COGNITIVE DISORDERS IN NEUROIMMUNOLOGICAL DISEASES

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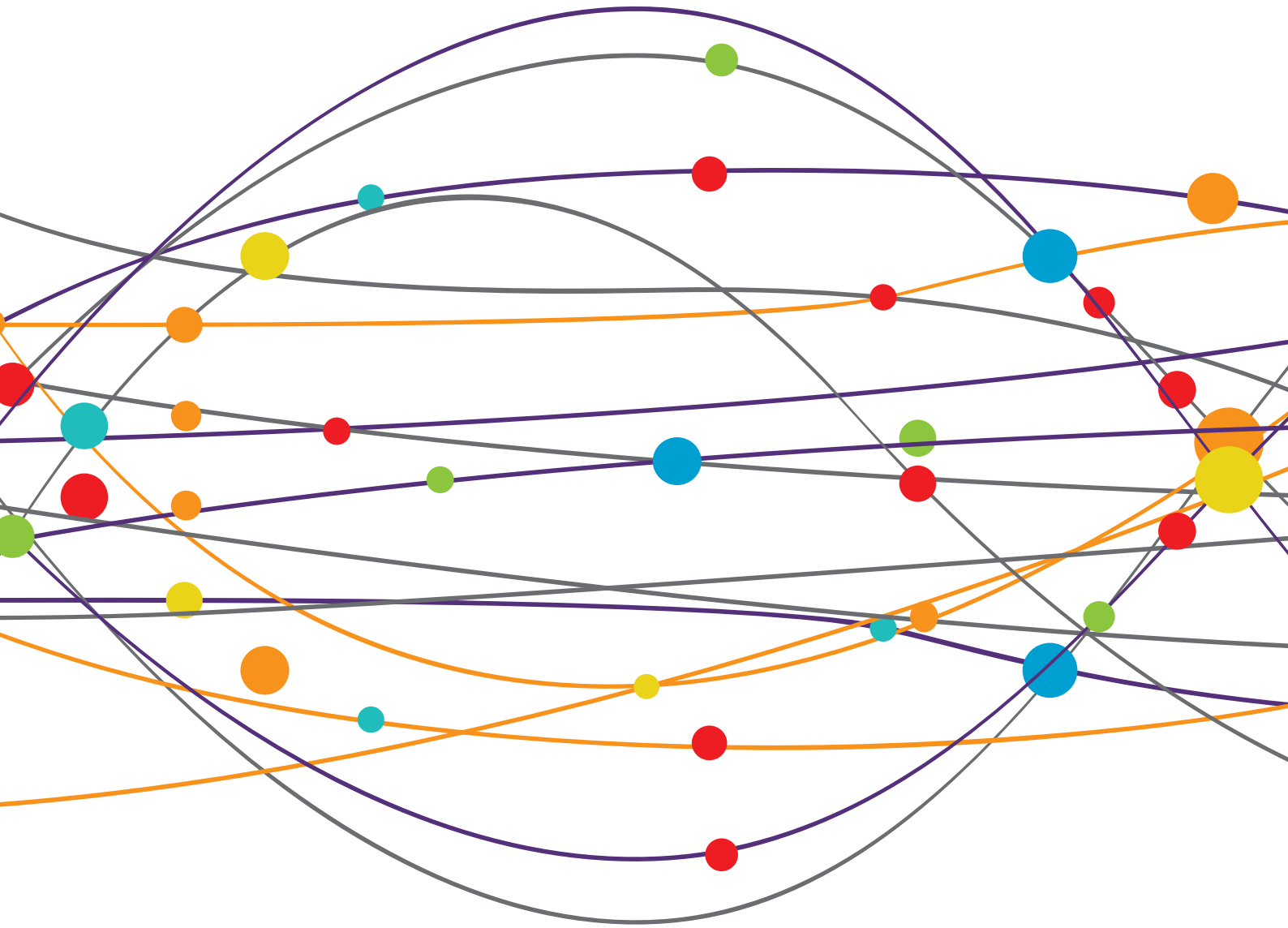

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COGNITIVE DISORDERS IN NEUROIMMUNOLOGICAL DISEASES

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EDITORIAL
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Editorial: Cognitive Disorders in Neuroimmunological Diseases

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Keywords: multiple sclerosis, cognition, neuromyelitis optic (NMO), limbic encephalitis, neuroimmunology

Editorial on the Research Topic

Cognitive Disorders in Neuroimmunological Diseases

In recent decades there has been a rapid growth in the discipline of neuroimmunology: A new area of research investigating how the immune system interacts with the brain, and affects brain function, for example with cognition (1). A convergence of multidisciplinary investigators helped to launch this fascinating research field by developing several groundbreaking lines of research. The close connection between the nervous, endocrine, and immune systems has become a major challenge for interdisciplinary research. Newly developed methods in neuropsychology, immunology, and imaging allow deeper insights into the mechanisms of neuroimmune interactions (2, 3).

In this Research Topic, the role of neuroinflammation on cognition and neuropsychology, basics of psychoneuroimmunology and neuropsychology including conditioned immunomodulation as well as diagnostic aspects such as measurement of cognition are presented (4). In addition, case reports can demonstrate cognitive problems, emotional disturbance, behavioral abnormalities and psychiatric symptoms beyond reviews and original papers. Beyond multiple sclerosis (MS), other neuroimmunological diseases as neuromyelitis optica (NMO) or limbic encephalitis are presented in this Research Topic.

In their review, Brochet and Ruet focus on the cognitive impairment in Multiple Sclerosis (MS) with regard to disease duration and clinical phenotypes. Whereas, the effect of disease duration seems to be confounded by the effect of age, the MS phenotype itself seems to play an important role in the cognitive profile of patients because of its specific pathological mechanism.

To characterize cognitive impairment in MS in a more detailed way, Yalachkov et al. demonstrate that MS patients exhibit an altered multisensory perception to sound-induced flash illusion, a multisensory perceptual illusion, and that their susceptibility to the perceptual illusion is negatively correlated with their neuropsychological test performance. This paradigm may serve as a screening test for cognitive deficits in MS patients.

Moving from MS to NMO spectrum disorders (NMOSD), Oertel et al. review the highly prevalent, but often unrecognized and insufficiently treated, cognitive burden in NMOSD patients; this consists mainly of problems in attention and memory and may reflect a clinical correlate changes which are independent of attacks.

Brain imaging can help to study the link between cognitive dysfunction and alterations of white matter connectivity using graph theory, as Bin Cho et al. report, in 14 NMO patients vs. 21 healthy controls. Overall white matter disruption and sub-network alterations as manifestation of network dysconnectivity seem to be crucial in pathophysiology of cognitive impairment in NMO.

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Beyond imaging, biomarkers could be of interest as Qiao et al. have investigated in patients with primary Sjögren's syndrome with and without NMOSD. Serological comparative proteomics including serum clusterin and complement factor H demonstrated significant differences between these entities.

Moving to limbic encephalitis, Hansen discusses long-term memory dysfunction in these patients: in translational transfer experiments of autoimmune encephalitis from humans to the mouse a critical impairment of synaptic long-term potentials in the hippocampus was induced by autoantibodies against the N-methyl-D-aspartate receptor (NMDAR) and alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor subunit GluA2 which might represent a potential cellular mechanism of mnestic dysfunction.

In line with this opinion, Wang et al. present in their original research that a decrease in amplitude of the NMDAR-mediated excitatory postsynaptic currents in the hippocampal neurons could be shown in rats treated with anti-NMDAR antibodies clinically linked to an impaired learning performance. Both papers suggest a significant role of neuroinflammation in exacerbating the memory impairment in anti-NMDAR disease which could be a potential target to delay or reverse memory impairment.

An autoimmune encephalitis could be mediated by antibodies to Metabotropic Glutamate Receptor 5 (mGluR5) Antibodies in Cerebrospinal Fluid, as Guevara et al. describe in a case report. This complex case tells us that antiinflammatory treatment should not be delayed for the antibody results when there is a reasonable index of suspicion of autoimmune encephalitis.

This is especially true as the exact target antigen of autoantibodies may not yet be known: Thanarajah et al. present an atypical case of autoimmune encephalitis with cerebellar and temporal dysfunction but without seizures associated with high levels of cerebrospinal fluid neuropil antibodies against a yet unknown epitope on the neuronal surface in the cerebellum, hippocampus, thalamus, and the olfactory bulb.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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CASE REPORT
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Encephalitis Associated to Metabotropic Glutamate Receptor 5 (mGluR5) Antibodies in Cerebrospinal Fluid

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A 68-years-old Hispanic man, complained of night sweats, low grade fewer, unexplained weight loss, and memory problems over 3 months. Abdominal tomography showed multiple intra-abdominal adenopathy and biopsy confirmed classic Hodgkin's lymphoma. He commenced treatment with chemotherapy. Three months later, he had acute onset of inattention, auditory hallucinations and alterations of anterograde memory. The patient developed psychomotor agitation, unresponsive to a combination of neuroleptics and benzodiazepines. Brain MRI showed a small established cerebellar infarction. Electroencephalogram was normal. Tests for toxic metabolic encephalopathy were negative. One oligoclonal IgG bands was found in the Cerebrospinal fluid (CSF), which was not observed in corresponding serum, but cell count and protein were normal. Extensive testing for infectious encephalitis was unremarkable. CSF testing for commercially available neural and non-neural autoantibodies was negative. The patient fulfilled the Gultekin diagnostic criteria for paraneoplastic limbic encephalitis and methylprednisolone IV 1g/d for 5 days was given. He recovered rapidly, with progressive improvement in memory and psychomotor agitation. After treatment commenced, results for antibodies to mGluR5 in CSF taken prior to treatment were returned as positive. mGluR5 is found on post-synaptic terminals of neurons and microglia and is expressed primarily in the hippocampus and amygdala. This case highlights the difficulties in diagnosing this type of encephalitis: the CSF did not show pleocytosis, the MRI showed only chronic change and the electroencephalogram was normal. The dramatic recovery after methylprednisolone help to better characterized the clinical spectrum of auto-immune encephalitis. Diagnosing anti mGlutR5 encephalitis may lead to potentially highly effective treatment option and may anticipate the diagnostic of a cancer. A high index of suspicion is needed to avoid missed diagnosis. In patients with unexplained encephalitis, testing for antibodies to mGluR5 in CSF and serum should be considered. When there is a reasonable index of suspicion of auto-immune encephalitis, treatment should not be delayed for the antibody results.

Keywords: encephalitis, metabotropic glutamate receptor 5, Hodgkin's lymphoma, Ophelia syndrome, limbic encephalitis

CLINICAL CASE

A 68-years-old Hispanic man with chronic depression and anxiety, complained of night sweats, low grade fewer, and unexplained weight loss over 3 months. He had also 3 months of difficulty managing finances and keeping track of appointments. Abdominal tomography showed multiple intra-abdominal adenopathy and biopsy confirmed classic Hodgkin's lymphoma, of nodular sclerotic variety. He commenced treatment with ABVD chemotherapy (adriamycin, vinblastine, bleomycin, and dacarbazine). Six months after first presentation of the cognitive problems, he had acute onset of disorientation, inattention, psychomotor agitation, confusion, delusional ideas of grandiosity, auditory hallucinations, and alterations of anterograde memory. His score was 20/30 on the Montreal Cognitive Assessment (MoCA) suggesting severe cognitive impairment. Two days later, the patient developed multiple episodes of psychomotor agitation and was unresponsive to a combination of neuroleptics and benzodiazepines. These neuropsychiatric changes were not attributed to the ongoing stable treatment with ABVD chemotherapy. Brain MRI showed a small established cerebellar infarction. Electroencephalogram was normal. Tests for metabolic encephalopathies were negative: complete blood cell count, calcium, magnesium, phosphorus, liver function tests, erythrocyte sedimentation rate, antinuclear antibody, C-reactive protein, thyroid-stimulating hormone, antithyroglobulin, antithyroperoxidase antibodies, cortisol, vitamin B_{12}, and laboratory tests for toxicology. Human immunodeficiency virus and rapid plasma reagin were negative. One oligoclonal IgG bands was found in the CSF, which was not observed in corresponding serum, but cell count and proteins were normal. CSF Gram stain and culture were negative. Extensive testing for infectious encephalitis was unremarkable (CSF PCR for *E. coli K1*, *H. influenzae, L. monocytogenes, N. meningitidis, S. agalactiae, S. pneumoniae, Cytomegalovirus, Enterovirus, Herpes simplex 1* and *2, Herpes 6, Parechovirus, Varicella zoster,* and *Cryptococcus neoformans*). The patient fulfilled the diagnostic criteria by Gultekin et al. for paraneoplastic limbic encephalitis (PLE) (1) and methylprednisolone one gram daily for 5 days was given. The patient recovered rapidly, with progressive improvement in memory and psychomotor agitation. CSF testing for commercially available neural and non-neural autoantibodies was negative (including against the N-Methyl-D-aspartate (NMDA) receptor, AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) receptor, and VGKC (voltage-gated potassium channel complex). No informative autoantibodies were detected in the CSF paraneoplastic evaluation. AGNA-1 [Anti-Glial Nuclear antibody (Ab)], Amphiphysin Ab, ANNA-1, 2 and 3 (antineuronal nuclear Ab), CRMP-5-IgG (Collapsin response-mediator protein-5), PCA-1, 2, and 3 (Purkinje Cell Cytoplasmic Ab). Further testing of CSF for antibodies to metabotropic glutamate receptor 5 (mGluR5) was positive on cell based assay and immunohistochemistry (2). This CSF sample was drawn before starting systemic steroids. At 30-days follow-up, the patient evolved oriented, attentive, without psychomotor agitation. MoCA was 30/30. He remains amnesic with respect to the hospitalization period, but with conservation of other memory modalities. Follow-up CT scan and EEG were unremarkable.

BACKGROUND

Glutamate is the major excitatory neurotransmitter in the central nervous system and glutamatergic neurotransmission is involved in most aspects of normal brain function. Dysfunction of glutamate receptors have recently been related to immune-mediated encephalitis (3, 4). The metabotropic glutamate receptors belong to a family of G protein-coupled receptors that have been divided into three groups based on their sequence homology, putative signal transduction mechanisms, and pharmacologic properties (3, 4). mGluR1 and mGluR5 constitute Group I metabotropic glutamate receptors. mGluR5 is found on post-synaptic terminals of neurons and microglia. mGluR5 signals via Gq/G11 coupling to activate phospholipase C, resulting in calcium mobilization and activation of protein kinase C, and are expressed primarily in the hippocampus and amygdala. The antibodies cause a decrease of mGluR5 cluster density at both synaptic and extrasynaptic locations (2), although the exact mechanism by which the antibodies alter the receptor density is unknown. Their location may explain the typical behavioral and memory problems in this mGluR5 antibody—associated encephalitis. Clinical correlates of mGluR5 antibodies have been reported in only 11 patients (2).

DISCUSSION

In the case of encephalitis with mGluR5 antibodies, Ophelia syndrome (neuropsychiatric abnormalities and coexisting Hodgkin's lymphoma) improvement with steroids is common (2). The resulting neuropsychiatric abnormalities can be diverse, ranging from mood and personality changes to anterograde amnesia, disorientation, headaches, involuntary movements, and fatigue (5, 6).

The diagnosis of anti-mGluR5 encephalitis is rare, but may increase as antibody testing become more widely available. The close link between the autoimmune response and Hodgkin's lymphoma or other malignancies may also contribute to its under-recognition, as neuropsychiatric changes may be attributed to treatment or psychological factors (2, 3, 5–7).

However, anti-mGluR5 encephalitis can occur without tumor: in the eleven previously reported cases of encephalitis with mGluR5 (2), the association with tumors occurred in six patients: five had Hodgkin's lymphoma and one had small cell lung cancer. In all five patients with Hodgkin's lymphoma, the neuropsychiatric disorders preceded the tumor diagnosis by 2–11 months (2), which also occurred in the present case.

This patient satisfied diagnostic criteria for PLE as he had intrathecal oligoclonal IgG band. Following the Gultekin criteria (1), we suggest that patients with unexplained psychiatric disorders should be tested for anti-mGluR5 encephalitis when there is at least one of the following abnormalities: CSF showing inflammatory changes (pleocytosis, oligoclonal bands, increased

immunoglobulin content or increased protein content in the absence of measured immunoglobulin); MRI showing unilateral or bilateral temporal lobe abnormalities on T_2 weighted images and EEG showing slow-or sharp-wave activity in one or both temporal lobes. Antibody status is not essential for early diagnosis and early treatment of autoimmune encephalitis should not be delayed, as antibody testing is not readily accessible in many institutions and result can take several weeks to obtain. The initial diagnostic approach should be based on neurological assessment and tests that are accessible to most clinicians (8). This does not imply that any manifestation of psychiatric disease should give occasion to testing for antineural antibodies, but that it should be done when clinically indicated.

This case highlights the difficulties in diagnosing auto-immune encephalitis as the paucity of positive diagnostic tests may have delayed or prevent the diagnosis of PLE. In this case, the MRI showed only chronic non-specific change. In the recent series of Spatola (2), six out eleven patients (55%) had normal MRI. Gultekin et al. (1) reported that 16 of 44 (36%) patients with PLE had a normal MRI signal. This patient had a normal EEG. Fifty percent of the patients reported by Spatola had also a normal EEG (2). CSF did not show pleocytosis, but all the 11 previous patients reported with antibodies to mGluR5 had pleocytosis (2). Whether this negative finding will be included in the clinical spectrum of this encephalitis or whether is related to a false-positive result for mGluR5 antibody testing and/or the possibility of epiphenom should be assessed after new publications of case reports and case series of this type of encephalitis. Thus, encephalitis with mGluR5 antibodies, along with other autoimmune encephalitis, may present with memory or behavioral deficits with non-diagnostic CSF, imaging and EEG.

This patient had an excellent response to treatment, which should not be delayed awaiting the antibody results.

CONCLUDING REMARKS

Diagnosing anti mGluR5 encephalitis is relevant and important, as it may lead to potentially highly effective treatment option and sometimes anticipates the diagnostic of a cancer. In patients with unexplained encephalitis, CSF and serum testing for antibodies to mGluR5 should be considered. Treatment should not be delayed for return of the antibodies when a clinical diagnosis of auto-immune encephalitis is made.

ETHICS STATEMENT

The study was conducted according to International Standards of Good Clinical Practice (ICH guidelines and the Declaration of Helsinki). The project was approved by the local Research Ethics Committees of Universidad de Chile Hospital, Santiago, Chile.

AUTHOR CONTRIBUTIONS

CG: conception, organization, execution, review, and critique; GF, CS, GA, PA, JE, AC, HA-L, and JdG: review and critique.

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ORIGINAL RESEARCH
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White Matter Network Disruption and Cognitive Dysfunction in Neuromyelitis Optica Spectrum Disorder

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Background: In neuromyelitis optica spectrum disorder (NMOSD), brain involvement is common and cognitive dysfunction is frequently found. The study investigated alterations of white matter (WM) connectivity using graph theory and correlations with cognitive dysfunction in patients with NMOSD.

Methods: We prospectively enrolled patients with NMOSD ($N = 14$) and age- and sex-matched healthy controls ($N = 21$). Structural connections between any pair of the 90 cortical and subcortical regions were established using diffusion tensor imaging and graph theory. Network-based statistics was employed to assess differences in WM connectivity between the NMOSD and healthy control groups. We further investigated the relationship between the topological network characteristics and cognitive test performances.

Results: WM network analysis showed decreased total strength of brain networks and two disrupted sub-networks in patients with NMOSD. The first featured six hub nodes in the rectus, hippocampus, calcarine, cuneus, and precuneus with the left-sided predominance. The second had six hub nodes in the orbitomiddle frontal, post-central, superior parietal, superior, and middle temporal, and caudate with the right-sided predominance. Compared to healthy controls, NMOSD patients showed poor performance on tests for attention/working memory and processing speed, visuospatial processing, and executive function, which were associated with significant decreases in nodal clustering coefficient, local efficiency, and regional efficiency in the disrupted sub-networks (all $p < 0.05$).

Conclusions: The data show the overall WM disruption and the relationship between poor cognitive function and sub-network alterations identified by the network analysis in NMOSD patients. We suggest that cognitive dysfunction is related to dysconnectivity of WM network including default mode network in NMOSD.

Keywords: neuromyelitis optica spectrum disorder, cognitive dysfunction, white matter network, diffusion tensor imaging, graph theory, multiple sclerosis

INTRODUCTION

Neuromyelitis optica spectrum disorder (NMOSD) is an inflammatory demyelinating disease of central nervous system (CNS), which is characterized by the presence of anti-aquaporin4 antibody (AQP4-Ab) and the predominant involvement of optic neuritis and myelitis. However, brain involvement is frequently observed, particularly in 52–89% of patients with AQP4-Ab (1). Moreover, brain abnormalities in NMOSD patients were identified even in the normal-appearing white matter (WM) and normal-appearing gray matter (GM), through diffusion tensor imaging (DTI), magnetization transfer, and volumetric MR imaging (2–4). Previous studies have shown multiregional WM disruption in NMOSD, using DTI parameters (5, 6). In addition, recent studies reported that cognitive deficits were observed in 57–67% of patients with NMOSD with a similar cognitive profile to that of patients with multiple sclerosis (MS), which features poor performance on tests of complex attention, executive function, speed of information processing, and/or memory (7–10). Brain volumetric MR studies of NMOSD reported the correlation between impaired cognition and WM damage focusing on WM atrophy (7, 11, 12). Still, unanswered question remains as to WM connectivity changes affect the cognitive functions in patients with NMOSD.

The human brain can be modeled as a complex structural network based on graph theory. According to this theory, brain networks can be described as graphs composed of nodes (neurons or brain regions) linked by edges (synapses or white matter connections), and large-scale white matter connectivity can be constructed using DTI (13). The healthy brain exhibits an efficient "small-world" property, which combines high levels of local clustering among nodes of the network (local segregation or specialization) and short average path lengths that represent global linkage of all nodes in the network (global integration) (13). There is growing evidence that normally efficient brain networks are affected in various diseases in a disease-specific manner (14, 15). To our knowledge, only one study has examined the altered topological organization of white matter connectivity in patients with NMO using graph theoretical analyses. Structural networks were constructed in each NMO patient and control group and regional efficiencies of the common hub nodes were compared (3). However, the clinical implication of WM network changes related to cognitive dysfunction remains unknown.

In this study, we investigated the topological organization of whole brain WM networks by comparing patients with NMOSD and healthy controls. Given multiregional WM damages were reported in previous studies in NMOSD, we adopted three hypotheses. The first is that there are topological alterations of WM networks in patients with NMOSD. The second is that there is a set of sub-networks that distinguish patients from healthy controls. The third is that topological characteristics of these sub-networks are related to impaired cognition.

MATERIALS AND METHODS

Subjects

A total of 15 patients with NMOSD and 22 healthy controls were enrolled in this prospective, single tertiary center study between January 2012 and December 2013. One patient from either group was excluded because of inadequate build-up of tractography due to head motion and blurring of MRI. Finally, 14 patients with NMOSD (13 females, one male; median age, 39 years; range 21–68) and 21 healthy controls (20 females, one male; median age, 30 years; range 22–56) were included. AQP4-Ab was measured with a cell-based indirect immunofluorescence assay as previously described (16). Thirteen patients (93%) had anti-AQP4-Ab and six patients (43%) met the diagnostic criteria for NMO (17). At the time of inclusion, all patients were in remission for more than 3 months and nine patients (64%) had received oral prednisolone at a median dose 10.0 mg/day (range, 2.5–10). Healthy controls did not have any history of medical, neurological, or psychiatric disorders. The present study was approved by the Institutional Review Board of Samsung Medical Center and written informed consent was obtained from all participants in accordance with the Declaration of Helsinki.

Neuropsychological Tests

All participants underwent a comprehensive neuropsychological test battery assessing all cognitive domains suggested by the Rao Brief Repeatable Neuropsychological Battery (BRBN) (18) or the Minimal Assessment of Cognitive Function in Multiple Sclerosis (MACFIMS) (19). For attention/working memory and processing speed, we used the Digit Span Test forward and backward, Trail Making Test part A, Paced Auditory Serial Addition Test (PASAT) at 3 and 2 s, and Digit Symbol Coding Test (DSCT). For visuospatial/perceptual processing, the Spatial Span forward and backward tests were used. We used the Korean version of Boston Naming Test (BNT) (20) to evaluate confrontational naming ability as language function and the Rey-Osterrieth Complex Figure Test (RCFT) to evaluate visuospatial function. In addition, the Korean version of California Verbal Learning Test (CVLT) (20) and the immediate recall, delayed recall, and recognition trials of RCFT were performed to evaluate memory (animal and

supermarket items), the Korean version of Controlled Oral Word Association Test (COWAT) using the three Korean letters, which were compatible with the English version of "F," "A," and "S" (21), and Trail Making Test (TMT) part B. Moreover, considering mood effects on neuropsychological testing, we evaluated the depressive symptomatology and anxiety with the Beck Depression Inventory-II (BDI-II) (22) and State-Trait Anxiety Inventory (STAI) (23), respectively. The time interval between neuropsychological tests and brain MRI was within a month.

Image Acquisition

All participants underwent a three-dimensional (3D) volumetric brain MRI scan. An Achieva 3.0-Tesla MRI scanner (Philips, Best, the Netherlands) was used to acquire 3D T1 Turbo Field Echo (TFE) MRI data using a sagittal slice thickness of 1.0 mm, over contiguous slices with 50% overlap and no gap, a repetition time (TR) of 9.9 ms, an echo time (TE) of 4.6 ms, a flip angle of 8° and matrix size of 240 × 240 pixels reconstructed to 480 × 480 over a field of view of 240 mm. 3D FLAIR MRI data were acquired in the axial plane with the following parameters: axial slice thickness of 1 mm, no gap; TR of 11000.0 ms; TE of 125.0 ms; flip angle of 90°; and matrix size of 512 × 512 pixels. In the whole-brain diffusion weighted MR imaging (DWI), sets of axial diffusion-weighted single-shot echo-planar images were collected with the following parameters: 128 × 128 acquisition matrix; 1.72 × 1.72 × mm^3 voxels; 70 axial slices; 220 × 220 mm^2 field of view; TE 60 ms, TR 7696 ms; flip angle 90°; slice gap 0 mm; b-factor of 600 s/mm^2. With the baseline image without diffusion weighting (the reference volume), DWI were acquired from 45 different directions. All axial sections were acquired parallel to the anterior commissure−posterior commissure line and perpendicular to the mid-sagittal plane.

Image Preprocessing and Network Construction

We used the automated anatomical labeling (AAL) template (24), which contains 78 cortical regions and 12 subcortical structures. For each subject, we non-linearly registered her/his T1 weighted image to the ICBM152 T1 template in the MNI space where the AAL template is defined, and linearly registered the T1 weighted image to her/his own DWI (FSL, http://www.fmrib.ox.ac.uk/fsl/). We then mapped the anatomically defined regions-of-interest (ROIs) defined in the AAL template to the individuals' diffusion space. We used the nearest neighbor interpolation method to preserve the discrete labels of the AAL temple during this inverse mapping.

To obtain streamline tractography, we first performed eddy-current correction on DWI by registering all volumes with non-collinear diffusion directions to the reference image using FSL. Then we employed the Fiber Assignment by Continuous Tracking (FACT) algorithm (25) with 45 degrees of angle threshold through the Diffusion toolkit along with TrackVis (26). This program performed tractography from all voxels (seed voxels) of WM whose fractional anisotropy (FA) value is over 0.2, except ventricles. We removed streamlines shorter than 20 mm in length.

Connectivity matrices were obtained by averaging FA values following neural tracts connecting between any two regions of interests (ROIs) (27). The FA values represent the WM integrity where the lower values may be associated with the damage on the WM in the patient group (28). Specifically, we collected all voxels passed by all streamlines which connect two ROIs, and averaged their FA values. The resulting matrix contains the mean FA values between all pairs of ROIs as its weight. This FA-weighted connectivity matrix may incorporate the WM damage in NMOSD better than mere streamline counts.

Network Topology Analysis

Network topological measures were computed using the Matlab routines of the Brain Connectivity Toolbox (29). Global topological measures included total strength (the summation of all weights in each subject's brain network), edge density (the number of non-zero edges over the number of all possible edges), clustering coefficients, characteristic path length, and small-worldness (29). The values of the last three measures are related to the edge density (30). Thus, we normalized them with the 100 randomly generated degree-strength preserved random networks (31). The clustering coefficient captures the level of local segregation. For each node, nodal clustering coefficient measures how strongly its neighbored nodes are connected each other. The global measure of clustering coefficient is the average clustering coefficient across all the nodes. The characteristic path length is the harmonic mean of all shortest paths capturing the global integration. The small-worldness captures the balance between global integration and local segregation. It is meaningful only when it is compared to the random networks; the characteristic path length is similar to that of the random network, while the clustering coefficient is far larger than that of the random network. Thus, it is given by the value of the normalized clustering coefficient over the normalized characteristic path length; when it is bigger than 1, the network is considered as a smallworld network.

We used nodal measures including nodal degree, nodal strength, nodal clustering coefficient, local efficiency, participation coefficients, and regional efficiency. All of them measure the centrality of a node in various aspects. The nodal degree and strength captures how many neighbors a node is connected with and how strongly it is connected with its neighbors in order. Specifically, the former is the number of edges that connected to the node, while the latter is the summation of the edges' strengths in terms of the number of streamlines. The local efficiency is similar concept with the clustering coefficient, which captures the efficiency of information communication between the neighbors of a certain node (32). The participation coefficient of a node captures its role in the modular organization (33). The brain network in general have a modular structure which consists of several modules whose intra-modular connectivity is denser than their inter-modular connectivity. The higher value represents that the node is connected with multiple modules and may have an important role in exchanging information between modules. The regional efficiency also captures the level of information exchanges in different levels. The regional efficiency summarizes

how efficiently information of a node can be exchanged with all the other nodes by averaging reciprocals of the shortest path lengths to all the other nodes (34).

STATISTICAL ANALYSES

Mann-Whitney *U* test and Chi-square test were performed to compare demographic variables and neuropsychological test scores between the NMOSD and healthy control groups. The Bonfferoni correction was performed over all 22 neuropsychological test scores for the multiple comparison correction.

For comparison of the global network measures, we used permutation-based ANCOVA, controlling for age, sex, and the duration of education. We re-populated the data sets *N-1* times by random re-assignment (permutation) of all subjects into one of two groups, where *N* is the number of permutations. We computed *F*-values for the original data set and *N-1* permuted sets through a simple ANCOVA, which formed a null distribution of group difference. Then we estimated the significance level of group difference by a fraction of the occurrence whose *F*-values were not less than the *F*-value of the original data set. We used 10,000 as *N*. We then employed the Bonferroni correction for 5 network measures as multiple comparison correction. For the survived network measures, we performed the correlation study with each score of neuropsychological test items of impaired performance using the partial correlation coefficients controlling for age, sex, and the duration of education.

For comparison of WM connectivity, we first performed two-sample *t*-test for each edge between the controls and the NMOSD patients, and then employed the network-based statistics (NBS) analysis for multiple comparison correction (35). NBS extracted sub-networks that consisted of significantly different connections between two groups. Specifically, significance levels of sub-networks were estimated based on how the size of the sub-networks was bigger than randomly formed sub-networks using permutation testing. In other words, a sub-network was defined as a set of connected edges whose representative statistics (i.e., *t*-statistics) was bigger than a certain threshold. A value of 2.5 was used as the initial threshold and 10,000 as the number of permutations. NBS works as multiple comparison correction, by controlling the family-wise error rate in the weak sense.

The relationships between the network topological characteristics of "hub nodes" in the sub-network and the clinical variables in patients with NMOSD were examined using Spearman correlation analysis. The hub nodes represented the brain regions most affected by the identified abnormal structural connectivity, and were defined when their degree exceeded the mean plus two standard deviations over all regions connected by the edges identified by NBS. The nodal measures for the correlation study with the neuropsychological tests include nodal strength, nodal clustering coefficient, participation coefficients, local efficiency, and regional efficiency. The clinical variables used in this analysis were part of cognitive function tests that exhibited differences between two groups with Bonferroni adjusted $p < 0.05$. Since the network measures often do not follow the normal distribution, Spearman partial correlation was used to control for the effects of age, gender, and level of education. The false discovery rate (FDR) procedure was performed for the multiple comparison correction over all found hub nodes (i.e., 12 nodes). While the correlation study of the global network measures showed how the global network organization affects the cognitive performance, the correlation study of the nodal network measures in the abnormal WM network may capture how the local topological changes due to the disease affect the cognitive function.

The demographic values and neuropsychological tests were analyzed through SPSS Version 20.0 (IBM Corp, Armonk, NY, USA). All other statistical analyses and visualization were performed using Matlab R2013a (Mathworks, Natick, MA, USA) and in-house software programs.

RESULTS

Demographic and Clinical Features

Demographic and clinical features of 14 NMOSD patients and 21 healthy controls are summarized in **Table 1**. Sex ratio, age, and duration of education did not differ between patients with NMOSD and healthy controls. In NMOSD patients, the most frequent involvement was the spinal cord ($N = 9$, 62.4%), followed by the optic nerve ($N = 6$, 42.9%), brainstem/cerebellum ($N = 6$, 42.9%), and cerebral hemisphere ($N = 2$, 14.3%). No patient showed gadolinium-enhancement in cerebral hemispheric lesions.

TABLE 1 | Demographics and clinical characteristics of study subjects.

	NMOSD ($n = 14$)	HC ($n = 21$)	*P*-value
Sex, female (%)	13 (92.9)	21 (95.5)	1.000
Age, years (range)	39 (21–68)	30 (22–56)	0.092
Education (%)			0.200
<9 years	2 (14.3)	0 (0)	
9–14 years	5 (35.7)	8 (38.1)	
≥15 years	7 (50.0)	13 (61.9)	
Positive AQP4 Ab (%)	13 (93)	NA	
Definite NMO (%)	6 (43)	NA	
Disease duration, years	3.4 (1.5–9.3)	NA	
Annual relapse rate	0.6 (0.5-1.7)	NA	
Anytime involvement (%)		NA	
Cerebral hemisphere[a]	2 (14.3)	NA	
Brainstem/cerebellum[a]	6 (42.9)	NA	
Spinal cord	9 (64.3)	NA	
Optic nerve	6 (42.9)	NA	
EDSS	3.0 (1.375–5.25)	NA	

NMOSD, neuromyelitis optica spectrum disorder; HC, healthy control; EDSS, Expanded Disability Status Scale.

Values are median (IQR, interquartile range) unless otherwise indicated; NA, Non Assessable.

[a] *Symptomatic or asymptomatic brain lesions on MRI for this study; frontoparietal white matter lesions in 2/14 (14.3%) and area postrema/cerebellum lesions in 6/14 (42.9%).*

Cognitive Dysfunction in NMOSD Patients

Compared to healthy controls, patients with NMOSD showed significant differences for the following tests scores: attention/working memory and processing speed (TMT-A, corrected $p = 0.001$; PASAT3, corrected $p = 0.004$; PASAT2, corrected $p = 0.007$; and DSCT, corrected $p = 0.001$), executive function (TMT-B, corrected $p = 0.022$; COWAT, corrected $p = 0.006$), and visuospatial processing (spatial span backward, corrected $p = 0.022$) (**Table 2**; **Table e-1**). Digit span forward and backward test in attention/working memory, RCFT copy in visuospatial function, and K-CVLT and RCFT in verbal and visual memory were not different between the NMOSD and healthy control groups.

The frequency of moderate depression or more as BDI $\geq$ 19 was higher in patients with NMOSD ($N = 5$, 35.7%), compared to healthy controls ($N = 1$, 5%), although BDI-II score was not statistically different between patients with NMOSD and healthy controls.

TABLE 2 | Neuropsychological test results from patients with NMOSD and healthy control group.

Neuropsychological test (possible maximum score)	NMOSD ($n = 14$)	Healthy control ($n = 21$)	*P*-value[a]
ATTENTION/WORKING MEMORY AND PROCESSING SPEED			
Digit span forward (12)	8.5 (6.75–10.25)	11 (8–12)	0.770
Digit span backward (12)	6 (5–8)	8 (6–11)	0.946
Trail Making Test A (sec)	31 (29.5–58.5)	21 (16–24.5)	**0.001**
PASAT 3" (60)	39.5 (26.75–49.75)	56 (50–59)	**0.004**
PASAT 2" (60)	26.5 (17.5–37.5)	43 (39–50.5)	**0.007**
Digit symbol coding test (90)	58 (31.5–71.5)	80 (78–90)	**0.001**
VISUOSPATIAL/PERCEPTUAL PROCESSING			
Spatial span forward (14)	8 (7–10)	11 (9.5–12)	0.110
Spatial span backward (12)	8 (6–8.5)	9 (8.5–10)	**0.022**
LANGUAGE			
K-BNT (60)	51 (46–54)	54 (51–56)	0.418
VISUOSPATIAL FUNCTION			
RCFT copy (36)	35 (33–36)	36 (34–36)	1.000
VERBAL MEMORY			
K-CVLT Immediate recall (36)	54 (44–59.5)	59 (52–62)	1.000
K-CVLT Delayed recall (16)	12 (10.75–14)	14 (11–14.5)	1.000
K-CVLT Recognition (16)	15 (12.75–16)	15 (15–16)	1.000
VISUAL MEMORY			
RCFT Immediate recall (36)	16.5 (7–22.75)	21 (18–27.25)	0.528
RCFT Delayed recall (36)	14 (9.75–23.5)	20.5 (16.25–24.75)	1.000
RCFT Recognition (24)	20 (18–21)	20 (19–22)	1.000
EXECUTIVE FUNCTION			
Trail Making Test B (sec)	83 (63.25–138.75)	53 (42.5–59)	**0.022**
Semantic generative naming	36 (25.75–38.75)	41 (36.5–45)	0.154
COWAT phonemic	26.5 (15.5–42)	52 (39–57.5)	**0.006**
EMOTION			
BDI-II (63)	11.5 (7.75–24.25)	5 (3–7)	0.066
STAI_state (80)	44.5 (34–59.5)	36 (32–44)	1.000
STAI_trait (80)	44.5 (34–63.25)	37 (29–44)	1.000

NMOSD, neuromyelitis optica spectrum disorders; PASAT, Paced Auditory Serial Addition Test; K-BNT, Korean version of the Boston Naming Test; K-CVLT, Korean-California Verbal Learning Test; RCFT, Rey-Osterrieth Complex Figure Test; COWAT, Controlled Oral Word Association Test; BDI-II, Beck Depression Inventory-II; STAI, State-Trait Anxiety Inventory. Data area expressed as median (interquartile range).

[a] P-values are results of Mann-Whitney U test adjusting through Bonferroni correction over 22 different test scores (bold indicates the significant results).

Correlations Between Cognitive Test Performance and Clinical Parameters in NMOSD Patients

No significant correlation was observed between cognitive test performances and clinical parameters, such as disease duration, annualized relapse rate, and EDSS scores, in NMOSD patients. The location of symptomatic lesions (brain, spinal cord, or optic nerve) was not associated with cognitive test performance. The dosage of oral prednisolone was not associated with depressive mood and cognitive performances in NMOSD patents. However, depressive mood was positively correlated with PASAT 2 and TMT-B scores ($\rho = -0.696$; $p = 0.037$ and $\rho = 0.729$; $p = 0.021$, respectively) in NMOSD patients, after controlling for age, sex, and education level.

Global Network Topology Analysis

Compared to healthy controls, the total strength was lower in NMOSD patients ($p = 0.009$), although no significant differences were found in edge density, normalized clustering coefficient, and normalized characteristic path length between the two groups (**Table 3**). Both brain networks of NMOSD patients and healthy controls showed the small-world characteristics (small-worldness > 1), which was not different between the two groups.

Disrupted WM Connectivity in NMOSD Patients

NBS analysis identified two disrupted sub-networks in NMOSD patients compared to healthy subjects (**Table 4**, **Figure 1**). All found connections in the sub-networks have weaker strengths in the NMOSD patients. The first sub-network consisted of regions located in the limbic and parieto-occipital lobes, predominantly in the left and posterior part of the brain. These regions were heavily connected to six hub nodes including

TABLE 3 | Global network topological measures.

Network topological measures	NMOSD	HC	*P*-value[a]
Total strength	275.03 ± 31.61	301.40 ± 22.50	**0.045**
Edge density	0.173 ± 0.011	0.175 ± 0.010	1.000
Clustering coefficient[b]	2.164 ± 0.190	2.132 ± 0.170	1.000
Characteristic path length[b]	1.073 ± 0.027	1.072 ± 0.016	1.000
Small-worldness[b]	2.015 ± 0.145	1.987 ± 0.145	1.000

NMOSD, neuromyelitis optica spectrum disorders; HC, healthy controls.

Data area expressed as mean ± standard deviation.

[a] P-values are results of the permutation test adjusting through Bonferroni correction over all 5 network measures (bold indicates the significant results).

[b] Normalized value.

TABLE 4 | The subnetworks of reduced connectivity in NMOSD patients identified through network-based statistics (NBS).

Subnetwork 1	T-stat [a]	Subnetwork 2	T-stat[a]
Lt. superior frontal, orbital part—Lt. middle frontal, orbital part	2.820	Rt. inferior frontal, orbital part—Rt. insula	3.450
Lt. superior frontal, orbital part—**Rt. rectus**[b]	2.798	Rt. inferior frontal, orbital part—Rt. putamen	2.507
Lt. rectus—**Rt. rectus**[b]	3.399	**Rt. middle frontal, orbital part**[b]—Rt. putamen	2.617
Lt. rolandic operculum—Lt. insula	2.996	**Rt. middle frontal, orbital part**[b]—Rt. temporal pole, superior temporal	3.550
Rt. rectus[b]—Lt. anterior cingulum	2.525	**Rt. middle frontal, orbital part**[b]—Rt. temporal pole, middle temporal	4.011
Lt. posterior cingulum—Lt. parahippocampus	2.794	**Rt. post-central**[b]—**Rt. superior parietal**[b]	2.949
Lt. hippocampus[b]—Lt. lingual	2.606	**Rt. post-central**[b]—Rt. supramarginal	4.064
Lt. hippocampus[b]—Lt. postcentral	2.662	**Rt. post-central**[b]—**Rt. caudate**[b]	2.770
Lt. hippocampus[b]—Lt. caudate	3.272	**Rt. post-central**[b]—Rt. pallidum	3.358
Rt. inferior frontal, triangular part—Rt. Lingual	2.839	Lt. superior occipital—**Rt. superior parietal**[b]	2.714
Rt. calcarine—Rt. Lingual	2.502	**Rt. superior parietal**[b]—**Rt. superior temporal**[b]	3.358
Lt. amygdala—Lt. fusiform	3.088	Rt. hippocampus—**Rt. caudate**[b]	3.040
Lt. calcarine[b]—Rt. Calcarine	2.521	Rt. paracentral lobule—**Rt. caudate**[b]	3.449
Lt. calcarine[b]—**Lt. cuneus**[b]	2.834	Rt. supramarginal—**Rt. superior temporal**[b]	3.568
Lt. calcarine[b]—Rt. Cuneus	2.587	**Rt. superior temporal**[b]—**Rt. middle temporal**[b]	3.843
Rt. cuneus—Lt. fusiform	2.564	Rt. middle occipital—**Rt. middle temporal**[b]	3.145
Lt. rolandic operculum—Lt. post-central	2.519	Rt. angular—**Rt. middle temporal**[b]	2.699
Lt. anterior cingulum—**Lt. precuneus**[b]	3.754	**Rt. middle temporal**[b]—Rt. inferior temporal	3.112
Lt. cuneus[b]—**Lt. precuneus**[b]	2.615	Rt. hippocampus—Rt. temporal pole, middle temporal	2.944
Rt. superior occipital—**Lt. precuneus**[b]	2.717	Rt. fusiform—Rt. inferior temporal	2.576
Lt. posterior cingulum—**Rt. precuneus**[b]	3.456		
Lt. cuneus[b]—**Rt. precuneus**[b]	3.838		
Lt. lingual—**Rt. precuneus**[b]	3.175		
Lt. superior parietal gyrus—**Rt. precuneus**[b]	2.641		
Lt. precuneus[b]—**Rt. precuneus**[b]	3.830		

[a] *T-statistics (T-stat) is a result of two-sample t-test. NBS was used as a multiple comparison correction, with 2.5 as a threshold value and 10,000 permutations.*
[b] *Hubs of the subnetworks 1 and 2 were marked as bold.*
NMOSD, neuromyelitis optica spectrum disorders; Lt., left; Rt., right.

the right rectus, left hippocampus, left calcarine, left cuneus, and bilateral precuneus. The hub nodes were heavily connected with the disrupted connections more than the average; in this manner, they represented the brain regions most affected by the identified abnormal structural connectivity. The second subnetwork included regions mostly within the right temporal, parietal, orbitofrontal lobes, and basal ganglia. These regions were heavily connected to six hub nodes: the right orbitomiddle frontal gyrus, right post-central gyrus, right superior parietal gyrus, right caudate, and right superior and middle temporal gyrus.

Cognitive Test Performance Associated With the Disrupted WM Connectivity in NMOSD Patients

We found that the total strength was positively associated with PASAT3 scores ($r = 0.712$, uncorrected $p = 0.014$) in NMOSD patients. Significant correlation was evident between topological characteristics of the hub nodes and cognitive test performances, which differed significantly between the two groups (**Table 5**). The nodal clustering coefficient of the right superior temporal gyrus was positively correlated with TMT-B test performance ($r = -0.893$, FDR adjusted $p = 0.014$). The reduced local efficiency of the left calcarine, cuneus, and right precuneus and the reduced regional efficiency of the left calcarine were associated with decreased performance on DSCT (all FDR adjusted $p < 0.05$). In addition, the participation coefficient of the right post-central gyrus was positively associated with PASAT3 scores ($r = 0.795$, FDR adjusted $p = 0.041$).

DISCUSSION

We demonstrate the dysconnectivity of overall WM network and two disrupted sub-networks in patients with NMOSD. Moreover, the strength of global WM network and several topological characteristics of hub nodes in sub-networks were closely related to cognitive dysfunction, especially for attention/working memory, processing speed and executive function.

In the global WM network, the total strength of patients with NMOSD was decreased, but other topological measures were not different, compared to healthy controls. This is not consistent to a previous study, where the structural brain networks of NMO patients exhibited a disrupted topological organization (abnormal small-world parameters) without changes in network strength and efficiencies compared to healthy controls (3). They previously investigated the WM networks of MS using similar

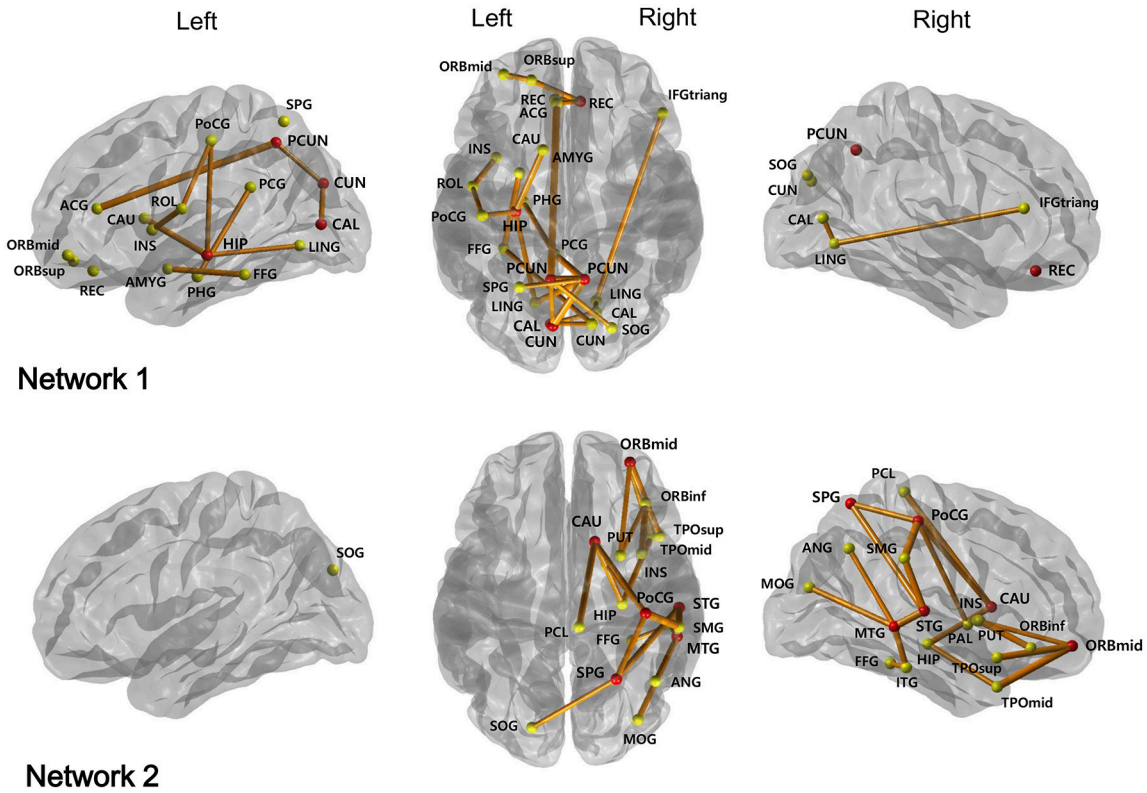

FIGURE 1 | Two subnetworks were identified by network-based statistics, which show the reduced connectivities in NMOSD patients compared to healthy controls. The red circles represent their hub regions representing the brain regions most affected by the white matter disruption, while the other yellow circles are the non-hub brain regions. ORBsup, Superior frontal gyrus, orbital part; ORBmid, Middle frontal gyrus, orbital part; REC, Rectus; ROL, Rolandic operculum; INS, Insula; ACG, Anterior cingulum; PCG, Posterior cingulum; PHG, Parahippocampal gyrus; CAL, Calcarine; CUN, Cuneus; HIP, Hippocampus; LING, Lingual gyrus; IFGtriang, Inferior frontal gyrus, triangular part; AMYG, Amygdala; FFG, Fusiform gyrus; PoCG, Post-central gyrus; SOG, Superior occipital gyrus; PCUN, Precuneus; SPG, Superior parietal gyrus; CAU, Caudate; ORBinf, Inferior frontal gyrus, orbital part; SMG, Supramarginal gyrus; PCL, Paracentral lobule; PUT, Putamen; PAL, Pallidum; STG, Superior temporal gyrus; TPOsup, Temporal pole: middle temporal gyrus; MTG, Middle temporal gyrus; MOG, Middls occipital gyrus; ANG, Angular gyrus; TPOmid, Temporal pole: middle temporal gyrus; ITG, Inferior temporal gyrus.

analytical method and found reduced network strength, global and local efficiencies (15); therefore, they suggested that greater damage to brain tissue in MS than in NMO could explain the different changes in WM structural brain networks (3, 15). Other studies using DTI and tract-based spatial statistics in NMO patients showed extensive cerebral WM tracts with decreased FA and increased mean diffusivity (MD) value, although patients had no brain lesions or small and non-specific lesions (5, 6). These suggested the possibility of widespread non-lesional pathology in normal appearing WM of NMO patients. In our study, although most of patients had normal brain MRI without severe tissue damage, the total strength was decreased, which may imply that the overall WM dysconnectivity results from the disruption of the two sub-networks by non-lesional change, without the alteration of architecture in global network.

More interestingly, we found 12 hub nodes in two disrupted sub-networks in NMOSD patients, of which 6 were regions in the default mode network (DMN); the bilateral precuneus, right rectus, and left hippocampus, right middle and superior temporal gyri (36). The concept of DMN comes from resting-state functional MRI, altered in MS and Alzheimer's disease (15, 36). Previously, the functional alteration of DMN regions, such as the left anterior/posterior cingulate, left medial frontal gyrus, right precuneus and right middle temporal gyrus was observed in NMO patients (37, 38). Other 6 hub nodes in our study were non-DMN regions; left calcarine, left cuneus, right post-central gyrus, right superior parietal gyrus, right orbitomiddle frontal gyrus, and right caudate. A recent study also showed the decreased regional efficiencies of non-DMN regions such as the right cuneus and left calcarine in NMO patients (3).

Decreased cognitive performance in our patients, compared to controls, was observed in attention/working memory and processing speed, visuospatial processing, and executive function and frequent depressive mood, which echo previous studies (9, 10, 39). The negative association between depression and attention/working memory, processing speed and executive function, was also consistent to the other studies (8, 9). Regarding the relationship between the cognitive function and topological characteristics of WM network, several interesting features in

TABLE 5 | The topological characteristics of hub nodes in association with cognitive test performance in patients with NMOSD.

Topological characteristics	Hub nodes	Neuropsychological tests	*R* values (adjusted *p*-values)[a]
Nodal clustering coefficient	Rt. Temporal_Sup	TMT-B	−0.8934 (0.0140)
Local efficiency	Lt. Calcarine	DSCT	0.9122 (0.0028)
	Lt. Cuneus	DSCT	0.8405 (0.0139)
	Rt. Precuneus	DSCT	0.7817 (0.0303)
Regional efficiency	Lt. Calcarine	DSCT	0.8430 (0.0263)
Participation coefficient	Rt. Post-central	PASAT3	0.7951 (0.0413)

NMOSD, neuromyelitis optica spectrum disorders; Temporal_Sup, superior temporal gyrus; TMT-B, Trail Making Test-B; DSCT, Digit Symbol Coding Test; PASAT3, Paced Auditory Serial Addition Test at 3 s.

[a] *Partial correlation coefficient analysis adjusting age, sex, and duration of education with FDR correction for the multiple comparison correction over all 12 hub nodes.*

our study are notable. First, the total strength of global WM network was associated with PASAT3 score, which indicates that the dysconnectivity of global WM network was related to decreased attention/working memory and processing speed. Second, topological characteristics of 5 hub nodes in the sub-networks were also associated with DSCT/PASAT3 scores or TMT-B score. Decreased local or regional efficiencies of right precuneus, the left calcarine and left cuneus were associated with poor performance on the DSCT, which suggests that the decreased local functional specialization or regional contribution to global integration in these regions were associated with decreased attention/working memory and processing speed. The precuneus, one of DMN regions, is known to be implicated in visuo-spatial imagery, episodic memory retrieval and self-processing operations, (40) and the cuneus and calcarine also include primary visual pathway contributing to visuospatial function (41). DSCT is highly correlated with symbol digit modality test (SDMT) (42), which is known as a very sensitive task of cognitive processing speed in MS and is also known that visual scanning efficiency explains ∼34% of digit symbol variance independent of perceptual motor speed (43). Therefore, decreased performance on DSCT in our patients might be attributed to decreased connection integrity of these regions regarding visuospatial function, as well as decreased processing speed. In addition, decreased clustering coefficient in the right superior temporal gyrus was associated with poor performance on TMT-B, which implies that the local functional specialization of this region was associated with executive function. That seems to be plausible since the right superior temporal gyrus, another DMN region, plays a crucial role in spatial awareness, visual search and probably maintaining working memory (44, 45). Finally, participation coefficient in the right post-central gyrus had positive correlation with PASAT3 scores, suggesting that the strength of connections to other modules in this region was associated with attention, working memory and processing speed. This may support the previous study, where post-central gyrus was one of lesions affecting processing speed, measured in the digit symbol subset (46).

The association between cognitive impairment and dysfunction of the DMN, such as precuneus and superior temporal gyrus, as well as non-DMN regions like the right sensory cortex and superior parietal gyrus, was reported in MS patients (47, 48). However, there have been no studies for the relatinoship between network connectivity and cognitive function in NMO, although it was reported that cognitive impairment could be attributed to damage in WM tracts (8, 11), and the reduced regional WM volume and brain atrophy were correlated with cognitive impairment (8). To our knowledge, only one study has examined the altered topological organization of WM connectivity in patients with NMO using graph theoretical analyses (3). Our study has several strengths compared to the previous study. First, we associated the changes in the topological measures with the clinical presentation. Thus, instead of mere difference in the topological measures, we investigated associations between topological network changes and specific cognitive functions with comprehensive neuropsychological testing. Second, our study presented statistically more valid observations. Although, the previous study reported the local changes in the nodal efficiency whose uncorrected *p*-values were <0.5, we employed the FDR procedures over the hubs nodes of the disrupted WM sub-networks for conceptualizing the rate of type I errors when conducting multiple comparisons.

We have several limitations. This is a cross-sectional study performed in a single center. We enrolled NMOSD patients who could have had a relatively mild disability to perform the cognitive tests, which may limit the generalization of this study to all NMOSD patients along with the small sample size. In addition, some of the confounders, such as fatigue and pain, which may influence the cognitive function, were not considered in this study. Moreover, DWI data is rather suboptimal with respect to fiber tracking; the voxel dimensions are anisotropic (2 mm vs. 1.72 mm), which can bias tracking accuracy in the longer z-direction. However, the bias might be insignificant, since it is only 16% longer than the other directions. Finally, our study inherited the limitation of

DTI and the deterministic tractography, including the issue of crossing-fibers and false positive connections (49). Other tracking method such as probabilistic tractography or Hough transform global tractography could be employed in the future research.

In conclusion, this study identified the alteration of overall WM network and the integrity disruption of sub-networks, including both DMN and non-DMN regions, in patients with NMOSD. Two disrupted sub-networks were associated with the decreased attention/working memory, processing speed and executive function, which suggests that the dysconnectivity of WM network contributes to the cognitive dysfunction in NMOSD.

AUTHOR CONTRIBUTIONS

EBC, CH, J-KS, and J-HM contributed to the conception and design of the study. EBC, CH, SWS, JC, J-HS, H-JC, JMS, STK, BJK, DLN, KHL, J-KS, and J-HM contributed to the acquisition and analysis of data. EBC, CH, J-KS, and J-HM drafted the text and figures. All authors contributed to the review and editing.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fneur.2018.01104/full#supplementary-material

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CASE REPORT
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Atypical Autoimmune Encephalitis With Neuropil Antibodies Against a Yet Unknown Epitope

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Autoimmune encephalitis often causes acute psychiatric symptoms and epileptic seizures. However, it is becoming increasingly clear that depending on the target antigen both symptoms and disease severity may vary. Furthermore, the identification and characterization of antibody subtypes are highly relevant for personalizing the treatment and to prevent relapses. Here we present an atypical case of encephalitis with cerebellar and temporal dysfunction but without seizures associated with high levels of cerebrospinal fluid neuropil antibodies against a yet unknown epitope on the neuronal surface in the cerebellum, hippocampus, thalamus, and the olfactory bulb. We treated the patient successfully with corticosteroids, plasmapheresis, and rituximab.

Keywords: encephalitis, cerebellitis, autoimmune, neuropil, plasmapheresis

INTRODUCTION

Autoimmune-mediated encephalitis is commonly associated with acutely emerging psychiatric symptoms and seizures refractory to antiepileptic treatment (1, 2). Evidence increases that, depending on the underlying immune processes and the target antigen, structures of the central nervous system beyond the limbic cortex can be affected, leading to a great spectrum of disease presentation and severity (3, 4).

New insights into the mechanisms underlying antibody associated encephalitis highlight the importance of early diagnosis and antibody characterization using both specialized serum/cerebrospinal fluid (CSF) panels and immunohistochemistry to initiate an effective, personalized treatment regime that addresses both the prevention of disease progression and relapses (5, 6). However, due to the varying clinical, imaging, and serum/CSF manifestations, the early diagnosis and the appropriate treatment still remain a great challenge.

Here we present an atypical case of encephalitis with cerebellar and temporal dysfunction but without seizures associated with high levels of CSF neuropil antibodies against a yet unknown epitope on the neuronal surface in the cerebellum, hippocampus, thalamus, and

the olfactory bulb. We treated the patient successfully with corticosteroids, plasmapheresis, and rituximab.

CASE STUDY

A 67-years old lady was admitted to our intensive care unit (ICU) after requiring intubation for respiratory failure secondary to aspiration pneumonia and decreased consciousness.

Past medical history revealed that 6 months earlier she had been admitted to her local hospital with features of delirium (disorientation, agitation, poor memory functioning) and a cerebellar syndrome with intention tremor, severe postural instability, and dysarthria.

In the local hospital, brain MRI showed a moderate temporo-parietal atrophy (medial temporal lobe atrophy score 2, **Figure 1**). Repeated EEGs did not reveal epileptic activity. Routine blood and CSF analysis (cell count, glucose and protein level) were normal and investigation for infectious pathogens (varicella zoster virus, herpes zoster virus, cytomegalovirus, mumps, rubella, borrelia burgdorferi, treponema pallidum) were negative. Screening for occult malignancy (CT of thorax and abdomen) showed a right ovarian enlargement, but the surgical biopsy did not reveal a neoplastic process. Suspecting an autoimmune pathogenesis, a 5-day course of intravenous high-dose prednisolone (1,250 mg per day) and a 5-day course of intravenous immunoglobulin (IVIg) (20 g IVIg per day) were applied, leading to complete remission of dysarthria and truncal ataxia and partial remission of the delirium. The patient was subsequently transferred to the rehabilitation facility, where the cerebellar symptoms and the level of consciousness deteriorated significantly. She required oropharyngeal intubation due to respiratory failure and was admitted to our ICU.

Upon admission, the patient showed a septic shock due to aspiration pneumonia requiring vasopressor drugs. Following antibiotic treatment (piperacilin/tacobactam and clarithromycin) the vital signs returned to the normal ranges. After sedation was stopped the patient was in a minimally conscious state. Pupillary and corneal reflexes were regular. Gag and cough reflexes were absent.

Brain MRI did not provide new findings. EEG on admission, and repeated EEGs during the stay did not display epileptic activity. The CSF work-up showed normal cell count, glucose and protein levels. Tau- and Phospho-Tau-protein level were slightly elevated while ß-Amyloid and Amyloid-ratio were decreased (Tau 622 pg/ml, Phospho-Tau 71 pg/ml, Amyloid ratio 0.05 pg/ml, Amyloid ß1-42 393 pg/ml). Identical oligoclonal bands were found in serum and CSF. Screening for common anti-neuronal and anti-neuropil antibodies was negative (VGKC, GAD65, NMDAR, GABABR, IgLON5, AMPAR2, DPPX, LGI1, CASPR2, Glycin-receptor, mGluR5, Amphiphysin, CV2/CRMP5, Ma2/Ta, Ri, Yo, Hu, Recoverin, Sox1, Titin, Zic4, DNER/Tr).

Increased Tau-protein level, decreased level of Amyloid-ratio and temporo-parietal atrophy suggested a pre-existing Alzheimer's disease. Still suspecting an autoimmune process, CSF and serum were incubated on mouse brain sections revealing strong antibody reactivity on neuropil and neuronal surface targeting a still unknown epitope in the cerebellum, thalamus, hippocampus, and the olfactory bulb (**Figure 2**). In the cerebellum, antibody reactivity was predominant on granular cells. Interestingly, the patient's CSF stained only a fraction of granule cells, which further underlines specificity as it is similarly known from other autoimmune encephalitides, such as NMDAR or GABAaR encephalitis.

The patient received intravenous high-dose prednisolone for 5 days and 10 courses of plasmapheresis. The clinical status improved significantly: She was able to follow commands and communicate and did not show psychotic symptoms. For long-term disease modifying treatment, we then introduced intravenous rituximab (500 mg). We recommended a clinical reevaluation and repeated screening for occult malignancy with CT of thorax and abdomen in a year.

DISCUSSION

Our case illustrates that autoimmune-mediated encephalitis may present with a broad spectrum of symptoms depending on the target antigen. Our patient developed a fast progressing condition with severe memory deficits, behavioral changes, ataxia and deterioration of consciousness, but no epileptic seizures. Correspondingly, we detected highly reactive antibodies to a yet unknown epitope on neuropil and neuronal surface in the hippocampus, cerebellum, and thalamus suggesting autoimmune mediated encephalitis although CSF workups did not detect lymphocytic pleocytosis.

In contrast to antibodies targeting intraneuronal antigens, that are not pathogenic and suggest a T-cell mediated process, antibodies against neuropil and neuronal surface proteins are often pathogenic (7), but cause reversible changes and limited neuronal death (8). The surface binding pattern of the antibodies in our case suggests pathogenicity involving auto-reactive B-cells and antibody-secreting plasma cells, even though the undetermined epitopic target precludes final proof. Remarkably, this type of encephalitis has been demonstrated to be more responsive to immunotherapy (5, 7, 9). Hence, a detailed classification of antibody-associated encephalitides may guide the individual therapeutic management and thereby help to improve the outcome. However, unknown and rare antibodies will be missed by most currently available commercial tests, particularly if only the serum is analyzed. Hence, a comprehensive CSF and serum workup using immunohistochemistry seems warranted.

So far, no evidence-based guidelines exist for the optimal therapeutic approach to autoimmune encephalitis associated with anti-neuropil antibodies. We applied corticosteroids, IVIg and plasmapheresis and found the strongest clinical improvement after combined treatment with corticosteroids and plasmapheresis. This observation is in line with a recent review on NMDAR-encephalitis (10), the most common anti-neuropil antibody associated encephalitis. In case of a relapse or delayed diagnosis, the escalation to second-line treatment with rituximab, cyclophosphamide, or both is recommended (11). We applied intravenous rituximab to selectively deplete circulating CD20-B-cells (12), and achieve long-term prevention to relapses. Due to its B-cell specificity rituximab has a better safety profile as compared to anti-metabolites, such as cyclophosphamide, which

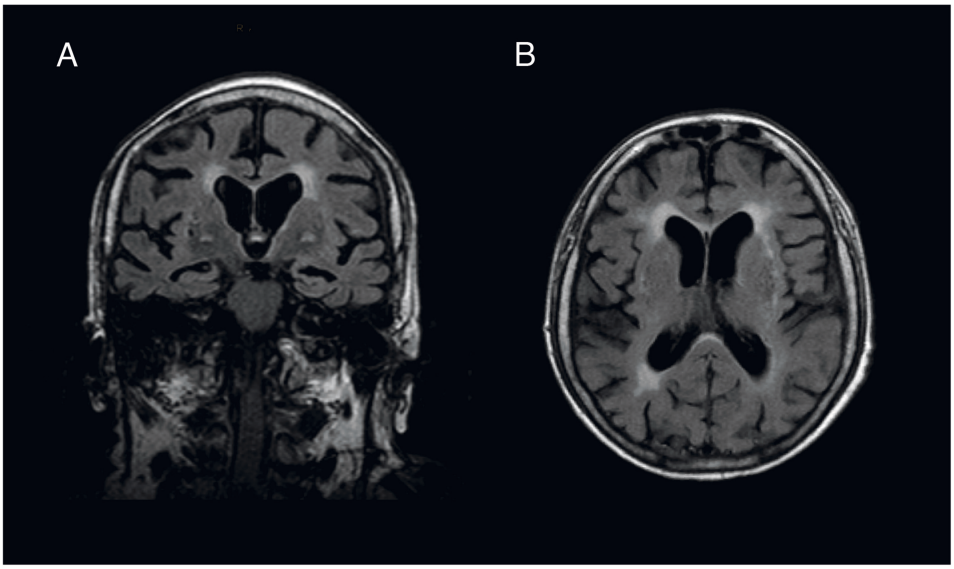

FIGURE 1 | Brain MRI. **(A)** Coronal and **(B)** transversal slice of T1-weighted brain imaging revealed moderate temporal lobe atrophy (medial temporal lobe atrophy score 2) and white matter lesions (Fazekas grade 2). We did not find any indication for inflammatory processes in FLAIR, T2- and Diffusion weighted sequences.

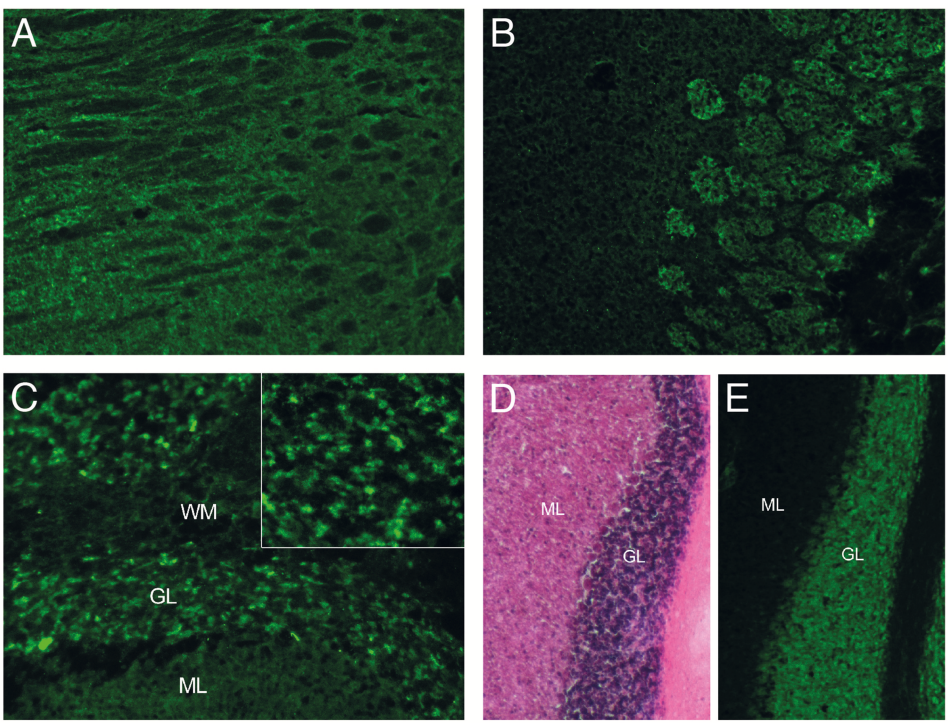

FIGURE 2 | Immunoreactivity of CSF. Indirect immunostaining of CSF with unfixed mouse brain sections showing signal to neuropil in the **(A)** thalamus, **(B)** olfactory bulb, and **(C)** cerebellum. CSF reactivity is restricted to a subset of granule cells (GL) in the cerebellum (C, insert), as compared to the dense neuronal layer seen with H&E staining **(D)** or the neuronal marker NeuN **(E)** of adjacent sections. (ML, molecular layer; WM, white matter).

inhibit cell-proliferation of both B- and T-cells and have more toxic side effects such as infertility, myelosuppression, and an increased malignancy risk (5, 13).

Our case demonstrates that autoimmune-mediated encephalitis needs to be considered in any condition with fast progressing central nervous symptoms even if the CSF

analysis does not reveal elevated cell counts or classical antibodies and brain imaging does not detect changes indicating inflammatory processes.

ETHICS STATEMENT

The patient gave written consent in accordance with the Declaration of Helsinki.

AUTHOR CONTRIBUTIONS

SET, CW, MS, MB, GF, and OO provided clinical care. SET and OO planned the case report and interpreted the data. HP analyzed the CSF data. SET drafted the manuscript. SET, CW, MS, MB, GF, and OO revised the manuscript. All authors approved the final version of the manuscript.

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Cognitive Impairment in Multiple Sclerosis With Regards to Disease Duration and Clinical Phenotypes

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The relationships between cognitive impairment that exist during the clinical course of multiple sclerosis (MS) remain poorly described. The effect of disease duration has been studied in a few longitudinal cohorts and some cross-sectional studies that suggest that cognitive deficits tend to extend with disease duration. However, the effect of disease duration seems to be confounded by the effect of age. At the pre-clinical stage, cognitive deficits have been observed in patients with radiologically isolated syndromes, and their profile is similar than in clinically isolated syndromes (CIS) and relapsing-remitting MS (RRMS). The frequency of cognitive impairment tends to be higher in RRMS than in CIS. In these phenotypes, slowness of information processing speed (IPS) and episodic verbal and visuo-spatial memory deficits are frequently observed, but executive functions, and in particular verbal fluency, could also be impaired. More frequent and severe deficits are reported in SPMS than in RRMS with more severe deficits for memory tests, working memory and IPS. Similarly to what is observed in SPMS, patients with primary progressive MS (PPMS) present with a wide range of cognitive deficits in IPS, attention, working memory, executive functions, and verbal episodic memory with more tests and domains impaired than RRMS patients. Altogether these data suggested that not only the duration of the disease and age play an important role in the cognitive profile of patients, but also the phenotype itself, probably because of its specific pathological mechanism.

Keywords: multiple sclerosis, neuropsychology, cognition, phenotypes, cognition

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INTRODUCTION

The relationships between cognitive impairment (CI) associated with multiple sclerosis (MS) that exist during the clinical course of the disease remain poorly described. When considering the prevalence of CI in the different phenotypes, the respective effects of disease duration and age (and consequently the accumulation of pathology) and of the clinical phenotypes (meaning the different pathological mechanisms underlying these phenotypes) have to be considered. These two dimensions overlapped largely, since in relapsing-onset MS the clinical phenotypes such as clinically isolated syndromes (CIS), relapsing-remitting (RR), and secondary progressive (SP) occur successively.

Methodological issues have to be taken into account when comparing the different studies. First, the NP tests could vary notably between studies. The number of tests, the domains studied, and the psychometric properties of the tests used could affect the results. Second, the definition of CI could also vary; for example, the number of NP scores need to be abnormal and different statistical thresholds were used. In this paper we provide details about the main studies, summarized in tables.

COGNITIVE IMPAIRMENT AND DISEASE DURATION

The impact of disease duration on CI has been a matter of debate for many years. This question has been addressed in a few longitudinal studies (1–4), but also in several cross-sectional studies taking disease duration as a covariate (5–8). **Table 1** summarizes the longitudinal studies. In a long-term controlled study, Amato et al. (3) and (4) examined 50 MS patients with short disease duration and 70 matched healthy controls (HC). After 10 years, impairment was confirmed for short-term verbal memory, abstract reasoning and linguistic abilities, but attention and short-term spatial memory were also involved (4). This study suggests that as the disease progresses, cognitive deficits tend to extend. Moreover, the proportion of patients who were cognitively preserved decreased over time from 74% at baseline to 44% after 10 years, while the proportion of patients with mild or moderate impairment tended to increase. Early cross-sectional studies concluded with a weak correlation between CI and disease duration (5, 6), or no correlation (7). In a large cross-sectional study including 1,500 MS patients evaluated by computerized NP testing, Achiron et al. (8) studied the effect of disease duration and observed that the proportion of CI increased over 25 years. In another study performed in 168 patients examining the different phenotypes using the Brief-Repeatable Battery of NP tests (BRB-N), an effect of disease duration was observed on all tests (9). A recent multi-center study in a large sample of 1,040 patients with MS tested using the BRB-N and the Stroop test, showed an association of CI with disease duration but also age and disability (10). However, when adjusting disease duration and clinical course to age and disability, the association with CI was no longer significant but it is quite obvious that age and disease duration are strongly associated.

COGNITIVE IMPAIRMENT ACCORDING TO CLINICAL PHENOTYPE

It is difficult to compare studies performed in different clinical phenotypes in different settings, with various NP batteries. Studies evaluating MS patients with different phenotypes using a similar methodology are necessary for comparing CI according to these phenotypes. However, the demographic characteristics of the different phenotypes, such as age and gender in particular, are different, and this needs to be taken into account by using appropriate controls.

Radiologically Isolated Syndromes (RIS)

At the pre-clinical stage, in subjects in whom lesions typical for MS were discovered on magnetic resonance imaging (MRI) performed in another purpose, the so-called RIS, CI has been observed with a similar cognitive profile than in RRMS affecting information processing speed (IPS) and memory (11, 12). So far, only small studies are available, and it is not possible to conclude on the prevalence of CI in RIS.

Clinically Isolated Syndromes

Two of the earliest studies conducted on CI in the MS spectrum were, in fact, in patients with optic neuritis, one of the most common type of CIS (13) and in CIS in general (14, 15). Many studies have been performed since, but only controlled studies with a healthy control group assessed with the same battery (or recent normative data of the same battery in the same country) are valid for evaluating the prevalence. **Table 2** summarizes results of the main studies. The prevalence varies from one study to another, according to selection characteristics (all CIS or CIS with dissemination in space and/or in time, disease duration, lesion load which reflects the duration of the pre-clinical stage, etc.). The study with the highest frequency (57%) (16) was performed in a population of selected CIS patients with dissemination in space on MRI, according to McDonald's et al. criteria (21). The number of NP scores studied was higher than in other studies, fulfilling the criterion of two impaired tests more easily. The study with the smaller prevalence (12.3%) (23) was the only one using the minimal assessment of cognitive function in multiple sclerosis (MACFIMS). Other studies using the BRB-N (**Table 2**) found frequencies between 18 and 34%, the two studies with the highest figures including patients with longer disease duration.

The profile of CI in CIS is characterized by slowness of IPS and episodic verbal and visuo-spatial memory deficits (10, 20, 22, 24). In patients with very short disease duration, isolated impairment of IPS was reported (19). In a recent study, a sample of 41 CIS patients, compared to a matched sample of healthy controls (HC), very limited CI was observed, significant only for IPS and visuo-spatial memory (25). In this group of patients, the lesion load was very small, suggesting that they were at a very early stage of the disease.

Executive functions (EF) and, in particular, verbal fluency could also be impaired (10, 20, 22). However, inhibition and switching seems to be preserved in CIS (24). Working memory (WM) impairment has been shown by event-related potential study (26) or eye-tracking oculomotor testing (27). **Table 3** presents the frequency of impairment of the different domains in the largest studies using BRB-N.

Relapsing-Remitting MS

The frequency of CI in RRMS has been measured in many studies, mainly in samples from neurological departments or specialized MS clinics with various mean disease duration, but these studies were rarely controlled. Few studies focused on the early stages of RRMS and on community-based samples. In a controlled study of consecutively enrolled and newly diagnosed RRMS patients [mean disease duration 24.33 (26.49) months (SD)] referred from community-based neurology practices, the frequency of CI was 45% ($\geq$2 scores $<$1.64 SD of HC scores) (28). A large multi-center study included 550 RRMS untreated

Abbreviations: MS, multiple sclerosis; CIS, clinically isolated syndrome; RRMS, relapsing-remitting MS; SPMS, secondary progressive MS; CI, cognitive impairment; HC, healthy controls; NP, neuropsychological; BRB-N, Brief-Repeatable Battery of NP tests; RIS, radiologically isolated syndrome; IPS, information processing speed; MACFIMS, Minimal assessment of cognitive function in multiple sclerosis; EF, executive functions; WM, working memory; DMT, disease-modifying therapies; PPMS, primary progressive MS; PASAT, paced-auditory serial addition test.

TABLE 1 | Controlled longitudinal studies on cognitive impairment in MS.

References	N MS patients	N HS	Phenotype/DD	Duration	NP tests	Outcome
Jennekens-Schinkel et al. (1)	33	18	13RR/20 progressive DD: 16±9.9 y	4 y	Specific battery	Variable deterioration in a few patients.
Kujala et al. (2)	42 (20 CP, 22 CI at baseline)	35	9RR/3SP/11PP DD:8.7±6 y	2.8 y [2–3.9]	Mild deterioration battery	2/20 CP and 17/22 CI deteriorated
Amato et al. (3)	49 (50 at baseline) 37/50 CP 4 mild CI 9 moderate CI	70	38RR, 6SP (44 RR at baseline)/5PP (6 at baseline) DD: 1.6 ± 1.6 y at baseline	4.53 ± 1.15 y	Specific battery	25/49 CP 16 mild CI 8 moderate CI
Amato et al. (4)	45 (50 at baseline)	65 (70 at baseline)	26RR, 14SP; 5PP	10 y	Specific battery	20/45 CP 15 mildly CI 10 moderately CI

N, number; CP, cognitively preserved; CI, cognitively impaired; RR, relapsing-remitting, SP, secondary progressive; PP, primary progressive; DD, disease duration; y, years.

TABLE 2 | Frequency of cognitive impairment (CI) in patients with CIS compared with matched HC or national normative data.

References	CIS *n*	HS *n*	% CI CIS	Definition CI	NP battery	DD
Feuillet et al. (16)	40***	30	57	≥2 tests ≤2SD	BRB-N + other tests	2.89/0.5 (1–3) months
Potagas et al. (17)	33	43	27.3	>2 scores <1.64 SD (5th percentile)	BRB-N	1.0 (1.5) years
Zipoli et al. (18)	61	Normative data	25	≥2 tests ≤2SD	BRB-N + Stroop	< 3 months
Khalil et al. (19)	44	Normative data	18.2	>1 test (z score <1.64) (5th percentile)	BRB-N	0.2 (0.1–0.8) years
Reuter et al. (20)	97*,**	55	20	≥2 scores <1.64 SD (5th percentile)	BRB-N	5.0 (1–16, 21) months
Vitterbo et al. (22)	100	Normative data	21	≥2 scores <1.5 SD	BRB-N	<12 months
Uher et al. (23)	81**	134	12.3	≥2 scores <1.5 SD	MACFIMS	<4 months
Ruano et al. (10)	167	Normative data	34.5%	≥2 scores <1.64 SD in ≥2 domains	BRB-N	1.4 (2.2) years

CIS, clinically isolated syndromes; CI, cognitive impairment; NP, neuropsychological; DD, disease duration; BRB-N, Brief-Repeatable Battery of neuropsychological tests; MACFIMS, Minimal Assessment of Cognitive Function in Multiple Sclerosis. SD, standard deviation.

**Oligoclonal bands in cerebrospinal fluid.*

*** >1 lesion brain or cord magnetic resonance imaging.*

****Dissemination in space on magnetic resonance imaging.*

with disease-modifying therapies (DMT), with an EDSS ≤4 and mean disease duration of 5.0 (5.3) years (SD), found CI in 34.9% of patients (≥2 scores <1.64 SD) (29). In another large multicenter study in specialized centers using a large battery [461 RRMS patients excluding patients referred for cognitive testing, mean disease duration of 75 months (24–210)], the prevalence of CI was 31% (≥2 scores <1.64 SD) (30). A population-based study in Sicily, showed CI in 36.9% of RRMS patients, with a mean disease duration of 8.0 ± 3.3 years, using the BRB-N and the Stroop test (at least three positive tests involving at least two different domains) (31).

Several studies compared the prevalence of CI in CIS and RRMS and are summarized in **Table 4**. Although the frequency tends to be higher in RRMS, the differences were not significant. One limitation of these studies is that age at onset was higher in the CIS samples than in the RR samples, suggesting possible selection bias (10, 19).

The cognitive profile in RRMS is very similar to the one observed in CIS, with deficits mainly in IPS, verbal and visuo-spatial memory impairment, and EF (studied by verbal fluency tests in the above studies). However, one study found a lower global cognitive index *z* score (meaning more impaired) in RR vs. CIS patients (19). Another study found a higher effect in RR than in CIS for verbal memory assessed by the selective Reminding Test (SRT) (17). Curiously, the largest study showed in logistic regression model an association between EF impairment and phenotype (CIS vs. RR), illustrating a more frequent verbal fluency impairment in CIS than RRMS (10).

Secondary Progressive MS

The epidemiology of secondary progressive MS (SPMS) has changed dramatically in the past 15 years, probably in relation to the availability of DMT. Epidemiological studies in patients studied in the 80s' reported that up to 75% of RRMS patients convert to SPMS after 30 years (32), although recent studies showed that the delay of conversion to SPMS was prolonged since the availability of DMT (33, 34), and, therefore, the proportion of converting patients, decrease. However, CI seems to be frequent in SPMS. Few studies estimate the frequency of CI in SPMS and compared the CI in SPMS with other phenotypes. In a study of 45 consecutively recruited SPMS patients, CI (≥2 tests <1.5SD of HC) was diagnosed in 55.6% (35), Another study using computerized testing found 80% of patients with CI out of 30 with SPMS (36). This figure was close to the results of another

TABLE 3 | Frequency of impairment of different cognitive domains in CIS (BRB-N).

References	N CIS	IPS	Memory	EF	WM
Reuter et al. (20)	97	SDMT 20%	SRT LTS 15% SRT DR 28% SPART 20% SPART DR 17%	WLG (P) 28% WLG (S) 20%	PASAT 3 22%
Vitterbo et al. (22)	100	SDMT 7%	SRT LTS 7% SRT DR 9% SPART 7% SPART DR 2%	WLG 22%	PASAT 3 7%
Ruano et al. (10)	167	IPS* (SDMT/PASAT) 41%	Verbal learning* (SRT) 27% Visuo-spatial* (SPART) 14.5%	EF* (WLG, Stroop) 42%	

*CIS, clinically isolated syndromes; IPS, information processing speed; EF, executive functions; WM, working memory. SDMT, Symbol-Digit modalities test; SRT, Selective Reminding test; LTS, Long-term storage; DR, delayed recall; SPART, Spatial Recall test; PASAT, Paced-Auditory Serial Addition test; WLG, Word List Generation test (P, phonemic; S, semantic). *At least one test score impaired.*

TABLE 4 | Frequency of cognitive impairment (CI) in patients with CIS compared with patients with RRMS.

References	CIS *n*	RR *n*	% CI* CIS	%CI* RR	Age CIS	Age RRMS	DD CIS (years)	DD RRMS (years)
Potagas et al. (17)	33	75	27.3	40.0	34.7 (8.7)	34.3 (8.9)	1.0 (1.5)	6.2 (4.9)
Khalil et al. (19)	44	80	18.2	21.3	33.9 (10.0)	37.0 (9.6)	0.2 (0.1–0.8)	8.1 (4.2–13.8)
Ruano et al. (10)	167	759	34.5	44.5	33.9 (9.8)	39.9 (10.2)	1.4 (2.2)	11.2 (8.4)

CIS, clinically isolated syndromes; RR, relapsing-remitting; DD, Disease duration; CI, cognitive impairment.
Definition of CI in each study (see **Table 1).*

study reporting 82.8% of CI (>33% of measures <5th percentile of HC), in a sample of 29 patients with SPMS tested by the BRB-N, as compared to 40.0% in a sample of 75 RRMS patients (17). The recent multi-center Italian study reported a high prevalence of CI in 79.4% out of 74 SPMS patients (10).

A study compared the cognitive performances in 28 patients with SPMS to 28 patients with primary progressive MS (PPMS) and 20 HC (37). The CI was not substantially different between the two phenotypes. In a larger study, 71 SPMS patients were compared with RRMS, PPMS and HC, and the results found more severe deficits in SPMS with high contrast estimates between SPMS and RRMS for memory tests, working memory (paced-auditory serial addition test, PASAT), and IPS (symbol-digit modalities test, SDMT) (38). Severity of CI was rather similar between SPMS and PPMS. In a study in 101 patients with various phenotypes, it was found that patients with SPMS were at least two-fold more frequently impaired than patients with RRMS and long disease duration in IPS, EF, verbal fluency, verbal episodic memory, working memory and visuo-spatial construction (39).

In the study cited above by Dackovic et al. (9), patients with PPMS or SPMS were more frequently impaired than those with CIS and RRMS in all cognitive tests in the BRB-N (9).

Primary Progressive MS

CI has long been considered rare in PPMS (40), which was considered to mainly affect the spinal cord. However, more recent studies have shown that this not the case. The MAGNIMS study analyzed a sample of 191 PPMS or transitional progressive MS patients recruited in specialized centers, 63 out of them being paired to HC. In this study, 28.6% of patients were diagnosed as cognitively impaired with rigorous criteria (≥3 tests <2SD) (41). Few studies have compared, with a similar methodology, selected samples of patients with RRMS and PPMS. They found more frequent impairment in PPMS than in RRMS patients. In one study, CI was diagnosed in 56.5% out of 23 PPMS patients (17). The larger Dutch study found that CI was more severe in PPMS than in RRMS but similar between SPMS and PPMS (38). The recent multi-center Italian study reported 91.3% of CI in 40 PPMS patients (10). However, these studies did not use separate control groups, and the greater mean age of patients with progressive MS could explain the differences with RRMS. In a recent study comparing PPMS and RRMS patients using a unique methodology and an adequate HC sample, PPMS patients presented a wide range of cognitive deficits in IPS, attention, working memory, EF, and verbal episodic memory, whereas the impairment in RRMS patients was limited to IPS, attention, and working memory in comparison with their respective matched HC (42). Besides the fact that CI in PPMS patients concerned more NP scores and more domains than in RRMS patients, PPMS patients had more severe cognitive deficits than RRMS patients. The differences on NP performance between PPMS and RRMS patients were observed after taking into account age and sex by using *z* scores based on the data from matched HC but also after controlling for EDSS. IPS was the most frequently impaired cognitive domain in both PPMS and RRMS patients, but the two cognitive domains, which differed between these two types of MS, were verbal episodic memory and EF with respect to the

frequency. In that study, patients with PPMS performed more poorly than HC on 16 of 23 NP scores (69.6%), whereas patients with RRMS exhibited lower NP performance on 5 of 23 scores (21.7%), compared with their matched HC.

CI IN DIFFERENT PHENOTYPES AND PATHOPHYSIOLOGY

In the long-term, CI evolution, considered as a whole, could be related to the progression of both gray matter (GM) and WM pathology. A longitudinal study conducted over 17 and a half years showed that CI progressed continuously paralleled by atrophy and lesion accumulation (43). These results are in agreement with another study of 202 long-standing MS patients in which CI was correlated with brain atrophy and diffuse white matter damage, irrespective of the phenotype (44). However, the role of GM pathology seems to be preponderant as suggested by a 13-year longitudinal study of 73 MS patients, showing that baseline disease duration and average GM magnetization transfer ratio (MTR) were the two only independent variables associated with cognitive deterioration (45).

For a better understanding of the role of the different mechanisms involved in CI, it could be worth studying specific cognitive domains separately and focusing on early stages when all mechanisms could be more easily disentangled. Indeed, when considering the two more frequent cognitive processes impaired at the early stage of the disease, IPS, and memory, different mechanisms could be discussed. It has been suggested that deficits in these two cognitive functions could progress differently in early RRMS patients, IPS being more impaired initially but progressing at a slower rate than memory (46). The different kinetics of CI evolution for these two functions may be explained at least in part by different mechanisms. At this early stage of the disease, the role of focal lesions, network disruption, or GM vulnerability has been suggested. IPS depends on the integrity of large-scale cortical integrative processes, which involve long-distance white matter projections which can be impaired due to diffuse demyelinating injury in patients (focal lesions) and the axonal pathology related to these lesions (28, 46). In early RRMS, the correlation of CI with lesion load is no longer significant when diffused white matter pathology [normal appearing white matter magnetization transfer ratio (28) or Diffusion tensor imaging (DTI) (47) metrics] is taken into account, suggesting that disconnection plays an important role in CI, mainly in IPS deficits. The involvement of several key brain regions has been shown, contributing to these deficits, such as the thalamus (48, 49), the cerebellum (50, 51), and default mode network (52).

Episodic memory impairment is associated with deep GM injury in the limbic system, in particular the hippocampi and the basal ganglia (53, 54). The growing role, along the disease course, of GM pathology spreading initially in regions with more vulnerability in deep GM and the ^{cortex} (55) and leading to progressive brain atrophy (43), could explain the deterioration of memory or other domains like EF.

However, atrophy is the late consequence of either Wallerian degeneration secondary to axonal transections in lesions or direct inflammation in the GM. DTI shows microstructural abnormalities in the GM that precedes atrophy. Experimental and human studies in CIS using DTI have shown that a selective vulnerability of some GM regions, for instance, hippocampus and some cortical areas, could occur (56). A role for inflammatory injury of the GM, in the hippocampi, associated with microglial infiltration leading to synaptic defects, has been demonstrated in an animal model of early MS (56). This early GM involvement seems to selectively affect some GM areas with more vulnerability, like the dentate gyrus of the hippocampi (57). Meningeal inflammation has been shown to be associated with inflammatory damage in the GM and seems to be more extended as long as the disease progress (58). This predominant GM pathology in progressive stages could explain the cognitive profile observed in these stages. For instance, EF impairment, which is more prominent in progressive stages, has been shown to be related with frontal cortical pathology (59). The importance of synaptic dysfunction in the development of CI has been recently underlined (60).

CONCLUSION

Although some similarities could be observed in the cognitive profile of the different phenotypes in MS, with predominant involvement of IPS and episodic memory, some differences could be observed. Memory and EF deficits seem to be more frequent as long as the disease progresses, although IPS seems to appear early and progress slowly. The highest prevalence of CI and its profile in the progressive forms of the disease as compared to the relapsing forms is in accordance with the recent pathological findings underlining the major involvement of the gray matter in these phenotypes (61).

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Cognitive Impairment in Multiple Sclerosis Is Reflected by Increased Susceptibility to the Sound-Induced Flash Illusion

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Objective: To determine whether the performance of multiple sclerosis (MS) patients in the sound-induced flash illusion (SiFi), a multisensory perceptual illusion, would reflect their cognitive impairment.

Methods: We performed the SiFi task as well as an extensive neuropsychological testing in 95 subjects [39 patients with relapse-remitting MS (RRMS), 16 subjects with progressive multiple sclerosis (PMS) and 40 healthy control subjects (HC)].

Results: MS patients reported more frequently the multisensory SiFi than HC. In contrast, there were no group differences in the control conditions. Essentially, patients with progressive type of MS continued to perceive the illusion at stimulus onset asynchronies (SOA) that were more than three times longer than the SOA at which the illusion was already disrupted for healthy controls. Furthermore, MS patients' degree of cognitive impairment measured with a broad neuropsychological battery encompassing tests for memory, attention, executive functions, and fluency was predicted by their performance in the SiFi task for the longest SOA of 500 ms.

Conclusions: These findings support the notion that MS patients exhibit an altered multisensory perception in the SiFi task and that their susceptibility to the perceptual illusion is negatively correlated with their neuropsychological test performance. Since MS lesions affect white matter tracts and cortical regions which seem to be involved in the transfer and processing of both crossmodal and cognitive information, this might be one possible explanation for our findings. SiFi might be considered as a brief, non-expensive, language- and education-independent screening test for cognitive deficits in MS patients.

Keywords: multiple sclerosis, cognitive deficits, screening test, sound-induced flash illusion, neuropsychological impairment

INTRODUCTION

Depending on the disease course, employed methods and cut-off-scores, prevalence rates of cognitive impairment in multiple sclerosis (MS) vary between 40 and 70% (1, 2). Due to financial, personnel and time limitations in the clinical routine, sometimes mild cognitive deficits in MS are not subjected to a further diagnostic investigation. An inexpensive, easy to apply language-independent screening tool for global cognitive deficits would facilitate the neuropsychological diagnostic process in MS.

The typical profile of neuropsychological impairment in MS encompasses slowed cognitive processing speed, episodic memory deficits, executive dysfunction, impaired verbal fluency, and visuospatial perception (3). Neuroimaging as well as neuropathological findings suggest that cortical regions as well as white matter tracts and deep gray matter areas which are involved in cognitive processing are often affected by MS (4). Interestingly, similar anatomical structures and functional circuits have been implied in the integration of perceptual information from different sensory channels, too (5–8). Therefore, lesions in these areas should result not only in neuropsychological impairment but also in an altered multisensory perception. In this study, we examined the principal utility of a multisensory perceptual task, the sound-induced flash illusion (SiFi) (9–11), as a marker for global cognitive impairment in MS.

The SiFi is a multisensory illusion, where two auditory beeps are presented to the subject with a short interval between them (12). A single visual flash is presented together with the first auditory beep. If the time interval between the two auditory beeps is less than 150 ms, healthy subjects perceive two instead of one visual flash, e.g., the inputs from the two different sensory modalities fuse and the subjects perceive a second, non-existing visual flash (**Figure 1**, bimodal illusion conditions) (9, 12). For interstimulus intervals longer than 150 ms the illusion is less frequently or not perceived at all. However, some patient groups seem to exhibit different perceptual patterns. For example, people with mild cognitive impairment (MCI) report seeing the second visual flash even at longer interstimulus intervals (up to 300 ms) (13). Similarly, older adults with a history of falling continue experiencing the illusion for time intervals of up to 270 ms (14).

Since both perception and cognitive processes depend strongly on viable brain connectivity, we hypothesized that neuronal damage as seen in MS would be associated not only with dysfunctional multisensory perceptual processes (and thus with increased susceptibility to the illusion) but also with cognitive impairment in MS patients. Hence, MS patients perceiving the illusion at interstimulus intervals long enough to disrupt the illusion for healthy subjects (i.e., $>$ 150 ms) should exhibit inferior performance on neuropsychological testing.

MATERIALS AND METHODS

Study Population

Fifty-nine multiple sclerosis (MS) subjects (43 relapse-remitting (RRMS) and 16 primary or secondary progredient (PMS) patients) and forty-one healthy controls (HC) were included initially. Five of them (four RRMS and one HC) did not complete the full experiment because they discontinued the task or did not show up for the second measurement. The data of the remaining 55 MS patients (39 RRMS and 16 PMS) and 40 HC were included in the analysis. Patients were recruited via the neurology department at the University Hospital in Frankfurt am Main, Germany. The diagnosis of MS was established according to the 2010 diagnostic criteria for MS (15). This study was carried out in accordance with the recommendations of the ethics committee of the University of Frankfurt Medical Faculty with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the ethics committee of the University of Frankfurt Medical Faculty.

The average age of the MS group (40 female, 15 male) was 43.1 years (SD 13.7). The RRMS subgroup consisted of 29 female and 10 male and the PMS subgroup of 11 female and 5 male subjects (mean age of RRMS patients 38.1 years [SD 11.3], mean age of PMS patients 55.4 years [SD 10.99]). The average visual acuity of the RRMS group was 0.81 for the left as well as the right eye (SD left 0.18, SD right 0.17). The average visual acuity of the PMS group was 0.70 for the left and 0.71 for the right eye (SD left 0.16, SD right 0.21). The mean number of years of education (YoE) was 13.2 (SD 0.9). The mean number of YOE for the RRMS patients was 13.3 (SD 0.8) and for the PMS 13.1 (SD 0.99). The average age of the HC group (30 female, 10 male) was 41.5 years (SD 14.7). The average visual acuity of the HC group was 0.86 for the left and 0.83 for the right eye (SD left 0.14, SD right 0.20). The mean number of years of education was 13.8 (SD 0.5). We ensured that no subject had a severe hearing loss. More detailed information on the basic demographic information can be found in **Table 1** and in Results.

Design and Data Collection

The study comprised two sessions which were conducted on two different days to reduce the effect of fatigue on performance. Demographics and basic sensory functions (visual acuity, hearing ability) were recorded during the first session. Visual function was measured using a Snellen eye chart. Auditory function was measured using a hearing test with an audiogram output (https://hearingtest.online/). The test files are based on the ISO 389-7:2005 international standard and use third octave band warble tones in order to minimize room and headphone resonance. We employed Sennheiser HD 201 headphones. Half of the participants was scheduled to complete a neuropsychological test battery during the first session consisting of pre-task Visual Analog Scale (VAS 1) on subjective performance capability, Rey Complex Figure Test (RCFT) (16), Symbol Digit Modalities Test (SDMT) (17), Verbaler Lern- und Merkfähigkeitstest (VLMT, a German adaptation of the Rey Auditory Verbal Learning Test) (18), Paced Auditory Serial Addition Test (PASAT) (19), Trail Making Test (TMT) (20), Regensburger Wortflüssigkeits-Test (RWT) (21), Wortschatztest (WST, a German vocabulary test) (22), Beck Depression Inventory (BDI) (23) and post-task Visual Analog Scale (VAS 2) on subjective performance capability to evaluate changes in fatigue related to the neuropsychological testing. During the second session, which was scheduled not more than 6 weeks after the first one, participants completed the

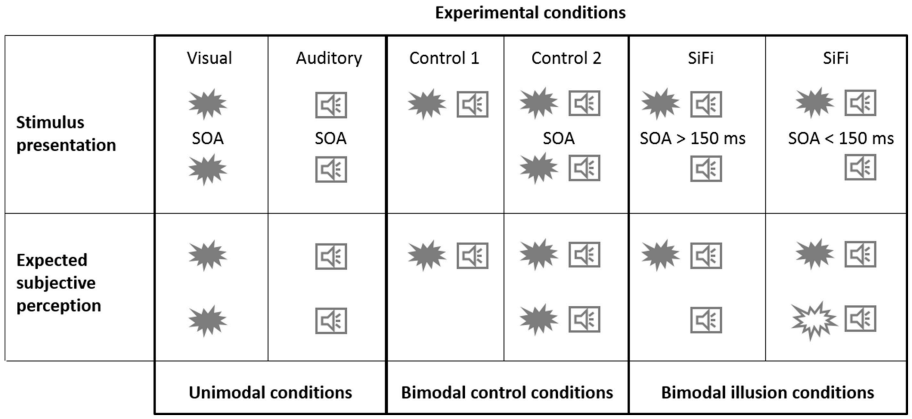

FIGURE 1 | Stimulus presentation. Experimental stimuli (illustrated here by a flash or a beep symbol) were presented either in only one (visual/auditory unimodal conditions) or in both of the sensory modalities (control/illusion bimodal conditions). The stimulus onset asynchrony (SOA) of two subsequent stimuli could amount to 0 ms (only one stimulus), 50 ms, 100 ms, 150 ms, 200 ms, 250 ms, 300 ms, or 500 ms. *Expected subjective perception*. In the illusion condition, if shortly after the first stimuli a second auditory beep is presented, some of the subjects perceive two instead of one visual flash, e.g., the inputs from the two different sensory modalities fuse and the subjects perceive a second, non-existing visual flash. Healthy subjects usually report perceiving this illusion if the time interval between the beeps is shorter than 150 ms. For interstimulus intervals longer than 150 ms the illusion is less frequently or not perceived at all by healthy subjects.

TABLE 1 | Main clinical characteristics for healthy controls (HC), relapse-remitting multiple sclerosis (RRMS), and progredient multiple sclerosis (PMS) patients.

Variable	HC mean (30 f, 10 m)	HC std. dev.	RRMS mean (29 f, 10 m)	RRMS std. dev.	PMS mean (11 f, 5 m)	PMS std. dev.
Age	41.45	14.69	38.10	11.34	55.44	10.99
Years of education	13.83	0.50	13.26	0.83	13.06	1.00
Vision left eye	0.87	0.14	0.79	0.18	0.72	0.19
Vision right eye	0.84	0.20	0.81	0.16	0.74	0.21
Disease duration (years)			6.81	7.39	13.21	12.70
EDSS			2.41	1.61	4.38	1.23
VAS relative score	−0.03	0.21	−0.17	0.33	−0.02	0.78
RCFT_IR (raw score)	22.60	5.44	17.71	7.94	14.78	7.48
SDMT (raw score)	54.65	10.61	46.00	12.14	33.13	9.68
VLMT total (raw score)	60.33	7.97	54.21	8.59	50.06	9.10
VLMT 5–7 (raw score)	0.60	1.57	1.10	2.17	0.94	1.84
PASAT (raw score)	9.41	6.73	16.56	11.22	26.00	16.77
TMT-A (raw score)	26.73	8.61	37.72	15.20	46.88	18.07
TMT B/A (raw score)	2.27	0.67	2.15	0.92	2.39	0.65
RWTp (raw score)	26.25	6.25	20.36	6.38	18.75	6.47
RWTs (raw score)	39.60	9.40	31.49	9.14	31.56	10.68
WST (raw score)	34.13	2.67	28.90	7.07	29.38	8.94
BDI	3.78	4.44	8.92	7.27	14.73	9.97
WST (z-score)	0.78	0.55	0.11	0.68	0.29	0.71

sound-induced flash illusion task (SiFi). The other half of the participants completed the tasks in reversed sequence (day 1: SiFi, day 2: neuropsychology).

For the SiFi, we employed a well-established experimental design already used in studying multisensory perceptual patterns in patients with mild cognitive impairment (13). The visual stimuli were presented on a 15.6″ laptop. The visual stimulus consisted of a white circular disk, subtending approximately 2° of visual angle. This disk was placed 8° of visual angle below the fixation cross. The presentation duration of the disk was 16 ms. The auditory stimulus consisted of a 16 ms, 3,500 Hz pure tone with a total rise- and decay-time of 20 μs at a sound pressure level of 68 dB(A). Auditory stimuli were presented using closed, circum-aural headphones (Sennheiser HD 201) to reduce any ambient noise.

We used a repeated measures design. Each trial began with a fixation cross presented at the center of screen. Participants were instructed to maintain fixation on the cross throughout the measurements. Experimental stimuli were presented either in only one or in both of the sensory modalities [factor "modality"

with levels "visual" (V), "auditory" (A), and "audio-visual" (AV)]. In the V-condition one or two flashes were presented and the subjects had to indicate how many flashes they perceived. In the A-condition one or two auditory beeps were presented and the participants had to indicate how many beeps they perceived. The stimulus onset asynchrony (SOA) of the two stimuli was varied (factor "SOA" with levels "0 ms" (only one stimulus), "50 ms," "100 ms," "150 ms," "200 ms," "250 ms," "300 ms," "500 ms"). The factor "modality" (V, A, AV) was blocked and the block order randomized between participants. Within each block, SOA was randomly permuted. Seventy trials of each unimodal conditions were presented: 35 trials with only one unimodal stimulus (SOA = "0 ms") and 35 trials with two unimodal stimuli and the remaining SOAs (5 trials for each of the SOAs "50 ms," "100 ms," "150 ms," "200 ms," "250 ms," "300 ms," "500 ms").

The AV-block comprised three different conditions: the illusion condition ("illusion" with 2 beeps and 1 flash) as well as two control conditions ("control 1": 1 beep and 1 flash; "control 2": 2 beeps and 2 flashes). The first flash and auditory beep were always presented at the same time and the SOAs between them and the second stimuli varied between 50, 100, 150, 200, 300, and 500 ms, as illustrated in **Figure 1**, upper row. Each of the "illusion," "control 1," and "control 2" conditions consisted of 35 trials. All 105 trials were randomly presented within the AV-block. Subjects were instructed to ignore the auditory stimuli and indicate how many visual flashes they perceived.

Responses were made via a response pad (LogiLink® Keypad). Participants were asked to emphasize accuracy over speed. The experiment was programmed in Presentation (Neurobehavioral Systems, CA, USA).

Statistical Analysis

SiFi Data

The average proportion of correct responses for each condition was calculated separately for each participant [numbers ranging between 0.0 (no correct responses) and 1.0 (all responses correct)]. These numbers were used as dependent variables. First, an ANOVA with repeated measures on the unimodal conditions was computed with factor "modality" with levels "visual" (V) and "auditory" (A); factor "SOA" with levels "0 ms," "50 ms," "100 ms," "150 ms," "200 ms," "250 ms," "300 ms," and "500 ms"; as well as factor "group" with levels relapse-remitting MS patients ("RRMS"), progredient MS ("PMS") and healthy controls ("HC").

During the next step of the analysis we computed an ANOVA with repeated measures on the bimodal conditions with factor "condition" with levels "illusion," "control 1," and "control 2," factor "SOA" with levels "50 ms," "100 ms," "150 ms," "200 ms," "250 ms," "300 ms," and "500 ms" as well as factor "group" with levels relapse-remitting MS patients ("RRMS"), progredient MS ("PMS"), and healthy controls ("HC").

To further disentangle the complex three-way interaction in the bimodal ANOVA, non-parametric Mann-Whitney-U-Tests were computed for comparisons of interest (e.g., comparing "RRMS vs. HC" or "PMS vs. HC" separately for SOAs 200 to 500 ms, s. below). The Bonferroni correction for multiple comparisons was applied.

Neuropsychological Tests

We concentrated on several cognitive processes which are usually affected in MS patients (3): information processing ability, speed and flexibility (measured by SDMT, PASAT, TMT-A and TMT B/A); learning capacity (measured by the total number of correctly recalled items in trials 1 to 5 of the VLMT [VLMT total]); memory loss due to forgetting over time (indicated by the "trial 7"–"trial 5" difference in the VLMT [VLMT 5–7]); visuospatial recall memory (measured by the Immediate Recall RCFT trial, RCFT_IR); phonemic and semantic verbal fluency {phonemic and semantic subtests of the RWT [RWTp and RWTs]}.

Premorbid intelligence and cognitive reserve were evaluated and included as covariates by means of calculating patients' years of education and vocabulary (WST-Z-scores) as suggested by Sumowski et al. (24). Depression was measured with BDI and included as a covariate, too. Task-related changes in fatigue were compared between the groups with a univariate ANOVA with "group" as a fixed factor and the relative VAS score as a dependent variable. The relative VAS score indicates how much the fatigue has increased during the neuropsychological testing relatively to the individual baseline score and was computed following the formula (VAS 2-VAS 1)/VAS 1.

For each of the neuropsychological variables a single univariate ANOVA was computed using the direct scores from the tests and with "group" ("RRMS," "PMS," "HC") as a fixed factor and "age," "years of education," "WST-Z-Score," and "BDI" as covariates, thus ensuring that we control for the confounding influence of premorbid intelligence, cognitive reserve, and depression on the neuropsychological performance. The threshold for statistical significance for these ANOVAs was corrected after considering multiple comparisons using the Bonferroni correction and set accordingly to $p < 0.005$.

To test whether cognitive performance of MS patients can be predicted by their multisensory perceptual patterns, we analyzed the data of MS subjects only. Their test results were converted to z-scores. These z-scores were calculated from normalized and normative values existing for each neuropsychological test and then used to compute a stepwise linear regression. The individual number of failed tests of each subject (i.e., tests with below average performance) as an indicator for global neuropsychological impairment was used as a dependent variable, while age, years of education, WST-Z-Score, BDI, the relative VAS-score and proportion of correct responses for the two SiFi illusion conditions with the longest SOA, namely 300 and 500 ms, were used as predictors, since the ANOVA analyses showed that these two illusion conditions differentiate between subgroups (s. below). The threshold for neuropsychological test failure was defined as one or more standard deviations below the reference mean.

To corroborate our findings, we conducted a complementary analysis using a global z-score of the broad neuropsychological test battery based on the procedure described in (25). First, domain-specific z-scores (Z-memory; Z-attention/executive; Z-fluency) were built according to the following formulae, where the different subtests were weighted by their z-score to balance the tasks: Z-memory = ("RCFT_IR" z-score + "VLMT total"

z-score + "VLMT 5-7" z-score)/3; Z-attention/executive = ("SDMT" z-score + "PASAT" z-score + "TMT-A" z-score + "TMT B/A" z-score)/4; Z-fluency = ("RWTp" z-score + "RWTs" z-score)/2. The global z-score was obtained by calculating the mean of the z-scores from the three cognitive domains and used as a dependent variable in a linear regression employing the entry method and the illusion condition with the longest SOA 500 ms (since the previous analysis step showed that SOA 500 ms is a significant predictor for the number of failed neuropsychological tests, see Results) as well as the clinical variables age, years of education, WST-Z-Score (indicator for vocabulary and cognitive reserve), BDI (indicative of depression), the relative VAS-score (indicative of task-related fatigue) and the individual disease duration in years.

RESULTS

Demographic Parameters

Groups did not differ significantly with regard to the female/male subjects' ratio (chi-square test, $p > 0.05$). The only significant group difference for the visual acuity was for the left eye between HC and PMS (HC > PMS, ANOVA *post-hoc t*-test, $p = 0.006$). However, visual acuity of MS patients did not correlate with their performance in the relevant illusion conditions (SOA 300 and 500 ms), nor with the number of neuropsychological tests with below average performance or the patient's global z-score in the neuropsychological test battery ($p > 0.05$ for all correlations for the left and the right eye).

ANOVA Unimodal Conditions

The repeated measures ANOVA employing the unimodal conditions demonstrated significant main effects of the factors "modality" and "SOA" as well as a significant interaction between them (**Table 2**). The close inspection of these results showed that subjects gave more correct responses in the auditory conditions across all SOAs and subgroups (main effect of the factor "modality"). Higher SOAs were associated with an increased number of correct responses (main effect of the factor "SOA"). This effect occurred more quickly for the auditory conditions with average correct responses reaching one of their highest values already at SOA 150 ms (interaction "modality x SOA"). The main effect of the factor "group" was not significant. Moreover, there was no significant interaction of any within-subjects factor with the between-subjects factor "group."

ANOVA Bimodal Conditions

The repeated measures ANOVA using the bimodal conditions demonstrated significant main effects of the within-subjects factors "condition" and "SOA" (**Table 3**). The highest accuracy was achieved in the condition "control 1," followed by "control 2" and "illusion" (main effect of the factor "condition", *post-hoc t*-tests $p < 0.05$). Higher SOAs were associated with an increased number of correct responses (main effect of the factor "SOA," *post-hoc t*-tests $p < 0.05$). Most importantly, there was a significant interaction "condition × group" ($p < 0.05$). To further disentangle this interaction, we performed *post-hoc t*-tests comparing PMS and RRMS with HC in the illusion as well as the two control conditions. PMS patients achieved far fewer correct responses in the "illusion" condition as compared to HC (average proportion of correct responses for PMS = 0.56 vs. 0.75 for HC, $p < 0.05$, Bonferroni correction for multiple comparisons) while there were no other significant group differences (**Figure 2**). The main effect of the between-subjects factor "group" was not significant.

The three-way ANOVA interaction "condition × SOA × group" did not reach significance. The close inspection of this complex interaction hinted, however, at possible group differences in the illusion condition at longer SOAs. Based on previous work demonstrating that healthy subjects stop perceiving the illusion at SOAs longer than 150 ms, but patients with mild cognitive impairment continue perceiving it even at longer SOAs (13), we compared the three groups separately using Mann–Whitney U tests on the SOAs of 200, 250, 300, and 500 ms in the "illusion" condition only. PMS subjects had a significantly lower average proportion of correct responses than healthy controls for all SOAs in the "illusion" condition. After applying a correction for multiple comparisons, the group differences for SOAs of 300 and 500 ms remained significant ($p < 0.05$, Bonferroni correction for multiple comparisons) (**Figure 3**).

TABLE 2 | Results from the unimodal repeated measures ANOVA with factors "modality" (levels "visual" and "auditory"), "SOA" (levels "0 ms," "50 ms," "100 ms," "150 ms," "200 ms," "250 ms," "300 ms," and "500 ms") as well as "group" (levels "RRMS," "PMS," "HC"). The Huynh-Feldt-correction for violation of sphericity was applied.

Main factor/interaction	Type III sum of squares	*F*-value	Significance
Modality	1.613	16.436	<0.001
SOA	49.797	190.400	<0.001
Group	0.158	0.441	n.s.
Modality × group	0.055	0.283	n.s.
SOA × group	0.198	0.379	n.s.
Modality × SOA	5.149	23.516	<0.001
modality × SOA × group	0.399	0.911	n.s.

"n. s.," not significant ($p > 0.05$).

TABLE 3 | Results from the bimodal repeated measures ANOVA with factors "condition" (levels "illusion," "control 1," and "control 2,") "SOA" (levels "50 ms," "100 ms," "150 ms," "200 ms," "250 ms," "300 ms," and "500 ms") as well as "group" (levels "RRMS," "PMS," "HC"). The Huynh-Feldt-correction for violation of sphericity was applied.

Main factor/interaction	Type III sum of squares	*F*-value	Significance (*p*-value)
Condition	24.494	86.416	<0.001
SOA	16.530	107.444	<0.001
Group	1.093	2.602	n.s.
condition × group	1.593	2.810	0.049
SOA × group	0.254	0.824	n.s.
condition × SOA	18.317	50.582	<0.001
condition × SOA × group	0.834	1.151	n.s.

"n. s.," not significant ($p > 0.05$).

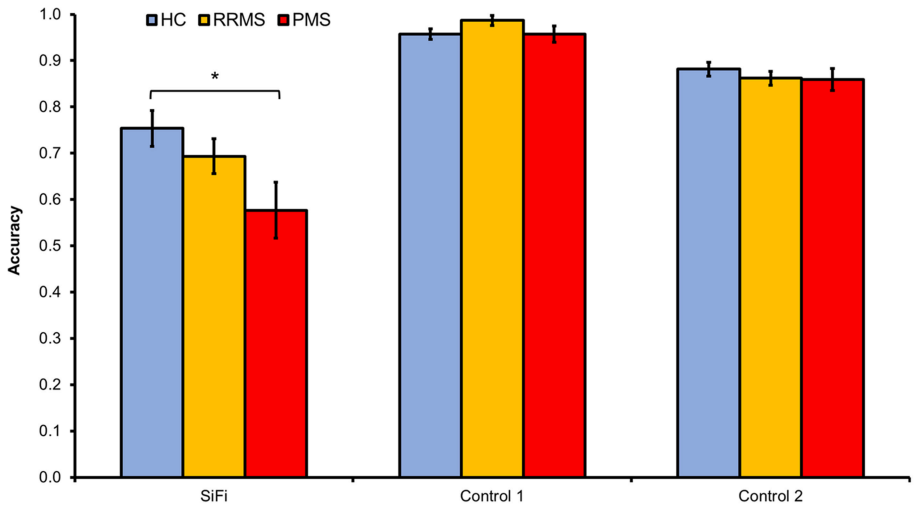

FIGURE 2 | MS patients' responses were less accurate as compared to those of HC in the illusion condition, i.e., MS reported more frequently than HC a second, non-existing visual flash in the SiFi condition, whereas there were no group differences in the non-illusion control conditions (ANOVA interaction "condition × group," $p < 0.05$). *post-hoc t*-tests revealed that this interaction was driven mainly by the poorer accuracy of PMS as compared to HC in the SiFi condition (average proportion of correct responses for PMS = 0.56 vs. *HC* = 0.75, $p < 0.05$, Bonferroni correction for multiple comparisons). Error bars represent standard error of the mean.

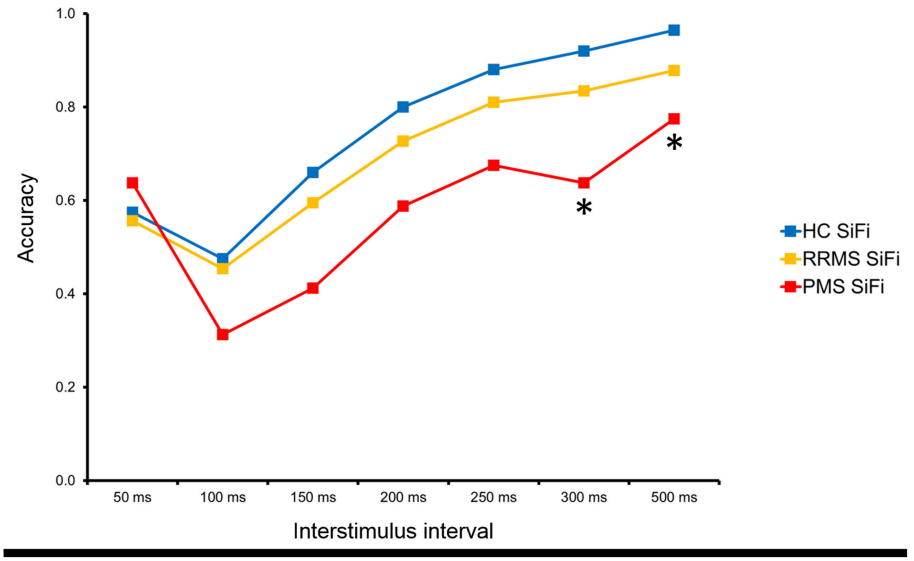

FIGURE 3 | The analysis of the separate interstimulus intervals revealed that PMS continued to perceive the SiFi more often than HC even at interstimulus time intervals of 300 and 500 ms (* = $p < 0.05$, Bonferroni corrected).

Neuropsychological Test Performance

Groups did not differ significantly on their relative VAS score ($p > 0.05$), indicating that fatigue did not increase group-dependently during the neuropsychological testing. The single univariate ANOVAs demonstrated at first significant group differences for RCFT_IR (HC > RRMS), SDMT (HC > RRMS > PMS), VLMT total (HC > RRMS, HC > PMS) and RWTp (HC > RRMS, HC > PMS) after controlling for age, years of education, vocabulary, and depression. However, after applying the Bonferroni correction for multiple comparisons only the group difference in SDMT remained significant (HC > RRMS > PMS, Type III sum of squares = 1327.001, *F*-value = 7.728, $p < 0.005$). The mean number of failed tests for the MS patients was 3.1 (min 0, max 8, SD 2.1). RRMS and PMS did not differ significantly on their number of failed tests ($p > 0.05$).

Cognitive Impairment and SiFi

The stepwise linear regression modeling the relationship between the individual number of failed tests as dependent variable and

TABLE 4 | Results from the stepwise linear regression (model "1") testing the relationship between the individual number of failed tests as dependent variable and age, years of education, WST-z-score, BDI, the relative VAS-score and proportion of correct responses for the two SiFi illusion conditions SOA 300 and 500 ms as independent variables.

Model	R	R square	F change	Significance	
1	0.374	0.140	7.988	***0.007***	
Variable	**B (unstandardized)**	**Std. error**	**t**	**Significance**	**Partial correlations**
Constant	5.076	0.826	6.143	<0.001	
Illusion SOA 500 ms	−2.577	0.912	−2.826	***0.007***	−0.374
Age			−1.770	0.083	−0.247
Years of education			−1.869	0.068	−0.261
BDI score			−0.836	0.407	−0.120
WST-z score			−0.347	0.730	−0.050
VAS relative score			−0.626	0.534	−0.090
Illusion SOA 300 ms			−0.172	0.864	−0.025

TABLE 5 | Results from the entry linear regression model (model "2") testing the relationship between the global z-score as dependent variable and the SiFi condition SOA 500 ms as well as age, years of education, WST-Z-score, BDI, the relative VAS-score and the disease duration as independent variables.

Model	R	R-square	F-change	Significance	
2	0.572	0.328	2.994	***0.012***	
Variable	**B (unstandardized)**	**Std. error**	**t**	**Significance**	**Partial correlations**
Constant	−3.824	1.222	−3.129	0.003	
Illusion SOA 500 ms	0.527	0.244	2.163	***0.036***	0.313
Age	0.011	0.006	1.970	0.055	0.288
Years of education	0.179	0.088	2.028	***0.049***	0.295
BDI score	0.009	0.008	1.157	0.254	0.174
WST-z score	0.052	0.107	0.486	0.630	0.074
VAS relative score	−0.096	0.123	−0.779	0.440	−0.118
Disease duration	−0.010	0.009	−1.136	0.262	−0.171

age, years of education, WST-z-score, BDI, the relative VAS-score and proportion of correct responses for the two SiFi illusion conditions SOA 300 and 500 ms revealed that only the average proportion of correct responses for the SOA 500 ms in the illusion condition contributed significantly to explaining the variance of the dependent variable "number of failed tests" in MS patients ($t = -2.826$, partial correlation $= -0.374$, $p = 0.007$, see **Table 4, Supplementary Figure 1**).

The second linear regression tested a model where the global z-score was used as a dependent variable and the illusion condition with the longest SOA 500 ms as well as age, years of education, WST-Z-score, BDI, the relative VAS-score and the individual disease duration were employed as independent variables. It revealed a significant result for the model ($R = 0.572$, R square 0.328, $p = 0.012$), whereby only the average proportion of correct responses for the SOA 500 ms in the illusion condition ($t = 2.163$, $p = 0.036$) and years of education ($t = 2.028$, $p = 0.049$) contributed significantly to explaining the variance of the dependent variable "global z-score" in MS patients, thus corroborating the results from the first analysis (see **Table 5** for details and **Supplementary Figure 1**).

DISCUSSION

MS patients performed worse than healthy control subjects in SDMT indicating worse general information processing ability and information processing speed. Within the MS group, performance of PMS patients was significantly lower than that of RRMS patients. These results are compatible with the findings reported in the literature (3).

Furthermore, perception of the SiFi differed across MS and HC groups. In particular, MS patients seemed to be more susceptible to the multisensory illusion than healthy control subjects. These findings resemble the results of Chan et al. (13), where patients with mild cognitive impairment (MCI) perceived the illusion more often than controls. Interestingly, MCI patients perceived the illusion for SOAs of up to 300 ms, while we demonstrated that PMS patients perceive the SiFi at even longer SOAs of 500 ms.

Essentially, increased susceptibility to SiFi was strongly correlated with the number of failed neuropsychological tests and the global z-score of the neuropsychological test battery used in MS patients. In the computed linear regressions, the perception of the illusion at the longest SOA of 500 ms contributed significantly to explaining the variance of the global cognitive impairment. Remarkably, MS patients who perceived the multisensory illusion at an SOA that was more than three times longer than the SOA at which the illusion was disrupted for healthy controls exhibited the most pronounced cognitive deficits.

One possible explanation for the fact that aberrant multisensory perception in MS patients predicted for cognitive deficits is a dysfunction of early cortical processes mainly involved in unimodal perception. Indeed, Shams et al. (26) demonstrated a sound-associated modulation of visually induced MEG activity in occipital and parietal scalp locations of healthy subjects as early as 35–65 ms from the onset of the visual stimulus. Furthermore, de Haas et al. (6) found that individual differences in proneness to the illusion in healthy subjects were strongly correlated with local gray matter volume in early retinotopic visual cortex. Participants with smaller early visual cortices were more prone to the illusion. Thus, it is possible that neuronal damage to early stages of the visual and/or auditory pathways of MS patients results in increased proneness to the illusion. This, however, would not explain the cognitive deficits. More importantly, dysfunctional unimodal visual and auditory systems —e.g., due to demyelination in MS—which are

significant enough to disrupt normal multisensory integration should have resulted in worse performance in our unimodal experimental trials, too. However, MS patients did not perform worse than HC in unimodal or bimodal control tasks. Moreover, Michail and Keil have recently shown that high cognitive load increases the susceptibility for the illusion in healthy subjects which hints at top-down cognitive influence on multisensory integration (8).

Therefore, we suggest that dysfunctional multisensory perception and cognitive deficits in MS share another common cause: impaired brain connectivity due to neuronal damage. In their original study Shams et al. demonstrated that illusion-associated MEG activity was also modulated in the occipital and parietal areas as well as anterior areas at a later (approximately 150 ms post-stimulus) onset (26). Furthermore, using MEG Keil et al. showed that sound-induced visual illusory perceptions were preceded by alpha and beta-band phase synchrony changes between several cortical areas (visual and auditory cortices, parietal and frontal cortical areas) (7) and beta-band phase synchrony is known to play an important role in the large-scale synchronization of functionally specialized brain regions (21). Similarly, Balz et al. employed MR spectroscopy and electroencephalography and found robust relationships between GABA concentration, gamma band oscillations and the SIFI perception rate in healthy subjects and suggested that the GABA level shapes individual differences in audiovisual perception through its modulating influence on gamma band oscillations (5). In an event-related potentials (ERP) study, Mishra et al demonstrated an early modulation of visual cortex activity at 30–60 ms after the second sound, which was larger in amplitude in subjects who saw the illusory flash more frequently (27). Further analysis found that short-latency ERP activity localized to auditory cortex and polymodal cortex of the temporal lobe and associated with gamma bursts in visual cortex determines the perception of the illusion. This suggests that the second sound triggers an interplay between auditory and visual cortical areas and results in perception of the illusory second flash (27). Thus, interactions between cortical areas seem to be crucial for viable multisensory perception.

Two mechanisms of MS-related dysfunction of these interactions leading to concomitant cognitive deficits are possible. First, predominantly white matter-related demyelinating and in the course of disease also axonal damage could impair the pathways connecting different brain areas, resulting in an insufficient interplay of the associative cortices, thus compromising crossmodal but also cognitive processes. Indeed, brain imaging studies have reported altered functional as well as structural connectivity in MS related to sensorimotor as well as cognitive symptoms (28–30). Second, direct damage to associative areas due to cortical lesions may impair their integrative function, thus disrupting both cognitive and multisensory processes. In the light of the recent development of MRI brain imaging it has become easier to visualize cortical lesions. Cortical lesions have been recently recognized as characteristic of multiple sclerosis, contributing to the MRI criteria for dissemination in space (31). Furthermore, extensive cortical damage at the onset of the disease is associated with florid inflammatory clinical activity and predisposes to a rapid occurrence of the progressive phase of MS (32). Moreover, cortical lesions are associated with cognitive and physical disability in MS (33, 34). Thus, MS-related demyelinating of white matter tracts which support the interplay between cortical regions in the healthy brain as well as cortical MS lesions might be the common underpinning of the observed findings: once the common neural pathways are disrupted, both crossmodal information transfer and cognitive processing are diminished.

An interesting question is how our findings relate to other works showing aberrant SiFi perception in neuropsychiatric disorders such as autism and schizophrenia. In autism, one study using the SiFi found a wider temporal binding window in autism spectrum disorder compared to controls (35), which implies a higher susceptibility for the illusion, similar to our findings in MS patients, while another study reported a narrower temporal binding window and thus a diminished susceptibility for the illusion in autism patients (36). Similarly, reduced perception of the SiFi has been reported for schizophrenia patients (37). An interesting observation has been made also by Brighina et al. who demonstrated that compared with controls, migraine patients are less prone to perceive the sound-induced illusion, especially during migraine attacks and/or if they had a migraine with an aura (38), which argues that a state of cortical hyperexcitability diminishes the effect of the illusion. Finally, Chan et al. showed that patients with MCI perceive the illusion more often than controls (13). These findings from clinical populations suggest that brain disorders characterized by an aberrant neural connectivity, such as autism and schizophrenia, exhibit also an altered SiFi perceptual pattern and that cognitive impairment due to neurodegenerative processes such as those seen in MCI might share a common neural basis with the SiFi.

Our findings not only show for the first time that multisensory perception in MS might be impaired but also imply that SiFi performance reflect cognitive deficits. We do not suggest that it should substitute SDMT, PASAT or other screening measures, as numerous studies have provided strong evidence for the utility of SDMT and PASAT in screening for cognitive deficits (3). Instead, we propose that SiFi can be employed complementary or alternatively in certain situations. Using SiFi in the clinical practice has several advantages. While the task takes approximately not more than 10 min, SiFi performance correlates with the global neuropsychological impairment, as indicated by the association between the susceptibility for the illusion and the performance in a broad neuropsychological battery measuring various cognitive deficits, e.g., information processing ability and speed, learning capacity, phonemic, and semantic verbal fluency, etc. The SiFi task is easy to use and does not require any special equipment apart from a laptop or mobile device and headphones. There are no additional costs and no extensive training for the staff is required. Furthermore, it is language- and education-independent. Importantly, there are no learning effects on the SiFi illusion which makes it particularly feasible in monitoring cognitively impaired patients or screening more often for

neuropsychological deficits in patients with active disease (10, 11).

Obviously, SiFi cannot replace neuropsychological tests. However, it offers an opportunity for neurologists—especially in outpatient care—to screen their MS patients for cognitive deficits with minimal time and resource investment. Patients exhibiting aberrant illusion perception (i.e., perceiving the SiFi at an SOA of 500 ms) could be referred to a more extensive neuropsychological investigation. Importantly, since SiFi is a multisensory illusion without significant learning effects, it can be applied multiple times during the course of disease, in particular for monitoring progression of cognitive deficits over time. According to our data, patients with progressive MS are particularly affected by the increased susceptibility to SiFi.

One limitation of the current study is the sample size. This, as well as the rather conservative analyses, i.e., controlling for multiple covariates in the ANOVAs and using the rigorous Bonferroni method, may have contributed to the fact that when directly comparing MS patients and HC in the neuropsychological testing and correcting for multiple comparisons, group differences remained significant only for the SDMT. Certainly, studies with larger patient samples are necessary before introducing SiFi to the clinical practice. Further aspects of MS-treatment can be additionally investigated in future studies with more patients: e.g., monitoring cognitive function under a particular disease-modifying therapy by using SiFi or monitoring rehabilitation for cognitive dysfunction.

Another possible limitation of the paradigm is the visual impairment typically seen in MS patients. One could argue that this would possibly confound the illusion measurements. However, in our study, the only significant group difference for the visual acuity was between the PMS patients and the HC for the left eye. Furthermore, there was no significant group difference in the unimodal (i.e., only visual or only auditory stimulation) control conditions and there was no significant correlation between the performance in the illusion conditions and the visual acuity. Last but not least, we believe that due to the nature of the visual stimulation (e.g., a very simple light flash on the computer screen from a reading distance) even MS patients with some visual impairment would not find it difficult to detect the stimulus.

An interesting observation was that the variable "years of education" was a significant predictor for the global neuropsychological performance in MS patients besides the illusion performance, as seen in the second regression model. This corresponds with other studies which reported a relationship between the highest degree of education and the cognitive performance of MS patients, suggesting that the formal education can exert a positive, possibly protective influence over neuropsychological functions, serving as a "cognitive reserve" (3). Future studies testing for the feasibility of the sound-induced flash illusion as a screening for global neuropsychological impairment should take these results into account.

Cognitive decline is recognized as a prevalent and devastating symptom of MS (3). To our best knowledge, our study is the first to show that MS patients exhibit altered multisensory perception in the SiFi task and that their susceptibility to the perceptual illusion is correlated with their number of failed neuropsychological tests. Thus, SiFi can be considered for further research as a screening test for global cognitive impairment in MS patients.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the ethics committee of the University of Frankfurt Medical Faculty with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the ethics committee of the University of Frankfurt Medical Faculty.

AUTHOR CONTRIBUTIONS

YY: design and conceptualized study, analyzed the data, drafted the manuscript for intellectual content, Interpreted the data, revised the manuscript for intellectual content; HB, DS: analyzed the data, interpreted the data, major role in the acquisition of data; CB, LF: analyzed the data, major role in the acquisition of data; MN, JK: design and conceptualized study, revised the manuscript for intellectual content; SF, MB, JG: design and conceptualized study, interpreted the data, revised the manuscript for intellectual content; CF: interpreted the data, revised the manuscript for intellectual content.

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A brief summary of this work has been presented as an ePoster at the 34th Congress of The European Committee for Treatment and Research in Multiple Sclerosis (ECTRIMS) in Berlin. The abstract of this poster has been published in *ePosters. (2018). Multiple Sclerosis Journal, 24(2_suppl), 738, https://doi.org/10.1177/1352458518798592.* We are thankful to Jason Chan for supporting us with the sound-induced flash illusion as well as to Leslie Kristin Held and Alexandra van Wijnen for their support in data acquisition.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fneur.2019.00373/full#supplementary-material

Supplementary Figure 1 | The binned scatter plots for the independent variable "SiFi SOA 500 ms" and the dependent variables "number of failed tests" and "z global score" from the two regression models are shown in the upper row. The lower row of scatter plots demonstrates the partial regression plots for the two significant predictors "years of education" and "SiFi SOA 500 ms" from the second regression model after controlling for the influence of the other variables.

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frontiers in Neurology

OPINION
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Long-Term Memory Dysfunction in Limbic Encephalitis

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Limbic encephalitis (LE) is an autoimmune disease defined by clinical criteria, such as seizures, psychiatric and in particular working memory abnormalities in conjunction with apparative criteria underlying structural or functional changes in the temporal lobe according to autoimmune encephalitis guidelines (1). Working memory encompasses a transient encoding of information in readiness for further processing within a time window of seconds during cognitive task operations based on neurophysiological mechanisms, such as short-term synaptic facilitation (2). On the contrary, long-term memory (LTM) serves to encode, consolidate, and finally store information for long intervals ranging from minutes to months or even life (3) through cellular mechanisms, such as long-term potentiation (LTP) (4).

In translational transfer experiments of autoimmune encephalitis from humans to the mouse, critical impairment of synaptic LTP in the hippocampus was proved by autoantibodies against the N-methyl-D-aspartate receptor (NMDAR) (5) and alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor subunit GluA2 (6). Synaptic LTP in the hippocampus is considered to be a mechanism of synaptic consolidation (7) serving to enable LTM storage within the hippocampus. Not only antibodies against glutamatergic receptors cause LTM dysfunction probably due to altered synaptic transmission and plasticity—LGI1-LE is also assumed to be based on a synaptic mechanism, as hippocampal AMPA 2 receptors are reduced via neutralization of LGI1-ADAM22 interaction by LGI1-antibodies (8). AMPA receptor reduction is functionally partially equivalent to enhanced AMPAR endocytosis as fewer AMPARs become available for synaptic transmission. LTP might in turn be impaired (9) and LTM formation as well consequently via this suggested process.

On the contrary, the pathophysiology of accelerated long-term forgetting (ALF) is still not well-understood. It occurs often in temporal lobe epilepsy patients (10), has been reported recently in LE, and is even more predominant in LE not associated with autoantibodies (11) (**Table 1A**). ALF can be assessed by neuropsychological tests assessing long-term free recall entailing a 1-week time period with not derogated free recall after half an hour (11). ALF is believed to be based on a shortage of memory consolidation (23), so that ALF in LE clearly depicts LTM dysfunction. Persistent deficits in memory retrieval (19) and recognition (14), anterograde (21) and retrograde (24), autobiographic (25), visuospatial, verbal, and episodic LTM deficits have been reported in LE patients (15, 19, 20, 22, 26, 27) (for reports on LTM dysfunction in adult LE patients see **Table 1A**, $n \geq 6$). These distinct facets of LTM deficits often occurred in LE patients in association with antibodies against membrane surface antigens, such as voltage-gated potassium channels (VGKC) with its subgroups of leucine-rich glioma-inactivated 1 (LGI1) and contactin-associated protein-like 2 (CASPR2), AMPARs as well as antibodies against intracellular antigens, such as glutamic acid decarboxylase 65 (GAD65) and Ma/Ta2. The time frame of LTM decline ranged from a month to years (15, 16, 19, 22, 28) and even decades, with dynamic fluctuations in memory capacity over time (18) (**Table 1A**).

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TABLE 1A | Reports in limbic encephalitis patients on memory including LTM dysfunction.

Antibodies	No. affected/all patients ($n \geq 6$)	Patient's age mean ± sd or median (range)	Neuropsychology tests	Neuropsychology results	MRI	References
Abs–	24/28	48 ± 15	DCSR, VMLT	Impaired verbal and figural memory	Mesiotemporal signal changes	(12)
Abs–, Abs+	23	44 (17)	VLMTe, RAVLT, DCSR	ALF in 31–67%	5% AHS, AE in 48%	(11)
Abs–	20–22/40	51 (10-73)	DCSR, VLMT	Verbal or figural deficit	Mesiotemporal signal changes	(13)
Abs–	1/6	–	CVLT	Anterograde amnesia, impaired retrieval and recognition		(14)
CASPR2	1/6	–	CVLT	Impaired retrieval and recognition	–	(14)
GAD65	16	–	VLMT, DCSR	Verbal and figural memory deficit	Mesiotemporal signal changes	(15)
GAD65	11	43.1 ± 11	VLMT, DCSR	45% verbal, 64% figural memory impairment	100% hippocampus and 73% amygdala affection	(16)
GAD65	7/12	30 (16-48)	VLMT, DCSR	Verbal and figural memory deficit	Greater amygdala volume within 12 months of disease vs. control	(17)
GAD65	5/7	35.3 ± 10.2	VLMT, DCSR	2/5 impaired verbal and figural memory	Signal hyperintensity in hippocampus in 2/5, atrophy of hippocampus in 3/5	(18)
GAD65	1/6	–	CVLT	Impaired retrieval and recognition	–	(14)
LGI1	30	66 ± 13	RAVLT, ROCF	Impairment of delayed recall	CA2/3, DG atrophy, reduced microstructure integrity	(19)
LGI1	27	66 ± 11	RAVLT, ROCF	Verbal and visual episodic memory impairment	Hippocampal hyperintensities 30% unilateral, 48% bilateral	(20)
Ma/Ta2	1/7	35.3 ± 10.2	VLMT, DCSR	Impaired verbal and figural memory	Unilateral amygdala enlargement, unilateral amygdala and hippocampus atrophy	(18)
Ma/Ta2	1/6	–	CVLT	Anterograde amnesia, impaired retrieval memory and recognition	–	(14)
NMDA, VGKC	1/7	35.3 ± 10.2	VLMT, DCSR	Impaired verbal and figural LTM	Hippocampal atrophy, amygdala enlargement	(18)
Paraneoplastic	11	41.5 ± 13.2	VLMT, DCSR	73% verbal and 64% figural memory impairment	64% hippocampus and 73% amygdala affection	(16)
VGKC	19	60.1 ± 15	WMS III	Impaired memory recall	Hyperintensities in temporal lobe	(21)
VGKC	15/18	55 (20-73)	VLMT, DCSR	Verbal memory impairment	Mesiotemporal signal changes 68% bilateral, 28% left, 11% right sided	(22)
VGKC	15/15	–	VLMT, DCSR	Verbal and figural memory deficit	Mesiotemporal signal changes	(15)
VGKC	12–14/15	59.9 (19-72)	VLMT, DCSR	Verbal or figural memory deficit	Larger volumes of amygdala and hippocampus within 12 months of disease vs. control	(17)

(Continued)

TABLE 1A | Continued

Antibodies	No. affected/all patients ($n \geq 6$)	Patient's age mean ± sd or median (range)	Neuropsychology tests	Neuropsychology results	MRI	References
VGKC	11/18	55 (20-73)	VLMT, DCSR	Figural memory impairment	Mesiotemporal signal changes 68% bilateral, 28% left, 11% right	(22)

Abs–, no antibodies; Abs+, antibodies; AE, amygdala enlargement; AI, autobiographic interview; ALF, accelerated long-term forgetting, AM, autobiographical memory testing; AMPA, alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; CASPR2, contactin-associated protein-like 2; CVLT, California verbal learning test; DCSR, Diagnosticum für Cerebralschädigung revised; GAD65, glutamic acid decarboxylase 65; LTM, long-term memory; MRI, magnetic resonance imaging; MTL, medial temporal lobe; NMDA, N-methyl-D-aspartate; RAVLT, Rey Auditory Verbal Learning Test; ROCF, Rey-Osterrieth Complex Figure Test, VGKC, voltage gated potassium channels; VLMTe, Verbaler Lern und Merkfähigkeitstest extended version; VMLT, Verbaler Lern und Merkfähigkeitstest; WMS III, Wechsler Memory scale III.

TABLE 1B | Proposal for revised criteria for limbic encephalitis.

We suggest using a novel memory criterion to diagnose limbic encephalitis.

The first criterion in both potential and definitive autoimmune encephalitis in the Graus criteria (1) should be amplified by specifying the term "working memory deficits" by "short- and/or long-term memory deficits."

TABLE 1C | Neuropsychological test battery for assessing LTM function in limbic encephalitis.

1. Figural/visuo-spatial learning and memory including long-term memory via the "Diagnosticum für Cerebralschädigung" (DCS-R) (40)
2. Visuocontruction via the "Rey-Osterieth Complex Figure Test"
3. Verbal memory including long-term memory and accelerated long-term forgetting via the "Extended version of the Verbal Learning and Memory Test" (extended version VLMT) (11)

Functional memory impairment seems to be based on the structural integrity of mesiotemporal brain structures, as memory function is known to correlate with reduced hippocampal subfield volumes, e.g., the CA1-4, dentate gyrus or subiculum in VGKC or paraneoplastic antibody positive-encephalitis (16, 29). LE involves frontal lobe structures (20) also, but the main underlying brain pathology on the macroscopic (17) and microscopic level (involving infiltrating lymphocytes) affects the amygdalohippocampal complex (30) indicating that dysfunctional LTM is more probable than impaired working-memory pathways. In particular, some LE forms are susceptible to LTM dysfunction as their disease-mediating antibodies against membrane receptors, such as AMPA- (31), NMDA- (32), metabotropic glutamate receptor 5 (mGluR5) receptors (33) are critically involved in hippocampal synaptic long-term plasticity and LTM formation (9, 34, 35). It is thus not surprising that some AMPAR-antibodies associated LE patients present with an amnestic syndrome, such as the unique clinical manifestation of autoimmunity (36). Antibody-mediated immunopathology involving distinct memory phenotypes fluctuates (18), but its pathogenic antigen-antibody interaction of glutamatergic receptors often take days to develop functional changes in receptor electrophysiology [neuronal incubation with antibody serum requires days: Ohkawa et al. (8)] and antibody-directed epitopes undergo post-translational changes in protein expression (37), indicating time preconditions to worsen LTM function. LTM dysfunction is not a unique feature of limbic dysfunction induced by autoimmunity, but can also be caused by viral encephalitis, such as herpes simplex encephalitis. The clinical features of viral encephalitis affecting the temporal lobe can resemble those of autoimmune-mediated limbic encephalitis (LE), but frequently start with a more fulminant onset, often with fever or aphasia. The diagnosis of viral encephalitis must be ascertained by detecting viral DNA in the cerebrospinal fluid via a polymerase chain reaction. The type of LTM impairment in herpes simplex encephalitis affecting either verbal memory (pattern a) and/or memory of names (pattern b) (38), and/or memory of living things (pattern c) (39) depends on structural lesions in the temporal lobe [involving the hippocampus (a) (38) or the lateral temporal lobe (b) (38) or antero-medial temporal cortex (c) (39)].

The occurrence of episodic LTM deficits in LE are often not followed by working memory disturbances (14), so that there may be patients suffering from LE who are not registered due to application of the Graus et al. criteria (1). Redefining the memory criteria in LE has been proposed to consider episodic LTM function in LE patients (14). We suggest an even more amplified and elaborated LTM-dysfunction criterion in addition to working memory performance to adapt LE criteria to include several aspects of episodic, semantic and visuospatial LTM and ALF. Thus, to diagnose limbic encephalitis, we suggest incorporating this aforementioned novel memory criterion within the existing criteria from Graus et al. (1) (**Table 1B**). Furthermore, we recommend utilizing specific neuropsychological tests (**Table 1C**) to detect subtle LTM deficits in LE patients.

This suggested framework provides a more realistic imprint of memory impairment in LE and might help us identify and treat LE patients with LTM disabilities. This is particularly important, as early immunosuppressive or other treatment options (e.g., tumor resection) are essential to improve or recover memory performance in LE patients.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

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Cognitive Impairment in Neuromyelitis Optica Spectrum Disorders: A Review of Clinical and Neuroradiological Features

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Neuromyelitis optica spectrum disorders (NMOSD) are mostly relapsing autoimmune inflammatory disorders of the central nervous system (CNS) with optic neuritis, myelitis, and brainstem syndromes as clinical hallmarks. With a reported prevalence of up to 70%, cognitive impairment is frequent, but often unrecognized and an insufficiently treated burden of the disease. The most common cognitive dysfunctions are decline in attention and memory performance. Magnetic resonance imaging can be used to access structural correlates of neuropsychological disorders. Cognitive impairment is not only a highly underestimated symptom in patients with NMOSD, but potentially also a clinical correlate of attack-independent changes in NMOSD, which are currently under debate. This article reviews cognitive impairment in NMOSD and discusses associations between structural changes of the CNS and cognitive deficits.

Keywords: neuromyelitis optica spectrum disorders, cognition, neuroinflammation, advanced imaging, MRI

INTRODUCTION

Neuromyelitis optica spectrum disorders (NMOSD) are inflammatory autoimmune conditions of the central nervous system (CNS) with a typically relapsing course and a strong female preponderance (1–6). Key clinical features comprise optic neuritis, myelitis, and brainstem syndromes (7–13). Approximately 80% of the patients with NMOSD have pathogenic serum autoantibodies against aquaporin-4 (AQP4), a bidirectional water channel protein predominantly expressed by astrocytes, which is present all over the CNS (7, 14–21). AQP4 appears to not only be important for the internal water balance but its downstream mechanisms also seem to be essential for synaptic plasticity and neuronal functioning due to the involvement of astrocytes, although the exact mechanism of action is still unclear (22). In a subgroup of AQP4 antibody (AQP4-IgG) seronegative NMOSD patients as well as in patients with recurrent optic neuritis and a few patients with multiple sclerosis (MS) an antibody against myelin oligodendrocyte glycoprotein (MOG-IgG) can be detected (23–33). Nowadays, MOG-IgG seropositive patients are mostly assigned to a separate disease entity called MOG-IgG autoimmunity [or MOG encephalomyelitis (MOG-EM)], although they are formally still part of the NMO spectrum (5, 34–36).

In clinical routine, detection of AQP4-specific antibodies in serum allows for discriminating NMOSD from its most common differential diagnosis, MS. The high specificity of AQP4-IgG together with various immunological studies has made clear that NMOSD are not a variant of MS but a separate disease entity (7, 37–39). Subsequently, disease-modifying drugs used in MS, for example interferon-beta, glatiramer acetate, or natalizumab, were found to be ineffective or even harmful for patients with NMOSD (40–45). In contrast, current treatment strategies for patients with NMOSD comprise immunosuppressive therapies with azathioprine or mycophenolate-mofetil and B cell targeting therapies with rituximab (46–54). For most NMOSD patients, these drugs effectively reduce the attack frequency and attack-related accumulation of disability. However, recent studies suggested attack- and lesion-independent "covert" tissue damage in patients with NMOSD, from which clinical implications are not yet entirely clear (55–64) but which presumably contributes to attack-independent symptoms as of which one appears to be cognitive impairment (CI). CI as attack independent symptom was further supported by a study by Saji et al. (65) who tried to proof a permanent interaction between astrocytes and AQP4-IgG which lead finally to dysfunctional synaptic plasticity and hence could be involved in CI in NMOSD AQP4-IgG positive patients. Furthermore, even though CI appears to be a persistent and progressive symptom it still seems to be underrepresented in clinical monitoring and disability scores and often not sufficiently treated (66–70). Over the last years, those neuropsychiatric symptoms in NMOSD came to the forefront, and comparable larger cohorts in observational studies and the application of advanced imaging techniques allowed for the investigation of incidence and structural correlates of these neuropsychological symptoms. This article reviews CI in NMOSD and discusses associations between structural CNS changes and cognitive deficits.

COGNITIVE IMPAIRMENT IN NMOSD

NMOSD show high rates of comorbidity with other physical and psychological conditions, including CI (67–69, 71). While cognitive dysfunctions have been commonly recognized as a burden in MS, this acknowledgment is still missing in NMOSD and hence studies investigating the link between NMOSD and CI are scarce (72, 73) despite NMOSD patients naming CI as one of their most relevant concerns (74) (see **Table 1**). In the few studies conducted, patients demonstrate a significant decrease of cognitive abilities and the prevalence of CI in different samples varies between 30 and 70% (71, 75–77). In addition to investigate cognition, each study has used its own assessment method to the end that one common disease specific cognitive test battery for patients with NMOSD is missing (71). Hence, meta-analyses such as the one of Meng et al. are difficult and are usually only able to analyse and make inferences from a small sample of studies (75). Currently, the most commonly used test battery appears to be the Rao's Brief Repeatable Battery of Neuropsychological tests (65, 76, 78). This battery assesses different aspects of cognition for example verbal memory, short and long term memory, processing speed, attention, concentration, and verbal fluency (65).

Studies investigating which aspects of cognition are most dysfunctional in patients with NMOSD depict ambiguous results: One of the first studies was conducted by Blanc et al. (79). They found alterations in attention, information processing and verbal fluency (79). The meta-analysis of Meng et al. concluded that patients with NMOSD perform generally worse than healthy controls and that patients are significantly worse in the areas of attention, language, memory, processing speed and executive function (75). Similar findings were made by Saji et al., who found that 57% of patients performed significantly worse in at least three cognitive tests compared to healthy controls (65). Furthermore, they found that deficits are predominantly overt in sustained attention, concentration, verbal memory, and speed of information processing (65). However, verbal fluency on semantic stimulation and spatial reasoning were intact (65). Opposed to these results, Vanotti et al. demonstrated a more pronounced dysfunction in the areas of attention and verbal fluencies (76). The variations across results are not only due to heterogeneity of samples including ethnic background, heterogeneity with regards to antibody status, and other interindividual differences, but also due to differences in assessment, the definition and percentage of CI in samples, correction for depression, as well as analysis of magnetic resonance imaging (MRI) and overall differences in study design (72). In particular, the heterogeneous antibody status and MOG-IgG could be a major confounder, as many studies had mixed samples for example Blanc et al. (17 AQP4-IgG seropositive patients/13 AQP4-IgG seronegative patients who were not tested for MOG-IgG) (79). Other studies for example Vanotti et al. or Liu et al. have not reported antibody status, and hence may have missed a possible association between antibody status and CI (76, 80). Further constraints entail that most of the studies recruited rather small sample sizes, ranging from 12 to 91 patients, and cover only a limited spectrum of ethnic backgrounds, with most studies being from Asian countries (71). Thus, while CI seems to be commonly present in a high percentage of NMOSD patients the specific domain of dysfunctional performance varies greatly between studies and samples. It appears that the most common cognitive dysfunction across all studies is decline in attention and memory performance. Moreover, the question as to the whether NMOSD pathobiology is causative for CI has not been clarified.

AQP4-IGG AND COGNITIVE IMPAIRMENT

Currently, attack-independent structural changes as part of the pathology in NMOSD are a matter of debate (56–59, 64, 81, 82). The continuous and sometimes deteriorating neuropsychological symptoms might point toward a smoldering disease process in NMOSD independent of clinical attacks, for instance directly caused by the pathogenic antibody AQP4-IgG (56–59). Hence, few studies have tried to investigate the interplay of AQP4-IgG, which usually are a persistent disease

factor, and cognitive impairment. Fan et al. investigated the link between AQP4-IgG and cognitive functioning, in particular spatial memory, in mice: They concluded that AQP4-IgG appear to inhibit neuronal plasticity and thus lead to a worsening in memory consolidation and spatial memory, as mice AQP4 knockout mice showed deficits in the acquisition and reversal phase in the Morris water maze (83, 84). This link is further supported by Skucas et al. (86) who found impaired long term potentiation and thus deficits in spatial memory in AQP4 knockout mice (83). While this finding would explain deficits in spatial memory, it provides no explanation for attention deficits, which are often observed in NMOSD patients. An attempt to explain the full extent of poor cognitive functioning in patients with NMOSD was made by Saji et al. who claimed that a unique dynamic between AQP4-IgG and astrocytes is the underlying mechanism belonging to CI (65). According to Saji et al. the unique dynamic exhibited by AQP4-IgG is leading to substantial diffuse cortical neuronal loss throughout the whole brain and hence may lead to neurodegeneration independent of clinical attacks (65). Thus, in contrast to MS where the disease causes demyelinating lesions, AQP4-IgG seropositive NMOSD results in pathological changes of the gray matter especially in the cortical layer II (65). These processes are suggested to include a disruption of glutamate and/or water homeostasis and thus excitotoxicity, release of soluble neurotoxic factors, which may trigger neurodegeneration by diffusing into the gray matter (GM) (65). This pathomechanism would further explain the GM atrophy observed by some studies in patients with AQP4-IgG seropositive NMOSD (80, 85). Other processes possibly induced by downstream mechanisms of AQP4 dysfunction are the activation of the innate immune system and hence activation of microglia as well as other autoimmune properties that could be involved not only in AQP4-IgG seropositive NMOSD but also as response to AQP4-IgG (37, 65, 86, 87). The hypothesis of an immune reaction, leading to pathological downstream mechanisms is further supported by Takeshita et al. (38). They found that, in cell cultures, AQP4-positive astrocytes produced interleukin-6 when exposed to AQP4-IgG. The cytokine interleukin-6 was found to disrupt endothelial cell functioning, which results in impaired blood barrier function (38, 88). On top of that, AQP4 expression seems to play an important role in regulating synaptic plasticity, which might be altered in NMOSD due to AQP4-IgG (88). Although the exact mechanism remains unclear, the existence of CI and other neuropsychological symptoms in AQP4-IgG seropositive NMOSD points toward an attack-independent pathology, potentially induced by the pathogenic antibodies themselves.

LINK BETWEEN COGNITIVE IMPAIRMENT AND DEPRESSION IN NMOSD

Depression is known to also be a common and insufficiently treated symptom in NMOSD: Whereas around 50% of moderately to severely depressed patients with NMOSD receive antidepressant medical treatment, only 50% of treated patients report satisfactory treatment responses (68). Nevertheless, only few studies have investigated the link between depression and CI in patients with NMOSD. On the one hand, studies found a strong association between poor cognitive performance and high levels of depressive symptoms (71, 76). On the other, the study of Blanc et al. reported no association between cognition and depression (79). These opposing results could be explained by small sample sizes, heterogeneous cohorts, and different ethnic backgrounds. Furthermore, as both CI and depression tend to have high prevalence in NMOSD, an association but not causation could be possible (67, 71). On top of that, even if a causal link could be proven, the direction of this association would still be questionable. Especially, since studies focusing on fatigue and quality of life are implying a role of these in depression as well as in cognition (89–91). Therefore, it is advisable to investigate the full spectrum of the disease and its psychological comorbidities instead of only exploring the link between depression and CI and thus eliminating possible confounders.

ASSOCIATION BETWEEN COGNITIVE IMPAIRMENT AND STRUCTURAL CHANGES

Numerous studies have described brain tissue alterations in MS [global atrophy, microstructural damage of normal-appearing white matter (WM) and GM)], but studies investigating MRI characteristics in NMOSD are still scarce (92–98). According to the current state of knowledge, up to 80% of AQP4-IgG seropositive NMOSD patients present with cerebral lesions in AQP4-rich sites for example the hypothalamus and periependymal regions and—in contrast to MS—cortical lesions are usually absent (99–105). Also, the few existing MRI studies on MOG-IgG seropositive encephalomyelitis suggest a high similarity with MRI features of AQP4-IgG positive patients with the occasional incidence of characteristic fluffy brainstem lesions (106–108). However, several groups recently reported seizures with cortical MRI involvement on MRI in MOG-IgG seropositive patients which is considered very rare in AQP4-IgG positive NMOSD (109, 110). Whereas, several studies exist describing transsynaptic damage after optic neuritis and myelitis, the existence of global atrophy, and diffuse tissue alterations of WM and GM in AQP4-IgG seropositive NMOSD is still a matter of debate (13, 58, 63, 64, 93, 95, 111, 113, 114).

In order to explain the cognitive dysfunction observed, studies have focused on cortical abnormalities (80, 115). While in MS cognitive dysfunction is linked to cortical lesions, no such correlation can be observed in patients with NMOSD (71, 79, 80, 115). In the study of Liu et al. (80) when comparing 54 NMOSD patients, 28 of which were cognitively preserved and 26 of which had CI, there was no association found between overall brain lesion load and CI (76, 80). Some studies found a correlation between WM and GM atrophy, and CI: Blanc et al. linked focal WM volumes of the brainstem, cerebellum, corticospinal tract, corpus callosum, longitudinal fascicle, and inferior longitudinal fascicle with general CI in

TABLE 1 | The most important original publications on cognitive impairment in NMOSD.

References	Country	Sample size	Portion of AQP4-IgG seropositive Patients	Neuro-psychological assessment	Main findings
Blanc et al. (79)	France	30 NMOSD vs. 30 MS vs. 30 HCs	17/30	BRB-N CTT DST Go-no-go SDMT	-57% CI Compared to HC reduced scores in PASAT, SDMT, phonemic fluencies, the direct & indirect digit span test -compared to MS no differences
Blanc et al. (116)	France	28 NMOSD vs. 28 HCs	12/28	BRB-N CTT DST Go-no-go	-54% CI compared to HC decreased global & focal WM Correlation of WM volumes and cognitive test performance -no Gm differences
Cho et al. (118)	Korea	14 NMOSD vs. 21 HCs	13/14	BRB-N MACFIMS PASAT Digit Span Test DSCT BNT RCFT CVLT TMT COWAT SST	-patients perform significantly worse in attention/working memory, processing speed, executive function and visuospatial processing - CI in patients associated with local efficiency, regional efficiency and nodal clustering coefficient of two disrupted sub-networks
Fujimori et al. (78)	Japan	12 NMOSD vs. 14 MS	12/12	BRB-N WAIS-III WMS-R	-impairment in perceptual organization, working memory and processing speed -compared to MS less CI
He et al. (89)	China	22 NMOSD (acute relapse) vs. 22 patients with depression vs. 22 HCs	Not reported	CLOX CVLT II DST-backward PASAT SDMT	-compared to patients with depression reduced scores on immediate and short-term memory, information processing speed, and attention -suggesting that CI in NMOSD is not only due to depression but also due to cognitive connectivity distortions
Hyun et al. (85)	Korea	91 NMOSD vs. 52 MS vs. 44 HCs	Not reported	COWAT DST PASAT RCFT SDMT Stroop SVLT	-compared to HCs reduced thalamic volume in NMOSD and MS (more severe in MS), a finding mainly observed in Asian patient populations -association between CI and volume of the thalamus
Kim et al. (77)	Korea	82 NMOSD vs. 58 MS vs. 45 HCs	Not reported	COWAT Digit span test HVLT-R PASAT RCFT SDMT Stroop SVLT	-29 % CI -compared to MS less cognitive dysfunctions, especially in verbal learning, verbal and visual memory tests
Liu et al. (119)	China	26 NMOSD vs. 26 HCs	Only 18 patients tested: 16/18	/	-patients showed abnormal small-world network properties -regional efficiency of patients decreased in the visual, sensorimotor and default mode networks
Masuda et al. (115)	Japan	16 NMOSD vs. 20 MS	15/16	CAT TMT WAIS-III WMS-R	-compared to MS better performance in verbal memory, general memory and delayed recall and larger superior temporal gyrus volume -left superior temporal gyrus volume correlated with scores on the delayed recall

(Continued)

TABLE 1 | Continued

References	Country	Sample size	Portion of AQP4-IgG seropositive Patients	Neuro-psychological assessment	Main findings
Moore et al. (71)	United Kingdom	42 NMOSD vs. 42 MS vs. 42 HCs	30/42	CVLT-II DKEFS DST Hayling phonemic and semantic SDMT	-67% CI substantial cognitive and psychiatric comorbidities in NMOSD -compared to MS greater psychological burden, but similar CI prevalence & profiles
Saji et al. (65)	Japan	14 NMO vs. 17 MS vs. 37 HCs	14/14	BRB-N	-57% CI compared to HC significant cortical neuron density decrease in layers II, III, and IV -cognitive deficits in sustained attention concentration, speed of information processing and verbal memory
Vanotti et al. (76)	Argentine	14 NMOSD vs. 14 MS vs. 14 HCs	Not reported	BRB-N SDMT Semantic fluency	-57% CI compared to HC dysfunctions in verbal fluencies & attention -compared to MS similar pattern of dysfunction

BRB-N, brief repeatable battery of neuropsychological tests; BNT, Boston naming test; CAT, cognitive abilities test; CLOX, clock drawing executive test; COWAT, controlled oral word association test; COWAT, Controlled Oral Word Association Test; CTT, color trails test; CVLT, California verbal learning test; DGM, deep gray matter; DKEFS, Delis-Kaplan executive function system; DSCT, digit symbol coding test; DST, drive self-test; HCs, healthy controls; GM, gray matter; HVLT-R, Hopkins verbal learning test–revised; MACFIMS, minimal assessment of cognitive function in multiple sclerosis; MS, multiple sclerosis; NMOSD, neuromyelitis optica spectrum disorders; PASAT, paced auditory serial addition test; RCFT, Rey-Osterrieth complex figure test and recognition trial; SDMT, symbol digit modalities test; SST, spatial span test; SVLT, Shiraz verbal learning test; TMT, trail making test; WAIS, Wechsler adult intelligence scale; WCST, Wisconsin card sorting test; WM, white matter; WMS-R, Wechsler memory scale–revised.

NMOSD patients (79). In particular, visual memory, verbal memory, speed of information processing, short term memory and executive functions were found to be impaired (116). Hence, both focal and global WM volume were linked to CI, but no GM atrophy was observed (116). This finding is in line with the work of Finke et al., who observed no changes in deep GM volumes in AQP4-IgG seropositive NMOSD (112). This is further underlined by so far unpublished results from our groups suggesting missing atrophy of the entire thalamic volumes in AQP4-IgG seropositive patients compared to HCs and only selective subnuclei atrophy in attack-related nuclei such as the lateral geniculate nucleus (117). Conflicting with the missing GM and thalamus atrophy was the study conducted by Hyun et al. (85). They described a significant link between volume of the thalamus and CI in patients with NMOSD, with reduced volumes in patients with poorer cognitive performance (85). Nevertheless, the different conclusions about thalamus volume could be due to the fact that Hyun et al. (85) measured their patients after a mean disease duration of 8 years where advanced degeneration has taken place, which potentially could not only be a confounder on its own but also could possibly lead to confounding through depression, advanced disability, and pain. Furthermore, the sample population appears to play an important role when comparing results in respect to GM atrophy, as reduced volume is mainly observed in Asian samples while studies examining a Caucasian sample fail to replicate these results (117). Hence, studies examining cortical volumes in NMOSD should be interpreted carefully.

Further studies investigating structural changes indicate that white matter network alternations could be the underlying reason for cognitive decline in some NMOSD patients (118, 119). One study by Liu et al. investigated the structural connectivity of 26 NMO-patients and 26 sex- and age-matched HCs with help of diffusion tensor tractography (DTI) (119). After performing network analysis, they found alternations in the small-world topology of the white matter structural networks, including abnormal parameters in path length, an increase in small-worldness as well as an increase in normalized clustering. Furthermore, they found an altered global network organization, which is in line with reduced cognitive efficacy observed in NMOSD patients. They suggest that in particular the reduced efficacy of the precuneus (PCUN), a hub belonging to the default-mode network, which is highly involved in cognitive processing, could partially contribute to CI in patients. A similar DTI study investigating white matter networks and cognitive dysfunction in NMOSD was performed by Cho et al. (118). They enrolled 14 NMOSD patients and 21 HCs, and could confirm the finding of Liu et al., that global network strength is decreased (118, 119). Furthermore, they indicated two disrupted sub-networks, each consistent of six hub nodes, including the PCUN. The disrupted networks were significantly linked to poor performance in attention, processing speed and working memory, as well as to visuospatial processing and executive functions. In particular, the local efficiency, regional efficiency and clustering coefficient of these two sub-networks appear to play a role in CI in NMOSD.

Hence, while several structural changes in GM as well as in WM networks seem to occur in NMOSD it appears to be rather difficult to link CI with one particular tissue alternation. On top of that these studies that have investigated the underlying structures of CI face limitations of which a mixed cohort with heterogeneous antibody-status of the patients is one of the most prominent (96).These limitations, in particular the heterogeneous antibody-status, with earlier

studies like Blanc et al. (79, 116) reporting a higher seronegative-seropositive ratio than current studies, hamper comparison between studies.

OUTLOOK

Cognitive impairment (CI) appears to be one of the more prominent progressive and attack independent symptoms of NMOSD. Hence, a sensitization of the treating neurologists as well as early and standardized screening tests are therefore necessary to improve the management and treatment of cognitive impairment and other neuropsychiatric symptoms in NMOSD. In the future, adequately powered studies investigating CI, its underlying pathobiological mechanisms as well as longitudinal changes and clinical impact should be a priority of NMOSD research.

With regards to NMOSD-specific pathology, continuous and sometimes deteriorating neuropsychological symptoms might point to covert disease activity in NMOSD independent of clinical attacks. In light of the ongoing discussion on attack-independent structural changes in NMOSD, we should therefore keep in mind that CI might represent a clinical correlate of underlying subtle microstructural CNS changes.

AUTHOR CONTRIBUTIONS

FO, JS, AB, and FP wrote the manuscript as well as read and approved the final version.

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Neuronal NMDAR Currents of the Hippocampus and Learning Performance in Autoimmune Anti-NMDAR Encephalitis and Involvement of TNF-α and IL-6

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Wang X, Ma C, Liu C-Y, Li G-J, Zhao D and Han D-F (2019) Neuronal NMDAR Currents of the Hippocampus and Learning Performance in Autoimmune Anti-NMDAR Encephalitis and Involvement of TNF-α and IL-6. Front. Neurol. 10:684. doi: 10.3389/fneur.2019.00684

Among autoimmune encephalitis, patients with anti-N-methyl D- aspartate receptor (NMDAR) encephalitis typically present epileptic seizures, memory deficits and psychiatric symptoms. However, the signal mechanisms leading to the functional disorders of autoantibodies are largely unclear. In this study, anti-NMDAR antibody was administered into dentate gyri against the NR1 subunit of the NMDAR. The purpose of the study examined the effects of pro-inflammatory tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) on neuronal NMDAR currents of the hippocampus in rats with anti-NMDAR encephalitis and we further determined the role played by TNF-α and IL-6 in modulating learning performance. In results, we observed a decrease in amplitude of the NMDAR-mediated excitatory postsynaptic currents (NMDAR-EPSCs) in the hippocampal neurons of animals treated with anti-NMDAR. In those rats with anti-NMDAR, we also observed impaired learning performance in the Morris water maze and spatial working memory test. Of note, cerebral infusion of TNF-α and IL-6 worsened NMDAR-EPSCs and this was accompanied with exaggeration of impaired learning performance. In conclusion, our findings suggest that the role played by neuroinflammation in exacerbating the memory impairment found in animals treated with anti-NMDAR. Anti-inflammation is a potential target in improving the memory impairment induced by anti-NMDA encephalitis.

Keywords: autoimmune disease, encephalitis, neuroinflammation, cytokines, memory deficits

INTRODUCTION

In patients with several identified paraneoplastic autoimmune encephalitis, autoantibodies are identified which are against cell surface and synaptic proteins (1, 2). In one of autoimmune encephalitis antibodies against the N-methyl-D-aspartate receptor (NMDAR, a glutamatergic receptor) are found (3, 4). It is noted that numerous functions including learning and memory, cognition and behavior are depend on synaptic plasticity regulated centrally by glutamatergic

transmission (5, 6). Thus, in association with the role of NMDARs in glutamatergic transmission and activity-dependent plasticity, in anti-NMDAR encephalitis sudden behavioral, memory, and personality changes are observed and these symptoms can progress to seizures, autonomic instability, and psychiatric symptoms. Irretrievable symptoms and death can occur without treatment; whereas a full recovery for about 80% of patients was reported after appropriate immunotherapy (7). Nonetheless, the mechanisms leading to the functional consequences of such autoantibodies in anti-NMDAR encephalitis are poorly understood.

In particular, defects in glutamate transmission are related to neuropsychiatric disorders, and NMDAR hypofunction is thought as a part of the pathophysiological mechanisms leading to schizophrenia (8). In human and rodent studies, sub-anesthetic doses of NMDAR inhibitors (i.e., phencyclidine and ketamine) have been reported to be psychotomimetic and they cause the stereotypic movements, autonomic instability, and seizures, all of which are characteristic of anti NMDAR encephalitis (9, 10). Pharmacological blockade or genetic knock-down of NMDARs can also alter learning performance, memory function, excitatory-inhibitory balance, and neurological behavior (11–13). Accordingly, it is important to study the consequences of NMDAR hypofunction and the mechanisms of antibody-mediated dysfunction in this disease to better understand pathophysiology of patient symptoms.

In addition, using cultured rats' hippocampal neurons pathophysiological role of anti-NMDAR antibodies was examined. Cerebrospinal fluid (CSF) obtained from anti-NMDAR encephalitis patients was applied to these neuronal cells and this produced a substantial and reversible loss of postsynaptic NMDARs leading to impaired NMDAR-mediated miniature excitatory postsynaptic currents (mEPSCs) after its short-term treatment (14). Also, a long-term potentiation (LTP) determining a classical NMDAR-dependent function was attenuated in mouse hippocampal slices bathed in CSF from anti-NMDAR encephalitis patients (15). These findings suggested that anti-NMDAR is likely to exert NMDAR inhibiting effects. Thus, in this study, we employed electrophysiological methods to examine the activity of mEPSCs in the hippocampal neurons of rats treated with anti-NMDAR.

Of note, a prior study provided evidence for an essential role played by anti-NMDAR antibodies *in vivo*, by demonstrating that anti-NMDAR plays a pathophysiologically relevant role *in vivo* (16). For example, in this prior study, CSF containing anti-NMDAR was stereotactically injected into the rat hippocampus. Substantial deficits in NMDAR-mediated synaptic transmission and plasticity were observed later *in vitro* after *in vivo* application of anti-NMDAR. In addition, in this prior study, Morris water maze experiments showed impairments in learning behavior associated with the hippocampus in the rats injected with anti-NMDAR. It is noted that pro-inflammatory cytokines (PICs) are elevated in the plasma and CSF in the patients with anti-NMDAR encephalitis and neuroinflammation has been reported to contribute to the severity of symptoms presented in patients with anti-NMDAR encephalitis (17–20). Since there is a close relation in neuroinflammation and anti-NMDAR encephalitis, PICs/chemokines have been suggested as biomarkers of this disease and potential therapeutic targets in encephalitis (19, 20). On the basis of those previous findings representative cytokines TNF-α and IL-6 were selected in this report. In this study anti-NMDAR antibody was administered into dentate gyri against the NR1 subunit of the NMDAR and we also examined the protein expression of NR1 in the hippocampus of control rats and rats treated with anti-NMDAR. We hypothesized that a chronic cerebral infusion of TNF-α and IL-6 worsens mEPSCs in the hippocampal neurons of rats treated with anti-NMDAR and this thereby amplifies impairment of learning performance.

MATERIALS AND METHODS

Animals

The guidelines of the International Association for the Study of Pain were followed for all animal protocols which were approved by the Institutional Animal Care and Use Committee of Jilin University. Adult male Sprague-Dawley rats weighting 200–250 g were housed in a temperature-controlled room (25°C) on a 12/12 h light/dark cycle and they had free access to food and water.

Antibody Injection

After the rats were anesthetized with sodium pentobarbital (45 mg/kg, i.p.), they were mounted on a stereotaxic frame (Stoelting Co.). A midline incision was made to expose the skull and one burr hole was drilled. Bilateral stereotaxic injection was performed. The injection of 5 μl of anti-NMDAR1 (50 ng/μl, dissolved in CSF; Merck Millipore, Billerica, MA, USA) into dentate gyri [coordinates: 5.2 mm posterior, ±4.3 mm lateral, 4.8 mm deep (relative to bregma)] was performed at each side with a Hamilton syringe connected to a syringe pump. In control rats, 5 μl of CSF was injected in the similar way. The injection was performed at a rate of 0.25 μl/min (over 20 min) via a perfusion pump. One to seven days following the injection learning behavior experiments and electrophysiological experiments were performed accordingly.

In a subset of animals, histological examinations were performed to examine the localization of the stereotactic injection into the dentate gyrus. In this procedure, 0.5 μl of 2% Evans blue was given through the dentate gyrus. Then, the animals were anesthetized with sodium pentobarbital and intra-cardiacally perfused with physiological saline followed by 4% of paraformaldehyde solution. The hippocampus was sectioned and the location of injection sites was verified by identification of blue dye according to the atlas of Swanson (21).

Administration of Drugs

After completion of antibody injection, drugs were given. The following procedures were performed as described in our previous publication (22). Animals were cannulated with an L-shaped stainless steel cannula aimed at the lateral ventricle (coordinates: 3.7 mm posterior to the bregma, 4.1 mm lateral to the midline, and 3.5 mm under the dura). The guide cannula was fixed to the skull using dental zinc cement and jewelers' screw. Then, the cannula was connected to an osmotic

minipump (Alzet pump brain infusion kit, DURECT Inc., Cupertino, CA) with polycarbonate tubing. The pumps were placed subcutaneously between the scapulae, and loaded with vehicle (CSF) as control or TNF-α (5 μg) and IL-6 (5 μg), respectively. Those agents were delivered for a period of 7 days (a rate at 0.25 μl/h). This intervention allowed animals to receive continuous intracerebroventricular (i.c.v.) infusion via the osmotic minipumps. After this procedure, animals were kept in individual cages to secure cannulation and brain infusion kit.

Western Blot Analysis

The tissues from individual rats were sampled for the analysis as described in our prior work (22). In brief, the hippocampus of the rats was removed. Total protein was then extracted by homogenizing the sample in ice-cold immunoprecipitation assay buffer with protease inhibitor cocktail kit (Promega Co. Madison, WI, US). The lysates were centrifuged and the supernatants were collected for measurements of protein concentrations using a bicinchoninic acid assay reagent kit. After being denatured, the supernatant samples containing 20 μg of protein were loaded onto gels and electrically transferred to a polyvinylidene fluoride membrane. The membrane was incubated overnight with primary antibodies (diluted at 1:500): rabbit anti-NR1 and anti-GluR2/3. The membranes were washed and incubated with an alkaline phosphatase conjugated anti-rabbit secondary antibody (1:1000). The primary and secondary antibodies were obtained from Abcam Co. or Antibodies online Com. Enhanced chemiluminescence was used to detect the immunoreactive proteins and the primary antibody recognized on the bands was visualized by exposure of the membrane onto an x-ray film. To show equal loading of the protein the membrane was stripped and incubated with anti-β-actin. After the film was scanned, the optical density of NR1/GluR2/3/β-actin bands was analyzed using the Scion Image software.

Electrophysiological Experiments

The rats were anesthetized with sodium pentobarbital (75 mg/kg, i.p.) and decapitated. Briefly, the brain was taken out and placed in ice-cold artificial cerebral spinal fluid (aCSF) solution. The aCSF perfusion solution contained 124.0 NaCl, 3.0 KCl, 1.3 $MgSO_4$, 2.4 $CaCl_2$, 1.4 NaH_2PO_4, 10.0 glucose, and 26.0 $NaHCO_3$ (in mM). A tissue block of the hippocampus was glued onto the stage of the vibratome and coronal slices (300 μm) were cut from the tissue block in ice-cold aCSF solution. Sixty minutes was allowed to incubate the slices in the aCSF at 34°C, saturated with 95% O_2-5% CO_2 before being transferred to the recording chamber.

A whole cell voltage-clamp mode was employed to record postsynaptic currents of hippocampal neurons. Borosilicate glass capillaries (1.2 mm OD, 0.69 mm ID) were pulled to make the recording pipettes. The resistance of the pipette was 4–6 MΩ as it was filled with the internal solution [contained 130.0 potassium gluconate, 1.0 $MgCl_2$, 10.0 HEPES, 10.0 EGTA, 1.0 $CaCl_2$, and 4.0 ATP-Mg (in mM) with pH 7.25 and osmolarity of 280–300 mOsm]. The slice was placed in a recording chamber perfused (at 3.0 ml/min) with the aCSF (containing 0 Mg^{2+}) saturated with 95% O_2-5% CO_2. An in-line solution heater was used to keep the temperature of the perfusion solution at 34°C. Whole cell recordings from hippocampal neurons were performed visually using differential interference contrast (DIC) optics on an upright microscope (BX50WI, Olympus) and a tight giga-ohm seal was subsequently obtained in hippocampal neuron viewed using DIC optics (23). A MultiClamp 700B amplifier digitized with a DigiData 1440A was used record signals were recorded. A liquid junction potential of −15.0 mV was corrected during off-line analysis (23) and 15 min was allowed after the recording reached a steady state.

At a holding potential of −70 mV, the mEPSCs were obtained in the presence of TTX (1 μM) and picrotoxin (10 μM). 2-amino-5-phosphonopentanoic acid (APV, a NMDA receptor antagonist in 50 μM) and 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, AMPA receptor antagonist in 20 μM) were used to block NMDAR- and AMPAR-mediated currents, respectively. Detection of events was accomplished by setting a threshold above the noise level (23) and the mEPSCs of the hippocampal neurons were analyzed off-line with a peak detection program (MiniAnalysis, Synaptosoft, Leonia, NJ).

Learning Behavior Experiments

The hidden platform task in the Morris water maze was used to examine learning behavior. The circular pool was consisted of polypropylene (diameter: 150 cm; water depth: 50 cm; and platform diameter: 7 cm). The pool was filled with opaque water and maintained at 21 ± 1°C. Four large black-and- white cues (a black cross on 10 cm of white square cardboard) were located at four different sites (east, south, west, and north) and 25 cm above the platform. On the day before the experiments, all rats were required to explore the water maze without platform for acclimatization. The platform was inserted below the water level (1–2 cm) and each rat was randomly assigned to one of four different platform locations on the first day. Then, each rat was given six consecutive trials to reach the platform from days 1 to 7. The starting points were chosen in a random fashion (six out of eight different positions). If a rat failed to reach the platform within 60 s, it was placed on the platform. In any case, a rat was allowed to stay on the platform for another 30 s, before it was moved back into the cage. The next trial was then started following a recovery time of 60 s in the cage. To analyze swimming path length and swimming speed the Ethovision Color software (Noldus Beijing, China) was used to track the animal.

In addition, as described in our publication (22) spatial working memory performance was assessed a week after antibody infusion, by recording spontaneous alternation performance in a Y-maze. The maze was made of gray-painted vinyl-chloride. Each arm was 50 cm long, 30 cm high, and 10 cm wide and converged at an equal angle. Each rat was placed at the center of the maze and allowed to move freely through it during an 8 min period. The numbers of arm entries were recorded for 8 min. An alternation was defined as entries into all arms. The percentage of alternation was calculated as (actual alternations /total entered-2) ×100.

Statistical Analysis

All statistical analyses were performed using SPSS (windows version 13.0). Experimental data (amplitude of mEPSCs and % spontaneous alternation performance were analyzed using one-way ANOVA and experimental data of water maze (swimming path length and swimming speed) were analyzed using two-way repeated ANOVA. As appropriate Tukey's *post hoc* analyses were utilized to determine differences between groups. All values were presented as mean ± standard error. Differences were considered significant at $P < 0.05$.

RESULTS

Protein Expression of NR1 and GluR2/3 Expression

In order to determine the effectiveness of anti-NMDAR after its administration, we examined the protein levels of NMDA receptor NR1 and AMPA receptor GluR2/3 in the hippocampus of control rats ($n = 6$) and rats treated with anti-NMDAR ($n = 6$). **Figure 1A** shows that NR1 was significantly downregulated after application of anti-NMDAR as compared with control rats ($P < 0.05$, between control and anti-NMDAR). However, anti-NMDAR did not alter the protein levels of GluR2/3 expression in the hippocampus ($P > 0.05$, between two groups).

Synaptic NMDAR and AMPAR Currents

First, we used whole-cell patch recordings of mEPSCs to assess NMDAR- and AMPAR- mediated currents in the hippocampal neurons (number of neurons = 12) of rats without treatment. **Figure 1B** demonstrates that the mEPSCs were examined at −70 mV in extracellular solutions containing 0 Mg^{2+}. TTX was used to block action potentials; and picrotoxin was used to block GABA receptor-mediated miniature currents. As shown in this figure (typical traces and averaged data), APV and CNQX effectively blocked NMDAR- and AMPAR-mediated currents.

Next, we examined the effects of anti-NMDAR on NMDAR activity using whole-cell patch recordings of mEPSCs. **Figure 2** demonstrates that amplitude of NMDAR-mediated mEPSCs in neurons of rats treated with anti-NMDAR (number of neurons = 15) was decreased as compared with the amplitude of NMDAR-mediated mEPSCs in CSF control rats (number of neurons = 10; $P < 0.05$, anti-NMDAR *vs.* control). However, frequency of NMDAR-mediated mEPSCs was not significantly changed by anti-NMDAR. In addition, the prior i.c.v. infusion of TNF-α and IL-6 significantly attenuated amplitude of NMDAR-mediated mEPSCs in neurons of rats treated with anti-NMDAR (number of neurons = 12 in each group; $P < 0.05$, TNF-α/IL-6 vs. CSF control), but no significant alteration was observed in neurons of control rats although TNF-α and IL-6 tended to decrease amplitude of NMDAR-mediated mEPSCs in control rats.

In contrast, anti-NMDAR was observed to have the minimal effects on amplitude of AMPAR-mediated mEPSCs in the hippocampal neurons. i.e., amplitude of AMPAR-mediated mEPSCs was 21 ± 3 pA in the hippocampal neurons of control rats; and 19 ± 3 pA in the hippocampal neurons of rats treated with anti-NMDAR ($P > 0.05$, control vs. anti-NMDAR; number of neurons = 8 in each group).

Learning Performance

As illustrated in **Figure 3A**, two-way repeated ANOVA shows that distance to locate the platform was decreased across the six training sessions in three groups of control rats [$F_{(3,32)} = 19.63$; $P < 0.001$]. There were no significant effects by session interaction for decreased distance to find the platform ($P = 0.75$). Likewise, in three groups of rats with anti-NMDAR distance to locate the platform was also decreased across the six training sessions [$F_{(3,32)} = 17.67$; $P < 0.001$]. No significant effects by session interaction was observed for decreased distance to find the platform ($P = 0.83$). In addition, the swimming path length cumulatively for six consecutive trials was greater in rats injected with anti-NMDAR as compared to control animals injected with CSF ($P < 0.01$, anti-NMDAR rats/$n = 12$ vs. control rats/$n = 10$). Note that TNF-α/or IL-6 amplified the swimming path length observed in rats anti-NMDAR ($P < 0.01$, TNF-α/IL-6/$n = 8$ in each group vs. CSF), but the minimal effects of TNF-α or IL-6 were observed in control rats. Also, to assess the possible effects of motor activity in rats we examined swimming speed and **Figure 3B** shows that there were insignificant differences in the swimming speed in those experimental groups ($P > 0.05$ among groups).

Moreover, spontaneous alternation performance was examined. In this experiment, as a measure of activity level the number of arm entries was determined by counting the number of arms entered in the maze for each animal during the test. Insignificant differences in the number of arm entries were found between control group and anti-NMDAR group. The number of arm entries for each group was 15.8 ± 2.6 in control rats ($n = 8$) and 16.2 ± 2.8 in rats with anti-NMDA ($n = 10$; $P > 0.05$ between two groups). Then, spatial working memory performance was examined. **Figure 3C** demonstrates that the percentage of spontaneous alternation was decreased in rats with anti-NMDAR ($n = 12$) as compared with control rats ($n = 10$; $P < 0.05$, between control and anti-NMDAR). A decrease of spontaneous alternation was greater in rats with anti-NMDAR after infusion of TNF-α or IL-6 ($n = 8$ in each group; $P < 0.05$, TNF-α/IL-6 vs. CSF). However, TNF-α/or IL-6 had insignificant effects on spontaneous alternation in control animals ($P > 0.05$, TNF-α/IL-6 vs. CSF).

DISCUSSION

Prior studies have shown that NMDAR antibody pathogenicity leads to neuronal surface receptor downregulation, subsequent impairment of NMDAR-mediated currents and behavior abnormalities (24, 25). Consistent with those findings, data of our current study specifically demonstrated that NMDAR-mediated mEPSCs in the hippocampal neurons of rats treated with anti-NMDAR were attenuated; and anti-NMDAR impaired learning performance in rats. In addition, after CSF containing anti-NMDAR was stereotactically injected into the dentate gyri of rats (16), substantial deficits in NMDAR-mediated synaptic transmission and plasticity were observed later *in vitro* after *in vivo* application of anti-NMDAR. In addition, in this prior study (16), Morris water maze experiments showed impairments in

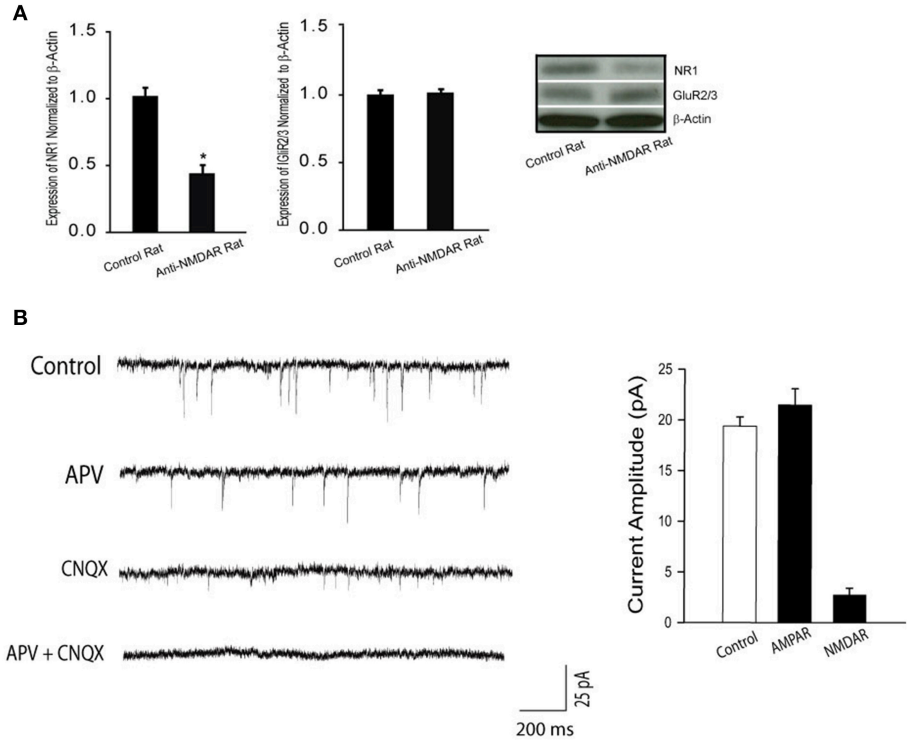

FIGURE 1 | (A) Protein expression NR1 and GluR2/3 in the hippocampus of control rats and rats treated with anti-NMDAR. Treatment of anti-NMDAR attenuated the protein levels of NMDA NR1 but not AMPA GluR2/3. *$P < 0.05$ vs. control rats. The number of rats = 6 in each group. **(B)** Typical traces and averaged data, showing NMDAR- and AMPAR-mediated mEPSCs. The mEPSCs were recorded in perfusion solution with TTX, picrotoxin, and 0 Mg^{2+} to isolate synaptic glutamate-mediated currents. APV, an NMDA receptor antagonist, blocks mEPSCs, allowing AMPAR-mediated currents observed. CNQX, an AMPA receptor antagonist, blocks mEPSCs, allowing NMDAR-mediated currents observed. As APV plus CNQX were applied, mEPSCs were completely blocked. Twelve neurons were used in this experiment. Under the same recording conditions, NMDAR-mediated mEPSCs were examined in the hippocampal neurons of control rats and rats with anti-NMDAR that were presented in **Figure 2**.

learning behavior in the rats injected with anti-NMDAR since the swimming path length cumulatively for consecutive trials was observed to be greater in rats injected with anti-NMDAR as compared to control animals injected with CSF. In the similar way, we injected anti-NMDAR antibody into the dentate gyri of rats in the current study and we found that consistent results demonstrating increases of the swimming path length in rats with anti-NMDAR. Our current results involved additional indication that the prior cerebral infusion TNF-α and IL-6 amplified the decreases of NMDAR-mediated mEPSCs in the hippocampal neurons with anti-NMDAR and this worsened learning performance. This result also provides potential evidence that TNF-α and IL-6 are engaged in the abnormalities in NMDAR-mediated mEPSCs and learning performance in anti-NMDAR encephalitis.

In patients with anti-NMDAR encephalitis, the autoantibodies are first found in the serum and CSF and then high antibody concentrations are seen in intrathecal fluid (3, 26). In general, functional NMDAR has two NR1 and two NR2 subunits, but anti-NMDAR is localized to the N-terminal extracellular loop of the NMDAR subunit NR1 (27). The N-terminal extracellular domain of NR1 is recognized in all patients' antibodies in studying an antibody-mediated pathogenesis (3). In the disease, the extracellular domain of the NR1 subunit of the NMDAR is directly targeted by autoantibodies (3, 4). Also, noticeable psychiatric and behavioral symptoms, rapid memory loss, seizures, abnormal movements, hypoventilation, and autonomic instability are presented in those patients (3, 4, 28). A study using *in vitro* and *in vivo* methods has further indicated the cellular mechanisms by which patients' antibodies result in a decrease in NMDAR density and function in cell surface and synaptic site (14). This is likely to lead to the learning, memory, and other behavioral deficits observed in patients with anti-NMDAR encephalitis. Nonetheless, the underlying mechanisms leading to antibody-mediated dysfunction in this disease are largely unknown.

Interestingly, a study showed that injection of patient's CSF into the rat hippocampus led to an NMDAR phenotype similar to key clinical features such as memory disturbance (16). In this prior study, the same results were found after anti-NMDAR1 was injected into the dentate gyrus of rats. Thus, in our current report, anti-NMDAR1 was given into the hippocampus of rats and we found that the protein levels of NR1 were downregulated in the hippocampus after injection of anti-NMDAR, suggesting

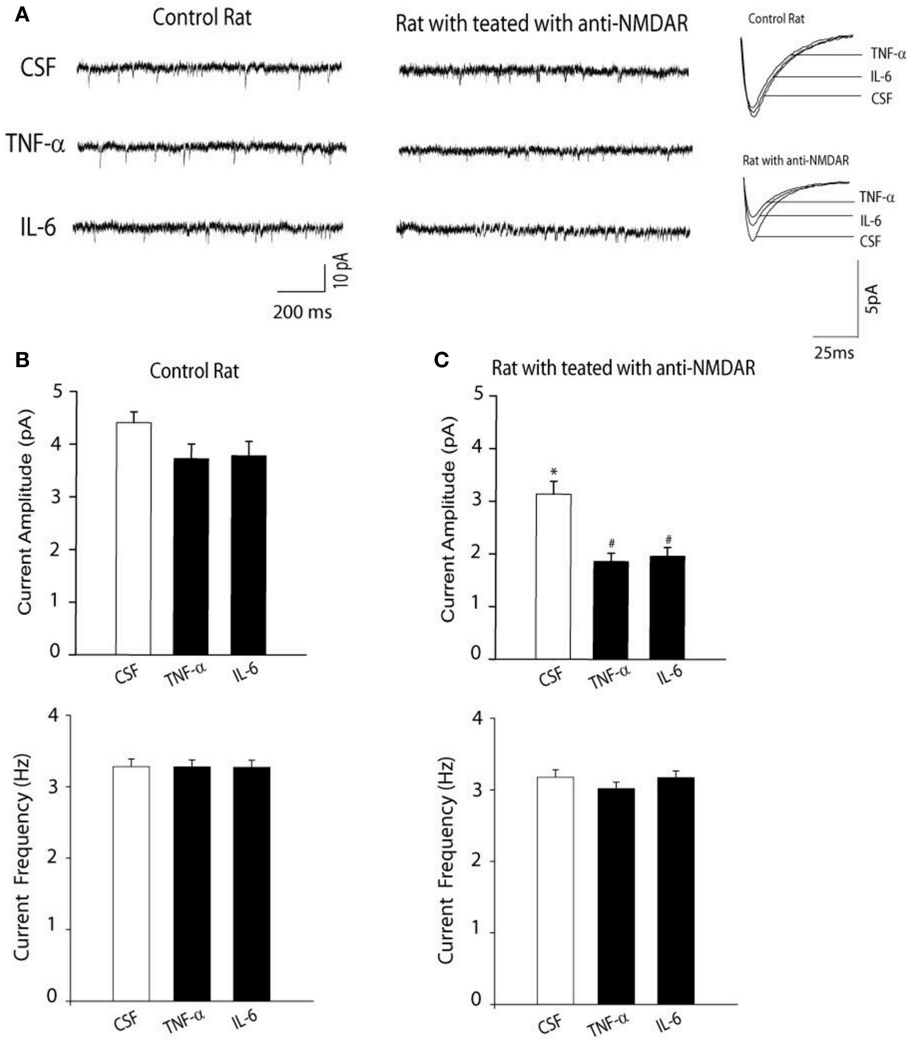

FIGURE 2 | (A) Representative traces and **(B,C)** averaged data showing amplitude of NMDAR-mediated mEPSCs was decreased in the hippocampal neurons of rats with anti-NMDAR. The frequency of NMDAR-mediated mEPSCs was not altered. The decreases in NMDAR-mediated mEPSCs became greater in the hippocampal neurons of rats with anti-NMDAR after the prior infusion of TNF-α or IL-6. The effects of TNF-α or IL-6 were observed to be insignificant in control animals. $^{*}P < 0.05$ vs. control rats with CSF; and $^{\#}P < 0.05$, TNF-α/IL-6 vs. CSF in rats with anti-NMDAR. The number of neurons = 10–15 in each group of experiments.

its effectiveness on attenuating NMDAR. In addition, we found that amplitude of NMDAR-mediated mEPSCs was decreased after injection of anti-NMDAR into the hippocampus. Of note, expression of AMPA GluR2/3 and AMPAR-mediated mEPSCs were not affected by treatment of anti-NMDAR. It should be noted that less expression of NR1 was observed in the current study it was likely that part of the NR1 subunits were already bound to the antibody administered.

Synaptic plasticity plays a role in regulating memory, learning, and cognition (5, 13). The proper synaptic localization and trafficking of the excitatory glutamate NMDA and AMPA receptors are necessary to modulate synaptic plasticity and these neurological functions (6). Using animal models the glutamate receptors are genetically or pharmacologically altered and the roles played by these receptors at the synaptic and cellular levels have been documented (29). In contrast, in human studies, indirect approaches are used to determine the role of these receptors in memory, learning, cognition and psychosis. For example, pharmacological trials (e.g., psychosis of NMDAR antagonists) (30) and analysis of brain tissue from patients with Alzheimer's disease or schizophrenia reveal several molecular pathways causing a downstream alteration of glutamate receptors (31).

Nonetheless, in animal studies, the Morris water maze hidden platform task was used to assess space learning, which is considered as a hippocampus-specific learning paradigm and depends on NMDAR activation (32). Considering that impaired spatial working memory was observed after granule cell-specific disruption of the NR1 gene (33), we further examined whether this behavioral task was likely affected in rats injected with

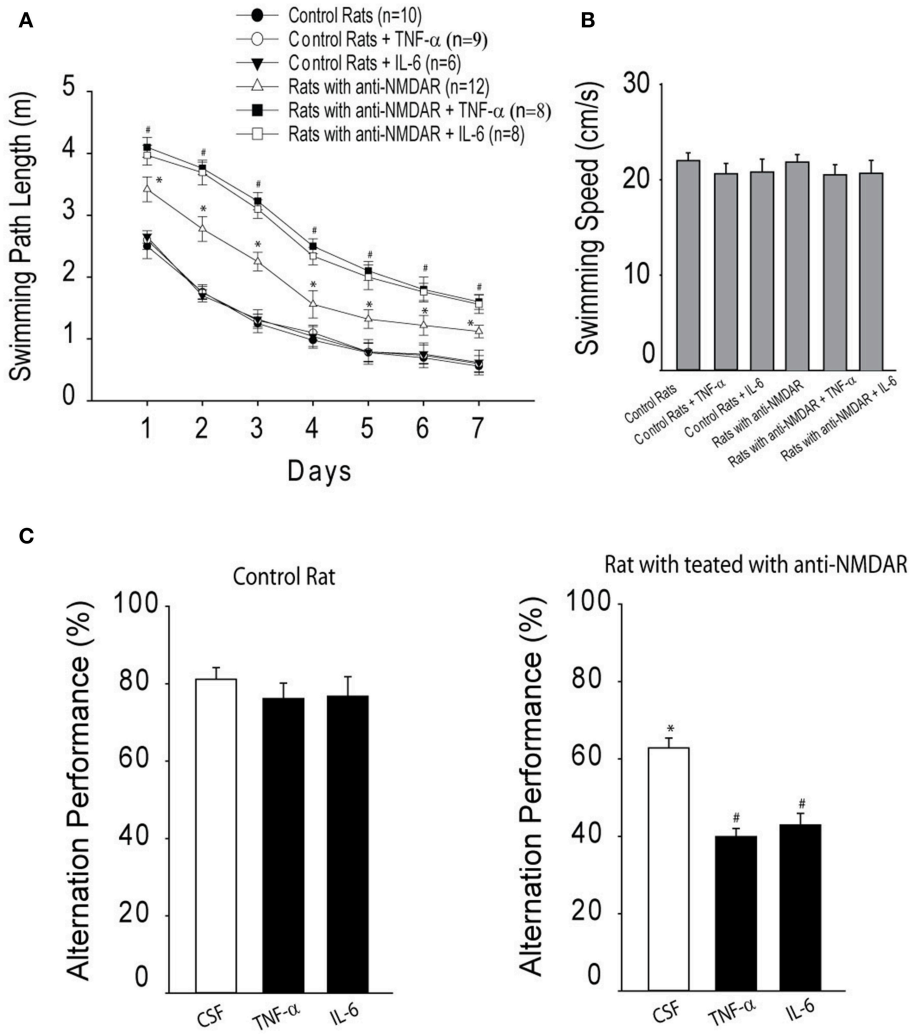

FIGURE 3 | (A) The cumulative swimming path length on 7 consecutive days in the Morris water maze for six experimental groups, showing increases of swimming path length in rats that have been injected with anti-NMDAR as compared to control rats. TNF-α or IL-6 amplified increases of swimming path length observed in rats anti-NMDAR, but not in control rats. $^{*}P < 0.05$ vs. control rats; and $^{\#}P < 0.05$, TNF-α/IL-6 vs. CSF control. **(B)** There were insignificant differences observed in the swimming speed in these different experimental groups ($P > 0.05$). **(C)** The effects of anti-NMDAR on spontaneous alternation performance in rats. The learning performance was examined after infusion of TNF-α or IL-6. $^{*}P < 0.05$, control rats vs. rats treated with anti-NMDAR. $^{\#}P < 0.05$, TNF-α/IL-6 vs. CSF control. The number of animals in each group is shown in the figure.

anti-NMDAR. Consistent with our data showing the decreased amplitude of NMDAR-mediated mEPSCs of anti-NMDAR rats, learning performance was impaired in these animals. In this experiment, we did not find any significant differences in the swimming speed in control rats and rats with anti-NMDAR, suggesting that motor activity has the minimal effects on learning performance in rats treated with anti-NMDAR.

Evidence has demonstrated that PICs (i.e., TNF-α) are engaged in memory function and synaptic plasticity in the hippocampus (34). Under physiological conditions, TNF-α can increase AMPA receptors into the cell membrane and this process is important for synaptic scaling (35). Synaptic scaling is a form of synaptic plasticity alleviating the neuronal excitability by adjusting the strength of all of the excitatory synapses of an individual neuron (36). However, at pathological concentration, TNF-α is injurious to memory and synaptic plasticity. For example, up-regulation of TNF-α is associated with deficits of memory and synaptic plasticity in Alzheimer's disease, and inhibition of TNF-α is effective for treating the disease (37, 38). Nevertheless, the mechanisms by which PICs impair synaptic plasticity and memory are mostly unknown in anti-NMDAR encephalitis although the studies have shown that some cytokines are elevated in CSF of patients with anti-NMDAR encephalitis and, in general, neuroinflammation contributes to the severity of anti-NMDAR encephalitis (17, 18). In the present work, we showed that a chronic infusion of TNF-α into the central nervous system decreased NMDAR-mediated EPSCs in the hippocampal neurons, suggesting that amplification of

TNF-α likely worsens dysfunction of NMDARs in anti-NMDAR encephalitis. Indeed, we found that chronic application of TNF-α amplified impairment of the learning performance in animals treated with anti-NMDAR.

IL-6 is an immune cell mediator in the periphery in involvement of the modulation of neurological functions (39). Under normal conditions, IL-6 expression is low in the brain, but it increases largely in neurological diseases such as stroke, brain damage and seizures (40, 41). Due to neuroinflammation, an increase in the levels of endogenous IL-6 in the brain contributes to pathogenesis of some neurodegenerative diseases (42, 43). A prior study has also shown that IL-6 decreases NMDA-induced cytosolic Ca^{2+} overload thereby inhibiting neuronal apoptosis and necrosis, suggesting a neuroprotection of IL-6 (44). Nonetheless, in our current study, a chronic infusion of IL-6 attenuated NMDAR-mediated mEPSCs in the hippocampal neurons of rats treated with anti-NMDAR and this worsened learning performance in animals. Thus, IL-6 may exert a bidirectional effect on synaptic plasticity and memory. Normalizing IL-6 production and its levels is likely a better strategy to treat numerous neurological disorders observed in anti-NMDAR encephalitis.

There are possible synaptic mechanisms by which cytokines can worsen the effects of anti-NMDAR on NMDA receptor currents. In a prior study, it was observed that TNF-α enhanced the frequency of spontaneous EPSCs, whereas IL-6 reduced the frequency of spontaneous IPSCs in neurons of isolated spinal cord slices (45). In contrast, IL-1β enhanced the frequency and amplitude of sEPSCs and reduced the frequency and amplitude of sIPSCs. In addition, TNF-α and IL-1β enhanced AMPA- or NMDA-induced currents, and IL-1β and IL-6 suppressed GABA- and glycine-induced currents (45). Those findings suggest that PICs induce central sensitization via distinct and overlapping synaptic mechanisms in superficial dorsal horn neurons either by increasing excitatory synaptic transmission or by decreasing inhibitory synaptic transmission. It is assumed that anti-NMDAR is likely to decrease central sensitization to a greater degree in synaptic sites of the hippocampal neurons via such synaptic mechanisms and application of TNF-α and IL-6 worsens its effects.

Study Limitations

A prior study demonstrated that the inhibition of TNF-α synthesis can significantly reverse hippocampus-dependent cognitive deficits induced by chronic neuroinflammation (46), suggesting that TNF-α is a critical mediator of chronic neuroinflammation-induced neuronal dysfunction and cognitive impairment. Our results showed that TNF-α and IL-6 are engaged in NMDAR-mediated currents and behavior abnormalities. However, it is needed in the further study examining the prevention of the observed effect of NMDAR antibody on the amplitude and amount of NMDA-receptors as well as cognitive functions after application of blockers of TNF-α and IL-6. In addition, a few issues need to be acknowledged. i.e., histological experiments designed to show the interaction of the antibody and NMDA-receptor and additional experiments for evaluation of LTP in rat hippocampus to show the electrophysiological correlation with learning disturbance.

In conclusion, our findings suggest that neuroinflammation plays a role in attenuating NMDAR-mediated mEPSCs and exacerbating the memory impairment observed in rats with anti-NMDAR encephalitis. Anti-inflammation should be considered in improving the memory impairment in anti-NMDA encephalitis.

ETHICS STATEMENT

All animal protocols were in accordance with the guidelines of the International Association for the Study of Pain and approved by the Institutional Animal Care and Use Committee of Jilin University.

AUTHOR CONTRIBUTIONS

XW and CM designed the studies, collected experimental and analyzed data, and drafted the paper. C-YL and G-JL have participated in performing experiments and assist in data analysis. DZ and D-FH designed the studies and participated in data analysis and reviewed the draft.

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Serum Clusterin and Complement Factor H May Be Biomarkers Differentiate Primary Sjögren's Syndrome With and Without Neuromyelitis Optica Spectrum Disorder

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Background: Neuromyelitis optica spectrum disorder (NMOSD) is a neurological complication of primary Sjögren's syndrome (pSS).

Objective: We aimed to explore potential serological differences between pSS patients with and without NMOSD.

Methods: There were 4 pSS patients with NMOSD and 8 pSS patients without NMOSD enrolled as the screening group for two-dimensional difference gel electrophoresis (DIGE) analysis. Then differential expressed protein spots between groups were identified by MALDI-TOF/TOF MS. The levels of the identified potential biomarkers were verified by ELISA in a second independent cohort including 22 pSS patients with NMOSD, 26 pSS without NMOSD and 30 NMOSD patients.

Results: Nine proteins were identified significantly differently expressed (more than 1.5-fold, $p < 0.05$) between these two groups. Serum levels of clusterin and complement factor H (CFH) were further verified by ELISA. Results showed that the serum clusterin was significantly higher in NMOSD with pSS than without (298.33 ± 184.52 vs. 173.49 ± 63.03 ng/ml, $p < 0.01$), while the levels of CFH were lower in pSS patients with NMOSD than without (24.19 ± 1.79 vs. 25.87 ± 3.98 ng/ml, $p < 0.01$).

Conclusion: This is the first study of serological comparative proteomics between pSS patients with and without NMOSD. Serum clusterin and CFH might be potential biomarkers for pSS patients with NMOSD and play important role in the pathogenesis of the disease but needs further verification.

Keywords: primary Sjögren's syndrome, proteome, neuromyelitis optica spectrum disorder, clusterin, complement factor H

INTRODUCTION

Primary Sjögren's syndrome (pSS) is a systemic autoimmune disease characterized by dry eyes and dry mouth. About 40% of pSS patients were found to be complicated with neuromyelitis optica spectrum disorder (NMOSD) (1). NMOSD is an inflammatory demyelinating spectrum disorder of the central nervous system characterized by severe attacks of optic neuritis and myelitis. However, the pathogenesis of pSS complicated with NMOSD has not been elucidated yet (2).

Generally, the autoimmune response appears to be more intense in the NMO group with SS (3). The prognosis of NMOSD patients complicated with autoimmune diseases was considered to be more poor than NMOSD patients without (4–6). Therefore, early diagnosis and treatment of pSS patients with NMOSD will improve their prognosis. However, the diagnosis of pSS patients with NMOSD, especially NMOSD, was confronted with problems that the specific serum biomarker, anti-aquaporin4 (AQP4) antibody was not sensitive enough (7). The similarity of sensitivity and specificity of AQP4 antibody in idiopathic NMOSD patients and NMOSD associated with autoimmune diseases is indicative of two distinct diseases with sequential or simultaneous incidences (8). Moreover, the symptoms of the central nervous system behave before mucosal symptoms and detection of anti-SSA/SSB antibody in pSS patients with NMOSD (9–11), which may result in ignoring the existence and treatment of pSS. Exploring serum biomarker of pSS with NMOSD will not only be useful for making its clinical decision but also help further illustrate the pathogenesis.

As we know, proteomics has been widely used in exploring diagnosis and prognosis biomarkers of many diseases. Previously, we have applied proteomics in establishing classification tree models and differential diagnosis biomarkers for autoimmune disease (12–15). Recently, we illustrated the pathogenesis of primary biliary cholangitis by using label-free proteomics (16). Among these researches, we found that the two-dimensional difference gel electrophoresis (DIGE) is a more sensitive and high-throughput based two-dimensional gel electrophoresis (2-DE) method (17).

In this research, we aimed to investigate serological differences between pSS patients with and without NMOSD by using DIGE, to established important biomarkers or primarily demonstrated the pathogenesis of pSS patients with NMOSD.

MATERIALS AND METHODS

Eligibility Criteria and Study Design

Serum samples were collected at Peking Union Medical College Hospital from June 2013 to December 2013. Participants met diagnosis criteria: pSS and NMOSD were diagnosed according to the revised version of classification criteria for pSS proposed by the American-European Consensus Group (18) and the Wingerchuk criteria (1, 19), respectively, and not receive immunosuppressive treatment could be included in this study. Four pSS patients with NMOSD and eight pSS patients without NMOSD were selected as the screening group for DIGE analysis. A second independent cohort including 22 pSS patients with NMOSD, 26 pSS without NMOSD, and 30 NMOSD patients was recruited for the verification of the DIGE results (**Table 1**). The Ethics Committee of the Peking Union Medical College Hospital approved this study, and all methods were performed in accordance with relevant guidelines and regulations. Written informed consent was obtained from all participants.

Sample Collection and Preparation for 2-DE Analysis

Blood samples were collected from the cubital vein under fasting conditions and dispensed into 5-mL pro-coagulation tubes with gel (Becton, Dickinson and Company, UK). Blood samples were centrifuged at 1,000 × g for 5 min within 6 h at 4°C. Sera were stored at −80°C until use.

High abundant proteins from the serum samples included for DIGE analysis were removed by applying the Agilent Human 14 Multiple Affinity Removal System Column (Agilent Technologies, CA, USA) according to the manufacturer's protocol. The 14 most abundant proteins (albumin, immunoglobulin G, α1-antitrypsin, immunoglobulin A, transferrin, haptoglobin, fibrinogen, α2-macroglobulin, α1-acid glycoprotein, immunoglobulin M, apolipoprotein A-1, apolipoprotein A-II, complement C3, and transthyretin) were removed by this method. Then protein concentrations of the depleted serum samples were detected with the Bradford assay (Bio-Rad, USA).

DIGE

Depleted serum samples from the patients of screening group were analyzed by DIGE, which was performed according to our previous research (14). Briefly, 50 μg of each sample were pooled and used as an internal standard that will run on each gel to control gel-to-gel variation. Then protein extracts from pSS without NMOSD, pSS with NMOSD, and the internal standard were labeled with DIGE fluors, Cy3, Cy5, and Cy2 (GE Healthcare, NJ, USA), respectively, according to the manufacturer's protocols (20). The Cy2, Cy3, and Cy5 labeled samples were loaded onto a 13 cm, pH 3–10 Immobiline Drystrips (GE Healthcare), with the setting of electrophoresis conditions as follow: 30 V for 12 h, 500 V for 1 h, 1,000 V for 1 h, 8,000 V for 8 h, and 500 V for 4 h. After equilibration, the strips were loaded on a 12.5% polyacrylamide gel for second separation with the setting of electrophoresis, 15 mA for 10 min, and then at 30 mA until the samples were 5 mm from the bottom of the gel. The gel was then scanned by Typhoon 9400 (GE Healthcare). DeCyder 6.5 sofeware (GE Healthcare) was used for image analysis. The dye intensity of protein spots from pSS patients with and without NMOSD were compared by Biological Variation Analysis module using a t-test, and p-values < 0.05 were considered as significant. Spots that were significantly different between groups (more than 1.5-fold) were selected and identified.

One milligram of internal standard was run on a 2-DE gel to gain all the selected spots. After destained, dehydrated and proteins digested, gel pieces of these spots were prepared for protein identification. A 4800 Plus MALDI TOF/TOF^{TM} Analyzer (AB SCIEX, USA) was used for protein identification.

TABLE 1 | Characteristics of all recruited patients.

Study group	Characteristics	Patients		
		pSS without NMOSD	pSS with NMOSD	NMOSD
Screening group				
	Total number	8	4	NA
	Sex ratio (F:M)	7:1	4:0	
	Average of age disease onset (years)	47.6 ± 15.7	32.5 ± 8.4	
	Average duration (months, median)	120	10	
	Dry mouth	8 (100)	4 (100)	
	Dry eyes	8 (100)	3 (75)	
	Objective xerostomia	8 (100)	4 (100)	
	Postive ocular tests	8 (100)	3 (75)	
	Positive salivary gland biopsy	8 (100)	4 (100)	
	ANA positive	8 (100)	3 (75)	
	Anti-Ro/SSA positive	8 (100)	4 (100)	
	Anti-La/SSB positive	6 (75)	1 (25)	
Verification group for CFH				
	Total number	22	22	NA
	Sex ratio (F:M)	21:1	8:3	
	Average of age disease onset (years)	50 ± 10.3	40 ± 11.2	
	Average duration (months, median)	48	36	
	Dry mouth	19 (86.3)	18 (81.2)	
	Dry eyes	17 (77.3)	16 (72.3)	
	Objective xerostomia	20 (90.1)	19 (86.4)	
	Postive ocular tests	20 (90.1)	19 (86.4)	
	Positive salivary gland biopsy	14/16 (87.5)	15/18 (83.3)	
	ANA positive	22 (100)	22 (100)	
	Anti-Ro/SSA positive	22 (100)	22 (100)	
	Anti-La/SSB positive	9 (40.9)	6 (27.3)	
Verification group for clusterin				
	Total number	26	22	30
	Sex ratio (F:M)	11:2	8:3	23:7
	Average of age disease onset (years)	47 ± 10.3	40 ± 11.2	40 ± 14.6
	Average duration (months, median)	46	36	38
	Dry mouth	23 (88.5)	18 (81.2)	0 (0)
	Dry eyes	19 (73.1)	16 (72.3)	0 (0)
	Objective xerostomia	24 (92.3)	19 (86.4)	NA
	Postive ocular tests	23 (88.5)	19 (86.4)	NA
	Positive salivary gland biopsy	17/19 (89.5)	15/18 (83.3)	NA
	ANA positive	26 (100)	22 (100)	5 (16.7)
	Anti-Ro/SSA positive	26 (100)	22 (100)	0 (0)
	Anti-La/SSB positive	10 (38.5)	6 (27.3)	0 (0)

pSS, primary Sjögren's syndrome; NMOSD, Neuromyelitis optica spectrum disorder; CFH, complement factor H.

Bioinformation including MS/MS queries were run on the MASCOT search engine 2.2 (Matrix Science).

ELISA Validation

ELISA was used to verify the reliability of DIGE results. In particular, we investigated the expression of clusterin and complement factor H (CFH). The validation experiments were performed using the commercially available ELISA Kit for human clusterin (Abcam, Cambridge, UK) and CFH (Abcam, Cambridge, UK). Twenty-six pSS patients without NMOSD, 22 pSS patients with NMOSD, and 30 NMOSD patients were used to determine the level of clusterin. And 22 pSS patients without NMOSD and 22 pSS patients with NMOSD were used to determining the level of CFH. For the quantification of clusterin and CFH, 10 μL of each sample was diluted 1:500 and 1:1,000 with sample diluent, respectively.

Serum samples and protein standards were added to pre-designated wells on the 96-well microtiter plate. The assay was performed according to the manufacturer's specifications. The absorbance was read by a microplate reader at 450 nm. And the assay results were analyzed using the GraphPad Prism software and SPSS 18.0 software package (IBM, Armonk, NY, USA).

Statistical Analysis

The data were expressed as the mean ± SD and were analyzed using SPSS statistical software (IBM, Armonk, NY, USA). Student's *t*-tests were used to compare pairs of means. The level of significance was set as $p < 0.05$. The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

RESULTS

Screening Protein Spots With Potential Value

Firstly, the proteins that present in all gels from one group were selected. Secondly, the abundance of these protein spots was compared between pSS patients with and without NMOSD, and a total of 206 protein spots were found significantly differently expressed in the screening group. Thirdly, the relative abundance of proteins spots with potential value was estimated 1.5 times higher or lower than expressed in the other group. Thirty-two of the 206 proteins spots were found to be of potential value (**Figure 1**). Among them, the abundance of 16 protein spots was increased in pSS without NMOSD, while the other 16 protein spots decreased.

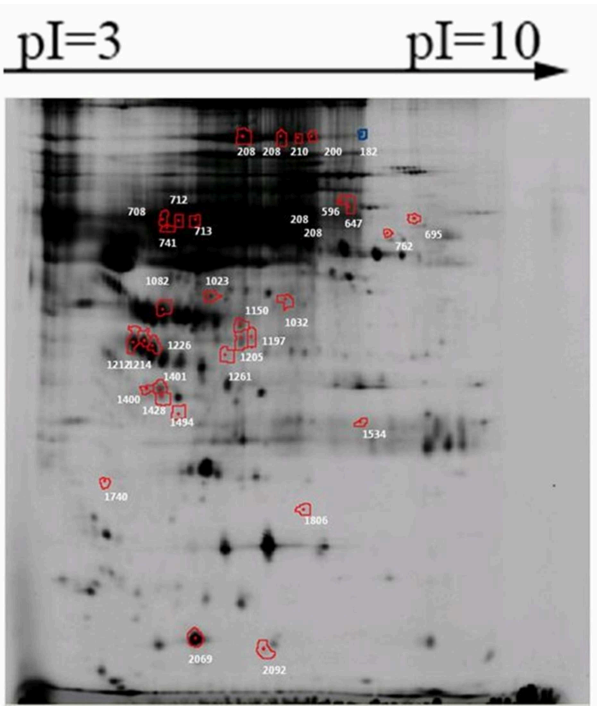

FIGURE 1 | Significantly differently expressed protein spots between pSS patients with and without NMOSD. Proteins of each spot represented were listed in **Table 2**.

Identification of Protein Spots With Potential Value by MALDI-TOF/TOF MS

Only 20 out of the 32 protein spots were found in 2-DE gels, and 9 candidate proteins were identified: complement factor H (CFH), hemopexin, alpha-1B-glycoprotein, putative macrophage-stimulating 1-like protein, CD5 antigen-like OS, HP protein, clusterin, keratin (type I cytoskeletal 9 OS), alpha-1-microglobulin (**Table 2**). Based on a review of related literature, CFH and clusterin might be related to immune diseases, and thus they were chosen for further verification.

Verification of Serum Clusterin and CFH

The levels of clusterin and CFH in sera were quantified by ELISA to confirm the altered expression that was revealed by proteomic analysis and to validate their potentials as biomarkers of pSS with NMOSD. The results of DIGE were validated firstly. In the screening group, the level of clusterin was the same with DIGE while the level of CFH was not. Serum levels of clusterin and CFH were then determined in the verification group by ELISA. Results showed that serum clusterin was higher in pSS without NMOSD than with NMOSD but not significant (307.26 ± 140.26 vs. 298.33 ± 184.52 ng/ml, $p = 1.00$). However, serum clusterin was significantly higher in NMOSD with pSS than without (298.33 ± 184.52 vs. 173.49 ± 63.03 ng/ml, $p < 0.01$). On the other hand, the levels of CFH were lower in pSS patients with NMOSD than without NMOSD (24.19 ± 1.79 vs. 25.87 ± 3.98 ng/ml, $p < 0.01$). ELISA results for serum levels of clusterin and CFH in the verification group are shown in **Figure 2**.

In statistical analysis, the period that onset of disease to diagnosis was significantly different between SS with and without NMOSD in the screening group, while the age on disease onset was not. The SS with NMOSD, without NMOSD, and the NMOSD patients of the confirmation group were matched for most of the clinical condition, especially in the age of disease onset. We, therefore, considered that the differences we found come from disease heterogeneity.

DISCUSSION

In this research, DIGE combined with MALDI-TOF/TOF MS was applied to compare the protein pattern of serum from pSS patients with and without NMOSD. There were 9 proteins found to be significantly differently expressed between groups and could be potential biomarkers for pSS with NMOSD. The serum levels of alpha-1B-glycoprotein, alpha-1-microglobulin, CD5 antigen-like OS, clusterin, hemopexin, type I cytoskeletal 9, and putative macrophage-stimulating 1-like protein were lower in pSS patients with NMOSD than without NMOSD, while CFH and HP protein OS were higher in pSS patients with NMOSD.

Based on literature review, clusterin and CFH, which may be relevant to related diseases, were further verified in this study.

Clusterin is a multifaceted protein functioning at the crossroads of inflammation and autoimmune diseases. The main form of clusterin is a secreted heterologous protein with a molecular weight of 80 kDa (21). In our study, serum clusterin was higher in pSS without NMOSD than with NMOSD but not significant. However, serum clusterin was significantly higher in NMOSD with pSS than without (**Figure 2**). Therefore, clusterin might be a potential biomarker that can differentiate NMOSD with and without pSS. Recent researches showed that clusterin could be found in saliva, tears, and salivary glands of pSS patients and participate in the pathogenesis of exocrine involvement (22–24), which suggest that increased clusterin might participate in the pathogenesis of NMOSD to complicated with pSS.

TABLE 2 | Characteristics of differential protein spots from pSS with NMOSD, pSS without NMOSD.

Spot No.	MW	pI	Protein name	Fold change (pSS with/without NMOSD)
208	143534.4	6.32	complement factor H	2.28
596	77007.2	6.81	Serotransferrin	1.77
708	52384.6	6.55	Hemopexin	−2.57
712	52384.6	6.55	Hemopexin	−1.77
713	54789.8	5.56	Alpha-1B-glycoprotein	−1.76
741	52384.6	6.55	Hemopexin	−2.36
762	66288.9	7.22	macrophage-stimulating 1-like protein	−2.25
1023	39602.5	5.28	CD5 antigen	−2.08
1082	31647	8.48	HP protein	3.4
1150	31647	8.48	HP protein	3.15
1212	53031.2	5.89	Clusterin	−2.4
1214	62254.9	5.14	Keratin	−2.41
1401	30329.6	6.01	Alpha-1-microglobulin	−1.77
1428	30329.6	6.01	Alpha-1-microglobulin	−1.53
2902	45860.8	6.13	Haptoglobin	−3.61
206			N/A	2.4
1226			N/A	−2.26
1400			N/A	−1.65
1740			N/A	−1.58
1806			N/A	−1.58

pSS, primary Sjögren's syndrome; NMOSD, neuromyelitis optica spectrum disorder; MW, molecular weight; pI, isoelectric point.

For the pathogenesis of NMOSD, binding of NMO-IgG to its target AQP4 initiates several immune processes. Often, the binding could readily activate the complement system, subsequently, amplify inflammation, and disrupt the blood-brain barrier, leading to astrocyte injury and causing secondary demyelination and neuronal loss (25). CFH is an important regulator in complement activation alternative pathways. It also acts as a co-factor for factor I to regulate the C3 and C5 convertases. Shi et al. (26) reported that engineered neural stem cells with CFH can attenuate inflammatory infiltration and immune-mediated damage of astrocytes. Moreover, Pohl et al. (27) reported that only T cells were seen in the lesions of destructive astrocyte from NMO patients. Of note, the T cells in the lesions showed signs of activation. Since T cell activation was regulated by the decreased expression of CFH in microglia, decreased CFH might be a risk factor for NMOSD. Interestingly, the levels of CFH were lower in pSS patients with NMOSD than without NMOSD (**Figure 2**), which is consistent with the assumption that decreased CFH is a risk factor for NMOSD.

There are three limitations in the current study. Firstly, observing the levels change of clusterin and CFH during treatment will support our finding to an extent. However, the rate of lost to follow-up is high that we could not perform this study. Secondly, comparing the levels change of selected proteins in cerebrospinal fluid among groups, which might act as an important confirmation of their pathogenesis role, were not done owing to ethical issues. Thirdly, the SS with NMOSD is a rare disease that limits the inclusion of related patients. The current sample size could help access a statically significant result, but not enough for generating a stable statistical result.

In conclusion, this is the first serum comparative proteomics focusing on the difference between pSS patients with and without NMOSD. Our results confirm that DIGE is a useful tool for such comparison. Increased clusterin and decreased CFH in pSS patients with NMOSD were potential biomarkers and related to the pathogenesis of these diseases. Further researches with larger sample size and more subgroup of patients are required

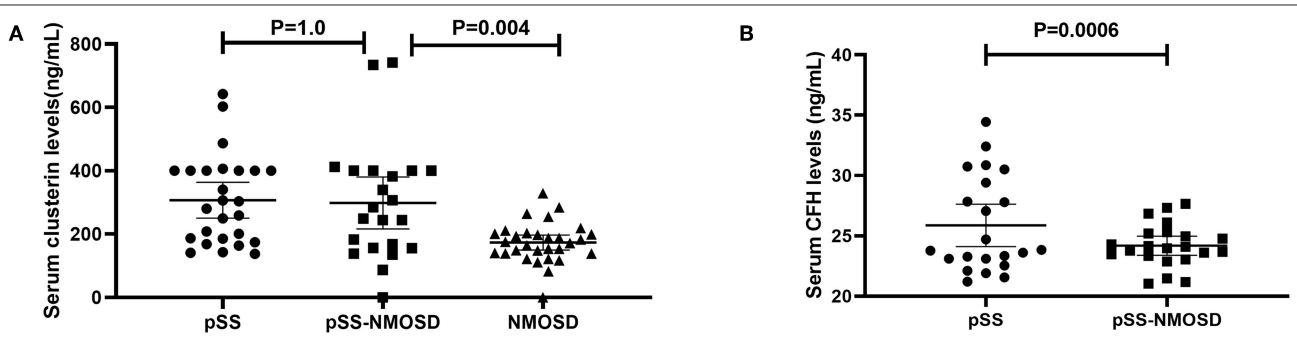

FIGURE 2 | Verification of clusterin and CFH by ELISA asssys. **(A)** validation of serum clusterin levels; **(B)** validation of serum CFH levels.

to verify the value of these candidate biomarkers and their clinical significance.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the Ethics Committee of the Peking Union Medical College Hospital with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the the Ethics Committee of the Peking Union Medical College Hospital.

AUTHOR CONTRIBUTIONS

YL and YZ designed the research. LQ and CD performed the experiments. QW, WZ, YF, and YX recruited patients. CD wrote the manuscript and YL approved the version to be published.

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