NATURE AND NURTURE, TWO SIDES OF THE COINS - WHERE WE ARE IN THE NEUROPSYCHIATRIC DISORDER RESEARCH

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ORIGINAL RESEARCH
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Increased Serum C3 and Decreased UA in Patients of Bipolar Disorder in Chinese Han Population

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The aim of this study is to explore the changes and clinical significance of serum C3, C4, hypersensitive C-reactive protein (hsCRP) and uric acid (UA) in patients of bipolar disorder (BD). In this case-control study, we recruited 141 BD patients from The Second Xiangya Hospital, Central South University, and 151 age and gender matched healthy controls (HC) from the health management central of The Second Xiangya Hospital. These patients were divided into two subgroups based on medicines use: 91 patients were treated with psychiatric drugs and 50 patients were drugs free, or four subgroups based on mood states: 54 patients in manic/hypomanic phase, 30 patients in depressive phase, 52 patients in euthymic phase and 5 patients in mixed phase. Serum levels of C3, C4, hsCRP and UA were measured in all subjects. The serum C3 levels in BD patients (0.9981 ± 0.1849 g/L) were significantly lower than that in HC group (1.0637 ± 0.2186 g/L), especially the drugs free subgroup and the euthymic subgroup (0.975 ± 0.153 and 0.983 ± 0.182 g/L), while the serum UA levels were significantly higher (354.6 ± 90.4 vs. 332.9 ± 88.7 μmol/L), especially the drug-treated subgroup and manic/hypomanic subgroup (361.56 ± 93.20 and 376.70 ± 88.89 μmol/L), and rates of hyperuricaemia (31.91 vs. 17.88%) were significantly higher in BD patients than in HC group. The serum C4 and hsCRP levels in HC group showed no significant difference with BD patients in whole or those subgroups. These findings suggested that the complement and purinergic systems of BD patients might be disrupted, the UA levels could be a potential marker in manic phase and the C3 might be the marker of therapeutic evaluation of BD patients.

Keywords: bipolar disorder, C3, C4, hypersensitive C-reactive protein, uric acid

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INTRODUCTION

Bipolar disorder (BD) is one of the severe mental disorders with a lifetime prevalence of 2.4% featured with the recurrence of depressive/manic episodes as well as mixed states (1). However, the etiology of BD remains uncertain. The immune-inflammatory has been suggested to play a role in the etiology of BD (2). In the previous investigation, the levels of inflammatory-associated cytokines were found increased both in the brain and peripheral serum in BD patients (3). C-reactive protein

(CRP) is a pentameric acute-phase protein that produced in the liver and secreted into the blood. CRP plays a prominent role in the innate immune system, as it increase rapidly in response to infection and inflammatory stimulation, then reduced sharply after the acute phase (4). Fernandes et al. (5) found that serum/plasma CRP levels are increased in BD patients than health control (HC) group regardless of mood states, particularly the CRP levels of those patients in manic/hypomanic phase were much higher than in depressive and euthymic phases. The complement system is one of the most important components of humoral immunity participating in both adaptive and acquired immune responses. CRP activates the classical complement pathway, and then stimulates phagocytosis via binding to Fc immunoglobulin receptors as an opsonin (4). Even though their importance in immune functions, complement factors have been barely investigated and levels of these factors among different stages of BD have not been compared. As inflammation responses often involve the complement system activation, we hypothesized that complement system factors might be related with the symptom severity of BD. Akcan et al.(6) found serum complement (C4, factor B, and sC5b-9) levels were significantly reduced in chronic BD patients when compared with HC group, while the peripheral blood mononuclear cell mRNA expression levels of C1q and C4 were significantly elevated. Thus, the detection of different components involved in this disorder might provide new possibilities of treatment, as it was reported that the adjunctive addition of anti-inflammatory medications with lithium has achieved complete remission in treating BD (7).

Recently, increasing evidences showed that BD might be closely associated with the dysfunction of adenosine and purinergic systems (8, 9). Adenosine is one of the purine nucleosides and appears to modulate some neurotransmitters, which has attracted our attention into understanding the pathophysiology of this disease that deep-rooted in human central nervous system (10). Uric acid (UA) is the product of xanthine or hypoxanthine catalyzed by xanthine oxidoreductase and the end product of purine nucleosides metabolism. The central UA levels have a strong positive association with peripheral levels (11), and the increased serum UA levels may be the sign of over-activation of purinergic system. A meta-analysis studied by Bartoli et al. (12) found that subjects with BD have higher UA levels than that in HC, especially in the manic/hypomanic and mixed phases. Recently, in a randomized, placebo-controlled study, a xanthine oxidase inhibitor allopurinol that used for gout and hyperuricemia was effective both in reducing serum UA levels and improving manic symptoms of BD patients (13).

The trait marker and state marker of each phase of BD has not been established. Therefore, the purpose of current study was to measure peripheral C3, C4, hsCRP, and UA levels in BD patients across the different mood states and look for whether levels of these indicators are correlated with the severity of mood symptoms. Besides, in order to find out if mood stabilizers could induce the changes of these parameters, we made a comparison between psychiatric drugs treatment and drugs free subgroup as well. In addition, considering this research has not been widely reported in Chinese Han population, we expect these results could provide potential diagnosis and treatment for BD patients in China.

MATERIALS AND METHODS

Subjects

One hundred and forty one BD patients were recruited from Department of psychiatry of the Second Xiangya Hospital, Central South University. Those patients were diagnosed by two senior psychiatrists according to criteria of the Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition (DSM-V). As we presented in the **Table 1**, 91 of 141 BD patients were taking psychiatric medication: 85(93.4%) were taking mood stabilizers, 68(74.7%) were taking antipsychotic medications and 12(13.2%) were taking antidepressants. The exclusion criteria for patients were any comorbid mental disorders, a history of traumatic brain injury, intellectual disability, serious somatic diseases, active or chronic inflammatory, autoimmune diseases, pregnancy or breast feeding. 151 age and gender matched HC were recruited from the health management center of The Second Xiangya Hospital at the same period after negatively screening for the presence of a past or current psychiatric symptoms. The severities of mood symptoms were assessed using the 17-item Hamilton Depression Rating Scale (HDRS) and the 11-item Bech-Rafaelsdn Mania Rating Scale (BRMS). Sociodemographic features of the group are demonstrated in **Table 1**.

This study was approved by The Ethics Committee of The Second Xiangya Hospital, Central South University. All the patients or their statutory guardians and HC were required to sign an informed consent forms.

Sample Collection and Processing

5 ml peripheral venous blood samples were drawn from fasting BD patients and HC in the morning and pro-coagulant tubes. Any sample with hemolysis was discarded and avoided repeatedly freezing-thawing. Peripheral serum were isolated after

TABLE 1 | Demographic data for BD patients and HC group.

	BD patients	HC	t/χ^2	*P*-value
Sex	N(%)	N(%)		
Male	61 (43.26)	67 (44.37)	0.036	0.906
Female	80 (56.74)	84 (55.63)		
Total	141	151		
Age(Mean ± SD)	29.11 ± 12.08	30.60 ± 11.04	1.101	0.272
MOOD STATES				
Mania/hypomania	54 (38.30)	/		
Depression	30 (21.27)	/		
Euthymia	52 (36.88)	/		
Mixed	5 (3.55)	/		
Drugs use(yes/no)	91/50	/		
HDRS(Mean ± SD)	18.04 ± 13.84	/		
BRMS(Mean ± SD)	22.80 ± 14.39	/		
Hyperuricemia	45 (31.91)	27 (17.88)	7.730	0.004

centrifuging at 3500 rpm for 10 min and stored at −80°C until analysis. All samples were measured after thawing to room temperature. The serum C3 and C4 levels were measured with immuno-scatter turbidmetric assay, in which C3 and C4 are combined with antibody to formed the complexes, hsCRP were measured with latex enhanced immunoturbidimetric assay in an automatic analyzer Abbott c8000, in which hsCRP are combined with monoclonal antibody attached on latex particle, and UA were analyzed by direct enzymatic method, in which UA was oxidized by uricase coupled with peroxidase in an automatic analyzer Hatichi 7600.

Statistical Analysis

Statistical analyses were performed with the SPSS (version 20.0). Data were presented as mean ± standard deviation (SD) for normal distribution variables (at Kolmogorov-Smirnov test) and median (quartile range) for non-normal distribution variables. Continuous variables were compared using Student's *t*-test or the Mann-Whitney U-test as appropriate. The rates of hyperuricemia (UA ≥ 420 μmol/L in male, UA ≥ 360 μmol/L in female) and gender distribution in these two study groups were compared with χ^2 test. When baseline characteristics among three groups were compared, normally distributed continuous variables were compared with the one-way ANOVA and skewed distributed with Kruskal-Wallis H test. Comparison between two groups were considered statistically significant if $P < 0.05$, Bonferroni correction was used to adjust our results for multiple comparisons.

RESULTS

Serum C3, C4, hsCRP, and UA Levels in HC Group and BD Patients

The mean age of the patients was 29.11 ± 12.08 years old, and 43.26% of the patients were males, and the HC was 30.60 ± 11.04 years and 44.37% were males. There were no significant difference in terms of age and gender between two study groups ($t = 1.101$, $df = 290$, $P = 0.272$; $\chi^2 = 0.036$, $df = 1$, $P = 0.906$). We enrolled 54 patients in manic/hypomanic state, 30 patients in depressive state, 52 patients in stable euthymia and 5 patients in mixed state. The serum C3, C4, hsCRP, and UA in BD patients and HC group were measured at the same time. Serum C3 levels in BD patients (0.998 ± 0.185 g/L) were significantly lower ($t = 2.76$, $df = 290$, $P = 0.003$) than that in HC group (1.064 ± 0.219 g/L), while the serum UA levels were significantly elevated ($t = -2.068$, $df = 290$, $P = 0.0195$) in BD patients (354.64 ± 90.37 vs. 332.96 ± 88.70 μmol/L). Meanwhile, the serum C4 and hsCRP levels showed no difference between two groups (0.240 ± 0.069 *vs.* 0.231 ± 0.070 g/L; 0.63 (0.33, 1.65) vs. 0.70 (0.26, 1.73) mg/L, respectively) as a whole (**Figure 1**). Both HDRS and YMRS

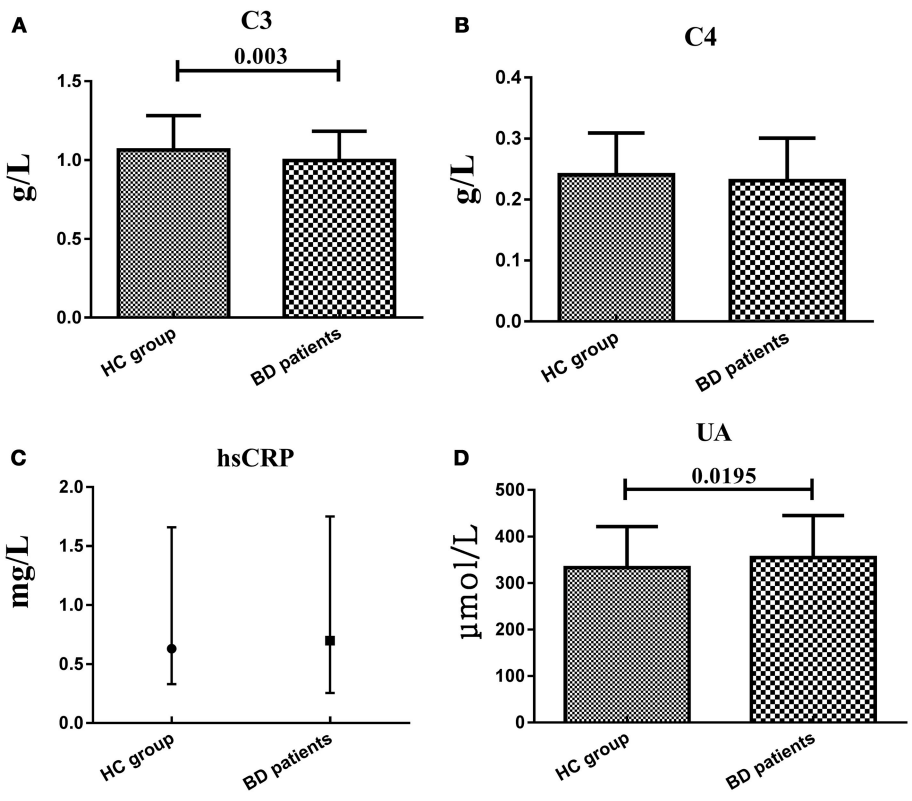

FIGURE 1 | Representative of the serum C3, C4, hsCRP, and UA levels in two groups. **(A,B and D)** were presented in mean ± SD and **(C)** was presented in median value and interquartile range.

scores showed no significant positive or negative correlation with serum C3, C4, hsCRP, and UA levels in BD patients.

Serum C3, C4, hsCRP, and UA Levels in Drugs Use/Free Group

Patients were evaluated in two subgroups in terms of previous treatment: never used any treatment or stopped treatment for at least 1 month before participating in this study, and continued their treatment for at least 1 month. Serum C3 levels in the drugs free subgroup (0.975 ± 0.153 g/L) were significantly lower ($t = 2.65$, $df = 199$, $P = 0.0045$) than that in HC group (1.064 ± 0.219 g/L), while the drugs treatment subgroup (1.011 ± 0.200 g/L) showed no significant difference with HC group. Serum UA levels in drugs treatment subgroup (361.56 ± 93.20 μmol/L) were significantly higher ($t = -2.384$, $df = 240$, $P = 0.009$) than that in HC group (332.96 ± 88.70 μmol/L), while the drugs free subgroup (342.04 ± 84.44 μmol/L) showed no significant difference with HC group. However, serum C4 and hsCRP levels in both drugs treatment and free subgroups showed no significant difference with HC group (**Figure 2**).

Serum C3, C4, hsCRP, and UA Levels in Different Mood States

Considering the small sample size, the patients in mixed state were not analyzed and compared with another three subgroups. There were no statistically significant difference of serum C3, C4, hsCRP, and UA levels between BD patients during different phases of illness, whereas instead of in another two subgroups, UA levels in manic/hypomanic phase (376.70 ± 88.89 μmol/L) were significantly higher ($t = 3.109$, $df = 203$, $P = 0.001$) than those of the HC (332.96 ± 88.70 μmol/L) and C3 levels in euthymic phase (0.983 ± 0.182 g/L) were significantly lower ($t = -2.406$, $df = 201$, $P = 0.0085$) than HC group (1.064 ± 0.219 g/L). Serum C4 and hsCRP levels in all these subgroups were not different with HC group (**Figure 3**).

Serum C3, C4, hsCRP, and UA Levels in Different Genders

To explain the effect of different genders on the levels of serum C3, C4, hsCRP, and UA levels, we compared these indicators between two genders within HC group or BD patients and between two groups from the same gender. Serum C3, C4 and hsCRP, levels showed no significant difference between males and females both in HC group and BD patients, nor the difference between two groups from the same gender. However, serum UA levels in female HC (285.02 ± 58.48 μmol/L) were significantly lower ($t = 9.328$, $df = 149$, $P = 0.000$; $t = 3.026$, $df = 162$, $P = 0.0015$, respectively) than that in male HC (393.06 ± 83.59 μmol/L) and female BD patients (317.02 ± 76.21 μmol/L), and the serum UA levels in male BD patients (403.97 ± 83.96 μmol/L)

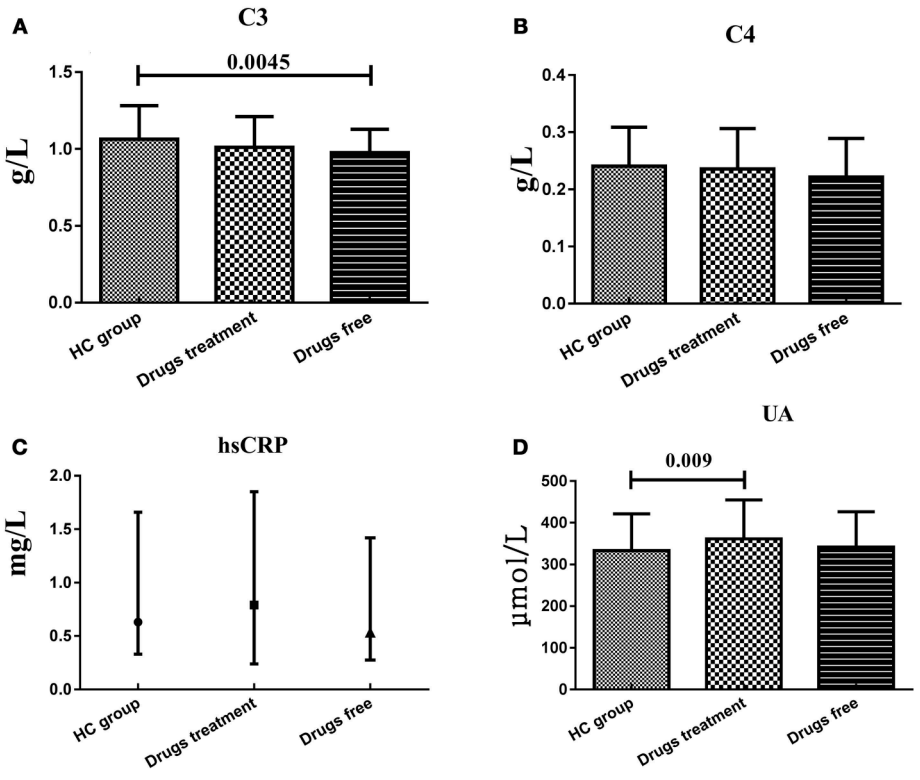

FIGURE 2 | Representative of the serum C3, C4, hsCRP, and UA levels in HC group and BD patients in drugs use and drugs free subgroups. **(A)** Statistical results showing the comparison of serum C3 levels between HC group and drugs treatment/free subgroup. **(B)** Statistical results showing the comparison of serum C4 levels between HC group and drugs treatment/free subgroup. **(C)** Statistical results showing the comparison of serum hsCRP levels between HC group and drugs treatment/free subgroup. **(D)** Statistical results showing the comparison of serum UA levels between HC group and drugs treatment/free subgroup.

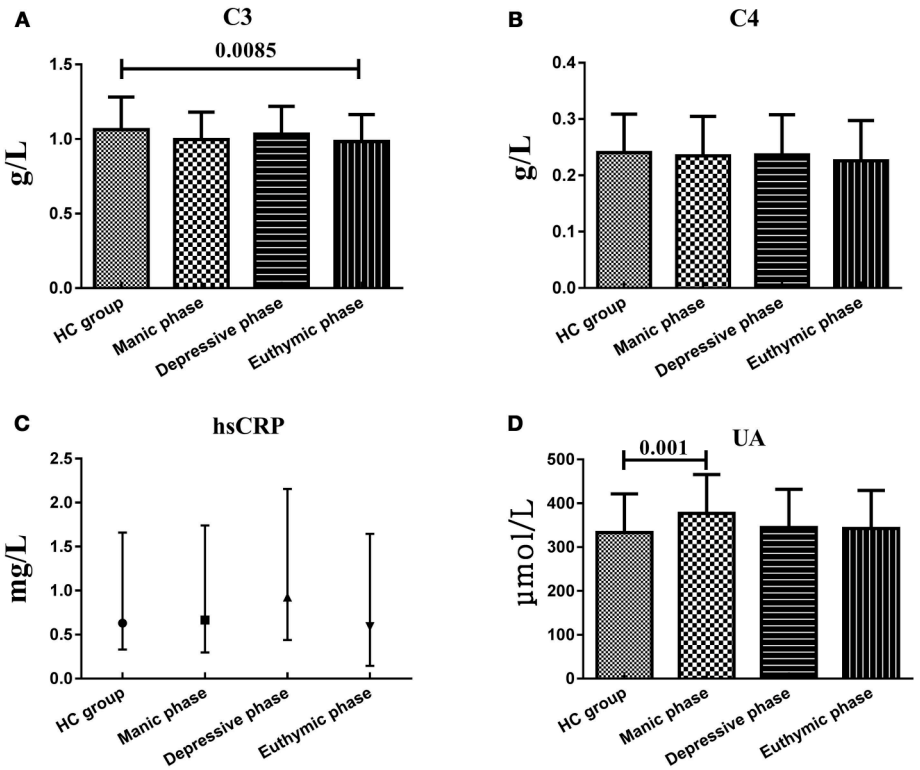

FIGURE 3 | Representative of the serum C3, C4, hsCRP, and UA levels in HC group and BD patients in different mood states. **(A)** Statistical results showing the comparison of serum C3 levels between HC group and patients in different mood states. **(B)** Statistical results showing the comparison of serum C4 levels between HC group and patients in different mood states. **(C)** Statistical results showing the comparison of serum hsCRP levels between HC group and patients in different mood states. **(D)** Statistical results showing the comparison of serum UA levels between HC group and patients in different mood states.

were significantly higher ($t = -6.423$, $df = 139$, $P = 0.000$) than that in female BD patients (317.02 ± 76.21 μmol/L) (**Figure 4**).

DISCUSSION

In psychiatric diseases, the mechanism of complement system has most widely been studied in schizophrenia and other mental disorders with plenty of contradictory results published previously, while the role of complement components in BD were scarcely reported. C3 is the most abundant complement in serum and the pivotal of connecting the classical and alternative complement pathways, and its serum levels was followed by serum C4 levels. So, the fluctuation of serum C3 levels is paralleled with total content of complement factors and the measurement of serum C3 levels could partly reflect the change of the whole complement metabolisms. C4 is activated by C1s and then hydrolyzed to C4a and C4b in the alternative pathways. In this primary study, we found that serum C3 levels in BD patients were significantly lower than HC, while C4 were not changed obviously. Therefore, we hypothesized that the complement system failed to maintain dynamic balance basically in the BD patients, but not knowing which one of the three pathways were disturbed, since there was no change in C4 levels. The significantly reduced serum C3 levels in drugs free subgroup of BD patients, but not in drugs treatment subgroup, illustrated that psychiatric drugs were able to increase serum C3 levels, and we can postulate that the decreasing C3 levels might be the cause of BD rather than the results. However, in a study reported by Akcan et al.(6) found that serum C4 levels were significantly reduced in chronic BD patients and the C4 mRNA expressions were elevated in a compensatory way. Furthermore, Santos and his colleagues failed to detect significant difference between HC and BD patients in euthymic state(14). In spite of these conflict results, this would be understandable when considering the huge discrepancy of sample size and the subjects recruited. In addition, there was no significant difference of serum C3 levels among these subgroups in different mood states, so in general, this indicator were not recommended as the state biomarker of BD patients.

Over the past decade, some authors have described the possible participation of purinergic system dysfunction in the pathophysiology of BD (15, 16). More recently, the increased serum UA levels in BD patients of manic state have been widely reported (8, 17). In accordance with these available data, our results confirmed that BD patients showed significantly higher serum UA levels and higher rates of hyperuricemia than HC group. In a large and multi-centric, nationwide population-based epidemiological investigation that spanning six years, Chung found that the BD patients had the increased risk of hyperuricemia that resulting in gout, indicating that the similar neurobiochemical mechanism were shared between these two

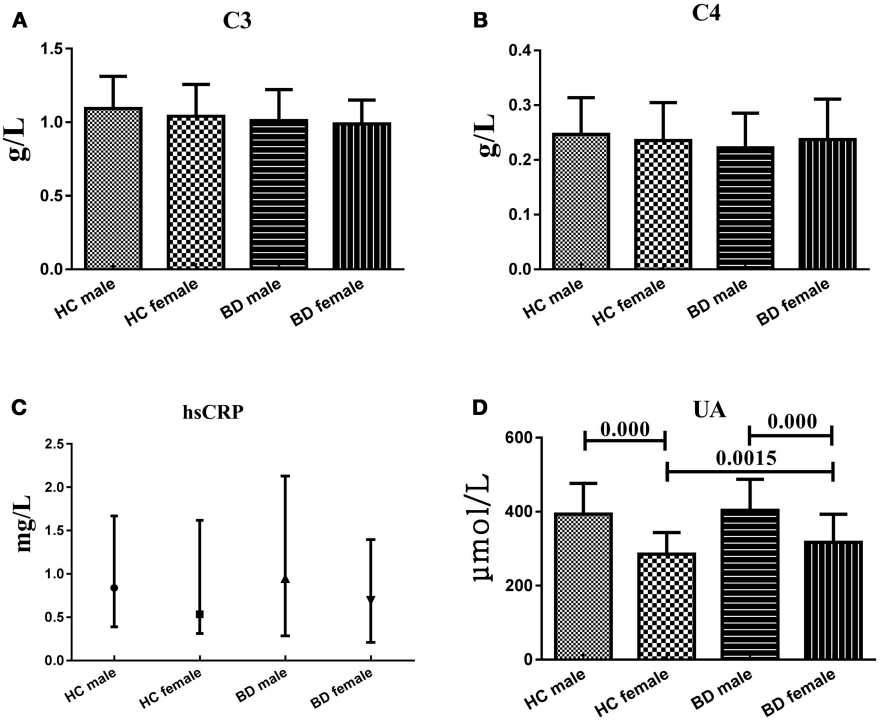

FIGURE 4 | Representative of the serum C3, C4, hsCRP, and UA levels in HC group and BD patients in different genders. **(A)** Statistical results showing the comparison of serum C3 levels between HC group and BD patients in different genders. **(B)** Statistical results showing the comparison of serum C4 levels between HC group and BD patients in different genders. **(C)** Statistical results showing the comparison of serum hsCRP levels between HC group and BD patients in different genders. **(D)** Statistical results showing the comparison of serum UA levels between HC group and BD patients in different genders.

diseases (18). Therefore, UA may be a promising biomarker in BD. Besides, the serum UA levels of BD patients in drugs treatment subgroup and in manic/hypomanic state were higher than HC group, but not the drugs free subgroup and patients in depressive and euthymic state. So, we agree that the routine measurement of serum UA levels might be helpful to identify patients in manic/hypomanic state who may benefit from adjunctive treatment with purinergic modulators (16). However, Albert reported that serum UA levels were higher in BD patients never exposed to mood stabilizers than HC group (8), which was contrary to our results. We speculated that the duration of drugs treatment maybe one of the critical factors should be considered seriously, and the short-term treatment might not able to reduce serum UA levels obviously. We were in a cautious attitude toward whether the increase of serum UA levels resulted from purinergic dysfunctions was the trait maker of BD, as we failed to detect significant differences among these BD patients in manic/hypomanic, depressive and stable euthymic states. In sum, serum UA level may represent key target in the search for clinically relevant biomarkers.

In the previous studies, pro-inflammatory and inflammatory cytokines were found elevated in manic episode of BD (19, 20), indicating inflammatory cytokines contribute to the pathophysiology of BD. According to our results, the serum hsCRP levels in BD patients showed no significant difference with HC group, and the differences among three mood states were moderate. It was reported that serum hsCRP levels were significantly higher in manic BD patients before treatment than HC group, and were significantly decreased after treatment. Therefore, serum hsCRP levels maybe a potential indicator in predicting treatment outcomes (20). However, the serum hsCRP levels in drugs treatment subgroup were not different from drugs free subgroup. A possible explanation is that the baseline hsCRP levels among individuals differs obviously from each other while this cross-sectional research didn't compare the serum hsCRP levels before and after treatment. A recent study about the structural volume change of a specific brain region along with cognitive function demonstrated that the orbitofrontal cortex had a significantly negative correlation with serum hsCRP levels after adjustment for age and gender. And authors speculated that persistent inflammation indicated by elevated serum hsCRP levels in euthymic phase may involve the pathogenesis or pathophysiology of alteration of the frontal pathway (21). Although not statistically significant, the results of the studies underlined above emphasize the role of hsCRP on BD.

In sum, this original research involved more subjects than most of previous studies and patients were grouped based on whether they were treated or not with psychiatric drugs and which mood states they are in. As for the gender factors, we failed to find any differences of serum C3, C4, and hsCRP levels between two genders within the HC group or BD patients, or between the two groups within the same gender. However, the serum UA levels in male HC group and male BD patients were both significantly higher than female HC group and female BD

patients, respectively. The significant gender difference within the same group could be explained by different lifestyle between males and females, like the diet, alcohol abuse, and cigarette smoking. The significantly higher serum UA levels in female BD patients than female HC group may attribute to the effect of estrogen on the UA metabolism.

Generally, some limitations and open questions requiring further research. The sample size of BD patients in mixed state was moderate, further research should involve more patients. In addition, we only measured the serum levels of those indicators, instead of the expression level. Even if we grouped these patient into drugs treatment subgroup and drugs free subgroup, the types of drugs, duration of administration and disease duration differed among each other. From this study, we could cautiously infer that UA could represent both a trait and state marker of BD, but whether a prognostic biomarker deserves further proof.

AUTHOR CONTRIBUTIONS

YL designed the study. HT and LX acquired the data, which YT and CL analyzed. XY, HT, and LX read and wrote the manuscript. All authors reviewed and approved for publication.

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REVIEW
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Depressive Syndromes in Autoimmune Disorders of the Nervous System: Prevalence, Etiology, and Influence

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Autoimmune diseases of the nervous system (ADNS) consist of a group of severely disabling disorders characterized by abnormal immune attack against protein components of the nervous system. This type of attack behavior may occur in the central or peripheral nervous system, and in the neuromuscular junction, resulting in neuronal damage, axonal injury, demyelination or destruction of the neuromuscular junction. While the neurological deficits of patients with ADNS have received significant research attention, the manifestation of depression tends to be ignored. In fact, depressive manifestation is common in ADNS and adds significant burden upon patients suffering from this disease. Here, we systematically reviewed the current literature to highlight the prevalence, etiology and influence of depressive manifestation in ADNS. Most autoimmune diseases of the nervous system are discussed in this paper, from multiple sclerosis, acute disseminated encephalomyelitis and autoimmune encephalitis to acute myelitis, neuromyelitis optica, Guillain-Barré syndrome and myasthenia gravis. Depressive symptoms usually develop as a comorbidity during the course of disease, but sometimes exist as a primary presentation of the disease. Psychosocial factors, long periods of disablement and chronic pain are the three most common causes of depressive symptoms in many chronic conditions, particularly in peripheral neuropathy. Furthermore, the higher prevalence of depressive symptoms in ADNS suggests that immunological dysregulation may contribute to the elevated morbidity of depression. Finally, structural lesions of the brain, and some medications for ADNS, are also thought to precipitate depressive states in ADNS.

Keywords: depression, anxiety, autoimmunity, nervous system disorder, etiology

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INTRODUCTION

Over the last few years, autoimmunity has been increasingly confirmed in a variety of neurological disorders involving both the central and peripheral nervous system. The concept of autoimmune disorders of the nervous system (ADNS) describes a broad spectrum of severely disabling disorders characterized by abnormal immune attack against protein components of the nervous system, which are mistaken as an invading antigen (1). Research has identified some of the novel autoantibodies involved, such as aquaporin 4 antibody (AQP4-Ab) and anti N-Methyl-D-Aspartate receptor antibody (NMDAR-Ab) (2). Such immune attack behavior may occur in the central or peripheral nervous system or the neuromuscular junction, resulting in neuronal damage, axonal injury, demyelination or destruction of the neuromuscular junction.

The complexity of autoimmune diseases, and the disabling effect of nervous system disorders in ADNS patients, requires timely and appropriate treatment, and careful clinical management. However, neurological deficit dominates the clinical profiling and outcome assessment of such patients. Depressive disorders or symptoms tend to be ignored, which may place a heavy burden on the patients affected (3). Indeed, various physical symptoms of the disease may be enhanced by a depressive condition, thus leading to poorer prognosis and a longer course of disease. Furthermore, overlapping neuropsychiatric presentations, such as fatigue and psychomotor retardation, could lead to an inadequate diagnosis and treatment of depression in affected patients. Depressive syndromes can also exert a strong negative effect upon a patient's quality of life and adherence to treatment. Conversely, severe physical symptoms can also reduce the level of depressive syndrome. Approximately 10–27% of patients with other types of neurological disorders also suffer from depression, including those with Parkinson's disease, Alzheimer's disease and post-stroke patients (4–6). However, these figures underestimate the noticeable likelihood of clinically significant depressive symptoms acting as a comorbid condition in some types of neurological disease, especially in patients with ADNS.

Depressed mood, lack of energy, pleasure loss, sleep disturbance and suicide ideation or attempts are the mainly depressive presentation. And mental retardation or agitation, loss of confidence are also frequently seen in ADNS. All these depressive symptoms can emerge with other psychiatric symptoms. The high incidence and negative influence of depressive syndromes in ADNS deserves increased clinical attention. Consequently, we systematically reviewed the current literature and aimed to highlight the prevalence and influence of depressive syndromes in ADNS. In psychology, different from primary depression, secondary depression describes the depressive state caused by other diseases including somatic disease-related depression and drug-induced depression. Therefore, we discuss the possible mechanisms underlying the elevated frequency of depression in ADNS.

Psychosocial factors and long duration are recognized as the basic causal factors of depression. Additionally, emerging neuroimmune inflammatory theories of depression support the doubtful causal connection between immunological dysregulation and comorbid depression in ADNS (7). Structural brain immune damage, as well as specific drugs for ADNS, is also thought to precipitate depressive states in patients with ADNS.

PREVALENCE AND PROFILE

Most autoimmune diseases of the nervous system are discussed in this paper, including multiple sclerosis, acute disseminated encephalomyelitis, autoimmune encephalitis, acute myelitis, neuromyelitis optica, Guillain-Barré syndrome and myasthenia gravis. For the convenience of description, autoimmune diseases of the nervous system discussed in this paper are divided into four parts according to the affected area; the brain, spinal cord, peripheral nervous system and neuromuscular junction.

Depressive Syndromes in ADNS Involving the Brain

Multiple sclerosis, acute disseminated encephalomyelitis and autoimmune encephalitis are grouped together because of their involvement with brain tissue, among which lesions in specific areas may correlate with changes in mental condition and behavior.

Multiple sclerosis (MS) is an acquired demyelinating disorder of the central nervous system (CNS) caused by an autoimmune response, affecting one in 1,000 individuals in high-prevalence areas and making MS the most frequent entity of neurological disability in young people (8). The association between MS and depression has been acknowledged for some time (9). Patients with MS have a higher risk of developing depressive syndromes which can emerge at any stage of the disease course. Depressive syndromes in MS are characterized by low mood along with predominant fatigue and sleep disturbance in the depressed background, but with a lower frequency of comorbid anxiety disorder(10). Some MS patients report psychiatric symptoms, such as hallucination, suicidal ideation and compulsive-obsessive symptoms after medication for MS or depression(11). About 15% MS patients present with depressive symptoms before neurological MS symptoms and one in four to half of the patients show depressive state within 1 year after MS onset (9, 12). 75% MS patients have a delay in diagnosis as a consequence of depressive symptoms(13). However, few studies have reported the incidence of depressive syndrome in MS patients. Furthermore, previous results relating to the prevalence of depression and anxiety in MS show significant discrepancy, ranging from 14 to 54%, and 1.24 to 36%, respectively (14–16). This could be explained by the heterogeneity of methodological issues and potential selection bias, such as differences in definitions, the diagnostic criteria used, and the size and source of the population studied. Moreover, some previously-published studies focused on lifetime depression rather than current depression, although the distinction between these two conditions was not always explicit. This may partly contribute to the differential results described in the present literature. Besides the elevated prevalence, a considerable number of studies show an increasing depressive symptom score in MS, compared with other chronic diseases, though the score shows no correlation with the level of neurological impairment (17). Furthermore, Boeschoten et al. found no statistical difference in depression severity and clinical profiles when evaluating different symptoms between depressed patients with MS ($n = 83$) and without MS ($n = 782$) (18). This finding showed that the criteria for simply identifying depression are also suitable for evaluating depression in MS.

Over the last decade, significant light has been shed on cognitive and psychiatric manifestations in MS, however these aspects have largely ignored acute disseminated encephalomyelitis (ADEM), an inflammatory demyelinating disorder of the CNS affecting the subcortical white matter and, to a lesser degree, the gray matter (19). ADEM is the most common cause of immune-mediated encephalitis and typically develops a monophasic course with various grouped symptoms

of fever, headache, meningitis, seizures, spasticity, and psychosis (20, 21). Psychiatric symptoms in patients with ADEM include depression, unspecific behavior changes and irritability. In addition, an increasing number of cases show that ADEM patients present with depression alone before the development of neurological symptoms (22–24).

Autoimmune encephalitis (AE) refers to a group of newly identified non-infectious encephalitis conditions which feature autoantibodies against neuronal cell-surface or synaptic proteins (25). Unlike other forms of infectious encephalitis, autoimmune encephalitis presents with pronounced psychotic and non-psychotic mood symptoms during the initial phase or during the course of the disease. Among the wide range of psychiatric symptoms presented, depressive syndromes are frequently seen in AE. These symptoms often show limited and transient effects in response to anti-depressive drugs and features can vary in different AE sub-groups. Anti-NMDAR encephalitis, arguably one of the best described subtype of AE, mainly manifests with depressive symptoms at onset, including depressed mood or depressed mood accompanied by anxiety, mood lability, apathy, sleep disorder and suicide attempts (26). Agitation and panic attack are another aspect of mood disorders in anti-NMDA receptor encephalitis and always accompanied by aggressive behavior. These emotional disturbances proceed to develop over the course of the disease. The depressive syndrome in AE with leucine-rich glioma inactivated 1-Ab (LGI1) or γ-amino-butyric acid B-receptor-Ab GABA(B)R-Ab can be psychotic. Depression can also develop with other psychiatric symptoms including visual or auditory hallucinations and obsessive disorders (27). In AE, the forebrain and limbic system and, in particular, the hippocampus, are severely affected (28, 29). The psychiatric presentation in AE suggests that the destruction of a specific protein, or a specific structure in the brain, may be correlated to depression episodes.

Depressive Syndromes in ADNS Involving Spinal Cord

Transverse myelitis (TM) and neuromyelitis optica (NMO) represent different types of autoimmune inflammatory demyelinating disorders of the CNS, and are characterized by predominant involvement of the spinal cord with no cerebral or only optic nerve lesions (30). The depressive comorbid conditions in TM and NMO have received less attention than MS, although many studies have highlighted its relationship with quality of life (31, 32). In fact, depressive morbidity is very common in these disease, with 17% of the patients with TM suffering from depression (33). Furthermore, TM patients with psychological morbidity are more disabled than those who do not have such additional morbidity (33). In one paper, patients with NMO were found to exhibit a similar prevalence (point prevalence 16%; lifetime prevalence 46%), along with features of cognitive impairments and depression, as MS patients, indicating a significant psychiatric burden (34). Another striking result was that nearly half of the NMO patients in this previous study reported recurrent depression and suicidality, which may be partly attributed to the psychological impairment experienced by these patients (34, 35). Fatigue and neuropathic pain, as the other common complaints, show overlapping interplay with the development of depression and may exacerbate the scale of depression (36). This is exemplified by Chavarro's research in which the scale of depression was moderately correlated with neuropathic pain, although this relationship was confounded by different levels of fatigue(37).

Depressive Syndromes in ADNS Involving the Peripheral Nervous System

Guillain-Barré syndrome (GBS) and chronic inflammatory demyelination polyradiculoneuropathy (CIDP) are both types of autoimmune-mediated peripheral neuropathy but develop on different clinical courses (38, 39). Previous research has demonstrated that the autoimmune response in GBS is triggered by molecular mimicry between microbial and nerve antigens and leads to demyelination and axonal damage (40). Strength and sensory deficits tend to depict the clinical profile when assessing patients with GBS and CIDP. A substantial number of patients with GBS or CIDP were observed to experience depressive episodes and increasing attention has been afforded to the burden of depressive syndromes and their influence on a patient's quality of life (41–43). In a population-based cohort study on the risk of psychiatric disorders in GBS, the hazard ratio (HR) of 4,548 GBS patients with regards to the development of psychosis was 4.320 (adjusted HR, 95% confidence interval (CI): 3.852–4.842, $p < 0.001$), and in depressive disorder was 4.834 ($p < 0.001$), in comparison to a control group (44). In other words, patients with GBS had a 4.8-fold elevated risk of developing depressive disorders. Data from the Dutch Society of Neuromuscular Disorders showed that nearly 6.7% of patients with GBS, and 9% of patients with GBS or CIDP, suffered depression (45). Furthermore, among the 49 most severely affected patients, anxiety (82%), depressive symptoms (67%) and brief reactive psychosis (25%) were observed (46). It is evident that among patients with GBS, the occurrence and severity of depressive syndromes are dependent on the severity of the neurological deficit. Depressive symptoms may be, to some extent, understood as a result of severe dysfunction in other words, the loss of movement and communication. In addition, depression, along with anxiety and fatigue, usually present as a residual symptom but not as the initial symptom, occurring during the recovery phase following the acute phase (47). This opinion was supported by another study which arrived at the conclusion that psychological distress and depressive symptoms were present at 3 months but improved significantly 3–6 months after disease onset (48). It is noteworthy that in a physical training study of patients with GBS and CIDP, simple physical exercises were shown to significantly relieve anxiety and depression (49).

Depressive Syndromes in ADNS Involving the Neuromuscular Junction

Myasthenia gravis (MG) is an autoimmune disorder featuring specific autoantibodies which target the acetylcholine receptor on the post-synaptic membrane of the neuromuscular junction. This disease is characterized by a chronic and fluctuating process

of muscle weakness and fatigue, causing multiple symptoms, such as eyelid ptosis, swallowing difficulties and limb weakness (50). The psychiatric symptoms of MG can be complicated and can coincide with other symptoms of MG, such as fatigue and shortness of breath, leading to inadequate recognition. Sometimes, these symptoms develop during the course of the disease, causing misdiagnosis and an unnecessary intensive drug treatment (51, 52). Although data are limited, affective disorder, particularly depressive disorder, appears to be the most frequent psychiatric manifestation in MG, with the frequency of depressive disorders ranging from 17 to 50% (53, 54). The large discrepancy in these figures could be attributed to heterogeneity in methodology and the different criteria adopted. More female patients have been observed to exhibit these presentations (57% in women vs. 35% in men) (55). In a Japanese cross-sectional study involving six neurological centers, it was reported that unchanged post-intervention status, dose of oral prednisolone, disease duration and MG composite were independent factors associated with depressive condition in MG (54). However, all of the assessments carried out in this study were performed by a neurologist without the assistance of a psychiatrist. Furthermore, it is uncertain whether the correlation of oral prednisolone dose was related to the side effects of prednisone, or to the higher MG severity associated with a higher prednisone dosage.

Depressive symptoms in ADNS are seldom reported in isolation and always emerge as a symptom cluster. Emotional disturbance frequently co-occur with fatigue, sleep disturbance and can be significantly strengthened by each other or somatic symptoms, such as pain. Depressive syndromes in ADNS involving brain tissues are more likely accompanied with other psychiatric symptoms including compulsive-obsessive disorder, visual or auditory hallucinations. Additionally, they show a complex auxo-action between each other or between physical symptoms and mental symptoms.

ETIOLOGY

The factors responsible for the high prevalence of depressive syndromes in ADNS are not fully understood and remain controversial. However, different etiologies of depressive syndromes might point to different treatment strategies, and therefore, affect both treatment success rate and patient outcome. Consequently, this topic deserves increased levels of attention.

Psychological Factors

All chronic disorders, including ADNS, may have psychological consequences during the clinical course of disease. Psychological factors can partly explain the elevated prevalence of depressive comorbidity in all types of chronic diseases, particularly in ADNS. Depression often occurs as a psychological reaction to the limitations of daily life caused by physical illness (56). The high-frequency and long-term disability of ADNS often acts as a traumatic stressor in patients (44). A large number of studies have searched for the possible psychological factors responsible for depression in ADNS, although it has proven difficult to conclude the subjective experience. Irrespective of the limitations, unpredictable course, feelings of helplessness, loss of pleasurable activities, recessive social relationships, significant stress, and inadequate treatment strategies, are all linked to depression to different extents (9, 57–59). The psychological elements may play the most basic role in the development of depression in both ADNS and other chronic diseases; a previous study, involving regression analysis, reported that psychological factors account for 40% of the variance of depression scores (57). Furthermore, psychological factors alone cannot explain the higher incidence of depression in ADNS.

Depression Related to Immunological Dysregulation

The relationships between immunological dysregulation and psychological function are a progressively important field of study at present for neuropsychiatric diseases. Many research studies into the underlying pathogenesis of major depressive disease have shown that immune activation, and the production of cytokines, may be involved in depression (60, 61). It has also been demonstrated that central or peripheral immune action can trigger depressive behavior by increasing the production of pro-inflammatory cytokines, such as interleukin-1 (IL-1) and IL-6, as well as activating cell-mediated immunity (62). In animal experiments, peripherally-administered pro-inflammatory cytokines IL-1β and TNF-α, as well as lipopolysaccharide (LPS) and synthetic compounds mimicking viral infection [Poly (I:C)], have induced "sickness behavior" is similar to depressive manifestation in human. And that the pro-inflammatory cytokines IL-1, IL-6, and tumor necrosis factor alpha (TNF-α), as well as C-reactive protein (CRP), may contribute to the initiation and progression of psychiatric diseases, such as depression (62–64).

Depression Secondary to Structural Brain Damage

The potential role of structural brain changes in the development of major depressive disorder is receiving increasing levels of attention from scientists; this is particularly driven by the recent advances in medical imaging technology. Several publications have reported that structural lesions in the brain may contribute to the high incidence of depressive syndromes in ADNS. This could be exemplified by a potential link between depression in MS and structural lesions associated with cerebral demyelination, although this association has not been confirmed as yet. Accumulating evidence shows that MS patients with depression tend to present with more severe atrophy of the frontal region and frontal white matter damage, compared to non-depressed MS patients. Goodstein and Ferrell, who reported three cases in which single and multiple depressive episodes preceded neurological symptoms, were the first to hypothesize that MS lesions cause symptoms rather than boost major depression by psychological factors (65). Subsequent studies aimed to specifically investigate this hypothesis. For example, Zorson et al. carried out a 2-years-long follow-up study in 90 MS patients and attempted to correlate their depressive symptoms with quantitative changes of regional and total lesion load, as well as brain parenchymal volumes. These authors found

that depressed MS patients showed an obvious reduction in brain parenchymal volumes in the temporal lobes which thus supported the hypothesis put forward by Goodstein and Ferrell (66). This result was also in line with Shen's research which concluded that depressive symptoms were mainly negatively associated with the degree of demyelinating lesions in the limbic system and frontal lobe (67). Shen et al. also noted that gray matter injury could describe clinical depression and disability better than white matter. More recently, Pravata et al. investigated the correlation between cortical and deep gray matter volume and depression. These authors noted that emotional behavior in MS could be convincingly explained by selective circumscribed cortical gray matter degeneration in the orbitofrontal and temporal lobes, which was similar to that seen in patients with major depressive disorder (68, 69). Furthermore, in a very recent paper, van Geest investigated lesion load and gray volume among MS patients with or without depression and held the opinion that depressed MS patients have more severe structural and functional disconnections than non-depressed MS patients (70). Though there is no definitive conclusion as yet, this does provide an underlying mechanism for depression in MS or in ADNS. Additionally, dysfunction at the hippocampal level, which is particularly evident in anti-NMDA receptor encephalitis, may also act on the structures upstream, thus causing emotive dysfunction. Hippocampal dysfunction may affect learning, thinking, memory and therefore frontal executive functions (27). The destruction of glutamatergic receptors and associated proteins in the forebrain and limbic system by anti-NMDA receptor antibodies may also provide direct evidence of an etiological correlation between autoimmunity and the subsequent risk of psychosis, including depression.

Drug-Related Depression

Some immunomodulatory drugs, such as interferon (IFN)-beta and steroids, may be blamed for the development of depression. IFN beta, as a form of disease-modifying drug, is commonly used in MS to reduce relapse rates and delay physical disability. Many cases have reported depression after IFN beta treatment (71); this has led to significant research efforts which have attempted to identify whether interferon shows neuropsychiatric toxicity and can induce depression. However, there is no definitive conclusion at present. Patten et al. endorsed the relationship between IFN and depression in his analysis of the incidence of depression in 1,995 patients receiving IFN and 824 patients receiving placebo (72); results showed that the number of patients included in the treatment group reporting depression was twice that of patients taking the placebo. Furthermore, 1.3% of patients developing depression went on to abandon the treatment. This finding was also supported by a range of subsequent studies (61, 73, 74). In contrast, some scientists arrived at a different conclusion and stated that there is no relationship between IFN and depression (75, 76). In fact, some authors have concluded that depression after IFN treatment may be better explained by a previous history of depression (77).

Furthermore, this, steroid may be partly responsible for the depression in MG (78). This hypothesis is exemplified by Suzuki's study in which 287 cases of MG were recruited to investigate the factors underlying depressive states in MG (54). These authors concluded that the dose of oral corticosteroids represented the major factor associated with depressive state in MG, followed by unchanged status, despite treatment and early disease stage. This possibility seems worthy of discussion, as exogenous corticosteroids have been associated with depression among the general population before. Although these links still remain unclear, the existence of a hypothesis relating to a possible relationship has led to the careful management of such patients (79).

The depressive state derived from diverse psychological factors present with more mood change, such as feelings of helplessness, anhedonia and lack of confidence. This status can be improved with the support from others or the improvement and stabilization of neurological symptoms. Organic depressive syndromes, depressive manifestation in ADNS involving brain tissue, show more psychotic feature. They are frequently occur along with other psychiatric symptoms and show poor reaction to the antidepressant. Depression related to immunological dysregulation and medication for ADNS often co-occur with behavior changes. In fact, there are no clear distinction between depressive presentations from different source. More than one element contribute to the development of depression and various components of depressive phenomenology can fluctuate over time.

INFLUENCE

Depression is one of the most important factors that can affect an individual's health. A substantial number of studies has shown that depression increases morbidity, mortality, reduces the quality of life of patients and increases the risk of complications and metabolic problems. Furthermore, the development of a psychiatric condition can increase medical costs, as well as the cost of caring for mental health. This form of adverse effect is strengthened in ADNS. Nowadays, the increased prevalence of depression in ADNS is gradually drawing our attention to the multifaceted burden of depression.

Reduction of Health-Related Quality of Life (HRQL)

A number of research studies have focused upon the reduction of health-related quality of life (HRQL) in patients with ADNS caused by depression. Neurological deficit usually accounts for 40–50% of the reduction in HRQL, while depression, along with fatigue, pain, and cognitive impairment accounts for the remainder. Significant research into the HRQL of MG has documented that psychosocial disorders, predominantly anxiety and depression, are negatively correlated with HRQL, based on multivariate linear regression analysis, apart from significant demographic predicators (older age and lower education) and the current status of myasthenia gravis. Interestingly, the Hamilton Anxiety Rating Scale was verified as a more significant prediction factor for a lower quality of life in both physical and mental aspects than the Hamilton Depression Rating Scale (80–82). Similarly, Shi's research demonstrated that anxiety, disability,

fatigue and depression were independent predictors of poor HRQL in NMOSD, and that anxiety was the best predictor of both the global and physical composite scores of HRQL, followed by disability, fatigue and depression (global composite, $r^2 = 0.76$, $P = 0.000$; physical composite, $r^2 = 0.71$, $P = 0.000$) (32). This result is in line with previous studies on the depressive condition in NMO (33, 37). Moreover, this type of negative effect is more complex when brain issues are affected. Despite the more severe conditions of ADNS with brain involvement, the burden of a lesion in the brain may modestly correlate to the development of cognitive disability and depression, which may also contribute to a poor quality of life. Taking MS as an example, and excepting the direct impact of the depressive state on the quality of life, depression, as well as fatigue, can cause deterioration in cognitive impairment and exert an indirect impact on the activities of daily living (83). However, the interactions between these factors are intricate. Scientific evidence shows that cognition dysfunctions in many aspects, including verbal memory, sustained attention and concentration and information processing speed, were all associated with depression and fatigue scores to differing extents (84, 85). Nunnari et al. reported that depression score is the most influential variable in terms of higher weight in regression models and the cognitive domains affected (85). Furthermore, symptoms, such as a lack of motivation, an inability to complete tasks, and sleep disturbance, overlap between fatigue, depression and cognitive impairment (86). Thus, it is suggested that recognizing and treating the common comorbidities of fatigue and depression is the first step in diagnosing cognitive dysfunction in a patient with MS (87). Due to the significant effect of anxiety and depression on the quality of life, emotional health should remain a significant clinical focus in patients with ADNS. Such patients should be treated aggressively, especially when cognitive dysfunction exists. Generally, depression is considered to be curable with pharmacological and cognitive-behavioral therapies. If the cognitive impairment persists after the successful treatment of depression, formal neuropsychological evaluation should be carried out.

Deterioration in Physical and Mental Symptoms

The relationships between physical and other perceived symptoms are also intricate. On the one hand, a depressive condition may contribute to the development and progression of other symptoms and cause further deterioration in these disorders. Depression has also been found to influence cognition in neurological and psychiatric disorders, thus contributing to disability and disease duration (83). Fatigue is the most common physical symptom in ADNS and can be enhanced by a depressive state. This is a very subjective feeling and defined as a reversible, motor and cognitive impairment with reduced motivation and a desire to rest (88). Many studies have found that fatigue is highly correlated with depression and physical impairment and that depressed mood and disability are significant predictors of fatigue in MS, GBS and MG patients (54, 89, 90). The feeling of fatigue will be enhanced in the presence of depression, thus leading to a higher score on the Fatigue Severity Scale (FSS) (17, 91). On the other hand, physical illness can in turn make depression quite probable. Depression symptomatology and prevalence are significantly increased in individuals who have a higher score on the fatigue severity scale (92). This finding is consistent among the general population in that fatigued individuals report a higher proportion of depression symptoms (93).

Delay of Diagnosis

Symptoms of physical disease may partially overlap with depressive symptoms, causing a delay in diagnosis of physical disease or an unnecessary intensive pharmacological treatment. Fatigue and a lack of energy, shortness of breath, and increased weakness of muscles are the prominent symptoms of MG but initially may not be recognized due to their coincidence with depressive symptoms. The comorbidity of psychological symptoms that appears during the course of disease may also be regarded as symptoms of MG. An incorrect understanding of this presentation may render a change of therapeutic strategy which is unnecessary. Psychological manifestation must be carefully treated because of the risk of deterioration in the underlying neurological disease. Furthermore, the incidence of therapeutic drug-related depression in ADNS can also reduce adherence to disease-modifying therapy.

CONCLUSION

Because of our limited understanding of the depressive syndromes experienced in patients with ADNS, there is a significant lack of attention and effective treatments at present with which to improve these unpleasant symptoms. In this article, we shed light on depressive syndromes associated with autoimmune disorders of the nervous system and demonstrate the prevalence and clinical profile of depressive symptoms in ADNS. We also discuss the potential mechanisms underlying the high incidence and prevalence of depression in ADNS.

Our study indicates that depression is a common comorbidity in ADNS with a frequency of 15–50% across different types of ADNS; this is higher than the general population and other chronic diseases. Therefore, neurologists should keep this in mind, especially with regards to patients presenting with psychiatric symptoms associated with unexplained neurological findings. The risk factors for depression vary across different disorders but share similar characteristics with major depressive disease. The high frequency of depression in ADNS highlights the need for further research with which to deepen our understanding of the origin of depression. More high-quality literature, with reduced heterogeneity, is now required. This paper uncovered some challenges or key questions for neurologists in diagnosis and treatment of ADNS. First, early discovery to the ADNS symptoms in the context of depressive presentation can be difficult. Second, timely and correct diagnosis as well as proper intervention to the depressive syndrome in ADNS can act as a puzzlement for neurologists. Third, how to address drug-related depression when the related medication is necessary. All these questions need the effort from our peers in this field to develop an integrative strategy

in providing appropriate treatment guidelines in future. We hope that this review will promote understanding of depressive syndromes in ADNS, and draw more attention to this clinical problem.

AUTHOR CONTRIBUTIONS

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

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Effect of Betahistine and Metformin on Antipsychotic-Induced Weight Gain: An Analysis of Two Clinical Trials

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Antipsychotic-induced weight gain is one of the most common adverse effects of antipsychotic treatment. However, there are no well-established interventions for the weight gain yet. In this study, we pooled the data from two clinical trials, which were originally examining the efficacy of betahistine and the efficacy of metformin in treating antipsychotic-induced weight gain and insulin resistance. A total of 67 people with schizophrenia or bipolar disorder treated with antipsychotics were assigned to 36 mg day^{-1} betahistine ($n = 13$) or 1,000 mg day^{-1} metformin ($n = 25$) or placebo ($n = 29$) treatment for 12 weeks, with evaluation at baseline and week 12. The primary outcome was the body mass index (BMI). After treatment, metformin group had a mean decrease in BMI of 1.46 ± 0.14 ($p < 0.001$) and insulin resistance index (IRI) of 4.30 ± 2.02 ($p < 0.001$). The betahistine group had no significant alteration in BMI or IRI. However, placebo group had a mean increase in BMI of 1.27 ± 0.77 ($p < 0.001$) and IRI of 0.45 ± 0.86 ($p < 0.001$). Between the two treatment groups, metformin significantly decreased weight, BMI, fasting glucose, insulin level, and IRI but not waist circumference when compared with betahistine. Moreover, metformin significantly decreased weight, BMI, waist circumference, fasting glucose, insulin level, and IRI when compared with placebo, whereas betahistine significantly decreased body weight, waist circumference, BMI, insulin level, and IRI but not fasting glucose when compared with placebo. In this study, we found that both metformin treatment and betahistine treatment were efficacious in improving antipsychotic-induced weight gain and insulin resistance, and metformin was more efficacious in preventing and revising the weight gain induced by antipsychotics.

Clinical Trial Registration: www.ClinicalTrials.gov, NCT00451399(Study 1), NCT00709202(Study 2)

Keywords: betahistine, metformin, BMI, insulin resistance, antipsychotic medication

INTRODUCTION

Antipsychotics, especially second-generation antipsychotics (1), could lead to serious metabolic adverse effects, including weight gain, insulin resistance, and glucose intolerance (2, 3). These adverse effects significantly increase the risk of stroke and coronary artery disease (4) and make patients 13 times more likely to discontinue medication (5). Interestingly, previous studies found that overall 23.5% of the patients with schizophrenia had metabolic syndrome (6), and 78.8% of the patients receiving antipsychotic medication showed more than 7% increase in body weight compared with baseline (7). In addition to schizophrenia, bipolar disorder is also commonly treated with antipsychotics, and individuals with bipolar disorder and schizophrenia have similar chances of showing weight gain and metabolic syndrome (8). Meanwhile, one study suggested that people with severe mental illness may not necessarily have a higher risk for cardiovascular diseases while other factors like dietary habits, health coverage, and family's support are possible explanations for higher risk (9). Therefore, antipsychotic-induced weight gain has been a major management problem for clinicians when treating schizophrenia and bipolar disorder (10).

Metformin, a biguanide, is commonly used for type II diabetes mellitus. It functions by inhibiting hepatic gluconeogenesis and improving the sensitivity of insulin in skeletal muscles (11). Previous work in our lab indicated that metformin can decrease antipsychotic-induced weight gain and insulin resistance (12–15), potentially by reducing insulin resistance (16) and suppressing appetite (17). Betahistine, an antagonist of histaminergic H_1 and H_3 receptors, has been widely used to treat vertigo symptoms in Meniere's disease. Interestingly, a previous study suggested that histaminergic H1 and H3 receptors are crucial to the potential mechanism of antipsychotic-induced weight gain (18). Additionally, previous studies found that the coadministration of olanzapine and betahistine in rats significantly reduced olanzapine-induced weight gain (19, 20), and clinical trials showed that the coadministration of olanzapine and betahistine significantly reduced weight gain in individuals with schizophrenia or schizoaffective disorder (21, 22).

Despite overwhelming evidence on the effects of metformin and betahistine in treating weight gain associated with many disorders, it remains unknown which medication is more efficacious in treating antipsychotic-induced weight gain and insulin resistance in people with schizophrenia or bipolar disorder. Therefore, we analyzed data from two studies to compare the efficacies of metformin and betahistine on improving antipsychotic-induced weight gain and insulin resistance in people with schizophrenia or bipolar disorder. We hypothesized that both betahistine and metformin could attenuate antipsychotic-induced weight gain and insulin resistance in these patients.

MATERIALS AND METHODS

Study Design

The study was designed using an independent double-blind, randomized, placebo-controlled 12-week clinical trial of metformin and an open label prospective cohort study of betahistine in weight gain and other metabolic changes. The first trial (NCT00451399) was to examine the efficacy of metformin in the treatment of antipsychotic-induced weight gain; the second trial (NCT00709202) originally was to examine the efficacy of betahistine in the treatment of antipsychotic-induced weight gain. The data from STUDY 2 have not been published elsewhere, though the primary outcomes of STUDY 1 have been published before (12).

The two studies were identical in terms of measurements of body weight, fasting glucose level, and other serum chemicals. In both STUDY1 and STUDY 2, participants had to gain more than 10% of their pre-drug body weight within the first year of treatment to be eligible for our study. Both studies were conducted in the Mental Health Institution of the Second Xiangya Hospital at the Central South University, China. In STUDY 1, 32 participants were randomly assigned to each group. After 12 weeks, 30 participants in metformin group completed the treatment, and 29 participants in placebo group completed the follow-up. In STUDY 2, there were 31 participants involved and 18 of them completed the treatment. After excluding 5 patients from metformin group who were not compliant and 5 patients from STUDY 2 whose course of illness was beyond 10 years, totally 54 patients from STUDY 1 and 13 patients from STUDY 2 were included for data analysis.

Participants

People aged 18 through 55 diagnosed with schizophrenia or bipolar disorder in accordance with the criteria set out in the Diagnostic and Statistical Manual of Mental Disorders-Fourth Edition (DSM-IV) were eligible for our study (23), and the Structured Clinical Interview of DSM-IV Axis I Disorders (SCID-1), clinical version (24), was used during the screening phase. All the participants were on a stable dose of antipsychotics for at least 3 months before the start of the study, and it remained at the same dose throughout the course of the study. To be qualified for STUDY 2, the participants had to meet the following criteria: (1) the participants should have gained 10% of their body weight within the first year of treatment or within the last year if the treatment is beyond a year; and (2) the participants should have taken one of the four antipsychotics—clozapine, olanzapine, risperidone, or quetiapine. All the participants had to be taken care of by a parent or another adult caregiver who monitored and recorded the intake of medication daily. All the participants were recruited from the outpatient clinic of the Mental Health Institute of the Second Xiangya Hospital, Central South University, China, between March 2015 and March 2017. Participants were excluded from the study if there was evidence of liver or kidney dysfunction, asthma, peptic ulcer, or pheochromocytoma; if they were taking prescription medication, which affects body weight or glycolipid metabolism; if they were taking thyroid replacement therapy or lipid-regulatory drugs, whose dose changed more than 50% over the last 3 months; or if they were pregnant or lactating. For STUDY 1, the inclusion and exclusion criteria have been previously described (12). For both studies, after a complete description of the study to the subjects, written informed consent was obtained in accordance with the

guidelines of the National Health and Medical Research Council. The study was approved by the Ethics Committee of the Second Xiangya Hospital.

Pharmacological Intervention

For STUDY 1, the participants were administered with the drug in a double-blind placebo controlled fashion. For the first 4 days, the participants took 250 mg of metformin or placebo at their evening meal, after which a second or third dose was added before breakfast and lunch, respectively, for another 80 days. Each participant was given a record sheet to log the number of trial medications taken daily. Only trihexyphenidyl for extrapyramidal symptoms or lorazepam for insomnia or agitation were allowed as needed. For STUDY 2, the participants took 6 mg of betahistine at their evening meal for the first 2 days after enrollment, then 6 mg of betahistine at lunch and evening meal for the third day, after which 6 mg more of betahistine was added each day till the 11th day. The participants took 18 mg of betahistine at lunch and evening meal at 11th day and for another 73 days. Any antipsychotics taken by the participants before enrollment remained at the same dosage throughout the course of the study.

Primary and Secondary Outcome Measurements

The primary outcome was body mass index (BMI). The secondary outcome was body weight, waist circumference, fasting glucose, insulin level, and insulin resistance index (IRI). To calculate the BMI, weight in kilograms was divided by height in meters squared. To calculate the IRI, insulin level (mIU/L) $\times$ fasting glucose (mmol/L)/22.5 was determined, in accordance with homeostasis model assessment (25).

Baseline data included related demographics, a comprehensive medical history, a physical examination with the measurement of weight and height, and a related laboratory test. Research nurses were blind to the type of treatment to perform all assessments. Related laboratory tests included fasting insulin and fasting glucose. Fasting blood samples were confirmed by the patients or their caregivers.

Follow-up visits were made at week 12 after starting the treatment, and all baseline evaluations including physical examination, laboratory test, and weight and height measurements were repeated. Serum insulin level was measured with a solid phase radioimmunoassay. Weight and height measurements were taken after removing their shoes and upper garments and donning an examination gown.

Statistical Analysis

All analyses were conducted by using the Statistical Package for Social Sciences, version 23 (SPSS Inc, Chicago, Illinois). Continuous variables were described as mean (SD) and confidence interval. Categorical variables were described by frequencies and percentages. We used *t*-tests, chi-squared analyses, and one-way analysis of variance (ANOVA) as appropriate. Fisher's exact test was used when necessary. For the comparison of the three groups at baseline, ANOVA was used to compare continuous variables with homogeneity of variance, Kruskal-Wallis test was conducted for the variables without homogeneity of variance, and chi-squared analysis was used for categorical variables.

For the follow-up data were all continuous variables, analysis of covariance (ANCOVA) was the main strategy used when we compared among the three groups, while the corresponding baseline values and those variables found to be significantly different at baseline were regarded as covariates. To compare the difference among the three groups, *post-hoc* tests (least significant difference procedure) were conducted to compare between the two groups. For the variables without homogeneity of variance, the ANOVA was conducted after ranking. The difference was considered statistically significant if a two-tailed *P*-value was < 0.05. The difference between baseline data and 12 weeks data was analyzed by a paired *t*-test.

RESULTS

Demographic and Baseline Outcome Measurements

Demographic and baseline outcomes were compared among the three groups (**Table 1**). The 25 patients (13 females and 12 males) of metformin group and 29 patients (15 females and 14 males) of placebo group were from STUDY 1, while the 13 patients (10 females and 3 males) of betahistine group were from STUDY 2. There were both schizophrenia and bipolar disorder participants in betahistine group, while only schizophrenia participants in metformin group and placebo group. No significant difference was found in the mean values of age, gender, medication, and IRI, while betahistine group had significantly higher values of illness duration, body weight, BMI, waist circumstance, fasting glucose, and insulin level than the other groups. Therefore, those seven outcomes in baseline were set as covariances in the data analysis. Betahistine group had a significantly longer course of illness than other two groups; therefore, we set the course of illness as the covariance in the data analysis. However, after ANCOVA, the course of illness was not statistically significant in any of the outcomes as a covariance.

Changes in Body Weight and Body Mass Index

After 12 weeks of treatment, statistical analysis showed that, in metformin group, the mean body weight and BMI value decreased significantly, while in placebo group, there's a significant increase of body weight and BMI; however, in betahistine group, no significant differences were found in the mean body weight or BMI value (**Table 2**). After 12 weeks of treatment, metformin was significantly superior to betahistine and placebo in terms of values of body weight and BMI, while betahistine was superior to placebo in terms of values of body weight and BMI (**Table 3**).

At the end point, patients in metformin group had a significant decrease in body weight by 5.79% compared with baseline, while body weight in betahistine group decreased by 1.32% without significance; however, patients in placebo group significantly gained 5.51% of their body

TABLE 1 | Demographic and clinical characteristics of 67 Participants across treatment groups at baseline[a].

Characteristics	Total ($n = 67$)	Betahistine ($n = 13$)	Metformin ($n = 25$)	Placebo ($n = 29$)	Test statistics[b]	*P*-value
Age	26.03 ± 5.11	26.23 ± 7.41	26.78 ± 4.30	25.84 ± 4.80	0.053	0.950
Gender(Male/Female)	(29/38)	(3/10)	(12/13)	(14/15)	2.683	0.260
Diagnose (schizophrenia/Bipolar disorder)	(59/8)	(5/8)	(25/0)	(29/0)	–	<0.001
Medication	(19/17/20/11)	(0/6/5/2)	(9/5/7/4)	(10/6/8/5)	–	0.177
Clozapine	19	0 (3.7)	9 (7.1)	10 (8.2)		
Olanzapine	17	6 (3.3)	5 (6.3)	6 (7.4)		
Risperidone	20	5 (3.9)	7 (7.5)	8 (8.7)		
Quetiapine	11	2 (2.1)	4 (4.1)	5 (4.8)		
Course, Mo	14.31 ± 15.41	35.54 ± 25.98	9.28 ± 2.51	9.14 ± 2.36	19.738	<0.001
Body weight	66.93 ± 9.04	76.25 ± 13.88	65.62 ± 5.78	63.88 ± 5.46	11.652	0.003
BMI	25.23 ± 2.33	28.38 ± 3.09	24.90 ± 1.10	24.09 ± 1.25	21.478	<0.001
Waist circumference	86.76 ± 8.52	99.47 ± 8.20	84.22 ± 5.78	83.25 ± 4.50	38.473	0.001
Fasting Glucose	5.22 ± 0.49	4.91 ± 0.52	5.44 ± 0.47	5.17 ± 0.41	5.961	0.004
Insulin	26.64 ± 11.48	26.42 ± 21.76	28.21 ± 8.08	25.39 ± 6.93	6.282	0.043
IRI	6.28 ± 3.03	5.88 ± 5.31	6.96 ± 2.56	5.87 ± 1.81	4.075	0.130

BMI, body mass index, which is calculated as weight in kilograms divided by height in meters squared; IRI, insulin resistance index, which is calculated as insulin level(mIU/L)×fasting glucose(mmol/L)/22.5.

SI conversions: To convert glucose from mg/dL to mmol/L, multiply by 0.0555; insulin from μIU/mL to pmol/L, multiply by a 6.945.

[a] *Data are presented as mean(SD).*

[b] *Test statistics: χ^2 Test and Fisher's exact test for categorical variables and analysis of variance for continuous variables.*

weight. Correspondingly, in metformin group, the mean BMI significantly decreased by 1.46, while the mean BMI decreased by 0.33 in betahistine group with no significance, and the mean BMI increased by 1.27 in placebo group significantly.

Changes in Waist Circumference

As shown in **Table 3**, at the end point, the mean waist circumference in metformin group significantly decreased by 1.52 cm, while placebo group had a significant increase of mean waist circumference by 2.29 cm. However, in betahistine group, the mean waist circumference increased with no statistical significance compared with baseline. Both metformin and betahistine were significantly superior to placebo in terms of waist circumference value. However, no significant difference was found between betahistine and metformin ($p = 0.058$).

Changes in Fasting Glucose, Insulin, and IRI

Over the 12-week period, there was a significant decrease in the values of mean fasting glucose level, insulin level, and IRI in metformin group; however, in betahistine group, no significant change in the value of fasting glucose level, insulin level, or IRI was found compared with baseline, while in placebo group, there was a significant increase in insulin level and IRI but not fasting glucose level (**Table 2**). At the end point, metformin was superior to both betahistine and placebo in terms of fasting glucose level, insulin level, and IRI, while betahistine was superior to placebo in terms of insulin level and IRI but not fasting glucose level (**Table 3**).

Adverse Events

There were no significant differences in the frequency and types of adverse events reported among the three groups. There were six serious adverse events that affected more than 5% of the entire sample (**Table 4**).

DISCUSSION

This study was designed to test the comparative efficacy of metformin and betahistine on preventing further weight gain or causing weight decrease in people with schizophrenia or bipolar disorder who had gained more than 10% weight in the first 3 years of treatment with antipsychotics. To our knowledge, this is the first research to compare metformin and betahistine in treating antipsychotic-induced weight gain. After a 12-week trial, we found that betahistine treatment effectively controlled antipsychotic-induced weight gain, while metformin significantly relieved antipsychotic-induced weight gain. Both metformin and betahistine were found to have a significant advantage when compared with placebo.

Current interventions to minimize antipsychotic-induced weight gain and metabolic syndrome include pharmacologic and non-pharmacologic ways (26). Pharmacologic interventions include switching to another antipsychotic, which has less weight gain effect, or adding an adjuvant. However, individuals who switched antipsychotics had significantly shorter times until discontinuation compared with individuals who continued with their baseline medication (27). Therefore, the risk of relapse should be carefully considered before medication switching (28). Meanwhile, non-pharmacological interventions usually consist of lifestyle intervention and cognitive behavior

TABLE 2 | Treatment outcomes for all 67 participants.

	Baseline	Endpoint	*P*-Value
Betahistine group (*n* = 13)			
Body weight, kg	76.25 ± 13.88 (67.86 – 84.643)	75.25 ± 12.90 (67.46 – 83.05)	0.223
BMI	28.38 ± 3.09 (26.51 – 30.25)	28.05 ± 3.19 (26.13 – 29.98)	0.245
Waist Circumference, cm	99.47 ± 8.20 (94.52 – 104.42)	98.65 ± 9.63 (92.83 – 104.46)	0.495
Fasting Glucose, mmol/L	4.91 ± 0.52 (4.60 – 5.23)	4.90 ± 0.61 (4.53 – 5.27)	0.920
Insulin, mIU/L	26.42 ± 21.76 (13.27 – 39.56)	20.34 ± 14.61 (11.51 – 29.17)	0.111
IRI	5.88 ± 5.31 (2.67 – 9.09)	4.48 ± 3.29 (2.50 – 6.47)	0.132
Metformin group (*n* = 25)			
Body weight, kg	65.62 ± 5.78 (63.25 – 68.01)	61.82 ± 6.26 (59.23 – 64.40)	< 0.001
BMI	24.90 ± 1.10 (24.45 – 25.36)	23.44 ± 1.31 (22.90 – 23.98)	< 0.001
Waist Circumference, cm	84.22 ± 5.78 (81.83 – 86.60)	82.69 ± 5.80 (80.30 – 85.09)	< 0.001
Fasting Glucose, mmol/L	5.44 ± 0.47 (5.24 – 5.63)	4.63 ± 0.65 (4.36 – 4.90)	< 0.001
Insulin, mIU/L	28.21 ± 8.08 (24.87 – 31.55)	12.87 ± 3.90 (11.26 – 14.47)	< 0.001
IRI	6.96 ± 2.56 (5.90 – 8.02)	2.66 ± 0.89 (2.29 – 3.02)	< 0.001
Placebo group (*n* = 29)			
Body weight, kg	63.88 ± 5.46 (61.80 – 65.96)	67.17 ± 5.28 (65.16 – 69.18)	< 0.001
BMI	24.09 ± 1.25 (23.61 – 24.56)	25.35 ± 1.16 (24.91 – 25.80)	< 0.001
Waist Circumference, cm	83.25 ± 4.50 (81.54 – 84.97)	85.54 ± 5.43 (83.47 – 87.60)	< 0.001
Fasting Glucose, mmol/L	5.17 ± 0.41 (5.01 – 5.33)	5.11 ± 0.40 (4.96 – 5.27)	0.081
Insulin, mIU/L	25.39 ± 6.93 (22.76 – 28.03)	27.62 ± 7.33 (24.83 – 30.40)	0.001
IRI	5.87 ± 1.81 (5.18 – 6.55)	6.32 ± 2.00 (5.56 – 7.08)	0.008

BMI, body mass index, which is calculated as weight in kilograms divided by height in meters squared; IRI, insulin resistance index, which is calculated as insulin level (mIU/L) × fasting glucose (mmol/L)/22.5.

SI conversions: To convert glucose from mg/dL to mmol/L, multiply by 0.0555; insulin from μIU/mL to pmol/L, multiply by a 6.945.

strategies (29). However, there is a significant heterogeneity in non-pharmacological interventions, and the majority of these interventions are associated with poor compliance. So, adding an adjuvant should be a chance to improve antipsychotic-induced weight gain and insulin resistance.

The potential clinical effect of betahistine on reducing antipsychotic-induced weight gain and its mechanism has gained more attention in recent years. The histamine system has played a crucial role in the regulation of energy homeostasis (18, 30, 31). Specifically, H1R antagonism has been recognized as the main mechanism for predicting weight gain induced by second-generation antipsychotics (SGAs) (18, 32, 33). Betahistine, as a H1R and H3R antagonist, can cross the blood-brain barrier, and it acts centrally by enhancing histamine neurotransmission in the hypothalamus (34). A previous animal study has suggested that co-treatment of betahistine could partially reverse olanzapine-induced body weight gain (19) and hypothalamic H1R pathway change (20). The clinical application of betahistine against weight gain has thus been the focus. In a multicenter randomized controlled trial (RCT) study of healthy women, Barak et al. (35) reported that in over 12 weeks of treatment with betahistine, there was a significant weight loss (35). Later, in 2016, their study showed that the coadministration of betahistine and olanzapine mitigated the weight gain induced by olanzapine in healthy women (21). In patients diagnosed with schizophrenia, Poyurovsky et al. (36) held a study for 6 weeks with the coadministration of betahistine and olanzapine, which demonstrated a increase in weight during the initial 2 weeks of the trial with no additional weight gain or minor reduction of body weight for the rest of the trial, and none of the patients gained 7% of the initial body weight (36). Another study by Poyurovsky et al. (37) showed that the reboxetine-betahistine combination produced a significant attenuation of olanzapine-induced weight gain (37), and the weight attenuating effect of this combination was two-fold higher than reboxetine alone (38). Another study carried out on female obese women demonstrated the beneficial effect of betahistine on improving dyslipidemia (39). However, a 1-day administration of betahistine in healthy women showed no difference in energy intake (40). Our research was inconsistent with most of the previous studies and for the first time showed that the treatment of betahistine could mitigate the increased insulin level and IRI induced by antipsychotics.

Metformin mainly increases the function of insulin in the liver and decreases the rate of hepatic glucose production (41). Metformin is regarded as the first line treatment for type 2 diabetes mellitus (42); besides, it was also used in non-diabetics against obesity. In the hypothalamus, metformin increases STAT3 signaling while it decreases NPY and AgRP expression, which suggests that metformin mediated food intake by affecting multiple appetite regulatory pathways (43, 44). Metformin also improves leptin sensitivity, which is an important adipocyte-derived hormone that regulates energy balance (45). Other researchers suggested that metformin could increase the secretion of GLP-1, a satiation signal secreted by the gut (46). In

TABLE 3 | The difference between baseline and end point of all treatment outcomes.

	Mean(SD) CI			*P*-value				
Assessment levels	**Betahistine (*n* = 13)**	**Metformin (*n* = 25)**	**Placebo (*n* = 29)**	**ANCOVA[a]**	**Partial η^2**	**Betahistine vs. Metformin**	**Betahistine vs. Placebo**	**Metformin vs. Placebo**
Body weight, kg	−1.00 ± 2.81 (−2.70, 0.70)	−3.80 ± 1.71 (−4.51, −3.10)	3.29 ± 1.92 (2.56, 4.02)	<0.001	0.752	0.002	0.001	<0.001
Waist circumference, cm	−0.82 ± 4.21 (−3.37, 1.72)	−1.52 ± 0.07 (−1.55, −1.50)	2.29 ± 1.58 (1.68, 2.89)	<0.001	0.628	0.058	<0.001	<0.001
BMI	−0.33 ± 0.96 (−0.91, 0.26)	−1.46 ± 0.70 (−1.75, −1.17)	1.27 ± 0.77 (0.97, 1.56)	<0.001	0.743	<0.001	0.009	<0.001
Fasting glucose, mmol/L	−0.013 ± 0.46 (−0.29, 0.26)	−0.81 ± 0.81 (−1.14, −0.48)	−0.05 ± 0.16 (−0.11, 0.01)	<0.001	0.575	<0.001	0.942	<0.001
Insulin, mIU/L	−8.00 ± 14.93 (−17.02, 1.03)	−15.34 ± 5.57 (−17.64, −13.05)	2.22 ± 3.12 (1.04, 3.41)	<0.001	0.741	0.002	<0.001	<0.001
IRI	−1.40 ± 3.12 (−3.28, 0.49)	−4.30 ± 2.02 (−5.14, −3.47)	0.45 ± 0.86 (0.13, 0.78)	<0.001	0.742	<0.001	<0.001	<0.001

ANCOVA, analysis of covariance; BMI, body mass index, which is calculated as weight in kilograms divided by height in meters squared; IRI, insulin resistance index, which is calculated as insulin level (mIU/L) × fasting glucose (mmol/L)/22.5.

SI conversions: To convert glucose from mg/dL to mmol/L, multiply by 0.0555; insulin from μIU/mL to pmol/L, multiply by a 6.945.

[a]P-value for the omnibus analysis testing for overall differences between the three groups on the continuous variables is based primarily on ANCOVA with baseline levels of the variables as covariates. When the overall omnibus analysis P-value was significant, the pair-wise comparisons were performed.

TABLE 4 | Adverse effects of three groups.

	No. (%)				
Adverse effect	**Total (*n* = 674)**	**Betahistine (*n* = 13)**	**Metformin (*n* = 25)**	**Placebo (*n* = 29)**	***P*-value**
Nausea	11(16.4)	0(0)	5(20.0)	4(13.8)	0.6
Extrapyramidal Symptoms	17(25.4)	2(15.4)	5(20.0)	8(27.6)	
Insomnia and agitation	13(19.4)	1(7.7)	5(20.0)	5(17.2)	
Somnolence	7(10.4)	1(7.7)	2(8.0)	2(6.9)	
Headache	6(9.0)	2(15.4)	2(8.0)	2(6.9)	
Dry mouth	6(9.0)	0(0)	2(8.0)	2(6.9)	

Fisher's exact test among three groups.

our previous study, it was found that lifestyle intervention and metformin alone and in combination were effective for reversing antipsychotic-induced weight gain, while metformin alone was more effective for inducing weight loss and improving insulin sensitivity than lifestyle intervention alone, and metformin remained effective and safe in attenuating olanzapine-induced weight gain and insulin resistance in drug naïve first episode patients (15). As a follow-up to our initial study, we found that the addition of metformin to antipsychotics was a potential treatment for dyslipidemia in people with schizophrenia (13) and amenorrhea in females with schizophrenia (14, 47). Multiple compounds have been investigated as add-on medications to cause weight loss, and metformin has the best evidence (26). However, in 2018, a meta-analysis that included six RCTs found that combining metformin and lifestyle interventions shows significant reduction in weight and BMI compared with metformin alone (48). Three metformin meta-analyses confirmed the significant effect of metformin in reducing BMI and improving insulin sensitivity (49–51). Our findings were consistent with most of these studies and meta-analyses.

In addition, our study showed that metformin significantly decreased body weight, BMI, fasting glucose level, insulin level, and IRI but not waist circumference when compared with betahistine. These findings suggested that the treatment with metformin could be more efficacious than betahistine in preventing and reversing the weight gain induced by antipsychotic agents in people with schizophrenia or bipolar disorder, while both treatments were found to have a significant advantage over placebo. Few studies had compared the effect of betahistine and metformin before. According to our study, although betahistine group failed to decrease the body weight significantly, it prevented further weight gain with a decreasing tendency. Therefore, we suggest metformin as the first consideration for antipsychotic-induced weight gain while betahistine as an alternative if metformin was not tolerated or adhered.

This study has some limitations. First, the data were collected from two independent studies, thus, the sample error was inevitable and STUDY 2 was not a randomized placebo controlled clinical trial. Second, this study was based on schizophrenia or bipolar disorder participants with four different antipsychotics: clozapine, olanzapine, risperidone, or quetiapine. Previous studies suggested that the type of antipsychotics affects the plasma adiponectin level and also affects body weight significantly (52, 53). However, we were unable to assess this effect by the type of antipsychotics because of the small sample size in our study. Third, we failed to test leptin level though it has been proven to play an important role in weight gain (54). Finally, the participants

were followed up for 12 weeks only, so we still cannot predict the long-term effects of metformin and betahistine. Further research including the well-designed RCT test to testify the findings or genetic variations, which might provide some explanation on individualized treatment response, should be carried out.

In conclusion, despite these limitations, this study has clearly shown that metformin could be more efficacious than betahistine in increasing insulin sensitivity and reversing the weight gain induced by antipsychotics with 12 weeks of treatment, while both could significantly improve the body weight and insulin sensitivity induced by antipsychotics. We suggest metformin as the first consideration for the treatment while betahistine as an alternative if not tolerated or adhered.

AUTHOR CONTRIBUTIONS

RW and ZJ designed and conducted the research. DK analyzed and interpreted data and drafted the article. RW, ZJ, and RL provided critical revision of the article, while GH, TS, ZJ, RL, LL, MS, YY, YW, XW, and YL collected and analyzed the data. XH analyzed the data and drafted the data. RW are responsible for the final approval of the version to be published.

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Brain-Derived Neurotrophic Factor Precursor in the Hippocampus Regulates Both Depressive and Anxiety-Like Behaviors in Rats

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Depression and anxiety are two affective disorders that greatly threaten the mental health of a large population worldwide. Previous studies have shown that brain-derived neurotrophic factor precursor (proBDNF) is involved in the development of depression. However, it is still elusive whether proBDNF is involved in anxiety, and if so, which brain regions of proBDNF regulate these two affective disorders. The present study aims to investigate the role of proBDNF in the hippocampus in the development of depression and anxiety. Rat models of an anxiety-like phenotype and depression-like phenotype were established by complete Freund's adjuvant intra-plantar injection and chronic restraint stress, respectively. Both rat models developed anxiety-like behaviors as determined by the open field test and elevated plus maze test. However, only rats with depression-like phenotype displayed the lower sucrose consumption in the sucrose preference test and a longer immobility time in the forced swimming test. Sholl analysis showed that the dendritic arborization of granule cells in the hippocampus was decreased in rats with depression-like phenotype but was not changed in rats with anxiety-like phenotype. In addition, synaptophysin was downregulated in the rats with depression-like phenotype but upregulated in the rats with anxiety-like phenotype. In both models, proBDNF was greatly increased in the hippocampus. Intra-hippocampal injection anti-proBDNF antibody greatly ameliorated the anxiety-like and depressive behaviors in the rats. These findings suggest that despite some behavioral and morphological differences between depression and anxiety, hippocampal proBDNF is a common mediator to regulate these two mental disorders.

Keywords: proBDNF, depression, anxiety, hippocampus, stress

INTRODUCTION

Depression and anxiety are highly debilitating mental disorders that severely affect patients' quality of life and put a burden on families and society. Globally, depression ranks as the largest contributor to global disability and nearly 300 million people suffer from anxiety. Around half of them have comorbidity of depression and anxiety (1, 2). However, studies on depression and anxiety

mechanisms and the invention of therapeutic drugs develop slowly. Clinical drugs take weeks to months to have therapeutic effects, while more than one-third of patients are still resistant to the treatment (3). Therefore, it is urgent to explore the underlying mechanism of depression and anxiety.

Brain-derived neurotropic factor (BDNF) is a member of the neurotrophin family of growth factors. It is widely expressed in different brain regions including the amygdala, hippocampus and neocortex (4). It is well known that BDNF exerts antidepressant effects in various experimental models (5–8). Notably, BDNF is first synthesized as the BDNF precursor (proBDNF), which is then intracellularly cleaved by proconvertases/furin or extracellularly processed by matrix metalloproteinases /plasmin to generate mature BDNF (9, 10). Besides being an intermediate during the synthesis of mature BDNF, proBDNF can act on its receptors and have opposite functions to mBDNF in regulating neuronal activity (11). In this regard, BDNF binds to its receptor TrkB to promote neuronal survival, differentiation and synaptic plasticity. In contrast, proBDNF induces neuronal apoptosis via activation of a receptor complex of p75 neurotrophin receptor ($p75^{NTR}$) and sortilin (12, 13). Furthermore, proBDNF negatively regulates dendritic complexity and depresses the synaptic transmission in the hippocampus (14). Thus, proBDNF in the hippocampus may have different biological functions in anxiety/depression than mature BDNF.

Accumulating evidence has shown that proBDNF signaling is involved in the disease progress of depression. For example, clinical studies have shown that proBDNF was decreased in the postmortem cerebellum and spleen of depressed patients (15). proBDNF and its receptors $p75^{NTR}$ and sortilin were upregulated in the serum of female depressed patients and positively correlated with depression scores (16, 17). Furthermore, the increase in proBDNF in the serum of depressed patients was reversed by long-term antidepressant treatment (16). Experimental studies have also shown that proBDNF and its receptors were increased with spine loss in the hippocampus of rats with depression, that has been induced by unpredictable, chronic, mild stress (18). Anti-proBDNF antibody (Ab-proBDNF) injection via intra-cerebroventricular and intraperitoneal approaches reversed the stress-induced depressive behavior (18). Moreover, proBDNF was upregulated in the medial prefrontal cortex while downregulated in the nucleus accumbens of learned helplessness rats (19, 20). The role of hippocampal proBDNF on regulating depressive behavior and the exact neuronal target for depression treatment are not yet clear. Moreover, the role of hippocampal proBDNF signaling in anxiety disorder is still unknown. Anxiety disorder, although sharing some symptoms with depression, is a different mental illness (21–23). In particular, anxiety usually precedes depression and eventually develops into depression (24). It was shown that hippocampal proBDNF was increased in carioca high-conditioned freezing rats, an anxiety disorder model (25). However, whether the increased hippocampal proBDNF is involved in anxiety disorders is still unknown.

This study aims to explore the possible role of hippocampal proBDNF in depression and anxiety. The rat model of anxiety was induced by complete Freund's adjuvant (CFA) intra-plantar injection and the depressed rat model was established by chronic restraint stress (CRS). The present study showed that proBDNF was upregulated in the hippocampus in these two animal models. Neutralizing the increased endogenous hippocampal proBDNF by the monoclonal Ab-proBDNF ameliorated the anxiety-like and depressive behaviors. Thus, the present study suggests that the hippocampal proBDNF is a common mediator that regulates depression and anxiety.

MATERIALS AND METHODS

Male Sprague Dawley rats (eight weeks old; weight, 250 ± 20 g) were purchased from Hunan SJA Laboratory Animal Co., Ltd. (Changsha, China). During the experiment, animals were kept in a room with a 12-h light/dark cycle and environmental temperature of 25°C with 50–60% humidity in the experimental animal center of The Second Xiangya Hospital. Rats were housed in standard cages with *ad libitum* access to food and water. All of the animals were habituated for at least 1 week before any manipulation. All of the procedures were approved by the Institution of Animal Care and Use Committee of The Second Xiangya Hospital and Use Committee, and conformed to the Guide for the Care and Use of Laboratory Animals.

Animal Models

Depression-like behavior was induced by CRS in our previous study (26). Rats were restricted in a transparent cone made of polyvinyl chloride films. The restraint was performed 4 h per day for 3 weeks, and animals could not move except for breathing. The time of the restraint was random from 10:00 to 14:00. Then, rats were released from the cone back to the home cage after the restraint. The control rats were handled by the same experimenter without any restraints. Another group of rats was used for CFA (Sigma-Aldrich, St. Louis, MO, United States) injection. First, 100 μl CFA was injected into the left footpad to induce anxiety-like behavior as described previously (27). Three weeks later, rats were subjected to a battery of behavioral tests for the assessment of nociceptive responses, anxiety-like behaviors and depression-like behaviors.

Nociceptive Behavioral Test

The mechanical allodynia induced by CFA intra-plantar injection was accessed by measuring the 50% paw withdrawal threshold (PWT), as described previously (28, 29). The 50% PWT in response to a series of von Frey filaments (Ugo basile, Gemonio, Italy) was determined by the up and down method. Briefly, rats were placed separately in plexiglass chambers with mesh floors for 30 min to habituate before the test. Eight von Frey filaments with bending forces ranging from 0.6 to 15 g were chosen. Firstly, the 2.0 g filament was applied perpendicular to the plantar surface of the paw for every trial. If a positive response (apparent withdrawal, licking, jumping) occurred, an "X" was recorded. Then, a weaker filament was applied. If no positive response occurred, we recorded an "O," and a stronger filament was applied. Each trial ended when a six-number sequence of Os and Xs was obtained. The maximum and minimum limitation for filaments forces was to 0.6 and 15 g. Finally, the 50% PWT

was achieved by an adjusted version of the formula presented by Chaplan (28).

Open Field Test

Animals were placed in the center area of an open arena (120 cm long*120 cm wide*40 cm high) and were free to explore the field for 5 min. All of the movements were tracked by the overhead camera. The total travel distance, the time and travel distance in the central square (80 cm wide*80 cm long) were analyzed by ViewPoint Video Tracking Software (ViewPiont Behavior Technology, Lyon, France).

Elevated Plus Maze Test

Animals were placed in the plus maze with a central area (10 cm long*10 cm wide), two open arms (50 cm long*10 cm wide) and two closed arms (50 cm long*10 cm wide*40 cm high). This maze was 50 cm above the ground. Animals were free to explore the 4 arms for 5 min. All of the movements were recorded by the overhead camera. The travel time and number of entries into the open/closed arms were analyzed by ViewPoint Video Tracking Software (ViewPiont Behavior Technology, Lyon, France).

Sucrose Preference Test

Animals were housed individually in cages, and provided with a bottle of water and a bottle of 1% sucrose solution. The position of the two bottles was switched to reduce the bias for place preference after 24 h. At the end of 48 h, bottles were removed and the liquid consumption was recorded. The percentage of sucrose intake was calculated as sucrose intake/total fluid intake * 100%.

Forced Swimming Test

Animals were placed in a plexiglass cylinder (20 cm diameter × 60 cm height), filled with 30 cm of water maintained at ∼25 ± 1°C and were forced to swim for 15 min to habituate. The next day, animals were placed in the water again for 5 min, and all of the movements were recorded by the overhead camera. The immobile time was assessed by ViewPoint Video Tracking Software (ViewPiont Behavior Technology, Lyon, France).

Western Blot

After being deeply anesthetized by sevoflurane, animals were decapitated and hippocampus tissue was collected on ice. Protein lysates were prepared as previously described (29). Then, 50 μg protein was loaded and separated by a 15% SDS-PAGE gel, and then transferred to a PVDF membrane (Millipore, Billerica, MA, United States) at 200 mA for 2 h. After incubation in 1% gelatin solution for blocking for 1 h at room temperature, rabbit anti-BDNF (Santa Cruz Biotechnology Cat# sc-546, RRID:AB_630940), rabbit anti-p75 (Abcam Cat# ab8874, RRID:AB_306827), rabbit-anti-sortilin (Abcam Cat# ab16640, RRID:AB_2192606), mouse anti-MAP2 (Boster Cat# BM1243), rabbit anti-synaptophysin(SYP) (Proteintech Cat# 17785-1-AP) and mouse anti-GAPDH (CMCTAG Cat# AT0002) were applied to the membrane at 4°C overnight. Then the membrane was incubated with HRP-conjugated goat anti-rabbit IgG (Sigma-Aldrich Cat# A0545, RRID:AB_257896) or goat anti-mouse IgG (Sigma-Aldrich Cat# A9044, RRID:AB_258431) at room temperature for 1 h. The immunoreactivity of the proteins on the membrane was detected with an enhanced chemiluminescence kit (Millipore Cat# WBKL S00 50) and x-ray film (Carestream, United States). The band intensity was quantified using Image J software (NIH, Bethesda, MD, United States).

Immunohistochemistry

After being deeply anesthetized by overdose chloral hydrate (400 mg/kg), animals were cardiac perfused with 100 ml normal saline and followed by 300 ml 4% ice-cold paraformaldehyde. The brain was harvested and post-fixed in 4% paraformaldehyde at 4°C overnight. Then the brain was dehydrated in 30% sucrose in phosphate buffer saline (PBS). The hippocampus region of the brain was sliced in a cryostat (CM1950, Leica Biosystems, Germany). Sections were washed in PBS and rinsed in 3% H_2O_2 for 30 min to remove endogenous peroxidase. Then, the sections were blocked by 5% BSA containing 0.01% Triton X-100 in PBS at room temperature for 2 h. Primary antibody anti-proBDNF monoclonal antibody which was generated by us and has been characterized previously was applied overnight at 4°C (29, 30). The sections were incubated in biotinylated goat anti-mouse immunoglobulin (Jackson ImmunoResearch Labs Cat# 111-065-003, RRID:AB_2337959) and followed by an ABC kit (Vector Laboratories Cat# PK-4000, RRID:AB_2336818). The glucose oxidase-DAB-nickel method was used for visualization (31). Finally, all of the sections were transferred onto gelatin-coated slides and dehydrated. The slides were cover-slipped with neutral balsam (ZSGB-BIO Cat# ZLI-9555) for visualization by optical microscopy (BX53, Olympus Corporation, Japan).

For confirmation of the Ab-proBDNF injection site, we performed the immunohistochemistry procedure mentioned above except for the application of the primary antibody.

Golgi Staining

The staining procedure was carried out using an FD Rapid GolgiStainTM kit (FD Neuro-Technologies Cat# PK-401). In brief, animals were deeply anesthetized by sevoflurane and decapitated. The brain was collected and rinsed in Golgi–Cox solution, a mixture of solution A and solution B, and kept for at least 2 weeks in the dark. Then the tissue was kept in solution C for cryo-protection for 3 days. The brain was sliced into 150 μm sections by a cryostat (CM1950, Leica Biosystems, Germany). The hippocampus sections were transferred onto gelatin-coated slides. A mixture of solution D and solution E was used to visualize the neuronal architecture. After dehydration, the slides were cover-slipped with neutral balsam for visualization by optical microscopy (BX53, Olympus Corporation, Japan). Dendrite branches were traced by Image J software (NIH, Bethesda, MD, United States) with the NeuronJ plugin (32). Then dendritic length and spine density were calculated. Neuronal arborization was analyzed by counting the number of crossings by dendrites of concentric circles originating at the soma with increasing radii of 20 mm, using the sholl analysis plugin in ImageJ (33).

Stereotaxic Surgery and Drug Infusion

The surgery was performed according to a standardized protocol (34, 35). After induction with 5% sevoflurane in the anesthesia chamber, rats were placed in a stereotaxic apparatus (68025, RWD Life Science, China) with continuous 2.5% sevoflurane inhalation. Two stainless steel guide cannulas were implanted bilaterally with the cannula tips 1.5 mm above the dentate gyrus (DG) area (AP-4.2 mm; ML ± 2.5 mm; DV-4.5 mm) (36). The guide cannula was secured with dental cement anchored to the skull. Stylets inserted into each cannula to maintain patency until the rats were subjected to delivery of drugs. The injection needle was inserted into the guide cannula, with its tip located 1.5 mm beyond the end of the guide cannula. Next, 1 μl monoclonal anti-proBDNF (29) antibody (1 μg/μl) was injected into the DG area in each hemisphere on day 22. The same volume of IgG (1 μg, 1 μg/μl) (CMCTAG Cat# AT1596) or normal saline was injected bilaterally into the same sites in the control group. The same dose of regents was administered repeatedly on day 28. From day 29 to 34, a series of behavioral tests was performed. Then rats were killed, and the brain was harvested for identification of the injection site or further analysis.

Statistical Analysis

Data are expressed as mean ± SEM. Statistical analyses were performed using an unpaired two-tailed Student's *t*-test, one-way analysis of variance or two-way analysis of variance where appropriate. *p*-values were accepted as significantly different at $p < 0.05$. The statistical program used was GraphPad Prism 6.0 (San Diego, CA, United States).

RESULTS

Different Behaviors in the Rats With Anxiety-Like Phenotype and Rats With Depression-Like Phenotype

Clinically, depression and anxiety result in different behaviors that can be substantiated via various behavioral tests: the self-rating anxiety scale, self-rating depression scale, or Hamilton depression scale tests. In rats, anxiety-like behavior can be induced by persistent inflammatory pain. It has been noticed that CFA intra-plantar injection rendered pain hypersensitivity as indicated by the decreased PWT (**Figures 1A,B**). Moreover, CRS-treated rats did not display mechanical hyperalgesia, whereas locomotor activity was not altered by CRS and CFA injection (**Figure 1C**). Both rats treated with CFA and those treated with CRS displayed decreased traveled distance in the central square in the OFT (**Figure 1D**) and reduced time spent in the open arms in the EPM (**Figure 1E**). In contrast, the SPT results showed that CFA-treated rats had comparable sucrose consumption with the controls whereas CRS-treated rats consumed less sucrose than both the controls and CFA-treated rats (**Figure 1F**). Similarly, in the FST, the immobility time of CRS-treated rats was longer than that of the controls and CFA-treated rats (**Figure 1G**). Thus, CRS-treated rats displayed anxiety- and depression-like behaviors, whereas CFA -treated rats only displayed anxiety-like behaviors.

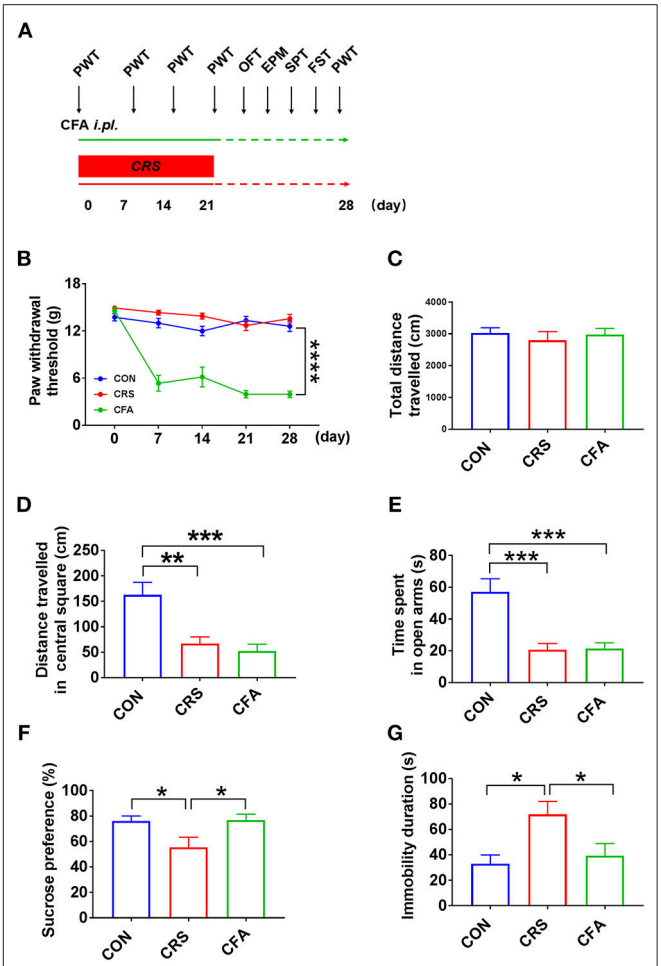

FIGURE 1 | Behavior analyses of CFA-evoked anxiety-like and CRS-evoked depressive behaviors **(A)** Time schedule of behavioral tests of rats injected with CFA or subjected to CRS for 21 days. **(B)** A significant decrease in the PWT in CFA-injected rats at 7, 14, 21, and 28 days after injection. CRS did not affect PWT. A repeated-measured analysis of variance (ANOVA), $^{****}p < 0.0001$ vs. CON, $n = 10$ for each group; all graphs represent values in mean ± SEM. **(C–G)** CRS and CFA did not affect the total travel distance in the OFT **(C)**. Distance traveled in central square in OFT **(D)** and time spent in the open arms in the EPM test **(E)** were significantly decreased following CRS or CFA injection; meanwhile, CRS decreased sucrose intake **(F)** and prolonged immobility time in the FST **(G)**. CFA, complete Freund's adjuvant; CRS, chronic restraint stress; PWT, paw withdrawal threshold; OFT, open field test; EPM, elevated plus maze; SPT, sucrose preference test; FST, forced swim test. $N =$ 9–10 in each group $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. control group (one-way ANOVA). All of the data are presented as mean ± SEM.

Different Granule Cell Morphology and Synaptic Integrity Changes Between Anxiety-Like and Depression-Like Behavior

It has been reported that depression and anxiety involve synaptic changes (37–39). Therefore, we examined whether there were synaptic changes in these two animal models. Rats with depression-like phenotype and rats with anxiety-like phenotype had similar spine density and dendritic length of granule cells in the DG area compared with the controls

(**Figures 2A,B,D**). However, sholl analysis showed a reduced complexity of granule neurons in rats with depression-like phenotype, and there was no difference in the dendritic arborization in rats with anxiety-like phenotype compared with the control group (**Figures 2C,E**). To assess the effect of CRS and CFA exposure on synaptic integrity, the expression levels of synaptophysin and microtubule-associated protein 2 (MAP2) in the hippocampus were measured. Synaptophysin was downregulated in the rats with depression-like phenotype but upregulated in the rats with anxiety-like phenotype. However, there was no significant change in MAP2 levels in the rats with anxiety-like phenotype and rats with depression-like phenotype as compared with the controls (**Figures 2F,G**). These results suggest that different morphologies and synaptic changes were responsible for depression and anxiety.

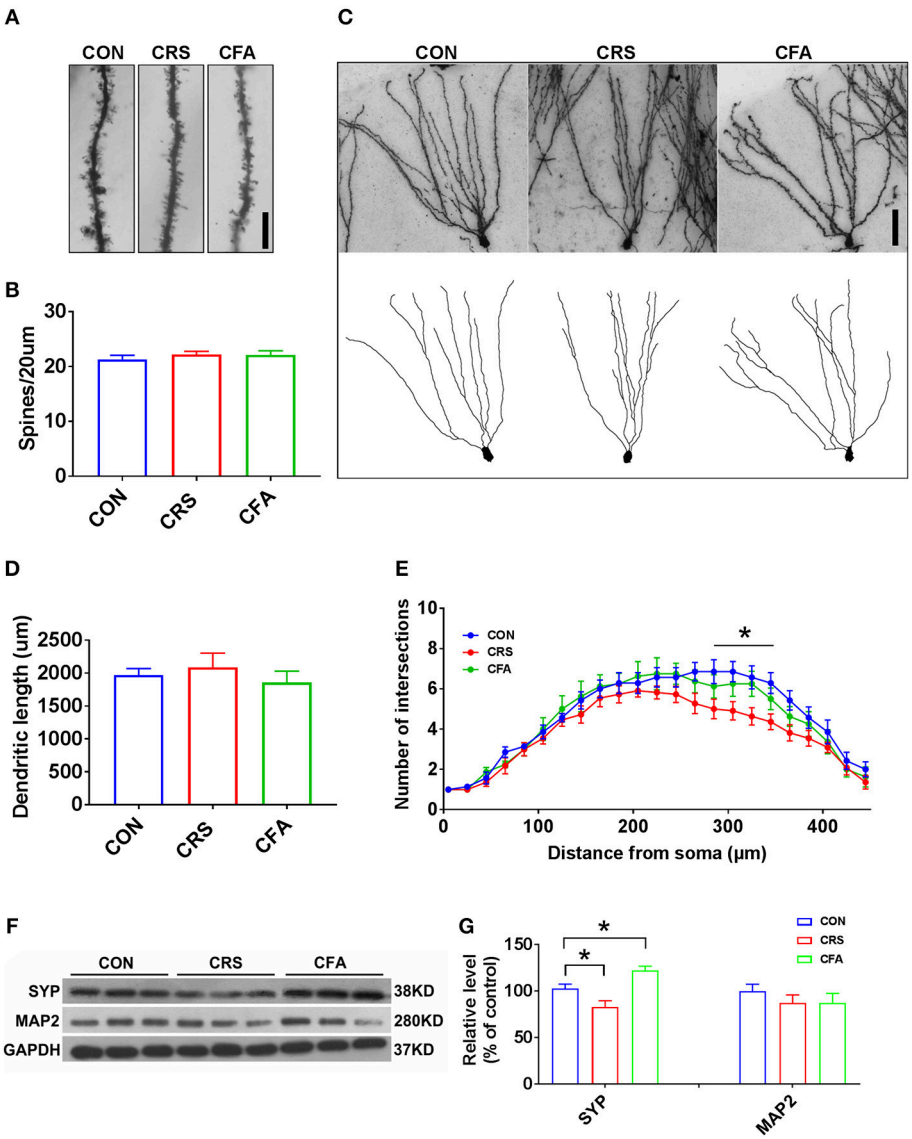

FIGURE 2 | Granule cell morphology and synaptic integrity changes between anxiety-like and depression-like behaviors. **(A,B)** Representative Golgi-Cox staining images of dendritic spines and spine density in the hippocampal DG granule cells in CRS-treated rats, CFA-treated rats, and the controls. $n = 10$ for each group, bar $= 10\,\mu m$. **(C)** Representative images of Golgi-Cox -stained granule cells in the DG area (top) and the reconstruction of its dendritic branches (bottom) from each group. Bar $= 50\,\mu m$. **(D)** There were no significant effects of CRS and CFA injection on the dendritic length of the DG granule cells. **(E)** Sholl analysis of dendritic length in DG granule cells. CRS reduced dendrite intersection in the region 285–345 mm away from the soma compared with the control group. A repeated measures analysis of variance (ANOVA), $^*p < 0.05$ vs. CON, $n = 10$ for each group; all graphs represent mean ± SEM. **(F,G)** Expression of SYP and MAP2 in the hippocampus was evaluated by Western blotting. Semi-quantitative analyses of SYP and MAP2 expression were performed. Note that the expression of SYP was significantly reduced in CRS-treated rats but was increased in CFA -treated rats. DG: dentate gyrus; CFA: complete Freund's adjuvant; CRS, chronic restraint stress; SYP, synaptophysin; MAP2, microtubule-associated protein 2. $^*p < 0.05$, vs. control group (one-way ANOVA). All of the data are presented as mean ± SEM.

Differential Expression of proBDNF and its Receptors in the Hippocampus in Rats With Anxiety-Like Phenotype and Rats With Depression-Like Phenotype

A recent study showed that chronic, unpredictable, mild stress upregulates proBDNF in the hippocampus of rats with depressive behavior (18). To further confirm the upregulation of proBDNF in depression and anxiety disorders, we examined the expression of proBDNF and mature BDNF in the hippocampus of these two rat models established in the present study. As shown in **Figures 3A,B**, proBDNF was found upregulated in the hippocampus. Interestingly, there was no significant change in BDNF levels in rats with depression-like phenotype or rats with anxiety-like phenotype as compared with the controls. These findings suggest that both depression and anxiety render the upregulation of hippocampal proBDNF. Surprisingly, the expression of hippocampal $p75^{NTR}$ was not significantly changed in the rats with CRS-evoked depression-like behavior but was greatly down-regulated in the rats with CFA-evoked anxiety-like behavior. In addition, there was no significant difference in sortilin expression among the rats with anxiety-like phenotype, the rats with depression-like phenotype and the control group. Furthermore, proBDNF expression was intensely upregulated in the hippocampus of rats with depression-like phenotype and rats with anxiety-like phenotype (**Figure 3C**). These results suggest that depression and anxiety may have discrepant expression of proBDNF signaling.

Intra-Hippocampal Injection of Ab-proBDNF Attenuated Anxiety-Like and Depressive Behaviors

The increased proBDNF in the hippocampus in the rats with anxiety-like phenotype and rats with depression-like phenotype may contribute to disease progress. In order to test this hypothesis, bilateral intra-hippocampal injection of monoclonal Ab-proBDNF (1 μg each side) was performed twice in the normal rats through the cannula. As shown in **Figures 4A,B**, intra-hippocampal injection of Ab-proBDNF was limited within the DG area, suggesting injection precision. Intra-hippocampal injection of Ab-proBDNF in the normal rats did not affect the total travel distance (**Figure 4C**) and the distance traveled in the central square in the OFT (**Figure 4D**). The EPM experiment also showed that injection of Ab-proBDNF into the hippocampus did not affect the time spent in the open arms (**Figure 4E**). These results suggest that intra-hippocampal injection of Ab-proBDNF did not change basal behavior.

As shown in **Figure 5A**, bilateral intra-hippocampal injection of Ab-proBDNF (1 μg each side) was performed twice, on day 22 and 28, after CRS or CFA exposure. Intra-hippocampal injection of Ab-proBDNF reversed the reduction of distance traveled in the central area and the decreased time spent in the open

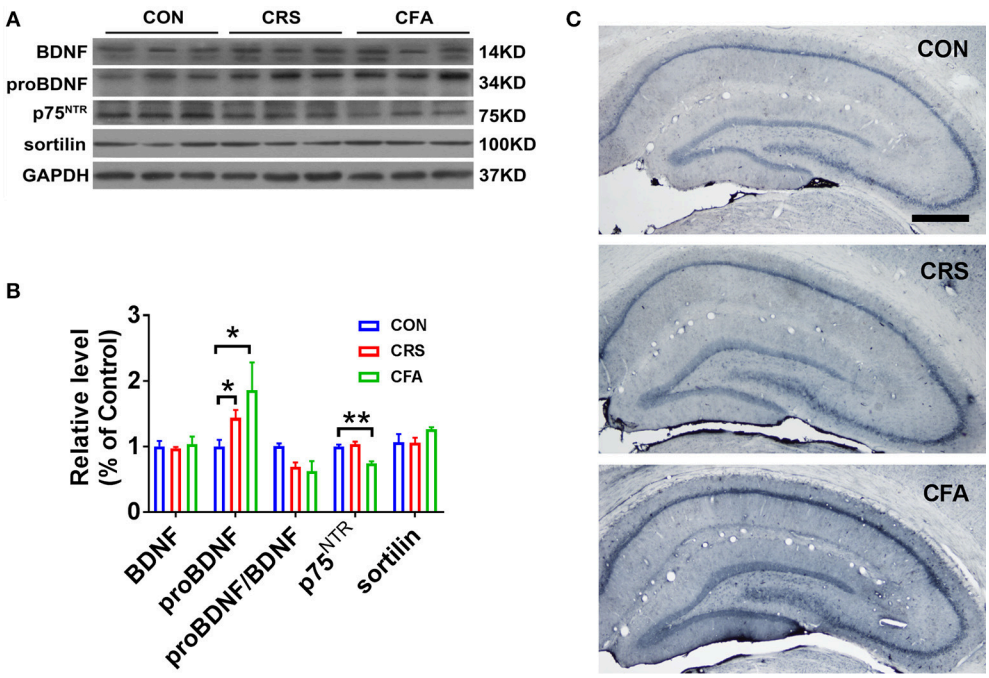

FIGURE 3 | Upregulation of proBDNF and its signaling in rats with anxiety-like phenotype and rats with depression-like phenotype. **(A)** Representative Western blot, and **(B)** semi-quantitative analyses of mature BDNF, proBDNF, proBDNF/ BDNF, $p75^{NTR}$, and sortilin in the hippocampus of rats with depression-like and anxiety-like phenotype. Note that the expression of proBDNF was increased significantly in the rats with depression-like phenotype and the rats with anxiety-like phenotype. **(C)** Immunohistochemistry of proBDNF in the hippocampus of rats with depression-like and anxiety-like behaviors. Note that intensive proBDNF was expressed in the hippocampus of the CRS rats and CFA rats. Bar = 100 μm. $^{*}p < 0.05$, $^{**}p < 0.01$, vs. control group (one-way ANOVA). All of the data are presented as mean ±SEM.

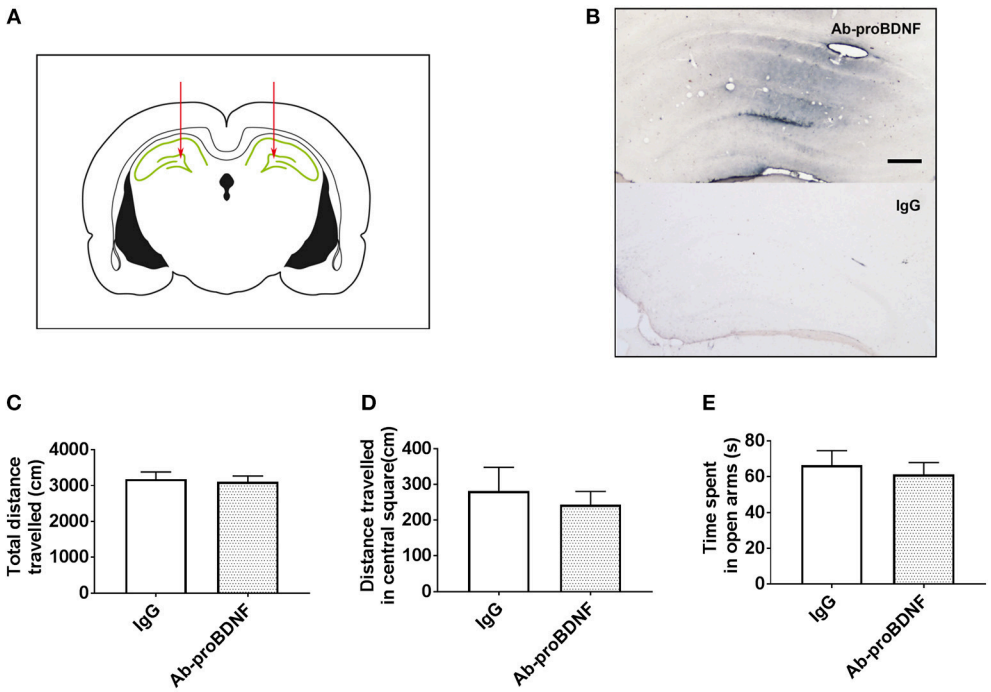

FIGURE 4 | Hippocampal Ab-proBDNF injection and its effect on the anxiety-like behavior in normal rats. **(A)** Schematic representation of the antibody injection sites into the bilateral DG area through the cannula. **(B)** Representative immunohistochemistry images of Ab-proBDNF (top) or IgG (bottom) detection by the application of biotinylated anti-mouse IgG and the glucose oxidase-DAB-nickel visualization system 1 week after injection. Bar = 100 μm. Hippocampal Ab-proBDNF injection did not affect the total travel distance**(C)** or distance traveled **(D)** in the central square in the OFT, or the time spent **(E)** in the open arms in the EPM test. Ab-proBDNF: monoclonal anti-proBDNF antibody. *N* = 9–10 in each group. All data are presented as mean ± SEM.

arms in the rats with anxiety-like phenotype (**Figures 5B–D**). Similarly, as compared with the vehicle injection group, injection of Ab-proBDNF also inhibited the decreased distance traveled in the central square and the decreased time spent in the open arms in the rats with depression-like phenotype (**Figures 5E–G**). Moreover, neutralizing the endogenous proBDNF in the hippocampus by Ab-proBDNF injection greatly increased the sucrose consumption in the rats with depression-like phenotype compared with vehicle injection rats (**Figure 5H**). Finally, FST results showed that the immobility time was significantly lower in the Ab-proBDNF treatment group than in the vehicle treatment group (**Figure 5I**). Taken together, intra-hippocampal injection of Ab-proBDNF relieved the anxiety-like behavior and exerted an anti-depressive effect.

DISCUSSION

Anxiety disorders and depression are the two most prevalent mental disorders worldwide (40). In clinical practice, anxiety disorders and depression can overlap; many patients with depression having experienced anxiety disorders earlier in life. Moreover, around 50% of patients with depression are also diagnosed with an anxiety disorder (2). However, depression also displays different clinical manifestations than anxiety. For example, patients with depression move slowly with flattened or dulled reactions, whereas people with anxiety tend to be more keyed up. In addition, anxiety patients display fear about the future whereas depressed people are less likely to be fraught with worry about future events (41). All of these clinical manifestations suggest that these two distinct mental disorders are closely related, and may share some common mechanisms.

In the experimental rat models, both depressive and anxiety-like behaviors displayed the same behaviors in the OFT and EPM tests, which showed a decreased travel distance in the central square of the OFT and a decreased time spent in the open arms of the EPM test. However, depressive behavior can be distinguished from anxiety-like behavior through SPT and FST examinations in which rats with depression-like phenotype had less sucrose consumption and a longer immobility time (42). Like the anxiogenic effect of neuropathic pain (43, 44), CFA injection induced persistent inflammatory pain, which developed the anxiety-like behavior 3 weeks after CFA injection. It is consistent with the studies reported previously (27, 45). Notably, the chronic inflammatory pain may affect locomotor activity. However, the total traveled distance by rats with anxiety-like behavior in the OFT did not change, thus indicating that the reduction in traveled distance in the central square was not due to the effect of inflammatory pain on locomotor activity. Similarly to results found in previous studies (26, 46–48), rats with depression-like phenotype also exhibited lower sucrose consumption in the SPT and a longer immobility time in the FST. These findings further confirmed that there are some overlapping

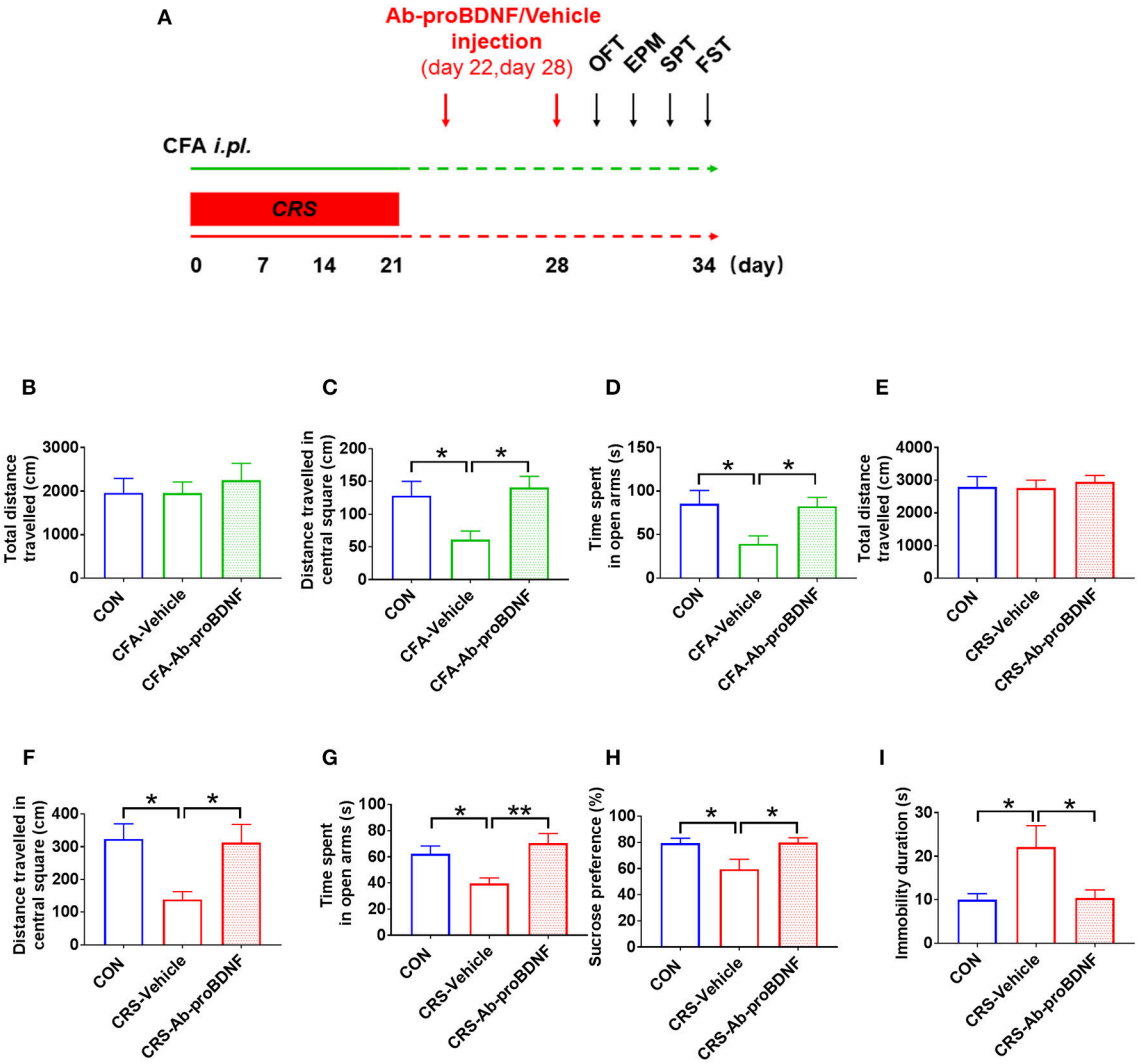

FIGURE 5 | Hippocampal Ab-proBDNF injection ameliorated the anxiety-like and depressive behaviors. **(A)** Time schedule of behavioral tests and Ab-proBDNF injection in the CRS-evoked rats with depression-like phenotype and CFA-induced rats with anxiety-like phenotype. Hippocampal Ab-proBDNF injection did alter the total travel distance **(B,E)**, but reversed the decreased distance traveled in the central square in the OFT **(C,F)** and time spent in the open arms in the EPM test **(D,G)** in CFA-induced rats with anxiety-like phenotype and CRS-evoked rats with depression-like phenotype. Ab-proBDNF injection reversed the decreased sucrose intake in the SPT **(H)** and the increased immobility time in the FST **(I)** in CRS-evoked rats with depression-like phenotype. Ab-proBDNF: monoclonal anti-proBDNF antibody. $N = 6$–8 in each group. $^{*}p < 0.05$, $^{**}p < 0.01$, vs. control group (one-way ANOVA). All data are presented as mean ± SEM.

mechanisms between depression and anxiety in experimental animal models; these yet need to be unraveled.

Mental disorders are often accompanied with changes in dendritic arborization and spine density in the neurons of the hippocampus and prefrontal cortex; this is seen in both humans and rodents (49–51). In the present study, the dendritic complexity was significantly decreased in rats with depression-like phenotype, which is consistent with the results of previous studies (52–54). However, the dendritic length spine density was not changed in the rats with depression-like phenotype, and any difference in dendritic complexity, dendritic length and spine density was not found in the rats with anxiety-like phenotype. Moreover, in the rats with anxiety-like phenotype, synaptophysin, a marker for synaptic density found in the hippocampus, was increased, which is supported by previous reports (55, 56). In contrast, hippocampal SYP was decreased in the rats with depression-like phenotype. Whereas, there was no difference found in hippocampal MAP2 among all three groups. These findings suggest that the CRS results in hippocampal dendritic retraction and CFA slightly stimulates synaptogenesis in the hippocampus, providing the neurobiological substrates responsible for the different behaviors related to depression and anxiety.

The precursor of BDNF, proBDNF has been reported to regulate depressive behavior under chronic stress (18). proBDNF was upregulated in the hippocampus, neocortex and medial

prefrontal cortex in rats with depression-like phenotype. In contrast, the expression of proBDNF was decreased in the nucleus accumbens in the rats with learned helplessness (19, 20), suggesting different expression patterns of proBDNF in different brain regions in the depression models. In the current study, the expression of proBDNF was also increased in the hippocampus of rats with depression-like phenotype. Furthermore, the expression of proBDNF signaling was also upregulated in rats with anxiety-like phenotype. Although intra-cerebroventricular injection of Ab-proBDNF could attenuate the depressive behavior (18), it is still unclear whether neutralization of the hippocampal proBDNF inhibits the anxiety-like and depressive behaviors.

In the present study, intra-DG injection of Ab-proBDNF in the control rats did not affect behavior as compared with the IgG control treatment, thus suggesting that the reduction of endogenous proBDNF in the hippocampus does not affect the behaviors under physiological conditions. This may be due to the low extracellular level of proBDNF, because only Ab-proBDNF can neutralize the extracellular proBDNF. In contrast to the lack of effect of Ab-proBDNF treatment on behaviors in control rats, Ab-proBDNF injection greatly inhibited the decreased sucrose consumption and increased immobility time in the FST. This indicates that hippocampal proBDNF contributes to the development of depression. Moreover, the intra-hippocampal Ab-proBDNF antibody also greatly protected against the anxiety-like behavior, as indicated by the EPM and OFT in both animal models. Collectively, these results indicate that hippocampal proBDNF is a common substrate that regulates depression and anxiety. Notably, in the present study, the injection site of Ab-proBDNF is mainly limited to the DG region. Previous studies have reported different alterations in the BDNF and proBDNF in CA1, CA3, and DG regions of the hippocampus within rodents with depression-like phenotype (20). Therefore, it would be greatly interesting to explore the role of proBDNF in CA1 and CA3 regions in depression and anxiety in a future study.

proBDNF exerts its biological effects through binding to its high affinity receptor $p75^{NTR}$ and co-receptor sortilin. It has been reported that $p75^{NTR}$ expression was increased in the rats depressed by unpredictable, chronic, mild stress (18). However, in the rats with CRS-evoked depression-like behavior, the expression of $p75^{NTR}$ was not altered. This contrast may be due to the different paradigms used to induce depression. Nonetheless, the expression of $p75^{NTR}$ was down-regulated in the rats with anxiety-like phenotype. This is consistent with the finding of a previous study, which showed that the deletion of $p75^{NTR}$ resulted in anxiety-like behavior (57). In addition, there was no significant difference in sortilin expression in the rats with anxiety-like phenotype compared with the control rats. Recent studies showed that sortilin-knockout mice displayed anxiety-like behavior, suggesting the involvement of sortilin in anxiety (58). As there was no significant change in sortilin in the rats with anxiety-like phenotype in the present study, this indicates that sortilin may not contribute to the development of anxiety induced by chronic pain.

In conclusion, the present study showed that depression and anxiety have both distinct and overlapping behaviors, and morphological hippocampal changes in rat models. Furthermore, the increased hippocampal proBDNF played an important role in regulating both depression and anxiety. Inhibition of the increased proBDNF by antibodies might be a potential therapy to treat depression and anxiety.

AUTHOR CONTRIBUTIONS

FZ: conducted the study, contributed to data collection, data analysis, and manuscript preparation and revision. LeL and J-LW: contributed to the conduction of the study, data collection, and data analysis. Z-LH and SW: helped collect and analyze the data, and revise the manuscript. LiL and J-MX: helped design the study and analyze the data. X-FZ and C-QL: contributed to the experimental design and revision of the manuscript. R-PD and Z-YY: designed and interpreted the work, contributed to data collection, and data analysis, and assisted with manuscript drafting and revision.

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REVIEW
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Treating the "E" in "G × E": Trauma-Informed Approaches and Psychological Therapy Interventions in Psychosis

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Despite advances in genetic research, causal variants affecting risk for schizophrenia remain poorly characterized, and the top 108 loci identified through genome-wide association studies (GWAS) explain only 3.4% of variance in risk profiles. Such work is defining the highly complex nature of this condition, with omnigenic models of schizophrenia suggesting that gene regulatory networks are sufficiently interconnected such that altered expression of any "peripheral" gene in a relevant cell type has the capacity to indirectly modulate the expression of "core" schizophrenia-associated genes. This wealth of associated genes with small effect sizes makes identifying new druggable targets difficult, and current pharmacological treatments for schizophrenia can involve serious side effects. However, the fact that the majority of schizophrenia genome-wide associated variants fall within non-coding DNA is suggestive of their potential to modulate gene regulation. This would be consistent with risks that can be mediated in a "gene × environment" (G × E) manner. Stress and trauma can alter the regulation of key brain-related pathways over the lifetime of an individual, including modulation of brain development, and neurochemistry in the adult. Recent studies demonstrate a significant overlap between psychotic symptoms and trauma, ranging from prior trauma contributing to psychosis, as well as trauma in response to the experience of psychosis itself or in response to treatment. Given the known effects of trauma on both CNS gene expression and severity of psychosis symptoms, it may be that pharmacological treatment alone risks leaving individuals with a highly stressful and unresolved environmental component that continues to act in a "G × E" manner, with the likelihood that this would negatively impact recovery and relapse risk. This review aims to cover the recent advances elucidating the complex genetic architecture of schizophrenia, as well as the long-term effects of early life trauma on brain function and future mental health risk. Further, the evidence demonstrating the role of ongoing responses to trauma or heightened stress sensitivity, and their impact on the course of illness and recovery, is presented. Finally, the need for trauma-informed approaches and psychological therapy-based interventions is discussed, and a brief overview of the evidence to determine their utility is presented.

Keywords: psychosis, schizophrenia, trauma, stress, psychological therapy, epigenetics, gene × environment

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INTRODUCTION

Early studies aimed at identifying underlying genetic components in schizophrenia highlighted both rare disruptions and common variants in a number of candidate genes such as Disrupted In Schizophrenia 1 (*DISC1*) (1, 2). These variants were identified in individuals with a diagnosis of schizophrenia and other major mental health conditions through pedigree analysis, though many schizophrenia candidate gene studies from this era suffered from lack of statistical power and reproducibility (3). Recent genome-wide association studies (GWAS) have significantly advanced our understanding of the genetic architecture of schizophrenia, revealing that risk for this condition is highly polygenic in nature (4). Comparing GWAS data on historical candidate gene variants did not provide supporting evidence for enrichment of GWAS signal at many of these previously identified loci (3, 5, 6), suggesting that implicated variants from early studies may not be representative of risk in the majority of individuals with this condition.

Schizophrenia GWAS studies have since identified 108 highly associated loci (4), and have provided some support for the utility of commonly used antipsychotic medications by highlighting variants at dopamine receptor genes, such as *DRD2*, to be associated with schizophrenia (7). However, currently available antipsychotic treatments can cause serious side effects in a number of individuals, such as increasing risk for metabolic and cardiovascular conditions (8–11).

Druggable target analysis of GWAS data has suggested a potential role for the repurposing of epilepsy medications and calcium channel blockers as treatment options to be explored (7). However, a recent omnigenic model of schizophrenia suggested that all expressed genes in a relevant cell type may have an effect on risk (12), and as such, the large number of implicated genes with small effect sizes, and the broad distribution of risk variants across the genome (13), has hindered the identification of new druggable targets.

That the large majority of schizophrenia GWAS single nucleotide polymorphisms (SNPs) fall in non-coding DNA (4), with enrichment in open chromatin and predicted transcriptional regulatory elements (12, 14, 15), would suggest that risk for this condition is likely to be mediated through gene-environment (G × E) interactions (16). Indeed, while polygenic risk scores (PRS) have typically been associated with increased odds for schizophrenia (4), a recent study examining polygenic risk with or without obstetric complications suggested that risk may be highly modified by the effect of early life stressors (17). Evidence supporting the effect of early life stress and trauma on psychosis risk is robust (18, 19), with an evident dose-response relationship between cumulative trauma exposure and increasing odds for experiencing psychosis (18, 20, 21). Similarly, studies have demonstrated that exposure to specific types of maltreatment in childhood can increase the odds of experiencing specific psychotic symptoms (21).

One in three cases of psychosis are predicted to be due to the effects of childhood trauma (18, 19), with complementary evidence demonstrating the beneficial and protective epigenetic effects of positive parental nurturing in early infancy and childhood (22, 23). As such, early experiences of trauma are one of the most preventable risk factors for psychosis and schizophrenia. Individuals with a history of psychosis or psychosis-like experiences are significantly more likely to have experienced trauma than those without a history of such experiences (24–26). Exposure to early trauma has been suggested to modulate developmental and molecular pathways such that an individual may become sensitized to future life stress (27–29). Indeed, high stress sensitivity has been correlated with increased rates of psychosis-like symptoms in the general population across 39 countries (30), and with significantly increased symptom intensity in individuals with first episode psychosis (FEP) compared to controls (31). In individuals with experiences of psychosis, exposure to childhood maltreatment has also been associated with significant increases in emotional and psychotic reactivity to small daily life stressors in adulthood (32). Such research would support the hypothesis that early life stress can predispose individuals to greater stress reactivity in adulthood, which would modulate risk for experiencing psychotic symptoms.

In addition to trauma as a risk factor for psychosis, there is a growing recognition that the experience of psychosis itself, and the distress associated with treatment methods, can result in new or further traumatization. Up to 69% of individuals that have experienced psychosis suffer post-psychosis trauma symptoms, while up to 39% develop clinical post-traumatic stress disorder (PTSD) in response to the events of their psychotic episodes, including distressing treatment-related events such as involuntary hospitalization (33–37). It is therefore clear that a significant subset of individuals with psychosis have endured repeated trauma, and may be re-traumatized during the course of illness and treatment (38, 39). Such experiences are likely to negatively impact treatment response and recovery. For example, individuals with "treatment resistant" schizophrenia report exposure to significantly higher rates of stressful life events and childhood trauma than those with medication-responsive symptoms (40, 41).

Stressful experiences can modulate the regulation of central nervous system (CNS) genes and genes involved in stress response (42–44), though preliminary studies have demonstrated that psychological therapy can normalize epigenetic marks over key CNS genes (45–47), suggesting that non-pharmaceutical treatments are also a plausible route to biological intervention in the context of mental health. Given this information, research into the provision and utility of psychological therapy for individuals with psychosis is currently being undertaken. Trauma-informed psychological interventions have proven safe and acceptable for use with those experiencing psychosis (48–50), and have been shown to be effective in reducing post-psychosis trauma (51). Similarly, Cognitive Behavioral Therapy (CBT) has been adapted for use with individuals experiencing psychosis (CBTp) and has proven to be both safe and effective for use in this population (52, 53), such that national guidelines in the UK now recommend this as a frontline treatment for those experiencing psychosis, or those at high risk (54). CBT for individuals with psychosis has been shown to result in sustained improvements in positive symptoms (55–58), as well

as improvements in depression, anxiety, quality of life, and functional outcomes, which were maintained at follow up (57, 58). Sustainable improvements in both positive and general psychosis symptoms have also been demonstrated in a trial of CBTp with individuals whose condition had previously been classified as medication-resistant (55).

This review aims to provide a brief outline of the genetic complexity of schizophrenia, the impact of trauma on brain development, function, and future mental health, and both the need and evidence base for trauma-informed approaches and psychological therapy-based interventions.

THE CURRENT LANDSCAPE OF SCHIZOPHRENIA GENETICS RESEARCH

Insights From Schizophrenia GWAS

The most recent advances in schizophrenia genetic research have come from large-scale GWAS, which have identified many common SNPs with small effect sizes, allowing the calculation of polygenic risk scores (PRS) based on hundreds of low effect alleles. The largest schizophrenia GWAS to date identified 128 variants at 108 genomic loci (4). However, these risk SNPs still only identify a very small percentage of risk, and the frequency of risk-associated alleles typically differ by <2% between case and control cohorts (59). The large majority of risk SNPs identified through GWAS reside in non-coding regions, which suggests that much of the identified genetic risk may be highlighting gene regulatory mechanisms. However, it is important to note that many such associated SNPs may have only reached significance as a consequence of being in linkage disequilibrium (LD) with truly causal SNPs, while conferring no risk-related functional alteration on their own. Despite this, a number of studies have confirmed that individual GWAS SNPs at key schizophrenia-associated loci reside in regulatory regions that are active in the CNS (60, 61), and that have functional consequences based on SNP genotype (62, 63).

It has previously been assumed that genetic variants that are associated with disease risk would be clustered at disease relevant gene loci, or around genes with relevant tissue-specific expression. However, it has been argued that enrichment of GWAS signal at specific disease relevant loci is weak for many complex traits and conditions (12). In the case of schizophrenia, work by Loh et al. demonstrated that 71–100% of all 1 Mb windows in the human genome contain one or more identified GWAS variant(s) which would influence schizophrenia risk (13). However, within such blocks in the cortex, Bryois et al. found that schizophrenia-associated SNPs were enriched in regions of open chromatin (15). Such findings provide further evidence consistent with a G × E model, in which our environment is likely able to modulate our neurochemistry, with potential effects on our risk for mental health conditions (16).

As stated, GWAS signal was found to be strongly enriched in regions of chromatin that are active in disease-relevant cell types. However, work by Boyle et al. demonstrated that, while genes with brain-specific expression patterns were more strongly enriched for schizophrenia-associated GWAS SNPs, SNPs within or near to genes with broad and non-specific expression profiles contributed more to the total schizophrenia heritability due to their larger number (12). Boyle et al. also noted weak enrichment for relevant functional ontologies associated with identified schizophrenia risk loci, which would be consistent with results from the Psychiatric Genomics Consortium (4).

Such findings have resulted in a hypothesized "omnigenic model" of complex conditions such as schizophrenia, in which a small number of variants have regulatory effects on core pathways central to schizophrenia, while a much larger number of variants influence expression of a peripheral gene set which can modify the action of the core gene set. This model would support the finding that variation affecting genes in core associated pathways explains only a small fraction of schizophrenia heritability, while the remaining heritability may be explained by many small variations across all other expressed genes (4, 12).

Uncovering Functional Mechanisms of GWAS Variants

Current research is beginning to integrate GWAS data with expression and methylation quantitative trait loci (eQTLs, meQTLs) to determine patterns of gene expression and altered regulation that may contribute to schizophrenia biology (64–67). Similar work is beginning to overlay GWAS data with chromatin profiles in relevant cell types in order to identify potential chromatin-modifying effects of GWAS SNPs.

Environmental stressors, such as childhood adversity, have been associated with altered methylation across CNS-expressed genes, such as FK506 binding protein 5 (*FKBP5*) and glucocorticoid receptors, which are known regulators of the stress response and have been associated with psychiatric conditions such as depression and PTSD (42–44). Further, methylation of the KIT ligand (*KITLG*) locus, which encodes the ligand for the receptor tyrosine kinase, KIT, has been shown to mediate the association between childhood trauma and cortisol stress response, and has been highlighted as a region of interest with regard to epigenetic programming of stress reactivity (68).

Multiple different types of early life trauma have been demonstrated to alter methylation at the glucocorticoid receptor in peripheral blood of individuals with diagnosed psychiatric conditions (42). Those that had been exposed to sexual abuse, physical abuse/neglect, and emotional abuse/neglect, had significantly higher levels of methylation across multiple CpG regions at the glucocorticoid receptor locus compared to those without such exposures. Further, a dose-response relationship was evident, in which exposure to multiple trauma types, or increased trauma severity, was associated with higher levels of methylation at this region (42). Methylation changes in response to trauma can also be mediated in a genotype-dependent manner, as in the case of *FKBP5* (43).

Schizophrenia GWAS SNPs are known to significantly overlap with eQTLs and meQTLs in the adult hippocampus, frontal cortex, and the fetal brain (69–71), suggesting that some associated variants may mediate risk by influencing gene methylation, potentially in a differential manner in response to environmental stimuli. Fetal brain meQTLs have been found

to be enriched in functional elements and were similarly demonstrated to be over-represented for schizophrenia GWAS variants and at schizophrenia-associated genomic loci (69). The majority of fetal brain meQTLs (83.46%) showed evidence of functionality in at least one of the adult brain regions tested in this study (pre-frontal cortex, striatum, and cerebellum), though such shared fetal and adult meQTLs that were enriched at schizophrenia-associated loci were found to have stronger effects in the fetal brain than in the adult brain. The remaining subset of fetal-specific meQTLs were also found to be enriched at genomic loci that had previously been associated with schizophrenia through GWAS (69). That many fetal meQTLs in the brain are preserved in adulthood may suggest a mechanism through which the effects of early environmental challenges can persist, influencing epigenetic risk and neurodevelopment in ways which may modulate the likelihood of experiencing psychiatric conditions (64).

Similarly, ATAC-seq (Assay for Transposable Accessible Chromatin) on post-mortem dorsolateral prefrontal cortex (DL-PFC) samples from 135 adults with a diagnosis of schizophrenia and 137 controls demonstrated that schizophrenia GWAS SNPs were enriched in accessible chromatin regions in the brain, with 1.2% of the GWAS SNPs in DL-PFC ATAC-seq peaks accounting for 8.55% of schizophrenia SNP heritability (15). ATAC-seq peaks from fetal brains were also enriched for schizophrenia association, demonstrating that such risk-related regulatory loci are accessible, and thus likely active, during early development (15). Taken together, such findings may provide a mechanism through which neurodevelopmental risk factors, such as maternal stress or immune activation, may have a lasting impact on the developing and adult brain by affecting gene expression through the modulation of methylation and chromatin accessibility.

Insights and Complications in Using Polygenic Risk Scores

Additional utility for the wealth of GWAS data has been found in the generation of PRS. PRS use data on variants across multiple associated loci in order to try to predict risk for a particular condition based on combined SNP genotypes. To date, multiple studies have demonstrated that increased polygenic risk for schizophrenia is significantly associated with increased odds ratios (OR) for schizophrenia-spectrum diagnoses, such as schizophrenia, schizoaffective disorder, first episode psychosis and psychosis not otherwise specified (4, 72, 73). The odds ratio is a measure of association between an exposure and an outcome. In this instance, increased overall genetic risk for schizophrenia is associated with a higher likelihood of schizophrenia-spectrum diagnosis. Notably, the landmark publication on recent schizophrenia genetics research identifying 108 schizophrenia-associated loci demonstrated that the highest polygenic risk scores in their analysis were associated with an odds ratio for schizophrenia of 7.8–20.3 (4). However, almost all studies on PRS and schizophrenia risk did not account for the influence or interaction with other known risk factors, such as early life stress. Further work has now begun examining to which risk-related traits the PRS may predispose, and how polygenic risk may interact with other such risk factors.

Higher schizophrenia PRS have been associated with increased levels of anxiety in a non-clinical adolescent population, but failed to demonstrate an association with psychotic experiences (74–76). This may suggest that schizophrenia risk in adolescence presents as, or contributes to, known phenotypes that are associated with schizophrenia in adulthood, such as anxiety or increased stress sensitivity. Higher schizophrenia PRS have also been associated with reduced hippocampal volume in FEP individuals and those classified as having an "at risk mental state" (ARMS) (73), as well as with multiple immune conditions (77), which provides supporting evidence for immune system mechanisms in schizophrenia.

The interaction of schizophrenia GWAS SNPs with environmental stressors, including early life complications (ELCs) such as complications during pregnancy, labor, and delivery, have further been shown to influence the effects of schizophrenia PRS (17). Ursini et al. constructed schizophrenia PRS using GWAS SNPs from meta-analyses of Psychiatric Genomics Consortium (PGC) data sets (17). Odds ratios for schizophrenia were calculated for each subset of PRS in comparison to the lowest polygenic risk quintile, in individuals with or without ELCs. For individuals in the highest polygenic risk quintile without ELCs, no statistically significant change in odds ratio for schizophrenia was observed. These findings were validated in separate Italian and German case-control cohorts, with the finding that five times more schizophrenia risk was explained by an individual's PRS in the presence of ELCs compared to such scores in the absence of ELCs. This relationship was seen for polygenic risk scores constructed using statistically significant GWAS SNPs, and also for PRS constructed with SNPs showing a trend toward genome-wide significance, but not for other putatively associated SNPs. The authors suggested the possibility that the high statistical significance of these SNPs identified by GWAS in a heterogeneous group of case samples may be due to their interaction with common environmental risk factors, which, in combination, would confer a strong effect on risk (17). Such work may in part explain the relatively low penetrance of common schizophrenia risk variants when assessed or inherited without the appropriate environmental context through which they exert their influence on mental health risk.

A study by Curtis further demonstrated that, while PRS were significantly associated with schizophrenia in the Common Mind Consortium data set, these measures were not robustly associated with expression of any of the 16,423 individual genes tested, nor with expression of specific schizophrenia-associated gene sets (78). Curtis suggested that common variants may instead be influencing indirect risk factors that may be far removed from core biological pathways, such as increasing risk for, or response to, schizophrenia-associated environmental factors. Indeed, Ursini et al. found that PRS for schizophrenia increased the likelihood of experiencing an ELC (17), and further PRS have been shown to significantly predict risk for multiple immune conditions (77). This could provide support for the hypothesis that polygenic risk for schizophrenia may be

identifying predisposition to other environmental risk factors for this condition (78), such obstetric complications, or altered immune function that may increase the likelihood of maternal infection.

Schizophrenia Risk Is Likely to be Driven by Environmental Challenge

Recent insights into the genomic underpinnings of schizophrenia have been successful in more clearly elucidating the complex polygenic architecture of this condition, and in providing support for the biological targets of currently available pharmacological treatments. However, schizophrenia GWAS signal is widely distributed across the genome, and does not necessarily converge on particular loci or novel molecular pathways (13). This has hindered the identification of new targets for improved pharmacological intervention. While PRS for schizophrenia have for the most part shown reliable increases in risk with increased genetic burden, emerging research in this area is beginning to highlight roles for environmental interaction with genetic risk, while raising the possibility that PRS may in part be predisposing to other schizophrenia-associated risk factors (e.g., obstetric complications) rather than the condition itself (17, 78). This may suggest that research should target a better understanding of these risk mechanisms, by which environmental stressors modulate biological pathways to explain observed alterations in schizophrenia.

EARLY LIFE STRESS AND SCHIZOPHRENIA RISK

Polygenic risk scores in schizophrenia point toward a potential for genetic risk to be mediated through environmental stress (17, 78). This section examines the evidence for one of the most significant and preventable risks for psychosis and schizophrenia (18, 19), the experience of early life stress and trauma, the mechanisms through which this may increase risk, and potential treatment options.

The experience of trauma can be defined as a highly stressful event that involves serious threat to one's physical wellbeing and to one's sense of self, as well as overwhelming one's capacity for coping (79). The Diagnostic and Statistical Manual of Mental Disorders 5th Edition (DSM-V) defines a traumatic event as involving actual or perceived threat of death, serious injury, or violence (80). Such experiences are likely to involve intense feelings of fear and helplessness (81), though these criteria are no longer required to define a traumatic experience in the DSM-V. Further, traumatic experiences can be encountered in a number of ways, including direct personal exposure to trauma, witnessing another person's experience of trauma, or in indirect ways such as coping with the trauma experience of a close associate, or repeatedly being exposed to the consequences of traumatic events in a professional role (such as emergency responders, or investigators of violent crime).

It is important to note that the studies covered in this review largely consider only experiences of direct personal trauma, and of trauma that is of an interpersonal nature, for the most part involving victimization. This includes experiences of multiple types of abuse (emotional, physical, sexual) and neglect, as well as experiences such as bullying. Other types of trauma experiences, such as natural disasters or sudden, life-threatening illnesses, were not included in this review. Further, the effects of exposure to pre- or peri-natal stressors are not covered in this review, nor are the effects of early stress that may be linked to poverty or other social factors.

Early Life Stress as a Risk Factor for Psychosis and Schizophrenia

Psychologically healthy individuals who report psychosis-like experiences have been found to have higher incidences of childhood trauma (24–26). Early experiences of trauma and adversity are well known risk factors for mental illness, with a World Health Organization (WHO) study of almost 52,000 individuals across 21 countries demonstrating that childhood adversity accounted for 30% of all adult mental health conditions (82). Similarly, a meta-analysis of studies assessing the link between childhood adversity and psychosis demonstrated that 78% of studies tested showed a positive association between childhood adversity and psychosis, and that 33% of the population risk for psychosis could be attributed to experiences of childhood adversity (18). A more recent study of 23,998 individuals examining the population attributable risk of childhood adversity on psychosis demonstrated similar results, finding 31% of psychosis cases to be attributable to early adversity (19).

Individuals at clinical high risk (CHR) for psychosis typically also report higher rates of childhood trauma. A meta-analysis of six studies on ultra-high risk (UHR) populations demonstrated a significant increase in exposure to childhood trauma (83). Loewy et al. further demonstrated that exposure to trauma is typically experienced in early life (before age 12) in clinical high risk populations, and occurs prior to the onset of clinical high risk symptoms (84).

Studies to assess the reliability of retrospective trauma reporting in individuals experiencing psychosis demonstrated that reports of trauma are consistent across time and are not significantly affected by current mental health status (85). Fisher et al. also demonstrated that retrospective trauma reporting was consistent with independent clinical case notes from abuse reports (85). However, a number of studies have also reported that minimization and denial of trauma is common in both case and control populations (86, 87), with minimization and denial of trauma being significantly increased in controls compared to individuals with psychosis in a recent study by Church et al. Despite this, individuals with experiences of psychosis still had significantly higher rates of self-reported childhood trauma (61% of case individuals reporting trauma, compared to 15% of controls) after correcting for minimization and denial scores in this study (87). Indeed, MacDonald et al. have suggested that current estimates of the relationship between childhood trauma and psychiatric conditions may be conservative due to the under-reporting

of trauma experiences. The authors further suggest that future studies investigating childhood trauma should measure minimization and denial scores in order to account for such biases (86).

While specific types of adverse experience have all been shown to increase overall psychosis risk (18), it has further been demonstrated that specific types of adversity show a correlation with specific psychotic symptoms (21, 88). For example, Bentall et al. demonstrated that childhood rape was associated with a significantly increased risk of hearing voices, but did not significantly alter risk of paranoia (21). On the other hand, growing up in institutional care was significantly associated with increased odds of experiencing paranoia, but did not significantly alter risk of voice hearing. Some incidences of trauma were also demonstrated to significantly increase the risk of both voice hearing and paranoia, such as in the case of physical abuse (21).

Furthermore, experience of trauma has been shown to have a cumulative, dose-response effect on psychosis risk (18–21, 89). For example, Shevlin et al. demonstrated that experiencing two or more traumas (in childhood and/or adulthood) was predictive of psychosis, with increased number of traumas also demonstrating an additive effect on risk (20). Similarly, the 2012 meta-analysis by Varese et al. highlighted that nine of the 10 studies that tested for dose response in their analysis demonstrated a positive correlation between increased number of adverse events and increased risk of psychosis (18). This pattern also remained when considering symptoms individually rather than diagnosis. Bentall et al. demonstrated the dose response effect of trauma for risk of paranoia and hearing voices, with a single adverse experience significantly increasing the risk for both, and additional traumatic experiences having an additive effect on risk (21).

A recent meta-analysis of 29 studies into the effect of childhood trauma on symptom severity in individuals with psychosis demonstrated that childhood trauma was significantly associated with increased severity of hallucinations and delusions (90). On the other hand, childhood neglect was significantly associated with increased negative symptoms and hallucinations (90). Total trauma scores, as well as sexual abuse, were significantly associated with increases in hallucinations, while total trauma and sexual abuse (but not neglect) were associated with increased delusions (90). Such work suggests that, as well as predisposing to psychosis, individuals with experiences of childhood trauma are also likely to experience more severe psychotic symptoms.

Stress Sensitivity and Sensitization as a Mechanism Mediating the Association of Trauma and Psychosis

Stress sensitivity is known to be correlated with the incidence of psychotic experiences (30). An individuals' stress response may be genetically influenced, but is also known to be modified by previous experience, in which early life stress can sensitize an individual to future stress (91, 92). Using World Health Survey data across 39 countries and 176,934 individuals, DeVylder et al. ranked individuals based on stress sensitivity into groups from 2 to 10 (least to most stress sensitive). Each increasing rank in stress sensitivity was associated with incremental increases in the prevalence of psychotic experiences, with stress sensitivity significantly increasing the odds of an individual reporting psychotic experiences (30). This association remained unchanged when carrying out the analysis either including or excluding those with a self-reported diagnosis of psychosis, and when separating the analyses for hallucinations and delusions. Such data would suggest that increased stress sensitivity is associated with increased risk of experiencing psychotic symptoms; both in the general population and in those with diagnosed psychotic conditions, and that this relationship is shared throughout many countries and cultures across the world (30).

As well as increasing risk, stress sensitivity has further been associated with the intensity of psychotic experiences in both case and control populations. For example, Reininghaus et al. demonstrated that increased sensitivity to multiple types of stressful situations was significantly associated with intensity of psychotic experiences in FEP individuals, ARMS individuals, and in healthy controls (31). When comparing groups, increased event-, activity-, and area-stress sensitivities were significantly associated with increased intensity of psychotic experiences in FEP individuals compared to controls.

In individuals with experiences of psychosis, exposure to childhood trauma was correlated with increased emotional and psychotic reactivity to small daily life stressors in adulthood, suggesting that early stress can sensitize individuals to future stress in a way that may modulate their risk for psychotic experiences, and may modulate the intensity of such psychotic experiences (32) (**Figure 1**). Supporting this finding, there is a known dose-response relationship between increased childhood adversity and more significant increases in daily life stress sensitivity in adulthood (93). Such work suggests a process of sensitization to stress, in which those exposed to early adversity may be sensitized to react more strongly in future to daily stressors. This sensitization process may then modulate risk for experiencing psychosis, or in individuals already experiencing psychosis, may contribute to increased severity of these experiences.

Cristóbal-Narváez et al. have provided evidence of this mechanism specifically in psychosis-like experiences, in a cohort of non-clinical adolescents (92). In this study, experiences of abuse and neglect were associated with psychotic-like and paranoid experiences, while exposure to bullying was associated only with psychotic-like experiences. Abuse, bullying, and neglect were significantly associated with negative affect. All types of childhood adversity tested were found to be associated with daily life stress reactivity, and adversity of an interpersonal nature (bullying, abuse, neglect, and loss) mediated the psychotic- and paranoid-like response to day-to-day social and situational stressors. In particular, individuals reporting higher exposure to bullying were shown to experience significantly higher levels of paranoia in response to social stress than those with lower rates of bullying (92).

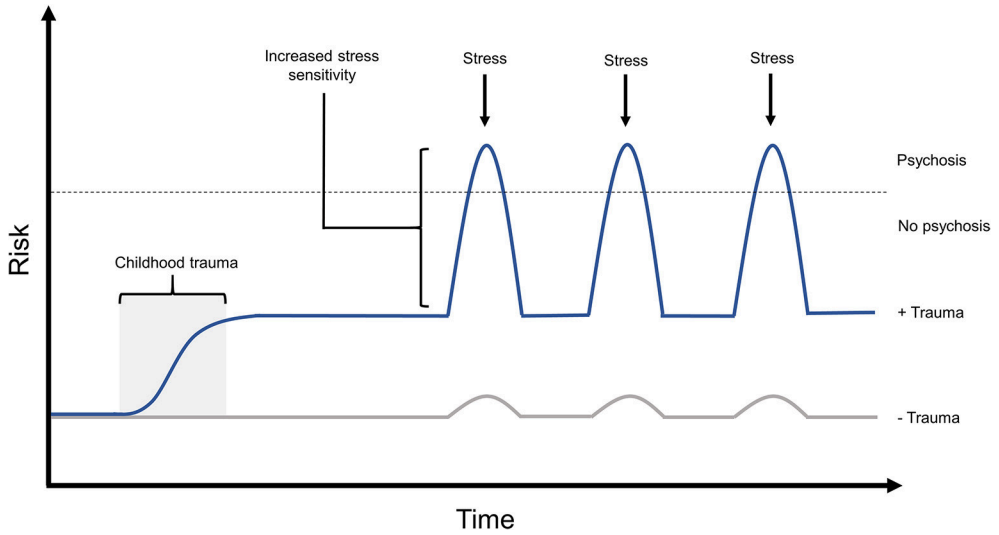

FIGURE 1 | The effects of childhood trauma on stress sensitivity and psychosis risk. Exposure to childhood trauma has been shown to be strongly associated with risk of experiencing psychosis. Such early adversity has also been demonstrated to increase stress sensitivity, both in non-clinical populations and in those experiencing psychosis, which is known to increase both risk and intensity of psychotic experiences. In this model, experience of early trauma is predicted to increase an individual's baseline risk for psychosis, while predisposing them to react more strongly to future stressors. This would further amplify psychosis risk when faced with later stressful experiences during adolescence or adulthood. Risk in this model can also be modulated by the individual's genotype, and allele-specific responses to environmental stressors, the effects of which are not shown in this diagram.

The Effects of Early Trauma on Brain Structure and Function

Childhood adversity was found to be associated with changes in brain structure and connectivity in non-clinical adolescent and adult groups (91, 94, 95), with such physical changes being shown to correlate with mental health-related traits that were likely to sensitize the individual to future life stress (91, 94). The traumagenic neurodevelopmental model of psychosis (**Figure 2**) posits that experiences of early life stress, combined with genetic risk, have the potential to alter the trajectory of brain development or neurochemistry in ways that may put an individual at higher risk of experiencing psychosis in response to future adversity (27, 29).

Studies have demonstrated the association between childhood trauma and alterations in brain structure and connectivity (95, 96). At present, a number of studies have further demonstrated that these trauma-associated structural changes result in increased stress sensitivity in adulthood (91, 94). For example, Gorka et al. demonstrated that childhood adversity was correlated with differences in hippocampal and medial prefrontal cortex gray matter volume in a sample of non-clinical individuals (91). Increasing childhood trauma scores were significantly associated with decreased gray matter volume in the left hippocampus and the medial prefrontal cortex (mPFC), but showed no association with gray matter volume in the right hippocampus or amygdala. Both childhood adversity and decreased gray matter were also correlated with increased anxiety in response to recent life stress. They further demonstrated that the association between decreased hippocampal and mPFC gray matter volume and increased anxiety in response to current stress was significantly mediated by trauma exposure, suggesting that early stress-induced alterations in brain structure can result in increased stress sensitivity in adulthood (91).

Similar studies in adolescents by Herringa et al. have shown that increased childhood maltreatment scores are predictive of lower resting state functional connectivity (rs-FC) between the hippocampus and subgenual cingulate in both male and female adolescents, and of lower rs-FC between the amygdala and subgenual cingulate in female adolescents (94). The effect of childhood maltreatment on the connectivity of these regions remained significant after correction for current life stress. Lower amygdala- and hippocampus-subgenual cingulate connectivity, as well as higher childhood maltreatment scores, was found to be predictive of higher rates of internalizing symptoms (found in depression and anxiety) in adolescents. In both male and female adolescents, experience of childhood maltreatment predicted lower total rs-FC, which in turn modulated the relationship between childhood maltreatment and internalizing symptoms (94). This work provides evidence for the hypothesis that childhood trauma can modulate brain connectivity, resulting in increased levels of internalizing symptoms in a non-clinical adolescent population. Evidence was not found to support reversal of this model (i.e., childhood trauma leads to internalizing symptoms, which results in altered connectivity), thereby suggesting that the effect of trauma on brain connectivity increases internalizing behavior, rather than such behavior affecting brain connectivity.

A recent review on this subject summarizes the evidence suggesting specific effects of different childhood trauma types on situation-relevant brain regions (97), such as alterations in auditory and language processing regions in those who were exposed to repeated verbal abuse (98, 99), and alterations in brain

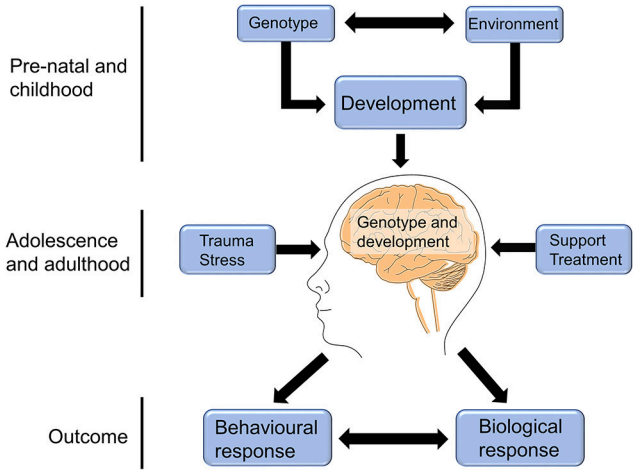

FIGURE 2 | The traumagenic neurodevelopmental model of psychosis. The traumagenic neurodevelopmental model of psychosis suggests that the interaction between an individual's genotype and environment would influence brain development in ways that could modulate psychosis risk in response to future life stress. For example, childhood trauma may negatively impact a child's brain development, resulting in molecular or structural changes that affect their response to adversity later in life. Experiences in adolescence and adulthood can then either result in further increased risk, such as in the case of chronic stress or further trauma experiences, or can reduce the individual's risk, in the case of receiving social support or appropriate treatment. The combination of genotype, brain development, and adolescent/adult life experiences can combine to influence the individual's molecular and biological response, as well as their behavioral response to future stress, which can in turn interact with and impact their risk of experiencing psychosis. This figure is reprinted, with alterations, from Heim et al. (28).

regions associated with processing sensory information from the genitals in those who had experienced sexual trauma (100). While such findings require replication, the authors suggest an evolutionary hypothesis in which these CNS alterations in response to trauma may be viewed as adaptive mechanisms to facilitate survival in a threatening environment (97).

In line with the traumagenic neurodevelopmental model, and adding another layer of complexity to this hypothesis, an individual's genotype has been shown to modulate the effects of childhood trauma on changes in brain structure. For example, Dannlowski et al. have demonstrated the effect of the oxytocin receptor SNP, rs53576, on ventral striatum gray matter volume in individuals with varying levels of childhood trauma (101). Individuals who were homozygous for the G allele of rs53576 showed marked decreases in ventral striatum gray matter volume in response to childhood trauma, compared to A allele carriers, who showed a small increase in gray matter volume at this region in individuals with the highest trauma scores (101).

Similarly, Morey et al. demonstrated a significant interaction between the genotype of the intergenic SNP, rs9373240, and childhood trauma in influencing caudate volume in military service members with experiences of childhood trauma (102). In individuals with no childhood trauma, the homozygous T rs9373240 genotype was not associated with caudate volume. However, in those reporting experiences of early trauma, rs9373240 TT individuals demonstrated significant association with caudate volume in a dose-dependent manner. Those reporting one type of trauma exposure showed significant association of SNP genotype with caudate volume, while those with experiences of two or more types of trauma showed much more significant association of rs9373240 genotype with caudate volume (102). The TT genotype of rs9373240 was significantly associated with increased caudate volume in individuals with experiences of childhood trauma exposure, but was not associated with PTSD diagnosis in this cohort.

Given the above studies, it is clear that early adversity is a substantial and preventable risk factor for psychosis, and that a significant proportion of individuals experiencing psychosis are likely to have adapted both behaviorally and biologically to respond differently to stress as a result of surviving early threatening experiences. Recent research demonstrates that increased stress sensitivity is a mechanism influencing psychosis risk and symptom severity (30, 31), and as such is likely to offer an additional route to intervention. For this reason, interventions such as psychological therapy, which aim to understand the impact of our life experiences on our behavior and current difficulties, and assist the individual in coping with present life stressors, have been gaining traction in the field of psychosis research and treatment. The following section considers the effects of stress and trauma on recovery, and the suitability of psychological interventions for individuals with experiences of psychosis.

TOWARD TRAUMA-INFORMED TREATMENT OPTIONS FOR INDIVIDUALS WITH PSYCHOSIS

Exposure to early life stress is known to influence symptom severity and outcomes with regard to pharmacological treatment with antipsychotic medications. The severity of early life trauma has been shown to be significantly correlated with symptom severity in individuals with psychosis (90). Similarly, those with "treatment resistant" schizophrenia displayed significantly higher levels of stressful life events, and scored more highly on measures of emotional trauma and general trauma, than those that respond to pharmacological treatment (40, 41). There is also suggestive evidence that childhood trauma can affect response to antipsychotic treatment in a genotype dependent manner. For example, multiple SNPs across the matrix metallopeptidase 9 (*MMP9*) gene have been implicated in antipsychotic treatment response, as measured by percentage change in Positive and Negative Symptoms Scale score (PANSS) (103). McGregor et al. found that the PANSS score change associated with SNP alleles in *MMP9* was modulated by exposure to childhood trauma. In particular, homozygotes for the A allele of rs13925 without trauma presented as significant responders to antipsychotic treatment, with a 10.5% reduction in PANSS scores compared to AG or GG individuals. However, individuals with the AA genotype that had experienced early life stress presented as non-responders to treatment, with an increase of 1.67% on the PANSS scale (103). This finding has also been demonstrated in other

mental health conditions, with early trauma exposure predicting poor response to treatment and longer time to recovery in individuals with major depressive disorder (104, 105). As such, current pharmacological treatment for individuals with psychosis may risk leaving traumatized individuals with an untreated and highly stressful environmental component, which is likely to continue to negatively impact both their neurochemistry and recovery. Complicating this matter, the experience of psychosis itself, and coercive elements of treatment such as involuntary hospitalization, have repeatedly been shown to induce trauma symptoms in up to two thirds of individuals experiencing psychosis, resulting in clinical PTSD in one in three such individuals (33–36).

Efforts to implement trauma screening in individuals with psychosis revealed that PTSD is drastically under-diagnosed in such populations (106). In a group of 2,608 individuals with a diagnosed psychotic condition, 0.5% had a previously identified PTSD diagnosis. After implementing trauma screening, this number increased to 16%, a striking 32-fold increase, demonstrating that 96.9% of PTSD cases in individuals with psychosis in this study had previously gone unreported and untreated.

Psychosis and Treatment as Traumatic Experiences

A large proportion of individuals with experiences of psychosis go on to suffer clinical PTSD, or PTSD-like trauma symptoms from this experience, the risk for which is modulated by previous experience of childhood trauma (38). Studies have demonstrated that 66–69% of individuals meet the symptom criteria for PTSD with regard to psychosis- and treatment-related trauma, and 30–39% meet the full criteria for post-psychosis PTSD diagnosis (33–36). Exposure to childhood trauma has been shown to increase risk of experiencing post-psychosis trauma by 27-fold (38). Such work clearly demonstrates that experiences of childhood trauma not only predispose to experiences of psychosis, but are also likely to hinder recovery from psychotic episodes by leaving individuals vulnerable to experiencing post-psychosis trauma (**Figure 3**).

Further work with individuals that had experienced either a single episode, or multiple episodes, of psychosis also yielded similar results (34). In these studies, up to half of first episode and multiple episode individuals met the DSM-IV criteria for having experienced a traumatic event with regard to their experience of psychosis (33, 34). Similarly, almost a third of first episode and over a quarter of multiple episode individuals met the DSM-IV criteria for having experienced trauma in relation to the mental health treatment they received (33, 34).

A study of 395 individuals with a range of psychotic disorders found 69% of individuals reporting that one or more of their hospitalizations had been "traumatic or extremely distressing" (39). Of the individuals in this study reporting treatment-related trauma, the most frequently reported traumatic experiences were involuntary hospitalization (reported by 62%), being placed in physical restraints (40%), and being forcibly medicated (37%). Similarly, 73% of individuals reporting treatment-related trauma also reported feelings of intense helplessness, fear, and horror, with 42% believing that they could be seriously harmed or killed during treatment. Indeed, Paksarian et al. found that 39% of individuals reporting treatment-related distress met the DSM-IV criteria for having experienced a traumatic event (39).

Such studies would advocate for psychological therapy interventions, and particularly trauma-informed interventions, in supporting both those with psychosis influenced by prior trauma, and those in recovery from psychosis who may be prone to ongoing mental health difficulties or at risk of relapse due to lingering trauma symptoms from the experience of psychosis itself, and from any traumatic treatment-related events.

Psychological Therapies for Individuals Experiencing Psychosis

Research into the utility of psychological interventions in psychosis have lagged behind that of other mental health conditions, such as depression and anxiety, likely due in part to the long-held view that psychosis and schizophrenia are largely biological conditions, or that individuals experiencing psychosis may struggle to fully engage with psychological therapy due to difficulties presented by ongoing symptoms (107). However, new research is beginning to demonstrate the safety and utility of psychological therapy for experiences of psychosis, particularly in the case of trauma-informed therapy and adaptations of cognitive behavioral therapy for psychosis.

Eye movement desensitization and reprocessing (EMDR), a form of therapy typically used to treat trauma and PTSD, has been shown to be safe and effective in significantly reducing trauma symptoms in individuals with diagnoses of concurrent psychosis and PTSD (48–50).

An assessment of the potential re-traumatizing effects of psychological intervention for individuals with trauma and psychosis compared symptom exacerbation, re-victimization, and adverse events in a single-blind randomized control trial comparing 108 individuals receiving trauma-focused treatment (TFT) with 47 individuals on a waiting list receiving treatment as usual (TAU) (49). In this study, TAU was administered in the form of multi-disciplinary care from the individual's community mental health team, typically involving antipsychotic medication, with further treatment and supportive counseling from multiple members of this team. In the treatment arm, 53 of the participants received prolonged exposure (PE) therapy, and 55 received EMDR. This treatment was delivered over the course of 10 weeks, in the form of 8 weekly sessions lasting 90 min each. As no significant difference in the outcomes was found between the PE and EMDR groups, the data was pooled to compare trauma-focused treatments vs. the waiting list condition. When comparing baseline to the post-treatment time point, those in the waiting list condition had significantly higher rates of self-reported PTSD symptom exacerbation compared to those receiving TFT, though this difference became non-significant at 6 months follow up. Similarly, those in the waiting list condition had significantly higher levels of general symptom exacerbation, and showed a trend toward reporting increased paranoid ideation post-treatment compared to those receiving TFT. No significant

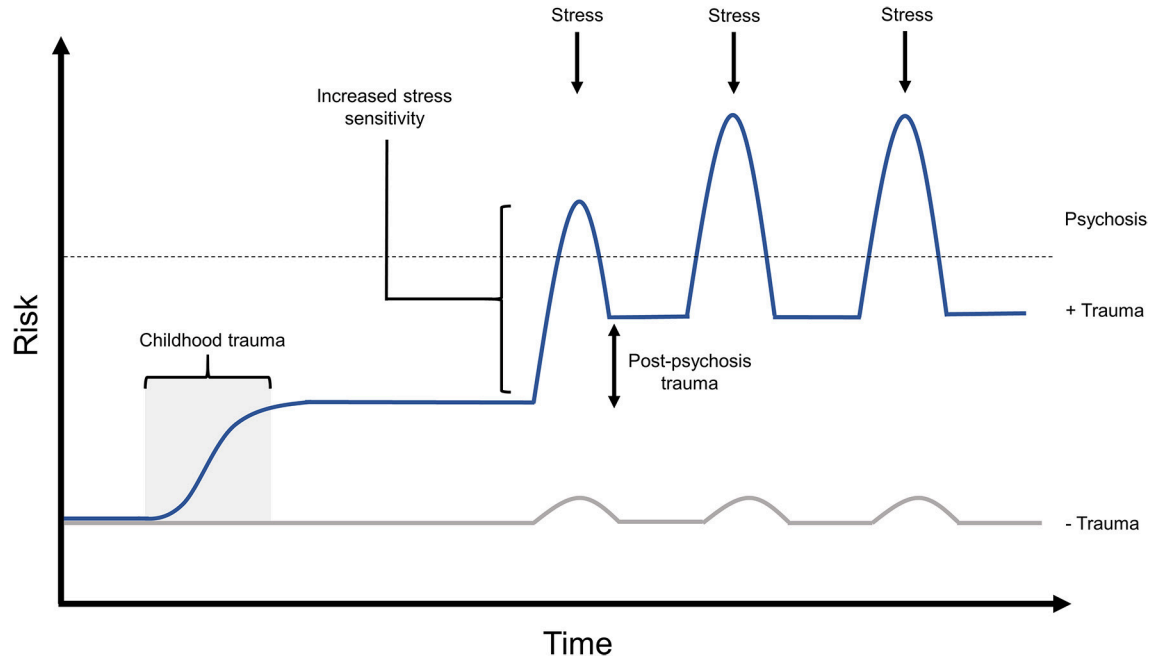

FIGURE 3 | The proposed effects of post-psychosis trauma on relapse risk. Up to two thirds of individuals have been shown to experience post-psychosis trauma, the risk for which is significantly increased by exposure to prior trauma. It is likely that lingering post-psychosis trauma symptoms, if left untreated, would hinder an individual's ability to recover from this experience, and to maintain recovery when faced with later stressors. In this model, childhood trauma would significant raise an individual's baseline psychosis risk, while predisposing them to experience post-psychosis trauma. After an experience of psychosis, in this example triggered by a stressor, the individual's psychosis risk is further amplified by untreated post-psychosis trauma symptoms. This may result in a lower threshold for experiencing further episodes of psychosis in response to future stress, potentially also with the capacity for increased symptom frequency or intensity in later episodes.

difference was found between the groups for clinician rated PTSD symptoms or depressive symptoms at the post-treatment time point. Those in the TFT group were found to be significantly less likely to experience adverse events during treatment and follow up, with a relative risk reduction of 27.9 and 34.6% post-treatment and at 6 months follow up, respectively. Similarly, the rates of re-victimization were significantly lower in the TFT group compared to waiting list at 6 months follow up, but not at the post-treatment time point (49). This work demonstrates that trauma-focused therapy is safe for use with individuals experiencing concurrent psychosis and PTSD, and is unlikely to cause distressing side effects or symptom exacerbation.

In a single-blind randomized control trial, 66 participants with FEP were randomly assigned to receive TAU or cognitive recovery intervention (CRI), a treatment aimed to reduce experiences of post-psychosis trauma (51). At 6 months follow up, those that had received CRI presented with significantly lower levels of post-psychosis trauma, with over two thirds reporting substantial improvement in trauma symptoms, compared to those receiving TAU, of which one third reported improvement. In particular, those reporting higher levels of pre-psychosis trauma benefited most from CRI (51), suggesting that individuals with prior trauma experiences may stand to benefit significantly from trauma-informed support. The authors note that failing to provide early cognitive therapy-based intervention to those experiencing first episode psychosis may put more than twice as many individuals at risk of lingering or worsening trauma symptoms following a psychotic episode (51).

In the UK, psychological therapy (particularly CBT) has been recommended as a frontline treatment for individuals both at risk of experiencing psychosis, and those experiencing psychosis (54). A recent single-blind randomized control trial involving 75 individuals compared the effects of antipsychotic medication alone, CBT alone, and a combination of antipsychotic treatment plus CBT (53). All treatment arms were found to significantly reduce symptoms, with total PANSS scores being significantly decreased in the combined treatment group compared to the CBT group alone. Reduction in PANSS scores was not significantly different between the treatment alone groups, or the antipsychotic and combined treatment groups. Morrison et al. found no difference in adverse events between the three groups, but reported fewer side effects in the CBT alone group (53). Such studies demonstrate the utility and feasibility of CBT as a treatment option for those with experiences of psychosis.

Similarly, an earlier single blind randomized control study in individuals not taking antipsychotic medications compared 74 individuals randomized to TAU or to TAU plus CBT (52). In this instance, TAU referred to regular and comprehensive care co-ordination and psychosocial interventions, with regular monitoring through PANSS assessment, as well as options for crisis management and family interventions. This trial further demonstrated the ability of CBT to significantly reduce

psychiatric symptoms (as measured by PANSS score) in those receiving this intervention, compared to those receiving TAU, with the authors reporting an effect size comparable to those identified in meta-analyses of psychiatric medication efficacy. Change in symptoms included significant improvements in total PANSS score and scores for positive symptoms, as well as improvements in some dimensions of delusional beliefs, voice hearing, and social functioning. However, cognitive therapy intervention was not found to have an effect on negative symptoms in this study (52).

An additional trial involving 385 individuals receiving CBT demonstrated that this intervention was effective in bringing about significant and sustainable improvements in delusional beliefs, voice hearing, anxiety, depression, and quality of life, which were maintained at 6 months follow up compared to pre-therapy baselines (57). Similarly, a meta-analysis of randomized control trials comparing CBT vs. TAU in the treatment of delusions demonstrated that cognitive therapy intervention significantly improved delusional beliefs, an effect which was maintained to an average follow up time of 47 weeks across the studies (56). However, CBT was not found to be significantly different in terms of effectiveness in comparison to other psychological interventions. A further meta-analysis of 12 studies found that CBT resulted in long-term beneficial changes in positive symptoms in individuals whose condition had previously been identified as medication-resistant (55). As well as evidence demonstrating the effectiveness of CBT in improving positive symptoms, a recent meta-analysis by Lutgens et al. also provided evidence for CBT and a number of psychosocial interventions in improving negative symptoms of psychosis (108). Lutgens et al. assessed 95 studies reporting negative symptom outcomes in order to determine the effectiveness of CBT and psychosocial interventions on these symptoms (108). Of the 95 studies, 26 studies assessed the efficacy of CBT. In this analysis, CBT was found to be an effective intervention for the treatment of negative symptoms compared to TAU in 59% of studies. However, in 12 studies that used an active control, no significant difference was found between groups. This would suggest that other active psychosocial interventions may also be beneficial for individuals experiencing negative symptoms of psychosis. Indeed, Lutgens et al. also found evidence to support the utility of skills-, exercise-, and music-based interventions in the treatment of these symptoms (108).

Despite a number of positive meta-analyses such as those above (55, 56, 58, 109), debate around the efficacy of psychological therapy for psychosis continues (110). Some studies have failed to find beneficial effects for CBT in the treatment of psychosis (111), or found only small effects (112), while others have reported variable outcomes which are influenced by other factors, such as the relationship between the client and therapist (113). It is also important to understand the potential barriers that may prevent an individual from accessing or benefiting from such interventions. For example, a recent study by Hazell et al. found that both clients and clinicians felt that ongoing psychotic symptoms might be one of a number of challenges in implementing such interventions, along with additional practical barriers, such as lack of resources within mental health services (107). Further research in this area will be necessary to improve access to, and utility of, psychological therapy for those experiencing psychosis. Additional work by Hazell et al. has suggested that symptom-specific treatment may be one route to overcoming the identified barriers (114).

With regard to randomized control trials, it has been suggested that the variability inherent in tailoring psychological therapy to the needs of each client may make such interventions difficult to assess in a trial setting, leading to overgeneralized criteria that do not accurately assess the efficacy of therapy in achieving the client's individual goals (115). On this subject, some have criticized the use of outcome measurements which are often based on overall symptom scales, with the assertion that the most appropriate uses for CBT are in, for example, targeting specific dimensions of an individual's delusional beliefs, aiming to reduce distress, and aiming to improve the individual's ability to cope with such experiences (116). Birchwood et al. suggested that the intricacies of such changes were unlikely to be captured by generalized symptom measurements, despite the fact that they may represent significant benefits for the client. In support of this, qualitative research into service user priorities identified that managing stress and anxiety were among the most commonly endorsed immediate treatment priorities for those experiencing psychosis, with improved coping and emotional wellbeing identified as top long-term treatment priorities (117). Similarly, research addressing personal experiences of CBT demonstrated that coping strategies were among the most commonly cited key components of CBT for those receiving this intervention for psychosis (118). This would suggest that improved ability to cope with stressors is considered to be among the most useful and valuable benefits by clients, regardless of actual change in symptom frequency or severity. However, measures of such benefits are often not included when assessing the efficacy of therapy in clinical trials.

Despite such debate (the remainder of which is beyond the scope of this review), research is ongoing with the aim of resolving such questions, and further establishing the effectiveness of CBT for individuals with psychosis. Preliminary research is also being carried out to assess the suitability of a number of other therapeutic modalities for use with individuals experiencing psychosis, including Cognitive Analytic Therapy (CAT) (119, 120), Compassion Focused Therapy (CFT) (121, 122), and others, with the hope of providing additional choices with regard to evidence-based treatment options for those seeking psychological therapy-based care.

LIMITATIONS

This work has a number of limitations that are of note. Firstly, this review aimed to cover a very wide range of topics from molecular biology to psychological therapy. Given the breadth of this article, there was insufficient space to present and deeply discuss the full range of supporting and opposing evidence for each topic. It did not systematically review the literature relating to control trials of psychological therapy

for psychosis, nor did it evaluate the quality of evidence in the studies reported. While the authors endeavored to fairly and accurately represent the available literature, this lack of systematic procedure means that the introduction of bias cannot be ruled out. Secondly, this review covers only very specific and extreme experiences of early life stress and trauma. As discussed above, the studies referenced in this review focus almost exclusively on direct personal experiences of victimization, centered largely around interpersonal abuse and violence. It is important in this case to note that additional non- victimization-based, or non-direct experiences, such as sudden and life-threatening illness, or witnessing another person's experience of trauma, may also constitute traumatic experiences and have the potential to contribute to future mental health risk. Similarly, the effects of pre- and peri-natal stressors, such as maternal stress, were not included, nor were additional factors which may contribute to early life stress and psychosis risk, such as low socioeconomic status. While there was not scope in this review to do so, the authors believe it is also of import to acknowledge the intersecting effects of numerous personal and social categorizations (e.g., race, sexuality, gender identity, socioeconomic status) in influencing one's likelihood of experiencing discrimination, disadvantage, or victimization that may result in trauma or repeated exposure to stress, thereby contributing to risk of developing related psychiatric symptoms.

SUMMARY AND CONCLUSIONS

Recent advances in the field of schizophrenia genetics have demonstrated the highly polygenic nature of this condition, with many common risk variants of low effect size spread widely across the non-coding genome (4, 13). That a significant number of identified risk variants fall within proposed regulatory regions (12, 15) would suggest that risk for schizophrenia or psychosis is likely to be modulated by environmental stressors in a genotype-dependent manner. Indeed, a significant number of schizophrenia GWAS SNPs have been shown to act as eQTLs or meQTLs, influencing gene expression or methylation in the human brain (69–71).

Childhood adversity is one of the largest preventable risk factors for experiencing psychosis (18, 19), and has been shown to significantly alter methylation across key stress response gene loci in humans (42–44). Exposure to childhood trauma has also been associated with alterations in brain structure and connectivity (91, 94, 95), with evidence to suggest that the effects of childhood trauma on brain structure can be influenced by genotype (101,

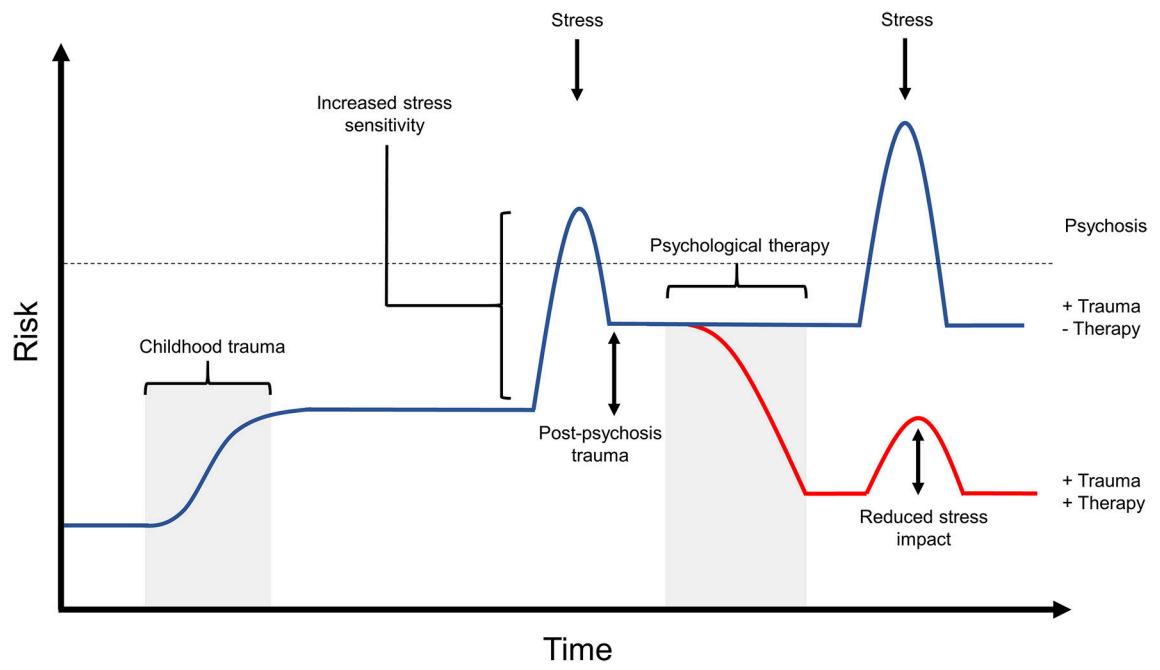

FIGURE 4 | The potential effects of psychological therapy on post-psychosis trauma and stress impact. Recent research into psychological therapy for psychosis is beginning to demonstrate the ability of such interventions to improve both psychosis symptoms as well as secondary issues in those experiencing psychosis, such as anxiety and depression. Similarly, psychological therapy is able to reduce distress associated with psychosis symptoms, and improves the individual's ability to cope with stressors. This diagram outlines one potential risk trajectory that may be associated with effective psychological therapy intervention. In this example, the individual has experienced early life trauma and post-psychosis trauma, which resulted in very high risk for experiencing relapse in response to further stress. If this individual receives successful therapy after their first psychotic episode, this could decrease risk of further episodes by working to resolve both early and recent trauma experiences, and by providing the individual with an improved ability to cope with stress. As can be seen in this figure, subsequent stress following therapy may have a smaller effect on risk due to the individual's improved capacity for coping. While not shown here, psychological therapy intervention may also be provided as a preventative measure to those at high risk for psychosis. Such intervention would similarly aim to improve coping and reduce the effect of stress, with the hope of preventing a psychotic episode in the future.

102). Such alterations in brain structure have been shown to be associated with increased stress reactivity (91), high levels of which is a known risk factor for psychosis (30), and has been shown to be associated with increased symptom severity in those experiencing psychosis or psychosis-like symptoms (32, 92). This traumagenic neurodevelopmental model of psychosis suggests that exposure to early life trauma can alter the trajectories of neurodevelopment and neurochemistry to influence response to future life stress in ways that would increase risk of experiencing psychosis (27, 29), and increase symptom severity in those with this condition (**Figure 1**). Given that over two thirds of people experience post-psychosis trauma symptoms (33, 34)—the likelihood of which is vastly increased by prior trauma exposure (38)—it is likely that lingering trauma symptoms and heightened stress sensitivity (32) will negatively impact the individual's ability to achieve or maintain recovery in the face of day-to-day or future stressors (**Figure 3**). Treating only with pharmacological interventions is unlikely to resolve such distress, or equip the individual with useful coping strategies, and as such, repeated experiences of stress will continue to act on the individual's mental health in a G × E manner.

Thinking in this G × E way about psychosis would advocate for the role of psychological therapies or other social support as ways to help a person cope with or resolve the stress in their life, that may be preventing them from achieving or maintaining recovery (**Figure 4**). Recent studies into the utility and effectiveness of psychological therapy for psychosis have demonstrated that trauma-informed therapies are safe and effective for use with such clients (48–50), and can reduce rates of re-victimization and post-psychosis trauma (49, 51). Similarly, meta-analyses assessing the effectiveness of CBT for psychosis have demonstrated that such interventions can provide sustainable improvements in positive symptoms (56, 58, 109), including in individuals that had previously been classified as "treatment resistant" (55).

A wealth of research, both biological and psychological, has demonstrated the effects of stressful life experiences on our epigenetics and subsequent risk of developing a mental health condition, though research into the epigenetic effects of positive experiences and their effect on mental health are less studied. Given the genetic data showing that common schizophrenia risk falls largely in regulatory regions which are likely to be responsive to environmental challenge or change, support that positively affects an individual's day-to-day environment or stress levels may be a feasible route to positive and therapeutic epigenetic change. Indeed, preliminary studies into the biological effects of therapy have demonstrated that psychological interventions can modulate biological mechanisms relevant to mental health, such as altering methylation over key CNS genes (45–47, 123), and modulating connectivity between brain regions that are relevant to psychosis in ways which correlate with clinical improvement in symptoms (124, 125). While further research is needed to address a number of questions and criticisms around the use of psychological therapy for psychosis, such interventions provide hope, particularly for those who find antipsychotic treatment unhelpful or intolerable, and for those with experiences of trauma, the ongoing effects of which are unlikely to have been adequately appreciated or addressed in regard to their mental health.

AUTHOR CONTRIBUTIONS

OG wrote the draft manuscript. VB and JQ provided feedback for revisions. All authors contributed to and approved the final manuscript.

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Long-Term NMDAR Antagonism Correlates Weight Loss With Less Eating

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Memantine hydrochloride is an uncompetitive N-methyl-D-aspartate (NMDA) antagonist for treatment of moderate-to-severe Alzheimer's disease. Several studies have shown that memantine can significantly correct the binge-like eating behavior in human and animal models. People with overeating behavior are more likely to be obese. Therefore, we suppose that memantine would be a good candidate for the treatment of obesity. In this study, memantine was shown to increase weight loss in obese mice induced by high fat diet. Memantine was shown to decrease food intake without inducing abdominal discomfort and anxiety, suggesting that this compound would be a good candidate drug for obesity control.

Keywords: memantine, obesity, weight loss, high fat food, NMDA (N-methy-D-aspartate receptor)

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INTRODUCTION

According to WHO 2018, worldwide obesity has nearly tripled since 1975 (http://www.who.int/mediacentre/factsheets/fs311/en/). Orlistat was the only available weight-loss medicine since 1999. Recently, four new anti-obesity drugs (lorcaserin, phentermine/topiramate, altrexone/bupropion and liraglutide 3.0 mg) for long-term use were approved in the USA. The new drugs were clinically effective, but their high price and risk of adverse effects shouldn't be ignored (1). Thus, new candidate drugs are still urgently needed.

Memantine hydrochloride, an uncompetitive N-methyl-D-aspartate receptor (NMDAR) antagonist, was approved in Europe (in 2002, marketed under the product names Ebixa and Axura) and the US (in 2003, marketed as Namenda) for moderate-to-severe Alzheimer's disease (AD) (2). Memantine is not only used for neurodegenerative diseases, but also for some neuropsychiatric syndromes, like binge eating disorder. Several studies have shown that memantine can significantly correct the binge-like eating behavior in human and animal models (3–6). As we know, our eating behavior can decide our whole day caloric intake. Eating behavior plays important role in obesity by modulating hormones such as letpin and ghrelin, which are related to BMI and body fat (7, 8). The imbalance of leptin and ghrelin affects the brain rewards system and promotes overeating (7, 8). People with overeating behavior are more likely to be obese (9–12). Therefore, we suppose that memantine would be a good candidate for the treatment of obesity. Further studies on whether and how memantine increases weight loss are needed.

The present study aimed to investigate the effects of memantine on weight loss. By taking advantage of the obesity mouse model, we firstly explored whether long-term NMDAR antagonism by memantine could systemically increase weight loss, and then tried to explain potential mechanisms.

MATERIALS AND METHODS

Animals

Male C57BL/6J mice were housed in standard cages (48 cm × 26 cm), with controlled temperature (22°C) and a 12 h light/12 h dark cycle. There were four mice in each cage. All procedures were carried out in accordance with the guidelines for the Care and Use of Laboratory Animals of Shanghai Jiao Tong University School of Medicine and approved by the Institutional Animal Care and Use Committee [Department of Laboratory Animal Science (DLAS), Shanghai Jiao Tong University School of Medicine] (Policy Number DLAS-MP-ANIM.01–05).

Long-Term NMDAR Antagonism by Memantine on Obesity

Six-week old C57BL/6J male mice were fed with high fat food (HF group) and standard control food (Ctrl group) for 5 months. The procedure was carried out as illustrated in **Figure 1A**. The high fat food (HF) contained a total of 45% kcal from fat (D12451, Research Diets, Inc., USA). At the end of the 5 months, the weight of mice in both groups was recorded. Then obese mice were divided into three groups, which were given saline, 5 and 20 mg/kg memantine, respectively. Memantine (Sigma, USA) dissolved in 0.9% saline was injected intraperitoneally for 17 days. During these days, the obese mice were continually fed with HF food. In order to investigate the effects of memantine on C57 mice fed with standard control food, memantine with different doses was also injected intraperitoneally. The body weight during the 17 days was recorded daily.

Food Intake Test

Eight-week old C57BL/6J male mice were made to fast for 24 h as previously described (13). The procedure was carried out as illustrated in **Figure 2A**. Then they were habituated to the test box (mouse cage with new material) for 20 min. After habituation, saline and memantine (5 and 20 mg/kg) were intraperitoneally injected in the home cage. Thirty minute later, standard food was presented in the test box and the food that was consumed during the next 20 min was recorded.

Conditioned Taste Aversion (CTA) Test

The CTA tests were performed as described previously with some modifications (14). The procedure was carried out as illustrated in **Figure 2D**. During the 1-week adaptation, 8-week old C57BL/6J male mice drank water once a day from two bottles (from 9:00 to 9:30 a.m.), but had free access to the standard control food. Water intake was recorded for each mouse by weighing both bottles before and after drinking time. Following the adaptation, each mouse was allowed to drink two bottles of 0.5% sodium saccharin solution (0.5% w/v) (Sigma-Aldrich) during the 30 min drinking time. Forty minute after drinking time, mice were given an intraperitoneal injection of saline, LiCl (0.15 M, Sigma-Aldrich), and memantine (5 and 20 mg/kg), respectively. On the day of the test, one bottle of 0.5% sodium saccharin solution and one bottle of water were inserted into each cage simultaneously. Fluid consumption was determined by weighting both bottles before and after drinking time. Aversion index (in %) = water intake (in grams) × 100%/[sodium saccharin intake (in grams) + water intake (in grams)].

Open Field Test

The test was carried out as previously described (15), in a square plexiglass apparatus (40 × 40 × 40 cm). A digital camera was set above the apparatus. Trace was recorded by the Ethovision video tracking system (Noldus Information Technology, Wageningen, Netherlands). Thirty minute before the test, 9-week old C57BL/6J male mice were intraperitoneally injected with saline and memantine (5 and 20 mg/kg). The mice were then gently placed in the apparatus and were left free to explore for another 60 min. After each trial, the apparatus was cleaned with 75% ethanol. In another 30 min test recorded by Tru Scan system (CoulBourn Instrument, USA), Cholecystokinin (CCK, 30 μg/kg, Tocris Bioscience, USA) and LiCl (150 mg/kg, Sigma-Aldrich, USA) were dissolved in saline and injected intraperitoneally 30 min before test.

Elevated Plus Maze Test

The protocols were followed as previously described (15). The black plastic elevated plus maze consisted of four 30 cm × 5 cm arms (two open without walls and two enclosed by 15.25 cm high walls). The maze was elevated 40 cm above the floor. Activity was recorded with a digital camera suspended from the ceiling. The test took place during the light phase. On the test day, mice were placed individually in the center of the maze facing the enclosed arms, and recorded for 5 min by the Ethovision tracking system (Noldus Information Technology, Wageningen, Netherlands). The maze was cleaned with 75% alcohol between trials. The time spent in the four arms was analyzed.

Statistical Analysis

Values were expressed as the mean ±S.E.M. Groups were compared using Student' s *t*-test or ANOVA. $P < 0.05$ was considered to be statistically significant.

RESULTS

Memantine Increased the Weight Loss of Obese Mice

After 5 months, the weight of the HF food diet group was significantly larger than that of the Ctrl food diet group (**Figure 1B**). To investigate the effects of memantine on obesity, memantine was administered intraperitoneally. The percentage of body weight during the 17 memantine injection days compared to the original body weight was analyzed. Compared to the saline group mice, the percentage of body weight in mice fed with HF food diet and administered with 20 mg/kg memantine showed a significant decrease during the memantine injection days (**Figure 1C**). Meanwhile, the percentage of body weight for

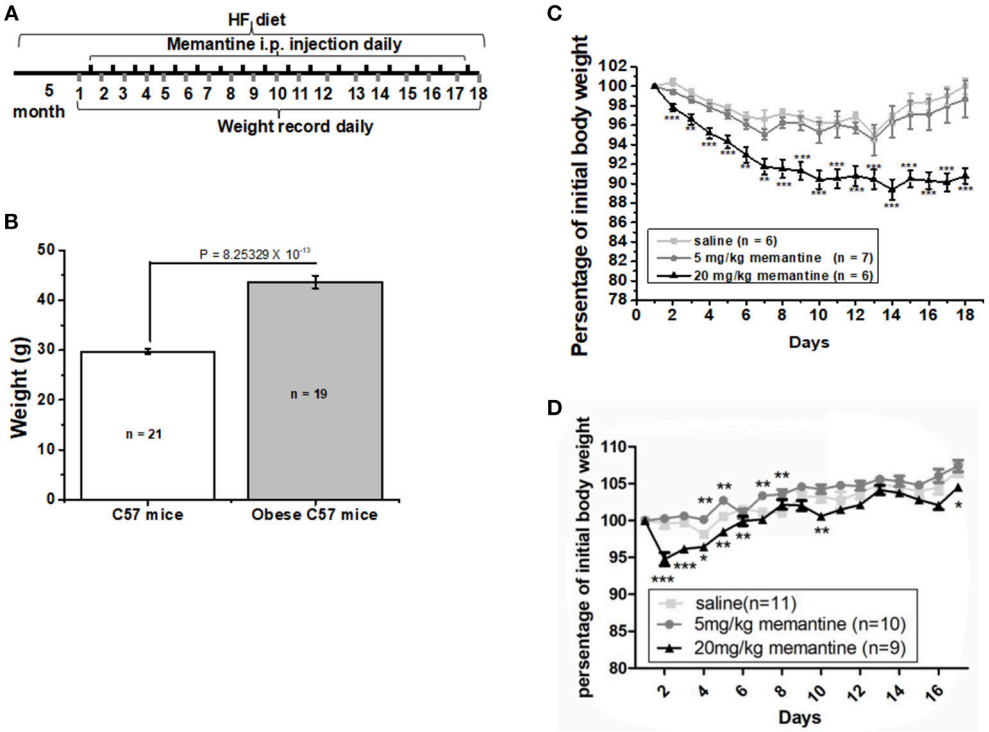

FIGURE 1 | Long-term NMDAR antagonist treatment by memantine decreased the weight of obese mice. **(A)** Schematic representation for memantine injection and weight recording. **(B)** The weight of HF food diet group mice was significantly larger than that of Ctrl food diet group mice (unpaired Student's *t*-test, $p = 8.25329 \times 10^{-13}$). **(C)** Memantine significantly decreased the percentage of body weight to original body weight during memantine injection days [Day, $F_{(17, 342)} = 5.577$, $P = 0.000$; Group, $F_{(2, 342)} = 107.155$, $P = 0.000$; Day*Group, $F_{(51, 342)} = 1.375$, $P = 0.058$, two way ANOVA; from the second day to the end, all $P < 0.001$ except the P_3 day $= 0.0028$; P_6 day $= 0.0023$; P_7 day $= 0.0027$, unpaired Student's *t*-test; **(D)** The percentage of body weight in mice fed with standard control food diet and administered with 20 mg/kg memantine showed a significant decrease during the first memantine injection days but did not differ in late injection days (Day, $F_{(15, 480)} = 33.133$, $P = 0.000$; Group, $F_{(3, 480)} = 73.964$, $P = 0.000$; Day*Group, $F_{(51, 342)} = 1.429$, $P = 0.041$, two way ANOVA). $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$].

5 mg/kg memantine group mice was similar to that of the saline group mice (**Figure 1C**). Compared to the saline group mice, the percentage of body weight in mice fed with standard control food diet and administered with 20 mg/kg memantine showed a significant decrease during the early memantine injection days but did not differ in later injection days (**Figure 1D**). By contrast, the percentage of body weight in mice administered with 5 mg/kg memantine injection was similar with that of saline group mice, while higher in some days (**Figure 1D**). These data showed the potential of memantine on obesity control.

Memantine Decreased Food Intake

In order to investigate whether memantine decreased the weight of obese mice by decreasing food intake, mice that fasted for 24 h were intraperitoneally injected with saline and memantine (5 and 20 mg/kg). The 5 mg/kg memantine group showed similar Ctrl food intake to the saline group (**Figure 2B**). However, the 20 mg/kg memantine group significantly reduced the Ctrl food intake compared to the saline group (**Figure 2C**). In order to find out whether memantine leads to severe side effect, like abdominal discomfort, CTA model was used. Compared to the saline group, the LiCl, and memantine groups saw a significant decrease in the intake of sodium saccharin solution (**Figure 2E**).

Memantine Increased Locomotor Activity Without Severe Side Effect

Open field test was performed to clarify whether memantine decreased food intake due to abdominal discomfort. Both 5 and 20 mg/kg memantine mice groups showed significantly increased locomotor activity compared to the saline mice group (**Figure 3A**). Memantine wasn't found to induce anxiety because mice injected with memantine spent more time in the center of the open field than the control group mice (**Figure 3B**). Besides, in the elevated plus maze test, mice injected with memantine spent similar time in the open arms compared to control group mice (**Figure 3C**). In order to explore the behavior under satiation and abdominal discomfort condition, CCK and LiCl were used. LiCl group mice covered significant less distance during the time in open field (**Figure 3D**), while CCK group mice showed a behavior similar to the saline group mice. These results suggest that no abdominal discomfort and no anxiety are induced by memantine.

DISCUSSION

Our results show that long term NMDAR antagonism by memantine significantly decreased the weight of obese mice. Our

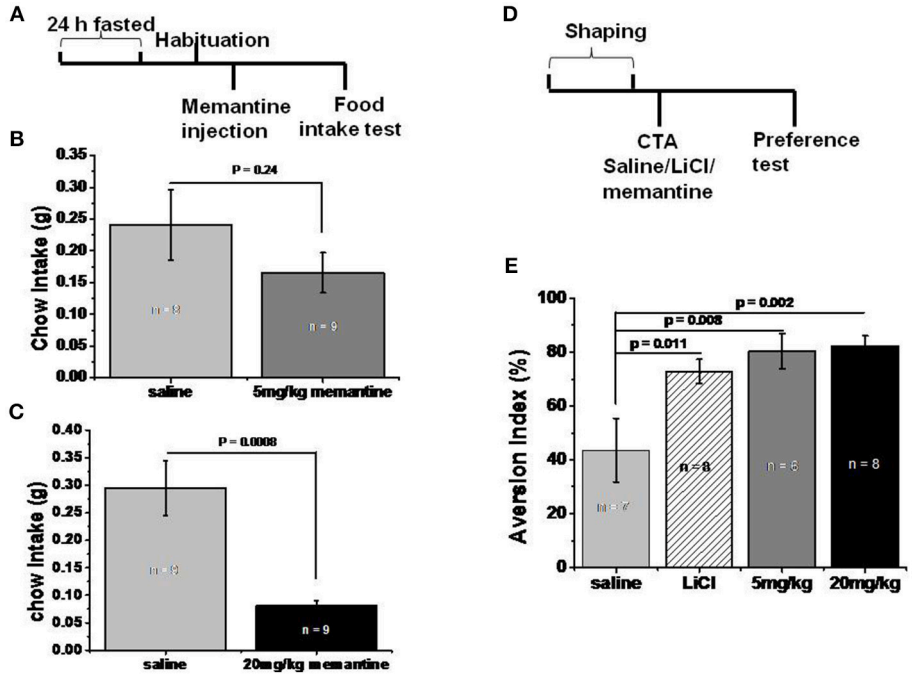

FIGURE 2 | Memantine decreased food intake. **(A)** Schematic representation of standard food intake test protocol by memantine. **(B)** 5 mg/kg memantine group showed similar Ctrl food intake with saline group (unpaired Student's *t*-test, $P = 0.241$). **(C)** 20 mg/kg memantine group significantly reduced the Ctrl food intake compared to saline group (unpaired Student's *t*-test, $P = 0.0008$). **(D)** Schematic representation of CTA behavioral protocol. **(E)** LiCl group and memantine group decreased the intake of sodium saccharin solution [group, $F_{(3, 28)} = 4.707$, $P = 0.01$, one way ANOVA], *post-hoc* analysis revealed LiCl, 5 and 20 mg/kg memantine group increased the aversion index to 72.88% ($P = 0.011$), 74.43% ($P = 0.008$), and 82.29% ($P = 0.002$) respectively, compared to 43.63% of saline group.

results are in accordance with clinical reports. By using an on-off-on design, Schaefer et al. found that memantine discontinuation and re-exposition were followed by a significant weight increase and a substantial weight loss (16). In a therapeutic trial in five obese women, Hermanussen et al. found that memantine could significantly suppress the appetite and binge-eating disorder and finally decrease the body weight within a few days (5). In our results, compared to the saline group mice, the percentage of body weight in mice fed with standard control food diet and administered with 20 mg/kg memantine showed a significant decrease during the early memantine injection days but was similar in later injection days. Meanwhile, the percentage of body weight in mice administered with 5 mg/kg memantine injection was similar with that of saline group mice, while higher in some days. There are few reports about whether memantine affects the weight of people with healthy weight. Under standard institutionalized diet, Venturelli et al. found that BMI decreased significantly in Alzheimer's Disease (AD), while in CTRL it remained unchanged with similar levels of daily energy expenditure. The combination of three factors, number of medications taken, albuminemia, and cortisolism, predicted ΔBMI in Woman with AD (17). Several studies have reported NMDAR signaling in the regulation of appetite (18–22). NMDAR signaling regulates food intake at several appetite-suppressing nodes, including the solitary tract nucleus (23–25), the parabrachial nucleus (26, 27), the ventromedial nucleus of the hypothalamus and the paraventricular nucleus of the hypothalamus (22, 28), and the lateral habenula (29). In another study, the central amygdala (CeA) region was shown to play an important role in appetite regulation (13). Further research needs to be carried out to elucidate which brain areas are involved in the mechanism of memantine on obesity. Because of the important role of peptides (like leptin and ghrelin) in appetite related brain areas like hypothalamus, the expression of these peptides in brain may change.

Our results showed that memantine decreased the weight of obese mice by suppression of food intake. In the CTA model, memantine had similar effects to LiCl. Traverso et al. reported that MK-801, another NMDAR antagonist, induced low intensity conditioned taste aversion (30). MK-801 was reported to virtually block all NMDAR activity and manifested unacceptable side effects (31). Differently, memantine preferentially blocks excessive (pathological/ extrasynaptic) NMDAR activity and its activity remains mostly normal (physiological/synaptic) due to an uncompetitive mechanism of action in conjunction with a relatively fast off-rate, resulting in a low affinity for the NMDAR (31). Combined with our findings that memantine decreased food intake in CTA model, we suppose that the mechanism of memantine that suppresses food intake may be different from that of MK-801.

In the CTA model, our results showed that memantine had similar effect to LiCl. As we know, LiCl can induce abdominal

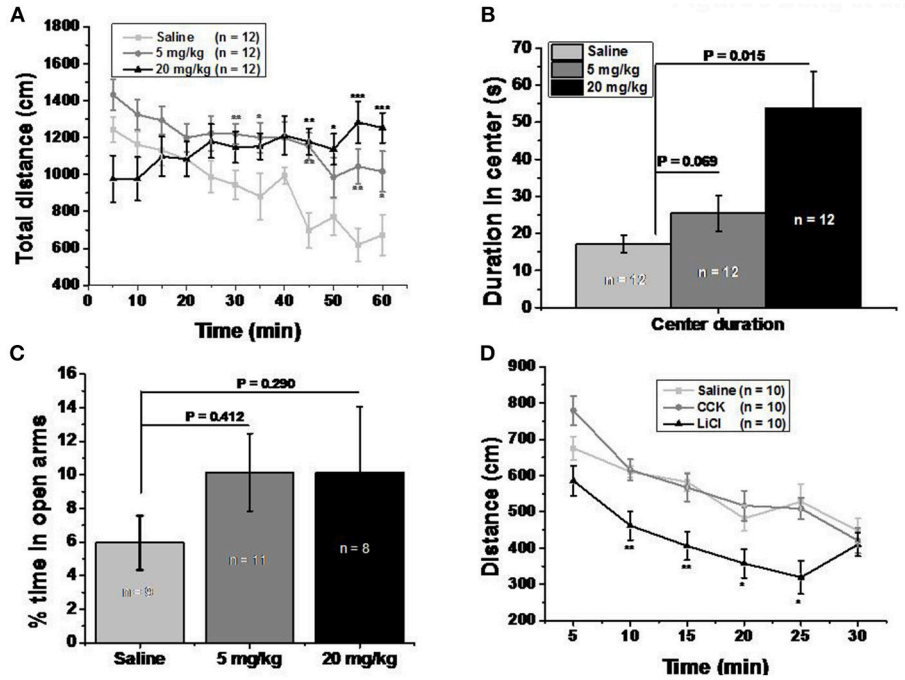

FIGURE 3 | Memantine increased locomotor activity without severe side effect. **(A)** Both 5 and 20 mg/kg memantine group increased the distance during the late period of open field test. [Group, $F_{(2, 360)} = 26.906$, $P = 0.000$; Time, $F_{(11, 360)} = 2.543$, $P = 0.004$; Group*Time, $F_{(22, 360)} = 2.810$, $P = 0.000$, two way ANOVA; saline group and 5 mg/kg memantine group, $P_{30} = 0.009$, $P_{35} = 0.047$, $P_{45} = 0.001$, $P_{55} = 0.004$, $P_{60} = 0.042$; saline group and 20 mg/kg memantine group, $P_{55} = 0.001$, $P_{50} = 0.02$, $P_{55} = 0.0004$, $P_{60} = 0.0008$, unpaired Student's *t*-test; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$]. **(B)** Mice injected with memantine spent more time in the center of open field than that of control group mice [group, $F_{(2, 29)} = 8.652$, $P = 0.001$, one way ANOVA; *post-hoc* analysis showed that saline group and 5 mg/kg memantine group, $P = 0.069$; saline group and 20 mg/kg memantine group, $P = 0.015$]. **(C)** Mice injected with memantine spent similar time in open arms with that of saline group mice [group, $F_{(2, 27)} = 0.64$, $p = 0.536$, one way ANOVA; *post-hoc* analysis saline group and 5 mg/kg memantine group, $P = 0.412$; saline group and 20 mg/kg memantine group, $P = 0.290$]. **(D)** LiCl group mice showed significant less distance during the time in open field [group, $F_{(2,180)} = 27.270$, $P = 0.000$; time, $F_{(5, 180)} = 20.181$, $P = 0.000$; group*time, $F_{(10,180)} = 0.316$; two way ANOVA; saline and LiCl group, $P_{10} = 0.002$, $P_{15} = 0.002$, $P_{20} = 0.039$, $P_{25} = 0.012$, unpaired Student's *t*-test; **(D)**].

discomfort. If memantine did induce abdominal discomfort, it would suppress the locomotor activity of mice in open field test. Interestingly, our results showed that LiCl group mice had less locomotor activity than saline group mice, while memantine didn't suppress but increased the locomotor activity in open field test. And memantine wasn't shown to cause anxiety. There are many studies about the effects of memantine on locomotion. When the time periods of open field test differ, the results can be different. In the 5 min open field test, the total distance traveled by rodents with and without memantine injection is similar (32). During the first 60 min open field test, Costa et al. found that the total distance increased following the increase of memantine doses (33). In the last 5 min of the 30 min open field test, Kotermanski et al. found that the total distance traveled by rats was increased following the increase of memantine doses (34). The duration of the period spent in the center of the open field could reflect the anxiety level in open field test, and the results among different studies differed. In the 5 min open field test, Camarasa et al. found that memantine-treated rats spent longer time in the center and shorter time in the periphery (35). When analyzed with different parameters or when the method of memantine intake differed, the anxiety level in the open field could differ (35, 36). Our results in plus maze showed that the percentage of duration in open arms (compared to duration in total arms) among different memantine groups was similar.

Foltin et al. reported that memantine decreased the food intake by enhancing the satiation (37). In our results, unlike LiCl suppressed locomotor activity, CCK group mice performed like saline group mice in open field test. We hypothesize that the decreased food intake and increased weight loss caused by memantine might be due to satiation.

Our results showed that memantine increased the locomotor activity in open field. It has been well known that exercise can improve health. In an elegantly designed study, Ross et al. (38) reported that the diet-induced and exercise-induced weight loss groups showed approximately 8% weight reduction, and had significant reductions in total fat mass, visceral fat and increased glucose disposal. However, when compared to the diet induced weight loss group, exercise training induced weight loss group had a greater reduction in total fat mass (39). In the sixth century B.C., Susruta advocated exercise as a treatment for diabetes (40). Muscle contractions and exercise increase energy consumption, glucose uptake (41, 42) and sensitivity of muscle to insulin (43). Adipose tissue and liver are also targeted by exercise. Adipose tissue is an active endocrine organ (44) that is dramatically influenced by exercise (45). Similarly, the liver helps

mediating the beneficial effects of exercise (46). So, increased locomotor activity by memantine might lead to improvements in glucose homeostasis and decreased markers of liver damage in obese mice. Further studies are needed. However, Zimmer et al. found that long-term administration of memantine could induce anxiety-like behavior (47).

CONCLUSION

Long term NMDAR antagonism by memantine increases weight loss in mice obesity induced by high fat diet. Memantine decreases food intake without inducing abdominal discomfort and anxiety, suggesting that this compound would be a good candidate drug for obesity control. However, the molecular mechanism and brain circuit involved in the regulation of weight loss by memantine need further study.

AUTHOR CONTRIBUTIONS

W-GL and FL designed the study and modified the manuscript. S-ND conducted the study and prepared the manuscript. Y-HY helped conduct the weight loss experiments and modify the manuscript. T-LZ and B-KM helped perform food intake experiments. H-RF and Y-ML helped finish open field tests and plus maze test.

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Brain mGluR5 in Shank3B$^{-/-}$ Mice Studied With *in vivo* [^{18}F]FPEB PET Imaging and *ex vivo* Immunoblotting

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Although several studies have found that metabotropic glutamate 5 receptor (mGluR5) may play an important role in autism spectrum disorders (ASD), the mechanisms remain unclear. Here, we used a Shank3 gene complete knockout mouse model (Shank3B$^{-/-}$) to explore the change in mGluR5 in the brain. To assess whether deletion of Shank3 in mice results in ASD-like behavior, we conducted a battery of behavioral experiments to characterize Shank3B$^{-/-}$ mice, including repetitive grooming behavior tests, three-chamber tests and resident-intruder tests. Wild-type C57/BL6 and Shank3B$^{-/-}$ mice underwent PET scans with [^{18}F]FPEB, which was highly specific to mGluR5. Mouse brains were extracted post-scan, and mGluR5 protein levels were assessed by immunoblotting. The binding potential (BPnd) of mGluR5 was rich in the hippocampus, thalamus, striatum, and amygdala. More importantly, Shank3B$^{-/-}$ mice showed significantly increased BPnd compared to the control mice in these brain regions. Immunoblotting revealed elevated mGluR5 levels in the hippocampus, thalamus, and amygdala but not in the striatum compared with control mice. These findings indicated that [^{18}F]FPEB could visualize mGluR5 in the mouse brain. The deficiency of Shank3 can impair mGluR5 expression in multiple brain regions. Future work is also needed to understand the reasons for different results between *in vivo* PET and *ex vivo* immunoblotting.

Keywords: autism spectrum disorders, Shank3, PET, mGluR5, immunoblotting

INTRODUCTION

Previous studies have found that metabotropic glutamate 5 receptor (mGluR5) may play an important role in autism spectrum disorders (ASDs). However, the mechanisms remain poorly understood. Genetic defects of SHANK3 (PROSAP2) are one of the most replicated findings in autism genetics (1, 2). Because mouse models can provide unique insights into the mechanisms underlying ASD, numerous lines of Shank3 isoform-specific mutant mice with deletions of different exons or point mutations have been reported (3–9). These studies have consistently demonstrated that the deletion of Shank3 in mice resulted in abnormal behaviors relevant to ASD. Among them, Shank3B$^{-/-}$ mice showed obvious repetitive behaviors and social interaction deficits (4). Therefore, Shank3B$^{-/-}$ mice were used as ASD animal models in the present study.

Shank proteins, which are composed of five protein-protein interaction domains, interact with more than 30 synaptic proteins, including cell adhesion proteins, cytoskeletal proteins, and ionotropic and metabotropic glutamate receptors (mGluRs) (10, 11). It is worth noting that the alteration of mGluR5 gene expression and function has been identified as a risk factor for ASD (12, 13). It has been reported that *in vitro* mGluR5 expression and function would be strongly affected when the expression level of Shank3 was downregulated (14). In addition, *in vivo* Shank3 deletion can impair mGluR5 functions (9, 10). To study the role of this protein further, we conducted *in vivo* positron emission tomography (PET) studies of mGluR5 binding using 3-18F-fluoro-5-(2-pyridinylethynyl)benzonitrile) ([^{18}F]FPEB) in Shank3 knockout (KO) and control mice. [^{18}F]FPEB is safe, well tolerated, and suitable for quantifying mGluR5 in humans (15–17). Since the results of PET might be inconsistent with the results of semiquantitative experiments *in vitro* (18, 19), we also performed *ex vivo* immunoblotting to further verify the characteristics of mGluR5 expression in Shank3 KO mice.

METHODS

Animals

In the present study, we used Shank3B$^{-/-}$ mice as ASD mouse models, which were obtained from Prof. Guoping Feng (4). Shank3B$^{-/-}$ mice and their wild-type control littermates were obtained by breeding heterozygotes with a C57BL/6J background. The animals were kept in a temperature-controlled room (22–26°C) under a 12-h light/dark cycle with free access to food and water. To acquire accurate results, animals were only used once in each test. All tests were conducted from 4 to 10 p.m.

Behavioral Tests

Repetitive Grooming Behavior

Habituated individual mice were introduced into a transparent box without a top (22 cm length × 22 cm width × 25 cm height), which was placed on a table with only the ceiling of the room visible to avoid the generation of fear. The testing room was lighted at ∼40 lux. The front-mounted video camera was placed 1 m away from the box and recorded a 40-min session, which included the mouse being introduced into the box and the initial 10-min segment of habituation that was not scored. The components of a grooming event included forelimb movement, rubbing the face and then the flanks, and finally the tail and genitals. The cumulative time spent grooming and the total number of grooming events during the final 30-min test segment were calculated by an observer blinded to the genotype.

The Three-Chamber Test

The test mouse was placed in the low-illuminated testing room for at least 1 h prior to the start of the experiment. A conspecific target mouse, matched for age and sex and unfamiliar to the test mouse, was habituated to being put inside a wire cage for 1 h each day for at least 5 days before the test. The social test apparatus was an opaque acrylic box with two pull-out doors and three chambers. Each chamber was identical in size (41 × 20 cm), with the dimensions of the entire box being 63 (length) × 43 (width) × 23 cm (height). There was a 10-cm gap between adjacent chambers that could be opened or closed with the removable doors. The transparent wire cage (12 cm in height and 9.5 cm wide) equipped with the novel, target mouse was placed 2 centimeters away from the edge of the testing chamber to allow an interaction between the mice.

The whole experiment was performed under low illumination and quiet conditions. The unfamiliar, target mouse was introduced into the wire cage in one side compartment, and an empty cage was placed in the opposite side compartment. The test mouse was introduced into the middle chamber and habituated for at least 5 min. The partitions were then removed, and the test mouse was permitted to explore all 3 compartments for 10 min. The entire process was recorded by a CCTV camera hanging 3 m above the apparatus. The relative positions of the empty cage and social cage were counterbalanced across test animals. The time spent in each compartment was recorded using the automated software SMART.

Resident-Intruder Test

The test mouse was placed individually in the test room to habituate for 1 h before the start of the experiment. A smaller, same-sex mouse selected as the target mouse was distinguished from the test mouse during the calculation of social behavior. The animals were fed in isolation for 3 days before the test day to motivate social behavior. The test was recorded by a CCTV camera for 10 min after the target mouse was introduced into the home cage of the test mouse. The specific episodes included sniffing (e.g., nose-to-nose, anogenital sniffing) and moving away from, following and pushing each other. The duration and frequency of these episodes initiated by the test animal toward the intruder animal were measured by a well-trained experimenter blinded to the genotype of the mouse.

PET Ligand and Imaging

PET imaging studies were conducted in six Shank3B$^{-/-}$ mice and six control mice. The animals were anesthetized using isoflurane (1.0–1.5% with oxygen flow of 1–1.5 L/min), and a tail vein was catheterized for radiotracer injection. Animal physiology was monitored using a system included with the imaging device (InterView^{TM} FUSION, Mediso). The center resolution of the field of view of the PET scanner was 0.7 mm. Radioactive [^{18}F]FPEB (150–200 Ci) was injected via the tail vein. After radiotracer injection, dynamic volumetric data were acquired for 10 min. Anatomical maps and data for attenuation correction were obtained during the PET studies (19). The mouse brain-atlas template from the Laboratory of Neuro Imaging (LONI) was applied for segmentation of regions of interest (ROIs). The ROIs selected for analysis were whole brain, olfactory bulb, cortex, striatum, hippocampus, thalamus, amygdala, hypothalamus, and cerebellum. The data were analyzed using PMOD3.2 (PMOD, Zurich, Switzerland) by MITRO Biotech Co., Ltd. Binding potential (BPnd) was determined for mGluR5 using muscular tissue data as an input function.

Western Blot Analysis

Brains were removed from Shank3B$^{-/-}$ mice and WT mice and sectioned into 1-mm-thick coronal sections at the end of the study. The tissues of each brain area were extracted from the sections according to the mouse atlas (*The Mouse Brain in Stereotaxic Coordinates*, second edition, by George Paxinos and Keith B.J. Franklin). The collected tissues were lysed in 100–300 μL of RIPA lysis buffer (10 mM Tris, 150 mM NaCl, 1% Triton X-100, 0.5% NP-40, and 1 mM EDTA at pH 7.4) containing a 1:100 (v/v) ratio of a protease inhibitor cocktail and a phosphatase inhibitor cocktail (Roche). We used the bicinchoninic acid protein assay (Pierce) to quantify total protein samples (20–40 μg). Then, the samples were resolved via sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride membranes. The primary antibodies were as follows: anti-β-actin (1:1,000, Cell Signaling Technology); anti-Shank3 (1:1,000, Abcam); anti-mGluR5 (1:1,000, Abcam); anti-NR2b (1:1,000, Cell Signaling Technology); and anti-homer1 (1:1,000, Abcam). All western blots were visualized using the enhanced chemiluminescence detection method (Advansta). The scanned images were quantified using ImageJ software (version 1.47).

Statistical Analyses

The data were analyzed with SPSS 21 (SPSS Inc., Chicago, IL, USA) or GraphPad Prism 7.0 and were expressed as the mean ± s.e.m. Comparisons between Shank3B$^{-/-}$ mice and control mice without regard to sex were conducted with the independent *t*-test or two-way analysis of variance (ANOVA).

RESULTS

Shank3B$^{-/-}$ Mice Display Core Behavioral Features of ASDs

To clarify whether deletion of Shank3 in mice results in ASD-like behavior, we conducted a battery of behavioral experiments to characterize Shank3B$^{-/-}$ mice. The grooming behaviors of animals were measured for analysis of repetitive stereotyped behaviors, as one of the core symptoms of ASD. We found that KO mice displayed a clear increase in time spent grooming and in the total number of grooming events compared with the WT mice (**Figures 1A,B**). Thus, Shank3B$^{-/-}$ mice showed self-injurious and excessive grooming behavior.

To assess defective social interactions, another core symptom of ASD, we measured the instinctual reaction of social interaction using the three-chamber test. The test mouse was free to explore the apparatus, and the preference to contact the target mouse placed inside the wire cage vs. the empty cage placed in the opposite chamber was assessed. In the test, KO mice favored contact with the empty cage, whereas the WT mice remained closer to the chamber containing the novel mice (**Figures 1C,D**). The observed abnormal initiation of social interaction in Shank3B$^{-/-}$ mice was an indicator of impairment.

In a subsequent trial, we tested the mice on social motivation using the resident-intruder test. Compared to the WT littermates, the KO mice showed a reduction in the time and frequency of social contact (**Figures 1E,F**). These data suggested that Shank3 mutant mice were indifferent in situations involving social interaction.

[^{18}F]FPEB Synthesis

The radiotracer [^{18}F]FPEB was prepared in an automated synthesis module, as described in a previous study (20) (**Figure 2**). The product was concentrated and rinsed with 10 mL of water. The final product was eluted with 3 mL of ethanol and collected into a product vial. The product solution was removed from the hot chamber and dried in a nitrogen blower. The solution was reconstituted with a small amount of ethanol, diluted with physiological saline, and sterilized by filtration through a 0.22-μm filter. The final product was in a sterile physiological saline solution with an ethanol concentration <7% (v/v).

In Vivo mGluR5 Expression in Shank3B$^{-/-}$ Mouse Brain

To investigate mGluR5 distribution in the brain, Shank3B$^{-/-}$ mice and control mice were administered [^{18}F]FPEB and PET-scanned for 10 min. PET data were quantified as binding potential (BPnd) in several brain regions by using the simplified tissue reference model with the muscular tissue as the reference region (**Figure 3**). The regions of interest included the olfactory bulb, cortex, striatum, hippocampus, thalamus, amygdala, hypothalamus, and cerebellum. The BPnd of mGluR5 was rich in the hippocampus, thalamus, striatum and amygdala (**Figure 4**). More importantly, Shank3B$^{-/-}$ mice showed significantly increased BPnd compared to the control mice in the hippocampus ($P < 0.01$), striatum ($P < 0.01$), thalamus ($P < 0.05$), and amygdala ($P < 0.05$) (**Figure 4**).

Deletion of Shank3 Reduces mGluR5 Expression in the Striatum

It has been proposed that Shank3 plays an important role in forming excitatory synapses via its multiple protein-protein interactions (21). Shank proteins are indirectly connected to group I mGlu receptors by Homer proteins. A previous study has shown that the protein levels of the scaffolding proteins (SAPAP3, homer, and PSD93) and glutamate receptor subunits (GluR2, NR2A, and NR2B) were reduced in striatal PSD fractions from Shank3B$^{-/-}$mice (4). However, the expression levels of mGluR5 in the striatum of Shank3B$^{-/-}$ mutants remained unknown. Our data showed that mGluR5 protein level was reduced in the striatum from Shank3B$^{-/-}$ mice. In addition, consistent with previous results, homer1 and NR2b were reduced in the striatum of Shank3B$^{-/-}$ mice (**Figure 5**).

mGluR5 Level Was Increased in Multiple Brain Regions of Shank3B$^{-/-}$ Mice

Depending on the brain region, Shank3 performed different functions at synapses (9, 10). Thus, we examined the expression of mGluR5 in multiple brain regions by western blot. mGluR5 was increased in the hippocampus, amygdala and thalamus of Shank3B$^{-/-}$mice (**Figures 6A–C**). However, the level mGluR5 did not change in the cerebellum, somatic cortex or prefrontal cortex of Shank3B$^{-/-}$ mice (**Figures 6D–F**).

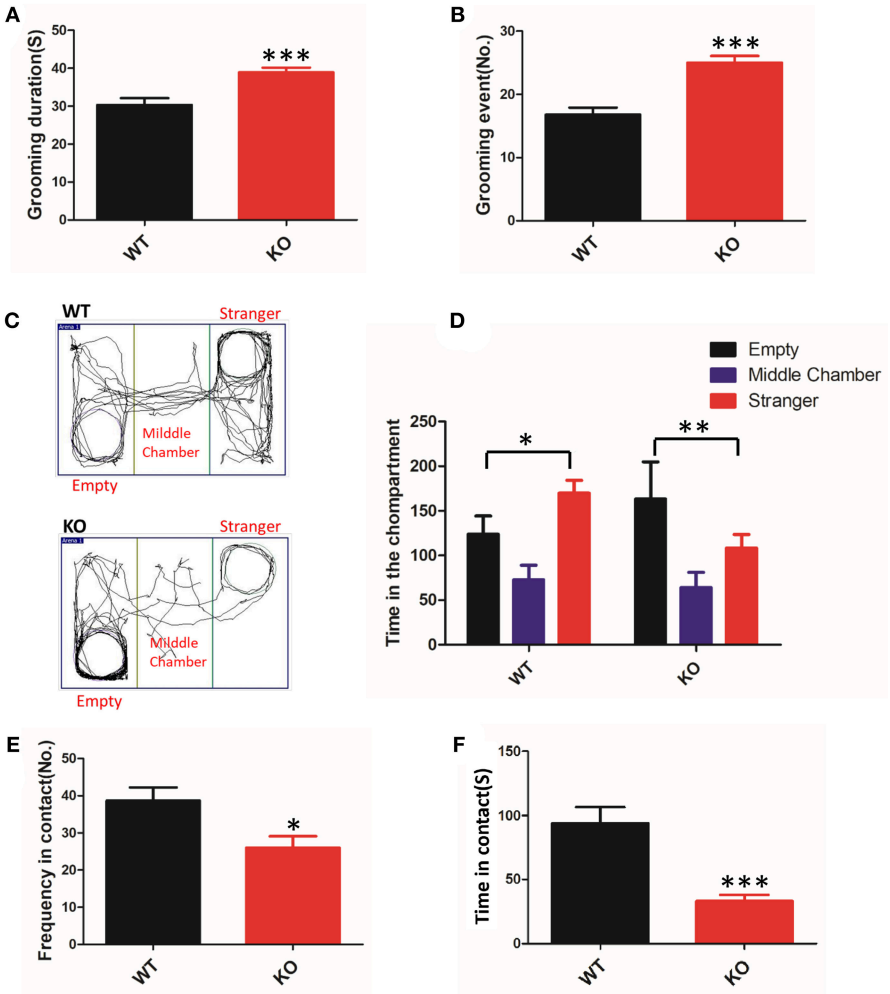

FIGURE 1 | ASD-like behaviors in Shank3B$^{-/-}$ mice. **(A,B)** Repetitive grooming was scored by the duration **(A)** and the total number of grooming events **(B)**. **(C)** Compared to the WT mice, the KO mice prefer to be in the chamber with the empty cage, as shown in the tracking map. **(D)** The KO mice spent more time in the chamber containing the empty cage and spent less time in the chamber associated with the unfamiliar mouse. **(E,F)** The resident-intruder interaction was evaluated by the frequency **(E)** and cumulative time of the social interactions **(F)**. The KO mice showed a clear reduction in social contact. *$p < 0.05$, **$p < 0.01$, ***$p < 0.0001$, compared to littermate WT mice. All data are displayed as the mean ± s.e.m. of 10–13 mice per group. Student's *t*-test was used for **(A,B,E,F)**; two-way ANOVA with Bonferroni's *post hoc t*-test for **(C)** and **(D)** were conducted for the statistical analysis.

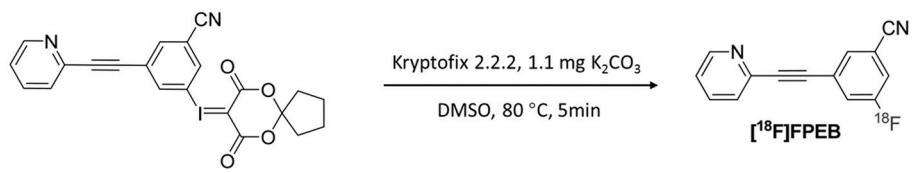

FIGURE 2 | Synthesis of [^{18}F]FPEB.

DISCUSSION

The present study investigated the changes in mGluR5 expression in Shank3B$^{-/-}$ mice and studied whether these changes could be imaged with the PET radioligand [^{18}F]FPEB. Previous studies have pointed toward the involvement of mGluR5 in the pathological process resembling autism caused by the complete knockout of Shank3 (9, 10). Our study,

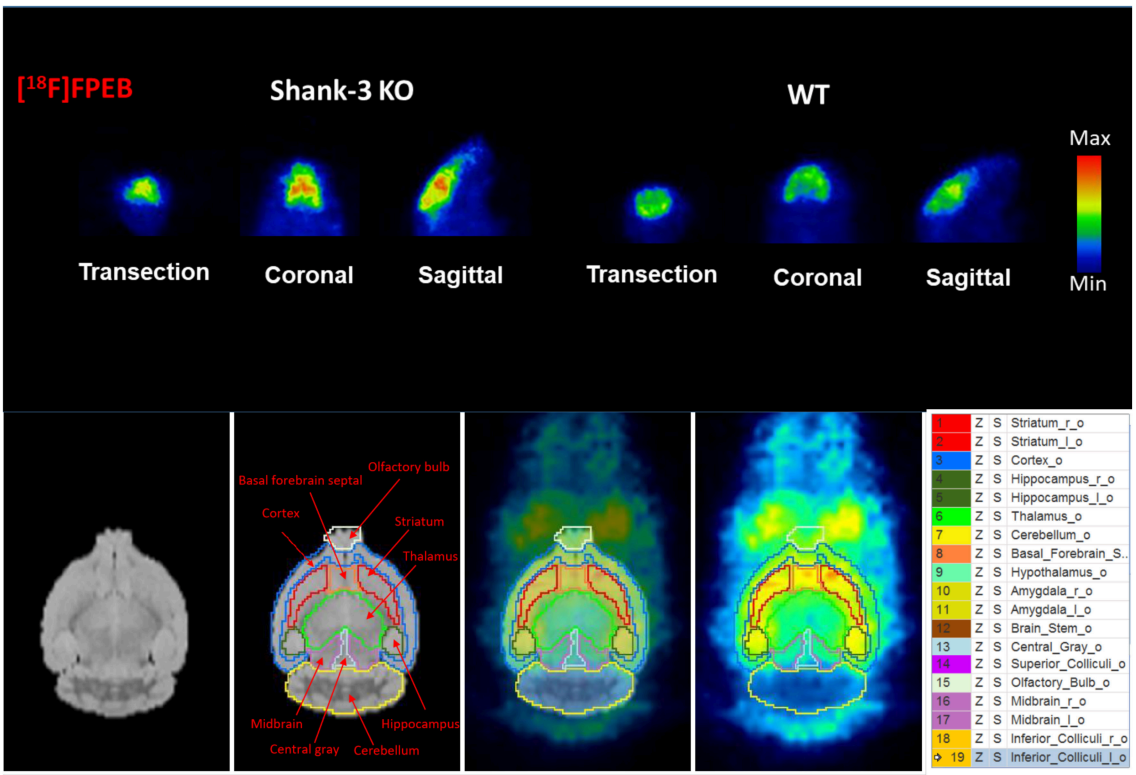

FIGURE 3 | Binding of [^{18}F]FPEB to Shank3B KO mice and their littermate control mice shows significant differences between the two groups.

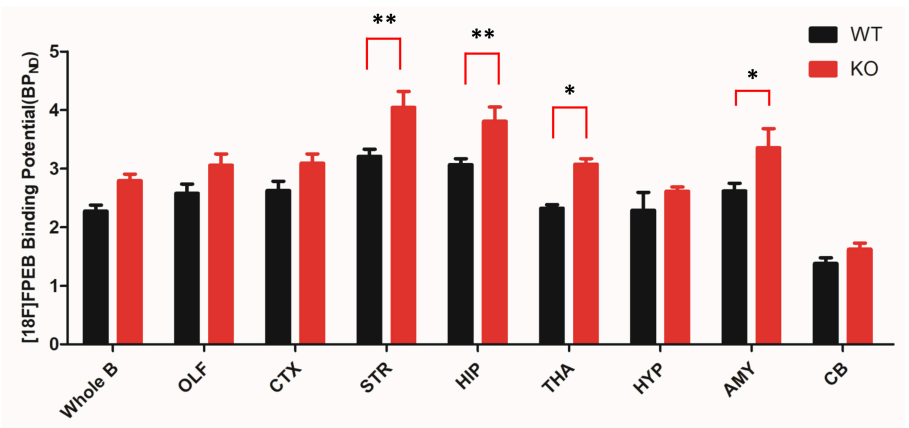

FIGURE 4 | Bar graph of binding of [^{18}F]FPEB to Shank3 KO ($n = 6$) and control mice ($n = 6$). Shank3 KO mice show significant increases in several brain regions compared to the control mice. STR and HIP: $^{**}p < 0.01$; THA and AMY: $^{*}p < 0.05$; whole brain, OLF, CTX, HYP, and CB showed no significant changes. OLF, olfactory bulb; CTX, cortex; STR, striatum; HIP, hippocampus; THA, thalamus; HYP, hypothalamus; AMY, amygdala; CB, cerebellum. $^{*}p < 0.05$, $^{**}p < 0.01$.

to some extent, confirmed previous reports of differences in mGluR5 expression between Shank3 KO mice and wild-type mice.

To our knowledge, this was the first *in vivo* study of mGluR5 in the Shank3 KO mouse model. Previously, the interaction between mGluR5 and autism had been investigated in Shank3Δ11$^{-/-}$ mice and Shank3Δ4-22$^{-/-}$mice; however, these were all *ex vivo* experiments (9, 10). In an earlier study, Verpelli et al. used RNAi to knock down Shank3 expression in neuronal cultures that specifically reduced the synaptic expression of mGluR5 but did not affect the expression of other major synaptic proteins (14). In addition, the reduced mGluR5 activity in Shank3-knockdown

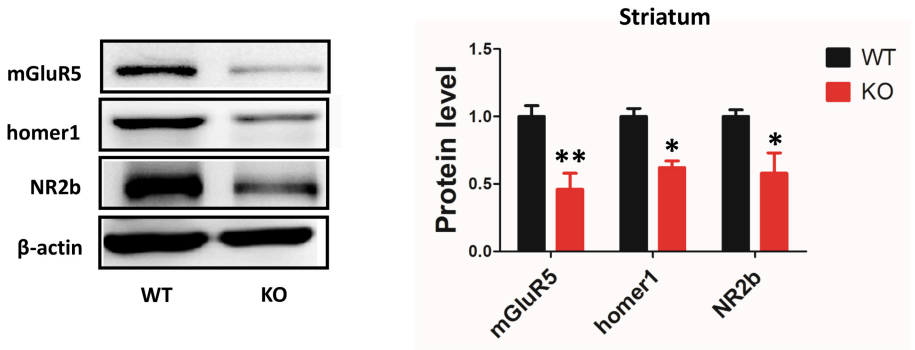

FIGURE 5 | Deletion of Shank3 reduces mGluR5 expression in the striatum. mGluR5, homer1 and NR2b were reduced in the striatum from Shank3B$^{-/-}$ mice. Each lane was loaded with 3 μg of protein, with β-actin as a loading control and normalized to wild-type levels. *$p < 0.05$, **$p < 0.01$, two-tailed *t*-test; all data are presented as the means ± s.e.m.; $n = 3$ samples per group.

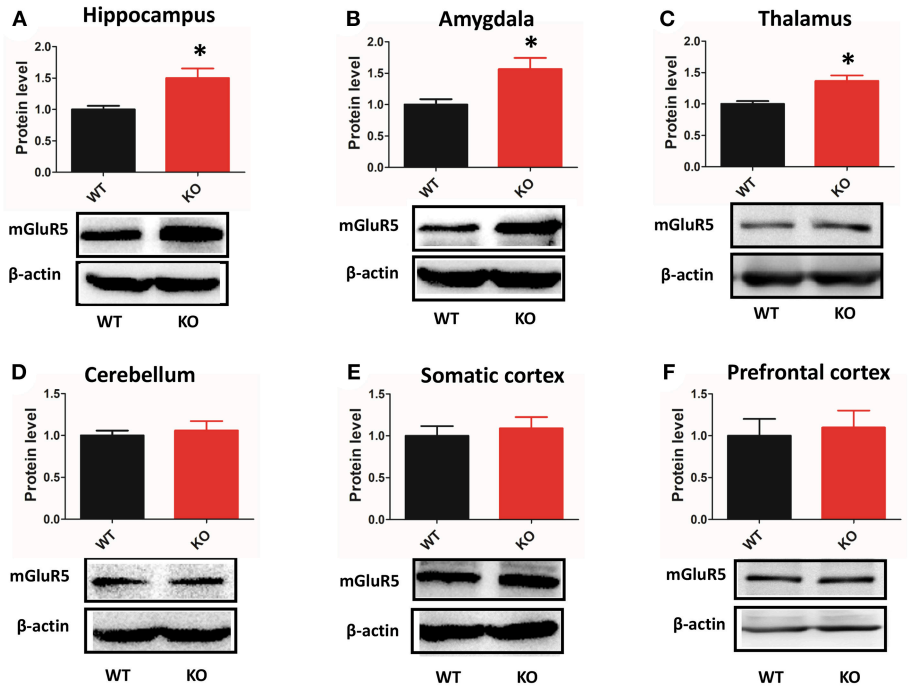

FIGURE 6 | Protein levels of mGluR5 are increased in multiple brain regions of Shank3B$^{-/-}$ mice. **(A–C)** mGluR5 is increased in the hippocampus, amygdala and thalamus of Shank3B$^{-/-}$ mice. **(D–F)** mGluR5 level did not change in the cerebellum, somatic cortex or prefrontal cortex of Shank3B$^{-/-}$ mice. Each lane was loaded with 3 μg of protein, with β-actin as a loading control and normalized to wild-type levels. *$p < 0.05$, two-tailed *t*-test; all data are presented as the means ± s.e.m; $n = 3$ samples per group.

neurons can be rescued by an allosteric agonist of mGluR5, such as CDPPB (14). However, subsequent study results from Shank3Δ11$^{-/-}$ and Shank3Δ4-22$^{-/-}$mice were wildly different (9, 10). Cinzia et al. found that the absence of Shank3 specifically reduced mGlu5/Homer interactions in the striatum and cortex, and the mGluR5 agonist CDPPB rescued ASD-like behavior in Shank3Δ11$^{-/-}$ mice (10). In contrast, the study by Wang et al. showed a marked decrease in Homer1b/c and increased mGluR5 in the PSD fractions from the striatum of Shank3Δ4-22$^{-/-}$mice, and some abnormal behaviors were normalized with the mGluR5 antagonist MPEP (9).

Our PET study showed high BPnd levels for [^{18}F]FPEB in the hippocampus, thalamus and amygdala, which was consistent with the expression pattern of mGluR5 in the human brain (22). More importantly, increased mGluR5 expression has been observed in these brain regions of Shank3B$^{-/-}$ mice compared to control mice. A recent pilot PET study, which also used [^{18}F]FPEB as a tracer, showed increased binding potential in

the postcentral gyrus and cerebellum of male individuals with autism (23).

Interestingly, the protein levels of mGluR5 assessed with immunoblotting could not be visualized with PET in any of the brain regions. For example, the protein level differences in mGluR5 in the striatum were not reflected in the changes in BPnd. This may be because of the limitation of performing PET on small animals. One such limitation is the spatial resolution of PET; the PET image resolution (1–3 mm) may not be sufficient for the small mouse brain (19).

In addition to the reduced protein level of mGluR5 in the striatum from Shank3B$^{-/-}$ mice, our data showed that homer1 and NR2b were reduced in the striatum. These genes converge on the NMDA receptor complex (24, 25), which has itself been associated with ASD (26). More specifically, mGluR5 potentiates the NMDA receptor, while homer ensures the appropriate cell surface localization of the NMDAR/mGluR5 complex.

CONCLUSION

[^{18}F]FPEB appears to be a good tracer with high specificity for mGluR5 in the mouse brain. Our data acquired from postmortem tissue and PET indicated that the deficiency of Shank3 can impair the expression of mGluR5 to varying degrees in different brain regions. However, the result of PET was inconsistent with the result of western blot in the striatum. Future work is also needed in order to understand the reasons for the different results observed between *in vivo* PET and *ex vivo* immunoblotting.

ETHICS STATEMENT

The experimental procedures were approved by the Animal Care and Use Committee of the FMMU and followed the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 80-23, revised 1996).

AUTHOR CONTRIBUTIONS

SheW and JW conceived and designed the experiments. GC and ShuW performed most of the experiments and analyzed the data. GC and YL wrote and refined the article. MW, YuZ, BG, and HY participated in the animal modeling and behavioral experiments. YaZ, SZ, and MZ assisted in laboratory work and figure preparation. WW supervised the acquisition of results.

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Primary Cilia–An Underexplored Topic in Major Mental Illness

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Though much progress has been made in recent years towards understanding the function and physiology of primary cilia, they remain a somewhat elusive organelle. Some studies have explored the role of primary cilia in the developing nervous system, and their dysfunction has been linked with several neurosensory deficits. Yet, very little has been written on their potential role in psychiatric disorders. This article provides an overview of some of the functions of primary cilia in signalling pathways, and demonstrates that they are a worthy candidate in psychiatric research. The links between primary cilia and major mental illness have been demonstrated to exist at several levels, spanning genetics, signalling pathways, and pharmacology as well as cell division and migration. The primary focus of this review is on the sensory role of the primary cilium and the neurodevelopmental hypothesis of psychiatric disease. As such, the primary cilium is demonstrated to be a key link between the cellular environment and cell behaviour, and hence of key importance in the considerations of the nature and nurture debate in psychiatric research.

Keywords: primary cilia, schizophrenia, bipolar disorder, neurodevelopment, brain

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INTRODUCTION

Recently Muñoz-Estrada et al. (1) published results from experiments on olfactory neuronal precursor cells obtained from human sufferers of schizophrenia (SCZ) and bipolar disorder (BD), linking primary cilia (PC) with major mental illness (MMI). Their work showed a general decrease in the percentage of cells with PC in subjects suffering from MMI. Furthermore, *in vitro* supplementation with lithium (a common pharmacotherapy for BD, mania and depression (2), and previously shown (3) to cause *in vivo* and *in vitro* PC elongation in mouse neuronal cells) was shown to have a positive effect on PC length. While their study (1) was conducted on samples obtained from a very limited number of patients suffering from a variety of MMI and on different treatment regimes, it highlights an area of psychiatric research that has been largely ignored.

PC are cellular protrusions originating from the centrosome's mother centriole, and are present on most mammalian cells (4, 5). Since they are linked with the centrosome, they need to be disassembled or retracted whenever the centrosome needs to perform its microtubule organizing centre functions, such as during cell division and migration (4, 6–9), making the exact role of the PC in these processes somewhat unclear. PC are largely regarded as cellular sensory antennae and signalling hubs, facilitating key developmental pathways such as Sonic Hedgehog (SHH) and

WNT signalling (10–12). More recently, proposals have been made that PC have an extracellular signalling role, as thanks to its biochemical autonomy from the rest of the cell membrane the cilium can express distinct proteins on its membrane as well as have a different concentration of various factors in its cytoplasm (13, 14). As such ciliary vesicles, can form distinct exosomic parcels, but their role, especially in mammals, is not yet clear, and arguments have been made that their primary function is to dispose of redundant ciliary content (13, 14). This article reviews various levels of evidence for the role of PC in MMI, focusing on the well-established developmental hypothesis of MMI (15, 16). PC play an important role in the development of the central nervous system (CNS) and in a wide variety of roles in the adult brain (10, 17). PC have been even called "neurons little helpers" in the context of neurodevelopment (18), and have recently been discussed in the context of the neuronal migration hypothesis of dyslexia (19). Nevertheless, as discussed later, PC retract during some cellular events, and might not be present on all types of neurons during early development (8, 20). The following sections will show why this elusive organelle should be considered an attractive target for psychiatric research.

GENETICS

Genomic and bioinformatics research has revealed that some PC genes are linked to MMI. Of course, the mere fact that a gene might be linked to both PC and MMI does not guarantee the involvement of PC in MMI, as proteins might function in different cellular compartments and in different cellular processes. Similarly, a different severity of mutation might be required to cause an effect on the PC or to precipitate MMI. Nevertheless, the fact that data from an RNA interference study looking at 41 MMI genes found that 23 affect cilia length (21) should at least prompt one to look at the correlation between PC and MMI genes. Here, we present evidence for two candidate genes which may potentially connect PC with MMI.

Previously (22), researchers described a region on chromosome 4p linked to MMI. One gene found in that region was CC2D2A (23), also known as MKS6 and JBTS9. It is involved in ciliogenesis (24), and in vesicles trafficking in the PC's transition zone (the region of the cilium that regulates the trafficking of proteins between the cilium and the rest of the cell, allowing the cilium to retain its distinct protein composition), and has been implicated in neural tube development and Sonic Hedgehog signalling (25–27). CC2D2A has been linked to a range of CNS developmental conditions linked to PC: Joubert syndrome (28–30), Meckel syndrome (31) and mental retardation (32, 33); there is also a potential link with Bardet-Biedl syndrome (BBS) (29). CC2D2A's link to MMI has not yet been thoroughly investigated (34), but MMI problems have been observed in individuals with Joubert syndrome, and AHI, also associated with Joubert syndrome, has been proposed as a marker for SCZ (35).

Disc1, a gene involved in the formation and regulation of cilia (36), has also been associated with MMI (37, 38). DISC1 associates with a variety of centrosomal components (39, 40), recruiting BBS proteins to the centrosome (41, 42), and acting as a switch between the processes of neuronal migration and proliferation. DISC1 also interacts with the dynein complex (43), which, together with the Intraflagellar Transport (IFT) complex A, is vital for retrograde transport within the PC (12). Moreover, one reported zebrafish DISC1 aberration caused a decrease in β-catenin levels (44) correlating to a decrease in canonical WNT activity.

As such, DISC1 is perhaps one of the strongest links between PC and MMI.

NEURODEVELOPMENT

PC associated CNS defects range from cerebellar hypoplasia through mental retardation to encephalocele and enlarged ventricles (45–47). Moreover, various neurodevelopmental defects have been associated with MMI, for which there is evidence of PC involvement. This is unsurprising, as PC are present from the earliest stages of CNS development through to the mature brain (17, 48, 49). The centrosome, with which PC are closely interlinked, is also a key player in CNS development (50, 51).

Defective neuronal migration has been reported in several studies relating to MMI, and is likely to contribute to reductions of grey matter in patients affected by MMI (15, 52–56). Additionally, BBS has been associated with cortical volume reductions in both a human and mouse study (57, 58). Molecular links between psychiatric pathways and PC, such as the interaction of DISC1, WNT signalling, the BBS complex and the centrosome exist in the context of cell migration and proliferation (41–43, 59, 60). For example, PC have been shown to be involved in several aspects of neuronal migration like radial glial scaffold formation and interneuron migration (8, 61) as well as galvanotaxic migration (62–64).

PC factors also influence important migratory processes of microtubule (65–67) and actin (68, 69) organisation. Further, CDC42, a molecule important for ciliary initiation (70–72) promotes actin skeleton remodelling (73) and cell polarity (74, 75) through non-canonical WNT signalling (51). Furthermore, CDC42 and actin skeleton remodelling have been associated with deficits in dendritic spine formation frequently reported in SCZ and BD (76–78). Issues relating to neuronal network health, such as synaptic connectivity and neurite number have also been highlighted in both mental illness and PC dysfunction (53, 54, 79–85).

Problems with neuronal differentiation have been associated with SCZ, and can result from DISC1 related changes in WNT signalling (86–88). Asymmetric PC membrane inheritance occurs during neocortical development, and is linked with the inheritance of the centrosome, which is important for proper neurogenesis (89–91), suggesting that PC function might be important for cell division and fate specification, further contributing to the aforementioned changes in cortical volume (57, 58).

Moreover, PC are known to be involved in other developmental aspects that could contribute to defective neurogenesis and CNS cell migration, often involving PC's

close association with the centrosome and the Golgi apparatus. These involve SHH (92–94) and Platelet Derived Growth Factor (95–98) signalling, and governance of cell migration (4, 8, 9, 50, 63, 99–102) and cell division (95–97, 103–110) through sensing extracellular cues. This last point is exemplified by the fact that serum withdrawal during cell culture is a ciliogenic condition (97, 103, 105, 109), indicating that cells may use their cilia to ensure that the extracellular conditions are right for mitosis initiation.

WNT SIGNALLING

The WNT pathway is one of the best studied MMI signalling pathways (111–114). Those affected by BD and SCZ have been found to express mRNA levels suggestive of attenuated canonical WNT signalling and enhanced non-canonical signalling, particularly the WNT/Ca^{2+} pathway (111); although a recent human cerebral organoid study showed an increase in canonical WNT signalling in the early developmental stages of brains with disrupted DISC1, suggesting that WNT changes might be context/age dependent (114). The changes in mRNA expression levels is a noteworthy finding since PC are known to facilitate the switch from canonical to non-canonical WNT signalling via Ca^{2+} signalling (103). PC modulates WNT signalling via the degradation of Dishevelled by Inversin at the basal body (51, 115, 116), repressing the canonical signalling pathway (117, 118) and promoting the planar cell polarity pathway (95). Curiously, different ciliary gene mutations perturb WNT signalling in different ways, with mutations in some genes being able to both increase and decrease β-catenin levels (119).

Moreover, WNT signalling affects motile cilia, and might affect PC by influencing basal body positioning on the apical membrane (51, 120–122). The importance of such an overlap and interaction between motile and primary cilia has been highlighted in hydrocephalus (96), where defects in both motile and primary cilia are known to be present (96, 123). Hydrocephalus-like changes have similarly been reported in SCZ (124). Motile cilia generate fluid flow, to which PC respond (125), which is crucial for establishing body asymmetry (126), and is detected by the polycystin receptors PC1 and PC2 (127–129), which facilitate Ca^{2+} entry (127). This flow-induced calcium signalling not only facilitates the switch from canonical to non-canonical WNT signalling (103) but also regulates the cell cycle (95), although recent experiments have started questioning whether flow sensing happens via Ca^{2+} signalling (130).

Nevertheless, there is some evidence disputing PC's role in WNT signalling (11, 119). There is evidence from both zebrafish and mice showing that disrupting PC does not affect WNT signalling (131–133). The role of PC in WNT signalling is further complicated by the fact that WNT signalling has a regulatory role in ciliogenesis (120, 134, 135). As such, the exact role of PC in WNT signalling, particularly in canonical WNT (11), requires further investigation, though as argued in this section, such an investigation might bring fruitful results if carried out in the MMI context.

FIBROBLAST GROWTH FACTOR

The importance of the Fibroblast Growth Factor (FGF) signalling system has been highlighted in SCZ research (136–138). This system can regulate neuronal differentiation via the Stat1 pathway, and neuronal proliferation and function via the ERK pathway (139). Moreover, FGF function has a positive effect on dopamine neuron survival and neurite outgrowth (139). Recently, bioinformatic and stem cell experiments investigated the role of the FGF receptor 1 (FGFR1) (136, 137). FGFR1 dysregulation can upregulate developmental pathways involved in neurogenesis and downregulate those involved in oligodendrogenesis (136), and data suggests that it can also lead to cortical maldevelopment (137), though the dysregulation of this pathway still awaits confirmation in a larger patient sample.

PC themselves do not seem to mediate FGF signalling, yet both motile and tethering cilia (a type of kinocilium, located on hair cells in the ear, with a microtubule structure similar to motile cilia (5, 140, 141)) length is affected by FGF (142–144). While it remains to be seen how ciliogenesis and PC length can be controlled by FGF in mammals, zebrafish and *Xenopus* studies suggest that FGF modulate the expression of Ift88 via FGFR1 (144). The IFT machinery is responsible for trafficking anterograde and retrograde cargo along the PC (145, 146) and IFT dysregulation can result in underdevelopment of certain organs, including the brain (145, 147). While the role of IFT in MMI requires further study, IFT27 [which together with IFT88 and IFT172 belongs to the IFT complex B (148)] has in one study been associated with BD (149), however the authors of that study note that this conclusion should be taken with caution due to the amount of variation present throughout the study. Since IFT172 has been identified as also being BBS20 (150) it is worthy to highlight that BBS is associated with such traits as reductions in hippocampal, white and grey matter volumes (57), traits often associated with MMI (151–154) and depression (155) belongs to the IFT complex B.

Therefore, if FGFR1 is proven to be implicated in a larger cohort of individuals with schizophrenia and in the regulation of human PC length, then there would be a mechanistic correlation between defective PC and SCZ. However, it would remain to be seen whether it was the PC dysfunction that contributed to SCZ or whether they were independent consequences of aberrant FGF signalling belongs to the IFT complex B.

PRIMARY CILIA AND DOPAMINE

The dopamine hypothesis is prominent in SCZ research (156, 157), and various dopamine receptors localise to PC (36) in a manner dependent on IFT and BBS components (158, 159). Type 1 and 2 receptors have been shown to localise to PC in neurons in regions such as the striatum, amygdala, and pituitary gland (36, 158, 160, 161). Type 5 receptors, mediating both chemical and mechanical signalling in the PC, were shown on mouse endothelial cell (162), and type 4 receptors have also been shown on non-neuronal cells (36).

While the relationship of dopamine signalling, PC and MMI has not been explored, there might be an overlap

between these during brain development. A possible explanation involves the dysregulation of WNT signalling important for the appropriate differentiation of dopaminergic neurons (163). The WNT pathway also regulates dopaminergic neural progenitor cell migration during electrotaxis (62); the health of PC has been shown to affect electrotaxis in fibroblasts (63), though studies in neurons are lacking. Moreover, dopamine signalling has been found to affect PC length in striatal neurons (160). As such, the implications of this interplay between PC and dopamine on MMI remain to be explored.

CILIA–NATURE AND NURTURE

The theme of this research topic compilation concerns neuropsychiatric disorders within the nature and nurture debate, and as such it is fitting to discuss how PC might fit within this debate. It is therefore valuable to reassess some of the aforementioned points within the context of some of the hypotheses of MMI.

The watershed hypothesis of MMI (164) suggests that the diseases might manifest themselves as the cumulative effects of smaller (potentially benign on their own) changes in physiological processes. PC dysfunction might contribute to small changes in several neurally important signalling pathways, not all of which have been mentioned here (165). These changes do not need to originate from serious mutations affecting a single gene (e.g., Disc1), but in themselves might be the result of several less severe changes in PC genes. Nevertheless, it must be remembered that some ciliary proteins might perform the majority of their work outside of the PC (166).

More importantly, genetic changes might, in themselves, not result in a pathological phenotype, but an environmental insult (or several) might be required to trigger the pathological process. This is known as the Two-Hit Hypothesis (167), and is of particular interest here due to the sensory role of the PC. There is a correlation between famine and SCZ (168–170), and there is experimental evidence that environmental stressors, such as maternal ethanol consumption, methylmercury exposure, and pentylenetetrazole (PTZ)-induced maternal seizures can cause neural damage to developing embryos, even at relatively low doses (171). This neural damage is associated with Heat Shock Factor expression level variability, which might be caused by oxidative stress damage (171). PC are involved in stress regulating pathways, such as ERK, but are also affected by the ERK response to oxidative stress and ischaemia (172, 173). Heat shock itself was found to cause ciliary absorption mediated via a reduced association of heat shock protein 90 with HDAC6, and was hypothesised to decrease PC mediated signalling during times of extracellular stress (174). Therefore, PC might provide a molecular link bridging the genetic and environmental components of MMI pathology.

TABLE 1 | Summary of the key points from each section, and avenues for future research related to each section.

Section	Key points	Future work
Genetics	There is an overlap between genes associated with MMI and PC. Disc1 is the gene with the strongest connection to both MMI and PC.	The extent to which PC genes are associated with MMI requires further study via GWAS. The large amount of genes associated with PC can be both a source of false positive (due to pure statistical chance) and negative (watershed hypothesis, or small frequencies of any one particular gene or SNP) results. Identified genes should also have a mechanistic link between MMI and PC before a role of PC in MMI can be deemed conclusive.
Neurodevelopment	PC are involved in a range of developmental processes, such as cell migration and proliferation. Defects in these processes are associated with MMI.	Developmental processes can be disrupted in a variety of ways, as processes such as cell migration and proliferation depend on a variety components. Moreover, a single protein might act at several cellular locations. It is important that defects in ciliary proteins that are found to play a role in MMI, do this in a way that is mechanistically related to the PC. Additionally, changes in brain PC should be studied via histological samples from both well-established MMI animal models, and post-mortem patient brains.
WNT signalling	WNT is a major signalling pathway that has been implicated in MMI. PC have been often presented as providing a switch mechanism for the different modalities of WNT signalling.	Direct evidence of PC role in MMI WNT aberrations is still lacking. As such, iPSC studies should look at WNT signalling changes in MMI patients, and assess if any changes are due to changes in PC function.
Fibroblast growth factor	FGF signalling has been highlighted in SCZ. FGF affects expression of Ift88, a component of the ciliary transport machinery.	The interplay between FGF, MMI and PC is still poorly understood. As such, the avenues for exploration are very wide.
Primary cilia and dopamine	Dopamine signalling has been of major interest in SCZ research. Several dopamine receptors have been found on PC, including neuronal PC. Moreover, dopamine signalling has been found to affect ciliary length.	The importance of dopamine signalling via PC remains to be explored in the context of MMI, iPSC experiments from patient samples could be of great help here. This should be explored in both the contexts of adult brain function, and neurodevelopment.
Cilia–nature and nurture	PC's main function is to receive extracellular signals, and as such defects in PC can cause cellular defects in responding to extracellular cues. PC presents a key point of interaction between nature and nurture.	This is a complex and exciting area, as we grow in appreciation of the interactions between genes and the environment. Investigators would need to both assess whether some PC defects predispose people to aberrant reaction to environmental stressors, and whether some mutations, while not disrupting PC function in a healthy environment, might cause PC defects, resulting in neurodevelopmental defects, when exposed to environmental stressors.

FUTURE DIRECTIONS

As noted, this manuscript explored several possible links between PC and MMI. Nevertheless, there is little literature directly exploring this topic. As such, we hope that this manuscript will encourage more research in this area. This section highlights some avenues that might be taken in this exploration.

PC length and frequency could be explored in histological specimens from animal models of MMI, and from human MMI patients. With the advances in microscopy and image analysis techniques [we have ourselves proposed such an analysis algorithm (175) for PC length], this is becoming a viable experimental strategy. Perhaps the biggest obstacle might be obtaining human post-mortem samples that would be of good enough quality to visualise PC.

This obstacle could be partly eliminated through the use of induced pluripotent stem cell technology, where human neurons (or other CNS cells) could be generated from tissues samples of MMI patients that could be ethically obtained during their lifetime. These cells could be subjected through a battery of tests, such as the study of their migration responses to a variety of cues. Such experiments would help to overcome several limitations highlighted in the text, e.g., the study of MMI and PC deficient neurons in electric fields. Moreover, using genetic editing technologies the effect of specific MMI associated mutations can also be investigated. These systems could also be used to evaluate the effects of environmental stressors on PC in MMI neurons, a link hypothesised in the previous section. The development of methods for growing cerebral organoids (114, 137), while perhaps raising ethical considerations, will allow for even more complex PC functions to be evaluated in a CNS-like environment.

Finally, while this paper has shown the involvement of PC in a range of signalling pathways. Yet, the evidence might not yet be strong enough to call the PC a signalling hub crucial for MMI. More research should be done to elucidate the role of PC in such key signalling processes for MMI as the dopamine and serotonin pathways (176) [5-HT6 receptors are predominantly expressed on PC (177, 178)], or to look at PC facilitation of pathways involved in the neurodevelopmental defects exhibited by those affected by MMI.

SUMMARY

This review has outlined why PC should be considered as an interesting area for MMI research (see summary in **Table 1**). It has demonstrated the involvement of PC in a wide variety of cellular processes, such as cell migration and proliferation, and as a signalling hub for various intracellular pathways related to MMI. PC have a unique ability to integrate information necessary for various developmental processes, and as such might be the missing link between the genetic and environmental causes of MMI.

PC and neuropsychiatric disorders are interesting fields for research, and much remains to be uncovered. While the arguments presented here show a correlation between PC and several different levels of biological processes associated with psychiatric disease as well as treatment, much yet remains to be experimentally proven. It is up to basic and clinical scientists to determine whether these are just correlations or if there is, indeed, a causative relationship between PC and MMI.

AUTHOR CONTRIBUTIONS

BL conceived the idea. MP wrote the manuscript with the support of BL.

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Decreased Serum Oxytocin and Increased Homocysteine in First-Episode Schizophrenia Patients

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Schizophrenia (SZ) is a debilitating and heterogeneous disease. We hypothesized that the oxytocin (OXT) system, inflammation and one-carbon metabolism would have a link with SZ. In this study, serum OXT, OXT receptor (OXTR), interleukin-6 (IL-6), high sensitivity CRP (hsCRP) and homocysteine (Hcy) levels were measured in 52 first-episode schizophrenia (FES) patients and 41 healthy controls (HC) from the Second Xiangya Hospital of Central South University. Meanwhile, the mRNA expressions of OXT and OXTR genes were determined by real-time quantitative PCR. Serum OXT and OXTR levels were significantly lower in FES patients (518.96 ± 22.22 and 174.60 ± 17.11 pg/ml) than the HC group (711.58 ± 40.57 and 252.15 ± 20.62 pg/ml). Serum IL-6 and hsCRP levels showed no difference between the two groups (1.82 ± 0.30 vs. 1.69 ± 0.36 pg/ml, 0.66 (0.22, 1.07) vs. 0.31 (0.13, 0.91) mg/L), but serum Hcy levels were significantly higher in FES patients (20.18 ± 1.83 vs. 15.24 ± 0.82 μmol/ml). The FES patients (0.27 ± 0.02 and 0.20 ± 0.02) have relatively higher mRNA expressions of OXT and OXTR genes than the HC group (0.16 ± 0.01 and 0.14 ± 0.01). In summary, our results suggested the possible function of the OXT system and Hcy in the pathogenesis of SZ.

Keywords: FES, OXT, OXTR, IL-6, hsCRP, Hcy

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INTRODUCTION

Schizophrenia (SZ) is a debilitating and heterogeneous disease with unknown mechanism. Oxytocin (OXT) is a nonapeptide, known to be synthesized in the hypothalamus and released into the blood stream from the axon terminals of the posterior pituitary. Increasing evidence has shown that OXT might be the potential therapeutic target for SZ patients in animal and clinical case-control research (1, 2). Therefore, several randomized controlled trials (RCTs) have investigated the efficacy of OXT on improving positive symptoms, negative symptoms and cognitive deficits in SZ patients (3–6). Alterations of OXT levels are reported in several studies; however, the endogenous OXT levels were still conflicting in SZ patients with higher (7) and lower (8) amounts in plasma and tantamount levels in CSF (9) when compared with healthy controls (HC). OXT receptor (OXTR), a single seven-transmembrane G-protein coupled receptor, is thought to mediate the actions of OXT. Meanwhile, the peripheral and central OXTR levels have only been scarcely reported.

At the gene expression level, there are several studies suggesting that single nucleotide polymorphisms (SNPs) of OXT and OXTR genes were associated with symptom scores in SZ patients (10, 11). Furthermore, variants in OXTR were nominally associated with severity of overall symptoms as well as with the improvement of the positive symptoms (12). In recent research, Yang et al. reported relatively higher mRNA expression of OXT and OXTR genes in the peripheral blood lymphocytes (13). In a post-mortem study, there was decreased OXTR mRNA expression in the anterior prefrontal cortex and caudate nucleus (14). Together, these data suggest that alteration in the OXT system may underlie the pathogenesis of SZ.

Numerous studies have demonstrated that the immune system and inflammation might be involved in the pathophysiology of SZ (15, 16). Interleukin-6 (IL-6) is a pleiotropic cytokine synthesized by activated monocytes and Th2 lymphocytes and is one of the most frequently studied cytokines in SZ. IL-6 has been suggested to mediate the microglial-induced inflammatory response to neurogenesis (17). Previous data showed higher serum and cerebrospinal fluid (CSF) IL-6 levels in SZ patients and increased mRNA expression of IL-6 in post-mortem brain of SZ patients (18–20). High sensitivity CRP (hsCRP) is a nonspecific marker of inflammatory state and is mainly synthesized by hepatocytes in response to proinflammatory cytokines. It has been reported that elevated hsCRP levels were associated with increased risk of SZ in a case-control study (21). Additionally, the elevation of hsCRP was suppressed by the medical treatment for SZ with acute agitation (22).

Homocysteine (Hcy) is a sulfur-containing amino acid involved in the one-carbon metabolism of methionine cycle. Recently, an increase in Hcy levels has been reported in several neuropsychiatric disorders including depression (23), bipolar disorder (24) and SZ (25). Fan et al. reported significantly higher serum Hcy in first-episode and drug-naïve SZ patients, which could be reduced after risperidone treatment (26). Furthermore, a positive correlation was found between plasma Hcy levels and scores of negative symptoms in SZ patients, but not with positive symptoms (27). It has also been estimated in a meta-analysis that a 5 μmol/L increase in plasma Hcy level may increase the risk of SZ by 70% (28).

Although the oxytocin (OXT) system, inflammation and one-carbon metabolism could have a link with SZ, some results were still conflicting. It was reported that antipsychotic treatment may influence OXT levels (29), but the exact mechanism is not known. Additionally, as far as we know, there is no report about the relationship of mRNA expression and serum levels of OXT and OXTR in the same patient. In animal experiments, OXT was decreased in older IL-$6^{-/-}$ mice (30), and Hcy was reported to increase OXTR expression (31). So, in this study, we determined the relative mRNA expression of OXT and OXTR genes in first-episode, unmedicated schizophrenia (FES) patients and HC. Meanwhile, the serum OXT, OXTR, IL-6, hsCRP and Hcy levels were measured.

MATERIALS AND METHODS

Subjects

A total of 52 FES patients were recruited from the Department of Psychiatry in the Second Xiangya Hospital of Central South University. These patients were diagnosed with FES by two senior psychiatrists according to the criteria of the Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition (DSM-V). All these patients were required to sign an informed consent form and were not treated with any antipsychotic drugs for a month at least before the research. Patients with comorbid mental disorders, a history of traumatic brain injury or intellectual disability, or serious somatic diseases, pregnancy or breast feeding were excluded. Meanwhile, 41 age- and gender-matched healthy controls (HC) were enrolled from the health management center of the Second Xiangya Hospital. Demographic features of FES patients and HC group are demonstrated in **Table 1**.

TABLE 1 | Demographic data for FES patients and HC.

	FES (N = 52)	HC (N = 41)	*P*-value
Gender (Male/Female)	31/21	23/18	0.733
Age (Mean ± SD)	20.71 ± 4.62	22.15 ± 4.11	0.104
Male	21.45 ± 5.19	22.91 ± 3.75	
Female	19.62 ± 4.08	21.17 ± 4.20	
PANSS			
Total	70.74 ± 19.39	/	
Positive	20.26 ± 5.35	/	
Negative	18.39 ± 4.38	/	
General	32.09 ± 10.01	/	

This study was approved by the Ethics Committee of the Second Xiangya Hospital of Central South University. All the patients or their statutory guardians and HC were required to sign an informed consent form.

Sample Collection

Blood samples of 52 FES patients and 41 healthy controls were collected from each participant at 08:00 a.m. after overnight fasting. Serum was isolated after centrifuging at 3500 rpm for 10 min, and peripheral blood mononuclear cells (PBMCs) were isolated and stored at −80°C until the biochemistry analysis or RNA was extracted without repeated freezing and thawing.

Measurement of Serum OXT, OXTR, IL-6, Hcy and hsCRP Levels

Serum OXT and OXTR as well as IL-6 levels were measured in duplicate by enzyme-linked immunosorbent assay (ELISA) using a comercially available kit (Cloud-Clone Corp). Serum Hcy and hsCRP were measured with latex enhanced immunoturbidimetric assay in a HITACHI 7600 020 automatic biochemical analyzer.

RNA Extraction and Real Time qPCR Analysis

The RNA was extracted from PBMCs in a MagNA Pure LC2.0 Automatic extractor with a MagNA Pure LC Total Nucleic Acid Isolation Kit (Roche Diagnostics, IN, USA). The complementary DNA (cDNA) was synthesized after extracting the RNA of all samples. The expression levels of OXT and OXTR mRNA were measured using real-time quantitative PCR. The primers of OXT and OXTR genes as well as reference genes refer to Yang et al. (13). All reactions were completed in triplicates with the Roche

LightCycler 480 (Roche) with the following cycling conditions (total reaction volume = 20 μl): 95°C for 10 minutes, followed by 40 cycles of denaturation at 95°C (10 seconds), annealing at 60°C (10 seconds) and extension at 72°C (20 seconds). The deltaCt (ΔCt) method was used to perform relative quantification, and the housekeeping gene β-actin was used as the reference gene.

Statistical Analysis

SPSS v21.0 (IBM, USA) statistical software was used to perform statistical analysis. Data were presented as mean ± standard error (SE) for normal distribution variables (at Kolmogorov-Smirnov test) and median (quartile range) for non-normal distribution variables. Comparisons between two groups were analyzed with unpaired Student's t-test or Mann-Whitney U-test as appropriate. $P < 0.05$ was considered statistically significant.

RESULTS

Serum OXT, OXTR, IL-6, hsCRP and Hcy Levels in HC Group and FES Patients

As we can see from **Figure 1**, serum OXT and OXTR levels in HC group (711.58 ± 40.57 and 252.15 ± 20.62 pg/ml, respectively) were significantly higher (t = -4.164 and t = -2.894, $P = 0.000$ and $P = 0.007$, respectively) than in FES patients (518.96 ± 22.22 and 174.60 ± 17.11 pg/ml, respectively). Serum IL-6 and hsCRP levels in HC group (1.69 ± 0.36 pg/ml and 0.31 (0.13, 0.91) mg/L) showed no difference (t = 0.283 and Z = -1.218, $P = 0.778$, $P = 0.223$) with FES patients (1.82 ± 0.30 pg/ml and 0.66 (0.22, 1.07) mg/L). Meanwhile, serum Hcy levels were significantly lower (t = 2.459, $P = 0.020$) in HC group (15.24 ± 0.82 μmol/ml) than in FES patients (20.18 ± 1.83 μmol/ml).

mRNA Expressions of OXT and OXTR Genes in HC Group and FES Patients

The mRNA expressions of OXT and OXTR genes were semi-quantitative and performed with real-time PCR. The expressions of OXT and OXTR mRNA were significantly lower ($P = 0.000$ and $P = 0.007$, respectively) in the HC group (0.16 ± 0.01 and 0.14 ± 0.01, respectively) than in FES patients (0.27 ± 0.02 and 0.20 ± 0.02, respectively). These results were shown in **Figure 2**.

DISCUSSION

The etiology of schizophrenia is still not known with certainty. One possible breakthrough is the OXT system, which presented potential therapeutic efficacy similar to antipsychotic drugs (APDs).

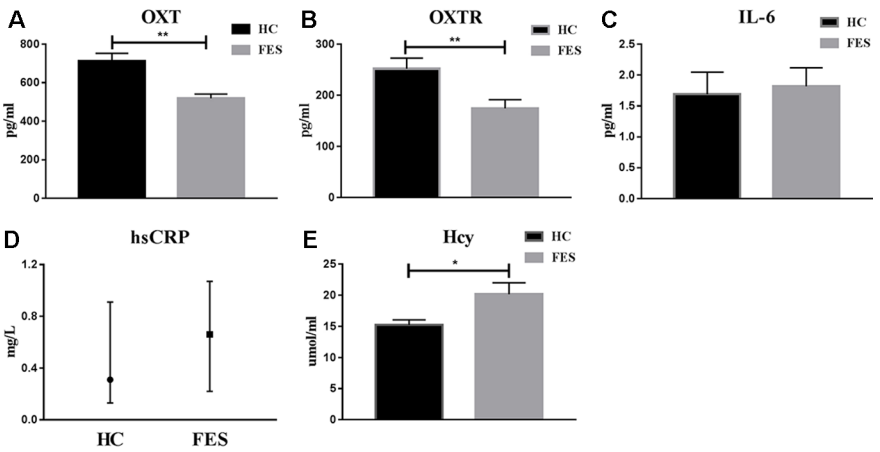

FIGURE 1 | Serum OXT, OXTR, IL-6, hsCRP and Hcy levels in HC group and FES patients. * means $P < 0.05$, ** means $P < 0.01$.

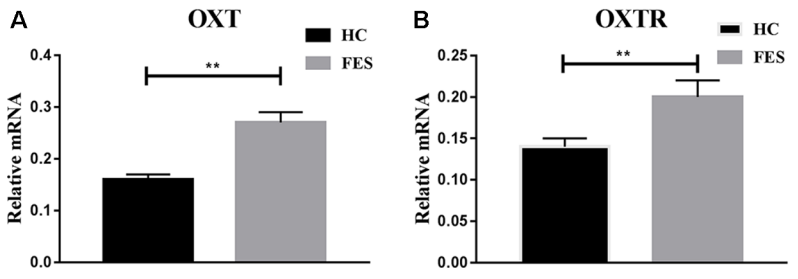

FIGURE 2 | mRNA expressions of OXT and OXTR genes in HC groups and FES patients. * means $P < 0.05$, ** means $P < 0.01$.

In this study, we found that serum OXT and OXTR levels were significantly lower in FES patients than in the HC group. This is consistent with two recent reports indicating diminished plasma OXT levels in SZ patients (8, 32). Based on these results, a pile of RCTs have investigated the efficacy of intranasal OXT on improving positive symptoms, negative symptoms and cognitive deficits in SZ patients (33–36). Although there is a minority of negative results (37, 38), most of these RCTs showed significant improvement in clinical symptoms of SZ patients using doses ranging from 10 to 40 IU for single-dose or twice daily (1). Meanwhile, we found that the mRNA expression of OXT and OXTR in FES patients was significantly higher than in the HC group in the peripheral blood lymphocytes, which is in accordance with a previous study by Yang et al. (13). However, Uhrig et al. reported downregulation of OXTR mRNA in the temporal cortex and a decrease in receptor binding in the vermis (14), but OXT mRNA expression in the brain areas has been scarcely reported. Thus, the relationship between peripheral and central mRNA expression deserves further exploration. It is worth noting that the increased serum OXT and OXTR levels were contrary to the downregulation of mRNA expression of OXT and OXTR genes. We speculate that the transcription of OXT and OXTR genes is working normally, but with the disrupted interpretation, transportation and release of OXT and OXTR proteins. Another possibility is that the elevated serum Hcy levels contributed to the increased OXT and OXTR.

Another goal of the study was to explore whether there are disturbances in the inflammation system of SZ patients. It has long been speculated that immuno-inflammatory disorders are associated with SZ. A meta-analysis performed by Miller et al. showed that some cytokine alterations (including IL-6) were significantly associated with SZ (39). HsCRP is one of the most frequently used nonspecific markers of inflammation state mediated by proinflammatory cytokines such as IL-6. In this study, our results showed that serum IL-6 and hsCRP levels in FES patients were slightly higher than those in the HC group with no significant difference. SZ is a heterogeneous disease with multiple pathogenic factors. Although the OXT system is abnormal in FES patients, the immuno-inflammatory indicators are normal in this study, suggesting that immune disorders might not be the main contributor of SZ. Despite the inconsistency of our observation with most of the previous studies, there are some reports indicating no difference between SZ patients and HC groups in IL-6 levels (40). Most studies reported higher serum hsCRP levels in SZ patients than HC groups (15, 41), which is contrary to our results. One possible explanation is that APD treatment raises serum hsCRP levels in SZ patients (42). Another speculation is that the OXT system might not obviously be related to inflammation in humans despite the positive effect of IL-6 on OXT secretion in mice (30).

Hcy alteration has been shown to be associated with many psychiatric disorders, including SZ. In this study, we found that serum Hcy levels were significantly higher in FES patients than in the HC group, which is in agreement with the majority of reported results (26, 43, 44). It was reported that more severe negative symptoms are associated with higher Hcy level, and there is a negative correlation between duration of untreated psychosis (DUP) and Hcy level (25). Usually, the elevation of serum Hcy level is considered to be a pathogenic factor for the development of SZ. However, the exact mechanism of increased serum Hcy levels in SZ patients is not definite, and it is speculated that poor nutrition, tobacco consumption, alcohol, coffee and polymorphisms in the enzymes of Hcy metabolism can all contribute to elevated Hcy levels (45, 46). Therefore, the one-carbon metabolism in FES patients seems to be a future direction to elucidate the psychopathology of SZ. Future research should focus on the expression of these parameters in the brain nuclei and finding out whether OXT administration, anti-inflammatory treatment and lowering serum Hcy levels could improve the symptoms of schizophrenia patients.

In summary, we found that serum OXT and OXTR levels were significantly lower in FES patients, while the mRNA expression of OXT and OXTR genes were significantly higher in FES patients. The serum Hcy levels were also significantly higher. These results suggested that the dysfunction of the OXT system and Hcy metabolism underlay the pathogenesis of SZ, and the negative results of inflammatory indicators must be interpreted with caution considering the moderate sample size.

AUTHOR CONTRIBUTIONS

CL designed the study. KH and XZ acquired the data, which HT and XY analyzed. YL wrote the article, which all authors reviewed and approved for publication.

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Altered Activity of SK Channel Underpins Morphine Withdrawal Relevant Psychiatric Deficiency in Infralimbic to Accumbens Shell Pathway

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Drug addiction can be viewed as a chronic psychiatric disorder that is related to dysfunction of neural circuits, including reward deficits, stress surfeits, craving changes, and compromised executive function. The nucleus accumbens (NAc) plays a crucial role in regulating craving and relapse, while the medial prefrontal cortex (mPFC) represents a higher cortex projecting into the NAc that is active in the management of executive function. In this study, we investigated the role of the small conductance calcium-activated potassium channels (SK channels) in NAc and mPFC after morphine withdrawal. Action potential (AP) firing of neurons in the NAc shell was enhanced *via* the downregulations of the SK channels after morphine withdrawal. Furthermore, the expression of SK2 and SK3 subunits in the NAc was significantly reduced after 3 weeks of morphine withdrawal, but was not altered in the dorsal striatum. In mPFC, the SK channel subunits were differentially expressed. To be specific, the expression of SK3 was upregulated, while the expression of SK2 was unchanged. Furthermore, the AP firing in layer 5 pyramidal neurons of the infralimbic (IL) cortex was decreased *via* the upregulations of the SK channel-related tail current after 3 weeks of morphine withdrawal. These results suggest that the SK channel plays a specific role in reward circuits following morphine exposure and a period of drug withdrawal, making it a potential target for the prevention of relapse.

Keywords: morphine, conditioned place preference, nucleus accumbens, medium spiny neurons, medial prefrontal cortex, small conductance calcium-activated potassium channels (SK channels)

INTRODUCTION

Drug addiction can be viewed as a mental health disorder caused by maladaptive neural plasticity (1). It involves long-term and persistent dysregulation of neural circuits, particularly in motivational systems and reward systems (2). Opioids, including morphine, are the first-line choice for the management of chronic pain and moderate-to-severe acute pain in both cancer and noncancer patients (3). However, repeated morphine exposure can lead to addiction, and relapse after morphine withdrawal occurs easily in the majority of individuals (3–5). Furthermore, increasingly higher doses of morphine are frequently used to overcome tolerance. This stratagem also exposes

patients to a higher risk of developing severe adverse reactions and side effects in the reward circuitry and can lead to withdrawal symptoms (6, 7).

The ventral tegmental area (VTA)–nucleus accumbens (NAc) circuit plays a crucial function in drug reward processing (8–10). The general understanding of the reward circuitry originates with the VTA, which is composed of 60–65% dopaminergic neurons (11, 12). Moreover, a previous study has shown that practically all known abused drugs increase DA release in the NAc core or shell, which exerts its effects *via* the activation of DA receptors located on medium spiny neurons (MSNs) (13). The NAc region of the ventral striatum is a main part of the reward circuitry, and is of great importance for various aspects of addiction, such as relapse and craving (9). MSNs are the predominant cell type in both the shell and core regions of NAc, and have specific features including spiny morphology, low basal firing rates *in vivo*, and lack of spontaneous activity *in vitro* (6, 14). MSNs in the NAc core appear to be indispensable for assigning motivational values to discrete stimuli associated with reward or aversion, and particularly to renewing these values as environments change (15). MSNs in the NAc shell drive behavioral reactions to repeated exposure to rewarding experiences, such as chronic opioid administration (15). The prelimbic (PL) and infralimbic (IL) cortices, the two subregions of the medial prefrontal cortex (mPFC), play essential roles in the neural circuits for memory extinction and influence the extinction of drug memories, thus reducing the risk of subsequent relapse (16, 17). While the PL projects to both the core and shell of NAc, the IL projects to the shell preferentially (18, 19).

Action potential (AP) firing plays a foremost role in the mechanism of storing and processing information by changing the strength of connections between neurons (20). The generation of signals in NAc core and shell neurons is believed to be related to certain behavioral events, such as the activation of primary reward, motivation, reinstatement/relapse induced by opioid exposure, and several categories of drug-related cues, together with several classes of conditioning occurrences (21–24). In neurons, small conductance calcium-activated potassium channels (SK channels) influence somatic excitability by contributing to afterhyperpolarization and modulate synaptic plasticity by coupling to N-Methyl-D-aspartate (NMDA) receptors (25). There are two homologous SK channels (SK2 and SK3) expressed in striatal neurons (24, 26). SK channels are gated solely by intracellular Ca^{2+} ions, and the activity of SK channel is regulated by protein phosphatase 2A (PP2A) and protein kinase CK2 (CK2) (27).

Numerous lines of evidence indicate that the activated opioid receptors in VTA contribute to the effects of morphine in NAc (28–31). Several studies have focused on the NAc core and shell firing during short-term morphine withdrawal *in vivo* (32, 33). A recent report demonstrated that the molecular neuro-adaptations in the NAc core and lateral dorsal striatum could potently enhance drug-seeking activity (34). In addition, the reduction in the function of the SK channel, which increases tonic firing in the NAc core after long-term alcohol self-administration and protracted abstinence, may indicate that it is a critical regulator of motivation after abstinence (34–36). However, it is still unclear whether the alterations of neuronal firing could persist after long-term morphine withdrawal, and whether it could change the expression or activity of the SK channel in these regions.

Our previous study demonstrated that the ability of the transient receptor potential vanilloid 1 (TRPV1) to regulate excitatory glutamatergic transmission in the NAc is enhanced during morphine withdrawal (37). To further comprehend the significance of these neuro-adaptations for morphine withdrawal, we assessed the effect of long-term morphine withdrawal on the SK channel in the NAc and mPFC. In this study, we investigated whether morphine withdrawal can alter the expression or activity of subtypes of SK channels in the NAc core and shell (ventral striatum) and mPFC during the withdrawal period. Our study linked SK channels induced electrophysiological changes in a specific brain region to an increase in drug-seeking behavior.

MATERIALS AND METHODS

Animals

Sixty Sprague–Dawley rats (male, 150–250 g body weight) were obtained from the Animal Care Committee of the Fourth Military Medical University (FMMU) (Xi'an, China). All experimental methods and procedures were carried out in compliance with the guidelines and regulations of Animal Care and Use Committee, FMMU, and this study was approved by the institutional ethical committee of Tangdu hospital, FMMU (Approval No. 2017LCYJ002). The rats were individually housed in cages at standard humidity (approximately 50%) and room temperature (20–23°C) with a 12-h light–12-h dark cycle (lights on at 8:00 AM). All tests were performed during the light phase. The animals were allowed to habituate to the laboratory conditions for 7 days before the beginning of conditioned place preference (CPP) pretest.

Drugs

Morphine was obtained from Shenyang No. 1 Medical Drugs Company (Shenyang, China) and saline was acquired from Disai Biological Pharmaceutical Company (Xi'an, China). Saline (0.9%) or morphine (10 mg·kg^{-1}) administrations were provided as subcutaneous (s.c). injections for seven consecutive days followed by 3 weeks of long-term withdrawal. The protocol of morphine administration was based on previous literature with CPP (37–39).

Conditioned Place Preference Procedure

CPP was carried out fundamentally as described previously (38, 39). Rats were trained to acquire CPP in the conditioning apparatus (Noldus Information Technology Co., Ltd, Netherlands). Briefly, the CPP training consisted of three different parts. 1) CPP pretest (day 1): the rats were individually placed in the intermediate compartment and allowed to move freely for 900 s in all compartments. Rats were excluded from the study for having an unconditioned side bias if they spent more than two-thirds of the total time in one of the compartments. 2) CPP conditioning period (days 2–8): morphine (10 mg·kg^{-1}) and saline were administered on alternating days to the

morphine-treated CPP rats, and they were immediately placed in the least preferred compartment for 45 min. 3) CPP test (day 9): the rat had access freely to all compartments for 900 s. The "preference time score" (PTS) was termed as time spent in the drug-paired compartments minus the time spent in the saline-paired compartments. The movement of each rat was recorded using a video camera on top of the compartments, and total time spent in each of the conditioning compartments was measured using ETHOVISION 3.1 software (Noldus Information Technology Co., Ltd., Netherlands).

Slice Preparation and *Ex Vivo* Electrophysiology

Rats from saline-treated groups or morphine-treated groups were anaesthetized with an intraperitoneal injection of chloral hydrate (15 mg·kg^{-1}, Aoxin Chemical Factory, Yangzhou, China) and decapitated. The brains were rapidly removed and submerged in ice-cold modified artificial cerebrospinal fluid (ACSF) containing 225 mM sucrose; 119 mM NaCl, 2.5 mM KCl, 4.9 mM $MgCl_2$, 0.1 mM $CaCl_2$, 26.2 mM $NaHCO_3$, 1.0 mM NaH_2PO_4, 1.25 mM glucose; 1 mM ascorbic acid; and 3 mM kynurenic acid. The brain was removed rapidly, and coronal slices (250–300 μm) containing the NAc shell or IL cortex were cut in the same modified ACSF as described previously (38). The slices were recovered at 32°C in carbogen-bubbled ACSF comprising 126 mM NaCl, 2.5 mM KCl, 1.2 mM $MgCl_2$, 2.4 mM $CaCl_2$, 18 mM $NaHCO_3$, 1.2 mM NaH_2PO_4, and 11 mM glucose, with pH 7.2–7.4 and 301–305 mOsm. During the trials, the slices were submerged and continuously perfused with carbogen-bubbled ACSF warmed to 32°C, and picrotoxin (50 μM; Sigma, USA) and CNQX (10 μM; Sigma, USA) were added to block GABA receptors and AMPA-type glutamate receptors. The trials were restricted to the GABAergic MSNs, which comprise more than 90% of the efferent neurons within the NAc core and shell, while other cell types can easily be set apart by a large soma or by very high rates of firing and a larger after hyperpolarization potential (AHP) (40–42).

Whole-cell recordings were attained using a Multiclamp 700B amplifier (Axon Instr., USA). Neurons were patched with a 3–5 MΩ micropipette, which was pulled using a P-97 puller (Sutter Instr., USA). The intracellular solution contained 130 mM KOH, 2.8 mM NaCl, 17 mM HCl, 20 mM HEPES, 105 mM methane sulfonic acid, 0.3 mM EGTA, 2.5 mM MgATP, 0.25 mM GTP, pH 7.2–7.4, 275–285 mOsm. A low level of the calcium-buffering agent EGTA was included in the pipette solution in order to sustain calcium-dependent potassium currents during whole-cell current and voltage clamp recordings (34). To measure firing, current pulses were applied using a patch amplifier in current clamp mode, and a sequence of seven to eight current pulses (300-ms duration, 20 pA apart) were applied every 30 s. The minimum current amplitude was adjusted for each neuron so that the first pulse was just subthreshold for spike firing. Depolarizing pulses were interspersed with a 33.3-pA hyperpolarizing pulse to examine the input resistance. Using the anterior commissure (AC), the lateral ventricles, and the dorsal striatum as landmarks, the NAc shell and IL cortex could be readily found in the experiments of patch clamping. The shape of NAc shell in coronal section is approximately like a ring. The distance from NAc shell to AC is about 4–13 mm. Individual neurons from NAc Shell or layer 5 pyramidal cells located in the IL subregion of the mPFC were visually identified using an upright infrared differential interference contrast microscope (BX51WI; Olympus, Japan).

Western Blotting

The protein expression of the SK2 and SK3 subunits was assayed in the NAc core and shell (ventral striatum) during drug withdrawal as described previously (26, 34, 43). Lysis buffer contained 50 mM Tris-HCl, 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, pH 8.0, supplemented with 1% protease inhibitor cocktail (P8340; Sigma Aldrich, USA). Antibodies used were SK2 C-terminus (cat. # APC-045, 1:800, Alomone, Jerusalem, Israel), SK3 N-terminus (cat. # APC-025, 1:800, Alomone, Jerusalem, Israel), β-actin (1:5,000, TA-09; ZSGB-BIO Co., Beijing, China). Fresh samples were lysed in lysis buffer (previously mentioned). Then, the protein concentration was determined using a bicinchoninic assay kit (Beyotime, Ltd., Haimen, China) according to the kit manufacturer's protocol. Equal quantities of protein from the NAc core and shell, dorsal striatum, or mPFC were resolved on 8% acrylamide SDS-PAGE gels and electrophoretically transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA). The membranes were blocked for 2 h in 5% skim milk diluted in PBS/tween [PBS containing Triton X-100 (PBST), 0.01 M PBS with 0.1% Tween 20] at 37°C with gentle shaking. The membranes were then incubated overnight with antibodies reactive against the SK2 C-terminus or SK3 N-terminus, overnight at 4°C in 4% skim milk. After being washed in PBST, the membranes were incubated with the HRP-conjugated secondary antibodies. Blots were developed with chemiluminescence (Chemi Doc XRS+; Bio-Rad, CA, USA). Band intensity was quantified and analyzed using ImageJ 4.0 (National Institutes of Health, Bethesda, MD, USA), and the expression of SK2 and SK3 was normalized to that of β-actin (1:5,000, TA-09; ZSGB-BIO Co., Beijing, China).

Immunohistochemistry

The animals were deeply anesthetized with an i.p. injection (15 mg·kg^{-1}) of chloral hydrate (Aoxin Chemical Factory, Yangzhou, China) and transcardially perfused with 100 ml of PBS, followed by 100 ml of PBS with 4% paraformaldehyde (PFA; Sigma-Aldrich, St. Louis, USA), pH 7.4. Brain tissues were carefully dissected, postfixed overnight with 4% PFA at 4°C, and cut into 30-μm sections using a vibratome (VT1000S; Leica, Wetzlar, Germany). For SK3 and NeuN immunostaining, sections were washed in 0.1 M phosphate buffer. The slices were then incubated in PBS with 0.2% Triton X-100 for 10 min, followed by washing with PBS (3×5 min), blocked in 1% normal horse serum in 0.1 M phosphate buffer for 30 min at room temperature, and subsequently incubated overnight at 4°C with the following primary antibodies: mouse monoclonal anti-NeuN (cat. # MAB377, 1:1,000; Millipore, Billerica, USA) and rabbit anti-SK3 monoclonal antibody (cat. # APC-025, 1:500, Alomone, Jerusalem, Israel) in PBS. Following washing in PBS (3×5 min), Cy2-conjugated anti-mouse IgG (1:200,

Jackson ImmunoResearch Laboratories, USA) and Cy3-conjugated anti-rabbit IgG (1:200, Jackson ImmunoResearch Laboratories, USA) were used for fluorescence detection. Nuclei counterstaining was performed using 4',6-diamidino-2-phenylindole dihydrochloride (DAPI; cat. D9542, Sigma). Fluorescence images were captured using a confocal microscope (A1; Nikon, Japan).

Statistical Analysis

The results of *ex vivo* electrophysiological recording were analyzed using pClamp 10.2 software (Axon Instr., USA) and Origin 9.0 software (Origin Lab, Northampton, MA, USA). For the reason that a different number of neurons was recorded for each rat, baseline spike firing and voltage clamp parameters (including baseline input/output slope, AP waveform and input resistance parameters, and tail currents) were averaged for all neurons achieved from a given animal, thus acquiring a specific value of each of these parameters for each individual rat. The data are expressed as means ± SEM for all the tests. Statistical analysis of results was presented using paired *t*-tests and ANOVA for the data of influences of morphine withdrawal on neuronal firing and AHP currents. Unless otherwise noted, all statistics were presented using a two-way repeated-measures ANOVA (RM-ANOVA). All tests of statistical significance were two-sided and the threshold of statistical significance was set at $p < 0.05$.

RESULTS

Nucleus Accumbens Shell Action Potential Firing was Enhanced After Morphine Withdrawal

We performed *ex vivo* whole-cell patch-clamp recordings to evaluate whether NAc shell AP firing was changed after 3 weeks of withdrawal from seven consecutive days of morphine administration (10 mg·kg^{-1}) *via* subcutaneous injections (**Figure 1A**). **Figure 1B** indicates that the morphine-treated group spent a significantly longer time in the drug-paired compartment, compared to the saline-treated group (t = 4.273, $p = 0.0003$, n = 12). *Ex vivo* electrophysiological experiments were carried out in current-clamp mode, where 300-ms depolarizing current pulses (both sub- and suprathreshold for firing) were used to elicit AP firing (**Figure 1C**). The resting membrane potential of each neuron was set to –90 mV before provoking the firing. The number of APs was significantly increased in NAc shell neurons after 3 weeks of withdrawal from

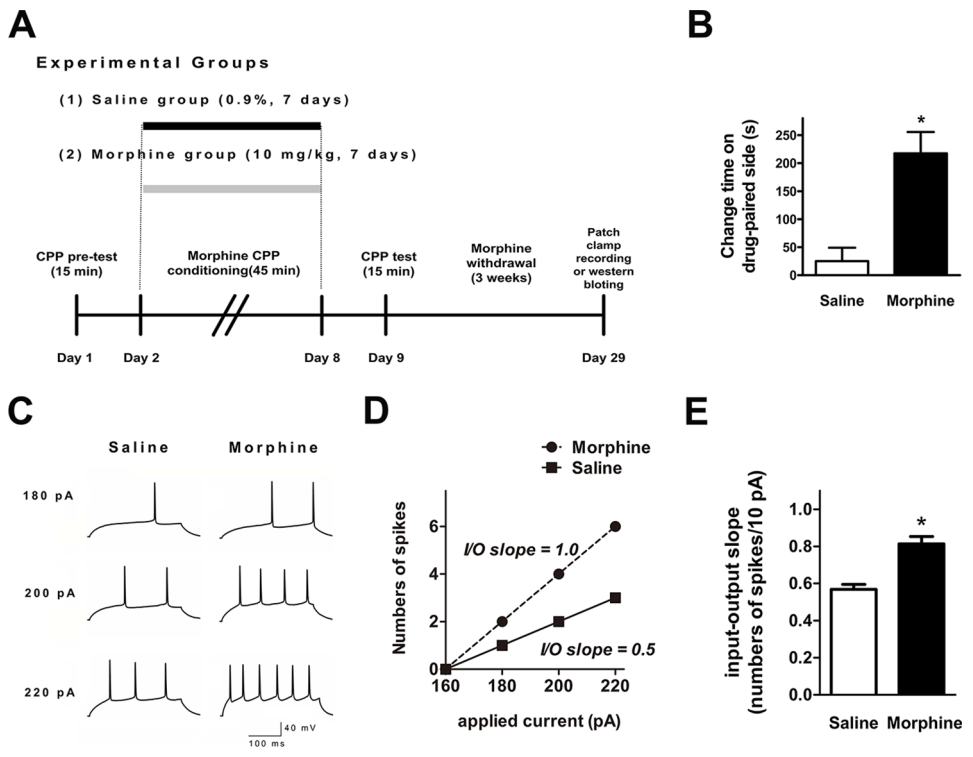

FIGURE 1 | Spike firing in the nucleus accumbens (NAc) shell was significantly enhanced after 3 weeks of morphine withdrawal. **(A)** Morphine-induced conditioned place preference of experimental groups over time. All animals were sacrificed 3 weeks after the final conditioned place preference (CPP) test. **(B)** For the CPP, the difference in the time spent in the less-preferred and drug-paired compartment between the preconditioning and postconditioning phase (n = 12). *$p < 0.05$ compared with the saline group. **(C)** Example traces of action potential (AP) generation evoked in response to depolarizing current steps in NAc shell neurons from saline mock-treated rats or morphine withdrawal rats. **(D)** Example input/output relationships (I/O slope) derived from the saline mock treatment group and morphine withdrawal group traces in (C). **(E)** Grouped data showing enhanced spike firing in NAc shell neurons from the saline mock treatment group versus morphine withdrawal group. The data are shown as means ± S.E.M., *$p < 0.05$ vs. saline.

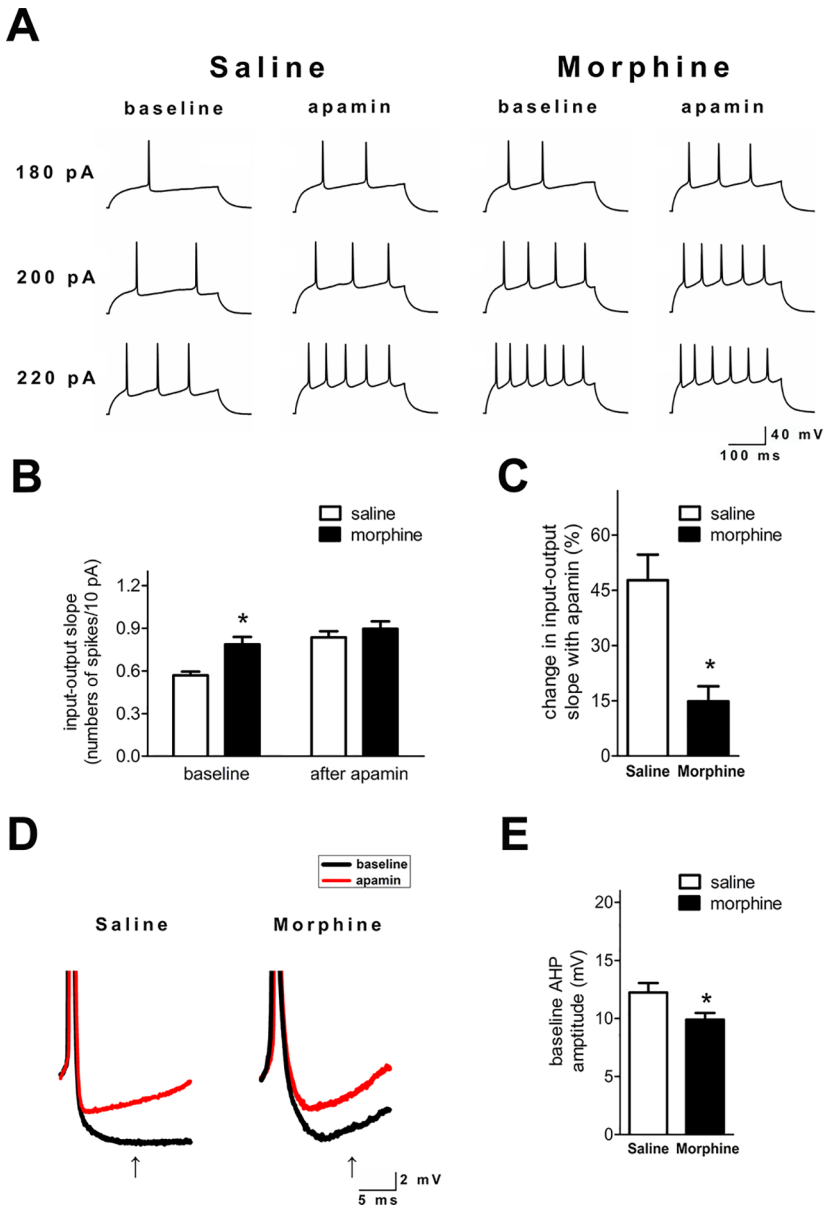

FIGURE 2 | SK inhibition enhanced neuronal firing in the NAc shell after 3 weeks of morphine withdrawal. **(A)** Examples illustrating that SK inhibition produced a greater enhancement of the firing rate in neurons from the saline mock treatment group versus the morphine withdrawal group. **(B)** Grouped data showing the changes of input/output slopes after apamin addition in both groups. **(C)** The relative proportions of apamin-induced changes in the I/O slope were significantly greater in neurons from the saline mock treatment group than those from the morphine withdrawal group. **(D)** Magnification of the AHP in **(A)** illustrating the amplitude of the AHP threshold at 15 ms after the AP threshold (arrow). **(E)** Grouped data showing that the amplitude of the AHP was significantly reduced in neurons from the morphine withdrawal group versus the saline mock treatment group. The data are presented as means ± S.E.M., $^*p < 0.05$ vs. saline.

morphine injection (**Figure 1C** and **D**). In order to describe the input/output relationship between spike firing and a series of depolarizing current pulses, we used the "input/output slope" (I/O slope) to analyze the data as done in a previous study (34). The slope was calculated from the number of spikes generated in the last subthreshold current pulse and the first three suprathreshold current pulses. NAc shell neurons from morphine-treated rats exhibited a significantly larger basal I/O slope than neurons from saline-treated rats (**Figure 1E**, saline: n = 12, 0.57 ± 0.03 AP/10 pA; morphine: n = 18, 0.81 ± 0.04 AP/10 pA, t = 5.176, $p = 0.0001$).

SK Inhibition Differentially Enhanced Firing in Nucleus Accumbens Shell Neurons From Morphine and Saline Control Rats

SK inhibition with apamin enhanced the *ex vivo* NAc shell firing in both groups (**Figure 2A**). However, the I/O slope

was significantly greater in NAc shell neurons from morphine withdrawal rats, but there were no significant differences between the two groups after exposure to apamin (**Figure 2B** and **C**; saline control: n = 14 from 10 rats; morphine withdrawal: n = 12 from 10 rats; apamin: $F_{(1,36)} = 9.818$, $p = 0.004$; group: $F_{(1,36)} = 17.89$, $p < 0.001$; apamin x group: $F_{(1,36)} = 6.336$, $p = 0.085$; two-way RM-ANOVA; $^*p < 0.05$ morphine withdrawal group versus saline control group before apamin). Thus, SK inhibition by apamin eliminated the difference in the I/O slopes of the two groups after apamin exposure, which suggests that basal alterations in firing reflect differential SK function. Specifically, a decrease in basal SK currents could enhance the neuronal excitability in rats after morphine withdrawal. Moreover, the magnitude of the slower component of the AHP at 15 ms after the AP threshold was significantly decreased in NAc shell neurons from the morphine withdrawal animals versus the saline control animals at baseline (**Figure 2D** and **E**; t = 2.334, $p = 0.035$; $^*p < 0.05$ morphine withdrawal versus saline control).

SK Currents in the Nucleus Accumbens Shell Were Reduced After Morphine Withdrawal

To directly test NAc shell SK function after morphine withdrawal, we used voltage clamp methods to isolate SK currents (34, 44). The neurons were held at –70 mV, then depolarized for 400 ms to voltages ranging from –40 to –10 mV (in 10-mV steps) prior to being brought back to –70 mV. A tail current was evident upon returning to –70 mV (**Figure 3A** and **A'**), which may reflect slow ion channel deactivation. Peak tail currents were significantly smaller in NAc shell neurons from the morphine withdrawal animals than in those from saline-treated controls, and SK inhibition with apamin nearly abolished the tail current in both groups (**Figure 3A'** and **B**; saline control group: n = 12 from 8 rats; morphine withdrawal group: n = 14 from 11 rats; baseline: saline 113.5± 16.2 pA, morphine 81.1 ± 8.2 pA; after apamin: saline 7.9 ± 2.0 pA, morphine 6.3 ± 2.2 pA; apamin: $F_{(1,34)} = 3.98$, $p = 0.054$; group: $F_{(1,34)} = 112.14$, $p < 0.001$; apamin x group: $F_{(1,34)} = 3.27$, $p = 0.0795$; two-way RM-ANOVA; $^*p < 0.05$ morphine withdrawal group versus saline control group before apamin). These imply that the peak tail current can be largely seen as SK-mediated currents (34, 44). Average basal tail current peak amplitudes at voltages from -40 to -10 mv in the saline control group were 42.2 ± 5.2, 36.0 ± 6.8A, 72.6 ± 8.6, and 65.1 ± 8.4 pA, and those in the morphine withdrawal group were 115.6 ± 13.6, 85.4 ± 7.6, 135.3 ± 15.4, and 100.5 ± 12.5 pA, respectively (**Figure 3C**; n = 16 for saline control, n = 20 for morphine withdrawal; voltage: $F_{(1,136)} = 131.78$, $p < 0.001$; group: $F_{(3,136)} = 414.49$, $p < 0.001$; voltage × group: $F_{(3,136)} = 18.76$, $p < 0.001$; two-way RM-ANOVA; $^*p < 0.05$ morphine withdrawal group versus saline control group).

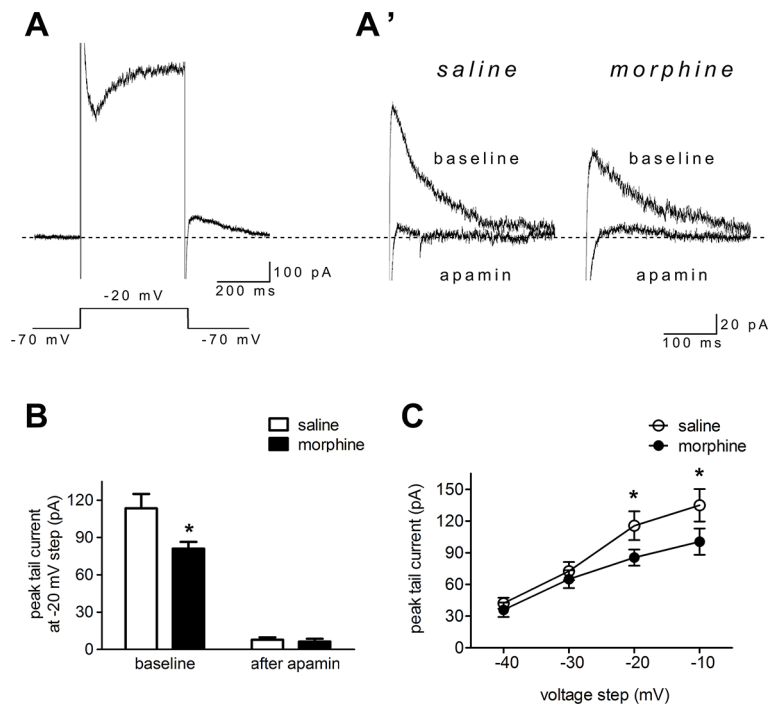

FIGURE 3 | Morphine withdrawal decreased SK currents in NAc shell neurons. **(A)** An example of an entire current response upon depolarization to –20 mV from a –70-mV holding potential in voltage clamp mode, with an apparent tail current after returning to –70 mV following the depolarization; **(A')** magnified example tail currents. **(B)** Grouped data showing that peak tail currents were reduced in neurons from the morphine withdrawal group versus the saline mock treatment group. **(C)** Peak tail currents (induced by depolarization to –40 to –10 mV) were significantly different in neurons from the morphine withdrawal group than in those from the saline mock treatment group. The data are shown as means ± S.E.M., $^*p < 0.05$ vs. saline.

Protein Expression of the SK2 and SK3 Subunits was Changed in Both the Medial Prefrontal Cortex and Nucleus Accumbens Core and Shell During Morphine Withdrawal

To verify whether the expression of SK channels in the mPFC and NAc core and shell (ventral striatum) changed during morphine withdrawal, we examine the expression of its SK2 and SK3 subunits, which are most abundant in the mPFC, NAc core and shell, and dorsal striatum (24, 26, 34). The results demonstrated that SK2 subunit expression was significantly decreased in the NAc core and shell of the morphine withdrawal rats compared to that of saline control rats (**Figure 4A** and **B**; saline: n = 12; morphine: n = 12; location: $F_{(1,66)} = 5.05$, $p = 0.0442$; group: $F_{(2,66)} = 45.56$, $p < 0.001$; location × group: $F_{(2,66)} = 12.53$, $p = 0.0012$; two-way RM-ANOVA, $^{*}p < 0.05$ morphine withdrawal versus saline control in NAc). In addition, no changes of protein expression of the SK2 subunit were observed in the mPFC and dorsal striatum (**Figure 4A** and **B**). Interestingly, the results showed that the expression of SK3 was significantly increased in the mPFC and reduced in the NAc core and shell of morphine withdrawal rats compared to that of saline control rats (**Figure 4C** and **D**; saline: n = 10; morphine: n = 12; location: $F_{(1,60)} = 4.43$, $p = 0.0570$; group: $F_{(2,60)} = 22.57$, $p < 0.001$; location × group: $F_{(2,60)} = 27.65$, $p < 0.001$; two-way RM-ANOVA, $^{*}p < 0.05$ morphine withdrawal versus saline control, $^{\#}p < 0.001$ morphine withdrawal versus saline control in mPFC, $^{*}p < 0.05$ morphine withdrawal versus saline control in NAc). Thus, morphine withdrawal was associated with enhanced protein expression of the SK3 but not the SK2 subunit in the mPFC, suggesting that enhanced SK3 expression likely contributed to the observed increase in SK currents after morphine withdrawal. **Figure 4E** shows the SK3/neuN immunostaining of layer 5 pyramidal neurons in the IL, which preferentially projects to the NAc shell (**Figure 4E**; saline control group and morphine withdrawal group). These data show there are a high proportion of SK3 positive neurons in Layer 5 of IL after morphine withdrawal or saline control.

Action Potential Firing and SK Currents Were Changed in the Infralimbic Cortex After Morphine Withdrawal

To verify whether the neuronal excitability and the function of the SK channel changed in layer 5 pyramidal neurons of the IL cortex after morphine withdrawal, electrophysiological experiments were performed. In current-clamp mode,

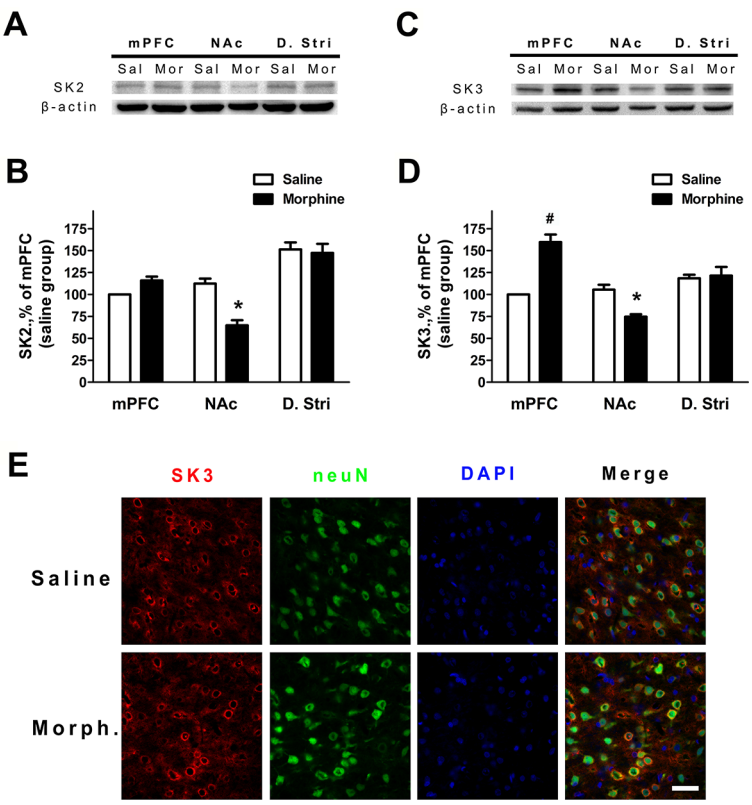

FIGURE 4 | The protein expression of SK2 and SK3 subunits was changed in different brain regions after 3 weeks of morphine withdrawal. **(A)** Representative Western blots showing the changes of SK2 subunit protein expression in mPFC, NAc, and dorsal striatum after morphine withdrawal. **(B)** Quantitative analysis of SK2 subunit protein expression in **(A)**, normalized to β-actin. The data are shown as means ± S.E.M., $^{*}p < 0.05$ vs. saline. **(C)** Representative Western blots showing the changes SK3 subunit protein expression in mPFC, NAc, and dorsal striatum after morphine withdrawal. **(D)** Quantitative analysis of SK3 subunit protein expression in **(C)**, normalized to β-actin. The data are shown as means ± S.E.M., $^{*}p < 0.05$ vs. saline. (E) SK3/NeuN/DAPI-positive neurons in the infralimbic (IL) cortex after 3 weeks of morphine withdrawal; scale bar = 50 μm.

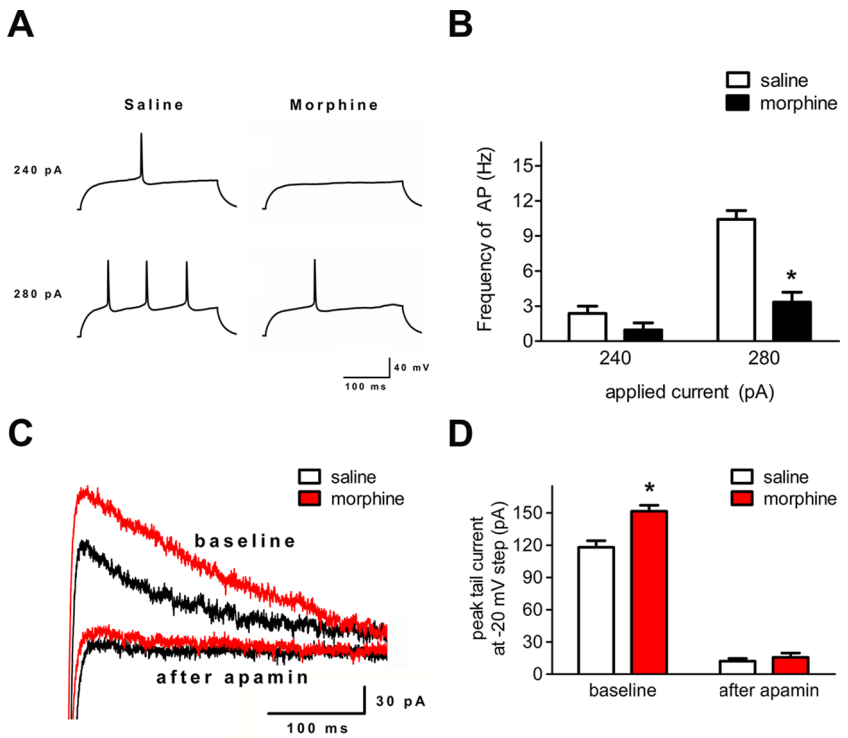

FIGURE 5 | Spike firing and SK currents were changed in layer 5 pyramidal neurons of the IL cortex after 3 weeks of morphine withdrawal. **(A)** Example traces of AP generation evoked in response to depolarizing current steps in pyramidal neurons from saline mock treatment rats or morphine withdrawal rats. **(B)** Grouped data showing reduced spike firing in neurons after 3 weeks of morphine withdrawal. The data are shown as means ± S.E.M., $^*p < 0.05$ vs. saline. **(C)** Examples of magnified tail currents induced by depolarized to –20 mV from a –70-mV holding potential, after returning to –70 mV following the depolarization. **(D)** Grouped data showing that peak tail currents were increased in neurons from the morphine withdrawal group versus the saline mock treatment group.

depolarizing current pulses (300 ms, 240–280 pA) were applied to elicit AP firing (**Figure 5A**). The number of APs was significantly decreased in IL neurons (**Figure 5A** and **B,** saline control group: n = 18 from 14 rats; morphine withdrawal group: n = 21 from 14 rats current: $F_{(1,52)} = 35.10$, $p < 0.001$; group: $F_{(1,52)} = 52.54$, $p < 0.001$; current × group: $F_{(1,52)} = 15.51$, $p < 0.001$; two-way RM-ANOVA, $^*p < 0.05$ morphine withdrawal group versus saline control group). Peak tail currents were significantly larger in IL neurons from the morphine withdrawal group than in those from the saline control group (**Figure 5C** and **D**; saline control group: n = 15 from 9 rats; morphine withdrawal group: n = 18 from 12 rats; baseline: saline 118.5 ± 14.1 pA, morphine 151.6 ± 10.2 pA; after apamin: saline 12.2 ± 3.4 pA, morphine 15.7 ± 4.9 pA; apamin: $F_{(1,38)} = 15.27$, $p = 0.0175$; group: $F_{(1,38)} = 643.43$, $p < 0.001$; apamin × group: $F_{(1,38)} = 9.94$, $p = 0.0344$; two-way RM-ANOVA, $^*p < 0.05$ morphine withdrawal group versus saline control group).

DISCUSSION

Our results show that chronic morphine withdrawal increases the intrinsic neuronal excitability of MSNs in the NAc shell. We identified a relationship between neuro-adaption and SK channels. Our data also showed that the increased AP firing was mediated by the slow component of the afterhyperpolarization current, which could be eliminated using the SK channel antagonist apamin. Furthermore, we investigated the protein expression of SK channel subunits in the NAc and mPFC. The data demonstrated that the expression of the SK2 and SK3 subunits was significantly reduced in the NAc after 3 weeks of morphine withdrawal, while it was not altered in the dorsal striatum. We further investigated the expression and function of SK channels in the mPFC, and the data showed that morphine withdrawal decreased the intrinsic excitability and SK current in layer 5 pyramidal neurons of the IL cortex.

Drug addiction represents a dramatic dysregulation of neural circuits that lead to reward deficits and stress surfeits, craving changes during the reward or stress process, and compromised executive function (45). Dopaminergic neurons located in the VTA and projecting to the NAc play a key role in the processing of reward-related stimuli, including those associated with drug abuse (46). The changes of craving and deficits in executive function involve the dysregulation of crucial afferent projections from the prefrontal cortex (PFC) and insula to the basal ganglia and extended amygdala (47, 48). The medial PFC plays an important role in the higher-order executive processes and sends highly organized projections to subcortical regions controlling motivation (49). A previous study showed that chronic withdrawal from repeated

morphine exposure elicits potentiation in both glutamatergic synaptic strength and intrinsic excitability of MSNs in the NAc shell (50). Moreover, a recent study reported that morphine-induced plasticity changes in IL cortex–NAc shell projections could regulate the reinstatement of morphine-evoked CPP (51). Our data confirmed that the I/O slope, which reflects the intrinsic excitability of the NAc shell, was enhanced after 3 weeks of morphine withdrawal (**Figure 1E**), and showed that AP firing and SK currents of the layer 5 pyramidal neurons in the IL cortex were changed after morphine withdrawal (**Figure 5B** and **D**). These findings confirm the presence of distinct neuronal changes in the IL cortex–NAc shell projections that may provide targetable molecular mechanisms for future pharmacotherapies. Furthermore, these observations may be evidence of a relationship between morphine-induced dysfunction of the higher-order executive processes in mPFC and morphine-induced changes of craving-related signals in the NAc.

Several studies have pointed out that numerous ion channel mechanisms are involved in opioid exposure or withdrawal (52–55). Our own previous study demonstrated that the ability of TRPV1 to regulate excitatory glutamatergic transmission in the NAc is enhanced during morphine withdrawal (37). Cognitive inhibition of craving is one of the cognitive control techniques involved in the higher-order executive function of the mPFC that enhance the patient's ability to cope with cravings and prevent relapse (56). Motivations or emotions generated by subcortical circuits involving the NAc may be powerfully modulated by the PFC (57, 58). Some studies also reported that mPFC inactivation also reduces morphine-induced DA release in the NAc, which regulates the neuronal excitability and function (59). Our results suggest that the decrease in SK channel function in the NAc shell after morphine withdrawal reflects the decreased levels of both SK2 and SK3 subunits. At the same time, the increased SK function in the mPFC after morphine withdrawal may be related to the enhanced levels of the SK3 subunit. Our study links the molecular changes in SK channel function in a particular brain region containing the reward and inhibition control circuitry, the NAc and mPFC, to dynamically balanced alterations of neuronal excitability during the drug-seeking period.

Opioids modulate the expression of genes involved in neuroplasticity through epigenetic changes and possible RNA modifications (54). Ultimately opioids perturb the intracellular signaling cascade and neural circuits, whose dysfunction is associated with long-term changes in craving (47). Several studies reported that the mRNA levels of voltage- and calcium-gated potassium channels increased in addicted rats (54). Previous evidence indicated that the changes of SK currents and neuronal excitability in the NAc represent a critical mechanism that facilitates the motivation to seek alcohol during abstinence (34). The data of the present study indicate that the protein expression of the SK2 and SK3 subunits was decreased in the NAc, while the protein expression of the SK3 subunit was increased in the mPFC after morphine withdrawal (**Figure 4B** and **D**). The present study thus adds more detailed information on the role of functional alterations in neuronal excitability and protein expression of SK channel subunits induced by morphine withdrawal. Moreover, our findings demonstrate that the function of the higher brain cortex, which participates in executive function, was altered due to a decrease in neuronal excitability *via* an increase in the SK current due to higher expression of the SK3 subunit.

Overall, the present findings offer new insights into the involvement of SK channel subunits in the NAc shell MSNs and layer 5 pyramidal neurons of IL cortex neurons. Understanding the molecular mechanisms active during morphine withdrawal is a crucial step on the path toward finding potential therapies for opioid relapse. Because ion-channel-mediated neuro-adaptation can facilitate drug-seeking behavior during morphine withdrawal, we explored the relationship between intrinsic excitability and SK function in the NAc and mPFC. For an optimal therapeutic strategy for addiction, all these factors and their interplay need to be taken into consideration. Further studies are needed to determine the pathophysiological role of SK channels in the process of reward and inhibition control.

ETHICS STATEMENT

Male Sprague–Dawley rats were obtained from the Animal Care Committee of the Fourth Military Medical University (Xi'an, China). All experimental procedures were carried out in accordance with the Institutional Animal Care and Use Committee guidelines at the Fourth Military Medical University and had received ethical approval from the institutional ethical committee of Tangdu hospital, the Fourth Military Medical University (Approval No. 2017LCYJ002).

AUTHOR CONTRIBUTIONS

X-LW and LQ designed the study. SG and JF designed the behavioral paradigm. JF and YL collected data for the behavioral paradigm. LQ, YZ, QW, and XW processed the brain tissue samples. YW, YZ, and NL collected and analyzed the data. X-LW and SH interpreted the data. X-LW, LQ, and YW wrote and edited the manuscript. All authors critically reviewed the content and approved the final version for publication.

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REVIEW
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Dysfunction in Serotonergic and Noradrenergic Systems and Somatic Symptoms in Psychiatric Disorders

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Somatic symptoms include a range of physical experiences, such as pain, muscle tension, body shaking, difficulty in breathing, heart palpitation, blushing, fatigue, and sweating. Somatic symptoms are common in major depressive disorder (MDD), anxiety disorders, and some other psychiatric disorders. However, the etiology of somatic symptoms remains unclear. Somatic symptoms could be a response to emotional distress in patients with those psychiatric conditions. Increasing evidence supports the role of aberrant serotoninergic and noradrenergic neurotransmission in somatic symptoms. The physiological alterations underlying diminished serotonin (5-HT) and norepinephrine (NE) signaling may contribute to impaired signal transduction, reduced 5-HT, or NE release from terminals of presynaptic neurons, and result in alternations in function and/or number of receptors and changes in intracellular signal processing. Multiple resources of data support each of these mechanisms. Animal models have shown physiological responses, similar to somatic symptoms seen in psychiatric patients, after manipulations of 5-HT and NE neurotransmission. Human genetic studies have identified many single-nucleotide polymorphisms risk loci associated with somatic symptoms. Several neuroimaging findings support that somatic symptoms are possibly associated with a state of reduced receptor binding. This narrative literature review aimed to discuss the involvement of serotonergic and noradrenergic systems in the pathophysiology of somatic symptoms. Future research combining neuroimaging techniques and genetic analysis to further elucidate the biological mechanisms of somatic symptoms and to develop novel treatment strategies is needed.

Keywords: somatic symptoms, mono-aminergic neurotransmitters, norepinephrine (NE), serotonin (5-HT), pathophysiology

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Somatic symptoms in psychiatric disorders are symptoms that have persistent bodily complaints but have found no explanatory structural, organic causes, or other specified pathology after a sufficient physical examination or investigation by physician or healthcare providers (1). The most common somatic symptoms include musculoskeletal pain, abdominal pain, fatigue, dizziness, ear, nose, throat symptoms and gastrointestinal symptoms. Somatic symptoms, including a range of physical symptoms, such as pain (e.g., stomachache, headache, and neuropathy), muscle tension, body shaking, difficulty breathing, heart palpitation, fatigue, and gastrointestinal symptoms, are commonly seen in individuals with major depressive disorder (MDD), anxiety disorders, and other psychiatric disorders (2). About 76% of patients diagnosed with depression had somatic symptoms, such as back pain, headache, stomach pain, migraine, and neuropathic pain (3, 4). Severity of

depression is positively associated with the frequency and severity of somatic symptoms (5, 6). Somatic symptoms were able to predict subsequent self-reported symptoms of depress in women patients with MDD (7). Somatic symptoms are also a common feature of anxiety disorders (8). Patients suffering from anxiety disorders often have somatic complaints, such as feeling jittery, muscular tension, stomachache, headache, and sweating (9). Young patients with anxiety disorders are more likely to report somatic symptoms than their healthy peers (10). Moreover, studies have found that somatic symptoms are related to acute stress disorder, posttraumatic stress disorder, and personality disorders (11).

A heightened awareness of certain body sensations may trigger somatic symptoms (12). Somatic symptoms may be a mechanism through which patients with depression or anxiety react to their emotional distress (13). Childhood neglect and adversity, childhood abuse, chaotic lifestyle, stress, alcohol abuse, and substance abuse are the risk factors for somatic symptoms. Women more likely present somatic symptoms than men. Antidepressant drugs, including selective serotonin (5-HT) reuptake inhibitors (SSRIs), dual 5-HT and norepinephrine (NE) reuptake inhibitors (SNRIs), and tricyclics are effective in treating somatic syndromes. The therapeutic effects of these drugs may be due to their effects on the 5-HT and NE systems. Hence, abnormal serotonergic and noradrenergic systems, which are indicated by low level of monoamine neurotransmitters, reduced production and/or release, pre- and/or post-synaptic receptor dysfunction, excessive self-inhibition, and decreased excitatory inputs, may play a predominant role in the pathophysiology of somatic symptoms. This narrative review paper was to summarize the role of serotonergic and noradrenergic systems in the development somatic symptoms.

5-HT AND NE NEUROTRANSMITTER SYSTEMS

5-HT is a monoamine neurotransmitter. Serotonergic neurons exist mainly in the dorsal and median raphe nuclei in the brainstem. 5-HT is released into the extracellular space from presynaptic nerve terminal, and is cleared primarily by neurotransmitter uptake, mediated by the 5-HT transporter. 5-HT receptors contain presynaptic autoreceptors and postsynaptic receptors. The 5-HT autoreceptors are key factors in the self-inhibitory mechanism of serotonergic neuronal activity. Activation of inhibitory 5-HT autoreceptors regulates 5-HT neuronal firing and maintains the homeostasis of the serotonergic system. 5-HT exerts its effects through its interaction with 5-HT receptors, including the 5-HT1 to 5-HT7 families, some of which have several subtypes (14, 15). The effects of 5-HT depend on the cell type and subtype of the receptor it acts on. A growing body of evidence has suggested the role of the serotonergic system in somatic symptoms. Most previous studies focused on 5-HT1, 5-HT2A, 5-HT2C, 5-HT3, 5-HT4, and 5-HT7 receptors.

NE is also a monoamine neurotransmitter in the brain. The primary source of NE is neurons in the locus coeruleus, which is situated at the floor of the fourth ventricle in the pontine brain (16). Other noradrenergic neurons include nuclei of the lateral tegmentum and the solitary tract. NE released from the locus coeruleus regulates brain function through various ways. The locus coeruleus receives afferent projections from the various brain regions, such as the insular cortex, the hypothalamus, the central amygdala, and the cerebral cortex (17–20). Adrenoceptors can be classified into two groups, the α-adrenergic family (comprising the α1 and α2 subtypes) and the β-adrenergic family (comprising the β1, β2, and β3 subtypes). NE exerts its action through either α1-, α2-, β1-, or β2-adrenoceptors in the central nervous system. α2-adrenoceptors located presynapticlly (autoreceptors) on the neural terminals inhibit NE release, whereas presynaptic β2-adrenoceptors enhance NE release upon activation. The release and effect of NE are modulated through interaction with these receptors. The action of NE in the synaptic cleft is ended largely through the neuron terminal reuptake by the NE transporter.

ANIMAL MODEL OF 5-HT AND NE IN SOMATIC SYMPTOMS

Rodent models of nociception demonstrate altered 5-HT and NE system function, and certain antidepressants enhance 5-HT and NE transmission. Both 5-HT and NE play a role in the descending inhibitory pathway, formed by projections descending from the brainstem or the midbrain to the spinal cord, which normally suppress painful inputs; thus, malfunction of these neurotransmitters may play a role in somatic syndromes, such as fibromyalgia and chronic headache (21).

It has been proposed that changes in 5-HT levels are part of pathophysiology of neuropathic pain. In mice deficient in 5-HT transporter (5-HTT-/- mice), the extracellular levels of 5-HT are increased in the brain; but the overall tissue concentrations of 5-HT are decreased. In contrast to wild-type mice, the 5-HTT-/- mice show absence of thermal hyperalgesia but present bilateral mechanical allodynia after an incomplete unilateral chronic constrictive injury of the sciatic nerve. The 5-HTT-/- mice also demonstrate higher levels of 5-HT in injured nerves and lower overall tissue levels of 5-HT than the wild-type mice (22, 23). Furthermore, the wild-type mice experience a longer period of thermal hyperalgesia and show higher levels of 5-HT in the sciatic nerves than the 5-HTT-/- mice after intra-plantar injection of Freund's complete adjuvant (23). These findings suggest that 5-HT participates in nociception transmission and in reducing spinal inhibition in bilateral mechanical allodynia.

Using a rodent model of neuropathy induced by inflammatory mediator and nerve injury, Liu found that the intrathecal administration of the 5-HT1A receptor antagonist, rather than the 5-HT2 or 5-HT3 receptor antagonist, significantly attenuates the increased anti-nociception induced by the administration of morphine in intra-periaqueductal gray. Therefore, the 5-HT1A receptor is involved in the spinal descending inhibition pathway that suppresses nociception transmission in rats with nerve injury or inflammation (24). Abbott et al. found that the intra-plantar injection of 5-HT2A receptor antagonists may lead to peripheral

analgesic effect (25). Another study found that neuropathic pain coincides with noradrenergic system disruption, as indicated by increased locus coeruleus bursting activity; enhanced expression of tyrosine hydroxylase, NE transporter, and α2 adrenergic receptors in the locus coeruleus; and hypersensitive α2 adrenergic receptors (26). Moreover, parathyroid hormone 2 receptor and TIP39 knockout mice display lower baseline nociceptive threshold and decreased inflammatory effect, and a blockade of α2-adrenoceptors could increase the thermal and tactile sensitivity in the knockout mice recovered from neuropathic injury (27). Antidepressant treatment of the SSRI fenfluramine was able to prevent mechanical allodynia, cold allodynia, and tonic pain in the model of neuropathic pain (28). Furthermore, treatment of venlafaxine (a serotonin-norepinephrine reuptake inhibitor (SNRI)), immediately after nerve injury, was able to inhibit the development of neuropathic pain; the antinociceptive effect of venlafaxine likely involves the α2-adrenoceptor (29).

Using a tail-flick rodent model, Eide et al. demonstrated that intrathecal injection of 5-HT1A and 5-HT1B receptor agonists could suppress the nociceptive tail-flick reflex at the spinal cord level, but none of them can change the temperature of tail skin (30). Dogrul et al. reported that the administration of the 5-HT7 receptor antagonist SB-269970 could inhibit the antinociceptive effect produced by systemic morphine administration, indicating that the spinal 5-HT7 receptor influences systemic morphine-induced antinociceptive actions (31). The authors also demonstrated that intrathecal injection of the 5-HT7 receptor antagonist abolishes the antinociceptive and antihyperalgesic effects produced by the systemic administration of paracetamol. Systemically administered paracetamol could stimulate 5-HT7 receptors at the spinal cord and activate descending serotonergic pathways (32).

Other animal models also suggest that somatic symptoms might be associated with impaired serotonergic and noradrenergic systems. In an exercise-induced chronic fatigue rat model, significantly increased levels of 5-HT and 5-HT transporter, decreased 5-HT1A mRNA expression (33), and significantly elevated 5-HT2A mRNA expression were found in various regions of the brain (34). Using an animal model with irritable bowel syndrome (IBS), a study found significantly upregulated expression of 5-HT7 receptor in the ileum and colon tissues compared with the control rats (35). Furthermore, treatment with 5-HT1A agonist/5-HT3 antagonist could inhibit the Bezold–Jarisch reflex and stress-induced defecation in this rat model; thus, agents that exert effects *via* 5-HT1A agonistic and/or 5-HT3 antagonistic activities might be beneficial for IBS treatment (36, 37). Another study also reported abnormal expression of colonic α2-adrenoceptors and NE reuptake transporter in different brain regions in a rat model of IBS (38).

NEUROIMAGING FINDINGS

Relatively few studies have examined 5-HT and NE system alterations in patients with somatic complaints using neuroimaging methods; published imaging studies primarily focused on 5-HT and NE receptors or 5-HT transporter and NET occupancies.

Several positron emission tomography (PET) studies have reported decreased 5-HT1A receptor binding in patients with panic disorder (39, 40), and the low 5-HT1A receptor binding may contribute to somatic symptoms associated with anxiety (41). Similarly, another PET study found a decrease in 5-HT1A binding in patients with chronic fatigue syndrome (42). Decreased 5-HT receptor binding may reflect a reduced number of 5-HT1A receptors or a decreased affinity of 5-HT or other ligands to the receptor. PET studies examining the relationship between 5-HT receptor/transporter binding and responses to noxious heat stimulation in healthy volunteers found a positive correlation between 5-HT2A binding and noxious stimulation (43). However, a negative correlation was observed between 5-HT transporter binding and response to tonic pain (44). Decreased 5-HT1A binding and changed 5-HT2A binding were detected in brain regions, including the hippocampus, amygdala, raphe nucleus, cingulate, insular cortex, prefrontal, parietal, temporal, and occipital cortices. These results suggest that the 5-HT neuronal function affects the activity of various brain regions. Abnormal 5-HT function in various brain regions may contribute to the development and modulation of somatic symptoms.

Shan et al. conducted a longitudinal MRI study to examine progressive brain changes in chronic fatigue syndrome. They found that white matter volumes in the left inferior fronto-occipital fasciculus was significantly reduced in patients with chronic fatigue syndrome (45). This result suggested that white matter abnormality in the inferior fronto-occipital fasciculus is associated with chronic fatigue syndrome. In addition, Chang et al. found subregions of the anterior cingulate cortex may play a role in the pathophysiology of chronic pain syndromes (46).

HUMAN GENETIC STUDIES AND NEUROPHARMACOLOGICAL STUDIES

Markoutsaki et al. reported an association between the population susceptibility of IBS and two single-nucleotide polymorphisms (SNPs) -1438 (G/A) and 102 (C/T) in the 5-HT2A receptor gene. They found that A allele and AA genotype of the -1438 (G/A) polymorphism in the 5-HT2A receptor gene show a significant association with IBS (47). Therefore, the carrier of A allele in this specific polymorphism in the 5-HT2A receptor gene might be a good candidate for IBS susceptibility.

Smith et al. conducted a study in 137 individuals that included patients with chronic fatigue syndrome, patients with mild fatigue, and those with no fatigue as controls. The study examined 77 polymorphisms in genes associated with 5-HT signaling (HTR1A, HTR1E, HTR2A, HTR2B, HTR2C, HTR3A, HTR3B, HTR4, HTR5A, HTR6, and HTR7), synthesis (TPH2), catabolism (MAOA), and transport (SLC6A4). Three biomarkers (-1438G/A, C102T, and rs1923884), located in the HTR2A gene of these polymorphisms examined, were identified to have a significant association with chronic fatigue syndrome. The HTR2A-1438 (rs6311) A allele, allele T of HTR2A C102T (rs6313), and C allele of HTR2A rs1923884 are more common in patients with chronic fatigue syndrome than

in controls. Furthermore, silico analysis revealed that the A allele of -1438 is located in the core of the Th1/E47 consensus sequence and creates an allele-specific binding locus for neurodevelopment-associated transcription factor Th1/E47. These results indicate that polymorphism in the HTR2A gene is involved in the pathophysiology of chronic fatigue syndrome (48). A previous study also reported that the promoter activity in cells or tissues was higher in the A allele carrier of HTR2A-1438 (rs6311) than in the HTR2A G allele carrier. The authors suggest that HTR2A-1438 A polymorphism plays a role in promoter activity (49).

Felippotti et al. examined the role of noradrenergic mechanisms in the locus coeruleus in postictal antinociceptive effects. They microinjected yohimbine (an α2-receptor antagonist) and propranolol (a β-receptor antagonist) into the unilateral locus coeruleus and found that both yohimbine and propranolol injection to the locus coeruleus area caused a distinct decrease in antinociceptive effects. The blockade effect of yohimbine was more prominent compared with that of propranolol, possibly due to the presynaptically located α2-adrenoceptors in locus coeruleus neurons. These effects are associated with the noradrenergic regulation in locus coeruleus, suggesting that both α2- and β-adrenoceptors in locus coeruleus are involved in the mechanism underlying postictal antinociception (50).

CLINICAL THERAPEUTICS

Drugs used to treat somatic symptoms include antidepressants, antipsychotics, antiepileptics, and natural products, such as St. John's wort (51). The effectiveness of these drugs has been reported by a limited number of studies (52–54). The proposed mechanisms include inhibition of spinal cord painful inputs, inhibition of prefrontal cortical areas that are involved in noxious activity, treatment of comorbid disease, and the direct effects on somatic symptoms.

Antidepressants are usually classified according to their impacts on neuronal synapses, such as inhibiting presynaptic transporters to block the reuptake of certain neurotransmitters, blocking certain neurotransmitter receptors, or the blockade of monoamine oxidase enzymes. Tricyclic antidepressants block the reuptake of NE and 5-HT neurotransmitters to achieve antidepressant therapeutic effects. However, tricyclic antidepressants also block M1, α1, and H1 receptors simultaneously, which can lead to diverse side effects, such as thirst, constipation, blurred vision, dizziness, orthostatic hypotension, sedation, lethargy, and weight gain in clinical applications. Other antidepressants include SSRIs such as fluoxetine, sertraline, paroxetine, and citalopram, SNRIs such as duloxetine and venlafaxine, and 5-HT receptor inhibitors such as mirtazapine. Evidence suggests that 5-HT and NE play an analgesic role in treating somatic symptoms through the spinal cord inhibitory descending pain pathway; however, their effects become aberrant in patients with somatic complaints (55–57). 5-HT and NE projection from brainstem descending the spinal cord could suppress painful inputs. The long-term administration of antidepressant treatments may enhance the efficacy of 5-HT synaptic transmission. Tricyclics enhance 5-HT synaptic transmission by increasing the sensitivity of postsynaptic 5-HT1A receptors, whereas SSRIs produce this effect by reducing the function of terminal 5-HT autoreceptors, thereby increasing the amount of 5-HT released. In addition, antidepressants may improve somatic symptoms, such as fatigue, anergy, or trouble sleeping, through their immunoregulatory effect (58–61).

The possible benefit of antipsychotics in somatic symptoms may be due to their analgesic effects (62), but the underlying mechanisms remains unclear. Their analgesic effect may be mediated by 5-HT antagonism (63), α2-adrenoreceptor stimulation (64), or other mechanisms. The mechanisms by which natural products such as St. John's wort treat somatic symptoms also remain unclear. The efficacy of St. John's wort on treating somatic complaints, including headache or gastrointestinal symptoms, is possibly secondary to the improvement in depression (65, 66). The effect of Hypericum extracts for somatic symptoms might be due to the inhibition of the reuptake of 5-HT, NE, and dopamine (67).

The application of antidepressants acting on 5-HT and NE systems for the treatment of somatic symptoms has been supported by many clinical trials and systematic reviews. For instance, a meta-analysis including 94 trials shows that antidepressants can substantially improve somatic symptoms (52). Another meta-analysis has shown that antidepressants appear to be effective in treatment of functional gastrointestinal disorders (68). In general, antidepressants have been used in the treatment of chronic pain syndromes, such as IBS (68, 69), chronic fatigue syndrome (70), fibromyalgia (71, 72), and other related somatic symptoms. Patients with fibromyalgia show low a threshold to pain that is caused by noxious stimuli, possibly due to the deficits in 5-HT and NE systems, which result in the failure to inhibit the painful inputs at the spinal cord level (73, 74). Jackson et al. summarized that antidepressants might be beneficial to treat 11 somatic symptoms including headache, chronic back pain, chronic facial pain, chronic pelvic pain, non-cardiac chest pain, fibromyalgia, IBS, tinnitus, chronic fatigue syndrome, interstitial cystitis, and menopausal symptoms (75).

Recent studies have found that the number of somatic symptoms in patients with depression who have not achieved remission show a significantly greater number of somatic symptoms than those who have achieved remission after 8 weeks of treatment with fluoxetine (76). Fluoxetine is also effective in improving somatic symptoms in adolescent patients with anxiety disorders and depression comorbid with severe somatic symptoms, such as stomachaches, restlessness, palpitations, blushing, sweating, muscle tension, and trembling/shaking (8). The relief of somatic symptoms may be due to the pharmacologic action to increase the levels of 5-HT in the synaptic cleft. In addition, another study reported that somatic symptoms markedly decreased in patients with depression after treatment with mirtazapine, a 5-HT receptor inhibitor (77). These results suggest that the 5-HT system dysfunctions are involved in the pathological mechanism of somatic symptoms.

Litoxetine is an antidepressant drug that combines 5-HT3 antagonism and 5-HT transporter inhibition to prevent the gastrointestinal and pain-augmenting side effects induced by SSRIs, such as sertraline (78). 5-HT3 receptor antagonists are effective in relieving symptoms, inhibiting urgency, and

prolonging the transit of small and large bowel in IBS patients with diarrhea. However, agonists of 5-HT3 receptor was able to activate intestinal motility and shorten transit times in IBS patients with constipation (79). Revexepride, a 5-HT4 receptor agonist, could be a safe and effective candidate treatment for gastroparesis, a chronic gastric disorder characterized by clinical symptoms such as abdominal pain, vomiting, nausea, early satiety, postprandial fullness, and bloating (80).

Dolasetron, a 5-HT3 alternative inhibitor, is efficacious in the treatment of fibromyalgia (81). Administration of 5-HT3 receptor antagonists can significantly decrease pain intensity in patients with fibromyalgia and neuropathic pain (55). Thus, specific antagonism of 5-HT3 receptors is considered a possible treatment method for fibromyalgia, a condition characterized by chronic fatigue and pain (81).

Furthermore, several drugs acting on the 5-HT1A, 5-HT1B, and 5-HT1D receptors have been evaluated for their efficacy in treating migraine. Peroutka et al. demonstrated that migraine drugs, including ergotamine, dihydroergotamine, and sumatriptan, show affinity for the 5-HT1A, 5-HT1B, and 5-HT1D receptors, suggesting that these 5-HT receptors are involved in the action of the these antimigraine drugs (82, 83). Peroutka et al. summarized that acute antimigraine drugs, such as ergotamine and sumatriptan, show great affinity for 5-HT1D receptors and low affinity for 5-HT1A receptors. It has also been suggested that sumatriptan may not work through 5-HT1B receptors (84). These 5-HT receptors are located on certain intracranial blood vessels. In theory, the reduction of 5-HT may associate with the increased production of pain-inducing or vasoactive substances in the perivascular space, which may lead to angiectasis and migraine. 5-HT1D receptor agonists may facilitate 5-HT release and inhibit noxious stimulation (82). This phenomenon possibly explains the high prevalence of migraine in patients with depression.

Drugs acting on the noradrenergic system also have been implicated in the treatment of somatic symptoms. The SNRI duloxetine (60mg/day) can effectively reduce overall pain, shoulder pain, back pain, and time in pain while awake in patients with depression (85, 86). Another SNRI, venlafaxine, is also effective in improving neuropathic pain, as suggested by a randomized, double-blind, 10-week crossover trial (87). In addition, several double-blind, placebo-controlled trials reported that depressive patients with fatigue symptoms experienced overall improvement as well as remission in fatigue-related complaints following the treatment of levomilnacipran extended-release, a type of SNRI antidepressant. These results suggest that SNRIs are effective in the treatment of somatic symptoms (88).

Tricyclics, dual-acting antidepressants seem to be more effective than SSRIs in treating somatic symptoms. A meta-analysis suggested that tricyclics are superior to SSRI antidepressants in the therapy of various somatic symptoms, such as headache, idiopathic pain, fibromyalgia, tinnitus, irritable bowel disorder, and chronic fatigue in patients with chronic depression (52). Amitriptyline, the most studied tricyclic medication, is effective in treating at least one of the following complaints: pain, sleep, morning stiffness, overall improvement, fatigue, function symptoms, and tenderness. Desipramine predominantly inhibits the reuptake of NE and, to a minor extent, inhibits the reuptake of 5-HT. Dinan et al. suggested that treatment of desipramine may alleviate IBS by blocking the abnormal function of central $\alpha 2$ noradrenergic receptors (89). Tricyclic antidepressants are effective in treating somatic symptoms, possibly because of their ability to block the reuptake of 5-HT and NE.

5-HT AND NE INTERACTION

Substantial interactions exist between the serotonergic and noradrenergic systems in the central nervous system. Both 5-HT neurons and noradrenergic neurons are active and affect each other in the locus coeruleus (90–92). In addition, the serotonergic system interacts with other neurotransmitter systems such as dopaminergic inputs from the midbrain corpus striatum (93) and glutamatergic and inhibitory γ-aminobutyric acid-ergic inputs from forebrain regions (94) and local interneurons (95–97).

Projections from 5-HT neurons to NE neurons are inhibitory. For instance, rats with damage in 5-HT neurons show a greater firing activity of NE neurons than intact animals (98). Previous studies also demonstrated that long-term administration with SSRIs might increase 5-HT transmission, presumably increasing the effectiveness of 5-HT projections to locus ceruleus and forebrain neurons (99). For instance, Szabo et al. found that the short-term administration of citalopram exerts no effect on the firing activity of NE neurons; however, the long-term treatment of citalopram could produce a progressive reduction of the spontaneous firing activity of NE neurons (100).

Other evidence suggests that the interaction between NE transporter (NET182C) and 5-HT transporter (5-HTTLPR) polymorphisms is associated with susceptibility and electroconvulsive therapy treating response in antidepressant treatment resistant depression patients. Patients with combined NET and 5-HT transporter polymorphism genotypes had poorer treatment responses (101). Moreover, functional and structural interactions with NE, 5-HT and dopamine systems that are known to have an impact on executive control processes (102, 103). Furthermore, researchers observed interactions between 5-HT transporter and a functional NET polymorphism, suggesting 5-HT and NE interplay in shaping goal-directed behavior (103, 104). Most interestingly, interactions of 5-HT transporter and NET polymorphism also influence cognitive and executive functioning, such as target accuracy and event-related potential, latency in n-back task (105).

In addition, studies have shown that mirtazapine can significantly increase the firing of 5-HT neurons and trigger a small but distinct increase in the firing of NE neurons (106, 107). Behavioral tests suggest that depletion of NE might block the effects of some SSRIs as well (108). These results have provided evidence that antidepressants selectively working on the serotonergic system may also indirectly influence the function of the noradrenergic system. In addition, blockade of the 5-HT2A receptor may potentiate the release of NE under the treatment of SSRI (109).

CONCLUSION

Somatic symptoms are highly prevalent in patients with depression, anxiety and some other psychiatric disorders. In this narrative review, we examined the potential role of serotonergic and noradrenergic systems in the development and treatment of various somatic symptoms. Antidepressants may play an important role in the therapy of somatic symptoms by regulating 5-HT and NE neurotransmitter systems at central and peripheral levels. Future research combining neuroimaging techniques and genetic analysis to further elucidate the biological mechanisms of somatic symptoms and to develop novel treatment strategies is needed.

AUTHOR CONTRIBUTIONS

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

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Opposite Expression Patterns of *Spry3* and *p75NTR* in Cerebellar Vermis Suggest a Male-Specific Mechanism of Autism Pathogenesis

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Autism is a genetically complex neurobehavioral disorder with a population prevalence of more than 1%. Cerebellar abnormalities, including Purkinje cell deficits in the vermis, are consistently reported, and rodent models of cerebellar dysfunction exhibit features analogous to human autism. We previously analyzed the regulation and expression of the pseudoautosomal region 2 gene *SPRY3*, which is adjacent to X chromosome-linked *TMLHE*, a known autism susceptibility gene. *SPRY3* is a regulator of branching morphogenesis and is strongly expressed in Purkinje cells. We previously showed that mouse *Spry3* is not expressed in cerebellar vermis lobules VI–VII and X, regions which exhibit significant Purkinje cell loss or abnormalities in autism. However, these lobules have relatively high expression of *p75NTR*, which encodes a neurotrophin receptor implicated in autism. We propose a mechanism whereby inappropriate *SPRY3* expression in these lobules could interact with TrkB and p75NTR signaling pathways resulting in Purkinje cell pathology. We report preliminary characterization of X and Y chromosome-linked regulatory sequences upstream of *SPRY3*, which are polymorphic in the general population. We suggest that an OREG-annotated region on chromosome Yq12 ~60 kb from *SPRY3* acts as a silencer of Y-linked *SPRY3* expression. Deletion of a β-satellite repeat, or alterations in chromatin structure in this region due to *trans*-acting factors, could affect the proposed silencing function, leading to reactivation and inappropriate expression of Y-linked *SPRY3*. This proposed male-specific mechanism could contribute to the male bias in autism prevalence.

Keywords: autism, cerebellum, *SPRY3*, *p75NTR*, pseudoautosomal region, *TMLHE*, carnitine

INTRODUCTION

Autism is a spectrum disorder whose core features include language delay, social deficits, and restricted interests and repetitive behaviours. In addition, there are significant co-morbidities including attention deficit hyperactivity disorder (ADHD), anxiety, intellectual disability, motor delay, and epilepsy, among others (1–3). Recently, the ESSENCE protocol was developed in response to the increasing diagnosis of autism associated with an expanding list of co-morbidities, including cases in which the co-morbidity may be the predominant clinical entity (1, 4). There is also a trend towards the increased diagnosis and inclusion of cases at the mild end of the spectrum (5). The combined effects of these trends may explain the current estimates of autism prevalence in school

age children, exemplified by two recent reports, which found 1.68% prevalence in the USA and 2.85% in Northern Ireland (6, 7).

Heritability estimates for autism are high, ranging from 38% (8) to more than 80% (9, 10), and there is an emerging consensus that the majority of the genetic risk is attributable to common genetic variants of small effect size acting in combination with rare or *de novo* variants of larger effect size (11–15).

A striking and unexplained feature of autism is the preponderance of affected males, with a sex ratio of between 3 and 4 to 1 consistently reported, including in recent large studies (6, 7, 16, 17). Earlier studies often reported more extreme male biases, particularly in milder cases (so-called high functioning autism or Asperger's syndrome), and there is continuing debate on the possibility of a female protective or "camouflage" effect that may result in their under-diagnosis (18–25). Currently, multiple genes and genomic variants are associated with autism with varying levels of confidence; however, the majority are autosomal and do not explain observed sex differences in prevalence (26). Rather than exhibiting sex-specific expression, autism genes may interact with normal regulatory pathways that are themselves sex-specifically regulated (27–29). This is conceptually similar to the proposal that autism genes operate against a background of sex-specific hormone profiles (30–32), and shifts the explanatory burden from the autism genes themselves to the normal sex-specific pathways with which they interact.

We previously analyzed the pseudoautosomal region 2 (PAR2)-linked *SPRY3* gene in autism because it is highly expressed in the cerebellum (33), a region consistently implicated in autism pathogenesis (34–39). *SPRY3* is expressed in Purkinje cells, a key cell type deficient in autism (40, 41), but we note that mouse *Spry3* is not expressed in the cerebellar lobules (VI–VII, X) homologous to those most affected in human autism (33, 41). If this expression pattern is recapitulated in the human, as suggested by a human *SPRY3* promoter–LacZ transgenic mouse strain (33), it suggests two alternative mechanisms by which *SPRY3* could be implicated in loss of Purkinje cells preferentially in these lobules. First, the normal absence of *SPRY3* expression in these lobules may increase their sensitivity to genetic or environmental "insults" that cause Purkinje cell loss. However, this would not explain the male bias. Second, the deregulation and inappropriate overexpression of *SPRY3* in these lobules may be pathogenic, and could provide a male-specific mechanism, as described below. SPRY3 is a receptor tyrosine kinase (RTK) signaling inhibitor that interacts with the TrkB neurotrophin receptor pathway (42), which is implicated in autism and social behavior (43–49).

The X-linked copy of *SPRY3* is adjacent to a known autism gene, *TMLHE*, and a proportion of *SPRY3* transcripts arise from upstream promoters in the X-linked *F8A3* and *TMLHE* regions (33). The *F8A2–F8A3* region contains an inversion polymorphism that could potentially affect the expression of flanking genes, including *SPRY3*. The Y-linked copy of *SPRY3* is epigenetically silenced in normal males (50), which could contribute to the male bias in autism due to X-linkage of the expressed gene copy. Alternatively, deregulation and reactivation of the silenced Y-linked copy could provide a male-specific pathological mechanism. A possible further mode of *SPRY3* deregulation is suggested by the fact that *SPRY3* is upregulated in the liver of piglets fed high levels of carnitine (51). Notably, the gene adjacent to *SPRY3*, *TMLHE*, encodes an enzyme in the carnitine biosynthesis pathway. As carnitine deficiency is implicated in autism causation (52), this suggests a mechanism whereby carnitine levels could impact on *SPRY3* regulation and autism.

In this study, we examined the expression of *SPRY3* and its functionally associated genes in cerebellum, and we analyzed genetic variation in predicted X and Y chromosome regulatory regions that may impact on *SPRY3* expression. We propose a pathogenic mechanism in autism involving *SPRY3* deregulation impacting on the BDNF–TrkB–p75NTR neurotrophin pathway.

MATERIALS AND METHODS

Online Bioinformatics and Other Resources

The following databases and online resources were used in this study: UCSC genome browser (https://genome.ucsc.edu/); GENSAT Brain Atlas of gene expression in EGFP Transgenic Mice (http://gensat.org/index.html); Allen Brain Atlases (http://portal.brain-map.org/; 53); GTEx Portal, v7, updated 09/05/2017 (https://gtexportal.org/home/); SFARI (Simon Foundation Autism Research Initiative; https://www.sfari.org/); AGRE (Autism genetic Resource Exchange; https://research.agre.org/program/descr.cfm). Other websites are listed under "Analysis of PsychENCODE data."

Whole Mount Immunohistochemistry of Mouse Cerebellum

All reagents were from Sigma, UK, unless otherwise stated. Adult male and female C57Bl/6J mice were humanely euthanized under permissions obtained following animal ethics and welfare review by UCC committees, under national and European legislation. Dissected mouse cerebellum was fixed in 4% Paraformaldehyde (PFA)-Phosphate-buffered saline (PBS) for 10 h, post-fixed in methanol–Dimethyl sulfoxide (DMSO) (4:1) overnight at 4°C, and then bleached in methanol–DMSO–30% H_2O_2 (4:1:1) overnight at 4°C. After 3 × 60 min wash in 100% methanol, it was frozen at −80°C and thawed at room temperature (RT) for six cycles in 100% methanol. After rehydrating with 50% methanol, 15% methanol, and PBS for 2 h each, it was digested by proteinase K (10 mg/ml; Sigma, UK) in PBS for 3 min at RT, washed in PBS for 3 × 2 h at RT, and incubated in PBS with 10% goat serum and 0.1% Triton X-100 overnight at 4°C. It was then incubated with anti-Spry3 primary antibody (Abcam, UK) in PBS containing 10% goat serum, 0.1% Triton X-100, 5% DMSO for 48 h at 4°C and washed twice in PBS containing 10% goat serum, 0.1% Triton X-100 for 20 min each, followed by incubation with secondary antibody in PBS containing 10% goat serum, 0.1% Triton X-100, 5% DMSO for 24 h at 4°C. It was then washed twice in PBS containing 10% goat serum, 0.1% Triton X-100 for 2 h each. Immunoreactivity was visualized by incubating the

cerebellum in freshly prepared DAB solution (Sigma, UK) for 3 min at RT. The stained cerebellum was imaged with a Nikon SMZ1500 microscope and Nikon DXM1200 camera.

Droplet Polymerase Chain Reaction Analysis of *F8A2–F8A3* Inversion Genotype

All reagents were from Sigma, UK, unless otherwise stated. Cultured cells were lysed in lysis buffer (0.1 M Tris, 0.2 M NaCl, 5 mM EDTA, 0.4% SDS, and 0.2 mg/ml proteinase K, pH 8.0) at 55°C. Cell DNA was precipitated by adding isopropanol and washed with 70% ethanol. DNA pellet was dissolved in water and digested with *Nru*I and *Bsp*EI (NEB, UK). Polymerase chain reactions (PCRs) were prepared in a total volume of 100 µl with 1× Go-taq buffer (Promega, UK), 25 mM $MgCl_2$, 250 µM dNTPs, 1 µM primers (Eurofins Genomics, Germany): F8A2-F1-2 5′-CAC ATGATGAAAGTGGGAGGA-3′, F8A2-R2-2 5′-GAATGCAACA AATCAGCAAGA-3′, and F8A2-R3-2 5′-TTCAGACCCATATAG TATTACTGGTGA-3′, 30 nM primer F8A2-R1-2 5′-GCATACAC TGCTAGGTGGGAATTCACAGCCACTGGAATGAC-3′, 200 ng digested genomic DNA, and 16 units Go-Taq DNA polymerase.

Emulsion step was carried out by adding PCR reaction dropwise over 30 s to 200 µl light mineral oil with 4.5% v/v Span 80, 0.4% v/v Tween 80, and 0.05% Triton X-100, in a 2 ml Corning Cryo-Tube stirring with a magnetic bar (8 × 3 mm with a pivot ring; VWR) at 1,000 rpm. Emulsions were stirred for 3 min before being overlaid with 30 µl mineral oil. The PCR conditions were 95°C for 120 s; 40 cycles of 95°C for 20 s, 60°C for 30 s, and 72°C for 15 s; 72°C for 5 min. Emulsions were disrupted using 600 µl hexane. Each clean PCR product (2 µl) was amplified in a total volume of 50 µl using primers F8A2-F1-2, F8A2-R2-2, and F8A2-R3-2 and reaction mix: 1× Go-taq buffer, 5 µl 25 mM $MgCl_2$, 250 µM dNTPs, 300 nM primers, and 1 unit Go-Taq DNA polymerase.

Lymphoblastoid Cell Lines and DNA Samples

Cell lines and DNA samples were randomly selected from AGRE (https://research.agre.org/program/descr.cfm) and SFARI (https://www.sfari.org/) resources. AGRE samples are from multiplex families, and SFARI samples are from simplex families. Further details are available from provider websites using sample reference numbers listed below. Additional autism DNA samples were obtained from Prof. David Skuse, University College London. Control DNA samples were from the Caucasian DNA panel from the Coriell Institute for Medical Research, USA. Cells were grown in T25 suspension cell flasks with RPMI-1640 medium supplemented with 10% FBS (Sigma, UK) at 37°C, 5% CO_2.

Cell lines used were as follows (double-underlining indicates samples with F8A2–F8A3 inversion; see Results section):

AGRE: 2095, 2325, 2396, 2479, 2609, 2615, 2659, 2664, 2718, 2815, 2838, 2853, 2880, 2883, 3126, 2742, 2831, 2327, 2628, 2678, 2326, 2791, 2487, 2328.

SFARI: SSC00317, SSC00591, SSC00636, SSC02727, SSC03440, SSC03459, SSC03537, SSC03774, SSC03989, SSC04232, SSC05124, SSC05350, SSC05435, SSC07444, SSC10172, SSC10210, SSC10777, SSC11067, SSC12271.

Long-Range PCR of β-Satellite Repeat

PCR reactions were prepared in a total volume of 50 µl with 25 µl 2× GoTaq Long PCR Master Mix (Promega, UK), 10 µl 300 nM primers (Eurofins Genomics, Germany) (Y-Chr BSR-Del-3F 5′-CACAGGCTGTAGTGCAGGTGATG-3′ and Y-Chr BSR-Del-4R 5′-CTGTGTTGTTGATCTGTCTAATGTTGACA TTA-3′), and 500 ng genomic DNA. The PCR conditions were 95°C for 120 s; 40 cycles of 93°C for 20 s, 60°C for 16 min; final extension of 72°C for 20 min.

Analysis of PsychENCODE Data

We obtained paired-end RNA-seq libraries of cerebellar vermis from 33 autism and 38 controls from PsychENCODE (54). Individual libraries contained 50–200 million reads. Human transcriptome sequence was obtained from the RefSeq database (55), downloaded from NCBI (Annotation Release 108). Raw reads were aligned to the set of human RefSeq transcript sequences using bowtie2 short read alignment program (56). Default parameters were used for local alignments. Reads mapping to only one location in the transcriptome were selected by removing the alignments with "XS:i" bowtie2 tag, which represents reads having more than one possible mapping to the reference. SAMtools version 1.3.1 (57) was used to obtain the sorted BAM alignment files, which were further used to predict the heterozygosity in *SPRY3* expressed sequences. *SPRY3* had a total mapped read count range of 653–4033. SAMtools mpileup (57) and BCFtools (58) were used to characterize variations in mapped reads at each coordinate in the *SPRY3* locus. The frequency of variants at each position was analyzed to estimate the likelihood of heterozygosity. For heterozygous genotypes, it is expected that the probability of finding a nucleotide matching the reference sequence at the single nucleotide polymorphism (SNP) position is 0.5, while for homozygous genotypes it is either 0 or 1.

RESULTS

Cerebellar Lobule Gene Expression Screen Identifies Opposite Expression of *Spry3* and *p75NTR* in Lobules VI–VII and X

We used whole mount immunohistochemistry of cerebellums from adult male and female C57Bl/6J strain mice and confirmed relatively low Spry3 expression in lobules VI–VII and X, as previously noted in mouse Allen Brain Atlas (ABA) and GENSAT data [**Figure 1A**, **B**, **F**; see also Ref. (33)]. We next sought to determine whether other genes share this expression pattern by visually inspecting the spatial expression patterns of genes in sagittal sections of the mouse ABA data as follows: i) 54 genes with biased expression in "Cerebellar cortex, Purkinje layer" under the "Fine Structure Search" option of the mouse ABA (**Supplementary Table 1**); ii) mouse homologues of 87 high-risk autism genes from SFARI (https://www.sfari.org/resource/sfari-gene/#bottom; **Supplementary Table 2**). Three of 54 cerebellar cortex-biased gene set (*Abhd3*, *Lrp8*, and *Plcβ4*) had lower expression in lobules VI–VII and X (**Figure 1G**, **H**, **I**),

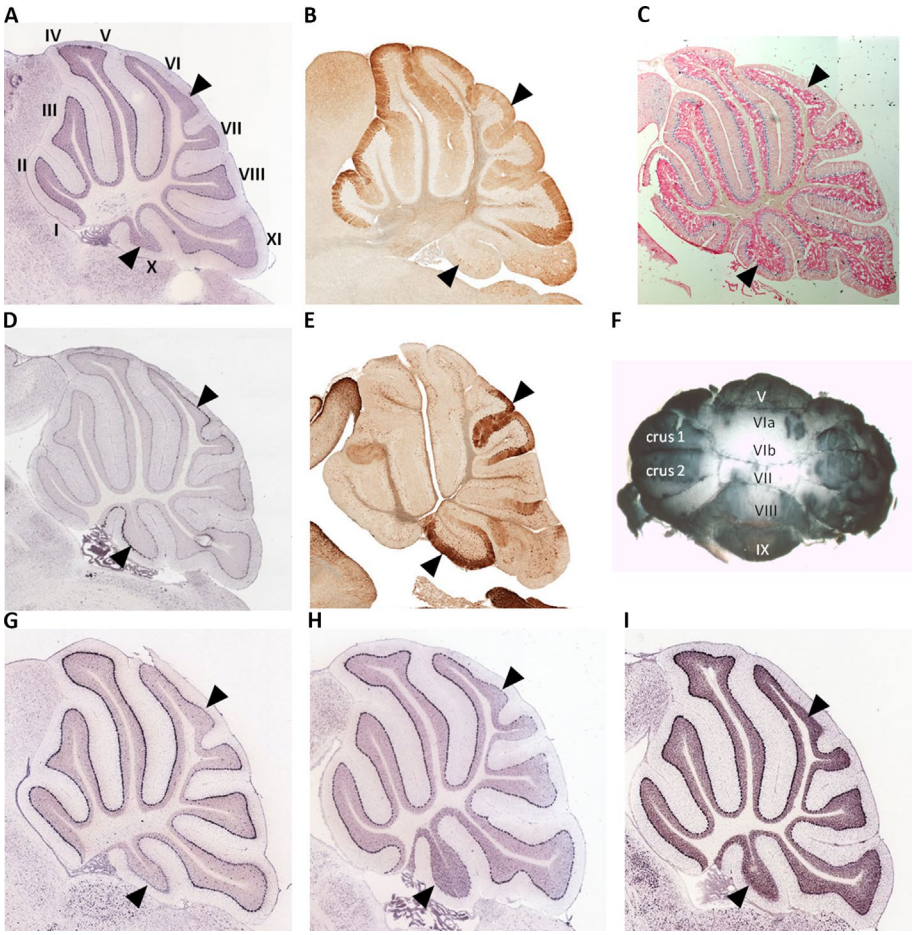

FIGURE 1 | Lobular expression of genes in adult mouse cerebellum. **(A, B)** *Spry3*. **(C)** Human *SPRY3* promoter–LacZ reporter transgenic mouse. **(D, E)** *p75NTR*. **(F)** Representative whole mount immumohistochemistry of adult female mouse cerebellum using anti-Spry3 antibody. **(G–I)** *Abhd3*, *Lrp8*, and *Plcβ4*. Images **(A, D)** and **(G–I)** were from Allen Brain Atlas; **(B** and **E)** were from GENSAT. Data for images **(A–C)** were previously published (31) and are included here for comparison with p75NTR expression. Roman numerals (I–X) in panel A indicate lobule identity. Arrowheads indicate lobules VI–VII and X with notable gene expression patterns.

reminiscent of the *Spry3* pattern, but none of 87 SFARI gene mouse homologues had this pattern; however, many of the latter had faint staining and were difficult to score.

Transcription factors (TFs) predicted to regulate human *SPRY3* (ZNF263, MAZ, PURA, EGR1, PAX6) are expressed in mouse Purkinje cells (33), and GTEx data confirm relatively high expression of these factors in human adult cerebellum (**Figure 2**). However, limited data on these genes in ABA and GENSAT did not allow us to determine whether their spatial expression patterns coincide with *Spry3* lobular expression.

We next examined spatial expression of *Spry1*, *Spry2*, and *Spry4*; neurotrophins (*Ngf*, *Bdnf*, *NTF3*, and *NTF4*); neurotrophin receptors (*p75NTR*, *TrkA*, *TrkB*, and *TrkC*); and *Bex3* (*Ngfrap1*), which encodes a p75NTR interacting protein (59), in cerebellum to determine possible lobular co-expression with *Spry3*. On ABA, *Spry1* and *Spry4* exhibit faint staining, whereas *Spry2* is widely expressed, including in cerebellar Purkinje cells, but does not exhibit a specific lobular expression pattern like *Spry3*. This is consistent with GTEx data in which *SPRY2* has relatively high expression in human cerebellum, whereas *SPRY1* and *SPRY4* exhibit no and low expression, respectively (**Figure 2**).

For the neurotrophins, expression data on GTEx indicated that *BDNF* and *NTF3* are relatively highly expressed in cerebellum, compared to other brain regions, whereas there was no expression of *NGF* and *NTF4* (**Figure 2**). However, on ABA, mouse *Ntf4* is expressed in Purkinje cells, whereas there is no *Bdnf* or *Ngf*, and barely detectable *Ntf3* expression. It is unclear if these species differences reflect biological differences or technical limitations.

The data for neurotrophin receptor expression were generally consistent between ABA (mouse) and GTEx (human) datasets. On ABA, *TrkB* is widely expressed in the brain, including in cerebellar Purkinje cells, and GTEx data also indicated its wide expression in brain including cerebellum (**Figure 2**). *TRKA* and *TRKC* exhibited low and moderate expression, respectively, on GTEx, and no expression was detected on ABA. Analysis of *p75NTR* expression in GTEx suggested that it is virtually absent from the central nervous system, apart from a marginal signal in cerebellum and high expression in the peripheral nervous system

FIGURE 2 | GTEx data for genes associated with *SPRY3* expression and regulation. Cerebellum samples are boxed to highlight expression relative to other tissues: brain–cerebellar hemisphere; brain–cerebellum. Note different scales on TPM (transcripts per million) Y-axis for each gene.

(spinal cord, tibial nerve; **Figure 2**). However, scrutiny of ABA and GENSAT data indicated wide expression of *p75NTR* in the mouse adult brain, but with restricted expression in cerebellar vermis Purkinje cells, largely restricted to lobules VI–VII and X (**Figure 1D**, **E**), the exact opposite of the *Spry3* lobular expression pattern. *Bex3* (and other *Bex* genes; data not shown) is expressed throughout the adult mouse brain (ABA), including in cerebellar Purkinje cells, and is highly expressed in human cerebellum (**Figure 2**).

Genetic Analysis of X Chromosome-Linked Regulatory Sequences Upstream of Human *SPRY3*

The unique genomic configuration of human *SPRY3* due to the evolution of the PAR2 in the hominin lineage suggests that sex-linked upstream elements could regulate expression of X-linked *SPRY3* and epigenetic silencing of Y-linked *SPRY3* (33). X-linked *SPRY3* transcription initiates in the *F8A3–TMLHE* region (33). *F8A3* is associated with inversions involving *F8A1* and *F8A2* (60, 61). The *F8A2–F8A3* interval contains regulatory elements approximately 20 and 60 kb centromeric of F8A3 (**Figure 3**). Inversion of this sequence might affect regulation of flanking genes including *F8A3* region-associated *SPRY3* transcription. We developed a single-molecule droplet PCR assay to determine the orientation of the *F8A2–F8A3* interval using a modified version of Turner et al. (62, 63) (**Figure 4**). We analyzed DNA from autism cases comprising 20 individuals from SFARI resource and 24 individuals from AGRE, representing simplex and multiplex families, respectively (see Materials and Methods for sample identifiers). There were 2/20 and 4/24 inversions compared to the reference sequence (GRCh38/hg38 assembly, December 2013, UCSC browser), which is similar to the 20% frequency (4/20 samples) found in non-autistic *F8* gene-associated hemophilia patients (64).

Analysis of Y Chromosome-Linked Regulatory Sequences Upstream of Human *SPRY3*

We hypothesised that Y chromosome sequences proximal to the Yq–PAR2 boundary act as a silencer of Y-linked *SPRY3*. Scrutiny of this region using the UCSC browser identified a predicted regulatory region that begins 60 kb upstream of the *SPRY3* PAR2 transcriptional start site (TSS) and extends a further 60 kb towards the centromere. This element comprises compositionally distinct regions of ~25 and ~35 kb and is flanked by a 50 kb sequence gap proximally, on the far side of which are the major Yq12 satellite sequences (**Figure 5**). The 25 kb region comprises ~10 kb of simple CATTC and CACTC repeats, while the remaining

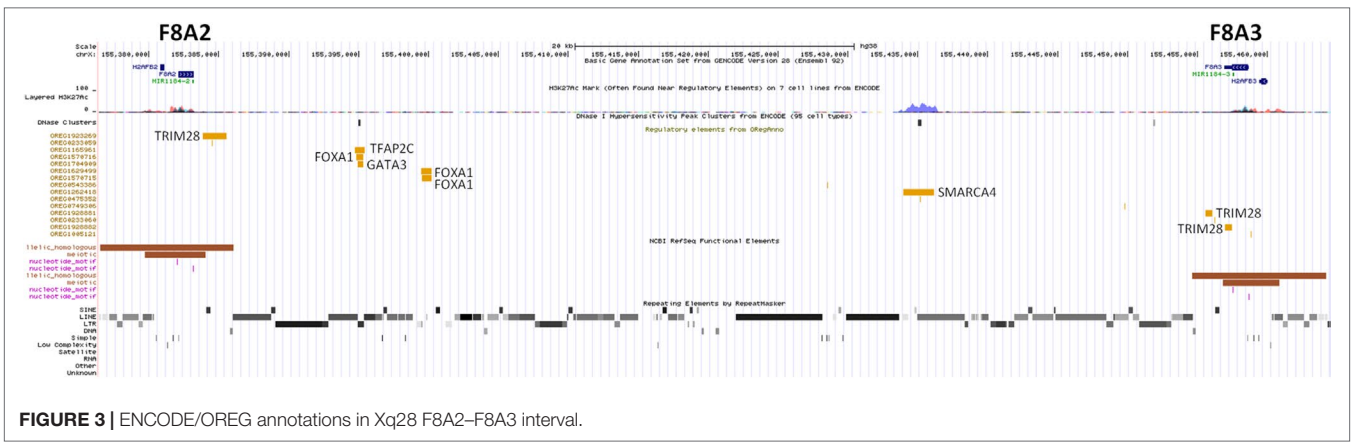

FIGURE 3 | ENCODE/OREG annotations in Xq28 F8A2–F8A3 interval.

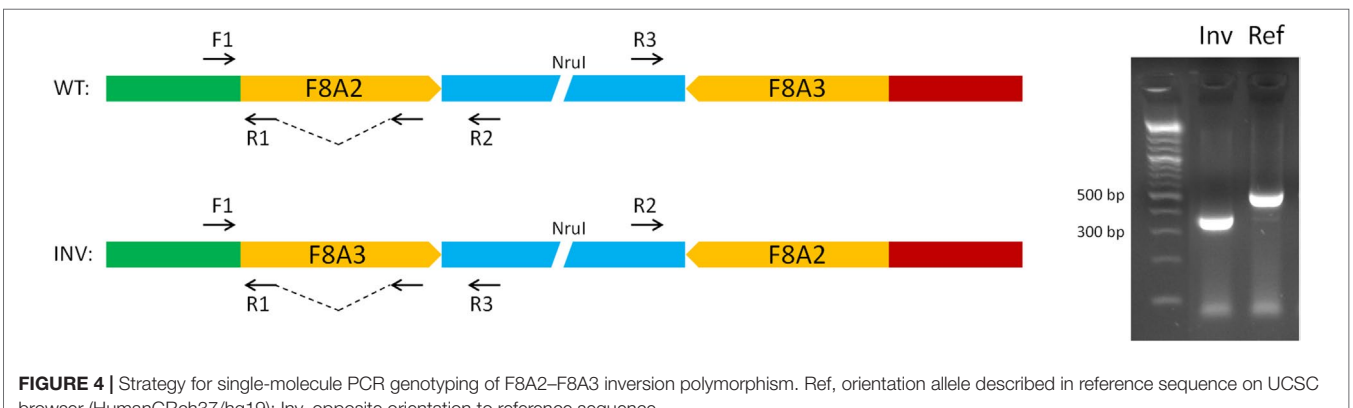

FIGURE 4 | Strategy for single-molecule PCR genotyping of F8A2–F8A3 inversion polymorphism. Ref, orientation allele described in reference sequence on UCSC browser (HumanGRch37/hg19); Inv, opposite orientation to reference sequence.

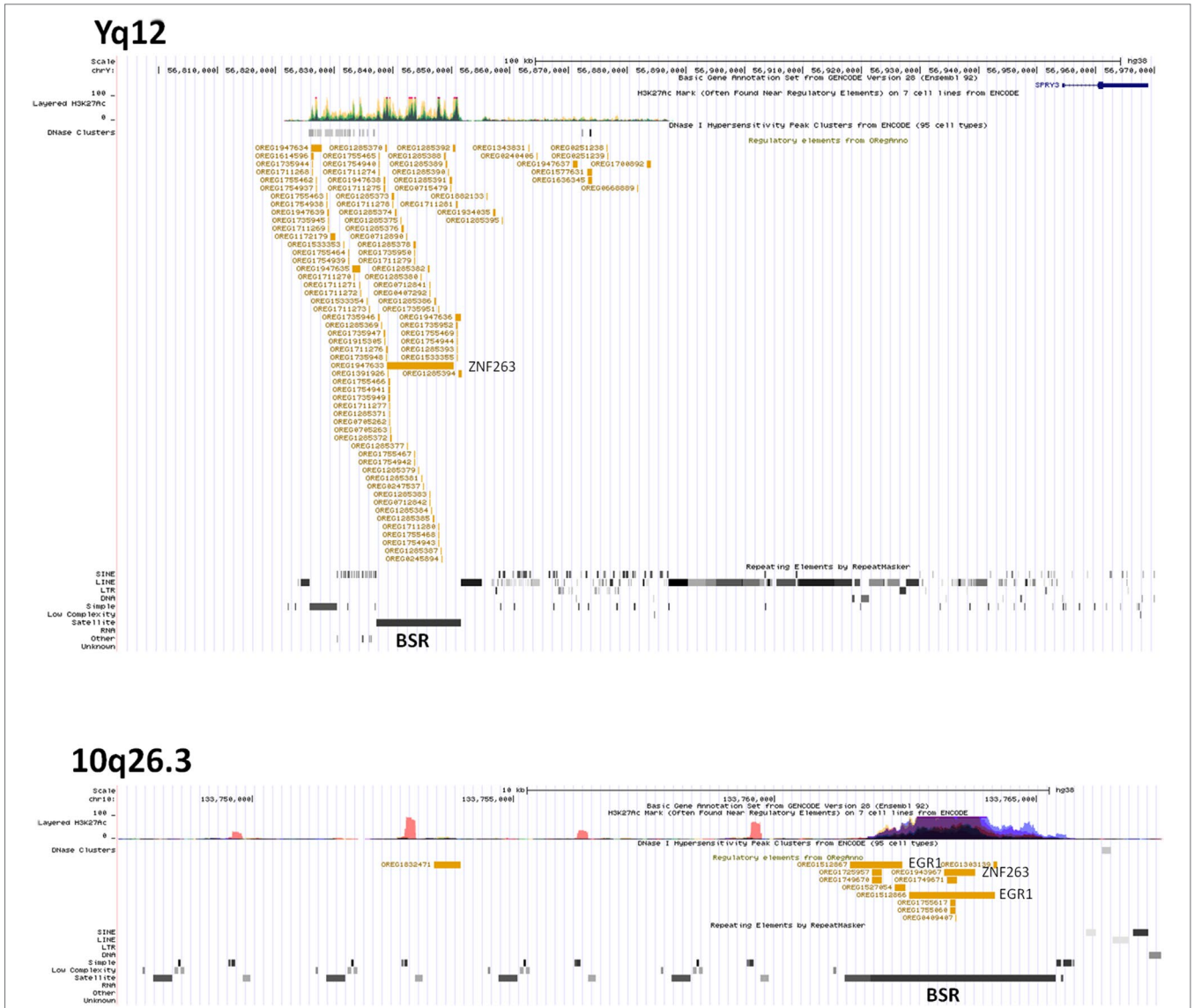

FIGURE 5 | ENCODE/OREG annotations in Yq12 and 10q26.3 regions showing major DNA factors binding in BSR regions (ZNF263, EGR1). Note different scales in each panel.

~15 kb is a beta satellite repeat (BSR). The entire ~60 kb region has multiple *DNase*I sensitive sites and CTCF binding sites (data not shown), and the BSR, similarly to the *SPRY3* core promoter AG-rich repeat, contains multiple ZNF263 binding sites. BLAST of the ~15 kb Yq12 BSR identified related sequences on chrs. 4, 10, 14, and 18 with 100% coverage and identity scores of 78–83%. The BSR-containing allele of the *FSHD* gene locus on chr. 4q35 (65) is not represented on the UCSC browser; but the structurally similar 10q26.3 locus is (66, 67). This exhibits a different pattern of chromatin modifications to the Yq12 BSR, increasing our confidence in the attribution of Yq12 annotations (**Figure 5**).

Inspection of the Yq12 region in the database of structural variants (DSV) using the UCSC browser indicated significant variability of the BSR, including independently reported deletions and duplications (**Figure 6A**). We attempted to use long PCR of genomic DNA using primers flanking the BSR to determine whether structural variants or length polymorphisms are associated with autism. Primer design was severely restricted due to the genomic architecture of the region, and the selected primer pair amplified a ~3.8 kb product from all male samples and no female samples (**Figure 6B**). Samples were as follows: 20 male autism (AGRE); 21 male autism (Skuse samples); 12 normal male and 4 normal female (Skuse and Coriell Caucasian panel). Cloning of this PCR product was problematic, but sequences obtained from both ends of a cloned partial product matched the expected 5′ and 3′ boundaries of the reference sequence (**Figure 6C**). However, this product was not amplified from BAC clone RP11-88F4, which covers the region, reducing confidence in the genomic location of the template for the ~3.8 kb amplicon (data not shown).

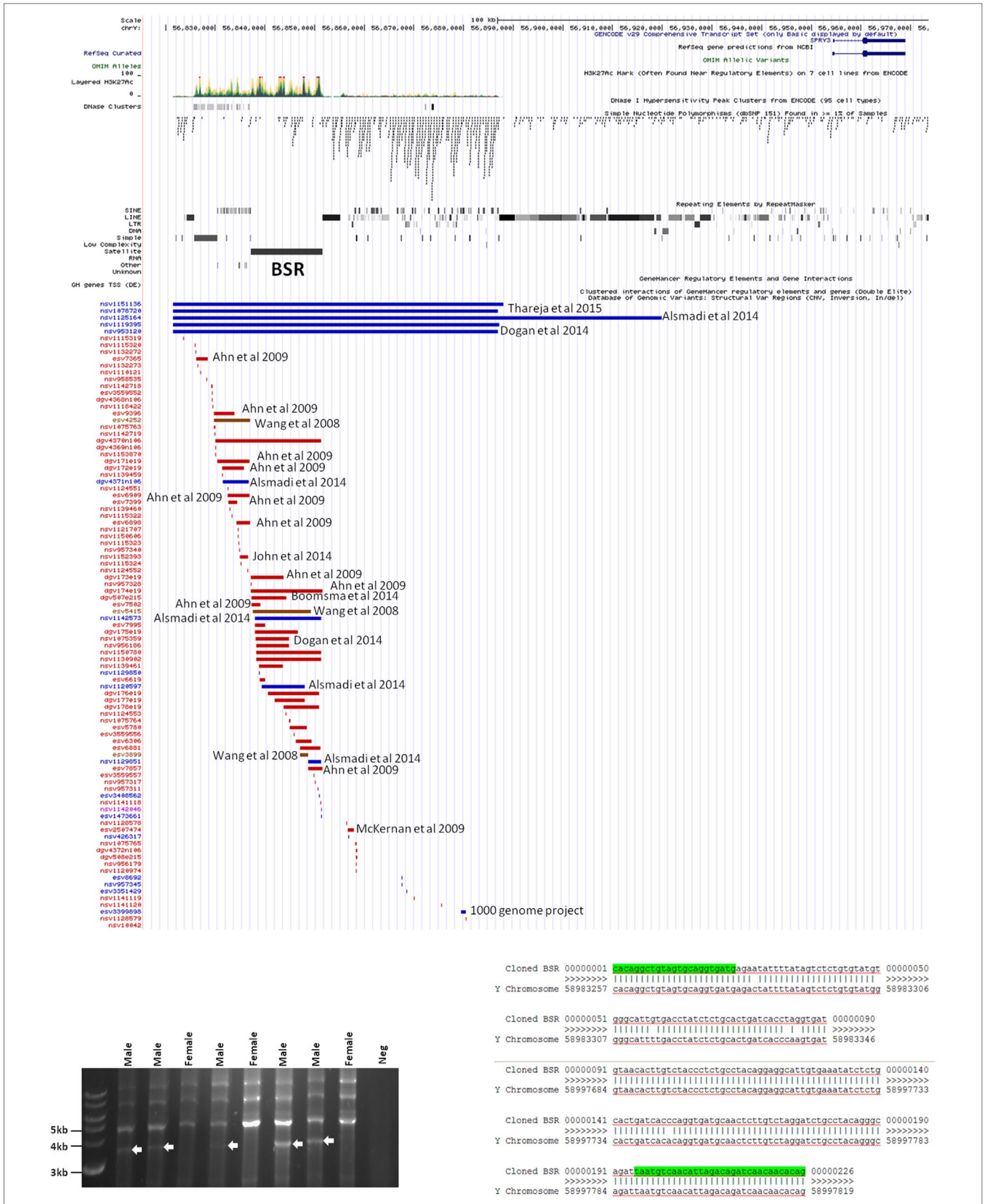

FIGURE 6 | (A) Map of genomic variants in Yq12 region. Details of annotated publication references are available on UCSC browser. **(B)** Male-specific ~3.5 kb PCR product amplified by BSR-specific primers (8 of 57 samples analyzed are shown). **(C)** Sequences of 5′ and 3′ ends of cloned PCR product.

Analysis of *SPRY3* Allele-Specific Expression in Cerebellum Using PsychENCODE Dataset

Genetic and structural analysis of the Yq12 putative regulatory region was inconclusive; therefore, we looked for loss of epigenetic silencing and reactivation of the Y-linked *SPRY3* allele in a comparison of RNA-Seq data from male autism and control cerebellum samples. We obtained paired-end RNA-seq libraries of cerebellar vermis from 33 autism and 38 control samples from PsychENCODE (54). Genotypes for X and Y chromosome (including PAR2) markers are not available for these samples; therefore, we used a statistical approach to analyze the level of heterozygosity of transcripts of the *SPRY3* and control genes. Heterozygous expression of *SPRY3* would be indicative of expression of both X- and Y-linked alleles due to pathological reactivation of the Y-linked copy. Genes flanking *SPRY3* were also examined. *TMLHE* was used as a negative control to estimate the level of variants due to technical noise (e.g., sequencing errors, substitutions during library preparation, and misalignments) since it is X-linked and no bona fide heterozygosity is expected. *SYBL1* was analyzed because it flanks *SPRY3* distally and is similar to *SPRY3* in having a Y-linked copy; however, its silencing is associated with a methylated CpG island, and it may be regulated differently to *SPRY3* (68). Autosomal genes (*NPTN* and *MCM6*) were used as positive controls, where we expected to identify heterozygosity. There was no significant difference in the heterozygosity plots of autism versus control samples for any of the genes analyzed (**Figure 7**; **Supplementary Figure 1**).

DISCUSSION

We have extended our previous work implicating *SPRY3* in autism (33) and provide evidence for a possible mechanism of chromosome Y-linked *SPRY3* gene deregulation underpinning male susceptibility. At the cellular level, we propose that *SPRY3* deregulation affects the functioning of the BDNF–TrkB–p75NTR neurotrophin pathway, leading to cerebellar Purkinje cell pathology. Our hypothesis can explain the specific lobular distribution of Purkinje cell loss in autism (lobules VI, VII, and X), as previously described (41). More speculatively, *SPRY3* deregulation could explain a reported, although currently unconfirmed, lung branching abnormality in autism (69).

The male bias in autism prevalence is not explained by known DNA susceptibility variants because the majority are autosomal, and a major sex-linked gene effect has not been identified (10, 14, 17, 26, 70–76). This suggests that autosomal variants interact with one or more sex-specific developmental or regulatory pathways, which could include sex hormones, or X or Y chromosome-linked gene-encoded regulators. This hypothesis requires that the majority (perhaps hundreds) of individual susceptibility variants converge on sex-specific mechanisms that underpin either a female protective effect or male susceptibility effect (FPE or MSE) (27, 29). The plausibility of FPE/MSE mechanisms is supported by mouse mutants of known autism genes, which exhibit sex-specific phenotypes (77–79), the presence of X-linked regulators expressed differently in males and females (80), and the influence of sex hormones such as testosterone and estrogen on normal and abnormal brain development and function (30, 31, 81–83).

However, recent studies did not detect a predicted Carter effect in autism because an increase in disease aggregation in families with a female proband was not observed, as would be expected if affected females require a higher mutation burden to overcome an FPE threshold (73, 84–86). This suggests that sex-specific departures from normal physiology, rather than normal sex-specific physiology *per se*, may underlie the male bias in autism (87). An alternative hypothesis to FPE/MSE is therefore the existence of one or more male-specific disease mechanisms

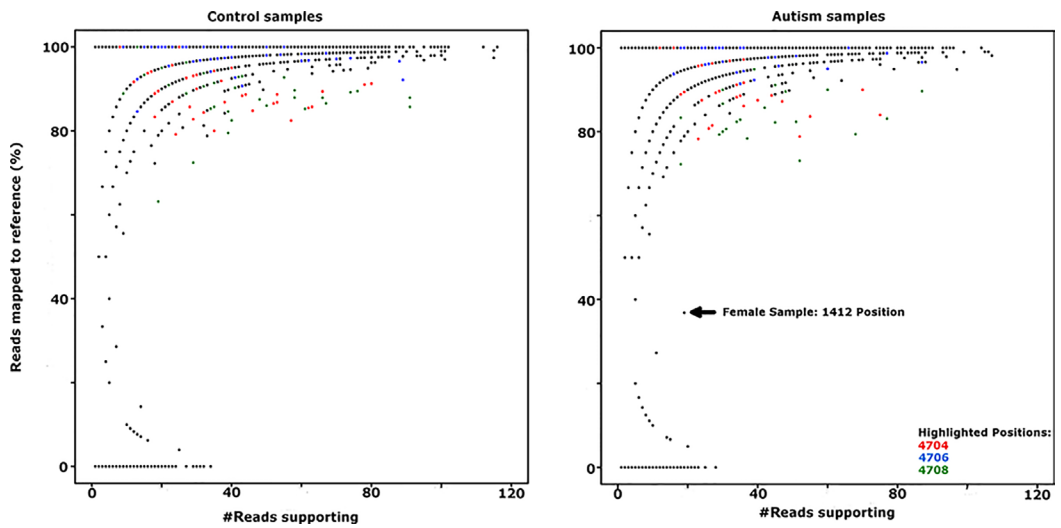

FIGURE 7 | Comparison of *SPRY3* (RefSeq ID: NM_005840) expression heterozygosity maps for autism and control samples. SNP positions 4704, 4706, and 4708 (highlighted in red, blue, and green, respectively) produced all possible genotypes in the majority of control and autism samples, suggesting a sequencing artefact at these positions, and were not counted as heterozygotes. Female sample 1412 position is included as indicative of a true heterozygous sample.

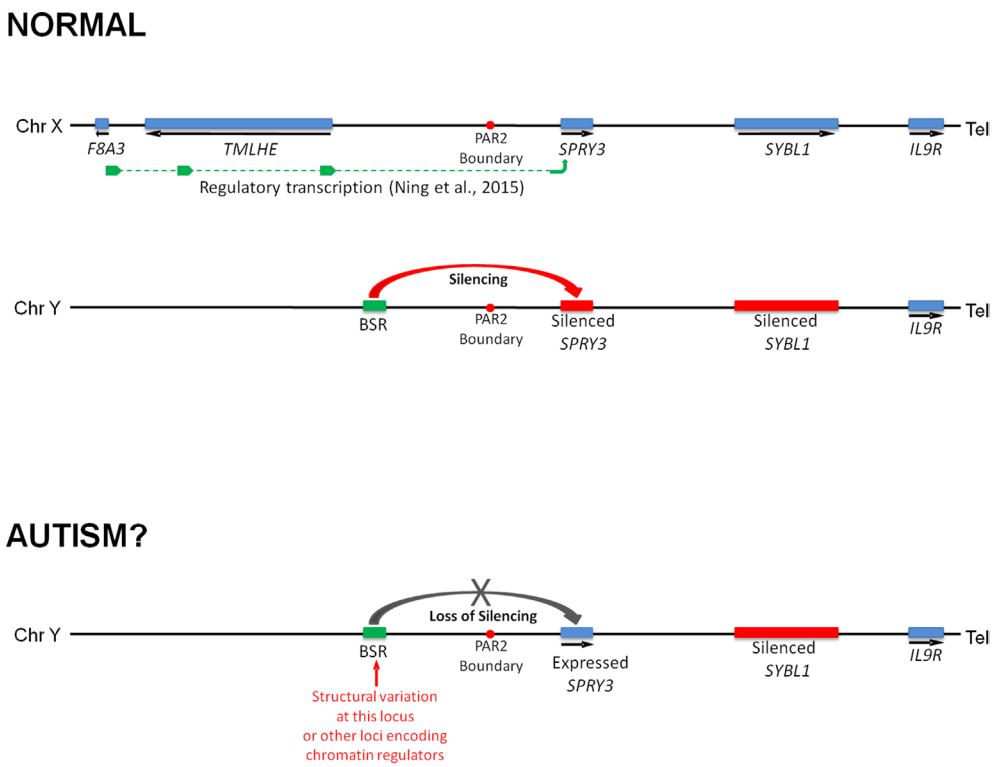

FIGURE 8 | Model of regulation of human *SPRY3* by X and Y chromosome-linked regulatory regions and deregulation in autism. We propose that regions including the *F8A2–F8A3* interval and associated transcription spanning the Xq28–PAR2 boundary, and Y-linked ENCODE/OREG-annotated regions including the BSR, may be deregulated by *cis*- or *trans*-acting factors. More specifically, we propose that cis-acting Yq12 genomic variants, including BSR length variants, may result in variable reactivation and expression of Y-linked *SPRY3*, leading to inappropriate lobular expression. Y-linked transcription may have altered lobular expression due to lack of putative Xq28-linked regulatory elements associated with the normal pattern of lobular expression from X-linked *SPRY3* or due to influence of Yq12 genomic elements.

(MDM). Such MDM would have to occur at a high frequency to explain the large male prevalence bias.

There are potentially two general mechanisms of *SPRY3* deregulation in autism. First, *trans*-acting effects of susceptibility variants at other loci encoding, for example, chromatin regulators could deregulate X- or Y-linked *SPRY3*. This category would also include environmental effects, for example, due to alterations in carnitine levels, to which *SPRY3* may be responsive (see below). Second, *cis*-acting *de novo* mutations or common variants in regulatory regions could cause aberrant expression of X- or Y-linked *SPRY3*. Previous genetic studies have not associated the Xq28 or PAR2 regions with autism (88). However, both the X and Y chromosome regions upstream of PAR2 are structurally complex and difficult to analyze, and therefore, autism-associated variants may have been overlooked.

The *F8A2–F8A3* interval has a common inversion polymorphism that may alter the orientation and distance from *SPRY3* of ENCODE/OREG-predicted regulatory sequences. The major regulatory factors that bind in this region (TRIM28, SMARCA4) are associated with autism (89–91); therefore, inversions or other rearrangements of this region may impact on expression of F8A2/F8A3 region-associated transcription or on flanking genes (*CLIC2*, *TMLHE*, *SPRY3*), potentially contributing to autism risk. It is unknown how frequently *de novo* inversions occur, and inversion alleles are not tagged by known SNPs. Therefore, we used single-molecule analysis to determine orientation of this inversion in a small number of autism and control DNA samples. We observed similar allele frequencies to those reported from an analysis of 20 hemophilia patients (64), with no evidence of a strong association with autism.

The Yq12 PAR2 region is poorly characterized due to the absence of genetic recombination and the highly repetitive DNA sequences that comprise much of the Yq and PAR2 boundary regions. However, our identification of a putative regulatory region in distal Yq12, 60 kb upstream of the *SPRY3* TSS, suggests a mechanism of silencing of Y-linked *SPRY3*. Similar to the *SPRY3* core promoter AG-rich repeat (33), a BSR in this region has multiple ZNF263 binding sites, suggesting a possible regulatory interaction with the *SPRY3* promoter. A BSR at chr. 10q26.3, also annotated in ENCODE, has a different pattern of chromatin modifications, increasing confidence in the Yq12 annotations. Interestingly, copy number variants (CNV) in the 10q26.3 region are associated with autism, although there is no evidence that this is due to the BSR (92; https://gene.sfari.org/database/cnv/10q26.3). Non-BSR sequences in the Yq12 region have abundant CTCF binding sites, a factor associated with gene imprinting and genome topology (93), further suggesting a regulatory function for this region in regulating Y-linked *SPRY3*.

There is extensive sequence and structural variation across the Yq12 putative regulatory region, including reported length polymorphisms of the BSR. However, BSRs are abundant in the genome (94, 95), and the majority are not annotated; therefore, caution is required when interpreting annotations arising from genome-wide studies underpinned by short sequence reads. We were unable to confirm Yq12 BSR variation using long PCR due to severe sequence constraints in primer design and instability of cloned PCR products. Other approaches, such as fiber FISH or single-molecule sequencing, anchored in unique sequences in PAR2, will be required to provide confirmation of BSR length alleles and to conduct genetic association studies. We also cannot exclude a role for somatic cell mutations, which are increasingly implicated in neurodegeneration (96). Somatic instability of the repeat-rich Yq12–PAR2 region could result in cell autonomous DNA rearrangements and deregulation of Y-linked *SPRY3*.

Notwithstanding significant technical difficulties in analyzing the Yq12–PAR2 boundary region, our data suggest a hypothetical mechanism whereby genetic (DNA sequence) or epigenetic (chromatin structure) variation could lead to reactivation and inappropriate lobular expression of Y-linked *SPRY3*. We sought to test this hypothesis by analyzing allelic expression of *SPRY3* in PsychENCODE cerebellum expression data, but we did not detect biallelic expression in male samples as would be predicted if the Y-linked copy is active. However, we lacked the sample genotypes and information about the exact cerebellar lobules sampled. Also, the pathological mechanism we propose may not be detectable in RNA-Seq data if reactivation of Y-linked *SPRY3* ultimately results in Purkinje cell death. Therefore, we do not consider this a definitive rejection of our hypothesis.

A possible further mode of *SPRY3* deregulation is suggested by its linkage with *TMLHE* and their overlapping regulatory sequences (33). TMLHE is an enzyme in the carnitine biosynthesis pathway, and carnitine deficiency is associated with autism (52, 97, 98). Intriguingly, there is evidence that carnitine levels modulate *SPRY3* expression in the pig (51). *TMLHE* mutations are rare but well-established autism susceptibility factors (52, 88, 97, 99, 100). Mouse *Tmlhe* is expressed in Purkinje cells; however, the lobular expression pattern is not restricted like *Spry3*. At least one mutation attributed to *TMLHE*-associated autism risk also affects *SPRY3* sequences (33), suggesting a possible role for deregulation of *SPRY3* in some reported cases.

Deficits in cerebellar vermis structure and Purkinje cell number and morphology have been reported frequently in autism at post mortem, using MRI imaging, and in mouse models (39, 82, 101–110). In a histological study of human brain tissues from autism cases, Skefos et al. (41) reported that Purkinje cell loss predominantly affects crus I and II (lobule VIIa), and they also noted a possible male-specific deficit in lobule X of the flocculonodular lobe. Following our previous study (33), and arising from our current observations, we show that mouse *Spry3* and *p75NTR* have opposite expression patterns in cerebellar vermis lobules VI–VII and X. *Spry3* is not expressed in these lobules, whereas *p75NTR* is strongly expressed [see also **Figure 2** in Ref. (111) and **Figure 1F** in Ref. (112)]. In a screen of 135 genes in the adult mouse (selected for high cerebellar expression or prior association with autism), we identified only three (*Abhd3*, *Lrp8*, and *Plcβ4*) with a somewhat similar lobular expression pattern to *Spry3*, and none that recapitulated the *p75NTR* expression pattern. This suggests that there are relatively few genes whose expression could explain the lobular pattern of abnormalities described by Skefos et al. (41). Our qualitative screen of the ABA mouse data was restricted to adult brain and may therefore lack sensitivity; however, we are reassured regarding its specificity by an independent report that *Plcβ4* is not expressed in lobules VI–VII and IX–X (113).

The opposite expression patterns of *Spry3* and *p75NTR* mirror the well-known opposite expression patterns of *zebrin II*/*aldolase C* and *Hsp25*/*Hspb1* on the anterior–posterior (AP) axis (114–118). The mechanisms responsible for patterning the anterior–posterior axis of the cerebellum include the autism gene *Engrailed-2* (*En2*; 118, 119), and intriguingly, *En2* has specific functions in the development of lobules VI–VII and X (118, 120), suggesting a further mechanism underpinning involvement of these lobules in autism (after 39).

A recent report suggests that lobules VI–VII (Crus I) in rodents are homologous to Crus I and II in primates (121). Therefore, if mouse *Spry3* and *p75NTR* expression patterns are conserved in human, as appears likely from a *SPRY3* promoter–reporter transgenic mouse (33), and scrutiny of the ABA (human) and GTEx databases, we can propose an MDM in which aberrant expression of human *SPRY3* in lobules VI–VII and X interferes with neurotrophin signaling, causing Purkinje cell pathology through a BDNF–TrkB–p75NTR mechanism. Spry1, 2, and 4 regulate receptor tyrosine kinase signaling including FGFR (122–125), whereas evidence from *Xenopus* and mouse indicates a regulatory loop involving BDNF- and TrkB-dependent expression of *Spry3*, and Spry3-mediated inhibition of BDNF–TrkB signaling (42). Therefore, it is possible that both SPRY3 and p75NTR proteins interact with BDNF–TrkB signaling, a pathway implicated in neuronal (including Purkinje) cell development and survival (126–130). Although *p75NTR* is not listed on the SFARI autism gene database, it is a compelling candidate for involvement in autism pathogenesis (128, 131, 132). There is extensive evidence of both pro- and anti-apoptotic functions for p75NTR, particularly in contexts with concomitant alteration of neurotrophin receptor signaling, including of TrkB (130, 133–138). SPRY3 regulates TrkB signaling (42); therefore, we speculate that inappropriate expression of SPRY3 in lobules VI–VII and X, in the context of TrkB and p75NTR expression, may affect Purkinje cell function or survival, although the exact mechanism would have to be established in relevant models, as neurotrophin signaling effects depend strongly on physiological context (136, 139).

We previously reported that a human *SPRY3* promoter–LacZ reporter transgenic mouse substantially recapitulated the mouse *Spry3* expression pattern (33). Similar to mouse *Spry3*, human *SPRY3*-LacZ is expressed in Purkinje cells throughout the cerebellum, except in lobules VI–VII. However, unlike mouse *Spry3*, human *SPRY3*-LacZ is expressed in lobule X. Therefore, sequences outside of the human core promoter, or *trans*-acting factors whose expression differs between mouse and human, may be required for regulation of *SPRY3* in this lobule. Interestingly, the deficits in lobule X identified by Skefos et al. (41) may be male-specific, which, in the context of our proposed mechanism of pathological over-expression of *SPRY3*, could indicate a role for deregulation

(reactivation) of the Y-linked copy in this lobule, which might have a different expression pattern compared to the X-linked copy due to the lack of cis-acting X-linked regulatory sequences, or the inappropriate influence of Y-linked sequences (**Figure 8**).

Finally, we note that Sprouty was originally described based on a branching phenotype of the apical airways of Drosophila (140), and mouse *Spry2* coordinates vascular and airway branching in the lung (141). *Spry3* is expressed in the mouse lung bronchial tree (our unpublished data) and in human lung (GTEx), suggesting that deregulation of *SPRY3* could potentially provide a mechanism underpinning a lung branching abnormality reported in autism patients (69).

In future work, we aim to deepen our understanding of *SPRY3* and *p75NTR* expression and functional interactions during brain and lung development in the human and mouse, including in autism mouse models. Transgenic under- or over-expression of *Spry3* in cerebellar lobules VI–VII and X in mice would provide an *in vivo* model of our proposed MDM in autism. Due to the unique genomic architecture and regulation of the human PAR2, and the difficulty in sourcing matched tissue samples from specific cerebellar lobules from normal and autism brains, and from other organs such as lung, the analysis of Y-linked *SPRY3* deregulation in the cerebellum and lung will be challenging, particularly if the pathology results in cell death. However, advances in single-molecule DNA sequencing techniques will facilitate detection of genomic variants in this region that may be associated with autism.

ETHICS STATEMENT

Access to human genetic data and biomaterials for this study was carried out in accordance with the procedures and recommendations of the UCC Office of the Vice-President for Research and Innovation, specifically for accessing AGRE and SFARI datasets and biomaterials, under their respective procedures. All subjects gave written informed consent in accordance with AGRE and SFARI protocols and procedures, consistent with the Declaration of Helsinki. Access to animal tissues for this study was pursued in accordance with the recommendations of the UCC Animal Experimentation and Ethics Committee under licencing from the Irish Health Products Regulatory Authority (https://www.hpra.ie/).

AUTHOR CONTRIBUTIONS

ZN and JW carried out all of the bench work and aspects of bioinformatics and data analysis. RK and PB carried out analysis of PsychENCODE data. TM conceived of, and supervised, the study and wrote most of the manuscript. All authors contributed to various aspects of manuscript preparation.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpsyt.2019.00416/full#supplementary-material

SUPPLEMENTARY TABLE 1 | Mouse Purkinje cell expressed genes whose spatial expression was examined in Allen Brain Atlas data. For consistency, the numbering scheme for the 54 genes identified as "cerebellum, Purkinje cell enhanced" in the Allen Brain Atlas "fine structure" search is maintained here, with inclusion of two blank rows (29 and 53).

SUPPLEMENTARY TABLE 2 | Human autism genes from SFARI "High confidence" and "Strong candidate" lists (https://www.sfari.org/resource/sfari-gene/#bottom) for which spatial expression of their mouse orthologues was examined in Allen Brain Atlas data.

SUPPLEMENTARY FIGURE 1 | Comparison of SPRY3 expression heterozygosity map with maps for TMLHE (X-linked), SYBL1 (PAR2-linked), and NPTN, MCM6 (autosomal) genes.

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Effects of Aspirin in Rats With Ouabain Intracerebral Treatment—Possible Involvement of Inflammatory Modulation?

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Bipolar disorder (BD) is a chronic and refractory disease with high probability of morbidity and mortality. Although epidemiological studies have established a strong association between BD and immune dysfunction, the precise etiology is still debatable, and the underpinning mechanism remains poorly investigated and understood. In the present study, manic-like symptoms of BD were induced in rats after intracerebroventricular administration of ouabain. Aspirin, a commonly used anti-inflammatory agent, was used to treat the induced manic-like symptoms and inflammation. Concentrations of a spectrum of inflammatory cytokines were examined by enzyme-linked immunosorbent assay in both plasma and brain tissues, and expression of Toll-like receptors 3 and 4 were determined in rat brains. Locomotor activity was monitored with open-field test to assess the effects of ouabain challenge and to evaluate the treatment efficacy of aspirin. Ouabain administration recapitulated many mania-like features such as increased stereotypic counts, traveling distance in open-field test, and decreased expression of brain-derived neurotrophic factor, interferon gamma, and Toll-like receptor 3, which were frequently found in patients with BD. These abnormalities could be partially reversed by aspirin. Our findings suggest that aspirin could be used as a promising adjunctive therapy for BD.

Keywords: mania, ouabain, cytokine, toll-like receptor, aspirin, animal model

INTRODUCTION

Bipolar disorder (BD) is a chronic, severe, and disabling medical condition, which frequently associates with high levels of morbidity and mortality (1). The lifetime prevalence is estimated as high as 2.4% (2). Numerous factors including genetics, oxidative stress, and environmental interaction predispose toward the pathogenesis of BD, but the precise pathological etiology remains enormously complex and debatable. Mood stabilizers and antipsychotics are first-line agents for BD with poor tolerance and high rates of treatment resistance. In addition, their effects are mostly palliative, which do not alter the overall prognosis.

Accumulating evidence has demonstrated that a high co-occurrence of inflammatory comorbidities with BD and immune dysfunction is emerging as a strong predisposition factor for this association (3–5). Many inflammatory comorbidities are associated with BD, such as systemic lupus erythematosis (6), autoimmune thyroiditis (7), psoriasis (8), Guillain-Barré syndrome, autoimmune hepatitis, multiple sclerosis (9), obesity, atherosclerosis and type II diabetes mellitus (10), rheumatoid arthritis (11), migraines, and inflammatory bowel disease (12, 13). In BP patients, levels of many circulating pro-inflammatory molecules were elevated, and inflammation was augmented in the brains (4). Accordingly, altered inflammatory cytokines in plasma have also been described during manic or depressive episodes (14). In addition, postmortem study has revealed increased transcripts of interleukin (IL)-1β, IL-1 receptor, myeloid differentiation factor 88, nuclear factor-kappa B subunits, and over-activated astrocytes and microglia in BP brains (15). In line with these observation, lithium and valproic acid have been demonstrated to exert mood stabilizing effects partially through immune system modulation (16, 17). As a result, BP itself is recently proposed as an inflammatory condition, and the recurrent mood episodes may represent fluctuating inflammatory states (13).

Toll-like receptors (TLRs) are important mediators of inflammatory responses and expressed on membranes or organelles of microglia. TLRs recognize endogenous molecules, referred to as danger-associated molecular patterns, which are typically sequestered from the immune system but released during tissue pathology (18). Among the TLR family, TLR3 and TLR4 in CNS are of particular interest, which can activate interferon (IFN) regulatory factor 3 and induce IFN-β production, followed by a phase of IFN-dependent gene expression, while the other family members of TLRs do not activate IFN regulatory factor 3 pathway (19). Furthermore, real-time PCR of TLRs 1–10-in cultured human astrocytes showed that TLR3 expression rapidly increased upon exposure to inflammatory cytokines IFN-γ, IL-1β, and IFN-β (20). Although increased peripheral TLR4 responses have been also reported in BD subjects (21), no further functional study was performed along this line.

Aspirin is a nonsteroidal anti-inflammatory drug, which suppresses the inflammatory response and reduces levels of inflammatory biomarkers such as C-reactive protein, tumor necrosis factor-α, and IL-6 (22). It can also reduce oxidative stress and protect against oxidative damage. There is some preclinical and clinical evidence suggesting beneficial effects for aspirin in mood disorders (23). Recently, anti-inflammatory regents including aspirin are proposed to have a moderate antidepressant effect in the treatment of BD (12), which is also confirmed in a phase IIA clinical trial (24). However, the underlying cellular mechanism remains unclear.

In the present study, ouabain was injected intra-cerebroventricularly to induce abnormally manic-like symptoms in rats (25, 26), which were followed with administration of aspirin. Animal behaviors were monitored before and after ouabain challenge and also after aspirin treatment. Concentration of inflammatory cytokines and expressing profiles of TLR3 or TLR4 were determined in plasma and brains of rats. Our data showed that ouabain injection reliably induced manic-like symptoms in rats with a spectrum of features similar with BD. Aspirin partially reversed abnormalities presented by the rats through upregulated expression of IFN-γ and TLR3 in brains. This study demonstrates that aspirin may have a beneficial impact in the treatment of BD.

METHODS

Animals

Thirty adult male Sprague-Dawley rats (250–350 g) were purchased from Hunan Slack Scene Of Laboratory Animal Co., Ltd (Changsha, China). During the experiment, rats were housed individually in a room with a 12-h light/dark cycle (light on at 7:00 am) and environmental temperature of 25°C with 50–60% humidity with *ad libitum* access to food and water in the experimental animal center of the Second Xiangya Hospital. All the animals were habituated for at least 1 week before any manipulation. All the described procedures were approved by the Institution of Animal Care and Use Committee of The Second Xiangya Hospital (protocol number: 2015.014) and adhered to the Guide for the Care and Use of Laboratory Animals. Every effort was made to minimize animal suffering and the number of animals used.

Surgical Procedure

Ouabain injection was performed as previously report (25, 27). Briefly, after intraperitoneal anesthetization with 10% chloral hydrate (3 ml/kg), animals were fixed in a stereotaxic apparatus (Narashiga, Japan). A longitudinal incision was made along the midline of scalp, and a 9-mm guiding cannula (27 gauge) was placed at the coordinates of 0.9 mm posterior to Bregma, 1.5 mm right from the midline, and 1.0 mm above the lateral brain ventricle (right side). Through a 2-mm hole made at the cranial bone, a cannula was implanted 2.6 mm ventral to the superior surface of the skull and fixed with jeweler acrylic cement.

Treatment

All animals were randomly assigned into three groups. Rats in the first and second groups received a single intra-cerebroventricular (ICV) injection of 5 μl of ouabain (10^{-3} M) dissolved in artificial cerebrospinal fluid (aCSF), whereas the animals in the third group received an injection of 5 μl aCSF after surgery. From the second day following the injection of ouabain or aCSF, rats in the first group were treated for 1 week with aspirin (50 mg/kg), while those in the second and third groups were treated for 1 week with equal volume of saline by gavage. Three groups were categorized as follows: ouabain ICV + aspirin IG (OUA + APC), ouabain ICV + saline IG (OUA + SAL), and aCSF + saline IG (aCSF + SAL).

Open-Field Test

Locomotor activities of the animals were assessed using the paradigm of open-field test as previously reported (25). The task was performed in a 43.2 × 43.2 × 30.5 – cm^3 box on two

occasions: immediately or 1 week after drug injection. The floor of the box was divided into nine equal rectangles with black lines. The animals were gently placed in the center area and free to explore the field for 5 min. All of the movements were tracked by an overhead camera. The total travel distance was analyzed by Computer-assisted Tracking Software (ENV-515-16, MED Associates, Inc). Rearing activity was monitored and videotaped automatically as main readout of stereotypic behavior. Rats were decapitated immediately after the last evaluation, and prefrontal cortices were dissected, snap frozen, and stored at -80°C until analysis.

Measurement of Cytokines and Brain-Derived Neurotrophic Factor

Enzyme-linked immunosorbent assay (ELISA) was used to determine the concentrations of various cytokines and brain-derived neurotrophic factor (BDNF) as previously reported (28). Blood samples were obtained from the rats and centrifuged at 1,000×g at 4°C for 15 min. The supernatant were carefully taken and stored at −20°C. Prefrontal cortices from each animal were homogenized in phosphate buffer solution followed by centrifuge at 5,000 × g for 5 min at 4°C. The pellets were discarded, and the supernatant were stored at −20°C. The expressing levels of C-reactive protein (CRP), IL-1β, IL-2, IL-6, IL-10, INF-γ, tumor necrosis factor alpha (TNFα), prostaglandin E_2 PGE-2, as well as BDNF and PGE-2 were subsequently measured by ELISA. Briefly, microtiter plates (96-well) were coated for 24 h with individual sample diluted with sample diluent, and a standard curve ranging from 0 to 320 pg/ml for each cytokine was plotted. The plates were then washed four times with sample diluent, followed by incubation with corresponding antibodies against each cytokine for 3 h at room temperature. After three times washing (with sample diluents), the plates were incubated with specified secondary antibody conjugated with peroxidase at room temperature for 30 min. Then peroxidase conjugated with streptavidin and reaction substrate was introduced for 5 min followed with the stop solution. Absorbance at 450 nm was read, and the amount of each cytokine was determined. Total protein was calculated by Lowry's method using bovine serum albumin as a standard. All the ELISA kits were purchased from Wuhan USCN Business Co., Ltd.

Expression of Toll-Like Receptors 3 and 4 in Frontal Cortex

Real-time reverse transcriptase PCR was used to determine the expression of TLR3 and TLR4 in frontal cortices of the rats. Standard protocols were used for total RNA extraction, complementary DNA (cDNA) synthesis, and PCR. cDNA sequence was obtained from Genebank at National Center for Biotechnology Information (www.ncbi.nlm.nih.gov). Primer sequences were designed using Operon Oligo Analysis Tool (https://www.eurofinsgenomics.com/en/resources/tools/pcr-primer-design/), and the sequence specificity was validated with the basic local alignment search tool. Primers were purchased from Invitrogen, and the specificity was verified by melt curve analyses. The following primers were used: TLR3, F: 5'-CTGGGTCTGGGAGCATTTC-3', R: 5'-GCGGGTCTTTCAGTAGGTG-3'; TLR4, F: 5'-ATGAGGACTGGGTGAGAAAC-3', R: 5'-ACCAACGGCTCTGGATAAAG-3'. PCR amplification of cDNA was performed using the SYBR Green PCR Kit (Thermo, USA). The quantity of PCR product was monitored in real time using the MyiQ Single-Color Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). Relative gene expressing abundance was calculated as the ratio of TLR3 or TLR4 against actin.

Western Blot Analysis

For immunoblotting, frontal cortices were homogenized with lysis buffer (Cell Signaling Technology) containing complete protease and phosphatase inhibitors (Roche) and centrifuged at 12,000×g for 15 min at 4°C. The protein concentration in supernatant was determined using bicinchoninic acid protein assay kit (Thermo Scientific, USA). Equal amounts of protein were fractionated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. The membranes were blocked and incubated with primary antibodies overnight at 4°C, incubated with horseradish peroxidase-conjugated secondary antibody (Invitrogen), and developed using an enhanced chemiluminescence kit (Millipore). The following primary antibodies were used: TLR3 (Bioworld 1:1,000, BS6749), TLR4 (Abcam, 1:500, Ab22048), and glyceraldehyde 3-phosphate dehydrogenase (Cell Signalling Pathway, 1:1,000, CST #5174). After three washing times with 0.01-M Tris-buffered saline, the membranes were probed with horseradish peroxidase-conjugated secondary antibodies at room temperature for 1 h and then developed with enhanced chemiluminescence kit (ECL-plus, Thermo Scientific, USA).

Statistical Analysis

Behavioral and biochemical data were presented as mean and standard error of the mean. The equality of variance in multiple data groups was assessed with Levene's test. Statistical difference was determined with one-way ANOVA, which was followed by least significant difference (LSD)-t test or Tamhane test. $P < 0.05$ was considered as statistical significance threshold.

RESULTS

Aspirin Facilitated the Amelioration of Hyperactivity Caused by Ouabain Intra-Cerebroventricular Injection

Previous studies have demonstrated that ICV injection of OUA in rats could lead to hyperactive locomotion, a phenotype compatible with the manic episode of BD patients. Open-field test is most widely used to assess this phenotype due to its supreme sensitivity and consistence compared with automated activity monitor (27). We hence performed open-field test to examine effects of aspirin to the behavioral phenotypes caused by OUA. Consistently, OUA immediately increased the traveling distance and stereotypic counts in OUA + SAL and OUA + APC groups after the ICV administration (**Figure 1**). Aspirin treatment modestly reduced stereotypic counts compared with the OUA + SAL

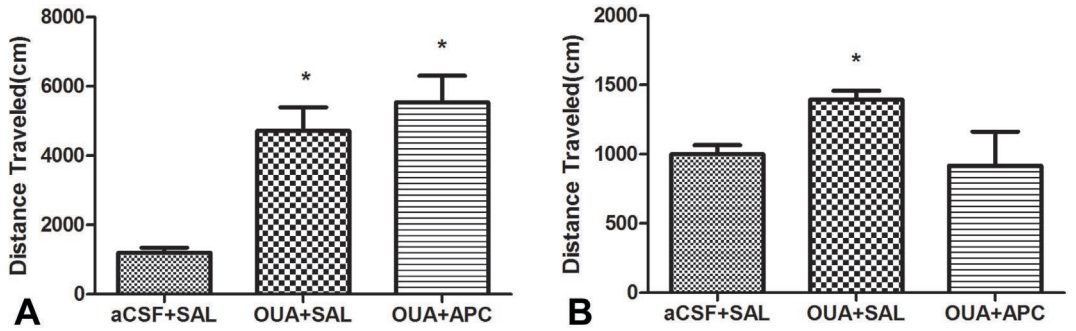

FIGURE 1 | Traveling distance was analyzed prior to and after aspirin treatment. **(A)** Data analysis for the first open-field test, $^{*}p < 0.05$ vs. aCSF + SAL group (ANOVA followed by Tamhane test, F = 16.653, $p < 0.001$). **(B)** Data analysis for the second open-field test, $^{*}p < 0.05$ vs. aCSF + SAL group (ANOVA followed by Tamhane test, F = 2.926, $p = 0.075$).

group (**Figure 2B**), which indicates an attenuation of increased stereotypic counts. In line with the reports by El-Mallakh et al., rats in the OUA + SAL group still presented hyperactivity during the second open-field test indicating that the effects of OUA are potent and can last at least for 1 week (**Figure 2A**). There was no effect on distance traveled between OUA + SAL and OUA + APC groups in the second open-field test, indicating that aspirin may only have modest effects, although the role of novel environment cannot completely be ruled out (**Figure 2A**).

Expressing Profiles of Brain-Derived Neurotrophic Factor and Pro-Inflammatory Cytokines

We next asked whether OUA injection could alter expression of BDNF and activate cascades of cytokines in both brain and periphery. In line with previous report (28), the expressing levels of cytokines CRP, IL-1β, IL-2, IL-6, IL-10, INF-γ, TNFα, as well as BDNF and PGE-2 in plasma did not show any difference among groups (data not shown). Notably, BDNF and INF-γ levels in prefrontal cortices of the aCSF + SAL group were higher than those in the OUA + SAL group, indicating that ICV administration of OUA decreased BDNF and INF-γ levels in brain tissue (**Figures 3A**, **C**). Intriguingly, INF-γ levels in brain tissue of aspirin treated rats increased compared with those in the OUA + SAL group, which indicated that aspirin reversed the reduction of INF-γ (**Figure 3C**). Apart from this observation, no difference was detected for the expressing profiles of cytokines such as CRP, IL-1β, IL-2, IL-6, IL-10, TNFα, and PGE-2 in brain tissues among groups (**Figures 3B**, **D–I**).

Expression of Toll-Like Receptors 3 and 4 in Prefrontal Cortex

TLRs are important modulators of immune homeostasis that facilitate the production of proinflammatory cytokines/chemokines including IFN-γ. Recently, a growing body of evidence indicates that TLR3 and TLR4 may be involved in psychosis with immune dysfunction including major depressive disorders (29) and BD (30). We therefore performed real-time reverse transcriptase PCR and Western blotting experiments to determine the expression of TLR3 and TLR4 in prefrontal cortices. We did not find any difference for the messenger RNA (mRNA) expression levels of TLR3 and TLR4 among the three groups; however, TLR3 protein levels in frontal cortex were overtly decreased in the OUA + SAL group, and aspirin treatment

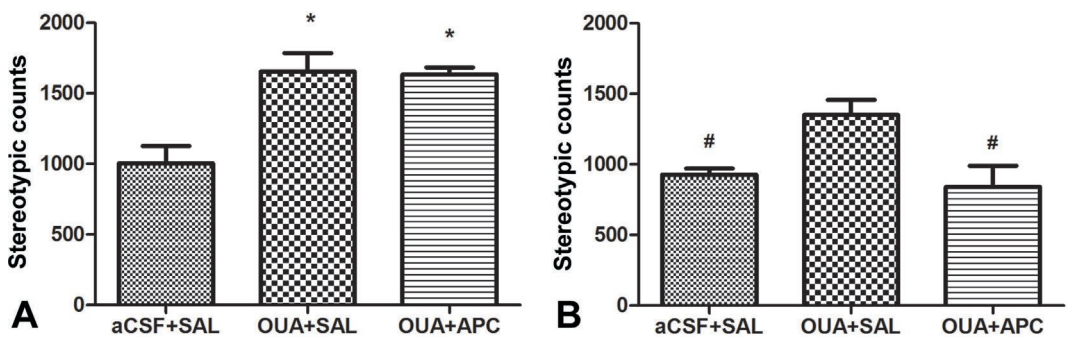

FIGURE 2 | Stereotypic counts of rats in three groups before and after aspirin treatment. **(A)** Data analysis for the first open-field test, $^{*}p < 0.05$ vs. aCSF + SAL group (ANOVA followed by LSD-t test, F = 11.779, $p < 0.001$). **(B)** Data analysis for the second open-field test, $^{\#}P < 0.05$ vs. OUA + SAL group (ANOVA followed by Tamhane test, F = 6.518, $p < 0.01$).

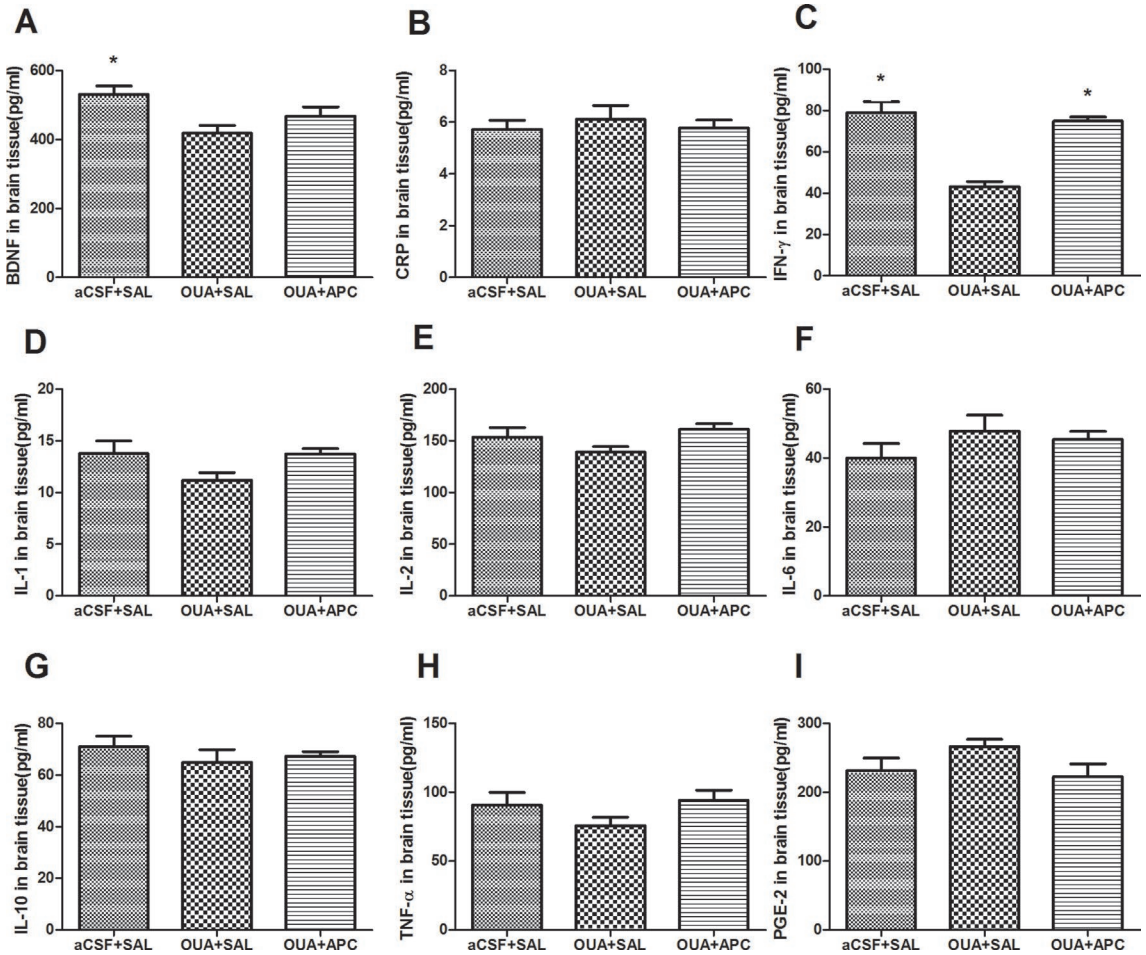

FIGURE 3 | Expressing levels of BDNF and various cytokines in brain tissue after ICV administration of OUA and aspirin treatment. **(A–C)**, BDNF, CRP and IFN-γ; **(D–G)**, IL-1β, 2, 6, and 10; **(H-I)**, TNF-α and PGE-2. *$P < 0.05$ vs. OUA + SAL group, according to ANOVA followed by the LSD-*t* or Tamhane test (BDNF: F=5.110, $p < 0.05$; INF-γ: F=29.687, $p < 0.001$).

significantly increased TLR3 protein levels compared with that of the OUA + SAL group (**Figures 4A, C**). These results indicate that aspirin could reverse the reduction of TLR3 protein levels caused by OUA injection. Interestingly, TLR4 protein levels did not show any difference between the three groups (**Figures 4A, B**).

DISCUSSION

BD is a refractory and relapsing illness with strong components of innate immune dysfunction. However, previous studies mainly focus on depression phase of BD, and little is known about the dysfunctional immunomodulation in manic phase of the disease. In the present study, we recapitulated manic-like behaviors in rats by ICV administration of ouabain, a potent Na^+/K^+-ATPase inhibitor. Ouabain injection successfully induced mania-like behaviors such as increased stereotypic counts and traveling distance that associated with aberrant expression of BDNF, INF-γ, and TLR3 in both plasma and prefrontal cortex. Importantly, aspirin treatment not only reversed the increased stereotypic counts of the rats but also increased expressing levels of BDNF, INF-γ, and TLR3 in rat plasma and brains. Our data suggest that inflammation may be closely involved in a subpopulation of BD patients and aspirin treatment could provide beneficial effects to BD patients with prominent inflammatory comorbidities.

In our study, ICV administration of OUA, a potent Na^+/K^+-ATPase inhibitor, caused mania-like behaviors such as increased stereotypic counts and traveling distance. Previous studies showed that ouabain-induced hyperlocomotion occurred immediately and could persist for 7 days after a single ICV administration (25, 26, 28, 31). In addition to the behavioral phenotypes, rats with manic-like symptoms also displayed decreased protein levels of BDNF, INF-γ, and TLR3 in brain tissue. Largely due to the potent modulation of inflammation and neurogenesis, BDNF is closely involved in neuroplasticity and has long been proposed as a potential biomarker for many mental disorders. In line with our findings, expression of BDNF in hippocampus and amygdala also decreased in rats after ouabain administration that could be prevented and reversed by lithium (31). Importantly, manic patients also exhibited decreased BDNF level in serum that was associated with acute mood

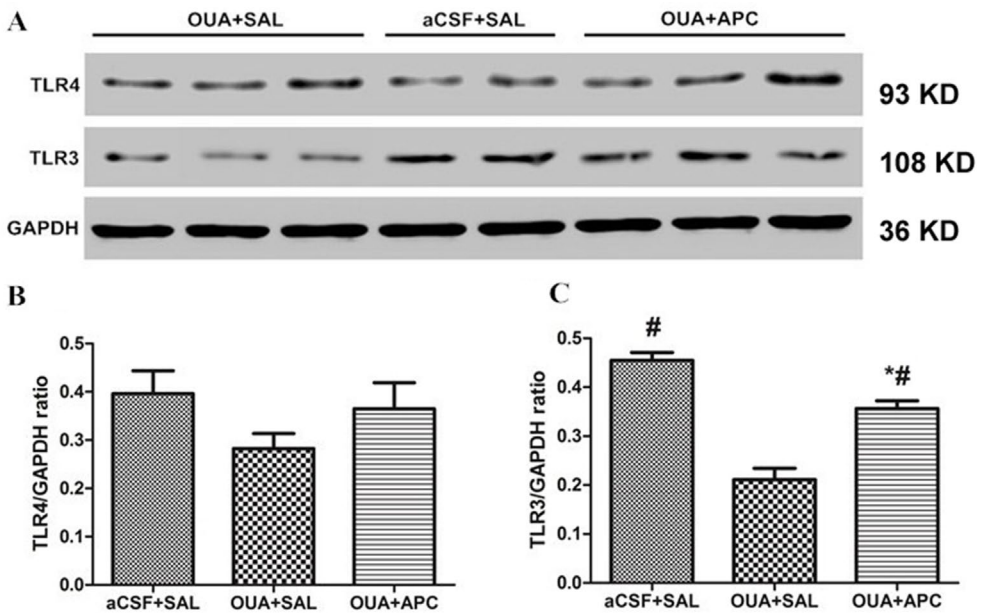

FIGURE 4 | TLR4 and TLR3 protein levels in brain tissue after ICV administration of OUA and aspirin treatment. **(A)** Western blotting results; **(B–C)** Statistical analysis. *$P < 0.05$ vs. aCSF + SAL group, #$p < 0.05$ vs. OUA + SAL group, according to ANOVA followed by the LSD-t test (TLR3 protein levels: F = 42.64, $p = 0.000$).

episodes in both medicated (32) and unmedicated patients (33). Interestingly, accumulating evidence has demonstrated that mood-stabilizing drugs, including lithium and valproate, have important neuroprotective roles in acute and chronic treatment (34), and one of the underpinning mechanisms is to increase BDNF levels in both serum and brain (35). As a response to treatment, manic patients often experience a sharp increase of BDNF in serum prior to the resolution of acute episode (36). This seems to support the concept that the main determinant related to lower BDNF levels in depression and mania is the presence of symptoms, not medication status. However, it must be noted that BDNF precisely modulates synaptic connection and signal transmission that helps to maintain structural and functional homeostasis of brain. It is sensitive in many neuropsychiatric conditions and thus lacks specificity to BD.

IFN-γ is a pleiotropic cytokine that induces antiviral, antiproliferative, and immune-modulatory effects in numerous inflammatory diseases (37). IFN-γ has been proposed to involve in the pathophysiology of BD, but the underpinning mechanism remains unclear. Previous clinical research revealed inconsistent expression of circulating IFN-γ in serum. Hope et al. did not find any alteration of IFN-γ in serum of BD patients (38), while upregulated expression of IFN-γ was also reported (39, 40). In the present study, we have detected significant decrease of IFN-γ in serum of BD patients. In agreement with our findings, stimulation of lymphocytes *in vitro* also led to a lower release of IFN-γ (41). Because IFN-γ can enhance neurogenesis in dentate gyrus of adult mice and promote spatial learning and memory performance, we propose a lower concentration of IFN-γ may have deleterious effects to the integrity of brain function of mice (42), although the precise underpinning mechanism needs to be elucidated further.

TLR3 is a key member of TLR family and plays an important role in the developmental patterning of innate immunity and autonomously regulates the establishment of neural network (43). Upon exposure to its specific ligand polyinosine:polycytidylic acid, TLR3 can rapidly cause growth cone collapse and inhibit neurite extension independent of nuclear factor kappa-light-chain-enhancer of activated B cells. In CNS, TLR3-mediated activation of astrocytes led to a marked induction of the enzyme indoleamine 2,3-dioxygenase, which acted as a local immune-suppressive factor (44). TLR3 also had a protective role in experimental autoimmune encephalitis due to increased expression of IFN-β (45). In cultured human astrocytes, TLR3 expression rapidly increased upon exposure to IFN-γ, IL-1β, and IFN-β (20). In our study, we did not detect any changes in TLR3 mRNA but a reduced expression of TLR3 protein. It should be noted that mRNA abundance is not always paralleled with the expression of corresponding proteins and discrepancy frequently occurs. For example, Abdi et al. has detected very low level of TLR3 mRNA but strong protein translation in human multiple myeloma cells. On the contrary, abundant mRNA transcripts of TLR5 were confirmed, but there is almost no protein expression (46). Similarly, Arvaniti et al. found that some B-cell chronic lymphocytic leukemia cells do not express TLR6 protein in spite of a high mRNA level and a high expression of proteins for TLR2 and TLR8 in spite of a low mRNAs (47). We assume that the reduced TLR3 protein could be subsequent outcome of reduced IFN-γ, although the precise mechanism needs to be further illustrated.

In the present experimental paradigm, we did not find any change of CRP, IL-1β, IL-2, IL-6, IL-10, INF-γ, TNFα, as well as BDNF and PGE-2 in rat plasma. Consistently, no alteration was detected for the concentration of CRP, IL-1β, IL-2, IL-6, IL-10, TNFα, and PGE-2 in rat brains challenged by our or aspirin. Similarly, Tonin et al. assessed concentrations of various cytokines (IL-1β, IL-6, IL-10, TNF-α, and cytokine-induced neutrophil chemoattractant 1) in distinct brain structures (hippocampus,

striatum, frontal cortex, and amygdala), serum, and cerebrospinal fluid (CSF) of rats subject to ouabain administration and only found decreased IL-6 in striatum (28). These findings highlight that despite the observed behavioral phenotypes, ouabain administration cannot produce overt alteration of most pro-inflammatory factors commonly occurring in BD patients. This may largely limit its usage as a generalized approach to mimic human BD. On the other hand, no detectable changes of cytokines in periphery also indicate that altered IFN-γ and TLR3 in rat brains are highly likely the outcomes of ouabain injection, although the influence of surgical procedure cannot be completely ruled out.

Anti-inflammatory treatment is rapidly arising as a new augmentation therapy for BD patients due to the high proportion of medical comorbid conditions. Aspirin is a particularly promising candidate, which has been well established in various clinical settings and is well tolerated even in long-term use. Importantly, it is well absorbed and brain penetrant. Low dosage of aspirin preferentially inhibits cyclooxygenase 1, which further blocks inflammatory cascades by conversion of arachidonic acid to prostaglandins and thromboxane A2. In clinical practice, compelling evidence has confirmed that aspirin can inhibit the production of pre-inflammatory cytokines, such as CRP and tumor necrosis factor-α (22). In addition, it can also downregulate oxidative stress and protect against oxidative damage (Mendlewicz et al., 2006) and acute cerebral infarction (48). Importantly, in patients with depression, aspirin successfully reduced oxidative stress (49) and promoted actions of antidepressants (50). In our study, we have demonstrated that aspirin successfully reduced manic-like behaviors in rats and elevated the expression of INF-γ and TLR3 in brain tissue. As a matter of fact, low-dose aspirin could produce a statistically significant duration-independent reduction in the relative risk of clinical deterioration in BD subjects treated with lithium (23). Epidemiological data also confirm that aspirin protects against depression in older men with elevated levels of homocysteine (51). Recently, a preliminary study of a phase IIA clinical trial has convincingly demonstrated a main effect of low-dose aspirin for BD patients treated with or without minocycline (24). Because low dosage of aspirin is mostly benign and affordable, an adequately-powered study in large-scale will help consolidate the concept of aspirin as an adjunctive treatment option for BD or even other relevant diseases such as schizophrenia and Alzheimer's disease. However, a major limitation of this experiment is that animals only received a single dose of aspirin that lasts for 1 week. Treatment with multiple dosages for longer time may help to precisely reveal roles of aspirin and inflammatory modulation in ouabain-induced manic-like symptoms in rats.

In summary, we have demonstrated that rats with ouabain ICV injection display reduced expression of BDNF, INF-γ levels, and TLR3 in brain tissues, and aspirin supplement can elevate the expression of INF-γ and TLR3. We propose that aspirin may be of potential benefit for adjunctive treatment of BD.

DATA AVAILABILITY

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

ETHICS STATEMENT

All the described procedures were approved by the Institution of Animal Care and Use Committee of The Second Xiangya Hospital (Protocol number: 2015.014) and adhered to the Guide for the Care and Use of Laboratory Animals.

AUTHOR CONTRIBUTIONS

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

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ORIGINAL RESEARCH
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Surgery Under General Anesthesia Alleviated the Hyperactivity but Had No Effect on the Susceptibility to PND in ADHD Rats

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Background: Attention-deficit hyperactivity disorder (ADHD) is a typical neuropsychiatric disorder characterized by inattention, impulsivity, and hyperactivity, particularly in children. Recent studies demonstrated a close relationship between the development of ADHD and surgery under general anesthesia. However, few studies illustrated if ADHD symptoms changed after surgery. Meanwhile, whether these individuals with natural neural impairment were sensitive to postoperative neurocognitive disorder (PND) still remain unclear.

Methods: Spontaneously hypertensive rats (SHR) were utilized as spontaneous ADHD animal model and Wistar-Kyoto (WKY) rats as non-ADHD animal model. We evaluated the variation of neurocognitive function and locomotor activity of the rats undergoing experimental laparotomy with general anesthesia by isoflurane. Neurocognitive function was assessed by fear conditioning test for contextual memory and Morris water maze (MWM) for spatial memory. Depressive-like behavior after surgery was detected by forced swim test, and open-field test and elevated plus maze test were utilized to evaluate locomotor activities and anxiety. Furthermore, we compared electroencephalogram (EEG) signal in ADHD and WKY rats under free-moving conditions. Afterward, *c-Fos* staining was also utilized to detect the excitatory activity of neurons in these rats to explore the neural mechanism.

Results: Locomotor activity of SHR assessed by average speed and number of line crossings in the open-field test decreased 1 week after surgery under general anesthesia, but there was no difference concerning anxiety levels between SHR and WKY rats after surgery. This phenomenon was also paralleled with the change in EEG signal (delta band 0~3 Hz). Surgery under general anesthesia had no effect on spatial and contextual memory, while it improved spontaneous depression in SHR. The expression of *c-Fos* was downregulated for at least 1 week in the nucleus accumbens (NAc) area of ADHD rats' brain after surgery.

Conclusion: ADHD rats were not sensitive to PND. Surgery with general anesthesia could partly improve the hyperactivity symptom of ADHD rats. This mechanism was related to the suppression of neural activity in the cerebral NAc of ADHD rats induced by general anesthetics.

Keywords: ADHD, postoperative neurocognitive disorder, surgery, general anesthesia, nucleus accumbens

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INTRODUCTION

Attention-deficit hyperactivity disorder (ADHD) is a typical and heterogeneous neuropsychiatric disorder characterized by impaired levels of hyperactivity, impulsivity, and inattention (1), which affects children in particular. In earlier studies, ADHD had been proved as a central nervous system (CNS) disorder induced by gene polymorphism and imbalance of excitatory and inhibitory neurotransmitters (2). Psychiatric Genomics Consortium genome-wide association study (GWAS), including 20,183 individuals with ADHD and 35,191 controls, provides large-scale data on how common genetic variations are associated with the ADHD (3). The etiology of ADHD was, however, not fully understood (4).

In 2016, the US Food and Drug Administration issued a warning regarding impaired brain development in children following exposure to certain anesthetic agents used for general anesthesia, namely, the inhalational anesthetics isoflurane, sevoflurane, and desflurane and the intravenous agents propofol and midazolam, in the third trimester of pregnancy (5). It indicated that there would be a close interaction between the development of this congenital neural disease and surgery under general anesthesia. However, few studies had illustrated if the symptom of ADHD changed following surgery (6). Meanwhile, whether these individuals with natural neural impairment were also sensitive to perioperative neural disorder still remained unclear. For instance, postoperative neurocognitive disorder (PND) was a severe complication that influences neural function, including compromised attention, memory, orientation, executive function, or language fluency after surgery (7). Hence, it is necessary to verify the impacts and safety of surgery and general anesthesia on ADHD patients.

In the current study, we utilized spontaneously hypertensive rats (SHR) as a natural ADHD model (8). Locomotor activity and neurocognitive behavioral performance were observed and electroencephalogram (EEG) signal under free-moving condition was monitored from preoperation to 1 week postoperation. We further compared the specific change in different nuclei among SHR and Wistar-Kyoto (WKY) rats after surgery. Such a research will help shed light on a new perspective for what the relations are between the surgery and ADHD.

MATERIALS AND METHODS

Animals

We used the SHR as spontaneous ADHD animal model and the Wistar-Kyoto Rats (WKY) as non-ADHD animal model. All animal procedures were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and experimental protocols were approved by the Ethics Committee for Animal Experimentation of the Fourth Military Medical University. Animals were habituated for 1 week before any intervention with access to water and food *ad libitum* and kept on a standard 12-h light/12-h dark cycle.

Experimental Design

All the rats were bought at the age of 6 weeks. After 1 week habituation, we divided both the SHR and WKY rats into surgery group and nonsurgery group and implemented the animal surgery and corresponding control procedure. Then, at the time point of 1 week postoperation, we evaluated the locomotive activity, contextual memory, spatial memory, EEG, and *c-Fos* expression of all rats. Except for EEG signal monitoring, rats in no-surgery group and surgery group were independently enrolled and treated nonconsecutively. We utilized different batches of rats at every individual experiment.

Experimental Laparotomy

Experimental laparotomy was performed on animals with general anesthesia by isoflurane to evaluate the variation of neurocognitive function and locomotor activity after surgery. Anesthesia was performed through a face mask (1.5 to 2.0% isoflurane, O2 1.0 L/min). Animals were placed on a heating pad during the surgery to keep the body temperature between 36.5 and 37.0°C. The abdominal hair was shaved, and the skin was sterilized. A 2-cm incision was performed on the midline of the abdomen. Approximately 5-cm small intestine was exteriorized from the peritoneal cavity, covered with gauze soaked with normal saline, and gently rubbed for 10 min. After the manipulation, abdominal muscle was closed continuously with 5-0 Vicryl sutures (Polysorb™, U.S.A.), followed by skin interrupted closure with 4-0 silk suture. Ropivacaine/lidocaine 0.2% (300 μl) was locally injected for postoperative analgesia to avoid the impact of pain to neurocognitive assessment. The surgery duration was controlled at approximately 30 min. Postoperative animals would recover in an incubator at 35°C for 30 min, then return to their home cages.

Neurocognitive Function Assessment

Behavioral tests were applied to assess locomotor activity, depression, anxiety, contextual memory, and spatial memory according to previous protocols with slight modifications (9–12).

Fear Conditioning Test

The fear conditioning test consists of three phases: habituation phase, training phase, and test phase. On training day, five times of foot shocks were delivered (current: 0.7 mA, 2 s; interval between each foot shock: 35–60 s). Twenty-four hours later, rats were kept in the same context for 5 min for assessment of contextual memory retrieval. The animals were considered freezing if no movement was detected for 2 s.

Morris Water Maze

The Morris water maze (MWM) was carried out as described before with modifications. The circular water pool (150 cm diameter and 80 cm high) was placed in a soundproof test room. The pool was filled with water (22 ± 1°C) to a height of 42 cm, and the pool was artificially divided into four conceptual quadrants (N, S, E, or W). Each quadrant was designed as a starting point in the subsequent sessions. A platform (20 cm diameter and 40 cm high) was set inside and fixed in the middle of S quadrant, submerged 2 cm below the water surface. In each session, animals were placed in the water at one of the four starting points. Rats underwent four trials per day at 50-min intervals, repeated for 5 training days. Each rat was given 90 s to search for the platform. If the rat failed to do so independently, it would be guided to the platform and left to stay there for 30 s

before returning to its home cage. In the memory trials, if the rat could not find the platform in 90 s, the session was finished and the maximum score of 90 s was recorded. Learning and memory trials were recorded using an overhead video camera (Sony DCR-SR85) at the center of the pool. The variables measured were the time to get the platform (or the time taken to cross the site of the platform) and the time spent on the platform quadrant (or spent in the quadrant where the platform was placed during training sessions).

Open-Field Test

Animals were placed in an open-field apparatus (120 cm long *120 cm wide *40 cm high) for free exploring the field for 5 min. All of the moving traces were recorded by an overhead camera. The total travel distance was analyzed by Video Tracking Software (ANY-maze, Stoelting Co., Ltd.). At the end of each session, the surface and side walls of the apparatus were cleaned with ethanol before and after each session to eliminate any olfactory cues that may affect the outcomes of subsequent sessions.

Elevated Plus Maze Test

The elevated plus maze apparatus consists of a central platform (10 cm long * 10 cm wide), two open arms (50 cm long * 10 cm wide), and two closed arms with protective walls of 40 cm high (50 cm long * 10 cm wide) that is 50 cm above the ground. Animals were placed in the central platform facing one open arm of the apparatus and were free to explore the arms for 5 min. The apparatus was cleaned with ethanol before and after each session. All of the traces were recorded by an overhead camera. The travel time and numbers of entry into the open/closed arms were analyzed by Video Tracking Software (ANY-maze, Stoelting Co., Ltd.).

Forced Swim Test

Animals were placed in a 60 × 20 cm Plexiglas cylinder containing 30 cm of water (maintained at 23–25°C) and forced to swim for 15 min. Animals were re-placed in the water on the next day for 5 min, and all of the behaviors were recorded by a front camera. The immobile time (floating and necessary movements to breathe) was assessed by Video Tracking Software (ANY-maze, Stoelting Co., Ltd.).

EEG Signal Monitoring

Electrodes were implanted on the scalps of animals at 5 weeks of age, and then they were allowed to recover for 5–7 days after electrode implantation. After rehabilitation for 1 week, EEG recording was performed at 6–7 weeks of age. Three EEG electrodes were implanted on the left and right frontal (± 2.0 mm lateral and 3.2 mm anterior from the bregma), parietal (± 3.5 mm lateral and 1.8 mm posterior from the bregma), and occipital cortex (± 2.0 mm lateral and 5.2 mm posterior from the bregma) of the scalps. The left occipital cortex electrode was used as a reference. One week after experimental laparotomy, rats in the same group were rehabilitation for another 1 week and then monitored EEG signal. With this setup, five times series of EEG recordings were obtained.

Immunohistochemistry

Animals were deeply anesthetized with overdose chloral hydrate (80 mg/kg, i.p.), cardiac perfused with ice-cold phosphate-buffered saline (PBS), and followed by 4% formaldehyde solution. The brains were harvested from the skull and then postfixed for at least 24 h. After postfixation, each brain was sliced coronally (30 mm thickness) using a microslicer (DSK-3000, Dosaka, Kyoto, Japan). The *Fos*-IR staining was performed using a previous protocol with adjustments (Ohno et al., 2009a, 2011). Briefly, brain slices were washed in PBS with 0.3% Triton X-100, incubated for 2 h in 2% normal rabbit serum, and then incubated again in the presence of goat c-Fos antibody and 2% normal rabbit serum (Santa Cruz Biotechnology Inc., Santa Cruz, CA) for 18–36 h. The sections were then washed in PBS and incubated with the biotinylated rabbit anti-goat IgG secondary antibody (Vector Laboratories, Burlingame, CA) for another 2 h. After incubating with the secondary antibody, brain sections were then incubated with PBS containing 0.3% hydrogen peroxide for 30 min. At last, the sections were cleaned with PBS and incubated for 2 h using an avidin-biotinylated horseradish peroxidase complex (Vectastain ABC Kit). The diaminobenzidine-nickel staining method was performed for visualization of *Fos* staining.

Statistical Analysis

Statistical analysis was performed using the SPSS version 20.0 program (SPSS Inc., Chicago, IL) or GraphPad Prism 7.0 software. For comparison of locomotor activity and neurocognitive tests between two groups, Student's t-test was used. A one-way analysis of variance (ANOVA) with *post hoc* Tukey's test was used when more than two groups were compared. Freezing time ratio was analyzed with Mann-Whitney U test when comparing two groups, whereas Kruskal-Wallis test with Dunn's *post hoc* test was used when comparing four groups. For spatial learning (escape latency), a two-way ANOVA was used to determine statistical significance among groups at different time points with Sidak's multiple comparison. $P < 0.05$ was considered significant.

RESULTS

Experimental Laparotomy Reduced Locomotor Activity in SHR and Had No Effect on Anxiety

We used both SHR and WKY rats to investigate the variation of locomotor activity and emotion after surgery, especially in ADHD individuals. Locomotor activities, assessed by number of line crossings in open-field test, were significantly higher in SHR than that in WKY rats (**Figures 1A**, **B**; ***$P < 0.0001$, SHR-No Surgery vs. WKY-No Surgery). Little has changed in WKY rats after surgery. However, locomotor activities in SHR were reduced 1 week after surgery, and this reduction could last up to 2 weeks postoperation (**Figures 1A**, **B**; **$P = 0.0002$, No Surgery vs. Surgery in SHR group, $P = 0.8502$, No Surgery vs. Surgery in WKY group). Compared with SHR, WKY rats spent less time in the open arm of the elevated plus maze, which indicated that WKY rats were more apt to be anxious in physiology (**Figures 1C**, **D**; *$P = 0.0237$, SHR-No Surgery vs. WKY-No Surgery, 26.90 ± 5.44 vs. 12.47 ± 5.56). So, we further detected anxiety by utilizing elevated plus maze test again 1 week postoperation. Results showed no alteration in the time ratio in the open arm

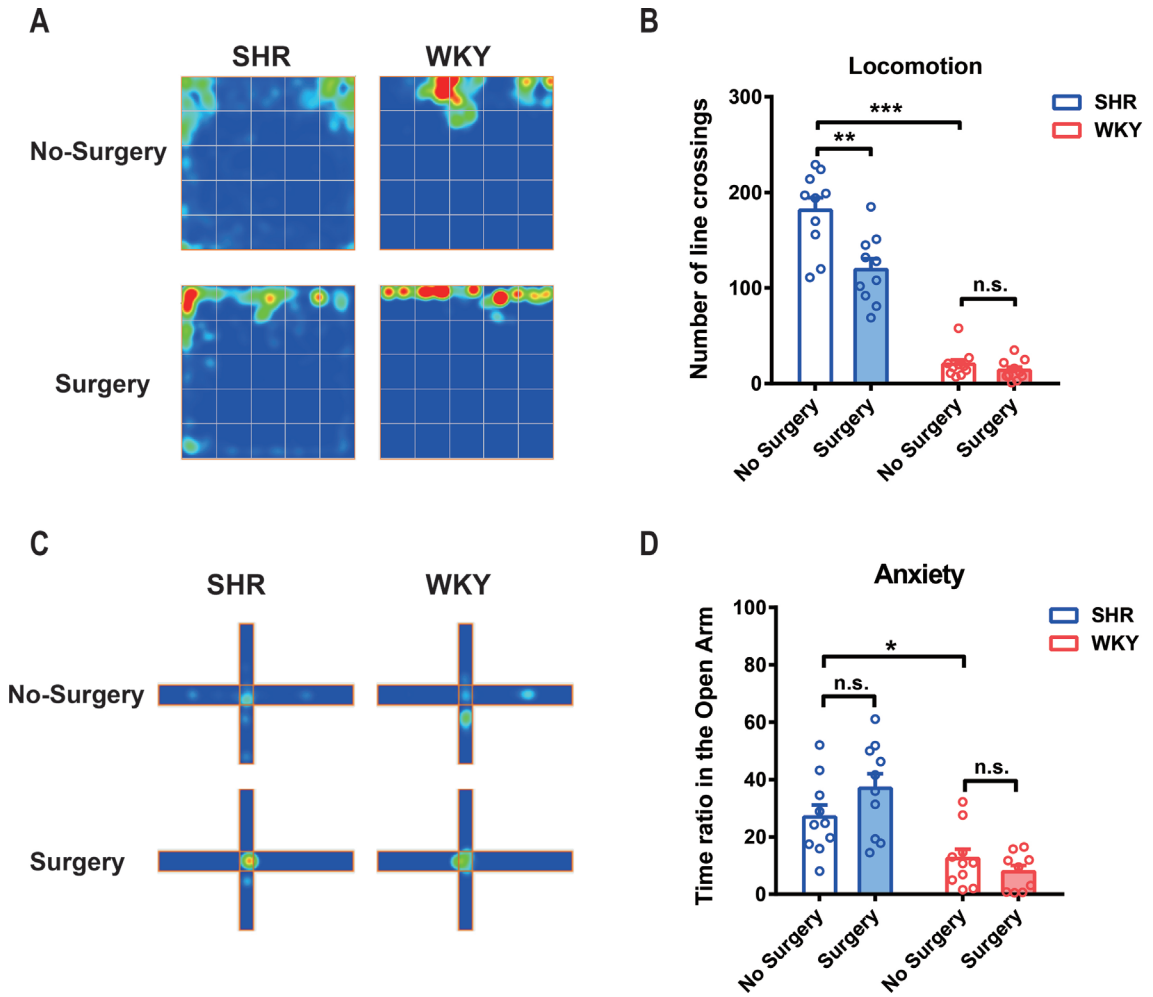

FIGURE 1 | Experimental laparotomy reduced locomotor activity in SHR rats and had no effect on anxiety. **(A)** Average heatmap of open-field test. **(B)** Statistical histogram of locomotor activity in the open field. Number of line crossings was calculated for assessing locomotor activities. SHR-No Surgery vs. WKY-No Surgery, ***$P < 0.0001$; SHR-No Surgery vs. SHR-Surgery, **$P = 0.0002$; WKY-No Surgery vs. WKY-Surgery, $P = 0.8502$, Two-way ANOVA, n = 10. **(C)** Average heatmap of elevated plus maze test. **(D)** Statistical histogram of anxiety situation in elevated plus maze. Time ratios in the open arm were calculated for assessing anxiety situation. SHR-No surgery vs. WKY-No Surgery, *$P = 0.0237$; SHR-No Surgery vs. SHR-Surgery, $P = 0.1398$; WKY-No Surgery vs. WKY-Surgery, $P = 0.6554$, Two-way ANOVA, $n = 10$.

between Surgery and No-Surgery group in both SHR and WKY rats (**Figures 1C, D**; $P > 0.05$, No-Surgery vs. Surgery). Therefore, 1 week after surgery, the hyperactivity symptoms but not the anxiety of SHR were alleviated.

Experimental Laparotomy Did Not Impair the Spatial and Contextual Memory, Whereas It Alleviated Spontaneous Depression in SHR

To testify whether ADHD individuals were more sensitive to PNDs, we utilized MWM test, fear conditioning test, and forced swim test to assess the spatial memory, contextual memory, and depression, respectively, in SHR and WKY rats. Our results showed experimental laparotomy did not cause any change in spatial memory in both SHR and WKY rats (**Figure 2A**, typical tracks of SHR and WKY rats in Morris Water Maze test. **Figure 2B**, No Surgery vs. Surgery in SHR, $P = 0.8820$; No Surgery vs. Surgery in WKY, $P = 0.9981$, $n = 10$ Two-way ANOVA, Tukey's multiple comparisons test. **Figure 2C**, No Surgery vs. Surgery in SHR, $P = 0.8735$; No Surgery vs. Surgery in WKY, $P = 0.5594$, $n = 10$, Two-way ANOVA), although SHR had a better performance in the retrieval of spatial memory on probing day under the condition without surgery (**Figure 2C**, SHR-No Surgery vs. WKY-No Surgery, $P = 0.0140$, $n = 10$, Two-way ANOVA). Meanwhile, surgery did not affect the contextual memory of SHR nor did it affect WKY rats (**Figure 2D**, No Surgery vs. Surgery in SHR, $P = 0.7314$; No Surgery vs. Surgery in WKY, $P = 0.6037$, $n = 10$ Two-way ANOVA). Subsequently, we observed that surgery could reduce the immobility time of SHR in the forced swim test, suggesting that surgery with general anesthesia could improve depression of SHR (**Figure 2E**; *$P = 0.0172$, No Surgery vs. Surgery in SHR). These results indicated that SHR were not susceptible to PND whereas surgery with general anesthesia was safe for ADHD patients.

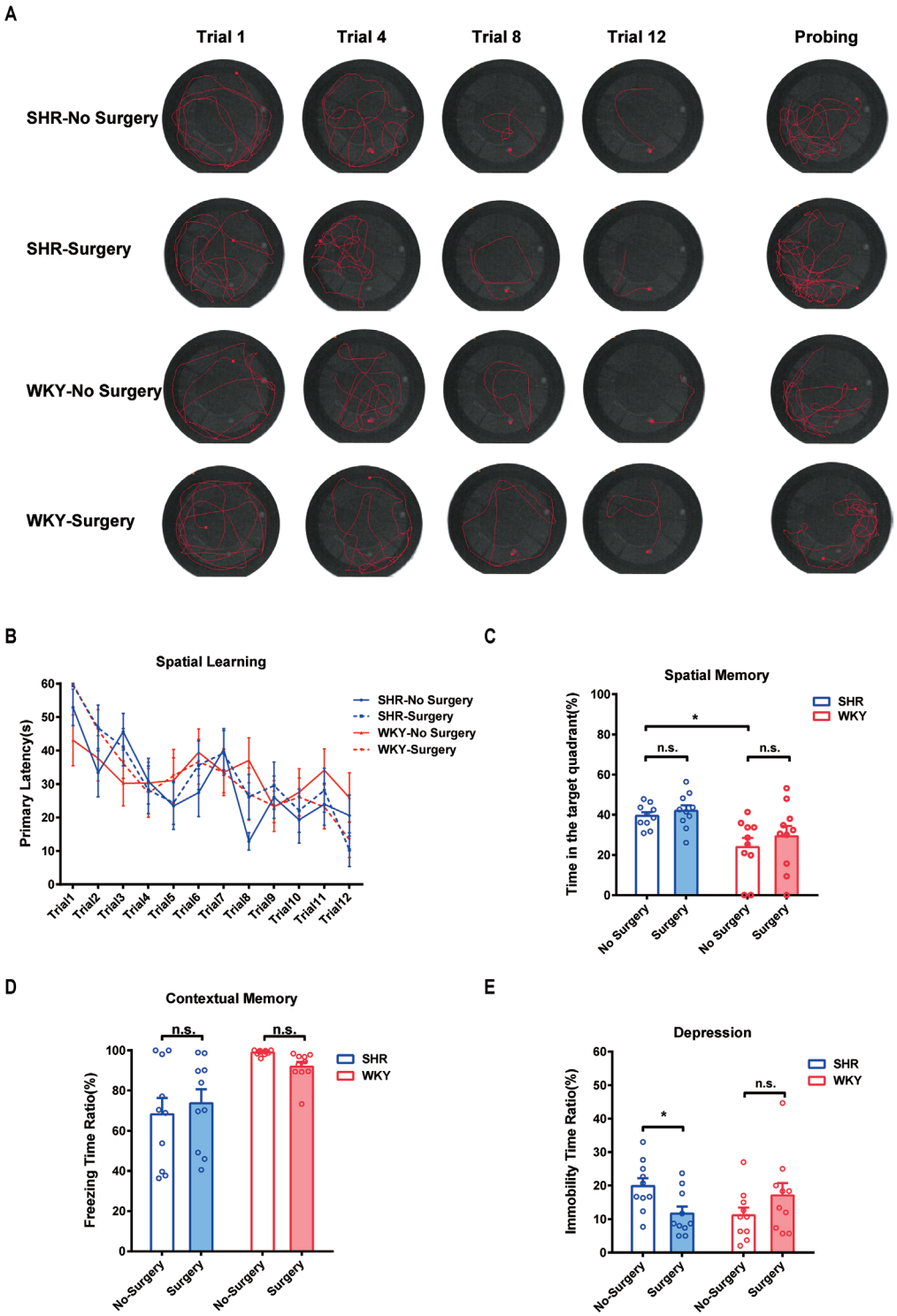

FIGURE 2 | Experimental laparotomy did not impair the spatial and contextual memory but alleviated spontaneous depression in SHR. **(A)** Typical track maps of MWM training and testing. **(B)** Statistical line graph of spatial learning speed in MWM. Primary latencies to the escape platform were calculated for assessing spatial learning speed, Two-way ANOVA, $P = 0.8820$, SHR-No Surgery vs. SHR-Surgery. $P = 0.9981$, WKY-No Surgery vs. WKY-Surgery. **(C)** Statistical histogram of spatial memory in MWM test. Time ratios in the target quadrant (%) were calculated for assessing spatial memory. $^*P = 0.0140$, SHR-No Surgery vs. WKY-No Surgery. $P = 0.8735$, SHR-No Surgery vs. SHR-Surgery. $P = 0.5594$, WKY-No Surgery vs. WKY-Surgery. **(D)** Statistical histogram of contextual memory in the fear conditioning test. Freezing time ratios (%) were calculated for assessing contextual memory. Two-way ANOVA, $P = 0.7314$, SHR-No Surgery vs. SHR-Surgery. $P = 0.6037$, WKY-No Surgery vs. WKY-Surgery. **(E)** Statistical histogram of depression in the forced swim test. Immobile time ratios (%) were calculated for assessing depressive situations. Two-way ANOVA, $^*P = 0.0172$, SHR-No Surgery vs. SHR-Surgery. $N = 10$ in each group.

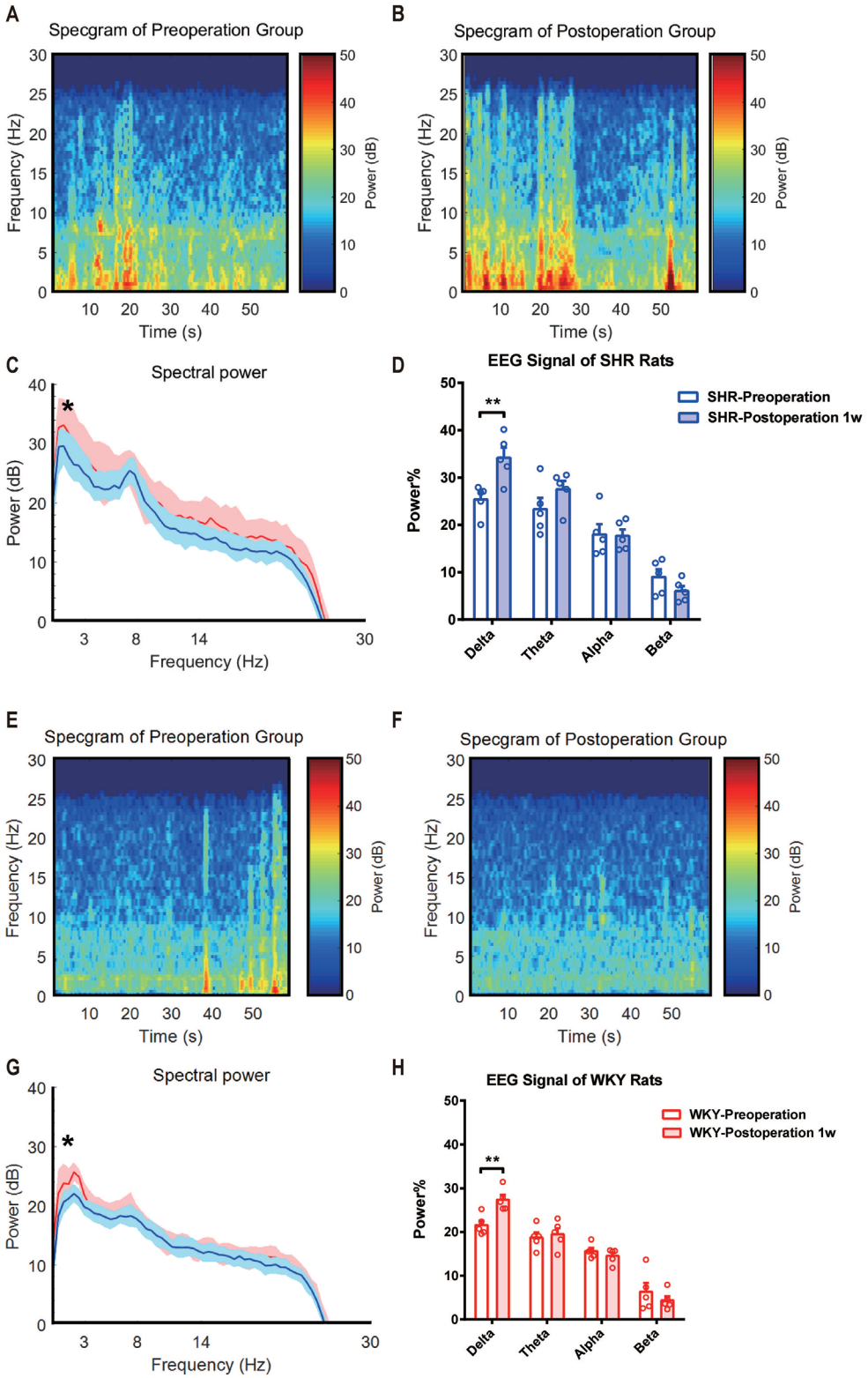

FIGURE 3 | Delta band power of EEG signal was significantly enhanced after surgery in both SHR and WHR rats. **(A, E)** Average EEG spectrum of SHR and WKY rats before surgery. **(B, F)** Average EEG spectrum of SHR and WKY rats at 1 week after surgery. **(C,G)** Statistical line graph of EEG signals power. $^{*}P = 0.0124$, SHR preoperation vs. SHR postoperation 1 week; $^{*}P = 0.0296$, WKY preoperation vs. WKY postoperation 1 week. **(D, H)** Statistical histogram of EEG signals power in different bands. $^{**}P = 0.0062$, SHR preoperation vs. SHR postoperation 1 week in delta band; $^{**}P = 0.0076$, WKY preoperation vs. WKY postoperation 1 week in delta band. Delta band (0~4 Hz). Two-way ANOVA, $N = 5$ in each group.

Delta Band of EEG Signal Was Significantly Enhanced 1 Week After Surgery in SHR

In earlier studies, it has been demonstrated that ADHD individuals performed an abnormal EEG signal (2, 13). Here, we monitored EEG signals of SHR and WKY rats under a free-moving condition before and 1 week after experimental laparotomy (**Figures 3A, B, E, F**). Compared with the preoperative signal, the power of EEG signal in Delta (0~3 Hz) band significantly enhanced 1 week after surgery (**Figure 3C, G**. Statistical line graph of EEG signals power. $^{*}P = 0.0124$, SHR preoperation vs. SHR postoperation 1 week; $^{*}P = 0.0296$, WKY preoperation vs. WKY postoperation 1 week.) and the band ratio of EEG in delta band also increased (**Figure 3D, H**. Statistical histogram of EEG signals power in different bands. $^{**}P = 0.0062$, SHR preoperation vs. SHR postoperation 1 week in delta band; $^{**}P = 0.0076$, WKY preoperation vs. WKY postoperation 1 week in delta band). No variation observed in Theta (3~8 Hz) band, Alpha band (8~14 Hz), and Beta band (14~30 Hz). These results suggested that the improvement of ADHD symptoms as hyperactivity induced by surgery was associated with the change in neural excitation.

The C-Fos Expression of SHR Significantly Decreased in Nucleus Accumbens After Surgery

To determine whether surgery under general anesthesia had a lasting impact on the function of neural circuits, we compared the neuronal excitability in nucleus accumbens (NAc)-related circuitry *via c-Fos* staining in both SHR and WKY rats. Before surgery, the expression of *c*-Fos in SHR was slightly higher than in WKY rats, but there was no statistical difference. After surgery, the expression of *c-Fos* in NAc region was significantly decreased, which would last for at least 1 week (**Figures 4A, B**; $^{*}P < 0.05$, No Surgery vs. Surgery in SHR; $^{**}P < 0.05$, No Surgery vs. Surgery in WKY rats). NAc nucleus was an important brain region associated with ADHD symptoms (8). The variation of neural activities in the NAc region induced by surgery was related to the behavioral improvement in ADHD individuals.

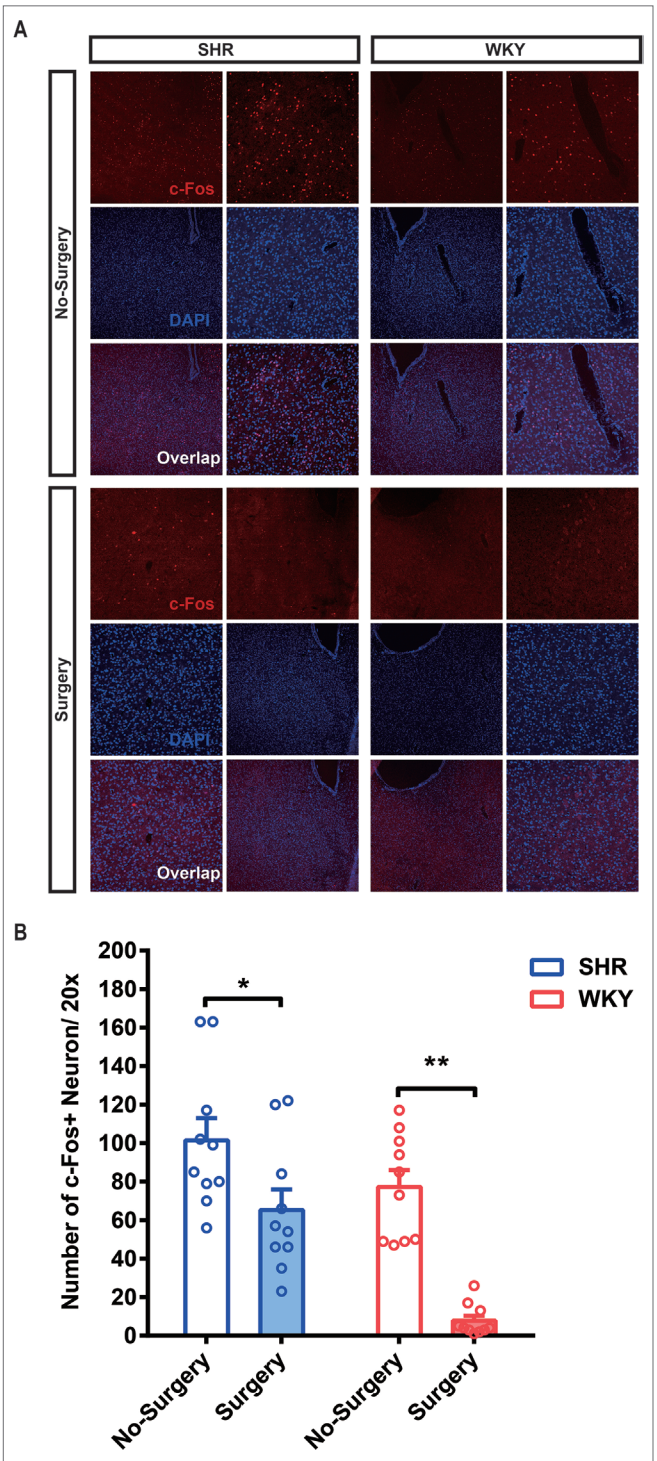

FIGURE 4 | The *c-Fos* expression of SHR and WKY rats was significantly alleviated in the NAc (nucleus accumbens) 1 week after surgery. (A) *c-Fos* staining in the NAc area of SHR and WKY rats brain tissue between No-Surgery group and Surgery group. (B) Statistical histogram of neuronal excitation. Number of *c-Fos*+ neuron per 20× vision was calculated for assessing neuronal excitatory.$^{*}P < 0.05$, SHR No-Surgery vs. SHR Surgery; $^{**}P < 0.01$, WKY No-Surgery vs. WKY Surgery. Two-way ANOVA, N = 10 in each group.

DISCUSSION

ADHD is one of the most common mental disorders affecting children, adolescents, and adults (14). In recent years, benefited from the combination of basic research and multicenter clinical research, the pathological mechanism and clinical therapeutics of ADHD gained great breakthroughs. In terms of a congenital nervous system disease, ADHD individuals not only suffered from typical symptoms, hyperactivity–impulsivity and inattention but also were sensitive to other neurocognitive disorders (15, 16). In the current study, we aimed to elucidate whether surgery under general anesthesia was safe for these ADHD individuals. Hence, we observed the locomotor activity, anxiety,

neurocognitive behaviors, depression, activity of specific neural circuits and monitored EEG signal in ambulatory state from preoperation to 1 week postoperation. According to the results, we demonstrated that laparotomy under general anesthesia was safe for spontaneous ADHD rodents, SHR. More importantly, the locomotor hyperactivity even partly improved in ADHD individuals 1 week after surgery, and there was no evidence that SHR were susceptible to perioperative neurocognitive disorders.

Scientists proceeded with researches about the etiology of ADHD from the aspect of genomics, epigenetics, neurology, psychology, and so on. In our previous study, we demonstrated that deficiency of tumor suppressor NDRG2 could lead to attention deficit and hyperactive behavior in children by inhibition of astroglial glutamate clearance (2). On the other hand, in children who were diagnosed as having ADHD, the functions of DA and NE transporters were overly or lowly activated, which was considered to cause symptoms of attention deficit, impulsiveness, and hyperactivity (17, 18). Such findings were a compelling reminder that the imbalance of excitatory and inhibitory neurotransmitter homeostasis in the neurotransmitter system was a crucial influential factor for ADHD development. Thus, we hypothesized that the imbalance of the neurotransmitter system caused by any stimulus was at risk of aggravating ADHD.

In the researches of perioperative neurocognitive disorder, scientists testified that an imbalance of excitatory and inhibitory homeostasis mediated by neurotransmitter receptor dysfunction also played an important role in pathogenic mechanisms of cognitive impairments induced by surgery or general anesthesia (19–21). On February 16, 2019, the GAS study clarified that general anesthesia in early infancy did not affect neurodevelopmental outcomes (22). However, whether surgery under general anesthesia was safe for patients with natural neural impairments still remained unclear. Postoperatively, those children with ADHD, comparing with the average person, inclined to exhibit a greater increase in maladaptive behaviors (23). Within another recent clinical research, after strabismus surgery, the relevant symptoms of the children with the ADHD trait were improved (6). Considering these discordances, it was necessary to explore and verify the impacts that surgery and anesthesia exerted on ADHD patients. It was the reason why we focused upon the cognitive performances and ADHD symptoms in this study.

Brain function of ADHD patients were usually deemed as being in the state of overactivation. In our results, the *c-Fos* expression in the NAc area was higher in SHR relative to WKY rats, and this phenomenon could be downregulated by surgery under general anesthesia. Consistent with our results, various changes in the dopaminergic neurotransmission had been shown in SHR (24). Neurons in the NAc area were always categorized as two subtypes, D1R neuron and D2R neuron. A previous study illustrated that neurotransmission mediated by dopamine D1 receptor was elevated in the SHR accumbens (8), which induced overexcitation and hyperactivity symptom. After surgery, the downregulation of neuronal activity in NAc was conducive to reconstructing a new balance in NAc-related circuits through the enhancement of neuroplasticity. For instance, the VTA-NAc-mPFC neuropathway, which was essential for arousal, food intake, memory, locomotor activity, and some other advanced brain functions (25), would be corrected by the de-excitation of NAc *via* surgery under general anesthesia. We would like to proceed with this research by using advanced neural techniques in further studies, such as DREADDs, optogenetic modulation, and so on.

The dynamics of the EEG spectral power was a reflection of brain functional activity. In the current study, we observed that comparing with the preoperation free-moving signal, the power of EEG signal in Delta (0~3Hz) band became significantly enhanced 1 week after surgery in both SHR and WKY rats, and the band ratio of EEG in delta band was also increased. It suggested that the intrinsic activity pattern of cerebral cortex turned into a new status with much slower wave oscillation after surgery. As we have known, EEG signal power in Delta band used to be generated and enhanced in general anesthesia or NREM sleep (26). In another study, we also found that hyperactivity of ADHD rats subjected to general anesthesia alone could be also improved for at least 1 week, which was paralleled by the variation in Delta band of EEG signal. However, the reasons why EEG change concentrated in Delta band and this phenomenon could last for a long term need further studies.

In conclusion, ADHD individuals were not sensitive to PND. Moreover, surgery with general anesthesia could partly improve the hyperactivity symptom of ADHD. This mechanism was related to the suppression of neural activity in NAc induced by surgery under general anesthesia.

DATA AVAILABILITY

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

We used the Spontaneously Hypertensive Rats (SHR) as spontaneous ADHD animal model and the Wistar Kyoto Rats (WKY) as non-ADHD animal model. All animal procedures were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and experimental protocols approved by the Ethics Committee for Animal Experimentation of the Fourth Military Medical University. Animals were habituated for 1 week before any intervention with access to water and food *ad libitum*, and kept on a standard 12 h light/12 h dark cycle.

AUTHOR CONTRIBUTIONS

PZ contributed to the animal model and behavioral experiments. FX contributed to acquisition and analysis of data and drafting of the manuscript. GZ contributed to animal experiments and drafting of the manuscript. XZ contributed to the EEG

experiments. AL contributed to the morphological experiments. HD contributed to the design of experiments and manuscript revision. LX contributed to the conception and design of the study.

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Association of *SGK1* Polymorphisms With Susceptibility to Coronary Heart Disease in Chinese Han Patients With Comorbid Depression

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There is a strong link between heart disease and depression, both of which are closely related to lifetime stress exposure. Serum/glucocorticoid-regulated kinase 1 (*SGK1*) is a stress-responsive gene with a pivotal role in both the heart and brain. To determine the role of *SGK1* polymorphisms (rs2758151, rs1743963, rs9493857, rs1763509, rs9376026, and rs9389154) in susceptibility to comorbid coronary heart disease (CHD) and depression, we conducted a hospital-based case–control study involving 257 CHD cases (including 69 cases with depression and 188 cases without depression) and 107 controls in a Chinese Han population. Six single-nucleotide polymorphisms (SNPs) in the *SGK1* gene were successfully genotyped by polymerase chain reaction–ligase detection reaction (PCR-LDR) assay. Our results showed no significant differences in *SGK1* genetic polymorphisms between CHD patients and controls, whereas significant associations were observed between *SGK1* SNPs (rs1743963 and rs1763509) and the development of depression in CHD patients (P = 0.018 by genotype, P = 0.032 by allele; P = 0.017 by genotype, P = 0.003 by allele, respectively). However, none of these associations remained significant after Bonferroni correction (P = 0.054 for rs1743963; P = 0.051 for rs1763509). Interestingly, both the GG genotype of *SGK1* rs1743963 and AA genotype of *SGK1* rs1763509 were associated with a higher risk of depression in CHD patients; for rs1763509, the Patient Health Questionnaire-9 (PHQ-9) scores in the carriers of the risk genotype for comorbid depression, AA, were significantly higher than in GG and AG carriers (P = 0.008). Notably, haplotype analysis indicated that haplotype GGA significantly increased the risk of depression in CHD patients (P = 0.011, odds ratio (OR) = 1.717, 95% confidence interval (CI) = 1.132–2.605), whereas haplotype AAG may be a protective factor for CHD patients with comorbid depression (P = 0.038, OR = 0.546, 95% CI = 0.307–0.972). It should be noted that only the significance of haplotype GGA survived after Bonferroni adjustment (P = 0.044) and that no significant differences were found for other *SGK1* SNPs

(rs2758151, rs9493857, rs9376026, and rs9389154) between CHD patients with and without depression. These findings, for the first time, elucidate the important role of *SGK1* variants in the comorbidity of CHD and depression.

Keywords: serum/glucocorticoid-regulated kinase 1, coronary heart disease, depression, polymorphism, stress

INTRODUCTION

Coronary heart disease (CHD) is among the most common chronic diseases, with a severe impact on human health and quality of life. It is also considered to be a psychosomatic disease. The poor prognosis and high sudden death rate associated with CHD may also result in the development of comorbid psychological complications such as anxiety and depression. Accumulating evidence shows that CHD patients suffer from depression to some extent and that the prevalence of depression in CHD patients is twice as high as in the general population (Follath, 2003). As a result, CHD with comorbid depression has become a concern worldwide. An increasing number of studies show that CHD and depression share common risk mechanisms, including inflammation (Shimohina et al., 2015), autonomic dysfunction (Drago et al., 2007), hypothalamus–pituitary–adrenocortical axis dysfunction (Lederbogen and Strohle, 2012), and enhancement of platelet aggregation activity (Tseng et al., 2010). Multiple genetic factors have also become the new focus of scientific studies. Emerging data suggest that genetic defects in 5-hydroxytryptamine (5-HT) (Golimbet et al., 2012), apolipoprotein E (ApoE) (Fritze et al., 2011), endothelial NOS (eNOS) (Salimi et al., 2012; Talarowska et al., 2012), and plasminogen-activator inhibitor-1 (PAI-1) (Lahlou-Laforet et al., 2006) may be related to the risk of CHD with comorbid depression.

A member of the serum/glucocorticoid-regulated kinase (SGK) family, serum/glucocorticoid-regulated kinase 1 (*SGK1*) regulates several ion channels and participates in many cellular reactions, including cell growth, proliferation, migration, survival, and apoptosis (Talarico et al., 2016). A recent study showed that *SGK1* contributes to the regulation of renal Na^+ reabsorption, K^+ secretion, and blood pressure (Valinsky et al., 2018). Given the close association between high blood pressure levels and risk of CHD, it is reasonable to speculate that *SGK1* is related to the risk of CHD. Additionally, *SGK1* plays a vital part in the regulation of neuronal activity, proliferation, and apoptosis and thus is a key determinant of susceptibility to mental illness. As the downstream target molecule of the glucocorticoid receptor (GR), *SGK1* is involved in the development of depression *via* the glucocorticoid signaling pathway. There is also growing evidence indicating that *SGK1* stimulates the production of pro-inflammatory cytokines and oxidants (Lang et al., 2010), which are also closely related to depression. Taking into consideration the complex relationships among *SGK1*, CHD, and depression, we hypothesize that *SGK1* may be a co-pathogenic gene underlying the comorbid mechanisms of CHD and depression. Thus, to further evaluate the role of *SGK1* single-nucleotide polymorphisms (SNPs) in susceptibility to CHD with comorbid depression, we carried out a case–control study involving 257 CHD patients with or without depression and 107 controls.

MATERIALS AND METHODS

Subjects

A total of 257 CHD patients were recruited at the outpatient clinic of the Jining First People's Hospital in Shandong Province, China. For all patients, the diagnosis of CHD was made by at least two experienced cardiologists and confirmed using coronary angiography results (significant coronary artery stenosis ≥ 50% in at least one of the three major coronary arteries or major branches). Those with valvular heart disease, severe autoimmunity disease, cancer, or severe liver and/or kidney disease were excluded. In addition, all CHD patients with or without depression were assessed by at least two experienced psychiatrists according to DSM-5 (5th edition of the *Diagnostic and Statistical Manual of Mental Disorders*) criteria for major depressive disorder, which is characterized by significant depressed mood and anhedonia. Then, the severity of depressive symptoms was scored by Patient Health Questionnaire-9 (PHQ-9), a nine-item questionnaire that is commonly used in outpatients. The scale uses a cutoff score for depression analysis of greater than or equal to 5 (Duko et al., 2018). The 107 age- and sex-matched healthy controls were adults without CHD who had undergone a series of assessments including clinical physical examination, radiographic chest examination, electrocardiogram, and evaluation of medical history. Our study received approval from the medical ethics committee of the Jining First People's Hospital, and informed consents were obtained from all participants.

Genetic Studies (DNA Isolation and Genotyping)

Genomic DNA was isolated from whole blood using a TIANamp Blood DNA Kit (TIANGEN, China) following the manufacturer's instructions. The genotypes of polymorphisms were identified by polymerase chain reaction–ligase detection reaction (PCR-LDR) assay. All primer sequences for both PCR and LDR are shown in **Table 1**. After identification using 1.5% agarose gel and a multiplex ligase detection reaction with an LDR probe, products were determined by direct sequencing with a DNA sequencer. To ensure the quality of genotyping, random DNA samples accounting for not less than 10% of the total subjects were genotyped twice. Genotyping quality assessment of the SNPs tested is presented in **Supplementary Table 1**.

TABLE 1 | Primers of target gene used in the PCR.

SNP	Ancestor allele	Primer sequence	Product size (bp)
rs2758151	C	F: 5′-ACGTTGGATGGGTAAAGGG AACTTCAGACG-3′ R: 5′-ACGTTGGATGGAAGAATCTT AGAGCTTCC-3′	108
rs1743963	A	F: 5′-ACGTTGGATGAGCCAGTGCT GGCCGGGAA-3′ R: 5′-ACGTTGGATGGTGGTAACTT GTAACTGCCC-3′	88
rs9493857	A	F: 5′-ACGTTGGATGGATTATTGTTG CAATGGAAGG-3′ R: 5′-ACGTTGGATGGTGATCATTTG ATTACTGC-3′	100
rs1763509	G	F: 5′-ACGTTGGATGGGAGTAGAGA GATGAGTTTC-3′ R: 5′-ACGTTGGATGTTACACTGAAA GAAGTATG-3′	120
rs9376026	C	F: 5′-ACGTTGGATGCTCAGTACTCTT AATGGATG-3′ R: 5′-ACGTTGGATGCACCTATTAGAT GTGTGGTC-3′	95
rs9389154	G	F: 5′-ACGTTGGATGGACCACTTACT AAAAGGAAGC-3′ R: 5′-ACGTTGGATGTCAGGCTTCCTT GAGTTTGG-3′	120

PCR, polymerase chain reaction; SNP, single-nucleotide polymorphism.

Statistical Analysis

Demographic and clinical characteristics of the study population were evaluated by *t*-test (for continuous variables) and Pearson's χ^2-test (for categorical variables). Genotype distributions and allele frequencies of CHD patients and controls were analyzed by Pearson's χ^2-test. To evaluate the quality of the genotyping data, the SHEsis online haplotype analysis software (http://analysis.bio-x.cn/myAnalysis.php) was applied to calculate the linkage disequilibrium and check Hardy–Weinberg equilibrium in controls based on Pearson's χ^2-test. Additionally, the SHEsis online haplotype analysis software was also performed for calculating the probability of obtaining a difference in the haplotype frequencies observed between patients and controls and for analyzing the haplotype frequencies and probabilities. Bonferroni adjustment was applied to correct for multiple comparisons. Odds ratios (ORs) and 95% confidence intervals (95% CIs) were also calculated. Differences in PHQ-9 scores among different genotypic individuals were assessed using one-way analysis of variance (ANOVA) or Student's *t*-test, when appropriate. All analyses were carried out using SPSS (version 17.0), and $P < 0.05$ was defined as statistically significant.

RESULTS

Basic Characteristics of Study Participants

The demographic and clinical characteristics of the study participants are described in **Table 2**. There were no significant differences between the CHD and control groups in terms of age, gender, body mass index (BMI), and smoking or drinking ($P > 0.05$). Then, CHD patients were further divided into CHD+D and CHD-D groups according to whether comorbid depression was present. There were still no significant differences concerning the basic characteristics between groups ($P > 0.05$).

Hardy–Weinberg Equilibrium Analysis

The locations of the *SGK1* gene and six SNPs are presented in **Supplementary Table 2**. The genotypes of all *SGK1* SNPs in control groups were in Hardy–Weinberg equilibrium based on the χ^2-test results (rs2758151: $\chi^2 = 0.020$, $P = 0.887$; rs1743963: $\chi^2 = 0.115$, $P = 0.734$; rs9493857: $\chi^2 = 0.472$, $P = 0.492$; rs1763509: $\chi^2 = 0.080$, $P = 0.778$; rs9376026: $\chi^2 = 2.909$, $P = 0.088$; rs9389154: $\chi^2 = 0.072$, $P = 0.789$), suggesting that the groups are representative of the population.

SGK1 Polymorphisms

Frequency distributions of genotypes and alleles of six SNPs in CHD patients and controls are shown in **Tables 3** and **4**. Our results suggest the absence of a significant relationship between *SGK1* SNPs and CHD risk. However, significant differences were found between CHD patients with and without depression in the genotype distribution and allele frequency of rs1743963 (A > G) and rs1763509 (G > A). For rs1743963, CHD patients with the GG genotype showed a significant susceptibility to depression ($\chi^2 = 7.988$, $P = 0.018$). Thus, the G allele may be a risk factor in the development of depression in CHD patients ($\chi^2 = 4.572$, $P = 0.032$). For rs1763509, the AA genotype and A allele were associated with a higher risk of depression in CHD patients ($\chi^2 = 8.118$, $P = 0.017$ by genotype; $\chi^2 = 8.974$, $P = 0.003$ by allele). However, the significance of the genotype distribution frequency could not be confirmed after strict Bonferroni adjustment ($P = 0.054$ for rs1743963; $P = 0.051$

TABLE 2 | Demographic and clinical characteristics of the study participants.

Variables	CHD (*n* = 257)	Controls (*n* = 107)	*P*-value	CHD+D (*n* = 69)	*P*-value	CHD-D (*n* = 188)	*P*-value
Age (years)	51.04 ± 6.854	49.84 ± 7.965	0.148[a]	51.26 ± 6.795	0.224[b]	51.24 ± 6.897	0.982[c]
Gender (M/F, *n*)	136/121	49/58	0.216[a]	32/37	0.940[b]	104/84	0.203[c]
Smoking (*n*, %)	89 (34.6)	32 (29.9)	0.383[a]	21 (30.4)	0.941[b]	68 (36.2)	0.392[c]
Drinking (*n*, %)	99 (38.5)	30 (28.0)	0.057[a]	22 (31.9)	0.585[b]	77 (41.0)	0.185[c]
BMI (kg/m^2)	23.73 ± 2.821	23.37 ± 2.332	0.245[a]	23.68 ± 2.543	0.403[b]	24.17 ± 2.938	0.218[c]

[a]CHD versus controls, [b]CHD+D versus controls, [c]CHD+D versus CHD-D. BMI, body mass index; CHD, coronary heart disease; CHD+D, CHD with depression; CHD-D: CHD without depression.

TABLE 3 | Genotype distribution of *SGK1* gene polymorphisms in CHD and control group.

SNP	Genotype	CHD (*n* = 257, %)	Controls (*n* = 107, %)	OR (95% CI)	χ^2	*P*-value
rs2758151	CC	75 (29.2)	30 (28.0)			
(C > T)	CT	131 (51.0)	54 (50.5)			
	TT	51 (19.8)	23 (21.5)		0.139	0.933
	CT + TT	182 (70.8)	77 (72.0)	1.058 (0.641–1.744)	0.048	0.826
rs1743963	AA	41 (15.9)	11 (10.3)			
(A > G)	AG	121 (47.1)	49 (45.8)			
	GG	95 (37.0)	47 (43.9)		2.667	0.264
	AG + GG	216 (84.0)	96 (89.7)	1.657 (0.816–3.361)	1.986	0.159
rs9493857	AA	14 (5.4)	3 (2.8)			
(A > G)	AG	85 (33.1)	36 (33.6)			
	GG	158 (61.5)	68 (63.6)		1.308	0.520
	AG + GG	243 (94.6)	104 (97.2)	1.997 (0.562–7.097)	0.666	0.414
rs1763509	GG	23 (9.0)	5 (4.7)			
(G > A)	AG	89 (34.6)	34 (31.8)			
	AA	145 (56.4)	68 (63.5)		2.197	0.333
	AG + AA	234 (91.0)	102 (95.3)	2.005 (0.742–5.422)	1.946	0.163
rs9376026	CC	176 (68.5)	66 (61.7)			
(C > T)	CT	74 (28.8)	32 (29.9)			
	TT	7 (2.7)	9 (8.4)		5.546	0.062
	CT + TT	81 (31.5)	41 (38.3)	1.350 (0.843–2.160)	1.568	0.211
rs9389154	GG	53 (20.6)	30 (28.0)			
(G > A)	AG	124 (48.3)	52 (48.6)			
	AA	80 (31.1)	25 (23.4)		3.402	0.182
	AG + AA	204 (79.4)	77 (72.0)	0.667 (0.397–1.120)	2.360	0.125

CHD, coronary heart disease; CI, confidence interval; OR, odds ratio; SNP, single-nucleotide polymorphism.

TABLE 4 | Allele distribution of *SGK1* gene polymorphisms in CHD and control group.

SNP	Allele	CHD (2*n* = 514, %)	Controls (2*n* = 214, %)	OR (95% CI)	χ^2	*P*-value
rs2758151	C	281 (54.7)	114 (53.3)			
(C > T)	T	233 (45.3)	100 (46.7)	1.058 (0.768–1.457)	0.119	0.730
rs1743963	A	203 (39.5)	71 (33.2)			
(A > G)	G	311 (60.5)	143 (66.8)	1.315 (0.940–1.838)	2.568	0.109
rs9493857	A	113 (22.0)	42 (19.6)			
(A > G)	G	401 (78.0)	172 (80.4)	1.154 (0.776–1.716)	0.501	0.479
rs1763509	G	135 (26.3)	44 (20.6)			
(G > A)	A	379 (73.7)	170 (79.4)	1.376 (0.936–2.023)	2.651	0.103
rs9376026	C	426 (82.9)	164 (76.6)			
(C > T)	T	88 (17.1)	50 (23.4)	1.476 (0.998–2.182)	3.834	0.05
rs9389154	G	230 (44.7)	112 (52.3)			
(G > A)	A	284 (55.3)	102 (47.7)	0.738 (0.536–1.015)	3.494	0.062

CHD, coronary heart disease; CI, confidence interval; OR, odds ratio; SNP, single-nucleotide polymorphism.

for rs1763509). Interestingly, when subdividing these samples into GG and AA + AG groups, statistical analysis showed that carriers with allele A were more likely to have comorbid depression (χ^2 = 4.238, P = 0.04, OR = 4.213, 95% CI = 0.961–18.466). However, the other four SNPs, rs2758151, rs9493857, rs9376026, and rs9389154, were not significantly related to the risk of depression in CHD patients (as shown in **Tables 5** and **6**).

Association of *SGK1* Polymorphisms With Severity of Depressive Symptoms

As shown in **Figure 1A**, no significant differences in PHQ-9 scores were observed among the three genotypes of rs1743963 (P > 0.05). For rs1763509 (**Figure 1B**), GG and AG carriers were combined because of the few GG carriers. The PHQ-9 scores in the AA carriers, the risk genotype for comorbid depression, were significantly higher than GG and AG carriers (10.63 ± 2.900 versus 8.62 ± 2.500, P = 0.008).

Haplotype Analysis

As shown in **Figure 2**, the LD block in the *SGK1* gene on chromosome 6 comprised rs1743963, rs9493857, and rs1763509, with a strong linkage (rs1743963/rs9493857: D' = 0.793, r^2 = 0.282; rs9493857/rs1763509: D' = 0.869, r^2 = 0.675; rs1743963/rs1763509: D' = 0.633, r^2 = 0.201). Haplotype frequencies indicated that there were no significant differences of haplotype distribution between CHD patients and healthy controls (as shown in **Table 7**). Interestingly, haplotype analysis of the CHD+D and CHD-D groups revealed that haplotype GGA

TABLE 5 | Genotype distribution of *SGK1* gene polymorphisms in CHD+D and CHD-D group.

SNP	Genotype	CHD+D (*n* = 69, %)	CHD-D (*n* = 188, %)	OR (95% CI)	χ^2	*P*-value[a]	*P*-value[b]
rs2758151	CC	21 (30.4)	54 (28.7)				
(C > T)	CT	33 (47.8)	99 (52.7)				
	TT	15 (21.8)	35 (18.6)		0.533	0.766	
	CT + TT	48 (69.6)	134 (71.3)	1.086 (0.594–1.983)	0.072	0.789	
rs1743963	AA	10 (14.5)	31 (16.5)				
(A > G)	AG	24 (34.8)	97 (51.6)				
	GG	35 (50.7)	60 (31.9)		7.988	**0.018**	0.054
	AG + GG	59 (85.5)	157 (83.5)	1.165 (0.538–2.524)	0.150	0.698	
rs9493857	AA	3 (4.3)	12 (6.4)				
(A > G)	AG	18 (26.1)	67 (35.6)				
	GG	48 (69.6)	109 (58.0)		2.924	0.232	
	AG + GG	66 (95.7)	176 (93.6)	0.667 (0.182–2.437)	0.100	0.752	
rs1763509	GG	2 (2.9)	21 (11.2)				
(G > A)	AG	19 (27.5)	70 (37.2)				
	AA	48 (69.6)	97 (51.6)		8.118	**0.017**	0.051
	AG + AA	67 (97.1)	167 (88.8)	4.213 (0.961–18.466)	4.238	**0.04**	
rs9376026	CC	48 (69.6)	131 (69.7)				
(C > T)	CT	20 (29.0)	51 (27.1)				
	TT	1 (1.4)	6 (3.2)		0.702	0.704	
	CT + TT	21 (30.4)	57 (30.3)	0.995 (0.546–1.812)	0.000	0.986	
rs9389154	GG	10 (14.5)	43 (22.9)				
(G > A)	AG	38 (55.1)	87 (46.3)				
	AA	21 (30.4)	58 (30.8)		2.524	0.283	
	AG + AA	59 (85.5)	145 (77.1)	0.572 (0.270–1.212)	2.165	0.141	

[a]P-value without adjustment; [b]P-value after Bonferroni adjustment for multiple comparisons; P-value < 0.05 has been bolded. CHD, coronary heart disease; CI, confidence interval; OR, odds ratio; SNP, single-nucleotide polymorphism.

TABLE 6 | Allele distribution of *SGK1* gene polymorphisms in CHD+D and CHD-D group.

SNP	Allele	CHD+D (2*n* = 138, %)	CHD-D (2*n* = 376, %)	OR (95% CI)	χ^2	*P*-value
rs2758151	C	75 (54.3)	207 (55.1)			
(C > T)	T	63 (45.7)	169 (44.9)	0.972 (0.657–1.438)	0.020	0.887
rs1743963	A	44 (31.9)	159 (42.3)			
(A > G)	G	94 (68.1)	217 (57.7)	1.565 (1.036–2.364)	4.572	**0.032**
rs9493857	A	24 (17.4)	91 (24.2)			
(A > G)	G	114 (82.6)	285 (75.8)	0.659 (0.400–1.086)	2.696	0.101
rs1763509	G	23 (16.7)	112 (29.8)			
(G > A)	A	115 (83.3)	264 (70.2)	2.121 (1.288–3.495)	8.974	**0.003**
rs9376026	C	116 (84.1)	313 (83.2)			
(C > T)	T	22 (15.9)	63 (16.8)	1.061 (0.625–1.803)	0.048	0.826
rs9389154	G	58 (42.0)	173 (46.0)			
(G > A)	A	80 (58.0)	203 (54.0)	0.851 (0.574–1.262)	0.647	0.421

P-value < 0.05 has been bolded. CHD, coronary heart disease; CI, confidence interval; SNP, single-nucleotide polymorphism.

significantly increased the risk of depression in CHD patients ($P = 0.011$, OR = 1.717, 95% CI = 1.132–2.605), while haplotype AAG may be a protective factor against comorbid depression in CHD patients ($P = 0.038$, OR = 0.546, 95% CI = 0.307–0.972). After Bonferroni adjustment, only the haplotype GGA remained significantly associated with the susceptibility to depression in CHD patients ($P = 0.044$) (**Table 8**).

DISCUSSION

The gene encoding human *SGK1* is located in chromosome 6q23.2. *SGK1* transcripts have been found in virtually all tissues tested (Raikwar et al., 2008). A key regulatory enzyme, *SGK1* was originally described as being involved in the hormonal regulation of several ion channels (Lang et al., 2011; Chraibi and Renauld, 2014). *SGK1* is linked to the regulation of Na^+ and K^+ transport in epithelial cells (Valinsky et al., 2018). Studies have shown that dysregulation of *SGK1* causes renal Na^+ retention and enhancement of cardiac output, followed by elevated blood pressure (Kawarazaki et al., 2012; Nakano et al., 2013). Several *SGK1* gene variants have been shown to affect blood pressure (Busjahn et al., 2002; Rao et al., 2013). Accumulating strong evidence indicates a direct connection between *SGK1* and cardiovascular development *via* involvement in the phosphatidylinositol 3-kinase (Catela et al., 2010) and ALK1 (Araki et al., 2018) signaling pathways. Notably, *SGK1* has been shown to contribute to cardiac remodeling and fibrosis,

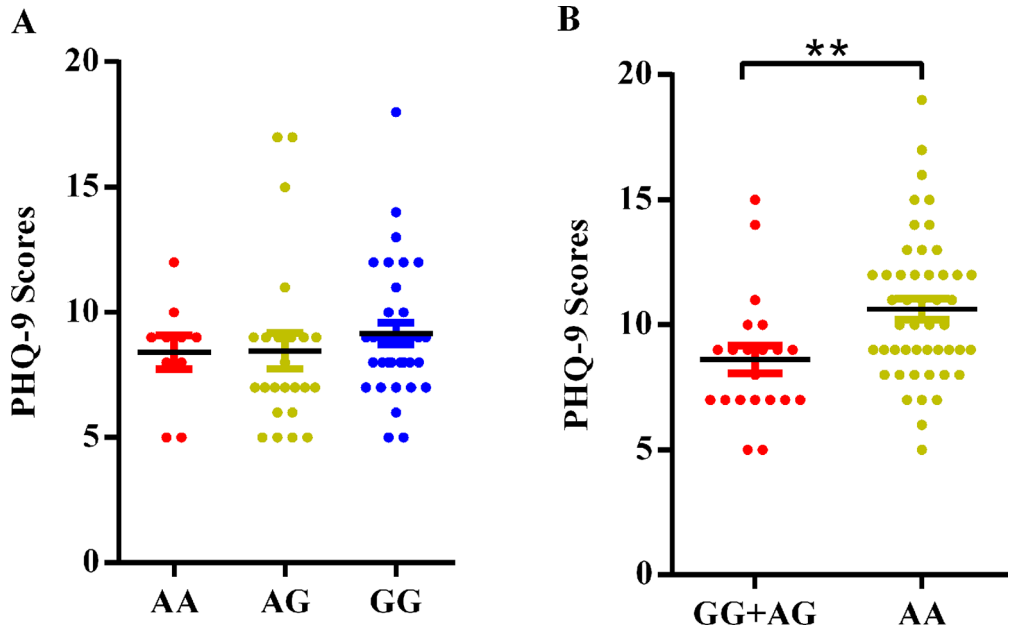

FIGURE 1 | Association of *SGK1* polymorphisms and PHQ-9 scores in CHD patients with comorbid depression. **(A)** rs1743963 and **(B)** rs1763509. **P < 0.01. CHD, coronary heart disease; PHQ-9, Patient Health Questionnaire-9.

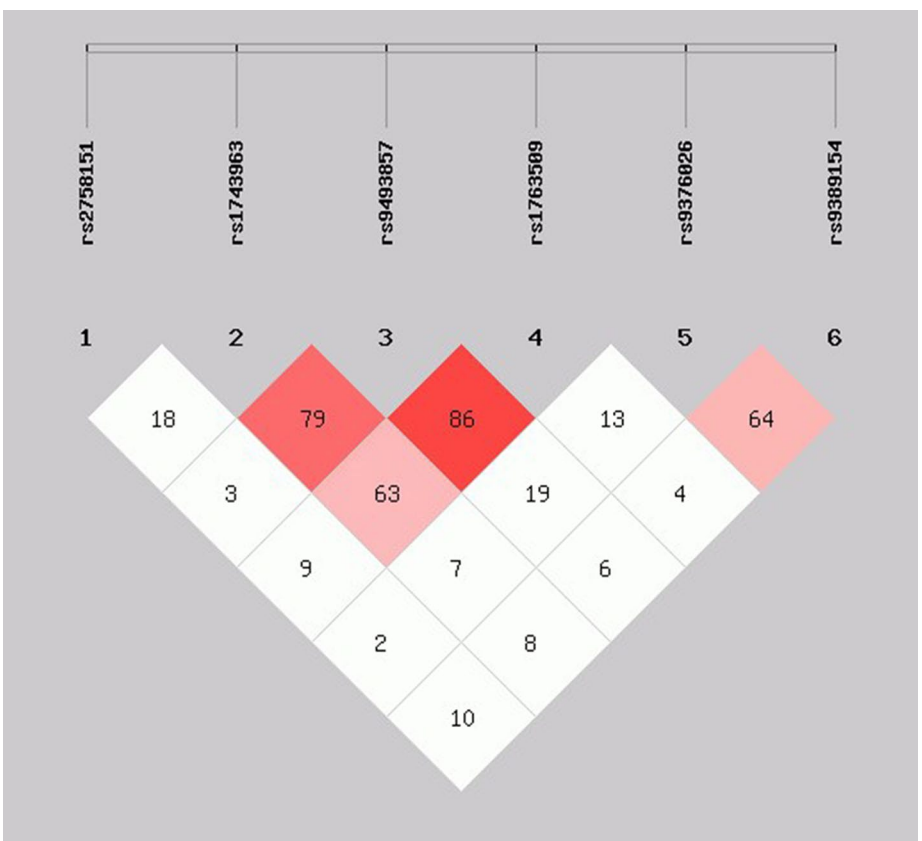

FIGURE 2 | Linkage disequilibrium pattern between three SNPs, rs1743963, rs9493857, and rs1763509, in CHD patients and healthy controls. CHD, coronary heart disease; SNP, single-nucleotide polymorphism.

TABLE 7 | Haplotype frequencies for *SGK1* polymorphisms in CHD and control group.

Haplotype (rs1743963/ rs9493857/ rs1763509)	CHD 2*n* = 514 (%)	Controls 2*n* = 214 (%)	OR (95% CI)	*P*-value
AAG	91.39 (17.8)	32.78 (15.3)	1.219 (0.787–1.888)	0.374
AGA	95.93 (18.7)	35.10 (16.4)	1.192 (0.778–1.827)	0.418
GGA	280.53 (54.6)	131.82 (61.6)	0.760 (0.542–1.066)	0.111
GGG	16.88 (3.3)	5.05 (2.4)	1.428 (0.521–3.913)	0.487

CHD, coronary heart disease; CI, confidence interval; OR, odds ratio. Haplotypes were omitted if the estimated haplotype frequency was <3%.

and development of heart failure. These findings suggest that *SGK1* regulates blood pressure and participates in cardiovascular development and occurrence of heart failure, indicating a potential link to CHD. In this regard, we consider that *SGK1* polymorphisms may be related to the occurrence of CHD.

Furthermore, *SGK1* participates in the regulation of dendrite growth (Lang et al., 2006) and long-term memory formation (Ma et al., 2006) and contributes to the pathophysiology of several neuronal diseases including Parkinson's disease, Alzheimer's disease, schizophrenia, and depression (Lang et al., 2010; Miyata et al., 2015). Animal experiments have shown that the mRNA level of *SGK1* in the hippocampus of mice increased significantly under acute cold water swimming stress (Bohacek et al., 2015), suggesting that *SGK1* is closely related to stress-related mental disorders. Accumulating evidence also suggests that *SGK1* participates in the occurrence of depression *via* the glucocorticoid (Sato et al., 2008) and brain-derived neurotrophic factor (BDNF) (Lang et al., 2007) signaling pathway. Similarly, decreased hippocampal neurogenesis and structural abnormalities have been reported to occur in depressed patients owing to the up-regulation of *SGK1* (Cattaneo and Riva, 2016). *SGK1* additionally contributes to the regulation of neuroexcitability, inflammation, and oxidative stress reactions (Lang et al., 2010), which may be involved in the pathogenesis of depression. In view of the complex relationships between *SGK1*, CHD, and depression, *SGK1* is likely to be a potential co-pathogenic gene underlying susceptibility to CHD with depression.

To test this hypothesis, a case–control study was carried out to identify the role of *SGK1* variants in susceptibility to comorbidity of CHD and depression. Six candidate intron variants located in the upstream of *SGK1* gene were selected. These SNPs were reported to have a tight link with multiple disorders, including blood pressure and renin response to dietary salt intake, and type 2 diabetes (Schwab et al., 2008; Luca et al., 2009; Rao et al., 2013; Chu et al., 2015), with the possibility to affect the process of splicing, processing, and editing of mRNA. Our study of 69 CHD cases with depression and 188 cases without depression found significant differences in the genotype distribution and allele frequency of rs1743963 (A > G) and rs1763509 (G > A). For rs1743963, CHD patients with the GG genotype showed a modest but non-significant susceptibility to depression (P = 0.054), and the G allele was found to be a risk factor for depression in patients with CHD (P = 0.032). Similarly, for rs1763509, the allele A was associated with a higher risk of depression in CHD patients (P = 0.003). Interestingly, when patients were divided into GG and AA + AG groups according to whether they carried allele A, CHD patients in AA + AG group are more likely to have comorbidity with depression. The PHQ-9 scores in the carriers of the risk genotype for comorbid depression, AA, were significantly higher than in GG and AG carriers. In support, Chu reported that SNP rs1763509 of *SGK1* was significantly associated with blood pressure response to the intervention of dietary sodium (Chu et al., 2015). Notably, single marker association analysis is sometimes not sufficient in complex diseases, whereas haplotype-based linkage disequilibrium mapping has been considered a more informative approach for genetic association studies. In the present study, strong linkage disequilibrium was observed between the three SNPs rs1743963, rs9493857, and rs1763509 in the intron region of *SGK1* gene, and haplotype analysis suggests that the haplotype GGA is likely to be involved in the development of depression in CHD patients after Bonferroni adjustment, which may affect RNA splicing, processing, and editing. Overall, our study demonstrates for the first time the importance of *SGK1* variants in the development of depression in CHD patients. Although many genome-wide association studies (GWASs) on depression or CHD (Li et al., 2019; Liu et al., 2019; Wong et al., 2019) have been published, none of these have identified *SGK1* as a risk factor.

The remaining three SNPs, rs2758151 (Rao et al., 2013), rs9376026, and rs9389154 (Chu et al., 2015), have been reported to be associated with blood pressure response to dietary salt

TABLE 8 | Haplotype frequencies for *SGK1* polymorphisms in CHD+D and CHD-D group.

Haplotype (rs1743963/ rs9493857/ rs1763509)	CHD+D 2*n* = 138 (%)	CHD-D 2*n* = 376 (%)	OR (95% CI)	*P*-value[a]	*P*-value[b]
AAG	16.53 (12.0)	73.74 (19.6)	0.546 (0.307–0.972)	**0.038**	0.152
AGA	24.57 (17.8)	72.42 (19.3)	0.894 (0.537–1.487)	0.665	
GGA	85.30 (61.8)	184.04 (48.9)	1.717 (1.132–2.605)	**0.011**	**0.044**
GGG	4.12 (3.0)	21.35 (5.7)	0.505 (0.172–1.477)	0.204	

CHD, coronary heart disease; CI, confidence interval; OR, odds ratio. Haplotypes were omitted if the estimated haplotype frequency was <3%. [a]P-value without adjustment; [b]P-value after Bonferroni adjustment for multiple comparisons; P-value < 0.05 has been bolded.

intake, and rs9493857 was found to regulate *SGK1* expression in response to stress (Luca et al., 2009). However, no significant differences were found between the genotypic and allelic frequencies of polymorphic sites of any of these four SNPs in our study. These negative results can be explained by the relatively small sample size, regional and racial biases, and no correction for potential population stratification, which are major limitations of the present study. Moreover, our study is also limited by the lack of a comparison group of subjects with depression but no CHD for the replication of positive results. Considering that the interactions between various genes and/or environmental factors play a part in the effects of *SGK1*, the association between *SGK1* polymorphisms and depression in CHD patients is likely to be confounded by various potential gene–gene and/or gene–environment interactions. Thus, additional association studies investigating *SGK1* diversity and susceptibility to depression in CHD patients are also required to replicate the associations. We are additionally unable to analyze the expression of *SGK1* and the functional consequence of these genetic variations. Thus, future studies are needed to further examine the effects of these SNPs on the expression of key components of *SGK1* signaling in the peripheral blood mononuclear cells of CHD patients with comorbid depression and thus confirm the relationship between *SGK1* and susceptibility to depression in CHD patients.

CONCLUSION

In conclusion, the present study supports the hypothesis that *SGK1* polymorphisms contribute to the susceptibility to depression in CHD patients of the Chinese Han population. To exclude the many environmental and geographical influences on study outcomes, replication studies with large samples are needed to verify the role of these *SGK1* polymorphisms in CHD patients with comorbid depression.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of medical ethics committee of Jining First People's Hospital guidelines with written informed consents from all subjects. All subjects gave written informed consents and the protocol was approved by the medical ethics committee of the Jining First People's Hospital.

AUTHOR CONTRIBUTIONS

PJ conceived and designed the study; HaixZ, XZ, XG, and YG were responsible for the sample collection; MY and HailZ conducted the experiments and had access to all the data in the study; WH analyzed the data and led the drafting of the manuscript; HaixZ, GL, YL, and GY provided critical revisions of the manuscript. WH and HaixZ contributed equally to the work. All authors approved the final version of the manuscript and agreed to be accountable for all aspects of the work.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2019.00921/full#supplementary-material

SUPPLEMENTARY TABLE 1 | Genotyping quality assessment of the SNPs tested.

SUPPLEMENTARY TABLE 2 | The information and location of SGK1 gene and these SNPs.

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