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Induction of Memory Deficit in Mice with Chronic Exposure to Cerebrospinal Fluid from Patients with Anti-*N*-Methyl-D-Aspartate Receptor Encephalitis

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Anti-*N*-methyl-D-aspartate receptor (NMDAR) encephalitis is now widely recognized and the patients with this disease show prominent psychiatric symptoms followed by seizures, respiratory failure, involuntary movement, autonomic instability, and amnesia. The anti-NMDAR antibody titer coincides with disease activity, and antibody-deprivation treatment ameliorates neurological symptoms. Previous studies have shown that clusters of NMDARs on the neuronal surface decrease in density upon incubation with the cerebrospinal fluid from patients (NMDAR-CSF), and that the induction of long-term potentiation, a cellular mechanism underlie learning and memory processes, was suppressed with NMDAR-CSF. In this study, we exposed mice to NMDAR-CSF in an attempt to reproduce the human symptoms in mice. CSF was continuously administered via a cannula placed in the lateral ventricle of the mouse that connected to an osmotic pump transplanted in the back of the mouse. From day 8-18, we evaluated the behavior of the mice using standardized tests that were performed serially. Mice exposed to NMDAR-CSF showed impaired spatial memory, as detected with the Morris water maze test. Brain tissue from mice with memory disturbances had decreased content of NMDAR protein in the hippocampal area shown by immunohistochemistry, which is consistent with the anti-NMDAR antibodies affect the expression and function of NMDARs, resulting in anti-NMDAR encephalitis-like symptoms. Also, the mice treated with the NMDAR-CSF did not show inflammatory cell infiltration or neuron loss in their brain tissue and this lack of nervous tissue destruction is encouraging as it is consistent with the idea that this disease can be treated through immunotherapy.

Keywords: amnesia; anti-NMDAR encephalitis; autoantibodies; Morris water maze test; mouse model
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Introduction

Anti-*N*-methyl-D-aspartate receptor (NMDAR) encephalitis exhibits a characteristic set of neurological and psychiatric symptoms including acute behavioral changes, seizures, memory deficits, involuntary movement, autonomic and breathing dysfunctions, and consciousness disturbance that necessitates intensive-care treatment. This predominantly occurs in young women with ovarian teratoma, and these patients express autoantibodies against the NMDA subtype of ionotropic glutamate (Dalmau et al. 2008; Florance et al. 2009; Titulaer et al. 2013). It is now recognized that anti-NMDAR antibody-mediated syndromes can present as partial and sometimes monosymptomatic neurological disease and rank among the most frequent forms of autoimmune encephalitis (Dalmau et al. 2011).

Anti-NMDAR antibodies are considered to be closely related with the pathogenesis of NMDAR encephalitis because the antibody titers parallel the disease activity at many occasions, and the removal of the antibodies leads to effective recovery from this condition (Dalmau et al. 2008).

Major symptoms that are initially observed in this disease, particularly in adult patients, are disturbance of memory and cognition (Titulaer et al. 2013). The NMDAR is essential for both memory and synaptic plasticity, and its involvement in cognition has been suggested by experiments in which NMDARs have been pharmacologically blocked or an NMDAR subunit has been knocked out

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(Herron et al. 1986; Morris et al. 1986). The administration of NMDAR antagonists, such as phencyclidine and ketamine, to normal individuals results in symptoms that closely resemble those seen in schizophrenia (Javitt and Zukin 1991; Krystal et al. 1994). Furthermore, NMDAR antagonists exacerbate symptoms in patients with schizophrenia (Lahti et al. 2001).

The epitope targeted by anti-NMDAR antibodies is located in the extracellular domain of the GluN1 subunit (Dalmau et al. 2008; Gleichman et al. 2012). Following the binding of anti-NMDAR antibodies, the receptor becomes cross-linked and internalized at the postsynaptic site, thereby suppressing NMDAR-mediated transmission (Hughes et al. 2010). Despite the understanding of these molecular processes, it is unclear whether the antibody (Ab)-induced dysfunction of NMDARs underlies the symptoms of anti-NMDAR encephalitis. Therefore, we previously tested whether the antibodies produced by patients with anti-NMDAR encephalitis suppress NMDAR-dependent long-term potentiation (LTP), which is a model of memory and learning. We found that the NMDAR-Ab-positive cerebrospinal fluid (CSF) of these patients reduced the induction of LTP in mouse hippocampal slices which was then reversed with anti-NMDAR antibody-deprived CSF, suggesting that the NMDAR antibodies in the patients' CSF are closely related to the memory disturbance of anti-NMDAR encephalitis patients (Zhang et al. 2012).

In mice that are deficient for GluN2A subunits of NMDARs, LTP induction at the CA1 synapses and spatial memory are defective, suggesting that NMDAR is closely linked to these phenomena (Sakimura et al. 1995). Also, spatial memory is reported to be impaired in mice whose hippocampal NMDARs are conditionally knocked out (McHugh et al. 1996; Tsien et al. 1996). Together, these findings suggest that the anti-NMDAR antibodies that are present in the patients' CSF may disrupt NMDAR-dependent LTP processes involved in memory formation and storage. Recently, it was shown that mice treated with anti-NMDAR antibody-containing CSF infused into the lateral ventricles of mice exhibited progressive memory deficits, and anhedonic and depressive-like behaviors (Planagumà et al. 2015). These symptoms paralleled with antibody-mediated reduction of NMDAR numbers which recovered after stopping the CSF infusion. We also examined the effects of anti-NMDAR antibodies on the behavior of mice that were chronically injected with CSF from anti-NMDAR-Ab-positive patients. Finally, we assessed the histological alterations in brain tissues of these mice.

Materials and Methods

Sample preparation

CSF was collected and pooled from two patients with typical clinical features of anti-NMDAR encephalitis (NMDAR-CSF) at the acute stage. CSF pooled from two patients who were diagnosed as having psychosomatic disease was used as a control (Cont-CSF).

For antibody detection, the full-length NMDAR GluN1 and GluN2A cDNAs were co-transfected into human embryonic kidney (HEK) 293 cells with a lipofectamine reagent (Invitrogen Japan, Tokyo, Japan) and cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum (FCS), which also contained 10 μM MK-801 (Wako, Tokyo, Japan) for neuroprotection. Twelve hours after transfection, HEK cells were fixed in 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS, pH 7.4) for 20 min. Non-specific binding was blocked with 10% goat serum/PBS, and cells were incubated with patient CSF (1:2-10) or sera (1:10-400) in 0.02% Triton X-100, 10% goat serum in PBS overnight at 4°C, and then with FITC-conjugated anti-human IgG (DAKO, Glostrup, Denmark; 1:50) for 1 h. SlowFade Gold anti-fade reagent (Invitrogen Japan) was then applied to the slides and the staining was observed under a fluorescence microscope (Axiovision, Zeiss). To confirm the localization of NMDAR antibody binding sites, double staining was done both with a patient's sample and a mixture of rabbit anti-GluN1 (PhosphoSolutions, CO; 1:10) and anti-NR2A (Frontier Science, Sapporo, Japan; 1:10) antibodies. Then, the primary antibodies were visualized by FITC-anti-human IgG and R-PE-anti-rabbit IgG and observed under a laser scanning microscope (Zeiss LSM 710). The antibody titers in the CSF of the two patients were ×2,560 and ×1,280.

Animals

All experiments were performed with C57BL/6 male mice (age: 8 weeks; weight: 22-24 g). Mice were housed in an environment with 12 h day-night cycles and allowed access to food and water *ad libitum*. Each mouse performed the novel object recognition (NOR) test. Only the mice that were judged to have abilities to perform further behavior tests were used in subsequent experiments, including the second NOR test. The mice were divided into 4 groups (intraventricularly injected with NMDAR-CSF and Cont-CSF, control mice without any treatment and mice who received intraventricular injection of saline). Each group comprised eight mice, and 32 mice were analyzed in total.

Animals were treated and maintained in accordance with the NIH principles for laboratory animal care (NIH publication 1984, No. 85-23, revised 1985).

All experiments and animal-care protocols were approved by Committees of Kanazawa Medical University with regard to ethics and animal care (Uchinada, Ishikawa, Japan).

Mouse selection using the novel object recognition test

The NOR test was performed as previously described (Siopi et al. 2012). The test was run 1 week before surgery for the selection of suitable mice for further studies. The test included three phases. During the adaptation phase, the mouse was placed in an empty chamber for 3 min in order to adapt to the environment. During the acquisition phase, the mouse was placed in the testing arena in which two identical objects were set in right and left corners of the box at a distance of 5 cm from the walls. The mouse was allowed to freely explore for 3 min. After a 24-h interval, the mouse performed the recognition phase of the test. During the recognition phase, one of the objects was replaced with a novel object, and the mouse was returned into the arena and allowed to explore the novel and familiar objects for 3 min. Between each phase, the mouse was returned to its home cage. Mice who spent at least 10 s with the two identical objects during the acquisition phase and spent more time with the novel object than with the familiar object during the recognition

phase were selected for the subsequent procedures.

Brain-infusion micro-osmotic pump implantation

A brain-infusion micro-osmotic pump (Model 1004, Durect Corporation, Cupertino, CA) was loaded with one of the prepared specimens and kept immersed in sterile saline at 37°C for 48 h before implantation. The mice were acclimatized for 1 week before surgery. Subsequently, the mice under pentobarbital anesthesia (35 mg/kg, administered intraperitoneally) were mounted on a stereotaxic frame (Narishige Co. Ltd., Tokyo, Japan) in order for the infusion cannula to be implanted into the left lateral cerebral ventricle. The stereotaxic coordinates were 0.4 mm posterior to bregma, 1.0 mm lateral to the lambda point, and 2.0 mm deep from the dural surface. The cannula was secured to the skull with dental cement and acrylate and connected to the micro-osmotic pump, which was placed subcutaneously at the back of the mice. CSF was continuously injected into the ventricle at a rate of 0.11 μL/h. The mice were returned to their home cage for a recovery period of 7 days. The behavior tests were then serially performed; spontaneous locomotor activity for 2 days, open-field test for 1 day, NOR test for 2 days, and Morris water maze test for 5 days. During this whole period, the mice were kept under continuous CSF infusion (Fig. 1). All experiments were carried out between 9:00 h and 11:00 h and between 14:00 h and 16:00 h. After the behavioral studies, some mice were randomly chosen to verify the location of the cannula tip. These mice were sacrificed under pentobarbital anesthesia, and methylene blue or thionine solution (2% in saline) was injected through the implanted cannula, followed by dissection of the brain. The diffusion of methylene blue in all ventricles was confirmed in all mice examined (Fig. 2). The behavioral testing was performed in mice that were injected with NMDAR-CSF, Cont-CSF, and saline.

Behavioral tests

Spontaneous locomotor activity: Spontaneous locomotor activity was measured using a PC-controlled digital counter with infrared sensors (ACTIMO-10, Bioresearch Center Ltd, Nagoya, Japan). Spontaneous locomotor activity was quantified as the number of times that the mouse interrupted the infrared beams in a cage over 24 h. The measurements were taken in the mouse's home cage in a temperature-controlled environment (22 ± 2°C), with 12-h day/night cycles and access to food and water *ad libitum*.

Open-field test: In this test, anxiety-like behavior was assessed in an open-field arena. The mice were allowed to walk around for 5 min, and their exploratory behavior was analyzed offline using SMART (Panlab SLU, Cornella, Spain). In the analysis, the arena was divided into two equally spaced concentric areas: the inner and outer zones. The relative length of time spent in the inner zone was calculated as an indicator of anxiety-like behavior.

Novel object recognition test: Details of this test were described above. Behavior was videotaped using an overhead hard-drive-based video camera. The novelty index was defined as the length of time spent with a novel object divided by the total time. In animals with an ability to discriminate the familiar object from the new object, the index should be > 50%.

Morris water maze test: The Morris water maze test was used to test the spatial memory of the animals (Szyndler et al. 2006). Training in the maze took place between 8:00 h and 14:00 h, which was during the light phase of the daily cycle. The Morris water maze comprised a large circular pool with a transparent Plexiglas platform submerged 1 cm below the surface of the water. The animals under-

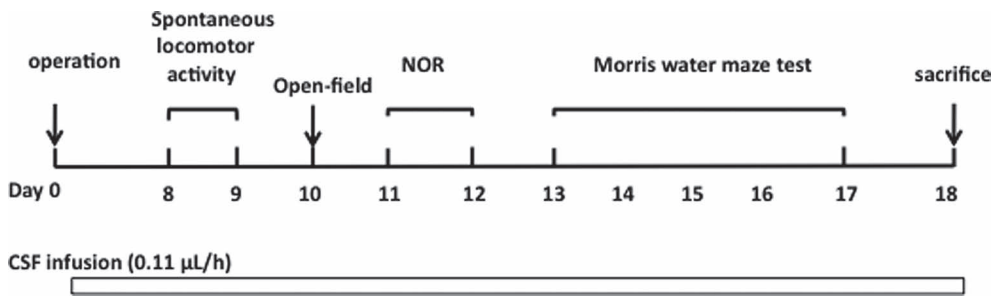

Fig. 1. Serial procedure of mouse behavioral tests.

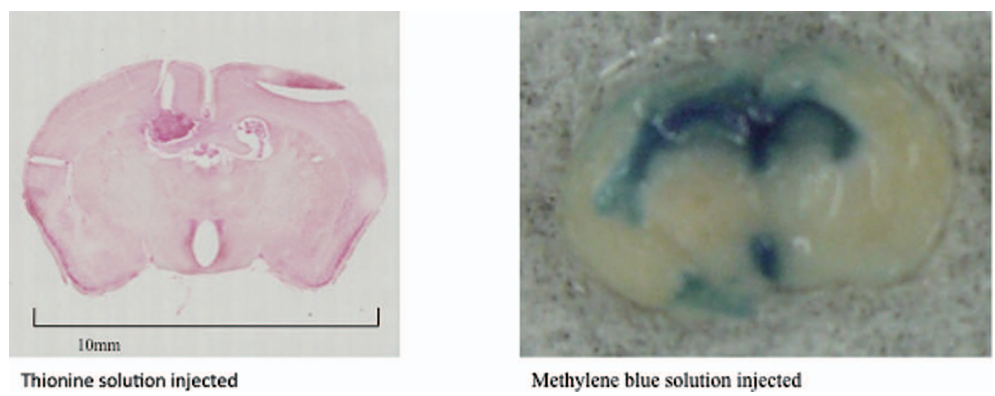

Fig. 2. Placement of ventricular cannule.
Mouse brain coronal section exhibiting cerebroventricular diffusion of thionine or methylene blue after ventricular infusion.

went four swimming trials per day for 5 consecutive days. A trial was started by placing mice into one of the four quadrant positions on the edge of the pool. Each trial was terminated as soon as the mouse reached the platform or the maximum trial time of 60 s had elapsed. After the last trial on the fifth day, the mice were subjected to the probe trial for 60 s with the platform removed. The swimming trajectory of each mouse was analyzed using SMART.

Histological study and immunohistochemistry

After the completion of the behavioral tests, the brain was rapidly removed and divided into parts for snap freezing and fixation in buffered paraformaldehyde. After confirming the correct injection site, paraffin embedded cerebral tissue blocks, consisting of tissue 2-mm rostral and 2-mm caudal from the injection site, were cut into 8-μm coronal sections, deparaffinized, and stained with hematoxylin-eosin. Serial sections from paraffin-embedded and also the equivalent portion of frozen sections were stained immunohistochemically using antibodies against NMDAR GluN1 (×100), GluN2A (×100) (both from Frontier Institute, Hokkaido, Japan), glial fibrillary acidic protein (GFAP) (×1,000) (MBL, Nagoya, Japan), and cell-surface markers CD4 (×20), CD8a (×50) (BD Biosciences, Tokyo, Japan); CD138 (×100) (BD Pharmingen, Tokyo, Japan); and CD68 (×100) (BioLegend, CA). Human IgG deposition was assessed with anti-human IgG (BD Biosciences) staining.

Western blotting

Using western blotting, NMDAR GluN1 protein levels were quantitatively compared in the brain samples from mice that were injected with either NMDAR-CSF or Cont-CSF. Samples containing the hippocampus were dissected and homogenized in lysis buffer (20 mM HEPES pH 7.9, 0.3 M NaCl, 0.1% Triton X-100, 10% glycerol) with a protease inhibitor cocktail (Roche, Germany), and centrifuged (12,000 rpm). The supernatants were electrophoresed by SDS-PAGE in 10%-20% Ready Gels (Bio-Rad, Tokyo, Japan) and transferred onto a polyvinylidene fluoride transfer membrane (PALL Life Sciences, FL). After blocking with a skim milk solution (5%), each membrane was incubated with a mouse anti-GluN1 antibody (1:20) and a rabbit anti-actin antibody (1:2,000; IMG-5142A; Imgenex). After reacting with HRP-conjugated secondary antibodies, bands were detected using a chemiluminescence substrate kit (Super-Signal West Femto kit, Thermo Scientific) and a detector (LAS-4000, FUJIFILM). The band intensities were quantified using Image J software and standardized to densities of the corresponding actin bands located in the same lanes. These standardized densities of the GluN1 bands were averaged for each experimental group.

Quantitative real-time PCR

The hippocampal area from three mice from each experimental group was rapidly dissected. The tissue was placed in liquid nitrogen and stored at −80°C until mRNA extraction was performed with an RNeasy Mini kit (QIAGEN, cat. no. 74104). Reverse transcription was performed using the SuperScript III First-Strand Synthesis SuperMix for quantitative real-time polynerase chain reaction (PCR) (Life Technologies) on an Applied Biosystems 7500 Real-Time PCR System with TaqMan probes (Applied Biosystems). The sets of probes and primers used in this analysis were as follows: Mm00599890_m1 (IFN-γ receptor 1: IFNγR1), Mm00438334_m1 (colony-stimulating factor 3: CSF3), Mm00439619_m1 (interleukin 17A:IL 17A), Mm00445235_m1 (chemokine (C-X-C motif) ligand 10: CXCL 10), Mm00441258_m1 (chemokine (C-C motif) ligand 3: CCL 3), Mm99999915_g1 (glyceraldehyde-3-phosphate dehydrogenase: GAPDH_mouse1). For each experimental group, two samples from three individuals, i.e., six samples, were subjected and relative levels of each of the mRNA expression were determined by RT-qPCR. The level of GAPDH mRNA serves as a loading control. Measurement was based on the comparative C_T ($\Delta\Delta C_T$) method for relative quantitation of gene expression.

Statistical analyses

Data are expressed as mean ± SEM. The statistical analysis of the results was performed using analysis of variance (ANOVA). One-way ANOVA was used for the analysis of data pertaining to locomotor activity, the open-field test, the NOR test, and the Morris water maze test, followed by the Newman-Keuls post-hoc test for multiple comparisons when the test prerequisites were fulfilled. When the prerequisite of the homogeneity of variances was not fulfilled, a non-parametric ANOVA on ranks (the Kruskal-Wallis test) was performed, followed by Dunn's test for multiple comparisons. Regarding the analysis of western blot, statistical differences were determined using the two-sided Student's *t*-test. Differences with a P value of < 0.05 were considered significant.

Results

Effects on spontaneous locomotor activity

In these experiments, we measured the number of times that mice interrupted the infrared beams over a 24-h period (Fig. 3a). Spontaneous locomotor activity was not significantly different between mice in the NMDAR-CSF group (11,449 ± 2,686 beam interruptions) and mice in the Cont-CSF group (11,096 ± 460 beam interruptions) (P = 0.9215).

Effects on the open-field test

The open-field test was used to assess anxiety-like behavior. The relative length of the times spent in the inner zone were calculated, and the results for the NMDAR-CSF-injected mice group was 13.52 ± 3.51% and Cont-CSF-injected mice group was 18.52 ± 6.18%, which did not differ significantly between two groups (P = 0.0577) (Fig. 3b).

Effects on the novel object recognition test

In this test, we measured the amount of time that mice spent with the two identical objects during the acquisition phase (d2) and the time spent on the novel object during the recognition phase (d1). The novelty indices [d1/(d1 + d2)] did not significantly differ between the NMDAR-CSF (0.67 ± 0.12) and Cont-CSF (0.76 ± 0.12) groups (P = 0.0985) (Fig. 3c), which indicated that CSF of patients with NMDAR encephalitis failed to impair nonspatial memory together with curiosity stimulation.

Effects on the Morris water maze test

Spatial memory was assessed using the Morris water maze. The results of the escape latency for the saline-, NMDAR-CSF-, and Cont-CSF-injected groups, respectively, were as follows; on day one: 49.96 ± 17.34 s, 51.34

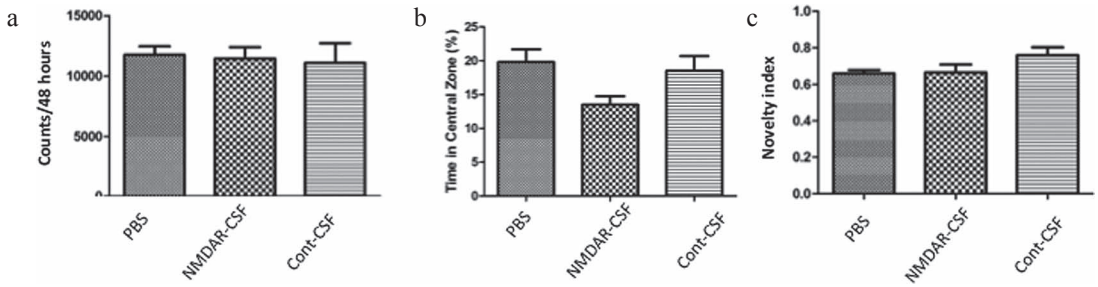

Fig. 3. Results of the behavioral tests.

a. Effects on spontaneous locomotor activity. The number of times that the mouse interrupted the infrared beams over 24 h. No differences in spontaneous activity were observed among the groups (NMDAR-CSF group, 11,449 ± 2,686; Cont-CSF group, 11,096 ± 460; and PBS group, 11,760 ± 1,976). *P*-value between the NMDAR-CSF and Cont-CSF groups was 0.9215.

b. Effects on the open-field test. The time spent in the inner zone was not significantly different among the groups (NMDAR-CSF group, 13.52 ± 3.51%; Cont-CSF group, 18.52 ± 6.18%; and saline group, 19.76 ± 5.43%; the *P* value for the comparison between the NMDAR-CSF and Cont-CSF groups was 0.0577).

c. Effects on the novel object recognition test. Mice with a total time spent on the two identical objects during the acquisition phase (d2) and mice with time spent on the novel object during the recognition phase (d1) were measured and are shown as the novelty indices [d1/(d1 + d2)]. The novelty index in NMDAR-CSF group was 0.67 ± 0.12; Cont-CSF group was 0.76 ± 0.12; and saline group was 0.66 ± 0.06, without statistically significant differences between the NMDAR-CSF and Cont-CSF groups (*P* = 0.0985).

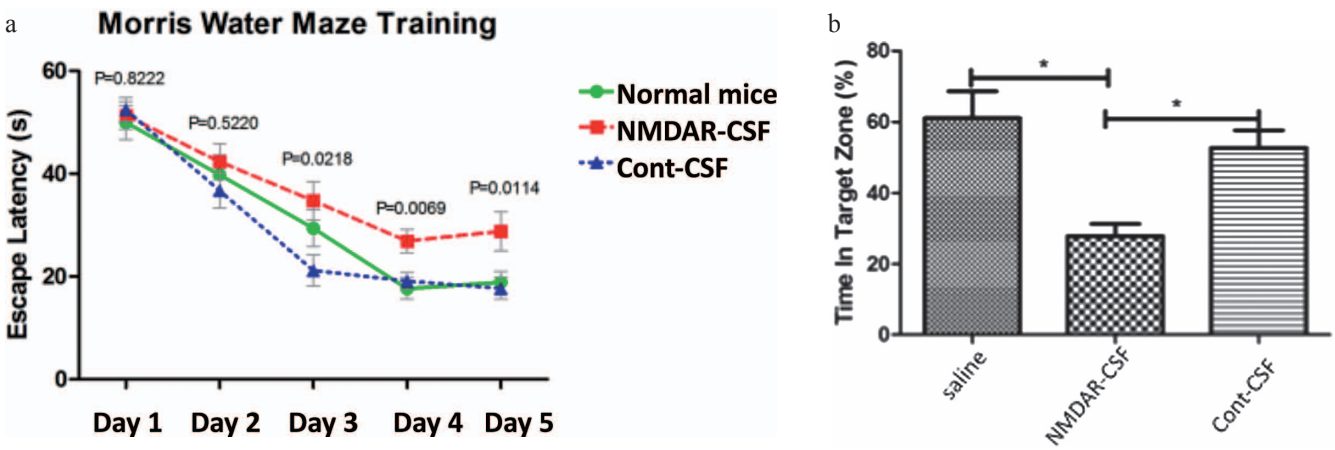

Fig. 4. Effects on the Morris water maze test.

a. Results of the escape latency for each group; the saline-, NMDAR-CSF-, and Cont-CSF-injected groups were compared. The difference in the latency to reach the platform increased daily, with a longer time observed in the NMDAR-CSF-injected group.

b. Time spent in the testing zone. The relative time spent in the target zone (%) was: NMDAR-CSF group, 27.89 ± 9.84; Cont-CSF group, 52.78 ± 13.86; and saline group, 61.11 ± 19.85. A significant difference was observed between the NMDAR-CSF and Cont-CSF groups (*P* = 0.0008), suggesting lesser abilities in spatial learning and memory formation in the NMDAR-CSF group.

± 15.36 s, and 52.44 ± 1.08 s; on day 2: 39.75 ± 20.22 s, 42.31 ± 19.59 s, and 36.69 ± 19.30 s; on day 3: 29.39 ± 19.06 s, 34.72 ± 21.38 s, and 21.19 ± 17.12 s; on day 4: 17.08 ± 10.21 s, 23.93 ± 12.15 s, and 19.09 ± 9.57 s; and on day 5: 18.89 ± 11.16 s, 28.78 ± 21.48 s, and 17.69 ± 11.94 s. The difference in the latency to reach the platform increased daily, with a longer time observed in the NMDAR-CSF-injected group (Fig. 4a).

A shorter time spent in the testing quadrant indicates lower abilities in spatial learning and memory formation. The relative time spent in the target quadrant was 27.89% ± 9.84% in the NMDAR-CSF group, 52.78% ± 13.86% in the Cont-CSF group, and 61.11% ± 19.85% in the saline group (Fig. 4b). A significant difference was detected between the NMDAR-CSF and Cont-CSF groups (*P* = 0.0008), indicating that spatial memory was impaired by CSF obtained from patients with NMDAR encephalitis.

Serial procedures of mouse behavioral tests are shown in Fig. 1 and the results of the behavioral tests are summarized in Table 1.

Table 1. Results of behavioral tests performed in mice injected with NMDAR-CSF and Cont-CSF.

Test Name	Saline	NMDAR-CSF	Cont-CSF	*P*-value
SP (Counts/48 hours)	11,760 ± 1,976	11,449 ± 2,686	11,096 ± 460	0.9215
OP (Time in central zone)	19.76 ± 5.43	13.52 ± 3.51	18.52 ± 6.18	0.0577
NOR (Novelty index)	0.66 ± 0.06	0.67 ± 0.12	0.76 ± 0.12	0.0985
MWM (Time in target zone)	61.11 ± 19.85	27.89 ± 9.84	52.78 ± 13.86	0.0008[$]

[$]*P*-value: NMDAR-CSF vs. Cont-CSF.
SP, spontaneous locomotor activity (Counts/48 hours); OP, open field test (Time in central zone); NOR, novel object recognition test (Novelty index); MWM, Morris water maze test (Time in target zone).

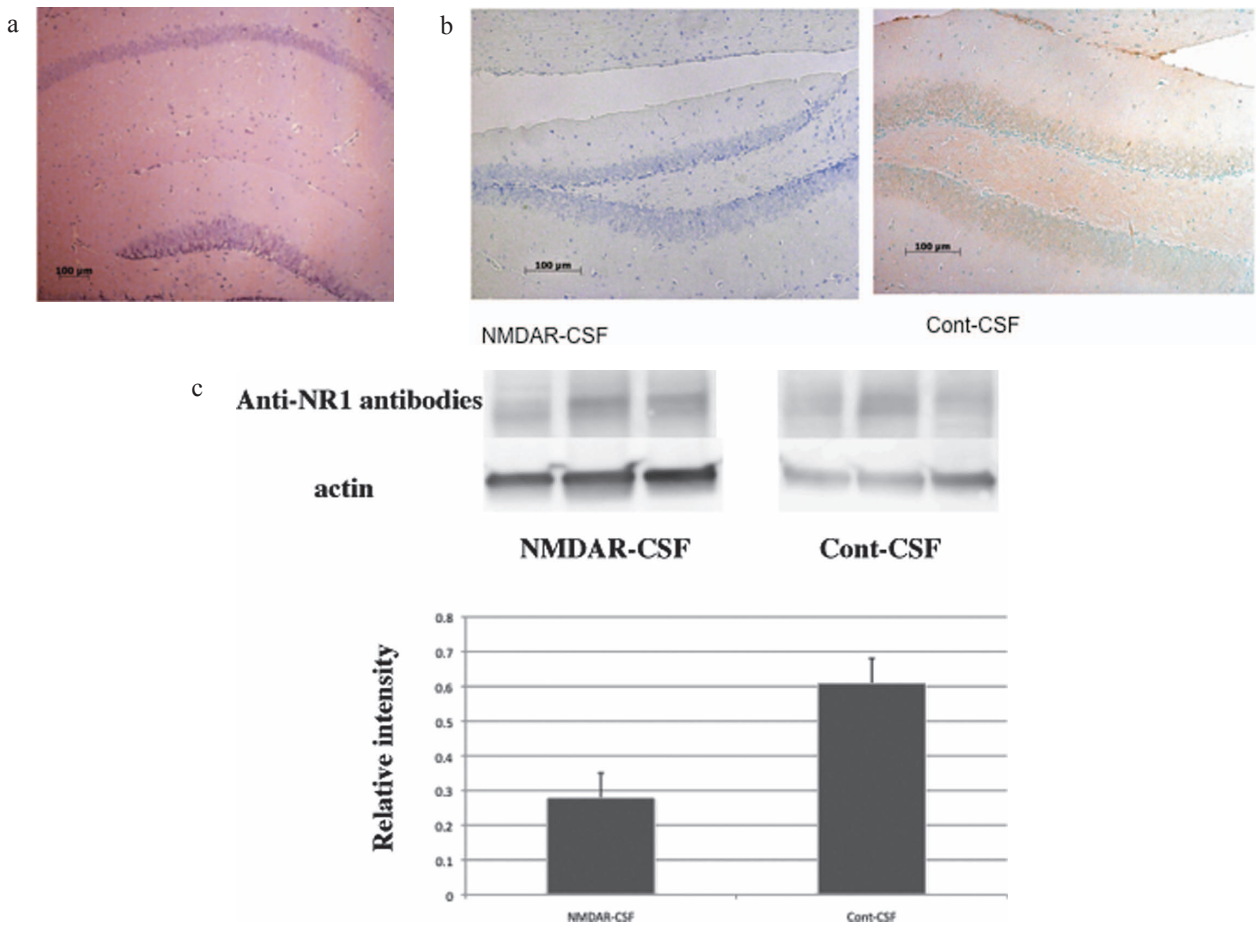

Fig. 5. Histological findings and western blotting of the mouse brain tissue.
a. Hematoxylin and eosin staining revealed that the brains of mice treated with NMDAR-CSF did not show mononuclear cell infiltration, neuronal cell loss, or astroglial proliferation in the parenchyma of the hippocampal area.
b. The staining intensity of anti-GluN1 antibodies in the hippocampus was lower in the brains of NMDAR-CSF-injected mice compared with that of Cont-CSF-injected mice.
c. Quantification of GluN1 protein levels in the brains of NMDAR-CSF-injected mice and Cont-CSF-injected mice shown as relative intensity are compared. Expression of GluN1 in the brain of NMDAR-CSF-injected mice was lower (0.28 ± 0.07) compared with that detected in Cont-CSF-injected mice (0.61 ± 0.19), though the difference was not statistically significant ($P = 0.082$).

Histological results

The mice treated with NMDAR-CSF and cont-CSF were sacrificed and histological alterations were assessed. There was a small amount of mononuclear cell infiltration on the CSF injected side of the lateral ventricles in mice, however, the brain parenchyma from mice that were injected with NMDAR-CSF, Cont-CSF, or saline did not show evidence of inflammatory cell infiltrations, neuronal loss, or astroglial proliferation (Fig. 5a).

Immunohistochemistry

Immunostaining with anti-CD4, anti-CD8, anti-CD68,

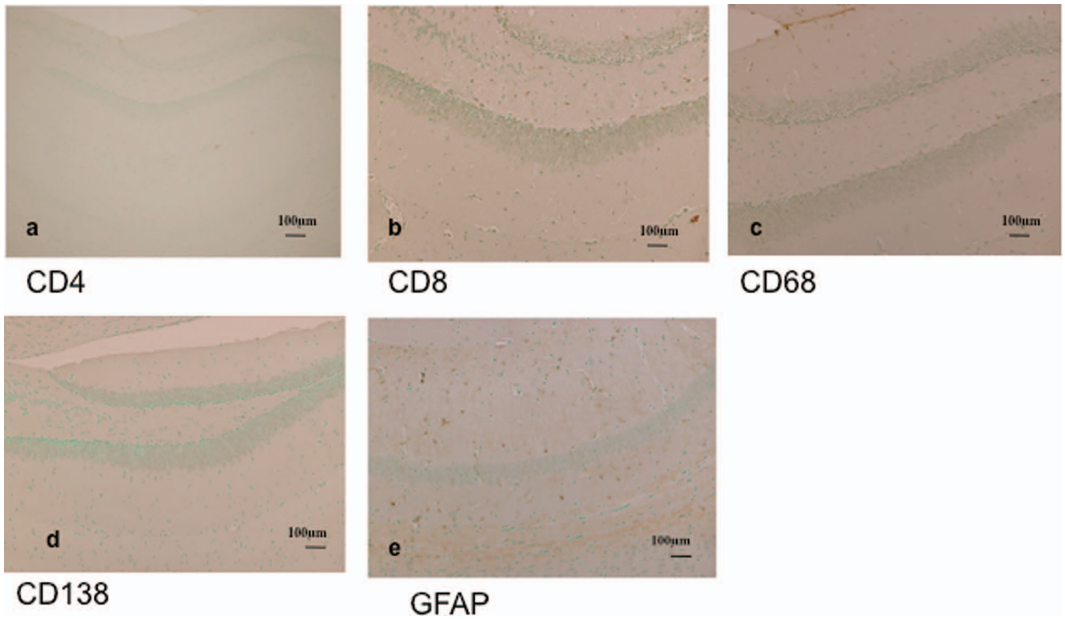

Fig. 6. Immunohistochemical staining.
Mouse brain sections treated with anti-NMDAR-CSF were immunostained with anti-CD4, CD8, CD68, CD138 and anti-GFAP antibodies without specific staining patterns (6a-e) which were similar as those with Cont-CSF treated mouse.

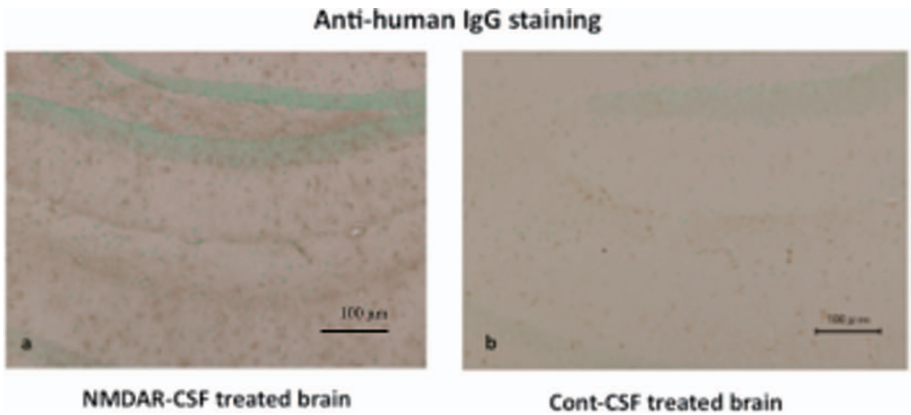

Fig. 7. Human IgG staining.
The deposition of human IgG exhibited a scattered pattern, without specific accumulation around hippocampus (7a, b).

and anti-CD138 was negative in the cerebral parenchyma of every mouse in each group and the staining with anti-GFAP showed a similar pattern between each group (Fig. 6a-e). Human IgG was not specifically accumulated at the hippocampus, rather it diffusely deposited in entire brain without specific binding sites, such as vessel walls or the neuronal surface (Fig. 7a-b). Compared with Cont-CSF-injected mice, staining intensities of anti-GluN1 antibodies in the hippocampus in NMDAR-CSF-injected mice were decreased (Fig. 5b).

Western blotting

Western blot analysis showed that GluN1 expression shown as relative intensity was lower in NMDAR-CSF treated mice (0.28 ± 0.07) than in Cont-CSF treated mice (0.61 ± 0.19), though the result was not statistically significant (P = 0.082) (Fig. 5c).

Quantitative real-time PCR

The brain tissues from animals in the NMDAR-CSF group exhibited increased expression of the chemokine IFN-γ-inducible protein of 10 kDa (CXCL10) compared with the Cont-CSF group (NMDAR-CSF, 5.68 ± 1.68 vs. Cont-CSF, 1.1 ± 2.06 shown as % of control). The difference was statistically significant (P = 0.04). IL-17 levels did not increase in any of the groups (Fig. 8).

We also measured the cytokine contents in NMDAR-CSF and Cont-CSF using a cytokine detection array kit. However, no significant difference was observed between these groups (data not shown).

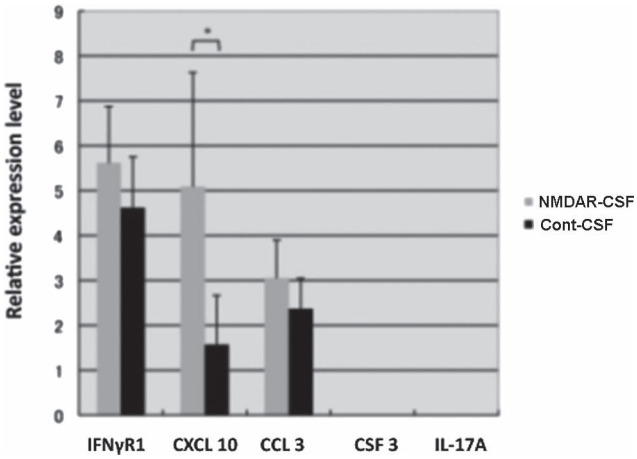

Fig. 8. Quantitative real-time PCR.
The brain tissue from mice injected with NMDAR-CSF exhibited increased levels of CXCL10 expression compared with that from the mice injected with Cont-CSF (NMDAR-CSF, 5.68 ± 1.68 vs. Cont-CSF, 1.1 ± 2.06, $P = 0.04$: shown as % of control). CXCL10 levels in the NMDAR-CSF group were significantly higher than the Cont-CSF group.

Discussion

In this study, we showed that the CSF of patients who are positive for anti-NMDAR antibodies affected the memory of mice, and memory loss is major symptom of patients with NMDAR encephalitis. In this study, the mice treated with NMDAR-CSF showed deterioration of spatial memory functions, as assessed by the Morris water maze test. We did not detect a clear difference between the NMDAR-CSF and Cont-CSF groups in any tests other than the Morris water maze test. The Morris water maze test was the last test (performed after 2 weeks from pump implantation), and the total amount of NMDAR-CSF that the mice received at the time of the Morris water maze test was higher than the cumulative amount received while they performed other tests (Fig. 1).

Recently, Planagumà et al. (2015) reported that mice treated with anti-NMDAR encephalitis patients' CSF into both lateral ventricles using two osmotic pumps for 14 days with four times the amount of CSF in our study, showed memory disturbances in the object recognition test and V-maze paradigms, and this memory disturbance recovered after the mice were deprived of the CSF. This study and our study confirm that the infusion of anti-NMDAR antibodies from patients with NMDAR encephalitis into mice affects learning and memory processes in those mice.

Previously, Irani et al. (2010) showed that NMDAR-Ab titers are related to the disease severity in many patients. Among studies of the functional roles of this antibody in NMDAR encephalitis, Hughes et al. (2010) showed that specific antibody-mediated internalization of NMDARs drastically reduced the density of NMDARs along the cell in culture and reduced the amplitude of miniature EPSCs in cultured rat hippocampal neurons. We have shown that CSF from patients that contains anti-NMDAR-Ab specifically suppresses LTP in the CA1 area in mouse hippocampus slices, which supports the clinical finding of a relationship between anti-NMDAR encephalitis antibodies and amnesia (Zhang et al. 2012). Using GluN1 knockout mice, Halene et al. (2009) showed that a GluN1 deficiency reduces behavioral inhibition and impairs social interactions, which may be related to negative symptoms in schizophrenia.

However, it had not been shown *in vivo* that the patients' antibodies affect neurological symptoms of anti-NMDAR encephalitis directly until recently. In general, it is difficult to reproduce disease features in experimental animals. Rats that were intrathecally injected with IgG from patients with anti-amphiphysin antibody-positive stiff-person syndrome, showed anxiety-like behaviors, as evaluated by the elevated plus-maze test and open-field test (Geis et al. 2012). Histological evidence related to antibody-mediated central nervous system disorders has been provided in rodent models of anti-aquaporin 4 antibody (AQP4)-mediated neuromyelitis optica (NMO) (Bradl et al. 2009; Kinoshita et al. 2009; Saadoun et al. 2010). However, these animals did not exhibit specific symptoms that are observed in NMO patients.

The pathological alterations in brains from patients with NMDAR encephalitis are very mild, with minimal evidence of inflammatory cell infiltration, neuronal loss, and no evidence of complement activation (Martinez-Hernandez et al. 2011). This contrasts with what is observed in patients with antibodies against intracellular antigens or even with what is observed in patients with other types of surface antigen antibody encephalitis (Bien et al. 2012).

Planagumà et al. (2015) showed that the IgG containing anti-NMDAR antibodies bound to the brain tissue, particularly in the hippocampus, and the presence of the antibodies coincided with the decline in memory function. The accumulation of the IgG resulted in a decrease in the density of NMDAR clusters. In their previous study, NMDARs were cross-linked with the antibodies and internalized inside the cells, which may lose the IgG binding capacity and IgG accumulation (Hughes et al. 2010). However, Planagumà et al. (2015) showed that the maximum accumulation of the IgG coincided with the maximum disturbance of memory function. We could not observe strong staining of human IgG specifically located around the hippocampus when a decrement in anti-NMDAR antibody staining was seen. We removed mouse brains 1 week after the last behavioral test, although the CSF infusion was continued until the time of dissection. This delay might be one of the reasons of losing the strong staining of human IgG around the hippocampus. However, we think that our mouse model of memory disturbance is more realistic because patients with anti-NMDAR encephalitis having long-term memory disturbance, and because we administered lower concentration of NMDAR-CSF with longer period, the acute damage to the NMDARs was avoided.

Rather the NMDARs were internalized and the neurons were not destroyed. The lack of gross morphological changes in the brains of patients could explain the full recovery of patients even after long periods of amnesia.

The quantitative RT-PCR of chemokine-related proteins performed in our study showed high levels of CXCL10 in the brains of mice that were injected with NMDAR-CSF. CXCL10 is secreted in response to IFN-γ and acts as a chemoattractant for monocytes/macrophages, T cells, NK cells, and dendritic cells (Luster et al. 1985; Lee et al. 2009). In our model, NMDAR-Ab-injected mice expressed a higher amount of CXCL10, without upregulation of inflammatory cytokines or inflammatory cell infiltration. It is possible that the stage of pre-inflammation or other factors may be required to induce the inflammatory process in NMDAR encephalitis.

The limitations of our study are as follows. The abnormal results were only detected with the Morris water maze test. The maze test was the last test and it occurred 10 days after the first test. The antibody-amount injected into the brain would be the highest, since the CSF was injected via osmotic pump throughout the entire period of behavioral testing. It is difficult to perform each of the behavioral tests under same condition that might affect the results of each test, if these results depend on the antibody dose. Also, we could not add the experiments with antibody-depleted CSF because we could not obtain a sufficient concentration of NMDA receptor protein to absorb the antibodies. Planagumà et al. (2015) showed that brain-bound IgG extracted from the mice treated with NMDAR-CSF could bind to the NMDARs. Taken together with our previous study showing that anti-NMDAR antibodies in patients' CSF specifically suppressed LTP (memory formation) in the CA1 area of mouse hippocampus slices (Zhang et al. 2012), our results suggest that the antibodies against NMDARs in CSF from the patients with anti-NMDAR encephalitis are directly related to the memory disturbance in this form of encephalitis.

In conclusion, we have shown that the CSF of patients who are positive for anti-NMDAR antibodies affected the memory function of mice, which is also a major symptom in patients with NMDAR encephalitis. The CSF containing anti-NMDAR antibodies is highly likely to induce memory disturbances in individuals with this disease by affecting NMDAR function in the absence of neuronal loss via a tissue inflammatory process. The prognosis for most of the patients with this disease is considered relatively favorable, which may be explained by the present findings that the patients' CSF impairs NMDAR function without major histological disruptions on the nervous tissue.

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Conflict of Interest

The authors declare no conflict of interest.

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