

Identification of the *N*-Methyl-D-Aspartate Receptor (NMDAR)-Related Epitope, NR2B, in the Normal Human Ovary: Implication for the Pathogenesis of Anti-NMDAR Encephalitis

Naoko Tachibana,^{1,*} Michiaki Kinoshita,^{2,*} Yuko Saito³ and Shu-ichi Ikeda²

¹Department of Neurology, Okaya City Hospital, Okaya, Nagano, Japan

²Department of Medicine (Neurology and Rheumatology), Shinshu University School of Medicine, Matsumoto, Nagano, Japan

³Department of Clinical Medicine, National Center Hospital of Neurology and Psychiatry, Kodaira, Tokyo, Japan

N-methyl-D-aspartate receptors (NMDARs) are one type of ionotropic glutamate receptors (GluRs) and are heterotetrameric cation channels composed of NMDAR1 (NR1), NMDAR2 (NR2A, 2B, 2C or 2D) and NMDAR3 (NR3A or NR3B) subunits. The main subunits are NR1 and NR2 and their combinations are classified into several diverse forms including NR1/NR1/NR2A/NR2A, NR1/NR1/NR2B/NR2B and NR1/NR1/NR2A/NR2B. NMDARs are physiologically related to synapse development and synaptic plasticity in the central nervous system. Anti-NMDAR encephalitis is a form of autoimmune limbic encephalitis mainly affecting young women, with various manifestations including initial psychiatric symptoms, subsequent unresponsiveness, intractable generalized seizure, dysautonomia and orofacial dyskinesia. This disorder is often accompanied by ovarian teratoma that is originated from oocytes. Anti-neural antibody for the NR1/NR2 heteromer of NMDAR has been identified as a disease-specific hallmark. It has been emphasized that neural components in ovarian teratoma act as a trigger to produce anti-NMDAR antibodies, although about half of the patients with anti-NMDAR encephalitis are not associated with ovarian teratoma. To identify NMDAR-related epitopes located outside of the brain, we performed immunohistochemical examinations of normal human ovary and testis using specific antibodies against NR1, NR2A and NR2B, respectively, and found expression of the NR2B epitope in the cytoplasm of oocytes. In contrast, the testis showed no immunohistochemical reactivity. Therefore, oocytes contain NMDAR-related epitopes including NR2B. The NMDAR-related epitopes in normal oocytes may cause an antigen-antibody reaction in certain pathological conditions. The presence of NR2B immunoreactivity in oocytes may account for the fact that anti-NMDAR encephalitis predominantly affects young females.

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Introduction

N-methyl-D-aspartate receptors (NMDARs) are one type of ionotropic glutamate receptors (GluRs), showing active involvement in synapse development, synaptic plasticity, and learning and memory. They are comprised of seven members: NMDAR1 (NR1), NMDAR2 (NR2A, 2B, 2C or 2D), and NMDAR3 (NR3A or NR3B) subunits, the main subunits being NR1 and NR2. They form different heterometric configurations (for example, NR1/NR1/NR2A/NR2A, NR1/NR1/NR2B/NR2B, NR1/NR1/NR2A/NR2B, NR1/NR1/NR2C/NR2C, NR1/NR1/NR2D/NR2D and NR1/NR1/NR2A/NR3A), which can respond to functional and spatiotemporal diversities of NMDARs

(Kutsuwada et al. 1992; Watanabe et al. 1992). Anti-NMDAR encephalitis is a recently established disease entity, showing the characteristic clinical manifestations of initial psychiatric symptoms and subsequent intractable seizures, dyskinesias and autonomic instability (Dalmau et al. 2007, 2011), and an anti-neural antibody for the NR1/NR2 heteromer of NMDAR has been identified as a disease-specific hallmark (Vitaliani et al. 2005; Dalmau et al. 2007; Iizuka et al. 2008): this disorder predominantly affects women of reproductive age and is often accompanied by ovarian teratoma. Mature- and immature-appearing neurons in ovarian teratoma ectopically express NMDARs, which seems to contribute to the production of antibodies to NMDARs. The disease was, therefore, considered to be a

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*These authors have contributed equally to this work.

Correspondence: Shu-ichi Ikeda, Department of Medicine (Neurology and Rheumatology), Shinshu University School of Medicine, Matsumoto 390-8621, Japan.

e-mail: ikedasi@shinshu-u.ac.jp

type of paraneoplastic encephalitis (Vitaliani et al. 2005; Dalmau et al. 2007), but in recent information about the rapidly increased number of patients with this encephalitis (Prüss et al. 2010; Dalmau et al. 2011), it is known that babies and children are also affected by anti-NMDAR encephalitis (Florance et al. 2009; Niehusmann et al. 2009; Titulaer et al. 2013). However, about half of diseased females were not associated with ovarian teratoma (Dalmau et al. 2008; Kamei et al. 2009; Irani et al. 2010). Additionally, a multi-institutional cohort observation study has revealed that 11% of 577 patients enrolled were male and that the vast majority of these male patients were found to have no underlying neoplasm (Eker et al. 2008; Novillo-Lopez et al. 2008; Wong-Kisiel et al. 2010; Tojo et al. 2011; Titulaer et al. 2013). Thus, the mechanism, which leads to the production of anti-NMDAR antibodies in patients, is still undetermined. We previously reported that the normal ovary itself expresses a NMDAR-related 2B epitope on the basis of immunohistochemical study of two autopsied patients aged 21 and 29, respectively (Tachibana et al. 2010), which might have provided a clue to understand the pathogenesis of this disease in female patients without tumors. To confirm this finding we further examined significant numbers of human ovaries and testes in this study.

Materials and Methods

The ovarian and testicular tissues were collected from autopsied samples from 11 females aged from 18 to 29 and 3 males aged from 19 to 34. Serial sections were prepared from formalin-fixed, paraffin-embedded blocks and a Ventana XT automated immunohistochemistry system (Ventana Medical System, AZ) was employed for the staining (Tachibana et al. 2010). The primary antibodies used and their dilutions were as follows: anti-gial fibrillary acidic protein (GFAP) antibody (Ventana, AZ, without dilution), anti-NR1 antibody (AB1516, Chemicon, Temecula, CA, $\times 100$), anti-NR2A antibody (clone A12W, Upstate, Lake Placid, NY, $\times 50$), and anti-NR2B anti-

body (Frontier Institute Co., Hokkaido, $\times 50$). For the application of anti-NR2A antibody deparaffinized sections in citrate buffer at pH 6.0 were pre-treated by microwave and for that of anti-NR2B antibody they were pre-treated by autoclave (121°C for 20 minutes). Positive control sections were prepared from human temporal lobe and cerebellum, and negative control sections were treated in the same way except that the primary antibodies were replaced with normal bovine serum. Our study protocol was approved by the Ethical Committee of Shinshu University School of Medicine.

Results

Anti-GFAP, anti-NR1 and NR2A antibodies showed no immunoreactivity (Fig. 1A and B), but an anti-NR2B antibody that recognizes the N-terminal portion of NR2B subunits of NMDAR produced positive immunoreactivity in all 11 ovarian tissues examined: coarse granular immunoreactivity was seen in the cytoplasm of oocytes in primordial follicles (Fig. 2A). Three tissue samples of the human testis did not show any immunoreactivities with anti-GFAP, NR1, NR2A, or NR2B antibody (Fig. 2B). Neurons in control temporal lobe and cerebellum sections were immunoreactive only with an anti-NR2B antibody, and astrocytes in the two control tissue block sections were immunoreactive with anti-GFAP antibody. No meaningful immunoreactivity was observed in negative controls (data not shown), which was very similar to that in our previous study (Tachibana et al. 2010).

Discussion

In the pathogenesis of anti-NMDAR encephalitis ovarian teratoma was initially considered to play an important role in the production of an anti-NMDAR antibody (Vitaliani et al. 2005; Dalmau et al. 2007). This anti-neural antibody causes selective and reversible capping and internalization of surface NMDARs in the brain, leading to

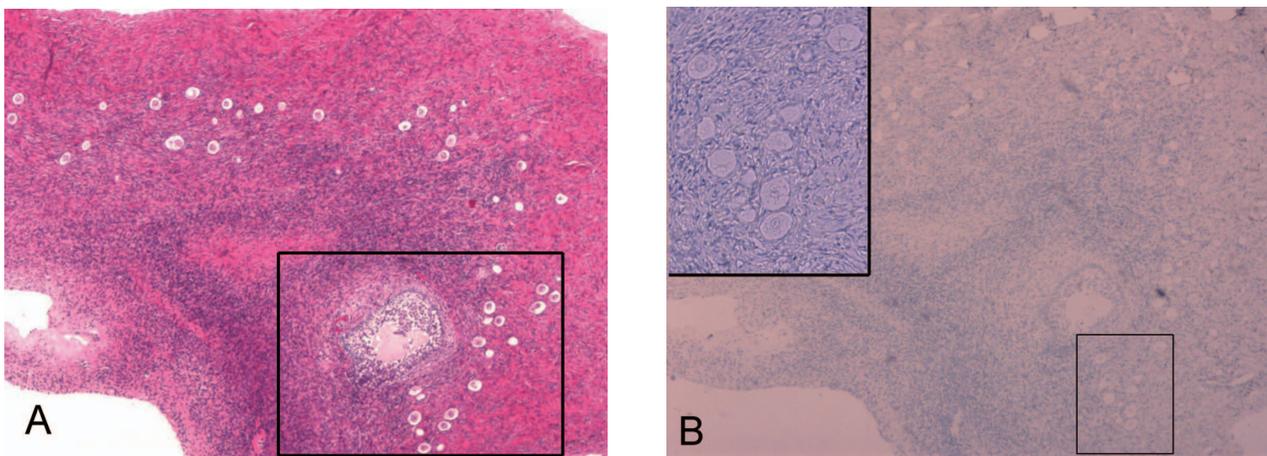


Fig. 1. Histological and immunohistochemical findings of ovary.

A: an ovary removed from a 24-year-old woman contains many primordial follicles with a single layer of flattened follicular cells. H&E $\times 30$. B: this is an enlargement of the framed area in A and the insert is an enlargement of the framed area: no oocytes in primordial follicles show immunoreactivity for anti-NR2A antibody. Immunoperoxidase staining $\times 80$.

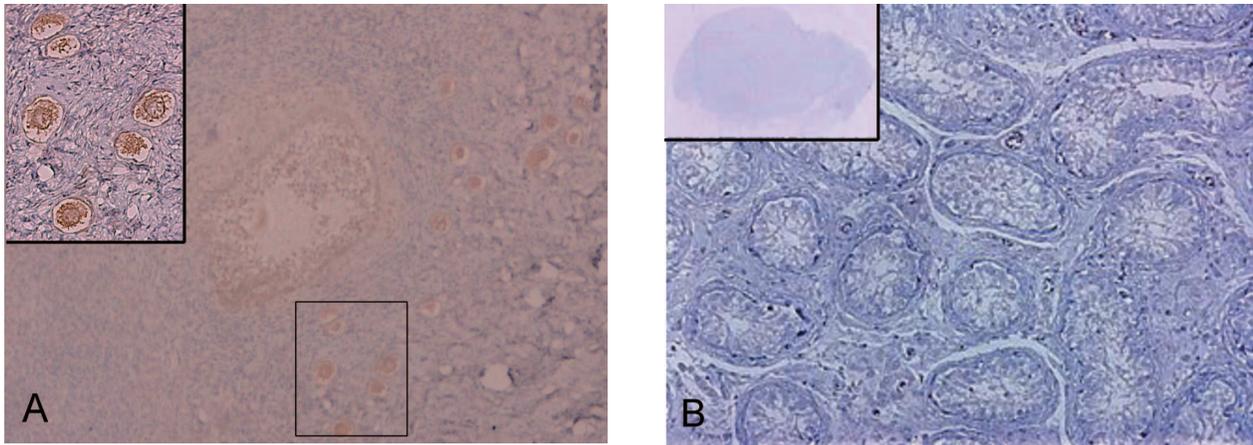


Fig. 2. Immunohistochemical findings of ovary and testis.

A: in the same case shown in figure 1 all oocytes in primordial follicles reveal positive immunoreactivity for anti-NR2B antibody. The insert shows an enlargement of the framed area. Immunoperoxidase staining $\times 80$, insert $\times 200$. B: The insert covers the whole area of the immunostained testicular tissue section removed from a 24-year-old man. No cellular components show immunoreactivity for anti-NR2B antibody. Immunoperoxidase staining $\times 80$, insert $\times 1.2$.

severe dysfunction of neocortical memory, learning and cognitive abilities (Hughes et al. 2010; Dalmau et al. 2011). Histopathological studies have shown that ovarian teratomas removed from patients with this encephalitis certainly contained many neurons having immunoreactivities for anti-NMDAR antibodies, mainly for an anti-NR2B antibody (Sansing et al. 2007; Seki et al. 2008; Tüzün E et al. 2009). However, recent studies on the basis of large numbers of patients with this disease have disclosed that ovarian teratomas were detectable in only about half of the affected females (Dalmau et al. 2008; Kamei et al. 2009; Irani et al. 2010; Titulaer et al. 2013), and added a significant number of male patients without tumors (Novillo-Lopez et al. 2008; Wong-Kisiel et al. 2010; Tojo et al. 2011; Titulaer et al. 2013). Another feature of this disease is that the vast majority of the patients are of reproductive age (Dalmau et al. 2011; Titulaer et al. 2013). Both points strongly suggest that NMDAR-related antigens are provided from tissues outside the brain, which led us to examine normal ovaries and testes.

Ovarian teratoma with NMDAR-related epitopes develops by neoplastic transformation of an oocyte and thus, it is expected that primordial oocytes have similar epitopes, although other epitopes than NR2B were not demonstrated in the present study. NMDARs are ionotropic glutamate receptors, structured as heteromeric channels comprising various combinations of NR1 (GluN1), NR2 (GluN2) and NR3 (GluN3) subunits (Kutsuwada et al. 1992; Watanabe et al. 1992). In hippocampus or cortex the most abundant NMDARs are composed of NR1 associated with NR2A or NR2B subunits (Wenthold et al. 2003; Groc et al. 2006) and seem to work in relation to learning and memory (Abe et al. 2004). However, it was described that, in experiments, surface NR2A-containing NMDARs could not be observed by conventional immunohistochemistry (Groc et al. 2006). The developmental change in the synap-

tic surface content of NR2A and NR2B subunits was also shown (Groc et al. 2006; Ewald et al. 2009) and the main expression of that is NR2B in the embryonic and early post-natal periods (Monyer et al. 1994). It is, therefore, understandable that primordial oocytes have only the immunohistochemical expression of NR2B. If so, it remains unclear how NMDAR-related antigens in oocytes contact inflammatory cells, including B lymphocytes and lead to the production of anti-NMDAR antibodies. In considering the pathogenesis of this disease the possibility of an infectious process has been given much attention, and prodromal infection, which was reported in 70-90% of patients with anti-NMDAR encephalitis (Dalmau et al. 2011), might secondarily cause inflammatory reactions on normal ovaries. Additionally, there is no information on the non-brain tissues expressing NMDAR-related epitopes in male patients. It is now controversial which of NR1 and NR2 subunits are targeted by patient anti-NMDAR IgGs. Gleichman et al. (2012) emphasized that NR1 epitope was crucial for the creation of immunoreactivity, while Mikasova et al. (2012) reported that the surface dynamics of synaptic NR2A and extrasynaptic NR2B subunits were significantly impaired by patient anti-NMDAR IgGs. The pathogenic process of anti-NMDAR encephalitis is considered to be more complicated than previously recognized. To prove our hypothesis, further studies including protein chemical analysis of oocytes are required.

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Conflict of Interest

We declare no conflict of interest.

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