a distributed lag model (with current and lagged values of the sex hormones). For example, changes in DRSP scores from the start of the cycle until the end of the luteal phase were significantly associated with lowered steady levels of progesterone combined with lagged changes in progesterone levels, indicating that when plasma levels of progesterone decline in the luteal phase (the week prior to DRSP measurements) severity of PMS is worse. These findings contrast with previous results that found the onset of PMDD to be associated with fluctuations in oestradiol and progesterone levels during the menstrual cycle, but not with their steady-state levels (Schmidt et al., 2017). Moreover, blocking the conversion of progesterone to its metabolite using the 5α-reductase inhibitor dutasteride mitigates symptoms of PMDD (Martinez et al., 2016). A recent study, which examined salivary progesterone, found that in women with PMS, progesterone levels declined rapidly 3 days prior to menstruation whereas mid-cycle progesterone concentrations were similar to those of asymptomatic participants (Lovick et al., 2017). Similar results were also reported by Andréen et al. (2006) who reported that participants who developed PMS symptoms had an increased severity score during the period when progesterone was stable and then further increased when progesterone levels declined rapidly by the end of the cycle. Animal studies also demonstrate reproducible depressive-like behaviors during various progesterone withdrawal protocols (Li et al., 2012).

Moreover, we found that lowered steady state levels of progesterone averaged over the cycle coupled with changes over time in oestradiol levels showed that the latter had a significant effect on DRSP scores, although this effect disappeared when progesterone changes over time were taken into account. This indicates that putative detrimental effects of oestradiol could at least in part be mediated (statistically) by progesterone levels. These results suggest that PMS is related to lower progesterone concentrations during the second half of the menstrual cycle, which is described as "corpus luteum insufficiency," and is considered to result from suboptimal pre-ovulatory follicular development (Dawood, 1994; Hinney et al., 1996). Deficient progesterone production is a condition that may lead to

TABLE 6 | Results of GEE analysis (repeated measurement) with the Daily Record of Severity of Problems (DRSP) total score and subscale scores as dependent variables.

Dependent variables	Exploratory variables	*B*	SE	Wald X^2	df	*p*
Sum DRSP (T1→T4)	Oestradiol (T1→T4)	0.022	0.025	0.80	1	0.370
	Progesterone (T1→T4)	−0.076	0.023	10.79	1	0.001
Sum DRSP (T1→T4)	Oestradiol (T1→T4)	−0.059	0.022	6.92	1	0.009
	Progesterone T1−(T2 + T3 + T4)	0.082	0.023	12.80	1	<0.001
Sum DRSP (T1→T4)	Prog_lag	0.084	0.039	4.76	1	0.029
Sum DRSP (T1→T4)	ΔProg_lag	−0.075	0.025	9.34	1	0.002
Sum DRSP (T1→T4)	Progesteron T2 + T3 + T4	−0.116	0.020	35.11	1	<0.001
	Prog_lag	0.118	0.037	10.49	1	0.001
Sum DRSP (T1→T4)	Progesteron T2 + T3 + T4	−0.081	0.023	12.52	1	<0.001
	ΔProg_lag	−0.074	0.025	8.87	1	0.003
Depression (T1→T4)	Oestradiol (T1→T4)	−0.059	0.021	7.74	1	0.005
	Progesterone T1−(T2 + T3 + T4)	0.096	0.022	20.01	1	<0.001
Depression (T1→T4)	Progesterone T1−(T2 + T3 + T4)	1.423	0.368	14.96	1	<0.001
	ΔProg_lag	−0.048	0.019	6.05	1	0.014
Physio-somatic (T1→T4)	Oestradiol (T1→T4)	−0.061	0.030	4.02	1	0.045
	Progesterone T1−(T2 + T3 + T4)	0.076	0.030	6.25	1	0.012
Physio-somatic (T1→T4)	Progesterone T2 + T3 + T4	−0.971	0.299	10.50	1	0.001
	Prog_lag	1.410	0.574	6.03	1	0.014
Anxiety (T1→T4)	Progesterone T2 + T3 + T4	−0.979	0.365	7.22	1	0.007
	Prog_lag	0.929	0.404	5.28	1	0.022
Eating-& Breast (T1→T4)	Progesterone T2 + T3 + T4	−0.517	0.131	15.50	1	<0.001
	Prog_lag	1.09	0.423	6.60	1	0.010
Eating-& Breast (T1→T4)	Oestradiol (T1 + T2 + T3 + T4)	−0.211	0.072	8.54	1	0.003
	Prog_lag	1.023	0.420	5.92	1	0.015
Progesterone (T1→T4)	Oestradial (T1 + T2 + T3 + T4)	0.167	0.040	17.74	1	<0.001
	Δoestradiol_lag	−0.383	0.101	14.26	1	<0.001
	Time	1.478	0.354	187.79	1	<0.001

T1→T4: denotes the repeated measurements over four time points. T1−(T2 + T3 + T4): computed as a z unit weighted composite score. T1 + T2 + T3 + T4: sum of the z scores of the four time points. T2 + T3 + T4: sum of the z scores of the three time points in the luteal phase. Prog_log: lagged progesterone values (1 week lag). ΔProg_lag and Δoestradiol_lag: delta values of current hormonal values – lagged hormonal values (1 week lag).

inadequate maintenance of a regular secretory endometrium and, therefore, may be associated with recurrent pregnancy loss and infertility, although up to 10% of fertile women also show corpus luteum insufficiency (Sonntag and Ludwig, 2012). This condition may be due to a functional inadequacy of the hypothalamic-pituitary secretion of gonadotrophins, or may otherwise occur in patients suffering from the polycystic ovary syndrome (PCOS) (Barthelmess and Naz, 2014). Whereas the former may be related to external factors, including exposure to environmental xeno-oestrogens, the latter is commonly associated with insulin resistance and metabolic disturbance.

The second major finding of this study is that the diagnostic criteria used to establish a diagnosis of PMS may determine to a large extent outcomes of biomarker studies. In this respect, we found that the diagnoses PMS, PeriMS and MCAS were externally validated by levels of sex hormones, whereas ACOG-based diagnosis of PMS was not associated with peripheral levels of sex hormones. Furthermore, the ACOG diagnosis performed less robustly in GEE analyses examining the effects of time and diagnosis on DRSP scores. It is interesting to note that both psychiatrists (PMS diagnosis) and gynecologists (ACOG) have developed overlapping but distinct sets of criteria for PMS. It seems clear, however, that a diagnosis of PMS based on ACOG criteria may not reflect the severity of PMS symptoms premenstrually, but merely screens a few symptoms premenstrually. In addition, we found that the diagnosis PMS was only predicted by steady state levels of progesterone, while the PeriMS and MCAS diagnoses were significantly related to both sex hormones. Furthermore, the PMS diagnosis may be less adequate because all DRSP scores during the cycle are significantly intercorrelated and thus using a 30% difference between the premenstrual and postmenstrual phases may fail to identify some "true" PMS cases, namely those who have high scores all over the menstrual cycle in addition to PMS.

Therefore, we have developed two new diagnoses based on DRSP scores during the cycle: (a) the peri-menstrual syndrome (PeriMS), which considered total DRSP scores on day 1 + day 2 + day 24 to 28 with a cut-off score at the 0.666 percentile to dichotomize the peri-menstrual DRSP score; and (b) menstrual-cycle associated symptoms (MCAS, using a cut-off score at the 0.666 percentile), which delineates a group of subjects with increased DRSP levels during the cycle. Our results show that changes in sex hormones during the menstrual

cycle and lowered steady state levels of these hormones determine increased peri-menstrual symptoms and increased ratings during the cycle, rather than "premenstrual" symptoms.

The third major finding of this study is that using factor analysis we were able to detect four interpretable factors in the DRSP data set, namely (a) a depressive dimension; (b) a physio-somatic component (with symptoms reminiscent of chronic fatigue); (c) increased appetite and craving combined with breast tenderness and swelling; and (d) an anxiety dimension. A previous factor analysis study also yielded four factors, namely (a) mood symptoms (depressed/sad/blue, mood swings, angry/irritability, anxious/tension/on edge, overwhelmed, sensitive to rejection, worthless/guilty, out of control, hopeless, conflicts/problems, less interest and trouble sleeping); (b) behavioral symptoms (lethargy/tired/fatigue, difficulty concentrating, sleepiness, craving specific foods, and increased appetite); (c) pain symptoms domain (two items including headache and joint/muscle pain); and (d) physical symptoms (four items including breast tenderness and breast swelling/bloating) (Wu et al., 2013). Thus, both factor analysis studies suggest the presence of at least three different dimensions, namely an affective, a behavioral (or physio-somatic) and a breast swelling dimension. Most importantly, we found that the peri-menstrual syndrome was characterized by increased scores on all four dimensions and that changes during the cycle in severity of those dimensions were significantly associated with steady state levels and (lagged) changes in sex hormones, mainly progesterone. This suggests that the four symptom dimesions measured with the DRSP are in part mediated by sex hormones.

An important question is how these sex hormones could exert their effect on the different DRSP dimensions. Progesterone receptors can be found throughout human brain including the caudate, hippocampus, hypothalamus and limbic system (Maggi and Perez, 1985). The limbic system, which modulates emotion and behavior, is influenced by circulating progesterone. For example, progesterone metabolites have antagonistic properties at GABA-A receptors and increase the metabolism and turnover of monoamines in the brain, which may lead to negative mood, including anxiety and depression (Panay and Studd, 1997). Changes in progesterone or its metabolites may induce GABA-A receptor dysfunctions that may increase susceptibility to develop PMS (Timby et al., 2016) and changes in estrogens or progesterone during the luteal phase may cause changes in dopamine receptors sensitivity (Wieck et al., 2003; Czoty et al., 2009; Seeman, 2012). Moreover, estrogen may impact depressive symptoms. For example, women with severe PMS show clinical improvements when cycles were absent during pregnancy to recur after birth as postnatal depression when estrogen levels fall (Studd, 2015). Previous functional magnetic resonance imaging studies during different menstrual phases showed effects of estrogen in attenuating arousal pathways in women and modulating the stress response (Goldstein et al., 2005, 2010).

Estrogen may inhibit food intake, whereas progesterone stimulates appetite (Hirschberg, 2012). Interestingly, some studies found low mean food intake during the mid-cycle of the menstrual cycle when estradiol levels are high, whereas the peak food intake occurs during the premenstrual period when progesterone levels are high (Buffenstein et al., 1995; Reed et al., 2008). Therefore, lower levels of estrogen in the luteal phase could explain increased appetite and craving in individuals with PMS. In addition, many physical symptoms associated with progesterone, including edema, weight gain, bloating, and breast tenderness, may be related to its mineralocorticoid-like effects, which enhance the renin–aldosterone cascade (Oelkers et al., 1974). Therefore, progesterone may compete for the mineralocorticoid receptor, leading to fluid and sodium retention during the luteal phase (Panay and Studd, 1997).

The classic-school allopathic approach to treatment of patients suffering from PMS recommends suppressing ovulation by a combined oral contraceptive. However, this approach does commonly not relief the symptoms, which is part is related to the type of progestagen used, with drospirenone possibly being preferable (Nevatte et al., 2013). For example, in a subgroup of patients, hormonal contraceptive pills, despite of suppressing the ovulation, may increase PMS-like symptoms (e.g., irritability, depression, anxiety, bloating, fatigue, and breast tenderness) (Oinonen and Mazmanian, 2002). Overall, progesterone treatment studies did not reveal an efficacy of progesterone to treat PMS or PMDD (Freeman et al., 1990; Ford et al., 2012). In addition, more than half of women who started taking hormonal contraceptives discontinue these drugs within the first year due to side effects including PMS-like symptoms (Berenson et al., 1997; Rosenberg and Waugh, 1998; Doyle et al., 2007). Other studies showed that cyclical and continuous progestogen treatment may induce PMS-like symptoms (Baker and O'Brien, 2012). Women with PMS may experience more PMS-like symptoms after administration of a gonadotropin-releasing hormone analog (GnRHa) followed by exogenous estrogen or progesterone administration (Schmidt et al., 1998).

Future research on the treatment of PMS should trial Clomiphene citrate (given the first 5 days of the cycle) and a mid-cycle injection of human Chorionic Gonadotrophin in subjects with peri-menstrual syndrome. Clomiphene citrate is an anti-oestrogen with complementary intrinsic oestrogenic activity, which is the treatment of choice for suboptimal follicular development. This medication should be given during the first 5 days of the cycle, and may be combined with a mid-cycle injection of human Chorionic Gonadotrophin (hCG), which increases endogenous progesterone secretion during the luteal phase. Nevertheless, some patients may experience adverse effects of Clomiphene and may prefer to combine injections of human Menopausal Gonadotrophin (hMG) on days 8 and 12 of the cycle, with a mid-cycle hCG injection. Anecdotal case studies using this approach have been published, but well-designed clinical trials are lacking so far. Hyperinsulinism and metabolic disturbance due to insulin resistance in PCOS patients can be treated with Metformin, but the Ayurvedic plant extract of *Momordica charantia* (bitter gourd) may be preferable because of its more favorable toxicological profile (Comhaire, 2014).

The results of the current study should be interpreted within its limitations. First, we enrolled a small sample ($n = 41$), although the power of the GEE, repeated measurements, analyses was adequate (> 0.8). Second, it would have been more interesting if we had sampled both sex hormones on a daily basis to be able to

perform group spectral analyses to examine associations between the cycles in DRSP ratings and hormones in the different study groups (Maes et al., 1995).

CONCLUSION

In conclusion, the cumulative effects of lowered steady state levels of progesterone in the luteal phase combined with (lagged) changes in progesterone in the luteal phase predict total DRSP scores as well as its four main dimensions (namely depression, physio-somatic symptoms, breast tenderness and appetite, anxiety) and, therefore, the diagnosis of peri-menstrual syndrome. Classical diagnoses of PMS are less adequate, whereas two new diagnoses developed in the current study are externally validated by the biomarkers, namely (a) a diagnosis of peri-menstrual syndrome denoting individuals with increased symptoms in the pre and post-menstrual period; and (b) a diagnosis of menstrual cycle-associated symptoms (MCAS) denoting subjects who experience increased DRSP symptoms all over the cycle. Therefore, future research should examine the associations of biomarkers with those two diagnoses and with changes over time in the DRSP (and its four dimensions), which provides more information on the steady state (increased scores all over the cycle) and cyclical nature (peri-menstrual) of the syndromes.

DATA AVAILABILITY STATEMENT

The datasets generated for this study will be made publicly available once the data set has been fully exploited by the authors. Requests to access the datasets should be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of the Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand (IRB No.611/60, COA No. 1111/2017). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

CR and MM designed the study. CR recruited and screened the participants. MM performed the statistical analyses. AC and FC contributed in a meaningful way to the intellectual content of this manuscript. All authors agreed upon the final version of the manuscript.

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Preventive Effects of Escitalopram Against Anxiety-Like Depressive Behaviors in Monosodium Glutamate-Teated Rats Subjected to Partial Hepatectomy

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Zhao B-B, Chen L-L, Long Q-H, Xie G-J, Xu B, Li Z-F, Wang P and Li H (2019) Preventive Effects of Escitalopram Against Anxiety-Like Depressive Behaviors in Monosodium Glutamate-Teated Rats Subjected to Partial Hepatectomy. Front. Psychol. 10:2462. doi: 10.3389/fpsyg.2019.02462

The reasons for the relationship between depression and chronic liver disease (CLD) are complex and multifactorial. Further research is needed to decipher the etiology and establish an optimal management approach for depression in patients, including the potential role of non-pharmacological treatments. monosodium glutamate (MSG)-treated rats are more likely to develop anxiogenic- and depressive-like behaviors, which could be related to the dysfunction of serotonergic system. In this study, partial hepatectomy (PH) was performed in MSG-treated rats and the histopathological changes were observed in orbitofrontal cortex (OFC) and liver. The effect of escitalopram, a widely used antidepressant, on neural and liver injury in this model was also examined. The MSG + PH-treated rats displayed decreased distances traveled in total, in center arena, and in the left side of arena in inner open field test (OFT), as compared to saline, saline + PH, and MSG-treated animals. The present study established that PH aggravated anxiety-like depressive behaviors in MSG-treated rats, concordant with damaged Nissl bodies (and neurites), decreased IBA-1 and Sox-2 expression in OFC and neurotransmitter disorder. Escitalopram treatment could alleviate these pathological changes as well as reduce hepatic steatosis and lipid metabolism.

Keywords: partial hepatectomy, MSG + PH-treated rats, orbitofrontal cortex, anxiety-like depressive behaviors, Nissl body and neurites, hepatic steatosis, neurotransmitters

INTRODUCTION

In recent years, mental health conditions, especially depression, have been recognized as a complication of chronic liver disease (CLD). The incidence of depression in patients with CLD is higher than that in general population. Such mental disorders may reduce life quality of patients, worsen clinical outcomes, reduce compliance and increase mortality of liver disease treatment (Popovic et al., 2015). Approximately 10–20% of hepatitis B virus (HBV) carriers become chronically infected, which can lead to fibrosis and even cirrhosis. Given the high prevalence of HBV infection in China and the high relapse rate of viral hepatitis, CLD patients typically experience panic, depression, and anxiety. Furthermore, participants with comorbid major

depression and liver disease showed higher rates of lifetime suicide attempts (33.2%) than general population [13.7%; odds ratio (OR): 3.1; confidence interval (CI): 1.3–7.6] (Le Strat et al., 2015). A meta-analysis also found that psychological distress was associated with liver disease mortality (Russ et al., 2015). Psychological factors that increase vulnerability to the temptation to use alternative medicines, such as herbs and plant preparations, have been revealed to be important for understanding toxic liver injury (Suh et al., 2013). In the presence of hepatic encephalopathy (HE) in patient with depressive symptoms, HE-directed therapies should be attempted before antidepressant drugs (Mullish et al., 2014; Telles-Correia et al., 2015). Nevertheless, the reasons for the relationship between depression and CLD are complex and multifactorial. Further research is needed to decipher the etiology and establish an optimal management approach for depression in these patients, including the potential role of non-pharmacological treatments.

The monosodium glutamate (MSG)-rat model is a well-characterized animal obesity model that is used to study many metabolic syndromes, such as lipid metabolism and insulin resistance (Dolnikoff et al., 2001; Caetano et al., 2017). Our earlier studies established a compromised liver regeneration model by applying the partial hepatectomy (PH) operation in MSG-treated rats. After undergoing PH, the MSG-induced animals displayed disorders of the neuroendocrine-immune network (NEIN) (Dolnikoff et al., 2001; Caetano et al., 2017) as well as liver injury (Li, 2014). The numbers of hypothalamic neurons were significantly reduced and increased apoptosis was observed. In addition, liver regeneration was suppressed in the MSG-rats after PH as compared to saline-treated rats. Recently, it was shown that MSG-treated rats are more likely to develop anxiogenic- and depressive-like behaviors, which could be related to the dysfunction of serotonergic system (Quines et al., 2014, 2015). Magnetic resonance imaging (MRI) studies revealed that increased functional connectivity of the lateral orbitofrontal cortex (OFC) Brodmann area 47/12 is related to depression. It is reported that changes in cortical connectivity within the OFC are critical to depression (Gao et al., 2015). In the present study, we performed PH in MSG-treated rats and observed the histopathological changes in OFC and liver. The effect of escitalopram, a widely used antidepressant, on neural and liver injury in this model was also examined.

MATERIALS AND METHODS

Reagents

Dopamine (DA), 5-hydroxytryptamine (5-HT), norepinephrine (NE), acetylcholine (Ach), epinephrine (Epi), glutamate (Glu), gamma-aminobutyric acid (GABA), 5-hydroxyindoleacetic acid (5HIAA), and 3,4-dihydroxybenzylamine hydrobromide (IS) were purchased from Sigma-Aldrich (St. Louis, MO, United States). Escitalopram was obtained from Forest Pharmaceuticals, Inc. (New York, NY, United States). Escitalopram was prepared as a 0.105 mg/ml stock solution in saline.

Establishment of MSG + PH Rat Model and Escitalopram Administration

Neonatal male Wistar rats were purchased from the Hubei Experiment Animal Research Center. Twenty-four rats were given subcutaneous injection of the MSG solution (in normal saline) at a dosage of 4 mg/g body weight (bw), and 16 rats were injected with the same volume of vehicle (saline) on days 2, 4, 6, 8, and 10 after birth. The pups were weaned on day 8 and caged at 6 weeks old in five groups, receiving saline or escitalopram via gastrogavage for 2 weeks. PH was performed during week 8 by excision of the left and median hepatic lobes (occupying about 68% of whole liver) according to the Solt-Farber method under ether anesthesia (Zhao et al., 2015). The groups (n = 8/group) received the following treatments: (1) saline daily via gastrogavage; (2) saline + PH; (3) MSG injection + saline; (4) MSG + PH; and (5) MSG +PH + escitalopram gastrogavage to the endpoint of the experiment (1 ml/100 g bw of the 0.105 mg/ml stock solution daily, corresponding to 10 mg/60 kg bw daily in humans). The rats were maintained in an air-conditioned (temperature 24 ± 1°C; 55 ± 5% relative humidity) animal room with controlled lighting (12 h light, 12 h darkness). They were provided with a commercial diet and water. On day 8 after PH, the rats were subjected to an open field test (OFT) before being sacrificed by CO_2 as phyxiation followed by cervical dislocation. The blood, liver, and OFC tissues were collected and then snap frozen or fixed in formalin. All animal handling and procedures were approved by the Institute of Animal Care and Use Committee of the Hubei University of Traditional Chinese Medicine.

Open Field Test

The inner OFT was conducted in an open arena (l, w, h: 50 cm × 50 cm × 40 cm) with the bottom and sides made of Plexiglas covered with black, nonreflecting material. The arena was placed in a quiet room. On day 8 after PH, the rats were individually placed in the center of the apparatus and left to move freely during a 3 min period with their movements being automatically recorded using a camera connected to a computer (ZH-ZFT, Zhenghua Biological Instrument Equipment Co., Ltd., Huaibei, China). The total distance moved (cm) and total distance traveled (cm) from the center area were analyzed.

Histological and Immunohistochemical Staining

After dewaxing and hydration of paraffin sections, the liver and cortex tissues were stained with hematoxylin and eosin (H&E). The sections of frozen liver tissue were stained with Oil Red O (ORO). Nissl staining was performed on the OFC samples for Nissl body observation. The OFC was also stained with anti-brain-derived neurotrophic factor (BDNF, sc-33904), anti-Sox-2 (sc-365964), and anti-ionized calcium-binding adapter molecule 1 (IBA-1, sc-32725; Santa Cruz Biotechnology, Santa Cruz, CA, United States). Five fields on each slide were randomly selected, viewed under a fluorescence microscope (Nikon TE2000-U, Nikon, Japan), and analyzed using Image Pro-Plus 6.0 software (Media Cybernetics, Silver Spring, MD,

United States). The minimal pixel number was set at 50 pixels. The average and cumulative optical density values, average area, and average diameter were analyzed. StreptAvidin Biotin Complex kits were purchased from Boster Biological Technology Co., Ltd. (Wuhan, China).

Lipid Metabolism Analysis

The serum levels of total cholesterol (TC), triglycerides (TG), low-density lipoprotein cholesterol (LDL), and high-density lipoprotein cholesterol (HDL)were measured using commercially available enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's instructions (Yuanye Bio-Technology Co., Ltd., Shanghai, China).

Neurotransmitter Levels

Neurotransmitter levels in the OFC were determined by a previously reported method with minor modification (Huang et al., 2014). Briefly, about 40 mg of OFC tissue was homogenized in a 1.5 ml Eppendorf tube after addition of 400 μl ice cold methanol (0.1% formic acid) and 10 μl IS (10 μg/ml, methanol). The homogenate was vortex-mixed for 1 min and then centrifuged at 18,000 × *g* for 10 min at 4°C. The supernatant was transferred and evaporated to dryness under a nitrogen stream. The dry residue was reconstituted in 100 μl of initial mobile phase (0.1% formic acidin water/acetonitrile, 98:2, v/v), and a 10-μl aliquot was injected into the liquid chromatography-tandem mass spectrometry (LC-MS/MS) system for analysis. The sample was run on an Agilent 1290 Infinity series connected to an Agilent 6420 triple quadrupole mass spectrometer equipped with an electrospray ionization (ESI) ion source (Agilent Technologies, Santa Clara, CA, United States). The analytes were separated on a Waters BEH C_{18} column (2.1 mm × 100 mm, 1.7 μm, Waters, Milford, MA, United States) at 30°C. The mobile phase consisting of 0.1% formic acid in water (Solvent A) and acetonitrile (Solvent B) was used with a gradient elution: 0–4 min, 2% B; 6 min, 80% B; 8–10 min, 90% B at a flow rate of 0.3 mL/min. The ESI-MS/MS conditions were set as following: gas temperature 350°C, gas flow 10 L/min, capillary 4000 V, and nebulizer pressure 30 psi. MS acquisition of NE, 5-HT, DA, Glu, GABA, Ach, Epi, and 5-HIAA spectra was performed in electrospray positive ionization multiple reaction monitoring (MRM) mode.

Statistical Analysis

Data are expressed as mean ± standard deviation (SD). Statistical analyses were performed using SPSS software (version 19.0; IBM, Armonk, NY, United States). Inter-group comparison was performed by one-way analysis of variance (ANOVA). $P < 0.05$ was considered statistically significant.

RESULTS

Anxiety-Like Depressive Behaviors in Model Rats

We first examined the effect of PH and escitalopram on the anxiety-like behaviors of neonatal rats. As shown in **Figure 1**, the rats treated with saline and no PH traveled a total distance of 2014.56 ± 441.06 cm in 3 min in the OFT experiments. The distances traveled in the center and the left side of open arena were 814.93 ± 150.30 and 180.46 ± 87.24 cm, respectively. The saline + PH-treated animals traveled a total distance of 1908.78 ± 330.96 cm, 712.74 ± 154.19 cm in the center, and 182.65 ± 82.59 cm

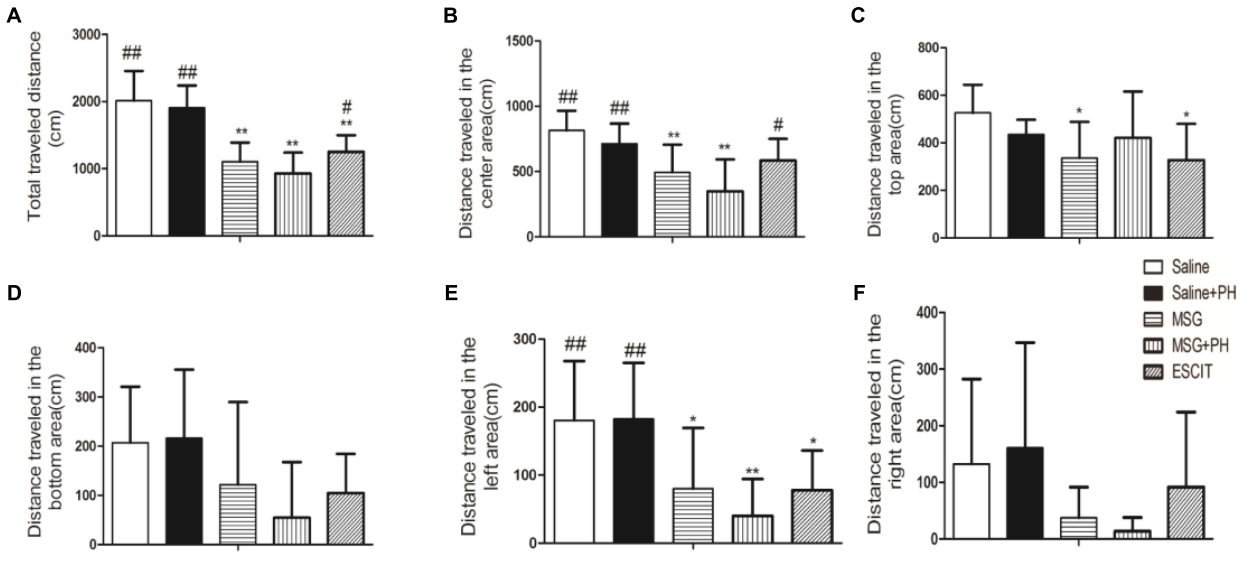

FIGURE 1 | Effect of escitalopram on the anxiety-like depressive behaviors in MSG + PH-treated neonatal rats. **(A)** Total traveled distance in the open arena; **(B)** distance traveled in the center area of the open arena; **(C)** distance traveled in the top area; **(D)** distance traveled in the bottom area; **(E)** distance traveled in the left area; and **(F)** distance traveled in the right area; $^{*}P < 0.05$, compared to the saline group; $^{**}P < 0.01$, compared to the saline group. $^{\#}P < 0.05$, compared to the MSG + PH group; $^{\#\#}P < 0.01$, compared to the MSG + PH group. $n = 8$ per group.

on the left side ($P > 0.05$ vs. saline-treated rats). The MSG-treated rats without PH traveled a total distance of 1103.14 ± 285.99 cm, 494.83 ± 210.80 cm in the center, and 80.05 ± 89.23 cm on the left, and these distances were significantly shorter than those traveled by saline-treated animals without PH ($P < 0.01$ or $P < 0.05$), suggesting that MSG treatment induced anxiety-like depressive behaviors of neonatal rats. In addition, the MSG-induced rats with PH showed even further decreases (but not significantly) in the total distance (932.05 ± 306.65 cm) as well as the distance traveled in the center of the arena (349.83 ± 243.47 cm) and on the left (40.23 ± 51.11 cm) compared to MSG-treated rats without PH (but all $P < 0.01$ vs. the saline group). These results confirmed the presence of anxiety-like depressive behaviors in MSG + PH-treated animals. The administration of escitalopram to MSG + PH-treated animals resulted insignificantly increased distances traveled in total (1252.42 ± 245.52 cm; $P < 0.05$), in the center (584.57 ± 165.68 cm; $P < 0.05$), and on the left side (77.93 ± 58.15 cm; $P > 0.05$) compared to those of the MSG + PH-treated animals. Therefore, we successfully established a liver regenerative model in MSG-treated neonatal rats with anxiety-like depressive behaviors, and escitalopram could partially alleviate the anxiety-like depressive behaviors in this model.

Histopathological Analyses of OFC

Representative H&E staining of the OFC is shown in **Figure 2**. The neurons of the saline-treated rats had an integral morphology and structure with normal synapses. Saline-treated rats subjected to PH had relatively integral neurons, yet some of the neurons exhibited shrunken soma and reduced synapses. In contrast, the neurons in the OFC of the MSG-treated animals were largely damaged, exhibiting a reduced size in the neuronal soma and disappearance of the synapses. These effects were aggravated in the MSG + PH-treated rats. Escitalopram administration partially rescued the cortical neurons, as evidenced by an increased volume of synapses and recovery of neuron morphology.

We next sought to investigate the protective effect of escitalopram on the cortical Nissl bodies and neurite outgrowth (**Figure 3**). As shown in **Figure 3A**, the saline-treated animals showed integral Nissl bodies in the OFC with long and thick neurites. The mean area was 869.82 ± 261.16, the mean diameter of Nissl bodies was 30.26 ± 3.31, and the IOD of stained tissue was 16018.16 ± 4968.46. PH decreased the volume of Nissl bodies (mean area 691.26 ± 90.89; IOD 12467.89 ± 3811.55; $P > 0.05$). The neurons exhibited abnormal morphology, including shortening and thinning of the neurites (**Figures 3A–C**). This was also significant in the OFC of MSG-treated rats with even weaker Nissl staining and the disappearance of some synapses. There were no significant differences between the MSG group and the saline group. In contrast, MSG + PH-treated animals showed significant differences in these parameters as compared to MSG-treated rats, indicating that PH significantly aggravated the neural injury in MSG-treated rats. PH in MSG-treated rats led to significantly decreased staining of the neurons (**Figure 3**) and the disappearance of almost all synapses, with a mean area of 586.59 ± 125.74, mean diameter of the Nissl bodies of 25.41 ± 2.66, and IOD of 6981.17 ± 2667.62. In contrast, we observed that administration of escitalopram largely protected the integrity of the Nissl bodies and the morphology of neurites (mean area of 1080.89 ± 35.22, mean diameter of the Nissl bodies of 32.45 ± 0.62, and IOD of 15849.69 ± 3466.60, all $^{*}P < 0.01$ compared to the MSG + PH group). These results demonstrated that escitalopram could attenuate the neural injury in this model.

IHC of OFC

We next examined the expression of IBA-1 (microglia marker), Sox-2 [neural stem cell (NSC) marker], and neurotrophic factors. As shown in **Figure 4**, the IOD for IBA-1 in the saline + PH group and MSG group were 3073.63 ± 912.00 and 4820.61 ± 2582.22, respectively, and these values were significantly higher than that in the saline group (1208.58 ± 1348.46; $P < 0.01$ or $P < 0.05$). The MSG + PH-treated rats displayed lower IBA-1 expression (1196.84 ± 418.61; $P < 0.01$) than the two groups that received only one individual treatment. The IOD for Sox-2 in the MSG group was 31666.59 ± 17705.65, which was significantly higher than that in the saline group (4812.43 ± 2985.14; $P < 0.01$). The MSG + PH-treated rats displayed lower expression of Sox-2 (3263.81 ± 2654.46; $P < 0.01$) than those treated with MSG only (31666.59 ± 17705.65, $P < 0.01$). There was no significant difference in BDNF expression among all the groups related to the saline-treated control group. Escitalopram treatment enhanced Sox-2 expression (24128.59 ± 34003.34; $P < 0.01$) compared to that in the MSG + PH group. These results demonstrated that MSG and PH led to decreased expression of microglial and NSC markers, but escitalopram treatment could partially restore the expression levels of these proteins.

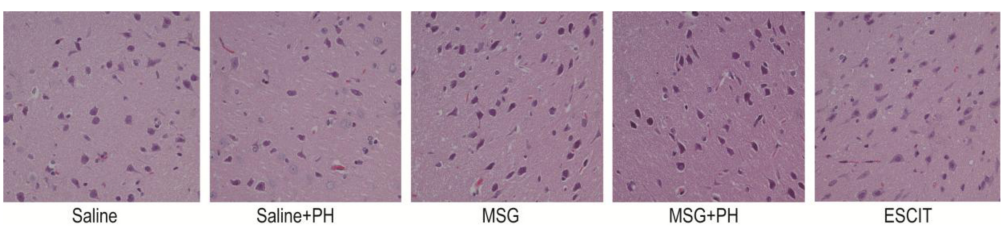

FIGURE 2 | Effect of escitalopram on OFC histological features in MSG + PH-treated neonatal rats. Sections were stained using H&E. Magnification, 400×.

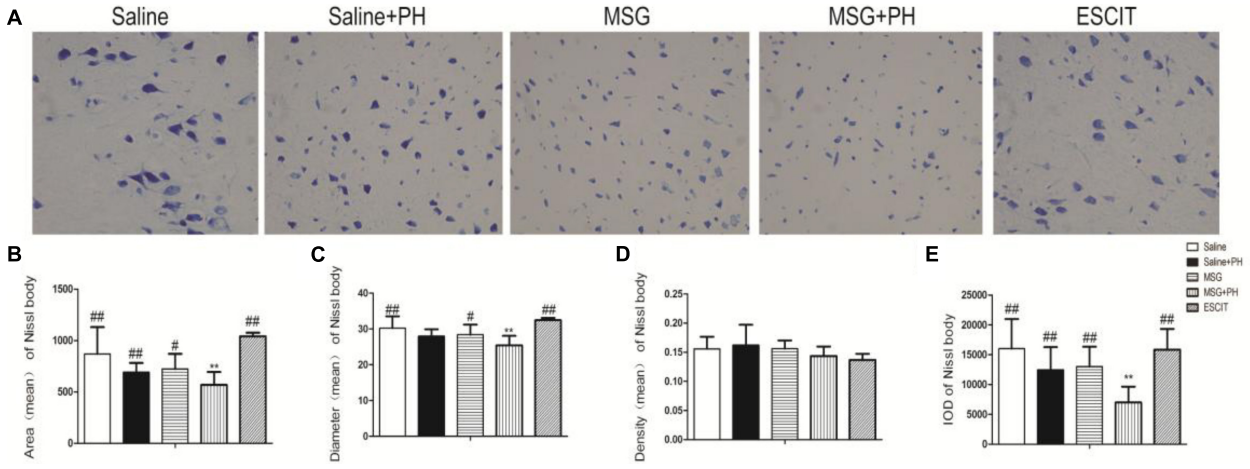

FIGURE 3 | Effect of escitalopram on the Nissl body morphology and neurite outgrowth in neonatal rats. **(A)** Nissl body staining (400×); **(B)** mean area; **(C)** mean diameter; **(D)** mean density; and **(E)** cumulative IOD. $^{*}P < 0.05$ compared to the saline group; $^{**}P < 0.01$ compared to the saline group. $^{\#}P < 0.05$ compared to the MSG + PH group; $^{\#\#}P < 0.01$ compared to the MSG + PH group. $n = 4\sim8$ per group, and five fields were recorded from each specimen.

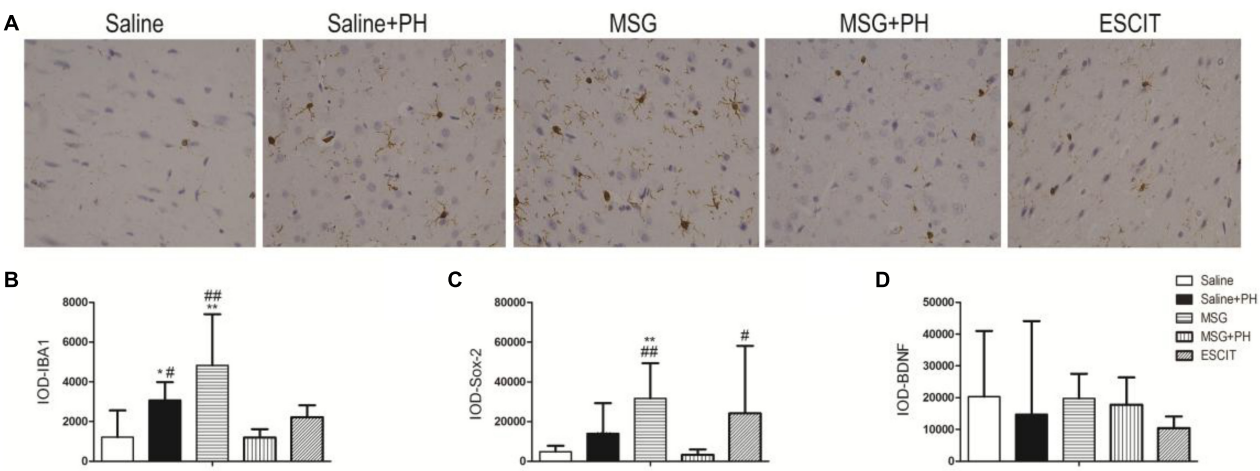

FIGURE 4 | Effect of escitalopram on the expression of markers for microglia and NSC as well as neurotrophic factors in the OFC of neonatal rats by immunohistochemistry. **(A)** OFC sections were stained using anti-IBA-1 antibody (400×). **(B)** IOD for IBA-1; **(C)** IOD for Sox-2; **(D)** IOD for BDNF. $^{*}P < 0.05$ compared to the saline group; $^{**}P < 0.01$ compared to the saline group. $^{\#}P < 0.05$ compared to the MSG + PH group; $^{\#\#}P < 0.01$ compared to the MSG + PH group. $n = 4\sim8$ per group, and five fields of each specimen were recorded.

Liver Histological Analysis

Representative images of liver histology using H&E staining are shown in **Figure 5**. Normal liver histology with typical lobular architecture was observed in saline-treated rats. Eight days after PH, the liver of the neonatal rats exhibited a similar initial architecture with only a few small fat vacuoles. In contrast, the liver lobes in the MSG group displayed diminished borderlines with loosening cytoplasmic structures in the hepatocytes. There were increased numbers of hepatic vacuoles and liver cells, especially those located centrally in the lobuli, which displayed considerable swelling with vacuolization and a balloon-like appearance similar to adipocytes. MSG + PH-treated rats exhibited more disturbances in the structure of the liver, as evidenced by the disappearance of borderlines and more balloon-like hepatocytes and vacuolization. The administration of escitalopram attenuated the changes in liver architecture, reducing vacuolization of the liver, although a few balloon-like hepatocytes were still observed.

The white areas were further analyzed in the H&E-stained slides. The unknown tiny particles were discarded by setting the minimum pixel value to 50 pixels, and the white portal areas were excluded by setting the maximum pixel value to 5000 pixels. As shown in **Figure 5**, the MSG + PH group had a significantly larger white area in the liver architecture, at 94256.37 ± 50510.38, compared with the saline group (43158.82 ± 12323.81; $P < 0.05$) and the saline + PH group (40799.50 ± 9360.42; $P < 0.05$). Escitalopram treatment significantly reduced the white area

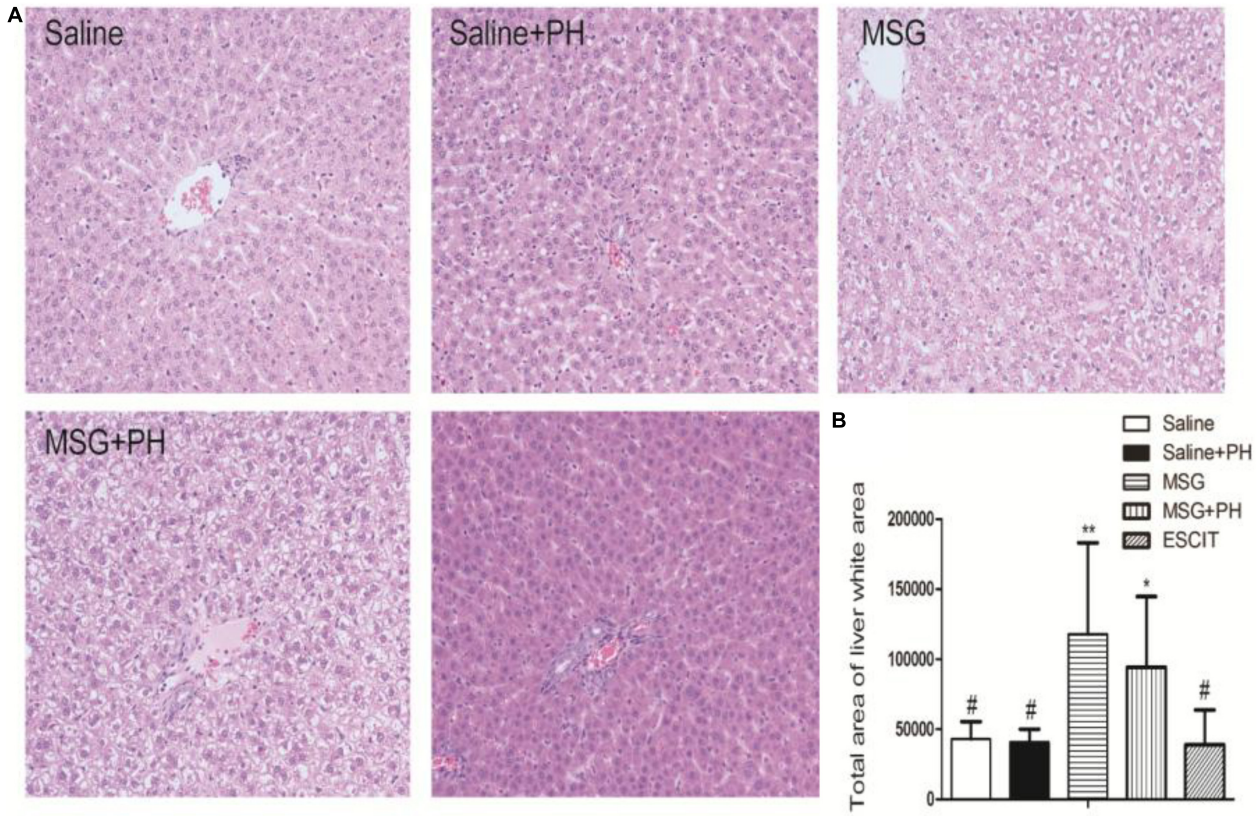

FIGURE 5 | Effect of escitalopram on liver histology in MSG + PH-treated neonatal rats. **(A)** Liver sections were stained using H&E (200×). **(B)** Statistical analysis of the liver white area for different groups of animals. *$P < 0.05$ compared to the saline group; **$P < 0.01$ compared to the saline group. #$P < 0.05$ compared to the MSG + PH group. $n = 8$ per group.

(38972.78 ± 24694.60; $P < 0.05$). These data suggest that the escitalopram was effective at alleviating liver injury and enhancing liver regeneration.

Analysis of Liver Steatosis

Hepatic steatosis was analyzed as follow in different groups. Frozen liver tissues were sectioned and stained with ORO solution. The saline group had an IOD for lipid deposition of 3214.23 ± 4843.85, and the saline + PH group had an IOD of 5559.96 ± 4475.62. The MSG group had an IOD of 26852.50 ± 13472.09. Compared with lipid deposition in the MSG + PH group (IOD of 57169.03 ± 46660.75), escitalopram administration during the recovery period after PH significantly alleviated liver steatosis (38179.23 ± 23306.88; **Figure 6**). These results demonstrated that MSG-treated rats had significant liver steatosis and PH further increased the level of steatosis. However, escitalopram treatment could protect against PH- and MSG-induced hepatic steatosis.

Lipid and Insulin Profiles

The serum lipid and insulin profiles were examined in the different groups. As shown in **Figure 7**, MSG treatment increased the serum levels of TC (6.21 ± 0.57 mmol/L), TG (1.83 ± 0.13 mmol/L), LDL (6.12 ± 0.52 mmol/L), and insulin (34.79 ± 3.09 mU/L) compared to the levels following saline treatment (TC: 5.42 ± 0.36 mmol/L, $P < 0.01$; TG: 56.68 ± 8.21 mmol/L, $P < 0.01$; LDL: 5.51 ± 0.38 mmol/L, $P < 0.01$; insulin: 31.87 ± 1.92 mU/L, $P < 0.01$). MSG + PH-treated rats had decreased serum levels of TC (5.63 ± 0.33 mmol/L; $P < 0.01$), TG (1.66 ± 0.08 mmol/L, $P < 0.05$), LDL (5.57 ± 0.29 mmol/L, $P < 0.05$), and insulin (31.18 ± 1.79 mU/L, $P < 0.01$) than those treated with MSG only. Interestingly, escitalopram treatment further decreased the serum levels of TC (2.63 ± 0.26 mmol/L), TG (0.85 ± 0.07 mmol/L), LDL (2.01 ± 0.28 mmol/L), and insulin (12.60 ± 1.62 mU/L) ($P < 0.05$ or < 0.01). Opposite changes were seen for the serum level of HDL in the saline (56.68 ± 8.21 mg/dl), MSG (45.44 ± 8.75 mg/dl; $P < 0.01$ vs. saline group), MSG + PH (57.14 ± 5.43 mg/dl; $P < 0.05$ vs. MSG group), and escitalopram-treated groups (120.78 ± 8.50 mg/dl; $P < 0.01$ vs. MSG + PH group). Therefore, the MSG-treated animals had abnormal metabolism and PH could aggravate the lipid deposition. In contrast, escitalopram could improve lipid metabolism. Inconsistently, there were lower serum levels of TC, TG, LDL, and insulin in the saline + PH and MSG + PH groups, as compared to the saline and MSG groups, indicative of compromised liver function in rats that received PH prior to complete liver regeneration.

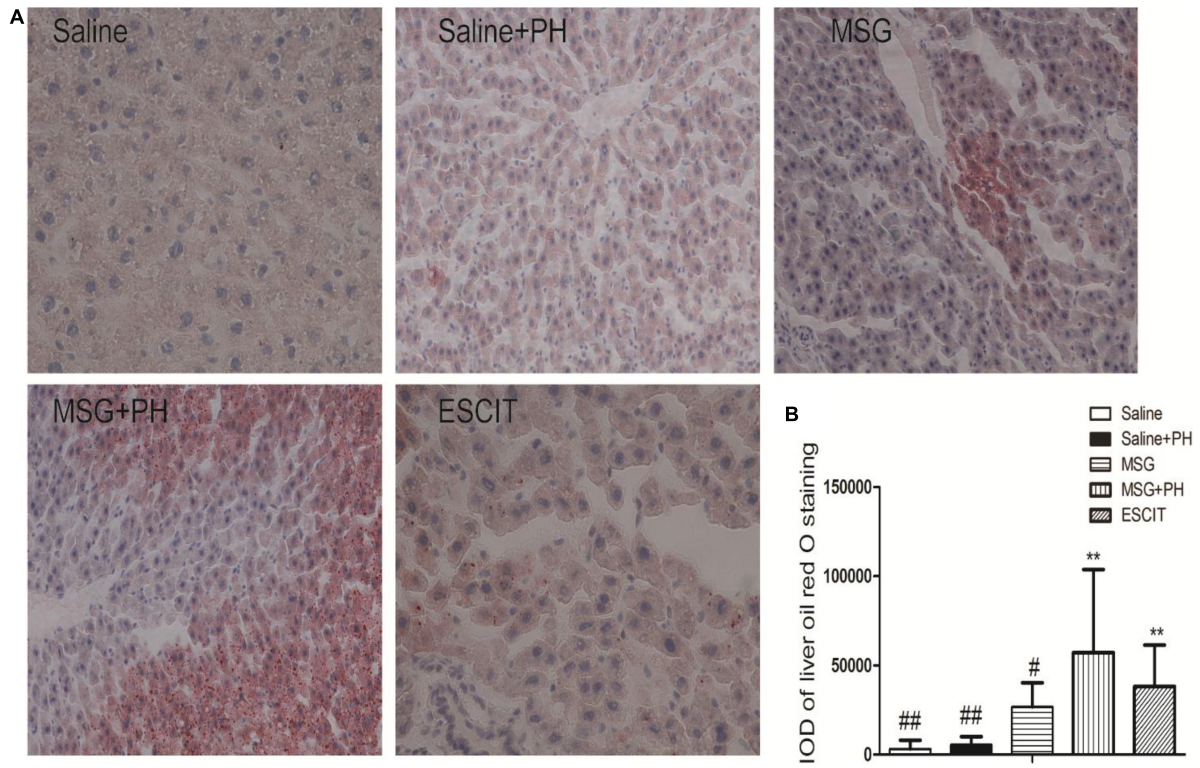

FIGURE 6 | Effect of escitalopram on lipid deposition in the liver of MSG + PH-treated neonatal rats. **(A)** Liver tissues were stained with ORO (400×). **(B)** Statistical analysis of the difference between different groups. $^{**}P < 0.01$ compared to the saline group. $n = 8$ per group. $^{\#}P < 0.05$ compared to the MSG + PH group; $^{\#\#}P < 0.01$ compared to the MSG + PH group. $n = 8$ per group.

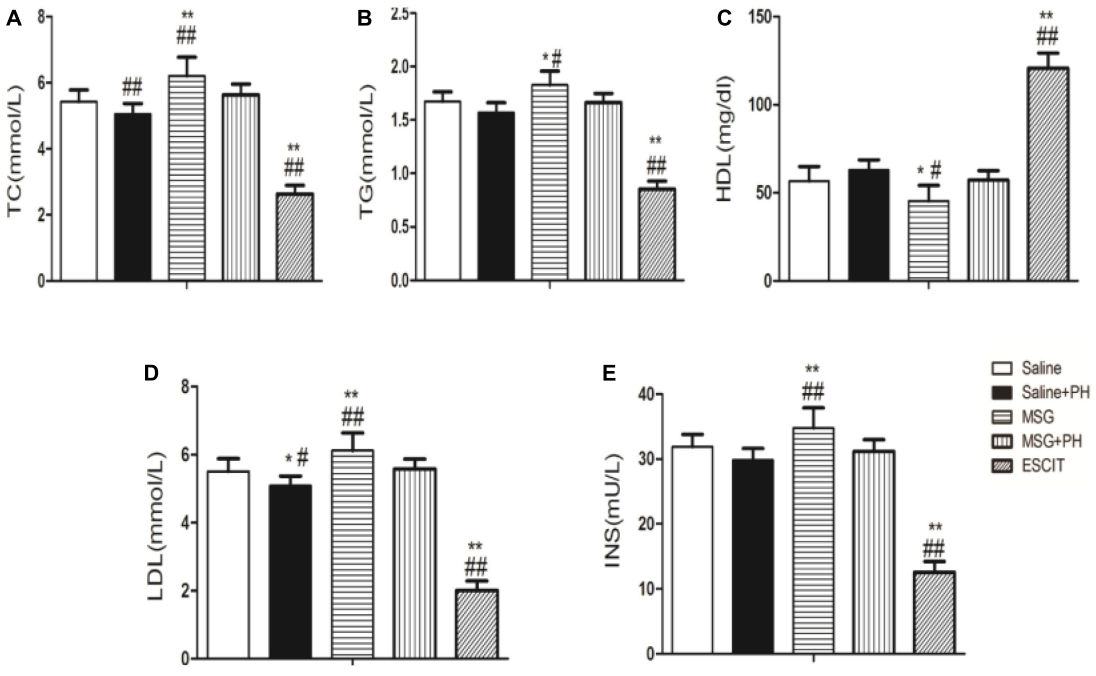

FIGURE 7 | Effect of escitalopram on serum levels of lipids and insulin in MSG + PH-treated neonatal rats. **(A)** TC; **(B)** TG; **(C)** HDL; **(D)** LDL; and **(E)** insulin levels.$^{*}P < 0.05$ compared to the saline group; $^{**}P < 0.01$ compared to the saline group. $n = 8$ per group. $^{\#}P < 0.05$ compared to the MSG + PH group; $^{\#\#}P < 0.01$ compared to the MSG + PH group. $n = 8$ per group.

OFC Neurotransmitter Profile

Lastly, the OFC neurotransmitter profile was investigated in the different treatment groups (**Figure 8**). The Epi level in the OFC of the MSG + PH group was 4.05 ± 0.90 ng/g, which was significantly lower than that in the saline group (5.78 ± 1.94 ng/g; $P < 0.05$). Compared to NE expression in the saline group, escitalopram treatment significantly increased the expression of NE (847.74 ± 191.77 vs. 1094.24 ± 367.91 ng/g; $P < 0.05$). The DA level in the OFC of the MSG + PH group was 2178.28 ± 257.76 ng/g, which was significantly lower than that in the saline group (2517.95 ± 381.10; $P < 0.05$). The GABA levels reduced in both MSG (26.11 ± 4.67 ng/g) and MSG + PH group (25.89 ± 3.77 ng/g), which significantly lower than that in the saline group (30.24 ± 4.95 ng/g). The OFC neurotransmitter profile showed that levels of a variety of neurotransmitters, including DA, GABA, and 5-HT, were decreased in rats treated with MSG and subjected to PH compared to those in MSG-treated animals not subjected to PH. Moreover, these changes could be alleviated by escitalopram treatment.

DISCUSSION

In the present study, we established a compromised liver regeneration model by performing PH in MSG-treated neonatal rats. The animals displayed anxiety-like depressive behaviors after MSG treatment, which was aggravated by PH. Transient lipid accumulation within hepatocytes preceding the peak proliferative phase is a characteristic feature of the regenerating liver (Kohjima et al., 2013). As evidenced by the liver HE and ORO staining, MSG-treated rats exhibited hepatic steatosis, and PH aggravated the degree of steatosis. However, administration of the antidepressant escitalopram could not only alleviate this anxiety-like depressive behaviors, but also reduce hepatic steatosis and improve liver function.

The neurobiological mechanisms of depression are complex, and the relationship between depression and CLD is multifactorial. Some factors, such as life stress and endocrine abnormalities, are thought to cause depression. In addition, genetic and environmental factors play important roles in the onset of depression in relation to epigenetics (Nabeshima and Kim, 2013). It involves signaling cascades within a complicated network, including the monoamine neurotransmitter system, the neuroendocrine system, neurotrophic factors, adult nerve regeneration, the neural immune system, and epigenetic modification. The hypothalamic-pituitary-adrenal axis (HPA) and the production of proinflammatory cytokines have also been implicated in the pathophysiology of depression (Leonard, 2006; Stepanichev et al., 2014; Jeon and Kim, 2016). Our earlier work revealed that, compared to the liver of normal rats subjected to PH, decreased mRNA and protein expression levels of transforming growth factor alpha (TGF-α) and epidermal growth factor receptor (EGFR) were observed in regenerated liver tissues. However, the expression of TGF-β1 and TGF-β type I receptors (TGFBRI and TGFBRII) were upregulated in the arcuate nucleus (ARN) of MSG-induced rats (Li, 2014). These cytokines would contribute to the compromised liver regeneration and disturbance of the NEIN. We have identified that the traditional Chinese medicine (TCM) formulation, Zuo Gui Wan (ZGW), can regulate the NEIN and consequently modulate liver regeneration (Li et al., 2005, 2006). In contrast, ZGW also can significantly increase the expression of TGF-α and EGFR, while decreasing the expression of TGFBRI and TGFBRII (Li et al., 2004; Li, 2014, 2017).

Escitalopram is an antidepressant of the selective serotonin reuptake inhibitor (SSRI) class and is mainly used to treat major depressive disorder or generalized anxiety disorder. Escitalopram was shown to have an antioxidant effect associated with an increase in GABA levels in frontal cortices and nucleus accumbens homogenates from rats exposed to chronic mild stress (Shalaby and Kamal, 2009). Our results revealed that MSG treatment decreased GABA levels and MSG + PH treatment

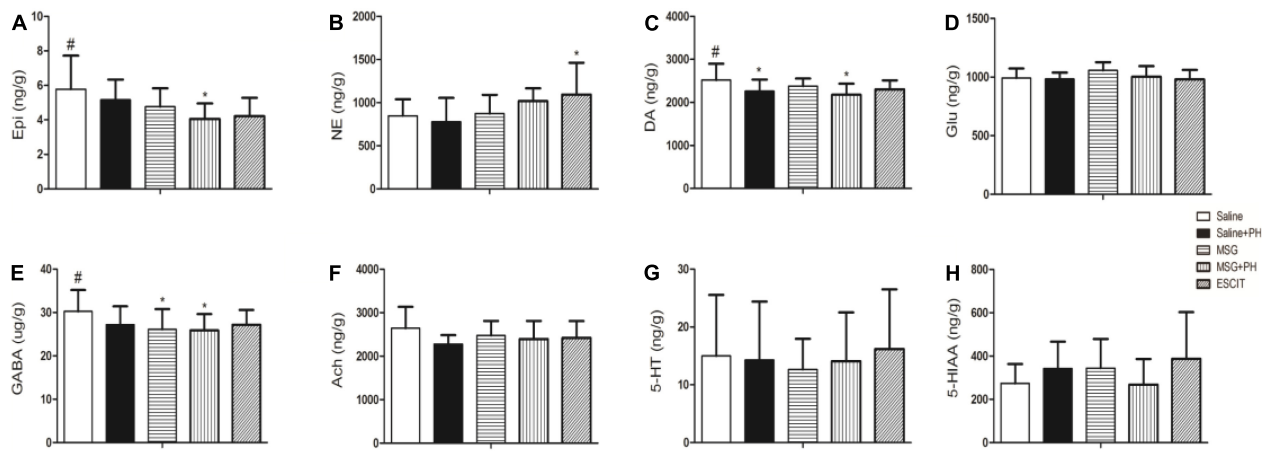

FIGURE 8 | Effect of escitalopram on OFC levels of neurotransmitters in MSG + PH-treated neonatal rats. **(A)** Epi; **(B)** NE; **(C)** DA; **(D)** Glu; **(E)** GABA; **(F)** Ach; **(G)** 5-HT; and **(H)** 5-HIAA levels in the OFC. $^{*}P < 0.05$ compared to the saline group. $^{\#}P < 0.05$ compared to the MSG + PH group. n = 4∼8 per group.

decreased both DA and GABA levels in the OFC of rats. Administration of escitalopram alleviated the disorder of the neurotransmitter profile in the OFC by increasing the level of NE. Escitalopram is also known to reduce inflammation in depression. Stress-related increases in proinflammatory cytokines may underlie the oxidative and nitrosative brain damage and the impairment of the 5-HT system (Maes, 2008). In a rat model of post-cardiac infarct depression, the antidepressant effect of escitalopram was shown to reduce circulating pro-inflammatory cytokines (tumor necrosis factor alpha [TNF-α], interleukin-1β, and prostaglandin E2) and improve depressive behavior without affecting sleep (Bah et al., 2011). A higher level of TNF-α might predict a non-response to treatment with escitalopram (Eller et al., 2008). As the cytokines and neurotransmitters affect neurogenesis in brain regions involved in depression and are functionally interconnected, the alteration of these profiles by MSG and PH might underlie the decreased expression of microglial and NSC markers (Lauterbach, 2016). The present study revealed that escitalopram could partially restore survival and proliferation of NSC while reducing microglial activation.

Escitalopram and its prodrug citalopram are extensively metabolized in the liver, mainly via the cytochrome P450 system (CYP 3A4, 2D6, and 2C19). Genetic polymorphisms of cytochrome P450 enzymes have been shown to influence the metabolism of escitalopram as well as treatment response (Tsai et al., 2010). As such, the maximum daily dose of escitalopram for patients with hepatic insufficiency is 10 mg. In addition, the efficacy of escitalopram in mild or moderate cases of depression has been disputed and may be outweighed by its side effects. To date, no serious liver injury has been reported with the use of escitalopram. We showed that PH led to liver regeneration in MSG-treated rats; however, the regeneration was compromised as evidenced by the greater hepatic steatosis compared with that in saline-treated animals subjected to PH. On the other hand, PH aggravated the histopathological changes in MSG rats. Depression and metabolic syndrome may have a common neuroendocrine and immune basis (Hess et al., 2004; Ghanei Gheshlagh et al., 2016; Song and Kim, 2016; Ohmori et al., 2017). Thus, the incidence of metabolic syndrome in patients with depression is relatively high, and conversely, patients with metabolic syndrome are more likely to suffer from depression (Kahl et al., 2015; Ghanei Gheshlagh et al., 2016). The two diseases are likely to be causal to some extent (Toker et al., 2008; Takeuchi et al., 2009; Foley et al., 2010; Lamers et al., 2013; Trief et al., 2014). We showed that although MSG treatment resulted in abnormal lipid metabolism and PH could aggravate lipid deposition, treatment with the antidepressant escitalopram could improve lipid metabolism.

In summary, our results clearly revealed that subtotal hepatectomy aggravated the anxiety-like depressive behaviors changes in the MSG rat model, and this effect was related to pathological changes in the OFC and disorder of neurotransmitters. The degree of hepatic steatosis was also aggravated by PH. The antidepressant escitalopram could alleviate the pathological changes in both OFC and liver in this model. Nevertheless, because depression is a multifactorial condition and closely affected by social events, further studies are needed to examine the efficacy of antidepressants in CLD patients with depression.

DATA AVAILABILITY STATEMENT

The data used to support the findings of this study are included within the article.

ETHICS STATEMENT

All relevant ethical safeguards have been met in relation to patient or subject protection, or animal experimentation.

AUTHOR CONTRIBUTIONS

B-BZ, PW, and HL designed and conceived the study. B-BZ, L-LC, Q-HL, G-JX, BX, and Z-FL carried out the experiments. BZ, LC, and QL wrote the manuscript.

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Corticotropin-Releasing Factor Receptors in the Locus Coeruleus Modulate the Enhancement of Active Coping Behaviors Induced by Chronic Predator Odor Inoculation in Mice

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Stress inoculation has been proved to induce active coping behaviors to subsequent stress. However, the specific neural mechanisms underlying this effect remain unclear. In this study, a chronic and mild predator odor exposure model was established to investigate the effect of predator odor stress inoculation on behaviors in novel predator odor exposure, open field test and forced swimming test (FST), and on the expression of CRF receptors in locus coeruleus (LC) and dorsal raphe nuclei (DRN). The results showed that predator odor stress inoculation increased the active coping of mice under the severe stress environment without changing the stress response to a new predator odor. Meanwhile, in LC, the CRFR1 expression was increased by predator odor stress inoculation. These results suggested that predator odor stress inoculation can be used as an effective training method to improve active response to later severe stress and the function of CRFR1 in LC might be a potential underlying biological mechanism.

Keywords: stress inoculation, predator odor exposure, active coping, corticotropin-releasing factor receptors, locus coeruleus, dorsal raphe nuclei

INTRODUCTION

Stress is a generalized set of physiological and psychological responses observed when an organism is placed under challenging circumstances (Greenberg et al., 2013; Wang et al., 2018). The adaptive and maladaptive responses to stressor are closely related to psychological health, and the intensity and duration of stress are important factors affecting the risk of mental disorders. Predator stress is a kind of instinctive response for maintaining the reproduction and survival of species (Philbert et al., 2015; St-Cyr and McGowan, 2015). The excessive predator stress has been documented to exert negative effects on behavior, emotion and cognition in the pattern of stress maladaptation (Apfelbach et al., 2005). For example, recent researches find that excessive predator stress may induce long-term anxiety and depressive behavior in rats (Lim et al., 2016; Wah et al., 2019; Wu et al., 2019). However, mild predator stress can lead to active coping behaviors in response to stress.

Previous study has been reported that chronic exposure of mild cat odor enhanced aggression, urinary attractiveness, and sex pheromones of mice (Zhang et al., 2008). To increase the adaptive response to stress, stress inoculation may be an effective way.

Stress inoculation originally refers to mild but not minimal nor severe stress exposure early in life enhances subsequent coping (Lee et al., 2016). Many studies have confirmed the effects of stress inoculation. Abraham and Gruss find that stress experience in early life improves cognitive and emotional processing of stressors later in life in Octodon degus (Abraham and Gruss, 2010). In primate studies, researchers find that exposure to stress in early life reduces subsequent indications of anxiety, increases exploration of novel situations, and decreases cortisol levels after stress (Lyons et al., 2009; Lyons and Parker, 2010). Some human studies have also found that stress experience in childhood and adolescent can effectively improve the ability to cope with similar stressors in adulthood (Khoshaba and Maddi, 1999; Mortimer and Staff, 2004; Forest et al., 2010; Bosnjak et al., 2019; Navaee and Kaykha, 2019). These findings suggest that stress inoculation training may be a good method to enhance the adaptive response to stress, which is of great significance for the prevention and treatment of stress-related mental diseases.

As the core neurohormone to initiate the stress response, corticotropin-releasing factor (CRF) has extensive neural connections with monoamine transmitters system (Henckens et al., 2016; Spierling et al., 2017), which is the most important stress system in brain, and plays an important role in stress response and coping strategies, but how are these systems involved in the process of stress inoculation is not clear. Many investigations indicate that the dorsal raphe nuclei (DRN) 5-HT system is compelling as a target of CRF given the established role of this system in stress coping (Lesch, 2001; Schindler et al., 2012). Two CRF receptor subtypes, CRF-R1 and CRF-R2, are distributed in DRN, these two receptors regulate the active and passive stress coping by a bimodal regulation of DRN-5-HT neuronal activity. Selective blockade CRF-R2, but not CRF-R1, decrease the passive responses induced by inescapable shock and swim stress (Hammack et al., 2003; Valentino et al., 2010). On the contrary, administration of CRF-R1 agonist into the DRN that inhibit the DRN-5-HT system and prevent learned helplessness produced by inescapable shock and swim stress, as well as facilitate an active escape response (Hammack et al., 2003, 2012; Valentino et al., 2010).

The locus coeruleus (LC), an important target of CRF neuronal projection, is critically involved in stress response and stress coping. CRF directly mediates the activation of LC-norepinephrine (NE) system during stress, and the CRF-R1 (only type of CRF receptor in LC) plays an important role in regulation of norepinephrine nervous system (Sánchez et al., 1999). Administration of CRF increases noradrenergic neurons activity and promotes NE release in LC (Curtis et al., 2012; Lavicky and Dunn, 2010; Page and Abercrombie, 2015). In forced swimming test (FST) the passive response is decreased by partial denervation of the LC-NE activity (Häidkind et al., 2003). In addition, antagonism of CRF receptors in the LC region is effective in attenuating immobilization stress induced defensive withdrawal in rats (Smagin et al., 1996). CRF micro-injection into LC increases an adaptive mechanism in an attentional set-shifting task with an inverted U-shaped dose-effect relationship (Snyder et al., 2012). These studies indicate that CFR in LC may also be involved in the regulation of coping strategies in stress.

The present experiments were designed to explore the stress inoculation effect of mild predator stress on the stress coping strategy, and its neurobiological mechanism was also discussed by investigated the expression of CRF receptor in LC and DRN. Firstly, the predator–prey interaction system between rats and mice was used to build an animal model of mild predator stress inoculation. Secondly, we tested the stress responses of animals in a similar predator stress, and whether there was a wider stress inoculation effect in a similar non-predator but threat stress condition. Furthermore, we attempted to explore the neurobiological foundation of predator stress induced stress inoculation effect by investigated the expression characteristics of CRF receptors in LC and DRN.

MATERIALS AND METHODS

Animals

Twenty-six male ICR mice were purchased from Weitong-Lihua Laboratory Animal Company, Beijing, China. They were individually kept in plastic cages (27 cm × 12 cm × 17 cm) under the 14:10 h (light: dark) light regime (light on at 6:00 PM) and at the temperature of 21 ± 0.2°C. Food (standard mouse chow) and water were provided *ad libitum*. They were used for experiments after 4 weeks of acclimation.

At the beginning of the experiment, the mice were randomly assigned to one of two groups, each with 13 individuals. These were housed in two separate rooms for testing the effects of exposure to rat urine and rabbit urine, respectively. The mice of the two groups did not differ in body weight at the beginning of the experiment. All experimental procedures were performed with the approval of the Institutional Review Board of the Institute of Psychology at the Chinese Academy of Sciences and according to the guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication number 80-23).

Urine Collection

The urine was collected as described before (Zhang et al., 2008). Rat urine for use as a predator odor was collected. We collected rabbit (Oryctolagus cuniculus) urine as a non-predator novel odor from four adult males (New Zealand white strain, raised on Rabbit Chow 5326 Laboratory Diet) individually housed in the animal unit under the 12:12 h (light: dark) light regime. Urine was collected by placing each rat or rabbit individually in a clean cage with a grid floor and setting a clean collecting pan (with plastic films) underneath. After the animals had urinated into the collecting pan, the urine was immediately collected into vials and placed in a freezer for storage at –20°C. Potential contaminated urine, such as that deposited with or next to feces, was rejected. We pooled urine samples from conspecific individuals and used these for treating the experimental subjects.

Predator Odor Exposure

To prepare the odor samples to be presented to test subjects, we used a micro-syringe to inject 5 μl rat urine, rabbit urine into a glass capillary (ID 1.5 mm, OD 2.0 mm, length 10 cm), which was then sealed with Bio-seal at one end. The samples stayed inside the micropipette in such a way that test subjects could not come into contact with the urine even if they occasionally touched the capillary. We hung the capillary at the side panel of each feeder, above the lid and just beyond the reach of the mice. The snout of the mouse could come no nearer than 1 cm. We renewed the urine in the capillary daily to keep the odor stimulus fresh for 31 consecutive days as stress inoculation training. After that, a set of behavioral tests were performed as follows.

Behavior Tests

Novel Odor Exposure

The novel predator odor exposure was designed to test whether early predator odor exposure (rat urine) induced habituation to the predator odor (ferret urine) in later life. For the novel odor exposure test, we provided the cattle urine samples and ferret urine samples as novel odor to rat urine-exposed mice and rabbit urine-exposed mice, respectively. We allowed 2 min between trials. The time spent in sniffing (within 1 cm of the rod) and licking each rod tip using stopwatches were measured. To control for experimenter bias, the experimenter was blind to the nature of the sample.

Open Field (OF) Test

Twenty-four hours after the novel odor exposure, the mice were tested in the open field. The open field test was designed to test anxiety-like behavior and active coping behavior (exploration) in mild stressful condition induced by early predator odor exposure. The apparatus was a plexiglas open field box (50 × 50 × 30 cm), the floor was divided into 25 equal squares, the three squares in the center of the open field were center grid, others were periphery grid. On each test, the mouse was placed in the corner of the open field, the observation lasted for 10 min. The latency of the mice left the corner where it was put in, the center grids and periphery grids which the mice crossed, the amount of time spent by each animal in either center grid or periphery grid, defecation score, urine score, rearing, and grooming were counted. Between each test in the interval, the box was cleaned by alcohol and then water.

Forced Swimming Test (FST)

The forced swimming test was designed to inspect whether the early stress experience (predator odor exposure) triggered the emergence of active coping behaviors in more intense stressful condition in later life. The protocol was adapted from previous studies (Xia et al., 2007). One day after the open field, the mice were tested in FST. At the beginning of the test, each subject was dropped into glass cylinders (height, 25 cm; diameter, 10 cm) containing 10 cm water, maintained at 24 ± 1°C. All animals were forced to swim for 6 min, each session was recorded by a video camera and the duration of test was measured during the final 4 min of the 6 min test. The immobility was defined that the mouse floating in the water without struggling and making only those movements necessary to keep its head above the water. The duration of immobility, swimming and climbing was recorded in this test.

Immunohistochemistry (IHC)

The IHC employed here was similar to that described in previous studies (Dermitzaki et al., 2007; Meng et al., 2011). After the behavior tests, mice were executed and brains was removed and routinely fixed in 0.01M PBS containing 4% paraformaldehyde (PFA), areas of interest (−3.08 to −7.08 mm from bregma for dorsal raphe nucleus and LC) were dissected and then post-fixed in 0.01M PBS containing 4% PFA for 6 h. Afterward the specimens were rinsed with 0.01M PBS (5 × 30 min, 4°C) and were dehydrated as follows: 3 × 30 min 70%, 90% and 96% ethanol, 3 × 30 min 100% ethanol and 3 × 30 min Roti-Histol. Brain samples were pre-embedded for 3 × 12 h in different Roti-Histol/paraffin solutions (2:1, 1:1, and 1:2) and 3 × 24 h in pure paraffin at 58°C. Then the samples were embedded in paraffin and mounted on standard cassettes and coronal paraffin sections (4 μm) were cut with a microtome (Leica 235) at room temperature, transferred onto coated slides (2 sections per slide) and dried on a heating plate at 58°C for 30 min.

The sections were decorated 3 × 5 min in Roti-Histol followed by 2 × 5 min in 100% ethanol and 5 min in 96%, 90%, and 70% ethanol. They were then washed 3 × 2 min in 0.05M PBS at room temperature. The sections were heated in a citrate buffer solution in a microwave oven. The slides were then washed in 0.05 M PBS (3 × 2 min) at room temperature, incubated in a blocking solution (3% hydrogen peroxide) for 10 min, and subsequently washed again (3 × 2 min) in 0.05M TBS at room temperature and incubated in a blocking solution (0.01M PBS containing 10% normal bovine serum and 1% BSA) for 20 min at room temperature. Afterward, the sections were incubated with anti-CRFR1 (sc-12381; 1:50, Santa Cruz Biotechnology) and anti-CRFR2 (sc-20550; 1:50, Santa Cruz Biotechnology) goat polyclonal IgG overnight at 4°C, washed in 0.05M PBS (3 × 5 min) at room temperature, then incubated in the Polink-2 plus Polymer HRP detection System (PV-9000) from Beijing Zhongshan Biotechnology Co. (Beijing, China). Finally, after washing in 0.05M PBS (3 × 5 min) at room temperature, slide-mounted brain sections were immunoreacted with 0.003% hydrogen peroxide in the presence of 0.05% 3,3′-dianino-benzidine (DAB). The slides were then dehydrated by serial alcohol rinsing as follows: 3 × 30 min 70%, 90%, and 96% ethanol, 3 × 30 min 100% ethanol, dewaxed in dimethylbenzene, and cover-slipped in the histofluid mountant.

Quantification and Statistical Analyses

The number of CRFR1/CRFR2 positive cells were counted using a light microscope (Olympus BX-51 with Camedia Master C-3040 digital camera) and image analysis software (Image-pro plus 6.0). For analysis, cell counts were averaged into a single score for each mouse. Results were presented as mean ± standard error for all measures. The group difference of behavior tests and LC and DRN CRFR1/CRFR2 expression in IHC between rat urine exposure group and rabbit urine exposure group were examined

by Student's t-test using SPSS 13.0 software. The significance level was defined as $p < 0.05$.

RESULTS

The Effects of Predator Odor Exposure on Behaviors in Novel Odor Exposure Test

The results were summarized in **Figure 1**, there was no difference in time spent in sniffing and licking rod tip within cattle urine samples (**Figure 1A**) or ferret urine samples (**Figure 1B**) between rat urine exposure group and rabbit urine exposure group.

The Effects of Predator Odor Exposure on Behaviors in Open Field Test

The results of open field test are showed in **Table 1**, predator odor exposure marginally significantly decreased the amount of time spent in either center grid ($t_{25} = 1.983$, $p = 0.059$). There were no changes in other behaviors in open field test between rat urine exposure group and rabbit urine exposure group mice.

The Effects of Predator Odor Exposure on Behaviors in Forced Swimming Test

As shown in **Figure 2**, the duration of immobility time in the FST of the rabbit urine exposure group mice was significantly longer than that of the rat urine exposure group mice ($t_{25} = 2.339$,

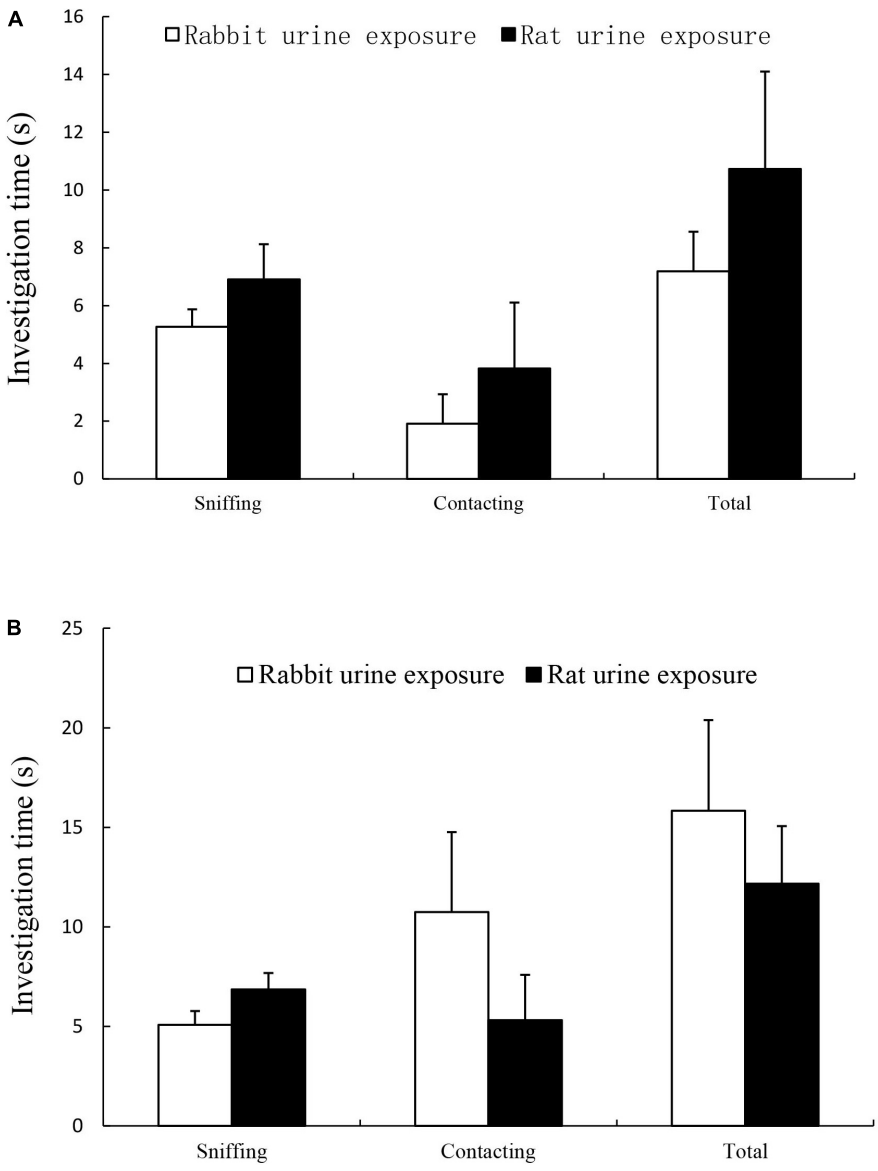

FIGURE 1 | Time spent on sniffing, licking and total (sniffing + licking) in rabbit urine exposure group and rat urine exposure group, including exposure to cattle urine samples **(A)** and ferret urine samples **(B)**. Data are expressed as the mean ± SEM.

TABLE 1 | Effects of predator odor exposure on behaviors in open field test.

Behavior pattern	Rabbit urine exposure mice	Rat urine exposure mice
Latency	11.77 ± 3.053	10.38 ± 1.704
Center grid	52.08 ± 4.972	49.23 ± 3.703
Periphery grid	214.5 ± 19.40	247.8 ± 13.53
Locomotion	266.5 ± 21.83	297.0 ± 15.85
Center grid/Locomotion	0.199 ± 0.014	0.166 ± 0.009*
Center time	68.91 ± 5.791	56.54 ± 6.166
Climbing	81.69 ± 5.481	86.38 ± 3.990
Rearing	42.23 ± 6.786	37.62 ± 5.677
Total	123.9 ± 9.423	124.0 ± 6.293
Grooming num	4.692 ± 0.702	3.462 ± 0.489
Grooming time	11.57 ± 1.878	8.632 ± 1.292
Urination	4.154 ± 0.750	2.923 ± 0.655
Defecation	6.462 ± 0.789	6.154 ± 0.807

*$p = 0.059$, compared to rabbit urine exposure mice.

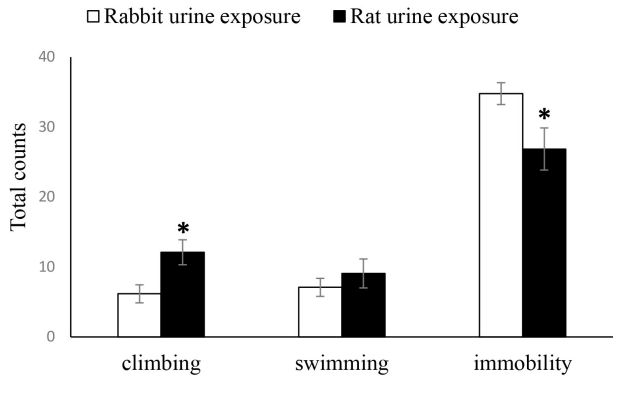

FIGURE 2 | Duration of immobility, swimming and climbing in rabbit urine exposure group, and rat urine exposure group. Data are expressed as the mean ± SEM (*$p < 0.05$ compared with the rabbit urine exposure group).

$p = 0.028$), inversely, there was a significantly increased duration of climbing time of the rat urine exposure group than that of the rabbit urine exposure group mice ($t_{25} = -2.697$, $p = 0.013$), and the duration of swimming time was not differ between the rat urine exposure group and rabbit urine exposure group mice.

The Effects of Predator Odor Exposure on Corticotropin-Releasing Factor Receptors Expression in Locus Coeruleus and Dorsal Raphe Nuclei

The expression of CRF receptors in each group was summarized in **Figure 3**. The rat urine exposure resulted in a significantly increase in the CRFR1 protein expression in the LC ($t_{22} = 2.23$, $p = 0.039$) compared with rabbit urine exposure group (**Figure 3A**). In the dorsal raphe nucleus, the rat urine exposure resulted in no significantly difference CRFR1 and CRFR2 protein expression compared of rabbit urine exposure group (**Figure 3B**).

DISCUSSION

In order to verify the effect of predator odor stress inoculation and explore its potential neurobiological mechanism, the current study examined the effects of predator odor exposure both on the behaviors and CRF receptor expression in DRN and LC. The results of present study showed that the responses to cattle and ferret urine were not significantly different between rat urine exposed mice and rabbit urine exposed mice in the novel odor exposure test. In addition, predator odor exposure didn't change the locomotor activity but moderately increased the anxiety-like behavior of mice in open field test. In FST, predator odor exposure significantly increased the duration of climbing and decreased the duration of immobility. The results of immunohistochemistry showed that the CRFR1 protein expression in the LC was significantly increased by predator odor exposure, but the expression of CRFR1 and CRFR2 in DRN was not effected by predator odor stress inoculation. These results suggested that predator odor stress inoculation can improve active response to subsequent severe stress and the function of CRFR1 in LC might be involved in this effect.

Firstly, the responses to ferret urine between rat urine exposed mice and rabbit urine exposed mice were not significantly different in the novel odor exposure, suggested that early predator odor exposure did not induce habituation to novel predator odor. The novel odor exposure was described in previous studies (Zhang et al., 2008; Zhang and Zhang, 2010), different predator and non-predator odors (ferret urine and cattle urine) were used for avoiding the habituation to the same odor used in predator odor exposure. In addition, exposure to rat urine moderately reduced the ratio of center grid and locomotion in open field test, which means that predator stress increased anxiety-like behavior in mice. Consistently, previous study has reported that rats exposed to a brief cat odor showed anxiogenic responses and decreased exploration in the hole board test (Zangrossi and File, 1992). Chronic exposure to rat odor significantly increased anxiety-like behaviors in mice (Calvo–Torrent et al., 1999). These studies support the results of the present study. However, Adamec et al. (2010) found that predator stress decreased anxiety-like behavior in the light/dark box test and risk assessment in the EPM, CRF-R1 antagonism blocked initiation and consolidation of predator stressor effects on anxiety, and decreased risk assessment in the EPM. These different results may attribute to the different kind of predator stressors and the different behavioral experimental paradigms.

Secondly, the present results also found that exposure to rat urine decreased the duration of immobility and increased the duration of climbing in FST, suggested that predator odor stress inoculation increased the active coping and decreased the passive coping in later stress. Previous research has also shown that rats exposed to predator stress in adolescent mice showed significantly reduced immobility in the FST in early adulthood (Kendig et al., 2011). Another study also found that chronic exposure of predator stress in juvenile decreased the immobile times of Wistar Kyoto rats in adulthood in FST (Chen et al., 2014). These results are consistent with the findings of the present study. Taking together, these findings suggested that mild predator stress

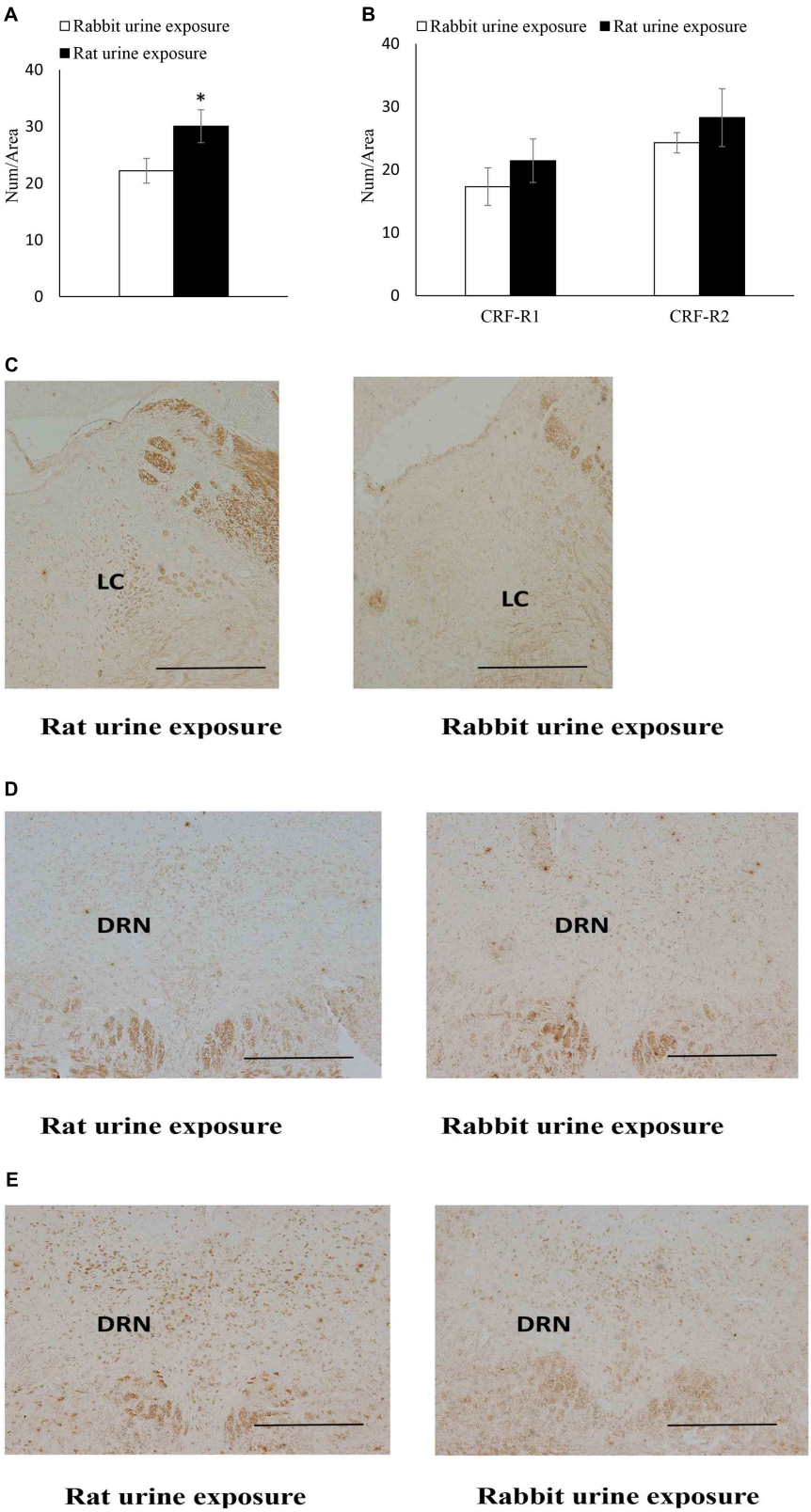

FIGURE 3 | Expression of CRF receptors in rabbit urine exposure group and rat urine exposure group, including CRFR1 expression in locus coeruleus **(A)** CRFR1, CRFR2 expression in dorsal raphe nucleus **(B)**, representative immunohistochemistry (IHC) figures of CRFR1 expression in LC **(C)** CRFR1, **(D)** CRFR2, and **(E)** expression in DRN. Data are expressed as the mean ± SEM (*$p < 0.05$ compared with the rabbit urine exposure group, Scale bar = 250 μm).

(rat urine) did not induce habituation to the predator stress later in life, indicated that the survival strategy of mice was intact. But the early stress experience triggered the emergence of active coping behaviors in more intense stressful condition (FST) later in life.

Thirdly, the present data indicated that predator odor exposure only significantly increased the CRF-R1 protein expression in LC, but the expression of CRF-R1 and CRF-R2 in DRN was not observably changed. These results suggested that LC CRF, rather than DRN CRF, involved in the enhancement of active coping induced by predator odor stress inoculation. Pobbeab et al. (2011) reported that selectively blocked 5-HT in DRN did not change behavioral responses of mice confronted with a predator, which supported the results of the present study. Previous study reported that predator stressor increased tonic LC discharge and decreased phasic auditory-evoked discharge, and this stress-induced alteration in LC discharge toward a high tonic mode was prevented by a CRF antagonist (Curtis et al., 2012). In addition to predator stress, researchers also found that repeated social stress decreases LC activity and CRF-R1 expression in LC (Chaijale et al., 2013). These studies have shown that the influence of CRF-R1 on LC neural activity played an important role in stress response, the mechanisms underlying this may explain how previous stress experience promoted the active coping behaviors. One mechanism through which this can occur was stress-induced CRF receptor redistribution, which was associated with changes in LC neuronal sensitivity to CRF (Reyes et al., 2008).

CONCLUSION

In summary, using the experimental paradigm of predator odor stress inoculation, evidences were provided that stress inoculation enhanced the sensitivity of the acute response system to stress. These changes were characterized by increasing the active coping strategy in severe stress condition. Meanwhile, the survival strategy of mice under the threat of predator was not affected. The neuro-modulation of CRF system in LC rather than in DRN constituted the potential neurobiological mechanism of this effect. These results reminded us that stress inoculation could be used as a training method to improve the adaptive response to secondary stress.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

ETHICS STATEMENT

The animal study was reviewed and approved by the Institutional Review Board of the Institute of Psychology at the Chinese Academy of Sciences.

AUTHOR CONTRIBUTIONS

WW and JZ designed the research. QW and YL performed the research and acquired the data. QW, YL, WW, and JZ interpreted and analyzed the data. QW and WW drafted, revised, and wrote the manuscript.

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ORIGINAL RESEARCH
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The Mediating Effect of Coping Style in the Relationship Between Depression and Disordered Eating Among Chinese Female Undergraduates

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The aim of the current study was to explore the relationship between depression and disordered eating in female undergraduates and the mediating role of coping style between depression and disordered eating. Self-report questionnaires assessing coping style, disordered eating, and depression were completed in 646 Chinese female undergraduates. The results illustrated that there were obvious differences in disordered eating among the undergraduates with various majors. The disordered eating in female undergraduates majoring in art was more serious than those in other majors. Depression and coping style were effective indicators to predict disordered eating. Moreover, depression could not only directly predict disordered eating, but also predict disordered eating through the mediating effect of coping style. These findings indicate that depression and negative coping style are associated with disordered eating. Coping style could mediate the effect of depression on disordered eating, as these may be an important target for early intervention programs for eating disorder (ED).

Keywords: depression, disordered eating, coping style, mediating effect, eating disorder

INTRODUCTION

Eating disorder (ED) refers to disordered eating caused by the interaction of psychosocial factors and specific cultural factors. According to the classification of DSM-V, ED can be classified into anorexia nervosa (AN), bulimia nervosa (BN), and binge eating disorder (BED) (American Psychiatric Association, 2013). The lifetime prevalence of AN, BN, and BED were 0.9%, 1.5%, and 3.5%, respectively (Hudson et al., 2007). In an eight-year study, 10–13% of young females met the diagnostic criteria for ED in DSM-V (Stice et al., 2013). ED seriously affects patients' psychosomatic health and has a higher mortality rate as well. 70% of ED patients suffered from comorbidity, such as mood disorders (>40%) and self-harm (>20%), and the risk of suicide was greatly increased (Anna and Linda, 2016). In a meta-analysis study, the weighted mortality rate (i.e., annual mortality per 1000 people) was 5.1% for AN and 1.7% for BN. More importantly, some patients died of suicide (Arcelus et al., 2011). Chinese researchers retrospectively analyzed the clinical data of 51 ED inpatients. The results showed that 33.33% of the patients had attempted suicide (Darong et al., 2002). The early manifestation of ED is disordered eating. Once individuals had disordered eating,

such as vomiting after overeating to control their weight and refusing to eat, they often further developed into ED (Ariel et al., 2019). Disordered eating on campus was a common problem (Ward and Hay, 2015). Both college and middle school students were at high risk for disordered eating (Xiaolu and Xiaoming, 2008). From mid-adolescence to mid-adulthood, the proportion of females who used extreme eating behavior to control their weight increased with age (Neumark-Sztainer et al., 2011). In addition to ED, disordered eating is often associated with other problems. For example, sexual risk behavior is closely related to disordered eating. Young females with disordered eating frequently changed their sexual partners and had a higher risk of unprotected sexual behavior (Fergus et al., 2019). Disordered eating was also a potential cause of schizophrenia (Youssef et al., 2018). Moreover, psychopathological symptoms presented a positive correlation with emotional and binge eating (Poínhos et al., 2018).

Eating disorder and disordered eating were associated with emotional state, especially depression (Lazarevich et al., 2016; van Strien et al., 2016). 80% of patients with AN or BN had mood disorders (Godart et al., 2015). Most of the emotional disorders associated with ED are depressive disorder. There might be a two-way relationship between disordered eating and depression (Holm-Denoma et al., 2014). Compared with the patients suffered from simple ED or ED combined with anxiety, ED combined with depression had more complex and severe symptoms (Hughes et al., 2013). Females with self-perceived weight problems had a higher risk of ED among those diagnosed with depression (Küçük et al., 2018). Although the causal relationship between depression and overeating remained to be studied, depression might be the risk, and maintenance factor of overeating (Brechan and Kvalem, 2015).

In addition to depression, there is a significant relationship between disordered eating and coping style. Maladaptive coping strategies were more likely to lead to disordered eating (Megan and Ann, 2019). Avoidant-oriented coping strategies were proved to partially mediate the link between stress and disordered eating (Iolanda et al., 2018). Coping style is also closely related to depression. For example, coping flexibility refers to an individual's ability to effectively adjust coping strategies according to the situation of stress. Higher coping flexibility was related to the lower depression risk (Kato, 2012). Coping style was related to suicide risk factors (i.e., depression, suicide ideation, and suicide behavior) (Adam et al., 2018). Depressive symptoms increased with the accumulation of stress events and developed corresponding coping strategies (Suzuki et al., 2018). Depressive patients tended to adopt negative coping strategies such as avoidance, especially when they encountered negative evaluation (David et al., 2017). The relationship among depression, coping style and disordered eating has aroused researchers' great interest. Negative coping strategies were triggered by depression and emotional eating behaviors (Raspopow et al., 2013). Depressive symptoms and avoidance coping strategy to control stress were associated with disordered eating (McGarrity et al., 2019).

In summary, ED is a common kind of psychological disorder that seriously endangers females' physical and mental health and even lives. Since disordered eating is the early manifestation of ED, if we identify and intervene the individuals who are still in the early stage of disordered eating, we could effectively reduce the incidence of ED. In previous studies, researchers have explored the relationship among depression, coping style and disordered eating, but most of them are the relationship between two variables, while the relationship between the three variables, especially the mediating effect, is still scarce. The relationship among depression, coping style and disordered eating needs to be further explored. In this study, we have two basic hypotheses. Firstly, depression is a vital predictor of disordered eating. Secondly, depression can not only directly predict the severity of disordered eating, but also indirectly affect the disordered eating through the mediating effect of coping style.

MATERIALS AND METHODS

Participants and Procedure

The occurrence of ED shows remarkable gender differences. Since more than 90% of patients with ED are young females, the subjects in this study were female undergraduates from 7 universities in Nanjing, China by cluster sampling. Two classes were randomly selected from each grade of each university. Of 650 eligible students, 646 (99.4%) consented, and took part in the current study, average age 19.4 years (SD = 1.1, range = 18–23). 254 (39.3%) participants came from rural area and 392 (60.7%) from urban area. 249 (38.5%) participants majored in science and engineering, 124 (19.2%) in medicine, 74 (11.5%) in art and 199 (30.8%) in liberal arts.

Assessment sessions took place in class during school time, supervised by members of the research team. The survey lasted half an hour and the questionnaires were collected on the spot. The research was approved by the Human Research Ethics Committee of NJUCM. Approval was also granted by each university. Information about the study was provided directly to the participants and informed consent and assent were obtained.

Measures

The eating disorder inventory (EDI; Garner et al., 1983) measures the severity of disordered eating. Sixty-four items are divided into eight sub-scales, including drive for thinness (DT), body dissatisfaction (BD), bulimia, perfectionism, interpersonal distrust (ID), mature fear (MF), interoceptive awareness (IA), and inefficiency, with varying response options scored 0–5. Higher score indicates more severe disordered eating. We used the Chinese version of EDI revised by Jianping W. The α coefficients of EDI subscales ranged from 0.68 to 0.87, and the cumulative contribution rate of 8 factors was 43.9% (Wei et al., 2005). In the current study, internal consistency α = 0.88.

The Simple coping style questionnaire (SCSQ; Yaning, 1998) contains 20 items assessing coping style which effectively reflect individual's coping style in the context of Chinese culture. Scores 0 (never) to 3 (always). Items 1–12 belong to positive coping and 13–20 belong to negative coping. If the average difference between positive coping and negative coping is greater than 0, it is positive coping and less than 0 is negative coping. The α coefficient of the full scale was 0.90 and the retest correlation

coefficient was 0.89 (Yaning, 1998). In the present study, total questionnaire internal consistency $\alpha = 0.76$.

The hospital anxiety and depression scale (HADS; Zigmond and Snaith, 1983) examines anxiety and depression symptoms, respectively. The total score of depression or anxiety can be regarded as the severity of symptoms. HADS had good reliability and validity (Olssøn et al., 2005). In the current study, internal consistency $\alpha = 0.82$.

Statistical Analysis

All analyses were performed using SPSS22.0. All statistical tests were two-sided and the significance level was set at $p < 0.05$. One-way ANOVA was used to compare differences between groups. Pearson's correlation was used to examine correlations among disordered eating, depression and coping style. The percentage bootstrap method of deviation correction was used to test the mediating effect.

RESULTS

Relationship Between Major and Disordered Eating

The participants were divided into groups according to their majors and the differences of disordered eating were compared. The relationship between major and disordered eating is shown in **Table 1**. As revealed in the table, there were obvious differences in disordered eating among various majors [$F_{(3,640)} = 3.42$, $p<0.05$, $\eta^2 = 0.17$]. The disordered eating of female undergraduates majoring in art was more serious than other majors. The results in the table also demonstrated that the DT [$F_{(3,640)} = 16.52$, $p<0.001$, $\eta^2 = 0.07$], BD [$F_{(3,640)} = 5.28$, $p = 0.001$, $\eta^2 = 0.02$], and bulimia [$F_{(3,640)} = 3.73$, $p<0.05$, $\eta^2 = 0.02$] of subjects majoring in art were more remarkable than other majors. See **Supplementary Table S1** for the original data of this study.

Correlations Among Disordered Eating, Depression, and Coping Style

The means and standard deviation (*SD*) of continuous variables are presented in **Table 2**. The correlations among disordered eating, depression and coping style are shown in **Table 3**. As revealed in the table, depression was positively related to disordered eating, whereas coping style was negatively related to disordered eating and depression.

TABLE 2 | Means and standard deviation (SD) of continuous variables.

Variables	*Mean*	*SD*
EDI (eating disorder inventory)	45.76	19.46
DT (drive for thinness)	5.05	4.60
BD (body dissatisfaction)	11.83	6.19
Bulimia	2.20	2.98
Perfectionism	5.27	3.58
ID (interpersonal distrust)	3.72	3.23
MF (mature fear)	8.26	4.36
IA (interoceptive awareness)	4.72	4.37
Inefficacy	4.70	4.10
Coping style	−0.09	1.35
Depression	5.31	3.07

The Mediating Effect of Coping Style in the Relationship Between Depression and Disordered Eating

The results of correlations analysis showed that the relationship among depression, coping style and disordered eating met the conditions of mediating effect test. The percentage bootstrap method of deviation correction and the method proposed by Hayes (2012) were used to test the mediating effect of coping style on the relationship between depression and disordered eating. The results showed that depression had a significant predictive effect on disordered eating ($\beta = 0.3095$, $t = 8.259$, $p < 0.001$). The direct predictive effect of depression on disordered eating was still significant when coping style was added as a mediator ($\beta = 0.2346$, $t = 5.752$, $p < 0.001$). Depression had a significant negative predictive effect on coping style ($\beta = -0.4225$, $t = -11.829$, $p < 0.001$), and coping style also had a significant negative predictive effect on disordered eating ($\beta = -0.1773$, $t = -4.347$, $p < 0.001$) (**Table 4**).

In addition, the upper and lower limits of the 95% confidence interval of bootstrap of the direct effect of depression on disordered eating and the mediating effect of coping style did not contain 0, indicating that depression could not only directly predict disordered eating, but also predict disordered eating through the mediating effect of coping style. The direct effect (0.2346) and intermediate effect (0.0749) accounted for 75.8% and 24.2% of the total effect (0.3095), respectively (**Table 5**). The mediating effect of coping style on depression and disordered eating is shown in **Figure 1**.

TABLE 1 | Comparison of scores of EDI among female college students of different majors.

Major	① (*N* = 249)	② (*N* = 124)	③ (*N* = 74)	④ (*N* = 199)	*F*	*LSD*
EDI	44.81 ± 18.78	42.19 ± 17.94	50.07 ± 23.80	47.66 ± 19.22	3.42*	③>①**, ③>②*, ④>②*
DT	4.23 ± 4.10	4.19 ± 4.29	8.13 ± 5.62	5.48 ± 4.47	16.52***	③>①***, ③>②***, ③>④*** ④>①**, ④>②*
BD	11.59 ± 6.19	10.33 ± 5.76	11.82 ± 5.51	13.07 ± 6.51	5.28**	③>①*, ③>②***
Bulimia	2.08 ± 2.65	2.04 ± 2.45	3.31 ± 5.00	2.07 ± 2.63	3.73*	③>①**, ③>②**, ③>④**

*EDI, eating disorder inventory; DT, drive for thinness; BD, body dissatisfaction; ①, medicine; ②, science and engineering; ③, art; ④, liberal arts; *p<0.05 (two-tailed), **p<0.01 (two-tailed), and ***p<0.001 (two-tailed).*

TABLE 3 | Pearson's correlation of various variables.

Variables	1	2	3	4	5	6	7	8	9	10	11
1. EDI	1										
2. DT	0.66***	1									
3. BD	0.60***	0.51***	1								
4. Bulimia	0.57***	0.41***	0.17*	1							
5. Perfectionism	0.50***	0.27***	0.11**	0.31***	1						
6. ID	0.37***	−0.08*	−0.01	0.10*	0.09*	1					
7. MF	0.47***	0.16***	0.14***	0.17***	0.15***	0.07	1				
8. IA	0.76***	0.37***	0.20***	0.46***	0.39***	0.35***	0.28***	1			
9. Inefficacy	0.64***	0.18***	0.20***	0.25***	0.18***	0.48***	0.19***	0.59***	1		
10. Coping style	−0.28***	−0.072	−0.03***	−0.18***	0.05	−0.31***	−0.11**	−0.30***	−0.43***	1	
11. Depression	0.31***	0.03	0.005	0.15***	0.08*	0.42***	0.06	0.35***	0.48***	−0.42***	1

****p < 0.001 (two-tailed), **p < 0.01 (two-tailed), and *p < 0.05 (two-tailed).*

DISCUSSION

Our results showed that disordered eating of female undergraduates majoring in art was more severe than other majors, such as medicine, science, and engineering. Previous studies have found that FAT (female athlete triad) was often associated with disordered eating and dancers were more likely to get FAT than runners and figure skaters (Nattiv et al., 2007). Our findings were consistent with previous studies, which showed that disordered eating was closely related to individuals' occupations.

Eating- and weight-related disturbances were key factors associated with depressive symptoms (Rawana et al., 2016). In this study, we also found a positive correlation between depression and disordered eating. Our study showed that depression could not only directly affect disordered eating, but also indirectly affect disordered eating under the mediating effect of coping style. Coping was a dynamic response to negative life events and subsequent impacts, helping to protect individuals from psychological and physical harms (Annemieke et al., 2019). As early as the end of the 20th century, researchers explored the relationship between stress levels, coping style, and problem-solving ability among bulimic and anorexic individuals. They found that both anorexics and bulimics reported higher levels of stress, lower levels of confidence in their ability to solve problems, a tendency to avoid confronting problems, a reluctance to share personal problems, and feelings of being driven (Soukup et al., 1990). In addition, undergraduates who used maladaptive coping styles showed higher levels of depression and poorer ability to adapt to the environment (Pinkasavage et al., 2015). In this study, we found a consistent result that negative coping style was associated with disordered eating.

This study provides a new perspective to prevent ED by exploring the mediating effect of coping style on depression and disordered eating. Because depression can positively predict disordered eating, when young women have emotional problems, psychological practitioners should pay attention to their eating behavior and evaluate it thoroughly at the same time, so

TABLE 4 | The mediating effect test of coping style.

Regression equation (N = 646)		Fit index		Coefficient significance	
Result variable	**Prediction variable**	R^2	F	β	t
Disordered eating	Depression	0.096	68.207***	0.3095	8.259***
Coping style	Depression	0.179	139.93***	−0.4225	−11.829***
Disordered eating	Coping style	0.122	44.5***	−0.1773	−4.347***
	Depression			0.2346	5.752***

****p < 0.001 (two-tailed). All variables in the model were brought into regression equation by standardized variables.*

TABLE 5 | Analysis of total effect, direct effect, and indirect effect.

	Effect value	Boot SE	Boot CI lower	Boot CI upper	Relative effect value
Total effect	0.309	0.042	0.246	0.391	
Direct effect	0.235	0.041	0.154	0.315	75.8%
Indirect effect	0.075	0.022	0.034	0.118	24.2%

Boot SE, Boot CI lower and Boot CI upper refer to the standard error of indirect effect estimated by percentile bootstrap method corrected by deviation, the lower limit and upper limit of 95% confidence interval, respectively.

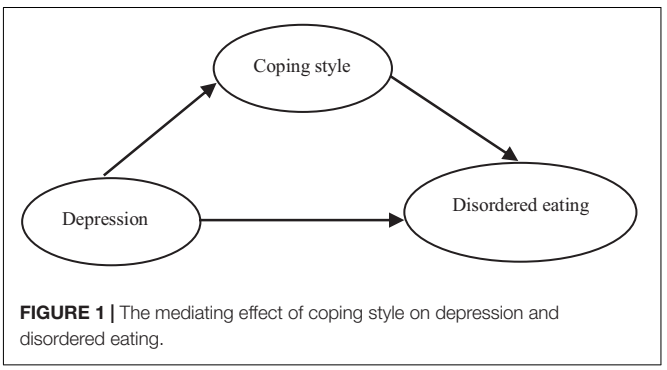

FIGURE 1 | The mediating effect of coping style on depression and disordered eating.

as to identify eating problems early. For individuals with disordered eating, we can focus on alleviating emotional problems to intervene eating problems. Since coping style plays a mediating role between depression and disordered eating, it can effectively alleviate the severity of disordered eating by teaching depressive individuals reasonable coping style and stress management strategies. Focusing on specific positive cognitive coping skills might be an essential way to reduce the frequency of binge eating (Nichole et al., 2012). As females began to adopt adaptive coping strategies, such as seeking support or acceptance from others, they became more confident in using coping strategies and their coping skills were strengthened in consequence (Blevins et al., 2017). In addition, it was useful to prevent and/or reduce disordered eating by teaching students problem-oriented, proactive skills to deal with daily problems related to college life and providing opportunities for repetitive exercises to enhance self-efficacy (Laura et al., 2012).

In the previous hypothesis, we proposed that depression could not only positively predict disordered eating, but also indirectly affect disordered eating through coping style. In this study, the hypothesis was verified. The innovation of this study is to verify the mediating effect of coping style between depression and disordered eating, which provides a theoretical basis for psychological intervention of disordered eating. To sum up, regulating depression and enhancing adaptive coping style can effectively alleviate disordered eating and promote the physical and mental health of female undergraduates, and may further reduce the incidence of ED. The limitation of this study is that we only studied the relationship among depression, coping style and eating behavior, and did not find the causal relationship among them. In addition, through exploratory analysis, we found that coping style plays a mediating role in depression symptoms and disordered eating, but the actual effect of changing coping strategies on preventing ED needs further empirical research.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, to any qualified researcher. Requests to access the datasets should be directed to ZZ, zzdoctor@126.com.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Human Research Ethics Committee of NJUCM. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

ZZ designed the project and wrote the manuscript. WH performed the questionnaire survey, analyzed the data, and reviewed the literature. YL and DW participated in the revision of the manuscript. SG revised the manuscript. FW revised the manuscript and offered the administrative support.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpsyg.2019.03011/full#supplementary-material

TABLE S1 | Raw data.

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The Modulation of Spatial Working Memory by Emotional Stickers and Facial Expressions

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This article aims to investigate the interaction effects of emotional valence (negative, positive) and stimulus type (sticker, face) on attention allocation and information retrieval in spatial working memory (WM). The difference in recognition of emotional faces and stickers was also further explored. Using a high-resolution event-related potential (ERP) technique, a time-locked delayed matching-to-sample task (DMST) was employed that allowed separate investigations of target, delay, and probe phases. Twenty-two subjects participated in our experiment. The results indicated that negative face can catch early attention in information encoding, which was indicated by the augmentation of the attention-related P200 amplitude. In the delay phase, the N170 component represents facial specificity and showed a negative bias against stickers. For information retrieval, the increase in the emotion-related late positive component (LPC) showed that positive emotion could damage spatial WM and consume more cognitive resources. Moreover, stickers have the ability to catch an individual's attention throughout the whole course of spatial WM with larger amplitudes of the attention-related P200, the negative slow wave (NSW), and the LPC. These findings highlight the role of stickers in different phases of spatial WM and provide new viewpoints for WM research on mental patients.

Keywords: spatial working memory, emotion, attention, stickers, faces

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INTRODUCTION

Working memory (WM) refers to the system responsible for the manipulation of information and temporary storage in the range of seconds and is important for a range of complex cognitive activities. Baddeley and Hitch (1992) hypothesized the existence of three major components in the WM system: (i) a phonological loop in the verbal WM system; (ii) a visuospatial sketchpad for the initial registration of non-verbal material in the visuospatial WM system; and (iii) a central executive component to determine which information should be made available for cognitive processing. These researchers also proposed the episodic buffer (Baddeley, 2000), which is assumed to play an attention-demanding role in binding together information from different sources.

As has been proposed by the processing efficiency theory (Eysenck and Calvo, 1992), negative emotions can have an impact on the phonological loop and central executive components, then further influence the cognitive process. Studies have suggested that spatial WM is vulnerable to negative emotion (Lavric et al., 2003; Weiland-Fiedler et al., 2004). For example, Lavric et al. (2003) found that anxiety can impair the performance of spatial WM in the *n*-back test. With the

development of positive psychology, attention has been focused on the function of not only negative emotion but also positive emotion in the course of spatial WM. Negative emotion and positive emotion were shown to modulate WM through distinctive neural circuits, in which negative emotion activated the right amygdala and positive emotion activated the substantia nigra (Osaka et al., 2013). There were evidences showing that positive mood can impair spatial WM and executive control (Storbeck and Maswood, 2016), which can be demonstrated by the increased level of brain activation in spatial WM tasks (Gray, 2001). Moreover, spatial WM and emotion can serve as variables to better understand the psychopathology of mental disorder. There was growing evidence that dysfunctional spatial WM abilities may be linked to functional deficits in psychiatric disorders (Purcell et al., 1998a,b; Wee et al., 2003, 2007). For patients of depression, altered prefrontal brain activity was found during spatial WM task (Schecklmann et al., 2011). For obsessive compulsive disorder (OCD) patients, they perform poorly on tasks than engaging spatial WM (Purcell et al., 1998a,b), and showed abnormal activity in the anterior cingulate cortex (Wee et al., 2003). For schizophrenia, relative to healthy subjects, aberrant physiologic activity in the dorsolateral prefrontal cortex (DLPFC) is detectable, which engaged in the maintenance of spatial information (Glahn, 2000). That is to say, the extent of WM impairment can be used as an indicator to mental illness. For emotional perception, the impaired function manifests in flat, blunted and inappropriate affect, and can be reflecting on in emotion processing deficits in identification, discrimination and recognition of emotional facial expressions (Mandal et al., 1998). For patients with depression, the adopt of maladaptive emotion regulation strategies led to deficits in cognitive control (Joormann and Gotlib, 2010). For patients with schizophrenia, the failure to activate limbic regions during emotional valence discrimination may explain emotion processing deficit (Gur et al., 2002).

In our daily life, facial expressions can serve as social communicative signals to allow us to better estimate a person's motivational state (Xu et al., 2015). The N170 component, which is shown in the temporo-occipital areas approximately 170 ms after the onset of a face stimulus, has been shown to be sensitive to facial stimuli (Bentin et al., 1996) and emotional facial expression (Schupp et al., 2004). Evidence shows a substantially stronger N170 for faces than non-facial objects, such as printed words (Marina, 2008) and cars (Dering et al., 2009). This kind of facial specificity can be explained by the expertise theory (Diamond and Carey, 1986), which argues that, as they possess a high degree of familiarity to faces, people are experts in recognizing facial information, thus inducing a stronger N170 component to faces than other non-facial stimuli. However, do stimuli which share similar features to faces also induce weaker N170 components than facial stimuli? With the rise in social media use, chatting online offers more flexibility for people to exchange information and provides a new way to communicate no matter where the users may be, during which non-verbal elements such as facial expressions, eye contact, and body movements are deprived. In order to compensate the lack of face-to-face communication between online environment, the use of stickers have become a common part in various forms of instant messaging use. A sticker can be simply textual, pictorial, or a combination of both, and can be either static or animated. It contains pictorial representations and can not only express the author's emotions, but also represent social situations (Lee et al., 2016) and enhance human interaction (Derks et al., 2008). In light of the facial specificity of the N170 component, we hypothesized that even though faces and these stickers share similar features, faces can still induce a stronger, face-specific N170 component than stickers.

Previous studies have indicated that faces are capable of reallocating attentional resources in spatial WM tasks (Moriya et al., 2014). However, the attentional capture mechanism of stickers has seldom been mentioned. Compared to faces, we find that most of the stickers are based on the faces of familiar celebrities modified by means of caricature and exaggeration to some extent, potentially strengthening the impact, comprehension and interpretation of information by emphasizing its intended positivity, negativity, or neutrality (Skovholt et al., 2014) and are abundant in social content (Chang and Lee, 2016). In online communication, stickers are always used as supplements to the text message and can express the underlying intention by enriching the information (Suvorov and Dolin, 2007). We hypothesize that stickers, which share similar characteristics to faces and sometimes act as our "facial expression" online, can also capture attention in spatial WM and may actually catch more attention than faces because participants may think further about the internal information they contain. For emotional perception, it is obvious that stickers share similar emotional features with faces and, to some extent, can even result in stronger emotional arousals than faces because of their exaggerated expressions and high popularity in online social networking. Previous studies have shown that stickers can be used to accurately classify the emotional content of text messages in many cases and are easier for conveying information than text imputing (Lee et al., 2016). How the emotional valence of stickers can influence spatial WM is one of the topics of our study. The negative emotional state may induce individuals to care more about anxiety responses unrelated to the current task, which would distract attention and consume the limited WM resources, resulting in prolonged response times (RTs) and low efficiency of cognition (Shackman et al., 2006). In light of the negative bias (Carretié et al., 2001), we further hypothesize that negative stickers have a stronger ability to capture attention than positive stickers. In this study, with the help of high temporal resolution event-related potential (ERP), we intended to explore the brain mechanisms of spatial WM under induced positive and negative emotion in faces and stickers at different phases of information encoding, storage and retrieval in healthy participants. A number of ERP studies (Josiassen et al., 1982; Mecklinger and Pfeifer, 1996; Hajcak et al., 2010; Leppänen et al., 2010) have shown the ability of the P200 component and negative slow wave (NSW) to reflect WM attentional allocation as well as emotional perception by late positive component (LPC). In information encoding, after the preliminary classification and evaluation of items such as letters or spatial locations, the amplitude of the P200 component can reflect the degree of representation for WM, which has a close connection to the allocation of early attentional resources

(Josiassen et al., 1982). Through the process of information storage, information is stored over time and represented in memory. Participants were instructed to engage in a sustained retention-rehearsal effort during the interval. Mecklinger found that there exists a bilateral posterior parietal occipital NSW component, which can reflect the storage and rehearsal of WM as well as the allocation of attention (Mecklinger and Pfeifer, 1996) and was thought to index early input operations, long-duration elaborative processes, and a rehearsal loop (Ruchkin et al., 2016). In information retrieval, parietal LPC have been found to be sensitive to emotion regulation (Hajcak et al., 2010) and to be associated with enhanced emotional processing (Leppänen et al., 2010). The LPC can also indicate sustained attention (Hajcak et al., 2010) and motivational significance (Schupp et al., 2000) to salient stimuli. The results of an ERP indicated that large LPC amplitudes are observed for positive emotions (Bublatzky et al., 2014) and that LPC amplitudes are decreased to aversive stimuli when compared to natural emotional states (Thiruchselvam et al., 2011). Previous studies indicated that mental diseases are often accompanied by attention deficit and emotional disorders (Mandal et al., 1998; Brandt et al., 2014). On the basis of our study, further research could be done on mental patients.

Delayed matching-to-sample task (DMST), which serves as a typical paradigm for spatial WM studies, can divide brain processing procedures into three parts: information encoding in the target phase, information storage and rehearsal in the delay phase, and information retrieval in the probe phase (Li et al., 2005). With the DMST, distinct brain processing procedures can be studied in separate phases. The main goal of this study was to examine the effect of the emotional states elicited from stickers and faces on attentional allocation in separate phases of spatial WM tasks as well as the interference of the interaction between emotion and stimulus types with spatial WM retrieval.

MATERIALS AND METHODS

Participants

Twenty-two participants (8 men and 14 women; aged 18–23 years; mean age = 21.5 years) took part in the experiment, all right-handed and with normal or corrected-to-normal vision and hearing capabilities with no history of illness affecting brain function or a history of major psychiatric illness in first-degree relatives. The sample size of electroencephalogram (EEG) experiment was calculated by G-power on the basis of relevant studies. The participants provided written informed consent to participate in this study, which was previously approved by the Ethics Committee of Hubei University. All participants received payment for their time.

Stimuli

For visual emotional stimuli, 64 facial affective stimuli pictures were selected from the Chinese Facial Emotional Picture System (Xu et al., 2011) and 64 affective sticker stimuli pictures were selected from WeChat, which is currently the most popular mobile instant messaging platform in China (Che and Cao, 2014). For each type of stimulus, 32 were classified as expressing positive (e.g., happiness) emotion and 32 were classified as expressing negative (e.g., anger, disgust, sad) emotions. All facial pictures were black and white (173 × 200 pixels, 24 bits). All sticker images were processed into 200 pixel width, black and white and without special body movement information using Adobe Photoshop. To pair the emotional valance of facial affective stimuli and affective sticker stimuli separately, an evaluation test was carried out. Sixty participants (27 male, 33 female, 19–22 years of age) were instructed to make an online evaluation on the emotional valence of the stimuli using a seven-point scale, where a value of 1 represented a very negative emotion, a value of 4 represented a neutral emotion, and a value of 7 represented a very positive emotion. A single sample *t*-test was conducted to eliminate data with no significant difference from the value of 4; pictures with high (value $>$ 6) and low (value $<$ 2) emotional valances were also removed. One positive facial stimulus ($p < 0.05$), one negative facial stimulus ($p < 0.05$), six positive Chinese sticker stimuli ($p < 0.05$) and four negative Chinese sticker stimuli ($p < 0.05$) were eliminated. The valence scores between positive and negative facial stimuli were significantly different (3.19 versus 5.19, $p < 0.05$) as were those for the sticker stimuli (2.58 versus 5.56, $p < 0.05$). Four types of stimuli (positive face, negative face, positive sticker, negative sticker) were paired separately with their equivalent emotional valances. For each type of stimulus, 26 pairs of images were produced to appear before and after the presentation of spatial WM tasks. The spatial WM task was a gray square, which was divided into nine equal pieces, four of which were randomly filled with black.

Procedure

The experiment was conducted in a quiet and bright room (Laboratory Room; Hubei University, China). All stimuli were generated and displayed with E-prime 2.0 and presented on a monitor (1680 × 1050 pixels; 60 Hz). Participants sat 70 cm from the screen and were asked to remember the location of the black squares in the target phase and to judge whether the location of the black square that appeared in the probe phase was congruent with the location of one of the four black squares in the target phase. When the emotional faces or stickers were presented, participants were asked to identify the type of emotion and perceive the expressed emotion just mentally without making any key response to insure the processing of the type of emotion. Then, after seeing both of the emotional stimuli, they were required to press the left mouse button if the two were congruent or the right mouse button if the two were incongruent as fast and accurately as possible. All visual stimuli were presented with a visual signal of 10.7 × 8.7° and were at a visual angle of 3.4° to the left or right of center.

Figure 1 shows the design of the experimental paradigm. At the beginning of each trial, a black fixation point appeared on a white background screen for 500 ms, and then an emotional picture (positive face, negative face, positive sticker or negative sticker) was presented on the screen for 1000 ms, followed by an interval of 300 ms. Then, the spatial WM task appeared for

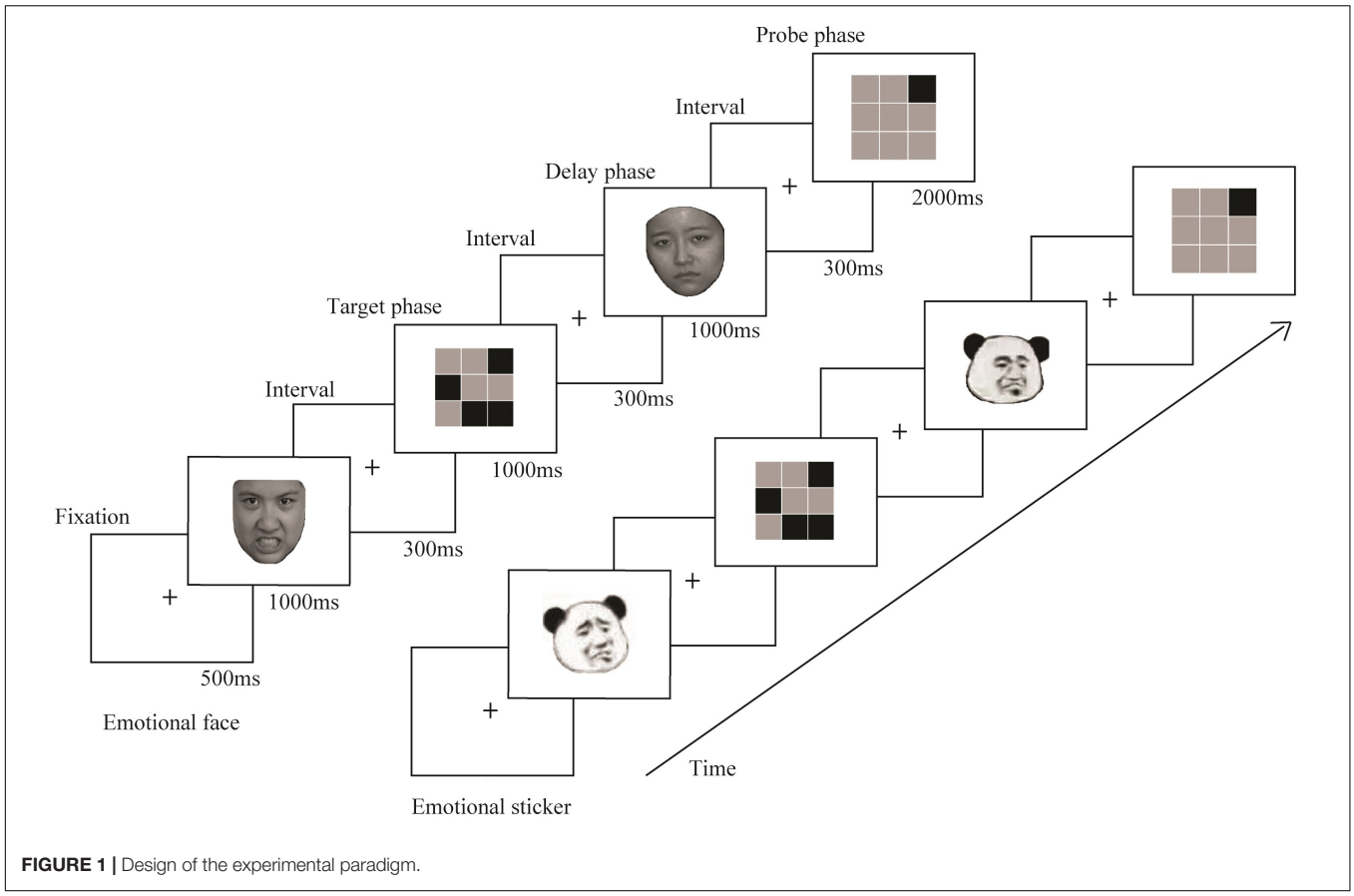

FIGURE 1 | Design of the experimental paradigm.

1000 ms. After an interval of 300 ms, an emotional picture that was of the same stimulus type and emotional valance as the picture before the task was presented for 1000 ms in the delay phase. Finally, the probe stimulus was presented after an interval of 300 ms. A maximum of 2000 ms was available for responding; after that time, the next trial started. There were 4 blocks that each consisted of 52 trials. The four kinds of stimuli were presented at random to each participant in all blocks. Prior to the formal experiment, a practice experiment was conducted to ensure that the participants were familiar with the procedures.

Apparatus

The EEG and behavioral data were recorded simultaneously. Stimulus presentation was controlled using E-prime 2.0. An EEG system (BrainAmp plus, Gilching, Germany) was used to record EEG signals through 32 electrodes mounted on an electrode cap (Easy-cap, Herrsching–Breitbrunn, Germany). All signals were referenced to FCz. Vertical eye movements and eye blinks were detected by deriving an electrooculogram (EOG) from a recording electrode positioned approximately one centimeter below the participant's right eye, and horizontal eye movements were measured by deriving the EOG from one electrode placed at the outer canthi of the left eye. All electrode impedances were maintained below 5 kΩ. All electrodes were referenced offline to the average of both mastoids. The EEG and EOG were sampled at a digitization rate of 500 Hz.

Data Analysis

Behavioral Data Analysis

The mean RTs were calculated based on the responses that fell within the average time period ±3 SD. The accuracy was the percentage of correct responses relative to the total number of target stimuli. The behavioral results for RTs and accuracy were analyzed using a 2 × 2 repeated-measures analysis of variance (ANOVA, Greenhouse–Geisser corrections with corrected degrees of freedom) with Emotion (negative/positive) and Stimulus type (face/sticker) as within-subject factors. The statistical significance level was set at $p < 0.05$ (Mauchly's sphericity test). The effect size estimates η_p^2 were reported.

ERP Data Analysis

Electroencephalogram data were analyzed using Brain Vision Analyzer software (Version 2.0, Brain Products GmbH, Munich, Bavaria, Germany). ERP data were analyzed offline for only those trials on which the performance was correct. EEG and EOG signals were epoched into periods of 1150 ms, from 150 ms before the stimulus onset to 1000 ms after onset, and baseline corrections were made from −150 to 0 ms relative to stimulus onset. Trials with artifacts were rejected with a criterion of ±80 μV. These trials were subject to automatic rejection from the analysis. Then, the remaining trials were averaged separately for each participant, each session and each stimulus type following digital filtering using a bandpass filter of 0.05–30 Hz.

The following six sites were chosen for statistical analysis: F3, F4, and Fz (frontal) and P3, P4, and Pz (central-parietal). The amplitudes of the frontal P200 (the maximum positive peak in the time window 165–235 ms) for the target phase, the central-parietal N170 (the maximum negative peak in the time window 150–200 ms) and central-parietal NSW (the mean amplitude in the time window 450–850 ms) for the delay phase, and the frontal LPC (the mean amplitude in the time window 450–850 ms) for the probe phase were measured. Repeated-measures analysis of variance (ANOVA) was conducted on each ERP component with three factors: stimulus type (sticker/face), emotion (positive/negative), and electrode locations (F3, F4, Fz for P200 and LPC/P3, P4, Pz for N170 and NSW). Greenhouse–Geisser Epsilon correction was applied to adjust the degrees of freedom of the F ratios as necessary. All statistical analyses were carried out using SPSS version 16.0 software.

RESULTS

Behavioral Results

The RTs and accuracy of each stimulus type are presented in **Table 1**. We performed 2 × 2 ANOVA with Emotion (negative/positive) and Stimulus type (face/sticker) as within-subject factors. For accuracy, the main effect of emotion [$F_{1,21}$ = 7.180, p = 0.014, η_p^2 = 0.255] was significant, accuracy (positive) > accuracy (negative). The main effect of stimulus type [$F_{1,21}$ = 0.041, p = 0.842, η_p^2 = 0.002] was not significant. There were no significant interactions between stimulus type and emotion [$F_{1,21}$ = 0.388, p = 0.540, η_p^2 = 0.018]. For RTs, the main effect of stimulus type [$F_{1,21}$ = 4.661, p = 0.043, η_p^2 = 0.182] was significant, RTs (face) > RTs (sticker). No main effect of emotion [$F_{1,21}$ = 7.33, p = 0.402, η_p^2 = 0.034] and no significant interactions between stimulus type and emotion [$F_{1,21}$ = 0.111, p = 0.742, η_p^2 = 0.005] were found.

ERP Results

For the target phase, a 2 (Emotion: positive, negative) × 2 (Stimulus type: face, sticker) × 3 (Electrode: F3, F4, Fz) ANOVA was performed. A significant main effect of stimulus type [$F_{1,21}$ = 5.824, p = 0.025, η_p^2 = 0.217] was revealed for the frontal P200 amplitude. The amplitudes for trials following the presentation of stickers (3.66 ± 1.23 μV) were significantly stronger than those of the faces (3.02 ± 1.25 μV). There were significant interactions between stimulus type and emotion [$F_{1,21}$ = 5.913, p = 0.024, η_p^2 = 0.220]. Further *post hoc* comparison results revealed that the P200 amplitude of the spatial task trials was stronger for the negative faces than for the positive faces (**Figure 2A**, p = 0.002) and stronger for the positive stickers than for the positive faces (**Figure 2B**, p = 0.008).

For delay phase, 2 (Emotion: positive, negative) × 2 (Stimulus type: face, sticker) × 3 (Electrode: P3, P4, Pz) ANOVA on central-parietal N170 and 2 (Emotion: positive, negative) × 2 (Stimulus type: face, sticker) x 3 (Electrode: P3, P4, Pz) ANOVA on central-parietal NSW components were performed. For the central-parietal N170 component, the main effect of electrode [$F_{2,42}$ = 32.988, p < 0.001, η_p^2 = 0.767] was significant, and an emotion × stimulus type interaction was revealed [$F_{1,21}$ = 5.608, p = 0.028, η_p^2 = 0.211]. *Post hoc* comparison results showed that in the negative emotion condition, the N170 amplitude was stronger for faces than stickers (**Figure 3A**, p = 0.008), and no difference was found for positive emotions (p = 0.775). In the sticker condition, the N170 amplitude was stronger for negative emotions than positive emotions (**Figure 3B**, p = 0.044), and no difference was found for the facial stimuli (p = 0.369).

For the central-parietal NSW component, the main effect of stimulus type [$F_{1,21}$ = 11.521, p = 0.008, η_p^2 = 0.354] and electrode [$F_{2,42}$ = 8.305, p < 0.004, η_p^2 = 0.454] was significant. The NSW amplitude for the stickers (−1.03 ± 0.29 μV) was significantly stronger than that of the faces (−0.52 ± 3.0 μV), as illustrated in **Figures 3C,D**. No significant interactions were found.

For the probe phase, 2 (Emotion: positive, negative) × 2 (Stimulus type: face, sticker) × 3 (Electrode: F3, F4, Fz) ANOVA on the frontal LPC amplitude resulted in a significant main effect of stimulus type [$F_{1,21}$ = 8.305, p = 0.003, η_p^2 = 0.454], and a larger amplitude of the LPC for stickers (2.66 ± 0.21 μV) than faces (2.42 ± 0.19 μV) was found. A main effect for emotion [$F_{2,42}$ = 8.305, p = 0.005, η_p^2 = 0.454] was also significant; a stronger amplitude of the LPC for positive emotions (2.66 ± 0.21 μV) than negative emotions (2.42 ± 0.19 μV) was also indicated, as shown in **Figure 4**.

TABLE 1 | Mean and standard deviation of accuracy and response times.

Stimulus type	Accuracy (%)		Response times (ms)	
	Positive	Negative	Positive	Negative
Sticker	0.84 (0.12)	0.81 (0.09)	712 (171)	717 (146)
Face	0.83 (0.12)	0.80 (0.10)	732 (165)	745 (167)

Standard deviations are given in parentheses.

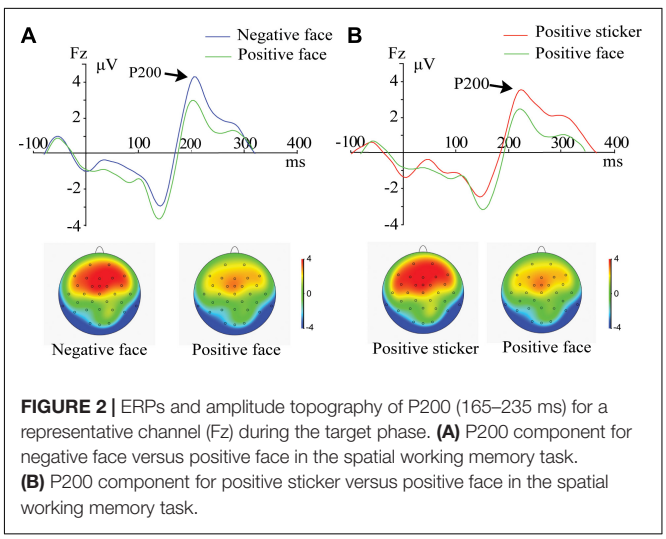

FIGURE 2 | ERPs and amplitude topography of P200 (165–235 ms) for a representative channel (Fz) during the target phase. **(A)** P200 component for negative face versus positive face in the spatial working memory task. **(B)** P200 component for positive sticker versus positive face in the spatial working memory task.

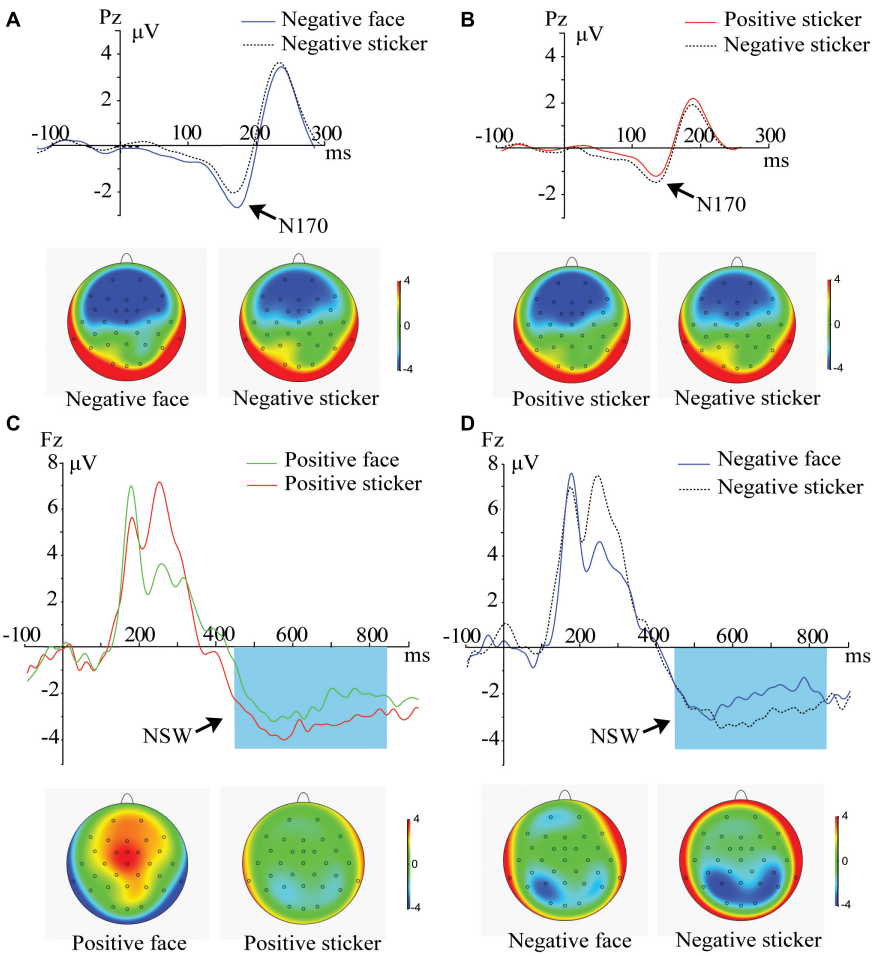

FIGURE 3 | ERPs and amplitude topography of N170 (150–200 ms) and the NSW (450–850 ms) for the representative channel (Pz, Fz) during the delay phase. Blue boxes show the averaged time interval for the NSW component. **(A)** N170 component for negative face versus negative sticker in the spatial working memory task. **(B)** N170 component for positive sticker versus negative sticker in the spatial working memory task. **(C)** NSW component for positive face versus positive sticker in the spatial working memory task. **(D)** NSW component for negative face versus negative sticker in the spatial working memory task.

DISCUSSION

The main aim of the present study was to examine the interaction effect of emotion and stimulus type on attention allocation and information retrieval in spatial WM, as well as the difference in recognition for emotional faces and stickers. With the use of DMST, we assessed stimulus and emotional effects during the encoding, retention and retrieval periods of spatial WM.

In the target phase, facial stimuli with negative emotions can capture more early attentional resources in information encoding. Behavioral results demonstrated that negative emotion can lead to less accurate task performance. Previous studies have reported similar negative biases in the attention-related P200 component (Carretié et al., 2001). The results led the authors to conclude that facial stimuli with negative emotions are more capable of capturing attention (Huang and Luo, 2006). During spatial WM, spatial attention is in demand and may result in competition between cognition and emotion for attentional resources (Postle et al., 2004). As suggested in processing efficiency theory, the effects of negative emotion on cognitive processing may be mediated by the effects on WM (Eysenck and Calvo, 1992). The negative emotional state may induce individuals to care more about anxiety responses unrelated to the current task, which would distract attention and consume the limited WM resources, resulting in prolonged RTs and a low efficiency of cognition (Shackman et al., 2006).

For the delay phase, a stronger NSW was found for the stickers than for the faces during the interval. Evidence has shown that the NSW is related to the preparation for making the response required in the probe phase (Ruchkin et al., 2010), and so an increase in the NSW amplitude represents the increase of task difficulty (Mecklinger and Pfeifer, 1996). In the present study, more effort might be required when the task is followed by sticker than a facial stimulus during the interval. The possible explanation is that the presence of a sticker increased the task difficulty relative to that of the face stimulus condition, thus inducing larger retention-related NSW amplitude. When pictures were presented, participants were required to perceive

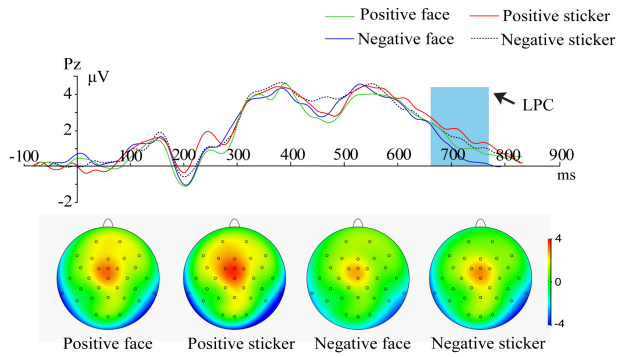

FIGURE 4 | ERPs and amplitude topography of the LPC (660–760 ms) for the representative channel (Cz) in all conditions (positive face, negative face, positive sticker, negative sticker) during the probe phase. Blue boxes show the averaged time interval for the LPC.

the emotion. However, a sticker is not always a direct labeling of emotional content; it can sometimes be ambiguous (Koto and Adriani, 2015) and thus may add to the task difficulty.

For the probe phase, a positive sticker induced a stronger LPC than a negative sticker. The increase of the LPC amplitude reflects the effect of positive emotion on the processes of information retrieval in WM. As has been proved by studies on visual processing (Ostaszewski et al., 1998), negative emotions induced participants to focus on details, while positive emotions lead participants to fix on the overall structure and ignore the details, thus damaging detail-demanding spatial WM and consuming more cognitive resources. Our results can also be supported by ERP studies, which suggested larger LPC amplitudes for positive emotions (Bublatzky et al., 2014) and decreased LPC amplitudes to aversive stimuli when compared to neutral emotional states (Thiruchselvam et al., 2011). The statistical results of the N170 component indicated that, although sharing similar facial expressions to facial stimuli, negative stickers still induced a weaker N170 component than negative faces, which illustrated the specificity of facial recognition in N170. Previous studies have provided evidence for the face specificity of N170 (Cauquil et al., 2000) and typically illustrated a smaller or absent N170 response for non-face stimuli such as printed words (Carmel and Bentin, 2002) and cars (Dering et al., 2009), which reflects the absence of category-specific effects on N170 (Itier et al., 2006) and is in agreement with our findings. At least two processing steps are required for facial recognition (Sergent et al., 1994; Martin and Amanda, 2002). The first step is linked to the structural encoding of facial features, which occurs prior to facial identification and could be modulated by physical features. The perception of facial traits has been shown to consume more cognitive resources than stickers, which may reflect the face-specific processing mechanisms in the human brain (Mccarthy et al., 1997). The second step, which is thought to be the identification of emotional expression, is the result of the configurations of various facial features (Bruce and Young, 1986) and depends on the outflow of early raw information (Ashley and Vuilleumier, 2004). In our study, for sticker stimuli, negative emotions can induce a stronger N170 component than positive emotions, which can be interpreted by the unconscious mobilization of attentional bias toward negative information (Vuilleumier and Schwartz, 2015). Negative emotional states have long been held to serve as social contextual information to elicit attentional bias and narrow the scope of people's attention and thinking (Murphy et al., 1999; Schmitz et al., 2009), in which anxiety is associated with the depletion of central executive resources and phonological resources, and can be associated with sub-vocal worry (Eysenck and Calvo, 1992). Some studies have proposed that the exogenous attention resources that have been automatically captured by negative emotions can no longer be used for other cognitive activities (Li et al., 2005), while others hold the view that visuospatial attention may be an overlapping area between negative emotion perception and spatial WM, which results in the impairment of spatial WM (Li et al., 2010a,b).

A notable finding of our study was the sustained larger attention-relevant P200 amplitude in the target phase, the stronger NSW component throughout the delay phase, and the larger LPC in the probe phase observed for stickers compared to facial stimuli. In light of this, the increased amplitude of P200 (Josiassen et al., 1982), NSW (Mecklinger and Pfeifer, 1996), and LPC (Hajcak et al., 2010; Weinberg and Hajcak, 2011) appear to have a connection with increased attention to stimuli. Moreover, the behavioral results indicated that sticker can lead to shorter RTs in task performance. Taken together, these results confirmed that stickers could capture attention throughout the entire spatial WM course. Stickers, which consist of abundant semantical and sentimental information, are widely used in daily communications to express people's feelings and create a new form of language for social media users. Moreover, stickers can also demonstrate tone, intent and feelings that normally cannot be conveyed in digital messages and act as non-verbal cues in personal communications (Alshenqeeti, 2016); that is to say, stickers might capture more attention than faces. However, on account to the common existence of attention deficit among mentally ill people, does this kind of sustained attention to stickers still occur in mental patients is yet to be investigated. Moreover, stickers can serve as "emotion indicators" that embed rich, culturally relevant meanings and can integrate reality, social context and the virtual environment (Dresner and Herring, 2010). Stickers act not only as an emotional expression in mobile messaging but also as cues that include social content and personality (Chang and Lee, 2016). Relevant emotional recognition studies on mental patients can also be further facilitated.

In summary, the present study adopted DMST to focus on distinct brain processing procedures in information encoding, storage as well as retrieval separately, and provided some electrophysiological evidence for the interaction effects of emotional valence (negative, positive) and stimulus type (sticker, face) on attention allocation and information retrieval in spatial WM. We found that negative emotions can cause lower accuracy at the behavioral level. For information encoding, face with negative emotion can catch early attention. For information storage and rehearsal, the N170 component represents facial specificity and shows a negative bias against stickers. For

information retrieval, positive emotions could damage in spatial WM and consume more cognitive resources. Moreover, stickers have the ability to catch attention on the entire course of spatial WM and lead to shorter RTs in task performance. However, several directions may be worth more research efforts. Firstly, in our study, emotional difference was only compared between negative and positive emotion for the value of amplitudes induced by certain ERP components. Further studies can add neutral emotion as baseline control condition to figure out whether there is any difference in quantity for interference or facilitating effect of emotion on spatial WM. Secondly, the sample size of this study was relatively small, which might cause failure to the discovery of interactions between emotional valence and stimulus type for NSW and LPC. Owing to this limitation, the results should to be considered preliminary, which need to be replicated in future studies with a larger sample size. Lastly, the interaction effects of emotional valence and stimulus type on attentional allocation and information retrieval in spatial WM was only studied on healthy participants preliminary. Previous studies indicated that mental diseases are often accompanied by attention deficit and emotional disorders (Mandal et al., 1998; Brandt et al., 2014). Whether the sustained attention to stickers and the impairment of positive emotion to spatial WM information retrieval that we found in our study occur to mental patients is yet to be investigated. On the basis of our study, future research can use psychopathology measures (e.g., anxiety, depression) to explore whether the sustained attention to sticker in spatial WM courses can still exist in mental patients.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of Hubei University. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

YL and WY wrote the manuscript. YL performed the experiments. SL analyzed the data. YR and WY conceived and designed the experiments. WY, SL, JC, and YR revised the manuscript and approved the final version.

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MINI REVIEW
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Effects of Electroconvulsive Therapy on Depression and Its Potential Mechanism

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Depression is one of the most common disorders causing mortality around the world. Although electroconvulsive therapy (ECT) is, along with antidepressants and psychotherapy, one of the three major treatments of depression, it is still considered as the last resort for depressed patients. This situation is partially due to limited studies and uncertainty regarding its mechanism. However, decades of increased research have focused on the effects of ECT on depression and its potential mechanism. Furthermore, these investigations may suggest that ECT should be a first-line therapy for depression due to its profound effects in relieving desperation in certain situations. Here, we outline recent clinical and preclinical studies and summarize the advantages and disadvantages of ECT. Thus, this review may provide some hints for clinical application.

Keywords: depression, ECT, neuroplasticity, Homer1a, cognition

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INTRODUCTION

Depression, the most prevalent mood disorder worldwide, has been indicated as an increasing social burden and as causing a significant proportion of mortality. Depression, occurring beyond or around and even among us, is the most common mental illness. Several hypotheses have been proposed for the cause of depression (Malhi and Mann, 2018). However, no single hypothesis can explain the full disorganization of depression or why therapeutic responses demonstrate individual differences (Malhi and Mann, 2018). Currently, there are two main treatments to fight depression, antidepressants and psychotherapy, while a third approach, electroconvulsive therapy (ECT), is regarded as a second- or third-line therapy that is usually resorted to in cases where medication and psychotherapy have failed (Kellner et al., 2016b; Karayagmurlu et al., 2019). However, most patients who were resistant to antidepressant or psychotherapy showed improvement after ECT was introduced. In other words, ECT may have a greater effect than the two routinely used methods in fighting depression (Husain et al., 2004; Spaans et al., 2015; Liang et al., 2017, 2018). Furthermore, the rate of failure in medication or psychotherapy is high and consistent (Schoeyen et al., 2015; Malhi and Mann, 2018). Therefore, further knowledge on how to treat patients resistant to these treatments is urgently needed.

Electroconvulsive therapy is a procedure that applies electrical stimulation to produce a generalized seizure. Though first introduced more than half a century ago, ECT is reserved as the last resort in treating severe mental disorders, such as depression or bipolar disorder (Husain et al., 2004; Schoeyen et al., 2015; Malhi and Mann, 2018). The reason for this difference between ECT and other remedies is partly due to its unfavorable procedure and side effects. Indeed, compared

with antidepressant drugs, induced seizures is required to deliver an electrical stimulus. Moreover, ECT recipients endure more headaches and muscle aches (Husain et al., 2004). However, when anesthesia is applied and improved apparatus, such as the unilateral device, is applied, ECT results are encouraging (Liang et al., 2018; Osler et al., 2018). Recently, extensive studies have suggested that ECT is highly effective in ameliorating depression symptoms with fewer unwanted effects (Liang et al., 2017, 2018; Karayagmurlu et al., 2019). Similar findings were also reported in animal studies using electroconvulsive seizures (ECS, an animal model for ECT) (Jonckheere et al., 2018; Alemu et al., 2019). Therefore, this review was aimed to interpret recent clinical and preclinical ECT studies and its potential mechanism on depression to provide clinicians and patients with greater appreciation of this approach to defeat depression.

SEARCHING STRATEGY AND SELECTION CRITERIA

We searched PubMed for studies published between June 1, 2010, and June 1, 2018, using the terms "depression," "depressive disorder," and "depressive disorder, major," with specifiers "therapy" as well as "ECT," "electroconvulsive shock," and "electroconvulsive seizure." In addition, we retrospectively pooled studies conducted by the CORE/PRIDE Work Group. We only included publications written in English and focused on publications from the past 5 years. We retrieved full-length articles for all the publications selected.

CLINICAL STUDIES OF ECT

Upon its introduction, more than half a century ago, ECT has been applied to benefit patients in clinical application, especially for mental disorders (Husain et al., 2004; Liang et al., 2018; Osler et al., 2018). Generally, only if medication and psychotherapy are unsuccessful is ECT considered. ECT was generally underused. Compared with ECT without anesthesia, modified ECT was found to be more durable and even favored in some circumstances deemed treatment-resistant after several rounds of medication and psychotherapy. Indeed, when compared to medication, ECT may not display performance inferior to medication, and might even have superior effects (Husain et al., 2004; Nuninga et al., 2018; Omori et al., 2019). Therefore, based on recent clinical studies, we illustrate what has been done with ECT compared with antidepressants. Differences between symptom assessment, medical imaging, and biological studies before and after ECT was applied will be discussed in this section. Furthermore, coordinated symptom assessment and medical imaging studies and predictions of remission and relapse are discussed and will be interpreted in this section.

Clinical Observation by Questionnaire Assessments

Electroconvulsive therapy is a favorable method to reduce depression symptoms regardless of whether antidepressants are involved (Husain et al., 2004; Kellner et al., 2016b). We first introduce some studies involving multi-site collaborations with relatively moderate or large samples. The Consortium for Research in ECT (CORE), supported by four collaborating clinical centers, showed a significant effect of bilateral ECT on unipolar depressed patients (Husain et al., 2004; Kellner et al., 2006). With 253 patients total enrolled without antidepressants, an acute series of ECT demonstrated a relatively high speed and rate response and remission (Husain et al., 2004). In addition, to prevent relapse, the CORE group conducted continuation ECT, indicating that continuation ECT can prevent remitted individuals from relapse and is non-inferior to combined pharmaceutical treatment (Kellner et al., 2006), and this effect seems to work regardless of racial differences (Williams et al., 2008). Of note, most CORE studies apply bilateral electrodes. Some studies sought to evaluate a unilateral electrode apparatus; the CORE group investigated the patients' response to bifrontal, bitemporal, and right unilateral electrode placement in ECT in 230 depressed patients who were randomly assigned to one of the three electrode placements. While all three groups showed significant clinical improvement, the bilateral electrode may induce a more rapid response, whereas bitemporal may perform the best with respect to symptom amelioration (Kellner et al., 2010). Though ECT is comparatively safe, studies conducted in a restricted population had assorted memory impairment that could be ascribed to ECT, especially with long-term use. Thus, a comparison of memorial practice with 24-week follow-up between continuation ECT and continuation pharmacologic intervention showed no statistical difference in memory performance (Smith et al., 2010).

Additionally, owing to the work of the CORE group, which showed the equal performance of continuation ECT and combination pharmacotherapy in preventing relapse following response to acute ECT, the Prolonging Remission in Depressed Elderly (PRIDE) group was established. The PRIDE/CORE group was established to investigate whether combined pharmacotherapy and ECT, personalized according to patient reaction, is more effective in preventing relapse in depressed older individuals than pharmacotherapy alone. For the phase I study, right unilateral ultrabrief pulse ECT combined with venlafaxine was introduced for the treatment of geriatric depression, showing that right unilateral ultrabrief pulse ECT combined with venlafaxine worked quickly and effectively against geriatric depression, with excellent safety and tolerability (Kellner et al., 2016b). Phase II participants were recruited from the remitted individuals of the PRIDE phase I study, and phase II was conducted using a novel Symptom-Titrated Algorithm-Based Longitudinal ECT (STABLE) regimen. As a result, the continuation ECT plus medication was preferable in clinical performance and did not show statistically different cognitive impairment from that of continuation medication alone (Kellner et al., 2016a), and STABLE resulted in overall net health benefits (McCall et al., 2018). As for health-related quality of life in elderly depressed patients who underwent ultrabrief-pulse ECT, an open-label study suggested that attaining remission was critical to acquiring better health quality (McCall et al., 2017).

In short, the CORE/PRIDE studies found that ECT was not inferior to and possibly had more preferable outcomes than antidepressants in certain circumstances. Although a bilateral electrode apparatus may perform better than a unilateral one, application of a special algorithm made the unilateral apparatus easier to implement in clinical practice. Continuation ECT meaningfully maintained remission and, combined with medication, may maximize the benefits of ECT without increases in memory loss (Omori et al., 2019).

In addition to CORE/PRIDE, some studies have also investigated the profile of ECT based on a clinical questionnaire assessment as to whether ECT is more likely to be inferred through subjective differences or bioinformatic measures through medical imaging or laboratory methods.

As CORE demonstrated that elder depressed individuals may respond better to bilateral ECT (Husain et al., 2004), Spaans et al. (2015) suggest that geriatric depression also responds rapidly to unilateral ECT. A double-blind, randomized controlled trial of ultrabrief- and brief-pulse unilateral EC found no differences between the two groups, and that if high dosage was applied in an ultrabrief pulse, less cognitive impairment was seen (Loo et al., 2014), and this pattern may apply to bilateral ECT as well (Martin et al., 2019). Similarly, high-dosage brief-pulse unilateral ECT may not be inferior to bitemporal ECT based on the 24-item Hamilton Depression Rating Scale (HAM-D) score, while the unilateral ECT group showed a more favorable cognitive portrait (Semkovska et al., 2016). According to a 17-year retrospective study of 1571 ECT recipients, psychiatric individuals who received ECT had lower mortality than those who did not, though nested social and physical parameters affected the results (Liang et al., 2017), and another multistate observation study showed the effect of ECT in reducing short-term readmission risk among those with server affective disorders (Slade et al., 2017). With respect to effects on memory, Brus et al. (2017) used national register-based information to inquire about the rate of subjective memory worsening (SMW) reported with ECT, suggesting that patients who were female, young, less cognitively impaired before ECT, treatment-resistant, and experiencing wider pulse width were more likely to be subject to SMW, and their SMW reported rate was not low. However, this study had such limitations as significantly heterogeneous interference, subjective report collection, and the details of the ECT procedure. Furthermore, many patients referred to ECT may complain of short-term memory deterioration (Nuninga et al., 2018) or no significant alteration compared to treatment with antidepressants (Bjoerke-Bertheussen et al., 2018; Osler et al., 2018), and for those who were afflicted with cognitive decline before ECT, evidence of cognitive improvement was presented (Socci et al., 2018). In relation to suicide, large-scale nationwide studies performed retrospectively indicate that in patients with unipolar disorder and bipolar depression, ECT had superior anti-suicidal effects (Liang et al., 2018). However, for adolescent and young adult females with a history of non-suicidal self-injury, lower odds of response and remission and great mean times of treatment were observed (Rootes-Murdy et al., 2019).

Apart from non-CORE/PRIDE, the studies above indicate that older age, male, and with baseline cognitive decline were predictors of a preferable response and less likelihood of a relapse (Socci et al., 2018; van Diermen et al., 2018). Contrariwise, depressed individuals who were young, female, and with a history of non-suicidal self-injury were more likely to be resistant to ECT (Socci et al., 2018; Rootes-Murdy et al., 2019). In addition, ultrabrief may not be superior to brief-pulse ECT in symptom improvement, but memory disturbance was less reported (Bjoerke-Bertheussen et al., 2018; Martin et al., 2019). Overall, ECT may benefit depressed patients regardless of heterogeneous backgrounds.

Clinical Observation by Medical Imaging Programs

Medical imaging programs are a powerful tool in clinical practice. Among these medical applications, magnetic resonance imaging (MRI) is more suitable for the measurement of ECT-related alteration. Regarding MRI, both structural and functional parameters were noted as useful for describing the profile of ECT. Therefore, we conducted MRI comparison at baseline and after ECT to illuminate the possible benefits of ECT. All the MRI studies were conducted in the last 5 years.

First, individuals with major depressive disorder (MDD) subject to brief-pulse bilateral ECT showed significant volume increases in the bilateral medial temporal cortices, inferior temporal cortices, and right anterior cingulate after ECT, and the increased ratio was correlated with the clinical improvement measured by the HAM-D (Ota et al., 2015). With respect to 12 treatment-resistant depressed patients who received brief-pulse bifrontotemporal ECT, bilateral medial temporal lobe and perigenual anterior cingulate cortex volume increases were archived after ECT, and left medial temporal lobe volume increase was associated with significant clinical improvement (Cano et al., 2017). The hippocampus, which plays an essential role in memory formation and emotional plasticity, was the primary focus of MRI changes. Small hippocampal volume at baseline predicted more profound symptom improvement, and the hippocampal and the amygdala volume increases with ECT were correlated with symptom improvement (Joshi et al., 2016). After a series of ECT with a predominately right placement of a unilateral electrode, the volume increases in the right hippocampal cornu ammonis (CA2/3), dentate gyrus (DG), and subiculum regions were correlated with depression reduction and the method of electrode placement (Abbott et al., 2014). In addition, the volume of the CA subfields, granule cell layer, molecular layer, and subiculum of the hippocampus increased in severe MDD patients, and is possibly attributable to neurogenesis (Cao et al., 2018). However, with 7.0 T MRI applied to detect hippocampal volume changes in MDD patients who underwent ECT, a large and significant increase was observed after ECT in only the DG of the hippocampal volume. Furthermore, the increase in DG volume was related to a decrease in depression scores (Nuninga et al., 2019). However, in a study of longitudinal MRI and clinical data from the Global ECT-MRI Research Collaboration (GEMRIC), the subcortical gray matter increase was found to be negatively associated with total ventricle volume, while total white matter

volume remained unchanged after ECT. In particular, the gray matter volumetric enlargements may not be predictive of a favorable outcome, though this was partially attributed to the heterogeneity among patients and the procedure and apparatus used (Ousdal et al., 2019). Moreover, a retrospective study revealed that though gray matter was enlarged, the changes may not correlate with psychopathology, age, gender, or number of ECT sessions (Sartorius et al., 2019), as did a longitudinal study of severe late-life unipolar depression (Bouckaert et al., 2016).

Although there remain controversies on the role of gray matter enlargement in clinical improvement and outcomes, the increased volume of gray matters in specific regions was relatively significant. As for a structural analysis, a functional algorithm may favor the use of MRI when examining the application of ECT in depression.

With regard to functional analysis, the hippocampal region remained hot. Abbott et al. (2014) found that hippocampal connectivity was enhanced after a series of ECT, and this change seems to correlate with symptom improvement and depends on how the electrode was fixed and located. Resting-state networks, believed to be neuronal activities of the brain in resting phase, showed that in patients with severe and treatment-resistant unipolar depression, the dorsomedial prefrontal cortex (including the dorsolateral prefrontal cortex, orbitofrontal cortex, and posterior cingulate cortex) and anterior cingulate cortex (including the dorsolateral prefrontal cortex, sensorimotor cortex, parahippocampal gyrus, and midbrain) had a great potential to predict the possibility of recovery from depression after ECT use (van Waarde et al., 2015). In addition, an enhanced feedforward cortical–subcortical connectivity from the fusiform face area to the amygdala was observed in MDD patients who underwent series ECT (Wang et al., 2017). Moreover, Wang et al. (2018) demonstrated that ECT may contribute to enhanced interactions in the intra- and inter-networks in MDD patients that result in symptom alleviation. Employed with perfusion MRI, ECT contributed to hippocampal cerebral blood flow increases and declines in specific regions relevant to seizure physiology. This balance was based on built-in functional neuroplasticity (Leaver et al., 2019). The fractional amplitude of low-frequency fluctuations (fALFF) can serve as a measure of the relative contribution of low-frequency fluctuations within a specific frequency band to the whole detectable frequency range. Qiu et al. (2019) revealed that the fALFF of post-ECT patients in the cerebellum anterior lobe, fusiform gyrus, and parahippocampal gyrus tended to be normalized compared with the fALFF of pre-ECT patients.

In short, according to the functional MRI studies mentioned above, ECT studies have brought renewed interest to the neuroplasticity of the brain, not only the hippocampus, but neural networks also showed more profound activity (Cao et al., 2018; Wang et al., 2018; Nuninga et al., 2019; Qiu et al., 2019).

Clinical studies have been conducted to provide evidence and suggestions for clinical practice. With respect to what is suggested by the results of the work above, we discuss the safety of ECT and the predictions of response and remission in ECT used to provide instructions for depressed individuals.

SAFETY AND EFFECTIVENESS OF ECT

Electroconvulsive therapy was shown to be a relatively safe method to treat depression and remedy treatment-resistant patients. Continuation ECT alone or continuation ECT combined with medication was favorable to remaining in a remission state after response to ECT. The CORE group revealed that ECT alone had rapid response and a high likelihood of remission with bilateral ECT in severe unipolar MDD patients (Husain et al., 2004) regardless of racial differences (Williams et al., 2008). In fact, the results of ECT exceeded those of medication of treatment-resistant bipolar depression as well (Schoeyen et al., 2015). Moreover, continuation ECT should be considered to prevent relapse (Kellner et al., 2006) without the cost of memory loss found with other treatments (Smith et al., 2010; Kellner et al., 2016a), and it was shown to have an overall net health benefit in older depressed individuals (McCall et al., 2018). In addition, besides depression, continuation ECT supported schizophrenia and schizoaffective disorder as well (Omori et al., 2019). As for electrode placement, bitemporal ECT showed rapid response, and unilateral placement may be inferior to bilateral in effects on depression (Kellner et al., 2010). However, high-dose unilateral ECT may not be inferior to bitemporal ECT and may achieve better cognitive performance (Semkovska et al., 2016). Additionally, regarding unilateral ECT, when proper dosage was applied, those receiving ultrabrief pulse suffered less cognitive decline than brief pulse without a difference in the effectiveness of depression alleviation (Loo et al., 2014; Martin et al., 2019). Moreover, the PRIDE Study showed that right unilateral ultrabrief-pulse ECT, combined with venlafaxine, performed with excellent safety and tolerability in treating geriatric depression (Kellner et al., 2016b) and improved health-related quality of life (McCall et al., 2017). In addition, ECT may reduce mortality among patients with psychiatric conditions (Liang et al., 2017) and short-term psychiatric inpatient readmissions whose symptoms were severe (Slade et al., 2017). In some urgent circumstances, suicide for example, ECT responded quickly and showed superior anti-suicidal effects in spite of unipolar or bipolar depression (Liang et al., 2018).

However, cognitive decline was noted in some depressed patients who received ECT (Brus et al., 2017), and recovery from this declination was suggested to take half a year (Nuninga et al., 2018). However, whether ECT contributed more memory loss than pharmaceutical treatment is still in dispute, but the majority support the view of no additional cognitive damage ascribable to ECT than antidepressants (Husain et al., 2004; Kellner et al., 2016a,b; Bjoerke-Bertheussen et al., 2018). Moreover, less cognitive withdrawal was seen in unilateral to bilateral (Semkovska et al., 2016) and ultrabrief pulse to brief pulse (Loo et al., 2014; Brus et al., 2017; Martin et al., 2019) when the dosage was properly applied. Nonetheless, some studies found no dementia in individuals who underwent ECT (Osler et al., 2018), and in geriatric depressed patients, ECT even improved cognitive function (Socci et al., 2018).

With respect to other common side effects that occurred during ECT treatment, headache and nausea/vomiting are believed to be the most common complaints (Kellner et al., 2006;

Karayagmurlu et al., 2019). Thankfully, in the view of severity and prevalence, the ECT recipients reported less headache and nausea/vomiting in recent studies (Husain et al., 2004; McCall et al., 2018; Socci et al., 2018). Furthermore, headache and nausea/vomiting usually recovery in a few hours without medical intervention. Lastly, anti-symptom therapy is safe if necessary.

Briefly, ECT was found to be relatively safe and effective in treating depression and may be superior to medication in symptom improvement, especially for those who failed to recover after rounds of medication (Husain et al., 2004; Schoeyen et al., 2015; Spaans et al., 2015; Slade et al., 2017). Continuation ECT should be employed to prevent relapse if possible (Kellner et al., 2006, 2016a). Unilateral ultrabrief-pulse ECT may result in less cognitive declination when advanced procedures are applied (Semkovska et al., 2016). Controversies over cognitive decline following ECT may partially be due to heterogeneous factors, such as aging and quantitative measurements. More studies should be conducted to investigate the relationship between ECT and memory plasticity.

Prediction of Response and Relapse to ECT, Bioinformatics

Comprehensive bioinformatics is assumed to be able to predict who is likely to benefit from ECT. It has been suggested that unipolar depressed patients with psychotic symptoms were more likely to respond to ECT and the elderly were more likely to reach remission (Husain et al., 2004; Socci et al., 2018; van Diermen et al., 2018). Additionally, the Maudsley Staging Method confirmed that shorter episode duration and more severe depressive symptoms predicted a favorable outcome (van Diermen et al., 2018). As a result of the overall prevalence of female depressed individuals, the number of female depressed patients who participated in ECT was greater than male (Liang et al., 2017; Slade et al., 2017; McCall et al., 2018). Interestingly, female ECT recipients may respond better in the postpartum period (Rundgren et al., 2018). In addition, estradiol withdrawal could be partially to blame, since estradiol has a protective role against depression in females (Schmidt et al., 2015) but boosts depression in young men (Stanikova et al., 2018). Furthermore, early improvement after two ECT sessions anticipated a final remission (Birkenhager et al., 2019). Based on structural and functional analysis, Abbott et al. (2014) suggested that increased hippocampal functional connectivity and volumes in MDD can predict the response to ECT, and functional-MRI-based resting-state networks predicted responses with great sensitivity and specificity (van Waarde et al., 2015). Moreover, the hippocampal subfield volumes at baseline anticipated clinical improvement when a machine learning algorithm was employed (Cao et al., 2018), and 7.0 T MRI indicated that the baseline DG may be a more specific predictor (Nuninga et al., 2019). In addition, a relatively small degree of structural impairment in the subgenual cingulate cortex seems to have responded strongly to ECT in a non-randomized prospective study (Redlich et al., 2016). In fact, with respect to treatment-resistant depressed individuals, ECT showed a rate of response and remission approaching that of antidepressants (Schoeyen et al., 2015). Njau et al. (2017) revealed that lower N-acetyl-L-aspartic acid (NAA) levels in the dorsal anterior cingulate cortex (dACC) at baseline predicted a better outcome for ECT recipients. Genetically, ECT recipients with high genetic risk load tended to be less responsive to ECT (Foo et al., 2019). On the other hand, homozygous catechol-*O*-methyltransferase (COMT) G/G genotype was more sensitive than mutant heterozygous genotype (A/G), as was Homer rs7713917 A > G (Benedetti et al., 2018). Lower promoter methylation rates of BDNF exon I were strictly associated with remission (Kleimann et al., 2015), and the CC genotype of BDNF polymorphism C270T contributed a useful response (Huuhka et al., 2007). In support of inflammation, higher levels of IL-6 at baseline anticipated profound depression relief, especially in females (Kruse et al., 2018), and the degree of change in serum matrix metalloproteinase-9 (MM-9) was associated with relapse following ECT (Shibasaki et al., 2018).

In short, in the elderly (Husain et al., 2004), psychotic symptom comorbidities (Husain et al., 2004), postpartum depression (Rundgren et al., 2018), shorter episode duration and more severe depressive symptoms (van Diermen et al., 2018), early depression alleviation (Birkenhager et al., 2019), lower NAA levels in the dACC at baseline (Njau et al., 2017), low genetic risk load (Foo et al., 2019), genetic or epigenetic modifications (Huuhka et al., 2007; Kleimann et al., 2015; Lin et al., 2015), and high baseline serum IL-6 levels (Kruse et al., 2018) and a serum MM-9 profile (Shibasaki et al., 2018) were more likely to benefit ECT recipients. Moreover, the subfield index of hippocampus and neural network plasticity (Abbott et al., 2014; Ota et al., 2015; van Waarde et al., 2015; Redlich et al., 2016; Cao et al., 2018; Nuninga et al., 2019) may provide precise evidence to predict the outcome. In addition, to the best of our knowledge, no study has reported an obvious discrepancy regarding the effectiveness of ECT between genders, as sex hormones may be linked with depressive formation, for estradiol-protected women with past perimenopausal depression (Schmidt et al., 2015) while exacerbating depressive symptoms in young males (Stanikova et al., 2018).

Here, we introduce the latest, crucial clinical studies, CORE/PRIDE for example, to demonstrate the profile of ECT utilization in clinical practice. We summarize the recent and major clinical studies in **Table 1**, which includes studies involving questionnaire assessment, medical imaging analysis, effectiveness and safety assessment, and bioinformatics for the prediction of response and relapse. According to the clinical observation studies by questionnaire assessment, ECT was a very useful tool to counteract depression and alleviate depressive symptoms. Continuation and advanced ECT was better able to maximize benefits and minimize side effects. Based on medical imaging programs, the structural and functional plasticity of the brain, especially the limbic system, was noted and probably contributed to symptom improvement. Assumedly, this neuroplasticity resulted from neurogenesis and is associated with ECT response and effectiveness. As for its safety and effectiveness, not only can ECT improve depressive symptoms, but its effects may be superior to those of classical psychotherapy and antidepressants in exclusively treatment-resistant depression. However, evidence of cognitive impairment is not conclusive; ECT was still

TABLE 1 | Summary of major ECT clinical studies.

Study design	Parameters	Major findings	References
TRD + previous medication + a-ECT ± c-ECT	Right unilateral + unknown	Increased BDNF and not significantly correlated with clinical improvement	Vanicek et al., 2019
TRD + previous medication + a-ECT ± c-ECT	Bifrontotemporal + brief pulse	Use of mood stabilizers and maintenance ECT to prevent relapse	Omori et al., 2019
MDE + a-ECT	Bitemporal + brief/ultrabrief pulse	Equal antidepression, better cognitive performance for ultrabrief pulse	Martin et al., 2019
MDE + a-ECT	Bitemporal + brief pulse	Early symptoms improvement predicted quicker remission	Birkenhager et al., 2019
MDE + a-ECT + MRI	Bilateral/unilateral + brief pulse	Gray matter volume increases not correlated with clinical improvement	Sartorius et al., 2019
MDD + a-ECT + MRI	Bitemporal + brief pulse	Enhanced functional plasticity in specific brain regions (normalized fALFF)	Qiu et al., 2019
Depression + MRI	Bifrontotemporal + unknown	Increase in DG volume, correlated with clinical improvement	Nuninga et al., 2019
MDD + a-ECT + polygenic risk score	Right unilateral + brief pulse	High polygenic risk score anticipated poor outcome	Foo et al., 2019
MDD + antidepressants + a-ECT + MRI	Bifrontal + unknown	Enhanced intra- and internetwork plasticity within the response	Wang et al., 2018
Depression + previous medication + a-ECT	Bilateral/unilateral + brief pulse	Shorter episode duration, more severe depression, and older age predicted ECT effectiveness	van Diermen et al., 2018
Postpartum depression and psychosis + ECT	Bilateral/unilateral + unknown	Postpartum depression and psychosis responded greatly to ECT	Rundgren et al., 2018
MDD + ECT + MRI	Bilateral/unilateral + unknown	Hippocampal volume increases not related with symptom improvement, greater increased indicated poor outcome	Oltedal et al., 2018
Depression + a-ECT/c-ECT	Bifrontotemporal + unknown	Bilateral ECT showed cognitive decline that recovered in 6 months	Nuninga et al., 2018
MDD + c-ECT	Right unilateral ultrabrief pulse	Overall net health benefit	McCall et al., 2018; Rundgren et al., 2018
TRD + a-ECT	Bilateral/unilateral + brief pulse	Baseline serum IL-6 level predicted response	Kruse et al., 2018
Severe MDD + a-ECT + MRI	Bilateral + unknown	Machine learning algorithm to the hippocampal subfield volumes at baseline to predict response	Cao et al., 2018
MDD + previous medication + a-ECT + MRI	Bifrontal + unknown	Enhanced the feedforward cortical subcortical connectivity from FFA to amygdala	Wang et al., 2017
Unipolar/bipolar depression + a-ECT + MRI	Unilateral + ultrabrief pulse/bilateral + brief pulse	Lower baseline NAA in the dACC predicted favorable outcome	Njau et al., 2017
Older unipolar MDD + previous therapy + a-ECT	Unilateral + ultrabrief pulse	Health-related quality of life improved regardless of cognitive impairment in short term	McCall et al., 2017
TRD + previous medication + a-ECT + MRI	Bifrontotemporal + brief pulse	Left MTL volume increase associated with hippocampal NAA decrease, Gla + Gln increase, and clinical improvement	Cano et al., 2017
Depression + a-ECT	Bitemporal/high dose unilateral + brief pulse	Non-inferior high dose unilateral and less cognitive decline	Semkovska et al., 2016
Depression + antidepressants + a-ECT + MRI	Bilateral/unilateral brief pulse	Small degree of structural impairment baseline in the subgenual cingulate cortex predicted better outcome	Redlich et al., 2016
Remitted a-ECT patients + c-ECT + medication	Unilateral + brief pulse	c-ECT + venlafaxine + lithium surpassed venlafaxine + lithium only	Kellner et al., 2016a
MDE + previous therapy + a-ECT	Unilateral + ultrabrief pulse	a-ECT + venlafaxine to effectiveness and safety	Kellner et al., 2016b
Depression + a-ECT	Bifrontal/bitemporal/unilateral + unknown	Bitemporal electrode responded quicker	Kellner et al., 2010
Remitted unipolar MDD patients + c-ECT	Bilateral + unknown	c-ECT equal to antidepressants to prevent relapse	Kellner et al., 2006
MDD + a-ECT	Bilateral + unknown	Rapid response and remission of ECT	Husain et al., 2004

MDD, major depressive disorder; MED, major depressive episode; TRD, treatment-resistant depression; ECT, electroconvulsive therapy; a-ECT, acute ECT; c-ECT, continuation ECT, NAA: N-acetyl-aspartate; MRI, magnetic resonance imaging; BDNF, brain-derived neurotrophic factor; fALFF, amplitude of low frequency fluctuations; FFA, fusiform face area; DG, dentate gyrus; dACC, dorsal anterior cingulate cortex; MTL, medial temporal lobe.

preferable in improving life quality, memory decline was not exclusive to ECT and was worse with antidepressants (Bjoerke-Bertheussen et al., 2018), and cognitive status was restored in the short term (Nuninga et al., 2018). In addition, for the depressed patients who had cognitive impairment at baseline, ECT may improve cognitive performance (Kellner et al., 2006; Osler et al., 2018). However, there were several social and physical factors, medical insurance coverage and physical tolerance for example,

that impede patients in receiving ECT. Therefore, ECT is a very useful tool to counter depression and should not be perceived as the last line for depressive treatment (Husain et al., 2004; Liang et al., 2017).

ECS in Preclinical Studies

ECS is an experimental animal model of ECT (Jonckheere et al., 2018). ECS successfully improved depression- or stress-associated performance by preventing depression- and stress-induced damage (Kyeremanteng et al., 2014; Luo et al., 2015; Schloesser et al., 2015; Jonckheere et al., 2018; Alemu et al., 2019). Notwithstanding the heterogeneity of clinical ECT research, major animal studies supported that ECS can induce neurogenesis, and the neuron or glial cell loss or malfunction was compensated by neurogenesis (Kaastrup Muller et al., 2015; Schloesser et al., 2015; Jonckheere et al., 2018). In brief, ECS-induced neurogenesis was the primary mechanism to encounter depression.

Behavioral analysis was recorded to demonstrate depressing activities in animal models and behavioral changes after undergoing ECS. Rats treated with chronic unpredictable mild stress (CUMS), which caused them to display depression-like behavior, exhibited decreased sucrose preference percentage (SPP) and impaired performance on the open field test (OFT), forced swim test, novelty suppressed feeding test (NSF), and Morris water maze, and ECS inversely increased SPP and OFT activities (Luo et al., 2011, 2014; Zhu et al., 2015a,b; Gao et al., 2016; Zhang et al., 2016). Similarly, in the Wistar–Kyoto (WKY) rat strain, a genetic model displaying depression- and anxiety-like behaviors and working memory deficit, ECS improved psychiatric and memory behaviors (Kyeremanteng et al., 2014).

Monoamine malfunction, hypothalamic–pituitary–adrenal (HPA) axis dysfunction, inflammation, disturbed neuroplasticity, and neurogenesis are believed to cause depression (Malhi and Mann, 2018). With respect to ECS, regulation of the HPA axis, neuroplasticity, and neurogenesis were mostly demonstrated in the animal model of depression-like behavior. Typical depression displayed a hyperactive HPA axis profile. Regarding depression, increased cortisol level is critical (Grinevich et al., 2012). Typical depression exhibited increased levels of adrenocorticotropic hormone (ACTH) and/or corticotropin-releasing hormone (CRH) without proper regulation by the hippocampus (Stetler and Miller, 2011). Additionally, downregulated glucocorticoid receptors were blamed to decrease the effectiveness of cortisol on target tissues (Zunszain et al., 2011; Chiba et al., 2012). The ineffectiveness of glucocorticoid inversely enhanced inflammation (Zunszain et al., 2011). Moreover, brain CRH was elevated following repeated ECS independent of depressive behavior. However, ECS did alleviate depressive symptoms (Kyeremanteng et al., 2014).

Regarding neurogenesis, adult hippocampal neurogenesis was essential to buffer stress responses and depressive behavior, while for neurogenesis-deficient mice, increased food avoidance in a novel environment after acute stress, increased behavioral despair in the forced swim test, and decreased SPP were shown compared with neurogenesis-normal mice (Snyder et al., 2011). In addition, Madsen et al. (2005) reported that ECS increased the number of new dividing cells in the frontal cortex, and the dividing cells were believed to be either oligodendrocytes or endothelial cells but not to express neurons. Furthermore, retrieved volume loss in the frontal cortex was compensated by cell proliferation (Madsen et al., 2005). The corticosterone-induced anxiety- and depressive-like behavior was rescued by the hippocampal neurogenesis of the adult-born neurons after ECS, while for the animals that were genetically deficient in neurogenesis of adult-born neurons, ECS cannot relieve behavioral deficiency. Thus, intact hippocampal neurogenesis was required for ECS to confront depression (Schloesser et al., 2015). Additionally, neuroplasticity triggered or enhanced by ECS administration significantly increased the number of mitochondria, synapses, and length of microvessels in depressed rats regardless of response (Chen et al., 2018). Employed with MAP6 KO mouse, a genetic model of depression, Jonckheere et al. (2018) demonstrated that synaptogenesis and neurogenesis compensated for behavioral deficits. ECS enhances the proliferation of adult hippocampal neuronal progenitors, and continuation ECS led to persistent behavioral and biological improvements. Moreover, volume increases in the hippocampus were specific after ECS applied to the animal model of depression-like behavior (Alemu et al., 2019). On the other hand, BDNF is a neurotrophin related to canonical nerve growth that plays a critical role in neurogenesis and neuronal plasticity. The expression of BDNF was significantly elevated in animal models of depression-like behavior after undergoing ECS (Zhang et al., 2016; Chen et al., 2018; Jonckheere et al., 2018). The effect of confronting depression needed to incorporate studies of mitochondria and synapses (Chen et al., 2018). However, in a genetic animal model of depression, though brain BDNF increased instantly after ECS was applied, it normalized after repeated BDNF. Contrariwise, repeated ECS consistently increased brain BDNF level in depression-free animals (Kyeremanteng et al., 2014). In conclusion, ECS has the ability to increase the levels of BDNF, though depression may mitigate against increases in BDNF.

With respect to cognitive performance, based on the CUMS animal models that reproduced depression, ECS deteriorated cognitive performance in animal models via (1) downregulating the ratio of hippocampal glutamate (Glu) and γ-aminobutyric acid (GABA) levels by promoting excessive expression of glutamic acid decarboxylase 65 (GAD65) (Luo et al., 2011); (2) dysregulating hippocampal synaptic plasticity, specifically downregulating long-term potentiation (LTP), postsynaptic density-95 (PSD-95), and phospho–response element binding protein (p-CREB) protein expression (Luo et al., 2014); (3) inducing inflammatory cytokine-mediated glutamate uptake dysfunction in the hippocampus (Zhu et al., 2015a); (4) promoting neuroinflammation and increasing the levels of Aβ1–40 and Aβ1–42 in the hippocampus (Zhu et al., 2015b); (5) dysregulating the NMDA receptor subunit 2B (NR2B)-(extracellular signal-regulate kinase) ERK-signaling pathway (Gao et al., 2016); and (6) up-regulating the ratio of the precursor of brain-derived neurotrophic factor (proBDNF)/mature BDNF (mBDNF) (Zhang et al., 2016). However, ECS caused different memory indexes between types of animal models (Luo et al., 2015). Moreover, contrary to

other CUMS cognitive performance, ECS improved memory function in CUMS compared with the baseline cognitive index (Luo et al., 2015). However, ECS under anesthesia with propofol, dexmedetomidine, or ketamine was able to rescue ECS-exacerbated dysfunction to improve memory performance (Luo et al., 2011, 2014; Zhu et al., 2015a,b; Gao et al., 2016; Zhang et al., 2016). In spite of the effectiveness of anesthesia plus ECS in enhancing cognitive performance without undermining ECS effectiveness, modified ECT caused less cognitive concern in clinical studies, and even older depressed patients with cognitive decline retrieved memory after undergoing ECT (Osler et al., 2018; Socci et al., 2018). Moreover, modified ECT, ECT programmed with anesthesia, is commonly applied in clinical procedures and is widely used (Kellner et al., 2006, 2016a,b; Semkovska et al., 2016; Bjoerke-Bertheussen et al., 2018), but anesthetic was not deemed essential to prevent memory loss because modified ECT still conceived cognitive impairment (Kellner et al., 2010; Loo et al., 2014; Bjoerke-Bertheussen et al., 2018). However, there was no comparison of cognitive characters between pure ECT and ECT programmed with anesthesia. Thus, it is unknown whether anesthetic in fact aids against ECT-induced cognitive impairment.

Recent research has investigated the profile of ECS. As shown in **Table 2**, ECS alleviated depression-like symptoms in general, and the major animal models worked on were CUMS, and among models, memory changes may vary.

THE POTENTIAL MECHANISM OF ECS

With regard to the mechanism of ECS on neurogenesis, the increased volume of specific regions of the brain with the application of ECS has been demonstrated. This increase was associated with improved behavior and neuroplasticity (Madsen et al., 2005; Kyeremanteng et al., 2014; Luo et al., 2015). Homer-1, or homer protein homolog 1, consists of two major splice variants, short-form (Homer1a) and long-form (Homer1b/c) (Shiraishi-Yamaguchi and Furuichi, 2007). Homer1 is widely expressed in the central nervous system and constitutes a major part of the postsynaptic density. Homer1 links metabotropic glutamate receptors (mGluRs) and regulates their downstream pathway. Homer1a is an instant splice variant induced by neuronal activity to compete for mGluRs with long-term Homer1b/c. The balance between Homer1a and Homer1b/c determined neuronal plasticity: If Homer1a is dominant, the neurons show homeostatic plasticity, while neurons tended to be activated when Homer1b/c was dominant (Shiraishi-Yamaguchi and Furuichi, 2007; Hu et al., 2010). Moreover, Homer1 is predominantly located in the CA1 region of the hippocampus. Homer1a was transcriptionally induced only upon neuronal stimulation, as in seizures, for example (Shiraishi-Yamaguchi and Furuichi, 2007; Kaastrup Muller et al., 2015). Elevated Homer1a in the hippocampus enhances α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor clustering and its synaptic transmission to aggregate the AMPA receptor-dependent excitatory postsynaptic potential (EPSC) without altering presynaptic glutamate release (Hennou et al., 2003). Moreover, Homer1a regulates the mGluR-IP3 signaling pathway and induces hyperpolarization in pyramidal neurons to establish reduced excitability and maintain homeostatic neuroplasticity (Hu et al., 2010). In general, Homer1a dominantly provides negative signals as feedback to enhanced excitivity of neurons. In agreement with region-specific theory, adaptive changes to chronic stress and morphological discrepancies are observed compared with normal animals and are attenuated following ECS (Kaastrup Muller et al., 2015). ECS-induced Homer1a helped to rescue the reductions in the total length of apical dendrites and the number of apical terminal branches of CA3c neurons. The balance between Homer1a and spinophilin contributed to the molecular compensation induced by ECS (Kaastrup Muller et al., 2015). Homer1a caused a short disruption in mGluR1/5 and Homer1b/c complex during its relatively short effect. The plasticity of the ratio of Homer1a/1b/c exhibited different neuronal activities in the respective regions and responded to different stimulations. For example, in reaction to chronic stress, though Homer1b/c overexpressed, its coupling with mGluR1/5 was reduced in the hippocampus. Initially, stress induced pro-activated AMPA receptors and decreased the activity of mGluRs. It has been reported that enhanced expression of Homer1a in the medial prefrontal cortex (mPFC) demonstrated an antidepressant effect, while decreased Homer1a enhanced depressive-like behavior (Serchov et al., 2015). Contrariwise, overexpression of Homer1a in the hippocampus promoted vulnerability to stress (Kaastrup Muller et al., 2015). Additionally, Homer1 regulated the HPA axis independent of mGluR1/5. Homer1a modulated mGluR1/5-mediated excitatory postsynaptic currents and interacted with the NMDA receptor to produce a rapid-acting antidepressant effect (Wagner et al., 2015). Homer1a expression is the final way of mediating the antidepressant effects of different antidepressant treatments. Though Homer1a counteracts Homer1b/c, the role of the different splice variants provided different ways of internal environment changes. However, questions remain: What is the upstream signal applied to Homer1? What is the profile of the receptor distribution of mGluR1/5 and AMPA in subsequent neuroplasticity? What causes the discrepancy of memory behavior between animals and depressed patients? Though mGluRs activated neurogenesis, the levels of BDNF and the role of BDNF after ECS or ECT are still matters of intense debate.

Therefore, **Figure 1** shows that ECS remodels neuroplasticity by mediating the balance between mGluR1/5 and AMPA receptors. ECS induced a fast-response antidepressant effect. Upon ECS application, the presynaptic glutamatergic neurons are activated while GABAergic neurons are inhibited. Glutamate is then released into the synaptic cleft and AMPAR is activated. Meanwhile, NMDAR is inhibited. Later, AMPAR releases BDNF to interact with TrkB. Next, activated Akt passes the signal to mTORC1 and promotes neurogenesis. On the other hand, Homer1 disrupts dysfunctional Homer1b/c and mGluR1/5 complexes and partially opens the BK channel by IP3R-released Ca^{2+}. The BK channel leads the hyperpolarization of the postsynaptic neuron to present the antidepressant effect.

TABLE 2 | Effect of ECS on depression in the preclinical studies.

Animal	Model	Test	Results	References
Male Wistar rats	Chronic restraint stress	FST	Normalized the volume of the hippocampal hilus to control levels; Normalized depression-like behavior in the FST.	Alemu et al., 2019
MAP6 KO model	Genetic	FST; NSF	Decrease the immobility time; increased the time spent climbing; Increased latency to eat.	Jonckheere et al., 2018
Male albino Swiss mice	Forced swim test	FST	Decreased the immobility time.	Socala et al., 2017
Adult male Sprague–Dawley rats	CUMS	SPT; MWM	Increased the percentage of sucrose preference, the total distance traveled, and the frequency of rearing.	Li et al., 2016
Male Sprague–Dawley rats	CUMS	SPT; MWM	Increased the values of SPP; Decreased space exploration time.	Zhang et al., 2016
Adult Sprague–Dawley rats	rECS	MWM	Increased the time reach the platform; Decreased the staying time and crossing time.	Zhu et al., 2015b
Adult male Sprague–Dawley rats	CUMS	SPT; MWM	Decreased the SPP of the rats with CUMS; Increased the SPP values of the rats.	Zhu et al., 2015a
Adult male Wistar rats and WKY rats	CUMS	SPT; MWM	Impaired WKY rats' memories but improved CUMS rats' memories; Elevated hippocampal BDNF and CREB proteins only in CUMS rats.	Luo et al., 2015
Adult male Wistar rats	CUMS	SPT; MWM	Exacerbated the memory damage; When administered in modified ECS, propofol improved memory.	Luo et al., 2014

MFB, medial forebrain bundle; DG, dentate gyrus; UPLC, ultra-performance liquid chromatography; FST, forced swim test; NSF, novelty suppressed feeding test; SPT, sucrose preference test; SPP, sucrose preference percentage; MWM, Morris water maze; CUMS, chronic unpredictable mild stress; rECS, Repeated electroconvulsive shock. MDD, major depressive disorder; MED, major depressive episode; TRD, treatment-resistant depression; ECT, electroconvulsive therapy; a-ECT, acute ECT; c-ECT, continuation ECT; NAA, N-acetyl-aspartate; MRI, magnetic resonance imaging; BDNF, brain-derived neurotrophic factor; fALFF, amplitude of low frequency fluctuations; FFA, fusiform face area; DG, dentate gyrus; dACC, dorsal anterior cingulate cortex; MTL, medial temporal lobe.

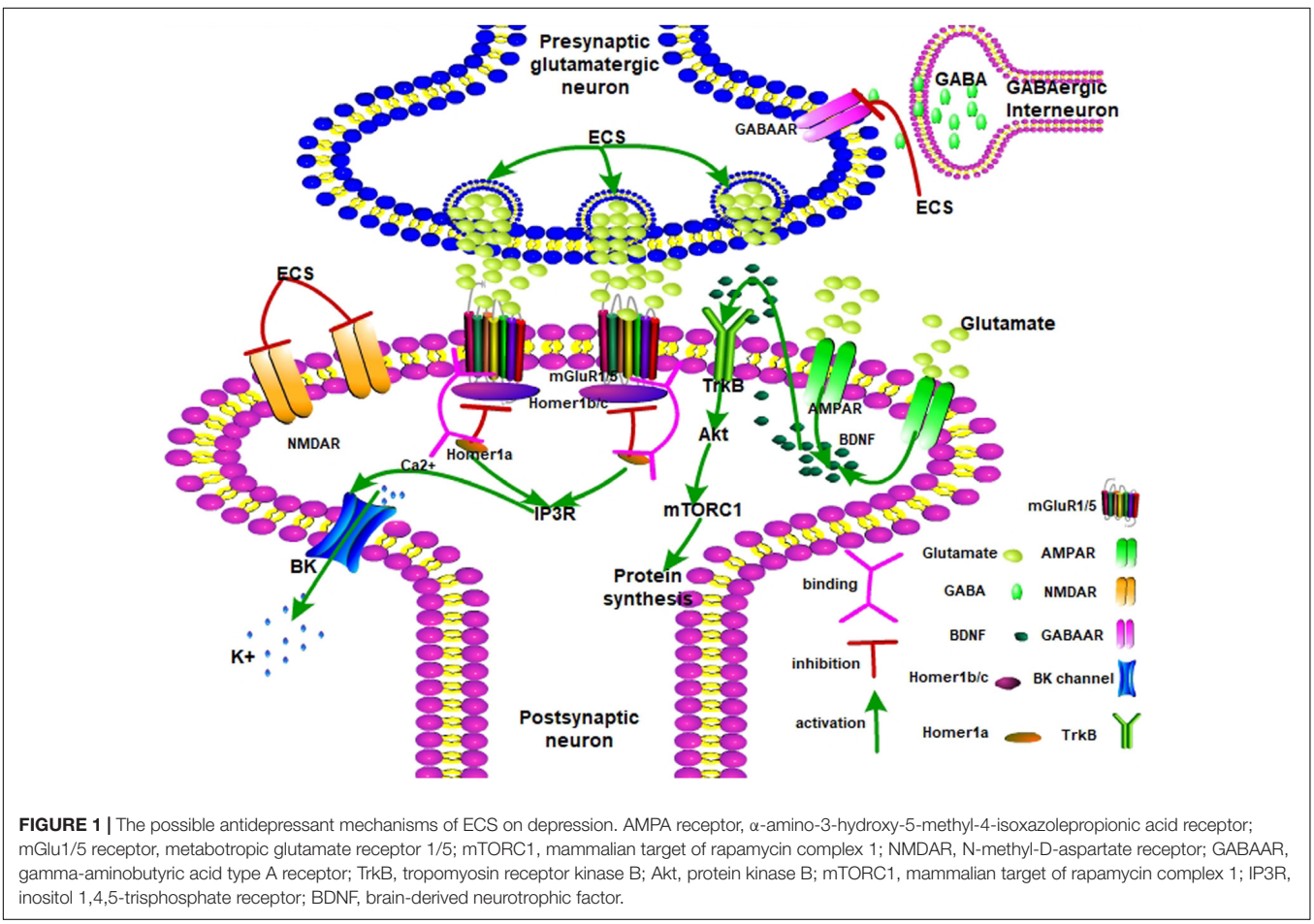

FIGURE 1 | The possible antidepressant mechanisms of ECS on depression. AMPA receptor, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor; mGlu1/5 receptor, metabotropic glutamate receptor 1/5; mTORC1, mammalian target of rapamycin complex 1; NMDAR, N-methyl-D-aspartate receptor; GABAAR, gamma-aminobutyric acid type A receptor; TrkB, tropomyosin receptor kinase B; Akt, protein kinase B; mTORC1, mammalian target of rapamycin complex 1; IP3R, inositol 1,4,5-trisphosphate receptor; BDNF, brain-derived neurotrophic factor.

DISCUSSION

With regard to clinical utilization of ECT, many studies favor its effectiveness and relative safety. ECT had been introduced to treat diseases for decades, leading more researchers to investigate modified current ECT and explore its clinical indication. Without question, ECT alleviated depressive symptoms. Previously, ECT is mainly applied to treatment-resistant depressed patients, though it is also employed for other mental disorders. ECT successfully improved some patients' symptoms independent of antidepressants (Husain et al., 2004; Loo et al., 2014), while when incorporated with certain antidepressants, sedatives for example, they mutually enhanced their antidepressive effects (Wang et al., 2018). Compared with ECT, continuation ECT is recommended for its ability to prevent relapse in those who were remitted following ECT (Kellner et al., 2006, 2016b). With respect to cognitive characters before and after ECT, no evidence indicates that ECT induces greater cognitive decline than antidepressants do (Kellner et al., 2006; Bjoerke-Bertheussen et al., 2018; McCall et al., 2018; Nuninga et al., 2018). However, memory impairment is common among depressed patients (Malhi and Mann, 2018). However, no studies have confirmed differences in cognitive profiles between patients, partially due to the heterogeneity of the clinical demonstrations. Moreover, although more advanced protocols were introduced, bioinformatics yielded predictions of the response of ECT that are scattered and lack comprehensive measurements (Husain et al., 2004; Redlich et al., 2016; van Diermen et al., 2018; Birkenhager et al., 2019; Foo et al., 2019; Nuninga et al., 2019; Omori et al., 2019). Additionally, even though ECT has been shown to be harmless and effective, the application rate is still low and it is considered the last resort in treating depression (Liang et al., 2017; Osler et al., 2018). Therefore, ECT should be more broadly used in treating depression, and more research focusing on how to predict responses in respective patients and how to enhance ECT currently in use is needed.

On the other hand, recent ECS studies have devoted greater effort to determining the mechanism behind the antidepressant effect. Similarly, ECS decreased depression-like behaviors and rescued molecular adaptive alteration responses to depression (Madsen et al., 2005; Kyeremanteng et al., 2014; Schloesser et al., 2015). However, there were discrepancies among model strains and behavioral reactions to exogenous stimulation (Kyeremanteng et al., 2014). Moreover, no single model can mimic depression in patients, due to the not fully disclosed mechanism of depression. In brief, the mechanism of depression remains under investigation, and while the effects of ECS on depression have been partially revealed, much remains unknown.

AUTHOR CONTRIBUTIONS

LZ, WX, HZ, ZC, ML, and FZ wrote the first draft. XY, LS, and XZ made major revisions to the logic of this article. BL, WY, and RC participated in the discussion of the manuscript. RC provided the critical revisions. All authors approved the final version of the manuscript for submission.

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Dissociable Posterior and Anterior Insula Activations in Processing Negative Stimulus Before and After the Application of Cognitive Reappraisals

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Although the role of the insular cortex in representing bodily and emotional feelings has been recognized, whether the mid-posterior and anterior parts of the insula act differentially in the encoding and regulation of emotional feelings is still unclear. In this functional magnetic resonance imaging (fMRI) study, we examined the effects of the creative cognitive reappraisals versus the non-creative ordinary reappraisals on the activation pattern of the mid-posterior and anterior insular cortex during the processing of unpleasant pictures by comparing the neural correlates for processing these pictures before and after the application of cognitive reappraisals. We found significant anterior insular activation after the application of cognitive reappraisals, especially the creative ones, in contrast to the significant mid-posterior insular activation before the application of the cognitive reappraisals or after the application of the non-creative ordinary reappraisals. This finding supports the posterior-to-anterior progression hypothesis with the mid-posterior insular cortex being used for the encoding of primary emotional feelings and the anterior insular cortex being used for the encoding of regulated or modulated emotional feelings.

Keywords: insular cortex, cognitive reappraisal, creative cognitive reappraisals, emotion regulation, effectiveness

INTRODUCTION

Cognitive reappraisal, which is a cognitive and linguistic strategy for altering the trajectory of emotional responses by reformulating the meaning of a situation, has been widely studied and applied in emotion regulation (Gross and John, 2003). Although previous studies have identified multiple subcomponents of cognitive reappraisal, which mainly include the cognitive brain mechanisms for cognitive control (i.e., the lateral, ventral, and medial prefrontal cortex as well as the anterior cingulate cortex) and emotional arousal and feelings (i.e., the amygdala and insula) (Ochsner and Gross, 2008), the neural correlates of the changes produced by the reappraisal regulation in the bodily and emotional feelings, which could be theoretically regarded as one of the two essential components that constitute a given emotionality in addition to the component

of motivations, induced by cognitive reappraisal are still unknown. There is converging evidence from the imaging of healthy (Critchley et al., 2004) and damaged brains (Wang et al., 2019) that demonstrates the critical role of the insular cortex in representing interoceptive attention and awareness of internal visceral responses, implying the insular cortex could be the key substrate for embodying the emotional feeling states. In particular, the insular activations have been widely observed during the application of emotion regulation strategies including both the explicit and implicit strategies (Diekhof et al., 2011; Langner et al., 2018; Picó-Pérez et al., 2019). More importantly, according to the posterior-to-anterior progression hypothesis on the hierarchical functional organization of the insular cortex (Craig, 2002, 2009), the posterior, middle, and anterior parts of the insula cortex are responsible for encoding primary bodily and emotional feelings, for mental re-representation of these feelings integrated with external context information, as well as for continuously updating introspective awareness of the present emotion and bodily states, respectively. Consistent with this theory, previous studies have demonstrated the double dissociation of activation in the mid-posterior and anterior insular cortex in the processing of sexual desire versus love (Cacioppo et al., 2012), primary versus secondary/modulated moral disgusts (Ying et al., 2018), and empathy and sympathy during childhood versus adulthood (with the latter showing increased frontalization and top–down modulation ability, Decety and Michalska, 2010).

The aforementioned posterior-to-anterior progression hypothesis (Craig, 2009) provides an ideal framework to formulize the neural encoding or representation of the changes in emotional feelings that accompany cognitive reappraisal applications. This is primarily because of the well-known role of the insular cortex in the representation of bodily and emotional feelings as well as the distinctive function of the posterior and anterior insula in the representation of the primary and the modulated bodily and emotional feelings. Prior to cognitive reappraisal application, it is possible that one's initial representation of emotional feelings toward a negative stimulus might be relatively more intensively represented in the posterior insular cortex, which is responsible for the primary mental representation of emotional feelings. However, after application of cognitive reappraisal, the mid-posterior insular activation reflecting primary emotional feeling toward the negative stimulus might "move forward" to the anterior insula since the reinterpretation of the negative stimulus could result in a updated format of the emotional feeling representation with integration of the new meaning implied by reappraisal; in other words, after the application of emotion regulation, the anterior insular cortex may be more involved in the encoding or updating of the introspective awareness of emotional and bodily feeling states. In supporting of this possibility, on the one hand, previous studies have identified the role of the posterior insular cortex in encoding and regulating bodily and emotional feelings, including the aversive ones (e.g., Gehrlach et al., 2019), of both physical and social stimulus (e.g., Gao et al., 2018). On the other hand, the role of the anterior insular cortex in emotion regulation, including cognitive reappraisals, has also been consistently proved by previous studies (Diekhof et al., 2011; Langner et al., 2018; Picó-Pérez et al., 2019). Our recent study, which used a novel emotion regulation approach of "sadness counteract anger" (i.e., the pre-induced sadness emotion could reduce the later anger and aggressive behavior) and "fear promote anger" (i.e., the pre-induced fear emotion could enhance the later anger and aggressive behavior), found that the underlying mechanisms for the efficiency of this approach could be related to the effects of the pre-induced sadness could enhance the activation level in the posterior insular cortex (and thus enhance one's internally orientated information processing tendency, which would reduce the externalized aggressive behavior), and the pre-induced fear emotion could enhance the activation level in the anterior insular cortex (and therefore facilitate an externalized angry expression), thus proving the possibility of making a double dissociation of the posterior and anterior insular cortex involvement during the application of different emotion modulation approaches (Zhan et al., 2018).

In order to explore how cognitive reappraisal could alter the emotional feelings embodied in the insular cortex, each trial of cognitive reappraisal of the target unpleasant picture consisted of the initial viewing of the target picture alone, the application of the cognitive reappraisal on the target picture, the re-watching of the target picture, and the final evaluation or appraisal of one's subjective emotion. We examined our key prediction on the dissociation of the anterior and mid-posterior insular cortex by comparing the first- and the third-time passive viewing of the negative target pictures (i.e., before and after the application of reappraisals) and by comparing the consequences of different types of cognitive reappraisals.

We used three different types of reappraisals. In the objective description (OD) condition, the participants read sentences that just objectively described the contents of the negative pictures without any attempt at reappraisal regulation (baseline condition). In the ordinary reappraisal (OR) condition, the participants read reappraisal sentences that adopted the commonly used, non-creative reappraisal strategies, such as "they are getting help" (explicitly positive) or "the tragedy would never happen again" (changed future circumstances), to downregulate the elicited unpleasant emotion (McRae et al., 2012). In the creative reappraisal (CR) condition, the participants read reappraisal sentences that had an unexpected and insightful perspective that were both novel and appropriate and allowed for the reinterpretation of the meanings or implications of the pictures (for example, to reinterpret the vomit in toilet as a woman who wanted to have a baby surprisingly found herself to be pregnant).

The final type of creative cognitive reappraisal strategy was developed based on our recent study that found a significant correlation between the creativeness rating of the reappraisal and its efficiency (Wu et al., 2017). We therefore proposed a creative cognitive reappraisal theory (Wu et al., 2019) that suggested that the previously applied ordinary cognitive reappraisal strategies were too common or "mediocre" to efficiently overcome one's emotional response bias, which is deeply rooted in one's information-processing tendency toward negative situations. This could resemble the situation in creative insight problem

solving where one's mental fixation on the old way of thinking could prevent one from finding novel and suitable solutions. We therefore suggested that truly creative and insightful cognitive reappraisal could greatly improve the unfavorable mental representation toward negative stimuli. Consistent with this hypothesis, our recent study found that creative or insightful reappraisal could produce a regulation effect size (Cohen's d, a measure of the effectiveness of emotion regulation) of 3.49, which was significantly higher than that reported in previous meta-analyses on cognitive reappraisal (all less than 0.95) (Augustine and Hemenover, 2009; Webb et al., 2012). Moreover, our previous study showed that the creative or insightful cognitive reappraisal resulted in an emotional rating that was above the neutral emotion level for standardized International Affective Picture System (IAPS) negative pictures (this means the CRs was able to turn the negative pictures to be perceived as positive) and that the application of this strategy was associated with robust positive activation in the amygdala, hippocampus, and ventral striatum, implying this change could be mediated by the enhanced activation in the reward circuits (Wu et al., 2019).

In this study, we compared the involvement of the mid-posterior and anterior insular cortex in the processing of standardized negative IAPS stimuli before and after the application of different types of cognitive reappraisal with different emotion regulation effectiveness. We predicted that cognitive reappraisal would alter the participants' emotional feelings, which are essentially represented and re-represented in the insular cortex, in a manner that was consistent with the posterior-to-anterior progression hypothesis of this area (Craig, 2009) and that this tendency would be associated with the effectiveness of the emotion regulation strategy. By this we mean the CR strategy, which was found to be more effective in downregulating the negative emotion arousal associated with the processing of unpleasant IAPS pictures, would be more capable in making such a posterior-to-anterior insular activation progression than the ORs and the objective descriptions (CD) conditions, which were found to be less effective in doing so.

MATERIALS AND METHODS

It should be noted that some of the results of this study have been published (Wu et al., 2019); however, this paper reports the results of different experimental data. As previously mentioned, each experimental trial of cognitive reappraisal of the negative IAPS pictures consisted of four information-processing steps: the first-time picture viewing (Step 1), the application of reappraisal on the target pictures (Step 2), the second-time picture viewing (Step 3), and the final emotion rating (Step 4). In our previous study, we analyzed and reported the imaging data in Step 2 (Wu et al., 2019). In this study, we compared the insular activation in Step 1 (before reappraisal) and 3 (after reappraisal) to test our key hypothesis. We have previously reported information on the participants, materials, and experimental procedure (Wu et al., 2019). Here, we briefly introduce these details.

Materials

The functional magnetic resonance imaging (fMRI) experiment used 75 negative IAPS pictures depicting the threat and attack scenario as well as disgust things and animals. Their mean valence rating was 2.56 (SD = 0.52), and their mean arousal rating was 5.43 (SD = 0.85), both on a nine-point scale. Given the difficulty in generating genuine insightful reappraisals on one's own (Wu et al., 2017), we made a list of ordinary and creative insightful reappraisal sentences for the negative IAPS pictures in advance and presented them to the participants to induce creative or ordinary reappraisal. Although studying the process of passively reading and comprehending cognitive reappraisals provided by the experimenter was not as ideal as investigating the process of the participants generating cognitive reappraisals by themselves, this triggering approach provided a reasonable solution to the difficulty of studying the neural correlates of insightful restructuring. By this we mean the participants' self-generation of the highly creative cognitive reappraisals are especially hard, if not impossible, and this made it difficulty to meet the technical requirement of an event-related fMRI, which needs a sufficient number of target mental events with accurate onset time (Luo and Knoblich, 2007), if we adopted a self-generated reappraisal paradigm.

We prepared three types of reappraisal interpretations for each of the 75 negative pictures; namely, objective description (OD), ordinary reappraisal (OR), and the creative reappraisal (CR). We obtained the CR sentences from the pool of 947 CR items toward the 75 negative IAPS pictures we mentioned earlier (Wu et al., 2017). The mean creativeness score (the extent to which the participant felt that the reappraisals were novel and unexpected) was 6.56 (SD = 1.57), the effectiveness score (the extent to which the description could improve one's emotional feelings) was 6.46 (SD = 1.71), and the appropriateness score (the extent to which the description was fit for the scene depicted in the picture) was 5.79 (SD = 1.85). **Figure 1** shows examples of the three types of reappraisals.

Participants

Thirty-one college students (17 females; age: 24 $\pm$ 2.01, range: 21–27) participated in this study as paid volunteers. We excluded data from eight participants due to technical or cognitive reasons (see Wu et al., 2019, for details). The experimental protocol was approved by the Institutional Review Board of the Institute of Biophysics, Chinese Academy of Sciences. Written informed consent was obtained from all participants before the experiment.

Procedure

The whole experimental procedure involved five experimental phases that included various types of follow-up evaluations and ratings performed 10 min or 3 days after the first phase (Wu et al., 2019). In the present paper, we have only reported the first phase that involved MRI scanning directly related to the goal of this study. In this phase, each of the 75 pictures and the corresponding reappraisal sentence were presented one by one in a random order. Regarding the exact procedure for each trial, the participants were first shown the target picture for

Sentence of OD for this picture: The toilet is blocked by unidentified yellow objects, and the water cannot go down because of black objects floating in it (scores of creativeness: M = 2.80, SD = 0.50).

Sentence of OR for this picture: While the vomit is a mess, fortunately, this is nothing serious, and she will get better after some rest (scores of creativeness: M = 4.80, SD = 1.32).

Sentence of CR for this picture: Although she just threw up, she has great joy in her heart because she will finally have a baby (scores of creativeness: M = 7.60, SD = 1.30).

FIGURE 1 | Example of three types of reappraisal.

2 s and were required to passively view it (Step 1). Next, after a 1- to 3-s unfilled delay, the picture was presented together with an OD/OR/CR sentence for 12 s (Step 2). The participants were required to attentively read these sentences and attempt to understand their meanings and implications for the target picture (this 12-s duration has been focally analyzed and reported in our previous study, Wu et al., 2019). Next, after a 2- to 6-s unfilled delay, the target picture was presented again without any reappraisals for 2 s (Step 3), and the participants were again required to passively view it, which was also followed by a 1- to 3-s unfilled delay. Finally, the participants were required to indicate their emotional valence associated with processing the target picture using two emotionality-rating tasks (Step 4, see Wu et al., 2019 for details of this rating procedure).

For each participant, the 75 pictures were randomly assigned to one of the three conditions (OD, OR, and CR) with 25 trials in each condition. For each given picture, each participant could only see one type of the OD, OR, or CR sentences to avoid possible interactions among different types of reappraisals for the same picture. Valence and arousal were not significantly different in the three conditions ($p_{all} > 0.05$). Prior to the start of the formal fMRI scanning experimental session, the participants received thorough instructions and were sufficiently trained using another set of similar materials with an identical procedure.

Image Acquisition and Preprocessing

We performed fMRI on a Siemens 3T Trio MRI scanner (Siemens Medical Systems, Erlangen, Germany). Functional scans were acquired using T2*-weighted gradient echo, echo-planar pulse sequences. The following acquisition parameters were used in the fMRI protocol: TR = 2,000 ms, TE = 30 ms, slice number = 32, flip angle = 90-degree, matrix size = 64 × 64, FOV = 220 mm × 220 mm, and voxel size = 3.4 mm × 3.4 mm × 3 mm. For each participant, functional data were acquired in three scanning sessions containing 416 vol per session. The total lasted for 2,496 s (1,248 scans, TR = 2), which separated into three equal-length scanning sessions. Stimulus were presented on an MR-compatible monitor using E-prime software (Psychology Software Tools). Participants were in a supine position with their heads snugly fixed by a belt and foam pads to minimize head motion.

Functional MRI data was subjected to preprocessing steps using SPM8 software package (Statistical Parametric Mapping, Wellcome Department of Cognitive Neurology, London, United Kingdom): slice timing, realigned to the first volume, co-registered to the T1 image, normalized to a standard template [Montreal Neurological Institute (MNI)], resampled to a spatial resolution of 3 mm × 3 mm × 3 mm, spatially smoothed with an 8 mm × 8 mm × 8 mm full-width-at-half-maximum (FWHM) Gaussian kernel and 128s high-pass filtering (0.011 Hz).

Functional MRI Data Analysis

A functional MRI data analysis was conducted using SPM8. For the first level analysis, general linear modeling (GLM) was conducted for the functional scans from each participant. Several regressors were created by convolving a train of delta functions, which consisted of a synthetic hemodynamic response function (HRF): the first-time passively viewing of three types of target pictures, which would be subsequently re-presented together with the corresponding CR, OR, or OD sentences respectively in Step 1 (defined as the event of CR1, OR1, and OD1); the processing of the three kinds of reappraisal materials (CR reappraisal sentence processing, OR reappraisal sentence processing, and OD description sentence processing, defined as the event of CR2, OR2, and OD2) in Step 2; as well as the second-time passively viewing of the three kinds of target pictures, which had just been paired with CR, OR, or OD sentences (defined as event of CR3, OR3, and OD3, respectively) in the Step 3. All events were entered as parametric regressors at the onset of picture display. Moreover, two picture rating events were also entered as out of interest regressors. Six parameters generated during motion correction were entered as covariates. Linear contrasts of the parameter estimates were made to identify the effects of the nine picture viewing events for each participant. These

images from all participants were then entered into a second-level group analysis conducted with a random-effects statistical model with the threshold of $p < 0.001$ (uncorrected) and 50 or more continuous voxels ($K \geq 50$).

Regions of interest (ROIs) in the insular cortex were defined using structural AAL templates for the left and right insular cortex in WFU_PickAtlas_3.0.3[1], which was used as an inclusive mask and overlapped with the functionally activated clusters based on certain contrasts (e.g., the contrast of "CR1 minus CR3" or that of "CR3 minus OR3"). Given the insular activation in processing unpleasant pictures were theoretically predicted by related theories and research results on emotional arousal and regulation, we took a relatively loose threshold, $p < 0.05$ (uncorrected), to get a comprehensive examination on possible insular activation changes associated with the application of different types of reappraisal approach. In addition to that, we extracted the percent signal changes in the bilateral posterior and anterior insular cortex based on a given contrast using the MarsBar[2]. We selected the contrasts, which we defined the insular ROIs in such a way that the contrast was independent of the targets contrast we were interested in. The percent signal changes within these ROIs were extracted for each condition separately for each participant and were further averaged across all participants to produce mean scores.

To investigate the relationship between insula activations and subjective ratings of pleasantness, we also correlated each participant's percent signal change within the given functional ROIs with subjective rating of pleasantness for the CR condition.

[1]http://fmri.wfubmc.edu/software/PickAtlas

[2]http://marsbar.sourceforge.net

RESULTS

The behavioral results showed that pictures paired with CR were rated as more pleasant than those paired with OR and OD. In addition, pictures paired with OR were rated as more pleasant than those paired with OD, implying that the emotional regulatory effectiveness of the three conditions was CR > OR > OD (Wu et al., 2019).

The brain imaging results found more anterior insular activations after the application of cognitive reappraisals or objective descriptions relative to the one before (OR3 > OR1, CR3 > CR1, OD3 > OD1, **Figures 2**, **5** and **Table 1**) as well as after the application of creative or ordinary reappraisals relative to that of objective descriptions (OR3 > OD3, CR3 > OD3, **Figures 3–5** and **Table 1**). In contrast, more posterior insular activations were found before the application of reappraisals relative to the one after (OR1 > OR3, CR1 > CR3, OD1 > OD3, **Figures 2**, **5** and **Table 1**) as well as after the application of objective descriptions relative to that of creative or ordinary reappraisals (OD3 > OR3, OD3 > CR3, **Figures 3–5** and **Table 1**). Additionally, we also provided the results of a whole brain analysis with the threshold of $p < 0.001$ (ucr), $K \geq 50$ in **Table 2**, but no super-threshold insular activation was observed at this threshold.

To further address our main hypotheses about the dissociable effects of the posterior and anterior insular cortex, we conducted

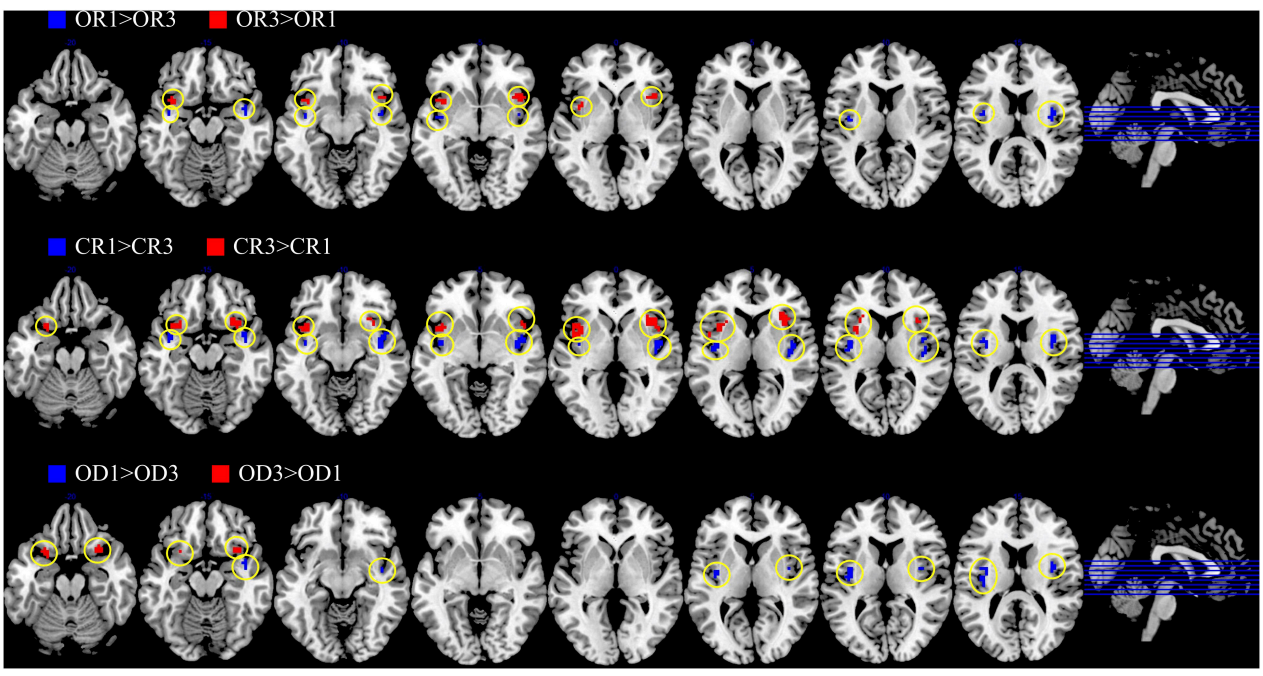

FIGURE 2 | The anterior and posterior insular activations observed before and after the application of different types of cognitive reappraisals (OR or CR) and objective descriptions (OD).

TABLE 1 | MNI coordinates of the peak insular activation observed in three contrasts between Step 1 and Step 3 for each condition (i.e., "CR 1 vs. CR3," "OR 1 vs. OR3," and "OD 1 vs. OD3") and three contrasts across condition in Step 3 (i.e., "CR 3 vs. OD3," "OR 3 vs. OD3," and "CR3 vs. OR3").

Brain regions	MNI coordinates			T	K
	x	y	z		
CR3 > CR1					
L anterior insula	−24	12	−18	3.155	119
R anterior insula	48	15	−3	3.153	59
R anterior insula	33	18	−15	2.779	30
CR1 > CR3					
L mid-posterior insula	−36	−6	18	5.090	61
R mid-posterior insula	42	3	−15	4.070	109
OR3 > OR1					
L anterior insula	−36	12	−15	2.589	31
R anterior insula	48	15	−3	2.485	31
OR1 > OR3					
R mid-posterior insula	42	0	−15	3.234	15
R mid-posterior insula	36	−3	15	2.809	14
L mid-posterior insula	−39	−3	15	2.651	18
L mid-posterior insula	−39	−3	−15	2.041	11
OD3 > OD1					
L anterior insula	−24	15	−21	3.880	15
R anterior insula	27	15	−18	3.199	22
OD1 > OD3					
L mid-posterior insula	−39	−9	12	4.209	52
R mid-posterior insula	42	0	−15	3.271	14
R mid-posterior insula	39	−3	15	2.617	13
CR3 > OD3					
R anterior insula	33	24	6	4.283	56
L anterior insula	−27	21	−3	2.847	41
OD3 > CR3					
L mid-posterior insula	−42	−9	−3	3.928	62
R mid-posterior insula	39	0	15	3.301	120
L mid-posterior insula	−33	0	15	2.856	23
OD3 > OR3					
L mid-posterior insula	−39	6	12	4.072	84
R mid-posterior insula	36	−15	6	2.823	37
R mid-posterior insula	45	0	−9	2.714	24
OR3 > OD3	No suprathreshold clusters				
CR3 > OR3	No suprathreshold clusters				
OR3 > CR3	No suprathreshold clusters				

(1) Threshold of voxel levels: T = 1.717, p < 0.05 (unc.), K = 10, inclusive mask = structural insula; MNI = Montreal Neurological Institute.

two kinds of repeated-measures analyses of variance (ANOVAs) on the percent signal changes in insular cortex as *post hoc* analysis. One type was 3 (reappraisal type: OD, OR, CR) × 2 (regulation phase: before and after reappraisal) repeated measure ANOVAs. The other was 2 (insula area: anterior insula [AI] and mid-posterior insula [PI]) × 3 (reappraisal type: OD, OR, and CR) × 2 (regulation phase: before and after reappraisal) repeated measure ANOVAs. The former 3 × 2 ANOVAs took the percent signal changes in the given insular ROIs as the function of the factors of the reappraisal type and regulation phase, whereas the latter 2 × 3 × 2 ANOVAs took the averaged percent signal changes in several left or right AI and PI ROIs as the function of the factors of the insula area, reappraisal type, and regulation phase.

The results of the former 3 (OD, OR, CR) × 2 (before and after reappraisal) ANOVAs are summarized in **Table 3**. As is shown in **Table 3**, in some insular RIOs, we found the significant main effects of the reappraisal type and/or reappraisal phase.

The 2 (AI, PI) × 3 (OD, OR, CR) × 2 (before and after reappraisal) ANOVAs were conducted on the mean percent signal change in the right lateral AI and PI regions (including the right AI ROIs that peaked at [x,y,z = 48,15,−3] and [33,30,6] as well as the right PI ROIs that peaked at [x,y,z = 36, −3,15] and [x,y,z = 42,0,−15]) and the left lateral AI and PI regions (including the left AI ROIs that peaked at [x,y,z = −36,12,-15] as well as the left PI ROIs that peaked at [x,y,z = −39, −3,15] and [x,y,z = −39,−3,−15]). The analysis results are reported below, starting with main effects and then two-way and three-way interactions.

For the right insular ROIs, we found significant main effects of the insula area (AI vs. PI), $F_{(1,22)} = 5.703$, $p = 0.026$, $\eta_p^2 = 0.206$, but no significant main effects of regulation phase (before vs. After reappraisal), $F_{(1,22)} = 0.814$, $p = 0.377$, $\eta_p^2 = 0.036$ and reappraisal type (CR vs. OR vs. OD), $F_{(1,22)} = 1.144$, $p = 0.328$, $\eta_p^2 = 0.049$, indicating that the right AI ROIs exhibited greater activation than the right PI ROIs across different reappraisal types and regulation phases.

Analysis of two way interactions revealed significant interaction between insula area and regulation phase, $F_{(1,22)} = 18.708$, $p < 0.001$, $\eta_p^2 = 0.46$, and insula area and reappraisal type, $F_{(2,44)} = 10.641$, $p < 0.001$, $\eta_p^2 = 0.326$, but no significant interaction between reappraisal type and regulation phase, $F_{(2,44)} = 0.141$, $p = 0.869$, $\eta_p^2 = 0.006$. More critically, we found a significant three-way interaction of insula area, regulation phase, and reappraisal type, $F_{(2,44)} = 3.778$, $p = 0.031$, $\eta_p^2 = 0.147$, indicating that, before regulation, the percent signal change of all regulation types (CR1, OR1, and OD1) in both AI and PI ROIs had no significant differences. However, a simple effect analysis using the Bonferroni correction indicated that the percent signal change after the application of CR in the right PI ROIs was significant lower than OD (i.e., CR3 < OD3, $p = 0.043$; OR3 vs. OD3 and CR3 vs. OR3, $p_{all} > 0.05$), but no significant differences were found between reappraisal types in the right AI ROIs (CR3 vs. OD3, OR3 vs. OD3, and CR3 vs. OR3, $p_{all} > 0.05$). Likewise, for the left insula, we also found a significant three-way interaction of insula area, regulation phase, and reappraisal type, $F_{(2,44)} = 3.696$, $p = 0.033$, $\eta_p^2 = 0.144$, indicating that, before regulation, the percent signal change of all reappraisal type (CR1, OR1, and OD1) in both AI and PI ROIs had no significant differences. However, simple effect analysis using the Bonferroni correction indicated that the percent signal change after the application of CR in the right PI ROIs was marginally significant lower than OD (i.e., CR3 < OD3, $p = 0.058$; OR3 vs. OD3 and CR3 vs. OR3, $p_{all} > 0.05$), but no significant differences were found between regulation type in the right AI ROIs (CR3 vs. OD3, OR3 vs. OD3, and CR3 vs. OR3, $p_{all} > 0.05$).

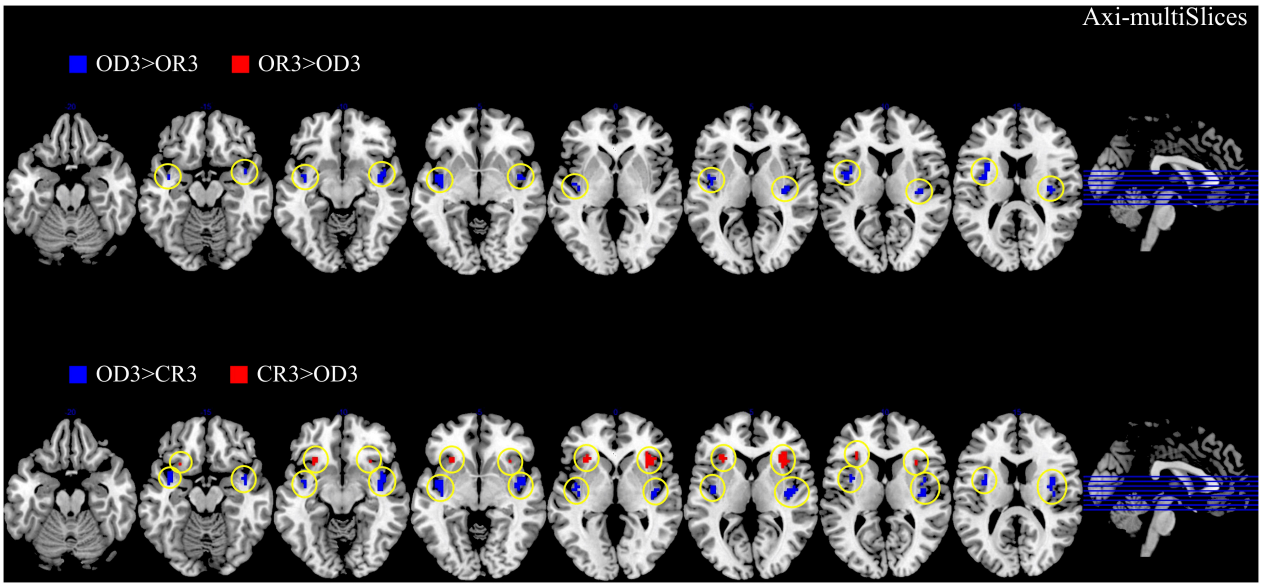

FIGURE 3 | The anterior and posterior insular activations observed after the application of creative reappraisals (CR) and objective descriptions (OD) (transverse plane).

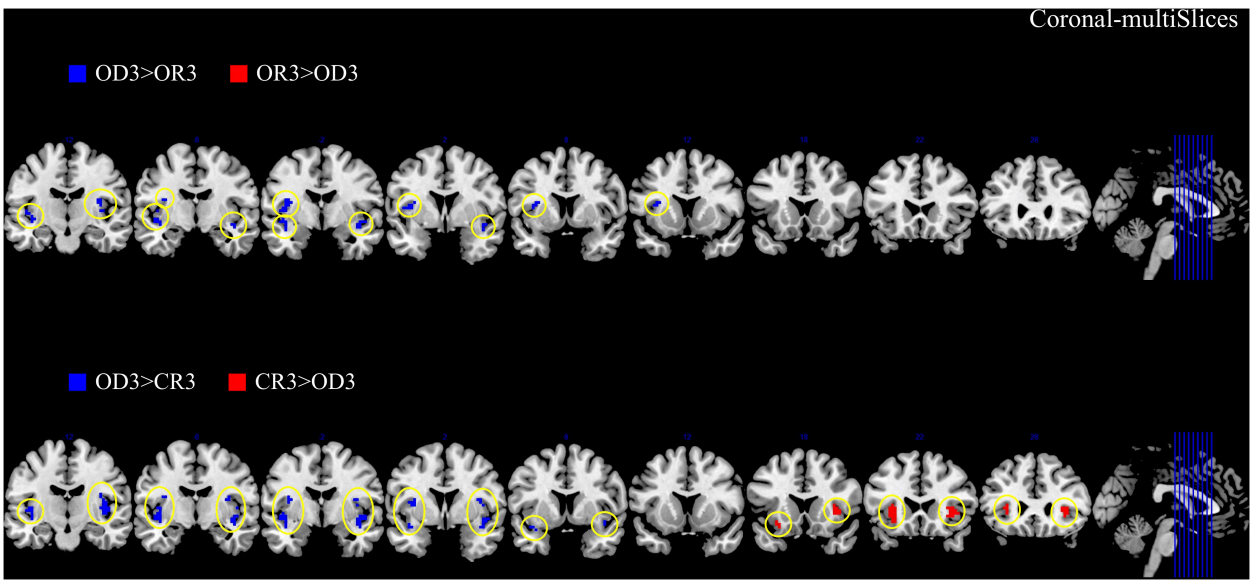

FIGURE 4 | The anterior and posterior insular activations observed after the application of creative reappraisals (CR) and objective descriptions (OD) (coronal plane).

We correlated each participant's percent signal change within the functional ROIs with subjective rating of pleasantness for the CR condition. However, no significant correlations were found between insula activation and the pleasantness ratings.

DISCUSSION

Given that the functions of the mid-posterior and insular cortex can be segregated, with the mid-posterior insular cortex playing a greater role in representing physiological reactivity and homeostatic states, and the anterior insular cortex playing a greater role in integrating homeostatic afferent activity from the dorsal posterior insula with emotional salience to form a global representation of the bodily feeling state (Craig, 2002, 2009), we made a key hypothesis in the present study that cognitive reappraisal would alter the emotional feelings (toward negative stimulus) represented in the insular cortex in a posterior-to-anterior progression manner (Craig, 2009). Consistent with this

hypothesis, we found that application of the cognitive reappraisals, especially the creative ones, could induce increased anterior insular activation and reduced mid-posterior insular activation.

Increased mid-posterior insular activations were found when the first-time viewing of the unpleasant pictures (in Step 1) was contrasted with the third-time viewing (in Step 3) (i.e., CR1 > CR3) or across the conditions

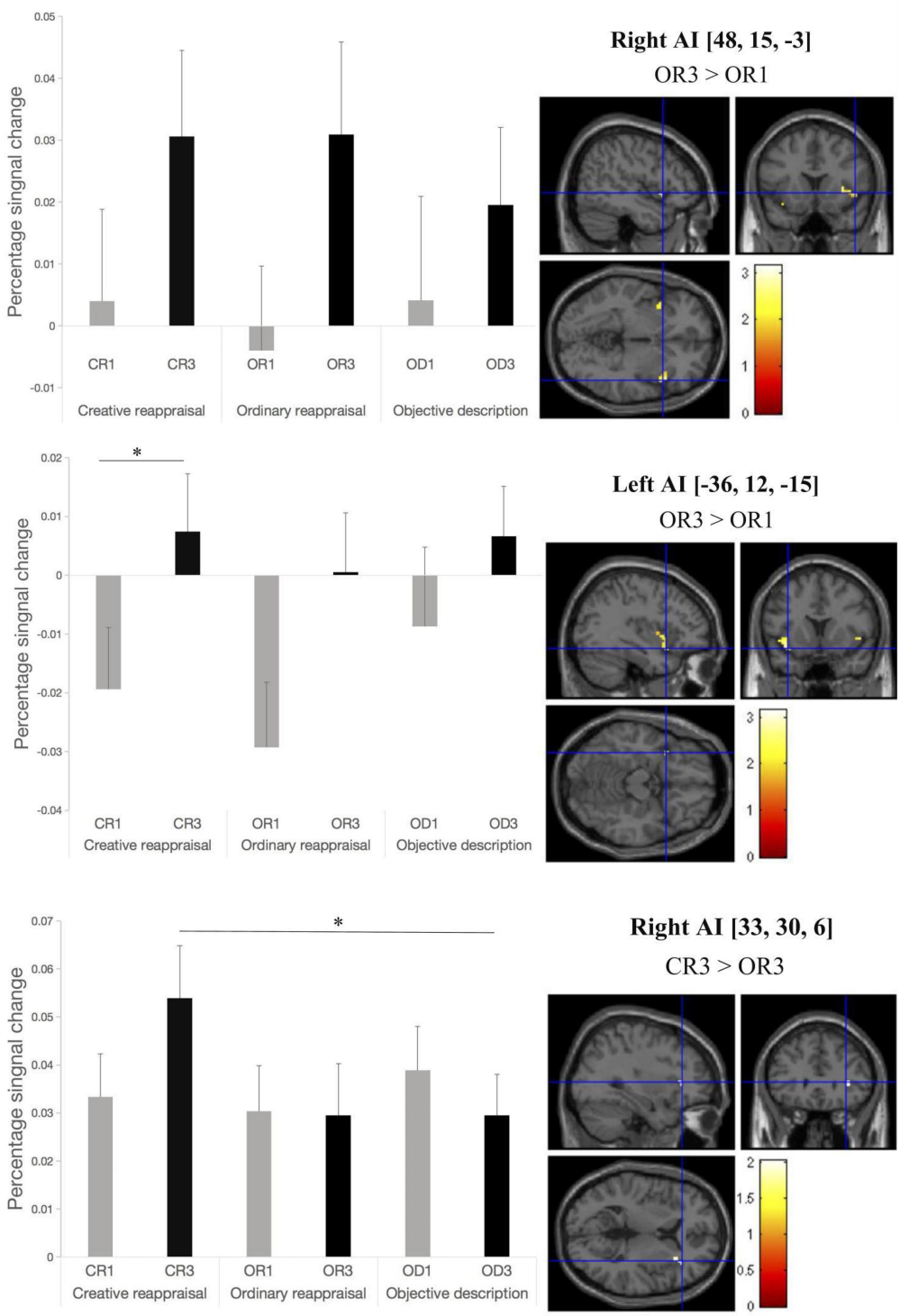

FIGURE 5 | Continued

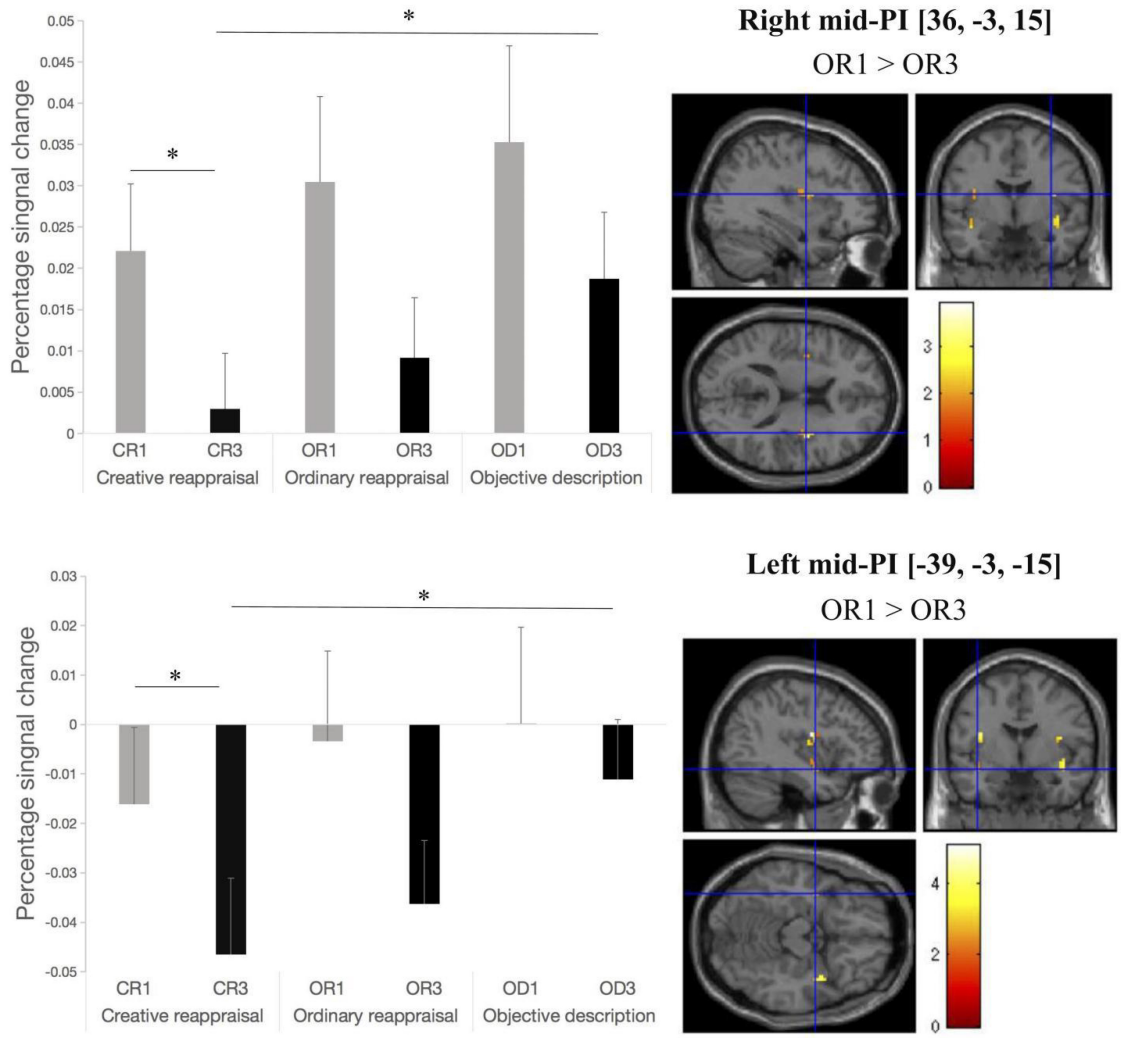

FIGURE 5 | Activation in mid-posterior and anterior insular ROIs before and after the application of cognitive reappraisal or objective descriptions. Note that all the differences that reached a significant level were marked as * ($p < 0.05$) or ** ($p < 0.001$) except for the contrast based on which we defined the ROIs. The location of the peak voxel in the given ROI and the contrast based on which we defined the ROIs were provided on the right top of each picture.

of different regulation strategies (e.g., OD3 > CR3). The mid-posterior insular cortex was more activated before the application of reappraisal regulation or in the OD condition relative to CR condition, and this could be related to the fact that participants had more intensive unpleasant emotional feelings before the reappraisal regulation or after the application of cognitive reappraisals in OD condition relative to CR condition. In support of this possibility, previous studies have generally demonstrated the function of the posterior insular cortex in encoding disgust sensory and emotional feelings (Gehrlach et al., 2019), such as unpleasant haptic sensations (Bonenberger et al., 2015), heat pain (Koenen et al., 2017), cold temperatures (King and Carnahan, 2019), and even some kinds of social emotionality like disgust (Britton et al., 2006; Ying et al., 2018).

In contrast, increased anterior insular activations were found when the third-time viewing (in Step 3) was contrasted with the first-time viewing of the unpleasant pictures (in Step 1) (i.e., CR3 > CR1) or the after the application of CRs relative to that of ODs. The anterior insular cortex activation has been widely observed in the processes of emotion regulation, and this includes both of the explicit regulations—the cognitive reappraisal (Morawetz et al., 2017) and expressive suppression (Giuliani et al., 2011)—as well as the implicit ones, such as the fear extinction and placebo (Diekhof et al., 2011; Picó-Pérez et al., 2019); this implies that the role of anterior insular cortex in emotion regulation could be either conscious and effortful (in explicit emotion regulation) or automatic and effortless (in implicit emotion regulation), which was in some sense inconsistent with the general impression that the anterior insular cortex, together

TABLE 2 | MNI coordinates of peak activation associated with three contrasts between Step 1 and Step 3 for each condition (i.e., "CR1 vs. CR3," "OR1 vs. OR3," and "OD1 vs. OD3") and three contrasts across condition in Step 3 (i.e., "CR3 vs. OD3," "OR3 vs. OD3," and "CR3 vs. OR3").

Brain regions	MNI coordinates			T	K
	x	y	z		
CR3 > CR1					
R Precuneus	6	−78	48	10.037	1492
R Middle frontal gyrus	42	33	36	9.585	1169
R Inferior parietal lobule	51	−42	45	8.640	783
R Caudate	21	−39	9	8.512	432
R Pyramis	45	−78	−33	7.413	445
L Middle frontal gyrus	−36	45	6	7.250	729
L Inferior parietal lobule	−48	−63	51	6.865	505
L Inferior temporal gyrus	−48	−3	−39	6.777	307
R Middle temporal gyrus	42	3	−39	6.192	52
R Inferior temporal gyrus	60	−39	−15	6.191	321
R Lentiform nucleus	15	12	−12	5.717	88
L Precentral gyrus	−54	12	6	5.217	50
L Medial frontal gyrus	−15	9	−15	5.181	65
R Precentral gyrus	54	12	9	4.778	65
CR1 > CR3					
L Inferior occipital gyrus	−39	−81	−6	14.980	5312
R Middle frontal gyrus	42	12	30	9.314	428
L Middle frontal gyrus	−39	−3	48	6.585	327
L Cerebellar tonsil	0	−51	−42	6.012	104
R Inferior frontal gyrus	48	30	9	5.834	56
OR3 > OR1					
R Precuneus	6	−75	48	8.448	1603
L Inferior parietal lobule	−57	−48	51	7.714	637
R Middle frontal gyrus	36	54	6	7.519	891
R Inferior parietal lobule	45	−45	42	7.237	676
R Middle temporal gyrus	45	6	−42	7.133	71
L Middle frontal gyrus	−21	45	−12	6.107	517
R Middle temporal gyrus	63	−21	−12	5.456	183
L Middle temporal gyrus	−45	3	−42	5.418	82
L Medial frontal gyrus	−15	9	−18	5.061	51
R Medial frontal gyrus	12	12	−15	4.881	56
L Middle frontal gyrus	−33	27	42	4.717	96
OR1 > OR3					
R Middle occipital gyrus	39	−87	6	13.733	5551
R Middle temporal gyrus	51	0	−21	6.684	62
R Inferior frontal gyrus	42	9	30	6.295	270
L Precentral gyrus	−39	−6	48	6.071	95
L Inferior frontal gyrus	−42	6	30	6.059	126
L Middle frontal gyrus	−36	33	−15	5.665	59
L Inferior semi-lunar lobule	−9	−78	−39	5.327	51
OD3 > OD1					
R Precuneus	12	−72	36	9.508	4707
R Middle temporal gyrus	45	6	−42	6.936	67
R Tuber	42	−81	−30	6.495	121
R Inferior parietal lobule	48	−42	39	6.374	577
L Inferior parietal lobule	−60	−45	48	6.169	342
R Superior frontal gyrus	24	27	51	4.742	124
Left cerebellum inferior semi-lunar lobule	−21	−87	−39	4.628	56
OD1 > OD3					
L Inferior occipital gyrus	−33	−84	−9	14.158	5055
L Precentral gyrus	−36	−6	51	7.320	430
R Inferior frontal gyrus	42	12	27	7.208	532
R Middle temporal gyrus	51	−6	−18	6.705	85
L Tonsil	0	−51	−42	6.429	76
L Inferior semi-lunar lobule	−9	−78	−39	5.747	119
CR3 > OD3					
Right cerebellum	15	−45	−24	6.253	139
L Precentral gyrus	−33	−24	57	5.812	83
R Middle frontal gyrus	33	57	6	4.975	75
L Superior frontal gyrus	−36	15	51	4.542	56
R Declive	12	−72	−9	4.235	61
OD3 > CR3					
R Precentral gyrus	39	−21	54	6.575	390
L Medial frontal gyrus	−3	60	0	5.947	75
OD3 > OR3	No suprathreshold clusters				
OR3 > OD3	No suprathreshold clusters				
CR3 > OR3	No suprathreshold clusters				
OR3 > CR3	No suprathreshold clusters				

Threshold of voxel levels: $T = 3.505$, $p < 0.001$ (uncorrected), $K \geq 50$ voxels.

with ACC, makes critical contributions to the function of cognitive control (Schmeichel et al., 2008; Gyurak et al., 2012). Regarding the function of the anterior insular cortex, a more fundamental possibility is it simply serves as the mechanism for making the mental representation or appraisal of the emotional responses or emotional arousal rather than for regulating emotion (Fullana et al., 2018; Picó-Pérez et al., 2019). This raises the question of what kind of function the anterior insular cortex actually served in the application of cognitive reappraisals in the present study. Did it simply mean the mental representation or emotional appraisal of the negative emotionality after watching unpleasant pictures? Or did it mean the regulation of that negative emotional arousal? And, if it did mean some regulation-related processing, did it embody the "processes" of emotion regulation or the "results" of it?

Firstly, we think our observation of anterior insular cortex activation in this study could be more related to the emotion regulation process rather than to the encoding or appraisal of the negative pictures because the anterior insular cortex was more activated after the application of reappraisals than before, and it was more activated after the application of CRs relative to that of ODs. If the "negative emotion appraisal account" was true, we might find more anterior insular cortex activation in the reverse contrasts. Secondly, we think the anterior insular cortex activation observed in the present could be more related to the "results" or "consequences" of the emotion regulation rather than the "process" of emotion regulation because the "process" of emotion regulation should

TABLE 3 | Summary of the anterior and posterior insular ROIs that demonstrated the main effects of regulation phases and/or reappraisal types in the 3 (reappraisal type: OD, OR, and CR) x 2 (regulation phase: before and after) repeated measure ANOVAs.

			Results of 3 (reappraisal type: OD, OR, and CR) x 2 (regulation phase: before and after) repeated measures ANOVA	
Regions	**Contrasts based on which the ROIs were defined**	**Location of peak voxels in ROI [x,y,z]**	**Main effects of regulation phases (before vs. after emotion regulation)**	**Main effects of reappraisal types (OD vs. OR vs. CR)**
Bilateral anterior insula (AI)	OR3 > OR1	Right AI [48,15,−3]	$F_{(1,22)} = 5.838$, $p = 0.024$, $\eta_p^2 = 0.210$; SEA: CR3 > CR1 ($p = 0.062$).	
		Left AI [−36,12,−15]	$F_{(1,22)} = 8.470$, $p = 0.008$, $\eta_p^2 = 0.278$; SEA: CR3 > CR1 ($p = 0.001$).	
	CR3 > OR3	Right AI [33,30,6]	$F_{(1,22)} = 3.521$, $p = 0.038$, $\eta_p^2 = 0.138$; SEA: CR3 > CR1 ($p = 0.073$).	$F_{(2,44)} = 4.124$, $p = 0.023$, $\eta_p^2 = 0.158$; SEA: CR3 > OD3 ($p = 0.011$).
Bilateral mid-posterior insula (mid-PI)	OR1 > OR3	Right mid-PI [36,−3,15]	$F_{(1,22)} = 7.568$, $p = 0.012$, $\eta_p^2 = 0.256$; SEA: CR3 < CR1 ($p = 0.011$).	$F_{(2,44)} = 3,744$, $p = 0.034$, $\eta_p^2 = 0.145$; SEA: OD3 > CR3 ($p = 0.036$)
		Left mid-PI [−39,−3,−15]	$F_{(1,22)} = 4.943$, $p = 0.037$, $\eta_p^2 = 0.183$; SEA: CR3 < CR1 ($p = 0.024$).	$F_{(2,44)} = 5.303$, $p = 0.009$, $\eta_p^2 = 0.194$; SEA: OD3 > CR3 ($p = 0.011$).
		Right mid-PI [42,0,−15]	$F_{(1,22)} = 18.575$, $p < 0.001$, $\eta_p^2 = 0.458$; SEA: CR3 < CR1 ($p = 0.001$), OD3 < OD1 ($p = 0.046$).	$F_{(2,44)} = 5.145$, $p = 0.012$, $\eta_p^2 = 0.190$.
		Left mid-PI [−39,−3,15]	$F_{(1,22)} = 13.430$, $p = 0.001$, $\eta_p^2 = 0.379$; SEA: CR3 < CR1 ($p = 0.003$), OD3 < OD1 ($p = 0.007$).	$F_{(2,44)} = 4.390$, $p = 0.018$, $\eta_p^2 = 0.166$.

SEA: simple effect analyses using Bonferroni correction.

had been mainly accomplished in the Step 2 of the experiment (the presentation of reappraisals, which was not the focus of the present study, Wu et al., 2019). Despite this, we still could not completely exclude the possibility that participants continued to make the emotion regulation when they saw the picture again, especially in the case of CR, which could take more intensive information processing to integrate the implication of reappraisal sentences with the scenes depicted by the unpleasant pictures. Thirdly, based on the first and second points of consideration, a further question we may ask is, if the anterior insular cortex activation we observed in the present study did embody—or embodied to a large extent—the "results" rather than the "process" of the emotion regulation; did it represent the encoding or appraisal of the emotional arousal toward the unpleasant pictures itself or to the newly established emotionality induced by the reinterpretation of cognitive reappraisals? To this question, two sides of considerations may help us to make a guess: (a) previous studies have proved the cognitive reappraisal exerted its emotional regulatory effects through an "indirect" approach that altered the semantic representation toward the unpleasant event (this could be mainly accomplished by the cognitive control regions together with the lateral temporal cortex) and finally changed the property of the emotional arousal embodied in emotional regions, such as the amygdala (Buhle et al., 2014), thus implying the importance and the involvement of "the mental representational change" in the application of the reappraisal strategy; and (b) our fMRI study on CRs found robust and positive reward circuit activations, including the ones in ventral striatum, amygdala, and hippocampus, during the application of creative cognitive reappraisal (relative to that of the ordinary and OD) in Step 2 (Wu et al., 2019), thus implying the importance and the involvement of "the positive mental representational change" (in at least the application of CRs). These two considerations reminded us that the activation of anterior insular cortex observed in the present study could not only be related to the encoding or appraisal of the emotional arousal toward the unpleasant pictures itself; rather, it could reflect the emotional appraisal of the newly established emotionality that integrated the content of cognitive reappraisals with the target pictures. This possibility was also consistent with, for example, Taylor and colleague's study that found the anterior insula was co-activated with the anterior cingulate cortex (ACC) to form an essential part of the salience network, which detected salient events and initiated appropriate control signals to regulate behavior and the homeostatic state (Taylor et al., 2009). It was also generally consistent with the findings of previous neuroimaging studies, which suggested that the anterior insular cortex was involved in cognitive control (Wager and Feldman Barrett, 2004; Ochsner and Gross, 2008; Aziz-Zadeh et al., 2009; McCrea, 2010) given the obvious need for executive functions, such as working memory capacity, inhibition, and category shift in emotion regulation, and the fact that the cognitive reappraisals have to be sustained against competing bias (Schmeichel et al., 2008; Gyurak et al., 2012).

In summary, we compared the involvement of the posterior and anterior insular cortex in processing negative stimuli before and after cognitive reappraisal regulation and found that reappraisal could alter the internal bodily and emotional

feeling representation in the insular cortex in a posterior-to-anterior progression manner. The more powerful an emotion regulation strategy was (e.g., in the case of CR condition relative to the OR and CD conditions), the more obvious posterior-to-anterior insular activation progression tendency the strategy would be able to make. Our study indicates a clear dissociation of the functions of the posterior and anterior insular cortex associated with the application of reappraisal strategies, which demonstrates the change of the internal bodily and emotional feeling representation patterns produced by the reappraisal strategy. This provides new evidence toward the systematic and comprehensive understanding of the cognitive brain mechanisms through which the cognitive reappraisal exerts its emotional feeling regulation effects.

The limitation of this study includes (a) the experimental design of passively viewing of pre-prepared cognitive reappraisal materials undermined the "ecological validity" of the study, and this approach was relatively rarely adopted in previous studies on cognitive reappraisals and it could also rarely occur in the real world psychotherapy practice; (b) we did not find any significant correlation between the activation of the insular cortex and the participants' subjective evaluation or appraisal of their emotional feelings, and this implied the activation in the insular cortex may just embody some elements of one's emotional feelings, and these elements could not directly impact the holistic subjective evaluation of emotions; and (c) we observed insular activation at a lenient threshold ($p < 0.05$, uncorrected). One possible reason for this was that all the six experimental conditions we compared were just the passive viewing of the same set of unpleasant IAPS pictures (before and after the application of three different kinds of reappraisals). This relatively minor between-condition difference might have made it difficult to obtain robust insular activation. However, given the function of insular cortex in processing unpleasant stimulus and that on participating cognitive reappraisals were well-documented by previous studies, we think the results of the present study were meaningful and reflected the dissociable function of the mid-posterior and the anterior parts of insula in representing the regulation effects on one's emotional feeling by reappraisal strategy. Further studies using more ecologically valid designs (such as have participants to make the cognitive reappraisals on their own) and more sensitive technologies or approaches to detect the information processing signals in insular cortex and to detect one's subjective emotional feelings, as well as their changes, are needed to further clarify these limitations.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Institutional Review Board of the Institute of Biophysics, Chinese Academy of Sciences. The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this manuscript.

AUTHOR CONTRIBUTIONS

TG and JL designed and supervised the study. TG collected the data. TT, ZZ, and XW analyzed the data. ZZ, JL, JF, and TT wrote the manuscript.

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Long-Term Physical Exercise and Mindfulness Practice in an Aging Population

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Previous studies have shown that physical exercise and mindfulness meditation can both lead to improvement in physical and mental health. However, it is unclear whether these two forms of training share the same underlying mechanisms. We compared two groups of older adults with 10 years of mindfulness meditation (integrative body-mind training, IBMT) or physical exercise (PE) experience to demonstrate their effects on brain, physiology and behavior. Healthy older adults were randomly selected from a large community health project and the groups were compared on measures of quality of life, autonomic activity (heart rate, heart rate variability, skin conductance response, respiratory amplitude/rate), immune function (secretory Immunoglobulin A, sIgA), stress hormone (cortisol) and brain imaging (resting state functional connectivity, structural differences). In comparison with PE, we found significantly higher ratings for the IBMT group on dimensions of life quality. Parasympathetic activity indexed by skin conductance response and high-frequency heart rate variability also showed more favorable outcomes in the IBMT group. However, the PE group showed lower basal heart rate and greater chest respiratory amplitude. Basal sIgA level was significantly higher and cortisol concentration was lower in the IBMT group. Lastly, the IBMT group had stronger brain connectivity between the dorsal anterior cingulate cortex (dACC) and the striatum at resting state, as well as greater volume of gray matter in the striatum. Our results indicate that mindfulness meditation and physical exercise function in part by different mechanisms, with PE increasing physical fitness and IBMT inducing plasticity in the central nervous systems. These findings suggest combining physical and mental training may achieve better health and quality of life results for an aging population.

Keywords: physical exercise (PE), mindfulness interventions, integrative body-mind training (IBMT), quality of life, secretory Immunoglobulin A (sIgA), cortisol, heart rate variability, skin conductance response

INTRODUCTION

Aging is associated with decline in processing speed, memory, motor control and mental flexibility, which greatly impacts individual well-being and health. Consequently, there has been an increasing interest in interventions that could (a) maintain and enhance physical and mental vitality and (b) reduce the risk of age-associated physical and mental disorders in aging populations (Mahncke et al., 2006; Hillman et al., 2008). Previous studies have shown that both physical exercise and forms of mindfulness meditation can influence aspects of attention and self-regulation (Okazaki et al., 2005; Tang et al., 2007, 2009, 2010, 2015a; Hillman et al., 2008; Ludwig and Kabat-Zinn, 2008; Wen et al., 2011). However, physical exercise (PE) and mindfulness meditation are vastly different from each other, as each intervention has unique training components and may work through different mechanisms to exert an impact on behavior, physiology and brain.

As one of the most widely adopted interventions, PE requires the engagement and control of body movement and coordination with the outside environment. Months to years of PE have been shown to improve cardiovascular function, physical health and cognitive performance, and are associated with larger gray matter volumes in certain brain regions such as the hippocampus (Okazaki et al., 2005; Hillman et al., 2008; Wen et al., 2011). In contrast, mindfulness meditation involves less physical activity compared to PE, but focuses more on training attention, self-awareness, and emotion regulation through various mental techniques and strategies (Tang et al., 2015a). Studies have shown that 5 days of mindfulness meditation using integrative body-mind training (IBMT) improves attention and reduces stress reactivity through changing the interaction between the anterior cingulate cortex (ACC) and the parasympathetic branch of the autonomic nervous system (Tang et al., 2007, 2009). Furthermore, it was found that about 10 h of IBMT over one-month period increases fractional anisotropy (FA), an index indicating the integrity and efficiency of white matter in the anterior corona radiata, an important white-matter tract connecting the ACC to other structures (Tang et al., 2010). In addition, several long-term meditation studies have shown increases in ACC activation and gray matter volume and other regions, such as the striatum and insula (Cahn and Polich, 2006; Ludwig and Kabat-Zinn, 2008; Chiesa and Serretti, 2010; Tang et al., 2015a; Tang, 2017).

Given that both mindfulness meditation and PE have produced documented brain changes, we first sought to investigate how neuroplasticity in these long-term trainings would differ from each other by comparing these two forms of training in older adults. In particular, we sought to determine how and why each might contribute to more successful aging. To accomplish these goals, our design stresses comparative effectiveness and comparative mechanisms of training rather than the more usual tests of whether a given training method is effective. While many comparative effectiveness studies have been performed, our study is unique because it involves an aging population with 10 years of either mindfulness meditation or physical exercise (PE) experience.

Based on our prior studies showing improvement in self-regulation and increases in ACC and striatum activity after short-term mindfulness training (Tang et al., 2007, 2009, 2010, 2012, 2015a), our first hypothesis was that there would be stronger resting state connectivity between ACC-Striatum and greater gray matter in striatum following IBMT than following PE. For physiological indices, we hypothesized that both long-term mindfulness meditation and PE should induce changes in immunology and stress hormone based on prior literature showing such physiological benefits following short-term training using these two interventions (Tang et al., 2007; Hillman et al., 2008). In our previous studies, we found that 5 days of IBMT improves secretory Immunoglobulin A (sIgA) level following additional 20 min training after stress (Tang et al., 2007), and the baseline sIgA level is greater after 4-weeks of IBMT compare to relaxation training (Fan et al., 2010, 2013). While PE may also improve the immune function, our second hypothesis was that the IBMT group would show greater or at least equal sIgA level when compare with the PE group.

Physiological indexes are important biomarkers for aging (Pumprla et al., 2002; Okazaki et al., 2005; Borresen and Lambert, 2008). Heart rate, heart rate variability (HRV), skin conductance response (SCR), respiratory amplitude and rate were used to assess autonomic nervous system (ANS) activity (Tang et al., 2009). Because IBMT changes the state of the body and mind through the interaction of central and autonomic nervous systems, and PE strengthens cardiovascular activity and physical fitness, our third hypothesis was that PE would have lower resting heart rate than IBMT, whereas IBMT would show better ANS regulation indexed by lower SCR, and greater high frequency HRV in comparison to PE.

Finally, to compare quality of life outcomes following 10 years of PE or IBMT, we used the widely validated World Health Organization Quality of Life Survey (WHOQOL-100) to evaluate physical and psychological domains (Power et al., 1999). Because IBMT improved mood and showed greater activation in reward associated brain structures (Tang et al., 2007, 2009, 2010, 2015a), we hypothesized that higher quality of life would be associated with IBMT.

MATERIALS AND METHODS

Study Population

Two groups of (IBMT:PE = 32:29; 17 males) healthy Chinese older adults (mean age = 64.25 years old, SD = 12.54) were randomly chosen from a longitudinal National Health Project in the local communities based on their willingness to participate in our behavior, physiology and brain imaging study. All participants were living independently in their own home with matched living environment and social-economic status and were free of psychiatric disability and dementia assessed by local clinicians using semi-structured clinical interviews. Participants were not habitual smokers or drinkers, did not take any psychotropic drugs, anti-depressants or cholinesterase inhibitors within the last 12 months. Participants also received an annual health/medical check in the community hospitals. The

TABLE 1 | Demographic information for participants.

Demographic factor	IBMT		PE		*P* value (two-tailed)
	Mean	*SD*	Mean	*SD*	
Age	64.38	13.95	64.13	11.19	0.94
Education	13.89	2.79	12.71	3.74	0.19

two groups were matched by level of education, age and sex, see **Table 1**. No sedentary control group was assigned, because (1) the majority of Chinese old adults exercise to some degree after retirement and (2) such a waitlist group would be unlikely to maintain good health over a 10-year period. All subjects provided written informed consent in accordance with the Declaration of Helsinki. The study protocol was approved by the Institutional Review Board of the Dalian University of Technology.

Interventions

China belongs to a collectivist culture and old adults after retirement often get together for shared daily activities in local communities (Goodwin, 1999; Nisbett, 2004; Markus and Conner, 2014). The participants were trained in small groups in local communities. Each group practiced PE or IBMT daily for an average of 1 h, usually 6–7 days per week, for a 10-year period. Although some were absent from some training sessions, the average training duration was about 3120 h over 10 years in total. The IBMT or PE instructor in the same community supervised the practice sessions. The instructors also met and had biweekly conversations with participants to monitor and motivate the two groups and gave appropriate feedback. We found Chinese collectivist culture helped facilitate group practice in old adults, and the first 3-month of practice was crucial for habit formation in either IBMT or PE group. After this period, participants per group seemed to maintain independent practices regularly. Three participants in IBMT or PE group dropped off the study and in total 55 participants (29 in IBMT group) completed.

Integrative Body-Mind Training

Mindfulness Meditation involves paying attention to the present and increasing awareness of one's thoughts, emotion, and actions without judging oneself. One form of mindfulness meditation, IBMT involves body relaxation, mental imagery and mindfulness training which are the common components in other mindfulness programs (Kabat-Zinn, 2013). All sessions started and ended with approximately 10 min of gentle and slow posture practice for the purpose of warming up and returning to normal states. Cooperation between body and mind is emphasized in facilitating and achieving a meditative state. The instructor helped participants find an appropriate and balanced body posture to achieve deeper levels of relaxed body and experience a quietness and mindfulness state. When the mind wanders, participants accepted and were open to these experiences without judgment. IBMT uses minimal effort to control thoughts, but instead establishes a state of restful alertness that allows a high degree of awareness of body, breathing, and external instructions. It stresses a balanced state of relaxation while focusing attention on the present moment (Tang et al., 2007, 2015a; Tang, 2017).

Physical Exercise

Training mainly involved aerobic walking. All walking sessions started and ended with approximately 10 min of stretching for the purpose of warming up and cooling down. The participants were encouraged to walk in their target heart rate zone following light to moderate intensity exercise, which was calculated based on the resting and maximum heart rates achieved during the baseline maximal graded exercise test (Strath et al., 2000). For example, if the participant was 60 years old, the target heart rate zone was from 80 to 112 beats per minute during moderate intensity exercise.

Outcome Measures

Brain Imaging

Brain imaging experiments were performed on a SIEMENS TrioTim 3 Tesla scanner (Siemens Medical System, Erlangen, Germany) at the MRI Center for Brain Research. The resting state session consisted of 180 contiguous echo planar imaging (EPI) volumes with TR/TE = 2000/30 ms; flip angle = 90^0, matrix = 64 × 64, axial, slice thickness = 4 mm. A high-resolution T1-weighted anatomical image was also obtained after the resting state.

Data preprocessing of the resting state image, including slice timing, realignment, coregistration, normalized and spatially smoothed steps (6-mm full width at half maximum Gaussian blur) using SPM 8[1]. Subsequent processing includes temporal band-pass filtering ($0.009 < f < 0.08$) typically performed by resting state fMRI data analysis (Fox et al., 2005).

To quantify (changes in) functional connectivity, subject specific (partial) Pearson's correlation coefficient maps were computed based on the temporal series from a spherical region of interest (ROI) (radius = 6 mm) centered at the coordinates ($x = 8$, $y = 7$, $z = 38$) within the dACC brain area. These correlations were adjusted for confounds using (six) parameters obtained by rigid body correction of head motion, global trend, and signal from non-gray matter (Fox et al., 2005; Castellanos et al., 2008). Following Z-transformation of the correlation coefficients, we identified regions showing a significant functional connectivity with the dACC across both groups. Within these regions, we tested for the orthogonal effect of group differences using a small volume correction (SVC) for multiple comparisons.

To quantify (changes) in functional anatomy, voxel-based morphometry (VBM) was applied to high-resolution (1 × 1 × 1 mm) T1-weighted whole-brain images, collected from every subject with TR/TE/TI = 2530/3.37/1100 ms, NEX = 1, flip angle = 7°, matrix size = 512 × 512, Sagittal, slice thickness = 1.33 mm. A unified segmentation/normalization framework in the SPM 8 and VBM toolbox[2] were used for VBM analysis (Ashburner and Friston, 2000).

[1]http://www.fil.ion.ucl.ac.uk/spm

[2]http://dbm.neuro.uni-jena.de/vbm

We examined regional gray matter differences between groups using a general linear model with age and gender as covariates. The voxel-wise threshold for activation was set at $P_{FWE} < 0.05$, corrected for the number of resolution elements in each of the regions of interest (ROI) by using the SPM small volume correction (SVC) procedure together with brain masks defined by the automated anatomical labeling toolbox (AAL)[3]. The brain masks defined the brain regions over each of which the SVC was performed. These brain regions included the insula, putamen and caudate, hippocampus, frontal, temporal and parietal cortex based on the physical exercise and mindfulness meditation literatures (Greenwood, 2007; Hillman et al., 2008; Tang et al., 2009, 2010, 2015a). Twenty-six subjects (13 in each group) met all criteria for participating in the analysis, including no metallic implants and usable data after motion correction.

Physiology

The physiological data were recorded and analyzed in 8 channels of the Procomp Infiniti System from Thought Technology (Tang et al., 2009). During the fitness sessions, participants' heart rate was monitored using a Polar E200 heart rate monitor. We first recorded the 5-min baseline (eyes open, labeled as Baseline 1 in **Figures 3A,B**) heart rate, SCR, abdomen and chest respiratory amplitude for each subject in the two groups. To attain steady physiological signals of SCR and HRV allowing measurement of habitual ANS activity/regulation, we then recorded two 10-min periods of rest (eyes closed) (labeled as 1 and 2 in **Figures 3A,B**), and 5-min post baseline (eyes open, labeled as Baseline 2 in **Figures 3A,B**).

Power spectral analysis of HRV was performed with a fast Fourier transform and Biograph software, and spectral components were identified and assigned, on the basis of their frequency: high frequency (HF; 0.16–0.45 Hz), low frequency (0.04–0.15 Hz), and very low frequency (VLF; 0-0.03 Hz). These components were obtained in absolute values of power (ms^2). HF components are reported in normalized units (nuHF), representing the relative value of the power of each component in proportion to the total power minus the VLF component (Okazaki et al., 2005; Tang et al., 2009). Forty participants (20 in each group) participated throughout the tests, 40 had usable heart rate and SCR data, 36 had usable chest respiratory data (18 in each group) and 35 had useable HRV data (18 in IBMT group) after movement artifacts were eliminated.

Salivary sIgA and Cortisol

Salivary sIgA and cortisol levels were assessed at three stages (rest, stress, and additional 20-min practice, labeled as before stress, after stress and additional training respectively in **Figure 4**). To control for variations of sIgA and cortisol levels over the circadian rhythm, saliva sample collection was performed from 2:00 pm to 6:00 pm. About 1-mL saliva samples were collected by one-off injectors and were encased in test tubes in succession, with the tubes placed into a refrigerator under-208C and then thawed 24 h later for analysis. The concentration of sIgA and cortisol was analyzed by radioimmunoassay at the Dalian Medical University. Intra- and inter-assay coefficients of variation were below 10%. To reduce error variance due to imprecision of the intra-assay, all samples from each subject were analyzed in the same run (Tang et al., 2009).

World Health Organization Quality of Life Survey (WHOQOL-100)

This scale was developed in 15 international centers using focus groups, pilot tests, and field tests. The final 100 items were grouped into one scale score assessing overall quality of life and general health perceptions, and 24 quality of life facets, grouped into six larger domains: Physical, Psychological, Independence, Social Relationships, Environment, and Spirituality. The international and multicultural aspects of this scale make it a very useful instrument. It has been used extensively in a variety of settings around the world, demonstrating excellent reliability and validity (Power et al., 1999).

RESULTS

The IBMT and PE groups did not differ significantly in age ($t_{53} = 0.08$, $p = 0.94$) and education ($t_{53} = 1.11$, $p = 0.27$). Following training, we assessed behavior, resting state brain activity and structural changes using fMRI and various physiological measures including HRV, SCR, sIgA and cortisol. Significant differences between the two groups are discussed in this section and in **Figures 1**–**5**.

Brain Imaging

Based on previous studies of long-term meditation effects, we chose dorsal ACC (dACC) as the seed region and performed a whole brain functional connectivity analysis using resting state fMRI (Raichle et al., 2001; Fox et al., 2005). A significant correlation between dACC and striatum was detected in the combined IBMT and PE groups ($N = 26$, 13 in each group). Crucially, within the striatum, there was a significant difference

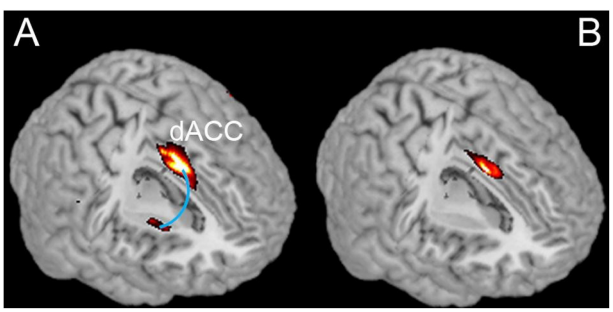

FIGURE 1 | Comparison of resting state dACC-Striatum connectivity and differences between IBMT and PE. **(A)** Shows the functional connectivity between dACC and Striatum detected in both groups combined. **(B)** Shows the differences between groups in terms of a significantly greater functional connectivity in the IBMT group relative to the PE group ($P_{FWE} < 0.05$, small volume corrected).

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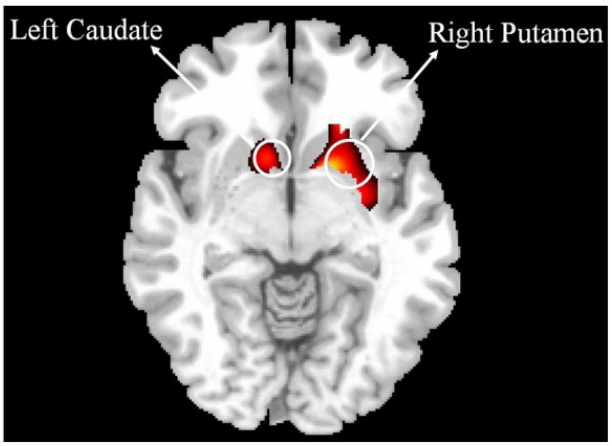

FIGURE 2 | Comparison of VBM gray matter between IBMT and PE. The voxelwise threshold for activation was set at $P_{FWE} < 0.05$, corrected for the number of resolution elements in each of the regions of interest (ROI) by using the SPM small volume correction (SVC) procedure together with brain masks defined by the automated anatomical labeling toolbox (AAL) (http://www.fil.ion.ucl.ac.uk/spm/ext). The brain masks defined the brain regions over each of which the SVC was performed. These brain regions included insula, putamen, caudate, hippocampus, frontal, temporal and parietal cortex. For better illustration, a liberal threshold ($P < 0.05$, uncorrected) was used.

in functional connectivity between the two groups ($P_{FWE} < 0.05$, small volume corrected); with a greater functional connectivity in the IBMT group see **Figures 1A,B**. When the statistical threshold was lowered (to an uncorrected level of $P < 0.001$), stronger functional connectivity between the dACC and insula was found in the IBMT group, relative to the PE group. In terms of structural differences, we found increased gray matter volume in striatum (left caudate and right putamen) using VBM in the IBMT group compared to the PE group ($P_{FWE} < 0.05$, corrected), see **Figure 2**. No other significant differences were found at this significance level. However, when the statistical threshold for VBM analysis was lowered to a descriptive level (uncorrected $p < 0.005$), PE showed increased gray matter in parietal and sensory-motor cortex relative to IBMT.

Physiology

We divided the data into four periods in **Figures 3A,B**: 5-min baseline with eyes open (labeled as baseline 1), two 10-min periods of rest with eyes closed (labeled as period 2 and 3), and 5-min post baseline with eyes open (labeled as baseline 2), and recorded physiological indexes including heart rate, SCR, abdomen and chest respiratory amplitude for each subject in the two groups. The ANOVAs showed lower resting (baseline) SCR ($F_{1,38} = 2.48$, $p = 0.123$), significantly lower SCR following the two 10-min resting (periods 1 and 2) and post baseline periods separately ($F_{1,38} = 8.42$, $p = 0.006$; $F_{1,38} = 5.25$, $p = 0.028$; $F_{1,38} = 5.58$, $p = 0.023$).

High-frequency HRV was greater in the IBMT group than in the PE group following the second 10-min resting period (period 2) and during post baseline ($F_{1,33} = 4.41$, $p = 0.043$; $F_{1,33} = 8.02$, $p = 0.008$), The IBMT showed better autonomic regulation of SCR and high-frequency HRV, see **Figures 3A,B**. Results of lower SCR and more high-frequency HRV demonstrated better ANS regulation, especially greater parasympathetic activity after 10 years of IBMT.

The PE group showed significantly lower resting heart rate ($F_{1,38} = 8.48$, $p = 0.006$) and greater chest respiratory amplitude than the IBMT group ($F_{1,34} = 5.41$, $p = 0.026$), indicating the training effects on cardiovascular system, see **Figure 3C**.

Immune Function

Repeated-measures ANOVAs were conducted with the factor of Group (IBMT and PE), and the within-subjects factor of Session (labeled as Before Stress, After Stress, and Additional 20-minute in **Figure 4**). The analyses revealed significant main effects for Group ($F_{1,\ 53} = 14.229$, $p < 0.01$) and Session ($F_{2,\ 106} = 32.31$, $p < 0.001$), as well as a significant interaction for Group × Session ($F_{2,\ 106} = 8.636$, $p < 0.01$). The long-term IBMT group showed higher basal sIgA level ($p < 0.05$) compared to PE before stress. After stress, sIgA in both groups increased, but IBMT was significantly higher than PE ($F_{1,53} = 9.628$, $p < 0.01$). After the additional 20-min practice, the sIgA in IBMT group continued to increase whereas it decreased in PE group, producing a significantly higher sIgA 20 minutes after stress ($F_{1,53} = 20.779$, $p < 0.001$). The IBMT group also showed marginally less basal cortisol concentration prior to stress than did PE ($p = 0.07$).

Quality of Life

Independent sample *t*-test showed significantly higher ratings in physical and psychological domains and in overall score of quality of life in the IBMT group compared to the PE group ($t_{53} = 3.02$, $p = 0.004$; $t_{53} = 4.17$, $p = 0.000$ and $t_{53} = 4.27$, $p = 0.000$); see **Figure 5**.

DISCUSSION

We observed similar default mode brain networks in both groups at resting state, as reported by previous studies (Raichle et al., 2001). Consistent with our first hypothesis – and with previous results summarized in the introduction – the IBMT group showed stronger dACC-Striatum functional connectivity at rest. This finding may relate to differences in parasympathetic regulation (Tang et al., 2009, 2019). In support of this view, the groups showed different physiological indexes of HRV and SCR indicative of parasympathetic reactivity and immune function, with the IBMT group showing generally greater HF-HRV and sIgA levels, and lower SCR (Tang et al., 2009, 2010). In addition, self-ratings in the quality of life scale were superior for the IBMT group.

In contrast, the PE group showed lower resting heart rate and greater chest respiratory amplitude compared to the IBMT group. These findings for the exercise group are in line with previous findings of exercise improving the cardiovascular system and health (Okazaki et al., 2005; Hillman et al., 2008). They also suggest different brain and physiological biomarkers for PE and IBMT, reflecting somewhat different underlying mechanisms

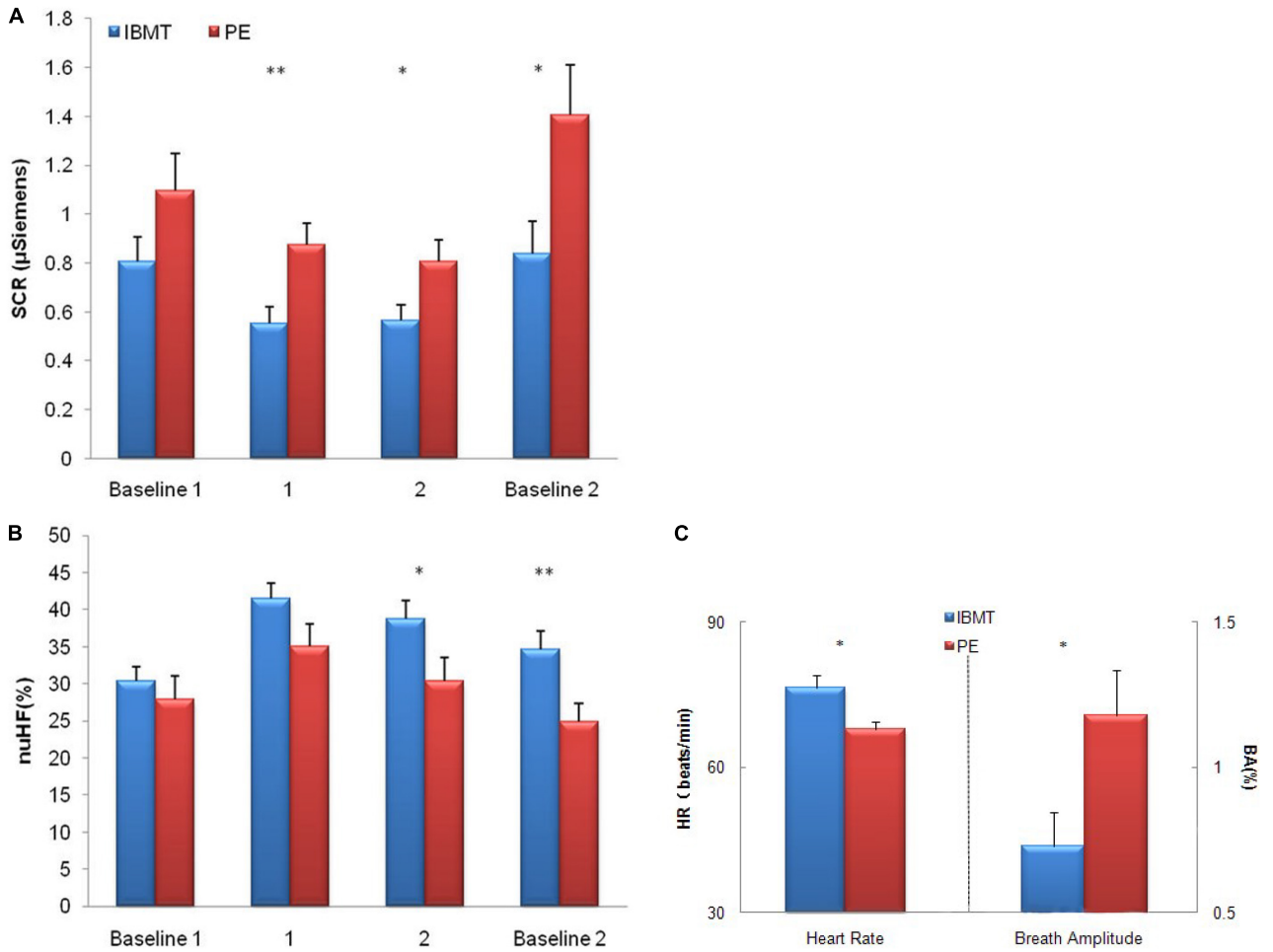

FIGURE 3 | Comparison of physiological indexes between IBMT and PE. **(A)** Skin conductance response (SCR) **(B)** High-frequency HRV **(C)** Heart rate and chest respiration amplitude in the IBMT and PE groups. For SCR, the lower score shows more parasympathetic activity. For high-frequency HRV, the higher score shows more parasympathetic activity. **(A,B)** The horizontal axis indicates the four test sessions: resting with eyes open (baseline 1), two periods of resting with eyes closed (labeled as 1, 2), and resting with eyes open (baseline 2). The vertical axis indicates SCR change and percentage of change in normalized units of high-frequency (nuHF) HRV, respectively. **(C)** The vertical axis indicates resting heart rate (HR) change and percentage of change of chest breath amplitude (BA), respectively. Error bars represent standard errors. $^{*}P < 0.05$, $^{**}P < 0.01$.

in long-term physical exercise in comparison with meditation. This may indicate the potential for integration of the two forms of practice.

Central Nervous System

Strong self-discipline and regulation are required to maintain 10 years of practice. In our previous study (Tang et al., 2009), we found increase in ventral midfrontal brain system control over parasympathetic activity after 5 days of IBMT. This beginning stage of practice requires ventral ACC coordination with the parasympathetic nervous system to change the participant's state. Members of the IBMT group also reported a happy and enjoyable experience during and after training. These reports may further suggest involvement of the striatal reward system (Tang, 2017).

Dopamine pathways serve the functions of reward (motivation), pleasure, and euphoria. The putamen and caudate play a key role in reinforcement and implicit learning and are thus associated with reward (Packard and Knowlton, 2002; Dahlin et al., 2008). Longitudinal shrinkage of the whole striatum with age is evident, even in a selected group of healthy adults (Raz et al., 2003). The IBMT group seemed to reverse this aging effect by showing larger putamen and caudate volume than the PE group, consistent with previous findings of practice-related neuroplasticity and improved connectivity of the striatum with the ACC (Draganski et al., 2004; Mahncke et al., 2006; Tang et al., 2009). These effects may be of practical importance in improving quality of life and brain health during aging.

With regular IBMT practice, the participants achieved greater satisfaction (as reported in their ratings), and this positive mood and reward experience may motivate the participants to maintain practice. It was reported that emotion and motivation associated with reward experiences serve to direct executive control and enhance overall behavioral performance (Pessoa, 2009). This also

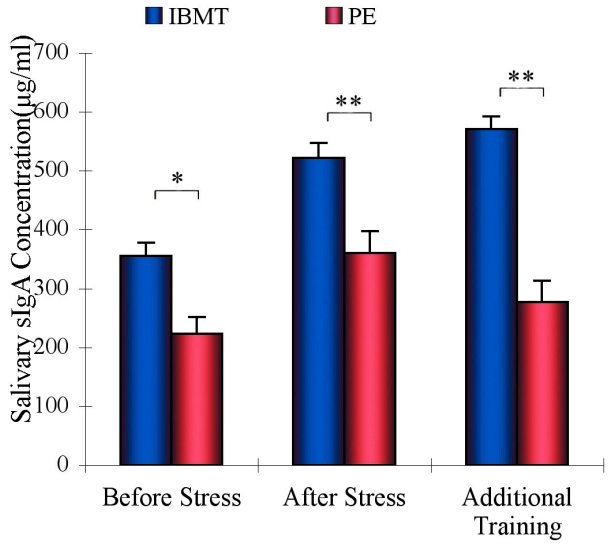

FIGURE 4 | Comparison of sIgA between IBMT and PE. IBMT (blue dots) and PE (red dots) groups. The IBMT group showed a significantly greater sIgA level than the PE group. *$P < 0.05$, **$P < 0.01$.

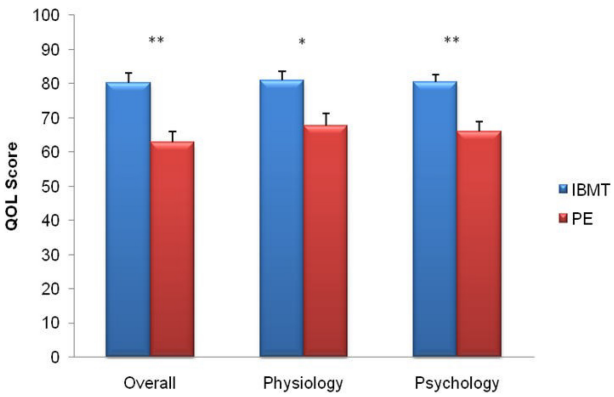

FIGURE 5 | Comparison of quality of life between IBMT and PE. The horizontal axis indicates Overall Score; Physiology; Psychology. The vertical axis indicates the quality of life (QOL) score. Error bars represent standard errors. *$P < 0.05$; **$P < 0.01$.

could help explain the persistence of long-term IBMT practice. For the PE participants, their motivator would be the desire for physical fitness, which may particularly motivate their long-term practice. It should also be noted that Chinese collectivist culture may also facilitate group practice in local communities where old adults easily got together for shared daily activities (Goodwin, 1999; Nisbett, 2004; Markus and Conner, 2014).

When the statistical threshold was lowered (to an uncorrected level of $p < 0.001$), stronger dACC and insula functional connectivity was found in the IBMT relative to the PE group. The dACC and insula are thought to work together in resting state (Dosenbach et al., 2007) and both have been shown to be involved in high-level attention and self-regulation (Posner et al., 2007; Sridharan et al., 2008). Moreover, the ACC and insula have been linked to self-regulation in connectivity studies (Posner et al., 2007; Sridharan et al., 2008). Our finding of increased functional connectivity between the dACC and insula is consistent with the distribution of Von Economo neurons in these two brain areas and their connectivity in the resting state (Allman et al., 2001; Dosenbach et al., 2007; Tang et al., 2015b). We propose that these two regions may provide an anatomical base for successful self-regulation (Tang, 2017) that may have close relationships to interoceptive inference (Seth and Friston, 2016).

Our current findings suggest that IBMT operates via central nervous system control of peripheral responses to enhance self-regulation. On the other hand, in our study PE appears to train the cardiovascular system more than IBMT. Thus, the two types of practice appear to involve distinct body-brain mechanisms. We failed to find significant gray matter changes in PE compared to IBMT ($P_{FWE} < 0.05$, corrected). However, when the statistical threshold for VBM analysis was lowered (by using an uncorrected $P < 0.005$), PE showed more gray matter in parietal and sensory-motor cortex than IBMT. The tendency for more sensory motor activity in PE might reflect more attention to the body during exercise and is also consistent with previous findings using relaxation training (Tang et al., 2009; Tang, 2017). These results are consistent with recent findings that continuous light-intensity physical activity contributes to brain plasticity (Spartano et al., 2019).

Physiology

Physiological measures of heart rate, SCR, respiratory amplitude and rate, and HRV are biomarkers of autonomic regulation (Pumprla et al., 2002; Borresen and Lambert, 2008; Tang et al., 2009). The IBMT group showed significantly better physiological reactions in lower SCR and higher abdominal respiratory amplitude than the PE group. These results reflected greater ANS regulation in the IBMT group. The greater high-frequency HRV in the IBMT group after training indicates successful inhibition of sympathetic tone and activation of parasympathetic tone in comparison with the PE group (Tang et al., 2019). In contrast, lower resting heart rate and increased chest respiratory amplitude were found in the PE group, congruent with physical exercise training which mainly engages cardiovascular system of the body (Pumprla et al., 2002; Borresen and Lambert, 2008; Hillman et al., 2008). These results were consistent with previous findings of endurance exercise effects on autonomic control of heart rate (Okazaki et al., 2005; La Rovere and Pinna, 2014).

Five days of IBMT improves attention and self-regulation by changing central and autonomic nervous system interaction, and 1-month of continuous IBMT practice shows accumulated effects in a dose dependent fashion (Fan et al., 2010, 2013; Tang, 2017). Further, about 10 h IBMT over 1-month period increases white matter connectivity in ACR, key regions associated with ACC and other areas (Tang et al., 2010). Months to years of aerobic exercise improve physical health and cognitive performance (Hillman et al., 2008). The current 10-year training did not examine changes after varying periods of time. Thus, we do not know when and how much training is optimal; these questions will require further research.

Immune Function

Secretory immunoglobulin A (sIgA), an index of mucosal immunity, plays an important role in host defense. The secretory immune system of the upper respiratory tract's mucosal tissues is considered as the body's first line of defense against pathogens. Salivary sIgA becomes a focus of interest in psychoimmunological research since it has been shown to be sensitive to variations in subjective and objective stress levels (Fan et al., 2010). Prior research had shown that an additional training session immediately after acute stress increased release of salivary sIgA in a group trained with 5-day IBMT in comparison to a control group given the same amount of relaxation training. Additionally, 4 weeks of increasing amounts of IBMT significantly increased basal sIgA level, suggesting further improvements in mucosal immune function. As we predicted, the long-term IBMT group had a greater sIgA level than the PE group. This higher immune function in an aging population may be of importance in maintaining health (Tang, 2017).

Our results and explanations must be considered in the context of several limitations. First, a relatively small sample of subjects with more females was recruited following the 10-years of training, so we could not answer the questions of sex-differences and dispositional influences (Killgore and Yurgelun-Todd, 2001; Gross, 2007; McRae et al., 2008; Tang et al., 2016). Future large trials will allow us to explore the sex-differences in behavioral, physiological and brain changes following longitudinal practice. Second, our sample was from a Chinese aging population; although our American adult results showed the same underlying mechanism for IBMT (Tang et al., 2012, 2015a; Tang, 2017). Replication of these findings would be helpful in a western aging population. Third, our design did not include no-training group. Since both PE and IBMT had already been shown to have benefits, it did not seem ethical to have a group with no activity for 10 years. However, this design may generate additional valuable information on how the IBMT and PE training postpone the normal aging process compared to a waitlist group. Moreover, future research should have assessments before and after the 10-years of training and examine differences at varying points during training since we do not know when and how much training is optimal. It should be noted that our previous research has reported mindfulness effects on attention, cognitive performance, emotional states and stress regulation. In the current study, we aimed to further explore physical and psychological changes of quality of life using widely validated WHO Quality of Life Survey. Future research should include multi-faceted questionnaires to fully evaluate the potential changes. Since our study findings are related to Alzheimer's Disease (AD) biomarkers, future intervention studies could clarify any differential response to the interventions in older adults with and without preclinical AD–and any effects of treatment on these AD biomarkers themselves in rigorous, randomized controlled trials. Finally, future studies should examine the benefits of combining PE and IBMT to determine if combining training methods would lead to further benefits. Overall, our study represents an important extension of previous research on the effects of physical exercise and meditation practice on the aging process.

In summary, the present findings suggest that the differences between long-term mindfulness practice and physical exercise may manifest in the functional architecture of this circuit including ACC, striatum and the parasympathetic branch of the autonomic nervous system. These differences are accompanied by higher quality of life ratings and immune function in the IBMT group. In contrast, PE group showed lower resting heart rate and increased chest respiratory amplitude, congruent with the notion that physical exercise training mainly engages and trains the cardiovascular system of the body. These results were also consistent with previous findings of endurance exercise effects on autonomic control of heart rate. Since the mechanisms of PE and IBMT are partially distinct, it is feasible to integrate physical and mental training to achieve greater health and well-being.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of DUT Institutional Review Board. The protocol was approved by the DUT Institutional Review Board. All subjects gave written informed consent in accordance with the Declaration of Helsinki.

AUTHOR CONTRIBUTIONS

Y-YT designed the study. YF, QL, and Y-YT performed research and analyzed data. Y-YT, L-HT, RT, KF, RK, MP, BT, KC, and ER interpreted the data and wrote/edited the manuscript.

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Chronic Psychological Stress, but Not Chronic Pain Stress, Influences Sexual Motivation and Induces Testicular Autophagy in Male Rats

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Spermiogenesis is an important physiological process of mammalian fertilization. The germ cells are susceptible to the harmful effects of either psychological or physiological stress, which could induce male infertility. Our previous studies have found that chronic psychological stress could decrease sexual motivation. However, molecular mechanisms underlying male reproductive toxicity induced by chronic stress remain elusive. Recently, autophagy is proven to be involved in regulating the survival of germ cells, which is related to apoptosis. Herein, we established a chronic psychological stress model and a chronic pain model (physiological stressor) to explore the roles of autophagy in germ cells. Thirty-two male Sprague-Dawley rats were randomly divided into four groups, including the control group, the chronic psychological stress group, the SNI-sham group, and the chronic pain stress group. After exposure to stress for 35 days, open field test and the unconditioned sexual motivation test were performed. Following the behavioral experiment, autophagy in the rat testis was detected by Western blot and immunohistochemistry. We found both chronic psychological stress and chronic pain stress reduced total travel distance, the frequency of central crossing and increased the sensitivity to mechanical pain. While chronic psychological stress, but not the chronic pain stress declined sexual motivation. Chronic psychological stress prompt the expression of LC3-II with the decreased expression of p62, indicating that chronic psychological stress induced autophagy in rat testis. However, there was no significant difference between the expression of LC3-II and p62 in male rats under chronic pain stress. Therefore, chronic psychological stress and chronic pain stress have common behavior changes, but due to its unpredictability, chronic psychological stress leads to a decline in sexual motivation in male rats and induced the autophagy in testicular tissues.

Keywords: chronic stress, chronic pain, testis, autophagy, sexual motivation

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INTRODUCTION

A declining trend in sperm concentration over the past 35 years with perceptions of the reasons of such deterioration in male reproductive health (Sengupta et al., 2017). Chronic stress triggers a series of cognitive dysfunction, metabolic syndrome, cardiovascular diseases, immune system dysfunction (Mulroney and Tache, 2010; Turdi et al., 2012; Lupien et al., 2018), and also affect the

reproductive system which could further cause male infertility (Ebbesen et al., 2009; Garcia-Diaz et al., 2015). Recent studies showed that chronic stress causes germ cell loss and apoptosis in testes, possibly as a result of testosterone decrease, affecting fertility (Almeida et al., 1998; Kanter et al., 2013; Retana-Marquez et al., 2014). However, apoptosis is closely related with autophagy which plays a role in various physiological functions such as intracellular pathogens clearance so as to protect the organism from various diseases. A study of autophagy in the reproductive system of rats found that defective autophagy and excessive apoptosis in the aggravation of testicular damage (Zhang et al., 2016). Therefore, we speculated that impaired autophagy after chronic stress exposure could have a potential correlation with reproductive health.

Chronic stress could induce autophagy in hippocampus, bone marrow, nerves, and synaptic regeneration, etc. However, few studies have focused on exploring changes in the autophagy activity of the reproductive system and the mechanism merit further investigation. In our daily life, the most common stressors could be simply divided into psychological stress and physical stress. Clinically, these two types of stress are prone to comorbidity, such as anxiety, depression and chronic pain. Therefore, comparing the effects of different sources of stress on reproductive system would supply a profound understanding of the pathogenic mechanisms of stress in male infertility.

Our previous study found that chronic unpredictable mild stress (CUMS) could trigger organic damage to testicular cells in male rats in correlation with alterations in sexual preference behaviors (Hou et al., 2014). In the present study, we aim to further investigate the effect on testicular germ cell autophagy and apoptosis by chronic psychological stress and chronic pain stress model.

MATERIALS AND METHODS

Animals

A total of 34 male and 4 female Sprague-Dawley rats (Shanghai Laboratory Animal Center at the Chinese Academy of Sciences, Shanghai, China) weighing 230 ± 10 g were housed under temperature- and humidity-controlled (22 ± 2°C, 50–60% humidity) rooms and supplied with food and water ad libitum. Thirty-two male rats were randomly divided into a control group, a chronic psychological stress group, a SNI-sham group, and a chronic pain group. The chronic psychological stress group and the chronic pain group were established by the chronic unpredictablemild stress model and the spared nerve injury (SNI) model, respectively. Rats were placed in the rooms 7 days prior to testing in order to ensure adaptation to the environment (12-h light, 12-h dark cycle, lights on 8 a.m.) and habituation to handling. Two male and four female Sprague-Dawley rats were prepared for the unconditioned sexual motivation test. All animal protocols were approved by the Animal Care and Use Committee for the Department of Psychology at Zhejiang Sci-Tech University in accordance with NIH guidelines for the care and use of laboratory animals.

Chronic Unpredictable Mild Stress Model

The experimental procedure was adapted from that described by Hou et al. (2015). Briefly, male rats were randomly separated into the control group (n = 8) which were remained undisturbed in their cages, whereas the rats in the stress group (n = 8) were housed separately and were exposed to CUMS for 35 days. Seven stressors (a tail clamp stimulus, wet bedding, electric foot shock, cold water immersion, food deprivation, water deprivation, and reversed light/dark cycle) were applied in a random order for 35 consecutive days during the light phase. The experiment was conducted in accordance with the National Animal Welfare Standards and codes of ethics.

Spared Nerve Injury Model

This study used the sciatic nerve branch selective impairment (spared nerve injury, SNI) model for neuropathic pain study (Richner et al., 2011). Briefly, the main steps are brief described as follows: (1) anesthetize rats and cut open the outer epidermis of the thigh, and exposing the sciatic nerve and its branches by blunt separation including fiphointestinal nerve, common peroneal nerve and tibial nerve. (2) Tightly knot the common peroneal and tibial nerve with 5.0 wire, the SNI-sham group involved only the exposure of the sciatic nerve and its branches without causing any damage. 3) The muscle layer is then closed, then the wound is stitched and disinfected.

Estrous Cycle Determination

The estrous cycle phases of female rats were determined as previously described (Hou et al., 2014). Briefly, vaginal secretion was collected with a cotton swab and placed on glass slides. The vaginal secretions of the four female rats were collected and observed twice per day under microscopy at 8 a.m. and 10 p.m. Determination of estrous cycle phase was based on the two complete estrous cycles observed before the test day by the characteristics of the vaginal secretions of female rats.

The Unconditioned Sexual Motivation Test

The apparatus for the test of the unconditioned sexual motivation was described as previously (Agmo, 2003; Hou et al., 2014). The time spent in incentive zones, the number of visits to the zones, and the total distance traveled were monitored and recorded by Noldus EthoVision XT (Noldus, Netherlands). First, the subjects were familiarized with the test arena for 3 days, 10 min/day, without incentive rats in cages. Before each test, the arena and the cages were carefully cleaned with 75% alcohol. Then, the incentives (one female and one male rat) were placed in their cages. About 5 min later, the rat was introduced into the middle of the arena and observed for 10 min. The unconditioned sexual motivation was quantified by the preference score [time spent in the female incentive zone/(time spent in the female incentive zone + time spent in the male incentive zone)].

Open Field Test

The uncapped open box (80 cm × 80 cm × 40 cm) is used as a field test device, and the central grid is limited to a range of 40 cm × 40 cm in the central position. Each rat was then placed in the center of the uncapped open box, allowing freely movement for 5 min. The behavior was recorded by a video system. At the end of each rat experiment, clean the waste from the field with a clean paper towel and spray 75% of the alcohol to remove the residual odor. The frequency of rearing and central crossing, the latency time in the central zone and total travel distance were analyzed by Noldus software EthoVision XT.

Von Frey Test

According to previous study (Richner et al., 2011; Pitzer et al., 2016), each rat was placed separately in a cage to accommodate for 15min. Then, Von Frey fibers with varying diameters were used to test the rat's sensitivity to a mechanical stimulus, and each bending force was measured five times. The number of withdrawals was recorded. When the number of withdrawals reached 40% (2 out of 5), paw withdrawal mechanical threshold (PWMT) was recorded. Pain sensitivity tests of both feet were performed on all rats per week.

Histology

Briefly, after blocking with 0.5% BSA (Bovine serum albumin) in PBST (Phosphate Buffered Saline with Tween 20), paraffin-embedded testis slices were incubated with LC3 antibodies at 4°C overnight (Anti-LC3, MBL Inc, #PM036). Biotin-conjugated secondary antibodies (CST, #8114) and ABC-DAB staining kit (Vecta ABC and DAB standard kit, #PK-400 and SK-4105) were used for antibody localization and the nuclei were counter-stained with hematoxylin. For TUNEL staining, tissues were dewaxed and detected with Fluorometric TUNEL System (Promega) according to the working manual.

Western Blot

Proteins were extracted from rat testes in RIPA buffer and separated by SDS-PAGE. The resolved proteins were transferred to PVDF membranes (Millipore). Non-specific reactivity was blocked in 5% bovine serum albumin for 2 h at room temperature. Diluted primary antibody (LC3B, SIGMA-ALDRICH, #L7543, diluted 1:1000; p62, CST, #5114, diluted 1:1000) was then incubated, followed by the appropriate secondary antibody. Protein detection was achieved with the ECL (Thermo Fisher Scientific). Relative protein level was calculated as a percentage of reference protein β-actin (CST, #4970, diluted 1:1000).

Statistics

All statistics were performed with the software program SPSS 17.0 (IBM Inc., Armonk, NY, United States). Data were presented as mean ± SEM. Comparison between groups was done for statistical significance by a *t*-test. Effects were considered significant for $p < 0.05$.

RESULTS

The Effect of Chronic Stress on Body Weight Gain and Anxiety-Like Behavior

From day 1 to day 35, the control group, the chronic psychological stress group, the SNI-sham group, and the chronic pain group were measured for body weight weekly (**Figure 1** and **Supplementary Figure S1**). The increase in body weight compared to day 1 was calculated. The data showed that the weight gain of the psychological stress group was significantly lower than that of the control group from the first week to the fifth week, but there was no significant difference in the weekly weight gain between the SNI-sham group and the chronic pain group. It is indicated that the chronic psychological stress model significantly reduces the weight gain of rats. In addition to the weight loss caused by surgical operation, SNI did not have a significant effect on weight gain.

The pre-test of open field experiment showed that there was no statistical difference in the total travel distance, the frequency of central crossing, number of rearing and the duration of the central zone in the four groups of rats (**Supplementary Figure S2**). However, after exposure to the chronic stress for 5 weeks, the post-test of open field (**Figure 2**) exhibited the total travel distance and number of rearing in the chronic psychological stress group was significantly reduced than the control group ($p < 0.01$), and latency time in the central zone was also significantly decreased ($p < 0.01$). Compared with the SNI-sham group, there was significant decline in the total travel distance ($p < 0.01$) and the frequency of central crossing ($p < 0.05$) in the chronic pain group, but not the number of rearing or the duration of the central zone.

The Effect of Chronic Stress on Pain Sensitivity

Before chronic stress, there was no difference in pain threshold among the four groups of rats (**Figure 3**). After 35 days of chronic

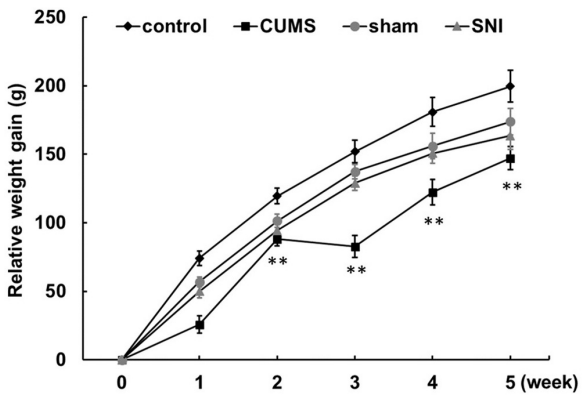

FIGURE 1 | Chronic psychological stress model significantly reduces weight gain in rats. Relative weight gains were body weight per week relative to body weight at week 0. Data are shown as mean ± standard error ($M \pm SEM$). $N = 8$. $^{**}p < 0.01$ vs. control group.

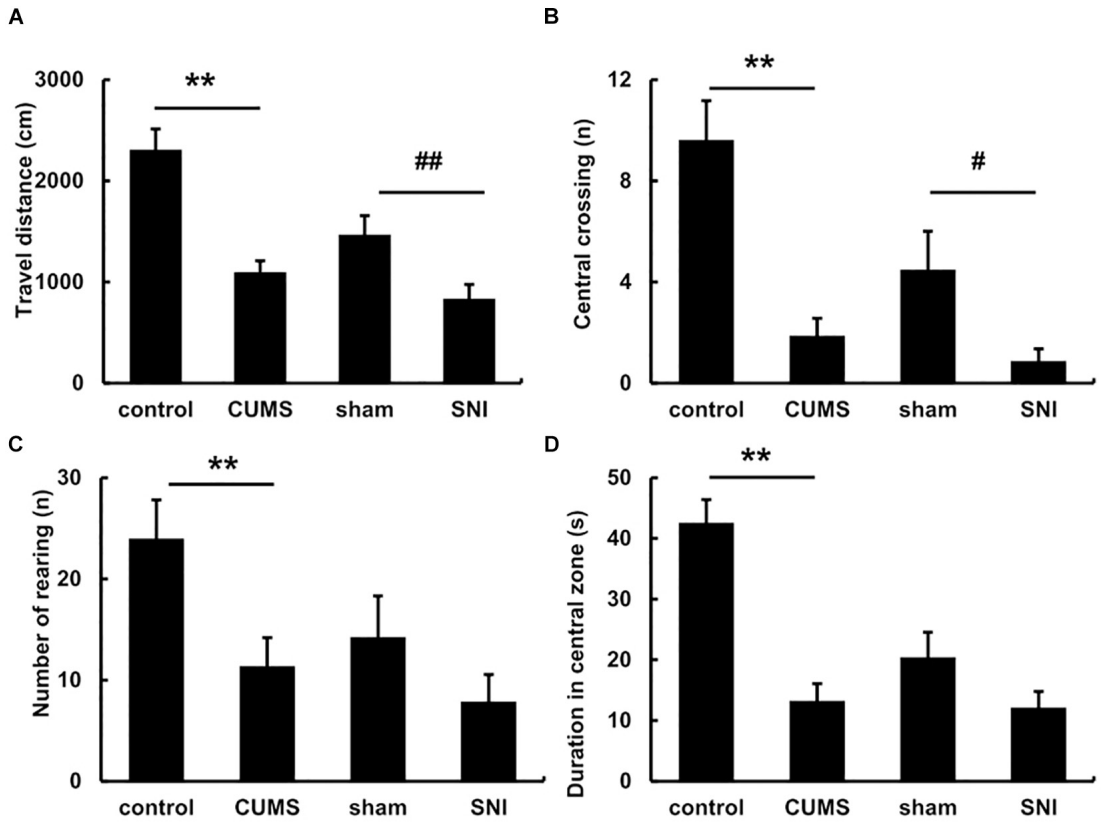

FIGURE 2 | The open field behavior change in the total travel distance **(A)**, the frequency of central crossing **(B)**, number of rearing **(C)** and the duration of the central zone **(D)** after exposure to chronic stress for 5 weeks. Data are shown as mean ± standard error ($M \pm$ SEM). $N = 8$. $^{**}p < 0.01$ vs. control group. $\#p < 0.05$ vs. SNI-sham group.

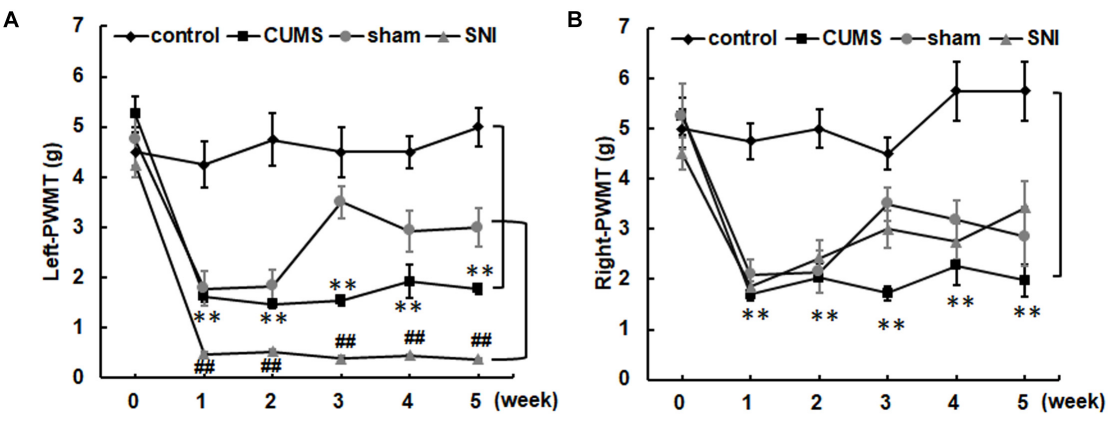

FIGURE 3 | The left **(A)** and right **(B)** paw withdrawal mechanical thresholds of the four groups. Data are shown as mean ± standard error ($M \pm$ SEM). $N = 8$. $^{**}p < 0.01$ vs. control group. $\#\#p < 0.01$ vs. SNI-sham group.

stress, the left and right PWMTs of the chronic psychological stress group were significantly lower than those of the control group ($p < 0.01$). However, chronic psychological stress and chronic pain stress caused the threshold of mechanical pain in the left paw of rats to be significantly lower than that in the control group and SNI-sham group ($p < 0.01$). The threshold of mechanical pain in the right paw of the chronic pain group was not significantly different from that of the SNI-sham group, while the left paw (injury paw) of the chronic pain group was significantly lower than that of the SNI-sham group ($p < 0.01$), indicating that the chronic pain model was successfully established.

The Effect of Chronic Stress on Sexual Motivation

The vaginal smear of the diestrus is mainly composed of leukocytes, while the estrus smear is primarily consisted of cornified cells (data not shown). The time between diestrus and estrus can comply with the requirements for experiments.

For the preference scores in **Figure 4**, when female rats came into estrus, the preference score of the chronic psychological stress group was significantly reduced compared with that of the control group ($p < 0.01$). In the psychological stress group, there was significant decrease between the preference scores of male rats for estrous and diestrous female rats ($p < 0.01$), while in the control group the preference score of male rats for estrous female rats was higher than that for diestrous female rats ($p < 0.05$). When female rats came into diestrus, there was conversely significant increase in preference score between the psychological stress and control groups. In addition, the chronic pain stress did not display any obviously influence on the alteration of sexual motivation.

For the time spent in incentive zones, the results showed that there was no significant difference between the time that male rats in the stress group spent in the male zone when female rats were in estrus and the time that male rats in the stress group spent in the female zone when female rats were in diestrus ($p > 0.05$). When female rats were in estrus, less time was spent in the female incentive zone in the chronic psychological stress group compare to the control group ($p < 0.01$) (**Figure 4**). Meanwhile, we found that there was no difference between the time spent near the female zone in the chronic pain group and that in the SNI-sham group when female rats were in estrus or diestrus ($p > 0.05$).

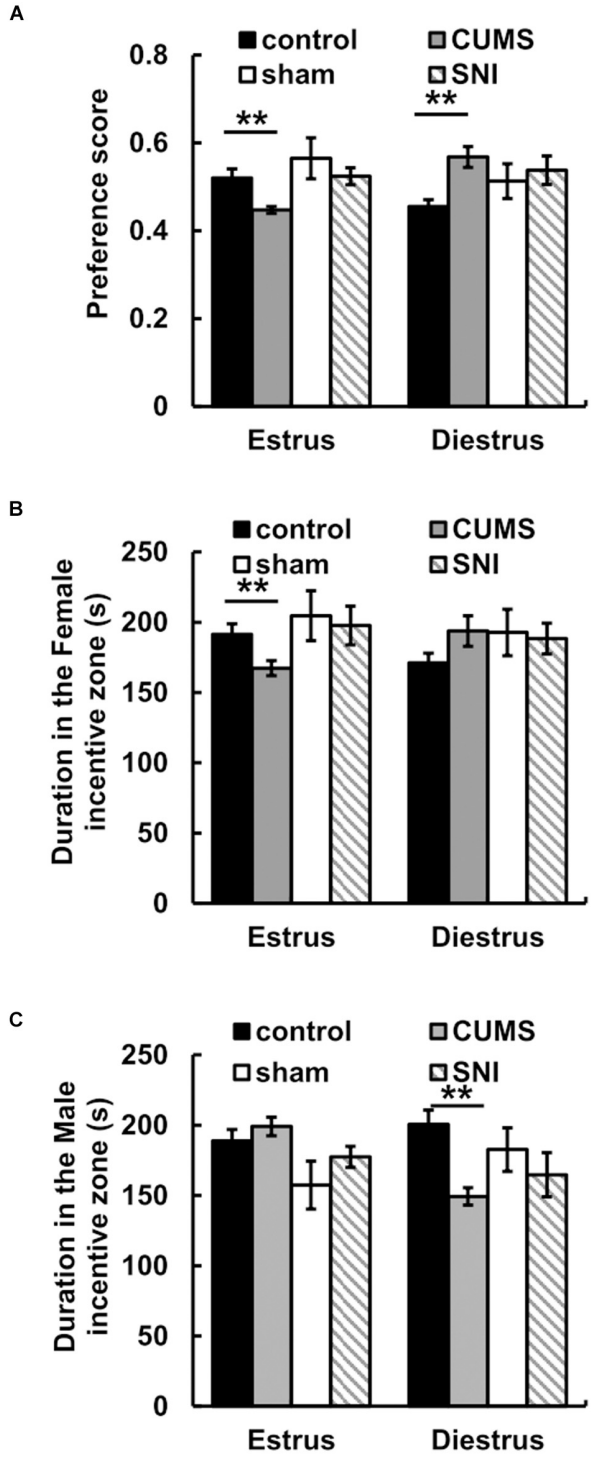

FIGURE 4 | The effect of chronic stress on preference scores of estrus and diestrus rats **(A)** and sexual motivation **(B,C)**. Data are shown as mean ± standard error ($M \pm$ SEM). $N \geq 7$. $^{**}p < 0.01$ vs. control group.

Chronic Stress Induced Autophagy in Testicular Tissues

The expression of LC3 was observed by immunohistochemistry (**Figure 5**). The percentages of LC3 positive cells in the testes of the four groups were calculated by Image J software. The results showed that the percentage of positive cells in the chronic psychological stress group was significantly higher than that in the control group, indicating that the autophagy of the chronic psychological stress group increased, but there was no significant difference between the SNI-sham group and the chronic pain group ($p > 0.05$). Western blotting also indicated that the expression of LC3-II in the chronic psychological stress group was significantly higher than that in the control group ($p < 0.05$). Similarly, there was no significant difference in LC3-II protein expression levels between the SNI-sham group and the chronic pain group (**Figure 5D**). The expression of p62 in the testis of the chronic psychological stress group was significantly lower than that of the control group ($p < 0.05$) but not between the SNI-sham group and the chronic pain group (**Figure 5E**).

Chronic Stress Induced the Testicular Cell Apoptosis

As shown in **Figures 6A–H**, tunel foci of both chronic stress model groups were more obviously than those of the control group and the SNI-sham group. Apoptosis occurred in three types of cells in the testis including Leydig cells, spermatogenic cells and supporting cells, which were mainly observed in the

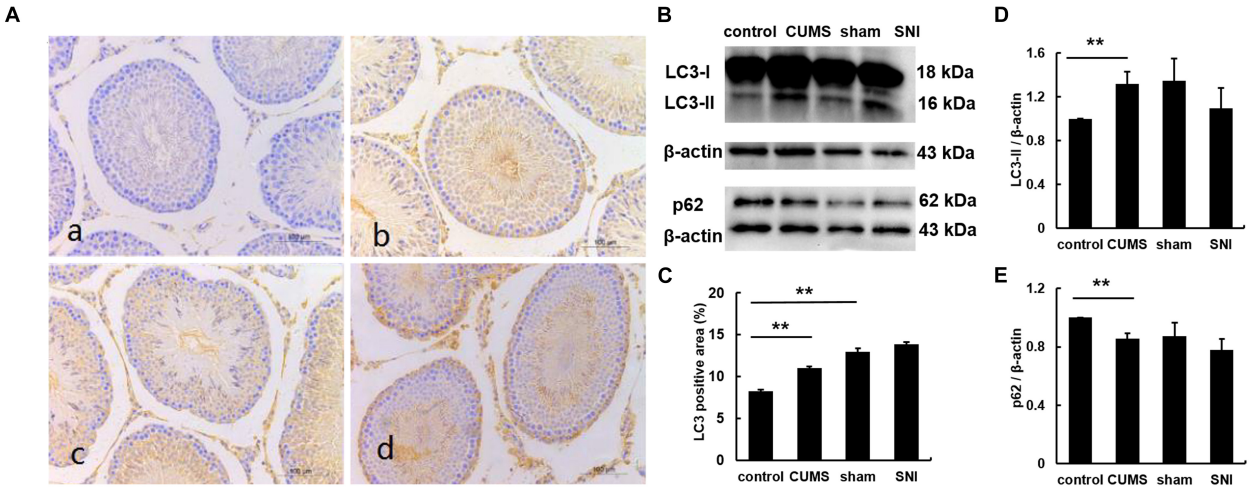

FIGURE 5 | The expression of LC3B protein in the testis tissues of four groups (a, control group; b, CUMS group; c, SNI-sham group; d, SNI group) by IHC staining **(A)**, and the LC3 positive area was quantified **(C)**. The expression of LC3 and p62 in the testis tissues of four groups **(B,D,E)** by western blot. Data are shown as mean ± standard error ($M \pm$ SEM). $N \geq 6$. $^{**}p < 0.01$ vs. control group.

both chronic stress groups. Descriptive statistics were performed on the number of testicular apoptotic cells in each field of the four groups of rats (**Figure 6I**). It was indicated that the number of testicular cell apoptosis in the chronic psychological stress group was significantly higher than that in the control group ($p < 0.01$). In addition, the number of apoptotic cells in the chronic pain group was also significantly higher than that in the SNI-sham group ($p < 0.01$).

DISCUSSION

Compare chronic psychological stress with chronic pain stress, two stress models were partly similar in behaviors, such as induced the sensitivity of mechanical pain threshold and anxiety-like behavior in rats. However, chronic psychological stress but not chronic pain stress attenuated male preference for the estrous female rats and generated autophagy and apoptosis in seminiferous tubules. Here, the results suggested that decreased sexual motivation under the psychological stress could not only be characterized as a kind of behavior disorder but also essentially impair male reproductive system.

Sexual motivation is one of the incentives, so we used an experimental device to detect this motivation and evaluated sexual motivation level with the preference score in rats (Agmo, 2003). In this study, the female preference scores of the rats in the chronic psychological stress group were significantly lower than those in the control group, indicating that chronic psychological stress attenuated the sexual motivation of the rats. On the other hand, the SNI-sham group and the chronic physiological stress group did not show significant differences in the female preference score and the latency time at the stimulation area, indicating that chronic pain did not affect male preference for the estrous female rats. Chronic unpredictable mild stress model due to its unpredictability mimicked different daily life stressors has a more obvious effect on behavior disorder than constant physical stress, so the outcome of the chronic psychological stress group showed that chronic psychological stress could lead to more serious stress related diseases including infertility (Hou et al., 2014; Demirci and Sahin, 2019).

Male reproductive function could be disrupted by various chronic stressors. It is reported that immobilization stress and forced swimming stress augment testicular toxicity and reduce the fertilization capacity in rats (Saki et al., 2009; Priya and Reddy, 2012). In the process of stress, autophagy is a very important metabolic pathway for the survival of the body. By studying the effects of chronic psychological stress and chronic pain stress on hippocampal autophagy, we found that autophagy was increased under chronic psychological stress while the autophagy in chronic pain group was almost constant (data not shown). Therefore, it could be speculated that different stressor participates in different ways.

An increase of LC3-II and accumulation of autophagic vesicles were found in spermatogonial cells disrupted by glutathione metabolism (Mancilla et al., 2015). Additionally, LC3-II expression was increased in 6 h, 12 h and 2 days after heat stress treatment in mouse testicular (Zhang et al., 2012). When male Sprague-Dawley rats were exposed to different doses of formaldehyde for four weeks, autophagy in testicular tissue in the high-dose treatment group was significantly increased (Han et al., 2015). In addition, intraperitoneal injections of different doses of cadmium chloride to rats for five weeks also induced an increase in LC3-II expression (Wang et al., 2017). The expression of LC3-II in the above studies is consistent with our results under the chronic psychological model, indicating that testicular LC3-II could be characterized as a common indicator of chronic stress including chemical, physical, and psychological stress.

However, the increase only in LC3-II does not indicate the activation of autophagy, because the increase in autophagosome LC3-II may be due to the fact that autophagy activation or

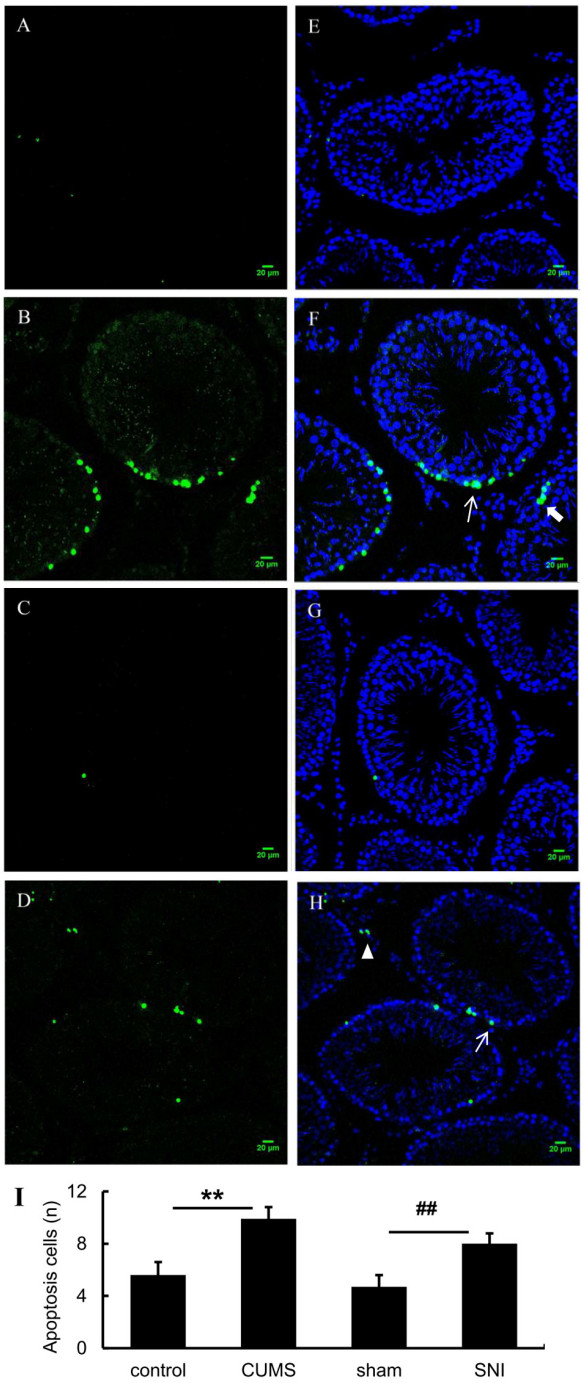

FIGURE 6 | TUNEL (green) positive apoptotic cells in rat testis induced by chronic stress (**A**, control group; **B**, CUMS group; **C**, SNI-sham; **D** group, SNI group). The nuclei were counter-stained with DAPI (**E**, control group; **F**, CUMS group; **G**, SNI-sham group; **H**, SNI group). Quantitative analysis of TUNEL staining **(I)** are shown as mean ± standard error ($M \pm SEM$). $N \geq 6$. $^{**}p < 0.01$ vs. control group. $\#\#p < 0.01$ vs. SNI-sham group.

inhibition of autophagy downstream degradation which would induce the accumulation of a large number of autophagosomes. Therefore, in the study of autophagy, the detection of autophagy substrate protein, p62, is often used as one of the hallmark autophagy degradants in autophagy (Sahani et al., 2014). p62 migrates not only to the phagocytic membrane, but also to the site of autophagosome formation. During autophagy, p62 can be selectively degraded by binding to LC3-II, so the accumulation of p62 often indicates that autophagy is inhibited. This is one of the reasons that p62 is used to detect autophagy flux (Pankiv et al., 2007). Here we found that the p62 protein in the testis of the chronic psychological stress group was significantly lower than that of the control group, but there was no significant difference in the p62 protein between the chronic pain group and the SNI-sham group. Combined with the results of the LC3 expression, it was indicated that chronic psychological stress triggered testicular autophagy in rats.

Under chronic pain stress, there was no significant difference between the rat testis LC3-II and p62 and the SNI-sham group, indicating that chronic pain did not affect the autophagy of rat testis. It was further found by immunohistochemistry that the expression of LC3B in the psychological stress group was significantly higher than that in the control group after DAB staining. There was no significant difference between the SNI-sham group and the chronic pain group, which was consistent with the results of Western blot. These data demonstrated that chronic psychological stress but not chronic physiology stress lead to a rise of autophagy in testicular tissues of male rats.

As known, some environmental toxicants, including heavy metals, pyrethroid pesticides, other endocrine disruptors, lifestyles, etc., can cause male reproductive toxicity (Sharpe, 2000; Wang et al., 2016), in which testicular histopathology is a necessary condition for assessing male reproductive toxicity. Previous studies have showed that chronic stress could lead to qualitative and functional damage to the testis, such as inhibition of spermatogenesis, specific stage of germ cell apoptosis, and testicular atrophy (Yin et al., 1997; Hjollund et al., 2004). In addition, apoptosis of testicular germ cell are different under chronic stresses. For example, primary spermatocytes and sperm cells are susceptible to heat exposure (Yin et al., 1997), and immobilization stress can enhance apoptosis of testicular spermatogenic cells (Wyllie et al., 1980). In the present study, according to the TUNEL test results, the number of apoptotic cells in the chronic psychological stress group was significantly higher than that in the control group, and the number of apoptosis in the chronic pain group was also significantly higher than that in the SNI-sham group, indicating that organic damage occurred in the reproductive system of two models. Compared with chronic psychological stress, chronic pain stress did not affect male sexual motivation and autophagy, but could affect germ cell apoptosis, indicating that the mechanisms of these two stress models acting on the reproductive system might be different.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

ETHICS STATEMENT

The animal study was reviewed and approved by the Animal Care and Use Committee for the Department of Psychology at Zhejiang Sci-Tech University.

AUTHOR CONTRIBUTIONS

GH and YS contributed conception and design of the study. YS, DH, LH, YB, BW, and YX performed the experiments. YS, DH, LH, and YB performed the statistical analysis. GH and YS wrote the manuscript. All authors contributed to manuscript revision, read and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpsyg.2020.00826/full#supplementary-material

FIGURE S1 | A timeline of the study. After adaptation for one week, 32 male rats were randomly divided into control group, CUMS group, SNI-sham group and SNI group. At day 0 and the end day, behavior tests were performed. And, weight gain and Von Frey test were measured per week.

FIGURE S2 | The open field behavior in the total travel distance (A), the frequency of central crossing (B), number of rearing (C) and the duration of the central zone (D) before exposure to chronic stress. N = 8, no significance.

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Screening Depressive Disorders With Tree-Drawing Test

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Objective: Diagnosis of psychiatric disease is still a major issue. Two key reasons are- there are variations in the opinions of the medical doctors and the presentation of a disease among the patients. Here we introduce a kind of mental projective test, tree-drawing test, trying to extract and analyze objective indexes in tree-drawing test in patients with depression.

Methods: The tree-drawing test was administered to 43 patients with major depressive disorders, 48 sub-threshold subjects, and 59 healthy subjects. Features of the drawing trees were analyzed using a kind of computer image recognition and data acquisition software. Quantitative indexes collected from pictures drawn by patients with major depression, patients with sub-threshold depression, and control subjects were compared using the ANOVA test.

Results: Five quantitative features (canopy area, canopy height, canopy width, trunk width, and total area of trees) were found to be statistically significant among the groups, while seven other features (trunk area, trunk height, root width, root height, root area, ratio of crown to trunk height, and ratio of crown to trunk area) showed no statistical significance. Further analysis with LSD-t test revealed that six quantitative indexes were significantly related to the depression symptoms, and six others were not statistically significant. Eleven quantitative indexes were not statistically significant when the depressive symptoms were compared with the subthreshold depression group, and the only index with statistical significance was canopy width.

Conclusion: Five quantitative indexes in the drawing tree are statistically significant in the depression patients were compared with those of the control subjects. Quantitative indexes of the tree-drawing test are of great value in assisting with the diagnosis of psychiatric disorders.

Keywords: tree-drawing test, affective disorders, depression, major depressive disorders, quantitative study, emotion

INTRODUCTION

Major depressive disorder is affecting more than 10% of populations worldwide, and the World Health Organization anticipated that it will be the first health problem in 2030 (Gu et al., 2018). However, diagnosis of depression (like many other psychiatric diseases) is still a major issue, and the two key reasons are: there are variations in the opinions of the medical doctors and the presentation of a disease among the subjects (Mistra et al., 2012). Current diagnoses, which are typically viewed as the "golden standard" (such as low mood, no interest, thought retardation, no motivation, thinking of suicide), depend on interview and self-rated questionnaires (Davison et al., 2009). Even though the criteria of diagnostic manuals, like DSM and ICD, is getting more and more detailed, some reports suggested that the diagnosis of depression is substantially underdiagnosed in primary care (Schwartz et al., 2019), or the rate of diagnosis of depression in non-depressed patients was estimated to be as high as 26.5% (Aragones et al., 2011). Some papers even suggested that clinician-based standardized diagnoses are not feasible, even not as good as self-reported questionnaire (Fisher et al., 2015). In addition, BD is often misdiagnosed as Major Depression Disorder (MDD), with approximately 40% of BD patients being initially diagnosed as MDD (Correia et al., 2009). These proposals suggest a need for definitive and truly objective physical or chemical index, or a judicious selection of MDD case-finding instruments depending on the study population and target periods of assessment (Owora et al., 2016).

So there are many seeks for new ways to diagnose depression, such as EEG based depression recognition (Li et al., 2019), or voice acoustics (Hashim et al., 2017). EEG is might be a good biomarker for depression, for example, a recent EEG study suggests that P1 amplitude to sad face showed potential as a state marker of depression (Ruohonen et al., 2020). Voice acoustic features extracted from reading speech demonstrated variable effectiveness in predicting clinical depression scores. Voice features were highly predictive of Hamilton depression. The methodology is feasible for diagnostic applications in diverse clinical settings as it can be implemented during a standard clinical interview in a normal closed room and without strict control on the recording environment.

Tree drawing is one kind of mental projective test too, which refers to the free expression of thoughts that can then be interpreted to reflect inside thoughts of the subject (Wang et al., 2014). Projective tests usually employ ambiguous stimuli, notably inkblots to evoke responses that may reveal facets of the subject's personality by projection of the internal thoughts, for example, Rorschach inkblot test is a kind of test that includes 10 irregular but symmetrical inkblots, and asks the subjects to explain what they see. The subject's responses are then analyzed in various ways, such as what was said, the time taken to respond, which aspect of the drawing was focused, etc. Other projective methods involve requiring the subjects to build wooden block structures, complete sentences, tree-drawings. The results are based on psychdynamic interpretation of the details of the drawing, such as size, shape, complexity of the tree. Projective test is a personality test designed to reveal hidden emotions and internal conflicts. The tree drawing test is easy to use and has less stress on the patients, and most importantly, the patients cannot easily hide their emotions, because they do not know which feature represents depression, unlike that in the questionnaire report.

Tree drawing test is a very useful tool in the differential diagnosis of mental health and the qualitative evaluation of treatment outcomes (Igimi et al., 2001; Morita et al., 2001). The tree-drawing test was developed in 1952, and quickly attracted the attention of researchers and has been widely used by clinicians (Hu and Chen, 2012). There were extensive researches of tree drawing test in the psychological field which demonstrated that tree drawing has a good ability to distinguish between pathological condition and normal condition (Kan and Guangxing, 2008; Kaneda et al., 2010). According to domestic and foreign literature reports, the tree-drawing test is reliable and valid (Chen and Xu, 2008a) and some scholars have used the tree-drawing tests in the diagnosis of neurosis, depression, and found some indexes are reliable markers for these mental disorders (Chen et al., 2011; Cai et al., 2012). The tree-drawing tests have been used in emotional test, psychological screening, and post-disaster relief (Chen and Xu, 2008b), and have been proved to be a useful tool for the diagnosis of emotional disorders (Inadomi et al., 2003).

Some studies have reported that certain common indexes of tree drawings are linked to emotional disorders. For example, some investigators reported that certain qualitative indicators of the tree-drawings differ in patients with mental health problems from that in healthy people, and they found eight drawing characteristics have a high level of diagnosis utility, and suggested that the tree drawing test is of some value for diagnosis of depression in adolescents (Inadomi et al., 2003). And it has been suggested that the values concerning the size of the tree, such as the height and width of the whole tree, height and width of the crown, and number of occupied areas (of the paper), were significantly lower in the depression (Murayama et al., 2016). However, these indicators are relatively primitive and existing studies have only addressed the correlation between the tree-drawing test and mental health problems. In this study, we used an impartial quantitative analysis to collect data from the tree-drawings. We used image scanning and computer image recognition technology to quantitatively collect the height and width of drawn trees, such that the size of the tree canopy, trunk, and roots were also determined from a scanned image. Therefore, we compared and analyzed the differences between the tree-drawing test indexes of patients with major depression in order to explore the in-depth putative diagnostic indexes of major depression, and also to evaluate the effectiveness in its clinical diagnostic application. This may help determine whether tree drawing tests can be introduced as an identifier of sub-threshold and depression in clinical psychology. In addition, it may be possible to use these quantitative indexes as an auxiliary diagnostic tool for the identification of various emotional disorders.

PATIENTS AND METHODS

Patients

Major depressive patients were clinically diagnosed patients, with stable symptoms and features, and they were recruited from the Department of General Psychiatry of Zhenjiang Mental Health Center from February 2017 to November 2018. These patients were newly admitted to and resided at the Center for depression. The inclusion criteria were: (1) patient met the DSM-5 mental and behavioral disorder classification for diagnosis of depression (Fan, 1993); (2) age 18–60 years old, no gender limits; (3) Hamilton Depression Scale (HAMD) score, 24 items ≥20 points (He, 1999; Zhang and He, 2015); (4) patients were selected between days 3 and 7 post-admission, i.e., during the symptomatic period. Exclusion criteria: (1) pregnant, lactating, or menopausal women; (2) psychoactive substance abuse and other severe psychiatric disorders; (3) patients with severe unstable physical diseases, diagnosed diabetes, thyroid disease, or hypertension. In this study, there were 43 patients with major depressive disorders, 14 males and 29 females, aged 18–49 years, with an average age of 34.3 ± 7.8. Patients with depression were hospitalized for 4–6 weeks. Medications used include: escitalopram, sertraline, and venlafaxine.

The sub-threshold depressive group and control group included subjects residing in the same region as the patients and were enrolled in this study at the same time. Enrollment criteria: (1) no obvious symptoms of psychiatric disorders (SCL-90, no positive factor), no previous history of mental illness; (2) match the gender of the experimental group; (3) did not receive any training in drawing. Exclusion criteria were the same as the patient group. The sub-group and the normal group were divided with the Hamilton score (**Figure 1**), and got 48 subjects in the sub-threshold group and 59 subjects for the control group. The sub-threshold group includes 23 males and 25 females, aged 18–55 years old (35.3 ± 8.6). The control group include were 26 males and 33 females, aged 18–50 years, with average age of 32.3 ± 8.9 years (**Figure 1**).

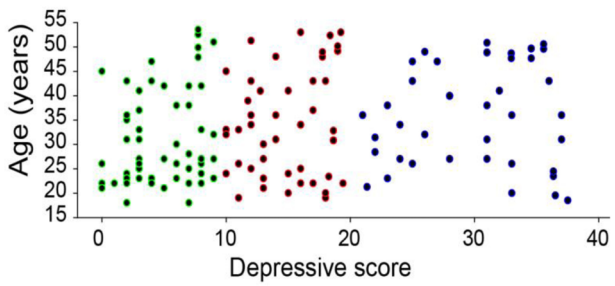

FIGURE 1 | The distribution of the subjects and their scores in Hamilton Depression Scale. The control group scored less than 10, while the sub-threshold group scored higher than 10, and the MDD group scored higher than 20. The average age for control was 32.3 ± 8.9 (n = 59), sub-threshold group was 35.3 ± 8.6 (n = 48), and MDD group was 34.3 ± 7.8 (n = 43). And the mean score from Hamilton was 4.44 ± 2.6, 14.5 ± 3.5, 28.1 ± 5.4 for the three groups, respectively.

Research Tools

Tree-Drawing Test

In order to make the experimental procedures standardized, we used the same procedure as reported before (Wang et al., 2014). Briefly, each participant was provided with A4 paper and a black or blue-black pen. Participants were instructed to draw a tree following these five rules: (1) The tree-drawing test is not a test of drawing technique and the drawing does not need to be aesthetically pleasant; (2) The tree does not need to appear life-like; (3) If you want to draw something that you are not capable of drawing, you can draw a circle and identify the intended object in writing with words; (4) Before you draw a tree, close your eyes and meditate for half a minute. Draw the tree that appears in the meditation. If there is no tree in the meditation, open your eyes and draw the tree that is most appealing to you; (5) After completing the drawing, write down your age, gender, and occupation at the bottom of the paper.

In addition, in the process of collecting the drawn trees, each drawing was inspected. If some features or indexes were not immediately identifiable, the drawing was discarded from the analysis. In all of the drawings collected, there were two drawings from control group and three drawing from the MDD group were discarded.

High-Definition Scanning

The Epson GT-1500 HD scanner was used to scan drawn trees and to save scanned images.

Data Collection

R&D image data scan acquisition software (**Figures 2**, **3**) was used to extract data. The project of developing software to analyze drawn trees was developed in 2015–2017, with technical support provided by the School of Computer Science of Jiangsu University. The software was licensed by the National Copyright Administration in July 2017 (Liu, 2017). The software automatically and accurately extracts tree length, width, height and area data, and calculates the proportion of each part.

Statistical Analysis

All data were shown as mean ± standard deviation, and analyzed using SPSS 17.0 statistical software. We used one-way ANOVA and LSD-t test for analysis.

RESULTS

Size of the Canopy, Trunk, Roots, and the Entire Tree

After the data from drawn trees were collected, statistical software was used to perform one-way ANOVA test. We first analyzed the ratios of the canopy area and trunk area to the root area, as it was reported before that the ratio between canopy and trunk as well as roots was different in the depression group. However, even though the ratio of canopy to root is higher than that of the depression group and sub-depression group (Pintea et al., 2014), they are not significantly different among the groups ($p > 0.05$, one-way ANOVA, **Figure 4**). The differences in the size

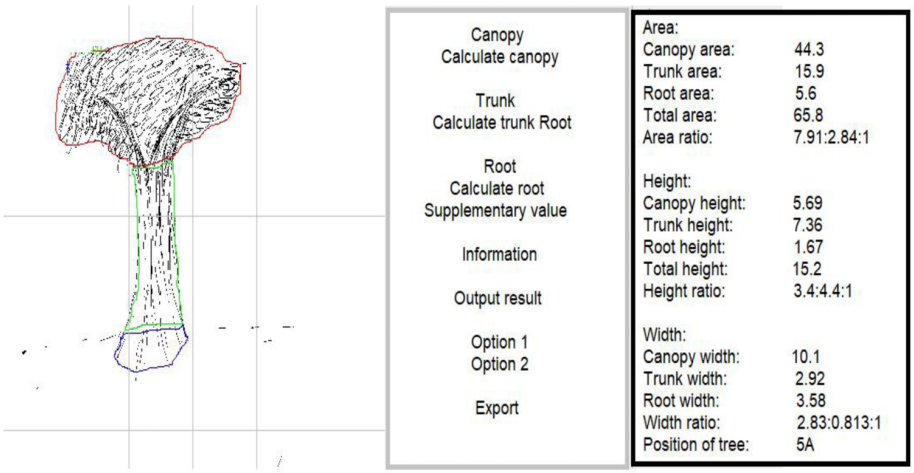

FIGURE 2 | Example of scanned tree in major depression group.

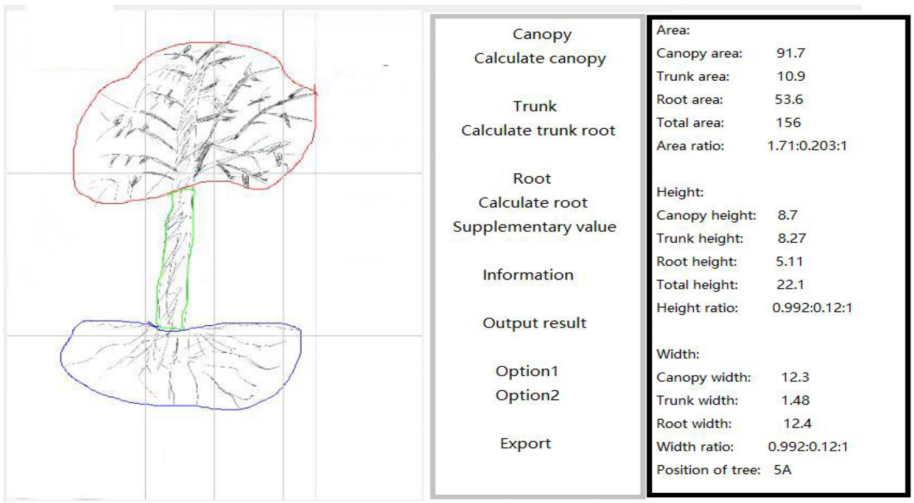

FIGURE 3 | Example of scanned tree in control group.

of canopy, trunk and total tree area among the three groups were statistically significant ($p < 0.05$, one-way ANOVA). There was no significant difference between the depression group and the sub-threshold group in canopy area, trunk area and total area of trees ($p > 0.05$, one-way ANOVA). However, there were significant differences between the major depressive group with the control group in canopy area, trunk area, and total area of drawn trees ($p < 0.01$, one-way ANOVA, **Table 1**).

Height of the Canopy, Trunk, Roots, and the Whole Tree

There were significant differences among the three groups in canopy height and in the height of whole trees ($p < 0.05$, one-way ANOVA, $n = 48 = 59$). The results of the analysis were post-tested. The difference in canopy height between the depression group and the sub-threshold group was statistically significant. The difference in canopy height between the depression group and the control group, as well as the difference between the sub-threshold group and the control group were statistically significant ($p < 0.01$). As for the total height of the tree, there were significant differences between the depression group and the sub-threshold group, as well as between the depression group and the control group. It was not significantly different between the sub-threshold group and the control group ($p > 0.05$, one-way ANOVA, **Table 2**). The ratios of canopy height, trunk height to root height were not significantly different either (**Figure 5**).

Width of the Canopy, Trunk, and Root

There were statistically significant differences among the three groups in the width of the canopy and the width of the trunk ($p < 0.01$, one way ANOVA, $n = 48$–59). *Post-hoc* results of the analysis showed that the difference in width of the canopy was

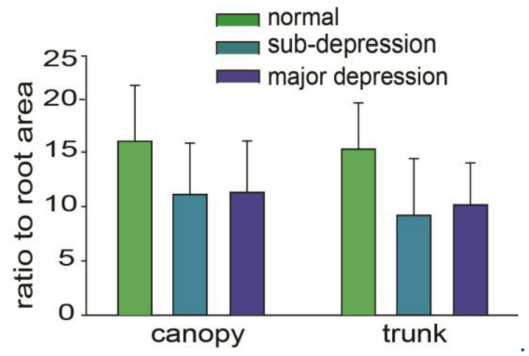

FIGURE 4 | Comparisons among the ratios of canopy area, trunk area to root area.

statistically significant between the three groups ($p < 0.01$). The depression group, the control group, and the sub-threshold group show a statistically significant difference in the width of the trunk as well ($p < 0.01$). It was not significantly different in the width of the trunk between the depression group and the sub-threshold group (**Table 3**). The ratios of canopy width, trunk width to the root width were not significantly different either (**Figure 6**).

Logistic Regression Analysis of Symptoms of Depression and Sub-Threshold

Quantitative indexes were selected for Logistic stepwise regression analysis. The respondent variable is "Y," $Y = 1$ is a patient with depression, $Y = 2$ is a sub-threshold patient, $Y = 3$ is a control subject; the independent variable is "X," X1–X11 represent 11 quantitative indexes of drawn trees. The independent variable assignment is shown in **Table 4**.

Logistic stepwise regression analysis revealed that there were seven quantitative indexes of the depression group in the regression equation, while there were four quantitative indexes of the sub-threshold group in the regression equation, $X^2 = 73.564$, $P = 0.000$, and the Logistic regression equation was statistically significant. The results are shown in **Table 5**.

Establish Regression Equations Based on Logistic Regression Analysis

From the regression coefficients in **Table 4**, the regression equations of the quantitative indexes for symptoms of depression and symptoms of sub-threshold can be obtained, and the Nagelkerke R2 coefficient test can be performed to explain the regression equation. The regression equation of drawn tree quantitative indexes for depressive symptoms is: $\ln(\frac{Y=1}{Y=3}) = 2.877 - 3.186\ X1 - 3.085\ X2 - 3.173\ X3 + 3.2\ X4 + 3.021\ X7 - 0.298\ X9 - 0.978\ X10$; The regression equation of quantitative indexes on sub-threshold symptoms is: $\ln(\frac{Y=2}{Y=3}) = 2.395 + 1.839\ X4 - 0.252\ X9 - 0.694\ X10 - 0.921\ X11$. The Nagelkerke R2 value is 0.395, indicating that the quantitative indexes are of an acceptable level.

DISCUSSION

Diagnosis of depression is still a major issue, and it is important to find more biomarkers for the diagnosis (Liu, 2017). The tree-drawing test is already in widespread use amongst psychiatric occupational therapists in Taiwan, but studies about objective standards are somewhat limited (Pintea et al., 2014), and many characteristics have been suggested to be related to many mental diseases (Gu et al., 2016). Results from this study show that there are statistical differences in the selected quantitative indexes of canopy area, canopy height, canopy width, trunk width, and total area of trees among the patients in the major depressive

TABLE 1 | Size (cm^2) of canopy, trunk, roots, and total tree in the groups.

Features	Canopy area	Trunk area	Root area	Total tree area
Depression	45.18 ± 42.34**	9.16 ± 9.64**	2.61 ± 7.59	56.76 ± 49.96**
Sub-threshold	55.17 ± 51.28*	16.80 ± 7.81*	4.39 ± 3.81	98.45 ± 23.43*
Normal	90.83 ± 61.96	19.33 ± 6.47	4.37 ± 12.46	114.52 ± 73.87
F	8.22	5.23	0.85	10.32
P	0.000	0.001	0.67	0.000

$^{**}p < 0.01$, $^{*}p < 0.05$, post-hoc results, compared with control.

TABLE 2 | Height (cm) of canopy, trunk, root, and whole tree in the groups.

Feature	Canopy height	Trunk height	Root height	Total tree height
Depression	6.22 ± 3.34**	5.01 ± 3.27	0.51 ± 0.99	11.74 ± 5.48**
Sub-threshold	7.56 ± 3.87*	6.66 ± 4.78	0.78 ± 0.74	13.46 ± 6.39*
Normal	8.72 ± 3.62	6.43 ± 3.02	0.47 ± 1.09	15.63 ± 5.41
F	6.16	1.95	0.844	4.754
P	0.003	0.21	0.237	0.007

$^{*}p < 0.05$, $^{**}p < 0.01$, post-hoc results, compared with control.

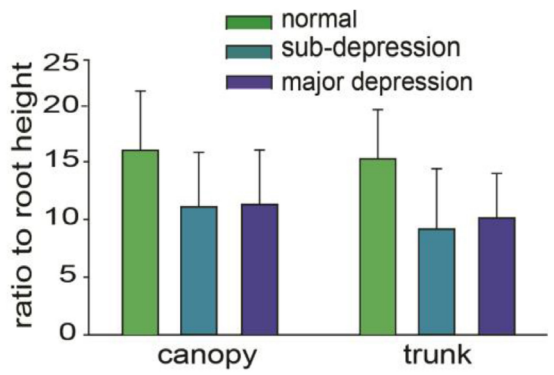

FIGURE 5 | Percentage of canopy, trunk, and root height in the total height. The differences were not statistically significant ($p > 0.05$, one way ANOVA).

TABLE 3 | Canopy, trunk, and root width (cm) in the groups.

Quantitative index	Canopy width	Trunk width	Root width
Depression group	7.53 ± 3.84**	1.67 ± 1.39**	1.29 ± 2.93
Sub-threshold group	10.54 ± 4.89*	3.38 ± 1.79	1.67 ± 2.32
Control	12.10 ± 4.78	3.73 ± 2.28	2.20 ± 4.82
F	14.28	11.75	0.966
P	0.000	0.000	0.517

*$p < 0.05$, **$p < 0.01$, post-hoc results, compared with control.

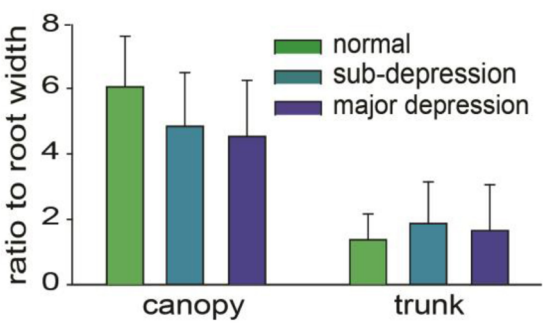

FIGURE 6 | Ratio of canopy width and trunk width to root width, the differences were not statistically significant ($p > 0.05$, one way ANOVA).

TABLE 4 | Variable assignment description ($n = 145$).

Variable	Quantitative index
X1	Canopy area
X2	Trunk area
X3	Root area
X4	Total area
X5	Canopy height
X6	Trunk height
X7	Root height
X8	Total height
X9	Canopy width
X10	Trunk width
X11	Root width

group, sub-threshold group and the control group. In addition they are co-related with the severity of the depression. However, there were no significant differences in trunk area, trunk height, root width, root height, root area, height of canopy to trunk, and area of canopy to trunk. These findings indicate that selected quantitative indexes from the tree-drawing test can be used as an auxiliary diagnostic tool to screen depressive disorders. This is consistent with previous reports that canopy mainly reflects the state of the patient's emotional state of the person, and the root of the tree mainly reflects the subject's unconscious instincts (Wang et al., 2014). Thus the tree drawing test can be used to diagnose depression through the size of the canopy, and also the ratio between crown and trunk (Li et al., 2011).

Analysis of Canopy of the Trees

The canopy is the part of the tree that is used to connect with the external environment and to exchange with the outside world. Therefore, the canopy was suggested to reflect mainly the conscious emotional state of the subjects (Kaneda et al., 2010). It is suggested that the canopy expresses the subjects' understanding and structure of his or her intimate relationship with family, relatives, and others. In addition, the canopy also describes the spiritual and emotional intellectual development of the subject, scope of interest, goals and aspirations, and their overall satisfaction (Kaneda et al., 2010). The height, width and area of the canopy in the depression group were much smaller than those of the control group. This is consistent with symptoms of depression, helplessness, feelings of uselessness, lack of interest, loss of interest, and pessimism. In this study, the total area of trees in the depression group was significantly smaller than that in the control group, and the difference was statistically significant. The results of this study also show that the height, width and size of the canopy in the depression group were smaller than those in the control group, and the difference was statistically significant. It has been pointed out that the size of trees directly reflects the current overall state of the individual. Tall trees indicate individuals with better development, high mental energy levels, sufficient self-confidence, etc. When trees are short, subjects are often found to have depression (Yan and Chen, 2011; Hu and Chen, 2012). In all, the results of the tree-drawing test in patients with depression reflect their personality traits, including depressed motivation, low self-esteem, lacking of vitality, lacking of self-control, dependence, withdrawal, disengagement, and self-destruction (Ji, 2011).

Analysis of Canopy of the Trees Trunk

The area, height and width of the trunk of the depression group were also smaller than those of the control group, and the differences in the three indexes were statistically significant. This is possibly due to the proposal that trunk is correlated with the inner emotional function of the person and represents the unconscious emotion state of the person (see **Figure 7**). The width of the trunk can be seen as an indicator of emotional

TABLE 5 | Logistic regression analysis of the quantitative indexes of tree drawings in depression and sub-threshold.

Group	Characteristic	B	S.E.	Wald	df	Sig	Exp(B)
Depression group	X1	−3.18	1.23	6.650	1	0.010	0.041
	X2	−3.08	1.22	6.326	1	0.012	0.046
	X3	−3.17	1.23	6.636	1	0.010	0.010
	X4	+3.20	1.23	6.731	1	0.009	0.009
	X7	+3.02	1.42	4.645	1	0.031	20.517
	X9	−0.29	0.13	4.912	1	0.027	0.742
	X10	−0.97	0.32	9.311	1	0.002	0.376
	Constant	+2.87	1.09	6.908	1	0.009	
Sub-threshold group	X4	+1.83	0.93	3.836	1	0.050	0.162
	X9	−0.35	0.32	5.86	1	0.054	084
	X10	−0.71	0.35	9.02	1	0.004	0.500
	X11	−0.85	0.47	4.42	1	0.026	0.398
	Constant	+3.25	1.29	4.76	1	0.018	

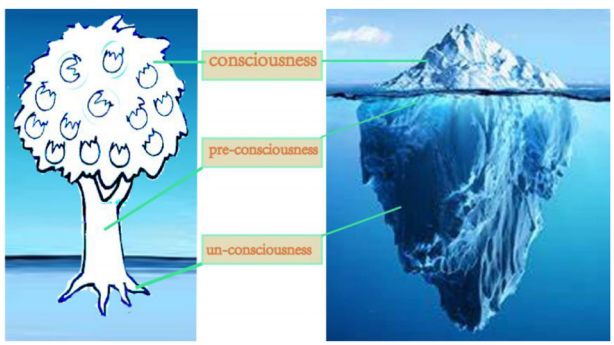

FIGURE 7 | Hypothesis about the representation of a tree-drawing with the consciousness.

depth, while the length represents the degree of domination of emotional mood (Ji, 2011). The core symptoms of depression patients include moodiness or depression, lack of interest, and loss of interest (Xie and Ye, 1994). The small trunk area of patients with depression reflects their negative mood and pessimism; the short trunk reflects their poor emotional control; the narrow width reflects their emotional vulnerability (Matt, 2015). An appropriate trunk area, height, and width might indicate a stable mood.

The results of our study also show that there are significant differences between the tree area and the trunk width of trees drawn by depressive patients and control subjects. Matt Rowley believes that the trunk is the most important part of a tree, connecting the roots and crowns, and feeding the branches and leaves, and that it is the foundation of a tree (Xie and Ye, 1994). In the tree-drawing test, the trunk is possibly the symbol of the inner self and symbol of emotion. Although disordered thinking is the most prominent manifestation of emotional disorder, it is also an important component and mainly manifested as emotional apathy: a cold and dull emotional response to surroundings, or emotional inversion, emotional dissonance, etc. Most MDD patients do not perceive their loss of emotional expression. Compared with that of the control group, the small trunk of depressive patients reflects the characteristics of affective disorders such as apathy, active requirements, reduced hobbies, and other emotional impediments. A wide trunk indicates long-term mood stability. The width of the trunk in patients with sub-threshold is smaller than that of the control group, indicating that their emotional response shows superficial and uncoordinated characteristics. Other studies have found that the ratio of canopy height to trunk height is different between patients with depression and healthy individuals (Pintea et al., 2014), however, our studies have not shown significant difference in the ratio of trunk height to the overall height of the tree, because the standard deviation is too high. Larger numbers of patients are needed for future studies to probe into this question.

Analysis of Canopy of the Tree Roots

There were not statistical differences in root area, width, and height among the three groups, as revealed by one-way ANOVA and LSD-t results. It is suggested that the root of the tree represents the instinct and unconsciousness of the subject (**Figure 7**). At the same time, some scholars believe that the root of the tree also reflects the cultural heritage of a person (Xie and Ye, 1994), or the traditional culture of the subject's living environment. The reason for the difference may be due to the fact that most Chinese are influenced by traditional culture and have oppressed instincts, and many people do not draw roots when painting trees. This led to too many missing data points in our statistical analysis. Some previous reports have extensively and profoundly compared both ancient and modern Chinese cultures and found that the vast majority of Chinese people have relatively conservative sexual morality and sexual behaviors (Kaneda et al., 2010).

Limits of This Study

The data in this study show that, of the 12 quantitative indexes studied, the only statistically significant difference is found in

canopy width between the major depressive and subthreshold groups, while the remaining 11 quantitative indexes were not significantly different. This is possibly due to the sample size of the groups. In the future we will add more subjects to test the ratios between the canopy, trunk and roots. In addition, the depressive subjects have had multiple episodes. In future studies, trees drawn by different episodes of major depressive patients should be compared.

In all, our data found that there are differences in the canopy size, width, and height in patients with major depression and those in the control group, indicating that quantitative indexes about the canopy in the tree-drawing test are meaningful in the diagnosis of mental disorders. As far as we know, this is the first report about the objective study of tree drawing, which may play a certain role in the diagnosis of emotional disorders. In addition, tree drawing may provide quantitative aids for clinical diagnosis, and provide a scientific basis for the development of emotional diagnosis norms.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the committee of Ethnic Jiangsu University. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

SG, FW, and WL designed the project, FL, RF, YLiu, GL, YLi, and MG did the experiments. FW, WL, and JH wrote the manuscript.

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