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Contents

Special Issue on Complex Mental Disorders

About the Cover

The suffering and disability that patients with mental disorders endure are poorly understood by people in general. Furthermore, the pathogenesis, objective diagnosis, and effective therapy of complex mental disorders remain to be established. In this special issue, we present a collection of articles that discuss genetics and neuroimaging in schizophrenia, bipolar disorder, major depressive disorder, and other major mental disorders, to shed light on this important topic. The cover image illustrates a tangled and disordered brain under both external and internal threat. Cover art by Yefei Li.

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·**Editorial**·

An update on research and approaches in biological psychiatry

Lingjiang Li[1,2,*]

[1]*Mental Health Institute of The Second Xiangya Hospital, National Technology Institute of Psychiatry, Key Laboratory of Psychiatry and Mental Health of Hunan Province, Central South University, Changsha 410011, China*

[2]*Shenzhen Kangning Hospital of Guangdong Province, Shenzhen 508003, China*

*Guest Editor of the Special Issue. E-mail: llj2920@163.com

Many studies have shown that, in terms of global burden, mental disorders have exceeded diseases of the cardiovascular and respiratory systems as well as malignant tumors[1]. Nevertheless, the pathological mental activities caused by the millions of neurons in the human brain are far less clear than diseases of other organs and systems. This is not only due to the limited approaches to exploring the human brain, but also the multiple mechanisms by which human mental activities are regulated, including the internal and external physical and social environment. Therefore, our knowledge of mental disorders is still nowhere near the boundaries of truth, especially in the three most important aspects: pathogenesis, objective diagnosis, and effective therapy. For example, environment and heredity may be the most important factors in the pathogenesis of mental disorders, while the mechanism by which the environment impacts the brain and mental disorders remains unclear, and genetic studies have not yet provided definitive specific evidence[2-5]. On the other hand, from the aspect of clinical diagnostic biomarkers, although some operable diagnostic criteria and series of psychological assessment tools have been developed, we are still in urgent need of thorough research to find objective, quantifiable, simple, and convenient markers to facilitate early identification and decrease the misdiagnosis of mental disorders. Considering the topic of treatment, clinical studies have shown that, even when all the currently available treatment approaches are tried, the cure rates of mental disorders remain very low, and refractory mental disorders remain a troublesome clinical dilemma.

To achieve breakthroughs in the above problems, one of the core scientific issues is to seek and establish stable biomarkers from genes to clinical phenotypes. Only in this way can we carry out in-depth research on pathogenesis, establish objective diagnostic markers, and develop effective treatment approaches to mental disorder[6]. In recent years, the development of new techniques and methods in neuroscience seems promising for exploring the essence of mental disorders[7]. Also, in China, funds for research and productive teams in the core scientific issues have been increasing. For this issue, we invited scholars in the fields of clinical psychiatry and basic neuroscience research in China and the USA to present their research and opinions regarding genetics and neuroimaging in schizophrenia, bipolar disorder, major depressive disorder (MDD), and other major mental disorders.

In five studies of schizophrenia, Chen *et al*.[8], Liu *et al*.[9] and Duan *et al*.[10] review the current status and discuss updates in genetic studies of schizophrenia[8], with emphasis on the significance of circadian rhythm-related genes[9], the genetic regulation and perturbation of neurons derived from induced pluripotent stem cells, and genomic encoding[10]. Using mRNA expression measurements in postmortem temporal cortex from Han Chinese and linkage disequilibrium analysis, Sun *et al*.[11] demonstrate that *GNB1L* (guanine nucleotide-binding protein, beta-1-like) is regulated by a *cis*-acting variant within the 3'-region of the gene. And, in light of the mRNA expression results, they further re-analyze the data of previously published case-control studies and show that the *GNB1L* high-expression allele is the risk allele for schizophrenia and bipolar disorder in the Han Chinese population. In a molecular imaging study of schizophrenia, Li *et al*.[12] reveal that patients with schizophrenia show reduced grey matter volume (GMV) in

the cerebellum and the visual, medial temporal, parietal, and middle frontal cortex compared with healthy controls. Another finding of this study is an association between a Val108/158Met polymorphism of the COMT (catechol-O-methyltransferase) gene and the GMV of the superior frontal gyrus, and this effect is mainly due to the gene's effect on cortical thickness rather than cortical surface area. The authors also show that a diagnosis × genotype interaction has an effect on the GMV of the left precuneus.

By reviewing the studies of MDD-related biological mechanism, Cai *et al.*[13] deduce that brain-derived neurotrophic factor (BDNF) dysfunction and increased apoptosis may be the final common cascade for MDD, and therapeutic strategies aimed at enhancing the BDNF system may prove to be an effective approach to achieving a rapid antidepressant effect. Wang *et al.*[14] review studies comparing the effects of monoaminergic and glutamatergic antidepressants on neuronal plasticity, and infer that the pathogenesis of depression may involve maladaptive neuronal plasticity in glutamatergic circuits, which may serve as a new class of targets to produce rapid antidepressant effects. Cai *et al.*[15] compare the GMV in unipolar and bipolar depression by magnetic resonance imaging and voxel-based morphometry, and show that both forms of depression have decreased GMV in the right inferior frontal gyrus, while only bipolar depression shows reduction of GMV in the right middle cingulate gyrus, which, they conclude, may be specific to this disorder. In a study of functional connectivity of hippocampal subregions in remitted late-onset depression (rLOD), Wang *et al.*[16] show that rLOD patients have decreased connectivity between the left cornu ammonis and the bilateral posterior cingulate cortex/precuneus and increased connectivity between right hippocampal subregions and the frontal cortex compared with healthy controls. Further correlative analysis reveals that the abnormal functional connectivity is positively associated with the longitudinal changes in scores in the Symbol Digit Modalities and Digit Span Tests.

Another four studies are concerned with other aspects of mental disorders. Chen *et al.*[17] review studies of the structural and functional changes in the hippocampus in amnestic mild cognitive impairment (aMCI), and conclude that a combination of advanced multi-modal neuroimaging measures can provide more precise and sensitive measurement of hippocampal changes than using only one of these measures. This may contribute to the exploration for biomarkers of the progression of aMCI to Alzheimer's disease. Peng *et al.*[18] investigate the point prevalence of the atypical features of 3 906 patients with bipolar disorder in 26 psychiatric services across China, and show that these features have potential impact on treatment practices for bipolar disorder in China. Zhang *et al.*[19] report on the application of Williams LifeSkills Training to improve trait anxiety, coping styles, and interpersonal support in Chinese male juvenile violent offenders, which may have significant practical value. Zhang *et al.* [20] also describe the mental health laws and regulations in different countries and districts, and outline their criteria and procedures for involuntary admission to psychiatric hospitals and to community services. This may help to standardize the legislation for involuntary disposition of patients with mental disorders in China and serve to improve mental-health care and treatment.

Although the studies in this issue are just a small part of the literature published by Chinese investigators in the field of biological psychiatry, we anticipate that our readers will be enlightened by these samples of current views.

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·Original Article·

Grey matter volume abnormalities in patients with bipolar I depressive disorder and unipolar depressive disorder: a voxel-based morphometry study

Yi Cai[1, 3, 4], Jun Liu[2], Li Zhang[1], Mei Liao[1], Yan Zhang[1], Lifeng Wang[1], Hongjun Peng[1, 5], Zhong He[2], Zexuan Li[1], Weihui Li[1], Shaojia Lu[1], Yuqiang Ding[1, 6], Lingjiang Li[1,7]

[1]*Mental Health Institute of the Second Xiangya Hospital, National Technology Institute of Psychiatry Key Laboratory of Psychiatry and Mental Health of Hunan Province, Central South University, Changsha 410011, China*

[2]*Department of Radiology, the Second Xiangya Hospital of Central South University, Changsha 410011, China*

[3]*Department of Mental Health and Psychiatry, Brain Hospital of Hunan Province, Changsha 410007, China*

[4]*Hunan University of Chinese Medicine Clinical Medical College, Changsha 410007, China*

[5]*Guangzhou Psychiatric Hospital, Affiliated Hospital of Guangzhou Medical College, Guangzhou 510370, China*

[6]*Key Laboratory of Arrhythmias, Ministry of Education, East Hospital, Tongji University School of Medicine, Shanghai 200092, China*

[7]*Shenzhen Kangning Hospital of Guangdong Province, Shenzhen 518003, China*

Corresponding author: Lingjiang Li. E-mail: llj2920@163.com

ABSTRACT

Bipolar disorder and unipolar depressive disorder (UD) may be different in brain structure. In the present study, we performed voxel-based morphometry (VBM) to quantify the grey matter volumes in 23 patients with bipolar I depressive disorder (BP1) and 23 patients with UD, and 23 age-, gender-, and education-matched healthy controls (HCs) using magnetic resonance imaging. We found that compared with the HC and UD groups, the BP1 group showed reduced grey matter volumes in the right inferior frontal gyrus and middle cingulate gyrus, while the UD group showed reduced volume in the right inferior frontal gyrus compared to HCs. In addition, correlation analyses revealed that the grey matter volumes of these regions were negatively correlated with the Hamilton depression rating scores. Taken together, the results of our study suggest that decreased grey matter volume of the right inferior frontal gyrus is a common abnormality in BP1 and UD, and decreased grey matter volume in the right middle cingulate gyrus may be specific to BP1.

Keywords: bipolar depressive disorder; unipolar depressive disorder; prefrontal cortex; cingulate gyrus; voxel-based morphometry

INTRODUCTION

Bipolar disorder (BP) is characterized by alternating episodes of mania and depression[1] and causes dysfunctions in cognition and emotion[2-4]. BP is a chronic, life-threatening illness affecting over 2% of the general population[5]. A World Health Organization report identified BP as one of ten disorders that most often result in permanent disabilities[6] and it has serious implications for morbidity and mortality[7]. In addition, patients suffering from BP are at high risk of drug abuse and suicide. Therefore, appropriate and timely diagnosis is critical in clinical practice. However, it is difficult for clinicians to diagnose BP. Wolkenstein *et al.*[6] reported that 60% of therapists do not correctly diagnose BP, only 20% of BP patients during a

depressive episode are correctly diagnosed within the first year of seeking treatment[8], and from onset to diagnosis the appropriate treatment averages 5 to 10 years[9, 10]. Nearly 60% of BP patients are misdiagnosed as unipolar depressive disorder (UD)[8]. UD is characterized only by episodes of depression, and its lifetime prevalence ranges from 10% to 30%[11]. Misdiagnosis of BP as UD can lead to inadequate treatment and devastating consequences[12]. Identifying objective biomarkers such as functional and structural brain abnormalities of BP may be helpful for its correct diagnosis.

Both BP and UD belong to the mood disorders. One prevalent hypothesis[13, 14] on the pathophysiology of mood disorders is a loss of top-down control over limbic structures, such as the amygdala, hippocampus, and thalamus[15-18], and cortical regions-of-interest (ROIs) include the inferior frontal gyrus, superior/middle frontal gyrus, and cingulate gyrus[2, 19]. Magnetic resonance imaging (MRI) is a noninvasive clinical tool to detect aberrant brain structures and functions, and has been used to reveal structural abnormalities in patients with BP and UD, albeit with heterogeneous and often conflicting results[20-22]. Arnone *et al*.[20] reported that UD is characterized by reduced brain volume in areas involved in emotional processing, including the frontal cortex, orbitofrontal cortex, cingulate cortex, hippocampus, and striatum. A meta-analysis[23] of studies in UD showed grey matter reductions in the rostral anterior cingulate cortex (ACC) and dorsolateral and dorsomedial prefrontal cortex. Studies in BP have increased in number in recent years, but the results remain contradictory[24]. Two meta-analyses of studies in BP revealed grey matter reductions in the ACC[25], and one meta-analysis reported grey matter reductions in the bilateral frontal cortices, cingulate gyrus, and left middle temporal gyrus, and increases in the basal ganglia[26].

ROI-based method was based on the anatomic knowledge and conventional MRI, by stepwise decreasing the regions of interest. It has potential biases. A number of studies have reported structural abnormalities and dysfunctions in depression. With ROI-based analysis, Liu *et al*.[27] reported that the right parahippocampal gyrus showed an abnormality specific to the BP group, while the right middle frontal gyrus, the right dorsal anterior insula, and the right posterior cingulate cortex showed abnormalities specific to the UD group. Another ROI-based analysis[28] reported that fractional anisotropy of the middle-anterior and middle-posterior cingulum bundle was associated with executive functioning and divided attention in patients with major depression. Voxel-based morphometry (VBM) allows automated voxel-by-voxel examination and avoids potential biases that may occur in ROI-based methods[29]. VBM has been shown to be useful for identifying structural changes associated with various disorders[30, 31]. Previous studies have reported that decreased grey matter volume overlaps in patients with BP[17, 32-34] and UD[35, 36]. While patients with BP and UD express different clinical symptoms, we hypothesized that the grey matter volumes would show altered and differential patterns in patients with BP and UD compared to healthy controls (HCs). To date, the majority of neuroimaging studies assessing patients with BP and UD have used ROI-based methods and compared their findings against those obtained in HCs, but few have examined possible differences in grey matter volumes between BP and UD groups directly using VBM.

The present study aimed to examine and compare the whole-brain grey matter volumes obtained with VBM among patients with bipolar I depressive disorder (BP1) or UD and HCs and to identify common and unique changes in grey matter volumes in patients with BP1 and those with UD. We also examined possible correlations of structural abnormalities with clinical characteristics to clarify their pathological mechanisms.

PARTICIPANTS AND METHODS

Participants

A total of 46 patients diagnosed with BP1 or UD (23 each) were enrolled in the study along with 23 HCs. All patients were recruited from the outpatient and inpatient units of the Department of Psychiatry at the Second Xiangya Hospital of Central South University, Changsha, China, between April 2010 and May 2011. HCs were recruited from the Health Examination Centre of the Second Xiangya Hospital during the same period. The three groups were matched for age, gender, and education. All were right-handed, aged between 18 and 45 years, and had completed >9 years of education. All patients underwent structured clinical interviews by two independent psychiatrists according to the Diagnostic and Statistical Manual of Mental Disorders-IV (DSM-IV)[37] and met the DSM-IV criteria for BP1 or UD.

Patients with UD who had experienced more than three depression episodes and had no family history of BP were considered for this study. Patients with any of the following were excluded: (1) head injury; (2) mental retardation; (3) neurological disorders; (4) history of alcohol, drug abuse, or smoking; (5) failure to meet screening criteria for MRI scan, including heart pacemaker or metal implants, claustrophobia, and pregnancy or breastfeeding; (6) Hamilton anxiety rating scale (HAMA)[38] score >14; (7) Bech-Rafaelsen mania scale (BRMS)[39] score >5; and (8) personal or family history of psychiatric disorders.

The study protocol was reviewed and approved by the Ethics Committee of the Second Xiangya Hospital. All participants were informed of the potential risks and benefits associated with study participation, and gave written informed consent.

Clinical Assessments

The following clinical criteria were used for psychometric assessment: the 17-item Hamilton depression rating scale (17-HAMD)[40], HAMA score, and BRMS. A score >17 on the HAMD scale, >14 on the HAMA scale, or >5 on the BRMS was considered as an episode of depression, anxiety, or hypomania respectively. Psychometric parameter assessment and demographic detail recording were carried out by two psychiatrists on the same day as MRI scanning.

Structural MRI

MRI examinations were conducted using a Philips Gyroscan Achieva 3.0 Tesla MRI Scanner (Philips, Best, The Netherlands) equipped with a SENSE-8 channel head coil. For each patient and HC, high-resolution T1-weighted anatomical images were obtained using a 3-dimensional rapid acquisition gradient echo sequence with the following parameters: repetition time = 7.5 ms, echo time = 3.7 ms, flip angle = 8°; field of view = 256 mm × 256 mm, slice number = 180, and voxel size = 1× 1 × 1 mm^3.

VBM Analysis

Data were analyzed using the default parameters of the SPM8 (Wellcome Department of Cognitive Neurology, London, UK) and the VBM8 toolbox (version 435; http://dbm.neuro.uni-jena.de/vbm8/) in the Matlab 7.8.0 environment (R2009b; Math Works, Natick, MA). Individual structural images were preprocessed with the VBM8 toolbox using the default parameters. T1-weighted images were corrected for bias-field inhomogeneities, spatially normalized to the Montreal Neurological Institute standard template space, and segmented into grey matter, white matter, and cerebrospinal fluid using the segmentation algorithm in SPM8[41], within a unified model including high-dimensional DARTEL normalization. Grey matter segments were modulated by the non-linear components only, which allows comparing the absolute amount of tissue corrected for individual brain size. The voxel resolution after normalization was 1.5 mm × 1.5 mm × 1.5 mm. The homogeneity of grey matter images was verified using the check data quality function. The resulting modulated and warped images were then smoothed with an isotropic Gaussian kernel of 8-mm full-width at half-maximum.

The grey matter volumes for clusters showing significant differences in *post-hoc* tests were obtained from each participant using self-developed software[42]. The level of two-tailed statistical significance was set at P <0.05 for all tests.

Statistical Analysis

All statistical analyses were performed with SPSS version 16.0 (SPSS Inc., Chicago, IL). One-way analysis of variance (ANOVA) with least significant difference (LSD) *post-hoc* tests were used to compare demographic and clinical data and the χ^2 test was used for gender comparisons. In addition, grey matter volumes in the three groups were compared using analysis of covariance (ANCOVA); the covariates in the statistical design for imaging data included grey matter volume, and LSD *post-hoc* tests were used to further investigate differences in grey matter volume as a significant main effect of group. Clusters >100 that survived an uncorrected threshold of P < 0.001 were considered significant. To evaluate whether clinical features were associated with brain regions that showed between-group differences in the voxel-wise statistics, correlation analyses between clinical features and brain regions were performed for all patients.

RESULTS

Demographic and Clinical Characteristics

The demographic and clinical characteristics of all three groups are shown in Table 1. There were no significant differences in age, gender, educational level, or BRMS

Table 1. Demographic and clinical characteristics of BP1, UD, and HC groups (mean ± SD)

Variables	BP1 (*n* = 23)	UD (*n* = 23)	HCs (*n* = 23)	F/x^2/*t*	*P*
Age (years)	25.65 ± 6.589	30.00 ± 7.293	28.2 ± 3.781	2.98	0.058
Gender (male/female)	16/7	13/10	13/10	1.095	0.578
Education (years)	10.91 ± 2.172	11.30 ± 2.835	11.78 ± 1.38	0.892	0.415
BRMS score	1.52 ± 0.593	1.43 ± 0.507	1.17 ± 0.984	1.434	0.246
HAMD score	28.52 ± 9.342	29.7 ± 6.197 [a]	6.04 ± 2.9	91.443	<0.001
HAMA score	8.26 ± 3.165	9.22 ± 1.704 [a]	3.87 ± 1.74	35.178	<0.001
Illness duration (years)	6.09 ± 3.667	4.35 ± 2.382	N/A	1.924	0.061
Age at onset (years)	19.7 ± 4.912	25.7 ± 6.825	N/A	−3.422	0.001
Taking lithium (Y/N)	6/17	N/A	N/A	N/A	N/A
Lithium dose (g)	0.542 ± 0.102	N/A	N/A	N/A	N/A
Taking citalopram (Y/N)	6/17	5/18	N/A	0.119	0.73
Citalopram dose (mg)	9.17 ± 3.76	10 ± 5	N/A	−0.316	0.759

[a]*Post-hoc t* tests ANOVA for comparison between BP1 and UD groups. BP1, bipolar I depressive disorder; BRMS, Bech-Rafaelsen mania scale; HAMA, Hamilton anxiety rating scale; HAMD, Hamilton depression rating scale; HCs, healthy controls; UD; unipolar depressive disorder.

scores among all three groups. As expected, we found increased HAMD and HAMA scores in the BP1 and UD groups compared to HCs (P <0.001). There were no significant differences in illness duration, HAMD, or HAMA scores between the BP1 and UD groups. The onset age of BP1 was younger than that of UD (P = 0.001).

Neuroimaging Studies

The volumes of the right inferior frontal gyrus (F = 9.95, P_{uncorr} <0.001) and right middle cingulate gyrus (F = 11.7, P_{uncorr} <0.001) were significantly different among the three groups. Cortical volumes in the right inferior frontal gyrus (Fig. 1A, Table 2) and the right middle cingulate gyrus (Fig. 1B, Table 2) were reduced in BP1 patients compared to the HCs. Besides, a reduction of cortical volume in the right inferior frontal gyrus (Fig. 1C, Table 2) was observed in UD patients compared to the HCs. In addition, compared to the UD patients, a significant reduction of cortical volume was found in the right middle cingulate gyrus (Fig. 1D, Table 2) in BP1 patients. We did not identify any regions with increased grey matter volume in the BP1 group relative to HCs, the UD group relative to HCs, or the BP1 group relative to the UD group. The grey matter volumes for each cluster are provided in Table 2.

Correlations

We performed Pearson correlation analyses for clinical characteristics and grey matter volumes in the right middle cingulate gyrus and the right inferior frontal gyrus in the BP1 and UD groups. We found that the grey matter volume in the right middle cingulate gyrus of BP1 patients was negatively correlated with HAMD score (r = –0.670, U = 21, P <0.001, Fig. 2A), and the grey matter volume in the right inferior frontal gyrus of UD patients was negatively correlated with HAMD score (r = –0.611, U = 21, P = 0.002, Fig. 2B). It should be noted that there was no correlation between the grey matter volume of this area and age, educational level, or illness duration.

DISCUSSION

To the best of our knowledge, this is the first study to examine and compare whole-brain volumetric differences among BP1, UD, and HC groups using VBM. The results showed significant differences in three main aspects. First, there was an evident reduction of the grey matter volume in the right inferior frontal gyrus in the BP1 and UD groups compared to HCs. Second, there was an evident reduction of the grey matter volume in the right middle cingulate gyrus

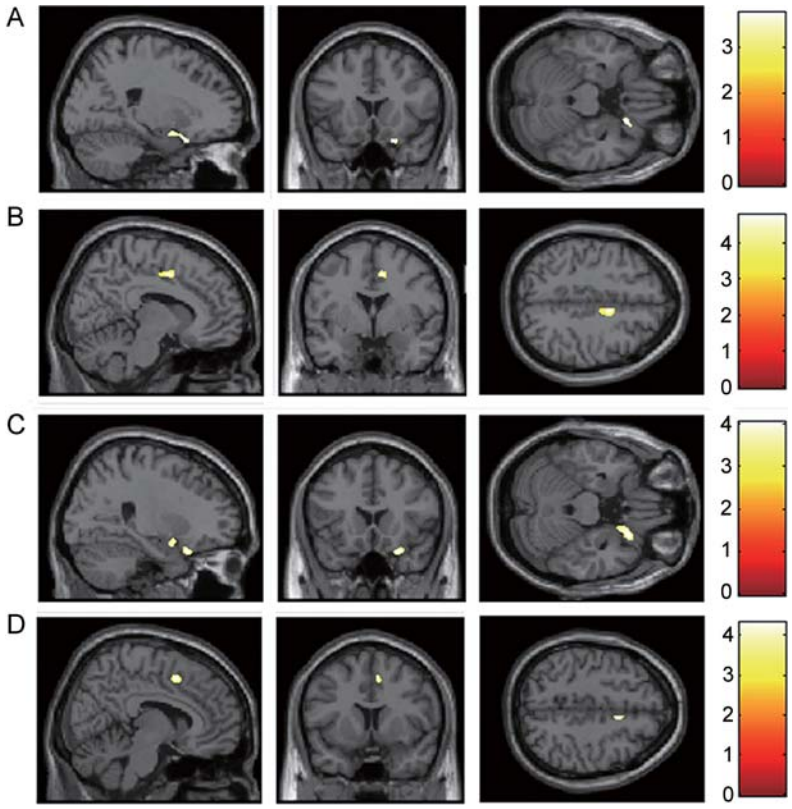

Fig. 1. Significant volumetric differences were found among the three groups (on sagittal, coronal, and axial planes). Patients with BP1 exhibited reduced volumes in the right inferior frontal gyrus (A) and right middle cingulate gyrus (B) compared to HCs, patients with UD had a reduced volume in the right inferior frontal gyrus (C) compared to HCs, and patients with BP1 exhibited a reduced right middle cingulate gyrus (D) volume compared to patients with UD (P <0.001 uncorrected, voxels >100).

Table 2. ANCOVA comparison of grey matter volumes among BP1, UD, and healthy control subjects

Brain regions	BA	Cluster size	Z-score	P_{uncorr}	MNI coordinate		
					x	y	z
Main effect of group							
Rt middle cingulate gyrus	24	243	3.9	<0.001	10	0	45
Rt inferior frontal gyrus	47	207	3.57	<0.001	24	18	–24
BP1<HCs							
Rt inferior frontal gyrus	47	596	3.57	<0.001	22	17	–23
Rt middle cingulate gyrus	24	520	4.4	<0.001	10	0	45
UD<HCs							
Rt inferior frontal gyrus	47	1330	3.8	<0.001	26	19	–24
BP1<UD							
Rt middle cingulate gyrus	24	198	4.03	<0.001	8	9	48

BA, Brodmann areas; BP1, bipolar I depressive disorder; HCs, healthy controls; MNI, Montreal Neurological Institute; Rt, right; UD, unipolar depressive disorder.

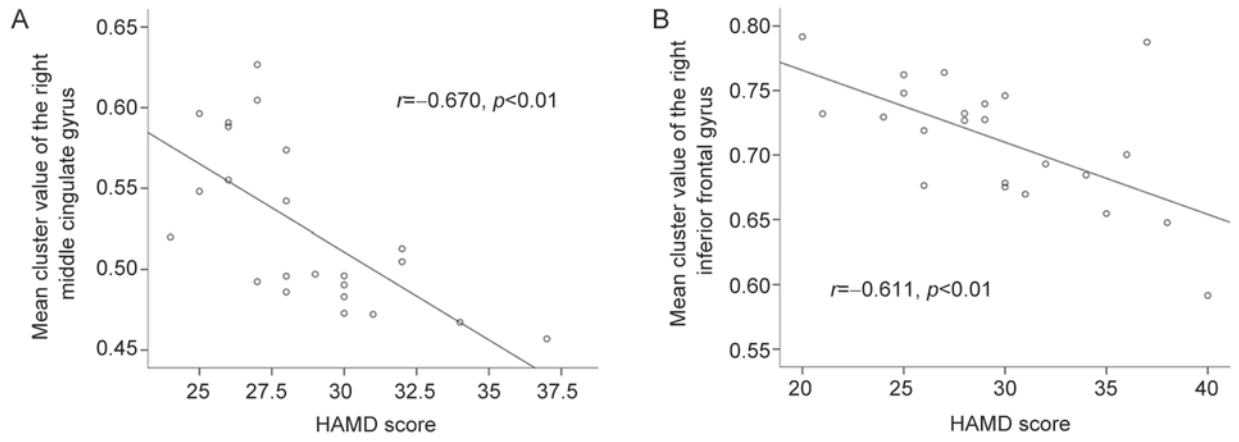

Fig. 2. Scatter plots showing negative correlations between grey matter volume in the right middle cingulate gyrus and HAMD scores in patients with BP1 (A) (*n* = 23), and between grey matter volume in the right inferior frontal gyrus and HAMD scores in patients with UD (B) (*n* = 23).

in patients with BP1 compared to the UD and HC groups. No grey matter volume abnormalities were detected in the cingulate gyrus in patients with UD compared to HCs. Finally, we found that the grey matter volume in the right middle cingulate gyrus of patients with BP1 was negatively correlated with the HAMD score, and the grey matter volume in the right inferior frontal gyrus of patients with UD was negatively correlated with the HAMD score. Along with the common and distinct changes in grey matter volume between the BP1 and UD groups, we found that patients with BP1 had a younger onset age than patients with UD. This result is consistent with a previous report[43].

It is interesting that the significant changes occurred in the right hemisphere. This is consistent with the known cortical asymmetry in BP[44]. Similarly, right hemisphere hyperactivity has been reported in depression[45]. Although we did not further assess this result, it is an interesting topic to explore in future studies.

The highest regional grey matter volume loss occurred in the inferior frontal gyrus. The inferior frontal gyrus (Brodmann area 47) belongs to the ventral lateral prefrontal cortex[44], an area critical in the integration of emotional information and the regulation of emotional intensity[45]. Our finding of a grey matter volume decrease in the right inferior frontal gyrus in the BP1 and UD groups relative to HCs is consistent with the results of previous brain imaging studies[12, 32, 46, 47]. For instance, Lyoo *et al.*[32] reported decreased grey matter density in the right inferior frontal gyrus in patients with BP1 compared with HCs, and Lopez-Larson *et al.*[46] also reported a reduction in the right inferior frontal gyrus volume in patients with BP. Two meta-analyses of functional neuroimaging studies and structural voxel-based MRI studies in adult patients with BP1 yielded similar results, showing lower neural activation and decreased grey matter in the inferior frontal gyrus in patients with BP1[12]. In addition, functional neuroimaging and magnetic resonance spectroscopy investigations in patients with BP suggested the existence of functional and biochemical abnormalities in the inferior frontal gyrus[48]. Meanwhile, a meta-analysis[23] reported grey matter reduction in the right inferior frontal cortex in patients with UD compared with HCs. The present results support the fact that the reduction of grey matter volume in the right inferior frontal gyrus is a common pathophysiology of patients with BP1 and UD. Grey matter volume reduction in the right inferior frontal cortex may be associated with depressive episodes.

Another striking finding in our study was that patients with BP1 also showed decreased grey matter volume in the right middle cingulate gyrus compared with the UD and HC groups. The cingulate gyrus plays a major role in the neurophysiological basis of complex emotional behaviors, and it is considered to be a part of a putative circuit involved in emotional expression and cognitive functions in humans[49-51]. The study finding of decreased grey matter volume in the right middle cingulate gyrus in BP1 is consistent with previous reports[25, 52-54]. Bora *et al.*[25]

reported grey matter reductions in the anterior cingulate gyrus in patients with BP1 in a meta-analysis of grey matter abnormalities, and Ellison-Wright and Bellmore[24] also reported a reduction in the cingulate gyrus volume in patients with BP1. Bearden *et al.*[55] found that reduced grey matter density in the cingulate gyrus was rescued in lithium-treated patients with BP1. In addition, a deficit in γ-amino-butyric acid, a major inhibitory neurotransmitter, was reported in the cingulate gyrus of patients with BP1[53]. Grey matter volume reduction in the right middle cingulate gyrus may be associated with bipolar disorder. In addition to the right inferior frontal gyrus, the right middle cingulate gyrus may be another key brain region implicated in BP1 pathophysiology. Our study did not find cingulate gyrus grey matter reductions in UD compared with BD and HCs, which is consistent with the results of Frodl *et al.*[56] but inconsistent with the findings of Bora *et al.*[23] and Lai[57]. This discrepancy may be associated with research methods and the heterogeneity of samples.

On the other hand, it should be noted that there were no differences in the grey matter volumes of the amygdala and hippocampus in the BP1 and UD groups relative to HCs. This could be attributed to the homogeneity of the study samples. Bora *et al.*[25] reported that grey matter volume in the amygdala was reduced in patients with UD who had co-morbid anxiety disorders and also in first-episode/drug-free subjects, while in the present study of BP1 and UD groups with HAMA scores <14, there was no significant difference between the two groups. Our result is consistent with a previous report of no significant grey matter volume loss in the hippocampus or amygdala in patients with depression[47]. Reduced grey matter volume in the hippocampus has been revealed in patients with Alzheimer's disease[58] but rarely in mood disorders. Our results suggest that the amygdala and hippocampus may not be key brain regions associated with UD or BP1.

Our findings suggest that reduced grey matter volume in the right inferior frontal gyrus is a common pathophysiological alteration in BP1 and UD, while decreased grey matter volume in the right middle cingulate gyrus is a distinctive change in patients with BP1. Therefore, the abnormal grey matter volume of the right middle cingulate gyrus may be an important neuroimaging marker of BP1, which may enable clinicians to distinguish BP1 from UD. Further work is required to understand the significance of these volumetric changes, including prospective studies and studies that integrate fMRI and DTI data.

The present study had a number of limitations. First, some patients had taken antidepressant medications and/or mood stabilizers, although the impact of medication intake on brain structures remains unclear. Sassi *et al.*[53] reported that lithium treatment might increase cingulate gyrus volume in patients with BP, so lithium treatment could have affected our findings. Second, the sample heterogeneity and variations in illness duration, number of episodes, and age at scanning might have influenced the results to some extent. Further studies with larger sample sizes are needed to verify our results and explore the pathophysiological mechanisms underlying BP1 and UD.

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·Original Article·

Altered functional connectivity networks of hippocampal subregions in remitted late-onset depression: a longitudinal resting-state study

Zan Wang[1], Yonggui Yuan[2], Feng Bai[1], Hao Shu[1], Jiayong You[3], Lingjiang Li[4], Zhijun Zhang[1]

[1]*Neurology Department, Affiliated ZhongDa Hospital, Neuropsychiatric Institute and Medical School of Southeast University, Nanjing 210009, China*

[2]*Department of Psychosomatics and Psychiatry, Affiliated ZhongDa Hospital, Medical School of Southeast University, Nanjing 210009, China*

[3]*Department of Psychiatry, Nanjing Brain Hospital Affiliated to Nanjing Medical University, Nanjing 210029, China*

[4]*Mental Health Institute, Second Xiangya Hospital of Central South University, Changsha 410011, China*

Corresponding authors: Zhijun Zhang and Yonggui Yuan. E-mail: janemengzhang@vip.163.com, yygylh2000@sina.com

ABSTRACT

The regional specificity of hippocampal abnormalities in late-life depression (LLD) has been demonstrated in previous studies. In this study, we sought to examine the functional connectivity (FC) patterns of hippocampal subregions in remitted late-onset depression (rLOD), a special subtype of LLD. Fourteen rLOD patients and 18 healthy controls underwent clinical and cognitive evaluations as well as resting-state functional magnetic resonance imaging scans at baseline and at ~21 months of follow-up. Each hippocampus was divided into three parts, the cornu ammonis (CA), the dentate gyrus, and the subicular complex, and then six seed-based hippocampal subregional networks were established. Longitudinal changes of the six networks over time were directly compared between the rLOD and control groups. From baseline to follow-up, the rLOD group showed a greater decline in connectivity of the left CA to the bilateral posterior cingulate cortex/precuneus (PCC/PCUN), but showed increased connectivity of the right hippocampal subregional networks with the frontal cortex (bilateral medial prefrontal cortex/anterior cingulate cortex and supplementary motor area). Further correlative analyses revealed that the longitudinal changes in FC between the left CA and PCC/PCUN were positively correlated with longitudinal changes in the Symbol Digit Modalities Test (r = 0.624, P = 0.017) and the Digit Span Test (r = 0.545, P = 0.044) scores in the rLOD group. These results may provide insights into the neurobiological mechanism underlying the cognitive dysfunction in rLOD patients.

Keywords: remitted late-onset depression; hippocampal subregional network; functional connectivity; functional magnetic resonance imaging; cognitive dysfunction

INTRODUCTION

Depression remains to be a health-care risk factor for older adults and a major cause of disability[1]. A subset of elderly depression, late-onset depression (LOD), of which the first episode occurs later in life (usually after 60 years of age), exhibits certain unique clinical features; thus, LOD patients are more likely to have associated medical comorbidity, greater cognitive deficits, and an increased risk of dementia conversion compared with groups with early-onset depression[2]. LOD patients show cognitive deficits mainly in the processing speed and executive domains[3]. This cognitive impairment can persist even after the remission of

affective symptoms[4]. However, the mechanism underlying the persistent cognitive impairment in remitted LOD (rLOD) patients is still unclear.

The hippocampus is a critical neuronal substrate for memory and emotion processing. Altered hippocampal morphology plays an important role in the pathophysiology of elderly depression. Structural studies have reported decreased hippocampal volume in patients with elderly depression relative to healthy volunteers[5-8]. Moreover, hippocampal atrophy is associated with reduced cognitive performance in LOD patients[8]. Importantly, the hippocampus, as a heterogeneous area, can be parcellated into several subregions including the cornu ammonis (CA), the dentate gyrus (DG), and the subicular complex (SUB)[9]. Recent neuroimaging findings have indicated that these hippocampal subregions are differentially influenced in depression[2, 10-12]. For instance, Cole *et al.*[12] demonstrated significant deformations of the subiculum and CA1 subfield extending into the CA2–3 subfields in patients with major depression. Similarly, Ballmaier *et al.*[2] observed notable atrophy of the bilateral anterior CA1–CA3 subfields and the subiculum in LOD patients. Therefore, these findings support the hypothesis that the cognitive deficits of LOD patients may be associated with regionally specific hippocampal abnormalities. However, it remains unclear how the functional coupling of hippocampal subregions changes in LOD, especially when the affective symptoms are relieved.

Resting-state functional connectivity MRI (R-fMRI) is a promising tool for investigating the intrinsic functional connections among anatomically distinct brain regions within specific networks[13]. The intrinsic hippocampal functional connectivity (FC) networks have been identified using the R-fMRI method under normal and pathological conditions, including Alzheimer's disease (AD)[14-16] and amnestic mild cognitive impairment[14, 17]. Specifically, a recent R-fMRI study from our lab reported impaired hippocampal FC networks in rLOD[18]. Importantly, the extent of network alterations was linked to inferior cognitive performance in rLOD patients. Furthermore, depressive symptoms and cognitive dysfunction have interactive effects on the hippocampal FC networks[19]. These cross-sectional studies suggest that aberrant hippocampal FC networks might contribute to cognitive impairment in LOD patients even when the depressive symptoms have remitted. However, it is still unknown whether these aberrant networks can predict the progression of rLOD. Therefore, it is important to measure the longitudinal changes in intrinsic FC patterns of the hippocampal subregions in rLOD and to investigate their association with cognitive changes, considering the hippocampal heterogeneity and its association with LOD neuropathology.

Together, in this study, we first identified the FC patterns of the hippocampal subregions in rLOD patients and healthy controls using the R-fMRI approach. Then, longitudinal changes of the hippocampal subregional networks were compared between rLOD patients and healthy controls. Finally, we investigated whether longitudinal changes of the hippocampal subregional networks are associated with cognitive deficits in rLOD patients.

PARTICIPANTS AND METHODS

Participants

Nineteen rLOD patients and nineteen healthy controls were recruited from the Affiliated Brain Hospital of Nanjing Medical University, China. All rLOD patients met the following inclusion criteria: (1) they had previously met the criteria for major depressive disorder in DSM-IV and had remitted for > 6 months before enrollment; (2) the age at first depression onset was > 60 years; (3) Hamilton Depression Rating Scale scores < 7, and Mini-Mental State Examination (MMSE) scores > 24; (4) duration of illness < 5 years and a medication-free period > 3 months prior to the assessment; (5) absence of other major psychiatric disorders, including abuse of or dependence on psychoactive substances; (6) absence of primary neurological disorders, including dementia or stroke; (7) absence of a medical illness that impairs cognitive function; (8) no history of electroconvulsive therapy; and (9) no gross structural abnormalities on T1-weighted images, and no major white matter changes such as infarction or other vascular lesions on T2-weighted MRI. This study was approved by the Research Ethics Committee of the Affiliated ZhongDa Hospital of Southeast University and written informed consent was given by all participants. All participants completed a battery of neuropsychological tests that covered multiple cognitive domains, including the auditory verbal memory test-delayed recall (AVLT-DR),

trail-making tests A and B (TMT-A and B), the symbol digit modalities test (SDMT), and the digit span test (DST).

The follow-up study was performed at an average of 21 months (12–32 months) after baseline. During the follow-up period, 5 patients relapsed with an episode of depression/mania. One healthy control was excluded due to excessive motion artifacts. Hence, 14 rLOD patients and 18 healthy controls underwent baseline and follow-up MRI scans, and the two groups were matched for the follow-up period. In addition, the follow-up neuropsychological tests and MRI scan parameters were identical to those conducted at baseline.

Magnetic Resonance Imaging Data Acquisition

The participants were scanned by a General Electric 1.5 Tesla scanner (General Electric Medical Systems, Milwaukee, Wisconsin) with a homogeneous birdcage head coil. Axial R-fMRI data (no cognitive tasks were performed, eyes were closed, and ears were occluded) were obtained with a single-shot gradient-recalled echo-planar imaging sequence: TR = 3000 ms; TE = 40 ms; FA = 90°; acquisition matrix = 64 × 64; FOV = 240 mm × 240 mm; thickness = 4.0 mm; gap = 0 mm; and 3.75 mm × 3.75 mm in-plane resolution. The R-fMRI scan lasted 7 min and 6 s.

Functional Image Preprocessing

R-fMRI data analysis was carried out using the Statistical Parametric Mapping software (SPM8, www.fil.ion.ucl.ac.uk/spm). The first ten volumes were discarded and the remaining images were corrected for timing differences and motion effects. Participants with head motion > 3 mm maximum displacement in the *x*, *y*, or *z* direction or 3° of angular motion were excluded. The resulting images (both baseline and follow-up data) were spatially normalized into a standard stereotaxic space using a 12-parameter affine approach and an echo-planar imaging template, and then resampled to 3 × 3 × 3 mm^3 voxels. The obtained images were smoothed with a Gaussian kernel of 8 mm × 8 mm × 8 mm. Further preprocessing included linear de-trending and temporal band-pass filtering (0.01–0.08 Hz), which were used to reduce the effects of low-frequency drift and high-frequency physiological noise. Finally, several nuisance variables, including six head-motion parameters, global mean signal, white matter signal, and cerebrospinal fluid signal were regressed out from the data.

Functional Connectivity Analyses

The six hippocampal subregions (bilateral CA, DG, and SUB; Fig. 1)[20] were defined using the Anatomy Toolbox in SPM. Basically, the Anatomy Toolbox provides a maximum

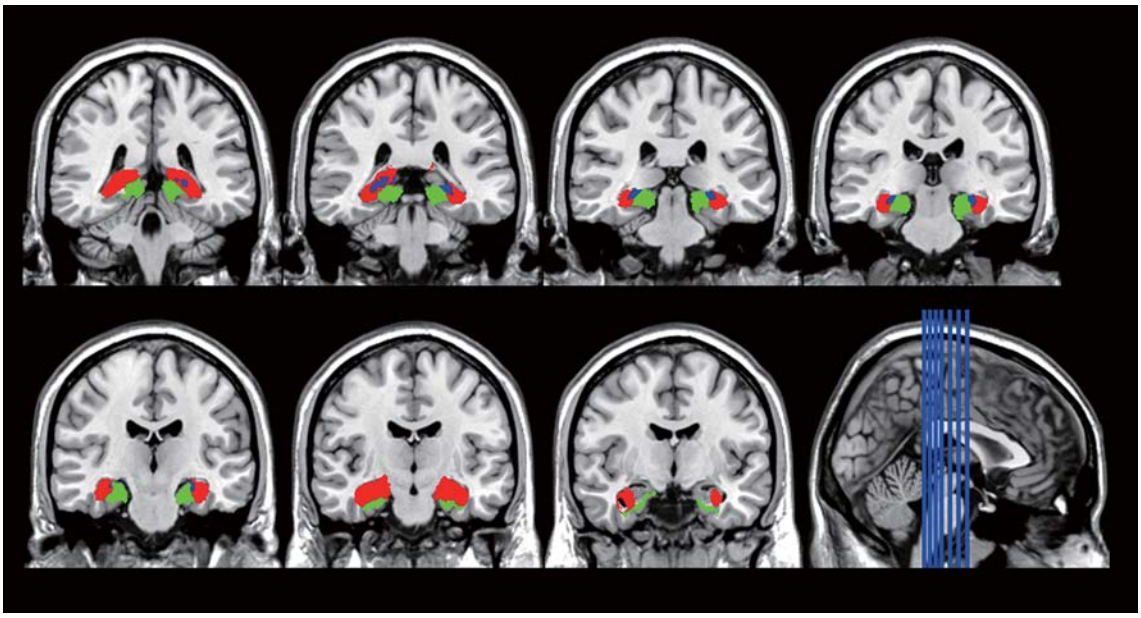

Fig. 1. Locations of hippocampal subregions. Red: CA (CA1-CA3); Blue: DG (fascia dentata and CA4); Green: SUB.

probabilistic map of each hippocampal subregion[21, 22]. This is a summary map of the hippocampal subregions that attributes each voxel to the most likely subregion with no less than 40% likelihood of cytoarchitectonic probability[21]. Then, the whole-brain FC maps for each subregion were computed as the Pearson correlation coefficients between the mean time course in the subregion and the time-course of every voxel of the whole brain. Fisher's *z*-transform was further applied to improve the normality of the correlation coefficients[23]. These analyses were performed using the Resting State fMRI Data Analysis Toolkit (REST) software (available at: http: //www.restingfmri.sourceforge. net).

Group-Level Analysis

Within-group: To determine the FC patterns of the hippocampal subregions in each of the four groups (baseline rLOD and healthy control groups and their follow-up), the FC maps were gathered in each group separately for random effect analysis using the one-sample *t*-test, corrected by the AlphaSim program based on Monte Carlo simulation (α = 0.05, single-voxel *P* value = 0.01, FWHM = 8 mm, with grey matter mask). To avoid potential interpretational confounds related to apparently negative connectivity resulting from correction for global signal changes[24], only positive functional connectivity was examined. Further, for each hippocampal subregion, we added the within-group FC patterns of the four groups together to produce a mask for between-group comparisons.

Between-groups: To determine whether the longitudinal changes in the FC strength of hippocampal subregions were significantly different between rLOD patients and healthy controls, a direct comparison between the change estimates (the FC maps at follow-up subtracted from those at baseline) between the rLOD group and the control group was conducted (networks of CA, DG, and SUB were performed separately). Multiple comparisons were corrected by the AlphaSim program (α = 0.05, single-voxel *P* value = 0.01, FWHM = 8 mm, with mask). These group-level analyses were performed using the REST software.

Statistical Analysis

One-way analysis of variance (ANOVA) was used to compare the neuropsychological performance among groups with statistical significance of $P < 0.05$. *Post-hoc* tests by Bonferroni correction were used to reveal the source of ANOVA difference. Further correlative analysis between R-fMRI data and neuropsychological performance was performed. First, the mean FC strengths of the clusters showing significant between-group differences were extracted for rLOD patients. Then, Pearson's correlation analysis between the extracted FC strength and neuropsychological performance was performed to investigate the cognitive significance of the altered FC strength in the rLOD group.

RESULTS

Sample Description

The demographic and neuropsychological characteristics for each group are shown in Table 1. The four groups did not differ significantly in terms of age, years of education, gender, and MMSE score (all $P > 0.05$).

Neuropsychological Data

Significant differences among the four groups were found in the scores on AVLT-DR, TMT-A, TMT-B, and DST (Table 1). *Post-hoc* tests further revealed that the rLOD patients had poorer performance in AVLT-DR, TMT-A, and DST than healthy controls at baseline. At follow-up, the performance in AVLT-DR and DST by rLOD patients was still worse than that by healthy controls. Moreover, from baseline to follow-up, the performance in TMT-A and -B improved significantly in the rLOD patients.

Functional Connectivity Patterns of Hippocampal Subregions

Each hippocampal subregional network involved diffuse subcortical, medial frontal, temporal, parietal, and cerebellar regions in both the control and rLOD groups at baseline and follow-up ($P < 0.05$, AlphaSim-corrected, Fig. 2). These FC patterns are similar to those in previous studies using the whole hippocampus as the seed region[14-16]. However, at both baseline and follow-up, nearly all hippocampal subregional seeds showed significantly stronger and more diffuse FC with regions typically located in the parietal cortex in the control group (Fig. 2); these parietal regions showed weaker FC with hippocampal subregions in the rLOD group. Further, in the rLOD group from baseline to follow-up, nearly all hippocampal subregions showed

Table 1. Demographic and neuropsychological data for all participants (mean ± SD)

	Baseline		Follow-up		F or χ^2	P^*
	rLOD (n = 14)	HC (n = 18)	rLOD (n = 14)	HC (n = 18)		
Age (years)	67.6 ± 4.0	71.4 ± 3.8	69.8 ± 3.9	73.2 ± 3.8	-	-
Education (years)	14.4 ± 2.1	15.2 ± 2.8	14.4 ± 2.1	15.2 ± 2.8	-	-
Gender (male/female)	7/7	10/8	7/7	10/8	-	-
Mean age at onset (years)	64.7 ± 4.1	-	64.7 ± 4.1	-	-	-
MMSE score	28.6 ± 1.9	28.3 ± 1.3	27.8 ± 4.0	28.6 ± 1.8	0.421	0.739
AVLT-DR[a,b]	5.9 ± 2.5	8.1 ± 1.9	5.0 ± 2.8	8.6 ± 2.7	7.837	0.000
TMT-A[a,c]	133.5 ± 96.7	70.0 ± 28.7	70.4 ± 25.5	75.7 ± 25.5	5.401	0.002
TMT-B[c]	237.7 ± 138.4	139.3 ± 39.2	157.7 ± 36.9	138.8 ± 55.5	5.935	0.001
SDMT	25.0 ± 15.0	34.3 ± 8.7	27.1 ± 15.8	33.2 ± 12.8	2.038	0.118
DST[a,b]	11.7 ± 2.1	13.2 ± 1.8	11.7 ± 2.1	13.6 ± 2.6	3.704	0.016

**P* values were obtained by one-way analysis of variance (ANOVA) analysis.

[a]*P* <0.05, baseline rLOD *vs* baseline HC; [b]*P* <0.05, follow-up rLOD *vs* follow-up HC; [c]*P* <0.05, baseline rLOD *vs* follow-up rLOD.

AVLT-DR, Auditory Verbal Learning Test-delayed Recall; DST, Digit Span Test; HC, healthy controls; MMSE, Mini Mental State Exam; rLOD, remitted late-onset depression; SDMT, Symbol Digit Modalities Test; TMT-A, Trail-making Test A; TMT-B, Trail-making Test B.

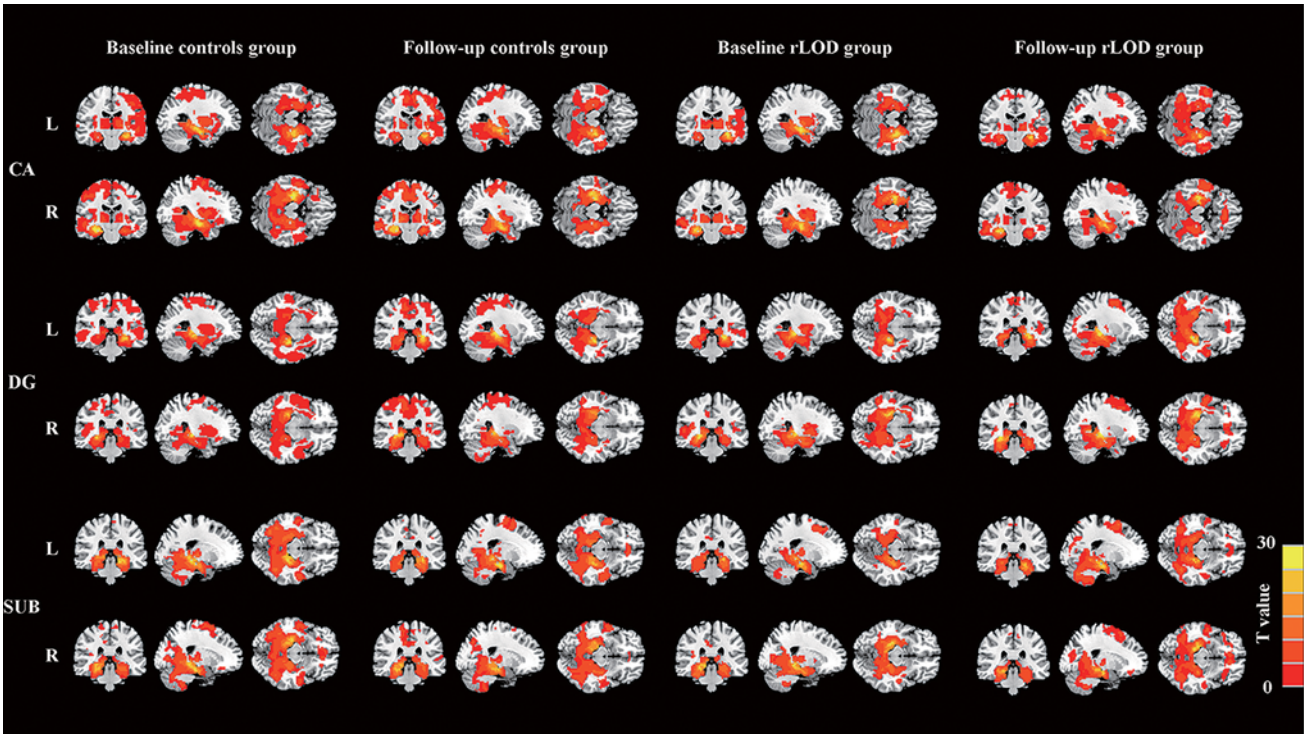

Fig. 2. Resting-state functional connectivity maps of the hippocampal subregions. Thresholds were set at *P* <0.05, corrected by Monte Carlo simulation.

stronger FC with regions mainly located in the frontal cortex.

Between-Group Differences in the Longitudinal Changes of Hippocampal Subregional Networks

With regard to the left hippocampal subregional networks, the rLOD group showed greater longitudinal deficits in the left CA network integrity, including connections with the bilateral posterior cingulate gyrus/precuneus (PCC/PCUN), compared with the control group (Fig. 3A). However, longitudinal changes in the left DG or SUB network did not show a significant between-group difference. Furthermore, within the rLOD group, longitudinal changes in the FC between the left CA and PCC/PCUN were positively correlated with the longitudinal changes in the SDMT and DST scores (Fig. 3B).

On the contrary, longitudinal changes in the right three subregional networks all showed significant differences between the rLOD and control groups. For the right three subregions (CA, DG, and SUB), the rLOD group showed greater increases in network strength, including connections with the frontal cortex (bilateral medial prefrontal cortex/anterior cingulate cortex, and supplementary motor area) compared with the control group (Fig. 3C1–3).

DISCUSSION

Using the R-fMRI approach, in this study we demonstrated altered hippocampal subregional networks during rLOD progression. From baseline to follow-up, the rLOD group showed greater decreases in the FC between the left CA and bilateral PCC/PCUN, but increased connectivity in right hippocampal subregional networks with the frontal cortex (bilateral medial frontal gyrus/anterior cingulate cortex, and supplementary motor area). Importantly, the impaired intrinsic connectivity was positively correlated with the deficits in cognitive performance within the rLOD group. These results may provide insight into the neurobiolgical

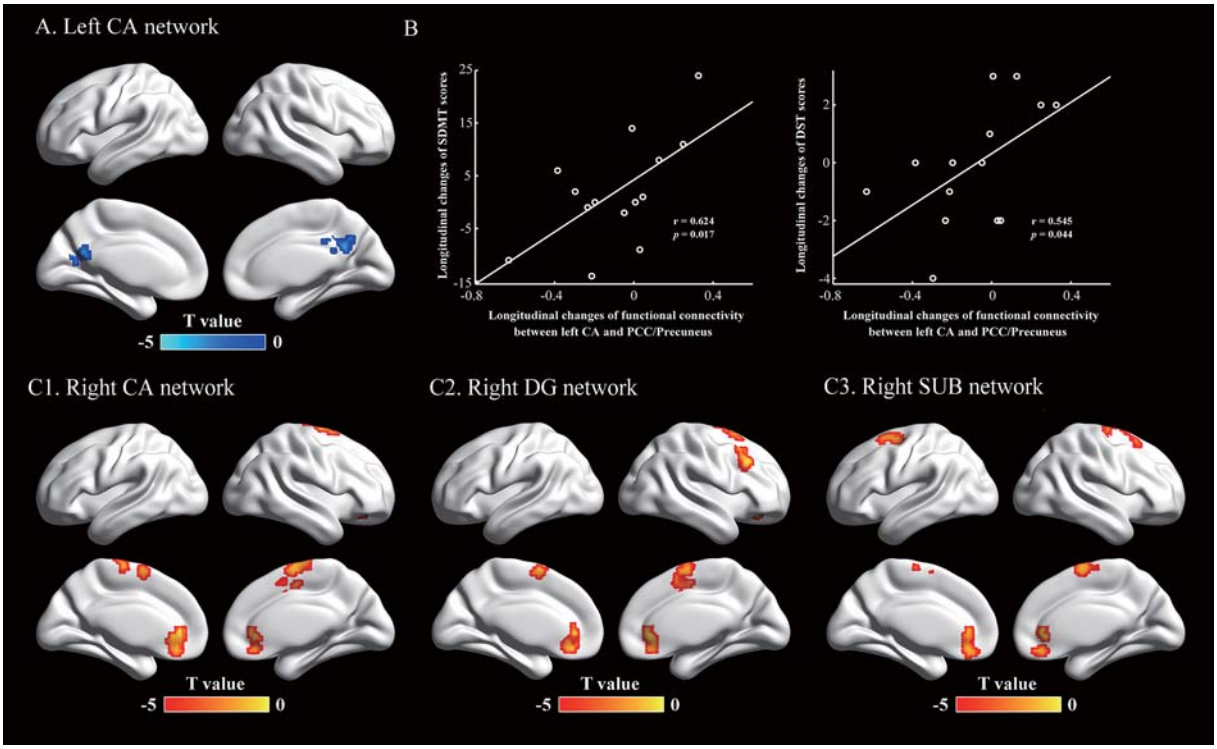

Fig. 3. Longitudinal changes in rLOD patients *versus* controls. Thresholds were set at P <0.05, AlphaSim-corrected. (A) Left CA network; (C1) right CA network; (C2) right DG network; (C3) right SUB network. (B) In the rLOD group, longitudinal changes in functional connectivity between the left CA and PCC/PCUN were significantly correlated with the longitudinal changes in the SDMT and DST scores.

mechanisms underlying the cognitive dysfunction in rLOD patients.

First, multiple domains of cognitive impairment in rLOD patients were detected at baseline. After follow-up, these patients still demonstrated significantly worse episodic memory (indicated by AVLT-DR) and attention (indicated by DST) than healthy controls. This is consistent with prior reports that remitted depression patients show persistent cognitive impairment[25, 26]. There is also supportive evidence that persistent cognitive impairment or a significantly elevated incidence of AD occurs in remitted depression patients[27], although we found that the rLOD patients showed greater improvement in executive function (indicated by TMT-A and -B) from baseline to follow-up. Furthermore, rLOD patients showed increased connectivity in the right hippocampal subregional networks from baseline to follow-up. Consistent with this finding, a previous study from our group using the same dataset observed increased right hippocampal volumes in rLOD patients from baseline to follow-up[28]; the lateralized enlarged volumes were positively correlated with increased SDMT scores. Therefore, given that antidepressant treatment may have a neuroprotective effect on the hippocampus[29-31], we speculate that the improvement in cognitive function (i.e., TMT-A and -B) and increased connectivity in the right subregional networks in rLOD patients may be, at least partly, associated with this neuroprotective effect. Further, this effect might be prolonged and so may postpone or even reverse hippocampal deterioration and its related cognitive deficits. As no rLOD patients took antidepressants during the follow-up period, future studies are essential to test this speculation.

Second, the rLOD group showed a greater decline in connectivity of the left CA to bilateral PCC/PCUN from baseline to follow-up. Previous postmortem studies have revealed complex neuronal abnormalities in distinct layers of the hippocampal subfields in depression, with the greatest changes in CA1, followed by CA2 and CA3[32]. Recent studies also reported subtle deficits in hippocampal subregions in LLD patients through shape analysis, i.e. deformations in CA (CA1–3)[12]. These data suggest that the CA subregion may be more sensitive to neuroplastic changes in depression. Furthermore, the PCC/PCUN has been considered an anatomical hub in the resting-state brain[33]. Structural and functional MRI studies have demonstrated that the PCC/PCUN is closely connected with medial temporal lobe structures (e.g. the hippocampus)[34-36]. Previous neuroimaging studies have revealed abnormalities of the PCC/PCUN in depressed patients, i.e., reduced fractional anisotropy in the PCC[37], hypoactivity in the PCUN[38], and decreased FC in the PCC/PCUN[39]. More importantly, a recent study reported that elderly patients with depression show widespread abnormalities, especially in the hippocampal cingulum tract connecting the hippocampus and the PCC/PCUN[40]. The authors also found that the abnormality of the hippocampal cingulum was associated with lower episodic memory scores. Interestingly, the greater longitudinal deficits (left CA-PCC/PCUN) were associated with greater impairment in cognitive function (indicated by SDMT and DST scores) in rLOD patients. The performances in SDMT and DST are specifically associated with activity in the medial temporal lobe as well as in frontal and parietal areas involved in associative recognition, visual-verbal memory and selective attention, and working memory[41]. Therefore, these findings suggest that altered functional coupling between the hippocampus and PCC/PCUN might be an important contributor to cognitive impairment in elderly depression, indicating their important role in the progression of connectivity disturbances in rLOD.

Third, interestingly, we found left-lateralized deficits of hippocampal subregional networks in rLOD patients. However, the mechanism is unclear. Previous studies using structural MRI have shown that decreased left hippocampal volume is significantly associated with later dementia onset among older depressed patients[42]. Therefore, it may be important to determine whether the left-lateralized deficits are associated with dementia onset, particularly in AD.

Several issues need to be further addressed. First, this longitudinal study had a relatively small sample size, so the findings cannot be considered categorical. Second, given that all patients had received various antidepressant treatments, the results could not exclude a potential medication effect. Third, magnetic resonance imaging was performed with a 1.5-Tesla scanner, which may not be suitable for subfield-level analysis. Thus, high-resolution 3T scanning would be necessary to validate our findings in future. Finally, it is necessary to validate these findings in a large cohort, as previous studies suggested only moderate test-retest reliability of R-fMRI measures.

In summary, in the present study we explored the longitudinal changes in the functional connectivity pattern of hippocampal subregions in rLOD patients, and these may have important clinical implications. Our findings suggest that the altered functional connectivity pattern of hippocampal subregional networks may be an important indicator for monitoring the progression of this disease.

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·Original Article·

Atypical features and treatment choices in bipolar disorders: a result of the National Bipolar Mania Pathway Survey in China

Daihui Peng[1], Ting Shen[1], Linda Byrne[2], Chen Zhang[1], Yueqi Huang[1], Xin Yu[3], Jingping Zhao[4], Marita McCabe[2], David Mellor[2], Yiru Fang[1]

[1]*Division of Mood Disorders, Shanghai Clinical Center for Mental Disorder, Department of Psychiatry, Shanghai Mental Health Center, Shanghai Jiao Tong University School of Medicine, Shanghai 200030, China*

[2]*School of Psychology (Burwood Campus), Deakin University, Melbourne, Australia*

[3]*Institute of Mental Health, Peking University, Beijing 100191, China*

[4]*Mental Health Institute, The Second Xiangya Hospital of Central South University, Changsha 410011, China*

Corresponding author: Yiru Fang. E-mail: yirufang@gmail.com

ABSTRACT

In this study, we examined the point prevalence rate of atypical features in bipolar disorder, and estimated the potential impact of these features on treatment practices in China. Using the atypical features criteria of the Diagnostic and Statistical Manual of the American Psychiatric Association (DSM-IV), we documented the atypical symptoms in 3 906 consecutive participants with bipolar disorder enrolled at 26 psychiatric services across China. We further assessed the association between atypical features and the treatment approaches, including the prescription of antidepressants. The overall point prevalence rate of atypical features was 9.1% among patients with various bipolar disorder subtypes. When the definition was broadened to include atypical features B, the overall rate increased to 11.8%. Interestingly, among patients with the mixed state and remission subtypes, there was a significant difference in the rates of antidepressant medication usage between patients who met and those who did not meet the criteria for atypical features B. These findings indicate a trend of using antidepressants for these two types of patients with atypical features. Further, for both mixed state and remission patients, treatment approaches were related to atypical features B. Our findings provide evidence to assist clinicians to readily recognize atypical features in bipolar subtypes and can propose treatments based on these diagnoses.

Keywords: atypical features; bipolar; treatment; antidepressant

INTRODUCTION

Bipolar disorder (BP) is characterized by recurring periods of high or low mood, thinking and activity, associated with hypomanic, manic, depressed, and mixed states[1], making it difficult to diagnose. This complexity and diversity of symptoms has led to a number of different prevalence estimates. When taking into account type I (BP-I), type II (BP-II), and sub-threshold types, prevalence estimates have ranged from 1.5% to 6% in the USA[2-6]. In China, estimates are less specific, as up to 91.7% of patients with mood disorders never seek medical help[7], but the latest investigations conducted in four provinces place the prevalence rate somewhat lower than that in the USA, with 0.1% for BP-I and 0.3% for BP-II[7]. This discrepancy is unsurprising — diagnosing BP is still challenging for clinicians worldwide.

A particularly confounding factor in the difficulty faced by clinicians when making a diagnosis of BP, is

the largely unacknowledged atypical features (ATFs), such as mood reactivity, hyperphagia, hypersomnia or weight gain, interpersonal rejection sensitivity, and leaden paralysis[1] that can manifest alongside the better-known basic characteristics. While ATFs were introduced into the fourth edition of the Diagnostic and Statistical Manual of the American Psychiatric Association (DSM-IV) after several studies on atypical depression[8-10] and other studies that have found overlaps between depressed patients with ATFs and BP-II[11-15], the atypical features of patients with BP have still not been well-studied. As a result, there is a lack of coherent findings related to ATFs in BP[13, 16, 17].

A further challenge faced by clinicians is how to best treat those patients they do diagnose with BP. Earlier studies reported that atypical depression symptoms may preferentially respond to antidepressants — for example monoamine oxidase inhibitors and tricyclic antidepressants[18-20] — and later a prominent meta-analysis indicated that antidepressants are effective in the short-term treatment of bipolar depression[21]. However, another study, the Systematic Treatment Enhancement Program for Bipolar Disorder, noted that the effects of maintained treatment showed no discernible difference between BP patients using antidepressants and those not using antidepressants[22]. At present, few treatment guidelines for BP recommend antidepressants as an adjunctive therapy to mood stabilizers, and no treatment guidelines recommend antidepressants as a monotherapy[23-26]. Despite these guidelines, a previous study reported that it is not uncommon to prescribe antidepressants for BP patients in China on a prolonged basis[27].

Given the relatively low diagnostic rates of BP and the reluctance of people in China to seek treatment for mood disorders as well as the lack of adherence to treatment guidelines, there is a pressing need to investigate the diagnosis and treatment of BP in China. The present report was designed to fill that gap by investigating the point prevalence of ATFs in BP and assessing their impact on treatment methods in China.

PARTICIPANTS AND METHODS

Study Design

To establish a database of BP clinical pathway in China, the National Bipolar Mania Pathway Survey (BIPAS) was carried out in 15 psychiatric hospitals and 11 psychiatry departments of general hospitals distributed throughout the mainland of China, between November 2012 and January 2013. A sample of 3 906 patients with a diagnosis of BP were recruited. Most of the patients were Han Chinese, their mean age was 34.75 ± 14.09 years, and 48.1% were female. Of these patients, 774 (19.8%) were in their first episode, 2109 (54%) were recurrent, and 1023 (26.2%) were at the stable/maintainance stage (see supplemental data for detailed information).

All inpatients and outpatients at the 26 services were consecutively screened and their medical records were reviewed. The patients were enrolled if they met the diagnostic criteria for BP as determined by the International Classification of Disease - 10 Edition (ICD-10). If patients met the criteria, socio-demographic and clinical features such as age at onset, first episode type, and psychiatric and medical histories were recorded. The current symptoms of participants were assessed by psychiatrists with at least six years of experience in both research and clinical practice, while medication data were independently reported by the patients, their family members, and their medical records when available. Both the research psychiatrists and assistants were trained before the study to ensure fidelity with protocols. Prior to inclusion in the study, all potential participants were informed of the parameters of this study, and provided written consent. All protocols and procedures were approved by the Ethics Committee of Shanghai Mental Health Center and the other participating institutions, and were conducted in accordance with the relevant national and international guidelines, as well as the Declaration of Helsinki.

Clinical Assessment

All patients enrolled in this study were diagnosed with subtypes of BP according to the ICD-10 criteria. Based on the DSM-IV definitions, ATFs were defined as mood reactivity plus two or more items of ATF B symptoms, which include hyperphagia, hypersomnia or weight gain, interpersonal rejection sensitivity, and leaden paralysis[1]. The researchers also examined the point prevalence by ATF B criteria, which refers to a patient having at least two of the four symptoms listed above[14].

Statistical Analysis

All socio-demographic and clinical features were gathered from surveys and observations and then summarized with descriptive statistics that allowed for further comparison between different BP subtypes using the χ^2 test. Statistical significance was set at $\alpha = 0.05$. To explore the relationship between ATFs and six subtypes of BP, multinomial logistic regression was used (see Table S1 for details). Because we focused on the point prevalence of the atypical depressive features in BP rather than the diagnosis of atypical depression according to DSM-IV, the variable ATF B was used for regression analysis. Several other confounding variables were included as well, to test if there was any significant difference or association: gender (male *versus* female), age at study entry, age at onset, current co-morbidity with mental disorders (no *vs* yes), current co-morbidity with physical disorders (no *vs* yes), mood state at onset (hypomanic/manic episode *vs* depressive episode), type of episode last time (mania, depression, mixed episode, rapid-cycling episode), and number of episodes in the past year. Using this information, the point prevalence of ATFs was individually computed among all of the subtypes present among the participants.

The χ^2 test was also used to explore the relationship between ATFs and the use of antidepressants (reported no or yes). The relationship between ATFs and different treatment approaches among all the subtypes of BP was also examined, to provide a clearer picture of treatments currently being used in China. In this study, ATF B was used as the main variable of ATFs, due to the possible impact of ATF B on treatment methods.

RESULTS

Demographic and Clinical Characteristics

Among the 3 906 patients with varying subtypes of BP, analysis showed no significant differences in terms of gender distribution, race, current comorbidity-mental disorders, or current comorbidity-physical disorders. However, some clinical characteristics showed significant differences (P <0.01) among the six subtypes of BP, notably mental health history and the number of episodes in the past year. Other variables, such as current state of episode, mood state at first episode, family history, age at entry, age at first episode, and episode type last time showed even higher significance (P <0.001; Table 1).

Distribution of Atypical Features in BP

We used multinomial logistic regression to explore the relationship between ATF B features and six subtypes of BP by comparing clinical variables among them. Using remission type as the internal reference index (P <0.05, see Table S1 for details), the results of these analyses showed that the ATF B features were significantly correlated with the subtypes of BP. First, we noted that the different subtypes exhibited different point prevalence rates of ATFs. The point prevalence rates of all atypical symptoms were relatively higher in patients exhibiting a mixed state (Table 2). Interestingly, rejection sensitivity was the most common symptom in all the BP subtypes (ranging from 42.5% in patients with recurrent mania to 64.3% in patients with mixed state). Second, based on the DSM-IV definition of ATFs, the overall rate of patients who met the criteria was 9.1%, with a specific breakdown as follows: 5.3% of manic patients without psychotic symptoms; 8.7% of those patients with psychotic symptoms; 7.4% of hypomanic patients; 11.3% of recurrent mania patients; 6.9% of remission patients; and most strikingly, 28.1% of mixed state patients. Finally, the overall rate of patients who met the ATF B symptom criteria was 11.8%. Specifically, this was less common in patients diagnosed with mania without psychotic symptoms (7.0%), but most common in mixed state patients (33.2%).

Association between Atypical Features B and Use of Antidepressants

The proportions of patients with and without ATF-B using antidepressant medication in each subgroup are shown in Table 3. Our analysis showed no significant differences between ATF B and non-ATF B patients in the hypomania, mania without psychotic symptoms, mania with psychotic symptoms, or recurrent mania subgroup in the use of antidepressants. In mixed state patients, those with ATF B symptoms were more likely to be using antidepressants (P <0.01) than those without ATF-B. A similar relationship was found in remission patients (P <0.001).

Impact of Atypical Features B Symptoms on Medication Approach

The current findings did not indicate any significant

Table 1. Demographic and clinical features

Characteristics		I	II	III	IV	V	VI	χ^2	*P*
Gender	Male	382	627	335	103	86	494	1.03	0.960
	Female	360	563	308	96	74	478		
Race	Han	733	1176	634	197	159	965	3.02	0.697
	Other	8	14	9	2	1	6		
Current state of episode	First	233	302	139	46	4	50	2652.39	<0.001
	Recurrent	460	842	479	112	147	69		
	Stable	49	46	25	41	9	853		
Mood state at first onset	Mania/Hypomania	466	673	397	89	111	553	31.13	<0.001
	Depression	296	517	246	110	49	419		
Co-morbidity, mental disorders (current)[a]	Yes	9	12	10	3	5	6	9.29	0.098
	No	733	1178	633	196	155	966		
Co-morbidity, physical disorders (current)	Yes	55	101	54	7	9	68	8.11	0.150
	No	678	1089	589	192	151	904		
Past history of mental health[b]	Yes	119	188	98	39	45	182	19.90	0.001
	No	623	1002	545	160	115	790		
Family history of mental health[c]	Yes	597	952	482	154	113	838	43.67	<0.001
	No	145	238	161	45	47	134		
Episode type (last time)	Mania/Hypomania	441	675	372	56	119	670	614.53	<0.001
	Depression	274	480	243	58	33	240		
	Mixed	22	28	18	78	3	51		
	Rapid-recycling	5	7	9	7	5	11		
Age at entry (years)		34.48 ±13.99	33.8 ±13.73	32.11 ±12.93	34.97 ±13.63	38.14 ±13.44	37.30 ±15.02	69.09	<0.001
Age at first episode (years)		27.64 ±12.0	26.85 ±11.21	25.55 ±10.86	28.69 ±11.7	27.99 ±9.94	28.48 ±11.9	35.76	<0.001
Episode number (past 12 months)		1.72 ±3.04	1.75 ±2.94	2.05 ±5.13	2.35 ±2.75	1.94 ±3.11	1.45 ±3.62	3.50	0.004

I, hypomania; II, mania without psychotic symptoms; III, mania with psychotic symptoms; IV, mixed state; V, recurrent mania; VI, remission. [a]Current psychiatric diseases other than BP; [b]Past psychiatric diseases other than BP; [c]Psychiatric diseases distributed in three generations of the family.

difference among most of the studied subtypes — hypomania, mania without psychotic symptoms, mania with psychotic symptoms, mixed state, and recurrent mania — in terms of medication used for treatment between patients that met the ATF B criteria and those who did not. However, in the remission subtype, the differences in treatment presented a significant correlation with the ATF B factor (*P* <0.001) (Table 4).

DISCUSSION

In the current study, a range of atypical symptoms was observed among all the currently known subtypes of BP. Using the DSM-IV criteria to define ATFs, the overall point prevalence was 9.1% across all patients. When the definition was broadened to include ATF B, the overall rate increased to 11.8%. We further examined the impact of ATF B symptoms on treatment practices for BP and its

Table 2. Percentage of atypical features in each subtype of bipolar disorder

	Mood reactivity[a]	Hyperphagia[b]	Hypersomnia[c]	Rejection sensitivity[d]	Leaden paralysis[e]	ATF B criteria[f]	DSM-IV atypical features[g]
I	42	7.7	5.3	48.9	6.7	10.1	7.4
II	39.9	7.7	2.2	45.9	1.4	7.0	5.3
III	45.3	10.3	5.4	49.9	4.0	10.6	8.7
IV	61.8	15.1	21.1	64.3	24.1	33.2	28.1
V	44.4	7.5	5.6	42.5	2.5	8.1	11.3
VI	43.8	7.3	7.1	49.3	13.5	15.8	6.9

I, hypomania; II, mania without psychotic symptoms; III, mania with psychotic symptoms; IV, mixed state; V, recurrent mania; VI, remission. [a]χ^2 = 121.57, P <0.0001; [b]χ^2 = 17.31, P <0.01; [c]χ^2 = 120.42, P <0.001; [d]χ^2 = 26.20, P <0.001; [e]χ^2 = 220.72, P <0.001; [f]χ^2 = 134.70, P <0.001; [g]χ^2 = 34.90, P <0.0001.

Table 3. Distribution of antidepressant use among each subtype of bipolar disorder

Subtypes		Using antidepressant (%)	Not using antidepressant (%)	χ^2	P
I	ATF-B met	9 (12)	66 (88)	2.66	0.10
	ATF-B not met	131 (19.8)	531 (80.2)		
II	ATF-B	11 (13.4)	71 (86.6)	0	0.99
	ATF-B not met	147 (13.4)	953 (86.6)		
III	ATF-B	14 (20.9)	53 (79.1)	2.46	0.12
	ATF-B not met	79 (13.8)	495 (86.2)		
IV	ATF-B met	22 (33.8)	43 (66.2)	6.52	0.01
	ATF-B not met	23 (17.9)	108 (82.1)		
V	ATF-B met	2 (15.4)	11 (84.6)	0.07	0.79
	ATF-B not met	13 (8.9)	133 (91.1)		
VI	ATF-B met	55 (36.4)	96 (63.6)	43.85	<0.001
	ATF-B not met	115 (14.1)	701 (85.9)		

I, hypomania; II, mania without psychotic symptoms; III, mania with psychotic symptoms; IV, mixed state; V, recurrent mania; VI, remission.

subtypes in China, and obtained two interesting findings: (1) among the mixed state and the remission patients, there were significant differences in the use of antidepressants between those who met and those who did not meet the criteria of ATF B; and (2) among remission patients, the medication type was associated with the presence of ATF B.

In this study, the point prevalence rate of ATFs was lower than that in previous reports. To date, only three comparable studies on BP have been conducted, and they reported prevalence rates using ATF B of 30%[17], 32.6%[13], and 38.8%[11], while we found the point prevalence rate to be 11.8%. However, when the investigation of bipolar depression used the DSM-IV criteria for ATFs, the prevalence rate increased to 44.2%[14]. To our knowledge, this is the first survey on atypical features in BP by a national database in China, so this may explain some of the discrepancies. Such differences may mainly result from the variations in the populations studied. For example, the three comparable studies enrolled all types of BP patients, but in this study, we, for the first time, investigated the point prevalence rate of ATFs in only six types of patients. This was to take into account the fact that atypical symptoms

Table 4. The comparison of treatment remedies among each subtype of bipolar disorders

Subtypes		A (%)	B (%)	C (%)	A+C (%)	A+B (%)	B+C (%)	A+B+C (%)	Others (%)	χ^2	*P*
I	ATF-B met	20	7	6	2	29	0	1	10	8.4	0.26
		(26.7)	(9.3)	(8.0)	(2.7)	(38.7)	(0)	(1.3)	(13.3)		
	ATF-B not met	142	41	79	18	203	19	15	145		
		(21.5)	(6.2)	(11.9)	(2.7)	(30.7)	(2.9)	(2.3)	(21.9)		
II	ATF-B met	10	7	4	2	37	1	4	17	3.2	0.85
		(12.2)	(8.5)	(4.9)	(2.4)	(45.1)	(1.2)	(4.9)	(20.7)		
	ATF-B not met	170	86	77	25	450	17	28	247		
		(15.5)	(7.8)	(7.0)	(2.3)	(40.9)	(1.5)	(2.5)	(22.5)		
III	ATF-B met	3	3	9	1	31	0	4	17	12.1	0.74
		(4.4)	(4.4)	(13.2)	(1.5)	(45.6)	(0)	(5.9)	(25)		
	ATF-B not met	78	45	31	8	242	12	28	129		
		(13.6)	(7.9)	(5.4)	(1.4)	(42.2)	(2.1)	(4.9)	(22.5)		
IV	ATF-B met	5	14	6	3	16	6	7	8	11.8	0.09
		(7.7)	(21.5)	(9.2)	(4.6)	(24.6)	(9.2)	(10.8)	(12.3)		
	ATF-B not met	14	26	12	3	41	2	6	27		
		(10.7)	(19.8)	(9.2)	(2.3)	(31.3)	(1.5)	(4.6)	(20.6)		
V	ATF-B met	3	1	0	0	3	0	2	4	12.3	0.51
		(23.1)	(7.7)	(0)	(0)	(23.1)	(0)	(15.4)	(30.8)		
	ATF-B not met	15	22	2	6	73	3	2	23		
		(10.3)	(15.1)	(1.4)	(4.1)	(50.0)	(2.1)	(1.4)	(15.8)		
VI	ATF-B	13	8	3	21	68	4	27	10	50.6	<0.001
		(8.4)	(5.2)	(1.9)	(13.6)	(44.2)	(2.6)	(17.5)	(6.5)		
	ATF-B not met	100	66	15	45	486	14	41	46		
		(12.3)	(8.1)	(1.8)	(5.5)	(59.8)	(1.7)	(5.0)	(5.7)		

I, hypomania; II, mania without psychotic symptoms; III, mania with psychotic symptoms; IV, mixed state; V, recurrent mania; VI, remission. A, mood stabilizer; B, antipsychotic drug; C, antidepressant; others, sedative and other anti-anxiety drugs.

make an important contribution to the recognition of BP: e.g., the characteristic of bipolarity predicted a higher likelihood of the diagnosis of BP in patients with major depressive disorder[28]. This decision was carefully thought out during the design of the study, as bipolarity is similar to the ATFs described in our study, and some evidence suggests that atypical symptoms are valuable in distinguishing BP-II disorder from depression[29, 30].

Among the atypical symptoms present in BP, the rate for those categorized as "rejection sensitivity" was the highest in our study. This finding is concordant with the basic clinical characteristics of BP, and underlies the relationship between rejection sensitivity and irritability and related symptoms[1, 28]. We also found that each of the atypical symptoms was most common among those patients exhibiting a mixed state; a not unexpected finding[31], since mixed state patients usually exhibit atypical depression symptoms, and atypical symptoms are among the core features of atypical depression in DSM-IV[1, 28]. Furthermore, some atypical symptoms such as hypersomnia have been assumed to indicate a balance of specificity and sensitivity for the diagnosis of BP-II disorder among depressed patients[30]. By extension, this suggests that we should focus on the ATFs during the diagnosis of

mixed states, as a failure to recognize BP in those treated for major depressive disorder is common in China[32].

In this study, we were also concerned about the impact of atypical symptoms on treatment approaches, such as the use of antidepressants. Several studies have highlighted the seriousness of the high prescription rate of antidepressants to treat bipolar disorder worldwide[33-35], and the mainland of China is no exception. In China, antidepressants are prescribed in many cases, even though their long-term use can have markedly negative outcomes, e.g., the risk of manic switch or suicide[34, 36]. If patients with BP have some non-specific symptoms, such as ATFs related to depression, treatment usually includes some clinical practices that are inconsistent with the guidelines. Our findings indicate that there is a tendency for the use of antidepressants among patients who meet the criteria of ATF B symptoms in the mixed state and the remission BP subtypes, both of which commonly have atypical symptoms. Earlier studies reported that atypical depression symptoms may respond to antidepressants, such as monoamine oxidase inhibitors and tricyclic antidepressants[18, 19], but more recent studies have suggested that there is a low rate of manic switch for serotonin reuptake inhibitors, and norepinephrine and dopamine reuptake inhibitors (bupropion). Both the previous and current clinical experience may contribute to the prescription of antidepressants, even if there is no consensus on their effects on BP during the acute[37] or maintenance periods[22].

Furthermore, we also examined the impact of atypical symptoms on treatment for BP. If compliance with the current treatment guidelines was usual in China, most clinicians would prefer a combination of drugs with different kinds of pharmacological action — atypical antipsychotics, mood stabilizers, and the like. The antidepressants should be carefully prescribed only for those patients with BP whose episodes are mainly in the depressive state. For example, for those patients with BP who are currently severely depressed for more than four weeks, bupropion may be the choice, when combined with a sufficient dose of mood stabilizer. However, our findings indicated that monotherapy is still unexpectedly common for all types of BP, although the presence of ATF B symptoms was associated with the treatment approach for remission BP patients. In addition, the antidepressants were still common for monotherapy or combination therapy. Unfortunately, we can draw few conclusions from this, as there is a lack of studies similar to ours.

Although this study offers several novel findings, there are limitations that should be considered. First, participating subjects were evaluated on a single appointment and most of the data were collected from their previous histories, which leaves room for errors in reporting or follow-up. Second, patients were recruited from specialized academic centers and may not be representative of the "real" situation of BP in mainland China. Third, BP was diagnosed according to ICD-10 but then DSM-IV ATFs were used. This was because ICD-10 is commonly used as the reference for diagnosis in China, and ATFs are only defined in DSM-IV criteria. Fourth, the treatment regimes prescribed to patients may be guided not only by disease-specific factors, but also clinician-specific factors. As we only focused on the ATFs without considering confounding factors such as age distribution, duration of disease, and co-morbidity of psychiatric diseases, there might be some potential confounding factors. We also did not assess the impact of different physicians on the treatment regimes. Despite these limitations, this study gives some guidance in refining further studies that can more effectively verify and expand the present results.

In conclusion, the current findings indicated that various atypical symptoms are commonly observed in all subtypes of BP (overall rate of 9.1% using the DSM-IV criteria for ATFs, 11.8% by the definition of ATF B). Of note, the ATFs were most common in mixed state patients. Likewise, among mixed state and remission patients, there was a higher frequency of antidepressant use when the patients met the criteria of ATF B; specifically, for remission patients, the treatment approaches were usually affected by whether or not ATF B symptoms were present. While this study reports on a survey built around information from a national database, it provides a framework of conclusions that are suggestive of larger issues with treating BP in the mainland of China. In particular, the findings highlight the need to strengthen the education of physicians on ATFs in BP, to help clinicians recognize different aspects of BP and depressive symptoms, and accordingly provide appropriate treatments.

SUPPLEMENTAL DATA

Supplemental data include detailed information of distribution of patients and one table, and can be found online at http://www.neurosci.cn/epData.asp?id=224.

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·Original Article·

Morphological changes in gray matter volume correlate with catechol-O-methyl transferase gene Val158Met polymorphism in first-episode treatment-naïve patients with schizophrenia

Ming-Li Li[1,2,*], Bo Xiang[2,*], Yin-Fei Li[1], Xun Hu[3], Qiang Wang[1], Wan-Jun Guo[1], Wei Lei[1], Chao-Hua Huang[1], Lian-Sheng Zhao[2], Na Li[1], Hong-Yan Ren[1], Hui-Yao Wang[1], Xiao-Hong Ma[1,2], Wei Deng[1], Tao Li[1,2]

[1]*The Mental Health Center and the Psychiatric Laboratory,* [2]*State Key Laboratory of Biotherapy,* [3]*Huaxi Biobank, West China Hospital, Sichuan University, Chengdu 610041, China*

*These authors contributed equally to this work.

Corresponding authors: Tao Li and Wei Deng. E-mail: litaohx@scu.edu.cn, mrdengwei@163.com

ABSTRACT

The catechol-O-methyltransferase (COMT) gene is a schizophrenia susceptibility gene. A common functional polymorphism of this gene, Val158/158Met, has been proposed to influence gray matter volume (GMV). However, the effects of this polymorphism on cortical thickness/surface area in schizophrenic patients are less clear. In this study, we explored the relationship between the Val158Met polymorphism of the COMT gene and the GMV/cortical thickness/cortical surface area in 150 first-episode treatment-naïve patients with schizophrenia and 100 healthy controls. Main effects of diagnosis were found for GMV in the cerebellum and the visual, medial temporal, parietal, and middle frontal cortex. Patients with schizophrenia showed reduced GMVs in these regions. And main effects of genotype were detected for GMV in the left superior frontal gyrus. Moreover, a diagnosis × genotype interaction was found for the GMV of the left precuneus, and the effect of the COMT gene on GMV was due mainly to cortical thickness rather than cortical surface area. In addition, a pattern of increased GMV in the precuneus with increasing Met dose found in healthy controls was lost in patients with schizophrenia. These findings suggest that the $COMT_{Met}$ variant is associated with the disruption of dopaminergic influence on gray matter in schizophrenia, and the effect of the COMT gene on GMV in schizophrenia is mainly due to changes in cortical thickness rather than in cortical surface area.

Keywords: schizophrenia; COMT; gray matter; imaging genetics

INTRODUCTION

Catechol-O-methyltransferase (COMT), which metabolizes the neurotransmitters dopamine (DA), epinephrine, and norepinephrine, has been reported to be related to schizophrenia[1-6]. Studies have also shown that alterations of DA signaling[7], as well as structural cortical maturation[8], are associated with a genetic susceptibility for schizophrenia. It has been suggested that DA plays an important role in the structural development of the cortex[9], and inherited disruptions of DA signaling can induce cortical dysmaturation, which has been found in patients with schizophrenia onset in adolescence and early adulthood[10, 11]. Furthermore, the catabolic action of COMT is important in regulating the levels of DA in the prefrontal cortex[12, 13], and the structure and function of this region have been confirmed to contribute to schizophrenia. These studies suggest that COMT plays a crucial role in the onset of schizophrenia and may act on the morphological changes of brain structure in this mental disorder.

The COMT gene is located on chromosome 22q11.22–23. A functional COMT polymorphism with a single nucleic acid change of guanine to adenine results in the replacement of valine (Val) by methionine (Met) (known as Val158Met, rs4680). This common polymorphism has been studied

extensively since the 1980s[14]. Compared with the Met158 allele, the Val158 allele is associated with greater COMT enzyme activity and greater DA degradation. The alleles are co-dominant as the heterozygotic genotype (Val158/Met158) and are associated with an intermediate level of the enzymatic activity of COMT[15, 16]. Met158 carriers with Val158/Met158 and Met158/Met158 have four- to five-fold lower COMT activity than carriers with Val158/Val158. Honea *et al*.[17] reported that the Val158 variant of rs4680 affects hippocampal and dorsolateral prefrontal gray matter volumes (GMVs) in healthy populations. Previous studies also identified an effect of the COMT Val158Met polymorphism on cortical activation or functional connectivity in schizophrenia[18-20]. In addition, as GMV is determined by cortical thickness and cortical surface area, alterations of GMV might be due to both of these measurements, or either one. However, few studies have probed the relationship between variants of the COMT gene and morphological changes, including GMV, cortical thickness, and cortical surface area in schizophrenia.

Therefore, this study was performed to explore the COMT Val158Met polymorphism underlying the abnormal gray matter morphological changes (GMV/cortical thickness/cortical surface area) in schizophrenia.

PARTICIPANTS AND METHODS

Participants

One hundred and fifty first-episode, treatment-naïve patients with schizophrenia and 100 healthy controls (HCs) were enrolled in this study. All patients were interviewed and assessed using the Structured Clinical Interview (SCID-I/P) of DSM-IV (Diagnostic and Statistical Manual of Mental Disorders, fourth edition)[21], and fulfilled the diagnostic criteria for schizophrenia or schizophreniform disorder in DSM-IV. Forty-nine patients diagnosed with schizophreniform disorder when they first participated in this study were followed up for at least 6 months to confirm a diagnosis of schizophrenia. Psychiatric symptoms were assessed with the Positive and Negative Syndrome Scale (PANSS)[22]. The HCs were recruited locally by advertisement and were screened for a lifetime absence of psychiatric illness using the Structured Clinical Interview (SCID-NP) in the DSM-IV Non-Patient Edition[23]. In addition, HCs were interviewed to ascertain that there was no psychiatric illness in their first-degree relatives. Participants with evidence of organic brain disorders, alcohol or drug abuse, or any other severe physical illness were excluded.

All participants were ethnic Han Chinese. This study was carried out in accordance with the Declaration of Helsinki and was approved by the Review Board of West China Hospital, Sichuan University. All participants provided written informed consent to their participation.

Genotyping

DNA was obtained from whole blood using the standard phenol-chloroform isolation method[24]. The Val158Met polymorphism of the COMT gene was genotyped as a restriction fragment length polymorphism after polymerase chain reaction amplification and digestion with NlaIII. COMT val allele has a G at position 1947, generating a 114-bp fragment after digestion with NlaIII, while COMT met allele has an A at this position, which resulted in digestion of the 114-bp fragment into two products of 96 and 18 bp. The amplicons were separated by agarose gel (3%) electrophoresis.

T1-Weighted Magnetic Resonance Imaging (MRI) Data Acquisition

Two hundred and fifty participants underwent MRI scans in the Department of Radiology at West China Hospital with a 3T MR imaging system (EXCITE, General Electric, Milwaukee, WI) with an 8-channel phase-array head coil. High-resolution T1 images were obtained from all participants using a 3D spoiled gradient echo sequence. The sets used in this protocol were as follows: TR = 8.5 ms; TE = 3.93 ms; flip angle = 12°; slice thickness = 1 mm; single shot; field of view = 24 cm × 24 cm; matrix = 256 × 256; voxel size = 0.47 × 0.47 × 1 mm^3. In total, 156 axial slices were collected from each brain. All scans were inspected for motion artifacts, and a neuroradiologist confirmed the absence of gross pathology.

MRI Data Preprocessing

Voxel-Based Morphometry

The non-parametric, non-uniformity intensity normalization technique in the MINC software package (http://wiki. bic.mni.mcgill.ca/index.php/MINC) was used to rectify the non-uniformity of the high magnetic-field signal in all

images. Then, the Diffeomorphic Anatomical Registration Through Exponentiated Lie algebra (DARTEL) toolbox in Statistical Parametric Mapping (SPM) 8 was used to process these images. The details are as follows: all structural MRI scans were realigned manually according to the anterior commissure-posterior commissure line and were segmented into probability maps of gray matter (GM), white matter (WM), and cerebrospinal fluid using the 'new-segment' routine implemented in SPM8. Flow fields and a series of template images were produced by running the 'DARTEL (create templates)' routine using imported versions of the GM and WM generated in the previous step. The flow fields as well as the final template images were used to generate smoothed (6 mm full-width at half-maximum isotropic Gaussian kernel), Jacobian modulated, and spatially normalized GM in Montreal Neurological Institute (MNI) space. Finally, GMVs were retained for subsequent statistical analysis.

Surface-Based Morphometry

FreeSurfer 5.1 (http://surfer.nmr.mgh.harvard.edu/fswiki) was used to reconstruct the cortical surface from the structural MRI scans[25]. After skull-stripping and intensity correlation, tissue intensity and neighborhood constraints were used to determine the GM-WM boundary for each hemisphere. Then, all the surfaces were checked following an automated topology-fixation procedure, and minor defects were manually corrected. The pial surface was created by GM-WM assessment as the starting point of a deformable surface algorithm. Spherical morphing and spherical registration were processed after that. Finally, cortical thickness and surface area were calculated using the methods developed by Fischl and Dale[26].

Statistical Analyses

Patients with schizophrenia and HCs were divided into the following subgroups according to the SNP genotypes: Val158/Val158, Val158/Met158, and Met158/Met158.

A full factorial model was used to obtain the main effects including the genotype effect and the diagnostic effect as well as diagnosis-by-genotype (D×G) interaction in the GMVs according to the genotype of the Val158Met polymorphism. Statistical significance was defined as $P < 0.001$ (uncorrected) with >200 linked voxels (cluster of voxels)[27].

Then, significant region-of-interest (ROI) masks of brain regions were obtained from the results of main effects or D×G interaction analysis of the GMVs. The masks were extracted using Xjview (http://www.alivelearn.net/xjview8/) and used to acquire their respective cortical surface area and cortical thickness in Freesurfer. The binary masks were registered onto an average image using 'bbregister' in Freesurfer, which is a boundary-based affine registration method that aligns images by maximizing the intensity gradient across tissue boundaries[28]. Then, the ROI labels were obtained from the masks and unwrapped back onto each participant's native image. Finally, the cortical surface area and thickness of each ROI were acquired by averaging the respective values from all the vertices included within the defined clusters for each participant.

RESULTS

Demographic Characteristics and Clinical Information

The demographic characteristics of participants are summarized in Table 1. All groups were of comparable age, gender, and years of education. No significant differences were found in PANSS scores among the three subgroups of patients.

The distribution of the COMT Val158Met polymorphism did not deviate from Hardy-Weinberg expectation in HCs ($P = 0.15$). The allelic and genotypic frequencies are presented in Table 2. There were no significant differences in the frequencies of genotypes and alleles between patients with schizophrenia and HCs.

Main Effects of Genotype and Diagnosis on GMV

Significant main effects of diagnosis were found for the bilateral cerebellar posterior lobe and vermis, right hippocampal-parahippocampal gyrus, lingual gyrus, bilateral calcarine, bilateral inferior and middle occipital gyri, bilateral superior temporal gyrus, right middle frontal gyrus, right supramarginal gyrus, bilateral precentral gyrus, left paracentral lobule, and bilateral supplementary motor area. Patients with schizophrenia showed reduced GMVs in these regions (Fig. 1). Moreover, a significant main effect of genotype was detected for the left superior frontal gyrus (MNI coordinates: x = −23, y = 66, z = 9; voxels = 1063). Compared with Met homozygotes, Val-allele carriers and Val homozygotes showed a decreased GMV in this gyrus.

Table 1. Demographics and clinical data

	Healthy Controls			Patients with Schizophrenia			Diagnosis	Genotype	Diagnosis × Genotype
	Met/Met	Met/Val	Val/Val	Met/Met	Met/Val	Val/Val	*F* (*P*)	*F* (*P*)	*F* (*P*)
Number	14	40	46		19	59		72	
Sex (M/F)	7/7	20/20	25/21		8/11	31/28		30/42	
Age (years)	27.07 (7.14)	25.13 (8.53)	25.54 (8.19)	24.84 (7.41)	24.20 (7.12)	24.29 (7.28)	1.65 (0.20)	0.35 (0.71)	0.09 (0.92)
Education (years)	12.86 (3.88)	12.01 (4.44)	12.97 (3.10)	11.68 (3.80)	11.83 (2.83)	12.68 (2.76)	1.22 (0.27)	1.95 (0.15)	0.29 (0.75)
Course (months)	-	-	-	6.89 (11.14)	9.12 (14.89)	10.41 (20.24)	-	0.33 (0.72)	-
PANSS_T	-	-	-	91.37 (12.29)	91.17 (21.47)	90.06 (17.50)	-	0.07 (0.93)	-
PANSS_P	-	-	-	22.95 (4.22)	26.53 (7.55)	26.17 (7.47)	-	2.06 (0.13)	-
PANSS_N	-	-	-	18.58 (6.07)	20.00 (8.49)	19.21 (11.06)	-	0.20 (0.82)	-
PANSS_G	-	-	-	49.84 (9.40)	47.90 (10.97)	45.39 (8.66)	-	2.05 (0.13)	-

Mean (SD). PANSS, Positive and Negative Syndrome Scale; PANSS_T, total score; PANSS_P, positive score; PANSS_N, negative score; PANSS_G, general psychopathology score.

Table 2. Genotype distributions and allelic frequencies of catechol-O-methyl transferase gene Val158Met polymorphism

	Genotype distributions			df	χ^2	*P*	Allele frequencies		df	χ^2	*P*
	Val/Val	Val/Met	Met/Met				Val	Met			
Patients	72	59	19	2	0.14	0.93	203	97	1	0.15	0.70
Controls	46	40	14				132	68			

However, no GMV difference was found between Val-allele carriers and Val homozygotes.

Diagnosis × Genotype Interaction in GMV

A significant D×G interaction in GMV was found. Our results showed that schizophrenic patients with the Met158/Met158 genotype had a decreased GMV in the left precuneus region compared to HCs (Fig. 2). Furthermore, the cortical thickness of the left cerebral parietal lobe in the precuneus region was reduced in schizophrenic patients with this genotype compared to HCs; however, there was no significant difference in the cortical surface area of this region between schizophrenic patients and HCs with this genotype (Fig. 3; Table 3).

Table 3. Differences in GMV, cortical thickness, and surface area of the left precuneus in patients with Met158 and controls with Met158

	Patients	Controls	*F*	*P* value
Volume	0.09 (0.02)	0.13 (0.04)	12.7	0.001
Thickness	2.94 (0.39)	3.23 (0.27)	5.66	0.02
Surface area	84.21 (26.38)	95.25 (24.16)	0.66	0.43

Mean (standard deviation); sex and age were included as covariates.

Effects of Val158Met Polymorphism on Brain Morphology in Healthy Controls and Patients with Schizophrenia

Since the D×G interaction effects occurred in the volume and cortical thickness in the precuneus, we estimated the effects of genotype on brain morphology in the HC and schizophrenic groups. In the HC group, we found a step-wise increase of GMV (P <0.01) and a trend of increasing cortical thickness (P = 0.06) in the precuneus,

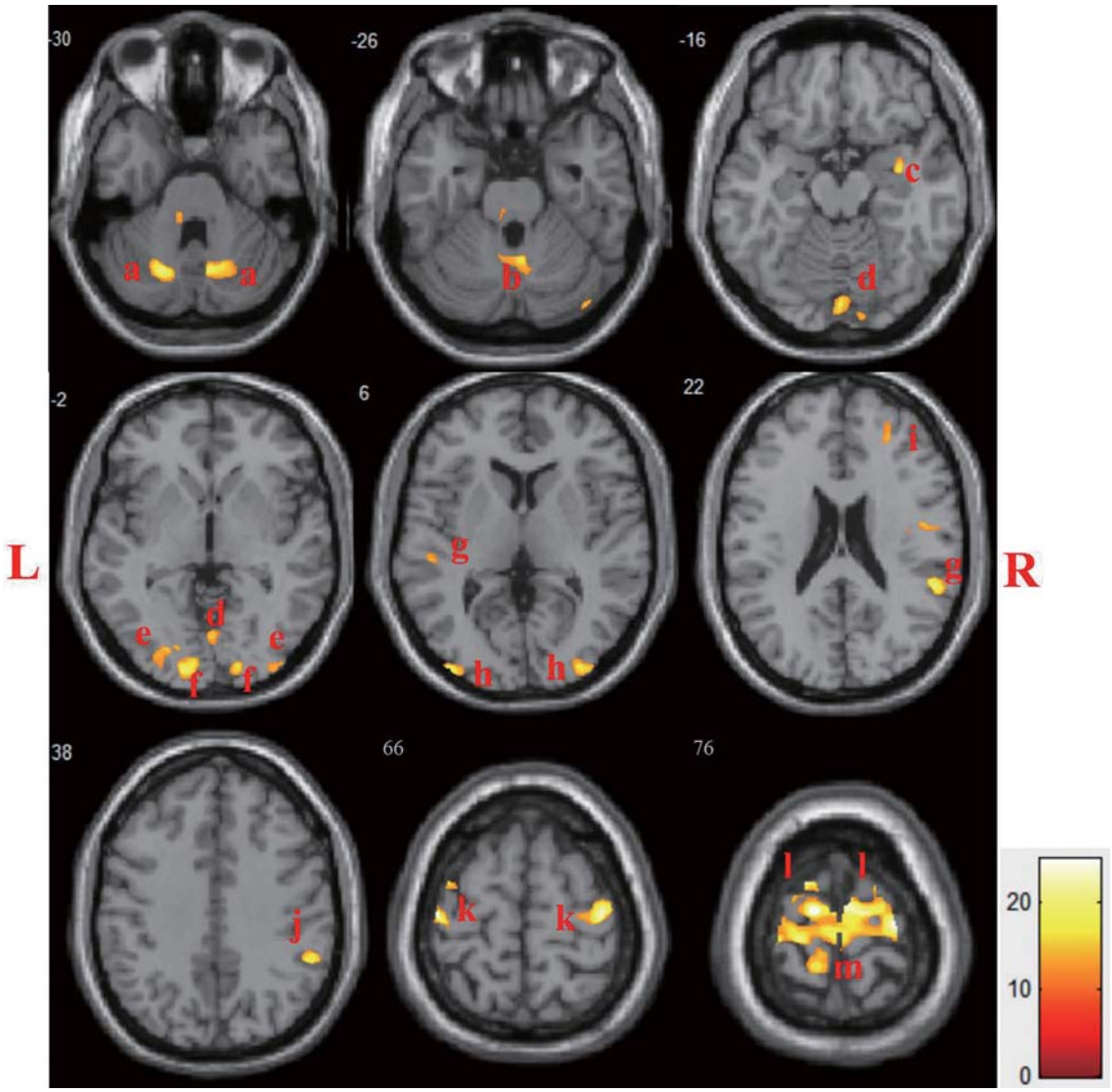

Fig. 1. Main effect of diagnosis on gray matter volume. Statistical inferences were made at *P* <0.001 (uncorrected) with >200 linked voxels. Patients with schizophrenia showed widespread reduced gray matter volume. a, posterior lobe of cerebellum; b, vermis; c, hippocampal-parahippocampal gyrus; d, lingual gyrus; e, inferior occipital gyrus; f, calcarine; g, superior temporal gyrus; h, middle occipital gyrus; i, middle frontal gyrus; j, supramarginal gyrus; k, precentral gyrus; l, supplementary motor area; m, paracentral lobule; L, left; R, right.

with increased dosage of the Met allele. However, contrary to the HC group, the schizophrenia group displayed no significant morphological differences in GMV (P = 0.27) and cortical thickness (P = 0.92) among patients with the Met158/Met158, Met158/Val158, and Val158/Val158 genotypes (Fig. 3). Thus, compared to HCs, patients with schizophrenia showed a loss of relationship between Met dose and GMV and thickness in the precuneus.

DISCUSSION

In the present study, the effect of the COMT Val158Met polymorphism on abnormal morphological changes (gray matter) in patients with schizophrenia was explored using the imaging genetic approach. We found a main effect of diagnosis on GMV, as GMV was decreased in the cerebellum and the visual, medial temporal, parietal,

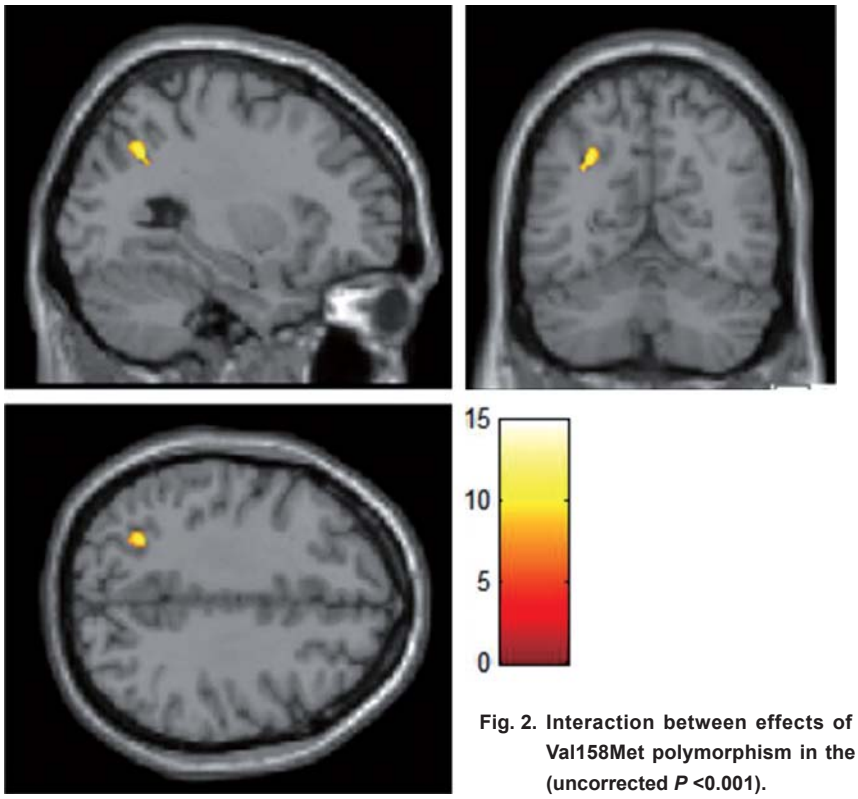

Fig. 2. Interaction between effects of diagnosis and COMT Val158Met polymorphism in the left precuneus region (uncorrected *P* <0.001).

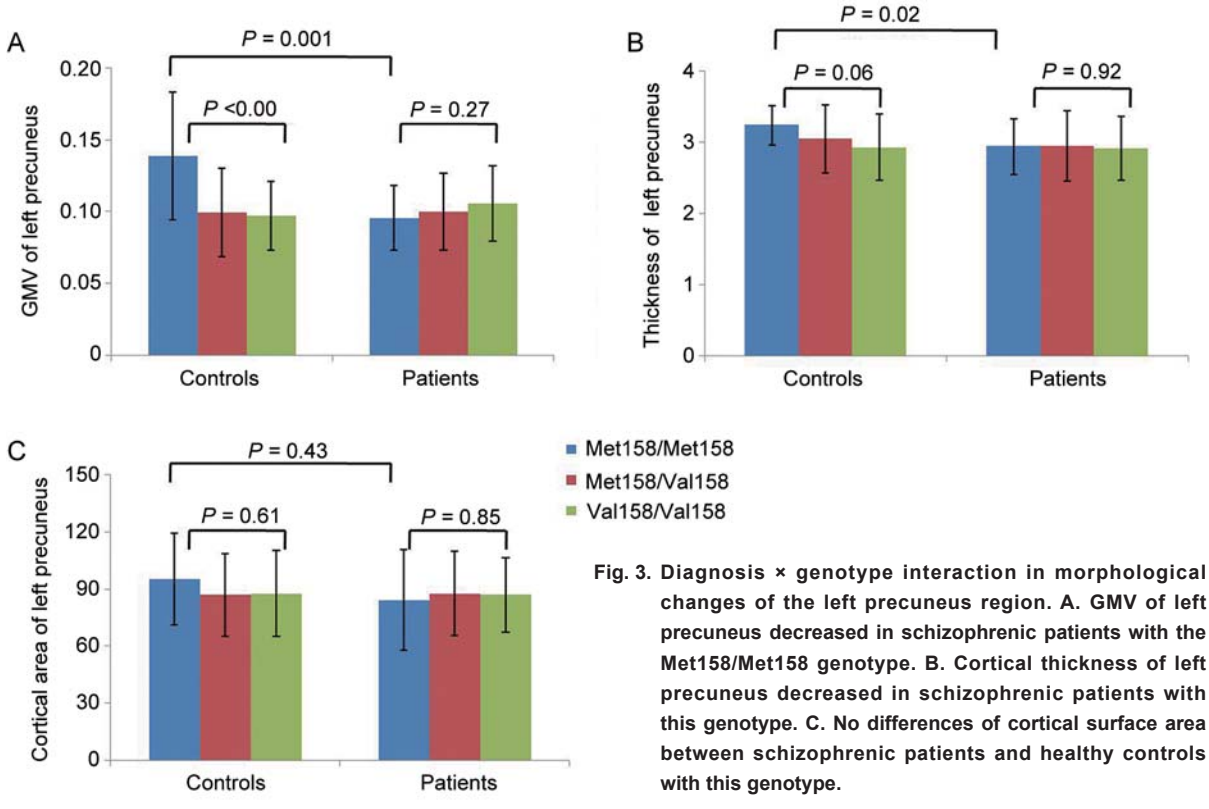

Fig. 3. Diagnosis × genotype interaction in morphological changes of the left precuneus region. A. GMV of left precuneus decreased in schizophrenic patients with the Met158/Met158 genotype. B. Cortical thickness of left precuneus decreased in schizophrenic patients with this genotype. C. No differences of cortical surface area between schizophrenic patients and healthy controls with this genotype.

and middle frontal cortex in schizophrenia. Furthermore, schizophrenic patients with the Met158/Met158 genotype had decreased GMV and reduced cortical thickness in the left precuneus compared with HCs of the same genotype.

We found that patients with first-episode schizophrenia showed widespread GMV reduction in the posterior lobe and vermis of the cerebellum, and the occipital cortex (inferior/middle occipital gyrus), lingual and calcarine gyrus, right hippocampal-parahippocampal gyrus, bilateral superior temporal gyrus, right middle frontal gyrus, and parietal lobe (supramarginal gyrus, precentral gyrus, paracentral lobule, and supplementary motor area). Two large meta-analysis studies[29, 30] highlighted deficits in the hippocampus-parahippocampus, superior temporal gyrus, and middle frontal gyrus in schizophrenia, and these abnormalities were further confirmed in MRI studies of first-episode schizophrenic patients[31-35]. GMV loss in the parietal cortex also occurs in schizophrenia. Abnormalities of the parietal lobe including the supramarginal gyri were reported in a meta-analysis carried out in the 2001[30]. Recent studies also found a decreased volume of the precentral gyrus in patients with first-episode schizophrenia[36]. In addition, among twin pairs discordant for schizophrenia, reduced GMV in the pre/postcentral gyrus was found in those with schizophrenia compared with their nonpsychotic twins, suggesting that the aberrant GMV in the pre/postcentral gyrus might be important in the development of schizophrenia[37]. Other than these regions, the cerebellum was traditionally supposed to be involved only in motor functions, but some studies have suggested its role in cognition and emotion[38] as well as in schizophrenia[39]. For example, abnormalities in the cerebellum and the occipital visual cortex have been detected in first-episode and/or drug-naïve patients[37, 40-43] using either the ROI approach or the voxel-based morphometry (VBM) method. In this study, the main effect of diagnosis on GMV suggested that the GM deficits in schizophrenia might involve widespread regions and circuits in the brain.

In line with previous studies[44], our results supported the idea that the COMT Val158Met variant might play an important role in the abnormal development of GM in schizophrenia. Moreover, our findings suggested that the effect of the COMT gene on GMV in schizophrenia is mainly due to cortical thickness rather than surface area. The radial-unit hypothesis of cortical development suggests that cortical thickness is different from surface area and both are characteristic features of human cortical development[45]. Cortical thickness and volume follow similar developmental patterns[46, 47]. Some studies have suggested that the deficit patterns of GMV and cortical thickness are similar in patients with schizophrenia[48], and that the aberrant cortical thickness might date back to adolescence in those patients[49]; although contradictory results have also been found. For example, Voets *et al*. found that differences in local surface area accounted for the GMV changes[50]. Moreover, although high heritability was detected in both the total cortical surface area and the average cortical thickness (0.89 and 0.81, respectively), no essential related genetics were found for them (genetic correlation = 0.08), which implied that GMV might be influenced by at least two distinct genetic sources[51]. These studies provided a newer and in-depth genetic view of the role of traditional GMV in pathological explanations.

In VBM method, regional GMVs are obtained by computing numbers of voxels, resulting in a mixture of thickness and cortical folding in the final measures, while in surface-based methods, GMVs are calculated based on cortical thicknesses and surface areas. However, compared with FreeSurfer, GMVs based on VBM provide more precise measures of subcortical structures such as the basal ganglia[52, 53] which may be one of the important etiological locations in schizophrenia. In addition, as suggested by Palaniyappan *et al.*[54], the differences in GMVs between two groups obtained using VBM are partially dependent on cortical thickness, surface area, and gyrification which are acquired using surface-based morphometric methods in Freesurfer software. Thus, in the present study, we first used VBM to explore the relationship between the Val158Met polymorphism and GMV, and then explored the anatomical properties of cortical thickness and surface area using part of the Freesurfer software. We found that compared with healthy carriers of the Met158/Met158 genotype, schizophrenic patients with this genotype had a decreased GMV and reduced cortical thickness in the left precuneus. The precuneus is located in the posteromedial parietal cortex and plays an important role in a wide spectrum of highly-integrated tasks, including visuo-spatial imagery, episodic memory retrieval, and self-processing operations[55]. These cognitive functions are widely impaired in schizophrenia[56-63]. Moreover, the precuneus has been

suggested to be the 'core node' of the default mode network[64], which has been hypothesized to be relevant to the pathological mechanism of schizophrenia[65-67]. Decreased volume and cortical thickness of the precuneus in schizophrenia have also been detected in previous studies[50, 54, 68-70], but are relatively rarely reported compared with the prefrontal and temporal lobes. A decreased volume of the precuneus has not been reported in drug-naive, first-episode schizophrenic patients[33, 35], but was found in patients with the Met158/Met158 genotype in this study. One explanation of this discrepancy might be high sample heterogeneity. Schizophrenia is a clinically and etiopathogenetically heterogeneous disorder, thus the association between abnormal morphology of the precuneus and schizophrenia might be concealed by the effects of population stratification[71]. So the significantly reduced volume in the precuneus might exist only within a particular genetic background, such as in the subtype of schizophrenic patients with the Met158/Met158 genotype.

As the D×G interaction had significant effects on GMV, we further evaluated the genotype effect on brain morphology between the different diagnostic groups. We found that the patterns of the Val158Met effect on the morphology of the precuneus differed in schizophrenic patients and HCs. HCs demonstrated a pattern of increased GMV and cortical thickness in the precuneus with dosage of the Met allele. Relative to Val homozygotes, a trend of increased brain volume, thickness, and density in Met-allele carriers and Met homozygotes has also been reported in the hippocampus, the parahippocampal gyrus, the medial temporal lobe and the anterior cingulate in HCs in previous studies[17, 71-74]. A possible explanation for these findings in HCs might be the DA effect on brain maturation. The expression of brain-derived neurotrophic factor in neurons can be regulated by DA[75]. Compared with the Val158 allele, the Met158 allele associated with lower COMT enzyme activity and reduced DA degradation would lead to relatively higher synaptic DA levels contributing to differential brain development.

But the pattern of increased GMV and cortical thickness in the precuneus with increasing Met dose did not occur in patients with schizophrenia, which suggested that the Met158 allele might lose its normal function in regulating the morphological development of the precuneus (especially GMV and cortical thickness) in schizophrenia. A disrupted gene-brain relationship (val158met polymorphism and adolescent cortical development) has also been reported in patients with childhood-onset schizophrenia and their non-psychotic siblings[76]. Otherwise, it is also possible that the COMT gene is just a genetic marker, while other genes linked to the COMT gene might contribute to abnormal brain morphology and function in schizophrenia. For example, the Val158Met polymorphism interacts with a P2 promoter region SNP (rs2097603) and an SNP in the 3' region (rs165599) in predicting an inefficient prefrontal working memory response[77]. Besides, other genetic variants, such as the DA D2 receptor[78], might also play an important role in regulating the DA systems.

There are strengths and limitations in the present study. We recruited first-episode, neuroleptic-naïve schizophrenic patients to explore the effect of the COMT Val158Met polymorphism on brain morphology, by which confounding factors such as illness chronicity and antipsychotic administration were excluded. Moreover, besides GMV, cortical thickness and cortical area were studied to clarify the effect of the COMT Val158Met polymorphism. However, several limitations must be borne in mind. First, only a modest proportion of the variance in GMV can be explained by surface-based morphometric methods[54], so GMV obtained by Freesurfer software might be more useful for exploring the relationship between GMV and surface anatomical properties. Second, considering the genotype effect on the structural maturation of the brain and that schizophrenia is a polygenetic disorder, a longitudinal neuroimaging genetic study exploring the relationship between genes and brain structural maturation could provide more information on the pathological mechanisms. Third, we explored only the effect of the COMT Val158Met polymorphism on morphological changes in schizophrenia. The effects on brain function need to be studied in future.

In summary, in a large sample, we found decreased GMV in the cerebellum and the visual, medial temporal, parietal, and middle frontal cortex in schizophrenia. In addition, our study is one of the largest imaging genetic studies to test the COMT effects on gray matter in schizophrenia in a Han Chinese population, and that the effect of the COMT gene on GMV in the precuneus in schizophrenia might be due mainly to changes in cortical thickness rather than changes in cortical surface area.

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·Original Article·

The schizophrenia/bipolar disorder candidate gene *GNB1L* is regulated in human temporal cortex by a *cis*-acting element located within the 3'-region

Yue Sun[1,2], Yu Tao[5], Jian Wang[1,4], David Saffen[1,3,5]

[1]*Institutes of Brain Science, Fudan University, Shanghai 200032, China*

[2]*School of Biological Sciences, Fudan University, Shanghai 200433, China*

[3]*Key Laboratory for Medical Neurobiology, Fudan University, Shanghai 200032, China*

[4]*Key Laboratory of Exploration and Utilization of Aquatic Genetic Resources, Shanghai Ocean University, Ministry of Education, Shanghai 201306, China*

[5]*Department of Cellular and Genetic Medicine, School of Basic Medical Sciences, Fudan University, Shanghai 200032, China*

Corresponding author: David Saffen. E-mail: saffen@fudan.edu.cn

ABSTRACT

22q11.2 deletion syndrome (DS) is a complex developmental disorder with a high incidence of psychiatric illnesses, including schizophrenia and mood disorders. Recent studies have identified *Guanine Nucleotide Binding Protein (G protein) Beta Polypeptide 1-Like* (*GNB1L*), located within the 1.5 Mbp 22q11.2 DS critical region, as a candidate liability gene for schizophrenia and bipolar disorder. In this study, we used mRNA expression measurements in Han Chinese postmortem temporal cortex and linkage disequilibrium (LD) analysis to show that *GNB1L* is regulated by a *cis*-acting genetic variant within the 3'-region of the gene. Significantly, this variant is located within an LD block that contains all of the common SNPs previously shown to associate with schizophrenia and bipolar disorder in Han Chinese and Caucasian populations. Contrary to our expectations, re-analysis of previously published case-control study data in light of our mRNA expression results implies that the *GNB1L* high-expression allele is the risk allele for schizophrenia and bipolar disorder in the Han Chinese population.

Keywords: *GNB1L*; schizophrenia; linkage disequilibrium; eQTLs; *cis*-regulatory variants

INTRODUCTION

Hemizygous deletion of 1.5 or 3 Mbp DNA within the 22q11.2 locus produces a complex spectrum of deficits, including facial, palatal, and conotruncal malformations, endocrine and immune deficiencies, and behavioral and cognitive abnormalities of variable severity[1, 2]. In addition, individuals with 22q11.2 deletions frequently suffer from psychiatric disorders, including schizophrenia[3-6] and bipolar spectrum disorders (bipolar I and II and schizoaffective disorders)[7-10]. The strong association of schizophrenia with 22q11.2 deletions has stimulated the search for genes within the deleted regions that contribute more widely to non-syndromic autism and schizophrenia.

The 3 Mbp deletion region at 22q11.2 contains ~60 genes and the 1.5 Mbp deletion region ~28 genes[11]. Because individuals carrying the 1.5 Mbp deletion collectively display most of the deficits observed in carriers of the longer deletion, the chromosomal segment affected by this deletion is inferred to comprise a critical

region for 22q11.2 DS[12]. It is proposed that deletions of dose-sensitive genes within the critical region produce developmental deficits *via* haploinsufficiency.

Among the many genes in the critical region, *GNB1L* has been linked to schizophrenia in two independent case-control studies[13, 14]. An additional study did not find statistically significant associations between *GNB1L* SNPs and schizophrenia in Japanese case-control samples, but did find evidence for low *GNB1L* mRNA expression in prefrontal cortex (Brodmann area 9) from schizophrenia patients[15].

The study by Williams and colleagues[14] identified two SNPs (rs5746832 and rs2269726) located within the 3'-region of *GNB1L* for which there was excess homozygosity in male schizophrenia patients. The study by Li and colleagues[13] identified 5 SNPs (rs5746832, rs5748427, rs5748432, rs2269726, and rs748806) also located within the 3'-region of *GNB1L* that are associated with schizophrenia and/or bipolar disorder in the Han Chinese (Shanghai) population. By contrast, a study by Ma and colleagues[16] failed to detect an association between three SNPs in the neighboring *TBX1* gene and schizophrenia in a Han Chinese-based case-control study. A summary of the results of Williams *et al*., Li *et al*., and Ma *et al.* is provided in Fig. S1.

GNB1L encodes a 327 amino-acid protein of unknown function[17]. Bioinformatics-based analysis of the amino-acid sequence of GNB1L protein predicts six WD40 motifs, homologous in structure (but not amino-acid sequence) to WD40 repeats found in the beta subunit of the human guanine nucleotide binding protein[17, 18].

GNB1L mRNA is ubiquitously expressed, with especially high levels in skeletal muscle, spleen, thymus, and testes[17]. The major mRNA transcript detected in northern blots is 1.4 to 1.5 kb in length. Expression is low in adult brain, but has higher levels in embryonic human and mouse brain[17, 19]. *GNB1L* knockout is lethal in early embryogenesis. Homozygous deletion of this gene causes reduced prepulse inhibition in adult mice[20], a phenotype associated with several psychiatric disorders, including schizophrenia[21]. These studies suggest that *GNB1L* haploinsufficiency caused by 22q11.2 DS may contribute to the pathology of schizophrenia.

In the present study, we used expression quantitative trait locus (eQTL) mapping and linkage disequilibrium (LD) analysis to further investigate the *GNB1L* SNPs previously shown to associate with schizophrenia.

MATERIALS AND METHODS

Brain Samples

Frozen sections of anterior temporal cortex from 36 Han Chinese individuals were obtained from the Chinese Brain Bank Center (CBBC; South-Central University for Nationalities, Wuhan, China). Written consent for tissue donation was given by relatives (on file at CBBC). The use of human autopsy tissue is not considered to be human-subject research and is internal review board-exempt under the NIH guidelines. A description of the demographics of the set of brain tissues, including gender, age, RNA integrity number (RIN), and cause of death, where available, is provided in Table S1.

Isolation of Genomic DNA, Total RNA, and cDNA Synthesis

Isolation of genomic (g) DNA and total RNA and preparation of cDNA were carried out as previously described[22]. Briefly, ~30 mg frozen brain tissue from each individual was used for gDNA isolation using QIAamp® Mini kits (Qiagen, Valencia, CA). About 100 mg of frozen tissue from each brain sample was used for isolation of total RNA. The frozen tissue was extracted with TRIzol® reagent (Invitrogen, Carlsbad, CA) followed by DNase I treatment (New England Biolabs, Ipswich, MA) and RNA purification using Qiagen RNeasy® Mini kits. The quantity of gDNA and total RNA was determined spectrophotometrically using a Nanodrop® spectrophotometer (Thermo Inc, Waltham, MA). The quality of isolated RNA was assessed by measuring RINs using Agilent® 2100 and software provided by the company. cDNA was generated from 5 μg total RNA in 20 μL reaction mixes using SuperScript® III First Strand kits (Invitrogen) and stored at −20°C until use. All procedures were conducted according to the instructions of the manufacturers.

Real-time PCR Quantification of *GNB1L* mRNA Expression

GNB1L mRNA levels in samples of anterior temporal cortex from the 36 Han Chinese individuals in our collection were quantified by real-time PCR using a Mastercycler®

ep Realplex Thermal Cycler (Eppendorf, Hamburg, Germany) and Thunderbird SYBR® qPCR Mix (Toyobo, Osaka, Japan) to detect PCR products. mRNA levels in each sample were normalized to the mRNA expression levels of three house-keeping genes: *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*), *cytochrome c-1* (*CYC1*), and *β-actin* (*ACTB*). Correlations of expression among the three house-keeping genes were quantified by linear regression (Fig. S2). Primers were designed using Oligo 6.0 (National Biosciences Inc., Plymouth, MN) and synthesized by Sangon Biotech (Shanghai, China): *GNB1L*, 5'-CGGCTATGAGGATGGATCG-3' (forward) and 5'-CTGGGAGTCAAAGTCAAGGTC-3' (reverse); *GAPDH*, 5'-TCAAGATCATCAGCAATGCC-3' (forward) and 5'-TGTGGTCATGAGTCCTTCCA-3' (reverse); *CYC1*, 5'-GAGCACGACCATCGAAAACG-3' (forward) and 5'-CGATATGCCAGCTTCCGACT-3' (reverse); *ACTB*, 5'-GAAGGTGACAGCAGTCGGTT-3' (forward) and 5'-GGGACTTCCTGTAACAACGCA-3' (reverse). Normalized mRNA expression (in ΔC_t units) was calculated by subtracting the cycle threshold (C_t) for the target gene from the geometric mean C_t of the three house-keeping genes: $\Delta C_{t(GNB1L)} = [C_{t(GAPDH)}\ C_{t(CYC1)}\ C_{t(ACTB)}]^{1/3} - C_{t(GNB1L)}$.

GNB1L mRNA Expression in European Brain Samples

Array expression data of "BrainCloud"[23] (Gene Expression Omnibus (GEO) Accession Number GSE30272) and "Four Brain Region"[24] (GEO Accession Number GSE15745) were downloaded from the GEO website (http://www.ncbi.nlm.nih.gov/geo/).

Genotyping and Imputation

Whole-genome genotyping of the 36 samples (~1.14 × 10^6 genotypes/sample) was carried out using Illumina HumanOmni1-Quad arrays (Illumina, San Diego, CA) by Genesky Biotech (Shanghai, China) following the directions supplied by the manufacturer. Quality control using the PLINK[25] (http://pngu.mgh.harvard.edu/~purcell/plink/) toolset consisted of eliminating SNPs with minor allele frequencies <0.05 and those with a genotype missing rate >5% and yielded ~700,000 genotypes per sample. SNPs of interest were examined *post hoc* for violation of Hardy-Weinberg equilibrium (*P* <0.05). Genotype data for "Four Brain Regions" (dbGAP Study Accession: phs000249.v1.p1) and "BrainCloud" (dbGAP Study Accession: phs000417.v1.p1) were obtained from dbGAP (http://www.ncbi.nlm.nih.gov/gap) and cleaned using the same quality control criteria.

To enable comparison of eQTLs in different studies, we imputed genotypes for *GNB1L* region SNPs into our Han Chinese genotype data and the "Four Brain Regions" and "Braincloud" databases using IMPUTE2[26, 27] with the assistance of SHAPEIT2[28] and GTOOL (http://www.well.ox.ac.uk/~cfreeman/software/gwas/gtool.html) with genotype data from the 1000 Genomes Project (Phase 1, Integrated Variant Set) as the reference. All imputed SNPs met the quality control criteria described above.

Expression Quantitative Trait Locus Analysis

Prior to eQTL analysis, *GNB1L* mRNA expression data from the Han Chinese samples (in C_t units), "BrainCloud", and "Four Brain Regions" array studies were adjusted for available biological and methodological covariates using linear regression in SPSS (Version 20.0). Values for biological (age and gender) and methodological (RIN) covariates for each of the 36 Han Chinese samples are listed in Table S1. Covariate values for individual samples for the "Braincloud" study were obtained from the GEO website and those for "Four Brain Regions" study from Supplementary Files in Gibbs *et al.*[24].

The residuals from the regression were retained as "adjusted *GNB1L* expression values" and used as a quantitative phenotype for linear regression analysis of correlation between mRNA expression and SNP genotype using the *--assoc* command within PLINK (see Section 11. "Association, Quantitative traits" in the Plink documentation). The empirical linear regression *P*-value for the correlation between *GNB1L* mRNA expression and the genotype for each SNP was determined by 10,000 random permutations of expression level values *versus* fixed genotypes. SNPs with permutation *P*-values <0.05 were identified as eQTLs for *GNB1L*.

Linkage Disequilibrium Analysis

The LD structure of human chromosome 22 in the neighborhood of *GNB1L* based upon genotype data for all studies was generated using Haploview[29] (version 4.2: http://www.broadinstitute.org/scientific-community/science/programs/medical-and-population-genetics/haploview/haploview). We used the proxy search option of SNAP

software (http://www.broadinstitute.org/mpg/snap/ldsearch.php/) to find pairwise LD constants for SNPs in the 1000 Genome project CEU (Utah residents with ancestry from northern and western Europe) and CHB/JPT (Han Chinese Beijing/Japanese in Tokyo) populations.

RESULTS

Identification of eQTLs in the *GNB1L* Region That Correlate with *GNB1L* mRNA Expression in Temporal Cortex from Han Chinese

To identify *cis*-eQTLs for *GNB1L* mRNA expression, we measured relative *GNB1L* mRNA levels in anterior temporal cortex samples from 36 Han Chinese and looked for correlations with genotypes for common SNPs within a ~134 kb region of chromosome 22 containing *GNB1L* and *TBX1* (Chr22: 19730592-19864252, GRCh37.3/hg19). In total, 44 genotyped and 28 imputed SNPs were examined. Linear regression analysis identified 8 SNPs with nominally significant correlations (permutation P <0.05; not corrected for multiple testing) between mRNA expression and SNP genotype (Fig. 1). P-values and coefficients of determination (R^2) for these SNPs are listed in Table 1.

The *C*-allele of rs2269726 (C>T) and the *G*-allele of rs11704083 (A>G) were identified as high-expression alleles for each SNP (Fig. 2). Inspection of the LD structure of these SNPs (Fig. 1 and Table S2) revealed that 7 SNPs located within the 3'-region of *GNB1L* (rs5993830, rs5748427, rs5748432, rs2269726, rs880194, rs748806, and rs3788301) were in moderate-to-strong LD (D' >0.8, r^2

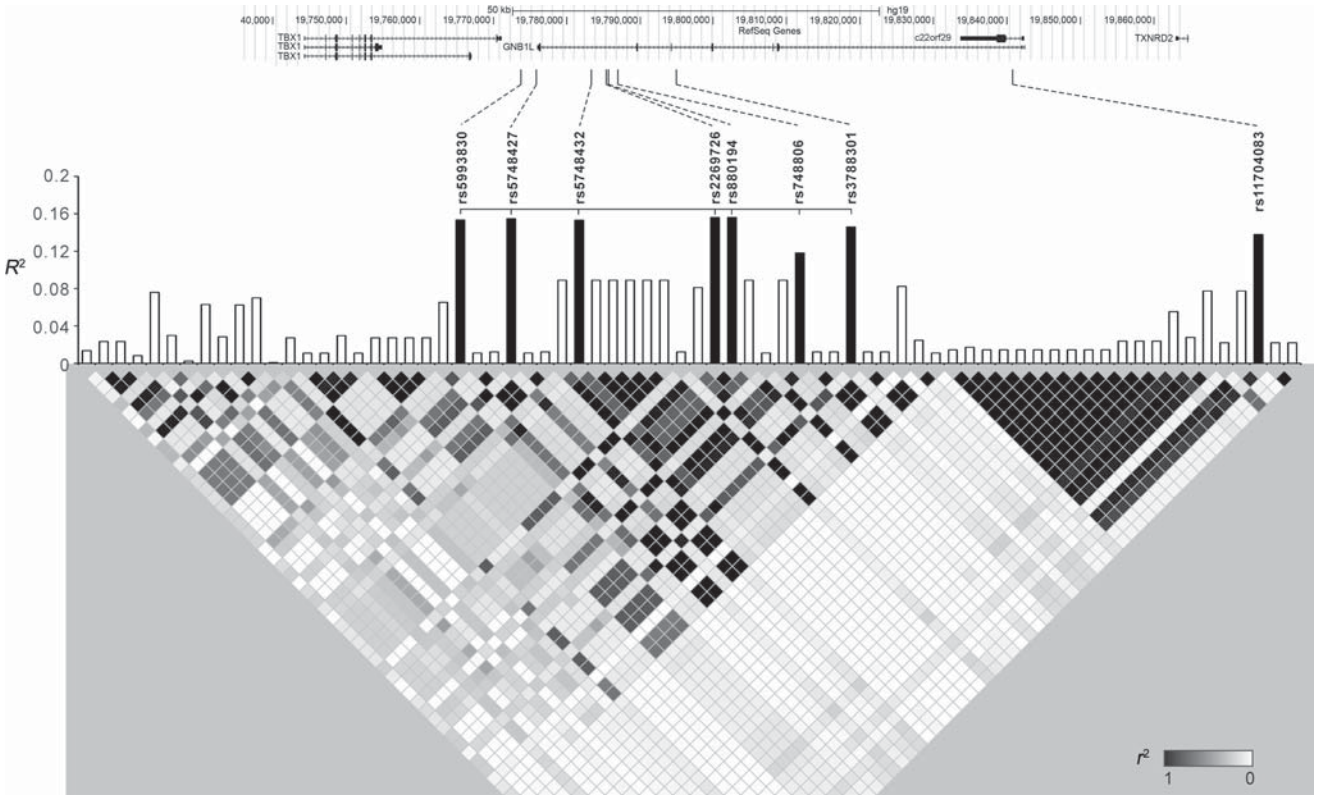

Fig. 1. SNPs in the *GNB1L* region showing correlations between genotype and *GNB1L* mRNA expression in temporal cortex from 36 Han Chinese. Top: Exon/intron structures for primary *TBX1* and *GNB1L* mRNA transcripts. Dashed lines indicate the locations of the 8 SNPs that correlate with *GNB1L* mRNA expression. Middle: Plot of coefficients of determination (R^2) from linear regression analysis of correlations between *GNB1L* mRNA expression and genotype for a set of genotyped and imputed SNPs in the set of 36 brain samples. Eight SNPs with nominally significant correlations with *GNB1L* mRNA expression analyzed by linear regression are plotted using solid bars. Bottom: LD plot showing values of the LD constant r^2 based on genotype data for 72 SNPs in the 36 Han Chinese individuals generated using Haploview.

Table 1. *GNB1L* SNPs that correlate with *GNB1L* mRNA expression in temporal cortex from Han Chinese

SNP	Position*	MAF** (major > minor alleles)	Linear regression			Origin
			R^2	*P*-value	Permutation *P*-value***	
rs5993830	19773308	0.400 (C>T)	0.154	0.0199	0.0206	Imputed
rs5748427	19775287	0.403 (G>A)	0.155	0.0177	0.0182	Genotyped
rs5748432	19783362	0.371 (G>A)	0.153	0.0201	0.0196	Imputed
rs2269726	19785006	0.375 (C>T)	0.156	0.0171	0.0163	Genotyped
rs880194	19785329	0.375 (C>T)	0.156	0.0171	0.0163	Genotyped
rs748806	19787736	0.471 (T>C)	0.118	0.0431	0.0415	Imputed
rs3788301	19795191	0.386 (G>C)	0.146	0.0235	0.0241	Imputed
rs11704083	19840413	0.403 (A>G)	0.138	0.0258	0.0248	Genotyped

*Chromosome 22 (Genome Build 37.3/HG19, reference assembly).

**MAF, minor allele frequency; based on genotype data from the set of brain samples from 36 Han Chinese.

***Permutation of mRNA expression levels was performed 10 000 times for each SNP.

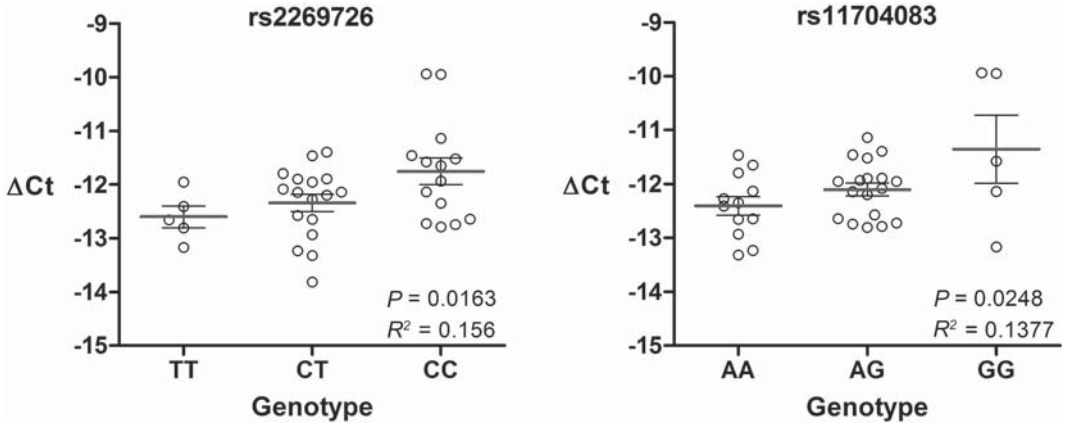

Fig. 2. Identification of high- and low-expression alleles for the 3'-region SNP rs2269726 and the 5'-region SNP rs11704083. Scatterplots show normalized *GNB1L* mRNA expression (in ΔC_t units) for 36 Han Chinese brain samples stratified according to genotype. Each dot represents one individual and the horizontal bars show the mean and standard error of the mean. Linear regression R^2 and *P*-value (10 000 permutations) for *GNB1L* mRNA expression *versus* genotype are listed. High expression alleles for rs2269726 and rs11704083 are the *C*-allele and the *G*-allele, respectively.

>0.53), while the 5'-region SNP rs11704083 was less tightly linked to the other SNPs (D' <0.4, r^2 <0.07).

Because these 7 SNPs had similar properties, we chose rs2269726, which has the highest R^2 and lowest *P* value for correlation with *GNB1L* mRNA expression, as the "index" SNP to represent the 3'-region LD block. Stepwise linear regression analysis showed that the 5'-region SNP rs11704983 combined with rs2269726 to produce a higher R^2 (0.251, P = 0.008), while the other 3'-region SNPs did not further increase the R^2 significantly. These results are consistent with the hypothesis that *GNB1L* mRNA expression in temporal cortex is controlled by at least two

independent *cis*-regulatory variants, one linked to SNPs in the 3'-region LD block and another linked to rs11704083.

GNB1L eQTLs in Caucasian Brain Samples

Currently, genome-wide genotype and mRNA expression data from two brain eGWAS studies are publicly available: the "BrainCloud" study[23] and the "Four Brain Regions (4BrainR)" study[24], both of which include a large number of samples from Caucasian individuals. To determine if correlations between *GNB1L* mRNA expression and SNP genotype are similar in Han Chinese and Caucasian brain samples, we downloaded individual-level genotype and expression data and searched for eQTLs in Caucasian individuals using the methods described above. To facilitate comparisons, we selected a subset of 44 SNPs that were genotyped or reliably imputed in all three studies. Only SNPs within the 3'-region LD block correlated with *GNB1L* expression in all three studies (Fig. 3). Although the 5'-region SNP rs11704083 also correlated with expression in most assays (except for the "4BrainR-PONS" and "BrainCloud" samples), the *P*-values were larger and the coefficients of determination smaller than SNPs in the Caucasian samples and our Han Chinese samples (Table S3).

In contrast to the results obtained with the Han Chinese brain samples, step-wise linear regression analysis of *GNB1L* mRNA expression in the Caucasian samples showed that rs11704083 did not significantly increase R^2 when combined with 3'-region SNPs (data not shown). These results suggest that either rs11704083 is an eQTL for *GNB1L* mRNA expression in the Han Chinese population only, or the observed correlation is a false-positive due to the relatively small number of Han Chinese brain samples analyzed. On the other hand, the finding that the 3'-region SNPs correlated with *GNB1L* mRNA expression in both the Han Chinese and Caucasian samples provides strong support for the existence of one or more *cis*-regulatory elements for *GNB1L* within the 3'-region LD block.

SNPs Tightly Linked to the Index SNP rs2269726 Correlate with Schizophrenia and/or Bipolar Disorder in Han Chinese and Caucasian Populations

Finally, we examined the relationship between linkage to rs2269726 and association with schizophrenia and/or bipolar disorder in Han Chinese and Caucasian case-control studies. We chose r^2 as the measure of linkage, since it more accurately correlated with *GNB1L* mRNA expression than D' or the midpoint of the 95% confidence interval for D' (data not shown). Estimates of r^2 in Chinese and European populations were based on genotype data obtained by the 1000 Genomes project for the "CHB/JPT" and "CEU" populations, respectively.

Only *GNB1L* SNPs with r^2 >0.649 with respect to rs2269726 associated with schizophrenia and/or bipolar disorder in Chinese[13] or European-based[14] case-control studies (Fig. 4). Because neither of these studies genotyped rs11704083 or any SNPs linked to it, we do not know its possible contribution to the risk of schizophrenia/bipolar disorder. Taken together, these observations strongly support the hypothesis that one or more regulatory variants that are tightly linked to rs2269726 contribute to schizophrenia and bipolar disorder.

Contrary to our expectations, the rs2269726 high-expression *C*-allele, rather than the low-expression *T*-allele, was identified as the schizophrenia risk allele in the Han Chinese case-control studies. The high-expression alleles of the rs2269726-linked SNPs rs5748427 (*G*), rs5748432 (*G*), and rs748806 (*T*) were also identified as schizophrenia risk alleles in the Han Chinese case-control study (Table 2, Fig. S3).

DISCUSSION

The results of this study confirm and extend the finding of Li and colleagues[13], who showed that *GNB1L* 3'-region SNPs associate with schizophrenia and bipolar disorder in the Han Chinese population. Specifically, we showed that each of the SNPs that correlated with schizophrenia and/or bipolar disorder in the study of Li and colleagues also correlates with *GNB1L* mRNA expression in both Han Chinese temporal cortex and Caucasian temporal and prefrontal cortex.

Because hemizygous deletion of a 1.5 Mbp segment of 22q11.2 containing *GNB1L* is strongly associated with schizophrenia and mood disorders, we had hypothesized that low expression of *GNB1L* mRNA in brain would also associate with schizophrenia and bipolar disorder. Surprisingly, however, re-analysis of the results of the Li *et al*. case-control association data in light of our mRNA

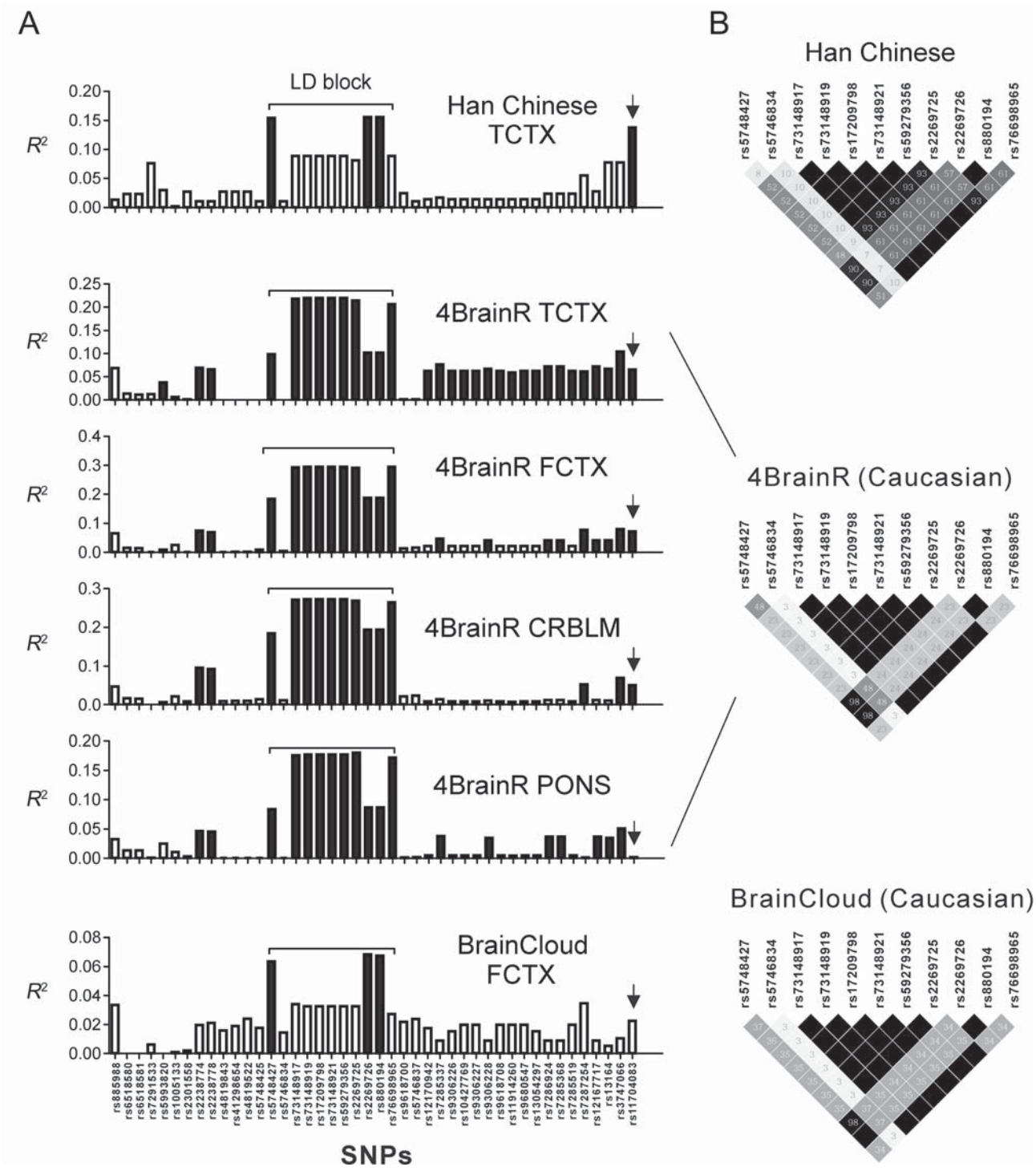

Fig. 3. SNPs located in the 3'-region LD block consistently correlate with *GNB1L* expression in Han Chinese and Caucasian brain eQTL studies. (A) Linear regression R^2 values for Han Chinese, "BrainCloud"[23], and "Four Brain Region"[24]. Solid bars indicate SNPs with nominally significant correlations with *GNB1L* mRNA expression. (B) Structure of 3'-region LD block generated using SNP genotypes for individuals in the indicated studies. Numbers within the small boxes within each plot are pairwise LD (r^2) values. TCTX, temporal cortex; FCTX, prefrontal cortex; CRBLM, cerebellum.

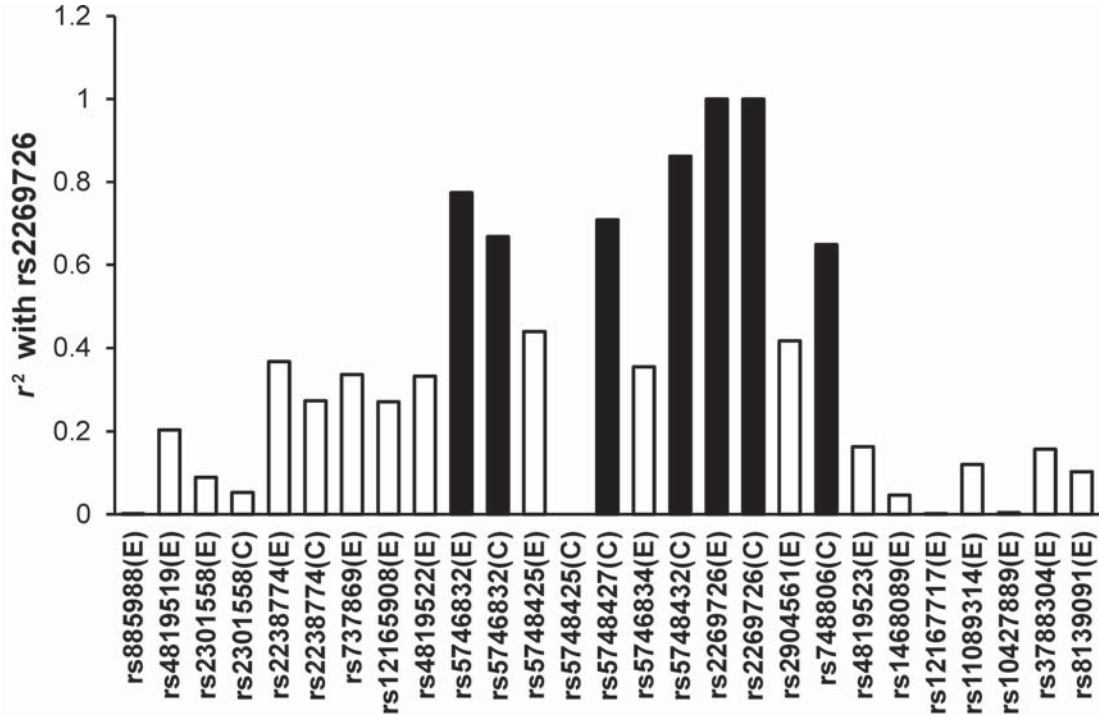

Fig. 4. Degree of linkage to rs2269726 predicts association with schizophrenia and/or bipolar disorder in Chinese and European samples for *GNB1L* SNPs. Values of LD constants (r^2) between rs2269726 and the listed SNPs estimated from data provided from the 1000 Genomes Project for CHB/JPT (C) or CEU (E) populations were obtained using the SNAP proxy SNP search program (Broad Institute, Cambridge, MA). SNPs that associate with schizophrenia are plotted as solid bars.

Table 2. Correspondence of the high-expression and risk alleles of *GNB1L* SNPs

Studies	Population	Tissue/Disease	Alleles	SNP				
				rs5746832	rs5748427	rs5748432	rs2269726	rs748806
This study	Han Chinese	Temporal cortex	High-expression	-	G	G	C	T
4BrainR [24]	Caucasian	Temporal cortex	alleles	A	G	G	C	T
	Caucasian	Prefrontal cortex		A	G	G	C	T
Braincloud [23]	Caucasian	Prefrontal cortex		A	G	-	C	-
Li *et al*.[13]	Han Chinese	SCZ	Risk alleles	-	G	G	C	T
	Han Chinese	BP		A	G	A	C	T

(-), not determined; 4BrainR, "Four Brain Region"; SCZ, schizophrenia; BP, bipolar disorder.

expression results implies that the *GNB1L* high-expression allele is the risk variant for these disorders in the Han Chinese population. This is the first report that high expression of a gene in the 22q11.2 DS critical region is associated with schizophrenia.

As noted above, an earlier study by Williams and colleagues[14] identified two SNPs in the 3'-region of *GNB1L*, rs5746832 and rs2269726, that associated with schizophrenia in two European population-based case-control studies (UK and Germany). The details of these associations differ from that reported for the Chinese population, in that associations were not found at the

allele-level, but rather as an excess of homozygosity for the above SNPs in males only. Based on our expression results, an association of homozygosity with schizophrenia can be interpreted as association with either high- or low-expression.

It should be mentioned that in the study by Williams and colleagues, both rs5746832 and rs2269726 were found to deviate significantly from Hardy-Weinberg equilibrium among male cases, but not male controls or female cases or controls. No evidence was found for duplicate samples or closely-related individuals among the male schizophrenia cases, suggesting that the excess homozygosity is related to the schizophrenia phenotype.

By contrast, no significant deviation from Hardy-Weinberg equilibrium or association with schizophrenia was found for rs5746832 or rs2269726 in a third European population (Bulgaria)-based case-control study. We also failed to find a statistically significant association between alleles or homozygous/heterozygous genotypes for rs2269726 in a large USA-based Caucasian case-control study (1189 cases/949 controls)[30] (data not shown). The interpretation of these divergent results is difficult, but we suggest that the contribution of *GNB1L* to schizophrenia may be stronger in specific populations.

In this study, we also identified a *GNB1L* 5'-region SNP, rs11704083, that correlated with *GNB1L* expression in 36 Han Chinese samples. This result, however, was not replicated in the two Caucasian population-based brain expression studies, "Braincloud"[23] and "Four Brain Regions"[24]. Although these results suggested that rs11704083 is a Han Chinese-specific eQTL, we are inclined to consider rs11704083 to be a false-positive correlation, since other SNPs within the 5'-region LD block did not show a significant correlation with *GNB1L* mRNA expression (see Fig. 1). By contrast, the finding that correlations between mRNA expression and genotypes were replicated in four independent studies strongly supports the conclusion that the 3'-region *GNB1L* SNPs are true eQTLs for this gene.

The fact that the same cluster of *GNB1L* 3'-region SNPs correlated with *GNB1L* mRNA expression in four different brain regions (Fig. 3), with the same alleles identified as the high-expression alleles in each region (Fig. S3), suggests that anterior temporal cortex is a suitable surrogate for other brain regions, such as the dorsolateral prefrontal cortex, which are more directly implicated in the etiology of schizophrenia.

In summary, we used measurements of mRNA expression in temporal cortex and linkage disequilibrium analysis to show that *GNB1L* is regulated by at least one *cis*-acting variant located within the 3'-region of the gene. In addition, we found that SNPs previously shown to associate with schizophrenia in Chinese and European case-control studies all share the property of being strongly linked to rs2269726. Finally, the high-expression allele of each of these SNPs was identified as the risk allele for schizophrenia and bipolar disorder in the Chinese population.

SUPPLEMENTAL DATA

Supplemental data include three tables and three figures, and can be found online at http://www.neurosci.cn/epData.asp?id=185.

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·Original Article·

Efficacy of Williams LifeSkills Training in improving psychological health of Chinese male juvenile violent offenders: a randomized controlled study

Simei Zhang, Hong Wang, Chen Chen, Jiansong Zhou, Xiaoping Wang
Mental Health Institute of the Second Xiangya Hospital, Central South University, Changsha 410011, China
Corresponding author: Xiaoping Wang. E-mail: xiaop6@126.com

ABSTRACT

This randomized controlled study was conducted to evaluate the efficacy of Williams LifeSkills Training (WLST) as a means of improving the psychological health of Chinese male juvenile violent offenders. Sixty-six participants were assigned randomly to receive the usual intervention plus 8 weeks of WLST (study group, n = 33) or only the usual intervention (control group, n = 33). We found that the study group exhibited significantly decreased State-Trait Anxiety Inventory (STAI X-1, X-2) STAX2 scores and Trait Coping Style Questionnaire (TCSQ) negative scores, and increased Interpersonal Support Evaluation List (ISEL) tangible scores from baseline to 9 weeks later (P <0.01). In addition, a between-group difference in changes of TCSQ negative score was observed at the end of week 9 (P <0.05). These findings suggest that WLST can improve trait anxiety, coping style, and interpersonal support in male Chinese juvenile violent offenders.

Keywords: violent offenders; juvenile; Williams LifeSkills Training; psychological health

INTRODUCTION

Male juvenile offenders are considered to have significant mental health concerns warranting attention. Studies have confirmed higher levels of problems such as depression, anxiety, bipolar disorder, dysthymia, and cyclothymia among young incarcerated male offenders than in the general population[1-4]. Not surprisingly, given their disturbed mood, suicide is a great risk in incarcerated youth and some data suggested that the prevalence of completed suicide is 2–4 times that among youth in the community[5]. Mental health problems in this population are associated with further offending and these problems continue into adulthood, resulting in failure to mature into reasonably healthy and well-functioning adults[6,7]. Psychological researchers have found similar problems and sequelae among incarcerated young people in China who suffer from more serious mental health problems, such as anxiety and depression[8].

Without effective interventions, young offenders' mental health symptoms and disorders are likely to worsen, resulting in an increased risk of recidivism. It is therefore clear that more effective strategies are needed to improve mental health care in this high-risk population. Researchers and experts have long-standing concerns regarding the provision of mental health interventions for incarcerated youth[9]. Most of the existing studies focus on families, schools, and community-based treatments, and find varying levels of effectiveness. Cognitive behavioral therapy, parent training, family therapy, and multi-systemic therapy have shown promise for achieving significant changes over the course of treatment[9]. In particular, cognitive behavioral therapy is generally considered to be the most effective intervention for improving mental health among offenders[10].

Williams LifeSkills Training (WLST)[11] is a highly-structured program for cognitive behavioral coping skills,

and has been used in carefully-conducted, randomized, controlled trials to reduce blood pressure, hostility, anger, depression, and anxiety, and to increase positive affect and satisfaction with social support in patients with coronary heart disease, stressed people in the community, and Alzheimer's disease family caregivers[12-15]. WLST also reduces hostility and maladaptive clinical decision-making in U.S. medical students[16] and overt aggression, hostility, and impulsivity in young Chinese male violent offenders[17]. Directly relevant to the current study, we recently reported that WLST significantly improves anxiety, depression, negative coping, social support, and self-esteem in a randomized controlled trial among Chinese medical students[18].

Few studies have estimated the effectiveness of intervention programs and mental health services among juvenile violent offenders. The prior dominant research in these young people has depended largely on investigating the psychological health and trends in youth violence. Based on the need for effective interventions among young violent offenders and the evidence-base of the WLST program, the goal of the present study was to evaluate its effectiveness in improving the mental health of incarcerated male offenders in China.

PARTICIPANTS AND METHODS

Participants

To evaluate the efficacy of WLST on psychological health and overt aggressive behavior (results reported in[17]), male juvenile violent offenders (14 to 24 years of age) from Hunan Reform School in Changsha, China, from March 2010 to March 2011, were enrolled in this study. Written informed consent was given by themselves or by their guardians. Recruitment occurred through participants' self-referral and prison guard referral. Educational lectures were held in a schoolroom of the reformatory to inform offenders about the purpose of our study. After gaining a good understanding of the purpose and content of the study, those who were willing to cooperate and complete the questionnaires were enrolled voluntarily and signed the informed consent forms. To reduce other potential confounders, participants suffering from neurological disease, serious somatic diseases (such as heart diseases, hepatic or renal diseases, or diabetes), infectious disease, and audio-visual impairments were excluded. Those who had intellectual disability, learning disorders, pervasive developmental disorders, attention-deficit/hyperactivity disorder, schizophrenia, major depression, bipolar disorder, or personality disorder were excluded as well. Participants did not receive any mental health service before this study.

Participant Protection and Ethical Approval

The current study was approved by the Ethics Committee of the Second Xiangya Hospital, Central South University in Changsha, and was performed in accordance with the Guidelines for Good Clinical Practice and the Declaration of Helsinki.

We evaluated all participants regarding their ability to independently sign informed consent. According to the General Principles of the Civil Law of the People's Republic of China, a citizen aged 18 or above is considered an adult who has full capacity for civil conduct, and may independently engage in any civil activities including deciding whether or not to participate in the present study. A citizen aged 16 to 18 and whose main income is his own labor is also regarded as a person with full capacity for civil conduct[18]. If a citizen was younger than 16 without full capacity for civil conduct, we contacted the guardian to obtain written informed consent.

In the current study, all potential participants aged 16–18 were found to be able to maintain their life by labor equal to citizens of the same age in society, and hence were qualified to have full capacity for civil conduct and provided written informed consent themselves. We gave particular consideration to informing participants of their rights and attended closely to issues of confidentiality.

Procedures

A randomized controlled study was performed. First, we collected demographic information of all participants with a self-administered questionnaire and assessed their baseline psychological health before the WLST. Then, they were computer-randomized in an equal ratio to the study group or the control group.

Participants in the study group received the usual intervention from the reform school plus 8 weeks of WLST, while participants in the control group received only the

usual intervention from the reform school (without WLST or any other cognitive-behavioral training) during the same period. After the 8-week training, all participants continued to receive the usual intervention for another week, and then received a final assessment at the end of week 9.

Usual Intervention

All participants continued to be treated by their officers in the reform school in the usual intervention with no cognitive-behavioral training, usually single educational training such as academic courses, and legal and health education.

WLST

WLST includes 10 core skill modules: (1) Being Aware (i.e. increasing awareness of and objectivity in distressing situations), (2) Making a Decision (evaluating one's reactions to those situations to decide whether to try to change one's reactions or to take actions to try to change the situations), (3) Getting Over It (changing one's reaction to distressing situations), (4) Problem Solving (using assertion to get others to change their behavior), (5) Assertion (problem solving to change distressing situations), (6) Saying "No" (saying No to reduce exposure to distressing situations), (7) Speaking Up (speaking clearly so others really listen), (8) Listening (listening skills to make sure you hear what others are saying), (9) Empathy (empathizing to increase understanding of others' behavior), and (10) Increasing Positives (increasing the positives in your interactions with others). In this study, WLST was delivered over the course of 8 weeks, with one 2-h session each week. The training included explaining the content, watching videos, role-playing, conducting group practice exercises, discussion, and assigned homework.

To ensure effective training, we divided the participants into small groups, each with 6–10 persons. All the sessions were conducted by the participants themselves under the supervision of two psychiatry post-graduates who had been trained by Dr. Virginia Williams and certified as medical practitioners qualified to deliver WLST in China. During the training of WLST facilitators at Central South University, adaptations were made to make the training consistent with Chinese culture and values. With a relatively fixed theme and content, each training session focused on one or two of the 10 core skills. The training theme and content were adjusted in the light of log entries regarding actual distressing situations reported by the participants in the training sessions.

Measures

Self-Administered Questionnaire

A self-administered questionnaire was used to obtain data for demographic information regarding age, years of education, childhood surroundings (city, small town, countryside), family income (high, >5000 RMB; medium, 2000–5000 RMB; low, <2000 RMB), physical health (very good, never or seldom get sick; good, sometimes get sick but not serious; not good, often ill or hospitalized), and major frustration (yes or no).

Psychosocial Outcome Measures

All participants were asked to complete a set of survey instruments both at baseline (before WLST) and at the end of week 9. The survey included the following questionnaires to assess psychosocial health.

Anxiety was assessed with the State-Trait Anxiety Inventory, which consists of 20-item questionnaires used to assess state anxiety (STAX1) and trait anxiety (STAX2). This inventory has been validated in a large number of studies of various populations. Scores range from 20 to 80, with higher scores indicating higher levels of anxiety[19].

Depressed mood was assessed by the Zung Self-rating Depression Scale (SDS), which comprehensively and accurately reflects the symptoms of depression and their severity. Scores range from 20 to 80, with higher scores indicating higher levels of depression[20].

Social support was assessed with Cohen's Interpersonal Support Evaluation List (ISEL)[21] which was translated into Chinese by the authors of the current study.

Coping style was measured with the Trait Coping Style Questionnaire (TCSQ) designed by Dr. Qian-Jin Jiang, and the results are presented as positive and negative coping scores[22].

The primary outcome of this study was difference between the two groups in changes of the total scores on all these scales from baseline to week 9, and the secondary outcome was changes in these scores within the two groups.

Statistical Analysis

SPSS 19.0 (SPSS Inc., Chicago, IL) was used for statistical analysis. Categorical variables are described using frequencies and percentages, whereas continuous variables are presented as mean and standard deviation (SD). Baseline between-group comparisons were performed using the *t*-test for continuous variables, and Pearson's χ^2 test for categorical variables.

Between-group comparisons of changes in STAX1, STAX2, SDS, TCSQ, and ISEL scores from baseline to the end of week 9 were performed using analysis of covariance (ANCOVA) with baseline values as a covariate. Inter-group changes in these scores from baseline to the end of week 9 were tested using repeated measures analysis of variance (ANOVA). The significance level was defined as P <0.05 (2-tailed).

RESULTS

Characteristics of Participants

Eighty-one male juvenile violent offenders were deemed eligible to participate in this study, but 14 refused to participate and one provided invalid data. The remaining 66 participants were assigned randomly to the study group (n = 33) and the control group (n = 33). During the intervention, two participants in the study group were withdrawn for missing 2 sessions and providing invalid data, respectively. At the end of week 9, 2 persons in the control group provided invalid data and were excluded from data analysis. Therefore, 31 participants were included in each group for final data analysis (Fig. 1).

All the participants were aged 16 to 23 years, being jailed when younger than 18. Among them, 14 (21.2%)

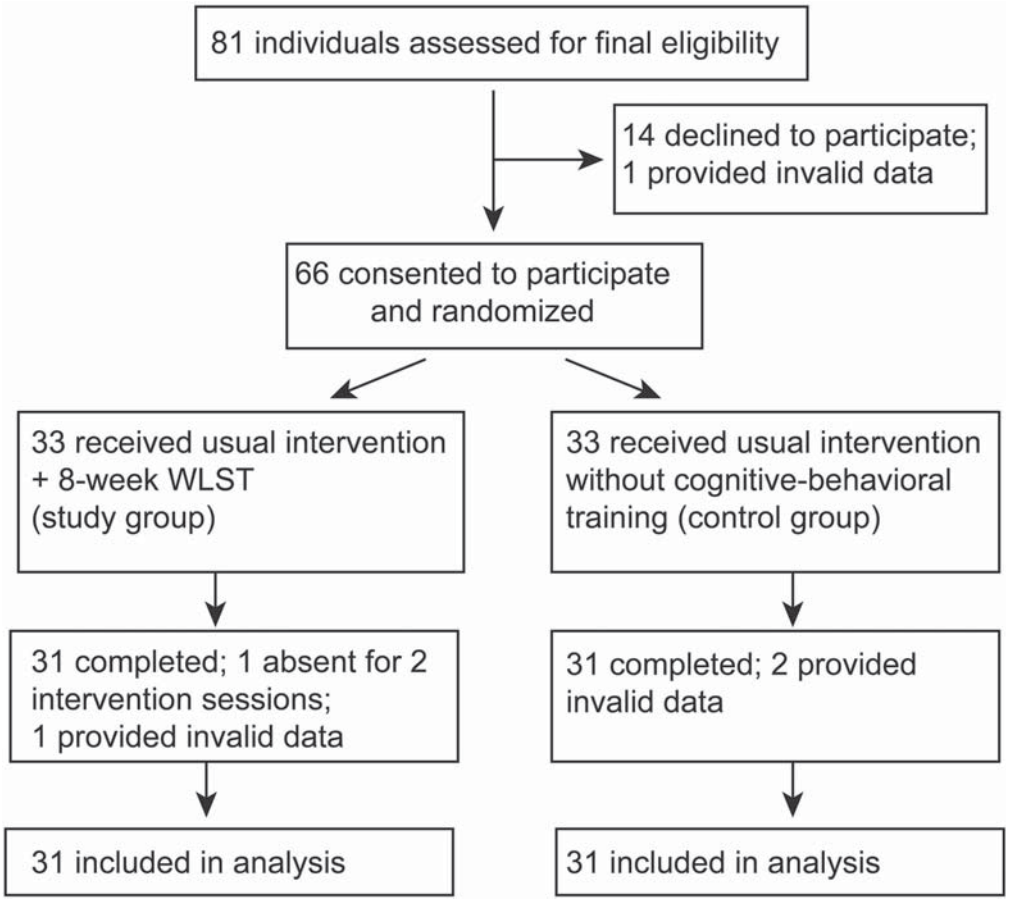

Fig. 1. Details of participant inclusion and exclusion.

came from cities, 20 (30.3%) from small towns, and 32 (48.5%) from rural areas. Their family incomes were <5000 RMB per month, and half had suffered major frustration. There were no significant differences in baseline characteristics between the two groups (Table 1).

Anxiety and Depression

To assess the effectiveness of WLST in treating anxiety and depression in young male violent offenders, we compared the mean scores at baseline and at the end of week 9. In the study group, there was a significant decrease in STAX2 [(F(1,30) = 17.663, P <0.001)]. In the control group, no significant difference was found. Further ANCOVA showed no significant between-group differences in changes of any of the anxiety or depression scores at the end of week 9 after controlling for baseline values. Between-group differences in changes of SDS scores were close to statistical significance (P = 0.057), with the WLST group decreasing more than the control group (Table 2).

Coping Style

The study group showed a significant decrease in TCSQ negative score at the end of week 9, compared with baseline [F(1,30) = 8.837, P = 0.006]. In the control group, no statistical difference was found. ANCOVA showed significant between-group differences in changes of TCSQ negative scores at the end of week 9 after controlling for baseline values (P = 0.031). In addition, there was no significant within-group or between-group difference in changes of the TCSQ positive score (Table 2).

Social Support

In the study group, there was a significant increase in ISEL tangible score at the end of week 9, compared with baseline [F(1,30) = 7.482, P = 0.010]. In the control group, no statistical difference was found. Further ANCOVA showed no significant between-group difference in changes of ISEL total and any subscale scores at the end of week 9 after controlling for baseline values (Table 2).

Table 1. Baseline demographics of participants

	Study group (n=33)	Control group (n=33)	T/χ^2	P
Demographic				
Age (years)	18.94 (1.87)	18.94 (0.86)	<0.001	1.000
Education (years)	7.67 (1.36)	7.32 (1.62)	0.946	0.348
Growth surroundings				
City	6 (18.2%)	8 (24.2%)	2.211[a]	0.331
Small Town	8 (24.2%)	12 (36.4%)		
Countryside	19 (57.6%)	13 (39.4%)		
Family Income				
High	0 (0.0%)	0 (0.0%)	0.363[a]	0.547
Medium	25 (75.8%)	27 (81.8%)		
Low	8 (24.2%)	6 (18.2%)		
Physical Health Status				
Very good	15 (45.5%)	10 (30.3%)	1.676[a]	0.433
Good	16 (48.5%)	21 (63.6%)		
Not good	2 (6.1%)	2 (6.1%)		
Suffered Major Frustration	18 (54.5%)	15 (45.5%)	0.545[a]	0.460
Sexual Activity	22 (66.7%)	18 (54.5%)	1.015[a]	0.314
Drug Abuse	7 (21.2%)	6 (18.2%)	0.096[a]	0.757

Data are expressed as mean (SD) or n (%). [a]Pearson's χ^2 test.

Table 2. Psychological outcome measures from participants before and after 9 weeks

Weighted score	Baseline mean (SD)	At the end of week 9 mean (SD)	Score change, mean (SD)[a]	Between-group difference in change score[b]	
				F	*P* value
STAX1					
Study group	43.19 (6.56)	41.74 (7.22)	-1.45 (6.07)	0.686	0.411
Control group	45.65 (4.86)	44.42 (7.88)	-1.23 (9.04)		
STAX2					
Study group	43.97 (5.76)	39.58 (5.85)	-4.39 (5.81)**	2.461	0.122
Control group	44.00 (3.82)	42.42 (8.24)	-1.58 (9.63)		
SDS					
Study group	43.19 (6.43)	41.77 (6.03)	-1.42 (6.30)	3.778	0.057
Control group	45.61 (6.06)	45.29 (4.99)	-0.32 (5.18)		
TCSQ positive					
Study group	30.90 (5.23)	31.35 (6.05)	0.45 (7.33)	1.239	0.270
Control group	30.45 (5.16)	29.71 (5.01)	-0.74 (3.92)		
TCSQ negative					
Study group	30.84 (8.00)	26.35 (6.47)	-4.48 (8.40)**	4.854	0.031
Control group	29.81 (4.89)	28.94 (5.01)	-0.87 (4.36)		
ISEL Tangible					
Study group	5.97 (1.47)	6.77 (1.28)	0.81 (1.64)**	0.163	0.688
Control group	6.39 (1.45)	6.71 (1.75)	0.32 (2.10)		
ISEL Belonging					
Study group	7.68 (2.36)	7.61 (1.52)	-0.06 (2.08)	0.026	0.872
Control group	6.77 (2.09)	7.23 (1.87)	0.45 (2.08)		
ISEL Appraisal					
Study group	6.84 (2.27)	6.68 (2.47)	-0.16 (2.41)	0.085	0.772
Control group	6.45 (1.95)	6.35 (1.76)	-0.10 (2.01)		
ISEL Self Esteem					
Study group	7.16 (1.90)	7.13 (1.78)	-0.03 (2.29)	1.132	0.292
Control group	6.58 (1.61)	6.48 (1.63)	-0.10 (1.56)		
ISEL Total					
Study group	27.65 (5.65)	28.19 (5.64)	0.55 (0.90)	0.257	0.614
Control group	26.19 (4.83)	26.77 (4.53)	0.58 (3.83)		

[a]Within-group differences analyzed using repeated ANOVA; ** P <0.01. [b]Between-group differences in change scores at the end of week 9 analyzed using ANCOVA controlling for baseline values.

DISCUSSION

In this 9-week study, we found that WLST significantly improved trait anxiety, negative coping skills, and tangible social support, with a close-to-significant trend toward a larger decrease in depression, among male juvenile violent

offenders. These effects are similar to those documented in prior clinical trials of WLST in Western populations[9-16] as well as in meta-analysis of interventions relevant to young offenders with mood disorders, anxiety disorders, or self-harm tendencies[23]. These findings reinforce our similar previous findings that WLST improves psychosocial health in Chinese medical students[18], as well as our prior study in this same incarcerated population of young male offenders in which WLST produced significant reductions in overt aggressive tendencies, hostility, and impulsivity[17].

It is likely that WLST produces these improvements because it trains participants in skills that not only help them to be more aware of negative thoughts and feelings in distressing situations, but also teaches them how to evaluate those thoughts and feelings to determine whether they need to take action to change the situation or to change their thoughts and feelings and how to undertake these actions. In addition, WLST enabled participants to improve their coping styles, resulting in positive problem-solving attitudes and skills with the potential to promote improved coping performance. Previous research has shown that a positive coping style reduces depression[24] and risk for behavioral problems among Chinese adolescents[25].

These findings suggest that WLST helped the participants to be aware of feelings early, gain control of bad feelings, and make smart choices about when to act, solve problems creatively, and get more enjoyment and meaning from life. During the WLST intervention, participants learned to use the 10 skills to deal with negative feelings and thoughts. They were able to understand their mood and the consequences, in turn, were to understand and change their current state, thus relieving negative mood and negative coping style.

Last but not least, we found that WLST was effective in enhancing tangible social support. This is likely to result from in-depth training in speaking, listening, and empathy skills that can help build supportive relations with and understand the behavior of others. Taken altogether, these improved skills in handling distressing situations and improving social relationships have the potential to significantly reduce recidivism rates, as well as help solve problems encountered in life.

There are some limitations in our study. First, the improvements reported here were at a single time point following a very short follow-up period. Future studies are suggested to extend the follow-up period to document not only maintenance of improvements in psychosocial factors but also reduced recidivism in this population at high risk for future offences. Second, this study had a relatively small sample size and the findings are preliminary. While previous studies evaluating WLST have found significant improvement in psychological health, these results need to be confirmed in a larger sample size. In addition, it will also be important to evaluate WLST effects in different types of offenders at different ages and different populations.

In conclusion, we revealed that WLST reduced anxiety and negative coping style and increased tangible interpersonal support, all of which are important aspects of psychosocial well-being. If the current findings are confirmed and extended in ongoing research, WLST could be implemented more broadly to improve mental health and reduce recidivism among juvenile offenders in China and elsewhere.

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·Review·

New hypothesis and treatment targets of depression: an integrated view of key findings

Shangli Cai, Shucai Huang, Wei Hao

Mental Health Institute and Key Laboratory of Psychiatry of Hunan Province, The Second Xiangya Hospital, Central South University, Changsha 410011, China

Corresponding author: Wei Hao. E-mail: weihao57@gmail.com

Major depressive disorder (MDD) is a common and devastating psychiatric disorder characterized by persistent low mood, cognitive disorder, and impaired social function. Despite its complex mechanisms, increasing evidence has identified the involvement of neurotrophic factors, inflammatory cytokines, the hypothalamus-pituitary-adrenal axis, and glutamate receptors in the pathophysiology of this illness. The present review synthesizes recent research achievements to define the network between different hypotheses of MDD and to understand which part is most pivotal for its pathogenesis. By integrating MDD-related signal pathways, we highlight brain-derived neurotrophic factor (BDNF) dysfunction and increased apoptosis as the final common cascades, and new therapeutic strategies aiming to enhance BDNF function have been shown to exert a rapid and effective antidepressant action.

Keywords: depression; BDNF; cytokines; hypothalamus-pituitary-adrenal axis; glutamate receptor

Introduction

Major depressive disorder (MDD) is a mental disorder characterized by prominent and persistent low mood, mental retardation, cognitive impairment, volitional decline, and somatic symptoms. MDD, which has a significantly high recurrence rate, can reduce the capacity of a patient to study, work, and engage in social skills, as well as increase the disability rate and suicide risk[1]. According to the statistics of the World Health Organization, there are 300 million patients with MDD[2]. It is estimated that by 2020 the disease burden caused by MDD will be ranked next to ischemic heart disease, becoming the second most common cause of disability and death[3].

Currently, the understanding of depression is mainly based on the monoamine-deficiency hypothesis, which proposes that the occurrence of depression is associated with deficiencies of three major monoamine transmitters, 5-hydroxytryptamine (5-HT), norepinephrine (NE), and dopamine (DA). By inhibiting their transporters, antidepressants block their reuptake, thereby increasing the transmitter concentration in the synaptic cleft and relieving the symptoms of depression.

However, the monoamine-deficiency hypothesis is being seriously challenged[4]. First, antidepressant treatment has an efficiency of only 60%–65% with a remission rate of ~30%[5, 6], while a high percentage of patients show no improvement, even after combination therapy with a variety of antidepressants. Second, although antidepressants rapidly increase the levels of monoamine neurotransmitters in the central nervous system (CNS) by blocking the transporters, it often takes two weeks or even longer for the onset of antidepressant efficacy. All this evidence indicates that monoamine-deficiency can only partly explain the pathogenesis of depression.

At the moment, large numbers of clinical and basic studies have provided new hypotheses for the pathogenesis of MDD. In this review, we begin with the classic monoamine hypothesis, and then review some new hypotheses of the mechanisms and therapeutic targets

in depression. We aim to present an integrated view of depression mechanisms and new thinking about the therapeutic strategies for the development of new drugs.

The Neurotrophic Factor Hypothesis and Related New Therapeutic Targets

Two major factors are related to the delayed efficacy of antidepressants. First, it takes two to three weeks for the adaptation of receptor sensitivity, such as the desensitization of presynaptic 5-HT_{1A} autoreceptors. So far, one of the main directions of antidepressant development is to inhibit the function of 5-HT_{1A} autoreceptors to facilitate their rapid desensitization[7, 8]. Second, the increased synthesis of cAMP response element-binding protein (CREB) and brain-derived neurotrophic factor (BDNF) often takes 2–3 weeks, which is coincident with the delayed onset of efficacy, suggesting that these are likely to be key mechanisms for the delayed efficacy of antidepressants[9]. More evidence has shown that decreased neurotrophic factors (NTFs), especially BDNF, and impaired synaptic plasticity may be the common pathways of depression[10].

NTFs are a class of small proteins with neurotrophic functions, and include nerve growth factor, BDNF, glial cell line-derived neurotrophic factor, insulin-like growth factor, and transfer growth factor[11]. The confirmed biological roles of NTFs include: maintaining neural survival in embryonic development and promoting differentiation, facilitating axonal growth, guiding nerve-growth direction, maintaining the survival of mature neurons, and accelerating neurogenesis[11].

Clinical and animal studies have shown reduced BDNF mRNA levels in the hippocampus of depressed animal models[12] and decreased levels of serum BDNF in untreated depressed patients[13]. Patients with depression often show atrophy or lack of neurons, particularly in the hippocampus and the cerebral cortex[14]. *In vivo* and *in vitro* animal experiments have shown increased BDNF levels in the limbic system and in plasma after long-term treatment with antidepressants[15]. Besides, administration of BDNF into the animal brain has antidepressant-like behavioral effects[16]. All these findings suggest that BDNF may be key in the treatment of depression. In fact, changes in the BDNF level have been widely used as a biomarker for depression. In addition, the BDNF Met allele is associated with an increased suicide risk in patients with depression[17, 18], especially in females as well as in early-onset[19] and elderly depression patients[20].

Therefore, NTFs are considered to be an important and new clue for understanding the pathogenesis of depression and the mechanisms of action of antidepressants[21]. In 2006, Duman *et al.*[22] proposed a neurotrophin hypothesis of depression, which claimed that NTFs promote synaptic growth and maintain neuronal survival, while their deficiency induces atrophy of brain structures and MDD. In addition, antidepressants exert their effect by enhancing the levels of NTFs in the brain, increasing synaptic plasticity, and promoting neuronal survival. As an important NTF, BDNF mainly acts on neurons in the hippocampus, cerebral cortex, cerebellum, and basal forebrain, which are associated with higher functions such as learning and memory. BDNF also promotes neural proliferation and differentiation and has an anti-apoptotic function, as well as regulating synaptic morphology, information transmission and plasticity, thereby improving the symptoms of depression[23, 24]. Interestingly, BDNF improves sleep architecture, especially slow-wave sleep, during antidepressant treatment, which reflects enhanced synaptic plasticity and the synchronization of neuronal circuits[25]. Decreased slow-wave sleep usually leads to reduced cognition and depressed emotion, which are commonly observed in depressed patients with sleep disorders[26, 27]. In addition, reduced synaptic plasticity and slow-wave sleep are commonly reported in populations carrying BDNF Val66Met polymorphisms, suggesting an association with functional defects of BDNF, and importantly, these patients are likely to be resistant to antidepressant treatments targeting BDNF[28, 29].

At present, the antidepressant effect of BDNF is not fully understood, so it is urgent to study the mechanisms of BDNF synthesis and release in order to develop new antidepressants. BDNF expression can be facilitated in two ways. One is to increase CREB-mediated BDNF expression, but it usually takes 2–3 weeks for the onset of antidepressant effects, which does not meet the demand for a rapid response. Another way is through direct action on membrane-binding receptors especially ion channel receptors, for example by N-methyl-*D*-aspartate receptor (NMDAR) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR) agonists. Drug

development based on this strategy is the major direction for future antidepressant agents. Moreover, the inhibition of factors that decrease BDNF expression and function can also indirectly maintain BDNF levels and function. Post-translational modification is an important procedure for BDNF maturation. After synthesis in the ribosomes, pro-BDNF is processed in the rough endoplasmic reticulum to become the mature and active form *via* a variety of proteins including furin, proconvertase, and P11-tPA-plasmin[30].

The Sigma-1 receptor (σ1R) is an important chaperone located at the endoplasmic reticulum-mitochondria junction and is involved in the maturation process of pro-BDNF[31] (Fig. 1). This association indicates that the σ1R may serve as a therapeutic target in the treatment of depression. In the brain, σ1Rs are mainly distributed in the dentate gyrus, thalamus, and hypothalamus; activation and up-regulation of this receptor facilitates neurogenesis and neural differentiation as well as having anti-apoptotic effects. Several studies have shown that activation of the σ1R enhances BDNF expression[32], while others claim that the agonists of this receptor actually promote the maturation

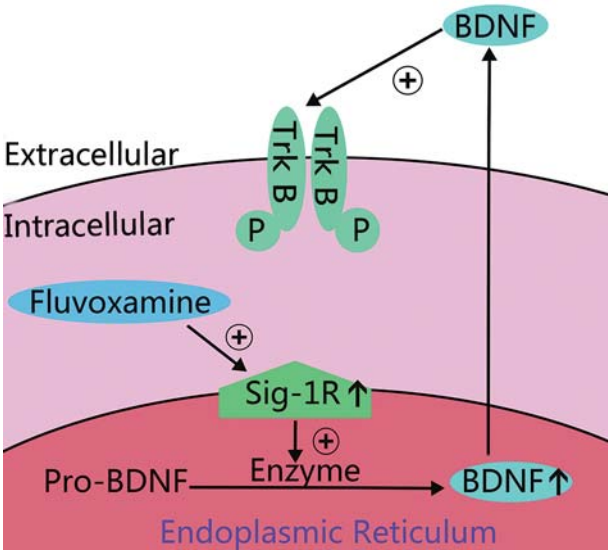

Fig. 1. Activation of the Sigma-1 receptor enhances the secretion of mature BDNF. The Sigma-1 receptor (Sig-1R) facilitates the maturation of pro-BDNF into BDNF and increases the secretion of mature BDNF, which could partially explain the fluvoxamine-mediated antidepressant effect. Increased BDNF secretion activates TrkB, which leads to enhancement of downstream signaling pathways.

of BDNF in the endoplasmic reticulum (i.e., the conversion of pro-BDNF to BDNF). Despite the increases of BDNF synthesis and secretion, its mRNA levels do not change. Moreover, an increase of mature BDNF is accompanied by decreased pro-BDNF levels after treatment with a σ1R agonist[33]. Clearly, the mechanism of BDNF upregulation by the σ1R is different from the antidepressant-mediated BDNF mRNA increase. In addition, the evidence below suggests that the σ1R is also involved in the pathogenesis of depressive disorders: (1) compared with healthy participants, the plasma level of the σ1R declines in patients with depression and increases after treatment with antidepressants[34]; (2) σ1R-knockout mice show a prolonged immobility time in the forced swimming test, an animal model of depression, indicating that deletion of σ1Rs exacerbates the severity of depression[35]; (3) administration of σ1R agonists reduces the immobility time in the forced swimming and tail-suspension tests in a dose-dependent manner, exhibiting a good antidepressant-like effect. In contrast, σ1R antagonists blocks its antidepressant-like effect[36]; and (4) the σ1R has a regulatory effect on monoamine neurotransmitters: σ1R agonists in rats up-regulate DA levels in the frontal cortex, increase the discharge of serotonergic neurons in the dorsal raphe nucleus, and enhance 5-HT release[37, 38]. σ1R agonists also enhance the antidepressant-like effects of NMDAR antagonists (amantadine and memantine) and other antidepressants such as fluvoxamine, venlafaxine, and buspirone[39].

In fact, many factors can affect BDNF synthesis, release, and function, such as changes in inflammatory cytokines and glutamate receptors, and hyperactivity of the hypothalamic-pituitary-adrenal (HPA) axis. All these have crucial regulatory effects on the expression and function of BDNF, and serve as new targets for antidepressants.

The Inflammatory Cytokine Hypothesis of Depression and Related New Therapeutic Targets

Cytokines are a class of signaling polypeptides secreted by the immune system, and are widely distributed in the immune and nervous systems. When the immune system responds to stressors such as disease, injury, infection, or psychosocial factors, cytokines are secreted to regulate body functions[40].

Much evidence indicates that changes in inflammatory cytokines are closely associated with the occurrence of depression, leading to the cytokine hypothesis of depression which proposes that depression is often associated with immune disorders, activation of the inflammatory response system, and elevated inflammatory cytokines under stress conditions. Under psychological stress or physical illness, the release of inflammatory cytokines can be dramatic, leading to significant increases in interleukin-1 (IL-1), IL-2, IL-6, tumor necrosis factor-α, and C-reactive protein[41-43]. Increased IL-1 and IL-2 can lead to apoptosis, attenuate neuronal differentiation, suppress synaptic transmission, and inhibit the induction and maintenance of long-term potentiation, which represents an impairment in learning, and finally result in MDD[44, 45]. The anti-inflammatory drug celecoxib, a specific inhibitor of COX-2, has a synergistic antidepressant effect. Combined treatment with celecoxib and antidepressant has shown an increased response rate and symptom remission rate compared with antidepressant alone[46] (Fig. 2).

Moreover, inflammatory cytokines are an important cause of glucocorticoid resistance, glutamate excitotoxicity, and reduced BDNF expression. Specifically, they block the functions of glucocorticoid receptors (GRs)[47], producing an effect similar to glucocorticoid resistance. Furthermore, by interfering with GR functions, inflammatory cytokines attenuate the negative feedback inhibition of glucocorticoid release mediated by GRs[47]; excessive secretion of glucocorticoids ultimately results in hyper-glucocorticoidemia, which reduces the BDNF levels in the

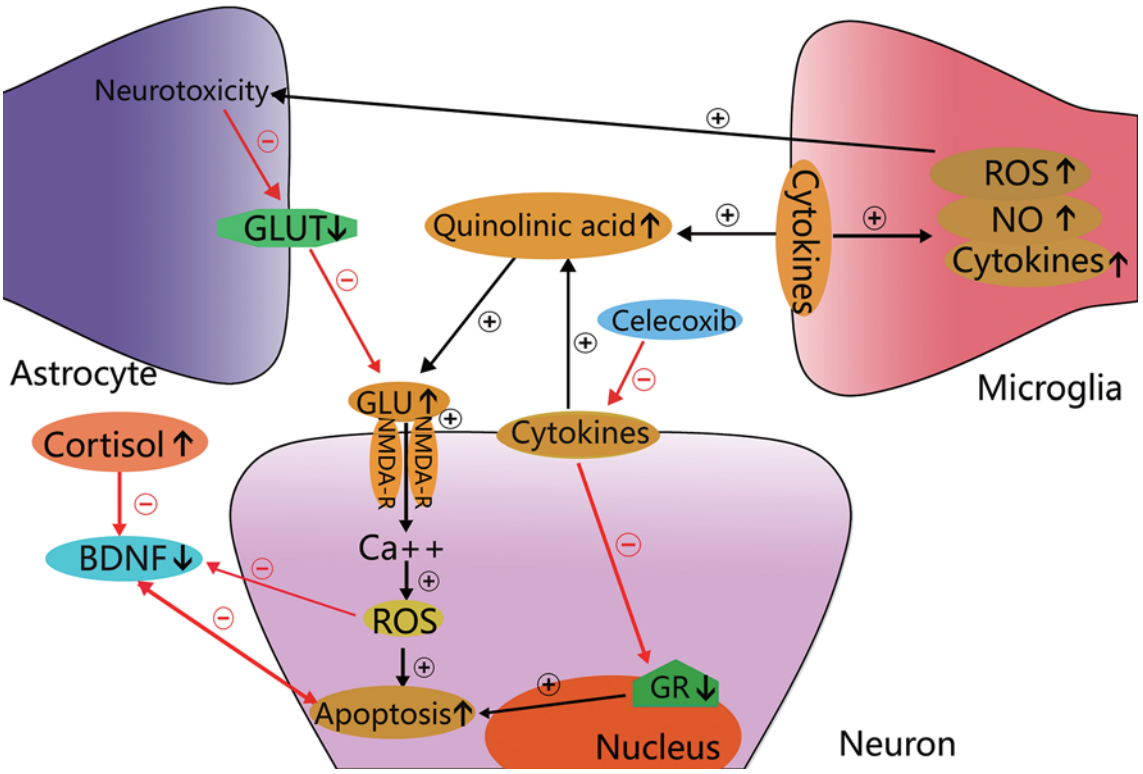

Fig. 2. Pro-inflammatory cytokines facilitate glucocorticoid resistance and glutamate excitotoxicity. The immune system activated by stressors releases excessive pro-inflammatory cytokines. The increased cytokines block glutamate receptors (GLUT), attenuating the negative feedback inhibition of glucocorticoid release. Excessive glucocorticoids ultimately reduce BDNF secretion, leading to apoptosis or degeneration in neurons. Specifically, activation of microglia and up-regulation of pro-inflammatory cytokines result in increased quinolinic acid, which contributes to excessive glutamate release into the synaptic cleft and less glutamate reuptake into astrocytes, ultimately leading to extrasynaptic NMDAR-mediated excitotoxicity. Celecoxib could exert adjunctive antidepressant effects by suppressing the pro-inflammatory cytokines.

brain, leading to apoptosis or degeneration of neurons[48]. Besides, the increased inflammatory cytokines in the CNS are closely associated with the elevation of glutamate as well as excitotoxicity mediated by NMDARs. When central microglial cells and inflammatory cytokines are activated, the activity of the tryptophan-degrading enzyme indoleamine 2,3-dioxygenase is enhanced, leading to reduced tryptophan, a precursor of 5-HT synthesis, and increased quinolinic acid synthesis[49]. Then the increased quinolinic acid enhances the release of glutamate, resulting in excessive glutamate in the synaptic cleft. Moreover, elevated quinolinic acid can directly activate extrasynaptic NMDARs, which are involved in excitotoxic injury. This scenario leads to the activation of extrasynaptic NMDARs and excitotoxicity characterized by Ca^{2+} overload[49]. Additional studies showed that the increases of inflammatory cytokines and glucocorticoids caused by stress lead to a reduction of central astrocytes, causing a decreased capacity of the glutamate transporters in astrocytes to transport intracellular glutamate and thus increasing extrasynaptic glutamate[50, 51]. Notably, the excitatory neurotoxicity caused by glutamate (mainly oxidative stress) can also lead to reduced BDNF expression, which is the core mechanism of the occurrence of depression[52, 53] (Fig. 2).

The Abnormal Glutamate Receptors Hypothesis and Related New Therapeutic Targets

Inotropic glutamate receptors, NMDARs and AMPARs, are closely associated with the occurrence of depression. Activation of these receptors permits the passage of cations such as Na^+ and Ca^{2+}. Moreover, there is a glycine-binding site at the outer membrane area of the NMDAR and a Mg^{2+}-binding site at the inner side of the channel. Under physiological conditions, the activation of AMPARs and NMDARs in the postsynaptic membrane (mainly NR2A receptor subtypes) is a crucial electrophysiological mechanism for synaptic plasticity as well as learning and memory. But under pathological conditions, particularly when stimulated by excessive glutamate, activation of extrasynaptic NMDARs (mainly NR2B receptor subtypes) results in a series of adverse events, including Ca^{2+} overload, oxidative stress injury, and apoptosis or degeneration[54]. In fact, recent research has revealed that the levels of AMPARs and NMDARs decrease in both depressed patients and depressive animal models after long-term stress, and this decline may be attributed to the over-activation of GRs and degradation of glutamate receptors[55, 56]. Decreased AMPARs in the postsynaptic membrane can result in the impairment of glutamate receptor-related cascades for cell survival and synaptogenesis and further induce MDD. All the above are key points of the abnormal glutamate receptors hypothesis of MDD.

Currently, the development of antidepressants is mainly focused on NMDAR antagonists. The key mechanisms of decreased BDNF induced by inflammatory cytokines are the quinolinic acid-mediated release of glutamate and the overactivation of extrasynaptic NMDARs. Therefore, blocking NMDARs to inhibit the over-activation of extrasynaptic NMDARs caused by glutamate and quinolinic acid is one of the important antidepressant mechanisms of NMDAR antagonists. More importantly, blocking NMDARs may enhance the activation of AMPARs *via* blocking NMDAR-mediated signal pathways (Fig. 3). This is because pretreatment with NBQX, an AMPAR antagonist, blocks the rapid antidepressant effects mediated by ketamine, an NMDAR antagonist[57], indicating that such antagonists are more likely to exert fast antidepressant effects by enhancing AMPAR signaling. But how does the activation of AMPARs lead to rapid antidepressant effects? Studies have shown that the fast antidepressant effects of NMDAR antagonists are dependent on the rapid synthesis of BDNF. Also, 4 h after treatment with ketamine for refractory depression, the plasma levels of BDNF rapidly and significantly increase; this is considered to be due to activation of the PI3K-AKT-mTOR pathway caused by AMPAR activation. Activation of mTOR deactivates eukaryotic elongation factor-2 kinase (eEF2K), which results in the activation of its substrate eEF2 and ultimately promotes the translation of BDNF[58, 59]. In addition, the increased BDNF can further act on mTOR through the TrkB-PI3K-AKT cascade to achieve positive feedback regulation of BDNF expression. In light of the importance of eEF2 in the facilitation of BDNF, inhibitors of eEF2 kinase are currently being developed, and preliminary results show that they have a rapid antidepressant effect by facilitating BDNF expression[60].

In addition to the PI3K-AKT-mTOR pathway, downstream pathways such as the PI3K-AKT-Wnt (GSK-3β-

catenin) pathway of TrkB and the BDNF receptor are also involved in the rapid antidepressant effects of ketamine. To exert an antidepressant effect, ketamine can both activate AKT and inhibit GSK-3β. Transgenic mice carrying a GSK-3 sustained-activation gene show complete resistance to the ketamine-mediated antidepressant effect[61]. The influence of GSK-3β activity on the efficiency of antidepressants is probably through its downstream molecule β-catenin. As a key signaling molecule in the Wnt pathway, β-catenin plays an important regulatory role in maintaining the genesis and proliferation of neurons as well as synaptic functions. AKT-mediated inhibition of GSK-3β reduces the phosphorylation levels of β-catenin and thereby blocks the degradation of phosphorylated β-catenin mediated by the ubiquitin-proteasome pathway, enabling a stable intracellular level of β-catenin that enters the nucleus to participate in the transcriptional activation of specific genes and the facilitation of neurogenesis. In addition, inhibition of the ubiquitin-proteasome degradation system is crucial for stabilizing the content of AMPARs, although this needs to be further confirmed. The latest research shows that the Wnt/β-catenin signaling pathway is crucial for the BDNF promotion of proliferation and differentiation of neural stem cells; treatment with IWR1, an inhibitor of the Wnt pathway, blocks the neuroprotective effects of BDNF[62].

Previous studies have shown that NMDAR antagonists have a promising effect on patients with treatment-resistant depression, but we need to be aware of their side-effects such as sedation and sensory gating disorders, especially the fact that NMDAR antagonists induce psychosis in healthy volunteers[63]. Furthermore, NMDAR antagonists including ketamine enhance the activity of acetylcholinesterase, thereby promoting the degradation of acetylcholine and leading to cognitive disorders[64]. Therefore, it will be very difficult to determine whether it is necessary to provide patients with long-term maintenance therapy of NMDAR antagonists.

Owing to the potential adverse effects of NMDAR antagonists, research has switched to studying NMDAR-related targets that can indirectly inhibit NMDARs. Two targets have received much attention: mGluR2/3 (metabotropic glutamate receptor 2/3 subtypes) and glycine binding sites. Currently, it has been confirmed that mGluR2/3 antagonists have antidepressant effects[65] that have many features in common with those of ketamine. This is because mGluR2/3 regulates glutamate release by negative feedback; hence, glutamate release in the synaptic cleft is largely enhanced after blocking mGluR2/3, and the increased glutamate further activates AMPARs and facilitates the activation of the downstream PI3K-AKT-mTOR pathway to exert antidepressant effects. Pretreatment with rapamycin (an mTOR inhibitor) or NBQX (an AMPAR antagonist) completely blocks the antidepressant effect of mGluR2/3 antagonists[66]. In addition, GLYX-13, a partial agonist of the NMDAR at glycine-binding sites, also has a strong antidepressant effect. If NMDARs are over-activated, GLYX-13 can exert its antidepressant effect mainly by blocking them; if NMDARs are insufficiently activated, it can enhance their function and facilitate long-term potentiation[67, 68].

The Hypothalamus-Pituitary-Adrenal Axis Hyperactivity Hypothesis and Related New Therapeutic Targets

As one of the important components of the neuroendocrine system, the HPA axis consists of three parts, the hypothalamic hypophysiotropic area, the pituitary, and the adrenal cortex. The hypothalamic hypophysiotropic area contains neurons that synthesize and release corticotropin-releasing hormone (CRH). The pituitary synthesizes and releases adrenocorticotropic hormone (ACTH), and the adrenal cortex is mainly responsible for the synthesis and release of glucocorticoids (mainly cortisol).

The HPA axis hyperactivity hypothesis is based on the postulate that enhancement of HPA axis activity is a key mechanism for depression when the body is exposed to stressors. The increased secretion of CRH, ACTH, and glucocorticoids has been reported in the cerebrospinal fluid of patients with depression[69]. High concentrations of glucocorticoids can have long-term adverse effects, which include: (1) imbalance of negative feedback in the HPA axis, including downregulation of negative feedback and dysfunction of GRs, disinhibition in the dexamethasone suppression test, and high concentrations of glucocorticoids in the blood; (2) excessive activation of GRs in its target cells in the CNS leads to neuronal apoptosis and degeneration[48] which is explained by the attenuation of BDNF expression and proliferation[70, 71]. In

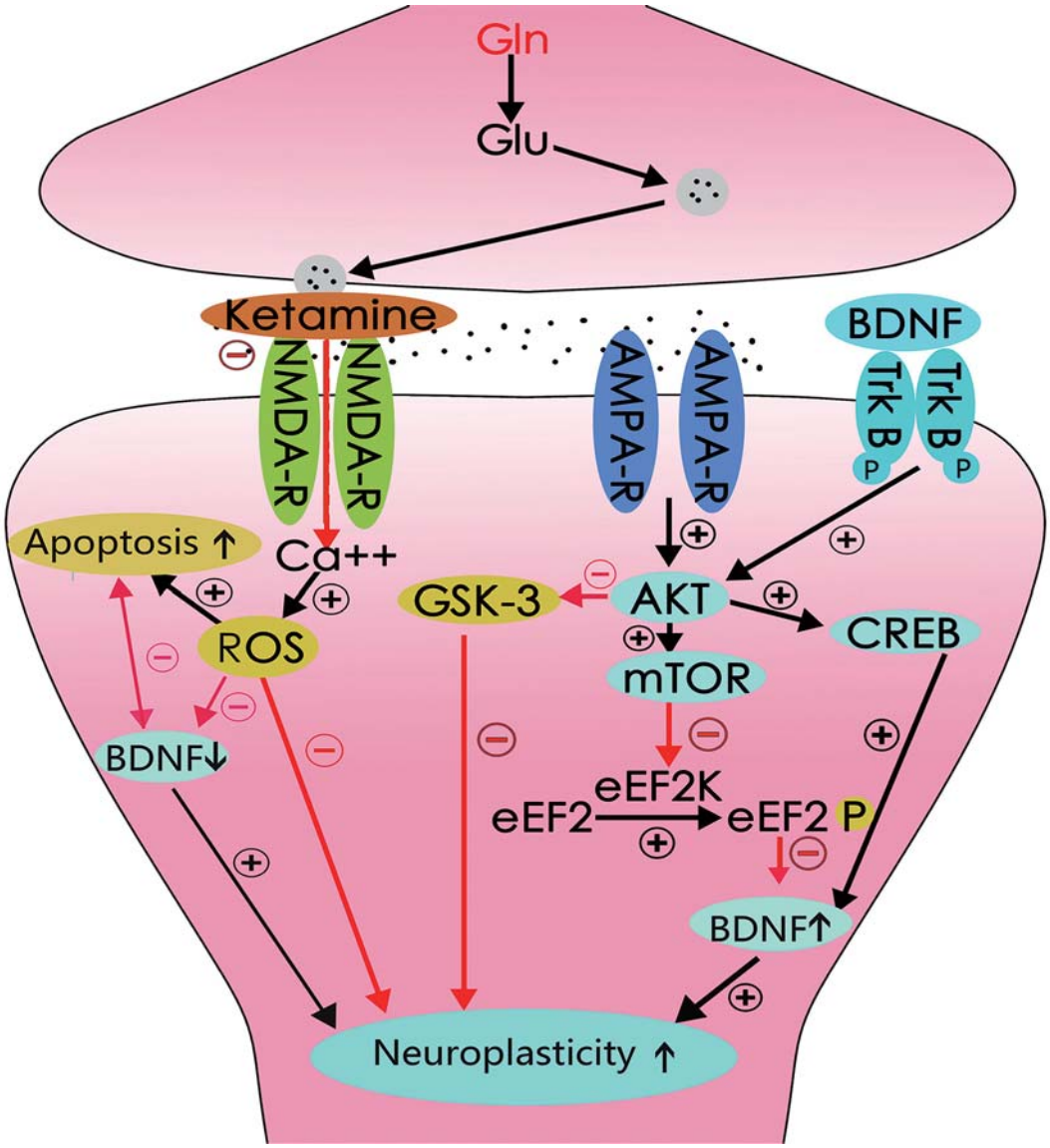

Fig. 3. Abnormal glutamate receptor hypothesis of MDD and mechanisms underlying the rapid antidepressant effects of NMDA receptor (NMDAR) antagonists. NMDARs and AMPARs are closely associated with depression. Blocking NMDARs to inhibit the over-activation of extrasynaptic NMDARs caused by glutamate and quinolinic acid is one of the important antidepressant mechanisms of NMDAR antagonists. More importantly, blocking NMDARs may enhance the activation of AMPARs *via* blocking NMDAR-mediated signal pathways. Activation of AMPARs results in enhancement of the PI3K-AKT-mTOR cascade and leads to inactivation of eEF2K and increased eEF2 phosphorylation, which ultimately promotes the eEF2-dependent translation of BDNF. Increased BDNF further achieves positive feedback regulation of BDNF expression through the TrkB-PI3K-AKT-mTOR and TrkB-PI3K-AKT-CREB pathways. In addition, suppression of GSK3 mediated by AKT contributes to the antidepressant actions of ketamine.

addition, the increased glucocorticoid levels enhance the expression of 5-HT transporters in the hippocampus, the frontal cortex, the amygdala, the dorsal raphe nucleus, and other brain regions in a GR-dependent manner, resulting in reduced 5-HT in the synaptic cleft and aggravation of depressive symptoms[72]. Accordingly, the strategy for MDD treatment is either restoring the negative feedback in the HPA or blocking the over-activated GRs. GR antagonists have demonstrated potential therapeutic properties for mood disorders, but risks also exist as the GR antagonist

mifepristone (RU-486) mediates significantly elevated cortisol and ACTH levels[73].

Recently, the macrophage migration inhibitory factor (MIF) has been found to be a key intermediate that links the activities of inflammatory cytokines and the HPA axis (Fig. 4). MIF is a pro-inflammatory factor that inhibits macrophage migration, and is present in many cell types, but it is mainly expressed in lymphocytes, macrophages, monocytes, and fibroblasts. In contrast to other pro-inflammatory factors that are inhibited by glucocorticoids, MIF expression can be induced by stimulation with glucocorticoids[74], and the induced MIF can reduce the sensitivity of immune cells such as lymphocytes and macrophages to glucocorticoids[75], thereby combating its anti-inflammatory and immunosuppressive effects. Evidence from clinical trials has shown that the MIF expression in leukocytes from patients with severe depression is 40% higher than that in patients with moderate depression[76], while in patients who are insensitive to antidepressants, the MIF mRNA levels are 48% higher[77]. In addition, depressed patients have reduced glucocorticoid sensitivity during and after stress events[76], which is likely related to the upregulation of MIF induced by glucocorticoids. After treatment with antidepressants, the inflammatory levels in the peripheral circulation as well as MIF expression levels in macrophages and lymphocytes are reduced[77]. Although MIF expressed by inflammatory cells (such as macrophages) in the peripheral circulation can act against glucocorticoids and inflammation, inhibition of MIF cannot stop depression; instead, it induces anxiety- and depression-like phenotypes in laboratory animals, leading to a decline in hippocampus-dependent learning and memory[78]. This indicates that high levels of MIF expressed by leukocytes in the peripheral circulation can facilitate the development of depression, and MIF expressed in the CNS is essential for neurogenesis, mood regulation, as well as learning and memory, which is contradictory to its role in inflammatory cells of the peripheral circulation.

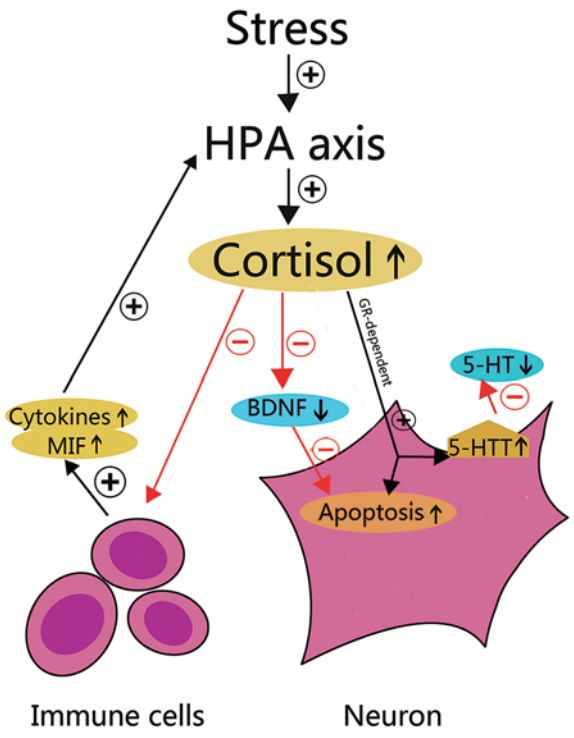

Fig. 4. Over-activation of the immune system and HPA axis synergistically disturb the normal physiological functions of neurons. Stress factors trigger excessive activation of the HPA axis, including the excessive secretion of CRH, ACTH, and glucocorticoids. Up-regulation of glucocorticoid release suppresses BDNF expression, leading to hypo-function of BDNF and attenuated synaptic plasticity. Excessive glucocorticoids result in decreased 5-HT levels in the synaptic cleft by overexpression of the serotonin transporter (5HTT). Interestingly, glucocorticoids stimulate MIF expression, which induces glucocorticoid resistance through inhibition of the response of immune cells to glucocorticoids.

MIF is also involved in mediating the pharmacological effects of antidepressants. Neuronal proliferation induced by fluoxetine can be blocked by MIF inhibition and MIF knockout[78], which suggests that MIF plays a role in the neurogenesis induced by antidepressants. Further studies have shown that the MIF-mediated cell proliferation is mediated by BDNF. Macrophage MIF induces BDNF gene expression both *in vitro* and *in vivo*, thereby restoring the reduced BDNF levels in patients with depression. This is likely to be the potential mechanism for antidepressants, electroconvulsive therapy, and long-term exercise in enhancing BDNF levels in the hippocampus, maintaining neuronal survival and promoting the growth and differentiation of newborn neurons[79].

The Circadian Rhythm Disorder Hypothesis and New Targets for Drug therapy

Substantial studies have shown that up to 80% of

depressed patients have varying degrees of sleep problems[80]. Their main symptoms include early wake-up, usually 2–3 h earlier than usual, and inability to sleep again after wake-up, which is important for diagnosis. Patients with depression might also have disorders in the sleep-wake rhythm. Furthermore, most patients with depression have diurnal mood variation, severe in the morning and mild at night. All the evidence indicates that the disturbance of biological or circadian rhythms is closely associated with the development of depression[81].

In mammals, the site of biological rhythm control is in the suprachiasmatic nucleus in the hypothalamus, which receives a variety of inputs, mainly light signal transduction from the retina, 5-HT produced by the raphe nuclei, and melatonin secreted by the pineal. Its main output is to the paraventricular nucleus of the hypothalamus, which relays signals to the HPA axis, the autonomic nervous system, and the pineal, to allow biological rhythms to regulate glucocorticoids, melatonin, and other hormones. Among these, CRH and cortisol produced by the HPA axis, and melatonin secreted by the pineal are most related to biological rhythms and sleep: CRH inhibits slow-wave sleep and triggers the sleep-wake transition; reduced CRH synthesis or CRH receptor blockade increases the duration of non-rapid eye movement (NREM) sleep and suppresses arousal[82]. Moreover, high concentrations of cortisol have effects similar to CRH, which include inhibiting NREM and prolonging arousal time. Meanwhile, it also reduces the latency of rapid eye movement (REM) sleep and increases the sleep density in REM sleep[82]. In contrast, melatonin has the opposite effects; it not only shortens the time to fall asleep and improves sleep quality, but also directly regulates the biological rhythms in the suprachiasmatic nucleus, especially the sleep-wake rhythm, through a melatonin receptor-GABA mechanism, thereby improving sleep rhythm disorders and endocrine disorders.

In as early as 1985, it was proposed that depression might be a syndrome with low melatonin[83], characterized by low levels of melatonin at night, abnormal dexamethasone suppression, and disorder of the 24-h cortisol rhythm. A cross-sectional study in 2012 further supported the low melatonin hypothesis[84] by showing that nocturnal melatonin levels in patients with depression are significantly lower than in controls. Other than the concentration changes, a change in the biological rhythm of melatonin is also an important factor. Although the abnormal rhythm in depression has high variability (phase advance, phase delay, or changes in amplitude), phase advance appears to be common in depressed patients[85] as manifested by shortened latency to REM sleep, earlier wake-up, and significantly elevated ACTH and cortisol levels at night (compared with normal controls). All these reflect a disturbance of circadian rhythms and enhancement of alertness in stress responses in patients with depression, while all these clinical manifestations are based on the advance in secretion of melatonin and cortisol, i.e., earlier reduction in melatonin levels and earlier increase in cortisol levels make it difficult to maintain sleep during the sleep period. This earlier wake-up is not only the start of a whole day of depressed mood, but also a strong indicator of relapse of depression[86].

Currently, reshaping the normal biological rhythms in depressed patients is a new approach to the treatment of depression. This includes non-drug treatments such as light therapy and sleep deprivation as well as drug treatments using melatonin and agomelatine. Melatonin inhibits ACTH-mediated cortisol secretion[87], decreases the cortisol release, and enhances the negative feedback in the HPA axis to restore this system[88]. It should be noted that the time of administration of melatonin is extremely important[89]: taking it before bedtime leads to an earlier sleep-wake cycle, which may improve the sleep difficulties and other symptoms, but aggravate the earlier wake-up symptoms; taking it at a relatively late time might postpone the sleep-wake cycle, which helps to improve the symptom of earlier wake-up. However, a number of studies have shown that melatonin can only improve sleep, but does not relieve the symptoms of depression[85].

Agomelatine has recently been added to the list of antidepressants. It is a novel antidepressant that works on melatoninergic (MT1 and MT2), 5-HT_{2B}, and 5-HT_{2C} receptors, which might function against anxiety and thereby improve sleep and regulate biological rhythms. Agomelatine regulates the sleep-wake rhythm[90], shortens the REM sleep latency, and reduces the REM sleep time and REM sleep ratio. The sleep symptoms, along with the depressive symptoms, are improved, which might be due to the overlap in antidepressant mechanisms and biological rhythm regulation. In addition, blocking the 5-HT_{2c} receptor indirectly enhances the DA and NE levels to

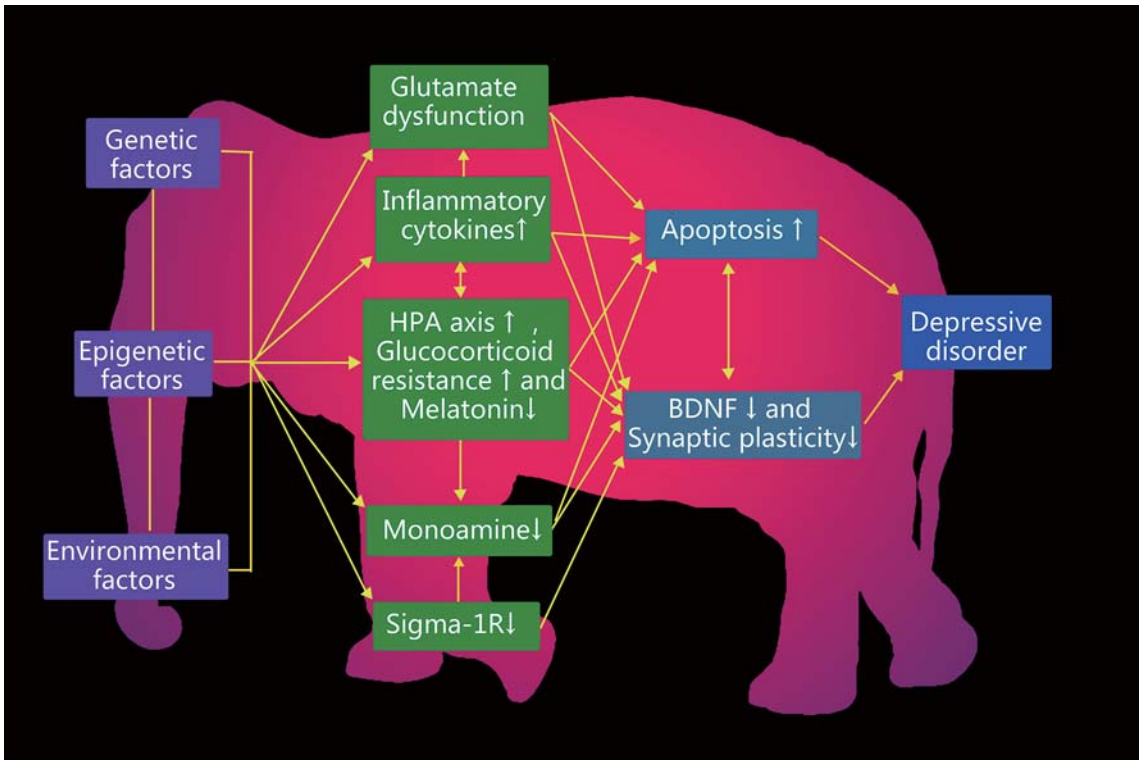

Fig. 5. Schematic of the pathogenesis of depression. The pathogenesis of depression is complex. Like the proverbial blind men exploring an elephant, different hypothesis have been proposed for the etiology and pathogenesis of depression. It is important to have an integrated view of the mechanisms. Genetic and stress vulnerabilities interplay to initiate a cascade of neurobiological changes that disrupt a dynamic system. The decrease BDNF with associated synaptic plasticity and increased apoptosis may play an important role in the onset and maintenance of depression, and may be considered as a common pathway in various hypotheses of depression.

improve depressive symptoms caused by the deficiency of monoamine neurotransmitters[85].

Summary and Outlook: Integrated View of the Pathogenesis of Depression

Like the proverbial blind men exploring different parts of an elephant, different hypotheses interpret the etiology and pathogenesis of depression from different viewpoints that are complimentary and mutually linked, rather than contradictory. It is important to have an integrated view of the mechanisms underlying depression. Genetic and stress vulnerabilities interplay to initiate a cascade of neurobiological changes that disrupt a dynamic system. As shown in Fig. 5, two factors, decreased BDNF with an associated decrease in synaptic plasticity and increased apoptosis may play an important role in the onset and maintenance of depression. Both are considered to be a common pathway in various hypotheses of depression. Although there is still much to elucidate, research progress in the pathogenesis of depression is promising for a cure of MDD.

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·Review·

Rapid-onset antidepressant efficacy of glutamatergic system modulators: The neural plasticity hypothesis of depression

Jing Wang[1, 2, 3], Liang Jing[1, 2], Juan-Carlos Toledo-Salas[1], Lin Xu[1, 2]

[1]*Key Laboratory of Animal Models and Human Disease Mechanisms of the Chinese Academy of Sciences and Yunnan Province, Kunming Institute of Zoology, Kunming 650223, China*

[2]*KIZ/CUHK Joint Laboratory of Bioresources and Molecular Research in Common Diseases, Kunming Institute of Zoology, Chinese Academy of Sciences, Kunming 650223, China*

[3]*Kunming College of Life Science, University of the Chinese Academy of Sciences, Beijing 100049, China*

Corresponding author: Lin Xu. E-mail: lxu@vip.163.com

Depression is a devastating psychiatric disorder widely attributed to deficient monoaminergic signaling in the central nervous system. However, most clinical antidepressants enhance monoaminergic neurotransmission with little delay but require 4−8 weeks to reach therapeutic efficacy, a paradox suggesting that the monoaminergic hypothesis of depression is an oversimplification. In contrast to the antidepressants targeting the monoaminergic system, a single dose of the N-methyl-*D*-aspartate receptor (NMDAR) antagonist ketamine produces rapid (within 2 h) and sustained (over 7 days) antidepressant efficacy in treatment-resistant patients. Glutamatergic transmission mediated by NMDARs is critical for experience-dependent synaptic plasticity and learning, processes that can be modified indirectly by the monoaminergic system. To better understand the mechanisms of action of the new antidepressants like ketamine, we review and compare the monoaminergic and glutamatergic antidepressants, with emphasis on neural plasticity. The pathogenesis of depression may involve maladaptive neural plasticity in glutamatergic circuits that may serve as a new class of targets to produce rapid antidepressant effects.

Keywords: depression; stress; neural plasticity; glutamatergic transmission; monoamine-based antidepressant; ketamine

Introduction

Depression is a common psychiatric disorder, with prevalence rates of 19% in Beirut and 16.2% in the United States[1]. Depression is also one of the leading causes of severe mental disability, suicide, and socioeconomic burden, and its diagnosis sometimes relies on self-reported symptoms in the major depressive inventory[2, 3]. The pathogenesis of depression is still not clear, and currently available antidepressants are far from satisfactory. Most antidepressants target the monoaminergic system[4]; however, 30%–40% of patients are resistant to monoamine-based antidepressants (remittance rates of 13% to 36.8% with citalopram[5]), and a clinically significant reduction of depressive symptoms in responders usually requires 4−8 weeks of daily treatment[6]. These limitations result in a large proportion of patients at high risk of suicide even after diagnosis. Thus, it is necessary to search for new antidepressants outside the monoaminergic system. Recent clinical studies have demonstrated that a single dose of the N-methyl-*D*-aspartate receptor (NMDAR) antagonist ketamine has rapid antidepressant efficacy within 2 h that can be sustained for over 7 days in patients with treatment-resistant depression[7, 8]. This finding suggests that depression could be directly associated with deficits in glutamatergic synaptic transmission and plasticity.

The monoaminergic hypothesis of depression, based on the efficacy of antidepressants that enhance monoaminergic transmission and signaling, has dominated the field of depression research for nearly half a century. However, the primacy of monoaminergic dysfunction in depression is inconsistent with the delay between enhancement of synaptic monoamines conferred by drugs such as specific serotonin re-uptake inhibitors, serotonin–noradrenalin re-uptake inhibitors, and monoamine oxidase inhibitors, and clinically significant antidepressant efficacy[9].

Many studies have revealed that these antidepressants have multiple pharmacological effects in addition to increasing monoaminergic function, such as enhancing adult hippocampal neurogenesis and rescuing stress-impaired NMDAR-dependent long-term potentiation (LTP)[10-12]. Neurogenesis enhances hippocampus-dependent memory and LTP, while stress impairs neurogenesis and alters hippocampus-dependent memory and LTP. Thus, the mechanisms underlying the clinical response to antidepressants may include effects on neural plasticity. This point of view is strongly supported by the rapid onset of antidepressant efficacy of ketamine in clinical trials, because NMDAR activation is critical for both LTP and hippocampus-dependent memory[8, 13, 14]. These developments suggest an alternative etiology for depression due to functional disturbances of neural plasticity in the glutamatergic system. In this paper, we review recent studies that implicate aberrant neural plasticity in depression and suggest that mitigation of these deficits underlies the efficacy of antidepressant medications. We predict that a

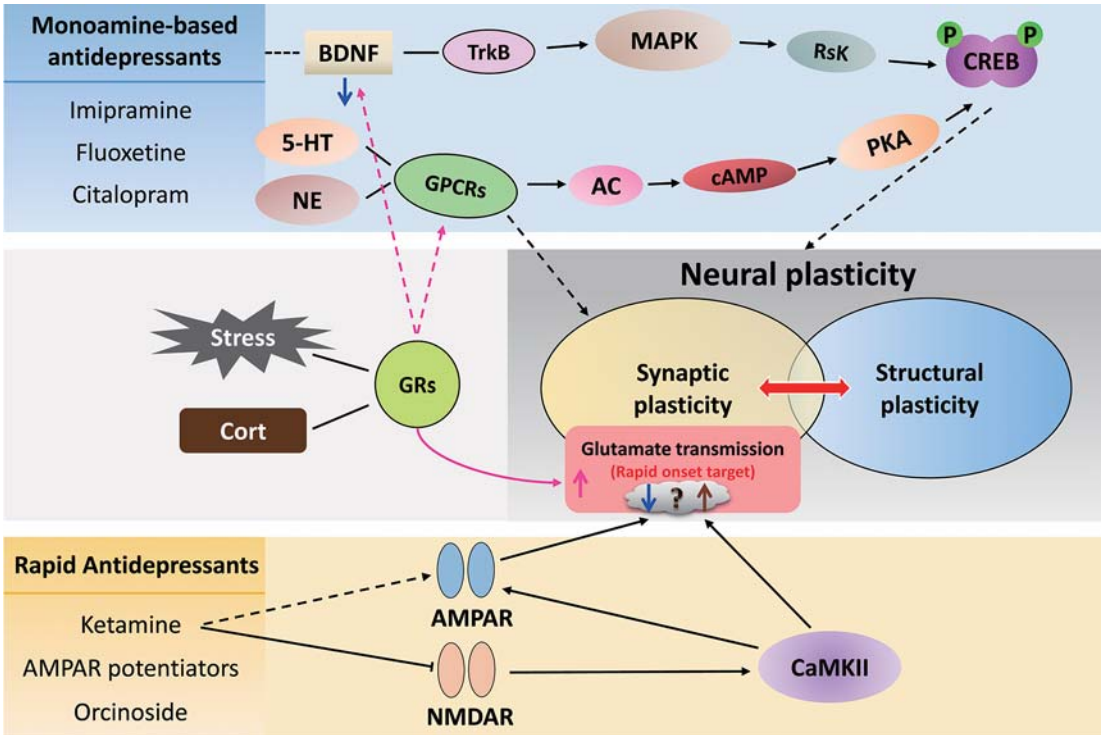

Fig. 1. Effects of stress and antidepressants on neural plasticity. Major signaling pathways critical for memory and neural plasticity are also regulated by stress and antidepressants. See text for details and references. Pink arrow, pathological status; solid arrows, direct action; dashed arrows, indirect action. Abbreviations: BDNF, brain-derived neurotrophic factor; TrkB, BDNF receptor; MAPK, mitogen-activated protein kinase; Rsk, ribosomal S6 protein kinase; CREB, cAMP response element-binding protein; 5-HT, 5-hydroxytryptamine (serotonin); NE, norepinephrine; GPCRs, G-protein coupled receptors; AC, adenylyl cyclase; cAMP, cyclic adenosine monophosphate; PKA, protein kinase A; Cort, corticosterone; GRs, glucocorticoid receptors; AMPAR, α-amino-3-hydroxy-5-methyl-isoxazole-4-propionic acid glutamate receptor; NMDAR, N-methyl-*D*-aspartate glutamate receptor; CaMKII, calcium-calmodulin-dependent kinase II.

new class of drugs targeting neurogenesis and synaptic plasticity within the glutamatergic system could produce rapid antidepressant responses (Fig. 1).

Dysfunctional Neural Plasticity in Depression

Neuroplasticity often refers to the functional and structural synaptic plasticity that is a prominent feature of glutamatergic neuronal circuits and crucial to adaptive behavioral responses and survival[15, 16]. Experience-dependent changes in glutamatergic synaptic transmission, notably LTP and long-term depression (LTD), are widely believed to encode hippocampus-dependent associative memories. However, both LTP and LTD are sensitive to stress-associated emotional states[17]. Dysfunction of synaptic plasticity is associated with many psychiatric disorders, such as depression, autism, and drug addiction[18-20].

Several brain regions critical for memory and emotion, most notably the hippocampus, amygdala and the prefrontal cortex (PFC), exhibit robust neural plasticity, which is disrupted in stress-induced animal models of depression. Moreover, hippocampal and amygdala functions are disrupted in clinical depression, suggesting a causal link between dysfunctional neural plasticity, memory, and mood regulation. Neuroimaging studies have demonstrated a reduced hippocampal volume in depressed patients that can be restored by antidepressant treatment[21-24]. In contrast, both the size and the activity of the amygdala, a structure critical for fear-associated memories, are increased in depressed patients[25-28]. Consistent with brain imaging studies, depressed patients exhibit deficits in hippocampus-dependent memory tasks and are more sensitive to stressful events[24, 29, 30]. Changes in neurotransmitter levels, receptors, and serotonin reuptake transporters have also been found in depressed patients, such as increased glutamate, decreased serotonin 1A receptors and impaired serotonergic neurotransmission[31-35]. However, due to the technical and ethical limitations of clinical research, most of this evidence has been obtained from studies of animal models of depression.

While the most-widely used animal models of depression have been established using chronic or acute stress, the relationship between stressful life experiences and depression risk in humans is complex. Nevertheless, stressful life-events do contribute to the development of depression[36]. Indeed, some individuals become depressed in response to stressors that may have no serious impact on others. Memory or synaptic plasticity related to beneficial or harmful experiences can be affected by stress. Both acute and chronic stressors have profound effects on the brain, causing increases of extracellular glutamate levels and changes in structural and functional plasticity.

Neurogenesis is maintained in the hippocampus throughout adulthood. At the structural level, chronic stress alters the neural morphology in the hippocampus and the medial PFC, including loss of dendritic spines and retraction of dendrites, and reduces neurogenesis in the dentate gyrus (DG)[37-40].

Certain types of stress impair NMDA-dependent LTP and facilitate LTD in the hippocampal CA1 region, processes that are critical for learning and memory. Stress can cause rapid glucocorticoid receptor-mediated alterations in presynaptic glutamate release and slower changes in postsynaptic glutamate receptor expression and function[41], which can affect LTP and LTD. In addition, stress may induce changes in functional plasticity by shifting the balance between synaptic and extrasynaptic glutamate receptors that are thought to contribute to potentiation and depression, respectively[42, 43]. Learned helplessness in rats is a widely-used behavioral model of depression. The underlying mechanism is associated with a marked increase in depolarization-evoked glutamate release in the PFC, which can be mitigated by monoamine-based antidepressants[44-46]. Moreover, antidepressants reduce the release of glutamate, possibly by decreasing phospho-activation of Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII)[47].

Antidepressants can enhance structural plasticity and neurogenesis. A few days (~5 days) of fluoxetine administration increases synaptic density in the hippocampal CA1 pyramidal cell layer, and 14 days of treatment has similar effects in the CA3 pyramidal cell layer[48]. In the olfactory bulbectomy model of depression, chronic treatment with the tricyclic antidepressant amitriptyline blocks the stress-induced decrease in spine density in hippocampal DG, CA1, and CA3 neurons[49]. In addition to the monoamine-based antidepressants, NMDAR antagonists rapidly reverse the low levels of synaptic proteins and spine loss in the medial PFC[50].

Rescuing neurogenesis in depression is crucial for the antidepressant effects. Reduced neurogenesis by both acute and chronic stress can be rescued by chronic administration of antidepressants, while abolishing neurogenesis by x-irradiation makes antidepressants behaviorally ineffective[51, 52]. Suppression of neurogenesis in the hippocampus by genetic manipulation or radiation in mice results in deficits in the hippocampal negative feedback control of corticosterone, and they develop anxiety and depressive-like behavior such as anhedonia[53]. The changes described in structural plasticity may result from or lead to alterations of functional plasticity, such as reduced LTP or enhanced LTD in the hippocampus[54, 55]. Thus, aberrant neural plasticity such as decreased neurogenesis in the DG, impaired LTP and facilitation of LTD, and dysfunction of glutamatergic neurotransmission may all contribute to the development of depression.

From Stressful Events to Depression: Clues from Maladaptive Neural Plasticity to Negative Memory

Dysfunctional learning and memory links neural plasticity with depression. Stressful events can change behavioral and cognitive patterns according to the learned helplessness cognitive model and Beck's cognitive model of depression[56, 57]. Memory dysfunction has been reported in depressed patients using paradigms such as the virtual reality spatial navigation task or paragraph recall, and the impairments are highly correlated with illness chronicity[58, 59]. In free-recall tests of biographical information, depressed patients show enhanced recall for negative events, a phenomenon termed mood congruent memory[29, 30]. This suggests that both hippocampal and the amygdala functions are altered in depression, and these changes are correlated with changes in functional neural plasticity.

There is compelling evidence that stress-related memory is essential to the development of depression, at least in animal models. Uncontrollable and unpredictable stress is commonly used to model depression in laboratory animals. These models include the forced-swim test (FST), tail suspension test (TST), learned helplessness, chronic mild stress, and social defeat[60]. Stress and the stress hormone corticosterone have profound impacts on both synaptic plasticity and memory, consistent with the role of memory in depression-associated behavioral changes. Stress or corticosterone treatment enhances the acquisition of spatial information[61], but also impairs the retrieval of memory[62]. Stress also enhances the acquisition of fear memory but impairs its extinction[63]. If stress occurs before a spatial learning task, it impedes both memory formation and retrieval[64]. However, briefly exposing the rats to acute stress, as measured by the T-maze, increases working memory performance, and the effects are acute, lasting <2 days[41]. Therefore, stress can either facilitate or impair memory formation and (or) retrieval depending on the timing and the severity, possibly due to the effects on neural plasticity[65]. At the functional level, stress blocks LTP induction and facilitates LTD induction, and the formation of fear memory and one-trial avoidance memory implicate possible stress-induced endogenous LTP in the amygdala and hippocampus, respectively[66, 67]. At the structural level, stress or corticosterone induces neuronal shrinkage in the hippocampus but enhances dendritic arborization in pyramidal and stellate neurons in the basolateral amygdala[68]. These changes may result from an overreaction of memory systems to stressful events. In other words, depression may result from over-activation of a highly conserved mechanism critical for survival (although stress-facilitated storage of pivotal information) which is one of the conserved mechanisms beneficial to survival competition but may lead to the development of depression. Antidepressants can mitigate the effects of stress on memory, and are associated with the restoration of neural plasticity. Fluoxetine treatment acts synergistically with extinction to erase conditioned fear in mice, possibly by enhancing LTP and brain-derived neurotrophic factor (BDNF) expression in the lateral amygdala. Indeed, this effect was not observed in $BDNF^{-/+}$ mice, while the effects of fluoxetine were mimicked by BDNF overexpression[69]. Antidepressants can also restore impaired memory performance in depression models by rescuing impaired LTP induction and by maintaining dendritic morphological complexity and hippocampal neurogenesis.

Changes in neural plasticity caused by chronic stress may alter cognition and behavior in humans, and this may lead to depressive symptoms. In depression, the encoding and retrieval of negative memories may be facilitated, and so come to dominate cognition and exert a prominent influence on behavior. Depressed patients may accumulate negative memories in a stressful environment

and ultimately are unable to update new memories due to dysfunctional neural plasticity. Antidepressants may restore maladaptive neural plasticity, allowing for normal memory function, as discussed below.

Monoamine-Based Antidepressants Restore Neural Plasticity

Monoamine-based antidepressants, fortuitously discovered from clinical observations in the 1950s, represent a milestone in the treatment of depression[6, 70, 71]. Moreover, they provide clues to the biological basis of depression. Iproniazid and imipramine were first developed for non-psychiatric conditions, but were discovered to have potent antidepressant efficacy[22]. It was found that these monoamine-based antidepressants either enhanced central 5-HT/norepinephrine (NE) transmission or depleted monoamine stores in the brain. The monoaminergic hypothesis has guided drug development for decades and resulted in the development of a myriad of antidepressants that share similar mechanisms and limitations. More specific drugs to inhibit 5-HT and NE reuptake have been developed, such as fluoxetine, citalopram, and tranylcypromine[22], all with similar limitations, most notably a 4−8-week delay for clinical effect. This delay likely increases the risk of suicide in the interim[72].

Brain-Derived Neurotrophic Factor

BDNF is critical for synaptic plasticity and memory. It is also the most important target for antidepressant efficacy. BDNF promotes synaptic and morphological plasticity, and regulates synaptic transmission and neuronal growth[73]. The neurotrophic hypothesis of depression is based on clinical and preclinical observations that include three lines of evidence: a low concentration of BDNF in the hippocampus of postmortem samples from depressed suicide victims[74], depression-related behaviors caused by impaired BDNF signaling in rodent hippocampus[75, 76], and the antidepressant effects of increased hippocampal BDNF[77, 78]. BDNF is critical for stabilizing synaptic plasticity. BDNF mRNA expression is reduced by stress, leading to impaired hippocampal synaptic plasticity[79]. Antidepressant effects mediated by slow changes in BDNF expression and downstream signaling, leading to morphological plasticity and enhanced neurogenesis, may explain the delay between drug administration and antidepressant efficacy. However, the neurotrophic hypothesis of depression is over-simplistic, as neuronal survival and plasticity depend on the connections to other cells and the activation of many additional signaling pathways.

CREB Signaling Cascade under Monoamine-Based Antidepressants

Since the 1990s, the cAMP response element binding protein (CREB) gene has been considered as a central “memory gene”. CREB activation and the ensuing CREB-dependent *de novo* protein synthesis are required to stabilize structural and functional changes in synaptic strength, which participate in long-lasting late-LTP and long-term memory[80-82].

The cAMP-PKA-CREB signaling cascade has been implicated in synaptic plasticity, memory, and antidepressant drug responses. Modulatory neurotransmitters such as 5-HT, NE, and dopamine increase the intracellular cyclic adenosine monophosphate (cAMP) concentration and activate cAMP-dependent protein kinase (PKA) through G-protein coupled receptors (GPCRs)[83]. Chronic antidepressant administration enhances the coupling of GPCRs to adenylyl cyclase, PKA activation, and expression of CREB[19]. Furthermore, the cAMP-PKA-CREB signaling cascade is critical for long-lasting forms of synaptic plasticity, notably late-LTP, and for long-term memory formation[84].

The mitogen-activated protein kinase (MAPK) pathway also regulates synaptic plasticity in the hippocampus, amygdala, and neocortex[85-88]. BDNF *via* its receptor TrkB activates the MAPK-RsK-CREB cascade, and this signaling cascade is altered by antidepressant treatment[89].

Activation of CREB promotes adult hippocampal neurogenesis, suggesting that antidepressants promote neurogenesis through activation of the cAMP-PKA-CREB pathway[90]. Postmortem studies on depressed patients have demonstrated reductions of CREB expression and phosphorylation in suicides[91, 92]. Animals overexpressing CREB in the hippocampus exhibit a shorter immobility time in the FST and fewer escape failures in the learned helplessness paradigm[93]. In addition, chronic antidepressant administration increases CREB phosphorylation and transcriptional activity[94, 95]. These studies provide a compelling rationale for the development

of new antidepressants targeting CREB activation and transcriptional activity.

Effects of Antidepressants Mediated by Glutamate Receptor Stimulation

Monoamine-based antidepressants interfere with NMDAR activation[96]. Chronic administration of antidepressants such as fluoxetine, desipramine, and reboxetine significantly reduces depolarization-evoked glutamate release in the hippocampus[97], and animal studies have shown that chronic antidepressant treatment reduces glutamatergic transmission and field potentials in rat frontal cortex[98, 99], thereby reducing NMDAR activation (which requires both synaptic glutamate and postsynaptic depolarization). Antidepressants also affect α-amino-3-hydroxy-5-methyl-isoxazole-4-propionic acid receptor (AMPAR) trafficking. Riluzole increases the surface expression of the AMPAR subunits glutamate receptor 1 (GluR1) and GluR2, and reversibly attenuates AMPAR-mediated synaptic currents in cultured cells[100, 101]. Fluoxetine regulates the phosphorylation state of AMPARs[102]. Cai *et al.* provided the first evidence that endogenous serotonin selectively potentiates temporoammonic-CA1 excitatory synapses *via* the activation of serotonin 1B receptors. This potentiation requires postsynaptic AMPAR expression and CaMKII-mediated phosphorylation of GluR1 subunits[103]. While many forms of LTP and LTD are triggered by stimulation of NMDARs, LTP and LTD expression depend on selective up- or down-regulation of AMRAR currents, respectively, due to changes in both channel ion permeability and postsynaptic surface expression. Therefore, changes in both AMPAR and NMDAR function and expression may relate directly to depression and the clinical effects of antidepressants. Thus, AMPARs as well as NMDARs are promising targets for antidepressant research and development.

Glutamate Receptors as Direct Targets for Rapidly-Acting Antidepressants

Glutamate was not acknowledged definitely as a neurotransmitter in the mammalian central nervous system until the early 1980s[104]. Prior to this, the conceptual framework of depression was dominated by the monoaminergic hypothesis, so most of the antidepressants developed for clinical therapy target monoaminergic transmission. However, stress affects glutamate release, clearance, metabolism, receptor function, and receptor expression, strongly implicating glutamatergic transmission and plasticity in the pathogenesis of depression[46]. Glutamate signaling is mediated by both ionotropic (AMPARs and NMDARs) and metabotropic glutamate receptors (mGluR1 to mGluR8). Excitatory synaptic efficacy is determined by the single-channel conductance, the number, and the stability of glutamate receptors at the postsynaptic membrane[46]. Acute stress enhances glutamate transmission by increasing the surface expression of NMDARs and AMPARs at the postsynaptic membrane, which dynamically regulates metaplasticity (e.g., LTP/LTD induction thresholds) at glutamatergic synapses[105]. Chronic stress further alters glutamatergic circuits and the survival of newborn glutamatergic neurons[106, 107]. Thus, disturbed glutamatergic neurotransmission is likely a core feature of stress-related mental illnesses. Drugs influencing basal transmission, plasticity, and metaplasticity may be effective antidepressants that act rapidly[46, 108, 109].

Ketamine Has Rapid-Onset Antidepressant Efficacy

The discovery of ketamine's fast antidepressive effects not only allowed exploration of new research of the brain circuits involved in depression but helped to develop new pharmaceutical approaches of a disease that for many decades showed no advance, and any new approach for psychiatric diseases should consider the past long-term mistakes to avoid them[110].

Preclinical and clinical studies have reported that low doses of the noncompetitive NMDAR antagonist and psychotomimetic ketamine have a rapid antidepressant action: a rapid (within hours) and sustained (up to 1 week) antidepressant effect on core symptoms can be induced by a single subanesthetic dose of intravenously-infused ketamine in patients with treatment-resistant depression[7, 8, 14, 111]. A recent report also emphasized that a single dose can have long-lasting effects on the FST behavior of rats[112]. This unanticipated finding suggests that ketamine could be an alternative treatment for depressed patients who have not previously responded to pharmacotherapy. Though ketamine is a hallucinogenic drug similar to lysergic acid diethylamide, it is relatively safe as an antidepressant because a low dose is used (0.5 mg/kg

antidepressant dose *versus* 2.0 mg/kg psychedelic dose)[8]. Furthermore, repeated ketamine infusion also has a sustained antidepressant effect, with no clinically significant psychotomimetic effects[113]. In contrast to most approved antidepressants which target the monoaminergic system, ketamine has a direct and rapid effect on the glutamatergic system and synaptic plasticity[47]. Ketamine rapidly increases the expression of synaptic proteins and the number of excitatory spine synapses in the PFC[50, 114, 115]. In the FST, ketamine reduces immobility (a behavioral endophenotype reversible by antidepressants) concomitant with a rapid increase in glutamate release, activation of AMPARs, and increased hippocampal BDNF concentration[116, 117]. From this evidence, a glutamatergic hypothesis of depression is proposed in which the glutamatergic system is the primary mediator of depression and serves as a final pathway in antidepressant therapy. By directly targeting NMDARs and rapidly restoring glutamatergic function, ketamine has fast antidepressant effects.

Targeting NMDARs

The NMDAR as an antidepressant target was first suggested by the findings that inescapable acute stress impairs hippocampal LTP and facilitates LTD. Thus, before the discovery of ketamine's antidepressant effect, several other NMDAR regulators had been evaluated for their antidepressant effect in animal models, such as MK-801 (a non-competitive antagonist), AP-7 (a competitive antagonist), and RO25-6981 (an NR2B antagonist)[118, 119]. While antidepressant effects occurred with these agents, they were not as sustained as those of ketamine[116]. Recent studies have found that the NMDAR agonist GLYX-13 has effects similar to ketamine but without the adverse effects, such as the psychotomimetic consequences and impaired cognition that limit its clinical use[120]. Further studies are required to determine how both agonists and antagonists of NMDARs exhibit antidepressant efficacy. One possibility is that agonists promote synaptic plasticity and improve cognition, while antagonists serve to protect neurons against stress-induced degeneration.

Targeting AMPARs

Glutamatergic synaptic strength is determined by AMPARs, as these receptors contribute to the majority of postsynaptic current. The function of AMPARs is rapidly modulated by phosphorylation at two sites on the GluR1 subunit, and both sites are implicated in the expression of synaptic plasticity and memory[121]. GluR1 phosphorylation is transiently increased by stress, possibly contributing to the stress-induced facilitation of memory[122]. In addition, the AMPAR potentiators LY392098 and LY451646 have antidepressant effects in the FST and TST[123-125]. Enhanced phosphorylation of GluR1 has been detected following fluoxetine, imipramine, and ketamine treatment, and ketamine rapidly increases postsynaptic AMPAR expression[103, 104, 115]. Moreover, the onset of ketamine effects requires AMPARs, as the antidepressant-like behaviors are attenuated by pre-treatment with the AMPAR antagonist NBQX in mouse models of depression[116]. Considering their importance in memory and synaptic plasticity, AMPARs may be another target for fast antidepressant effects.

Orcinoside

Orcinoside is a small compound extracted from a traditional Chinese herb that has been used to treat depression-like symptoms, lack of mental energy, and memory defects for over 100 years. A fruitful collaboration by the Kunming Institute of Zoology and Kunming Institute of Botany, Chinese Academy of Sciences, has identified orcinoside as a potent antidepressant[126].

Conclusion and Future Prospects

Depression is a functional illness. Stress-induced effects on neural plasticity and memory appear critical for disease pathogenesis as evidenced by the actions of stress, monoamine-based antidepressants, and glutamate receptor modulators on memory pathways. The NMDAR is necessary for many forms of neural plasticity and memory, and thus may be a feasible target for a new class of antidepressants with more rapid efficacy than currently achieved using monoaminergic modulators. Indeed, the antidepressant efficacy of the NMDAR antagonist ketamine may provide proof of principle. A key question is why the efficacy of ketamine, while rapid, also wears off in time. Moreover, how different changes in synaptic plasticity and metaplasticity contribute to the development and (or) amelioration of specific depressive behaviors requires much future study.

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·Review·

Genetic studies of schizophrenia: an update

Jingchun Chen[1], Fei Cao[2], Lanfen Liu[3], Lina Wang[3], Xiangning Chen[1]

[1]*Virginia Institute for Psychiatric and Behavioral Genetics, Virginia Commonwealth University, Richmond, VA 23298, USA*

[2]*Department of Neurology, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430022, China*

[3]*Department of Psychiatry, Shandong Mental Health Center, Jinan 250014, China*

Corresponding author: Jingchun Chen. E-mail: jchen@vcu.edu

Schizophrenia (SCZ) is a complex and heterogeneous mental disorder that affects about 1% of global population. In recent years, considerable progress has been made in genetic studies of SCZ. A number of common variants with small effects and rare variants with relatively larger effects have been identified. These variants include risk loci identified by genome-wide association studies, rare copy-number variants identified by comparative genomic analyses, and *de novo* mutations identified by high-throughput DNA sequencing. Collectively, they contribute to the heterogeneity of the disease. In this review, we update recent discoveries in the field of SCZ genetics, and outline the perspectives of future directions.

Keywords: schizophrenia; genome-wide association study; copy-number variant; *de novo* mutation; sequencing; genetics

Introduction

Schizophrenia (SCZ) is a severe neuropsychiatric disorder with a lifetime prevalence of ~1% worldwide[1]. Clinically, SCZ is characterized by symptoms traditionally classified as positive (delusions and hallucinations), negative (flattened emotions and social withdrawal), and impairments of cognitive functions[2-4]. The age at onset is typically in late adolescence or early adulthood[5]. This disorder has a considerable impact not only on patients' health and well-being, but also on society and health services worldwide[6].

SCZ is a complex genetic disorder with a relatively high heritability, exceeding 60% in two national family studies[7,8] and 80% in twin studies[9]. After decades of frustration, genetic studies of SCZ have made significant progress in recent years with the application of genome-wide association studies (GWASs) and next-generation DNA sequencing technologies. In these studies, a number of genes have been identified with common risk alleles, rare copy-number variations (CNVs), and *de novo* mutations (DNMs). From these studies, we have learned that the genetic risk for SCZ is highly polygenic: many genes contribute to the development of the disorder but the contribution from individual genes is relatively small. These studies also reveal the complexity of the genetic architecture that includes structural variations (CNVs), common and rare single-nucleotide variations (SNVs), and DNMs. In this brief review, we update recent progress on genetic studies of SCZ, focusing on the common SNVs discovered by GWASs, as well as CNVs and rare SNVs and DNMs discovered by high-throughput DNA sequencing.

Common Variants Contributing to SCZ

SCZ is now established as a heritable disorder by family and twin studies[7-9]. In the early years, the search for genes involved in SCZ by linkage and candidate gene studies did not produce replicable and consistent results[10,11]. From 2009 onwards, a number of creditable candidates were identified, largely by GWAS, a linkage disequilibrium-based technique designed to find links between genetic variations and diseases in a homogeneous population without *a*

priori knowledge of the disease. The variants discovered by GWASs are common variants, conventionally defined as those with allele frequencies ≥1%. This is largely due to the design of genotype chips used in GWAS, a design intended to test the "common diseases – common variants" hypothesis. Table 1 summarizes the top findings from GWASs in recent years. Of the loci identified, the region encompassing the major histocompatibility complex (MHC) on chromosome 6p[12] is the most significant and consistent. Many markers in this region reach genome-wide significance ($P \leq 5.0 \times 10^{-8}$). The association signals cover an interval of ~6 million base-pairs, including all three classes of MHC regions that encode for >50 genes[13,14]. Due to the high linkage disequilibrium and complex genomic structure, it is difficult to determine whether one or multiple genes in this region are involved in the disorder. It has long been speculated that the immune system is involved in SCZ, so the finding that the genetic effects are enriched in the MHC region or even in regions outside the MHC that are also involved in acquired immunity is consistent with this hypothesis. After the first GWAS reporting on the MHC region, a total of 30 loci across the whole genome were reported to be associated with SCZ by 2013[15-25], including the genes for transcription factor 4 (TCF4), neurogranin (NRGN), and DPYD/MIR317 that are known to play crucial roles in brain development. Of these GWASs, only two used Chinese subjects, one used Japanese subjects and the rest used Caucasian subjects. Most recently, the Psychiatric Genomics Consortium (PGC) SCZ group published the largest SCZ GWAS, identifying 108 independent loci across the genome, including all but 5 loci reported before[26].

GWASs, as noted above, are designed to discover associations between common variants and diseases. The successful discovery of many risk loci for SCZ provides evidence that its genetic architecture is polygenic by nature, and that individual genes have limited effects on its development. The common variants identified so far have a low genotypic relative risk individually (odds ratios (ORs) 1.1- to 1.5-fold). But collectively, these variants account for >50% of the heritability[27]. As we examine these variants closely, it is clear that most do not have known functions, and many of them are not located in protein-coding genes,

Table 1. The 25 most common variants identified by GWASs

Index SNP	Chr	Position (hg19)	Allele	OR (95% CI)[a]	*P*-value[a]	Genes	References
rs115329265	6	27143833-30174131	AG	1.21 (1.17-1.24)	3.48×10^{-31}	MHC class II including HIST1H2BJ, PRSS16, NKAPL, and TRIM26	[12,15,16, 19-21,25,26]
rs1702294	1	97792625-98559084	TC	0.89 (0.87-0.91)	3.36×10^{-19}	DPYD, MIR137 (micro-RNA),	[19,25,26, 108]
rs11191419	10	104423800-105165583	AT	0.91 (0.89-0.93)	6.2×10^{-19}	ARL3, AS3MT, C10orf32, CNNM2, CYP17A1, INA, NT5C2, PCGF6, PDCD11, SFXN2, TAF5, TRIM8, USMG5, and WBP1L	[19,25,26, 108,109]
rs2007044	12	2321860-2523731	AG	0.91 (0.89-0.93)	3.22×10^{-18}	CACNA1C	[24-26,108]
rs4129585	8	143309503-143330533	AC	1.09 (1.07-1.11)	1.74×10^{-15}	TSNARE1	[25,26]
chr7_2025096_I	7	1896096-2190096	DI3	0.92 (0.90-0.94)	8.2×10^{-15}	MAD1L1	[25,26,110]
rs4391122	5	60499143-60843543	AG	0.92 (0.90-0.94)	1.1×10^{-14}	ZSWIM6	[25,26,110]

To be continued

Continued from previous page

rs2851447	12	123448113-123909113	CG	0.92 (0.89-0.94)	1.86×10^{-14}	ABCB9, ARL6IP4, C12orf65, CDK2AP1, MPHOSPH9, OGFOD2, PITPNM2, RILPL2, SBNO1, SETD8	[25,26]
chr2_200825237_I	2	200715237-200848037	I2D	0.91 (0.89-0.93)	5.65×10^{-14}	AC073043.2, C2orf47, C2orf69, and TYW5	[25,26]
rs10791097	11	130714610-130749330	TG	1.08 (1.06-1.10)	1.09×10^{-12}	SNX19	[25,26]
rs11693094	2	185601420-185785420	TC	0.93 (0.91-0.95)	1.53×10^{-12}	ZNF804A	[26,85]
rs7893279	10	18681005-18770105	TG	1.13 (1.09-1.16)	1.97×10^{-12}	CACNB2	[25,26]
rs12129573	1	73766426-73991366	AC	1.08 (1.06-1.10)	2.03×10^{-12}	LRRIQ3	[25,26]
rs6704768	2	233559301-233753501	AG	0.93(0.91-0.95)	2.32×10^{-12}	C2orf82, EFHD1, GIGYF2, KCNJ13, and NGEF	[25,26]
rs55661361	11	124610007-124620147	AG	0.93 (0.91-0.95)	2.8×10^{-12}	ESAM, MSANTD2, NRGN, and VSIG2	[16,26]
rs9636107	18	52747686-53200117	AG	0.93 (0.91-0.95)	3.34×10^{-12}	TCF4	[16,17,19,25, 26,108]
chr11_46350213_D	11	46342943-46751213	I2D	0.91 (0.88-0.93)	1.26×10^{-11}	AMBRA1, ARHGAP1, ATG13, CHRM4, CKAP5, CREB3L1, DGKZ, F2, HARBI1, MDK, and ZNF408	[26,111]
rs11682175	2	57943593-58502192	TC	0.93 (0.91-0.95)	1.47×10^{-11}	FANCL and VRK2	[17,26]
rs2535627	3	52541105-52903405	TC	1.07 (1.05-1.09)	4.26×10^{-11}	GLT8D1, GNL3, ITIH1, and ITIH3	[24,26]
rs111294930	5	151941104-152797656	AG	1.09 (1.06-1.12)	1.06×10^{-10}	GRIA1	[25,26]
rs2905426	19	19374022-19658022	TG	0.93 (0.91-0.95)	3.63×10^{-10}	CILP2, GATAD2A, HAPLN4, MAU2, NCAN, NDUFA13, PBX4, SUGP1, TM6SF2, and TSSK6	[25,26]
rs77149735	1	243503719-244002945	AG	1.32 (1.20-1.44)	3.73×10^{-9}	AKT3 and SDCCAG8	[24,26]
rs59979824	2	193848340-194028340	AC	0.94 (0.92-0.96)	8.41×10^{-9}	PCGEM1	[19,26]
rs10503253	8	4177794-4192544	AC	1.07 (1.05-1.10)	1.06×10^{-8}	CSMD1	[19,26,108]
rs7819570	8	89340626-89753626	TG	1.08 (1.05-1.11)	1.22×10^{-8}	MMP16	[19,26]

Chr, chromosome; CI, confidence interval; OR, odds ratio; SNP, single-nucleotide polymorphism. [a]All ORs and *P*-values are from the most recent PGC study[26].

suggesting that most of the identified variants are not causal. Therefore, even though GWASs have identified >100 variants associated with SCZ, we still cannot be certain to what extent common variants contribute to the etiology of SCZ. Another lesson learned from GWASs is that a stringent threshold requires a huge sample size to reliably identify risk genes. From the incremental PGC studies[19,25,26], it is clear that when the sample size reaches a critical value, every addition of 1 000 samples leads to the identification of 2–3 more new loci. The same trend is observed in studies of other complex diseases and traits, such as obesity, height, and blood lipids[28-30]. This trend demands collaborative work from many investigators to ensure the successful discovery of risk genes for complex diseases. In the case of SCZ studies, the PGC includes almost all existing samples of Caucasian ancestry worldwide.

Since we have good reasons to believe that there are other unidentified risk genes for SCZ, and that continuing to increase the sample size is not sustainable in the long run, other approaches must be considered. Based on this rationale, we and others have explored alternative approaches to discover risk genes for SCZ. With a two-stage design, by integrating data-mining and functional analyses of a selected number of candidates from GWAS datasets, we found that two markers in the CMYA5 gene are associated with SCZ[31]; one of the markers (rs10043986) changes a proline to leucine in the protein sequence. Using a convergent functional genomics approach, which utilizes poly-evidence scoring and pathway analyses, Ayalew *et al.* identified several genes involved in SCZ, including *DISC1* and *TCF4*[32]. By combining gene expression profiling and GWAS data, Luo *et al.* showed that *CAMKK2* is differentially expressed in SCZ and controls, and a marker in the gene, rs1063843, is associated with the expression and diagnosis of SCZ in a large GWAS dataset[33].

Copy-Number Variations and SCZ

Changes of DNA copy involving insertion/deletion (indel) or duplication (dup) are known as CNVs. Indels can be as small as a few hundred base-pairs or as large as an entire chromosome, and about one-quarter of the human genome harbors CNVs[34]. As a result, CNVs can change the dosage of one or more genes in the regions covered by CNVs, and therefore exert a profound effect on the expression of the genes. Most studies of CNVs in SCZ are based on the analyses of signal intensity from GWAS microarrays. The assumption is that most parts of the genome (except for the X and Y chromosomes) have two copies, and the signal intensity of markers along the chromosome is approximately the same when smoothed across a reasonable number of markers or genomic distance. When the signal intensity changes consistently and continuously for a reasonable genomic interval (hundreds of kilobase-pairs or more), a copy-number change can be inferred. Typically, these inferred CNVs need to be verified experimentally *via* real-time quantitative PCR or other techniques.

Over the years, many CNVs have been found to increase the risk for SCZ. Most of these involve multiple genes, while some involve a single gene or do not have known genes in the interval. For example, 2p16.3 del only affects the NRXN1 gene[35-42], and 7p36.3 dup only affects the VIPR2 gene[38,43,44]. CNVs that alter the expression of multiple genes include 1q21.1 del/dup (34 genes)[36-39,41,45-47], 3q29del/dup (21 genes)[38-40,44,48,49], 15q13.3del (12 genes)[38,39,44,46,47,49], 16p13.1dup (11 genes)[40,41,50,51], and 22q11.2del/dup (53 genes)[38,39,44,46,47,51-56], *etc*. (see Table 2 for more details). For the involved genes, the effect is more pronounced[34,57-59]. The large 22q11.21 locus (3 Mb), also known as DiGeorge and velo-cardio-facial syndrome critical region, was first reported to be associated with SCZ in the 1990s[60], and this was verified in many later studies[38,39,44,46,47,51-55]. Most of the studies show that deletion of this region increases the risk of SCZ. A recent study indicates that a duplication of this same region is protective against SCZ[56], demonstrating a dosage effect on the development of SCZ. As this region contains >50 genes, it is still not clear which are involved and how they contribute to SCZ[61,62]. There is evidence that the total burden of rare CNVs (numbers of CNVs per individual in combination with the number of genes per CNV) is increased in SCZ patients at both the whole-genome and specific loci levels[37,46,47].

In general, CNVs have relatively low frequencies (typically less than 1 in 1 000 individuals), but account for a substantially higher risk (OR 2.7 to infinity)[63]. Although it is uncertain which gene(s) are responsible for the effects, as multiple genes are involved in most CNVs, evidence shows that the risky CNVs most likely affect genes involved

Table 2. Top CNVs in SCZ

CNV	Position (Mb)	Size (Mb)	Types	CNV frequency		OR (95% CI)	*P*-value	Genes	References[a]
				Cases	Controls				
1q21.1	144.6–148.0	3.4	Del/dup	0.00176 (20/11372)	0.00021 (10/47311)	8.3 (3.7–19.9)	2.2×10^{-8}	34	[36,37,**38**,39,41,45-47]
2p16.3	49.9–51.5	0.02–0.42	Exon del	0.00182 (23/12627)	0.00022 (10/45284)	8.2 (3.8–19.4)	5.5×10^{-9}	1 (NRXN1)	[35-37,**38**,39-42]
3q29	197.2–198.8	0.84–1.6	Del	0.00080 (6/7539)	0.00003 (1/39747)	17.0 (1.4–1198.4)	9.7×10^{-3}	19	[38-40,**48**,49]
3q29	196.8–196.9	0.05	Dup	0.00121 (10/8280)	<0.00013 (0/7431)	Inf (1.6–Inf)	1.0×10^{-2}	2	[39,**44**]
7q36.3	158.5–158.8	0.12–0.36	Exon dup	0.00191 (14/7322)	0.00047 (7/14814)	4.0 (1.5–11.9)	2.0×10^{-3}	1 (VIPR2)	[**38**,43,44]
15q11.2	20.3–20.8	0.5	Del	0.00551 (26/4692)	0.00192 (79/41115)	2.7 (1.5–4.9)	6.0×10^{-4}	4	[40,**47**,49,51]
15q11.2-13.1	20.3–26.4	4.1–9.0	Mat dup	0.00053 (4/7578)	0.00007 (3/41367)	7.3 (1.2–50.0)	1.0×10^{-2}	13–24	[49,**112**]
15q13.3	28.7–30.3	1.5	Del	0.00193 (21/10866)	0.00020 (9/45913)	9.9 (4.3–24.4)	2.0×10^{-9}	12	[**38**,39,44,46,47,49]
16p11.2	29.4–30.1	0.7	Dup/Del	0.00313 (31/9859)	0.00027 (8/29589)	11.6 (5.6–29.3)	1.5×10^{-12}	29	[37,**38**,39,44,49,55, 113-115]
16p13.1	14.6–18.7	1.16	Dup	0.00299 (13/4332)	0.00091 (32/35047)	3.3 (1.3–7.9)	7.1×10^{-3}	11	[40,41,**50**,51]
17p12	14.1–15.4	0.93–1.31	Del	0.00151 (8/5292)	0.00015 (6/39213)	9.9 (3.4–28.5)	5.0×10^{-5}	15	[40,**51**]
17q12	34.8-36.2	1.4	del/Dup	0.00073 (5/6882)	0.00018 (2/11255)	4.2 (1.3-Inf)	1.8×10^{-2}	18	[**39**,116]
22q11.2	17.1–20.2	1.4–2.5	Del	0.00307 (35/11 365)	<0.00002 (0/45 361)	Inf (35.9–Inf)	$<1.0 \times 10^{-16}$	53	[**38**,39,44,46,47,51-55]
22q11.2	18.9-21.9	1.5–3.0	Dup	0.014% (3/21 138)	0.085% (22/25 867)	0.2 (0.1–0.6)	8.6×10^{-4}	31	[**39**,56]

[a]For each CNV case-control frequency, ORs and *P*-values are from the bolded study. CI, confidence interval; CNV, copy-number variant; Del, deletion; Exon del, exonic deletion; Dup, duplication; Exon dup, exonic duplication; Inf, infinite; Mat dup, maternally-derived duplication; OR, odds ratio.

in specific brain functions[64]. In addition, most CNVs are not SCZ-specific; in contrast, many have effects in multiple neurodevelopmental disorders[65].

De Novo Mutations in SCZ

It is known that SCZ patients have a reduced reproductive rate[66,67], which is a negative selection pressure in evolution. However, the incidence rate of SCZ remains stable worldwide at ~1%. Therefore, there must be a genetic mechanism to supply causal factors to balance the negative selection. DNMs, defined as mutations arising sporadically either in the germ-line of the parents or at an early stage of embryonic development so that the mutations are only detected in affected individual but not in the parents, was proposed many years ago as a mechanism to offset the

negative selection[68]. This proposition was based on the epidemiological observation that paternal age is associated with an increased risk of SCZ[69-71]. This hypothesis did not receive much attention in genetic studies until recently, when high-throughput DNA sequencing of SCZ families provided direct evidence that affected offspring have excess DNMs across the genome[72,73]. Further studies of DNMs in individual genes[74,75], a particular set of genes[76,77], or the exome[78-80], also provided evidence that DNMs are enriched in SCZ patients. Direct measurement also indicates that SCZ patients have a higher mutation rate[76].

It should be pointed out that DNMs can be CNVs[48,81], but the overwhelming majority are SNVs. Due to their low frequencies, DNMs are mostly classified as rare mutations. The difference between DNMs and other rare mutations is that DNMs occur only in the offspring, not in the parents. Since the frequencies of DNMs are extremely low, most tests of association are performed at the level of selected genes (gene sets or pathways). Tests can also be conducted on the basis of functional classification of the DNMs: coding *versus* non-coding sequences, *versus* non-synonymous, and neutral *versus* deleterious. Detailed analyses of DNMs in affected offspring reveal an excess of missense and disrupting mutations in protein-coding sequences, especially those involved in synaptic functions[77,79]. While most DNMs are unique events, some are recurrent[79]. Analyses of rare mutations reach the same conclusion that the polygenic burden of rare disruptive mutations is excessive in SCZ patients[80]. The converging results from DNMs, CNVs and SNVs support the notion that these mutations are likely enriched in the same pathways and thus play similar pathological roles in the etiology of SCZ.

Conclusions and Future Directions

Some important conclusions can be drawn from the above description. First, SCZ is a polygenic and heterogeneous disorder and its genetic basis involves defects in many genes. These defects can include common SNVs, common and rare CNVs, and rare and recurrent DNMs. Overall, more than one hundred common variants and many more rare variants (including both CNVs and DNMs) are associated with SCZ. The discovery of these loci confirms that a substantial number of genetic defects may be required for the manifestation of the disease and each individual gene has a limited effect. Currently, it is not clear what proportion of these variants is common and what proportion is rare. Given that the number of genes involved is likely to be more than hundreds, the genes responsible in individual patients may or may not overlap. This genetic heterogeneity not only imposes great challenges to the discovery of risk variants, but also demands individualized treatment for optimal effect. This explains why the commonly-used antipsychotics have very different effects on different patients.

Second, many of the common variants, CNVs, and DNMs identified in recent years are not specific to SCZ. Polygenic scores calculated from risk variants for SCZ can predict bipolar disorder[12], suggesting some sharing of genetic risks between these disorders. Further examination of bipolar disorder, major depression, autism, and attention deficit and hyperactivity disorder indicates broader sharing of genetic liabilities among these disorders[82-84]. For example, ZNF804A and TCF4, loci first identified by SCZ GWASs, are associated with bipolar disorder as well[83,85]. MIR137 targets multiple genes involved in SCZ, bipolar disorder, and autism[86-88]. While the extent of sharing between these disorders may differ, the pleiotropic effects seem to extend beyond these traditional psychiatric disorders[89-92]. The extent and the identity of the variants specific to SCZ remain unknown.

Third, most variants identified so far are non-functional and non-causal. In the largest SCZ GWAS that reported 108 independent loci, 15 loci had no known genes nearby, and 36 loci had >3 genes[26]. For each of these loci, tens to hundreds of SNVs are involved. This implies that most of the variants showing association signals are most likely not causal. The functional variants at these loci remain unknown and much effort is needed to discover their mechanisms, and thus to improve our understanding of the biological mechanisms involved in disease etiology[93]. Rare variants (including CNVs and DNMs) may be pathologically causal. Unfortunately, due to the extremely low frequencies of these rare variants, only a few are reported in cases[36,78-80]. For example, the largest exome sequencing project reported a significantly higher rate of rare (frequency <0.1%), disruptive mutations in cases compared to controls among gene sets that had previously been associated with SCZ[80]. Those variants found only or overwhelmingly

in affected individuals present a realistic opportunity to establish causal relationships in functional and animal model studies.

Fourth, although we cannot be sure of their relative proportions, both common and rare variants contribute to the development of SCZ. Given that SCZ is negatively selected in evolution but maintains a stable incidence rate, we would argue that rare mutations are more likely the driving force in SCZ, and DNMs are the main counter-event to balance negative selection[94-96]. This note is consistent with the polygenic nature and heterogeneity of SCZ.

Based on what we have learned from these recent studies, future studies require extensive collaboration among investigators and across disciplines. Collaborations and consortia of investigators are necessary to assemble the sample sizes required to detect common variants with small effects and rare variants with low frequencies. SCZ GWASs organized by the PGC are successful examples of the discovery of common variants and rare CNVs associated with the disease[23,25,26,46,97]. The same model has been adapted for exome sequencing to discover rare variants[80]. Multi-disciplinary collaboration is also a current trend. Geneticists need to work more closely with clinicians, statisticians, informaticians, and computer programmers to improve the processing, integration, and analysis of large genetic, phenotypical/clinical, and genomic datasets. This is because more and more studies use the systems biology approach to collect data, and more and more studies incorporate data and information from different fields. These studies produce ever-increasingly large datasets that require specialized techniques and expertise to process and analyze. To some extent, the success of a study depends on the capability and efficiency of data processing, integration, curation, and analysis.

Looking forward, to understand the genetic mechanism of SCZ, we should focus on the following areas. The first is functional studies aiming at the discovery and understanding of causal variants at identified loci. As described above, many loci have been identified by GWASs, most of which should contain genuine variants contributing to the development of SCZ. Therefore, the time is ripe to pursue functional studies to understand the mechanism. Since most of the loci discovered by GWASs encompass large genomic intervals and contain multiple genes, deep sequencing of a substantial number of subjects is a necessary first step to discover the causal variants at these loci. Deep sequencing can provide a catalog of variants with potentially deleterious functions. By combining functional genomics analyses with molecular, cellular, and animal model studies, we hope to demonstrate that a variant, or a group of variants, causes functional changes of a gene, leading to changes of the properties of neurons such as migration, communication, and differentiation. While it may be difficult to prove the causality of the variants in SCZ, we can reasonably interpret the effects of the variants if they have functional consequences at the level of neurons or lead to behavioral and cognitive changes in animal models similar to SCZ patients. Since a single variant may not be sufficient to cause observable changes resembling SCZ, cellular and animal models accommodating multiple variants should be explored.

The second direction is to continue the search for new risk loci. There is a good reason to believe that more loci await discovery. Given the small effect sizes of common variants and modest effects at best even for rare variants or DNMs, further searching for new loci requires a significant increase of power, to which there are several approaches. The simple approach is to increase sample sizes. Since most of the well-studied Caucasian samples have been included in PGC studies, there is not much room to expand the sample size, and the collection of new samples takes time, so this is not sustainable in the long run. For samples from other understudied ethnicities, organizing consortia and collaboration will be the most effective approach to discover novel risk loci. Integration of information from independent sources, including genome-wide functional genomic data such as biological pathways, gene expression, and DNA/chromatin modification, is another approach to improving power. These functional data can be used to exclude unlikely genes/loci, effectively reducing the number of tests needed for unbiased searches across the genome, thus improving the power to discover novel loci. For rare variants, pathway- and network-based analyses are essential. Furthermore, refinement of phenotype and the use of endophenotype and multiple related phenotypes to purify samples provide another approach to improving power. As discussed above, SCZ is genetically heterogeneous, so if we combine clinical information and functional endophenotypes (such as cognitive functions and immunological responses) to screen samples and define

biologically-based disease subgroups, we can reduce the heterogeneity within the group and thus improve power. A benefit of having a biologically-defined phenotype is that it can provide insights into the underlying mechanism and provide options for treatment of the disease.

A third direction is to study gene-environment interactions. It has long been speculated that the immune system is involved in SCZ. As the immune system is the primary defense against environmental pathogens, infection with pathogens leads to the activation/dysfunction of immune responses, which can modulate the risk of developing SCZ. This is consistent with increased incidence in individuals who are migrants, have an urban upbringing[98], are exposed to maternal infection during pregnancy[99,100], and suffer childhood infection[101]. While the specifics of the interaction remain largely unknown, some studies have implicated specific genes that can serve as a model for studying gene-environment interactions[102,103]. Another well-known fact is that there is a small but consistent difference of incidence between males and females. This difference can be seen as a special case of gene-environment interaction where sex hormones are the most likely mediators. The study of gene-sex interaction can follow the models of other sexually dimorphic diseases[104]. In SCZ, the RELN gene has been shown to have sexually different effects[105-107]. Other environmental factors, such as stressful/traumatic life events and substance use may also alter the risk of developing SCZ. Systematic examination of the interactions between these environmental factors and genetic variants would provide insights into how environmental factors modulate and mediate the risk of SCZ, thus improving our understanding of the pathology of this disorder.

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·Review·

Involuntary admission and treatment of patients with mental disorder

Simei Zhang[1], Graham Mellsop[2], Johann Brink[3], Xiaoping Wang[1]

[1]*Mental Health Institute of The Second Xiangya Hospital, Hunan Province Technology Institute of Psychiatry, Key Laboratory of Psychiatry and Mental Health of Hunan Province, Central South University, Changsha 410011, China*

[2]*Waikato Clinical School, University of Auckland, New Zealand*

[3]*University of British Columbia, Vancouver, British Columbia, Canada*

Corresponding author: Xiaoping Wang. E-mail: xiaop6@126.com

Despite the efforts of the World Health Organization to internationally standardize strategies for mental-health care delivery, the rules and regulations for involuntary admission and treatment of patients with mental disorder still differ markedly across countries. This review was undertaken to describe the regulations and mental-health laws from diverse countries and districts of Europe (UK, Austria, Denmark, France, Germany, Italy, Ireland, and Norway), the Americas (Canada, USA, and Brazil), Australasia (Australia and New Zealand), and Asia (Japan and China). We outline the criteria and procedures for involuntary admission to psychiatric hospitals and to community services, illustrate the key features of laws related to these issues, and discuss their implications for contemporary psychiatric practice. This review may help to standardize the introduction of legislation that allows involuntary admission and treatment of patients with mental disorders in the mainland of China, and contribute to improved mental-health care. In this review, involuntary admission or treatment does not include the placement of mentally-ill offenders, or any other aspect of forensic psychiatry.

Keywords: involuntary admission; involuntary treatment; mental-health legislation; compulsory admission; commitment criteria

Introduction

From an ethical perspective, the involuntary admission and treatment of patients with mental disorders are often discussed from the perspective of personal liberty. However, influenced by an increasing emphasis on individual rights, the autonomy of patients with mental disorders has been growing in importance. This viewpoint may undermine the original purpose of involuntary admission and treatment, which is to provide adequate mental-health care to those individuals whose mental disorders interfere with their rational ability to consent or decline treatment. The United Nations Convention on the Rights of Persons with Disabilities adds a new perspective on non-discrimination and equality. Given this context, the legal framework for involuntary admission and treatment, and/or commitment laws pertaining to persons with mental disorder has been reformed in many countries[1, 2].

Involuntary admission and treatment generally have been accepted as a necessary measure to protect patients, others, and society. However, it remains a controversial and complex ethical and legal issue, and sometimes it is difficult to balance the rights of patients with the rights of the public. A number of international human rights documents are available to provide context and guidance. These include the Principles for the Protection of Persons with Mental Illness (or MI Principles, 1991), the European Convention for the Protection of Human Rights and Fundamental Freedoms (1950), The Declaration of Hawaii

(1983), and the Ten Basic Principles for Mental Health Law published by the World Health Organization (WHO)[3]. Many countries also stipulate a number of relevant provisions for involuntary admission and treatment that govern their national or regional mental-health care systems. The principles and procedures of involuntary admission and treatment vary among countries because of different cultures, traditions, economies, and human resources.

Criteria for Involuntary Admission

The formulation of a clear criterion for involuntary admission or treatment is a complex and cumbersome process. According to the checklist for Involuntary Admission and Treatment developed by the WHO, the criteria for detention in most countries include similar conditions: the patient must be suffering from a severe mental disorder; and compulsory treatment is necessary in the interest of the patient's health or safety, or the protection of other persons. However, these criteria are not included in all legal frameworks (Table 1).

It is worth noting that there is a difference in procedures for involuntary placement in France: the need for treatment criteria being present only in the HDT (Hospitalisation à la Demande d'un Tiers) procedure, but not in the HO (Hospitalisation d'Office) procedure[2]. According to Norwegian legislation (the Mental Health Care act of 1999 and its precursors), involuntary admission of a patient may be conducted when a patient who suffers from a psychotic disorder is a danger to himself/herself or others and/or there is a need to admit the patient to ensure that he or she receives necessary treatment. Paragraph 5 of the Danish law concerning the involuntary criteria stipulates that besides being psychotic, a patient has to be either dangerous to himself/herself or others or have a prospect of recovery if treated involuntarily.

Presence of A Mental Disorder

The basic requirement in all countries is that the patient

Table 1. Criteria or conditions for involuntary admission

Region	Country/District	Mental disorder +Danger	Mental disorder +Need for treatment	Mental disorder +Danger/mental disorder+Need for treatment	Mental disorder +Danger+Need for treatment
Europe	UK			Yes	
	Austria	Yes			
	Denmark			Yes	
	France	Yes			
	Germany	Yes			
	Italy		Yes		
	Ireland			Yes	
	Norway				Yes
Americas	Canada				Yes
	USA	Yes			
	Brazil			Yes	
Australasia	Australia			Yes	
	New Zealand			Yes	
Asia	Japan	Yes			
	The mainland of China	Yes			
	Taiwan region				Yes
	Hong Kong Special Administrative Region			Yes	

suffers from a mental disorder[4-6], but the type and severity of mental disorder that qualify a person for involuntary admission vary across jurisdictions. Some countries allow involuntary admission only for "severe mental disorder (illness)"; others stipulate specific mental disorders, such as "psychotic illness"; while the remaining countries use a broader definition of mental disorder. Thus, despite the availability of detailed international classification systems (e.g., the ICD-10 or DSM-5), the definition of "mental disorder" varies across jurisdictions. A specific ICD-10 diagnosis is rarely required, but words that cover a variety of psychiatric phenomena, mostly related to the broad concept of psychosis, are used. Whether the criteria should include mental retardation, substance abuse, or personality disorders is often contentious[7].

As described in Table 2, the 2007 Mental Health Act of the UK defines mental disorder as “any disorder or disability of the mind”. However, the Royal College of Psychiatrists of the UK has opposed having a personality disorder, in and of itself, as a criterion for involuntary admission, largely because of the unresponsiveness to available treatments. The laws of Austria, Germany, and the UK use broad concepts, but mental deficiency without psychotic symptoms, noncompliance, substance abuse, sexual promiscuity, and sexual psychological disorders are excluded from the criteria[8]. In Norway, the term "serious mental disorder", as stipulated by the Supreme Court's interpretation, includes active psychosis or deviant states of mental deficiency where the reduction in functioning is as substantial as that seen in psychosis.

In Canada, a “person with a mental disorder” means a person who has a disorder of the mind that requires treatment and seriously impairs the person’s ability to react appropriately to their environment, or to associate with others. For example, in British Columbia, involuntary admission and treatment require that the person has a disorder of the mind that causes serious impairment of the person’s ability to react appropriately to their environment, and requires care, supervision, and control in, or by, a designated facility to prevent substantial mental or physical deterioration, or for the protection of the person or others. In the Mental Health Act amendment of 1998, mental retardation was removed from the definition of mental disorder. In the USA, the state must prove that the person

Table 2. Psychiatric diagnoses for involuntary admission

Region	Country/District	Definition of psychiatric/medical diagnosis
Europe	UK	Any disorder or disability of the mind
	Austria	Not defined
	Denmark	Psychosis
	France	Not defined
	Germany	Wide diagnostic criteria
	Italy	Not defined
	Ireland	Mental illness, severe dementia, significant intellectual disability
	Norway	Serious mental disorder
Americas	Canada	Mental disorder
	USA	Not defined
	Brazil	Not defined
Australasia	Australia	Wide diagnostic or serious mental disorder
	New Zealand	Severe mental disorder
Asia	Japan	Not defined
	The mainland of China	Severe mental disorder
	Taiwan region	Severely ill
	Hong Kong Special Administrative Region	Not defined

suffers from a mental illness or disorder, which is often defined as a substantial disorder of emotional processes, thought, or cognition that grossly impairs judgment, behavior, or the capacity to recognize reality. Detention or involuntary commitment might be permitted for persons with any kind of mental retardation, epilepsy, alcoholism, or harmful drug addiction.

In Australia, involuntary inpatient treatment requires the presence of a mental illness/disorder as defined in the relevant state legislation. There are state differences in the name, specificity, severity, consequences of symptoms, and exclusions. Most Australian jurisdictions use the term "mental illness". In the majority of jurisdictions the definitions of the terms are detailed and similar to that of New South Wales where mental illness is a "condition that seriously impairs, either temporarily or permanently, the mental functioning of a person and is characterized by the presence of (a) delusions; (b) hallucinations; (c) serious disorder of thought form; (d) a severe disturbance of mood; (e) sustained or repeated irrational behavior indicating the presence of any one or more of the above… symptoms "(according to the Mental Health Act 2007, S.4). Some South Australian jurisdictions use the term "serious mental disorder". In New Zealand, Section 2 of the Mental Health (Compulsory Assessment and Treatment) Act (1992) defines mental disorder as "an abnormal state of mind (whether of continuous or intermittent nature)" characterized by delusions or disorders of mood, perception, volition, or cognition. The criteria appear to exclude persons with only a personality disorder[9].

Japan's Mental Health and Welfare Law (1995) and Hong Kong's Mental Health (Amendment) Ordinance (1997) do not define specified diagnostic categories for involuntary admission. In Hong Kong Special Administrative Region, the "mentally incapacitated person (MIP) who does not demonstrate mental illness plus abnormally aggressive or seriously irresponsible conduct cannot be detained in a mental hospital or correctional services department (CSD) psychiatric centre"[10].

In the mainland of China, a "severe mental disorder" requires severe symptoms that result in serious impairments in social adaptation (or other types of functioning) and awareness of objective reality or of one's medical condition, or result in an inability to deal with one's own affairs[11]. In the Taiwan region, only those persons whose disoriented and unusual thoughts and behavior render them unable to manage their own affairs, or who are clearly likely to injure others or themselves, can be subjects of involuntary admission. Also included in the class of severely ill are those who, due to disoriented and unusual thought and behavior, have actually injured others or themselves

Serious Likelihood of Immediate or Imminent Danger

Generally, preventing harm to oneself or to others is an important requirement of mental-health legislation[3]. The "dangerousness criterion" (threatened or actual danger to oneself or to others) is the most common additional criterion, while in some laws it is the only criterion justifying or permitting someone to be treated involuntarily[12]. However, this is not an essential prerequisite in all the jurisdictions reviewed here. Table 3 summarizes the diversity of dangerousness criteria for involuntary admission to mental-health care.

The dangerousness criteria are sufficient on its own for involuntary admission in Finland, Greece, Ireland, Portugal, and the UK, though it is not the only essential prerequisite in the UK, Denmark, Ireland, Australia, New Zealand, or Hong Kong Special Administrative Region. In the above countries or regions, the need for treatment is stipulated as an alternative criterion. And in Italy, Spain and Sweden, danger to oneself or to others is not considered as a criterion. In addition, in some countries such as Iceland, Portugal, and Spain, a lack of insight by the patient is a requirement[12].

In some Canadian jurisdictions, the dangerousness criterion is offered as an alternative, but in other jurisdictions there is a deterioration criterion. Four jurisdictions (Ontario, Nunavut, Northwest Territories, and Quebec) continue to limit danger to physical or bodily harm. In British Columbia, the word "dangerous" is not mentioned in the involuntary admission criteria, which include the need for care, supervision, and control in or by a designated facility to "prevent the person's...substantial mental or physical deterioration" or "for the protection of the person...or the protection of others". Since California adopted a standard in 1969 stipulating that a person had to be dangerous to self or to others to be considered for involuntary commitment,

Table 3. Dangerousness criteria for involuntary admission

Region	Country/ District	Danger level specified	Danger to oneself	Danger to others	Danger to oneself or to others
Europe	UK	No			Yes
	Austria	Yes			Yes
	Denmark	Yes			Yes
	France	Yes			Yes
	Germany	Yes			Yes
	Italy	No	No	No	No
	Ireland	Yes			Yes
	Norway	Yes			Yes
Americas	Canada	Yes			Yes
	USA	Yes			Yes
	Brazil	Yes		Yes	
Australasia	Australia	Yes			Yes
	New Zealand	Yes			Yes
Asia	Japan	Yes			Yes
	The mainland of China	Yes		Yes	
	Taiwan region	Yes			Yes
	Hong Kong Special Administrative Region	No			Yes

most states in the USA have passed similar acts. Some states even specify suicidal behavior, harmful attacks, *etc*., and provide clear time-frames for such behavior. Hence, the presentation of a risk of harm "as a result of mental illness" is essential for involuntary admission. To be a candidate for involuntary civil commitment in Florida, a person must be deemed at risk of inflicting serious bodily harm on another person in the near future, as evidenced by recent behaviors causing, attempting, or threatening such harm.

All the Australasian jurisdictions have a broad harm/ danger criterion; for example, in South Australia, "the person requires treatment for the person's own protection from harm (including harm involved in the continuation or deterioration of the person's condition) or for the protection of others". Harm is not limited to physical or bodily harm. In addition, most Australian states have a deterioration alternative — for example, Queensland requires a risk that the person may (a) cause harm to himself or herself or someone else; or (b) suffer serious mental or physical deterioration.

Section 2 of the mental-health code of New Zealand requires the person to have an abnormal state of mind posing a serious danger to the health or safety of oneself or of others, or the capacity of that person to take care of himself or herself is seriously diminished. Thus, the involuntary criteria do not only rely on measures of "dangerousness", but have provisions for those persons with mental disorders who have no ability to care for themselves in the community[9].

Japan's Mental Health and Welfare Law (1995) introduced two types of involuntary psychiatric admissions: compulsory admission by two or more designated physicians and admission for medical care and protection; only the former requires the patient to be likely to cause danger to themselves or others unless admitted to a hospital. Such a person shall be admitted to a national or prefectural mental hospital or other designated institution[13]. The Article 30(2) of China's 2012 Mental Health Law also provides two legal conditions for involuntary admission: the

patient has already injured himself/herself or others, or has the potential to commit the said act[14]. "Dangerousness" as an alternative condition for involuntary admission in the Taiwan region, has a definition similar to that in the mainland of China, as is only to be implemented when a severely ill person is "clearly likely to injure" others or self, or who has already acted injuriously.

Need for Treatment

Prior to 1969, most legal frameworks stipulated a specific need for treatment as a standard criterion for compulsory admission[2]. The MI Principles (Principle 16) of the WHO Resource Book on Mental Health, Human Rights and Legislation, states that involuntary admission may be considered "in the case of a person whose mental illness is severe and whose judgment is impaired, failure to admit or retain that person is likely to lead to a serious deterioration in his or her condition or will prevent the giving of appropriate treatment that can only be given by admission to a mental health facility…"[7].

In Europe, there have been many objections to this by organizations, individuals, and mental-health services users. In Italy, if a person needs urgent treatment and the treatment cannot be provided outside the hospital, involuntary hospitalization is authorized. This is very different from the dangerousness criteria used in most other jurisdictions. The 2007 Mental Health Act in the UK has a requirement that the patient cannot be detained for treatment unless appropriate treatment is available. Italy, Spain, and Sweden also stipulate the need for treatment as a criterion.

In North America, five Canadian jurisdictions (British Columbia, Saskatchewan, Manitoba, Nova Scotia, and Newfoundland and Labrador) have a specific need for treatment requirement, but eight do not, including the two largest provinces, Ontario and Quebec[15]. In the USA there is a strong tendency to replace "need for treatment" with a "dangerousness criterion".

In Australasia, most Australian jurisdictions have a requirement that psychiatric treatment is needed before a person can be involuntarily admitted. In Western Australia, the individual must have a mental illness requiring treatment.

In Asia, Japan's Mental Health and Welfare Law (1995) stipulates that family members can initiate involuntary admission if the need for treatment can be demonstrated[13]. In the mainland of China, Article 30(2) of Mental Health Law does not mention the need for treatment, but is ambiguous about the enforceability of hospitalization when there is no appropriate medical treatment available[14]. The Mental Health Law in the Taiwan region expressly requires that the person needs full-time hospitalization[16, 17].

Procedure for Involuntary Admission

Mental-health legislation usually specifies the procedure for involuntary admission. Although these procedures are heterogeneous, they all include the following sections (see Tables 4–6).

Who Should Make the Application?

Who should make the application for involuntary admission is a matter of debate. The person may be a family member, a close relative or guardian, a mental-health practitioner, or another state-appointed person (e.g., a social worker in the UK). In some countries, family members are not involved in the application at all. These differences may be affected by different cultures and processes[7].

Required Qualifications and Numbers of Assessors for the Applicability of Involuntary Admission Criteria

As an additional safeguard to protect the rights of those being detained involuntarily, the issues of who and how many assessors should determine the psychiatric/medical criteria for involuntary admission or treatment are important. The MI Principles of the WHO recommend that two medical practitioners conduct the assessment separately and independently. Generally, multiple assessments by additional qualified assessors are likely to decrease the possibility of abuse and provide the greatest protection for patients. In some countries, the clinicians who make the evaluation (such as psychiatric social workers, psychiatric nurses, and psychologists) need to be specifically trained and accredited. However, this is not possible or practical in low-income countries with a shortage of psychiatrists and general medical professionals[7].

All member states of the European Union (EU) require psychiatrists to perform the assessment upon patient admission to a psychiatric facility, although regulations for preliminary assessment or emergency assessment

Table 4. Psychiatric /medical assessment for involuntary admission

Region	Country/ District	Psychiatrist mandatory for initial assessment	Number of assessor	Deciding authority
Europe	UK	Yes	2	Med
	Austria	Yes	2	Non-Med
	Denmark	No	1	Med
	France	No	2	Non-Med
	Germany	No	1	Non-Med
	Italy	No	2	Non-Med
	Ireland	Yes	2	Med
	Norway	No	1	Non-Med
Americas	Canada	Yes	2	Med
	USA	Yes	2	Med
	Brazil	No	1	Non-Med
Australasia	Australia	Yes	2	Med
	New Zealand	No	2	Non-Med
Asia	Japan	Yes	1–2	Non-Med
	The mainland of China	Yes	1	Med
	Taiwan region	No	At least 2	Non-Med
	Hong Kong Special Administrative Region	Yes	1	Non-Med

Non-Med, non-medical; Med, medical.

differ. For example, only Austria, Greece, Ireland, the Netherlands, Portugal, Spain, and the UK require the initial assessor to be a trained psychiatrist. In the HO-procedure of France and some Federal States of Germany, any physician is allowed to make the psychiatric assessment[17].

In Norway, general practitioners or other physicians not working in a psychiatric hospital may conduct the assessment for involuntary commitment; however, a psychiatrist (or a physician and clinical psychologist approved for this) finally decides whether the patient's admission should be voluntary or involuntary after the patient arrives at the acute psychiatric unit[18].

Most EU countries require more than one expert to make the decision. However, in Belgium, Denmark, Germany, and the Netherlands, only one expert is required[17].

In most Canadian provinces, a physician in the community can authorize a short-term (24–72 h) admission. For example, in British Columbia, a person may be admitted involuntarily and treatment may commence based on a Mental Health Act certificate from a physician that is approved by the Director of a hospital, but a second certificate must be completed by a psychiatrist within 48 h.

In Brazil, a physician needs to be duly authorized for assessment by the Regional Medical Board (CRM) in which the facility is situated.

Section 8 of the 1992 Mental Health (Compulsory Assessment and Treatment) Act of New Zealand allows any medical practitioner to examine the person, assess if the person may be suffering from a mental disorder, and issue a certificate which initiates a more demanding process. Then a psychiatrist approved by the Director of Area Mental Health Services performs a more definitive assessment of the person.

In the Taiwan region, at least two specialist physicians are required to check the diagnosis of a severely ill person for involuntary diagnostic criteria. In the mainland of China, the diagnostic assessment can be conducted only by a

Table 5. Procedural regulations for involuntary admission (1)

Region	Country/District	Involuntary admission and treatment legally defined as different modalities	Detailed regulation of coercive measures	Compulsory outpatient treatment possible	Mandatory inclusion of patient counsel
Europe	UK	Yes	No	No	No
	Austria	Yes	Yes	No	Yes
	Denmark	Yes	Yes	No	Yes
	France	No	No	No	No
	Germany	Yes	Yes	No	No
	Italy	No	No	No	No
	Ireland	No	No	No	Yes
	Norway	No	Yes	Yes	Yes
Americas	Canada	No	Yes	No	No
	USA	No	Yes	Yes	Yes
Australasia	Australia	Yes	Yes	Yes	No
	New Zealand	Yes	Yes	Yes	Yes
Asia	Japan	No	Yes	No	No
	The mainland of China	No	Yes	No	No
	Taiwan region	No	Yes	Yes	No
	Hong Kong Special Administrative Region	No	No	No	No

registered psychiatrist[11].

Independent Authority and Periodical Review

In order to improve the physical and mental health of psychiatric patients, the 17th Principle for the Protection of Persons with Mental Illness and the Improvement of Mental Health suggests setting up an independent agency as a review mechanism. Such a mechanism could include medical, psychiatric, and other professional expertise to confirm the appropriateness of involuntary admission. The independent authority would make decisions to admit or retain a person as an involuntary patient according to the procedures designated by the relevant law, and to review all the patients at reasonable intervals.

Involuntary patients could apply to such a review body for release or review of their voluntary status within a reasonable time as specified by the relevant domestic legislation. A patient or his/her personal representative unsatisfied with the result would have the right to lodge a complaint in a higher court [according to Principles for the Protection of Persons with Mental Illness and the Improvement of Mental Health Care, 1991 (General Assembly resolution)].

In Europe, to restrict the physicians' discretion and medical paternalism, many countries require that the final decision of involuntary placement be transferred to a non-medical authority, such as a judge, prosecutor, or other representative of the legal or medical systems, or another agency that is independent of the medical system[12]. The 2007 Mental Health Act of the UK empowers the Mental Health Review Tribunal (MHRT) to safeguard patient interests by reviewing hospital decisions involving involuntary commitment and the current discretionary time-limit for review, and to permit automatic referral by hospital managers to the MHRT.

Other EU member states confer these rights on psychiatrists or other health care professionals. Legislated time intervals for re-evaluation or re-decision differ considerably.

Table 6. Procedural regulations for involuntary admission (2)

Region	Country/ District	Maximum between psychiatric assessment and involuntary admission	Maximum of short-term detention	Decision-making authorities for short-term detention	Maximum length of initial placement	Re-approval
Europe	UK	14 days	72 h	Police or physician plus social worker	Assessment order: 28 days; Treatment order: 6 months	28 days; 6 months
	Austria	4 days	48 h	Psychiatrist	3 months	3 months
	Denmark	24 h (D) 7 days (T)	Not separately defined	Psychiatrist	Not defined	3, 10, 20, 30 days, then monthly
	France	24 h (HO-procedure)	48 h	Mayor (Paris: police)	Not defined	HDT-procedure: 15 days, then monthly HO-procedure: 1, 3, 6 months
	Germany	24 h–14 days	24 h (15 Federal States) 3 days (1 Federal State)	Municipal public affairs office or psychiatrist	Preliminary detention: 6 weeks; regular placement: 1 year, in obvious cases 2 years	Preliminary detention: 6 weeks;regular placement: 6 months (defined by Federal State of Saarland only)
	Italy	2 days	48 h	Public health department	7 days	7 days
	Ireland	24 h	Not separately defined	Psychiatrist	21 days	21 days, 3, 6, 12 months
	Norway	3 days	Not separately defined	Psychiatrist	3 months	Every 3 months
Americas	Canada	14 days	48 h	Psychiatrist	30 days	30 days × 2, then 90 days, then every 180 days
	USA	15 days	90 days	Local court	Temporary: 90 days Not defined: more than 90 days	Temporary: not defined Not defined: annually
Australasia	Australia	24 h	72 h	Judge	Less than 3 months	8 weeks; then annually
	New Zealand	14 days	Not available	Judge	14 days	5 days
Asia	Japan	Not defined	Emergency: 72 h Temporary: 1 week	Prefecture governor	4 weeks	Not defined
	The mainland of China	Not defined	Not defined	Psychiatrist	Not defined	Not defined
	Taiwan region	2 days	5 days	Mayor	60 days	60 days
	Hong Kong Special Administrative Region	7 days	7 days	Judge	28 days	Not defined

In Norway, a patient can complain to the Supervisory Commission about involuntary admission. This commission usually consists of a lawyer (acting as a judge), a physician (not affiliated with the hospital), and two other members (who have received psychiatric treatment, or are relatives of the patients) to fully represent the interests of the patient. If the Commission finds that a patient does not meet the involuntary admission standards, it can overthrow the involuntary admission decision made by psychiatrists[18].

In British Columbia (Canada), a Review Panel composed of three or more people determines whether patients meet the standards for involuntary admission, has decision-making powers to decertify or to continue involuntary hospitalization, and determines if a person younger than 16 continues to meet the criteria set out in the Act for a "person with a mental disorder", which includes the need for psychiatric treatment.

The Review Panel consists of a medical practitioner, a lawyer, and some other person(s) who is not a medical or legal professional, with the lawyer being the chairman of the panel. A patient or anyone on behalf of the patient can apply for a hearing.

Usually review panels make a decision directly after the hearing, or within 48 h. If the majority of the panel members are of the opinion that the involuntary admission standards specified in the law are not met, the panel must cancel the patient's involuntary hospitalization.

In most states of the USA, it is a judge who decides, but there are also many states that allow the respondent to request a jury trial. Except for temporary admission without a hearing, “not sure hospital” reviews must be conducted at least annually.

In Brazil, the patient's family members or legal representative may apply to the State Prosecutor upon mandatory hospitalization. State Prosecutors convene a multi-disciplinary team that includes a medical professional, preferably a psychiatrist, to conduct a mental-health assessment to decide whether there is a need to continue the involuntary admission.

In New Zealand, a District Court Judge must decide whether there is a mandatory medical situation within 14 days after a lawsuit filed by a patient. According to the New Zealand Mental Health legislation, patients may challenge their compulsory status through a variety of means, including judicial review, and appeal to a Mental Health Review Tribunal. This Tribunal consists of a lawyer, a psychiatrist, and a lay community member. At every step, a person challenging their compulsory treatment order is entitled to free legal representation. Under this Act, compulsory assessment of a person proceeds in a stepwise process with the initial period of compulsory assessment being only 5 days prior to the completion of a reassessment. Then, before a family court judge determines whether a compulsory treatment order should be made, there are two further periods of 14 days of compulsory assessment prior to a hearing, which is reviewed on a 6-month basis prior to an indefinite order being considered. Any compulsory treatment order made under the Act is deemed to be an order for compulsory assessment or treatment in the community, unless a case can be made to the presiding judge that such assessment or treatment can only be effectively undertaken as an inpatient. Section 4 of the Mental Health (Compulsory Assessment and Treatment) Act (1992) is clearly intended to exclude persons with personality disorders from compulsory treatment under the Act[9].

Every county in Japan has its own Psychiatric Review Board. The prefectural governor appoints the members of the board of directors, including the designated physicians, jurists, and other learned and experienced persons. The board of directors has two main functions. First, to evaluate the necessity for mandatory admission, and second, to give full consideration to patient and guardian requests for discharge and improvements in medication and treatment, so as to decide whether to continue medication or how to improve treatment[13].

In the mainland of China, according to Article 32 of the law, reassessment is conducted by the original medical facility or another medical institution with the appropriate legal qualifications within three days of receiving the results of the original diagnostic assessment. If the assessment was done by another medical institution, two registered psychiatrists have to be appointed to a face-to-face assessment of the patient. The medical institution must release its evaluation immediately[11].

Maximum Length of a Compulsory Admission

There are no clear rules about the maximum duration for (initial) involuntary admission in Denmark, France,

Portugal, and Spain. In the rest of the EU countries, the first-time compulsory treatment duration varies from 7 days to 2 years[17].

In Norway, the referring physician must have seen the patient in person within 10 days prior to the compulsory hospitalization. After a patient arrives in a hospital's acute ward, a psychiatrist (or a physician and a clinical psychologist approved for Mental Health Act decision-making) is required to evaluate, within 24 h, the necessity for compulsory hospitalization[18].

In British Columbia, an initial Medical Certificate, completed within the past 14 days, gives authority for 48-h mandatory psychiatric care. A second Medical Certificate must be completed within 48 h of admission. If the second certificate confirms the need for involuntary hospital care, the treatment period is extended for 30 days from the date of hospital admission. The treating psychiatrist reviews the need for compulsory treatment after 30 days and the first Renewal Certificate stipulates treatment for an additional one month. A second Renewal Certificate can extend compulsory treatment in a hospital for an additional three months. A third or subsequent Renewal Certificate can extend compulsory psychiatric treatment for an additional six months. All initial and renewal certificates are subject to appeal by patients or their representative.

In Brazil, the period a patient can be confined in a hospital, based upon the judgment of a mental-health expert submitting a report to a prosecutor, is 72 h. The procedure also applies when patients are discharged from hospital.

In the mainland of China, according to Article 44, the law does not specify the duration of mandatory treatment or the time interval for re-evaluations. It only regulates that if medical institutions think patients no longer meet the compulsory medical conditions, patients shall be discharged from the hospital[19].

Emergency Admission

Some jurisdictions provide for emergency, short-term detention (from 24 to 72 h), immediately, at night, or during weekends. For example, Belgium allows for ten days, whereas some EU member countries have emergency short-term detention standards that are different from those governing normal involuntary admission.

The laws of almost all EU member countries distinguish between emergency short-term detention and regular compulsory detention. Only in Denmark, Finland, and Ireland, do the laws make no distinction. The duration of emergency short-term detention varies from 24 h to 10 days[2].

Mental-health legislation may combine involuntary admission and treatment into one procedure, or treat them separately. Under the "combined approach", patients admitted involuntarily may be treated without their consent. Under a fully "separate" approach, the treatment of an involuntarily admitted patient requires a separate procedure for determining if such treatment is necessary[7]. This distinction is partly due to the influence of the international human rights standards: "Principles for the Protection of Persons with Mental Illness and for the Improvement of Mental-health Care" [17].

The legal frameworks of Austria, Denmark, Germany, the UK, Sweden, the Netherlands, and Luxembourg define involuntary commitment and involuntary treatment as distinct modalities.

A number of Canadian jurisdictions allow involuntary patients to refuse treatment. For example, in Ontario, even a patient who is judged to be incompetent may refuse treatment. Refusal is considered to be in the best interest of the patient if there is no prior wish for treatment. In addition, in British Columbia the Mental Health Act (Appendix 14) states that the aim of an involuntary admission is to treat a patient’s mental disorder. Treatment is defined in the Act as "safe and effective psychiatric treatment and includes any procedure necessarily related to the provision of psychiatric treatment".

Canadian jurisdictions authorize (usually by the treating physician) involuntary treatment for the involuntarily admitted patient. British Columbia, Saskatchewan, and Newfoundland and Labrador, like all Canadian jurisdictions, do not allow patients admitted involuntarily to refuse treatment. However, patients in British Columbia may request a second opinion regarding the appropriateness of treatment. It is noteworthy that Section 31 of the Mental Health Act of British Columbia states that any person admitted to a psychiatric hospital for court-ordered treatment (those found by the courts to be unfit to stand

trial, or not criminally responsible for a criminal act) is deemed to have consented to treatment, with no further certification required to compel treatment. Other provinces do allow treatment refusal but it can be overruled. Persons who have a mental illness (whether or not they are competent) and are thought to pose a risk of harm to themselves or others can be treated without consent[20]. In New Zealand, committed patients can refuse treatment in some circumstances. This can be over-ridden by a second (psychiatrist's) opinion.

In the mainland of China, the new mental-health legislation does not allow involuntary patients to refuse psychopharmacological treatment. Moreover, there is no form of mandatory outpatient treatment specified in the law[21].

Involuntary Treatment in the Community

Involuntary or compulsory treatment in the community is a mechanism by which treatment is delivered in the "least restrictive environment"[22]. Early involuntary community programs were seen as a means to provide a less restrictive alternative to hospitalization and to increase individual autonomy. It is not clear if community compulsory care offers advantages or disadvantages in terms of outcomes, such as subsequent service use, social functioning, quality of life, or cost-effectiveness[23].

In England and Wales, Community Treatment Orders (CTOs) are included in the 2007 revision of the act. The legislation has a provision for conditional discharge, which requires the service user, still defined as an inpatient, to accept treatment in the community for extended periods[24].

In Norway, Community Care with Special Provisions (CCC) was permitted in 1961 under the law, but it can only be initiated following compulsory treatment in a hospital until 2001 when revisions were made to allow initiation of CCC without a prior hospital stay. So far, we consider CCCs to be "a least restrictive form" of coercive care.

Among the 13 Canadian jurisdictions, nine have some form of compulsory community treatment. These include six jurisdictions that have Community Treatment Orders (CTOs) provisions, two others that have conditional leave, and Quebec where court-ordered treatment can continue after discharge.

In the USA, CCC was introduced because of the enactment of "Kendra's law" in New York in 1999. Now, 42 states allow for CCC; in some jurisdictions, a compulsory order can only be issued after a period of hospitalization, in some cases after several hospitalizations[25]. All Australian jurisdictions have CTO provisions. New Zealand is one of the few jurisdictions in which a compulsory CTO can be made without the person first being admitted to a hospital.

In Japan, community support services are not mandatory, and the government has not taken the initiative to remove economic and social obstacles to the development of community support services. In the mainland of China, the mental-health law mandates that different levels of government develop and support community-based mental-health services.

Conclusion

There are many different ways of approaching involuntary admission and treatment, and they are part of modern psychiatry all over the world. Since the commitment law came into force, the involuntary commitment rate (annual number of compulsory admissions per 100 000 population) has increased, but the involuntary placement quotas (percentage of all psychiatric admissions) have remained more or less stable during the past decades, or have even decreased in some countries with financial austerity and a limited number of hospital beds[12]. While decreases are seen across Europe, the general number of psychiatric beds has increased in the mainland of China[26].

Some researchers have reported a positive correlation between rates of involuntary admission and the number of psychiatric beds, whereas areas that give priority to comprehensive outpatient care have less frequent involuntary commitments. A range of factors, such as gender, age, employment status, poverty, perceived dangerousness, and attitudes may be important in determining the manner by which jurisdictions utilize involuntary admission and coercion[27-34] .

Involuntary admission and treatment have advantages and disadvantages. In general, there is no doubt that the legislation of involuntary placement pays more attention to the psychiatric patients' right. This will prevent unnecessary involuntary admission and treatment. On

the basis of the law, the independent authority is obliged to review the patient's status at regular intervals. On the other hand, many countries have devoted much effort to minimizing the potential side effects of involuntary admission and treatment[34] . This was followed by an increasing shift from inpatient to outpatient psychiatric care. A diagnosed psychiatric disorder, imminent danger to self or to others, a causal link between the disorder and the danger, and the need for treatment, are the most frequently used determinants set out by the legal requirements for compulsory disposition. For example, according to the general trend towards evidence-based or guideline-supported procedures in mental-health care, the inclusion of standardized risk assessments could improve the assessment of the danger criterion. However, few countries currently stipulate the application of standardized risk assessment procedures as a mandatory part of a psychiatric examination. Ambiguities and lack of specificity of some provisions of the law create practical difficulties for implementation.

The mental-health legislation of the mainland of China, without detailed related administrative enactments, came into effect on May 1, 2013. This wide-ranging mental-health law on involuntary admission and compulsory treatment reformulated the key principles of the WHO. The main advantage offered by this law is to legalize the involuntary placement process, and provide suitable treatment to psychiatry patients. Meanwhile, there are some disadvantages, such as the operational problems that the introduction of a new law always entail. Besides, the regulation of involuntary admission and treatment detailedly stipulates the dangerous criterion, but with a lack of standard strategy and procedure to assess the risk of patients. Whether or not it may face challenges in practice, national data on involuntary admission and treatment will be collected to more precisely determine both the patterns and the types of diagnoses that are most commonly used in cases of involuntary placement. This wide-ranging law will fundamentally transform the provision of mental-health services in the mainland of China. Even after contentious debate, it still has the above problems and is far from satisfactory in being able to protect the legal rights and interests of involuntary patients. Like all nations, China still has a long way to go before finding the right balance between protection and control.

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·Review·

Path from schizophrenia genomics to biology: gene regulation and perturbation in neurons derived from induced pluripotent stem cells and genome editing

Jubao Duan[1,2]
[1]*Center for Psychiatric Genetics, NorthShore University HealthSystem, Evanston, IL, USA*
[2]*Department of Psychiatry and Behavioral Neuroscience, The University of Chicago, Chicago, IL, USA*
Corresponding author: Jubao Duan. E-mail: jduan@uchicago.edu

Schizophrenia (SZ) is a devastating mental disorder afflicting 1% of the population. Recent genome-wide association studies (GWASs) of SZ have identified >100 risk loci. However, the causal variants/genes and the causal mechanisms remain largely unknown, which hinders the translation of GWAS findings into disease biology and drug targets. Most risk variants are noncoding, thus likely regulate gene expression. A major mechanism of transcriptional regulation is chromatin remodeling, and open chromatin is a versatile predictor of regulatory sequences. MicroRNA-mediated post-transcriptional regulation plays an important role in SZ pathogenesis. Neurons differentiated from patient-specific induced pluripotent stem cells (iPSCs) provide an experimental model to characterize the genetic perturbation of regulatory variants that are often specific to cell type and/or developmental stage. The emerging genome-editing technology enables the creation of isogenic iPSCs and neurons to efficiently characterize the effects of SZ-associated regulatory variants on SZ-relevant molecular and cellular phenotypes involving dopaminergic, glutamatergic, and GABAergic neurotransmissions. SZ GWAS findings equipped with the emerging functional genomics approaches provide an unprecedented opportunity for understanding new disease biology and identifying novel drug targets.

Keywords: schizophrenia; genomics; open chromatin; microRNA; iPSC; neurons; genome editing

Introduction

Although schizophrenia (SZ) symptoms can be improved by current medications, there is a need for more effective treatments. Most available antipsychotic drugs are still based on the blockade of dopamine D2 receptors (DRD2s), a mechanism discovered over 50 years ago[1]. Recent SZ genome-wide association studies (GWASs) have identified >100 significant genome-wide susceptibility loci with common variants associated with disease[2-7], providing an unprecedented opportunity to understand new disease biology and identify novel drug targets. The genome-wide approach has also implicated multiple rare and large recurrent copy number variations (CNVs) of larger effect size in an increasing risk for developing SZ[8-10]. Although large-scale exome sequencing in SZ has not identified specific rare/low-frequency genetic variants or genes associated with SZ[11, 12], these studies still revealed biological insights consistent with SZ GWAS and CNV studies. This review summarizes the leading biological insights from these genetic findings and discusses conceptual and technical challenges and opportunities in understanding the disease biology underlying the exciting genetic discoveries.

Success of SZ GWAS

In the past five years, we have witnessed the success of SZ GWAS[2-7], an unbiased approach to interrogate the entire genome for SZ risk loci. SZ GWASs have demonstrated

the polygenic nature of SZ, each gene contributing a small to moderate effect[3]. With 36,989 SZ cases and 113,075 controls[7], SZ GWAS yielded unparalleled increases of independent genome-wide significant risk loci, from the previously reported 7 to the current 108 independent SZ risk loci. These discoveries not only establish the significant genome-wide association with the *DRD2* locus[7], which is central to the classical dopaminergic hypothesis of SZ pathogenesis, but also identify the enrichment of associations with genes involved in neuronal calcium signaling, dendritic spines, and post-synaptic densities[6, 7], highlighting the importance of glutamatergic neurotransmission. Most loci represent new disease biology.

Albeit the success of SZ GWAS, challenges remain. Besides the "missing" heritability, a substantial proportion of genetic risk remains unexplained; one major challenge is to understand the causal molecular mechanisms underlying these associations. This has been hampered by the fact that each risk locus often spans multiple genes and contains many equally-associated single-nucleotide polymorphisms (SNPs) (i.e., due to linkage disequilibrium, LD; r^2 >0.8) with SZ (Fig. 1). This makes it difficult to determine the causal variant and what gene(s) is affected. For instance, the GWAS association at the *DRD2* locus[7] spans not only *DRD2*, but also the adjacent *NCAM1*, a gene important for neurite outgrowth. Thus, whether SZ GWAS at this locus supports the classical dopaminergic hypothesis remains uncertain. Similarly, the SZ risk locus at chr10[6] (Fig. 1) contains hundreds of equally-associated SNPs, and spans >10 genes of which three have synaptic functions: *INA*, *CALHM1*, and *NEURL*. It thus remains to be tested whether the causal variants at this locus influence

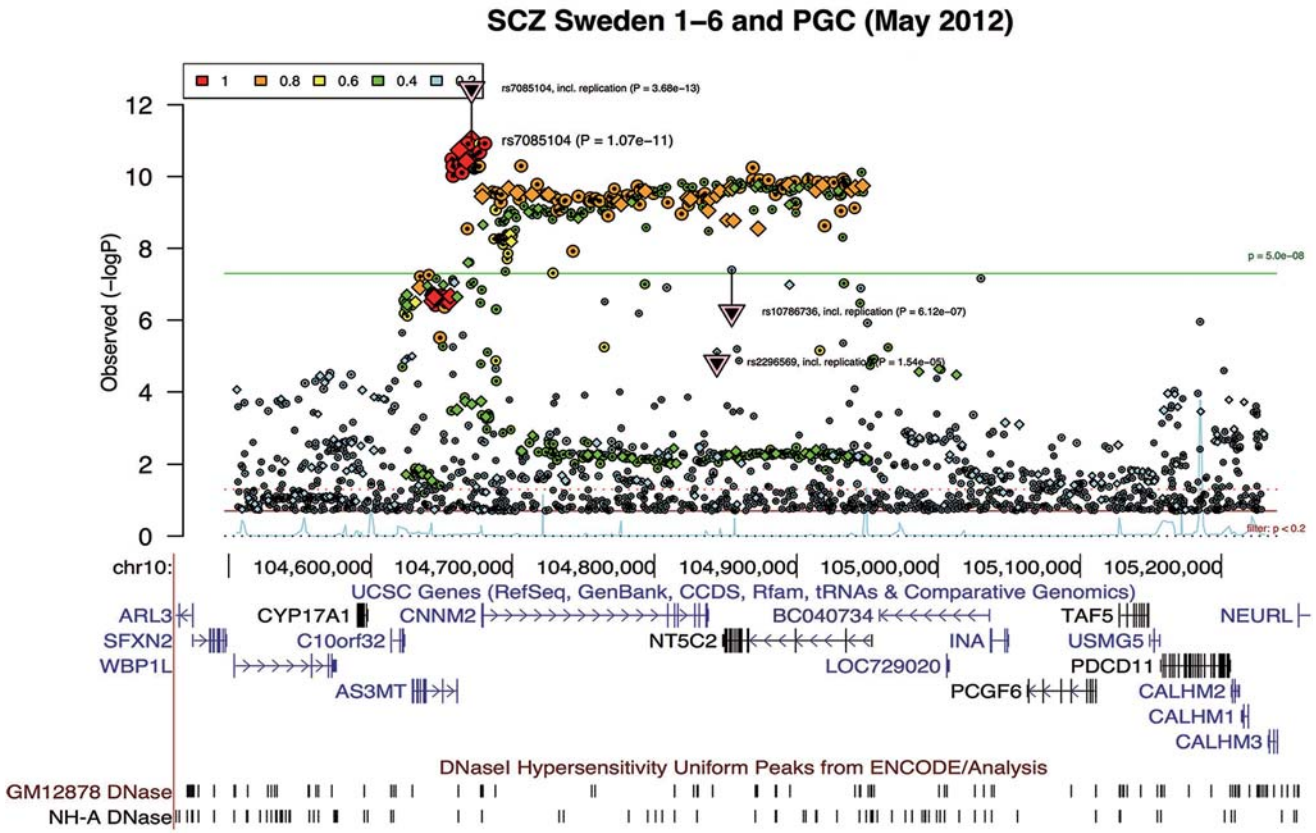

Fig. 1. Schizophrenia GWAS locus at chr10 spans hundreds of significant genome-wide SNPs in strong linkage disequilibrium (LD) and multiple candidate genes. Regional association plot and the LD information (color-coded r^2) were downloaded from Ricopili (http://www.broadinstitute.org/mpg/ricopili/)[6]. UCSC genome browser (hg19) gene track and open chromatin (DNaseI hypersensitive sites) tracks are shown below the association plot.

genes with synaptic functions. Therefore, despite the exciting GWAS findings, there is a need to identify which of the GWAS-implicated variants are functional and causal, as well as which genes are affected and how.

SZ GWASs Implicate Abnormal Synaptic Plasticity and Glutamatergic Neurotransmission

The exact pathophysiology of SZ remains unclear. Although multiple major neurotransmitter systems (dopaminergic, glutamatergic, and GABAergic) may be involved, SZ GWASs suggest a major role for glutamatergic neurotransmission, neuronal calcium signaling, and morphological changes (dendritic spines and post-synaptic densities)[6, 7]. This is not to say that other neurotransmitter systems are not important for SZ pathogenesis; for instance, one of the genome-wide significant SZ GWAS loci spans *DRD2*, a gene central to the classical dopaminergic hypothesis of SZ[1]. However, compared with other neurotransmitter systems, many more GWAS-implicated genes are involved in glutamate neurotransmission. Out of the 108 SZ risk loci, eight contain genes related to synapses or excitatory neurotransmission (Table 1). Indeed, multiple lines of evidence suggest that SZ is a neurodevelopmental disorder with impaired frontal cortical development[11-16]. Whole-exome sequencing[11, 12] and CNV studies[10] also support the role of abnormal synaptic plasticity and glutamatergic neurotransmission in SZ. Exome sequencing in 2 536 SZ cases and 2 543 controls demonstrated that rare disruptive mutations are enriched in gene sets associated with the voltage-gated calcium channel and the signaling complex formed by the activity-regulated cytoskeleton-associated scaffold protein of the postsynaptic density[11]. Another large-scale exome sequencing of SZ trios has shown that *de novo* mutations are over-represented in glutamatergic postsynaptic proteins comprising activity-regulated cytoskeleton-associated protein and N-methyl-d-aspartate receptor complexes[12]. In terms of CNVs, there is also an increased burden of the largest CNVs (>500 kb) in genes present in the postsynaptic density[10].

Table 1. SZ GWAS genes associated with synapses

Chr	*P*-value	Gene	Synaptic function
4	3.441E-08	*CLCN3*	Chloride channel; synaptic plasticity
5	1.305E-09	*GRIA1*	AMPARs; synaptic plasticity
7	2.358E-14	*ELFN1*	Postsynaptic protein
10	5.523E-17	*CALHM1*	Calcium channel; neural excitability
		INA	Transport to axons and dendrites
		NEURL	Neurogenesis
12	1.298E-17	*CACNA1C*	Calcium channel; neurotransmission
16	1.075E-09	*GRIN2A*	Mediator of synaptic plasticity
17	1.04E-09	*SRR*	Activator of NMDARs
22	8.076E-12	*CACNA1I*	Calcium channel; synaptic plasticity

These genetic findings converge with previous pathophysiological evidence of abnormalities of synaptic neurotransmission in SZ. SZ patients show reduced cortical grey matter volume and thickness, as well as reduced functional cortical connectivity[17-20]. Reductions in dendritic spine density are thought to directly contribute to these abnormalities[17, 18, 21, 22]. Specifically, reduced spine density on cortical pyramidal neurons has been reported in SZ[15, 22, 23], and cognitive function in humans has been intimately linked to dendritic spine morphology and density[15, 22, 23]. Dendritic spines, mushroom-shaped protrusions, are the sites of most of the excitatory synapses on pyramidal neurons in the mammalian forebrain[24, 25]. Spine plasticity contributes to the neural circuit remodeling that is crucial for postnatal cognitive development[26, 27]. Altered synaptic plasticity and abnormal synaptic neurotransmission provide a basis for prioritizing synaptic genes for mechanistic studies of SZ biology. The biological insights from GWASs and other SZ genetics findings further inform the cellular phenotypes to characterize in disease modelling.

Gene Regulation as A Causal Molecular Mechanism Underlying the SZ Genetic Findings

Variations in expression are expected to be as influential as changes in protein structure in shaping human-specific brain functions[28, 29]. In SZ, the best case for the importance of gene expression regulation is the gene dosage effect of SZ-associated rare CNVs of high penetrance[8-10]. For most CNVs, although it remains uncertain which gene deletion or duplication is the “driver” of the SZ disease phenotype, it is clear that a 2-fold expression difference as

a result of heterozygous deletion or a 1.5-fold expression difference as a result of heterozygous duplication can produce pronounced disease phenotypes. Recent SZ GWAS and exome sequencing further highlight the pivotal role of gene regulation in the causal mechanisms of SZ. Most risk variants are noncoding, and only ~10% of the >100 SZ GWAS risk loci have associations possibly explained by protein-coding SNPs[7], implying that most SZ causal variants may influence the expression of nearby (*cis*) genes. Exome sequencing of large SZ samples also suggests a limited role of rare coding variants in disease etiology[11, 12], further strengthening the importance of rare noncoding variants.

Transcriptional Regulation

Gene expression is regulated at the transcriptional and post-transcriptional (RNA decay and protein synthesis) levels, so noncoding variants can influence gene expression through both transcriptional and post-transcriptional regulatory mechanisms[30-32]. Compared to RNA decay, transcription remains the predominant mechanism determining individual expression variation[31]. Expression quantitative trait locus (eQTL) mapping can identify variants associated with gene expression[33, 34]. SZ risk loci (under a polygenic model and using SNPs with P <0.5) are enriched for *cis*-eQTL[35], thus likely conferring disease risk through influencing transcript abundance. However, *cis*-eQTL mapping in a sizable postmortem brain sample only identified eQTLs that could explain two genome-wide significant SZ loci[7]. The eQTL study may be improved by a larger brain sample, but it will still be limited by the well-known confounding factors associated with using postmortem brain tissue[36] and suboptimal cell types or developmental stages[37-39]. After all, eQTL analysis is still an association-based indirect test rather than directly pointing to specific functional variants.

It has been a challenge to interpret the functional noncoding sequences and predict specific regulatory variants. Classical comparative genomics predicts the regulatory function of a sequence based on its evolutionary conservation, however, sequence conservation and function are often discordant[40]. The recent ENCODE Project and Roadmap Epigenomics Program[41-43] provide rich empirical resources of chromatin state marks and transcription factor binding sites (TFBSs) in 349 cell and tissue samples for the bioinformatic annotation of functional noncoding sequences[44]. These genome-wide chromatin marks can help to predict promoters, enhancers, insulators, and TFBSs. One of the most commonly used chromatin marks is DNaseI hypersensitive sites (DHSs) of chromatin, also called accessible or open chromatin[44]. Mammalian DNAs are tightly coiled and compacted in the form of chromatin, which is a characteristic structure of repeating units of nucleosomes, each with ~200 bp of DNA winding around histone proteins[45]. The extent of chromatin compaction affects the ability of transcription factors and other protein regulators to access the regulatory sequence. Accessible or open chromatin is associated with active transcription. The chromatin state is a dynamic process with multilevel control[46], in which transcription factor binding has been suggested to be the primary driving force. Open chromatin is also correlated with epigenomic histone modifications associated with active enhancers and promoters (e.g., H3K4me1 and H3K4me3)[47-51]. In addition, >60% of methylation-eQTLs[52] are within open chromatin. A major determinant of transcription is chromatin accessibility[41, 51], and open chromatin overlies >97% of *cis*-regulatory sequences[41, 51, 52]. Open chromatin is thus a versatile index of regulatory sequence elements, and a powerful assay for screening *cis*-regulatory variants. Like other common diseases, SZ-associated variants are enriched in ENCODE-annotated open chromatin[7, 43, 51, 53]. An effort to identify functional non-coding elements in the brain (PsychENCODE program by NIMH) may complement the ENCODE-annotated chromatin state marks by providing information more relevant to neuropsychiatric disorders, thus facilitating the illumination of more specific SZ-risk variants with regulatory potential. However, the accuracy of functional annotation based on physical location in open chromatin is limited by the assay resolution (~600 bp)[40], sequence context-dependent buffering[54], and the lack of disease-relevant cell/tissue types[39]. Empirical testing of the functionality of putative regulatory variants of interest in disease-relevant cell/tissue types thus remains necessary.

Post-transcriptional Regulation

The importance of post-transcriptional regulation, namely mRNA stability and protein translation control, is becoming increasingly appreciated in understanding the dysregulation of synaptic development and function related to neuro-psychiatric disorders[55]. Dysregulated synaptic protein

synthesis is linked to the abnormal synapse formation, axon arborization, and plasticity in autism[55-57]. In support of this is the widespread and extensive lengthening of 3' UTRs (untranslated regions) that are targeted by miRNAs in the mammalian brain[58]. Targeting the dysregulation of protein synthesis opens up a novel approach for the effective treatment of some neuropsychiatric disorders[55-57, 59].

One of the biological insights from SZ GWAS is the enrichment of noncoding RNAs in top-ranking association hits of PGC (Psychiatric Genomics Consortium) SZ GWAS[6, 7]. A major player is microRNA, small (~22-nt) noncoding RNA that binds to the 3'-UTR of mRNAs, promoting RNA decay and/or repressing mRNA translation (protein synthesis). miRNA dysfunction has been suggested in neurodevelopmental disorders such as autism and SZ[9, 60-65]. Recent SZ GWASs have further strengthened evidence for an etiological role of miRNAs in SZ. Among >100 GWAS-implicated SZ-risk loci[2-7, 66], 24 loci span a total of 33 miRNA genes of which 15 are expressed in the brain (based on BrainSpan-http://www.brainspan.org). Three (MIR124-1, MIR132 and MIR137) are known to regulate neurogenesis, dendritic plasticity, and synaptic function[63, 67-80] and MIR132 also shows reduced expression in SZ postmortem frontal cortex[81, 82]. The predicted (by TargetScan) target genes of these brain-expressed miRNAs are enriched for gene ontology terms associated with neuron development, differentiation, neuron projection, axon guidance, synapse, calcium ion transport, learning and memory, and/or locomotion. There is also a 3-fold enrichment of glutamate receptors among the brain-expressed target genes of these miRNAs. These miRNA-mediated functional gene networks fit well with the known SZ-relevant cellular phenotypes such as reduced synapse density, abnormal circuit connectivity, and synaptic transmission[14-16]. Although most common SZ risk variants or their LD proxies from SZ GWAS may not directly involve the fine regulation of target gene expression, rare genetic variants in miRNA targeting sites may post-transcriptionally tune the expression of genes pathophysiologically important to SZ such as *DRD2*[32].

Rare regulatory variants conferring a risk for SZ remain to be identified. In this regard, the analytic approach for testing association with rare regulatory variants may need conceptual improvements; for instance, variants in predicted or empirically proven promoters/enhancers may not be simply aggregated together with variants in transcriptional insulators for association tests, because of possible differential effects on the direction of expression. It is clear that gene regulation may play an important role in SZ pathogenesis, but the available eQTL catalogs and ENCODE-based functional annotations have not yet provided power, cellular specificity, or developmental diversity to provide clear mechanistic hypotheses for biological follow-up. It is thus imperative to empirically identify which SZ-risk variants alter chromatin states and ultimately affect gene expression in an experimental model relevant to SZ.

iPSC-Derived Neurons as A Model for Studying Regulatory Variants in Psychiatric Disorders

iPSC-derived neurons are a good model for studying psychiatric disorders for the following reasons: (1) regulatory variants are often cell-type- and developmental stage-specific[37-39], and (2) iPSC neuron differentiation provides an experimental model pathophysiologically relevant to SZ[14, 83-88]. Other alternatives, such as human postmortem brain tissue and genetically-modified model organisms[89-91] have provided insights into SZ pathophysiology, but also have limitations. The postmortem brain is not a living tissue and thus does not capture changes at early neuronal developmental stages[88]. Furthermore, gene expression in postmortem brain is well-known to be confounded by tissue variability and environmental factors[36], and postmortem brain is not amenable to genetic modification. Animal models often do not faithfully reflect the human pathophysiology, especially for brain disorders. Moreover, animal models may not elicit the expected functional impact of human variations[92] because regulatory variants are often species-specific[93]. Although the immune hypothesis remains vital for SZ, which is indeed supported by SZ GWAS findings (the strongest SZ risk locus is at the major histocompatibility complex), after all, schizophrenia is a brain disorder. An abnormal function or expression level of a gene in peripheral blood may predispose an individual to the risk of SZ; however, the phenotypic expression of SZ is closely associated with dysfunction of the brain, manifesting as various abnormal cellular or physiological phenotypes such as reduced functional cortical connectivity[17-20] and reductions in dendritic spine density[17, 18, 21, 22]. iPSC-derived neurons thus provide a unique model to resolve more

disease-relevant cellular and molecular phenotype changes as a result of genetic perturbation.

iPSC Generation and Characterization

Most of the commonly-used source tissues or cells for iPSC production are skin biopsies and blood cells, and there are multiple ways of delivering the pluripotent re-programming factors (Oct3/4, Klf4, Sox2, and c-Myc), which have been reviewed extensively elsewhere[94]. The optimal way to generate iPSC lines would be free of virus integration, e.g., by Sendai virus[95], that has been used by the NIMH stem cell center (http://nimhstemcells.org) to establish a resource of iPSC lines for psychiatric research. As iPSC clones can vary substantially, it is necessary to characterize the pluripotency and other biochemical and epigenetic properties of different iPSC clones for the same individual. Besides the confirmation of morphology and positive immunofluorescence staining of pluripotent stem-cell markers (e.g., TRA-1-60, OCT4, NANOG, and SSEA4) (Fig. 2A), full pluripotency of an iPSC line is often confirmed by PluriTest assay (www.pluritest.org)[96] of transcriptome data (Illumina HT-12v4 array) and by the capacity to form embryoid bodies that spontaneously differentiate into the three germ layers (ectoderm, endoderm, and mesoderm) (Fig. 2B). More extensive characterization includes aberrant genetic/epigenetic modification[97], reprogramming-induced point mutations[98] and CNVs[99],

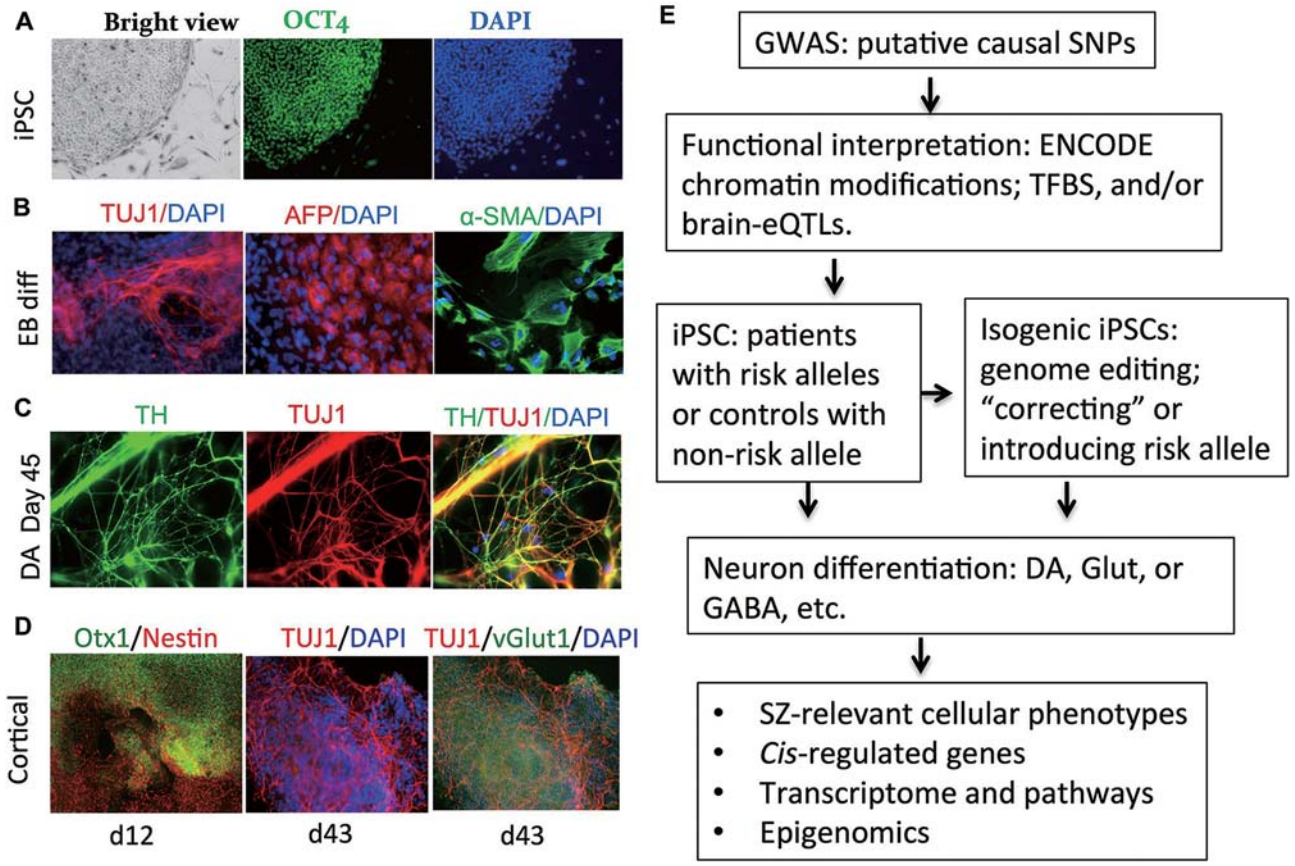

Fig. 2. Human iPSC-derived neurons as a model for studying regulatory variants of SZ. (A) Human iPSCs characterized by positive immunofluorescence staining for the pluripotent stem cell markers TRA-1-60, OCT4, NANOG, and SSEA4. Only OCT4 staining is shown. DAPI stains the nuclei. (B) Germ layers from embryoid bodies stained positive for TUJ1 (βIII-tubulin), AFP (α-fetoprotein), α-SMA (α-smooth muscle actin), markers specific for ectoderm, endoderm, and mesoderm cells respectively. (C) iPSCs subjected to dopaminergic neuronal differentiation. Neurons are TUJ1$^+$, and DA neurons are TH$^+$. (D) iPSCs subjected to cortical neuronal differentiation. Neuron progenitor cells are nestin$^+$ with cortical identity (Otx1$^+$). Neurons are TUJ1$^+$, and glutamatergic neurons are vGlut1$^+$. (E) Flowchart for the functional characterization of regulatory variants implicated by SZ-GWAS. Photomicrographs for DA neuron differentiation are from Shi *et al.* J Biol Chem, 2014[32].

or whole-genome sequencing. Because of the technical and financial limitations of scaling-up iPSC production and characterization, generating iPSC lines has often been restricted to small samples, with an emphasis on modeling specific mutations of relatively large effect, e.g., SZ-associated rare CNVs.

iPSC-Neuron Differentiation

It is important to determine what would be an appropriate neuronal subtype to derive from iPSCs to model the genetic perturbation of SZ-relevant molecular and cellular phenotypes. iPSCs can be differentiated into multiple major types of neurons (dopaminergic, glutamatergic, and GABAergic)[100] that are relevant to SZ pathophysiology. Dopaminergic neuronal differentiation from iPSCs is the most developed method with relatively high purity[86]. We have achieved ~80% dopaminergic neuronal differentiation efficiency[32] using a floor-plate-based midbrain DA neuronal differentiation method[86]. We observed neurons with DA characteristics, i.e., tyrosine hydroxylase $(TH)^+$ and pituitary homeobox 3 $(PITX3)^+$ at day 30 after plating iPSCs and denser TH^+ staining at day 45 (Fig. 2C)[32]. We also found a dynamic expression change of DRD2 that is inversely correlated with the expression of two miRNAs (miRNA-9 and miRNA-326) that target to the 3'-UTRs of *DRD2*, suggesting a pathophysiologically and developmentally relevant post-transcriptional regulation of *DRD2* by both miRNAs[32]. Consistently, we also found an inverse correlation of *DRD2* and the two miRNAs in multiple brain regions during brain development[32].

iPSC can also be efficiently differentiated into cortical excitatory neurons, mimicking a process of human cortical development[84, 85]. Given the emerging evidence of dysfunctional frontal cortical development[11-16] and abnormal glutamatergic neurotransmission in SZ[6, 7], iPSC-derived cortical excitatory neurons may be a very important experimental model for studying SZ disease biology. The cortical neurogenesis from iPSCs lasts ~2 months after neuronal induction, which is similar to the ~70-day period of cortical neurogenesis in humans[84]. The cortical neuronal differentiation from iPSCs is reproducible[85]. iPSC-derived neurons include early-born (~day 35) deep-layer $Tbr1^+$/$CTIP2^+$ neurons and later-born (~day 70) upper-layer $Brn2^+$/$Cux1^+$/$Satb2^+$ neurons, most of which are $vGlut1^+$ (~70%)[84]. We have observed ~100% neuron progenitor cells (NPCs; $nestin^+$) with cortical identity ($Otx1/2^+$) at ~day 12, and a substantial number of $vGlut1^+$ neurons at ~day 43 after neuron induction (Fig. 2D).

The most difficult type of neuron to derive from iPSCs is the GABAergic interneuron. Parvalbumin (PV) GABAergic interneurons have been shown to be relevant to SZ pathogenesis[101]. However, the above-mentioned cortical neuron differentiation procedure does not produce PV^+ interneurons[84]. Recently, human pluripotent stem cells (hPSCs) were successfully differentiated into GABAergic interneuron with mature physiological properties along a prolonged intrinsic timeline of up to 7 months, mimicking endogenous human neural development[102]. These neurons express ventral telencephalic GABAergic neuronal lineage markers (ASCL1, DLX1, and DLX5) with increasing expression intensity over time. About 75%–86% of neurons express GABAergic markers (GAD1, SLC32A1, and SLC6A1), and about 53%–78% of neurons express VGAT from 5 to 30 weeks post-differentiation[102].

A common challenge of using iPSC-neurons as a model is the heterogeneity of neuronal culture. There has been a lack of specific cell-surface markers for purifying live neurons[103]. For cortical neurons, although a new method is reported to yield 100% excitatory neurons in a much shorter time than typical cortical neuron differentiation, it requires genetically-modified iPSCs and only ~20% of neurons are $vGlut1^+$[104]. Nonetheless, with 70%–80% purity of dopaminergic neurons, glutamatergic neurons, or GABAergic neurons derived from iPSCs or hPSCs, we believe the iPSC-derived neuronal model will provide an invaluable tool for studying causal molecular mechanisms underlying the genetic findings in SZ. Alternatively, because iPSC can be quickly transformed into NPCs with high purity, and NPCs show transcriptome profiles similar to the developing brain, iPSC-derived NPCs have been proposed as a suitable model for studying the developmental aspects contributing to SZ[88].

CRISPR-Mediated Genome Editing Empowers the Study of Regulatory Variants in iPSC-Neurons

For common disease variants, directly comparing the differential expression between iPSC-neuron cultures of different subjects carrying risk alleles *versus* non-risk

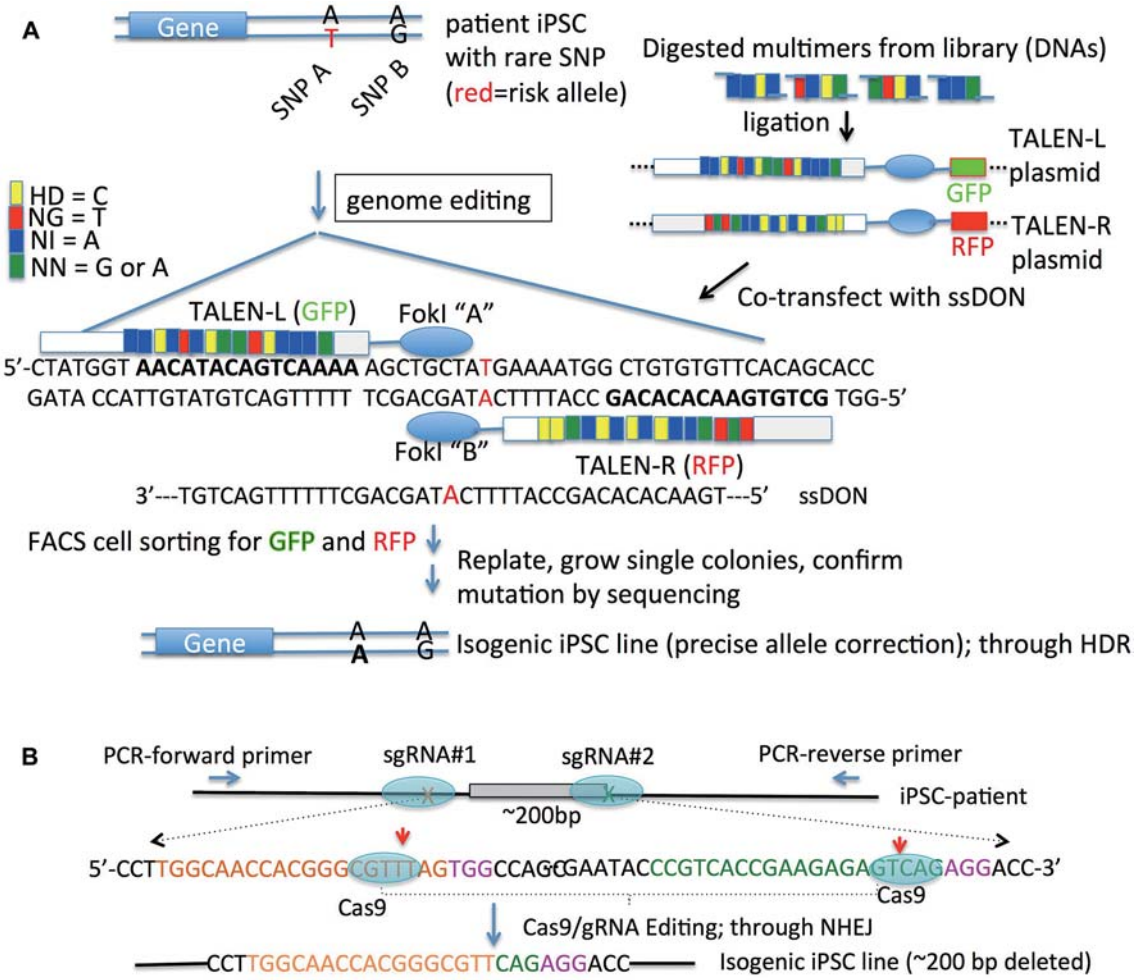

Fig. 3. Genome editing to create isogenic patient-specific iPSCs. (A) TALEN plasmids are used for precise risk allele correction (T to A for SNP A) through ssDON (single-stranded DNA oligonucleotide)-mediated homology-directed repair. (B) CRISPR/cas9-mediated an exon deletion (~200 bp) using paired sgRNAs (#1 and #2) for targeting. An isogenic iPSC line is generated with ~200 bp DNA being deleted through non-homologous end-joining (NHEJ) of the double-strand breaks of DNA. In each sgRNA, the specific target sequence (20 nt, green or orange) must immediately precede a 5′-NGG adjacent motif (PAM; pink). The red arrow indicates the expected cut site (~3 bp upstream of the NGG PAM sequence) of Cas9 nuclease. Flanking PCR primers can be designed for a rapid screen of iPSC clones carrying the deletion allele.

alleles would require a technically and financially prohibitive number of iPSCs. This is because common variants often have small effects and at the same time, there is substantial variation, in particular the variable genetic background, between iPSC lines[103]. Furthermore, although the iPSC-neuron provides a model to directly test regulatory effects relevant to SZ, the associated high cost and labor prevent scaling up. A prominent solution is the emerging genome-editing technology. Genome editing enables the generation of isogenic iPSC-neurons that differ only at the SNP site of interest, a powerful design that can overcome possible confounding effects of variable genetic backgrounds when comparing differences between cells carrying risk *versus* non-risk alleles[105-113]. With a genome-editing strategy to generate isogenic iPSC-neurons, a workflow combining bioinformatics prediction and empirical iPSC-neuronal disease modelling (Fig. 2E) will help us to link the genotype to cellular phenotypes for causal regulatory variants implicated by SZ GWAS and by future whole-genome sequencing.

Major genome-editing systems include zinc finger nuclease (ZFN), transcription activator-like effector nuclease (TALEN)[105-113] and Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/CRISPR-associated (Cas) nuclease-mediated genome editing[114-119]. ZFN has the lowest editing efficiency and the most tedious laboratory procedure. TALENs comprise a FokI nuclease domain and DNA-binding domain that can be engineered to recognize specific DNA sequences. TALEN-mediated editing substantially improves the editing efficiency, and presents higher specificity than regular CRISPR/cas9 editing because of the requirement of a dimer of DNA-binding domains to target the specific sequence flanking the variant site to be edited (Fig. 3A). Although an improved version of TALEN editing using GFP (green) and RFP (red) fusion proteins allows rapid screening for edited iPSC clones[108], constructing TALENs is still a tedious procedure (Fig. 3A) and the editing efficiency is often lower than that of CRIPSR/Cas9. CRISPR/Cas9 editing system is noteworthy for its simplicity, high efficiency, and facility for multiplexing[114-119]. With CRISPR/Cas9, a sequence-specific guide RNA (gRNA) leads Cas9 to create a DNA double-strand break at a target site, which in turn triggers DNA damage repair through non-homologous end-joining (NHEJ) or homology-directed repair (HDR). NHEJ results in small insertions or deletions (indels), while HDR introduces precision allele editing. For studying regulatory variants, one could either disrupt the regulatory sequence flanking the risk allele, create a deletion of the putative regulatory sequence using paired gRNAs, or carry out precise risk-allele "correction" with low editing efficiency (<2%) (Fig. 3). Although it has high editing efficiency, the regular CRSIPR/cas9 editing system is reported to often generate off-target editing, which can be monitored by Surveyor nuclease assay[120] or whole-genome sequencing. Improved CRISPR-mediated editing systems, such as modified Cas9 nuclease and Cas9 nickase, can substantially reduce off-target editing thus providing enhanced editing specificity[121], but it often reduces on-target editing efficiency[114]. A better understanding of the biochemical mechanism of each genome-editing system will lead to the improved design of editing tools to increase the editing specificity while retaining the high editing efficiency.

Genome-modified isogenic iPSC-neurons are thus a powerful approach to compare the functionality of different alleles of single genetic variants on the same genetic background, and using patient-specific iPSCs may further assure a genetic background maximizing the expressivity of a causal variant. However, because of the polygenic nature of complex diseases like SZ, the same single causal variant may still elicit variable functionality in genome-modified isogenic iPSCs derived from different patients with different genetic backgrounds. One way to overcome this limitation would be to characterize the functionality in isogenic iPSC lines derived from more than one patient, and when necessary, combine this with "rescue" experiments to validate the specificity of the observed functional effect.

Conclusion and Outlook

Gene expression regulation contributes substantially to phenotypic variation[28]. Although not sufficient, studying the regulatory effect of a risk allele on cellular phenotypes relevant to SZ is essential for understanding the causal role of a specific regulatory risk variant[122]. Because a simple cellular model such as iPSC-neurons has reduced system "buffering" to genetic or environmental perturbations compared to the whole organism[123], common SZ risk variants with a small population effect size may still elicit moderate or even strong effects on molecular/cellular phenotypes[124-129]. The use of isogenic iPSC-neurons as a disease-relevant experimental model is also expected to enhance the sensitivity of detecting phenotype differences by minimizing the confounding effects of variable genetic backgrounds[123]. Neurons derived from patient-specific iPSCs[14, 83-87] have been used to study SZ-relevant cellular phenotypes such as reduced synaptic density, and abnormal circuit connectivity and synaptic transmission[14-16].

Emerging technology and conceptual innovation will enhance the power of the iPSC-neuron as a model in understanding the disease biology underlying most genetic findings. First of all, defining a sensitive and specific functional assay of regulatory effect on gene expression for neuronal cells is critical. The allele-specific effect on open chromatin as measured by DNaseI HS sites can be an effective functional readout of functional screening for regulatory variants. The most recently developed Assay for Transposase-Accessible Chromatin using sequencing (ATAC-seq)[130] provides a much simpler alternative method

that requires very few cells for mapping open chromatin, which fits well with studying neuronal cells. Secondly, there is a need for high-throughput genome editing to systematically assay the regulatory effects of a large number of putative regulatory variants. Although high-throughput reporter gene assays can directly examine allelic effects on promoter/enhancer activity of short synthetic regulatory sequences[131, 132], the assayed "function" is not in the context of native genomic architecture. CRISPR/cas9-mediated genome editing of iPSCs can be used for high-throughput loss-of-function gene screening in regular human cells by sgRNA-guided exon knockout[119] or disruption of non-coding sequences; however, it is still difficult to scale up with a large number of iPSCs, and in particular, the laborious and costly production of iPSC-neurons[123]. The concept represented by the iCRISPR genome editing platform to create compound mutants may have the potential to support high-throughput genetic analysis in iPSCs[133]. Finally, there is still a lack of high-throughput functional assays of neuronal morphology and synaptic properties. Conceptual and technical innovation to develop such functional assays will be fundamental for translating the genetic findings into clinically "actionable" disease biology.

The use of human iPSC-derived cortical neurons enables us to observe molecular and cellular phenotypic alterations more relevant to SZ. However, such an "in-dish" model has limitations in capturing the cortical ultrastructure and synaptic transmission *in vivo*[123]. Any interesting finding or failure to observe an expected phenotype in iPSC-neurons may thus require cross-platform validation, e.g., in mice[134, 135]. Albeit the limitations mentioned above for mouse models and the different cortical organizations between mice and human brain[135], the basic cellular phenotypes are conserved in the two species and studying abnormal brain development and function in mice has been and remains a powerful approach for modeling genetic perturbations in neuropsychiatric disorders[134]. Indeed, future animal modeling of SZ will benefit from the genetic discoveries from GWAS and from studying iPSC-derived neurons. GWAS provides more specific gene targets for constructing more disease-relevant animal models, while functional genomics in cellular models will inform the causal molecular mechanism of a genetic variant, e.g., whether the risk allele reduces or increases gene expression. This information will thus guide whether knockout (KO) or knock-in (KI) of a target gene in mice is a more appropriate disease model. Furthermore, the current genome-editing technology has been successfully applied to animal disease modeling where one can introduce a specific genetic mutation more efficiently and with higher precision than traditional time-consuming KO/KI animal modeling. Moreover, brain disease modeling is rapidly evolving; for instance, human iPSCs have been recently used to derive 3-D "mini-brains"[136], which may ultimately allow us to understand SZ disease biology in a faithful neurodevelopmental model.

Finally, the identification of specific functional risk variants and their *cis*-regulated risk genes in broad genomic regions implicated by SZ GWAS and by future whole-genome sequencing studies holds the promise of benefiting SZ pharmacogenomics. New SZ GWAS findings provide an opportunity for developing more effective drug targets as an alternative to the classical antipsychotic drugs that mainly target DRD2[1].

SZ genetic findings may also help to predict which individuals may be more responsive to a particular drug, thus improving the effectiveness of commonly-used antipsychotic drugs. In this regard, iPSC-neurons carrying a specific SZ causal risk variant as a model may serve as a platform for screening drugs that are most effective in a specific subpopulation. Ultimately, the path from SZ genomics to biology will lead to a deeper understanding of the molecular pathogenic mechanisms[122], which will facilitate more precise SZ risk prediction and developing more effective and individualized treatments.

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·Review·

Can multi-modal neuroimaging evidence from hippocampus provide biomarkers for the progression of amnestic mild cognitive impairment?

Jiu Chen[1], Zhijun Zhang[1], Shijiang Li[2]

[1]*Department of Neurology, Affiliated ZhongDa Hospital, Neuropsychiatric Institute and Medical School of Southeast University, Nanjing 210009, China*

[2]*Department of Biophysics, Medical College of Wisconsin, Milwaukee, WI, USA*

Corresponding authors: Zhijun Zhang and Shijiang Li. E-mail: janemengzhang@vip.163.com, sjli@mcw.edu

Impaired structure and function of the hippocampus is a valuable predictor of progression from amnestic mild cognitive impairment (aMCI) to Alzheimer's disease (AD). As a part of the medial temporal lobe memory system, the hippocampus is one of the brain regions affected earliest by AD neuropathology, and shows progressive degeneration as aMCI progresses to AD. Currently, no validated biomarkers can precisely predict the conversion from aMCI to AD. Therefore, there is a great need of sensitive tools for the early detection of AD progression. In this review, we summarize the specific structural and functional changes in the hippocampus from recent aMCI studies using neurophysiological and neuroimaging data. We suggest that a combination of advanced multi-modal neuroimaging measures in discovering biomarkers will provide more precise and sensitive measures of hippocampal changes than using only one of them. These will potentially affect early diagnosis and disease-modifying treatments. We propose a new sequential and progressive framework in which the impairment spreads from the integrity of fibers to volume and then to function in hippocampal subregions. Meanwhile, this is likely to be accompanied by progressive impairment of behavioral and neuropsychological performance in the progression of aMCI to AD.

Keywords: Alzheimer's disease; amnestic mild cognitive impairment; hippocampus; episodic memory; functional magnetic resonance imaging; structural magnetic resonance imaging; diffusion tensor imaging; multi-modal MRI biomarker

Introduction

Mild cognitive impairment (MCI) is considered to be an intermediate cognitive state between normal aging and dementia[1]. Based on the profile of cognitive impairment, MCI can be further classified into several subtypes including amnestic MCI (aMCI) or non-amnestic MCI and single-domain MCI or multi-domain MCI. Single-domain aMCI involves only a deficit in memory, whereas in multi-domain aMCI the impairments involve memory and at least one other cognitive domain. Accordingly, single-domain non-amnestic MCI is diagnosed if the impairment involves a single non-memory domain, whereas the multi-domain form refers to deficits in multiple non-memory domains[1, 2]. The aMCI subtypes are considered to be the most likely to progress to AD[2]. Thus, early and comprehensive detection of the transitional stages of disease progression is of great importance for intervention and delay of the clinical symptoms of dementia.

In general, episodic memory deficit is typical in AD and involves the abilities to retain, recall, and encode information related to personal events and experiences occurring at specific times and places[3, 4]. The hippocampus plays a pivotal role in processing episodic memory[5] and is one of the earliest regions affected by AD neuropathology[6]. Structural and functional changes in the hippocampus are

valuable indicators of possible progression from aMCI to AD. Neuroimaging techniques for evaluating hippocampal function are useful in the assessment of disease progression and trajectory and the related pathology[7]. Previous studies have indicated that hippocampal atrophy is the most prominent structural hallmark of progression from aMCI to AD[8, 9]. Therefore, it is reasonable to assume that structural and functional changes in the hippocampus are important in understanding the origins of impaired memory function in AD.

A biomarker is a parameter that can be considered as an indicator of normal biological processes, pathogenic processes, or pharmacological reactions to therapeutic drugs[10]. While most people with aMCI progress to AD, others develop non-AD (i.e. Parkinson's disease, vascular dementia, *etc.*), remain in the aMCI state, or revert to normal. Any increase in the ability to predict the outcome of aMCI will be invaluable for counseling patients, advising on disease-modifying treatments, and programming clinical trials[11]. Accordingly, there is a strong need for specific and sensitive tools or biomarkers as aMCI progresses to AD, with an emphasis on early diagnosis and disease-modifying treatments. To date, however, no available biomarkers can distinguish aMCI converters from non-converters.

In recent years, most attention on aMCI has been focused on two main research questions—how to predict the progression of aMCI to AD, and which measures protect against conversion to dementia in disease-modifying clinical trials. In this review, we provide a brief summary of recent hippocampal research in aMCI patients, with special emphasis on the combination of multi-modal MRI biomarkers based on neurophysiological, neuroimaging, and neuroanatomical data.

Multi-Modal Neuroimaging Techniques in the Hippocampus of aMCI Patients

aMCI, the assumed pre-stage of AD, has been extensively described with regard to the underlying pathology of AD. Therefore, most current studies have probed for potential surrogate markers of disease progression. Multi-modal neuroimaging techniques are powerful tools for creative exploration of the epidemiology, diagnostic sensitivity, progression, and therapeutic targets for aMCI. Numerous candidate measures have also been used in cross-sectional and longitudinal neuroimaging studies. Recently, advanced analytical techniques have become available, further improving our ability to discover disease-associated pathological changes and clinical correlations *in vivo*.

Structural MRI in the Hippocampus of aMCI Patients

Structural magnetic resonance imaging (sMRI)-based image analysis has the potential to automatically track brain atrophy at multiple time-points. sMRI has demonstrated that fine-scale anatomical changes are associated with cognitive decline and occur in a spreading pattern that mirrors the advance of pathology[12]. Therefore, the extent of brain degeneration in aMCI can be quantified using structural techniques such as MRI alone.

Using different image analysis techniques in cross-sectional studies, researchers have revealed marked structural changes in the hippocampus of aMCI patients. Mostly, the hippocampal gray matter (GM) volume in aMCI patients is smaller than controls, but larger than in AD, and has a left-right asymmetric pattern of atrophy[13-16], although inconsistent findings have also been reported[17]. Recently, Fouquet and colleagues have also demonstrated that memory-encoding deficits appear to be specifically linked to atrophy of the hippocampal CA1 subfield, while the episodic retrieval impairment seems to be caused by a more distributed tissue loss in aMCI[18]. Using semi-automatic segmentation of hippocampal subfields, Pluta and colleagues have demonstrated that the volumes in aMCI are reduced in CA1 and the dentate gyrus (DG), as well as the head and tail subfields of the hippocampus compared to controls, and significant differences are found in the bilateral hippocampal volumes[19]. Notably, based on the above studies, variability of atrophy has been found between the left and right hippocampus and in different subregions of the hippocampus in different aMCI studies. Nonetheless, the complementary findings highlight the fact that the deficits in aMCI seem to be in a left-right asymmetric pattern and are associated with different hippocampal subregions. This raises the possibility of a distinct sequential progression of atrophy of hippocampal subregions in aMCI patients. Furthermore, the variation suggests that the hippocampus needs to be divided into more precise subregions based on structure and function.

In longitudinal studies, the importance of subicular and CA1 volume as a biomarker for aMCI conversion has been studied using different techniques in different numbers of patients. Most of the studies found that smaller hippocampal volumes, predominantly in the subicular and CA1 areas, are related to an increased risk for conversion from aMCI to AD[20-24], despite some inconsistent findings[25, 26]. Furthermore, hippocampal volumes are associated with the presence of one or two APOE ε4 alleles in aMCI, which can accelerate the accumulation of subsequent clinical pathology of AD[27-29]. Interestingly, Arlt and colleagues have shown that loss of GM in the left hippocampus is correlated with poorer performance on the Boston Naming Test, Mini-Mental Status Examination, and trail-making test B in aMCI over time[30]. Notably, although these studies used different analysis techniques and follow-up times (1, 2, 3, or 6 years), they consistently indicate that the hippocampal atrophy is greater in aMCI converters than non-converters, and further suggest that hippocampal subregions, especially CA1 and the subiculum, can better predict the conversion of aMCI to AD. Therefore, more attention should be paid to the interaction between hippocampal volume and APOE genotype, which may contribute to the varied results.

In addition, to precisely distinguish aMCI from controls, aMCI converters from non-converters, and aMCI from AD, several highly accurate quantitative studies have been performed. For example, Pennanen and colleagues have reported that the overall classification by total hippocampal volume between AD patients and controls is 90.7% (sensitivity, 85.4%; specificity, 94.9%), and that between AD and aMCI patients is 80.5% (sensitivity, 77.1%; specificity, 83.1%)[31]. Jack and colleagues have also reported that the odds ratio for progression to sporadic AD over 1.2–4.8 years is 1.75 in aMCI patients with smaller hippocampal volumes when compared to those with larger hippocampal volumes[32]. In a 3-year donepezil/vitamin E/placebo study of aMCI, Jack and colleagues further demonstrated a higher hippocampal atrophy rate (5.44%) in the placebo group than in the others[23]. Using hippocampal atrophy data from different time points (0, 6, 12, 18, 24, and 36 months), Leung and colleagues demonstrated that the rates in aMCI patients accelerated by 0.22%/ year^2, and the acceleration of hippocampal loss may contribute to the progression of aMCI to AD[33]. However, all of the studies were based on data-driven methods, which do not generate a precise cut-off value that separates MCI converters from non-converters. In addition, most of these studies based their MCI diagnosis on clinical grounds alone, without usng a neuropsychological score as a cut-off. Further, these studies were based on a relatively small sample size, which may influence the statistical power. This can also explain some of the discrepancies and may lead to overestimation of the classification rate.

Diffusion Tensor MRI in Hippocampus of aMCI Patients

Diffusion tensor imaging (DTI) is a noninvasive and quantitative MRI technique that can assess the orientation and integrity of white matter (WM) tracts by measuring the diffusion of water molecules in the living human brain[34]. Most studies use DTI measures of mean diffusivity (MD) and fractional anisotropy (FA) as markers of cerebral integrity, although the decomposition of individual eigenvalues to axial and radial diffusivity is becoming more common. MD provides a measure of translational diffusion, and FA provides a measure of the directionality of diffusion. In intact tissues, MD is restrained by barriers to free diffusion and FA is determined by the parallel organization of the tissue[35].

The number of studies using the DTI approach to examine WM integrity in aMCI has greatly increased over time. Furthermore, DTI has been considered to be sensitive in exploring hippocampal changes[36]. For example, Fellgiebel and colleagues have demonstrated that aMCI patients show increased MD and decreased FA in the left hippocampus, with no significant changes in the right hippocampus[37]. However, Mulle and colleagues evaluated the cross-sectional discriminative accuracy of hippocampal volume and DTI measures with regard to aMCI and demonstrated increased MD and reduced FA in the bilateral hippocampus, particularly on the left side[38]. This could be used as a sensitive cross-sectional marker for detecting subtle hippocampal abnormalities related to aMCI[38]. Using the tract-based technique, Zhou and colleagues examined pathways connecting the hippocampal region to the posterior cingulum and the hippocampal region to the whole brain in a sample of aMCI patients and healthy controls. They demonstrated a significant reduction in the

number of fibers in the pathways from the hippocampus to the whole brain only in the aMCI patients[39], which indicates that the neurobiological changes associated with aMCI may be detectable, especially at the neuronal and axonal levels.

In a recent cross-sectional study, Cherubini and colleagues used the DTI method to detect microstructural changes in aMCI and observed damage (MD increase) in the bilateral hippocampus relative to controls, but no differences in FA values[40]. Lee and colleagues have demonstrated that aMCI patients show reduced FA in the fornix, which is associated with decreased anterior CA1 and antero-medial subiculum thickness[40]. Furthermore, both reduced fornix FA and hippocampal volume are linked to reduced episodic memory, but only hippocampal volume predicts episodic memory in models including both hippocampal and fornix predictors[41]. Converging findings suggest that aMCI patients present with microstructural damage in the hippocampus (increased MD, reduced FA, or both), which may be explained by increased intercellular space and elevated extracellular water content as well as by degenerative neuronal loss and Wallerian degeneration. However, it remains unknown whether the detectable DTI changes in WM are due to amyloid or neurofibrillary tangle formation, microvascular disease, or other as yet unknown processes.

In addition, the DTI technique has recently been used to discover relationships between brain-wide WM integrity and cognitive ability in old age[42]. A higher MD of the hippocampus is associated with worse memory performance, while FA of the hippocampus is not associated with memory performance[43]. However, none of these studies assessed the relationship between microstructural damage in the hippocampus and memory performance in aMCI. Numerous studies have also demonstrated that the hippocampal subfields are differentially affected by pathological damage, and DTI parameters may be more sensitive and quantifiable measures of early degeneration in AD than conventional MR imaging techniques[44]. Another important consideration is that a few studies using DTI measures report no differences in FA values of the hippocampus in aMCI patients. This indicates that changes in anisotropy in the whole hippocampus as an ROI may not be easily detected by DTI during aMCI, as atrophy occurs in different subregions from shape analysis[45], and may only be apparent at a later stage of disease progression. Furthermore, most studies were performed with small sample sizes, so statistical power was decreased, and they differed in disease characteristics (e.g. level of cognitive impairment), both of which may lead to increased variability. Also, the diagnostic criteria for aMCI are not uniform and longitudinal follow-up is generally lacking. The use of ROIs instead of whole-brain voxelwise or tract-based approaches may provide differential sensitivities for group differences in DTI measures. Finally, differences in scanning parameters such as voxel size, may introduce differences into partial volumes, causing measurement differences between studies.

Functional MRI in the Hippocampus of aMCI Patients

Functional magnetic resonance imaging (fMRI) is a noninvasive technique that does not require the injection of a contrast agent. This method reflects synaptic activity through changes in blood flow and the oxyhemoglobin: deoxyhemoglobin ratio[46]. fMRI also measures the interregional temporal correlations between spontaneous blood oxygenation level-dependent (BOLD) fluctuations and neurophysiological activity in spatially separated, but functionally related brain regions at rest or during task performance[47]. The fMRI approaches mainly include resting-state fMRI (R-fMRI) and task-based fMRI. R-fMRI experiments are relatively free of subject compliance and training demands[47], making this approach especially attractive to dementia researchers. In task-activation fMRI, the neuronal response depends on the type of task, the severity of cognitive impairment, and the participant's attention and motivation[48]. It is difficult to study the functional connections between spatially isolated brain regions that are activated by task-specific fMRI approaches.

There is increasing evidence that alterations in synaptic function occur very early in the disease process of AD, possibly long before the progression of clinical symptoms and even significant neuropathology[49]. It follows that fMRI may be a particularly useful technique for detecting changes in brain function that are present very early in the progression of AD. In this section, we review fMRI data regarding functional abnormalities of the hippocampus in aMCI.

Resting-State Functional MRI

Recently, R-fMRI has attracted increasing attention and

has also been extensively used on the hippocampus of aMCI patients. Numerous R-fMRI cross-sectional studies have demonstrated altered hippocampal connectivity with cortical and subcortical regions in aMCI patients, who can be characterized by abnormalities in resting-state hippocampal connectivity. Recently, in our research group, Li and colleagues have demonstrated that the regional coherence of R-fMRI signals within the hippocampus decreases significantly in aMCI patients. These data can be used to differentiate between aMCI patients and controls[50]. Bai and colleagues selected the whole hippocampus as the ROI and found that aMCI patients show significantly lower hippocampal functional connectivity (FC) with the prefrontal, temporal, and parietal lobes, and the cerebellum[51]. Bai and colleagues further divided the bilateral hippocampus into six subregions as seeds and have indicated that decreased FC of these subregions is related to episodic memory declines in aMCI patients. The sensitivity is 83.3% and the specificity 91.7% when differentiating aMCI patients from controls, and the sensitivity is 83.3% and the specificity 83.3% when differentiating progressing from stable aMCI patients[52]. In a recent fMRI study, Xie and colleagues selected the whole hippocampus as the ROI and have also shown diminished hippocampal FC with the right frontal, left temporal, and insular regions. These findings may be associated with the impairment of episodic memory and cognitive decline in the early stage of AD[53]. Based on these studies, the complementary findings consistently emphasize that the impairment of episodic memory in aMCI patients may be directly associated with decreased FC of some of the hippocampal subregions within the temporal cortex.

Furthermore, our research group has been advancing the FC study of the hippocampus in aMCI patients by improving different methodologies. Our studies have routinely divided the hippocampus into more precise subregions, established the association of FC with neuropsychological performance, and increased the statistical power by using large samples[52, 53]. A longitudinal aMCI study from another research group has also indicated that the left hippocampus has significantly reduced connectivity with the right parahippocampal gyrus and bilateral hippocampus. The right hippocampus shows significantly decreased connectivity with the left cuneus, right parahippocampal gyrus, right posterior cingulate cortex (PCC), and left hippocampus after 3 years in aMCI patients[53]. Moreover, long-delayed memory scores are significantly positively correlated with connectivity within the left hippocampus itself. In addition, MMSE scores are positively correlated with the connectivity between the left inferior temporal gyrus and the right hippocampus[54].

In contrast, Qi and colleagues have used the FC analysis method and found that impairment and compensation coexist in the disease progression of aMCI[55]. Accordingly, other recent studies have also shown both increased and decreased hippocampal connectivity in aMCI patients. For example, Bai and colleagues have shown that the hippocampus has increased connectivity with more diffuse areas of the brain in aMCI patients. Those regions associated with increased FC with the hippocampus have a significantly negative correlation with episodic memory performance[51]. Wang and colleagues have found that baseline increases are followed by longitudinal decreases in the left and right hippocampal FC in aMCI patients, suggesting that the initially reported enhanced connections are a compensatory process[54]. Moreover, Das and colleagues have also reported increased FC between the entorhinal cortex (ERC) and the anterior hippocampus in aMCI patients[56]. Xie and colleagues have shown increased FC with the left PCC, the left caudate, and the right occipital gyrus. This suggests that aMCI patients can recruit network resources, primarily from the frontoparietal regions, to compensate for the losses due to the degenerative process of the disease. The increased connectivity between the hippocampus and PCC offsets the disruption of the hippocampal–temporal connectivity in early aMCI[53]. Numerous studies have demonstrated and proposed that decreased input from the ERC to CA3/DG due to reduced integrity of the perforant pathway, which is particularly vulnerable to early AD pathology, is related to the hyperactivity reported in CA3 neurons in aging rodents and humans[57, 58]. Furthermore, this hyperactivity may relate to increased connectivity with outputs to the ERC. Therefore, the increased FC may have allowed more input from those regions into the hippocampus. Converging evidence suggests that patients with aMCI can use different brain areas and employ unique strategies for storing and recalling information, presumably as a compensatory mechanism for cognitive decline[59]. Furthermore, this increased connectivity attempts to bolster

stabilization of the resting-state network as neuropathology spreads in aMCI and may represent an attempted compensatory response to AD neuropathology.

The above studies, however, did not explore full-scale information from the whole brain, manually draw ROIs, use small and nearby ROIs, or provide results in coarsely-divided subregions of the hippocampus (head, body, and tail). Even though the body region can be divided into CA and DG subfields in the anatomical images, this segmentation is susceptible to confounding effects arising from signal variations caused by non-neuronal sources. Furthermore, all of these studies are limited by the spatial resolution of BOLD fMRI data (3 mm^3), whereas ≤2 mm^3 is commonly regarded as the appropriate resolution for detailed functional studies of the hippocampus[60]. In addition, transient brain and body states may influence fMRI measures at the time of imaging. Such transient states include multi-domain psychological (attention and sensory processing of irrelevant stimuli) and physiological states (arousal, sleep deprivation, or substances with pharmacologic central nervous system activity). Particularly, these studies were performed in a relatively small cohort regarding the unrelated samples (i.e. patients with diagnosis by mistake) that may have influenced the statistical power or sensitivity resulting in the identification of additional abnormal areas.

Task-Based Functional MRI

Recent task-based fMRI data from aMCI patients are also beginning to uncover relationships between abnormalities of functional activity in the hippocampus and functionally-connected cortical and subcortical regions. These data will contribute to our understanding of fundamental memory processes in the human brain and how they are perturbed in memory disorders.

Using a task involving the repetitive presentation of faces, Johnson and colleagues have shown that aMCI patients do not display the same slope of hippocampal hypoactivation with face repetition as that seen in older controls[61]. Using a face-name associative paradigm, Petrella and colleagues have found no differences in hippocampal activation during encoding, but reported left hippocampal hypoactivation during the retrieval condition (forced-choice recognition) in aMCI patients compared to controls. Furthermore, hippocampal hypoactivation is correlated with the clinical severity of memory loss in aMCI patients[62]. Using an item-based old/new recognition retrieval task, Johnson and colleagues have demonstrated right hippocampal hypoactivation in aMCI patients compared to controls[7]. In contrast, several studies have shown greater hippocampal activation in aMCI patients relative to controls. Using a visual encoding task, Dickerson and colleagues have shown that both hyperactivation in relatively more impaired aMCI patients and greater activation within the hippocampal formation are correlated with better memory performance[63]. In a separate study using the associative face-name encoding task, Dickerson and colleagues found hippocampal hyperactivation in very mild aMCI relative to controls[64]. The reason may be that the aMCI patients in that study were very mildly impaired based on the Clinical Dementia Rating, MMSE, and neuropsychological data, as well as performance of the fMRI memory task similar to controls. By separating the aMCI spectrum into two subgroups, the milder end and the more impaired end, Celone and colleagues demonstrated hyperactivation in the bilateral hippocampus in very mild aMCI, but hypoactivation in more impaired aMCI in an associative face-name encoding paradigm[65]. Using an item-based task with words, the first event-related subsequent memory study indicated that aMCI patients have activation in the rostral left hippocampus to a greater degree than controls[66]. These aMCI participants were at the more impaired end of the spectrum based on MMSE scores, but neuropsychological data indicated milder impairment compared to controls. In addition, the aMCI participants performed similarly to control participants in the fMRI memory task. Additional studies using event-related fMRI tasks[67] will be useful in determining whether increased activation of the hippocampus in aMCI is specifically associated with successful memory, as opposed to a general effect that is present regardless of success, possibly indicating increased effort. Using a visual scene-encoding task, Miller and colleagues also reported that greater hippocampal activation predicts a greater degree and rate of subsequent cognitive decline after controlling for baseline degree of impairment, age, sex, education, hippocampal volume, and APOE status[68]. Yassa and colleagues used a continuous recognition task that taxes the pattern-separation ability between similar studied and unstudied lure test items, and found hyperactivation localized to the CA3/DG subfield in aMCI patients[69]. Using

the encoding and retrieval phases of a memory task, de Rover and colleagues have reported that the bilateral hippocampal activation in aMCI patients and controls relies upon load, with the former activating significantly more than controls at low loads and significantly less at higher loads[70]. Using an incidental emotional memory test and fMRI measures, Parra and colleagues have shown that the recognition pattern in hippocampal activation is similar in aMCI patients and controls, but the extent of activation is stronger in the former[71]. Recently, in a likelihood-estimation meta-analysis of 28 fMRI studies, Nellessen and colleagues indicated that aMCI patients show enhanced right hippocampal activation during memory encoding and reduced activation in the left hippocampus during retrieval tasks[72].

Taken together, the evidence suggests that the variability in fMRI data from aMCI patients probably relates to the complex relationships between the severity of clinical impairment and their performance in the memory task used as the fMRI paradigm[73, 74]. One explanation is that these studies do not require participants to perform below a particular cutoff on neuropsychological memory tests, and therefore, some patients perform relatively well on neuropsychological testing, despite clinical manifestations of impaired memory in daily life. In addition, there may be two phases of decreased and increased hippocampal activation in the progression of aMCI. The phase of increased hippocampal activation may reflect a compensatory response to the pathology of AD. It is also probable that hyperactivation in the hippocampus reflects cholinergic or other neurotransmitter upregulation in aMCI patients[75]. Further research is needed to clarify these relationships and provide a deeper understanding that will be pivotal to our interpretation of imaging data.

A number of other factors are also likely to contribute to the variability in hippocampal activation. Some of the differences between studies may be due to the use of memory tasks that require different abilities and psychological resources. Examples of such differences are those between paired-associate and item-based memory, encoding and retrieval, and visual and verbal material. Further differences could stem from imaging analysis techniques (e.g. ROI *versus* voxel-based whole-brain methods) and the dependent variables that decide the level of activation (e.g. extent and magnitude of activation and voxel-based measures that include both extent and magnitude), and differences in age, sex, education, and APOE genotype[63]. Future studies will be crucial to clarify the contributions of these and other confounding factors to the variability in fMRI measures, if such techniques are to be converted into biomarkers for clinical trials.

More recently, the hippocampal neuroplasticity in aMCI patients has attracted increased attention and has been extensively investigated in fMRI studies. Using an auditory-verbal fMRI task, Rosen and colleagues have shown increased verbal memory scores and increased left hippocampal activation in a cognitive-training aMCI group compared to a control group. These data suggest that the hippocampus in aMCI may retain sufficient neuroplasticity to benefit from cognitive training[76]. In a randomized, controlled, single-blind fMRI study, Hampstead and colleagues demonstrated that, prior to mnemonic strategy training, aMCI patients show attenuated hippocampal activity compared to controls during both encoding and retrieval. After training, aMCI patients show increased activity during both encoding and retrieval, while there are no significant differences between the aMCI and matched-exposure controls in the right hippocampus during retrieval. This suggests that mnemonic strategy training promotes hippocampal function in a partially restorative way[77].

Combination of Structural MRI, Diffusion Tensor MRI, and Functional MRI Approaches in the Hippocampus of aMCI Patients

Recently, the combination of multi-modal MRI approaches to examine the hippocampus of aMCI patients has begun to show efficiency in asessing the progression of disease in precisely separated aMCI converters and non-converters, and in establishing objective and quantitative biomarkers for the conversion of aMCI to AD[78, 79].

Combining structural MRI and DTI techniques, Muller and colleagues have reported decreased volume in the left hippocampus and significant increases in MD in the bilateral hippocampus in aMCI patients compared to controls. These deficits are associated with poor verbal memory performance, and the data further suggest that a combination of macro- and microstructural parameters can enhance the early detection of neurodegenerative processes[80]. Subsequently, this research group compared the diagnostic accuracy of structural MRI and DTI techniques and reported that the left and right hippocampus

have different predictive power in these techniques[38]. By combining structural MRI and fMRI approaches in a visual object encoding task, Hamalainen and colleagues showed that aMCI patients have more atrophy in anterior parts of the left hippocampus and greater activation of the caudal hippocampal formation than controls. This information suggests that increased activation of the caudal hippocampus is compensatory, due to the initial atrophy in the anterior hippocampus[81]. Combining cortical volume and thickness measures with cognitive tests to predict the conversion of aMCI to AD, Liu and colleagues found decreased hippocampal volumes in progressive aMCI patients compared with controls and stable aMCI patients. However, this combination does not improve the accuracy of the measurements[24]. Combining structural MRI and fMRI methods, de Rover and colleagues showed that the functional activation deficit in aMCI is accompanied by structural atrophy in the hippocampus, which suggests that the decrease in hippocampal activation may contribute to the decreased amount of GM[70]. Palesi and colleagues combined structural MRI and DTI methods and reported that aMCI patients have reduced volumes and increased MD relative to controls, and verbal memory in MCI is correlated with the FA of total WM in the hippocampi and hippocampus-precuneus/PCC tracts. Both DTI and hippocampal volume measurements can reveal early signs of AD in aMCI patients[82]. In a meta-analysis that compared DTI to hippocampal measurements, Clerx and colleagues revealed that the effective size of the hippocampal MD is better than that of hippocampal volume in distinguishing aMCI from controls, and MD values are more discriminatory than FA values[83]. Douaud and colleagues examined both volumetric and microstructural abnormalities and found that progressive aMCI patients have significantly smaller GM volume in the left CA region and significantly higher MD in the left hippocampus compared to stable aMCI patients[84]. These findings highlight the benefit of using information about microstructural damage and traditional GM volume to detect early, mild abnormalities in aMCI patients prior to clinical progression to AD[84]. Another recent study combined structural MRI and fMRI methods and demonstrated that aMCI patients show GM volume loss in the bilateral hippocampus and significant decreases in the amplitude of low-frequency fluctuation (ALFF) in the left hippocampus. The GM volume and ALFF are correlated, suggesting that the anatomical and functional deficits are linked[85].

Based on these studies, the combination of multi-modal MRI approaches in the hippocampus can improve the precise classification of aMCI patients and controls and promises early detection of neurodegenerative processes. These results, however, are not consistent due to the differences in sample size, different research centers, and the ambiguous clinical stages of disease progression. Furthermore, sequential associations have not been established between macrostructural (volume and cortical thickness), microstructural (fiber integrity), and functional changes in the hippocampus (functional activation and functional connectivity), and neuropsychological performance. Further research is needed to establish a predictive model by combining these parameters of multi-modal MRI as predictors.

Perspectives

Numerous neuroimaging studies have indicated differences between “converters”, “stable” and “improved” groups of patients when analyzed retrospectively. Unfortunately, few studies have specifically addressed the issue of integrating different MRI approaches for efficient and quantitative diagnostics. The development of accurate and sensitive tools for the early diagnosis and monitoring of disease progression requires the discovery of new biomarkers in the asymptomatic and prodromal stages of AD. A universal and in-depth understanding of the interactions between biomarkers can be achieved using statistics and by selecting the proper model. Combination of structural and functional neuroimaging biomarkers in the progression of aMCI, the conversion of aMCI to AD, and the early diagnosis of AD, could lead to the standardization of imaging protocols and quantitative metrics. Before combinations of multi-modal MRI approaches can be considered as biomarkers and translated into clinical trials, the issues described in the following section need to be resolved.

Establishing More Precise Hippocampal Sub-regions Based on Structure and Function

The hippocampus is not an anatomically uniform structure; rather, it can be divided into subregions that perform different functions. Some studies have reported that the

progression in different subregions may be sequential in aMCI patients[19, 45]. However, previous studies have only provided results in coarsely-divided subregions. Therefore, more precisely-defined hippocampal subregions are needed for future studies, and they will facilitate the discovery of new multi-modal MRI biomarkers for the conversion of aMCI to AD.

Elucidating the Effects of APOE Polymorphism on Hippocampus in aMCI Patients

It is well known that the APOE genotype is associated with structural and functional changes in the early stages of AD. APOE is encoded by a polymorphic gene localized on chromosome 19 and exists as three alleles designated ε2, ε3, and ε4. Although these alleles differ by only single amino-acid substitutions, these small changes have profound functional consequences at both the cellular and molecular levels. APOE ε3 is the normal isoform, while ε4 and ε2 can be dysfunctional[86]. Recently, our research group reported that ε4 and ε2 have opposing effects on brain morphology across the spectrum of cognitive aging[15]. Because of this, it is important to investigate the effects of APOE genotype on the hippocampus in the progression of aMCI to AD.

Clarifying the Timing of Decline or Progression of aMCI to AD or Dementia

Another issue is that the ability to detect change is based on the period of observation that is predicted for the decline or progression of aMCI to AD or dementia. An in-depth understanding of the role of biomarkers in the prediction of decline in aMCI will require both short (1 to 2 years) and long-term (many years or even decades) periods of observation. Additional cross-sectional and longitudinal studies are needed to clarify the dynamic hippocampal changes in structure and function for the conversion of aMCI to AD over time.

Defining the Sequence of Hippocampal Changes Based on Multi-Modal MRI Measures in aMCI Patients

In the coming years, when many of the above issues have been resolved, we believe that a combination of advanced multi-modal MRI measures will provide more sensitive measures of hippocampal changes than the measures on their own in the progression of aMCI to AD. Furthermore, we propose a sequential and progressive framework for the progression of aMCI to AD (Fig. 1). First, the impairment changes from fiber integrity to volume and then

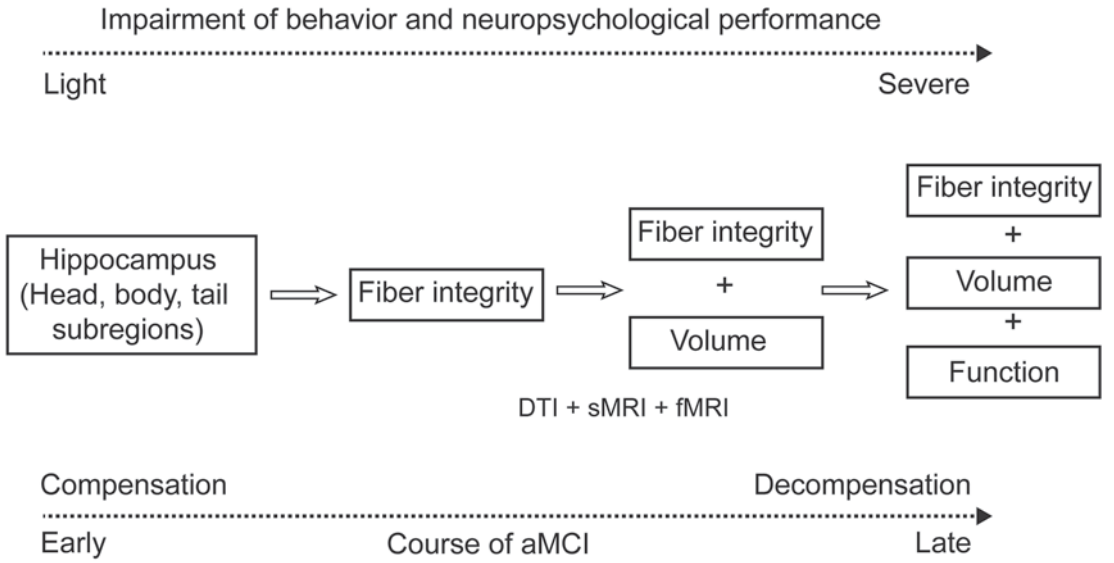

Fig. 1. Sequential and progressive structural and functional changes during the progression of aMCI to AD. The impairment changes from fiber integrity to volume and then from volume to function in hippocampal subregions. These changes accompany the progressive impairment of behavioral and neuropsychological performance in disease progression. fMRI, functional magnetic resonance imaging; DTI, diffusion tensor imaging; sMRI, structural magnetic resonance imaging.

from volume to function in the hippocampal subregions. In addition, these changes accompany the progressive impairment of behavioral and neuropsychological performance as the disease progresses. Early during the course of aMCI when the deficits of memory, fiber integrity, and volume are less prominent, there may be increased hippocampal connectivity, which could reflect an inefficient compensatory mechanism. Later during the course of aMCI, when the impairment of memory, fiber integrity, and volume is exacerbated, the hippocampal connectivity may be disrupted due to decompensation. Finally, longitudinal studies are pivotal to determine whether this hypothetical framework of the physiological, anatomical, functional, and behavioral progression of aMCI is supported by trajectories in individuals and groups of individuals.

In conclusion, we believe it is important in future studies to provide detailed demographic, clinical, neuropsychological, and behavioral memory performance data as well as multi-modal MRI data in the hippocampus and its subregions to help clarify the similarities or differences between samples of aMCI patients and the conversion of aMCI to AD over time.

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·Review·

Genetics and epigenetics of circadian rhythms and their potential roles in neuropsychiatric disorders

Chunyu Liu[1,2], Michael Chung[2]

[1]*State Key Laboratory of Medical Genetics of China, Changsha 410078, China*

[2]*Department of Psychiatry, University of Illinois at Chicago, Chicago, Illinois, USA*

Corresponding author: Chunyu Liu. E-mail: liucy@uic.edu

Circadian rhythm alterations have been implicated in multiple neuropsychiatric disorders, particularly those of sleep, addiction, anxiety, and mood. Circadian rhythms are known to be maintained by a set of classic clock genes that form complex mutual and self-regulatory loops. While many other genes showing rhythmic expression have been identified by genome-wide studies, their roles in circadian regulation remain largely unknown. In attempts to directly connect circadian rhythms with neuropsychiatric disorders, genetic studies have identified gene mutations associated with several rare sleep disorders or sleep-related traits. Other than that, genetic studies of circadian genes in psychiatric disorders have had limited success. As an important mediator of environmental factors and regulators of circadian rhythms, the epigenetic system may hold the key to the etiology or pathology of psychiatric disorders, their subtypes or endophenotypes. Epigenomic regulation of the circadian system and the related changes have not been thoroughly explored in the context of neuropsychiatric disorders. We argue for systematic investigation of the circadian system, particularly epigenetic regulation, and its involvement in neuropsychiatric disorders to improve our understanding of human behavior and disease etiology.

Keywords: epigenetics; circadian rhythms; neuropsychiatry

Introduction

Circadian rhythms are endogenous biological cycles ~24 h in length. They are found in most living organisms, and can be adjusted by factors called *zeitgebers,* or "time-givers", including light[1], temperature[2], diet[3], odor[4], and gravity[5], light being the dominant cue. Maintaining a rhythmic daily life is critical for surviving the recurrent environmental changes. These rhythms can be easily observed in behaviors such as sleeping and eating, but also, less visibly, affect crucial biological systems such as metabolism[6,7] and the cardiovascular system[7].

Multiple lines of evidence have suggested the potential roles of circadian rhythms in neuropsychiatric disorders such as sleep disorders, anxiety, mood disorders and addiction. Meanwhile, studies in animal models have identified several regulators and effectors of the endogenous clock. These core clock genes are known to comprise transcriptional-translational auto-regulatory complexes. However, these remain insufficient to explain all observations, especially the contribution to human behavioral traits and disorders. Further identification of the molecular components of circadian systems and their regulatory relationships is an important step for understanding neuropsychiatric disorders and for developing better diagnostics and treatment.

This review describes current findings on the genetic and epigenetic determinants of the circadian system in the context of neuropsychiatric disorders. By reviewing the literature, we highlight the complexity of circadian regulation beyond the classic core clock genes. Such complexity involves many genetic and epigenetic factors.

Since epigenetic mechanisms are important mediators of environmental factors and regulators of rhythmic gene expression, we therefore propose that developing comprehensive genome-wide and epigenome-wide data from multiple sample sources will improve our understanding of the circadian regulatory system and its role in neuropsychiatric disorders.

Clock Genes, Rhythmic Expression, and Regulatory Networks

Circadian rhythms in vertebrates are controlled by a conserved brain region in the anterior hypothalamus called the suprachiasmatic nucleus (SCN), made up of about 20 000 neurons. The SCN serves as a central regulator of circadian rhythms throughout the rest of the brain[8] and the body[9]. At the same time, peripheral tissues, even cultured cells[10;11] have their own local, autonomous clocks that can be self-sustaining, but they may be synchronized by signals from the SCN[12].

Clock genes underlying circadian rhythms can be broadly defined as genes that show diurnal variation of activity or function, typically showing rhythmic changes of transcript abundance, as such measures are more accessible than other molecular phenotypes, such as protein levels and activity. Although an increasing number of genes have been found to demonstrate the circadian characteristics of clock-controlled genes (CCGs), a small set of genes is denoted here as core "classic clock genes (CGs)". The CGs include Period (*PER*), Timeless (*TIM*), Clock (*CLK*), Cycle (*CYC*, a *Drosophila* gene, with the mammalian homolog *ARNTL* or *BMAL1*), Cryptochrome (*CRY*), *REV-ERBalpha,* retinoic acid related-orphan receptor alpha (*RORalpha*), D-box-binding protein (*DBP*), thyrotrophic embryonic factor (*TEF*), hepatic leukemia factor (*HLF*), *E4BP4* (*also known as NFIL3*), deleted in esophageal cancer 1 (*DEC1*), *DEC2,* Neuronal PAS domain-containing protein 2 (*NPAS2*), and Double Time (*DBT, a Drosophila* gene, with the mammalian homolog casein kinase 1e, *CSNK1E*). These genes were mainly identified by the screening of mutants of fruit flies, mice, and hamsters[13]. These few CGs make up a group of auto-regulatory loops and present rhythmic expression of their own and their regulatory target transcripts. The CGs have been frequently called clock genes in the literature but as will be discussed in this review, these CGs only represent a small set of a much broader network of clock genes.

Most of the CGs encode proteins that function as transcription factors to drive the rhythmic expression of their target genes. Some of the CG proteins form heterodimer complexes, such as PER-CRY, CLK-BMAL1, and TIM-PER. They not only regulate the expression of many other genes that carry E-box promoters, but also their own expression. In contrast, *DBP, HLF, TEF*, and *E4BP4* regulate through D-box promoters[14,15]; while the REV-ERB alpha and ROR family members bind to the REV-ERB/ROR response element (RRE)[16]. cAMP response elements (CREs) are also central regulatory motifs that mediate rhythmic expression[17]. These regulatory systems have been thoroughly reviewed[13,18-20].

In addition to CGs, hundreds of non-CG genes are transcribed rhythmically. They are part of the broadly-defined clock genes. In fact, one would expect that genes carrying E-box, D-box, RRE, and CRE promoters could be potential clock genes[21,22]. Certainly, these genes could include both the drivers and passengers of a large circadian regulatory system, though most of the cause-effect relationships remain to be discovered. Increasing numbers of CGs have been identified through genome-wide expression profiling studies, mostly in mice, as summarized in Table 1. Just to name a few, 2%–10% of genes are expressed in a circadian manner in various mouse tissues[23-28]; in the mouse SCN, 337 genes were found to be expressed cyclically, and 335 were in the liver[26]. Another mouse study detected 575 genes in the liver and 462 in the heart with circadian expression[27].

Human studies have also revealed time-dependent expression in blood and brain. A 2010 study on gene expression induced by food-intake identified expression changes associated with biopsy time for 8 197 genes in blood (false discovery rate (FDR) 2.6%)[29]. This is the largest human circadian study thus far, with 40 individuals sampled at 14 time-points each, for a total of 560 blood samples. In this study, Li *et al.* (2013) reported circadian expression in human postmortem brain. They used time of death to represent time-points in the 24-h cycle, turning individual differences into differences of expression at different time-points. They analyzed 12 000 transcripts in six brain areas (dorsolateral prefrontal cortex, anterior cingulate cortex, hippocampus, amygdala, nucleus

Table 1. Genome-wide studies of the genetics and epigenetics of circadian rhythms

Author, Year	Species	Tissues	Measure	Strains	Microarray/Methods
McDonald *et al.* 2001[153]	*Drosophila*	Head	Expression	clock mutant (Clk) flies	Affymetrix array
Hughes *et al.* 2012[135]	*Drosophila*	Brain	Expression	Wild-type Canton-S flies, y w, per0, and per0 in a Canton-S background	RNA-Seq, 100-bp paired-end reads. On average, each sample >40 million readsa
Kornmann *et al.* 2001[24]	Mouse	Liver	Expression	C57BL/6 X 129/SV	ADDER (Amplification of Double-stranded cDNA End Restriction fragments) differential display
Ueda *et al.* 2002[154]	Mouse	SCN and liver	Expression	Balb/c	Affymetrix U74Av2 and U74A oligonucleotide array
Akhtar *et al.* 2002[23]	Mouse	Liver	Expression		Custom-made cDNA microarray
Panda *et al.* 2002[26]	Mouse	SCN and liver	Expression	Male C57BL/6J and clock mutant	Affymetrix mouse (U74A) high-density arrays
Storch *et al.* 2002[27]	Mouse	Liver and heart	Expression	C57/Bl6	Affymetrix U74Av2 oligonucleotide array
Zvonic *et al.* 2006[155]	Mouse	Liver, brown and white adipose tissue	Expression	AKR/J	Mouse430a2
Oster *et al.* 2006[156]	Mouse	Adrenal gland	Expression	C57BL/6J	Mouse4302
Lemos *et al.* 2006[157]	Monkey (*Macaca mulatta*)	Adrenal gland	Expression		Affymetrix U133-A GeneChips.
Zvonic *et al.* 2007[158]	Mouse	Calvarial bone	Expression	AKR/J	Mouse430a2
Yang *et al.* 2007[159]	Mouse	Prefrontal Cortex	Expression	C57BL/6J	Mouse4302
Maret *et al.* 2007[160]	Mouse	Whole brain	Expression	C57BL/6J, AKR/J, DBA/2J	Mouse4302
Bray *et al.* 2007[161]	Mouse	Atrium and ventricle	Expression	Cardiomyocyte-specific circadian clock mutant	illuminaMousev1
Miller *et al.* 2007[25]	Mouse	Liver, skeletal muscle, gastrocnemius	Expression clock mutant	Male C57BL/6J and	custom-made genome arrays
Storch *et al.* 2007[28]	Mouse	Retina	Expression	Male CBA/CaJ	Affymetrix mouse 430.2 arrays
Yan *et al.* 2008[32]	Mouse, human, and monkey	14 tissue	Expression	22 datasets	
Hughes *et al.* 2012[12]	Mouse	Liver	Expression	ClockΔ19 mouse Arrays	Affymetrix Mouse Exon 1.0 ST
Zambon *et al.* 2003[208]	Human	Skeletal muscle	Expression		Affymetrix U95a
Leonardson *et al.* 2010[29]	Human	Blood	Expression		Custom array
Li *et al.* 2013[30]	Human	6 brain areas	Expression		Affymetrix U133-A or U133Plus-v2 GeneChips
Lim *et al.* 2014[31]	Human	Dorsolateral	Gene Expression and DNA methylation		Illumina Infinium HumanMethylation450k Bead Chip; Illumina HiSeq prefrontal cortex with 101 bp pairedend reads and 150-M reads for the first 12 samples. Remaining samples, 50-M reads

accumbens, and cerebellum) from 55 controls and 34 major depressive disorder (MDD) patients[30]. Among the healthy controls, >417 transcripts in each region showed 24-h oscillation (nominal P <0.05), while 169 genes had an FDR of <0.5 for combined P values across regions. These 169 genes are considered to be common circadian genes in the brain[30]. Another study on human postmortem dorsolateral prefrontal cortex samples from 536 individuals used RNA-Seq and global statistics to show rhythmic expression without naming specific clock genes[31].

Most of these cases of rhythmic expression are tissue-specific, suggesting tissue-specific regulatory network. Only 28 cycling genes are shared between mouse SCN and liver[26], while 37 are shared between mouse liver and heart[27]. Another analysis of 21 microarray data sets from 14 mouse tissues found that the expression of 41 out of 19 168 genes showed consistent circadian oscillation across multiple tissues[32]. This alerts us to the fact that studies of the circadian regulatory system should take tissue-specificity into account.

It should be noted that the number of cyclically-expressed genes reported is related to the experimental design, statistical method, and significance cutoff. With the liberal significance criteria (P <0.05) used in the human brain study by Li *et al.*, some of the CGs still did not show rhythmicity[30]. However, these findings are not definitive, since many pre- and post-mortem factors could have destroyed rhythmic expression patterns and produced false-negatives. Moreover, as transcription factors are typically expressed at low levels, major circadian regulators, which are often transcription factors, could be missed by some of the techniques used in genome-wide studies when sensitivity is not sufficient.

After considering experimental artifacts, the fact that many genes other than CGs are rhythmically expressed and that some CGs do not show cycling expression in genome-wide studies could have many implications, including the possibility that our current list of clock genes may not be exhaustive. Given the complexity of the system, it is likely that we have not yet identified the complete set of genes regulating and responding to circadian rhythms. Several studies have proposed novel genes as important central regulator genes. For example, a 2008 study of mutant mice proposed that the *NR3C1* and *FKBP/HSP90* complexes are central to the control of circadian gene expression by environmental cues[32]. *CHRONO*[33,34] and *UBE3A*[35] were found to be essential in regulating circadian rhythms in mice in three 2014 studies. Genes involving protein translation, including rRNA, also showed rhythmic expression[36]. Furthermore, an siRNA screen of a human osteosarcoma cell line, targeting 17 631 known and 4 837 predicted human genes, discovered ~343 clock genes or modulators[37]. These data suggest that the underlying organization of circadian rhythms has not yet been completely described.

Rhythmic expression is certainly not the only aspect of circadian rhythms. Protein abundance and post-translational modifications, such as phosphorylation and ubiquitination, have also been shown to have daily oscillations in *Neurospora crassa*, the fruit fly, and multiple tissues in mouse, rat, and hamster, as reviewed by others[38-41]. Similar circadian molecular mechanisms may exist in humans, but remain to be explored. From chromatin to transcripts, mRNA to proteins and to protein modifications, circadian rhythms encompass a complex regulatory system, including the epigenomic components discussed below.

Genetics of Sleep-Related Traits

While circadian patterns can be observed from the molecular level all the way up to the organismal behavior level, the sleep-wake cycle and other sleep-related traits are probably the most salient outputs of the circadian clocks. These traits are known to be heritable[44], and the underlying genes can be identified directly by genetic methods using human population data, without relying on knowledge of specific clock genes. The heritability of sleep measures, including timing, duration, and quality, varies between 12.4% and 29.4%[42]. A study of 410 normal adults has identified a polymorphism in *CLK* associated with morningness-eveningness preferences[43]. Furthermore, in a GWAS of 4 251 individuals, Allebrandt *et al.* (2013) identified an intronic variant (rs11046205) in the *ABCC9* gene associated with sleep duration (P = 3.99e-8)[44].

On the other hand, the circadian system is not the only regulator of sleep. Energy homeostasis and its interactions with circadian rhythms also contribute to maintenance of the sleep-wake cycle[45,46]. While homeostasis and other factors play significant roles in sleep regulation, sleep has

been used as the major model to study the genetics and regulation of circadian rhythms, although it should be noted that the genetics of the sleep-wake cycle is not necessarily all about circadian rhythms.

Healthy people vary in their preferences for sleep timing and length; some are often classified as morning "lark" or evening "owl" chronotypes. If these variations do not impair the quality of life, they are considered normal. Sleep-wake behaviors in humans and animal models offer opportunities to understand circadian regulation. In humans, variable sleep traits or disorders provide avenues for studying the molecular bases of sleep regulation, and, by extension, circadian rhythms. In animal models, one can take advantage of better-controlled environmental factors to study their contribution to circadian regulation. For example, manipulating the lighting environment and feeding pattern have been shown to induce circadian and related genomic and epigenomic changes[47].

Circadian Disruptions Are Implicated in Neuropsychiatric Disorders

Disruption of circadian rhythms is associated with or implicated in many traits or diseases, including metabolic syndrome[48], obesity[49], diabetes[50], inflammatory diseases and autoimmune disorders[51], cancer[52], drug efficacy and toxicity[53], cardiovascular disorders[54], and mental disorders[55,56].

Among neuropsychiatric disorders, sleep-wake disorders, anxiety, mood disorders, and addiction have the strongest connections to altered circadian rhythms. While the connection between circadian rhythms and sleep-wake disorder is self-evident, circadian rhythms have been implicated in other psychiatric disorders based on biological and clinical observations. Specifically, many of these disorders exhibit co-morbidity with sleep disturbance and their treatments often elicit responses that are related to candidate clock genes and behavioral or clock gene expression changes in animal models. Additional links between circadian rhythms and neuropsychiatric disorders can be found in several candidate gene association studies. Major evidence is summarized in Table 2. A few examples of ***indirect evidence*** linking circadian rhythms to non-sleep-related neuropsychiatric disorders are as follows: abnormal sleep is co-morbid with many disorders[57]. Persistent sleep disturbances have been found to increase the risk of developing anxiety[58] and depression[59;60]. Insomnia and substance abuse disorders promote the risk of each other[60,61]. Melatonin is important in synchronizing circadian rhythms[62], and agomelatine that targets the melatonergic system is an antidepressant[63]; agomelatine has been shown to increase the relative amplitude of an individual's rest-activity cycles[64]. Ketamine, a drug with rapid-acting antidepressive effects, influences the recruitment of the CLK-BMAL1 complex to E-box promoters and alters the expression of CGs[65]. Another antidepressant, escitalopram, has been reported to restore the disrupted rhythmic expression of several CGs in a study of blood samples from 12 MDD patients and 12 controls[66]. Animal models with disrupted clock genes show behavioral changes similar to mood disorders[67] or schizophrenia[68]. Therefore, circadian rhythms have been of interest in the study of these disorders. However, the question of causation largely remains to be addressed.

A multi-system hypothesis has been formulated to explain the connections between circadian rhythm disturbance and addiction, as well as anxiety. Based on a literature review, Gorwood highlighted the cortisol-melatonin-vasopressin interaction for anxiety, as this interaction nicely bridges stress-response and circadian systems[69]. Drugs of abuse may influence the interwoven molecular networks of circadian rhythms, stress-response, reward circuitry, neuroplasticity and memory, and ultimately lead to the development of addiction, as well as withdrawal symptoms. The paraventricular nucleus in the hypothalamus has been proposed to be the location where circadian and stress signals converge, and where multiple clock genes, neuropeptides, and stress-response genes interact[70]. Such interactions between circadian systems and stress-response systems may play an important role in many psychiatric disorders, including but not limited to addiction[71].

The dopamine D2 receptor (*DRD2*) is another interesting candidate linking the circadian system and the reward pathway, as it mediates the photic response to regulate circadian rhythms, and the most important reward pathway is dopaminergic[72]. Several candidate gene studies have found significant associations between *DRD2* variants and different kinds of addiction (alcohol, cocaine, heroin, and nicotine)[73,74]. However, a meta-analysis[75] and

Table 2. Studies that implicate circadian rhythms in psychiatric disorders

	Mood disorder (MDD and bipolar disorder)	Anxiety	Addiction
Sleep disturbance comorbidity, or as a risk factor	Risk factor for developing MDD[59,60]. Abnormal circadian rhythms in hormone levels, body temperature, sleep, and behavioral patterns reported in patients with MDD[162-164]; degree of circadian misalignment correlated with severity of depression[165]; sleep disturbance in manic and depressive phases, even possibly euthymic phases[166,167].	Risk factor for developing anxiety[58]; children daily regularity predicts anxiety levels >10 years later[168]	Bi-directional relationship: addiction disrupts circadian rhythms; sleep and mood problems increase chance of addiction[60;169]
Therapy	Seasonal affective disorder (SAD) patients respond to light therapy[170]; sleep deprivation treats depression[171]; sleep deprivation may switch a depressed patient into hypomania or mania[172]; agomelatine increases relative amplitude rest-activity cycles[64]; lithium lengthens circadian period in hamsters[173].	Agomelatine affects circadian rhythms[64], also effective in treating anxiety disorders[176,177].	
Chronotype	Mood disorders associated with chronotype[178,179]		Evening chronotype associated with substance abuse[180;181]
Social rhythms	Disrupted social rhythms[182-184]	Lower daily regularity in anxiety patients[185]	
Seasonal pattern	Seasonal changes in bipolar disorder[186,187]		Case report of SAD with cyclical cocaine craving[188]
Hormone	Rhythmic melatonin level[189,190]	Cortisol level is rhythmic[191]	Cortisol secretion patterns linked to addiction[192]
Clock gene genetic association	17 studies of candidate genes[193]	One candidate gene study[194]	Six genetic studies[195-200]
Clock genes in animal models	Knockout of REV-ERB alpha increases midbrain dopamine production and induces mania-like behavior by regulating tyrosine hydroxylase gene expression in a mouse study[201]. Clk mutant shows mania[67]	Per1 and Per2 expression levels in nucleus accumbens regulate anxiety levels in knockout mouse models and mice experiencing chronic social defeat stress[202]. Clk mutant less anxious[67]	14 studies of Clock, per1, and per2 mutant mice showed behavior changes in response to drug[203]
Clock gene expression in response to drug treatment	Ketamine influences expression of clock genes[65]; escitalopram restores disrupted rhythmic expression of several clock genes[66]; lithium affects expression of multiple clock genes[174,175,204]; valproic acid changes phase and amplitude of *PER2* expression in cultured cells[117].	Anxiolytic medications reduce mPer1 expression in mice[205]	Chronic methamphetamine treatment desynchronizes clock gene expression between striatum and SCN[206]; ethanol and drugs of abuse alter clock gene expression in SCN and other brain regions[203]
Clock gene expression changes in patients	Expression changes of clock genes in MDD brain[30] and blood[207].		
Review	Gonzalez 2014[193]	Philip Gorwood, 2012[69]	Logan RW *et al*. 2014[203]

a GWAS[76] of alcoholism reported inconsistent results, suggesting that the *DRD2* contribution may be small, if at all. A *DRD2* variant has also been reported to be associated with anxiety disorders with co-morbid alcohol-use disorder[77]. But the finding is also weak and requires replication.

Circadian Genetics of Neuropsychiatric Disorders

Genetic studies may capture direct evidence that specific clock genes are involved in neuropsychiatric disorders if mutations or variants of clock genes are associated with the risk of disorders. Genetic association is an important venue leading to the translation of clues from animal models to clinical relevance.

Sleep-Wake Disorders

Sleep-wake disorders impair the quality of life, affect learning, memory, and mood. These disorders have a clear genetic basis. Family and twin studies of insomnia report heritability ranging between 21% and 58%[78-80] [see Palagini *et al.* (2014) for a thorough review[81]]. Travel across time zones, sleep deprivation, and shiftwork all disturb sleep patterns. Insomnia, hypersomnia, and narcolepsy are major sleep-wake disorders[82]. Some of these are common, like insomnia (~10% of adults have severe insomnia that cause daytime consequences[83]), and some are rare, like narcolepsy (affecting ~1 in 3 000)[84]. Jet-lag and shiftwork-related sleep problems are common in specific occupations, like flight attendants, nurses, and soldiers.

Mutations in genes responsible for some specific, mostly rare forms of sleep disorders have been discovered. A mutation (R192H) in *GABRB3* has been found in patients with chronic insomnia. Since *GABRB3* encodes a subunit of a chloride channel that serves as the receptor for gamma-aminobutyric acid (GABA), a major inhibitory neurotransmitter of the mammalian nervous system, a decrease in GABAergic inhibition may contribute to insomnia[85]. Interestingly, hypersomnia was also recently linked to GABA(A) receptor regulation[86]. Another excessive sleeping disorder, narcolepsy, is mostly caused by a deficiency in hypocretin (*HCRT*), an excitatory neuropeptide[87].

Mutations in *PER2* and *CSNK1D* have been reported in Advanced Sleep Phase Syndrome (ASPS) patients. A rare autosomal dominant mutation of *PER2* has been found to be responsible for ASPS in members of a Utah family[88]. CKIdelta (*CSNK1D*) was found to have a missense mutation responsible for ASPS[89].

Knockout or mutation of many other genes, including *IA2*[90], has been found to change sleep-related behaviors in mice, and has been reviewed elsewhere[91]. These genes could be candidates for human sleep disorders, but mutations have not been detected in humans so far.

The search for genes of the common forms of sleep disorders has produced positive and negative results. A GWAS of insomnia with 2 267 samples did not detect any significant genome-wide association[92]. However, several other sleep disorders have yielded significant genome-wide signals in GWASs with hundreds of cases, including restless legs syndrome (*MEIS1*, *BTBD9*, *PTPRD*, *MAP2K5*, *SKOR1*, *TOX3*, *BC034767*, *MAP2K5*, and *LBXCOR1*)[93-96] and narcolepsy (*TRA-alpha* and *TRAJ10*)[97].

It should be noted that a circadian defect is not the only cause of sleep-wake disorders. Cardiovascular, neurological, and pulmonary diseases, substance use and medication, irregular metabolism, and bad habits can all disturb sleep. One can certainly argue that some of the genes associated with sleep disorders may not be involved in circadian regulation at all. In fact, among the classic clock genes, CGs, only *PER2* has been found to carry a mutation [c.1984A>G (p.Ser662Gly)] responsible for a sleep disorder[88]; all the other associated genes are outside of the CGs. Most of them do not have any known connection with circadian regulation, or have not been studied for rhythmic expression. How sleep disorder-associated genes are related to circadian rhythms remains to be investigated. It may turn out that some of these non-CG genes are also actual clock genes, participating in circadian regulation.

Non-Sleep-Related Neuropsychiatric Disorders

Genetic variants of candidate genes of CGs have been tested for association with bipolar disorder[98,99], depression[100], seasonal affective disorder[101,102], or anxiety disorders[77], alcohol use[103], heroin addiction[104], bipolar disorder and schizophrenia[105], major depression and bipolar disorder[106], and depression and sleep disorder[107]. Though some positive associations were reported, most findings were weak and not replicated.

Genetic associations of ~360 selected clock genes

were systematically assessed in 14 psychiatric GWAS data sets based on relaxed thresholds for significance by McCarthy *et al.* (2013)[108]. Bipolar disorder, schizophrenia, attention deficit hyperactivity disorder, and MDD as a group of disorders and lithium-responsiveness have been shown to have association signals enriched in 18 core clock genes and genes reported to be rhythmically expressed in more than six mouse tissues. This is the first GWAS evidence supporting potential genetic contributions of the circadian system to neuropsychiatric disorders, although the selection of clock genes in this study may be debatable and replication is warranted.

It is important to note that the CGs did not appear as any of the top GWAS signals of these psychiatric disorders, despite the fact that circadian disruption has been strongly implicated in such disorders. Genetic studies of depression, anxiety, and addiction have yielded largely negative results[109-113]. The study by McCarthy *et al.* suggested collective weak contributions from clock genes to the susceptibility of various disorders. This has several implications. First, it is possible that other non-CG genes associated with disease are also part of the circadian system, but have not yet been identified as such. Second, clock genes may be more relevant to specific subtypes or endophenotypes of those diseases; therefore, subgroups of those disorders may provide better association with clock genes. Last, the circadian system may contribute more to disease risk through a non-genetic route, such as epigenetics.

Epigenetic Factors Regulate Circadian Rhythms

When genetics has had limited success in providing direct evidence to link circadian rhythms to neuropsychiatric disorders, epigenetics naturally attracted attention as the critical regulator (for gene expression) and mediator (for environmental factors). Then the first questions are whether epigenetic factors regulate circadian rhythms as they should in theory, and what these circadian epigenetic factors are.

The circadian system is dynamic and flexible, and is tightly regulated by the interactions between internal molecular systems and environmental cues. The environmental factors, including light, food, temperature, stress, hormones, drugs, and age, act through epigenetic factors to shape the phenotypes. Gene expression, as the molecular representative of circadian rhythms, is known to be regulated by genetic variants and epigenetic factors. Epigenetic factors include DNA methylation, histone modification (e.g., methylation, acetylation, phosphorylation, and citrullination, biotinylation, ribosylation, ubiquitination, and palmitoylation), and non-coding RNAs. Studies on histone acetylation, DNA methylation, non-coding RNA, and RNA modification have shed light on their roles in regulating the expression of clock genes, and ultimately, circadian phenotypes. With such studies, we could look for circadian epigenetic factors, and further study their contribution to neuropsychiatric disorders.

Acetylation and Deacetylation

The epigenetic mechanism that *CLK* uses to regulate circadian rhythms is histone acetylation and deacetylation. Etchegaray *et al.* (2003) showed in a mouse liver study that histone acetyltransferase (HAT) p300 works with the Clock/Bmal1 complex to regulate histone H3 acetylation at the promoters of the Cry and Per genes to influence their expression[114]. Doi *et al.* (2006) further showed that CLK itself possesses HAT activity, which can be enhanced by its partner BMAL1, when bound to E-box[115]. CLK is also involved in acetylating other non-histone substrates including BMAL1. Acetylated BMAL1 recruits CRY1 to the CLK-BMAL1 complex and represses transcription[116].

Histone deacetylase (HDAC) has a function opposite to that of HAT, and is also an important regulator of circadian rhythms and memory formation, as well as metabolism. It removes acetyl groups from ε-N-acetyl lysine on histones, allowing the histones to wrap the DNA more tightly. The HDAC inhibitor valproic acid and trichostatin A were found to increase H3 acetylation and affect *Per2* expression in an *in vitro* study[117].

A mouse model has shown that Hdac3, one of the Hdac subtypes, is recruited by nuclear receptor corepressor 1 (Ncor1) and is involved in repressing *Bmal1* expression, thus affecting circadian rhythms and metabolism[118]. Hdac3 recruitment also fluctuates rhythmically in the mouse liver, in conjunction with Rev-erb-alpha and Ncor, to form a Hdac3/Rev-erb-alpha/Ncor complex[119]. It is to be expected that the transcription of many genes oscillate with the fluctuation of HDAC3-related histone modification, or Rev-Erb-alpha/NCoR1-related signaling pathways.

Another member of the HDAC family, an NAD(+)-dependent protein deacetylase, SIRT1, also works directly with clock genes. SIRT1 binds CLK-BMAL1 and promotes deacetylation and degradation of the PER2 protein in mice[120]. SIRT1 is also a metabolic sensor, as it requires binding of its coenzyme NAD+ for its HDAC enzymatic activity. Thus, through SIRT1, metabolic states are linked to the circadian system. In addition, *SIRT1* has been implicated in aging and neurodegeneration[121,122], synaptic plasticity, and memory formation in mouse studies[123,124].

The lysine-specific demethylase JumonjiC and ARID domain-containing histone lysine demethylase 1a are also major binding partners of CLK-BMAL1. This can inhibit HDAC1 function and enhance transcription by CLK-BMAL1 in a demethylase-independent manner. The CLK-BMAL1 complex plays a conserved circadian regulatory role across insect and mammalian species[125].

DNA Methylation

The role of DNA methylation in circadian regulation is supported by a human study in which plasma homocysteine levels and the global DNA methylation level showed 24-h variation in the blood of 15 males and 15 females[126]. Homocysteine level has been linked to DNA methylation in many studies[127]. An epigenome-wide study using methyl-DNA immunoprecipitation (MeDIP-chip) in mice showed that altered day-length changed gene expression profiles and promoter DNA methylation in the SCN, suggesting that DNA methylation regulates the circadian clock in the SCN[128]. Moreover, a study in mice showed that sleep deprivation can change the DNA methylation and hydroxymethylation of hundreds to thousands of CpG sites near genes involved in neuritogenesis and synaptic plasticity, the cytoskeleton, signaling, and neurotransmission[129]. Direct evidence supporting the roles of DNA methylation in regulating circadian rhythms came from a human study, which used global statistics to show evidence of significant 24-h rhythmicity of DNA methylation, as well as its correlation with rhythmic gene expression in human dorsolateral prefrontal cortex[31].

Non-coding RNA

MicroRNAs (miRNAs), probably the most intensively studied class of non-coding RNAs (ncRNAs) so far, may contribute to the regulation of circadian rhythms. Dicer is the major enzyme in miRNA biogenesis, and Dicer-deficient mice and cells show shorter circadian cycles due to faster translation of *PER1* and *PER2* proteins. It has been proposed that microRNAs miR-24, miR-29a, and miR-30a specifically target *PER1* and *PER2,* thus determining the period of the cycle[130].

Studies in mice have also implicated two other miRNAs, miR-134 and miR-132, in circadian regulation. miR-134 is brain-specific, and regulated by *SIRT1*[124]. It is involved in the regulation of CREB and BDNF levels, proteins that are important in many neuronal functions and activities[124]. miR-132 is a direct link between light and chromatin remodeling: it is induced by photic entrainment cues *via* the mitogen-activated protein kinase (MAPK)–*CREB* signaling pathway[131] and regulates chromatin remodeling and translation[132].

Other ncRNAs have strong potential in regulating circadian rhythms too. Rhythmic expression has been reported for 112 long non-coding RNAs (lncRNAs) in the rat pineal gland, which is the source of melatonin[133], while melatonin is an important hormone timing circadian rhythms. A study of *Neurospora* gene frequency (frq) demonstrated that lncRNAs regulate circadian rhythms through anti-sense expression[134]. RNA-Seq of *period*-null *Drosophila* has identified several ncRNAs with diurnal expression, including a family of small nucleolar RNAs (snoRNAs)[135]. It should be noted that some ncRNAs, particularly lncRNAs, evolved fast and are species-specific[136,137]. These findings in non-humans only suggest possible epigenetic mechanisms that may occur in humans. The actual genes in humans remain to be discovered.

RNA Modification

Post-transcriptional RNA processing and modification may be relevant to clock function. A recent study in mice and cultured human cells showed that n^6-methyladenosine RNA-methylation, one of the most common RNA modifications, is involved in circadian clock regulation[138].

Studies have also shown that diet affects the epigenetic regulation of circadian function. In a study of Japanese macaques, a maternal high-fat diet *in utero* disrupted the regulation of expression, and increased individual variations in fetal hepatic *Npas2*, one of the CGs. Such disruption was associated with altered histone acetylation (H3K14ac) but not DNA methylation at the *Npas2* promoter region. These changes of gene expression and histone modification were

reversed by postnatal diet[139]. Exposure to different lengths of light per day changes the SCN and neuronal *Per1* gene expression and behavior after birth in mice[140], suggesting possible epigenetic modification induced by early-life environmental effects, although epigenetics was not part of the study.

The epigenetics of circadian systems is a new, emerging research field, leaving a lot to be investigated. Most studies have been performed in mouse models, and only a few in humans. Since differences in epigenetic regulation between mice and humans during preimplantation development have been reported[141], findings from mice and other species may not translate to humans directly. Moreover, many epigenetic factors, such as hydroxymethylation, lncRNAs, and most of the histone modifications other than acetylation, have not been studied in the context of circadian regulation in humans. Even for those factors studied, the findings are still fragmentary, and do not form one coherent picture of the regulatory system. It is not known whether these factors work independently or interactively to regulate each of the clock genes, or the circadian system as a whole, and how. For these reasons, we advocate a more comprehensive epigenomic study of the circadian system in humans.

Circadian Epigenetics in Neuropsychiatric Disorders

Although plenty of data have implicated circadian rhythms in the risk of neuropsychiatric disorders, and that epigenetic factors are important regulators of these rhythms, only very limited studies have been performed to explore the epigenetics changes in neuropsychiatric disorders.

Sleep-Related Disorders

A few studies have been published on gene expression changes in disturbed sleep. The epigenetic regulation of those changes remains largely unknown as only one candidate gene study exists for DNA methylation.

Möller-Levet *et al.* studied gene expression profiles and reported that 711 genes were up- or down-regulated in the blood of people suffering from insufficient sleep. The number of genes with a circadian expression profile was also reduced from 1 855 to 1 481[142]. This same research group also studied the blood transcriptome in desynchrony of sleep-wake timing and circadian rhythms, and identified a dramatic reduction of rhythmic transcripts (6.4% to 1.0%) caused by desynchrony[143]. The chromatin modification and expression regulation pathways were consistently implicated by the differentially-expressed genes in these two sleep studies.

Bollati *et al.* (2010) studied the effects of daytime and nighttime shiftwork on global DNA methylation and the methylation of the promoters of three candidate genes (glucocorticoid receptor, tumor necrosis factor alpha (TNF-α), and interferon-gamma) using peripheral blood DNA from 100 shift-workers and 50 day-workers in Northern Italy. A small but significant difference in methylation was detected between morning and evening type shift-workers in the TNF-α promoter. However, no significant methylation difference was detected when comparing shift-workers to day-workers[144]. It is notable that all the reported associations or correlations were weak.

Non-Sleep-Related Neuropsychiatric Disorders

Prader-Willi syndrome (PWS) is the first neuropsychiatry disorder with evidence of disrupted circadian epigenomics. PWS is a genetic disorder featuring obesity, intellectual disability, and sleep abnormalities. This disorder is frequently co-morbid with psychiatric problems[145]. It is caused by a deletion on the paternal chromosome 15q11-q13, considered to be caused by loss of snoRNAs[146], which are processed products of a lncRNA gene, 116HG. A study of mice lacking 116HG showed altered expression of several clock genes and energy use in the brain[147].

Another pilot study connecting the genetics and epigenetics of clock genes to psychiatric disorders is on miRNA. The precursor of miR-182 was found to carry an SNP rs76481776 that is associated with late insomnia in MDD patients (corrected P <0.00625), in a study of 359 MDD patients and 341 control individuals. *CLK* is one predicted target of miR-182 and the regulatory relationship was validated by *in vitro* assays[148]. This relationship between an SNP, the expression of an miRNA, and its targets warrants further investigation. However, it does suggest that we should pay more attention to subtypes or endophenotypes rather than diagnostic classification, when studying the genetics and epigenetics of those disorders.

Clearly, circadian epigenomics has not received sufficient attention in the study of neuropsychiatric disorders although the circadian rhythms have been one of the major phenotypes to study in these disorders.

Future Perspectives

Based on the studies reviewed above, we see that knowledge of the regulatory systems of circadian rhythms may provide an opportunity to understand psychiatric disorders. However, we still have limited understanding of the broader network context of such circadian regulatory systems, particularly the epigenetic aspects of such regulation. Genes involved in circadian rhythms remain to be discovered and organized into network systems. Epigenomics need to be integrated in order to complete the circuitry regulating expression, and connected with environmental factors. More importantly, considering such regulation and networks in the context of neuropsychiatric disorders would provide new perspectives on the link between circadian and human behaviors, therefore allowing better understanding of the disorders.

Understanding the complex biological systems that produce them is necessary to decipher complex traits such as neuropsychiatric disorders[149]. Circadian regulation is a complex biological system. Environmental factors and internal biological infrastructure work together: light acting through photoreceptors, food working through SIRT1-related pathways, and psychological stress through the hypothalamo-pituitary-adrenal neuroendocrine system, all of which regulate an organism's internal clock. Serotonergic, dopaminergic, and maybe other neurotransmitter systems interact with circadian regulatory networks to influence human behavior and disorders.

Our current knowledge about circadian rhythms is largely derived from studies of candidate genes, their biochemistry, genomics, and epigenomic regulation. There are very limited genome-wide, systematic studies in humans (Table 1). Genome-wide studies are critical for obtaining an unbiased understanding of biological systems. It is important to put existing knowledge into a biological network, to re-assess all the interactions and signaling connections. Novel components of circadian controls, from environmental cues to the downstream effectors, will be discovered. Several papers have advocated the use of systems biology to study circadian rhythms, and to construct the regulatory system of circadian rhythms through integration of multiple -omics[150-152].

A complete circadian regulation system should contain every member of the circadian regulation cascades, from the core clock modulators to the downstream effector genes. It should also represent regulators at different levels, from environmental cues to epigenetic factors, RNA and protein modifications. Relationships among these nodes, genes, and their interactions are critical parts of the system. The use of such systems holds the key to understanding circadian rhythms and their role in neuropsychiatric disorders.

Regulatory systems are spatiotemporally-specific. This suggests that tissue selection is important for the study of circadian rhythms and psychiatric disorders. The brain is the critical organ/tissue for understanding neuropsychiatric traits or disorders, including those that are circadian-related, particularly as circadian regulation is tissue-specific. However, other than imaging studies, it is almost impossible to study circadian dynamics in the live human brain. Excessive assumptions have to be made in the analyses of human postmortem data. Different individuals could differ by variables other than the time of death. Model animal brains, cultured or induced neuronal cells derived from stem cells, and human blood are a few alternatives that could provide multiple time-point data around the clock. However, each model system has its own limitations. Complementary use of these different models may help us build a comprehensive understanding of the regulatory network and its relevance to human circadian-related traits and disorders.

Genomic and epigenomic studies of patient and control samples should also take diurnal variations into account. Hundreds or even more genes have variable gene expression or epigenetic markers within 24-h. In the past, the time when data or material was collected has rarely been recorded and incorporated into analyses. As a result, artifacts may have been introduced into some published data unless the sample collection was done at a similar time of day. Circadian studies in healthy humans will provide critical baseline information for other studies when time of day data are not available.

The findings from genetic and epigenetic studies could lead to novel drug targets. Belsomra, a hypocretin receptor antagonist, was recently approved by the Food and Drug Administration (USA) as a new drug to treat insomnia. Hypocretin has been connected to the sleep-wake cycle since the discovery of a mutation responsible

for narcolepsy, though it is not considered one of the CGs. We may have many other drug targets buried in the list of components with rhythmic expression or epigenetic regulation of circadian rhythms, the complete circadian regulation systems. Epigenetic drugs have great potential in treating neuropsychiatric disorders. Theoretically, it will be much easier to use drugs to modify the epigenome than to correct mutated genes.

Understanding the genetics and epigenetics of circadian-related traits and diseases will lead to better and more precise diagnosis of circadian-related disorders. Ultimately, this will improve the quality of life for people suffering from disorders due to jet-lag or shift-work, when we are able to develop epigenetic interventions to ease the pains and discomfort.

Through our review, it is clear that epigenetics may play important roles in regulating circadian rhythms and associated neuropsychiatric disorders, but related studies are lacking today. The circadian cycle is a highly environment-dependent biological process. The circadian cycle is one of the best models to study environmental impact and gene-environment interactions. Much effort should be placed on this interesting research field. Using circadian-related phenotypes and biomarkers, we may have an exceptional opportunity to access the dark kernel of psychiatric disorders.

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