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Structural and functional alterations in MRI-negative drug-resistant epilepsy and associated gene expression features

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ABSTRACT

Neuroimaging techniques have been widely used in the study of epilepsy. However, structural and functional changes in the MRI-negative drug-resistant epilepsy (DRE) and the genetic mechanisms behind the structural alterations remain poorly understood. Using structural and functional MRI, we analyzed gray matter volume (GMV) and regional homogeneity (ReHo) in DRE, drug-sensitive epilepsy (DSE) and healthy controls. Gene expression data from Allen human brain atlas and GMV/ReHo were evaluated to obtain drug resistance-related and epilepsy-associated gene expression and compared with real transcriptional data in blood. We found structural and functional alterations in the cerebellum of DRE patients, which may be related to the mechanisms of drug resistance in DRE. Our study confirms that changes in brain morphology and regional activity in DRE patients may be associated with abnormal gene expression related to nervous system development. And SP1, as an important transcription factor, plays an important role in the mechanism of drug resistance.

1. Introduction

Epilepsy is a chronic brain disease that causes dysfunction of the central nervous system due to abnormal discharges of neurons in the brain. It is mainly characterized by recurrent seizures, which have a great negative impact on the patient's quality of life and mental and psychological well-being (Xue-Ping et al., 2019). The International League Against Epilepsy (ILAE) refers to this type of failure to achieve sustained seizure freedom after standardized treatment with 2 antiepileptic drugs as drug-resistant epilepsy (DRE) (Scheffer et al., 2017). Approximately 50 million people worldwide suffer from epilepsy seizures, and 1/3 of these patients are DRE (GBD 2016 Neurology Collaborators, 2019). More importantly, a definite etiology was found in only 44.1 % of DRE, and MRI abnormalities were found in 49 % of DRE (Alturaifi et al., 2024). Current study suggests that the risk factors associated with drug resistance are related to earlier age of onset, ineffectiveness of the first antiepileptic drug, abnormal EEG at diagnosis, and neurologic dysfunction or mental retardation at diagnosis (Berg, 2009). However, these risk factors for DRE do not explain the pathophysiologic mechanisms of resistance.

Neuroimaging technology, as a new and promising research tool, is increasingly being used to identify abnormalities in brain structure and brain function in a variety of brain disorders (Li et al., 2022; Wang et al., 2023). It is currently accepted that the thalamo-cortical circuit is critical in the onset and development of epilepsy, and that diminished thalamic inhibition of the cortex is an important mechanism for seizures (Warsi et al., 2022). DRE that is effectively treated with DBS exhibits strong structural connectivity of the thalamus and cerebellum, and the cerebellum may desynchronize the brain through extensive structural connectivity (Remore et al., 2023). There is evidence of an 87.2 % prevalence of DRE in combination with cerebellar degeneration (Ibdali et al., 2021a). Earlier studies using voxel-based morphometry (VBM)

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analysis found significant cortical and subcortical atrophy in epilepsy (Bonilha and Halford, 2009a; Bonilha et al., 2010). Regional Homogeneity (ReHo) is used as a method of resting-state functional magnetic resonance (rs-fMRI) data analysis due to the highly synchronized degree of neuronal activity across voxels within a brain region when it is activated. Reho can be used to localize local spontaneous neural activity (Zang et al., 2004). However, many studies have been analyzed only in comparison with healthy controls, which is not objective enough to explain the mechanisms of drug resistance in DRE. In addition, fewer studies have examined both brain structure and function in DRE. In order to discover important brain regions with altered brain structure and function in DRE, VBM and ReHo can be chosen to be studied together.

Although MRI can help us understand the functional and structural changes in the brain in DRE, it cannot reveal the specific changes in it at the cellular and molecular level. Studies have shown that epilepsy has a high genetic risk (International League Against Epilepsy Consortium on Complex Epilepsies, 2014). ILAE has identified 16 risk loci in a genome-wide association analysis of epilepsy, with significant enrichment of genes at the loci and antiepileptic drug targets (International League Against Epilepsy Consortium on Complex Epilepsies, 2018). In recent years, transcription-neuroimaging association analysis has emerged as a promising analytical modality for studying the molecular basis of brain imaging phenotypes (Fornito et al., 2019). Allen Human Brain Atlas (AHBA) in which densely sampled genetic data from the brain are recorded, transcriptional-neuroimaging association studies can identify genes associated with neuroimaging changes in brain disorders in relatively small samples (Ji et al., 2021). Currently, a variety of neuropsychiatric disorders have been analyzed in this way to understand the relationship between neuroimaging features and differences in gene expression in the brain. However, there are not many current DRE findings on this imaging genetics, and there is a lack of real DRE gene expression data for validation. The presence of a persistent seizure-inducing etiology and low antiepileptic drug responsiveness are two widely recognized hypotheses for the onset and development of DRE (Chen et al., 2023). In more than half of epilepsy disorders, no clear etiology of seizures and recurrences has been identified (Chen et al., 2023). Indeed, the surgical area of MRI-negative DRE is still not optimally determined (Říha et al., 2022). Seizure type presenting as generalized tonic clonic seizures (GTCS) accounted for 64 % of DREs in one health center, which greatly increases the difficulty of preoperative clinical assessment of the epileptogenic region (Navab et al., 2022). Another study indicated that the histopathology of surgically resected patients with MRI-negative DRE was mostly cortical dysplasia (Arya et al., 2016). This suggests that MRI-negative DRE is highly likely to have morphologic alterations in brain tissue structure that cannot be detected visually. Besides, neuroinflammation is involved in epileptogenesis and loss of neurons, which is associated with peripheral innate and adaptive immune cells crossing the damaged blood-brain barrier (BBB) (Li et al., 2023; Vezzani et al., 2019). Neuroinflammation can keep neurons in a state of hyperexcitability, and a sustained inflammatory response may lead to drug resistance in epilepsy (Vezzani et al., 2019). Therefore, we may be able to discover some molecular genetic information related to pathological alterations within the brain from the periphery, thereby revealing the mechanism of brain structural and functional alterations in DRE.

In this study, we assessed structural and functional brain alterations in patients with DRE and drug-sensitive epilepsy (DSE) by analyzing whole-brain gray matter volume (GMV) and ReHo, and used transcriptional-neuroimaging association analysis to determine gene expression associated with structural alterations. Finally, we utilized gene expression data in blood for validation. We hypothesized that (1) there is abnormal cortical morphology and spontaneous neural activity in patients with DRE compared to patients with DSE, and (2) the structural brain changes in patients with DRE are associated with underlying gene expression.

2. Materials and methods

2.1. Subjects

A total of 47 patients with epilepsy and 27 sex- and age-matched healthy controls (HC) were included in this study. The enrolled epileptic patients were followed up for 12 months. According to the diagnostic criteria for ILAE[2], patients who achieved three times the longest seizure-free duration or 12 months seizure-free (the longer one was used as a criterion) after the standardized use of antiepileptic drugs were defined as the drug-sensitive group (DSE) ($n = 25$). Patients who applied two correctly selected and tolerated antiepileptic drugs but still failed to achieve sustained seizure-free were defined as the drug-resistant group (DRE) ($n = 22$). The seizure type of all included subjects with epilepsy was GTCS or GTCS + Complex Partial Seizure.

Exclusion criteria for all epileptic patients: (i) T1 structural image and T2 Flair image revealed any lesions or structural abnormalities such as hippocampal sclerosis. (ii) Subjects had a history of epilepsy surgery. (iii) history of other traumatic brain injuries, neurological or psychiatric disorders, and drug abuse; (iv) contraindications to MRI scanning such as metal implantation in the body and claustrophobia. The study was approved by the Ethics Committee of the First Affiliated Hospital of Hainan Medical University, and all subjects signed an informed consent form.

2.2. Image acquisition

MRI data were acquired using a 3T magnetic resonance MR750 scanner. Functional magnetic resonance image acquisition was performed with an echo planar imaging sequence with the following parameters: repetition time (TR) = 2000 ms, echo time (TE) = 30 ms, flip angle (FA) = 90°, field of view (FOV) = 24 cm × 24 cm, matrix = 64×64, layer thickness = 4 mm, gap = 0.4 mm, and 255 whole brain images were acquired each time. T1-weighted structural images were acquired with the T1–3DFSPGR sequence with the following parameters: repetition time (TR) = 6.012 ms, echo time (TE) = 1.968 ms, flip angle (FA) = 9°, matrix = 256 × 256, field of view (FOV) = 25.6 cm × 25.6 cm, layer thickness = 1 mm, and 152 consecutive layers were scanned in axial position. During the scanning process, all subjects tried to relax as much as possible, closed their eyes, and did not perform any specific thinking activities while remaining awake.

2.3. Voxel-based morphometry analysis

Voxel-based morphometry analysis was performed using the SPM8 toolbox. First, the T1-weighted structural images were corrected and reoriented along the horizontal and sagittal directions of the anterior commissure-posterior commissure (AC - PC). Next, the corrected images were segmented into gray matter, white matter, and cerebrospinal fluid. The gray matter images were normalized to Montreal Neurological Institute (MNI) space and resampled with voxel size of 1.5 × 1.5 × 1.5mm^3. Third, the gray matter volume (GMV) was obtained after regressing the total intracranial volume (TIV) of each image. Finally, the obtained images were smoothed with a 3 × 3 × 3mm^3 FWHM Gaussian kernel and then analyzed statistically, gaussian random field (GRF) correction, two-tailed, voxel level $P < 0.005$, cluster level $P < 0.05$.

2.4. Resting-state fMRI preprocessing

The resting-state MRI data were preprocessed using Data Processing & Analysis for Brain Imaging (DPABI, version 6.1, http://rfmri.org/dpabi). The processing steps were as follows. (i) The first 5 time points were removed due to the inhomogeneity of the magnetic field. (ii) Slice-time correction was performed. (iii) We implemented strict preprocessing to reduce motion artifacts, including alignment correction and motion estimate regression. We added head motion scrubbing

regressors to reduce the influence of head movement. Following, the spikes were defined that the framewise displacement of volumes exceeds 0.2, and we generated a regressor model for each spike (Power et al., 2012). To further ensure data quality, subjects were excluded if their head motions were >3 mm or 3° in any direction or >10 % of volumes identified as spikes. (iv) Images were spatial normalized to MNI space with a voxel size of 3 × 3 × 3mm^3. The participants were excluded if they have obvious structural damage. (v) Friston 24-parameter head motion, white matter, and cerebrospinal fluid signals were regressed out. (vi) Using a band-pass filter (0.01–0.08 Hz) to decrease the effect of the low-frequency drifts. No spatial smoothing was applied because the ReHo analysis was performed voxel by voxel.

2.5. ReHo analysis

ReHo was calculated using the DPARSF toolbox. The homogeneity of the time-series signals of 27 voxels was reflected by calculating the Kendall's coefficient for each voxel and its neighboring 26 voxels. The calculated Kendall's coefficient was used as the ReHo values for the center voxel, and a ReHo map was obtained for each study subject. The obtained ReHo maps were smoothed with a 6 × 6 × 6 mm^3 FWHM Gaussian kernel. After *z* transformation for statistical analysis, GRF correction, two-tailed, voxel level $P < 0.005$, cluster level $P < 0.05$.

2.6. Gene expression data processing

The gene expression data was obtained from Allen Human Brain Atlas transcriptomics dataset (AHBA, http://human.brain-map.org/). The dataset encompasses 3702 spatially unique tissue samples from six neurotypical postmortem adult brains (mean age: 42.5 years, range: 24–57 years, 5 male, 1 female, 3 Caucasian, 2 African-American and 1 Hispanic). Within these brain samples, information from 58,692 probes was compiled across cortical, subcortical, brainstem, and cerebellar regions to assess transcriptional expression measurements of 20,737 genes. Since four donors in the dataset lacked tissue samples from the right hemisphere, we only performed further analysis in the left hemisphere. According to the default recommended six-step processing pipeline from Allen Institute (Arnatkeviciute et al., 2019), we processed the gene expression microarray data from brain tissue samples as follows: (i) Gene-to-probe annotations was performed using the *Re*-Annotator toolbox, which resulted in a newly re-annotated set of 45,821 probes corresponding to 20,232 unique genes. (ii) Intensity-based filtering was applied to eliminate probes that were below background noise levels in >50 % of the samples. We excluded samples pertaining to the brainstem and cerebellum due to insufficient number of probes in the cerebellum and brainstem, limiting accurate characterization of regional gene expression. (iii) Probe screening was performed by prioritizing the highest correlation criterion with RNA sequencing data from two of the six brains, selecting a single probe representing each gene, ultimately resulting in 31,977 probes and 15,746 genes. (iv) Each tissue sample in the left cerebral hemisphere was assigned to the 246 ROIs atlas (Fan et al., 2016) using a 2 mm distance threshold. (v) Given the individual differences and outliers, gene expression measurements were normalized using a scaled robust sigmoid for all probes per sample and for all samples per subject. This normalization approach provides an estimate of the relative expression of each gene in different regions while accounting for donor-specific variation in gene expression. (vi) Finally, all samples from six donors in a certain brain region were averaged to generate a 123 brain region × 10,027 gene expression matrix for subsequent analysis. The code for gene expression analysis can be found at https://github.com/BMHLab/AHBAprocessing.

2.7. Gene expression–neuroimaging association analysis

Using partial least squares regression (PLSR), structural and functional alterations can predict relevant gene expression data. The two-sample *t* values for whole-brain GMV/ReHo between the two groups were assigned to 246 smaller regions (Atlas) (Fan et al., 2016). Considering that only gene data from the left hemisphere were included, a normalized gene expression matrix of 123roi × 10027 genes was finally obtained. In the PLSR model, the response and predictor variables are the GMV/ReHo two-sample *t* value matrix (123 × 1) and the gene expression matrix (123 × 10,027), respectively. The PLSR results were analyzed using a non-parametric technique (5000 times) to test whether the explained variance of the first component of the PLSR was significantly greater than the variance expected by random. The first component of the PLSR can be considered as the component with the most relevant gene expression value for epilepsy drug resistance or epilepsy disease. To determine the corrected weight of each gene for the first component of the PLSR, the bootstrapping method was used, and then the weight was divided by the estimated standard error to get the Z-score value of the corrected weight. In the subsequent analysis, after sorting the Z-score values, the significant (false discovery rate [FDR] correction, $P < 0.05$) top-ranked (top and tail) related genes were extracted by *t*-test. Considering the spatial autocorrelation, a spin test based on spherical rotations (5000 times) was used to obtain the null distribution of correlation coefficients for each gene and a map of GMV/ReHo two-sample *t* values to control for spatial continuity and hemispheric symmetry (FDR correction, $P < 0.05$).

2.8. Enrichment analysis

Considering that our validation data came from peripheral blood, we chose non-brain-specific databases for gene annotation and enrichment analysis. GMV- and ReHo- associated genes include both positively and negatively associated, and the top 1500 most associated genes of each were taken as the gene enrichment list. For the enrichment results of differential genes to be more discoverable, we utilized multiple databases. (i) The Kyoto Encyclopedia of Genes and Genomes (KEGG) (https://www.kegg.jp) database includes diverse array of biological entities into systems, genomic data, chemical compounds, and health information that can provide a wealth of biological information. (ii) The Gene Ontology (GO) knowledgebase (http://geneontology.org) is an extensive repository focusing on gene functions and gene products, encompassing proteins and noncoding RNAs. GO annotations encompass genes from diverse organisms across the tree of life. (iii) TRRUST (www.grnpedia.org/trrust) is a database of manually annotated transcriptional regulatory networks. The database includes both the target genes corresponding to the transcription factors and the regulatory relationships among the transcription factors. (iv) Pattern Gene Database (PaGenBase) (https://bioinf.xmu.edu.cn/PaGenBase/) is a database that provides pattern genes information for 11 organisms. Using this database it is possible to determine the cell specificity and tissue specificity of genes.

Besides, we analyzed protein-protein interaction (PPI) networks in exploring disease mechanisms in epilepsy. By this approach, interacting protein clusters can be engaged in parallel biological processes or executing distinct biological functions collectively, allowing for the deduction of evolutionary orthologies, as well as the functions and attributes of proteins yet unexplored (Pizzuti and Rombo, 2014). PPI enrichment was analyzed using several databases, including STRING8, BioGrid9, OmniPath10, InWeb_IM.11. These databases are rich in protein interaction information. Generation of a PPI network includes subset of proteins that form physical interactions at least with one other member. Molecular Complex Detection (MCODE) is a new clustering algorithm that allows for tightly connected regions in a PPI network corresponding to known molecular complexes if a PPI network contains between 3 and 500 proteins (Bader and Hogue, 2003).

This study utilized Metascape (http://metascape.org/) for enrichment analysis. Metascape analysis provides automated meta-analysis tools to understand either common or unique pathways in 40 independent knowledge bases. The Metascape allows putting in any number of

gene lists to get consistent biological pathways and protein complexes. The first component of PLSR was used for enrichment analysis, FDR correction, $P < 0.05$.

2.9. Validation analysis

To validate the results of the transcriptional-neuroimaging association, we collected blood from 21 subjects and extracted total RNA from peripheral blood single nucleated cells of 7 patients with DRE, 7 patients with DSE, and 7 patients with HC using Trizol reagent. RNA sequencing was performed on a NanoDrop ND-1000 (NanoDrop, Wilmington, DE, USA). After the mRNA was subjected to quantitative detection and purity testing, its fragment size was screened and purified using magnetic beads to construct a cDNA library. Finally, we used illumina NovaseqTM 6000 to sequence it bipartite according to standard operation.

After obtaining clean data, the differentially expressed genes in different comparison groups were identified by using fold change ≥2 (i.e. absolute value of $\log_2$FC ≥1) and *P*-value <0.05 as the threshold criteria for screening differentially expressed genes list. Differential genes were analyzed for enrichment analysis, as before, FDR correction, $P < 0.05$.

3. Results

3.1. Demographic information and clinical characteristics

There was no significant difference between DRE, DSE and HC in terms of gender and age ($P > 0.05$). The difference between DRE and DSE in duration was statistically significant ($P < 0.05$). There was no statistically significant difference in age of onset ($P > 0.05$), Table 1, Supplementary Table S1.

3.2. GMV analysis

VBM analysis revealed that DRE had significantly reduced GMV in the 8, 9, Crus2 and 7b regions of the left cerebellum compared to DSE. DRE had significantly reduced GMV in the 8, 9, Crus1 and Crus2 regions of the left cerebellum compared to the HC group (GRF correction, two-tailed, voxel level $P < 0.005$, cluster level $P < 0.05$, voxel size > 20), Table 2, Fig. 1.

3.3. ReHo analysis

ReHo analysis showed that DRE had significantly reduced ReHo in the Crus1 and Crus2 regions of the left cerebellum compared to DSE. DRE had significantly increased ReHo in the right precuneus compared to the HC group. DSE had significantly reduced ReHo in the bilateral medial superior frontal gyrus and left anterior cingulate gyrus compared with the HC group (GRF correction, two-tailed, voxel level $P < 0.005$, cluster level $P < 0.05$, voxel size > 20), Table 3, Fig. 2.

Table 1
Demographic Information and Clinical Characteristics.

	DRE ($n = 22$)	DSE ($n = 25$)	HC ($n = 27$)	*P* value
Age(year)	29.32±9.44	28.08±12.97	30.48±11.34	0.751[a]
Gender(M/F)	10/12	8/17	15/12	0.217[b]
Duration(year)	15.5(8.75, 20.5)	5(3, 10)		0.005[c]
Age of onset(year)	12.5(8, 19.25)	15(11, 30)		0.100[c]

Note: DRE, Drug Resistant Epilepsy; DSE, Drug Sensitive Epilepsy; HC, Healthy Control; F, Female; M, Male.
[a] One-way ANOVA.
[b] Pearson Chi-square tests.
[c] Mann-Whitney U test.

Table 2
GMV differences between DRE, DSE and HC.

Groups	Regions	MNI coordinates			Voxels	*t* value
		X	Y	Z		
DRE vs DSE	**Clusrer 1**					
	Cerebellum_8_L	−27	−44	−51	2087	−3.755
	Cerebellum_9_L	−14	−44	−55	649	−6.629
	Cerebellum_Crus2_L	−28	−76	−47	242	−3.352
	Cerebellum_7b_L	−31	−71	−47	224	−3.123
DRE vs HC	**Clusrer 1**					
	Cerebellum_8_L	−33	−58	−48	2506	−4.555
	Cerebellum_Crus1_L	−17	−82	−24	510	−3.347
	Cerebellum_9_L	−17	−42	−52	395	−3.325
	Cerebellum_Crus2_L	−42	−58	−46	340	−3.372
DSE vs HC	None					

Note: DRE, Drug Resistant Epilepsy; DSE, Drug Sensitive Epilepsy; HC, Healthy Control; MNI, Montreal Neurological Institute.

3.4. Transcription-neuroimaging association analysis

In the transcription-neuroimaging association analysis, each comparison group produces respectively a corresponding mean brain map and a weighted map. The PLS1 of the ReHo *t* map of DSE vs HC was not involved in the enrichment analysis because it didn't survive by spin test, Fig. 3.

3.4.1. Gene expression associated with drug resistance

The GMV significance *t* map of DRE vs DSE and AHBA gene expression were analyzed by cross-sample spatial correlation analysis to explore the molecular biological mechanisms related to drug efficacy. KEGG and GO enrichment showed significant enrichment of GMV-related genes in biological processes such as nervous system development, adaptive immune system and neuronal system, Fig. 4A. TRRUST enrichment analysis showed that GMV-related genes were regulated by specificity protein (SP1) and other transcription factors, Fig. 4B. PaGenBase enrichment analysis showed that GMV-related genes exhibit cellular specificity in dorsal root ganglion (DRG) cells and cardiac myocytes, Fig. 4C.

Equally, we repeated the above analysis process in the PLS1 of ReHo significance *t* map. KEGG and GO enrichment revealed interesting results, with ReHo-related genes being significantly enriched in biological pathways such as neuron projection development, neuronal system and regulation of nervous system development, Supplementary Fig. S2A. TRRUST enrichment analysis similarly identified SP1 as the most significant transcription factor, Supplementary Fig. S2B. The most striking result of PaGenBase enrichment analysis was consistent with that of GMV, showing cell specificity in DRG cells, Supplementary Fig. S2C.

3.4.2. Gene expression related to the mechanism of epilepsy

PLS1 s gene lists derived from the *t* map of DRE vs HC and the *t* map of DSE vs HC were used for enrichment analysis. Co-enrichment of multiple gene lists can yield common significant results in the comparison groups, thus exploring the common molecular biological mechanisms of DRE and DSE. KEGG and GO enrichment showed that GMV-related genes were significantly enriched in biological processes such as neurodevelopment, adaptive immunity, and signaling. Besides, protein-protein interaction network (PPI) enrichment generated 11 Molecular Complex Detection (MCODE) networks. Among them, MCODE_3, MCODE_4 and MCODE_9 are the most noteworthy, which are mainly related to potassium ion, response to wounding and cardiovascular related functions, Fig. 5A-B.

3.5. Validation analysis

3.5.1. Gene expression associated with drug resistance

Subjects information for the validation analysis is in Supplementary

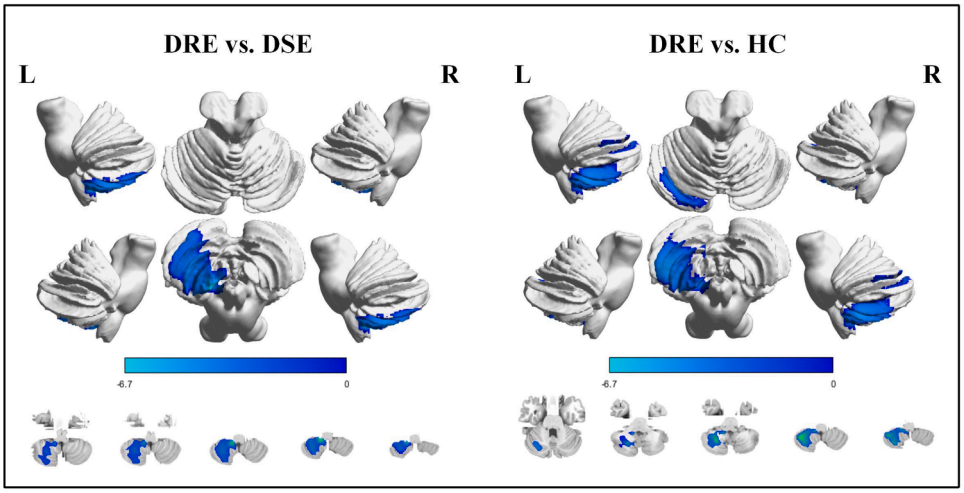

Fig. 1. GMV differences between DRE, DSE and HC. Red shows increased and blue shows decreased. DRE, drug resistant epilepsy; DSE, drug sensitive epilepsy; HC, healthy control.

Table 3
Differentially expressed genes in different groups.

Groups	Regions	MNI coordinates			Voxels	*t* value
		X	Y	Z		
DRE vs DSE	**Clusrer 1**					
	Cerebellum_Crus1_L	−37	−60	−36	74	−3.982
	Cerebellum_Crus2_L	−22	−81	−36	59	−3.974
DRE vs HC	**Clusrer 1**					
	Precuneus_R	14	−49	22	46	3.739
DSE vs HC	**Clusrer 1**					
	Cingulum_Ant_L	−7	45	−1	62	−3.700
	Frontal_Sup_Medial_L	−7	52	7	41	−3.185
	Frontal_Sup_Medial_R	10	48	1	46	−3.772

Note: DRE, Drug Resistant Epilepsy; DSE, Drug Sensitive Epilepsy; HC, Healthy Control; MNI, Montreal Neurological Institute.

Table S2. Subjects included for validation adhered to the inclusion and exclusion criteria above. Differentially expressed genes in different groups based on threshold criteria, Supplementary Fig. S1. Similarly, we enriched real transcription data from DRE and DSE. KEGG and GO enrichment analysis revealed abnormalities in the biological process of pallium development, which seemed to match the nervous system development of GMV-AHBA enrichment, Fig. 4A. We compared the gene symbols (136 for nervous system development and 101 for neuronal system) with the 7 gene symbols for pallium development and found an overlap of one neurodevelopment-related gene: *TUBB2A*, Supplementary Table S3. The expression of this gene was significantly down-regulated in DRE compared to DSE in blood. We have also focused on some of the biological pathways in ReHo-AHBA enrichment, including neuron projection development, regulation of nervous system development, and neuronal system, Supplementary Fig. S2A. In the same way, We compared the gene symbols (183 for neuron projection development, 118 for regulation of nervous system development and 115 for neuronal system) with the 7 gene symbols for pallium development and found an overlap of two neurodevelopment gene: *SEMA6B* and *TUBB2B*, Supplementary Table S3. Gene expression of *SEMA6B* and *TUBB2B* was significantly down-regulated in DRE compared to DSE in blood.

The TRRUST enrichment analysis obtained the matching results with GMV-AHBA and ReHo-AHBA, and the significant difference genes of DRE and DSE were regulated by SP1, Fig. 4B and Supplementary Fig. S2B. PaGenBase enrichment analysis found DRG, which is consistent with GMV-AHBA and ReHo-AHBA, Fig. 4C and Supplementary Fig. S2C.

3.5.2. *Gene expression related to the mechanism of epilepsy*

In the validation data, we used the same method. KEGG and GO enrichment analysis revealed immune-related biological pathways such as adaptive immune response and B-cell mediated immunity. Among the

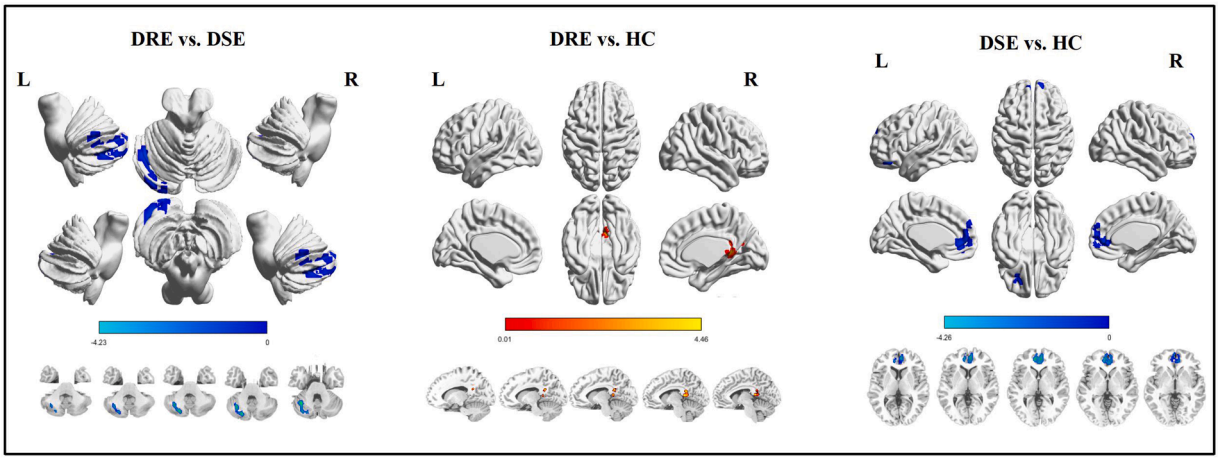

Fig. 2. ReHo differences between DRE, DSE and HC. Red shows increased and blue shows decreased. DRE, drug resistant epilepsy; DSE, drug sensitive epilepsy; HC, healthy control.

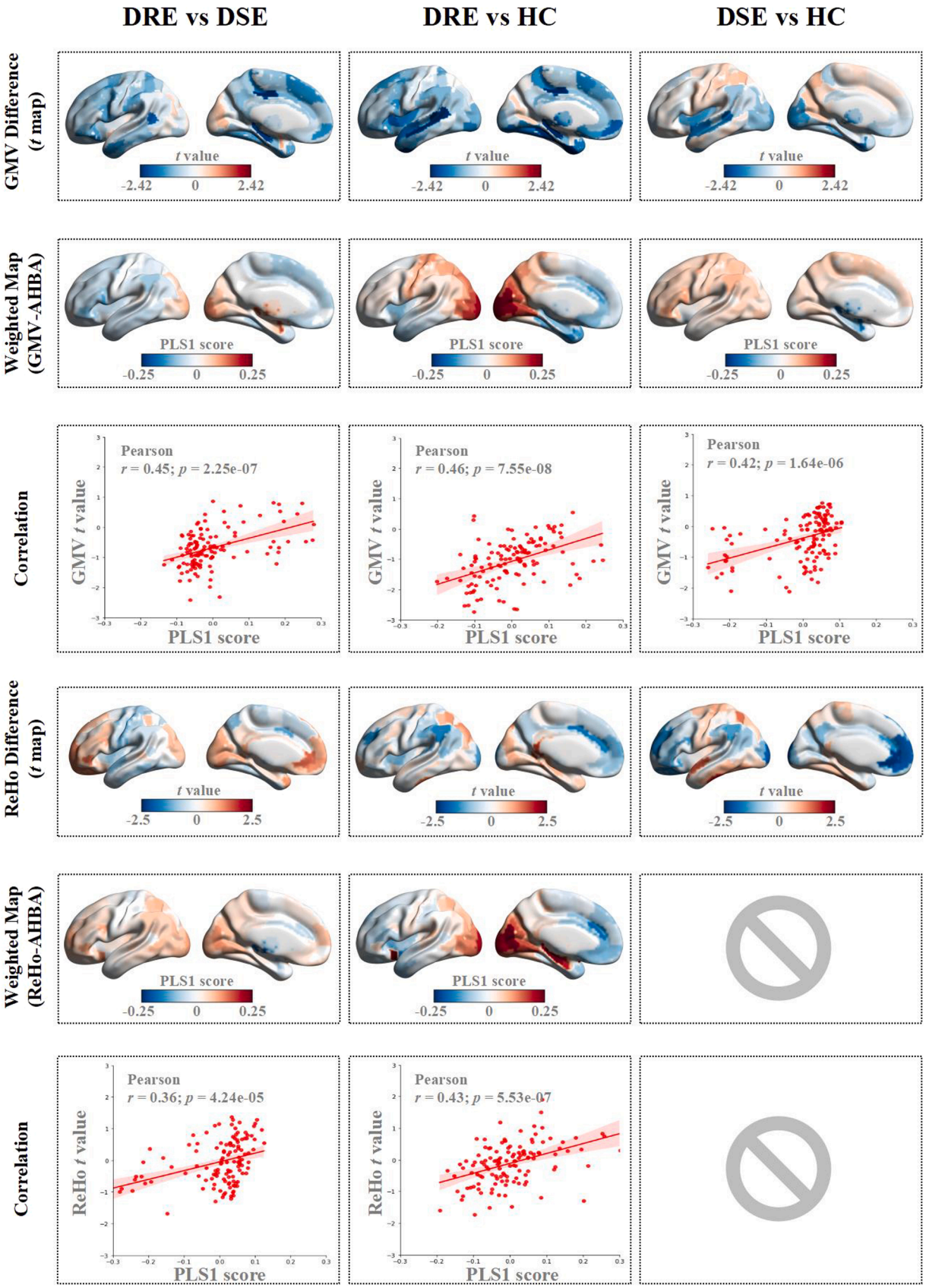

Fig. 3. All brain maps, gene expression weighted maps and their correlation maps. DRE, Drug Resistant Epilepsy; DSE, Drug Sensitive Epilepsy; HC, Healthy Control. ReHo, GMV, Gray Matter Volume; Regional Homogeneity; AHBA: Allen Human Brain Atlas.

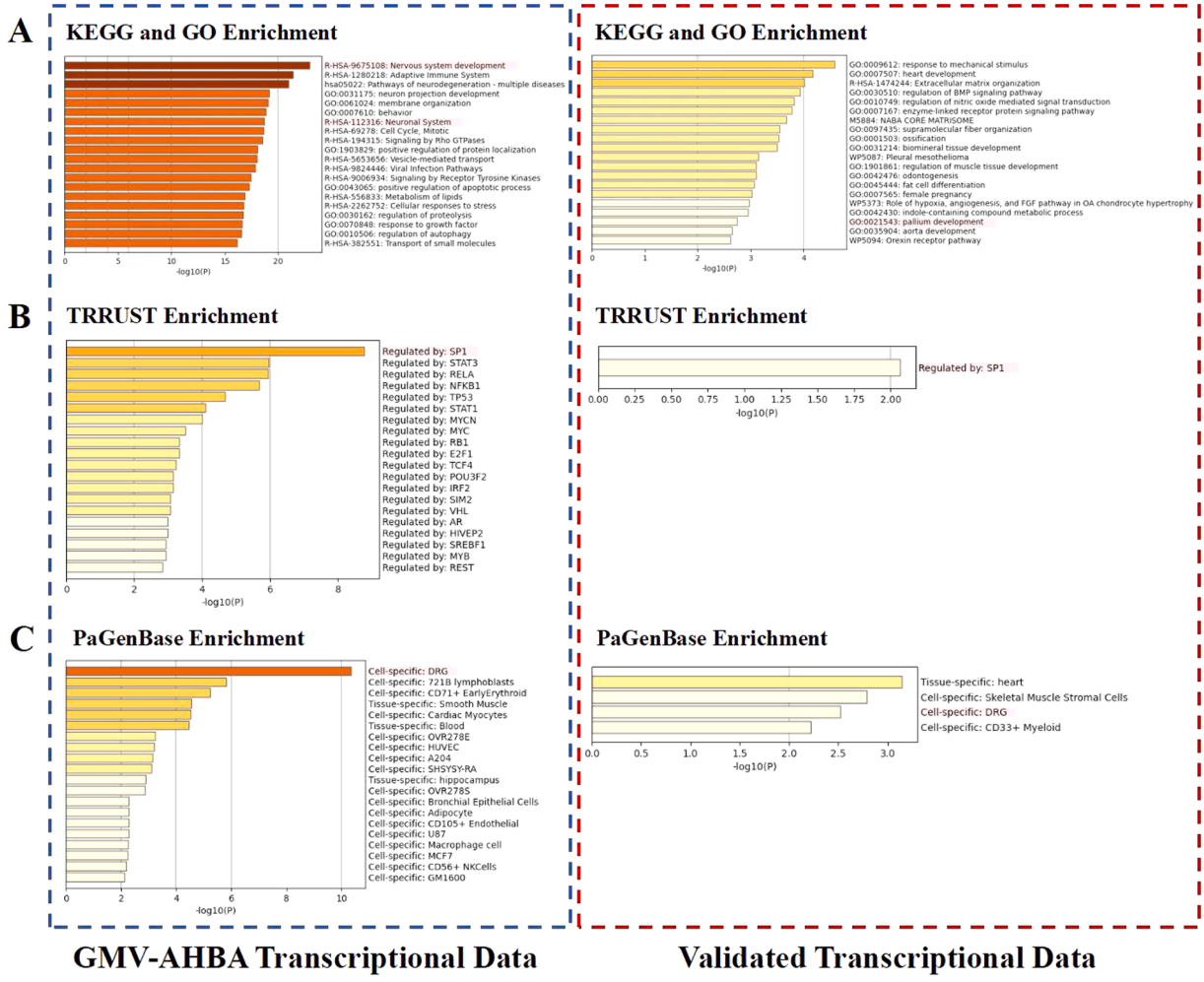

Fig. 4. Enrichment analysis of GMV-AHBA transcriptional data and real transcriptional data in DRE vs DSE. (**A**) indicates KEGG and GO enrichment of GMV-AHBA transcriptional data and validated transcriptional data. (**B**) indicates TRRUST enrichment of GMV-AHBA transcriptional data and validated transcriptional data. (**C**) indicates PaGenBase enrichment of GMV-AHBA transcriptional data and validated transcriptional data. The colorbars represent -log10(P) with FDR correction. GMV, Gray Matter Volume; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; TRRUST: Transcriptional Regulatory Relationships Unraveled by Sentence-based Text mining; PaGenBase: Pattern Gene Database.

biological processes of adaptive immunity in GMV-AHBA and validation data, 171 gene symbols and 26 gene symbols, respectively, were compared to each other to discover an overlapping gene: *HLA-DRB5*, Supplementary Table S3. The expression of this gene was significantly up-regulated in DRE and DSE compared to HC. The PPI enrichment yielded four MCODE networks, of which MCODE_1, MCODE_3 and MCODE_4 were NABA CORE Matrisome, response to wounding and vasculature development, respectively, Fig. 5A-B. Of these, MCODE_3 and MCODE_4 validated MCODE_4 and MCODE_9 for PPI of GMV-AHBA.

3.6. Subgroup analysis

To examine the impact of different epilepsy syndromes, we performed subgroup analyses of TLE and IGE to replicate previous results of GMV and ReHo. After categorization, 27 TLEs (DRE: 16, DSE: 9) and 22 IGEs (DRE: 6, DSE: 16) were compared with HC, respectively. Due to the small number of IGE, we calculated the deviation (z score) from HC for each IGE patient and finally averaged at the group level.

Obviously, the results of the differences in GMV and ReHo for TLE were generally consistent in tendency with the previous results, Supplementary Table S4, Figs. S3 and S4. The cerebellum still appears as an important region. At IGE, in GMV and ReHo, we examined the deviation between DRE/DSE and HC, and compared the mean maps of the deviations of DRE and DSE. The cerebellum remained a stable result in GMV, but ReHo was not, even though this was not statistically valid, Supplementary Figs. S5 and S6.

4. Discussion

In this study, we analyzed GMV and spontaneous brain functional activity in patients with epilepsy to characterize structural and functional alterations related with the features of drug resistant from a multimodal perspective. Combining magnetic resonance imaging and brain transcription data to study potential gene expression in patients, validated with real gene expression data in the blood. First, we found DRE alterations in the left cerebellum in both VBM and ReHo analysis, suggesting that the cerebellum as may play an important role in the process of epilepsy drug resistance. Second, gene expression enrichment analyses associated with drug resistance revealed abnormal biological processes of nervous system development, transcription factor SP1, and DRG cells, which were validated in the validation data. And overlapping neurodevelopment-related genes: *TUBB2A, SEMA6B* and *TUBB2B* was identified. In addition, we enriched the genes related to the mechanisms of epilepsy, found abnormalities in the biological processes of adaptive immunity, had the same findings in the validation data, and identified

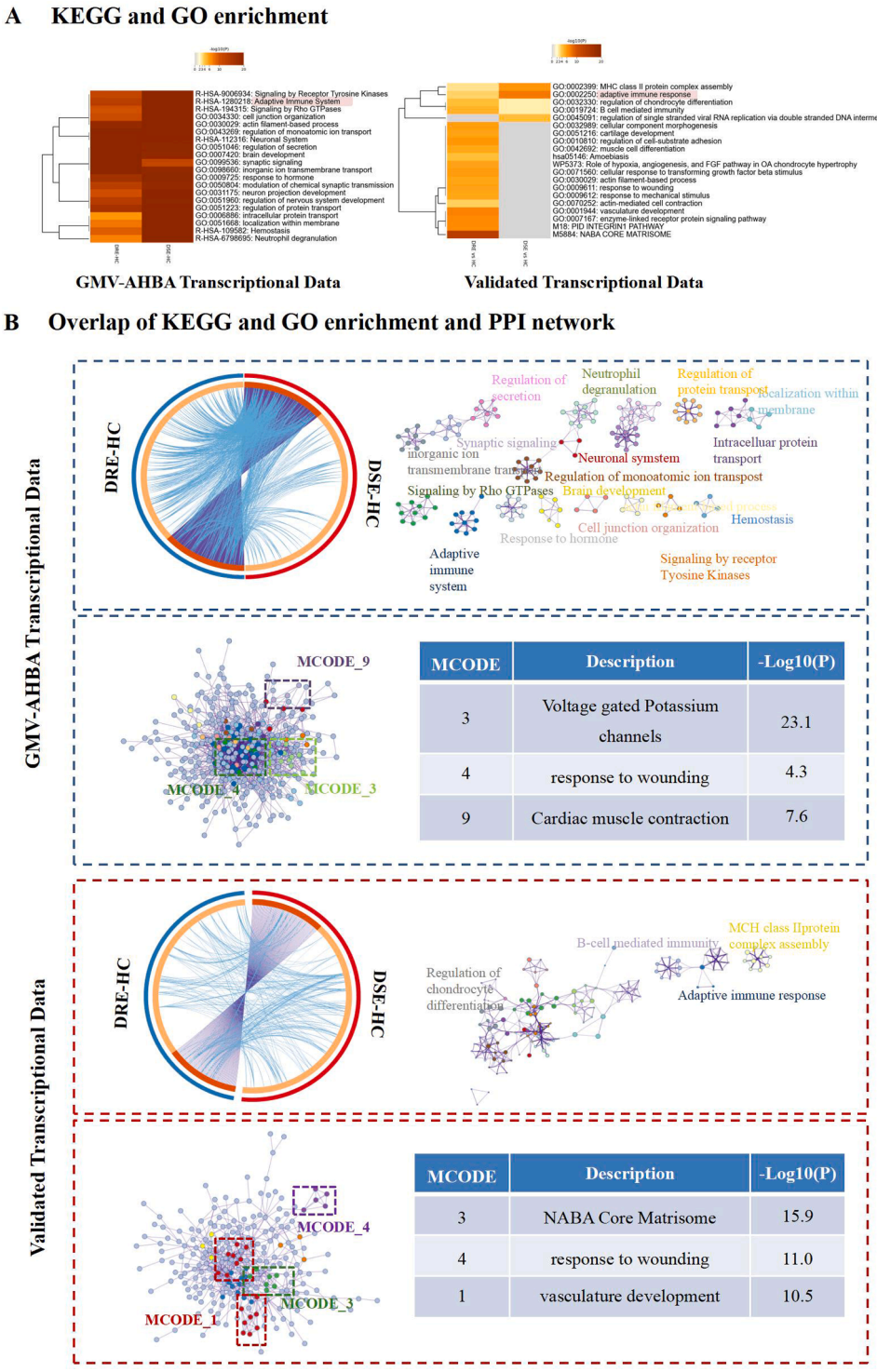

Fig. 5. Enrichment analysis of GMV-AHBA transcriptional data and real transcriptional data in intersections of DRE vs HC and DSE vs HC. (**A**) indicates KEGG and GO enrichment of GMV-AHBA transcriptional data and validated transcriptional data. (**B**) indicates Overlap of KEGG, GO enrichment analysis and PPI network. The colorbars represent -log10(P) with FDR correction. GMV, Gray Matter Volume; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; PPI: Protein-Protein Interaction network; MCODE: Molecular Complex Detection.

an overlapping gene: *HLA-DRB5*. Two MCODEs were validated in the PPI analysis, response to wounding and vasculature development.

The role of the cerebellum in epilepsy has gained increasing attention in recent years and is closely associated with motor symptoms, cognitive dysfunction, and anxiety-depressive disorders in epilepsy (Bostan and Strick, 2018; Fastenrath et al., 2022). It has been found that TLE patients with hippocampal sclerosis have cerebellar volumetric atrophy, and even progressive cerebellar atrophy with the course of the disease (Hao et al., 2020; Hellwig et al., 2013; Bonilha and Halford, 2009b). Another study supports this idea that repeated seizures may cause progressive damage to the cerebellum (Marcián et al., 2018). Patients with newly diagnosed TLE have cerebellar volume differences from patients with chronic epilepsy, suggesting that the cerebellum may be a susceptibility factor for poor seizure control (Park et al., 2015).

Neuronal loss and glial cell proliferation are the main pathologic changes in cerebellar atrophy, and this loss of neuronal cells is predominantly in Purkinje cells, and the reduced density of Purkinje cells may lead to de-suppression of excitatory neurons in the cerebellum, which can instead be a trigger for seizures (Crooks et al., 2000). Our findings also identified reduction of GMV in the cerebellum of patients with DRE, suggesting that structural alterations in the cerebellum may be involved in drug resistance or induced seizures, which is consistent with previous results.

In recent years, the nodal stress hypothesis has been proposed (Zhou et al., 2012), which suggests that central nodes (nodes with many connections to other brain regions), as regions of the brain that efficiently process and integrate information, are highly susceptible to pathological factors, and that in the presence of pathological factors central nodes show high metabolic activity and increased associations with other brain network nodes. As a result, central nodes are more susceptible to structural damage (atrophy) than nodes that are not central (Fornito et al., 2015; Vanasse et al., 2021). It is well known that epilepsy is a disease of transient malfunction of the central nervous system caused by synchronized abnormal discharges of neurons in the brain (Liu et al., 2023). Thus, a study looking for patterns of associated brain network atrophy in common epilepsy types based on this hypothesis found that the central nodes of TLE and idiopathic generalized epilepsy (IGE) are more prone to epilepsy type-associated atrophy, and that this structural damage is more likely to be seen in the cortical nodes in TLE, and more likely to be seen in the subcortical-cortical nodes in IGE (Royer et al., 2022). One large sample study of TLE also found atypical patterns of interhemispheric cortical atrophy and cross-sectional regional atrophy compared with healthy individuals (Ark et al., 2022). However, it is not clear whether the mechanism of drug resistance in epilepsy is related to some specific node atrophy. Our structural results provoked further reflection. The fact that our DRE and DSE groups, which both included IGE and TLE, did not yield significant results for hippocampal atrophy as in other TLE studies was expected, even though the seizure type of all our subjects was GTCS (Sainburg et al., 2022). However, the cerebellum has not been included in previous studies of the distribution of epileptic atrophy, and the cerebellum is an important node in the complex mechanisms of drug resistance that may have been overlooked. Another study supports this result, with a very high prevalence of drug-resistant patients with cerebellar degeneration (Ibdali et al., 2021b). Therefore, a pattern of brain network atrophy related to drug resistance may be superimposed on top of the epilepsy-related pattern of brain network atrophy. A stressor-threshold model of selective neuronal vulnerability has been proposed in neurodegenerative diseases, where chronic perturbations of the disease brought about by the persistence of pathologic factors cause stressors to accumulate in selective vulnerable neurons. When stressor tolerance is not balanced with the current load of disease-related stressors, it triggers dysfunction and progressive changes in thresholds (Saxena and Caroni, 2011). We speculate that the development of epileptic drug resistance may also be due to the accumulation of stressors, which leads to "suprathreshold manifestations", and further mechanisms may depend on molecular level studies.

In addition to the structural changes, the intrinsic functional activity of the cerebellum is diminished and affects the altered remote brain function (Liu et al., 2021). The Deep Cerebellar Nuclei (DCN) play a key role in efferent projections from the cerebellar cortex to the cerebral cortex and subcortical structures, primarily the fastigial nucleus, globose nucleus, emboliform nucleus, and dentate nucleus (Jiang et al., 2020). Some studies have observed increased connectivity from the dentate nucleus to the thalamus in patients with epilepsy, suggesting that dysfunction of cerebellar regulation of the thalamo-cortical circuit affects the excitatory-inhibitory balance between the cerebellum and the brain thereby inducing seizures (Gong et al., 2021). Applying electrical stimulation to the DCN in epileptic patients can effectively suppress seizures (Streng et al., 2023). Although functional changes in the cerebellum of DRE patients were also observed in our results, we did not find ReHo changes in the same cerebellum in our comparison with healthy controls, suggesting that functional changes in the brain are more complex than structural changes in DRE patients. Notably, brain regions with overlapping structural and functional changes were found in our results, with cerebellar Crus 2 regions having both reduced GMV and reduced ReHo. By extracting the mean time series of significant clusters, we did not find significant correlations between cerebellar structural and functional outcomes. Previous studies have found that altered functional activity in the Crus 1 and Crus 2 regions of the cerebellum can modulate the default mode network (DMN) (Halko et al., 2014). The Cerebello-frontal network plays an important role in the speed of information processing in the brain, and impaired cerebellar function may be compensated by increasing the functional activity of the frontal lobes, thus optimizing the speed of information processing in the brain (Arleo et al., 2023). Epilepsy is a brain network disorder, and its development is closely related to changes in the brain network (Sisodiya et al., 2020; Pang et al., 2023; Schaper et al., 2023). Some studies have identified many alterations in DRE resting-state brain networks at the network level, such as the DMN and the dorsal attention network (Bernhardt et al., 2016; Bacon et al., 2023). Seizures in the DRE may affect the consistency of the DMN, showing reduced functional connectivity in the medial prefrontal lobe and increased functional connectivity in the precuneus (Jiang et al., 2018). In our study, increased ReHo in the right precuneus was observed in patients with DRE compared to healthy controls, whereas patients with DSE had reduced ReHo in the bilateral medial prefrontal and left anterior cingulate gyrus compared to healthy controls. The precuneus, medial prefrontal lobe, and anterior cingulate gyrus, the core brain regions of DMN, showed different DMN alterations in both drug-resistant and drug-sensitive patients (Andrews-Hanna et al., 2010). This may be related to complex pathologic mechanisms involving the cerebellum or other brain regions.

To further investigate the mechanisms of drug resistance in epilepsy, we found abnormalities in nervous system development in an enrichment analysis of GMV-related genes and ReHo-related genes. Abnormalities of pallium development were found in the validation data, which certainly validated the results of the transcription-neuroimaging association analysis and were consistent with those observed in the clinic. Most patients with refractory epilepsy requiring surgical treatment in epilepsy centers are patients with temporal lobe epilepsy with hippocampal sclerosis and patients with extratemporal epilepsy with cortical developmental malformations (Tavakol et al., 2019). Focal cortical dysplasia is highly epileptogenic, with abnormal neurodevelopment and cell growth leading to structural lesions in the brain, which induce abnormal synaptogenesis and hyperexcitable circuits that promote epilepsy (Lee et al., 2023). Further matching of genes encompassing both nervous system development and pallium development led to a common gene, *TUBB2A*, which has been found to cause epilepsy and developmental delays in infants, but what role it plays in the mechanisms of drug resistance in DRE in adults is not yet known (Cai et al., 2020). Additionally, we identified *TUBB2B* and *SEMA6B*. *TUBB2B* and *TUBB2A* are from the same gene family, and genetic variants in *TUBB2B* can affect neurodevelopmental disorders and brain malformations, which can lead to epilepsy (Schröter et al., 2021). *TUBB2B* has been demonstrated to be the most common known gene responsible for polymicrogyria, a common epileptogenic cortical malformation (Akula et al., 2023). One study reviewed 29,031 patients with neurodevelopmental disorders and found that *SEMA6B* is an important gene that causes the disease to become dominant (Torene et al., 2024). Particularly, *SEMA6B* was validated as a dominant gene for progressive myoclonic epilepsy (Courage et al., 2021; Hamanaka et al., 2020). We gained more confidence for our previous hypothesis that there are visually difficult to detect structural alterations in the brains of MRI-negative DRE. Whether or not all patients with DRE have some underlying problems with the development of the nervous system needs to be further investigated. In addition, we enriched for the transcription factor SP1 from the differential genes of DRE and DSE in the validation

data, validating the results of GMV-AHBA and ReHo-AHBA. SP1 causes prolonged seizure activity by mediating peroxiredoxin 6 (Prdx6) upregulation in astrocytes in the CA1 region of the hippocampus of rats with chronic epilepsy (Kim et al., 2022). SP1 is also involved in epileptogenesis by activating small nucleolar RNA host gene 1 (SNHG1) (Zhao et al., 2020). Beyond that, we performed cell type enrichment in another public database and came up with one overlapping result: DRG. This may seem difficult to explain, as epilepsy is usually thought of as a brain disorder. However, it has been found that transient receptor potential melastatin 3 (TRPM3), a gene associated with seizures, is expressed in both the central nervous system and the DRG (Lines et al., 2022). Another study found that Perampanel, a drug used for refractory seizures, was also found to inhibit both hippocampal mossy fiber neurons and DRG neurons in animal experiments, resulting in an anti-seizure effect (Taniguchi et al., 2022). Despite studies supporting the correlation between DRG and epilepsy, the role of DRG in epilepsy remains ambiguous. In summary, we believe that the drug resistance mechanism and grey matter volume atrophy of DRE may be related to the abnormal expression of genes related to the nervous system development. From our results, SP1 plays an important role in the resistance mechanism and should be further confirmed in animal experiments of DRE in the future. Although the enrichment of ReHo-related genes also supports our main findings, we believe that GMV is more robust to use for transcription-neuroimaging association analysis. ReHo, as an indicator for observing regional brain activity, may have some uncertainty.

We tried to find the molecular mechanisms of the epilepsy disease itself. The validation data affirmed the abnormality of the biological process of adaptive immunity. One study found that over time the body's adaptive immune system responds to recurrent seizures with peripheral immune cells entering the brain through the damaged blood-brain barrier (Xu et al., 2018). Many of the same inflammation-related genes as in epileptic rats are significantly upregulated in the human hippocampus after status epilepticus, and sustained expression of these adaptive immune system genes leads to cytoarchitectural damage and aberrant synaptogenesis, which can lead to seizure recurrence (Erisken et al., 2022). In our results, GMV-related genes and real transcriptional data have an overlapping gene in the biological process of adaptive immunity, *HLA-DRB5*,which is significantly up-regulated in expression in the patient group, which supports the above article. However, there are no studies on this gene in relation to epilepsy. Interestingly, this seems to subtly link to the nodal stress hypothesis and stressor-threshold model we mentioned earlier. Whether such immune- or inflammation-related gene expression is characterised by a distribution pattern in brain tissue is unclear, and whether there are more significant immune- or inflammation-related gene expression abnormalities at important nodes remains to be further investigated. Subsequently, we calculated GMV-related PPI finding significant MCODE including voltage gated potassium channels, response to wounding, and cardiac muscle contraction. The latter two results were verified in validation data. Response to wounding is actually a series of immune responses and processes that echo the immune-related outcomes described above. Notably, significant results related to cardiovascular function were obtained for PPI in both sets of transcriptional data. Epilepsy-related cardiac dysfunction is an important cause of sudden death in patients with epilepsy (Pasini and Michelucci, 2023). Several studies have found that alterations in calcium, sodium, and potassium ion channels are all involved in sudden cardiac death in epilepsy (Tevoufouet et al., 2014; Frasier et al., 2018; He et al., 2021). Furthermore, one study identified potassium channel genes associated with TLE through bioinformatic analysis, and by downregulating these potassium channel genes affected the balance between excitatory and inhibitory neurons leading to epilepsy (Zhang et al., 2023). An association between excitation/inhibition imbalance in epilepsy and ion channel-related genes has also been observed at the macro level (Duma et al., 2024). However, most of the antiepileptic drugs currently in clinical use are blockers targeting sodium, chloride, and calcium channels, and future studies targeting potassium channels and transport may provide new breakthroughs in the development of new antiepileptic drugs.

There are some limitations of this study. First, the small sample size and lack of subgroup analysis from epilepsy types in this study, and studies with larger sample sizes are needed to further validate our results. Second, because the magnetic resonance data and the gene expression data came from different subjects, and we only included gene samples from the left cerebral hemisphere when performing the data analysis, this may lead to bias in our experimental results. Finally, although we designed three identical subgroups within the real transcriptional data in blood, they were not the same subjects as those analyzed in the MRI data, which gives some heterogeneity to our results.

In conclusion, we designed three groups and found structural and functional alterations in the cerebellum of DRE patients, which may be related to drug resistance mechanisms in epilepsy. To the best of our knowledge, we are the first study to validate the results of transcription-neuroimaging association analysis in epilepsy. Our study confirms that changes in brain morphology and regional activity in DRE patients may be associated with abnormal gene expression related to nervous system development. And SP1, as an important transcription factor, plays an important role in the mechanism of drug resistance. Furthermore, abnormal expression in adaptive immunity was found in gene expression analysis of mechanisms of epilepsy. Abnormalities in protein interactions in gene expression related to cardiovascular function revealed the pathological mechanism of sudden cardiac death in epilepsy patients in terms of genetics. Our study affirms the results of transcription-neuroimaging association analysis as reliable. Combined with validated transcriptional data, it could provide evidence to reveal the molecular genetic mechanisms of structural changes in the brain.

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Ethics approval and consent to participate

The study was approved by the Medical Ethics Committee of the First Affiliated Hospital of Hainan Medical University. Informed consent was obtained from all patients included in this study.

CRediT authorship contribution statement

Ting Liu: Writing – original draft, Methodology, Funding acquisition, Data curation, Conceptualization. **Sheng Wang:** Writing – original draft, Formal analysis, Data curation. **Yingjie Tang:** Writing – original draft, Visualization, Methodology, Formal analysis. **Sisi Jiang:** Writing – review & editing, Methodology. **Huixia Lin:** Visualization, Data curation. **Fei Li:** Visualization, Data curation. **Dezhong Yao:** Writing – review & editing, Funding acquisition. **Xian Zhu:** Writing – review & editing, Conceptualization. **Cheng Luo:** Writing – review & editing, Methodology, Funding acquisition, Conceptualization. **Qifu Li:** Writing – review & editing, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare no competing interests.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.neuroimage.2024.120908.

Data availability

The data of this study are available from the corresponding authors upon request. They are not publicly available due to privacy or ethical restrictions.

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