INTERNAL ✷ MEDICINE

Expression of Various Glutamate Receptors Including N-Methyl-D-Aspartate Receptor (NMDAR) in an Ovarian Teratoma Removed from a Young Woman with Anti-NMDAR Encephalitis

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Abstract

A 21-year-old woman developed psychiatric symptoms, progressive unresponsiveness, generalized seizures, severe dyskinesia, marked fluctuation of blood pressure, and hypersalivation after a flu-like episode. Anti-glutamate receptor (GluR)ε2 and anti-N-methyl-D-aspartate receptor (NMDAR) antibodies were positive in both her serum and CSF. After she recovered five months later she underwent surgery to remove a right ovarian teratoma. Immunohistochemical examinations of her teratoma disclosed abundant expression of various GluRs including NR2B subunit of NMDAR, GluR1, and GluR2/3. These immunoreactivities of GluRs were seen not only in small areas of neural tissue identified as anti-glial fibrillary acidic protein (GFAP)-immunoreactive areas but also in other large areas of undifferentiated neuroepithelial tissue without GFAP immunoreactivity. Our findings strongly support the recent idea that neural elements in ovarian teratoma play an important role in the production of antibodies to NMDARs in anti-NMDAR encephalitis. Additionally, the study of control ovaries clearly showed NR2B-related immunoreactivity in the cytoplasm of oocytes, indicating that the normal ovary itself has expression of NMDARs. This finding might provide a clue to understand the pathogenesis of this disease in female patients without ovarian teratoma.

Key words: limbic encephalitis, paraneoplastic syndrome, ovarian teratoma, glutamate receptor, N-methyl-D-aspartate receptor (NMDAR)

Introduction

A unique limbic encephalitis that predominantly affects young females and exhibits various manifestations including initial psychiatric symptoms, and subsequent central hypoventilation, intractable seizures, dysautonomia and prominent orofacial dyskinesia has been noted (1-3). Recently a causative relationship between such encephalitis and ovarian teratoma has been proposed (4-6) and in patients with this disorder a new anti-neural antibody for the NR1/NR2 heteromers of N-methyl-D-aspartate receptor (NMDAR) (NMDAR complex composed of NR1+NR2A or NR2B) has been identified as a disease-specific hallmark (2). This disease is, therefore, called anti-NMDAR encephalitis.

Ionotropic glutamate receptors (GluRs) are subdivided into three major subtypes: N-methyl-D-aspartate (NMDA)-type, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)-type and kainate (KA)-type (7). NMDA-type GluRs (NMDARs) have heterotetramer complex structures composed of NMDAR subunits (8). NMDAR subunits have the two nomenclatures from rats and mice, and NR1, NR2

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A, and NR2B in rat-derived nomenclature have almost homologous sequences with GluRζ1, GluRε1, and GluRε2 in mice-derived nomenclature, respectively.

In this study we examined the immunohistochemical expression of GluRs in the ovarian teratoma obtained from a young woman with anti-NMDAR encephalitis, and showed the characteristics of GluR expression in the tumor.

Case Report

A 21-year-old woman developed orthostatic fainting, appetite-loss and insomnia after a flu-like episode. During the subsequent two weeks, a progressive psychiatric state with emotional instability and abnormal behavior appeared. At admission she showed confusion and agitation, but physical and neurological findings were unremarkable. Routine laboratory data were normal except for an increased number of leukocytes in peripheral blood (12,900/μL). Brain MRI showed no specific findings, but lumbar puncture revealed lymphocytic pleocytosis (124 cells /μL) with normal glucose and protein concentrations. Bacterial and viral studies, including PCR for herpes simplex virus, were all negative. She soon started to experience recurrent generalized tonic-clonic seizures, severe dyskinesia in face and arms, hyperthermia, marked fluctuation of blood pressure, and hypersalivation. The symptoms were not relieved by methylprednisolone pulse therapy (1 g/day for 3 days) and subsequent intravenous administration of immunoglobulin (400 mg/kg/day for 5 days), and her convulsions were unresponsive to conventional anti-epileptic drugs. She was finally treated with intravenous administration of thiopental sodium (100 mg/hour) and mechanical ventilation. After two months she was released from mechanical ventilation, and her symptoms gradually subsided. Five months after admission, her cognitive function had recovered, and a pelvic CT disclosed a right ovarian tumor of 52 mm in diameter. She underwent unilateral salpingo-oophorectomy, and a mature teratoma was found. After this operation she returned to her university.

Materials and Methods

Analysis of anti-neural antibodies in serum and CSF

Using GluRε2-cDNA from mice and immunoblotting technique, IgG and IgM-antibodies to whole molecules of GluRε2 (NR2B) were examined (9). Recombinant B18 cells expressing cDNA of GluRε2 and non-recombinant A1 cells were cultured for 48 hours with doxycycline (1 μg/mL). Supernatants of cell extracts were subjected to SDS-PAGE, and the gels were transferred to nitrocellulose membranes. Each membrane was cut into 20 strips after overnight blocking with the blocking buffer (0.02 M Tris HCl, 0.16 M NaCl, 0.05% bovine serum albumin). The strips of B18 and A1 were reacted with patient serum (diluted 20-fold with blocking buffer) or CSF (diluted 15-fold with blocking buffer) for

48 hours at 4℃, and were stained by alkaline phosphatase-labeled second antibodies (IgG or IgM) (Jackson ImmunoResearch, West Grove, Philadelphia, PA, U.S.A.). The presence of antibodies against GluRε2 was judged by a positively stained band with molecular size around 180 kDa, which was found only on the B18 strip and not on the A1 strip.

Detection of anti-NMDAR antibody was carried out as follows: cDNAs encoding NR1 (GluN1) and NR2B (GluN2B) (Gene Bank accession number NM-008169, NM-008170, NM-008171, respectively) were ligated into the expression vectors and transfected into human embryonic kidney (HEK) 293 cells in the media containing 10 μM MK-801 using Lipofectamine (Invitrogen). Twelve hours after transfection, HEK-293 cells were fixed in 4% paraformaldehyde in 0.1M phosphate-buffered saline (PBS, pH 7.4) for 20 minutes. After non-specific binding was blocked with 10% goat serum in PBS, these cells were incubated with patient sera (1:40) or cerebrospinal fluid (1:2) overnight at 4℃ and then with FITC-conjugated rabbit anti-human IgG (BD Biosciences) for 30 min at room temperature. *SlowFade* gold anti-fade reagent (Molecular Probes) was applied to the slides and the staining was observed under a fluorescence microscope. NMDAR expression on the cell surface was confirmed with the rabbit antibodies against each of the NMDAR subunits, NR1, NR2A and NR2B.

Immunohistochemical examination of ovarian tumor

Serial sections were prepared from a formalin-fixed, paraffin-embedded block of the ovarian teratoma and a Ventana XT automated immunohistochemistry system (Ventana Medical Systems, AZ) was employed. The primary antibodies used, dilutions, and the pretreatment procedures were as follows: anti-glial fibrillary acidic protein (GFAP) (Ventana, AZ, without dilution), anti-phosphorylated neurofilament (SMI-31, Sternberger Monoclonals, Baltimore, MD, ×2000), anti-human synaptophysin (Dako, Glostrup, Denmark, × 100), anti-NR 1 (AB1516, Chemicon, Temecula, CA, ×100), anti-NR2A (clone A12W, Upstate, Lake Placid, NY, ×50, microwave treatment in citrate buffer), anti-NR2B (Zymed, South San Francisco, CA, ×50), anti-GluR 1 (AB1504, Chemicon, Temecula, CA, ×5, microwave in citrate buffer), and anti-GluR 2 / 3 (AB1506, Chemicon, Temecula, CA, × 10, microwave in citrate buffer). GluR1 and GluR2/3 are pharmacologically classified into the AMPA type. Positive control sections were prepared from blocks including two ovarian tissues obtained from 21- and 29-year-old females at autopsy, and human temporal lobe and cerebellum. Negative control sections were treated in the same way except that the primary antibodies were replaced with normal bovine serum.

Prior to the study, detailed informed consent was obtained from the patient following a clear explanation of the purpose of the study. Our study protocol was approved by the local ethics committee.
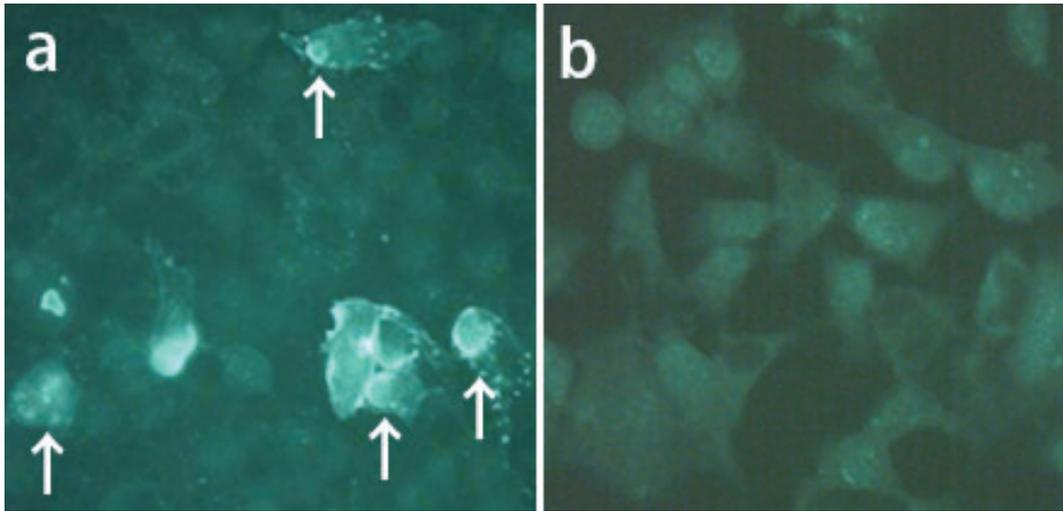
Figure 1. Immunohistochemical demonstration of antibodies against NMDAR. The serum of the patient showing positive immunoreactivity against heteromers of NR1 and NR2B subunits of NMDAR. a: serum of the patient, b: serum of a control patient without this disorder. Arrows indicate positively stained HEK cells. Immunofluorescence staining (×400).

Results

Detection of anti-neural antibodies in the serum and CSF of our patient

IgM-antibodies to whole molecules of GluRε2 were detected in serum obtained on day 50, and IgG-antibodies to whole molecules of GluRε2 were seen in CSF taken the same day. Both serum and CSF specifically reacted with HEK-293 cells expressing heteromers of NR1/NR2B (Fig. 1).

Histopathological and immunohistochemical findings of ovarian tumor

The capsular layers in this cystic tumor contained squamous epithelium, exocrine and sebaceous glands, hair follicles, fat, and neural tissues (Fig. 2-A and C). GAFP-immunoreactive areas showed a band-like or small dot-like distribution in the mural tumor tissues (Fig. 2-B and D), and among them many fibrous structures that were positively stained by an anti-phosphorylated neurofilament antibody were seen (Fig. 2-E, F and G). Other neural tissues were widely distributed in the areas with no expression of GFAP: they contained many small cells that were labeled by anti-phosphorylated neurofilament and anti-synaptophysin antibodies, and some of them showed epithelial pseudo-rosette formation (Fig. 3-F and G). A small number of anti-phosphorylated neurofilament antibody-positive fibrous structures were also observed in these areas with no immunoreactivity for GFAP (Fig. 2-A, B and H). Extensive areas of this tumor, including both mature neural tissues with expression of GFAP and immature neuroepithelial tissues without expression of GFAP, were specifically immunolabeled by anti-NR 2B and GluR 1, and GluR 2/3 antibodies

(Fig. 3-C, D and E), while anti-NR1 and NR2A antibodies did not produce any significant immunoreactivities (Fig. 3-A and B). In two normal appearing ovaries anti-GFAP, anti-phosphorylated neurofilament, anti-synaptophysin, anti-NR1 and NR2A antibodies showed no immunoreactivities (Fig. 4-A), but anti-NR 2B, GluR 1 and GluR 2/3 antibodies produced faint immunoreactivity: this was most clearly seen on the sections stained by an anti-NR 2B antibody (Fig. 4-B), where the cytoplasm of oocytes was specifically immunolabeled (Fig. 4-C). Neurons and astrocytes in positive control sections were immunoreactive for some or all anti-NR2B, GluR1 and GluR2/3 antibodies (Fig. 4-D and E). However, no significant immunoreactivity was observed in negative controls.

Discussion

Limbic encephalitis is a disorder ascribed to diverse causes, and several antibodies against neural surface antigens were identified in autoimmune or paraneoplastic limbic encephalitis (10). In Japan, young female patients with acute non-herpetic limbic encephalitis have been studied as acute juvenile female non-herpetic encephalitis (AJFNHE) (3). Neurological manifestations in these Japanese females consist of prominent psychiatric symptoms, seizures, dysautonomia, and involuntary movements, and the vast majority of them have a history of prodromal flu-like symptoms. This clinical picture of AJFNHE closely resembles that of the recently proposed anti-NMDAR encephalitis (1, 2), where antibodies to NR1/NR2 heteromers of NMDAR play an important role (4-6). Although antibodies against GluRε2 (NR2 B) have been frequently found in the sera and CSF of patients with AJFNHE (3), they have been also detected in patients with Rasmussen's syndrome and epilepsia partialis continua (EPC) (8). The presence of antibodies against
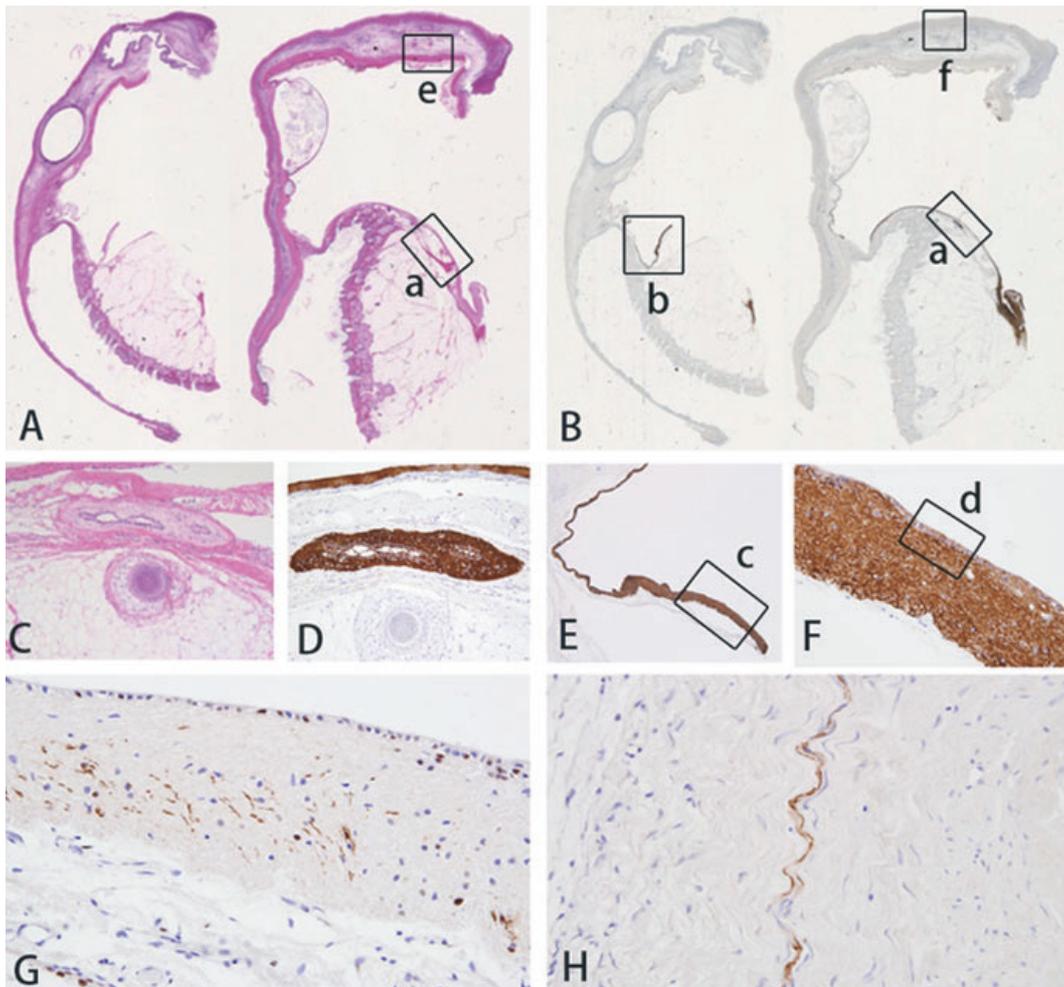
Figure 2.   **Histopathology of ovarian teratoma. A&B: Low magnification of the tumor. A: Hematoxylin and Eosin staining (×3), B: Immunoperoxidase staining with anti-GFAP antibody (×3). GFAP-positive immunoreactivity is seen in small localized areas of the tumor. C and D are higher magnifications of the framed area "a". The presence of hair follicle and neural tissue is noted and the latter is strongly immunolabeled by anti-GFAP antibody. C: Hematoxylin and Eosin staining (×50), D: Immunoperoxidase staining with anti-GFAP antibody (×60). E: Higher magnification of the framed area "b". F: Higher magnification of the framed area "c". Band-like distribution of neural tissue on the tumor can be identified as GFAP-immunoreactive area. E (×10), F (×70). G: Higher magnification of the framed area "d". Many anti-phosphorylated neurofilament antibody immunoreactive fibrous structures (possibly axons or dendrites) are seen in this area. Immunoperoxidase staining with anti-phosphorylated neurofilament antibody (×220). H: Higher magnification of the framed area "e". An anti-phosphorylated neurofilament antibody immunoreactive axon-like structure is visible. Immunoperoxidase staining with anti-phosphorylated neurofilament antibody (×250).**

GluRε2 in patients with anti-NMDAR encephalitis is now recognized to be less specific for the disease (11, 12).

In a large series of patients with anti-NMDAR encephalitis more than half of them were reported to have ovarian teratoma (2) and thus, the pathogenetic significance of ovarian teratoma in this encephalitis has been investigated. Dalmau et al reported that all five tumors obtained from the diseased patients showed mature- and immature-appearing neurons with expression of NR2B and /or NR2A (5), which suggests that ectopically expressed NMDARs in ovarian teratoma contribute to the production of antibodies to NMDARs. A patient with this disorder who promptly recovered after early removal of an ovarian teratoma has been reported (13). In the pathogenesis of anti-NMDAR encephalitis the antibody immune-response is thought to be more relevant than cytotoxic T-cell mechanisms (14): patients' NMDAR antibodies cause a specific, titer-dependent, and reversible decrease in NMDAR surface density and synaptic localization, especially in hippocampus (15). The loss of this subtype of GluRs eliminates NMDAR-mediated synaptic function, resulting in the learning, memory, and other behavioral deficits seen in patients with anti-NMDAR-
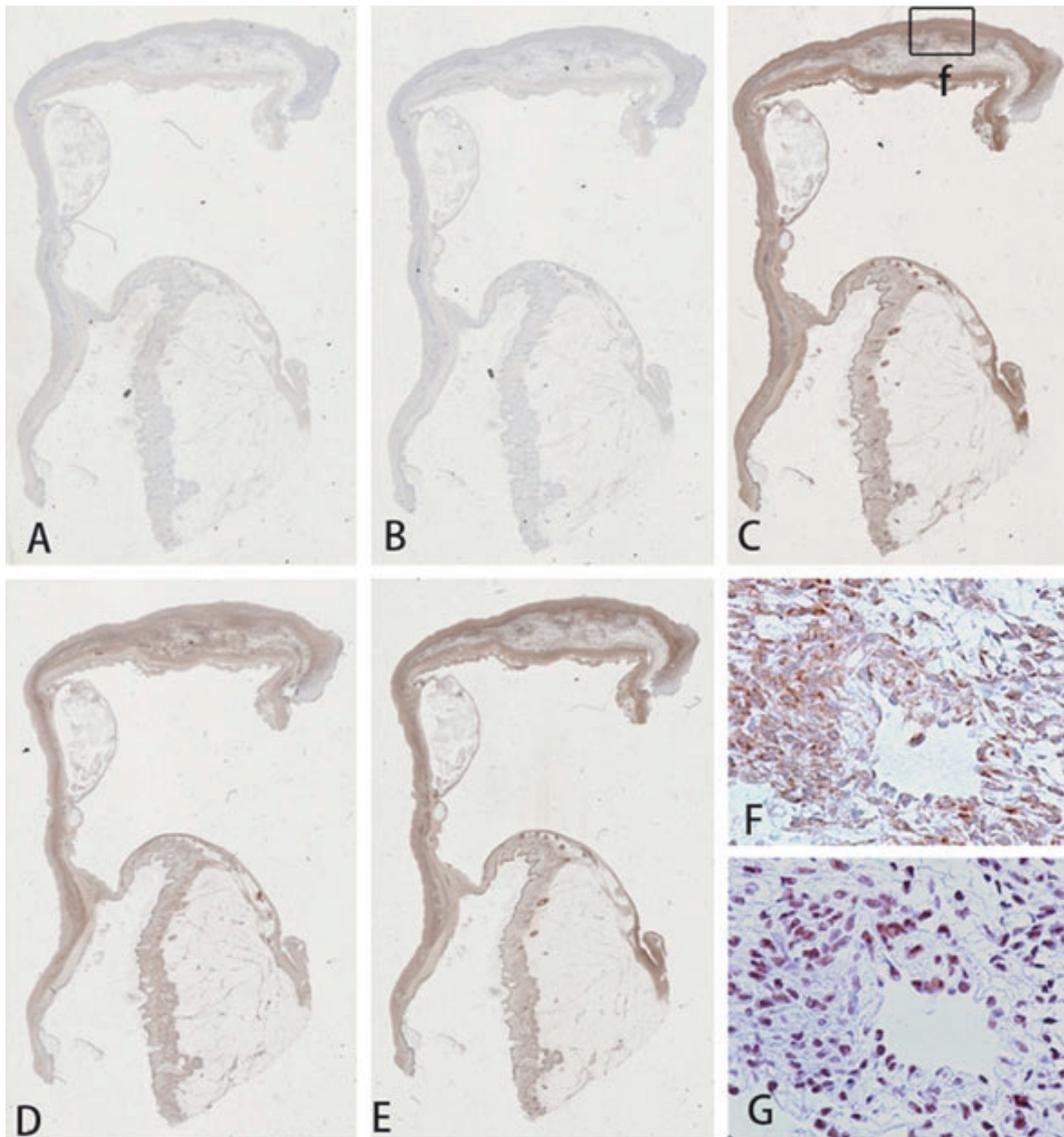
Figure 3. Immunohistochemical expression of NMDAR epitopes within ovarian teratoma. A: anti-NR1, B: anti-NR2A, C: anti-NR2B, D: anti-GluR1, E: anti-GluR 2/3, F&G: Higher magnification of the framed area "f". Neither anti-NR1 nor NR2 antibody is immunoreactive for the ovarian tumor, while anti-NR2B, GluR1 and 2/3 antibodies disclose strong immunoreactivity on an extensive area of this tumor. It is notable that the framed area "f" is not immunolabled by anti-GFAP antibody (see Fig. 1-B) but that this part is apparently immunoreactive for all the anti-NR2B, GluR 1 and GluR2/3 antibodies. This area consists of many small cells, some of which contribute to the pseudo-rosette formation. The vast majority of these small cells are immunolabeled by both anti-phosphorylated neurofilament (F) and anti-human synaptophysin antibodies (G). Immunoperoxidase staining (A to E, ×3; F&G, ×250).

encephalitis. Thus, immunosuppressive therapy including corticosteroid, plasma exchange and intravenous immuno-globulin has been used for the treatment of this disease (2). Recently rituximab, an anti-CD20 monoclonal antibody, is expected to expedite the recovery of the patients with this disease (16, 17).

In the present study we immunohistochemically examined an ovarian teratoma removed from a young female patient with antibodies to GluRε2 (NR2B) and NR1/NR2 het-eromers. In mural tissues of the tumor, well-differentiated neural tissues with GFAP-immunoreactivity showed expres-sion of GluRs including NR2B, GluR1, and GluR2/3. Addi-tionally, immature neuronal tissues without GFAP-immunoreactivity also showed expression of these GluRs. The most notable finding in this study is that the former tis-sue was very limited within the tumor but the latter was more extensively distributed than previously recognized. Al-though NR1 and NR2A epitopes were undetectable in our teratoma tissues, the lack of immunoreactivity for NR1 might be attributed to technical reasons (11), because ex-pression of NR2B, which is one subunit of NR1/NR2 het-eromers in the NMDAR complex, was clearly seen in the
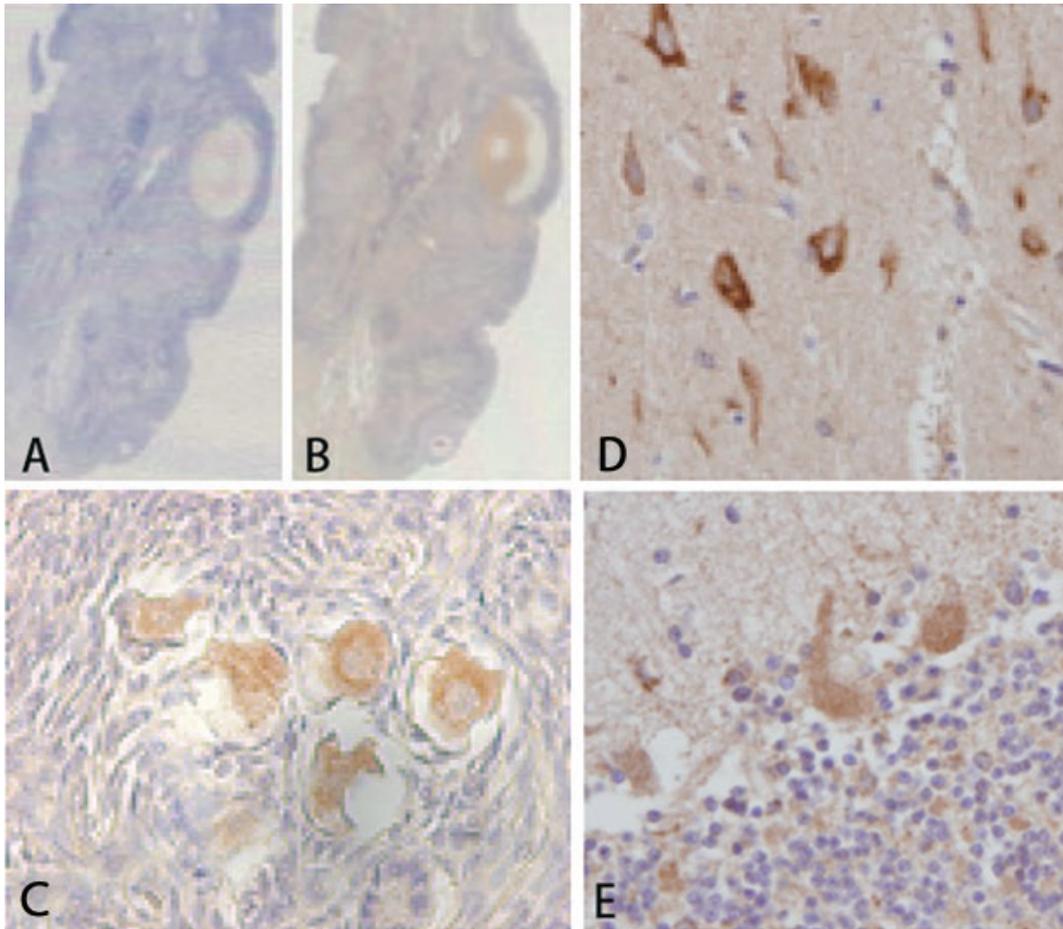
Figure 4. Immunohistochemical findings of controls. A&B: Low magnification of the control ovary obtained from a 21-year-old woman. A: Immunoperoxidase staining with anti-GFAP antibody (×2). No immunoreactivity is seen. B&C: Immunoperoxidase staining with anti-NR2B antibody. Some areas show slightly positive immunoreactivity (B, ×2) and among them the cytoplasm of oocytes is specifically immunolabeled (C, ×200). D: Immunoperoxidase staining of human temporal lobe with anti-NR2B antibody. Neurons and astrocytes are positively stained (×180). E: Immunoperoxidase staining of human cerebellum with anti-GluR 2/3 antibody. Purkinje cells are positively stained (×180).

cell surface of neural tissues examined. On the basis of our immunohistochemical findings ovarian teratoma seems to have abundant expression of various GluR-related epitopes including that of NMDAR, supporting the paradigm whereby the preceding flu-like illness causes inflammation in ovarian teratoma (14), which then leads to the triggering of abnormal antibody production targeting NMDARs (4, 5). Moreover the strong expression of GluR1 and GluR 2/3 (they are pharmacologically classified into AMPA) within the tumor might cause other antibodies against other GluRs than NMDAR: a few anti-AMPA encephalitis cases with antibodies to GluR 1 and GluR 2 were recently reported (18, 19), but they were not accompanied with ovarian teratoma, and their clinical pictures were different from those of the patients with anti-NMDAR encephalitis. The significance of the expression of GluR1 and GluR2/3 within the tumor, therefore, remains undetermined in considering the pathogenesis of autoimmune or paraneoplastic limbic encephalitis.

It is now widely accepted that the presence of ovarian teratoma is a serious predisposing factor for the development of anti-NMDAR encephalitis, but this tumor could not be found in about 40% of adult patients with the disease (2), the number of patients with the latter condition being increasing (20). Raising the recognition of this unique encephalitis has also disclosed that children or adolescents also encounter this disease (21), although the frequency of associated ovarian teratoma in them was much lower in comparison with that in adults (21). Although a few male cases (2, 22, 23) with anti-NMDAR encephalitis including a 20-month-old boy (17) were reported, the vast majority of the patients with this disease are females. The present study has added the possibility that the ovary itself has expression of NMDARs, since NR2B-related immunoreactivity was apparently observed in the cytoplasm of oocytes in control ovaries. The mechanisms that initiate this disorder are still incompletely understood in patients without ovarian teratoma and further studies are required.

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