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Correlations between the level of antibody against peptide of glutamate receptor NR3B subunit in the CSF and cognitive comorbidities of patients with epilepsy

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Abstract. – OBJECTIVE: Autoimmune epilepsy is an under-recognized condition, and the mechanisms of antibody-mediated epileptogenesis are unknown. The N-methyl-D-aspartate (NMDA) receptor subunit 3 peptide B (NR3B) modulates Mg^{2+} sensitivity and Ca^{2+} mobilization of glutamate responses in the central nervous system (CNS). The levels of antibodies against NR3B (NR3B Ab's) in the cerebrospinal fluid (CSF) and the correlations between NR3B Ab's and cognitive comorbidities of epilepsy patients remain unclear.

PATIENTS AND METHODS: CSF samples were collected from 36 patients with consecutive epilepsy and 17 healthy controls. The levels of NR3B Ab's in the CSF were measured by ELISA. The cognitive function was assessed by Montreal Cognitive Assessment (MoCA) and Mini-Mental State Examination (MMSE).

RESULTS: The results showed that the levels of NR3B Ab's were significantly higher in patients with epilepsy than those in the controls ($p<0.01$). Thirteen of 36 patients had higher levels of NR3B Ab's exceeding mean+ 2SD of all patients, and the scores of MMSE and MoCA of these 13 patients were significantly lower than the other 23 patients and controls ($p<0.01$; $p<0.001$). However, there were no significant differences in the scores of MMSE and MoCA between the 23 patients and the controls. Correlation analysis indicated a significant negative correlation between the levels of NR3B Ab's and the scores of MMSE (correlation coefficient: $r=-0.543$; $p<0.01$) or the scores of MoCA (correlation coefficient: $r=-0.548$; $p<0.01$).

CONCLUSIONS: We suggest that some patients with epilepsy may have immune process after onset and the presence of NR3B Ab's may be associated with cognitive comorbidities in patients with epilepsy.

Key Words
Epilepsy, NMDA Receptor, Autoantibody, Cognitive Function.

Abbreviations

NMDA: N-methyl-D-aspartate; NR3B: N-methyl-D-aspartate receptor subunit 3 peptide B; CNS: the central nervous system; CSF: the cerebrospinal fluid; MoCA: Montreal Cognitive Assessment; MMSE: Mini-Mental State Examination; AEDs: antiepileptic drugs; LGI1: leucine-rich glioma inactivated-1; CASPR2: contactin-associated protein like 2; GAD: glutamic acid decarboxylase; ELISA: enzyme-linked immunosorbent assay.

Introduction

Epilepsy is one of the most common neurological disorders affecting children and adults. About 65 million people currently carry the diagnosis of epilepsy all over the world[1]. Cognitive comorbidities, such as learning difficulties, attention-related problems and execution-related problems, are very common in epilepsy. The assumption based on a serious of studies is that epilepsy damages the brain and thus leads to functional deterioration and behavioral alterations. Although most of the patients are treated successfully with antiepileptic drugs (AEDs), nearly one-third of newly diagnosed patients with epilepsy without identifiable etiology continue to have chronic recurrent

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epileptic seizures[2]. A recent retrospective study found that a handful of patients with unidentifiable but intractable epilepsy had an autoimmune condition[3]. It has long been suggested that inflammatory pathways are involved in epileptogenesis[4]. This hypothesis has been reinforced by the identification of specific autoantibodies directed against neuronal targets. The targets of the major epilepsy-associated antibodies include the extracellular domains of neuronal proteins such as leucine-rich glioma inactivated-1 (LGI1), contactin-associated protein like 2 (CASPR2), the N-methyl-D-aspartate (NMDA) receptors, glutamic acid decarboxylase (GAD) and more recently the $GABA_A$ receptor[5-7]. The study of autoantibodies has provided new insights into the relationship between neuroinflammation, autoimmunity and seizure generation, giving rise to the concept of 'autoimmune epilepsy'. NMDA receptors are multi-subunit receptors formed from the assembly of NR1 with NR2 and/or NR3 subunits. It has been confirmed that anti-NMDA-NR1 antibodies and anti-NMDA-NR2A/B antibodies are present in subpopulations of patients with epilepsy[8,9]. In humans and animal models the anti-NMDA-NR1 antibodies and anti-NMDA-NR2A/B antibodies at high concentration can kill neurons by activating NMDA receptors and inducing 'excitotoxicity', damage the brain, cause dramatic decrease the expression of NMDA receptors in hippocampal neurons, and also decrease the cluster density and synaptic localization of the NMDA receptors. Such changes can impair glutamate signaling and lead to various neuronal/behavior/cognitive/psychiatric abnormalities[10-12]. NMDA receptors subunit 3 peptide B (NR3B) regulates the function of NMDA receptors by negatively modulating Mg^{2+} sensitivity and Ca^{2+} permeability and alleviates the glutamate-mediated excitotoxicity of NMDA receptors[13,14]. In addition, the NR3B protein was found to be expressed in the neurons of the hippocampus (CA1, CA3, dentate gyrus) and the cerebral cortex[15,16]. The $NR3B^{(-/-)}$ mice showed a significant impairment in motor learning or coordination, increase of anxiety-like behavior and decrease of social interaction in a novel environment[17]. Matsuno et al[18] found that a naturally occurring null variant of NR3B is a risk factor of schizophrenia. We speculated that the antibody against NR3B (NR3B Ab's) in the cerebrospinal fluid (CSF) of patients with epilepsy may be involved in epileptogenesis and the level of NR3B Ab's is related with the cognitive comorbidities of patients with epilepsy. To certify our assumption, we first detected the protein level of NR3B Ab's in the CSF by enzyme-linked immunosorbent assay (ELISA). Then, the correlation between the level of NR3B Ab's and the cognitive comorbidities of patients with epilepsy were further analyzed.

Patients and Methods

Patients and Controls

Cerebrospinal fluid (CSF) samples were collected from 36 patients with epilepsy (15 male and 21 female) admitted to the Affiliated Hospital of Xuzhou Medical University and 17 age- and gender-matched healthy people who were devoid of neurological disease or a family history of epilepsy enrolled as control. The median (range) age of epilepsy onset was 19.5 years (7-65 years), and the median (range) age at CSF examination was 26 years (15-67 years). Before participation in this work, informed consent was obtained from the patients or guardians both in the epilepsy group and control group. In addition, medical records including demographics, seizure classification, epilepsy duration, AED usage, EEG, imaging findings and the disease severity of each epilepsy patient, were carefully reviewed by a qualified clinician who was blinded to their NR3B Ab status at the time of the review. This study was approved by the Ethics Committee of our institution.

Cognitive Evaluation

Cognitive function was conducted during two consecutive days using two different screening tools. A trained staff performed the Mini-Mental State Examination (MMSE) on the first day, followed by the Montreal Cognitive Assessment (MoCA) on the second day. This method was used to avoid fatigue given the similarity of questions across tools as well as to ensure a standardized procedure for the evaluation of the participants. The MMSE contains five groups questions with a maximum score of 30 points. The cutoff (for illiteracy, <17; for 6^{th} grade education, <20; and for secondary school education and more, <24) suggests cognitive impairment[19,20]. Items assessed in the tool are registration, orientation, delayed recall, attention/concentration, visual-spacial ability and verbal comprehension. The modified MoCA is a 30-point assessment tool comprising 11 questions. According to the manual, we added one point for an individual who has 12 years or fewer of formal education. A cutoff $\leq$ 25 points indicate cognitive impairment[21]. Items evaluated

in this tool are attention and concentration, executive functions, memory, language, conceptual thinking, calculation, visual constructional skills, and orientation. If the patients had normal MMSE results but abnormal MoCA results, they were considered to have mild cognitive impairment.

Detection of NR3B Ab's by ELISA

CSF samples collected from patients and controls were centrifuged and the supernatants were frozen immediately and stored at -80°C. The level of NR3B Ab's was detected using ELISA. Briefly, a Maxisorp microtiter immunoplate (Nunc, Roskilde, Denmark) was coated with 100 µl of amino acids 351-395 of human NR3B peptide (R&D Systems, Minneapolis, MN, USA) per well, which was diluted to 20 mg/l using coating buffer (Na_2CO_3, pH 9.6). In parallel, another plate was coated with 100 µl per well of phosphate-buffered saline (PBS) with 1% bovine serum albumin (BSA) to detect nonspecific control binding to bovine serum albumin (BSA). The plates were incubated overnight at 4°C and then washed with 1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS). After blocking unreacted sited of the plate with phosphate-buffered saline (PBS) containing 5% bovine serum albumin (BSA), CSF was added (100 µl/well) and incubated overnight at 4°C. After washing vigorously, 100 µl of horseradish peroxidase (HRP)-conjugated goat anti human IgG (1:1000, Jackson ImmunoResearch, West Grove, PA, USA) were added and detection was performed with 100 µl of horseradish peroxidase (HRP) substrate (Kirkegaard and Perry Laboratories, Gaithersburg, MD, USA). Preliminary experiment suggested that assays using undiluted CSF were the most sensitive and specific. Optical densities (OD) at 405 nm were measured using a microplate reader (Bio-Rad, Hercules, CA, USA). All tests were done in duplicate. The well containing phosphate-buffered saline (PBS) with 1% bovine serum albumin (BSA) (no CSF; reagent blank) was measured for nonspecific binding, and this OD was subtracted from the OD value of each sample. Antibody level was expressed in OD unit. Findings were considered positive if the final value of NR3B Ab's was higher than the average OD + 2 standard deviation (SD) of the average of all the controls tested in the same assay.

Statistical Analysis

All statistical analyses were performed using the SPSS statistical 18.0 software (SPSS Inc., Chicago, IL, USA). The measurement data are expressed by mean ± SD, Student's *t*-test and one-way analysis of variance followed by Dunnett's Post-Hoc test of ANOVA were used to assess differences between groups. Categorical variables were used to describe the composition, and the x^2-test was used to compare the groups. The effects of various risk factors on cognitive function were analyzed by multiple linear regression analysis. The correlation analysis of the two variables was detected using Spearman correlation analysis. If $p<0.05$, there was statistically significant difference.

Results

Description of Participants

The clinical characteristics of the study population were summarized in Tables I-III. As shown in Table I, 10 female (59%) and 7 male (41%) patients were included in the control group, ages ranged from 18 to 38 years. According to the levels of NR3B Ab's in the CSF, the 36 patients were divided into two groups: NR3B Ab-positive patients (Table II) and NR3B Ab-negative patients (Table III). Totally, 21 female (58%) and 15 male (42%) patients were included in the epilepsy group; ages ranged from 15 to 67 years. The dis-

Table I. Control group.

No./sex	Age (years)	Cognitive function	
		MoCA	MMSE
1/F	30	29	30
2/F	26	30	30
3/M	18	30	30
4/M	32	30	30
5/F	35	28	29
6/F	37	29	29
7/F	29	30	30
8/M	35	30	30
9/M	29	30	30
10/F	18	30	30
11/M	38	28	29
12/F	33	29	29
13/M	27	30	30
14/F	20	30	30
15/F	30	30	30
16/M	29	30	30
17/F	36	29	29

Table II. NR3B Ab-positive patients.

No./sex	Age (years)	Epilepsy features			Current AED	Cognitive impairment	
		Type	Duration (years)	Severity		MoCA	MMSE
1/M	25	Generalized, Idiopathic	15	C	VPA	18	19
2/M	21	Generalized, Symptomatic	13	I	LTG+PHT	16	16
3/M	28	Generalized, Symptomatic	3	C	NI	24	25
4/F	18	Generalized, Idiopathic	11	C	NI	24	25
5/F	27	Generalized, Cryptogenic	16	C	PHT	21	22
6/M	38	Generalized, Idiopathic	20	I	CBZ+VPA+PB	16	17
7/M	67	Generalized, Symptomatic	2	C	VPA	15	15
8/M	35	Partial+SGTCS, Cryptogenic	22	I	CBZ+PHT+VPA	22	23
9/F	29	Generalized, Idiopathic	20	C	NI	27	27
10/F	18	Generalized, Idiopathic	1	C	NI	24	24
11/F	29	Partial, Symptomatic	2	I	CBZ+LEV	19	19
12/F	28	Partial+SGTCS, Cryptogenic	1	C	OXC	27	28
13/M	17	Generalized, Symptomatic	1	C	VPA	24	25

C, controlled; I, intractable; CBZ, carbamazepine; LEV, levetiracetam; LTG, Lamotrigine; OXC Oxcarbazepine; PHT, Phenytoin; PB, Phenobarbitone; VPA, valproic acid; NI, no information available; SGTCS, secondary generalized tonic-clonic seizures.

ease duration of patients ranged from 0.5 to 22 years. 20 patients (56%) had generalized epilepsy, and 16 patients (44%) had focal epilepsy. The disease was classified into three categories based on etiology: 11 patients (31%) had symptomatic epilepsy (either primary neurologic disorders or systemic disorders), 18 patients (50%) had idiopathic epilepsy, and 7 patients (19%) had cryptogenic epilepsy. Five of the 36 patients (14%) had intractable epilepsy.

Levels of NR3B Ab's in the CSF of the Patients with Epilepsy and the Controls

We compared the levels of NR3B Ab's in the CSF of patients with epilepsy and the controls. In epilepsy patients, NR3B Ab's levels significantly increased compared to the control group (p=0.003). Thirteen of 36 patients (36%) had higher levels of NR3B Ab's exceeding mean+ 2SD of all patients (Figure 1); these patients known as NR3B Ab's positive patients. The OD value of NR3B Ab's in other 23 patients was lower than mean+ 2SD, these patients known as NR3B Ab's negative patients. The detailed information of the 13 NR3B Ab's positive patients and the 23 NR3B Ab's negative patients including gender, age, features of epilepsy (type, duration and severity) and current AEDs were listed in Table II and Table III, respectively.

Correlations of NR3B Ab's Levels with Cognitive Comorbidities

To determine the correlations between the levels of NR3B Ab's and cognitive comorbidities, firstly, the cognitive status of each patient with epilepsy was measured using MMSE and MoCA.

Table III. NR3B Ab-negative patients.

No./sex	Age (years)	Epilepsy features			Current AED	Cognitive impairment	
		Type	Duration (years)	Severity		MoCA	MMSE
1/M	21	Generalized, Cryptogenic	7	C	CBZ	26	27
2/F	52	Partial, Cryptogenic	1	C	OXC	26	26
3/F	19	Generalized, Idiopathic	1	C	NI	27	27
4/M	25	Partial+SGTCS, Idiopathic	1/2	C	VPA	28	28
5/F	48	Generalized, Idiopathic	2	C	NI	22	23
6/M	22	Partial+SGTCS, Symptomatic	3	C	CBZ	26	26
7/M	28	Partial, Symptomatic	3	I	OXC+LEV	21	21
8/F	23	Generalized, Idiopathic	3	C	VPA+TPM	26	27
9/M	16	Partial, Idiopathic	5	C	VPA+LEV	26	26
10/F	35	Partial, Idiopathic	4	C	OXC	26	26
11/F	25	Generalized, Idiopathic	1/2	C	NI	28	28
12/F	20	Partial, Idiopathic	5	C	LTG+LEV	27	27
13/M	29	Partial, Idiopathic	1	C	NI	27	27
14/F	19	Partial, Symptomatic	7	C	LTG+CBZ	26	27
15/M	30	Partial+SGTCS, Idiopathic	3	C	PB	26	26
16/M	29	Generalized, Symptomatic	1/2	C	VPA	25	25
17/F	15	Generalized, Idiopathic	1	C	NI	26	27
18/F	26	Generalized, Idiopathic	1/2	C	NI	29	30
19/F	21	Generalized, Idiopathic	8	C	CBZ+PHT	29	29
20/F	17	Generalized, Symptomatic	1	C	VPA	30	30
21/F	42	Partial, Cryptogenic	7	C	NI	22	22
22/F	23	Generalized, Symptomatic	7	C	NI	24	25
23/F	37	Partial+SGTCS, Cryptogenic	1/2	C	VPA	28	28

C, controlled; I, intractable; CBZ, carbamazepine; LEV, levetiracetam; LTG, Lamotrigine; OXC Oxcarbazepine; PHT, Phenytoi; PB, Phenobarbitone; VPA, valproic acid; NI, no information available; SGTCS, secondary generalized tonic-clonic seizures.

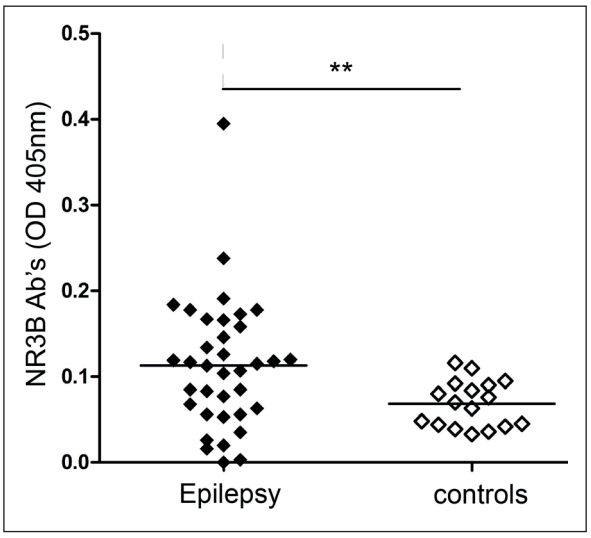

Figure 1. The detection of NR3B Ab's by ELISA. The levels of NR3B Ab's were significantly higher in the CSF of epilepsy patients than that in the controls. The calculated cutoff was 0.122; ** $p<0.01$ vs. controls.

Cognitive impairment was present in 14% of individuals (mean score: 24.8, SD 3.8) when using the MMSE; in 44% (mean score 24.4, SD 3.8) when using the MoCA. Interestingly, the score of MMSE in theNR3BAb's positive patients was significantly lower than that in NR3B Ab's negative patients (21.9±4.3 *vs.* 26.4±2.2; $p<0.01$; Figure 2A). The score of MoCA in theNR3BAb's positive patients was also decreased compared with the NR3BAb's negative patients (21.3±4.2 *vs.* 26.1±2.2; $p<0.001$; Figure 2B). As NR3B Ab's might be a marker of epilepsy, we further observed the correlation between levels of NR3B Ab's and cognitive comorbidities. The correlation between the OD value of NR3B Ab's and the score of MMSE was -0.543 ($p<0.01$), and between OD value of NR3B Ab's and the score of MoCA was -0.548 ($p<0.01$). There were significant negative correlations between the levels of NR3B Ab's and the cognitive function results. A scatter plot of standardized scores correlations was shown in Fig-

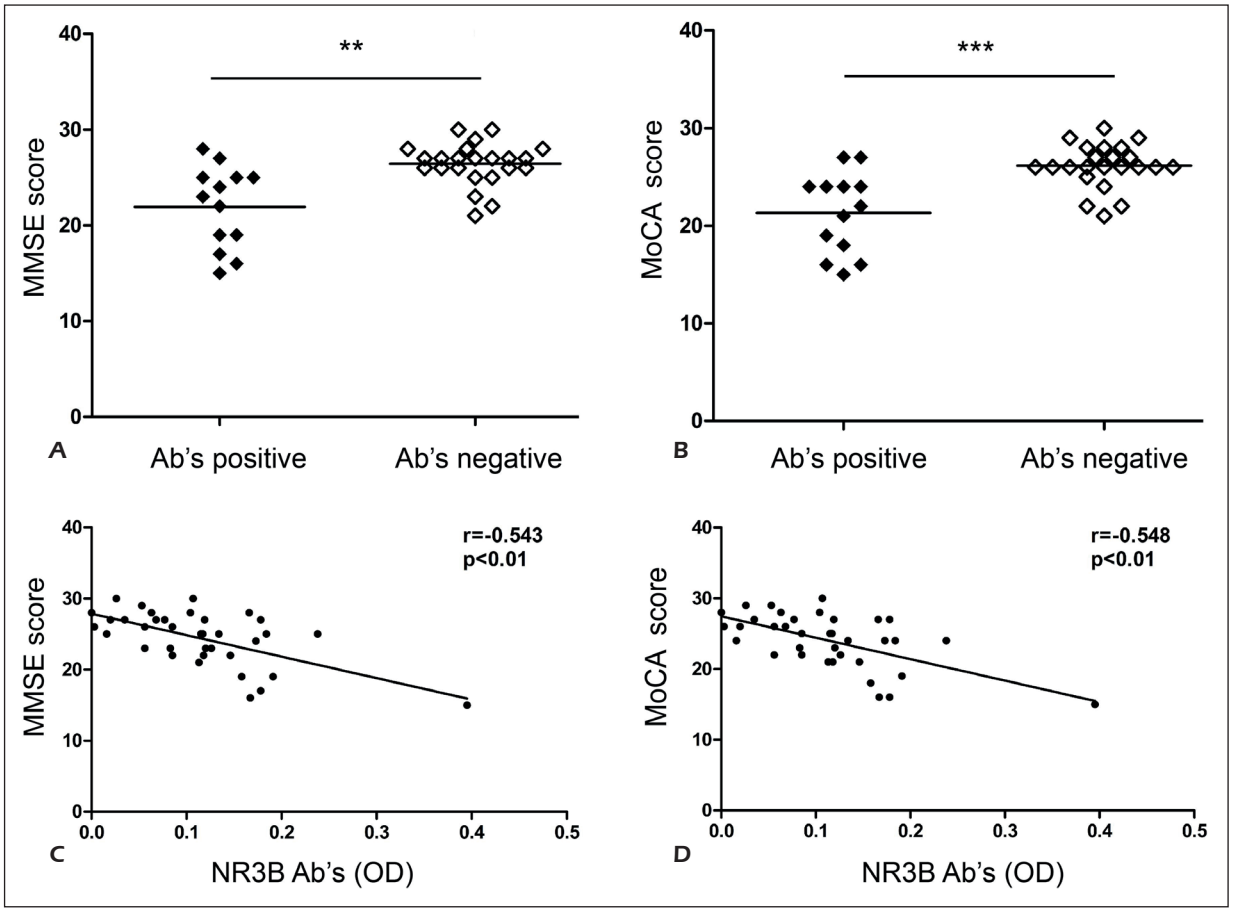

Figure 2. The evaluation of cognitive impairment and the correlational analyses of the NR3B Ab's with the scores of MMSE or MoCA. (**A**-**B**) Both the score of MMSE and MoCA in theNR3B Ab's positive patients were significantly lower than those in NR3B Ab's negative patients. **$p<0.01$, ***$p<0.001$ vs. NR3B Ab's negative patients' group. (**C**-**D**) A significant negative correlation was observed between the levels of NR3B Ab's and results of MMSE or MoCA. ** $p<0.01$.

Table IV. Multiple linear regression analysis with MoCA score as the dependent variable.

Variables	Unstandardized coefficients (B)	SE	Standardized coefficients (β)	p	Adjusted R^2
Age	-0.128	0.036	-0.369	0.001*	
Gender	1.320	0.819	0.173	0.118	
Duration	-0.047	0.080	-0.077	0.560	
Severity	4.085	1.362	0.375	0.006*	
Type 1	1.547	0.847	0.203	0.079	
Type 2	0.360	0.479	0.084	0.458	
NR3B Ab	-2.338	1.007	-0.298	0.028*	0.653 (p<0.001)*

MoCA, Montreal Cognitive Assessment; SE, standard error of B; *p<0.05; Type 1, epilepsy type divided by the electroencephalogram (EEG) findings and semiology; Type 2, epilepsy type divided by the etiology.

ure 2C and Figure 2D. In addition, a multiple linear regression analysis of the MoCA and MMSE scores as the dependent variables was performed to evaluate the contribution of factors, such as gender, age, disease duration, disease severity, epilepsy type, and CSF levels of NR3B Ab's, to the scores (Table IV, Table V). The regression model was significant (p<0.001) and accounted for 65.3% of the variance in the MoCA scores and 63.9% of the variance in the MMSE scores. Moreover, the CSF NR3B Ab's levels (β=-0.298, p=0.028), age (β=-0.369, p=0.001), and severity of disease (β=0.375, p=0.006) were significantly correlated with the MoCA score. The CSF NR3B Ab's levels (β=-0.282, p=0.040), age (β=-0.389, p=0.001), and severity of disease (β=0.399, p=0.004) were significantly correlated with the MMSE score.

Discussion

In this study, for the first time, the antibody against peptide of NR3B subunit in the CSF of patients with epilepsy was detected to elucidate the contribution of immunological mechanisms to the cognitive comorbidities. We found higher CSF levels of NR3B Ab's in epilepsy patients, and levels of NR3B Ab's showed negative correlation with cognitive comorbidities of epilepsy patients. These data suggest that some patients with epilepsy may have NR3B Ab's antibodies in the CSF and the antibody probably associated with cognitive comorbidities. Autoimmune encephalitis is now an established neurological diagnosis in patients presenting with combinations of neuropsychiatric features, seizures, movement disorder and autonomic symptoms. Recent studies of epilepsy have drawn attention to the relationship between neuroinflammation, autoimmunity and seizure generation, giving rise to the concept of 'autoimmune epilepsy', a new classification of epilepsy by the International League Against Epilepsy (ILAE)[22]. Epilepsy-associated antibodies including LGI1, CASPR2, NMDA receptors, GAD and $GABA_A$ receptors have been reported[5-7]. When autoimmune epilepsy is suspected on clinical grounds, CSF evaluation and comprehensive screening for neural autoantibodies are indicated. Autoantibodies to the NMDA

Table V. Multiple linear regression analysis with MMSE score as the dependent variable.

Variables	Unstandardized coefficients (B)	SE	Standardized coefficients (β)	p	Adjusted R^2
Age	-0.133	0.036	-0.389	0.001*	
Gender	1.362	0.825	0.181	0.110	
Duration	-0.026	0.080	-0.042	0.753	
Severity	4.289	1.372	0.399	0.004*	
Type 1	1.300	0.854	0.172	0.139	
Type 2	0.319	0.483	0.075	0.513	
NR3B Ab	-2.182	1.014	-0.282	0.040*	0.639 (p <0.001)*

MMSE, Mini Mental State Examination; SE, standard error of B; *p<0.05; Type 1, epilepsy type divided by the electroencephalogram (EEG) findings and semiology; Type 2, epilepsy type divided by the etiology.

receptors were first identified in a case series of 12 young females with malignancy that developed severe encephalopathy with specific clinical features, including psychiatric symptoms, seizures, cognitive and autonomic dysfunction, movement disorder and decreased level of consciousness[23]. Many studies[24] since have reported cases that include males, children and patients with no underlying malignancy. NMDA receptor antibodies have been also identified in patients with new-onset epilepsies. And that study pointed out that anti-NMDA receptors encephalitis accounts for a relevant proportion of otherwise unexplained new-onset epilepsies[25]. Though many studies have explicated that over expression of anti-NR2A antibodies and anti-NMDA-NR2 antibodies may play roles in epileptic seizure pathogenesis[8,10-12,23], the levels of NR3B Ab's in the patients with epilepsy remain unclear. The NR3B subunit forms hetero-oligomers with NR1 and NR2 and further increases the functional diversity of NMDA receptors[26,27]. It has been shown to reduce Mg^{2+} sensitivity and Ca^{2+} permeability and lower the glutamate-mediated excitotoxicity of the NR3B-containing receptors[13,14,28,29], which were initially considered to be dominant-negative modulators of NMDA receptors. The NR3B$^{(-/-)}$ mice showed a significant impairment in motor learning or coordination, increase of anxiety-like behavior and decrease of social interaction in a novel environment[17]. Matsuno et al[18] found that a naturally occurring null variant of NR3B is a risk factor of schizophrenia. In our study, the NR3B Ab's were detected in the CSF of 36 patients with epilepsy. We found that NR3B Ab's exist in various types of epilepsy and the levels of NR3B Ab's were significantly higher in the CSF of patients with epilepsy than that in the healthy individuals. We speculated that the NR3B Ab's are produced in the CNS by some unknown mechanism and are involved in epileptogenesis or are produced as a result of the autoimmune processes elicited by the seizures themselves. Cognitive comorbidities were significantly higher in the NR3B Ab's positive group, with a higher percentage of patients exhibiting intractable epilepsy. Multiple linear regression analysis revealed that the CSF levels of NR3B Ab's were positively associated with the severity of cognitive impairment. Notably, we did not observe a significant relationship between the epilepsy type, based on the electroencephalogram (EEG) findings and the aetiology (generalized/focal, p=0.083; symptomatic/idiopathic/cryptogenic, p=0.576). Additionally, there was an association between the NR3 Ab's and intractability of the disease (controlled/intractable, p=0.047). Furthermore, the disease duration in the NR3B Ab's positive patients was longer (10 ± 8 years *vs.* 3 ± 3 years; p= 0.024). Altogether, the presence of NR3B Ab's in the brain may directly or indirectly be associated with poorer outcomes in epilepsy patients. Though of some limitations, the present study found an increase in NR3B Ab's levels in the CSF of patients with epilepsy.

Conclusions

We demonstrated the possible association between the levels of NR3B Ab's in the CSF and cognitive comorbidities of patients with epilepsy. We hypothesize that some epilepsy patients, particularly with an unknown cause, have a primary or secondary immune process after the onset of epilepsy. Thus, anti-NR3B antibodies play a role in the pathogenesis of patients with epilepsy.

Ethics approval

This study was approved by the Ethical Committee of the Affiliated Hospital of Xuzhou Medical University.

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Authors' contributions

Hongbin F and Xinyu L designed the study and wrote the manuscript. Peng H performed the experiments. Peng H, Rui Y, Qingyun L, Xiao W was involved in part of the ELISA. Qingwei L critically reviewed the manuscript. Meng Z performed statistical analysis of longitudinal data.

Conflict of Interests

The authors declare no conflicts of interest.

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