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Unlocking the link: how hippocampal glutathione–glutamate coupling predicts cognitive impairment in multiple sclerosis patients

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Cognitive impairment is a common symptom of multiple sclerosis and profoundly impacts quality of life. Glutathione (GSH) and glutamate (Glu) are tightly linked in the brain, participating in cognitive function. However, GSH–Glu couplings in cognitive brain regions and their relationship with cognitive impairment in relapsing–remitting multiple sclerosis (RRMS) remains unclear. Forty-one RRMS patients and 43 healthy controls underwent magnetic resonance spectroscopy to measure GSH and Glu levels in the posterior cingulate cortex, medial prefrontal cortex and left hippocampus. Neuropsychological tests were used to evaluate the cognitive function. The Glu/GSH ratio was used to indicate the coupling between GSH and Glu and was tested as a predictor of cognitive performance. The results show that RRMS patients exhibited reduced hippocampal GSH and Glu levels, which were found to be significant predictors of worse verbal and visuospatial memory, respectively. Moreover, GSH levels were dissociated from Glu levels in the left hippocampus of RRMS patients. Hippocampal Glu/GSH ratio is significantly correlated with processing speed and has a greater predictive effect. Here we show the hippocampal Glu/GSH ratio could serve as a new potential marker for characterizing cognitive impairment in RRMS, providing a new direction for clinical detection of cognitive impairment.

Key words: cognitive impairment; glutamate; glutathione; magnetic resonance spectroscopy; relapsing–remitting multiple sclerosis.

Introduction

Multiple sclerosis (MS) is an immune-mediated inflammatory demyelinating disease of the central nervous system. Nearly 40–70% of MS patients develop cognitive impairment (Eijlers et al. 2018), with the most commonly affected aspects of cognition including processing speed and episodic memory (Benedict et al. 2020). Cognitive impairment can occur even in the earliest "pre-clinical" phase and progress insidiously, leading to a deterioration in patients' quality of life and negatively affecting their participation in work and social activities (Kavaliunas et al. 2019; van Gorp et al. 2019). Unfortunately, objective assessment of cognitive function is still limited in routine clinical practice (DeLuca et al. 2020). Advances in neuroimaging techniques have enhanced our understanding of brain structural and functional alterations in MS patients with cognitive impairment. Thus, identifying useful neuroimaging markers of cognitive function could support drug development and rehabilitation strategies.

The pathogenesis of MS is characterized by a cascade of pathobiological events, ranging from focal lymphocytic infiltration and microglia activation to demyelination and axonal degeneration (Ciccarelli et al. 2014). Multifocal immune-mediated destruction of myelin and oligodendrocytes leading to progressive axonal loss is a primary cause of neurological disability in MS (Andravizou et al. 2019). In addition to immune-mediated inflammatory responses, certain neurodegenerative processes, such as glutamatergic dysfunction and oxidative stress, also contribute to the disease's pathogenesis and progression (Rajda et al. 2017). Furthermore, among the immune-mediated processes of MS, activated microglia and macrophages, as well as mitochondrial dysfunction, are thought to contribute to the production of reactive oxygen species (ROS) (Carvalho et al. 2014). High amounts of ROS can induce oxidative injury, which is considered a potential therapeutic target (van Horssen et al. 2011). Glutathione (GSH), an endogenous antioxidant, plays a crucial role in protecting against oxidative damage and is consumed during protective antioxidant and detoxification processes (Rae and Williams 2017). Previous magnetic resonance spectroscopy (MRS) studies (Choi et al. 2010; Choi et al. 2017; Choi et al. 2018) have reported lower GSH levels in the frontoparietal region of MS patients, indicating the presence of oxidative stress. Of note, GSH depletion is thought to be correlated

with cognitive impairment in rats (Gonzalez-Fraguela et al. 2018). Moreover, reduced GSH levels in the hippocampus and prefrontal cortex are associated with cognitive impairment in dementia (Mandal et al. 2015). Such findings suggest that GSH levels could predict the degree of cognitive impairment. However, whether changes of GSH levels in brain regions involved in cognition are related to cognitive impairment in relapsing–remitting multiple sclerosis (RRMS) patients is unknown.

Glutamate (Glu) is the main excitatory neurotransmitter in the central nervous system, plays a significant role in synaptic plasticity, participating in learning and memory (Cox et al. 2022). Lower Glu levels measured by MRS were found in the sensorimotor and parietal regions of the brain in MS patients (Nantes et al. 2017). Other MRS studies have reported that decreased hippocampal Glu levels are closely correlated with memory impairment in RRMS (Muhlert et al. 2014; Gao et al. 2018). Glu acts as a precursor for the generation of GSH, while GSH serves as a reserve pool for Glu and molds synaptic Glu activity (Sedlak et al. 2019). The intake of Glu by microglia is related to GSH synthesis (Persson et al. 2006). Additionally, decreased GSH levels caused by oxidative stress have been shown to relate to Glu release from activated microglia (Barger et al. 2007). In patients with schizophrenia, GSH levels in the anterior cingulate cortex were found to closely correlate with Glu levels, and the strength of this correlation was significantly reduced compared with healthy controls (HCs) (Dempster et al. 2020). Given such findings, further research is warranted to identify the links between GSH and Glu levels in brain areas associated with cognitive function of RRMS patients and the relationship with cognitive impairment.

Demyelination is the major pathological hallmark of MS which occurs in both white and gray matters. Extensive cortical demyelination has been observed in the frontal, temporal, the cingulate gyrus and the hippocampus, which may be the pathological course leading to neurological disability and particularly cognitive impairments in MS patients (Bø et al. 2003). Previous studies of postmortem MS brains have established that hippocampal demyelination disrupts the maintenance of excitatory synapses and activation of neuronal signaling cascades that regulate learning and memory (Dutta et al. 2011). Additionally, hippocampal volume loss (González Torre et al. 2017), microstructural damage (Planche et al. 2017) or impaired structural connectivity (Llufriu et al. 2019) were shown correlated with memory impairment. Therefore, this study aimed to investigate the levels of GSH and Glu in the medial prefrontal cortex (mPFC), left hippocampus, and posterior cingulate cortex (PCC), which are considered critical regions associated with cognitive impairment in MS patients (Louapre et al. 2014; Rocca et al. 2018). Given evidence of oxidative damage and glutamatergic dysfunction may be interdependent (Volterra et al. 1994), we hypothesized that GSH–Glu coupling would be altered in RRMS. Furthermore, we predicted there might be a relationship between GSH levels or Glu–GSH coupling and cognitive impairment.

Materials and methods

This is a prospective case-control study and performed at one institution. This study was approved by the Ethics Committee of our hospital. All participants provided written informed consent prior to enrollment. Prior to any experimental procedure, participants filled in several neuropsychological tests to assess cognitive function. Subsequently, participants were positioned inside the MR scanner and underwent a series of sequence scans.

Participants

A total of 41 RRMS patients (Expanded Disability Status Scale (EDSS) scores range from 0 to 4) were enrolled in this study. EDSS was used to quantify clinical disability of MS patients. The EDSS scale ranges from 0 (normal) to 10 (death due to MS) in half-point increments and with the score based on a neurologist's examination (Kurtzke 1983). The inclusion criteria for patients were as follows: a confirmed diagnosis of RRMS according to 2017 McDonald criteria (Thompson et al. 2018) and neurologically stable with no history of relapse or steroid treatment within 3 months of enrollment. The exclusion criteria for patients were as follows: MRI-contraindicated, with severe depression or a history of antidepressant use, with visible lesions on the voxel of interest (VOI) in MRS, and a history of other central nervous system disorders. For the HC group, 43 age- and sex-matched individuals were enrolled from the local community. All participants were right-handed and had no history of drug abuse, neurological or mental disorders, or brain injury. Drinking, smoking, and caffeine consumption were prohibited for 12 h before MR scanning.

Cognitive testing

All participants underwent the following neuropsychological tests: (i) Auditory Verbal Learning Test (AVLT, Chinese version) was used to assess verbal memory (Zhao et al. 2012); (ii) Rey–Osterrieth Complex Figure Test (ROCFT) was used to assess visuospatial memory (Shin et al. 2006); (iii) Symbol Digit Modalities Test (SDMT) was used to assess information processing speed (Van Schependom et al. 2014); (iv) Trail Making Test (TMT, Parts A and B) was used to assess executive function (St-Hilaire et al. 2018); (v) Stroop Color and Word Test (SCWT, Stroop card 1, 2, and 3) was used to assess attention (Periáñez et al. 2021). H.L. performed the testing and analyzed in a blinded manner. The tests were performed in a fixed order and took about 1 h to complete. More details of neuropsychological tests are provided in the Supplementary Methods.

MRI protocol

All participants underwent MRI scanning on a Philips Achieva 3.0 T scanner. Three-dimensional T1-weighted imaging (3D-T1WI) sequence (repetition time [TR] = 8.1 ms; echo time [TE] = 3.7 ms; slice thickness = 1 mm; field of view = 24×24 cm^2; and voxel size = $1 \times 1 \times 1$ mm^3) was collected as a spectroscopic voxel localizer. The point-resolved spectroscopy (PRESS) sequence (TR = 2000 ms; TE = 35 ms; bandwidth = 2000 Hz; 32 averages) was acquired to measure metabolite levels. Unsuppressed water spectra with four scan averages were acquired for spectral quantification. Chemical Shift Selective Suppression was used for water suppression. FASTMAP shimming of the VOI was performed automatically prior to each acquisition. The VOIs for MRS were placed in the PCC (voxel size: $3 \times 3 \times 2$ cm^3), mPFC (voxel size: $2 \times 3 \times 3$ cm^3), and left hippocampus (voxel size: $4 \times 2 \times 2$ cm^3). For the PCC, the voxel was placed posterior and superior to the splenium of the corpus callosum, and was angled to arrange along the corpus callosum, the posterior edge of the voxel does not exceed the parieo-occipital sulcus in the sagittal section. For the mPFC, the posterior edge of the voxel and the genu of corpus callosum were aligned in the sagittal section. In the axial and coronal sections, both the PCC and the mPFC voxels were centered on the longitudinal medial fissure. The hippocampus voxel consisted largely of the parahippocampal structure, and was aligned with the body of the hippocampus in the sagittal plane (Fig. 1). Besides, T2-fluid-attenuated inversion recovery (FLAIR) sequence (TR = 11 000 ms; TE = 125 ms; slice thickness = 3 mm;
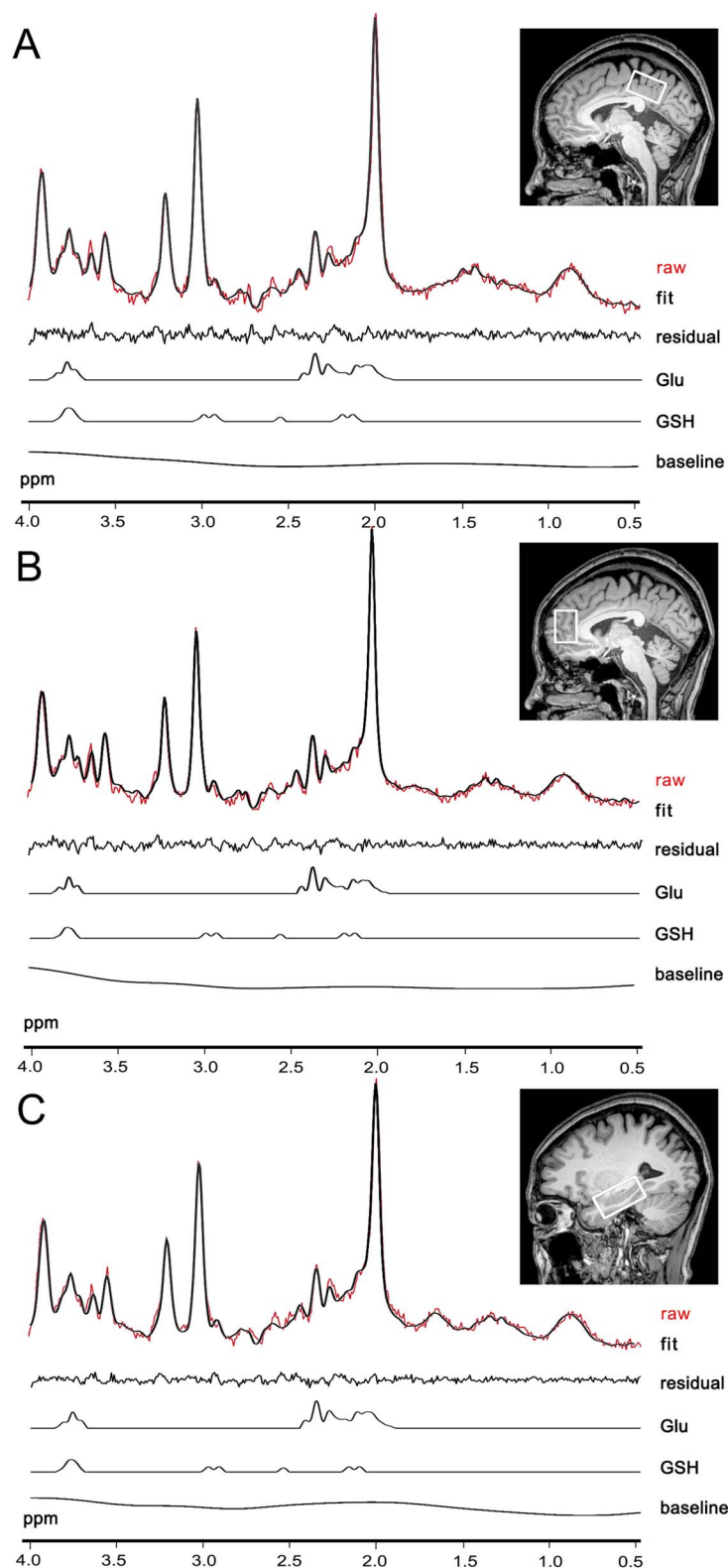
Fig. 1. Representative 1H MR spectrum from PCC(A), mPFC (B), and left hippocampus (C) acquired with the PRESS sequence at 3T, the corresponding LCModel spectral fit, fit residual, baseline, GSH and Glu fits.

field of view $= 24 \times 20$ cm^2; and voxel size $= 1 \times 1 \times 3$ mm^3) was obtained to evaluate white matter (WM) lesions.

Volumetric data processing

The lesion prediction algorithm as implemented in the lesion segmentation tool (LST 3.0, www.statistical-modelling.de/lst.html) (Ribaldi et al. 2021) was applied to segment the WM

lesions in the T2-FLAIR image. Hypointense lesions on the T1-weighted volume were marked and filled before segmentation using the "lesion-filling" function in LST, which reduces the impact of lesion-associated segmentation bias on gray matter (GM) and WM segmentations (Chard et al. 2010). Then, the GM, WM, and total intracranial volumes (TIV) were computed with the Computational Anatomy Toolbox

Table 1. Demographics, clinical and cognitive data of RRMS patients and HCs.

Characteristics	Patients ($n = 41$)	HCs ($n = 43$)	P
Gender (male/female)	15/26	13/30	0.537
Age (years)	38.22 ± 10.17	40.63 ± 12.83	0.345
Education (years)	11.78 ± 2.98	12.63 ± 4.07	0.278
EDSS (median (range))	2 (0–4)	—	—
Disease duration (years)	6.29 ± 3.74	—	—
AVLT	47.63 ± 7.96	60.44 ± 9.95	<0.001*
ROCFT	77.68 ± 13.40	86.51 ± 10.96	0.001*
SDMT	43.71 ± 10.47	56.30 ± 12.42	<0.001*
SCWT	146.49 ± 33.75	122.23 ± 30.35	0.001*
TMT-A	46.17 ± 12.55	33.35 ± 11.41	<0.001*
TMT-B	113.83 ± 26.33	98.49 ± 26.87	0.01*

Note: Data are presented as mean ± SD. *$P < 0.05$. The cognitive data are raw values. RRMS = relapsing–remitting multiple sclerosis; HCs = healthy controls; EDSS = Expanded Disability Status Scale; AVLT = Auditory Verbal Learning Test; ROCFT = Rey-Osterrich Complex Figure Test; SDMT = Symbol Digit Modalities Test; SCWT = Stroop Color and Word Test; TMT-A = Trail Making Test, Part A; TMT-B = Trail Making Test, Part B.

Table 2. Between-groups differences in the volumetric measurements.

Characteristics	Patients (N = 41)	HCs (N = 43)	P
TIV (cm^3)	1327.15 ± 90.98	1357.17 ± 134.96	0.238
WM volume(cm^3)	467.58 ± 47.73	473.97 ± 49.22	0.548
GM volume(cm^3)	598.80 ± 52.03	613.69 ± 62.53	0.240
Left hippocampus volume(cm^3)	4.58 ± 0.66	4.69 ± 0.88	0.520
Lesions volume (cm^3)	6.68 ± 12.88	—	—

Note: Data are presented as mean ± SD. HCs = healthy controls; TIV = total intracranial volume; WM = White matter; GM = Gray matter.

(CAT12: http://www.neuro.uni-jena.de/cat/) within Statistical Parametric Mapping software (SPM12, http://www.fil.ion.ucl.ac.uk/spm/software/spm12/) (Ashburner and Friston 2005). The left hippocampus volume was quantified using FMRIB's Integrated Registration and Segmentation Tool (FIRST) in the FMRIB Software Library (FSL) (Patenaude et al. 2011).

Spectral quantification

LCModel 6.3 (http://s-provencher.com/lcmodel.shtml) (Provencher 1993) was used to quantify the levels of Glu, GSH, glutamate plus glutamine (Glx), creatine-plus-phosphocreatine (tCr), N-acetylaspartate (NAA), myo-inositol (mIns), and choline-containing compounds (Cho). Representative LCModel fits, Glu and GSH fits from each VOI are shown in Fig. 1. Spectra with Cramer–Rao lower bounds (CRLB) exceeded 20%, signal to noise ratios (SNR) less than 10 (Baek 2023) or line-width (FWHM) exceeded 0.1 ppm were excluded from further analysis (Lind et al. 2020). Some GSH levels were excluded from analysis due having a CRLB > 20% (PCC: one patient; mPFC: two patients and six HCs; left hippocampus: five HCs). Gannet Co Register was applied to generate the VOI masks and register these masks to the T1-weighted anatomical image. Then, individual T1-weighted images were segmented using SPM 12, and Gannet Segment was applied to obtain GM, WM, and CSF fractions of the VOIs. Corrections for T1 and T2 relaxation times and tissue composition were applied using previous methods (Gasparovic et al. 2006). Additional details are provided in the Supplementary Methods.

Statistical analysis

A two-tailed *t*-test was used to compare age, educational level, cognitive performance, volumetric measurements, spectral quality, and metabolite levels in each VOI between groups. Gender was compared between the two groups using the Chi-square test.

The Pearson correlation coefficient was used to explore the relationship between Glu and GSH levels in RRMS patients and HCs. Correlations between metabolite levels in each VOI and cognitive performance in groups were examined using partial Pearson correlation analyses, adjusting for age, educational level, gender, disease duration, and lesion volume. *P* values were corrected for multiple comparisons using the false discovery rate (FDR). Correlation coefficients were compared between groups using Fisher's *r*-to-z transformation. When a significant relationship between metabolite levels and cognitive performance was found, hierarchical linear regression was then conducted to evaluate the extent to which metabolite levels predicted cognitive test performance. Cognitive performance served as the outcome variable. In block 1, we entered age, educational level, gender, disease duration, and lesion volume as covariates. In block 2, each metabolite was entered individually as a predictor and the change in R^2 (ΔR^2) was obtained.

Results
Comparison of participant characteristics

There were no significant group differences in age ($P = 0.345$), gender ($P = 0.537$), or educational level ($P = 0.278$). Compared with HCs, RRMS patients performed significantly worse on all cognitive tests (all $P < 0.05$, Table 1). There were no significant group differences in all volumetric measurements (all $P > 0.05$, Table 2).

Comparison of GSH, Glu, and Glu/GSH ratio between groups

Metabolite levels in the PCC, mPFC, and left hippocampus in the two groups are presented in Table 3. No significant group difference was observed in the CRLB of Glu and GSH. Compared with HCs, RRMS patients showed significantly lower levels of GSH ($P = 0.002$) and Glu ($P = 0.006$) in the left hippocampus. Furthermore, the Glu/GSH ratio, which reflects the coupling between GSH

Table 3. GSH and Glu levels, and Glu/GSH ratio in different VOIs in RRMS patients and HCs.

	RRMS Patients	HCs	P
PCC			
GSH	1.71 ± 0.21	1.68 ± 0.19	0.51
GSH CRLB (%)	8.48 ± 2.29	9.14 ± 2.61	0.22
Glu	8.13 ± 0.98	8.21 ± 0.76	0.71
Glu CRLB (%)	5.51 ± 1.36	5.60 ± 0.93	0.72
Glu/GSH	4.87 ± 0.55	4.92 ± 0.51	0.66
mPFC			
GSH	1.69 ± 0.45	1.77 ± 0.36	0.42
GSH CRLB (%)	11.46 ± 3.97	11.00 ± 3.29	0.58
Glu	8.22 ± 1.22	8.53 ± 1.47	0.31
Glu CRLB (%)	6.51 ± 1.86	6.56 ± 1.75	0.91
Glu/GSH	5.03 ± 0.99	4.90 ± 0.73	0.51
Left hippocampus			
GSH	1.30 ± 0.27	1.66 ± 0.62	0.002**
GSH CRLB (%)	14.27 ± 4.04	13.37 ± 3.14	0.27
Glu	6.29 ± 0.84	6.90 ± 1.14	0.006**
Glu CRLB (%)	8.00 ± 1.53	8.663 ± 2.68	0.19
Glu/GSH	5.02 ± 1.12	4.46 ± 1.15	0.03*

Note: Data are presented as mean ± SD. **$P < 0.01$. *$P < 0.05$. RRMS = relapsing–remitting multiple sclerosis; HCs = healthy controls; GSH = glutathione; Glu = glutamate; PCC = posterior cingulate cortex; mPFC = medial prefrontal cortex; VOI = voxel of interest.

Table 4. MRS data quality measures in RRMS patients and HCs.

	RRMS (N = 41)	HCs (N = 43)	P
PCC			
Line-width(ppm)	0.033 ± 0.009	0.032 ± 0.009	0.75
SNR	29.83 ± 2.88	30.14 ± 2.55	0.60
mPFC			
Line-width(ppm)	0.050 ± 0.014	0.051 ± 0.009	0.73
SNR	22.07 ± 3.78	21.79 ± 2.81	0.70
Left hippocampus			
Line-width(ppm)	0.065 ± 0.014	0.066 ± 0.012	0.64
SNR	18.49 ± 2.31	18.19 ± 2.26	0.55

Note: Data are presented as mean ± SD. RRMS = relapsing–remitting multiple sclerosis; HCs = healthy controls; PCC = posterior cingulate cortex; mPFC = medial prefrontal cortex; SNR = signal-to-noise ratio.

and Glu, was significantly higher in the left hippocampus of RRMS patients ($P = 0.03$). No significant group differences were observed in GSH or Glu levels or Glu/GSH ratio in the mPFC or PCC (Fig. 2). No significant differences were observed in spectral quality (i.e. linewidth and SNR; Table 4) or tissue volume fractions (Table 5) between groups. Comparison of other metabolites levels between groups are shown in Supplementary Results and Table S1.

Correlations between GSH and Glu levels in the VOIs in RRMS patients and HCs

Pearson correlation analysis showed positive correlations between GSH and Glu levels across all three VOIs in HCs (PCC: $r = 0.532$, $P < 0.001$; mPFC: $r = 0.721$, $P < 0.001$; left hippocampus: $r = 0.451$, $P = 0.004$). In the RRMS group, GSH levels in mPFC and PCC were positively correlated with Glu levels ($r = 0.575$, $P < 0.001$; $r = 0.460$, $P = 0.003$, respectively). However, there was no correlation between hippocampal Glu and GSH levels in the RRMS group ($r = 0.233$, $P = 0.142$) (Fig. 3). No significant between-group difference was observed in the correlation between the levels of Glu and GSH in PCC ($z = 0.420$, $P = 0.675$) and mPFC ($z = 1.080$, $P = 0.280$).

Correlations between GSH, Glu, and Glu/GSH ratio and cognitive performance

As shown in Table 6, Glu and GSH levels in the left hippocampus of RRMS patients were positively correlated with ROCFT scores

($r = 0.516$, $P = 0.003$, FDR corrected) and AVLT scores ($r = 0.426$, $P = 0.030$, FDR corrected), respectively. SDMT scores showed significant associations with GSH levels ($r = 0.377$, $P = 0.035$, FDR corrected) and the Glu/GSH ratio ($r = -0.415$, $P = 0.035$, FDR corrected) in the left hippocampus. In addition, TMT-A scores were significantly associated with GSH levels ($r = -0.381$, $P = 0.036$, FDR corrected) and the Glu/GSH ratio ($r = 0.474$, $P = 0.012$, FDR corrected) in the PCC. Significant between-group differences were observed in the correlation coefficients of TMT-A scores with GSH levels ($z = 2.000$, $P = 0.046$) and the Glu/GSH ratio in the PCC ($z = 2.130$, $P = 0.033$). There was no significant correlation between metabolite levels in the mPFC and any cognitive performance measure in RRMS patients. For HCs, no significant correlations were observed. No significant correlations were observed between other metabolite levels in all VOIs and any cognitive performances in RRMS patients (see Supplementary Results and Table S2 for details).

As shown in Table 7, ROCFT and AVLT scores were significantly predicted by Glu and GSH levels in the left hippocampus, respectively, of RRMS patients ($\Delta R^2 = 0.198$, $P = 0.001$; $\Delta R^2 = 0.103$, $P = 0.010$). Furthermore, SDMT scores were significantly predicted by GSH levels and the Glu/GSH ratio in the left hippocampus ($\Delta R^2 = 0.100$, $P = 0.023$; $\Delta R^2 = 0.121$, $P = 0.012$, respectively). Both GSH levels and the Glu/GSH ratio in the PCC were statistically significant predictors of TMT-A scores ($\Delta R^2 = 0.120$, $P = 0.024$; $\Delta R^2 = 0.186$, $P = 0.004$, respectively). Regardless whether it was
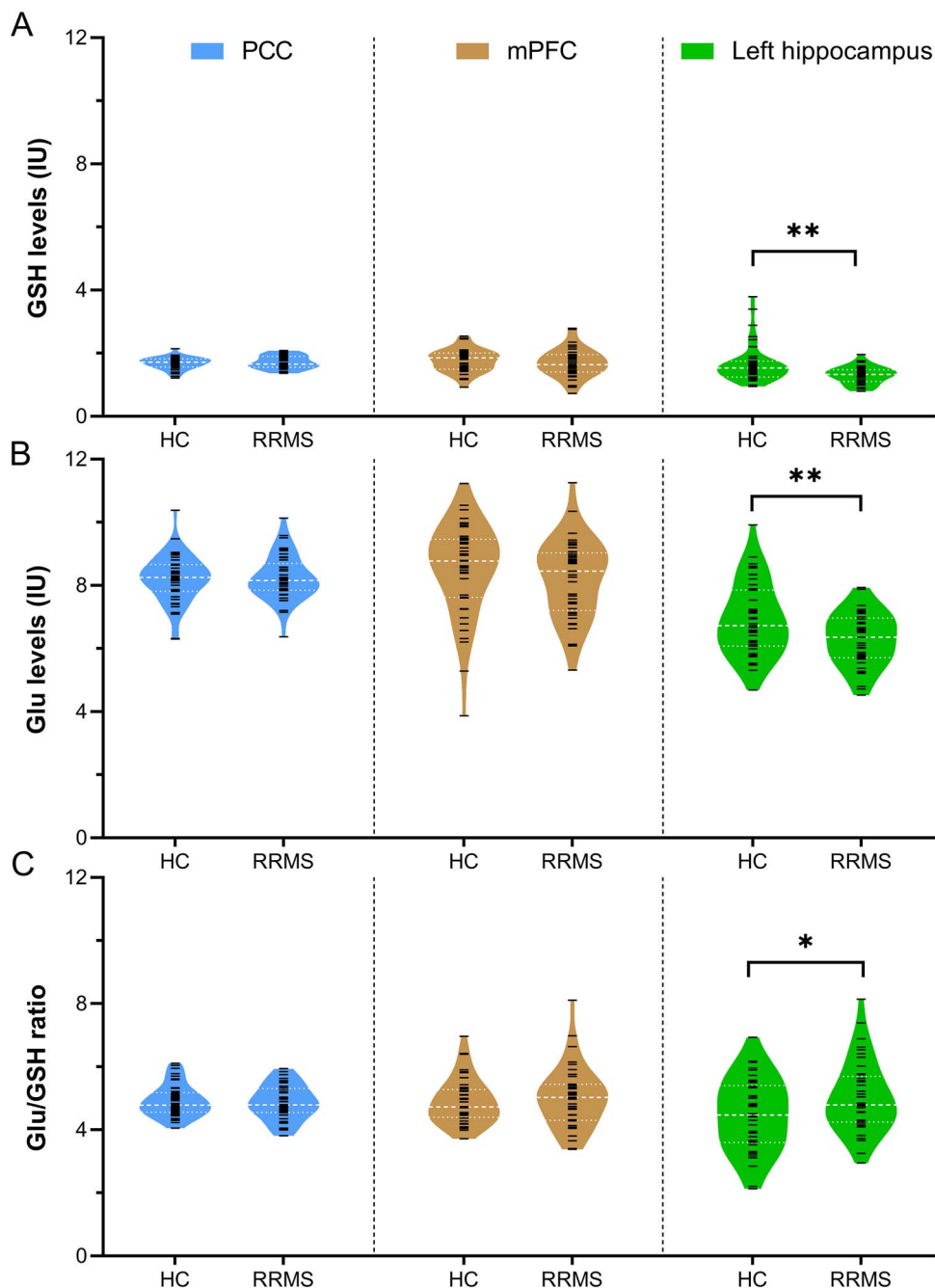
Fig. 2. Comparison of the levels of GSH and Glu, and Glu/GSH ratio between groups. Significantly lower levels of GSH ($P = 0.002$) (A) and Glu ($P = 0.006$) (B) in the left hippocampus were shown in patients with RRMS compared with HCs. Glu/GSH ratio was significantly higher in the left hippocampus of RRMS patients ($P = 0.03$) (C). No significant group differences were observed in GSH or Glu levels or Glu/GSH ratio in the mPFC or PCC. ** indicates $P < 0.01$. * indicates $P < 0.05$.

measured in the PCC or left hippocampus, the predictive effect of the Glu/GSH ratio was greater than that of GSH level, as indicated by the ΔR^2 values.

All in all, RRMS patients showed significantly lower hippocampal Glu ($P = 0.006$) and GSH ($P = 0.002$) levels, which were statistically significant predictors of ROCFT and AVLT scores, respectively ($\Delta R^2 = 0.198$, $P = 0.001$; $\Delta R^2 = 0.103$, $P = 0.010$). Moreover, there were no significant correlations between hippocampal Glu and GSH levels in the RRMS group ($r = 0.233$, $P = 0.142$). The Glu/GSH ratio in the PCC and left hippocampus showed greater predictive effect of TMT-A ($\Delta R^2 = 0.186$, $P = 0.004$; and SDMT scores ($\Delta R^2 = 0.121$,

$P = 0.012$) than that of GSH level ($\Delta R^2 = 0.120$, $P = 0.024$; $\Delta R^2 = 0.100$, $P = 0.023$, respectively).

Discussion

To the best of our knowledge, this is the first in vivo ^{1}H-MRS study investigating the coupling between GSH and Glu levels in RRMS and its association with cognitive impairment. In our study, RRMS patients showed significantly reduced hippocampal GSH and Glu levels, which were found to be significant predictors of worse verbal and visuospatial memory, respectively. Moreover, GSH levels

Table 5. Tissue compositions of the MRS voxel.

Region	Tissue fraction (%)	HCs (N = 43)	RRMS (N = 41)	P
PCC	WM	36.52 ± 4.09	36.15 ± 4.62	0.69
	GM	55.98 ± 3.93	56.21 ± 4.01	0.80
	CSF	7.49 ± 2.10	7.64 ± 3.08	0.80
mPFC	WM	31.37 ± 2.94	31.34 ± 3.23	0.78
	GM	57.38 ± 2.79	57.57 ± 3.07	0.96
	CSF	11.25 ± 1.65	11.10 ± 1.23	0.64
Left hippocampus	WM	44.33 ± 4.07	44.47 ± 4.06	0.87
	GM	51.61 ± 3.45	51.41 ± 3.51	0.79
	CSF	4.07 ± 1.74	4.12 ± 1.17	0.86

Note: Data are presented as mean ± SD. RRMS = relapsing–remitting multiple sclerosis; HCs = healthy controls; PCC = posterior cingulate cortex; mPFC = medial prefrontal cortex; WM = White matter; GM = Gray matter; CSF = cerebral spinal fluid.

Table 6. Correlations between GSH and Glu levels, and Glu/GSH ratio and cognitive performances in RRMS patients.

		GSH-P	Glu-P	Glu/GSH-P	GSH-M	Glu-M	Glu/GSH-M	GSH-H	Glu-H	Glu/GSH-H
AVLT	r	−0.092	−0.044	0.153	0.188	−0.032	−0.253	0.426	−0.001	−0.330
	p	0.598	0.799	0.379	0.294	0.852	0.163	0.010*[f]	0.996	0.049*
ROCFT	r	0.356	0.186	−0.203	−0.180	−0.137	−0.118	0.231	0.516	0.064
	p	0.036*	0.278	0.242	0.315	0.424	0.521	0.176	0.001*[f]	0.711
SDMT	r	−0.143	−0.145	0.072	0.164	0.321	0.001	0.377	−0.140	−0.415
	p	0.413	0.400	0.683	0.363	0.057	0.995	0.023*[f]	0.417	0.012*[f]
SCWT	r	−0.341	0.013	0.389	−0.103	−0.025	0.315	0.184	−0.130	−0.228
	p	0.045*	0.942	0.021*	0.570	0.885	0.079	0.282	0.448	0.180
TMT-A	r	−0.381	0.020	0.474	−0.017	−0.130	0.087	−0.036	−0.266	−0.139
	p	0.024*[f]	0.908	0.004*[f]	0.926	0.449	0.636	0.835	0.117	0.418
TMT-B	r	0.012	0.050	0.051	0.058	−0.185	−0.150	0.163	0.015	−0.134
	p	0.946	0.774	0.770	0.749	0.279	0.413	0.342	0.933	0.436

Note: *P < 0.05. Correlations that survived false discovery rate correction are marked by the letter "f." P indicates posterior cingulate cortex. M indicates medial prefrontal cortex. RRMS = relapsing–remitting multiple sclerosis; GSH = glutathione; Glu = glutamate; AVLT = Auditory Verbal Learning Test; ROCFT = Rey-Osterrich Complex Figure Test; SDMT = Symbol Digit Modalities Test; SCWT = Stroop Color and Word Test; TMT-A = Trail Making Test, Part A; TMT-B = Trail Making Test, Part B.

Table 7. Regression models for different cognitive trials in RRMS patients[a].

	Model 1		Model 2					
cognition	R^2	p	Added Variable	β(95%CI)	R^2	ΔR^2	p
AVLT	0.431	0.001*	GSH-H	0.353(2.721 to 18.284)	0.534	0.103	0.010*
ROCFT	0.256	0.056	Glu-H	0.482(3.248 to 12.175)	0.454	0.198	0.001*
SDMT	0.295	0.026*	GSH-H	0.348(1.961 to 25.307)	0.395	0.100	0.023*
	0.295	0.026*	Glu/GSH-H	−0.367(−6.036 to −0.806)	0.417	0.121	0.012*
TMT-A	0.173	0.241	GSH-P	−0.366(−41.582 to −3.173)	0.293	0.120	0.024*
	0.173	0.241	Glu/GSH-P	0.451(3.585 to 17.376)	0.359	0.186	0.004*

Note: [a]Model 1 (columns 2 and 3) included covariates (age, educational level, gender, lesion volume, and disease duration) of cognitive function. Model 2 (columns 5–8) added the levels of metabolites after considering the control variables. The ΔR^2 represents the degree of additional variation that can be explained by the metabolite level after adjusting for other variables. *P < 0.05. P indicates posterior cingulate cortex. H indicates left hippocampus. RRMS = relapsing–remitting multiple sclerosis; GSH = glutathione; Glu = glutamate; AVLT = Auditory Verbal Learning Test; ROCFT = Rey-Osterrich Complex Figure Test; SDMT = Symbol Digit Modalities Test; TMT-A = Trail Making Test, Part A.

were dissociated from Glu levels in the left hippocampus of RRMS patients and the predictive effect of the Glu/GSH ratio was greater than that of GSH levels.

Reduced hippocampal GSH and Glu levels in RRMS

Compared with HCs, RRMS patients had decreased GSH levels in the left hippocampus. As previously reported, oxidative stress induced by excessive ROS production could play an important role in the early, active phase of MS (Carvalho et al. 2014). GSH modulates neuroprotection from oxidative stress and is consumed during this antioxidant process (Rae and Williams 2017). Notably, there is evidence of disrupted GSH metabolism in the cerebrospinal fluid of MS patients (Calabrese et al. 2002) and in MS animal model (Mohamed et al. 2003). Our

results are consistent with previous MRS studies demonstrating significantly reduced GSH levels in the hippocampus of patients with Alzheimer's disease (AD) and mild cognitive impairment (MCI) (Mandal et al. 2015). Depletion of GSH is thought to be responsible for elevated oxidative stress, which is also a vital player in the pathogenesis and progression of AD and MCI (Song et al. 2021).

Consistent with the earlier MRS research (Gao et al. 2018), lower Glu levels were observed in the left hippocampus of RRMS patients. Decreased Glu levels were found to co-occur with demyelination in a cuprizone mouse model of MS (Orije et al. 2015), indicating that a dysfunctional glutamatergic system may be attributed to the damage of myelin in MS. In our study, lower levels of GSH and Glu were found in the left hippocampus, but not the PCC and mPFC. The hippocampus is susceptible to damage in
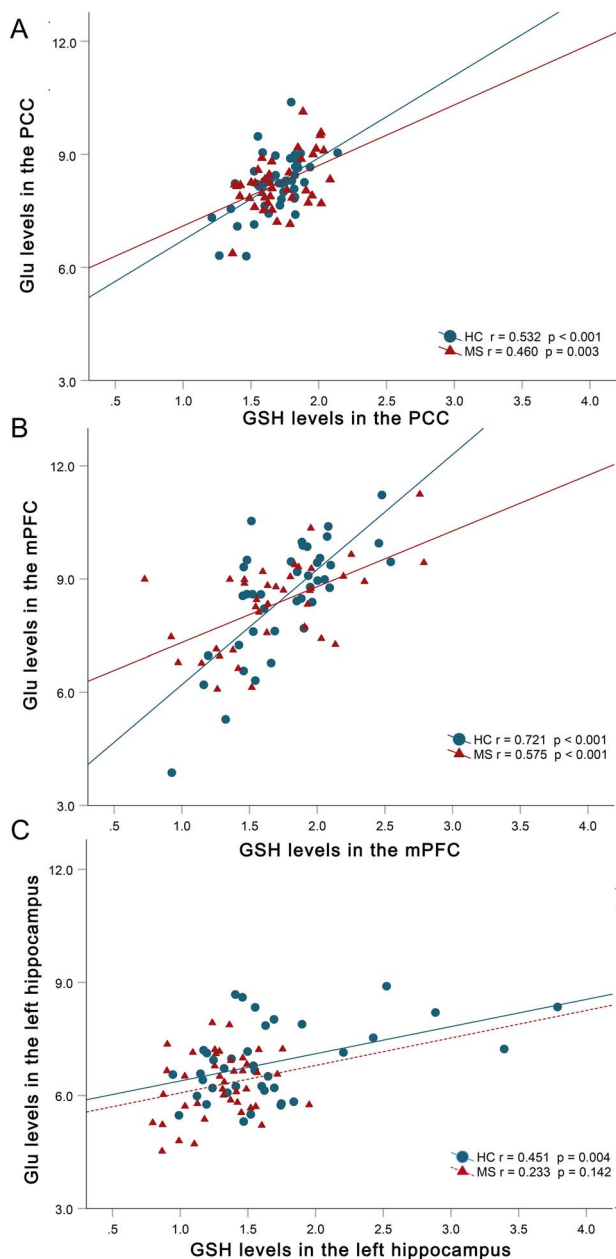
Fig. 3. Correlations between the levels of GSH and Glu in the three regions (PCC, mPFC, and the left hippocampus) in RRMS patients and HCs. GSH levels in PCC (A) and mPFC (B) were positively correlated with Glu levels in both groups. A positive correlation was noted between the levels of GSH and Glu in the left hippocampus in the HC group (C). However, no correlation was found between hippocampal Glu and GSH levels in the RRMS group (the dotted line represents no significant correlation).

the pathological process of MS and previous pathological studies have reported extensive demyelination, synaptic abnormalities, and neuronal damage in this brain region (Rocca et al. 2018). Changes in GSH and Glu levels in the hippocampus could reflect oxidative stress and glutamatergic abnormality in RRMS patients, whereas changes in GSH and Glu levels may be brain-region specific.

Hippocampal GSH and Glu levels correlate with cognitive function

Decreased visuospatial memory and verbal memory were significantly predicted by lower hippocampal Glu and GSH levels, respectively. Our findings suggest that decreased Glu and GSH levels could contribute to a neurophysiological process that aggravates the influence of pathological factors, leading to decline in cognitive function. Consistent with our findings, reduced hippocampal Glu levels were found to be closely correlated with worse visuospatial memory in RRMS patients (Muhlert et al. 2014) and animal models of amnesia (Shimizu et al. 1998). The hippocampus has crucial functions in plasticity and neurogenesis, both of which underlie the pathological episodic memory deficits that often occur in MS (Rocca et al. 2018). The involvement of the hippocampus in learning and memory depends heavily on Glu-mediated signaling pathways in hippocampal subfields (Tamminga et al. 2012), and its volume is associated with episodic memory (Travis et al. 2014). GSH depletion could influence redox signaling pathways and lead to neural disconnection and synaptic dysfunction, which are associated with learning and memory impairment (Gonzalez-Fraguela et al. 2018). Additionally, moderately decreased GSH levels may cause dendrite disruption in the hippocampal CA1 layer (Fernandez-Fernandez et al. 2018), which is responsible for verbal memory. Taken together, the above-mentioned findings suggest that abnormalities of hippocampal GSH and Glu levels could be used as predictors of visuospatial and verbal memory performance in RRMS patients.

Glu/GSH ratio as a predictive biomarker of cognitive impairment

In HCs, significant correlations between Glu and GSH levels were observed in three VOIs. These results support the hypothesis that the glutamatergic system and antioxidants in the normal human brain are mechanically linked. Under normal physiological conditions, excessive Glu can lead to neurotoxic oxidative stress, whereas the concomitant increase in GSH may provide neuroprotection. However, due to insufficient GSH synthesis and glutamatergic dysfunction in MS patients, the regulation between GSH and Glu may be disrupted, resulting in reduced GSH–Glu covariance. This dysregulation may underlie neurotoxic damage in MS patients. In our study, no correlation was found between hippocampal Glu and GSH levels in RRMS patients. A similar dissociation has been reported in early psychosis patients (Xin et al. 2016). This finding suggests that the accompanying GSH response does not occur when there is a demand due to glutamatergic dysfunction. Such patients may be more susceptible to neurotoxic damage and show greater cognitive decline. Considering that GSH and Glu levels may not be independent in MS, we used the Glu/GSH ratio as a predictor of cognitive performance. The hierarchical regression analysis showed that both GSH levels and the Glu/GSH ratio in the hippocampus or PCC of RRMS patients were significant predictors of processing speed and executive function, with the Glu/GSH ratio having a greater predictive effect. These results suggest that a variable combining GSH and Glu has increased predictive power compared to Glu or GSH levels alone. These findings are attributed to the links between GSH and Glu and suggest that disrupted GSH-Glu coupling in the hippocampus may impact cognitive function. Although GSH-Glu coupling was not disrupted in the PCC of RRMS patients, the correlation coefficients of TMT-A scores with Glu/GSH ratios differed significantly between RRMS patients and HCs. The Glu/GSH ratio could serve as an imaging marker sensitive to cognitive impairment in RRMS. Recent evidence has demonstrated that N-Acetylcysteine (NAC), a GSH precursor with antioxidant properties, may be beneficial in MS and improve cognition and attention in the MS patients (Monti et al. 2020). Our results suggest that treatments with such as NAC may be efficacious particularly in patients who demonstrate have

a lower ability to synthesize GSH in response to glutamatergic dysfunction. Given the evidence that oral NAC administration in patients with MS increases GSH level in the brain (Holmay et al. 2013; Samuni et al. 2013), we consider MRS could be a viable tool for monitoring the redox abnormalities of MS and therapeutic effects of NAC.

Limitations and future directions

This study is subject to several limitations. First, although patients with macroscopic lesions in VOIs were excluded, smaller lesions only detectable by MRI with special sequences or higher fields could have contaminated the measurement of metabolites. Second, the MRS data were obtained by a short-TE PRESS sequence rather than MEGA-PRESS sequence. However, previous studies have proved that GSH can be accurately and reproducibly quantified without the use of spectral editing techniques (Wijtenburg et al. 2014; Wijtenburg et al. 2019). Due to the challenges in separating Glu from Gln at 3T, Glu is actually Glx (the combined signal of Glu + Gln). Previous study recommends that a separate PRESS-35 be used when studying Glx and Glu, rather than using Glx or Glu metrics from the MEGA-PRESS data (Bell et al. 2021). Third, to reduce the overall acquisition time, 32 averages were selected in the PRESS parameter instead of 64 averages of the specified voxel size recommended at 3T. Fourth, the same relaxation times for the water signal of MRS VOI were used for HCs and MS patients. Finally, we did not separate RRMS patients into sub-groups, such as cognitively impaired and cognitively preserved. Future studies with larger samples should be conducted to compare sub-groups patients with different symptom profiles and create a more accurate and useful predictive model incorporating MRS-based metabolites levels and clinical characteristics. Furthermore, no cross-sectional design can identify causal relationships between neurometabolites abnormalities and cognitive impairment: this will need longitudinal studies of subjects with high vulnerability to developing cognitive impairment, and interventional (therapeutic) trials.

Conclusions

In summary, our findings provide preliminary evidence that disrupted hippocampal GSH–Glu coupling may contribute to cognitive impairment in RRMS, better than the effect of GSH alone. Hippocampal Glu/GSH ratio may be a potential noninvasive imaging biomarker for predicting cognitive impairment in RRMS, providing a new way for clinical detection of cognitive impairment. Future studies will further explore the main causes of this uncoupling and provide a new therapeutic direction for the treatment of RRMS patients with cognitive impairment.

Abbreviations

AD, Alzheimer's disease; AVLT, Auditory Verbal Learning Test; CHESS, Chemical Shift Selective Suppression; Cho, choline-containing compounds; CRLB, Cramer-Rao lower bounds; CSF, cerebral spinal fluid; EDSS, Expanded Disability Status Scale; FDR, false discovery rate; FLAIR, fluid-attenuated inversion recovery; Glu, Glutamate; Glx, glutamate plus glutamine; GSH, Glutathione; GM, Gray matter; HC, Healthy control; MCI, mild cognitive impairment; mIns, myo-inositol; mPFC, Medial prefrontal cortex; MRS, magnetic resonance spectroscopy; MS, Multiple sclerosis; NAA, N-acetylaspartate; NAC, N-Acetylcysteine; PCC, Posterior cingulate cortex; PRESS, point-resolved spectroscopy; ROS, Reactive oxygen species; RRMS, Relapsing–remitting multiple sclerosis; SCWT, Stroop Color and Word Test; SDMT, Symbol Digit Modalities Test; SNR, signal to noise ratios; tCr, creatine-plus-phosphocreatine; TMT-A, Trail Making Test, Part A; TMT-B, Trail Making Test, Part B; TIV, total intracranial volume; VOI, Voxel of interest; WM, White matter.

CRediT statement

Fuyan Li (Data curation, Formal analysis, Validation, Writing—original draft), Wei Zong (Formal analysis, Investigation, Supervision, Writing—original draft), Chenxi Xin (Investigation, Software, Visualization), Fuxin Ren (Funding acquisition, Methodology, Software, Validation), Ning Li (Formal analysis, Investigation, Methodology), Honghao Li (Formal analysis, Investigation, Resources), Xiao Li (Data curation, Methodology, Supervision), Lili Wu (Methodology, Visualization), Zongrui Dai (Data curation, Methodology), Weibo Chen (Data curation, Methodology), Muwei Li (Data curation, Formal analysis), Fei Gao (Conceptualization, Funding acquisition, Project administration, Writing—review & editing), Guangbin Wang (Conceptualization, Project administration, Supervision, Validation, Writing—review & editing).

Supplementary material

Supplementary material is available at *Cerebral Cortex* online.

Data availability

All data presented in this study are available on reasonable request from Dr. Fei Gao (feigao@email.sdu.edu.cn).

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