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ORIGINAL RESEARCH

The Effects of Variation in the $GABA_A$ Receptor Gene on Anxious Depression are Mediated by the Functional Connectivity Between the Amygdala and Middle Frontal Gyrus

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Background: γ-aminobutyric acid (GABA) and its main receptor, the $GABA_A$ receptor, are implicated in major depressive disorder (MDD). Anxious depression (AD) is deemed to be a primary subtype of MDD. The amygdala and the dorsolateral prefrontal cortex (DLPFC) are key brain regions involved in emotional regulation. These regions contain the most $GABA_A$ receptors. Although the GABAergic deficit hypothesis of MDD is generally accepted, few studies have demonstrated how $GABA_A$ receptor gene polymorphisms affect the functions of specific brain regions, in particular, the amygdala and the DLPFC.

Methods: The sample comprised 83 patients with AD, 70 patients with non-anxious depression (NAD), and 62 healthy controls (HC). All participants underwent genotyping for polymorphisms of $GABA_A$ receptor subunit genes, followed by a resting-state fMRI scan. The HAMD-17 was used to evaluate the severity of MDD. ANOVA was performed to obtain the difference in the imaging data, $GABA_A$ receptor multi-locus genetic profile scores (MGPS), and HAMD-17 scores among three groups, then the significant differences between AD and NAD groups were identified. Mediating effect analysis was used to explore the role of functional connectivity (FC) between the amygdala and DLPFC in the association between the $GABA_A$ receptor gene MGPS and AD clinical features.

Results: Compared with the NAD group, the AD group had a higher $GABA_A$ receptor MGPS. AD patients exhibited a negative correlation between the MGPS and FC of the right centromedial (CM) subregion, and the right middle frontal gyrus (MFG). A negative correlation was also observed between the MGPS and anxiety/somatic symptoms. More importantly, the right CM and right MFG connectivity mediated the association between the $GABA_A$ receptor MGPS and anxiety/somatic symptoms in patients with AD.

Conclusion: The decreased FC between the right MFG and right CM subregion mediates the association between $GABA_A$ receptor MGPS and AD.

Keywords: anxious depression, GABAA receptor, multi-locus genetic profile scores, dorsolateral prefrontal cortex, amygdala subregion

Introduction

Major depressive disorder (MDD) is a highly heterogeneous affective disorder. MDD can be divided into several subtypes, with anxious depression (AD) being the most common. In addition to depression, the AD subtype manifests as nervousness, restlessness, an inability to relax, difficulty concentrating, and a fear of losing control. Previous studies have shown that 50–65% of patients with MDD satisfy the diagnostic standard for AD.[1,2]

In a previous study, compared to non-anxious depression (NAD), AD was found to be characterised by unique features, including difficulty achieving remission, greater side effects from medications, and a higher recurrence frequency.[3]

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Additionally, AD patients exhibit a poor response to antidepressants[4–6] and higher rates of suicidal ideation[5] and suicidal behaviour.[7,8] These unique clinical features and the associated poor prognosis of patients with AD suggest that there may be differences in the biological mechanisms of AD and NAD.[9] An understanding of the biological mechanism of AD is crucial for the early judgement of patient prognosis and the design of personalised interventions for MDD patients.

γ-aminobutyric acid (GABA) is a primary inhibitory neurotransmitter in the central nervous system of mammals. It plays a role in the regulation of physiological and psychological processes.[10] GABA inhibits neurotransmission through $GABA_A$ and $GABA_B$ receptors. The current study focuses on the $GABA_A$ subtype because of its well-established connection with depression and anxiety.[11,12] Many studies have shown that nucleotide polymorphisms of *GABRA2*,[13] *GABRA3*,[14–16] *GABRA6*[17] and *GABRG2*[18,19] are associated with anxiety.[20] However, the exact mechanism by which these genes affect emotion regulation has not been elucidated.

At present, several scholars are attempting to evaluate the possible genetic connection between these four $GABA_A$ receptor genes and MDD, although there were limited researched in this area. Gottesman described an "endophenotype" as a measurable biological change that connects disease symptoms with pathogenic genes and pathways. Endophenotypes can be neurobiochemical, neuroanatomical, etc., and are an embodiment of the biological attributes of a disease.[21] The functional connection or activation value based on resting-state functional magnetic resonance imaging (rs-fMRI) is considered a good endophenotype for research into the pathogenesis of depression.[22,23] Focusing on endophenotypes could be a more effective strategy for examining the roles of certain genes in various diseases. Past research has revealed that resting brain imaging features can serve as biological markers of depression subtypes and can predict treatment efficacy.[24]

Human neuroimaging studies suggest that the $GABA_A$ receptor is an important neuromodulator in the limbic system. Imaging studies have demonstrated the presence of associations between brain regions rich in $GABA_A$ receptors and symptoms of anxiety and depression. For example, previous studies have described a reduction in the volume of the amygdala in patients with MDD.[25] The amygdala, which plays a role in the regulation of fear and anxiety, is particularly rich in $GABA_A$ receptors.[26] In addition, emotions such as fear and anxiety can be regulated by the $GABA_A$ receptor.[27,28] Unlike other brain regions, the amygdala contains three subregions, the laterobasal (LB), centromedial (CM), and superficial (SF) subregions; each subregion regulates different emotional and cognitive functions.[29,30] The dorsolateral prefrontal cortex (DLPFC), another critical brain region involved in emotional regulation, has been widely demonstrated to exhibit abnormally low activity levels in patients with depression.[31,32] The DLPFC is an important component of the executive control network (CEN) and is involved in goal-directed cognition and attention allocation. It also plays a role in top-down emotional cognitive regulation[33,34] and the inhibition of excessive activation of primitive instinctive emotions in low-level brain regions, such as the anterior cingulate(ACC) and amygdala.[35]

Studies suggest that the amygdala, through bottom-up mechanisms, facilitates perceptual processing by directing or biasing attention.[36,37] Concurrently, a growing body of literature indicates that cognitive processes such as distraction or reappraisal can regulate emotional responses through top-down mechanisms.[38] The manner by which these two regions interact has become increasingly central to models of psychopathology. Similar to the amygdala, the DLPFC is a structurally and functionally heterogeneous brain region consisting of two main brain subregions, the middle frontal gyrus (MFG) and the superior frontal gyrus (SFG).[39–41] Positron emission tomography (PET) imaging has shown that, in unmedicated patients with MDD, GABA levels are reduced in several cortical regions, including the prefrontal cortex.[42] Similarly, reduced expression of the GAD67 protein or gene has been found in the DLPFC of patients with depression.[43] These dysfunctions in the GABAergic system in the DLPFC region will not only affect the activity of the brain region itself but also the relationships between the DLPFC and other brain regions.

In our previous study, AD patients showed lower functional connectivity (FC) between the right CM subregion and the right MFG as compared to NAD patients, highlighting the diverse neuropathological mechanisms underlying the interaction between the amygdala and DLPFC in emotional processing.[44] However, the pattern and extent of the involvement of the amygdala and its interaction with the DLPFC, in mediating negative emotions through the $GABA_A$ receptor remains largely unknown.

Research on the roles of single nucleotide polymorphisms (SNPs) in MDD has not yielded definitive answers. Therefore, new avenues are needed to identify biological markers of depression and avoid missing important rare sites.[45] In recent years, the multi-locus genetic profile score (MGPS) has been used to study the influences of multiple genes on psychiatric

disorders.[46] The MGPS method summarizes several gene polymorphic differences in order to synthesise relevant signals. In a recent study, this method was used to explore the relationship between the hypothalamic-pituitary-adrenal (HPA) axis and dopamine pathway-related SNPs and MDD.[47–49] Based on the contribution of the $GABA_A$ receptor to MDD and the evidence indicating that dysfunction of amygdala subregions might be the neurobiological mechanism of AD,[50] as evidenced by recent studies,[44,51] we hypothesised that dysfunctional connectivity of the amygdala subregions may mediate the connection between the MGPS of the $GABA_A$ receptor and the clinical symptoms of patients with AD.

Material and Methods

Selection of the Research Subjects

A total of 182 patients were recruited for this research from the Nanjing Brain Hospital Affiliated with Nanjing Medical University between September 2014 and December 2017. The Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition Text Revision (DSM-IV-TR) and the Mini International Neuropsychiatric Interview (MINI) were used to diagnose MDD.[52] All patients were in a current episode. The inclusion criteria for MDD patients included: right-handed, Han nationality, age >18 years, age <55 years. Potential study subjects must not have undergone repetitive transcranial magnetic stimulation (rTMS), electroconvulsive therapy (ECT), or any other physical therapy in the six months prior to undergoing MRI The exclusion criteria included: a history of alcohol and drug abuse, organic brain illness, serious physical illness, neurological illness, comorbid psychiatric diseases, and any contraindications to MRI. Further, patients' medical records were consulted to exclude patients who had received a diagnosis of bipolar disorder, patients in a mixed episode, and those with psychiatric symptoms and atypical symptoms, since these symptoms are considered to be associated with bipolar disorder. The patients who met the above criteria were included in this study. The 17-item Hamilton Rating Scale for Depression (HAMD-17)[53] was used to evaluate the severity of MDD. HAMAD-17 scores ranged from 0–50 on the day of scanning. Only patients who scored at least 17 were included in the study. The HAMD-17 anxiety/somatic factor was calculated as the total of the following six items: mental anxiety (item 10), somatic anxiety (item 11), gastrointestinal symptoms (item 12), general somatic symptoms (item 13), hypochondria (item 15), and insight (item 17).[3] Scores on the anxiety/somatic factor ranged from 0–18. In addition to this factor, the HAMD-17 scale can be categorised into four independent symptom types – weight loss, psychomotor retardation, cognitive impairment, and sleep disorder; every category consists of relevant items.[54] Examination of these four categories can provide more comprehensive clinical insight into the patients.

MDD patients were divided into an AD group and an NAD group as per the multi-dimensional criteria outlined in recent reports.[55,56] Specifically, AD was defined as MDD plus HAMD-17 anxiety/somatic score ≥7, as previously described.[57,58] All MDD patients with anxiety/somatic factor scores < 7 were included in the NAD group.

The same inclusion criteria were applied to the healthy controls and all potential participants were screened for medical conditions. Healthy controls with a first-degree relative with a DSM-IV axis one psychiatric disorder were not eligible for participation. Ultimately, 64 healthy controls from the local community were enrolled in the study. All participants underwent blood collection and MRI scanning (For patients, the MRI scanning was finished after hospitalization within 2 days).

Informed consent was obtained from all participants, and the Medical Research Ethics Review Committee at the Brain Hospital Affiliated with Nanjing Medical University approved this research.

Selection Criteria for SNPs

The candidate SNPs of the $GABA_A$ receptor gene met at least two of the following standards: (1) shown to have an association with MDD or anxiety disorders previously, (2) are in the 3′ untranslated region (UTR3), 5′ untranslated region (UTR5), or coding sequence (CDS) area of the $GABA_A$ receptor gene, and (3) have a minor allele frequency (MAF) >5% in the Han Chinese population. All relevant information on the candidate SNPs, including the variant type, chromosome, alleles, gene, and MAF, was obtained from the Golden Path database (NCBI build 35: http://genome.ucsc.edu/). After filtering, 22 SNP loci were selected as candidate gene loci: *GABRA2* (rs11503014, rs1442062, rs279858, rs279871), *GABRA3* (rs12688128, rs201877630, rs7391474, rs750841), *GABRA4* (rs12506608, rs1400685, rs2229940, rs6447520), *GABRA5* (rs140682,

rs140685), *GABRB3* (rs8041610, rs13303016, rs20318), *GABRD* (rs2229110, rs28398772, rs28408173), *GABRG1* (rs976156), and *GABRG2* (rs424740).[20,59–63] The 22 loci satisfied the above criteria and were chosen for subsequent analysis.

DNA Extraction and Genotyping

DNA was extracted from whole venous blood samples obtained from the study subjects. The blood samples were treated with ethylenediaminetetracetic acid (EDTA), an anticoagulant, utilizing an EZ Gene Blood DNA Mini-prep Kit (Bio Miga, San Diego, CA). The Illumina standard genotyping scheme (Illumina, United States) was used to perform genotyping on an Illumina HiSeq X-10 Sequencing System. The DNA extraction and genotyping processes have been described in our previous study.[64] PLINK v1.07 software was utilized to perform data quality control, including examination of the personal genotyping call rate, MAF, and Hardy-Weinberg equilibrium (HWE). None of the SNPs had a missing call rate > 0.1 or MAF < 0.05. Five SNPs did not conform to the HWE law. After performing gene quality control, the 17 SNPs that met the required standards were considered for follow-up analysis. These SNPs included rs11503014, rs1442062, rs279858, rs279871, rs12506608, rs1400685, rs2229940, rs140682, rs140685, rs13303016, rs20318, rs2229110, rs28398772, rs28408173, rs976156, rs424740, and rs201877630.

Calculation of the $GABA_A$ Receptor MGPS

Given the small effect that each SNP has in the pathogenesis of MDD, the MGPS was used in this study to calculate the effect of all risk genes. Each participant was assigned a MGPS based on the mutant genotypes of SNPs they carried. The MGPSs of different SNP combinations in the study subjects were calculated using specific assignment principles as follows: wild-type homozygote was coded as "0", heterozygote as "1", and mutation homozygote as "2". The approach has been detailed in previous research.[48,49]

MRI Acquisition

Bold images were obtained using gradient-recalled echo-planar imaging (GRE-EPI) (repetition time [TR] = 3000 ms; echo time [TE] = 40 ms; flip angle = 90°; 32 slices, slice thickness = 4 mm; field of view [FOV] = 240 mm × 240 mm; matrix size = 64 × 64; voxel size = 3.75×3.75 mm) on a 3T Siemens Verio scanner (Siemens, Erlangen, Germany). Each scan lasted 6 min and 45s. T1-weighted images were acquired with the following parameters: TR = 1900 ms; TE = 2.48 ms; flip angle = 9°; 176 slices, slice thickness = 1 mm; FOV = 250×250 sq.mm; matrix size = 256×256. The total time was 4 min and 18s. During data acquisition, the participants were required to remain still and in a relaxed state with their eyes closed. A homogeneous birdcage head coil was used. Scans were performed at the Nanjing Brain Hospital Affiliated with Nanjing Medical University.

Pre-Processing and Analysis of Rs-fMRI Data

The first six volumes of data from each participant were removed to eliminate the initial instability in the signal due to the influence of the machine. Thereafter, slice timing correction was performed, and the images were spatially normalized to the standard Montreal Neurological Institute space. The images were resampled into 3-mm isotropic voxels and the data were spatially smoothed with a Gaussian kernel (full width at half maximum 4 mm) and a temporal band-pass filter (0.01–0.08 hz). Several nuisance signals (including the Friston head motion parameters, global mean, and noise from cerebrospinal fluid and white matter) were regressed out of the data. The fMRI data pre-processing was conducted using the Statistical Parametric Mapping (SPM12, www.fil.ion.ucl.ac.uk/spm) toolkit in MatlabR2013b. After pre-processing, head movements ≥ 2 mm in any direction of x, y, and z and ≥ 2° in any angular dimension were excluded. In total, nine patients developed bipolar disorder, seven had poor imaging quality, and 13 had large head movements. Therefore, these samples were excluded from the analysis. Ultimately, imaging data from a total of 153 patients and 62 healthy controls were retained for further analysis.

Region of Interest (ROI) Definition and FC Analysis

The ROIs of the amygdala were defined anatomically using probability maps based on cell structures from the JuBrain Cell Structure Atlas.[30] According to anatomical and functional definitions, the amygdala can be divided into three subregions:

the CM, LB, and SF subregions. The SPM Anatomy Toolbox (www.fz-juelich.de/inm/inm-1/DE/Forschung/_docs/SPMAnatomyToolbox/SPMAnatomyToolbox-node.html)[65] was used to extract six subregions of the amygdala as ROIs; see Figure 1. According to previous research, the DLPFC consists of two brain areas, the SFG and MFG. The mask of the DLPFC was constructed using the WFU_PickAtlas_3.0.4 toolbox (NITRC: WFU_PickAtlas: Tool/Resource Info); see Figure 2. Then, the above anatomical masks, including the amygdala subregions and DLPFC (including the MFG and SFG), were applied in the DPARSF toolbox[66] when calculating the FC values. To obtain the time series, the BOLD signal from all voxels in each ROI was averaged and extracted. Subsequently, Pearson's correlations were calculated between the extracted time series in each ROI. Finally, the correlation coefficients were submitted to Fisher's Z transformation for normalisation. After the above steps, a 4 by 6 FC matrix was obtained for each participant.

Statistical Analysis

One-way analysis of variance (ANOVA) was conducted using SPSS19.0 software to compare the average age and years of education of the AD/NAD and HC groups. The Chi-square test was utilized to examine gender differences. The HAMD-17 total score and its factor scores were analysed by *t*-tests between the AD and NAD groups. The chi-square test was utilized to compare the genotypes and allele frequencies between the MDD patients (AD, NAD, and MDD) and the HC group. Estimated odd ratios (ORs) and 95% confidence intervals (95% CIs) were calculated for the MDD patients (AD, NAD, and MDD) and the HC group (Bonferroni corrected, $p < 0.05/17$). ANCOVA analysis was used to examine the differences in the FC between the amygdala and DLPFC (Bonferroni correction, $p< 0.05/10$) and the MGPS among the three groups. As the years of education differed between the three groups, in order to reduce the effects of this variable on the results, it was included as a covariate. Although age and gender were not significantly different between the three groups, they were still included as covariates to eliminate any potential effects. Post-hoc *t* tests were performed to analysis the difference between each paired groups in terms of FC and MGPS. Pearson correlation analyses were

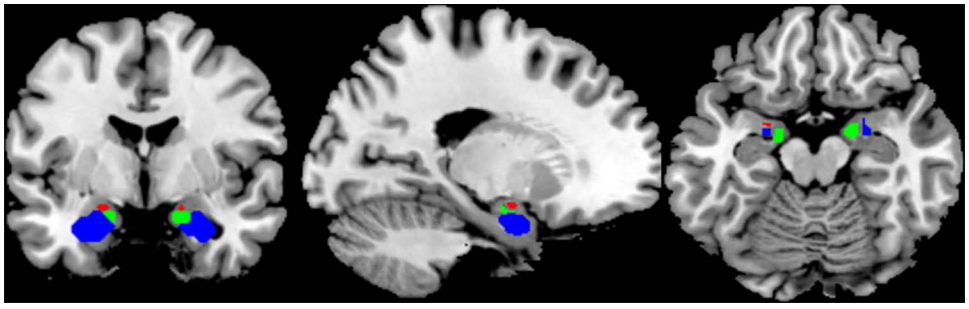

Figure 1 The amygdala subregions.
Notes: The centromedial (CM; red), laterobasal (LB; blue) and superficial (SF; green) subregions.

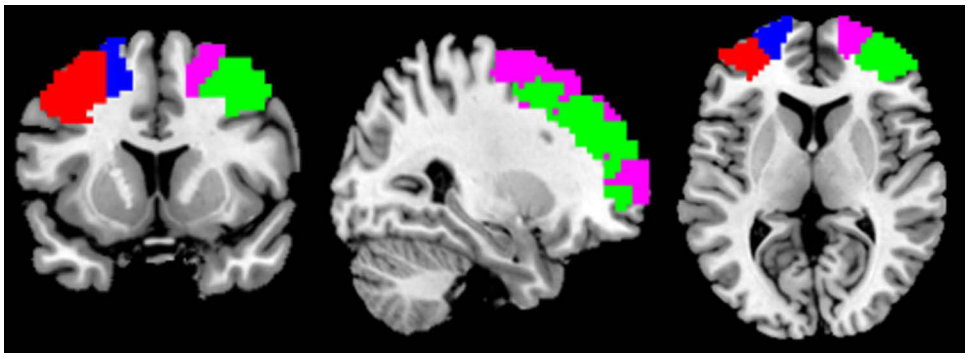

Figure 2 The ROI of the dorsolateral prefrontal cortex (DLPFC).
Notes: The right superior frontal gyrus (purple), right middle frontal gyrus (green), left superior frontal gyrus (blue), left middle frontal gyrus (red).

performed between each pairs of FC, total HAMD and its factors, FC between the amygdala and DLPFC in AD group. Finally, mediation analysis was conducted to evaluate the possible connections between the MGPS, amygdala subregions, and clinical symptoms. The established patterns are displayed below:

$$Y = cX + c1U1 + c2U2 + c2U2 + e1 \quad (1)$$

$$M = aX + a1U1 + a2U2 + a2U2 + e2 \quad (2)$$

$$Y = c'X + bM + b1U1 + B2U2 + b2U2 + e2 \quad (3)$$

where X, Y and M are the independent variable (MGPS), the dependent variable (clinical symptoms), and the mediator (abnormal FC of the amygdala subregions), respectively, with each value entered separately. The direct influence is the influence of X on Y, independent of the influence of M on Y (path c′). The indirect influence, or the influence of X on Y via M, is assessed as the product of the influence of X on M and the influence of M on Y, controlling for X (ab with 95% bootstrap CI). The overall influence of X on Y is the total of the direct and indirect influences (path c). To analyse the above patterns, simple moderation analysis was performed using the PROCESS Marco in the R package (with gender, age, and years of education as covariates).

Results

Demographic and Clinical Characteristics of the Sample

The participants' demographic characteristics and clinical symptoms are shown in Table 1. There were no significant differences in gender, antidepressant use, and age between the groups ($p > 0.05$). In contrast, the years of education of the three groups were dramatically different ($p < 0.05$). In relation to clinical symptoms, the HAMD-17 except anxiety/somatic factor displayed no significant difference between the AD and NAD groups ($p > 0.05$). While the HAMD-17 and anxiety/somatic factor were significantly different between the two groups of patients ($p < 0.05$).

The Associations Between $GABA_A$ Receptor Gene Polymorphisms and AD

Among the 17 SNPs studied, two SNPs (rs140682, rs140685) were significantly associated with AD (rs140682 [OR=0.549; 95% CI: 0.335–0.9; p=0.021; $\chi 2$=5.713] and rs140685 [OR=1.9; 95% CI: 1.16–3.113; p=0.011;

Table 1 The Demographic and Clinical Characteristics of Three Groups

Measure (mean±SD)	AD (n=83)	NAD (n=70)	HC (n=62)	*p*-value
Age, year[a]	34.7±10.45	31.67±9.65	32.92±9.87	0.17
Sex, male:female[b]	33/50	33/37	28/34	0.63
Education, year[a]	13.12±3.17	14.06±2.80	15.45±2.76	0.00
HAMD score[c]	24.29±4.22	19.42±3.92	–	0.00
Weight[c]	0.76±0.85	0.64±0.83	–	0.40
Psychomotor retardation[c]	7.60±1.33	7.27±1.49	–	0.15
Cognition[c]	2.47±1.80	2.01±1.56	–	0.09
Sleep[c]	3.50±1.88	3.50±2.04	–	0.98
Anxiety/somatic[c]	9.08±1.65	4.46±1.30	–	0.00
HAMD-17-anxiety/somatic[c]	15.20±4.12	14.95±3.78	–	0.18
Antidepressants[b]				0.89
SSRI	21	17		
SNRI	13	9		
NaSSA	10	7		
Treatment-naive	39	37		

Notes: [a]indicates *p* values for one-way ANOVA; [b]indicates *p* values for chi-square test. [c]indicates *p* values for independent two-sample t-tests.

Abbreviations: SD, standard deviation; AD, anxious depression; NAD, non anxious depression; HC, healthy control; HAMD, Hamilton Rating Scale for Depression.

$\chi 2$=6.589]), two were associated with NAD (rs140682 [*OR*=0.539; 95% CI: 0.322–0.9; *p*=0.022; $\chi 2$=5.642] and rs140685 [*OR*=2.009; 95% CI: 1.204–3.353; *p*=0.008; $\chi 2$=7.238]) and two were associated with MDD (rs140682 [*OR*=0.544; 95% CI: 0.345–0.858; *p*=0.009; $\chi 2$=6.958] and rs140685 [*OR*=1.948; 95% CI: 1.236–3.071; *p*=0.005; $\chi 2$=8.422]). However, these associations were no longer statistically significant after Bonferroni correction. In summary, no single SNP was associated with AD (Table 2).

Table 2 Association Between $GABA_A$ Receptor Gene Polymorphism and AD

SNP	Risk Allele	OR value	95% CI	*P* value	χ^2
MDD					
rs11503014	G	0.941	0.442–2.005	0.875	0.025
rs1442062	A	1.248	0.492–3.167	0.666	0.218
rs279858	C	0.746	0.494–1.127	0.176	1.938
rs279871	T	1.342	0.887–2.024	0.175	1.935
rs12506608	C	1.091	0.721–1.650	0.752	0.164
rs1400685	T	0.186	0.021–1.707	0.169	2.731
rs2229940	T	0.954	0.622–1.467	0.912	0.046
rs140682	T	0.544	0.345–0.858	0.009*	6.958
rs140685	T	1.948	1.236–3.071	0.005*	8.422
rs13303016	G	0.991	0.591–1.663	1	0.001
rs20318	A	1.036	0.651–1.651	0.906	0.022
rs2229110	C	0.968	0.631–1.485	0.913	0.022
rs28398772	T	1.181	0.625–2.233	0.642	0.263
rs28408173	T	1.033	0.684–1.560	0.917	0.024
rs976156	C	0.979	0.646–1.485	1	0.010
rs424740	T	0.758	0.495–1.161	0.223	1.624
rs201877630	T	3.422	0.438–26.734	0.367	0.815
AD					
rs11503014	G	0.945	0.411–2.175	0.894	0.018
rs1442062	A	1.297	0.474–3.552	0.633	0.257
rs279858	C	0.731	0.465–1.150	0.206	1.838
rs279871	T	1.368	0.869–2.153	0.205	1.833
rs12506608	C	1.266	0.804–1.995	0.356	1.037
rs1400685	T	0.848	0.483–1.491	0.663	0.329
rs2229940	T	0.792	0.491–1.282	0.388	0.909
rs140682	T	0.549	0.335–0.901	0.021*	5.713
rs140685	T	1.902	1.160–3.113	0.011*	6.589
rs13303016	G	0.966	0.548–1.703	1	0.014
rs20318	A	1.091	0.654–1.817	0.796	0.110
rs2229110	C	0.882	0.549–1.416	0.629	0.272
rs28398772	T	1.270	0.638–2.531	0.607	0.465
rs28408173	T	1.123	0.715–1.766	0.646	0.254
rs976156	C	1.026	0.649–1.621	1	0.012
rs424740	T	0.740	0.462–1.187	0.226	1.562
rs201877630	T	3.433	0.415–28.401	0.407	0.688
NAD					
rs11503014	G	0.937	0.391–2.246	0.884	0.021
rs1442062	A	1.188	0.411–3.432	0.796	0.101
rs279858	C	0.766	0.477–1.229	0.281	1.226
rs279871	T	1.306	0.814–2.096	0.281	1.226
rs12506608	C	0.901	0.557–1.455	0.714	0.184
rs1400685	T	0.777	0.427–1.414	0.444	0.685

(*Continued*)

Table 2 (Continued).

SNP	Risk Allele	OR value	95% CI	*P* value	χ^2
rs2229940	T	1.185	0.726–1.932	0.535	0.461
rs140682	T	0.539	0.322–0.900	0.022*	5.642
rs140685	T	2.009	1.204–3.353	0.008*	7.238
rs13303016	G	1.024	0.564–1.860	1	0.006
rs20318	A	0.974	0.571–1.661	1	0.009
rs2229110	C	1.084	0.664–1.767	0.804	0.103
rs28398772	T	1.074	0.515–2.238	0.855	0.036
rs28408173	T	0.930	0.578–1.496	0.809	0.090
rs976156	C	0.924	0.572–1.493	0.807	0.103
rs424740	T	0.781	0.477–1.278	0.378	0.969
rs201877630	T	3.407	0.39–29.735	0.452	0.566

Notes: *Showed the result was significantly difference. In the above analysis, gender, age, and educational years as covariates.
Abbreviations: SNP, Single nucleotide polymorphism; OR value, Risk ratio; 95% CI, 95% confidence interval.

Associations Between the MGPS and Clinical Traits in AD Patients

When including gender, age, and years of education as covariates, ANCOVA analysis revealed a significant difference in the MGPS among the three groups ($F = 3.74$, $p = 0.025$). Least Significant Difference (LSD) test was used in pairwise comparison, The MGPS was found to be significantly higher in the AD group than in the NAD group ($\mu = 0.83$, $p = 0.037$) and the HC group ($\mu = 1.02$, $p = 0.013$). There was no significant difference between the NAD group and the HC group ($\mu = 0.19$, $p = 0.654$) (Figure 3). Then, the correlations between the MGPS and the clinical symptoms of AD and NAD patients were investigated. The results indicated that the MGPS was significantly correlated with the anxiety/somatisation score ($r = 0.223$, $p = 0.043$), but not with any other clinical symptoms in the AD group ($p > 0.05$) (Figure 4). There were no correlations between the MGPS and clinical symptoms in the NAD group.

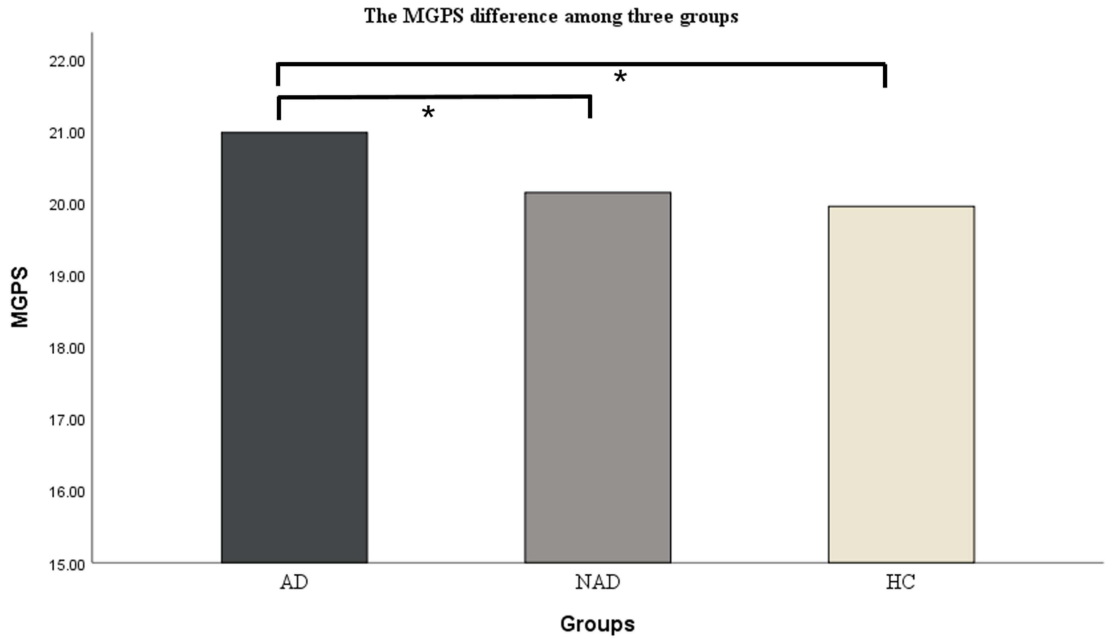

Figure 3 Comparison of the MGPS among the three groups.
Note: *statistically significant *p*-value ≤ 0.05.
Abbreviations: AD, anxious depression group; NAD, non-anxious depression group; HC, healthy control group.

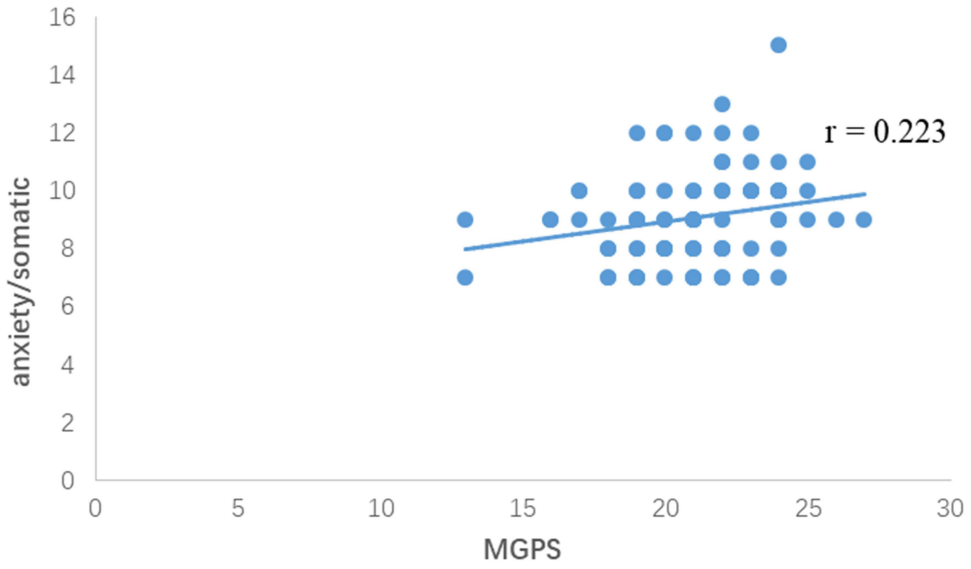

Figure 4 Correlation analysis between the MGPS and the anxiety/somatic factor in the AD group.
Notes: N=83. The x-axis is the MGPS and the y-axis is the anxiety/somatic score (r = 0.223, p = 0.043).

Differences in the Amygdala Network Between the Three Groups

There were significant differences among the three groups in the FC between the right CM and right MFG (F = 9.37, p = 0.000125), and between the right LB and right MFG (F = 9.46, p = 0.00016). Subsequently, pairwise comparisons revealed that AD patients showed decreased FC between the right CM and right MFG (p<0.001), and between the right LB and right MFG (p <0.001), as compared to the NAD group. Decreased FC was also found between the right LB and right MFG in the AD group, as compared to the HC group. No other significant differences in FC were observed in the pairwise comparisons (Table 3).

Table 3 The Difference of FC Among the Three Groups

ROI	DLPFC	FC_AD	FC_NAD	FC_HC	F	P
CM_L	SFG_L	−0.09±0.07	−0.09±0.07	−0.07±0.08	0.201	0.871
	SFG_R	−0.15±0.12	−0.13±0.09	−0.19±0.15	1.132	0.323
	MFG_L	−0.20±0.19	−0.13±0.11	−0.16±0.13	2.203	0.112
	MFG_R	−0.22±0.16	−0.16±0.17	−0.25±0.20	3.522	0.033
CM_R	SFG_L	−0.12±0.08	−0.14±0.09	−0.15±0.11	0.541	0.582
	SFG_R	−0.09±0.05	−0.11±0.07	−0.12±0.15	0.270	0.851
	MFG_L	−0.22±0.17	−0.20±0.19	−0.23±0.19	0.522	0.592
	MFG_R	−0.13±0.11	−0.06±0.49	−0.01±0.08	9.371	<0.001*
LB_L	SFG_L	−0.04±0.02	−0.06±0.03	−0.06±0.05	0.196	0.821
	SFG_R	−0.12±0.07	−0.11±0.15	−0.16±0.12	1.172	0.312
	MFG_L	−0.13±0.11	−0.10±0.08	−0.12±0.10	0.253	0.771
	MFG_R	−0.19±0.20	−0.15±0.11	−0.19±0.18	0.872	0.422
LB_R	SFG_L	−0.12±0.08	−0.14±0.11	−0.14±0.12	0.211	0.811
	SFG_R	−0.08±0.04	−0.09±0.04	−0.11±0.09	0.493	0.612
	MFG_L	−0.13±0.11	−0.11±0.08	−0.11±0.09	0.410	0.671
	MFG_R	0.06±0.48	0.18±0.16	0.17±0.15	9.462	<0.001*

(*Continued*)

Table 3 (Continued).

ROI	DLPFC	FC_AD	FC_NAD	FC_HC	F	P
SF_L	SFG_L	−0.04±0.05	−0.08±0.06	−0.08±0.05	0.83	0.439
	SFG_R	−0.08±0.05	−0.09±0.05	−0.11±0.13	0.451	0.631
	MFG_L	−0.13±0.11	−0.10±0.08	−0.11±0.09	0.323	0.722
	MFG_R	−0.15±0.10	−0.13±0.07	−0.11±0.08	0.572	0.563
SF_R	SFG_L	−0.10±0.08	−012±0.10	−0.13±0.11	0.471	0.630
	SFG_R	−0.15±0.11	−0.13±0.15	−0.18±0.20	1.554	0.212
	MFG_L	−0.17±0.16	−0.16±0.13	−0.20±0.16	0.711	0.491
	MFG_R	−0.15±0.11	−0.11±0.09	−0.17±0.13	1.952	0.142

Note: *indicate $p < 0.001$.
Abbreviations: ROI, Region of Interest; AD, anxious depression; NAD, non-anxious depression; HC, healthy control; FC. Functional connectivity; SFG, superior frontal gyrus; MFG, middle frontal gyrus; CM, centromedial; LB, laterobasal.

Correlations Between the MGPS and the FC Between the Amygdala and DLPFC

Partial correlation analysis was conducted between the MGPS and the measures of FC that were significantly different between the AD and NAD groups (including the FC between the right CM and the right MFG and the FC between the right LB and the right MFG). The results indicated that the MGPS was significantly negatively correlated with the FC between the right CM and right MFG ($r = -0.24$, $p = 0.034$), even when gender, age, and years of education were included as covariates (Figure 5).

Correlations Between the FC of the Amygdala Subregions and Clinical Symptoms

In AD patients, a negative correlation was observed between the FC of the right CM and right MFG and the anxiety/somatic factor ($r = -0.36$, $p = 0.001$). No other correlations were observed between measures of FC and measures of other clinical features ($p > 0.05$); see Figure 6.

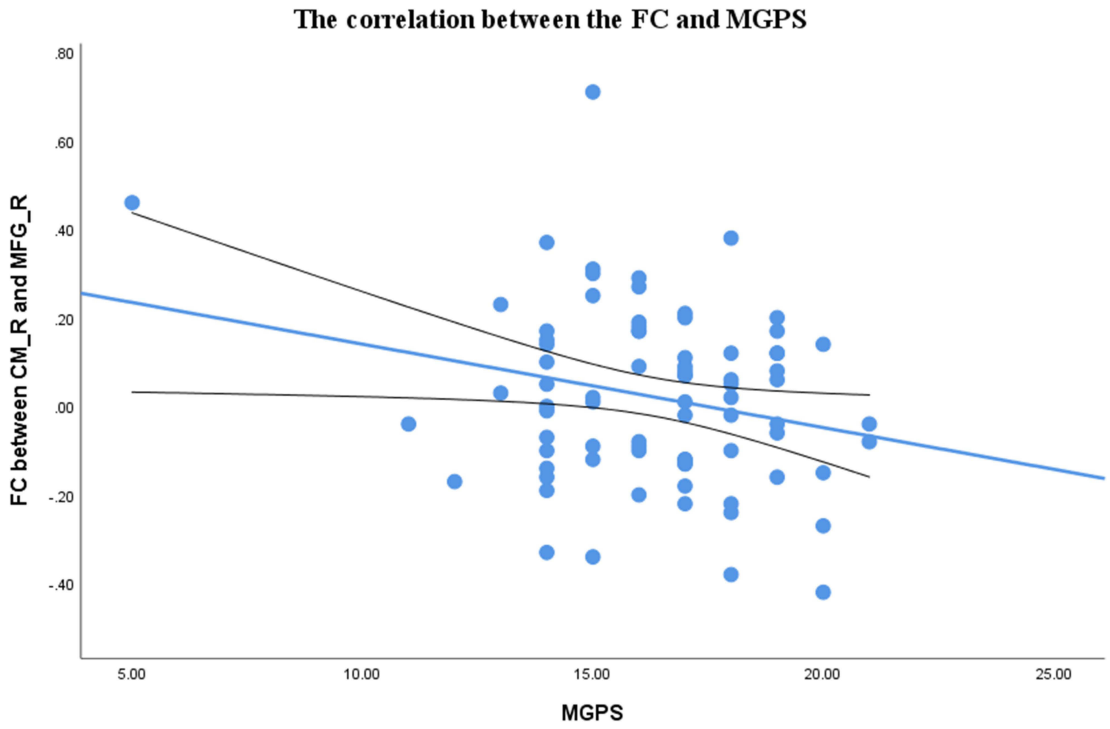

Figure 5 Correlation analysis between the MGPS and FC in the anxious depression group.
Notes: N=83. The x-axis is the MGPS and the y-axis is the FC between the right CM and right MFG ($r = 0.241$, $p = 0.034$).

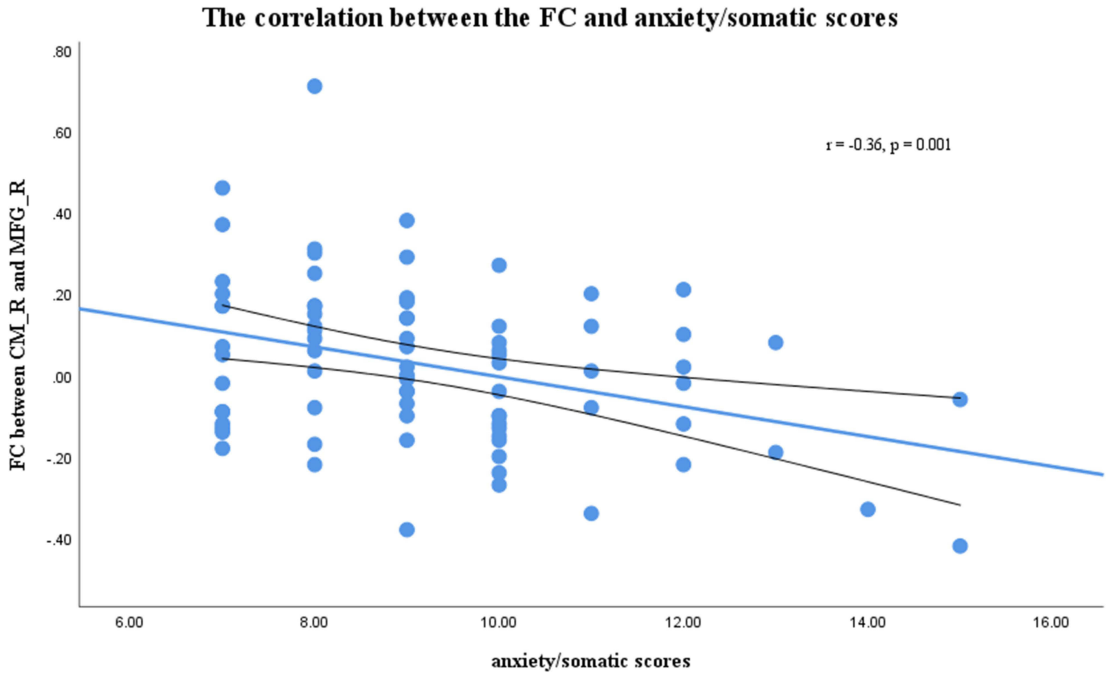

Figure 6 Correlation analysis between FC and clinical symptoms.
Notes: N=83. The x-axis is the anxiety/somatic factor and the y-axis is the FC between the right CM and right MFG (r = 0.360, p = 0.001).

Mediating Effect Between the MGPS, Amygdala Subregion Network, and Clinical Symptoms

The analysis of the mediating effect among the three factors showed a significant total influence (c =0.168, 95% CI: 0.00585–0.34, p=0.034). Test coefficient a (p = 0.035) and test coefficient b (p = 0.005) were statistically significant. Therefore, the confidence interval of ab was reported. The bootstrap method was used to test coefficient ab (ab = 0.068, p=0.034, 95% CI: 0.00283–0.14). The indirect effect was significant. The direct effect, c'= 0.11, p =0.17, was not statistically significant. Further, the effect of c′ was significant, indicating that there was a mediating effect rather than a direct effect. This suggests that the FC between the right CM and the right MFG plays a mediating role in the associations between the $GABA_A$-MGPS and the anxiety/somatic factor. The results indicated that FC could explain 32.19% of the association between the MGPS and clinical symptoms; see Figure 7.

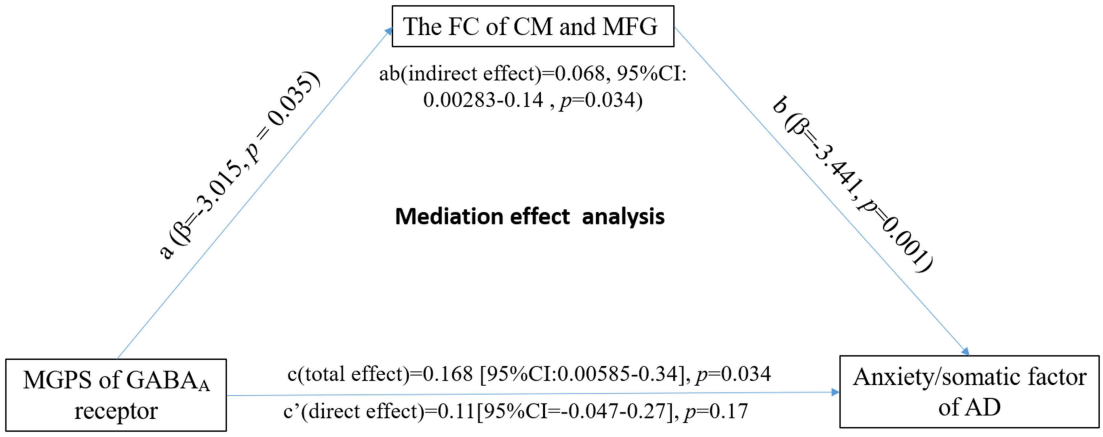

Figure 7 Analysis of the mediating effect in patients with anxious depression.
Notes: c, c′ and a, b are coefficients [95% confidence interval].

Discussion

The results of the current research revealed, for the first time, a mediating role of the FC between the right CM and right MFG in the relationship between the $GABA_A$ receptor MGPS and anxiety/somatic symptoms in AD patients. These results suggest that the FC between the right MFG and right CM could be the potential neuropathological basis of AD. These results provide new insight into the associations between genetic factors, abnormalities in brain function, and clinical symptoms.

This study did not identify any significant associations between single SNPs and AD. Further, the associations between MDD and both rs140682 and rs140685 did not survive Bonferroni correction for multiple comparisons. Thus, the MGPS was the focus of this research. Previous studies have reported inconsistent findings regarding the connection between $GABA_A$ receptor subunit gene polymorphisms and depression and anxiety symptoms. A comprehensive genomics study of 119 SNPs in 24 GABAergic and glutamatergic genes aimed to elucidate the connections between genotypes, the brain transcriptome, and MDD or suicidal behaviour. However, no SNPs were found to be associated with depression or suicidal behaviour.[62] In another study of the associations between anxiety spectrum disorder and $GABA_A$ receptor gene polymorphisms, 26 SNPs of four $GABA_A$ receptor genes were included in the association analysis. Although two SNPs were found to be associated with anxiety, these two loci could not be verified in new participants.[20] These two studies suggest that single SNP loci of the $GABA_A$ receptor do not play essential roles in the pathogenesis of anxiety and depression symptoms.[20,67] Such findings are consistent with previous studies indicating that genetic susceptibility to depression is related to the accumulation of multiple genetic variations or polygenic loci.[68]

The current study also found no associations between single SNPs and AD. Research suggests that depression is related to the joint effects of numerous genes operating with particular biological effects.[69] As such, the MGPS method has recently been employed to investigate the role of polygenes in psychiatric disorders, including MDD.[46]

In the present study, the FC between the right MFG and right CM was found to be decreased in the AD group and was negatively correlated with the HAMAD-17 anxiety/somatic factor. This finding suggests a reduction in the connection between the amygdala and medial PFC. Therefore, anxiety symptoms may reflect prefrontal dysfunction and amygdala hyperactivity, consistent with previous studies.[70–72]

Previous studies have reported that the interaction between the medial PFC (including the MFG) and the amygdala (especially the CM subregion) plays a crucial role in enhancing the visual processing of emotional stimuli and in the selective attention process.[73] Several studies have shown that the PFC (including the MFG) regulates negative emotions through the top-down inhibition of amygdala output.[74] He et al[75] found that MDD patients with severe anxiety exhibit a widespread reduction in FC in the amygdala network, especially in the frontoparietal system. Tang et al[76] reported that MDD patients exhibit hypoconnectivity between the CM and the brainstem/cerebellum. Our results are similar to those of He et al, indicating that reduced FC between the right MFG and the right CM may constitute the underlying neuropathology and a potential biomarker for AD.

The current results also demonstrated that the MGPS of the $GABA_A$ receptor was negatively correlated with the FC of the right MFG and right CM. This finding can be interpreted as follows.

Input from the medial PFC to the amygdala plays a clear role in anxiety states in humans and animals. Pyramidal neurons of the medial PFC send excitatory projections to the amygdala and are controlled by the GABA interneuron network.[77,78] Unlike the LB, the CM is a striatal-like framework composed almost entirely of inhibitory neurons, including local interneurons and projective neurons.[79] The CM acts as an integrated centre that translates emotionally relevant sensory information about the external and internal environment into behavioural and physiological responses. This process is regulated by top-down inhibition through GABAergic neurons.

Past research has shown the degree of GABA receptors in the medial prefrontal lobe of animals and humans or the level of the immune marker of its transporter is decreased.[80,81] Therefore, we speculate that the loss of GABAergic neurons in the MFG leads to a decrease in its top-down regulation of the amygdala, especially in the CM region. The CM contains abundant $GABA_A$ neurons,[82] which receive inputs from the LB and PFC neurons. The activity of the CM, which is responsible for information output and physiological responses, is downregulated by the prefrontal regions (including the right MFG).[75,83,84] Hence, we speculate that patients with AD have increased levels of the MGPS of the $GABA_A$ receptor

in the MFG brain region. This translates to a decrease in the receptor, which results in a weakening of top-down regulation in the CM region of the brain. This could cause the emergence and growth of anxiety symptoms in AD patients.

The current study discovered important associations across three variables of interest: the MGPS of the $GABA_A$ receptor gene, CM amygdala-MFG connectivity, and the anxiety/somatic factor in MDD. Modelling of the paths between these three variables can shed light on the possible associations underlying these pathways. Indeed, the reduction in CM-MFG connectivity may serve as a potential mediator in the association between the MGPS and clinical features. The CM is a subregion of the amygdala that plays a critical role in regulating anxiety,[28] which is induced by the $GABA_A$ receptor located in the CM region.[85] An optogenetic study revealed that stimulation of the basolateral terminals in the CM produces anxiolytic-like effects, while their inhibition elicits the opposite effect.[86] Therefore, reduced amygdala-PFC connectivity may serve as an endophenotype of the $GABA_A$ receptor,[86] wherein the high cumulative effects predisposes individuals to a range of neuropsychiatric disorders by decreasing inhibitory control.[87–89] This novel interpretation aligns with the associations between reduced $GABA_A$ receptors and various neuropsychiatric diseases.

There are several limitations of this research that should be noted when interpreting the findings. First, this study used a combination of imaging and genetics to explore the role of various factors in AD. Although this method can increase the effect of samples to a certain extent, the overall sample size of this study was still small. Second, the number of years of education differed significantly among the groups, which may have affected the conclusions. Third, this study did not recruit drug naïve patients, which could restrict generalisation of the outcomes. Finally, the MGPS method assumes that each risk SNP may play an equal role within the whole genome, but this may not be the case in reality. This method also ignores the individualized effects of each SNP and the interaction effects with each other. Further, this study did not identify any single SNPs associated with AD, which may have affected our analysis of the associations between the MGPS, FC, and clinical symptoms. Nonetheless, the present research still offers an effective beginning point for future genetic imaging investigations of AD.

In conclusion, the current results suggest that reduced FC between the right MFG and the right CM may represent a possible neuropathological mechanism underlying the pathophysiology of AD. The cumulative effect of $GABA_A$ receptor subunit mutations is related to the susceptibility to AD. Furthermore, a decrease in FC between the right MFG and the right CM subregion mediates the association between the $GABA_A$ receptor MGPS and AD. These findings offer new insight into the pathogenesis and biomarkers for AD.

Author Contributions

All authors made significant contributions to the reported work, whether in the conception, study design, execution, acquisition of data, analysis and interpretation. All authors took part in drafting, revising, or critically reviewing the article; gave final approval of the version to be published; agreed on the journal to which the article has been submitted; and agreed to be accountable for all aspects of the work.

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Disclosure

The authors declare that this study was performed without any business or financial relationships that could be interpreted as potential conflicts of interest.

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