seizures or status epilepticus, the pathophysiology of PLEDs is still a subject of debate and it remains unclear whether they can be characterised as ictal phenomena (Fitzpatrick and Lowry, 2007). Here, we report a man with chronic right hemispheric PLEDs for more than 14 months with elevated levels of anti-*N*-methyl-d-aspartate (NMDA) receptor antibodies and granzyme B in his cerebrospinal fluid (CSF). We administered two courses of steroid pulse therapy, which partially resolved the PLEDs and improved subtle neurocognitive deficits. In the light of these findings, the pathophysiology of chronic PLEDs is briefly discussed.

Case study

The patient, a 54-year-old man, experienced monthly seizures with loss of consciousness from the age of 10. Phenytoin reduced the patient's seizure frequency to one a year. The patient graduated from a university, lived alone, and was employed as an office worker. An EEG recorded when the patient was 43 revealed no epileptic discharge, but registered sporadic, diffuse theta waves (4-7 Hz) and high-voltage slow waves (3 Hz) with right fronto-centro-parietal dominancy. After being seizure-free for a number of years, the patient discontinued his medication.

When the patient was 52 years old, a cluster of seizures culminating into status epilepticus occurred and he was brought to a hospital. Left hemiparesis was observed between ictus and he was intubated and sedated for four days. Seizures recurred after extubation and a 1,000 mg of valproate was started. MRI, 12 days after admission, revealed cortical thickening and hyperintensity on fluid-attenuated inversion recovery (FLAIR) in the right temporal, parietal, and occipital lobes (*figure 1A*). Seizures were controlled by administering 200 mg of carbamazepine and 3,000 mg of levetiracetam. During the follow-up period, right hemispheric PLEDs emerged on an EEG taken five months after the status.

The patient was referred to our hospital 19 months after the status with major complaints of sleepiness and loss of appetite. Upon admission, the man appeared untidy, and put all of his valuables and what appeared to be useless rubbish into a dirty sack. He also presented with mild left hemispatial neglect. Laboratory tests were normal. Cessation of valproate improved the patient's sleep patterns and appetite. An EEG showed intermittent PLEDs prevailing for more than 50% of the total EEG recording (*figure 2A*). In addition, a hypermotor seizure occurred during the EEG, at which time PLEDs disappeared several seconds before the onset of a clinical seizure (*figure 2C*). FLAIR and diffusion-weighted MRI sequences revealed no significant abnormalities. However, fluorodeoxyglucose positron emission tomography (FDG-PET) showed right temporo-parieto-occipital hypometabolism, which was consistent with the source area of the PLEDs, as estimated by magnetoencephalography (MEG) (*figure 1B, 1C, and 1D*). No signs of inflammation were found in the CSF test and a systemic workup aimed at tumour identification, including whole-body FDG-PET and serological tumour markers, was negative. In addition, autoantibodies associated with systemic lupus erythematosus and thyroiditis tested negative. Upon further examination, we found that anti-NMDA receptor (anti-GluR2B and anti-GluR1) antibodies, as well as granzyme B, were strongly elevated in the CSF, compared with disease controls (Takahashi *et al.*, 2009).

Based on these findings, two courses of steroid pulse therapy (1,000 mg/day of methylprednisolone for three days) were performed, which improved the patient's left hemispatial neglect (*figure 3*) and caused the patient's PLEDs to dissolve into periodic delta activity (*figure 2C*).

Written informed consent was obtained from the patient for this case report.

Discussion

Chronic PLEDs are usually found in patients with prolonged partial seizure disorders, and are associated with sustained structural brain abnormalities (Westmoreland *et al.*, 1986). In agreement with previous reports, the patient in this case presented with chronic epilepsy, however, the patient demonstrated no structural brain abnormalities.

As previously mentioned, it is still unclear whether PLEDs represent ictal activity (Fitzpatrick and Lowry, 2007). Based on the finding that the onset of PLEDs is accompanied by ipsilateral hypermetabolism in PET studies, some have argued that these discharges represent ictal activity, comparable to that of partial status epilepticus (Handforth *et al.*, 1994). Others, however, consider that PLEDs reflect acute cerebral damage and are not necessarily related to seizures (García-Morales *et al.*, 2002). It has been proposed that ictal/interictal differences should be considered as a continuum rather than a discrete dichotomy, with PLEDs stretching across the entire ictal-interictal continuum (Chong and Hirsch, 2005). Because PLEDs accompanied by rhythmic discharges (PLEDs plus) are more frequently followed by seizures than those without rhythmic discharges (PLEDs proper), the former are placed towards the ictal end and the latter towards the interictal end of the spectrum (Reiher *et al.*, 1991).

A couple of points should be discussed regarding the nature of PLEDs observed in the present case. First, the

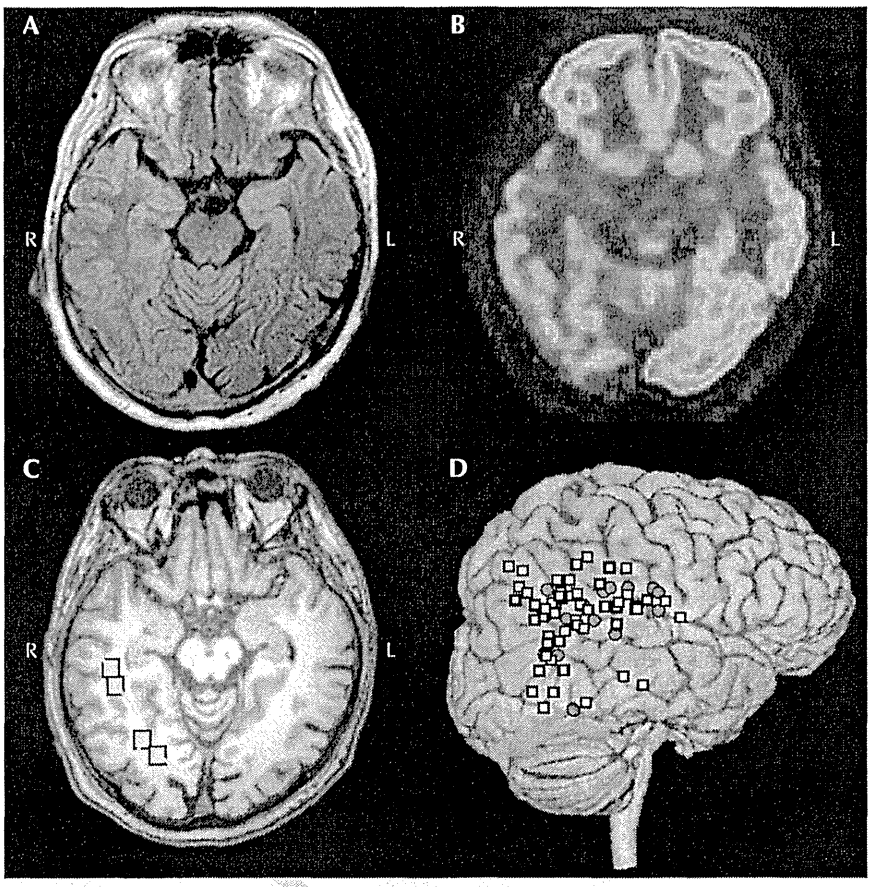

Figure 1. (A) Axial fluid-attenuated inversion recovery MRI of the brain, acquired 12 days after the onset of status epilepticus. Thickening and hyperintensity were observed in the right temporo-parieto-occipital cortex. (B) Fluorodeoxyglucose positron emission tomography and spike dipoles of periodic lateralised epileptiform discharges (PLEDs) estimated by magnetoencephalography, superimposed on T1-weighted MR images obtained upon referral to our hospital (C, D).
Yellow squares represent dipoles with a goodness of fit of more than 90%. Green circles represent dipoles with a goodness of fit of more than 80%. The source area of PLEDs roughly coincided with the hypometabolic region.

patient presented with mild left hemispatial neglect. Meador and Moser (2000) described left hemispatial neglect to be a possible sign of negative seizures and reported a patient with PLEDs in the left parietal region who experienced negative symptoms including right hemiparesis, aphasia, apraxia, and severely depressed mood, which were improved with phenobarbital. Because the negative symptom in the present case was far milder than the aforementioned case, it might be better interpreted as a functional impairment caused by neuropathology underlying chronic epileptiform discharges.

The present case exhibited clinical seizures. However, observed PLEDs were unaccompanied by rhythmic discharges. Moreover, PLEDs disappeared several seconds before the onset of a clinical seizure. This pattern is different from the reported progression of PLEDs proper to PLEDs plus to seizures, and supports the hypothesis that the recorded PLEDs were interictal discharges (Reiher *et al.*, 1991).

In the present case, PET studies disclosed hypometabolism rather than hypermetabolism in the right temporo-parieto-occipital region, which roughly coincided with the localisation of the source dipole of the PLEDs estimated by MEG. This finding is similar to a previous study of a patient who experienced recurrent PLEDs due to a metastatic brain tumour and was investigated using MEG and PET (Hisada *et al.*, 2000). Hypometabolism is indicative of an interictal brain state during PET. However, lack of concurrent EEG recordings restrict the significance of the PET findings as conclusive evidence that observed PLEDs were interictal phenomenon, since PLEDs might have resolved during a PET scan.

By combining clinical, EEG, and PET findings together, the chronic PLEDs recorded in this case apparently

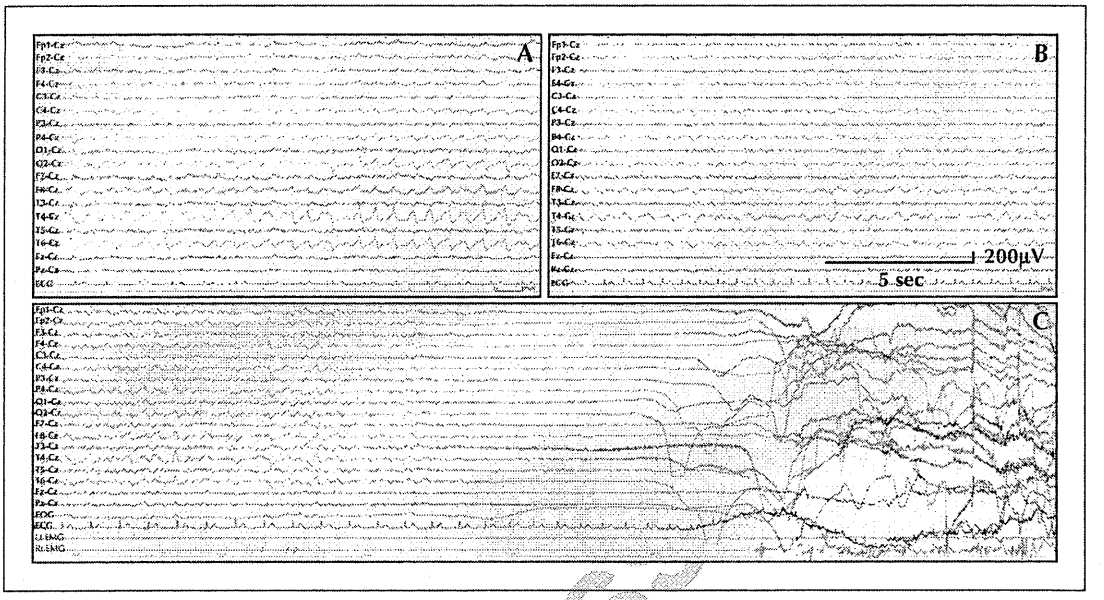

Figure 2. (A) EEG tracing 19 months after the status epilepticus. Right hemispheric periodic complexes (1.0-1.5 Hz) were observed with the highest voltage of the sharp-wave components recorded at T4 and T6 using the international 10-20 EEG system. Because the A2 reference was contaminated by periodic lateralised epileptiform discharges (PLEDs), the midline (Cz) reference was used. (B) EEG after two courses of steroid pulse therapy; sharp-wave components of the PLEDs disappeared and only right hemispheric periodic delta waves could be observed. (C) Seizure onset; the interval between PLEDs became longer and finally disappeared about 10 seconds before the onset of the clinical seizure.

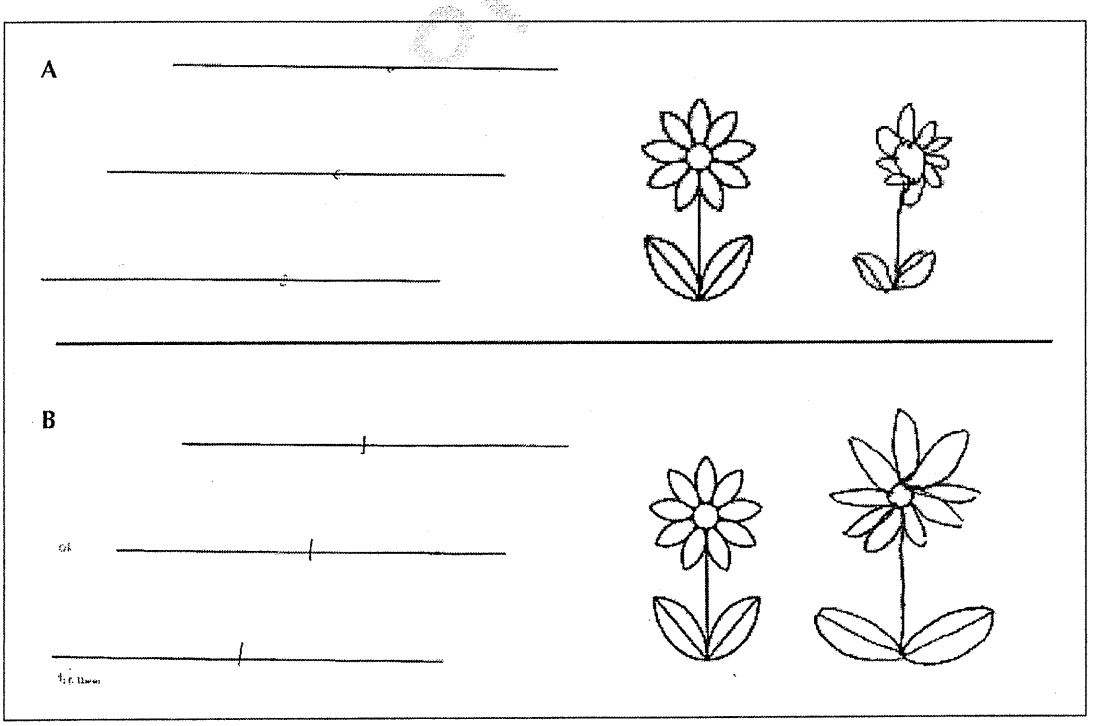

Figure 3. Improvement of left hemispatial neglect after steroid pulse therapy. The patient was asked to mark the centre of three horizontal lines (left) and to copy a picture of a flower (right).
(A) Test result before the two courses of steroid pulse therapy. (B) Test result after the steroid pulse therapy.

correspond to the interictal end of the ictal-interictal continuum.

What is notable in this case was the positive finding of anti-NMDA receptor antibodies in the CSF. Anti-NMDA receptor antibodies have received attention lately because of the recent establishment of anti-NMDA receptor encephalitis as a clinical entity associated with ovarian teratoma (Dalmau *et al.*, 2011). Rasmussen's encephalitis is also accompanied by anti-NMDA receptor (anti-GluR2B) antibodies (Takahashi *et al.*, 2009). Moreover, although rare, PLEDs can be accompanied by the presence of anti-NMDA receptor antibodies (Labate *et al.*, 2009) and Rasmussen's encephalitis (Fitzpatrick and Lowry, 2007).

The causative role of anti-NMDA receptor autoantibodies in the induction of status in the present case is equivocal; although epileptic status and cortical thickening are common in patients with encephalitis, the clinical course in the current case was dissimilar to that of patients with anti-NMDA receptor or Rasmussen's encephalitis. In addition, since the patient had been affected by epilepsy for a long period of time, the withdrawal of antiepileptic medication may have caused the status.

Granzyme B is a serine protease secreted chiefly from cytotoxic T lymphocytes, which induces DNA fragmentation and apoptosis in target cells. Granzyme B in the CSF of patients with Rasmussen's encephalitis is reported to be elevated, and is considered to be involved in the autoimmune pathophysiology of the disease (Takahashi *et al.*, 2009). The existence of anti-NMDA autoantibodies and granzyme B in the CSF may be a sign of cytotoxic T-cell-mediated neuronal injury and fragmentation of neuronal molecules including glutamine receptors, which could cause the production of autoantibodies against them (Takahashi *et al.*, 2009). In the present case, the effectiveness of steroid pulse therapy indicates that reversible autoimmune processes were involved in the pathology that caused both chronic epileptiform discharges and the subtle neurocognitive deficit. □

Acknowledgements and disclosures.

We acknowledge Grants-in-aid for Scientific Research I Nos. 21591342, 23591238 and 24591537; Comprehensive Research on Disability Health and Welfare; Research on Rare and Intractable Diseases; Intramural Research Grant (22-3) for Neurological and Psychiatric Disorders of NCNP; and grants from The Japan Epilepsy Research Foundation.

The authors have no conflicts of interests to disclose.

References

Chatrian GE, Shaw CM, Leffman H. The significance of periodic lateralized epileptiform discharges in EEG: an electrographic, clinical and pathological study. *Electroencephalogr Clin Neurophysiol* 1964; 17: 177-93.

Chong DJ, Hirsch LJ. Which EEG patterns warrant treatment in the critically ill? Reviewing the evidence for treatment of periodic epileptiform discharges and related patterns. *J Clin Neurophysiol* 2005; 22: 79-91.

Dalmau J, Lancaster E, Martinez-Hernandez E, Rosenfeld MR, Balice-Gordon R. Clinical experience and laboratory investigations in patients with anti-NMDAR encephalitis. *Lancet Neurol* 2011; 10: 63-74.

Fitzpatrick W, Lowry N. PLEDs: clinical correlates. *Can J Neurol Sci* 2007; 34: 443-50.

García-Morales I, García MT, Gálan-Dávila L, *et al.* Periodic lateralized epileptiform discharges: etiology, clinical aspects, seizures, and evolution in 130 patients. *J Clin Neurophysiol* 2002; 19: 172-7.

Handforth A, Cheng JT, Mandelkern MA, Treiman DM. Markedly increased mesiotemporal lobe metabolism in a case with PLEDs: further evidence that PLEDs are a manifestation of partial status epilepticus. *Epilepsia* 1994; 35: 876-81.

Hisada K, Morioka T, Nishio S, *et al.* Magnetoencephalographic analysis of periodic lateralized epileptiform discharges (PLEDs). *Clin Neurophysiol* 2000; 111: 122-7.

Labate A, Irani SR, Vincent A, *et al.* Anti-NMDA receptor encephalitis: a video case report. *Epileptic Disord* 2009; 11: 267-9. doi: 10.1684/epd.2009.0268.

Meador KJ, Moser E. Negative seizures. *J Int Neuropsychol Soc* 2000; 6: 731-3.

Reiher J, Rivest J, Grand'Maison F, Leduc CP. Periodic lateralized epileptiform discharges with transitional rhythmic discharges: association with seizures. *Electroencephalogr Clin Neurophysiol* 1991; 78: 12-7.

Takahashi Y, Mine J, Kubota Y, Yamazaki E, Fujiwara T. A substantial number of Rasmussen syndrome patients have increased IgG, CD4+ T cells, TNF alpha, and Granzyme B in CSF. *Epilepsia* 2009; 50: 1419-31.

Westmoreland BF, Klass DW, Sharbrough FW. Chronic periodic lateralized epileptiform discharges. *Arch Neurol* 1986; 43: 494-6.

Case Reports in Neurology

Case Rep Neurol 2014;6:291–296

DOI: 10.1159/000371442
Published online: December 24, 2014

1662–680X/14/0063–0291$39.50/0
www.karger.com/crn

Karger Open access

Anti-Glutamate ε2 Receptor Antibody-Positive and Anti-N-Methyl-D-Aspartate Receptor Antibody-Negative Lobar Encephalitis Presenting as Global Aphasia and Swallowing Apraxia

Yuki Hayata[a] Kensuke Hamada[a] Yasuhisa Sakurai[a] Izumi Sugimoto[a]
Toru Mannen[a] Yukitoshi Takahashi[b]

[a]Department of Neurology, Mitsui Memorial Hospital, Tokyo, and [b]National Epilepsy Center, Shizuoka Institute of Epilepsy and Neurological Disorders, Shizuoka, Japan

Key Words
Anti-glutamate receptor antibodies · Aphasia · Lobar encephalitis · N-methyl-D-aspartate receptor encephalitis · Swallowing apraxia

Abstract
Background: Little is known about the difference between anti-N-methyl-D-aspartate receptor (NMDAR) antibody-positive encephalitis and anti-glutamate receptor (GluR) antibody-positive encephalitis. ***Objectives:*** To characterize anti-GluR antibody-positive encephalitis. ***Methods:*** We report a 33-year-old man with nonparaneoplastic anti-GluR ε2, ζ1 and δ2 antibody-positive and anti-NMDAR antibody-negative encephalitis, using neuropsychological tests and imaging studies including magnetic resonance imaging and single photon emission computed tomography (SPECT) with a ^{99m}Tc-ethylcysteinate dimer. ***Results:*** The patient exhibited global aphasia and swallowing apraxia (inability to transfer food to the pharyngeal cavity without sialorrhea). He was treated with 3 courses of corticosteroid pulse therapy and had recovered markedly 3 weeks after onset. Magnetic resonance diffusion-weighted images revealed hyperintensity in the bilateral frontal and left parietal cortices. Seven months later, a small area of hyperintensity in the left supramarginal gyrus remained. SPECT revealed hypoperfusion in extensive regions of the bilateral frontal lobes and left supramarginal gyrus. Thirteen months later, blood flow reduction was restricted to diffuse

Yasuhisa Sakurai, MD, PhD
Department of Neurology
Mitsui Memorial Hospital
1, Kanda-Izumi-cho, Chiyoda-ku, Tokyo 101-8643 (Japan)
E-Mail ysakurai-tky@umin.ac.jp

KARGER

Case Rep Neurol 2014;6:291–296
DOI: 10.1159/000371442

areas in the frontal lobes. ***Conclusions:*** Frontal lobar encephalitis without medial temporal involvement, marked cognitive impairment with a relatively preserved level of consciousness, and a favorable response to corticosteroid therapy, with nearly reversible cortical damage, may characterize anti-GluR antibody-positive encephalitis.

Introduction

Autoimmune encephalitis associated with antibodies against N-methyl-D-aspartate-type glutamate receptors (anti-NMDAR encephalitis) was first reported by Dalmau et al. [1] in 2007. Clinical features are acute limbic encephalitis with personality and behavioral change, catatonia, memory loss, seizures, dyskinesia, and autonomic dysfunction [2–5]. The disease most often involves young women and accompanies ovarian teratoma. However, 40% of patients do not have a detectable tumor, and men are also affected [2]. The clinical manifestation of nonparaneoplastic anti-NMDAR encephalitis (without ovarian teratoma) is similar to that of paraneoplastic anti-NMDAR encephalitis (with ovarian teratoma) [6]. In many cases, the medial temporal lobes and cerebral cortex are affected on MRI [2]. On the other hand, patients with anti-glutamate receptor (GluR) ε2 (NR2B) antibody have been reported to show an association with nonherpetic acute limbic encephalitis (NHALE), Rasmussen's encephalitis, and chronic forms of epilepsia partialis continua [7, 8]. Although anti-NMDAR antibody and anti-GluR ε2 antibody could be simultaneously detected in NHALE [6, 9], some patients had anti-NMDAR antibody but did not have anti-GluR antibody [10], and others vice versa [11]. Little is known about the clinical difference between anti-NMDAR antibody-only encephalitis and anti-GluR antibody-only encephalitis.

We herein report a man with anti-GluR antibody but without anti-NMDAR antibody, presenting with predominantly frontal lobar encephalitis, and discuss the clinical significance of this type of non-paraneoplastic anti-GluR encephalitis.

Case Report

A left-handed, 33-year-old man, an office worker who had graduated from university, presented with progressive speech disturbance in February 2013. He was noted to have difficulty finding words in the office and made grammatical errors on writing e-mails. He was referred to and admitted to our hospital 2 days after disease onset. On neurological examination, the patient exhibited fluctuating consciousness disturbance and difficulty saying words: he could only say 'yes'. He did not obey some simple verbal commands such as eye closing or tongue protrusion. When asked to perform dictation, he repeatedly wrote down a character that was a part of our oral command. He was diagnosed with global aphasia. Once he had raised his arms, he kept them raised until we forced him to stop (catalepsy). No neck stiffness or Kernig's sign was noted.

Laboratory findings were unremarkable. Lumbar puncture showed cerebrospinal fluid (CSF) lymphocytic pleocytosis (cells: 51/mm^3, protein: 35 mg/dl), and an elevated IgG index (1.32, normal <0.7). HSV-IgM, HZV-IgM, and HIV antibodies were all negative. Antibodies to N-terminals of NMDA-type GluR including GluN2B (ε2, NR2B) and GluN1 (ζ1, NR1) [7], and those to the N-terminal of GluD2 (δ2) were all positive, and the antibody to the NMDAR NR1/NR2 complex (Dalmau's method) [1] was negative in both the CSF and serum. Magnetic resonance imaging (MRI) performed 2 weeks after onset revealed hyperintensity in diffusion-weighted images in the bilateral frontal and left parietal cortices (fig. 1). Electroen-

cephalography (EEG) showed diffuse semirhythmic 1- to 2-Hz δ waves with a small amount of 10-Hz α waves superimposed on the δ waves in P-O, which was similar to the 'extreme delta brush' in anti-NMDAR encephalitis [12].

On admission, the patient was able to take meals. However, oral intake decreased gradually, and he could not swallow purposely at 6 days after onset. Even if the patient put water in his mouth, the swallowing reflex did not occur, and water finally leaked out through the corners of his mouth. Although sialorrhea was not noted, he choked on his saliva at 10 days after onset, suggesting that voluntary oral transport of the trapped saliva was interrupted. As a result, he was dehydrated and required an intravenous drip. The patient exhibited bulging eyes, became mute and inattentive (he sometimes turned his eyes away while the doctor talked to him), with forced grasping that was more pronounced in the left hand. He was restless in the evening, and tried to leave the bed despite the infusion tube, and so required sedation with intravenous haloperidol. Suspecting herpetic encephalitis, we first administered intravenous acyclovir (1,500 mg/day) from 5 to 7 days after onset, which was ineffective and discontinued because of drug-induced acute renal injury. From 7 to 9 days after onset, he developed a transient partial seizure on the right side of his face and extremities.

Taking the possibility of an autoimmune mechanism into account, we then administered a total of 3 courses of intravenous corticosteroid pulse therapy (methylprednisolone at 1,000 mg/day for 3 days) from 10 to 25 days after onset, and subsequently gave him oral prednisolone at 30 mg/day that was tapered and discontinued for 6 weeks. Immediately after the first pulse therapy, the patient was able to repeat a syllable following the doctor's example and responded properly to yes-no questions, such as 'Are you Mr. (patient's name)?' He was able to take meals by himself 16 days after onset and spoke some sentences correctly 20 days after onset. EEG performed 23 days after onset showed a moderate amount of 10-Hz α waves with occasional 6- to 7-Hz θ waves.

SPECT with a ^{99m}Tc-ethylcysteinate dimer (ECD-SPECT) performed 3 weeks after onset revealed, for the mean cerebral blood flow (CBF), a reduced blood flow in both hemispheres [early picture (EP) method, left 32.8, right 32.2 ml/100 g/min] with the Patlak plot method and, for the regional CBF, significant hypoperfusion (uncorrected $p < 0.001$, by Statistical Parametric Mapping version 2) in the bilateral frontal convexity and mesial frontal gyri, and the left supramarginal gyrus (fig. 2).

Neuropsychological Assessment

The patient's cognitive function was evaluated for the first time 21 days after onset. The Mini-Mental State Examination (MMSE) score was 24.7/30: mental arithmetic (serial 7) and auditory comprehension (3-step command) were impaired. The Frontal Assessment Battery (FAB) score was 13/18. The Western Aphasia Battery (Japanese edition) conducted 27 days after onset revealed that spontaneous speech was dysfluent (5/10), with stuttering, halting, and occasional phonemic paraphasia and phonetic distortion (e.g., [handan] → [hannan]), suggesting slight apraxia of speech. Auditory comprehension (9.35/10), repetition (9.2/10), naming (9.3/10), reading (9.2/10), and writing (9.85/10) were minimally impaired. Overall, his language profile was rated as slight Boca's aphasia with apraxia of speech. The Wechsler Adult Intelligence Scale-III conducted 4 weeks after onset revealed a nearly normal cognitive function: verbal IQ 84, performance IQ 80, and working memory 76. The digit span forward score was 4. The Wechsler Memory Scale-Revised conducted 33 days after onset revealed a normal memory function (verbal memory 110, visual memory 101). However, retrograde amnesia for the 3 weeks from onset to recovery remained.

The patient returned to work 2 months after onset. The MMSE score at this time was 29/30, the FAB score was 16/18 with word fluency of 7 words/min. Stuttering, halting speech, phonemic paraphasia, and phonetic distortion disappeared 3 months after onset. However, the working memory remained lower (digit span forward, 5) after 1 year. MRI performed 7 months after onset revealed a small area of hyperintensity in the left parietal cortex (fig. 1). In the follow-up SPECT performed 13 months after onset, the reduced blood flow recovered to the normal range (EP, left 46.9, right 46.9 ml/100 g/min) and the regional hypoperfusion was restricted to the diffuse areas of the frontal lobe (fig. 2).

Discussion

The patient presented with global aphasia, swallowing disturbance, abnormal behavior [catalepsy (maintaining a forced posture) and nocturnal delirium], and partial seizure. Global aphasia was characterized by scanty speech and motor perseveration in writing, which resolved to apraxia of speech a few days after corticosteroid pulse therapy.

The swallowing disturbance was voluntary in nature: he had difficulty transporting food to the pharyngeal cavity. In contrast, he swallowed saliva automatically; therefore, he did not exhibit sialorrhea. This automatic-voluntary dissociation in swallowing is characteristic of apraxia. It was clear that the patient did not initiate bolus transfer with a lack of lingual movement during the oral stage. Therefore, the symptom can be diagnosed as swallowing apraxia [13].

It is noteworthy that our patient presented with frontal lobar encephalitis. As described earlier, nonherpetic anti-NMDAR encephalitis usually involves the limbic cortex and is associated with several psychiatric symptoms [2–5]. On the other hand, the clinical features of anti-GluR antibody-positive encephalitis (GluR encephalitis) are similar to those of NMDAR encephalitis with ovarian tumor, except that paraneoplastic NMDAR encephalitis necessitates a longer hospitalization period [6]. It is suggested that in these patients with GluR encephalitis, anti-NMDAR antibody was also positive.

One problem is that in many reported cases of nonparaneoplastic anti-GluR encephalitis, anti-NMDAR antibody was not examined. Thus, the clinical difference between anti-NMDAR encephalitis and anti-GluR encephalitis remains unknown. It is suggested that our patient with lobar encephalitis without medial temporal involvement, marked cognitive impairment with a relatively preserved level of consciousness, and a favorable response to corticosteroid therapy, with nearly reversible cortical damage, characterizes anti-GluR antibody-only encephalitis.

It should also be noted that the lesion was difficult to detect on MRI, whereas the extent of the lesion was easily identifiable on SPECT. This discrepancy suggests that neuronal damage was too mild to produce cytotoxic edema, and only a small area of the left supramarginal gyrus remained permanently injured. This reversible cortical damage may be another characteristic of anti-GluR antibody-only encephalitis.

Disclosure Statement

The authors declare that they have no conflict of interest.

References

1 Dalmau J, Tuzun E, Wu HY, Masjuan J, Rossi JE, Voloschin A, Baehring JM, Shimazaki H, Koide R, King D, et al: Paraneoplastic anti-N-methyl-D-aspartate receptor encephalitis associated with ovarian teratoma. Ann Neurol 2007;61:25–36.
2 Dalmau J, Gleichman AJ, Hughes EG, Rossi JE, Peng X, Lai M, Dessain SK, Rosenfeld MR, Balice-Gordon R, Lynch DR: Anti-NMDA-receptor encephalitis: case series and analysis of the effects of antibodies. Lancet Neurol 2008;7:1091–1098.
3 Dalmau J, Lancaster E, Martinez-Hernandez E, Rosenfeld MR, Balice-Gordon R: Clinical experience and laboratory investigations in patients with anti-NMDAR encephalitis. Lancet Neurol 2011;10:63–74.
4 Irani SR, Bera K, Waters P, Zuliani L, Maxwell S, Zandi MS, Friese MA, Galea I, Kullmann DM, Beeson D, et al: N-methyl-D-aspartate antibody encephalitis: temporal progression of clinical and paraclinical observations in a predominantly non-paraneoplastic disorder of both sexes. Brain 2010;133:1655–1667.
5 Titulaer MJ, McCracken L, Gabilondo I, Armangue T, Glaser C, Iizuka T, Honig LS, Benseler SM, Kawachi I, Martinez-Hernandez E, et al: Treatment and prognostic factors for long-term outcome in patients with anti-NMDA receptor encephalitis: an observational cohort study. Lancet Neurol 2013;12:157–165.
6 Takahashi Y, Yamazaki E, Nishimura S, Tsunogae H, Niwa K, Dalmau J, Imai K, Fujiwara T: Acute limbic encephalitis and NMDA type-glutamate receptor (in Japanese). Rinsho Shinkeigaku 2008;48:926–929.
7 Takahashi Y, Kubota Y, Yamasaki E, Matsuda K: Rasmussen encephalitis and non-herpetic acute limbic encephalitis (in Japanese). Rinsho Shinkeigaku 2008;48:163–172.
8 Takahashi Y, Mori H, Mishina M, Watanabe M, Kondo N, Shimomura J, Kubota Y, Matsuda K, Fukushima K, Shiroma N, et al: Autoantibodies and cell-mediated autoimmunity to NMDA-type GluRε2 in patients with Rasmussen's encephalitis and chronic progressive epilepsia partialis continua. Epilepsia 2005;46(suppl 5):152–158.
9 Takahashi Y: Epitope of autoantibodies to N-methyl-D-aspartate receptor heteromers in paraneoplastic limbic encephalitis. Ann Neurol 2008;64:110–111, author reply 111–112.
10 Ishiura H, Matsuda S, Higashihara M, Hasegawa M, Hida A, Hanajima R, Yamamoto T, Shimizu J, Dalmau J, Tsuji S: Response of anti-NMDA receptor encephalitis without tumor to immunotherapy including rituximab. Neurology 2008;71:1921–1923.
11 Yamamoto F, Yamaguchi T, Tamaoka A: Case report; a case of anti-glutamate receptor antibody positive limbic encephalitis with positive various autoantibodies (in Japanese). Nihon Naika Gakkai Zasshi 2013;102:2057–2059.
12 Schmitt SE, Pargeon K, Frechette ES, Hirsch LJ, Dalmau J, Friedman D: Extreme delta brush: a unique EEG pattern in adults with anti-NMDA receptor encephalitis. Neurology 2012;79:1094–1100.
13 Logemann JA: Evaluation and Treatment of Swallowing Disorders, ed 2. Austin, Pro-Ed, 1998.

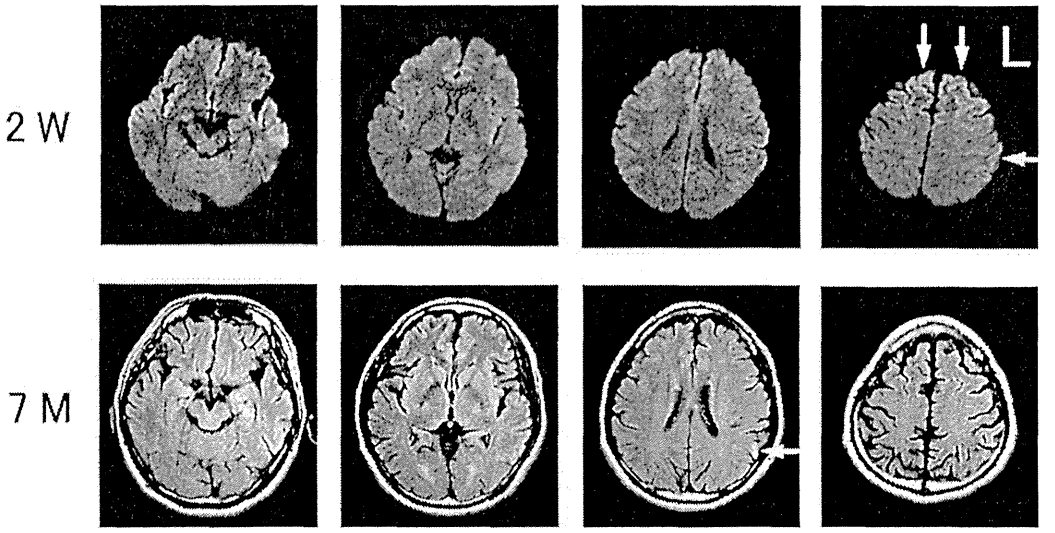

Fig. 1. MRI 2 weeks after onset (2 W) and 7 months later (7 M). Diffusion-weighted axial imaging performed 2 weeks after onset (upper panels) revealed hyperintensity in the bilateral frontal and left parietal cortices (arrows). Fluid-attenuated inversion recovery axial images obtained 7 months after onset (lower panels) revealed a high signal intensity in a small area of the left parietal cortex (arrow).

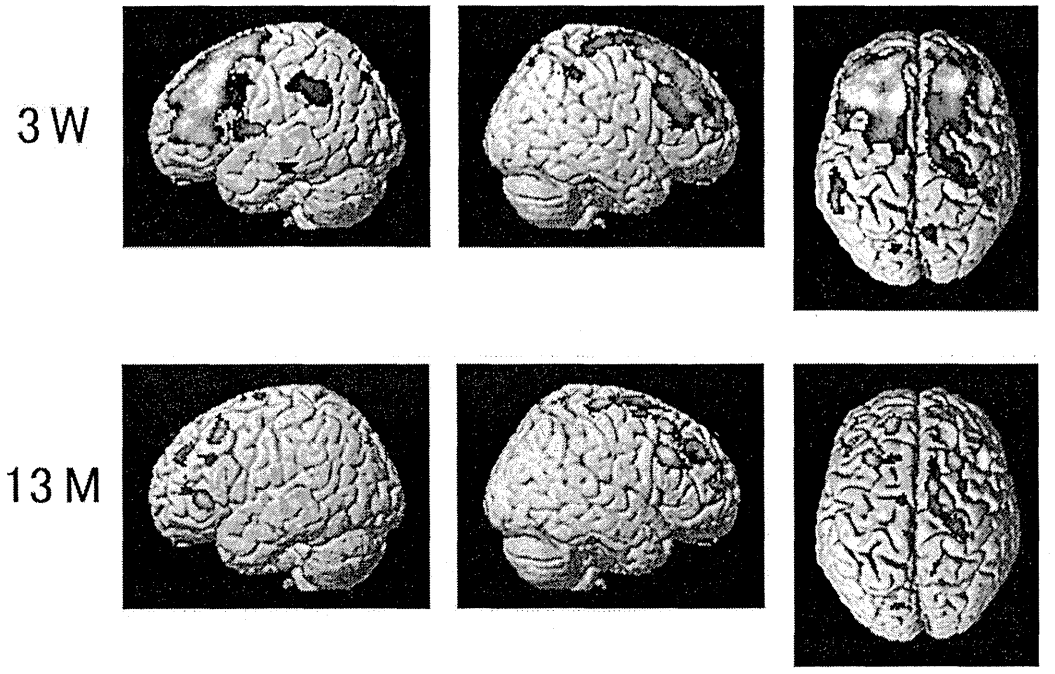

Fig. 2. ^{99m}Tc-ECD-SPECT images 3 weeks (3 W) and 13 months (13 M) after onset. ^{99m}Tc-ECD-SPECT using a 2-sample t test [patient vs. healthy subjects aged between 20 and 39 years (n = 28), uncorrected $p < 0.001$] in Statistical Parametric Mapping version 2 revealed hypoperfusion in the bilateral frontal convexity, mesial frontal gyri, and left supramarginal gyrus 3 weeks after onset (upper panels). Regional blood flow reduction was restricted to diffuse areas in the frontal lobes 13 months later (lower panels).

ELSEVIER

Brain & Development 36 (2014) 645–652

BRAIN & DEVELOPMENT
Official Journal of the Japanese Society of Child Neurology

www.elsevier.com/locate/braindev

Review article

Anti-NMDAR autoimmune encephalitis

Kazushi Miya [a], Yukitoshi Takahashi [b], Hisashi Mori [c,*]

[a] *Department of Pediatrics, Graduate School of Medicine and Pharmaceutical Sciences, University of Toyama, Toyama 930-0194, Japan*
[b] *Division of Pediatrics, Shizuoka Institute of Epilepsy and Neurological Disorders, Shizuoka 420-8688, Japan*
[c] *Department of Molecular Neuroscience, Graduate School of Medicine and Pharmaceutical Sciences, University of Toyama, Toyama 930-0194, Japan*

Received 14 August 2013; received in revised form 7 October 2013; accepted 10 October 2013

Abstract

The *N*-methyl-D-aspartate receptor (NMDAR) is involved in normal physiological and pathological states in the brain. Anti-NMDAR encephalitis is characterized by memory deficits, seizures, confusion, and psychological disturbances in males and females of all ages. This type of encephalitis is often associated with ovarian teratoma in young women, but children are less likely to have tumors. Anti-NMDAR encephalitis is a neuroimmune syndrome in patients with autoantibodies recognizing extracellular epitopes of NMDAR, and the autoantibodies attenuate NMDAR function through the internalization of NMDAR. Following the initial symptoms of inflammation, the patients show the various symptoms such as memory loss, confusion, emotional disturbances, psychosis, dyskinesis, decrease in speech intelligibility, and seizures. About half of these patients improved with immunotherapy including high-dose intravenous corticosteroids and intravenous immunoglobulins is administrated to these patients, but the patients who had no improvement with these therapy require further treatments with rituximab or cyclophosphamide. It is necessary to detect anti-NMDAR antibodies at early stages, because the prognosis of these patients may be improved by early treatment. Recovery is slow, and the patients may have some disturbances in their motor function and cognition. The pathologic mechanism underlying the development of anti-NMDAR encephalitis has been elucidated gradually, but the optimal treatment has not yet been clarified. Further studies are required to clarify in detail the mechanism underlying anti-NMDA encephalitis and to develop effective treatments.

Keywords: Encephalitis; *N*-Methyl-D-aspartate receptor; Autoantibody

1. Introduction

The *N*-methyl-D-aspartate receptor (NMDAR) is critically involved in normal neural network formation, synaptic plasticity, and higher brain functions such as learning and memory [1,2]. A highly active NMDAR is composed of multiple glutamate-binding GluRε (NR2, GluN2) subunits and a glycine/D-serine-binding GluRζ1 (NR1, GluN1) subunit [3]. The hyperactivation of NMDAR has been shown to mediate acute neuronal death and chronic neurodegeneration [4]. In contrast, the hypoactivation of NMDAR is involved in the development of psychiatric states [5,6]. The NMDAR subunits are widely distributed throughout the brain including the limbic system. In situ hybridization analyses, the GluRζ1 (NR1, GluN1) subunit mRNA distributes ubiquitously in the brain. The GluRε1 (NR2A, GluN2A) subunit mRNA is expressed postnatally and widely in the brain. The GluRε2 (NR2B, GluN2B) subunit mRNA is found throughout the entire embryonic brain, but its expression becomes restricted to the forebrain at postnatal stages. The GluRε3 (NR2C,

* Corresponding author. Address: Department of Molecular Neuroscience, Graduate School of Medicine and Pharmaceutical Sciences, University of Toyama, Sugitani 2630, Toyama 930-0194, Japan. Tel.: +81 76 434 7230; fax: +81 76 434 5015.
E-mail address: hmori@med.u-toyama.ac.jp (H. Mori).

0387-7604/$ - see front matter © 2013 The Japanese Society of Child Neurology. Published by Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.braindev.2013.10.005

GluN2C) subunit mRNA appears postnatally and predominantly in the cerebellum. The GluRε4 (NR2D, GluN2D) subunit mRNA is abundantly expressed in the diencephalon and the brainstem at embryonic and neonatal stages [3].

Limbic encephalitis is an inflammation of the limbic system, which includes the hippocampus, thalamus, hypothalamus, and amygdala. The symptoms of limbic encephalitis are memory deficits, seizures, confusion, and psychological disturbances. In 1960, the disease was first described as subacute encephalitis affecting the limbic areas by Brierley et al. [7]. Subsequently, it was mainly reported as paraneoplastic limbic encephalitis associated with lung carcinoma and malignancies in the ovary, breast, stomach, uterus, kidney, bladder, and colon [8]. The report suggested that limbic encephalitis is caused by autoimmunity against limbic system antigens, similarly to Eaton–Lambert syndrome. In 2001, Buckley et al. reported the cases of two patients with limbic encephalitis in whom the antibodies to voltage-gated potassium channels (VGKCs) were detected in their serum samples [9]. Later, the true target antigen of the antibodies to VGKCs has been shown to be leucine rich glioma inactivated 1 (LGI1) and contactin associated protein 2 (CASPR2) [10]. Furthermore, anti-NMDAR NR2 subunit autoantibodies were detected in some patients with acute encephalitis including those with limbic encephalitis in 2003 [11]. From these reports, the role of immunity and inflammatory processes in epilepsy or encephalitis became the focus of interest.

In 2007, the concept that anti-NMDAR encephalitis associated with ovarian teratoma, a severe, potentially lethal, treatment-responsive disorder, is mediated by autoantibodies against NMDAR was proposed by Dalmau et al. [12]. However, an increasing number of cases have been reported for both men and women from children to adults of advanced age, with and without tumors [13–18]. Recently, the spectrum of the neuroautoimmune syndromes has greatly expanded by the discovery of new antigen-specific antibodies. These syndromes are suggested to categorize (1) classical paraneoplastic syndromes associated with antibodies to intracellular antigens such as Hu, Ma2, collapsin-responsive mediator protein-5 (CRMP5), Yo or amphiphysin and (2) autoimmune encephalitis associated with antibodies to cell surface or synaptic antigens such as NMDAR, gamma aminobutyric acid receptor (GABAR-B), alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR), LIG1 or CASPR2, based on the underlying immunopathogenesis and responsiveness to immunotherapy [19,20]. In this report, we present the case of a girl with anti-NMDAR encephalitis and review the clinical presentations, diagnosis, and evidence supporting autoimmune mechanisms of this syndrome.

2. Case presentation

A 7-year-old previously healthy Japanese girl had a cough and low-grade fever. 5 days later, she sought her mother frequently and complained of anxiety. A week later, she was brought to our hospital owing to

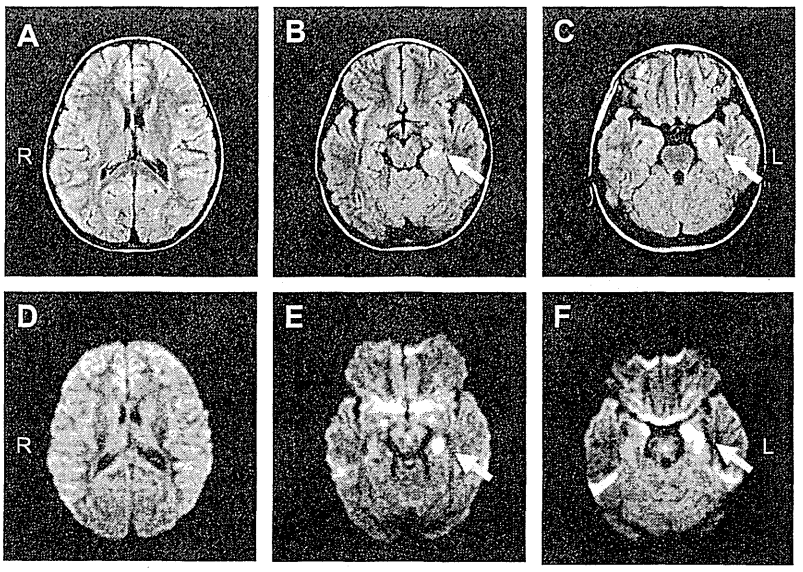

Fig. 1. MR images of a patient with anti-NMDAR encephalitis. Axial FLAIR images (panels A–C) and diffusion-weighted (DW) images (panels D–F) of a 7-years-old girl with anti-NMDAR encephalitis. Arrows in panels B, C, E, and F show hyperintensities in the left medial temporal lobe on FLAIR and DW images.

vomiting and her state of confusion. She had a temperature of 38.0 °C without focal neurologic deficits or meningeal signs. Her white blood cell count and serum C reactive protein level were slightly elevated. Cerebrospinal fluid (CSF) analysis revealed a lymphocytic pleocytosis of 296 nucleated cells/mm^3 with 90% lymphocytes and normal glucose and protein levels. Treatments with acyclovir and dexamethasone were started for presumed viral encephalitis. Her blood and CSF bacterial cultures showed negative results. Viral cultures and polymerase chain reaction analysis of CSF for herpes simplex virus also showed negative results. Thus, these treatments were stopped. Brain computerized tomography (CT) and magnetic resonance imaging (MRI) findings were normal. Electroencephalography (EEG) revealed diffuse slowing of waves, but no epileptic discharges.

Two days after admission, her state of confusion worsened and she spoke incomprehensible words, "blue, blue, blue" or "green, green, green" to her parents. She was treated with intravenous methylprednisolone, which did not improve her neurological states. She refused to take anything by mouth, necessitating nutritional support using a nasogastric tube. She demonstrated orofacial dyskinesias and involuntary movements of the right upper extremity on arousal. A week later, a second brain MRI with fluid attenuation inversion recovery (FLAIR) and diffusion-weighted imaging (DWI) revealed hyperintensities in the left medial temporal lobe (Fig. 1). A course of intravenous immunoglobulins (2 g/kg) was completed with no response. She began to develop symptoms of dyskinesias, stereotyped motor automatisms, and spastic rigidity. We had to administer sedatives and antipsychotic medications, because she was awake during nighttime and slept during daytime and her serum creatinine kinase level increased owing to her involuntary movements.

Two months later, she showed gradual improvements in her motor and cognitive functions. 3 months after her admission, she could take food by mouth and walk a short distance by herself. After her discharge, her serum was found to be positive for anti-NMDAR antibodies (Fig. 2) and we diagnosed her as having anti-NMDAR encephalitis. Presently, she goes to school cheerfully, but has some cognitive problems, such as mild memory disturbance and learning disabilities.

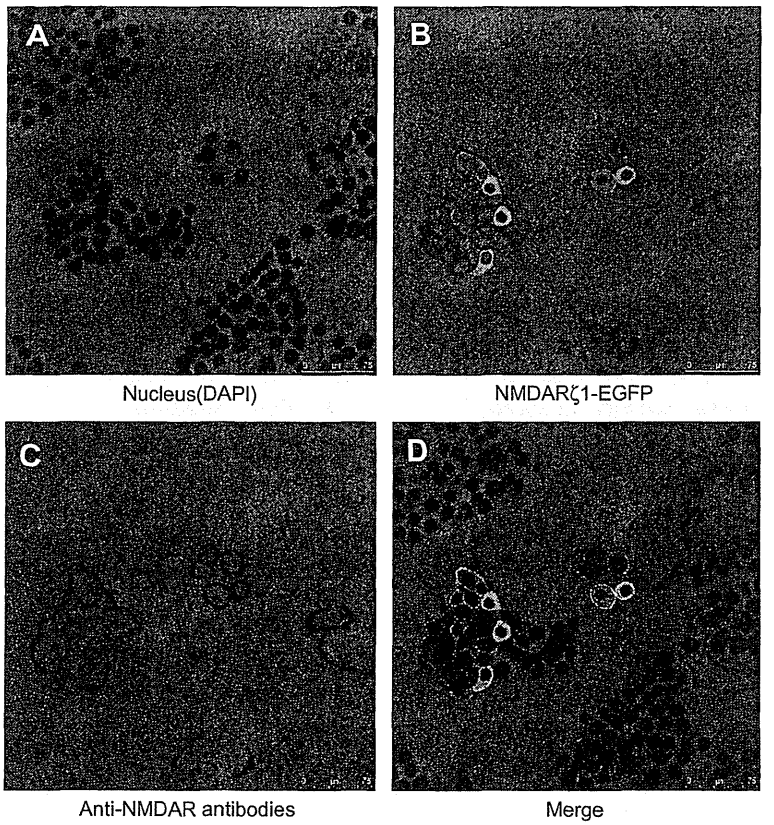

Fig. 2. Immunostaining of HEK 293T cells transfected with NMDAR ζ1-EGFP/ε2 subunits with serum from the 7-years-old girl with the anti-NMDAR encephalitis. Signals of nuclei DAPI (blue, A), fluorescence signals of NMDAR ζ1-EGFP (green, B), and immunofluorescence signals of autoantibodies in serum (magenta, C) from the patient. Panel D shows the merged image of these signals. Scale bar: 75 μm.

3. Clinical features

The exact incidence of anti-NMDAR encephalitis is unclear. The antibodies to NMDAR were detected in 4% of patients with encephalitis by a multicenter, population-based prospective study in England [21]. In another study, anti-NMDAR antibodies were detected in 1% of patients with encephalitis of unknown etiology admitted to the intensive care unit [15]. In a study in the United Kingdom and Europe, 11.1% of patients suspected of having encephalitis were positive for anti-NMDAR antibodies [22]. In the California Encephalitis Project, 4% of patients with encephalitis of uncertain etiology aged <30 years were positive for anti-NMDAR antibodies [23]. From these studies, anti-NMDAR encephalitis is not rare, and may be misdiagnosed as an unidentified encephalitis or a psychiatric disorder [16,24–26]. Initially described as paraneoplastic encephalitis associated with ovarian teratoma, young female patients often had ovarian teratoma [12,17,22]. The other tumors were sex-cord stromal tumor, neuroendocrine tumor, teratoma of the mediastinum, small cell lung cancer, and lymphoma [17,18,22]. Recent reports of the expression of the NMDAR in some tumors and its role in tumor invasion are interesting [27–29]. Many cases in children are less likely to have tumors [13,14,18]. Anti-NMDAR encephalitis commonly occurs in young females, but has been reported in males and females of all ages (from 8 months to 85 years) [13,16,18,22].

Prodromal symptoms such as fever, headache, upper respiratory symptoms, vomiting, and diarrhea are observed in 48–86% of patients within 2 weeks before hospital admission [14,17,30]. The initial symptoms of anti-NMDAR encephalitis are evenly distributed between psychotic and neurologic. However, the severity and sequence of the symptoms such as memory loss, confusion, emotional disturbances, psychosis (delusions and hallucinations), dyskinesis, decrease in speech intelligibility, and seizures vary [14,17,18]. During the course of the disorder, 76–77% of patients have seizures, most commonly tonic–clonic seizures [14,17]. Patients presenting with psychosis are often treated with antipsychotic agents [16,24–26]. Sequentially, dyskinesis (especially orofacial), involuntary movement, spastic rigidity, echolalia, ataxia, refractory seizures, and decreased level of consciousness are observed [14,17,30,31]. Some patients develop drastic involuntary movements and spastic rigidity, and have high levels of creatine kinase [17,31]. Days–weeks later, autonomic instability often causes cardiac arrhythmia, hypotension, and central hypoventilation, requiring intubation or pacemakers [14,17]. Patients show gradual improvement in motor and cognitive functions. The median duration of hospitalization is in the range of 2–2.5 months (range, 1–14 months) [17,30].

Results of conventional investigations including examination of CSF, brain imaging, and EEG are nonspecific for anti-NMDAR encephalitis. CSF analysis revealed lymphocytic pleocytosis in many cases (68–91%), oligoclonal banding and increased CSF protein level within the first few days after the onset of neurological symptoms [17,22,30]. EEG demonstrated epileptic discharge or slowing of waves [17,22,30]. A unique electrographic pattern "Extreme delta brush" may be associated with a more prolonged illness [30,32]. There are some reports that EEG showed generalized rhythmic delta activity with a nonconvulsive status epilepticus [33,34]. In MRI, few patients showed hyperintensities on T2-weighted sequences or FLAIR images of the medial temporal lobes, corpus callosum, or cerebral cortex [17,18,22,30]. The results of brain PET were limited, but all the patients showed abnormal frontotemporal, occipital, and cerebellar hypermetabolism [22,35]. The identification of anti-NMDAR antibodies is critical for the diagnosis of anti-NMDAR encephalitis, because other clinical examination results are nonspecific.

4. Detection of anti-NMDAR antibodies

The laboratory approach to the detection of anti-NMDAR antibodies involves indirect or direct examinations. Indirect immunofluorescence on cryopreserved sections or primary cell cultures of the rodent brain may be a good screening test in patients suspected of having autoimmune encephalitis regardless of having autoantibodies for brain antigens or not [12,22]. The lysates of human embryonic kidney (HEK) cells ectopically expressing NR1 or NR1–NR2 heteromers and the peptide of the NMDAR subunit were used in in vitro enzyme-linked immunosorbent assay (ELISA) [17]. A cell-based assay is an immunoassay of culture cells (i.e., HEK cells) transfected with the complementary DNA (cDNA) representing the single or assembled NR1–NR2 subunits [12,36]. The cell-based assay is a more specific and sensitive evaluation system for detecting autoantibodies recognizing conformational extracellular epitopes of NMDAR [12,36].

Rapid quantitative evaluation systems for detecting autoantibodies against extracellular epitopes of NMDAR are necessary, because paraneoplastic anti-NMDAR encephalitis has a better prognosis after tumor resection and immunotherapy (corticosteroids, intravenous immunoglobulins, or plasma exchange) [12,17,18]. Thus, the establishment of cells stably expressing functional NMDAR is desirable. However, Ca^{2+} influx through NMDAR activated by glutamate and glycine present in a culture medium is toxic to non-neurons [37]. We reported a method to rapidly analyze the presence and function of autoantibodies against NMDAR using cultured cells (HEK293T) that stably expressed mutant NMDAR with decreased Ca^{2+}

permeability on a heterologous cell surface [36]. The level of the anti-NMDAR antibody in serum of the patients is significantly higher than that in the CSF [13,22,38].

5. Treatment and prognosis

A randomized controlled trial of the treatment for anti-NMDAR encephalitis has not been reported. When the patients were diagnosed as having anti-NMDAR encephalitis, the immunotherapy including high-dose intravenous corticosteroids, intravenous immunoglobulins, plasma exchange, cyclophosphamide, azathioprine, mycophenolate mofetil, tacrolimus, methotrexate, and monoclonal antibodies (e.g., rituximab) was used in sequence or in combination [14,17,18]. Although a few patients recovered to their normal state with supportive care alone, most of the patients required further treatments such as tumor resection and immunotherapy [17,18,39]. Thus, Dalmau et al. proposed that the tumor (an ovarian teratoma or a testicular tumor) should be removed when present [39]. When tumor is not present, they prefer the first-line therapy with intravenous immunoglobulins, methylprednisolone, plasma exchange [40],

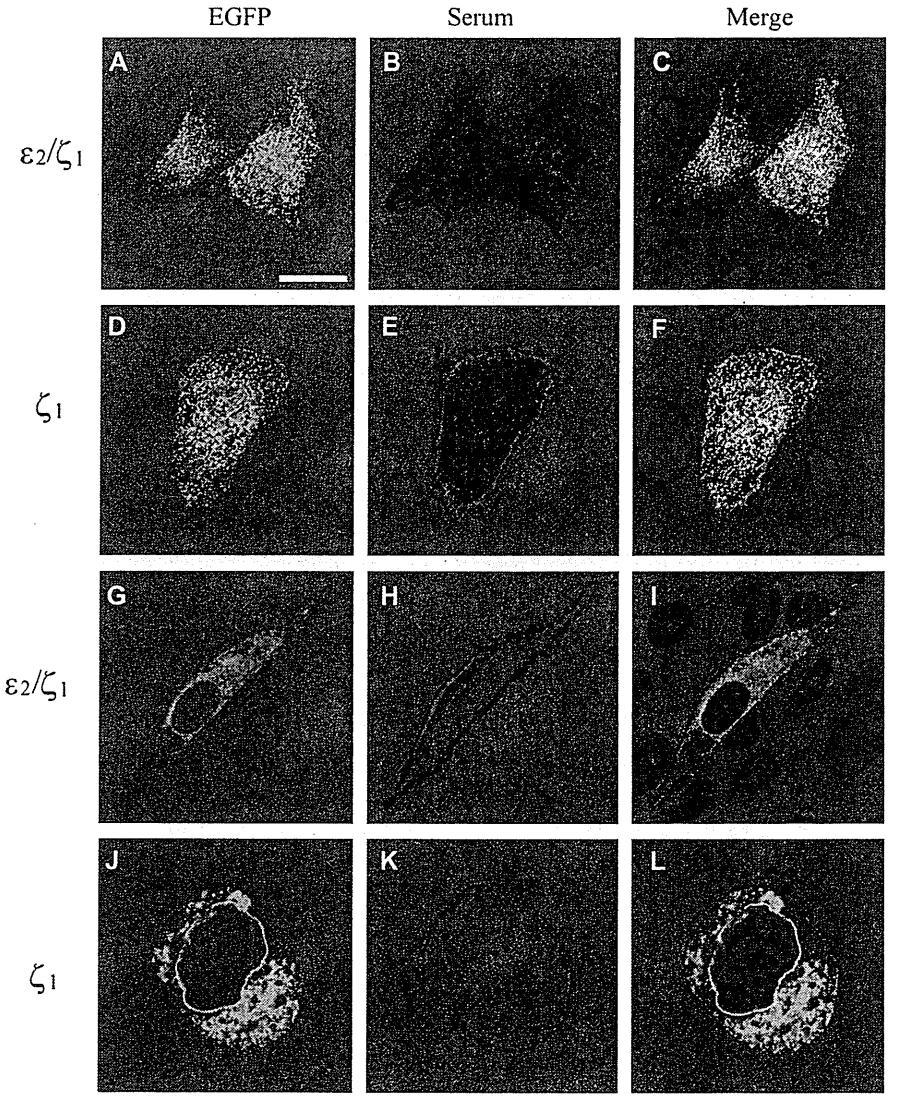

Fig. 3. Internalization of cell surface NMDAR subunits in Chinese hamster ovary (CHO) cells induced by patient's antibodies (reproduced from [36]). The ε2/ζ1-(A–C and G–I) and ζ1- (D–F and J–L) subunit-transfected CHO cells were incubated with the patient's antibodies at 37 °C (A–F) or 4 °C (G–L) then fixed and observed by confocal laser microscopy. The fluorescence signal of EGFP (green; A, D, G, and J) and the immunofluorescence signals of the antibodies in the serum (magenta; B, E, H, and K) samples from patients were detected and merged with the signal of DAPI (blue; C, F, I, and L). Scale bar: 25 μm.

or their combinations. If no response is observed after 10 days, they prefer to start the second-line therapy with rituximab [41] or cyclophosphamide [42]. The patients who did not improve with the first line immunotherapy may have better outcome due to the second line immunotherapy [18]. For patients showing a good response, the treatment shifts to supportive care and tumor surveillance. The level of anti-NMDAR antibodies in CSF and serum usually decreases when patients show substantial clinical recovery [15,17,22,43].

Recovery may take 2 years or longer, and the patient may not always return to their former levels of motor function and cognition [14,17,18,44,45]. In a cohort of 252 patients, 81% experienced complete or near-complete recovery (Modified Rankin scale scores of 1–2) and 10% (14/252) died [18]. Relapses have been reported to occur in 20–30% of patients [14,17,18,22,46], and the occurrence is higher in patients without immunotherapy [18,46]. This finding suggests the benefit of early immune suppression and tumor resection.

6. Pathology

The clinical features of anti-NMDAR encephalitis correspond to the state caused by the change in the activity of NMDAR. Anti-NMDAR encephalitis is considered to be antibody-mediated because anti-NMDAR antibodies are detected in the serum or CSF of most patients, anti-NMDAR encephalitis has a better prognosis after tumor resection and immunotherapy, and antibody levels are related to clinical outcomes [15,17,22,43]. Furthermore, the reversibility of the disorder, irrespective of the duration of symptoms, suggests an immune-mediated neuronal dysfunction rather than irreversible degeneration [17,47].

These features indicate that anti-NMDAR antibodies do not mediate neuronal death by hyperactivation of NMDAR or complement or cytotoxic T-cell mechanisms, but that these antibodies recognize extracellular epitopes of NMDAR and change NMDAR functions. The mechanisms underlying the pathogenic effects were proposed, including attenuation of NMDAR function by internalization and degradation of NMDAR by anti-NMDAR antibodies associated with paraneoplasms, such as ovarian tumors [36,39,47]. The internalization of NMDAR with anti-NMDAR antibodies was suggested by a biochemical study and a study using primary cultured neurons [36,47]. We detected the immunofluorescence signals of autoantibodies in the cytoplasm in addition to the membrane in cells expressing NMDAR (Fig. 3) [36]. The antibodies in patients with anti-NMDAR encephalitis lead to the loss of surface NMDAR by antibody-mediated internalization, resulting in the attenuation of NMDAR function [39,47]. In anti-NMDAR encephalitis, autoantibodies crossreacting with NMDAR are produced against tumors or pathogens (*Mycoplasma pneumonia* [14], influenza viruses A and B, *Chlamydophilia pneumoniae*, *Bordetella pertussis*, *Bordetella parapertussis* [15], and Epstein–Barr virus [48]). Anti-NMDAR antibodies may be produced in subarachnoid space, because the patients with NMDAR encephalitis had significant B-cell expansion in CSF and infiltration of plasma cells around vessels or in CSF [17,49–51]. This intrathecal production of autoantibody may be important factor associated with poor prognosis or resistant to first-line immunotherapy with intravenous immunoglobulins, methylprednisolone, plasma exchange. On the other hand, it is also considered that the leakage of antibodies from vessels into the brain occurs in the patients,

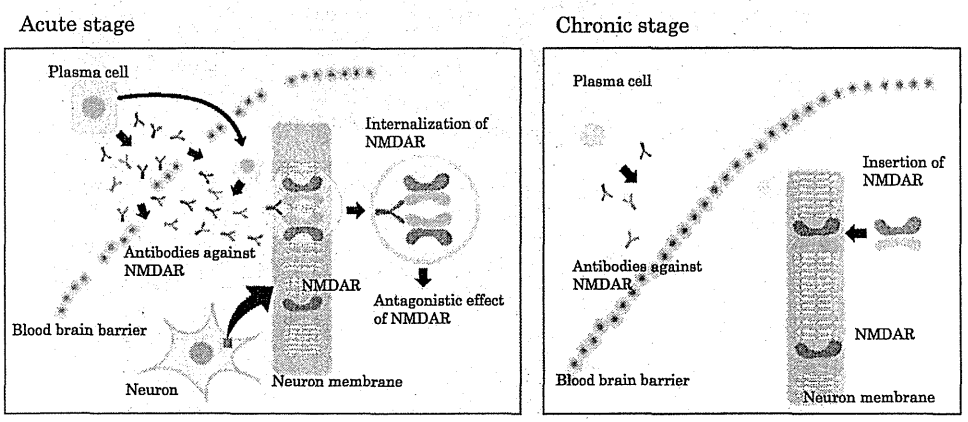

Fig. 4. Schematic models of the possible mechanism underlying the development of anti-NMDAR autoimmune encephalitis. At the acute stage, autoantibodies against NMDAR leak into CSF through the disruption of the blood–brain barrier. Intrathecal production of anti-NMDAR antibodies are also suggested. Anti-NMDAR antibodies induce the internalization of NMDAR. The decrease in the expression level of neuronal surface NMDAR results in neuronal hypoactivity. At the chronic stage, after the level of anti-NMDAR antibodies produced by plasma cells decreases and the blood–brain barrier is restored, the level of anti-NMDAR antibodies in CSF decreases. The NMDAR is expressed on the neuronal surface again, and neuronal functions recovers.

because there are some reports that the anti-NMDAR antibody levels in serum are significantly higher than that in CSF [13,22,38]. Finally, anti-NMDAR antibodies in CSF disrupt the interactions between EPHB2R and NMDARs [52], which results in the attenuation of NMDAR function through the internalization of NMDAR (Fig. 4). At the chronic stage, after the level of anti-NMDAR antibodies produced by plasma cells decreases and the blood–brain barrier is restored, the level of anti-NMDAR antibodies in CSF decreases. The NMDAR is expressed on the neuronal surface again, and neuronal functions recovers (Fig. 4).

7. Conclusion

Anti-NMDAR encephalitis with central nerve or psychiatric symptoms is detected in a significant percentage of patients with acute encephalitis. The early and precise examination for the presence of anti-NMDAR antibodies is necessary, because the prognosis of these patients may be improved by early treatment. The pathologic mechanism underlying the development of anti-NMDAR encephalitis has been elucidated gradually in in vitro studies, but the optimal treatment has not yet been clarified. Further studies are required including those of additional cases and animal models of anti-NMDAR encephalitis to clarify in detail the mechanisms underlying the development of anti-NMDA encephalitis.

Acknowledgments

We thank Dr. Shiho Takano for establishment of cell-based assay detecting anti-NMDAR antibodies. This work was supported by a grant from the Ministry of Health and Labour Sciences Research Grants for Comprehensive Research on Disability Health and Welfare (H24).

References

[1] Bliss TV, Collingridge GL. A synaptic model of memory: long-term potentiation in the hippocampus. Nature 1993;361:31–9.
[2] Komuro H, Rakic P. Modulation of neuronal migration by NMDA receptors. Science 1993;260:95–7.
[3] Mori H, Mishina M. Structure and function of the NMDA receptor channel. Neuropharmacology 1995;34:1219–37.
[4] Lancelot E, Beal MF. Glutamate toxicity in chronic neurodegenerative disease. Prog Brain Res 1998;116:331–47.
[5] Gunduz-Bruce H. The acute effects of NMDA antagonism: from the rodent to the human brain. Brain Res Rev 2009;60:279–86.
[6] Coyle JT, Tsai G, Goff D. Converging evidence of NMDA receptor hypofunction in the pathophysiology of schizophrenia. Ann NY Acad Sci 2003;1003:318–27.
[7] Brierley J, Corsellis J, Hierons R, Nevin S. Subacute encephalitis of later adult life. Mainly affecting the limbic areas. Brain 1960;83:357–68.
[8] Newman NJ, Bell IR, McKee AC. Paraneoplastic limbic encephalitis: neuropsychiatric presentation. Biol Psychiatry 1990;27:529–42.
[9] Buckley C, Oger J, Clover L, Tuzun E, Carpenter K, Jackson M, et al. Potassium channel antibodies in two patients with reversible limbic encephalitis. Ann Neurol 2001;50:73–8.
[10] Irani SR, Alexander S, Waters P, Kleopa KA, Pettingill P, Zuliani L, et al. Antibodies to Kv1 potassium channel-complex proteins leucine-rich, glioma inactivated 1 protein and contactin-associated protein-2 in limbic encephalitis, Morvan's syndrome and acquired neuromyotonia. Brain 2010;133:2734–48.
[11] Takahashi Y, Mori H, Mishina M, Watanabe M, Fujiwara T, Shimomura J, et al. Autoantibodies to NMDA receptor in patients with chronic forms of epilepsia partialis continua. Neurology 2003;61:891–6.
[12] Dalmau J, Tuzun E, Wu HY, Masjuan J, Rossi JE, Voloschin A, et al. Paraneoplastic anti-N-methyl-D-aspartate receptor encephalitis associated with ovarian teratoma. Ann Neurol 2007;61:25–36.
[13] Dale RC, Irani SR, Brilot F, Pillai S, Webster R, Gill D, et al. N-methyl-D-aspartate receptor antibodies in pediatric dyskinetic encephalitis lethargica. Ann Neurol 2009;66:704–9.
[14] Florance NR, Davis RL, Lam C, Szperka C, Zhou L, Ahmad S, et al. Anti-N-methyl-D-aspartate receptor (NMDAR) encephalitis in children and adolescents. Ann Neurol 2009;66:11–8.
[15] Pruss H, Dalmau J, Harms L, Holtje M, Ahnert-Hilger G, Borowski K, et al. Retrospective analysis of NMDA receptor antibodies in encephalitis of unknown origin. Neurology 2010;75:1735–9.
[16] Day GS, High SM, Cot B, Tang-Wai DF. Anti-NMDA-receptor encephalitis: case report and literature review of an under-recognized condition. J Gen Intern Med 2011;26:811–6.
[17] Dalmau J, Gleichman AJ, Hughes EG, Rossi JE, Peng X, Lai M, et al. Anti-NMDA-receptor encephalitis: case series and analysis of the effects of antibodies. Lancet Neurol 2008;7:1091–8.
[18] Titulaer MJ, McCracken L, Gabilondo I, Armangué T, Glaser C, Iizuka T, et al. Treatment and prognostic factors for long-term outcome in patients with anti-NMDA receptor encephalitis: an observational cohort study. Lancet Neurol 2013;12:157–65.
[19] Bien CG, Vincent A, Barnett MH, Becker AJ, Blumcke I, Graus F, et al. Immunopathology of autoantibody-associated encephalitides: clues for pathogenesis. Brain 2012;135:1622–38.
[20] Davis R, Dalmau J. Autoimmunity, seizures, and status epilepticus. Epilepsia 2013;54:46–9.
[21] Granerod J, Ambrose HE, Davies NW, Clewley JP, Walsh AL, Morgan D, et al. Causes of encephalitis and differences in their clinical presentations in England: a multicentre, population-based prospective study. Lancet Infect Dis 2010;10:835–44.
[22] Irani SR, Bera K, Waters P, Zuliani L, Maxwell S, Zandi MS, et al. N-methyl-D-aspartate antibody encephalitis: temporal progression of clinical and paraclinical observations in a predominantly non-paraneoplastic disorder of both sexes. Brain 2010;133:1655–67.
[23] Gable MS, Sheriff H, Dalmau J, Tilley DH, Glaser CA. The frequency of autoimmune N-methyl-D-aspartate receptor encephalitis surpasses that of individual viral etiologies in young individuals enrolled in the California encephalitis project. Clin Infect Dis 2012;54:899–904.
[24] Tsutsui K, Kanbayashi T, Tanaka K, Boku S, Ito W, Tokunaga J, et al. Anti-NMDA-receptor antibody detected in encephalitis, schizophrenia, and narcolepsy with psychotic features. BMC Psychiatry 2012;12:37.
[25] Steiner J, Walter M, Glanz W, Sarnyai Z, Bernstein HG, Vielhaber S, et al. Increased prevalence of diverse N-methyl-D-aspartate glutamate receptor antibodies in patients with an initial

diagnosis of schizophrenia: specific relevance of IgG NR1a antibodies for distinction from N-methyl-D-aspartate glutamate receptor encephalitis. JAMA Psychiatry 2013;70:271–8.
[26] Barry H, Hardiman O, Healy DG, Keogan M, Moroney J, Molnar PP, et al. Anti-NMDA receptor encephalitis: an important differential diagnosis in psychosis. Br J Psychiatry 2011;199:508–9.
[27] Stepulak A, Luksch H, Gebhardt C, Uckermann O, Marzahn J, Sifringer M, et al. Expression of glutamate receptor subunits in human cancers. Histochem Cell Biol 2009;132:435–45.
[28] Li L, Hanahan D. Hijacking the neuronal NMDAR signaling circuit to promote tumor growth and invasion. Cell 2013;153:86–100.
[29] Prickett TD, Samuels Y. Molecular pathways: dysregulated glutamatergic signaling pathways in cancer. Clin Cancer Res 2012;18:4240–6.
[30] Armangue T, Titulaer MJ, Malaga I, Bataller L, Gabilondo I, Graus F, et al. Pediatric anti-N-methyl-D-aspartate receptor encephalitis – clinical analysis and novel findings in a series of 20 patients. J Pediatr 2013;162:850–6.
[31] Turner MR, Irani SR, Leite MI, Nithi K, Vincent A, Ansorge O. Progressive encephalomyelitis with rigidity and myoclonus: glycine and NMDA receptor antibodies. Neurology 2011;77: 439–43.
[32] Schmitt SE, Pargeon K, Frechette ES, Hirsch LJ, Dalmau J, Friedman D. Extreme delta brush: a unique EEG pattern in adults with anti-NMDA receptor encephalitis. Neurology 2012;79:1094–100.
[33] Goldberg EM, Taub KS, Kessler SK, Abend NS. Anti-NMDA receptor encephalitis presenting with focal non-convulsive status epilepticus in a child. Neuropediatrics 2011;42:188–90.
[34] Kirkpatrick MP, Clarke CD, Sonmezturk HH, Abou-Khalil B. Rhythmic delta activity represents a form of nonconvulsive status epilepticus in anti-NMDA receptor antibody encephalitis. Epilepsy Behav 2011;20:392–4.
[35] Morooka M, Kubota K, Minamimoto R, Furuhata M, Abe T, Ito K, et al. 18F-FDG and 11C-methionine PET/CT findings in a case with anti-NMDA (NR2B) receptor encephalitis. Clin Nucl Med 2012;37:400–2.
[36] Takano S, Takahashi Y, Kishi H, Taguchi Y, Takashima S, Tanaka K, et al. Detection of autoantibody against extracellular epitopes of N-methyl-D-aspartate receptor by cell-based assay. Neurosci Res 2011;71:294–302.
[37] Anegawa NJ, Lynch DR, Verdoorn TA, Pritchett DB. Transfection of N-methyl-D-aspartate receptors in a nonneuronal cell line leads to cell death. J Neurochem 1995;64:2004–12.
[38] Suh-Lailam BB, Haven TR, Copple SS, Knapp D, Jaskowski TD, Tebo AE. Anti-NMDA-receptor antibody encephalitis: performance evaluation and laboratory experience with the anti-NMDA-receptor IgG assay. Clin Chim Acta 2013;421:1–6.
[39] Dalmau J, Lancaster E, Martinez-Hernandez E, Rosenfeld MR, Balice-Gordon R. Clinical experience and laboratory investigations in patients with anti-NMDAR encephalitis. Lancet Neurol 2011;10:63–74.
[40] Pham HP, Daniel-Johnson JA, Stotler BA, Stephens H, Schwartz J. Therapeutic plasma exchange for the treatment of anti-NMDA receptor encephalitis. J Clin Apher 2011;26:320–5.
[41] Ikeguchi R, Shibuya K, Akiyama S, Hino S, Kubo H, Takeda T, et al. Rituximab used successfully in the treatment of anti-NMDA receptor encephalitis. Intern Med 2012;51:1585–9.
[42] Kashyape P, Taylor E, Ng J, Krishnakumar D, Kirkham F, Whitney A. Successful treatment of two paediatric cases of anti-NMDA receptor encephalitis with cyclophosphamide: the need for early aggressive immunotherapy in tumour negative paediatric patients. Eur J Paediatr Neurol 2012;16:74–8.
[43] Seki M, Suzuki S, Iizuka T, Shimizu T, Nihei Y, Suzuki N, et al. Neurological response to early removal of ovarian teratoma in anti-NMDAR encephalitis. J Neurol Neurosurg Psychiatry 2008;79:324–6.
[44] Leypoldt F, Gelderblom M, Schottle D, Hoffmann S, Wandinger KP. Recovery from severe frontotemporal dysfunction at 3 years after N-methyl-D-aspartic acid (NMDA) receptor antibody encephalitis. J Clin Neurosci 2013;20:611–3.
[45] Poloni C, Korff CM, Ricotti V, King MD, Perez ER, Mayor-Dubois C, et al. Severe childhood encephalopathy with dyskinesia and prolonged cognitive disturbances: evidence for anti-N-methyl-D-aspartate receptor encephalitis. Dev Med Child Neurol 2010;52:78–82.
[46] Gabilondo I, Saiz A, Galan L, Gonzalez V, Jadraque R, Sabater L, et al. Analysis of relapses in anti-NMDAR encephalitis. Neurology 2011;77:996–9.
[47] Hughes EG, Peng X, Gleichman AJ, Lai M, Zhou L, Tsou R, et al. Cellular and synaptic mechanisms of anti-NMDA receptor encephalitis. J Neurosci 2010;30:5866–75.
[48] Xu CL, Liu L, Zhao WQ, Li JM, Wang RJ, Wang SH, et al. Anti-N-methyl-D-aspartate receptor encephalitis with serum anti-thyroid antibodies and IgM antibodies against Epstein–Barr virus viral capsid antigen: a case report and one year follow-up. BMC Neurol 2011;11:149.
[49] Martinez-Hernandez E, Horvath J, Shiloh-Malawsky Y, Sangha N, Martinez-Lage M, Dalmau J. Analysis of complement and plasma cells in the brain of patients with anti-NMDAR encephalitis. Neurology 2011;77:589–93.
[50] Camdessanche JP, Streichenberger N, Cavillon G, Rogemond V, Jousserand G, Honnorat J, et al. Brain immunohistopathological study in a patient with anti-NMDAR encephalitis. Eur J Neurol 2011;18:929–31.
[51] Dale RC, Pillai S, Brilot F. Cerebrospinal fluid CD19(+) B-cell expansion in N-methyl-D-aspartate receptor encephalitis. Dev Med Child Neurol 2013;55:191–3.
[52] Mikasova L, De Rossi P, Bouchet D, Georges F, Rogemond V, Didelot A, et al. Disrupted surface cross-talk between NMDA and ephrin-B2 receptors in anti-NMDA encephalitis. Brain 2012;135:1606–21.

THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 289, NO. 49, pp. 33904–33915, December 5, 2014

FBXO22 Protein Is Required for Optimal Synthesis of the *N*-Methyl-D-Aspartate (NMDA) Receptor Coagonist D-Serine*

Received for publication, October 12, 2014 Published, JBC Papers in Press, October 21, 2014, DOI 10.1074/jbc.M114.618405

Elena Dikopoltsev[‡], Veronika N. Foltyn[‡], Martin Zehl[§], Ole N. Jensen[¶], Hisashi Mori[‖], Inna Radzishevsky[‡], and Herman Wolosker[‡1]

From the [‡]Department of Biochemistry, Rappaport Faculty of Medicine and Research Institute, Technion-Israel Institute of Technology, Haifa 31096, Israel, the [§]Department of Pharmacognosy, University of Vienna, 1090 Vienna, Austria, the [¶]Department of Biochemistry and Molecular Biology, University of Southern Denmark, 5230 Odense M, Denmark, and the [‖]Department of Molecular Neuroscience, Graduate School of Medicine and Pharmaceutical Sciences, University of Toyama, Toyama 930-0194, Japan

Background: Serine racemase produces D-serine, which is required for optimal NMDA receptor activity.
Results: FBXO22 interacts with and activates serine racemase by preventing its targeting to membranes.
Conclusion: The data suggest an atypical role of FBXO22 in regulating D-serine synthesis unrelated to its effects on the ubiquitin system.
Significance: The results provide a new mechanism affecting D-serine synthesis with implications for the regulation of NMDA receptors.

D-Serine is a physiological activator of NMDA receptors (NMDARs) in the nervous system that mediates several NMDAR-mediated processes ranging from normal neurotransmission to neurodegeneration. D-Serine is synthesized from L-serine by serine racemase (SR), a brain-enriched enzyme. However, little is known about the regulation of D-serine synthesis. We now demonstrate that the F-box only protein 22 (FBXO22) interacts with SR and is required for optimal D-serine synthesis in cells. Although FBXO22 is classically associated with the ubiquitin system and is recruited to the Skip1-Cul1-F-box E3 complex, SR interacts preferentially with free FBXO22 species. *In vivo* ubiquitination and SR half-life determination indicate that FBXO22 does not target SR to the proteasome system. FBXO22 primarily affects SR subcellular localization and seems to increase D-serine synthesis by preventing the association of SR to intracellular membranes. Our data highlight an atypical role of FBXO22 in enhancing D-serine synthesis that is unrelated to its classical effects as a component of the ubiquitin-proteasome degradation pathway.

The NMDA receptor (NMDAR)[2] is a main excitatory receptor in the nervous system and is involved in a wide array of processes, including synaptic plasticity, learning, and memory, and also in neurodegenerative diseases (1). NMDARs display unique regulatory mechanisms requiring binding of glutamate along with a coagonist (glycine or D-serine) for the receptor/channel activation (2). Accumulating evidence demonstrates that D-serine, a D-amino acid present in the mammalian brain, is the major ligand at the coagonist site of the receptors and mediates several NMDAR-dependent processes (3–9).

D-serine is synthesized from L-serine by the enzyme serine racemase (SR) (10, 11). In addition to producing D-serine, SR generates pyruvate by catalyzing the α,β-elimination of water from L-serine (12, 13). SR KO mice display about 90% decrease in brain D-serine and exhibit deficits in NMDAR-dependent synaptic plasticity and spatial learning (14–16). These mice are also resistant to α,β-mediated neurotoxicity *in vivo* and are less susceptible to stroke damage (17, 18), indicating that SR may be involved in neurodegeneration. In this framework, SR inhibitors may provide a novel neuroprotective strategy in neurodegenerative conditions.

Until recently, D-serine was thought to be exclusively released by exocytosis from astrocytes, a type of glia cells that ensheath synapses (4, 6, 19). However, recent data demonstrate that neurons are a main site of D-serine production and storage in the brain and that they regulate their own NMDARs by releasing D-serine (5, 8, 20–24). Astrocytic SR is activated by interaction with a number of proteins, including Grip-1 (25), Pick-1 (26), and Disc-1 (27). Disc-1 binds to and stabilizes SR in glia cells but has no effect on neuronal SR (27). Astrocytic SR is inhibited by interaction with inositol phospholipids and also by *S*-nitrosylation (28, 29). In neurons, NMDAR stimulation promotes translocation of SR from the cytosol (where it normally resides) to the membrane, a process that appears to involve atypical palmitoylation of the enzyme (30). NMDAR-stimulated translocation to the membrane inactivates SR, providing a feedback inhibition of neuronal D-serine synthesis that may work as a failsafe mechanism to prevent NMDAR overactivation in vicinal neurons or synapses (30).

* This work was supported by the Israel Science Foundation, by the Legacy Heritage Fund, by the Allen and Jewel Prince Center for Neurodegenerative Disorders of the Brain, and by the Herbert N. Goldman Medical Research Fund (to H. W.).
[1] To whom correspondence should be addressed: Dept. of Biochemistry, Rappaport Faculty of Medicine and Research Institute, Technion-Israel Institute of Technology, Haifa 31096, Israel. Tel.: 972-4-8295386; Fax: 972-4-8295384; E-mail: hwolosker@tx.technion.ac.il.
[2] The abbreviations used are: NMDAR, NMDA receptor; SR, serine racemase; SCF, Skp1·Cullin·F-box; GNPDA, glucosamine 6-phosphate deaminase; P1, crude nuclear fraction; E16, embryonic day 16; DIV, day(s) *in vitro*; FIST C, F-box intracellular transduction C-terminal domain; CHIP, C terminus of Hsc70-interacting protein.

Here we sought to identify new SR-interacting proteins that might regulate SR. We found that FBXO22, an F-box motif-containing protein (31), interacts with SR and regulates its subcellular localization and activity in cells. The FBXO22 effect on D-serine dynamics is unrelated to its role as a component of the SCF ubiquitin ligase complex. Our data imply a non-canonical role of FBXO22 in regulating D-serine synthesis via changes in SR intracellular targeting.

EXPERIMENTAL PROCEDURES

Materials—DMEM, glutathione-agarose beads, anti-Myc-agarose matrix, red anti-FLAG M2 affinity gel, red anti-HA affinity gel, MG132, rabbit anti c-Myc (catalog no. C3956), mouse anti-c-Myc (catalog no. M4439), mouse anti-β-tubulin (catalog no. T7816), rabbit anti-KDM4A (catalog no. HPA007610), mouse anti-FBXO22 (catalog no. WH0026263M1), and mouse and rabbit anti-FLAG antibodies (catalog nos. F1804 and F7425, respectively) were purchased from Sigma-Aldrich. The A172 glioblastoma cell line was obtained from the ATCC. Mini-complete protease inhibitor mixture was purchased from Roche. [^{35}S]methionine/cysteine was purchased from PerkinElmer Life Sciences. Mouse anti-FBXO22 (catalog no. sc-100736), rabbit anti Cul1 (catalog no. H-213), rabbit anti-HA (catalog no. sc-805), and mouse anti-KDEL (catalog no. sc-58774) were purchased from Santa Cruz Biotechnology. Mouse anti-β-actin (catalog no. 691001) was purchased from MP Biomedicals. Anti-rabbit and anti-mouse peroxidase-conjugated antibodies, anti-rabbit Cy3 or Cy2, anti-mouse Cy3 or Cy2, and normal goat serum were obtained from Jackson ImmunoResearch Laboratories. Mouse anti-HA (catalog no. MMS-101P) was purchased from Covance. Mouse anti-Cul1 (catalog no. C32620) and mouse anti-Skp1 (catalog no. 610530) were from BD Biosciences. Mouse anti-histone H4 (catalog no. ab17036) was obtained from Abcam. Rabbit anti-H3K9me3 (catalog no. 491008) was purchased from Invitrogen.

Basal medium Eagle, minimum essential medium, FBS, penicillin/streptomycin, penicillin/streptomycin/amphotericin, trypsin, and soybean trypsin inhibitor were obtained from Biological Industries (Kibbutz Beit Haemek, Israel). Lipofectamine 2000 was purchased from Invitrogen.

Protein Identification by Mass Spectrometry—Immunoprecipitation of HA-tagged mouse SR (HA-SR) from transfected SH-SY5Y neuroblastoma cells and mass spectrometric analysis of coimmunoprecipitated proteins was performed as described previously (30). Briefly, HA-SR-transfected SH-SY5Y cells were lysed by sonication with 20 mM Tris-HCl (pH 7.4), 1 mM EDTA, 50 mM KCl, protease inhibitor mixture, 2 mM pyrophosphate, 1 mM NaF, and 1 mM orthovanadate. Then Triton X-100 (0.3%) was added to the samples and kept under rotation for 10 min at 4 °C. After removing cell debris by a 10-min centrifugation at 1400 × *g*, the suspension was centrifuged at 200,000 × *g* for 1 h to obtain cytosolic and membrane fractions. Immunoprecipitation of HA-SR from the cytosolic fraction was carried out with anti-HA affinity matrix. The immunoprecipitate was washed extensively for 2 h by seven changes of high-stringency buffer consisting of 50 mM Tris-HCl (pH 7.4), 0.5 M NaCl, 1% Triton X-100, and 0.1% SDS. Following SDS-PAGE, the bands were excised at the 36- to 42-kDa range, and proteins were reduced, carbamidomethylated, and digested in-gel with trypsin (modified sequencing grade from Promega). The tryptic peptides were extracted from the gel and desalted using C18 STAGE tips. Capillary LC-MS/MS analysis was performed on an UltiMate 3000 LC system (Dionex) coupled to an electrospray ionization (ESI)-Q-TOF mass spectrometer (Q-Tof micro, Waters) using a custom-made reverse-phase analytical column. The mass spectrometer was operated in an automated data-dependent acquisition mode where each MS scan (*m/z* 350–1500, 1-s scan time) was followed by three MS/MS scans (*m/z* 50–1500, 1-s scan time) of the most intense peptide ions (z = 2–4). The MassLynx raw files from the Q-TOF micro were processed using ProteinLynx Global Server 2.0.5 (Waters) and exported in Micromass pkl format for automated peptide identification using an in-house Mascot server v2.2.03 (Matrix Sciences). The search was performed against the taxonomy-filtered (Mammalia) SwissProt database (version 56.0, 63,150 sequences after taxonomy filter) and a corresponding decoy database. The following parameters were applied: enzyme, trypsin; maximum missed cleavages, 3; fixed modifications, carbamidomethyl (C); variable modifications, oxidation (M), Phospho_STY (STY), and PhosphoIntact (STY); peptide mass tolerance, 0.25 Da; fragment mass tolerance, 0.6 Da; mass values, monoisotopic; instrument type, ESI-QUAD-TOF. With default Mascot search parameters (significance threshold, $p < 0.05$; ion score cutoff, 15) the estimated false discovery rate was 0.0% (number of matches above identity threshold in search of real/decoy database, 115/0). Using these parameters, we identified the doubly charged tryptic peptide VVAEELENVR (*m/z* 579.32) covering position 87–96 and the doubly charged tryptic peptide STFVLSNLAEVVER (*m/z* 782.45) covering position 19–32 of human FBXO22. The given peptides were assigned to the spectra with Mascot ion scores of 60 and 77, respectively, and the identification was verified by manual *de novo* sequencing and homology search.

Recombinant Proteins—Sequence-verified constructs of FBXO22a and b were subcloned into the pGEX4T-2 vector. The GST-FBXO22 fusion constructs were introduced in codon plus BL21 bacteria and induced by isopropyl 1-thio-β-D-galactopyranoside (0.3 mM) at 30 °C for 3 h. Bacteria were pelleted by centrifugation for 10 min at 5000 × *g* at 4 °C. The pellet was resuspended in PBS supplemented with 100 mM NaCl, 2 mM DTT, and 0.4 mM PMSF, and cells were disrupted by sonication. After addition of Triton X-100 to 1%, insoluble material was removed by centrifugation at 40,000 × *g*. Then GST-FBXO22 fusion proteins were purified by binding to glutathione-agarose beads, followed by extensive washes with cold PBS. Proteins were eluted and dialyzed against PBS plus 6% glycerol. GST-α-synuclein and GST-CHIP (C terminus of Hsc70-interacting protein) constructs (received from Prof. S. Engelender, Technion Institute of Technology) were produced in the same fashion. His-SR was purified from BL21 bacteria as described previously (13).

In Vitro Binding Experiments—Purified His-SR (0.4 μg/ml) was incubated with GST recombinant proteins bound to glutathione-Sepharose beads (0.8 μg/ml GST-FBXO22a, 1.5 μg/ml GST FBXO22b, or 30 μg/ml GST-α-synuclein). The binding buffer consisted of 20 mM Tris-HCl (pH 7.4), 0.2% Triton