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Review Article

Anti-NMDA receptor encephalitis: a review of mechanistic studies

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Abstract: NMDA receptors (NMDARs) are ion channels gated by glutamate, the major excitatory neurotransmitter in the central nervous system. Anti-NMDA receptor (anti-NMDAR) encephalitis is an autoimmune disease characterized by the presence of autoantibodies against the NMDAR GluN1 subunit. Here we briefly review current advances in the understanding of the mechanisms underlying the pathogenesis of anti-NMDAR encephalitis. The autoantibodies bind to and cross-link the endogenous NMDARs, disrupt the interaction of NMDARs with receptor tyrosine kinase EphB2 leading to internalization and reduced function of NMDARs. Hypofunction of the NMDARs results in impairment in long-term potentiation and deficit in learning and memory, leads to development of depression-like behavior, and lowers the threshold for seizures. Recent development of active immunization models of anti-NMDAR encephalitis provides insight into the inflammation process and paves the way for further studies that may lead to better treatment.

Keywords: NMDA receptors, encephalitis, autoimmune antibodies

Introduction

NMDA receptors (NMDARs), along with AMPA receptors (AMPARs) and kainate receptors, are ion channels gated by glutamate, the major excitatory neurotransmitter in the central nervous system [1]. In this mini review, we start with a summary of NMDARs highlighting the pharmacological and genetic evidence for the roles played by NMDARs. Then we introduce the anti-NMDAR encephalitis. In the main text, we discuss the current advances in our understanding of the disease, focusing on the cellular and cognitive neuroscience mechanisms underlying the effect of the NMDAR antibodies. We look into three approaches broadly used in the current research: the *in vitro* models where the effect of patient autoimmune antibodies on the cells and tissues from normal animals can be examined, the *in vivo* passive transfer models where the patient antibodies are transferred to experimental animals, and the *in vivo* active immunization models where the experimental animals are immunized with antigens to produce NMDAR antibodies and reproduce the anti-NMDAR encephalitis.

NMDARs and anti-NMDAR encephalitis

NMDARs play important roles in synaptic development, synaptic plasticity, learning, memory, and cognition [1]. Unlike AMPARs and kainate receptors, NMDARs require co-agonist glycine (or D-serine endogenously) for activation. They are permeable to calcium and blocked by magnesium at resting membrane potentials [1]. Because activation of NMDARs requires presynaptic release of glutamate and synchronous post-synaptic membrane depolarization, NMDARs are unique in their capacity to act as coincidence detectors.

NMDARs are tetramers composed of subunits from GluN1, GluN2 (A-D) and/or GluN3 (A and B), with GluN1 being obligatory [2, 3]. The glycine binding site is conferred by GluN1 (or GluN3), whereas the glutamate binding site is conferred by GluN2. NMDAR subunits are transmembrane proteins that follow a similar

topology on the membrane: a large N-terminal extracellular region with three trans-membrane-spanning domains (TM1, 3, 4), a membrane re-entrant loop (TM2), and an intracellular C-terminal domain [4]. The large N-terminal extracellular region is further divided into the amino-terminal domain (ATD) (also called N-terminal domain, NTD, and will be referred to as ATD/NTD throughout the review) and the ligand-binding domain (LBD).

NMDAR subunit composition within each receptor determines the biophysical and pharmacological properties (such as affinity to glycine, glutamate, sensitivity to zinc, magnesium, and other pharmacological agents), localization in specific brain regions, developmental dynamics, subcellular localization (pre- vs post-synaptic; synaptic vs extra-synaptic), intra-cellular signaling pathways and synaptic function [2, 3]. NMDARs are dynamic entities and are trafficked to and from the plasma membrane through exocytosis and endocytosis, respectively [5], and their surface distribution and synaptic content are highly dependent on their lateral diffusion [6]. Whereas the receptors composed of two GluN1 subunits and two identical GluN2 subunits were thought to be the predominant receptors (diheteromers), recent progress favors the idea that a lot of (if not the majority of) native NMDARs are tetramers composed of two GluN1 subunits with two different GluN2 subunits (or a combination of GluN2/GluN3) (triheteromers) [2, 3].

The terminology of diheteromer and triheteromer only indicates the number of different subunits in an NMDAR, not the total number of subunits in the whole receptor, which is believed to be four consistently. The best known triheteromer is GluN1/GluN2A/GluN2B, which is widely expressed in the adult forebrain, where it represents a major synaptic NMDAR population. The other combinations such as GluN1/GluN2A/GluN2C, GluN1/GluN2A/GluN2D, and GluN1/GluN2B/GluN2D have also been suggested to function in certain brain regions [2, 3]. GluN3-containing NMDARs are currently viewed as atypical and unconventional. There is evidence that GluN1-GluN2-GluN3A receptors do exist in hippocampal and cortical neurons. The GluN2 in the GluN1-GluN2-GluN3 triheteromer can be GluN2A or GluN2B in neurons, or GluN2C in oligodendrocytes [7]. The available evidence suggests that GluN3-containing receptors function to destabilize synapses and are important in synapse pruning [7].

Pharmacological and genetic inhibition of NMDARs

Both pharmacological and genetic inhibition studies have demonstrated that NMDARs are essential for the induction of long-term synaptic plasticity at various synapses and for learning and memory. In animal models, all non-competitive NMDAR antagonists (e.g. phencyclidine (PCP), ketamine and MK-801) could produce aspects of cognitive impairment in schizophrenia [8]. Similarly, in otherwise healthy human subjects, pharmacological inhibition of NMDARs induced cognitive and behavioral dysfunction [9]. For example, ketamine administration induced both positive and negative symptoms of schizophrenia [10]. Genetically, global knock-out of GluN1 gene in mice resulted in neonatal death [11], a discovery consistent with the vital function of NMDARs. Inducible, reversible, and specific GluN1 knockout in the hippocampal CA1 region demonstrated that NMDARs are critical for the consolidation period of memory formation [12]. Mice with specific ablation of GluN1 in the hippocampal CA3 region were impaired in spatial memory retrieval [13]. Furthermore, selective ablation of GluN1 in parvalbumin-positive interneurons caused impairment in hippocampal synchrony, spatial representations, and working memory [14]. In summary, the roles of NMDARs in synaptic plasticity and learning and memory have been well documented.

Anti-NMDAR encephalitis

Anti-NMDAR encephalitis is an autoimmune disease characterized by the presence of antibodies against the NMDAR subunit GluN1. The disease has a strong female predominance (female to male ratio, 4:1) and the median age of the patients is 21 years (range 2 months to 85 years; 37% of patients are younger than 18 years old). Children with the disease show more neurological symptoms such as seizure, movement disorders, insomnia, irritability and confusion, while adults demonstrate more psychiatric symptoms such as psychosis and abnormal behaviors [15, 16]. Tumors, particularly ovarian teratomas that often contain nervous system

tissue and express NMDARs and herpes simplex encephalitis, are two known risk factors for anti-NMDAR encephalitis [15, 17, 18]. Thus 58% of women ages 18-45 years with anti-NMDAR encephalitis have ovarian teratoma [15]. Readers interested in the clinical aspects of the anti-NMDAR encephalitis are referred to several excellent recent reviews [15-18].

Mechanistic studies in in vitro models

Conceptually, NMDAR antibodies could affect the synaptic transmission by altering the NMDAR channel biophysics and/or the number of the NMDARs at the synapses and extra-synaptic locations. It is now firmly established that NMDAR antibodies cross-link NMDARs and cause them internalization, leading to a reduction of NMDAR-mediated (but not AMPAR-mediated) synaptic currents [19]. Despite the profound effect on NMDARs, there is no evidence demonstrating that patients' antibodies alter the localization or expression of other glutamate receptors like AMPARs or synaptic protein PSD-95, change the number of synapses, dendritic spines, dendritic complexity, or affect cell survival in *in vitro* or *in vivo* models [19], which are all consistent with the lack of gross brain damage from MRI imaging studies [15]. Since this seminal study [19], progress has been made on several fronts: the elucidation of antibody binding on the GluN1 extracellular sites [20, 21], the discovery of NMDAR-EphB2 interaction in antibody-mediated NMDAR endocytosis [22-24], the understanding of receptor dynamics at the single molecule level [25], the suggestion of involvement of additional synaptic receptors such as dopamine D1R and D2Rs [26, 27], and the recent implication of NMDARs in the oligodendrocytes [28].

Antibody-mediated changes in NMDAR dynamics and internalization

After it was learned that patient antibodies cross-linked and internalized the NMDARs, the progress first came in the field when the anti-NMDAR antibody binding site was mapped on the specific region of the GluN1 [20]. Since patient NMDAR antibodies do not recognize the denatured proteins on the Western blot, it was necessary to use the GluN1/GluN2 expression in the HEK293 cells to assay the antibody binding [29]. Through analysis of binding autoantibodies to GluN1 with mutations in various regions, it was determined that the epitopes for most GluN1 antibodies are located in the ATD/NTD of GluN1 that contains N368/G369 [20, 21]. The antibody binding site on the ATD/NTD could be close to, or partially overlapping with, the sites on the GluN1 that interact with the EphB2, the receptor tyrosine kinases for the ephrin B class [30]. NMDARs and EphB2 are known to interact, and ephrin B2-dependent activation of EphB2 results in the direct interaction between the extracellular domains of EphB2 and the NMDAR GluN1 subunit that stabilizes NMDARs in the synapses [31].

It was reasonable, then, to ask the question of whether NMDAR antibodies disrupt NMDAR-EphB2 interaction [22]. Using a unique combination of high-resolution nanoparticle and bulk live imaging approaches, Mikasova and colleagues demonstrated that in cultured hippocampal neurons, patient antibody IgGs disrupted the interaction between NMDARs and EphB2 [22]. Patient cerebrospinal fluid (CSF) reduced the surface content of both GluN2A- and GluN2B-NMDARs, and prevented a chemically induced long-term potentiation (cLTP) of glutamate synapses [22]. Quantum-dot technique was used to demonstrate that with pretreatment of the patient IgGs, the GluN2A-NMDAR lateral diffusion was significantly increased compared to the control [22]. The lateral diffusion of GluA1-AMPAR was only slightly increased. The lateral diffusion of a metabotropic GABA receptor ($GABA_AR$) and a potassium channel Kv1.3, which worked as control synaptic proteins, was not affected by NMDAR antibody treatment [22]. Inclusion of ephrin B2 with the patient NMDAR antibodies abolished the effect of antibodies on the NMDAR lateral diffusion, the calcium entry, and the NMDAR surface expression [22].

The NMDAR-EphB2 interaction is now known to involve positively charged residues in the GluN1 ATD/NTD region (N273 and R337 among the six key amino acids in the hinge region) and the phosphorylated, negative charge extracellular region of EphB2 [30]. Activation of EphB2 by ephrin B2 resulted in the phosphorylation of Y504, an extracellular tyrosine (p*Tyr) residue in EphB2. Phosphorylation of EphB2 Y504 was necessary and sufficient for the NMDAR-EphB2 interaction and synaptic localization of the NMDARs [32]. These new

findings should help us in designing research to understand how patient antibodies cause dysfunction of the NMDARs at the synapses and extra-synapses because NMDAR antibodies disrupt NMDAR-EphB2 interaction, and both the antibody-binding epitope and the key residues critical for NMDAR-EphB2 interaction are located in the hinge region of GluN1 ATD/NTD [30].

Super-resolution microscopy and single-molecule imaging

Super-resolution microscopy is used to study the effect of patient antibodies on the nanoscale distribution of NMDARs in cultured neurons [25]. The single-molecule localization microscopy, particularly the stochastic optical reconstruction microscopy (STORM), was a powerful tool to study receptor dynamics at high resolution *in vitro*. Under control conditions, NMDARs are organized in small nanosized clusters (nano-objects) along the dendrite as revealed by STORM [25]. Treatment of cultured neurons with NMDAR antibodies decreased the number of surface NMDAR nanoobjects per unit length of dendrite [25], a finding consistent with previous studies using conventional confocal microscopy. NMDAR antibodies reduced both total and synaptic GluN2A-NMDARs and GluN2B-NMDARs in cultured neurons, with a more dramatic decrease in GluN2B-NMDARs. The patient antibodies induced a time-dependent increase in the receptor content of the surface NMDAR nano-objects in synapses and extra-synapses. The increase in the receptor content of the surface NMDAR nano-objects in synapses and extra-synapses were due to an increase in the receptor content of the GluN2B nano-objects, as the size of the GluN2A nano-objects remained unchanged or slightly decreased. Activation of EphB2 by soluble ephrin-B2 abolished the effect of the patient antibodies on the change of nano-objects.

Monte-Carlo simulation supported the experimental results and a new model emerged pertaining to the cross-linking and internalization of NMDARs by NMDAR antibodies [25]. Previously, studies have shown that human NMDAR IgG antibodies lead to slower diffusion of GluN1/GluN2B heterodimers [22]. On the surface, there seems to be consistency in the increased immobility of GluN2B-NMDARs [22] and increased receptor content of the GluN2B nano-objects [25]. A deeper perusal, however, reveals some discrepancies. In the quantum-dot imaging study, the higher mobility of GluN2A was associated with higher loss through internalization [22]. On the other hand, in the STORM super-resolution microscopy, increased nano-size in GluN2B correlated with higher level of cross-linking, which preceded subsequent internalization [25]. How the patient antibodies differentially affect GluN2A vs GluN2B is still an open question. Given that GluN1/GluN2A/GluN2B is common in the hippocampal and frontal region, it becomes ever more important to resolve the issue [2, 3].

Beyond NMDARs and neurons

The NMDARs and Dopamine D1 receptors (D1R) physically and functionally interact in synapses [33, 34]. Two groups have now studied whether patient antibodies against NMDAR affect D1R. NMDAR antibodies purified from patients with anti-NMDAR encephalitis or schizophrenia reduced D1R surface dynamics (measured by instantaneous diffusion coefficients) in quantum-dots single molecule imaging [26]. CSF from patients with anti-NMDAR encephalitis significantly decreased D1R and increased D2R clusters on the cultured hippocampal neuronal cells, but did not change the clusters of synaptic protein PSD-95 [27].

Another recent mechanistic study extended our knowledge of the antibody effect beyond neurons. Oligodendrocytes are a major glial cell type in the brain that myelinate the neuronal axons and are known to express all the NMDAR main subunits. Activation of NMDARs in oligodendrocytes promotes the insertion into the cell surface of glucose transporter GLUT1, which transports glucose [35]. Do NMDAR antibodies impair the NMDAR function in the oligodendrocytes? CSF of patients with anti-NMDAR encephalitis, and a specific human monoclonal antibody against NMDAR, both significantly reduce calcium entry into oligodendrocytes after NMDAR activation. When the CSF was deprived of the NMDAR antibodies by GluN1 pre-absorption, CSF lost its effect on the NMDAR-mediated calcium entry. Moreover, the patient CSF also impaired the NMDAR-mediated GLUT1 insertion [28].

Long-term potentiation (LTP) in in vitro models

LTP at the Schaffer collateral-CA1 synapses in hippocampal slices is one of the best-studied

models for NMDAR-dependent synaptic plasticity [28]. Patient antibodies (in the form of CSF) suppressed LTP in this region and the control CSFs (an artificial CSF, CSF from a non-encephalitis control patients, or CSF that was depleted of the NMDAR antibodies) did not suppress the LTP [36]. The suppression of LTP by the patient antibodies *in vitro* has been confirmed by passive transfer of patient antibodies experiments (see below).

Although the *in vitro* approaches provided unprecedented levels of resolution and precision in defining the antibody binding sites on the GluN1, in dissecting the roles of NMDAR-EphB2 interaction, in tracing the molecular dynamics, and in unraveling the roles played by other synaptic receptors, they cannot answer the question of how patient antibodies induce pathogenesis in the body, which require more *in vivo* approaches. Another limitation of the *in vitro* models was the lack of opportunity to test behaviors. The development of *in vivo* models may allow researchers to overcome this drawback.

In vivo passive transfer models: LTP, memory and behavioral defects, and seizures

Passive transfer of patient antibodies into animals can overcome some limitations observed in *in vitro* approaches. First, the *in vivo* passive transfer models provide a way to confirm the detrimental effect of the patient antibodies on the number and function of NMDARs. So far, the models have provided convincing evidence that chronic transfer of patient antibodies indeed decreased endogenous NMDARs in synapses, reduced NMDAR-mediated currents or calcium-entry, and impaired NMDAR-dependent LTP. The passive transfer models also confirmed the clinical observation that the transfer of patient antibodies do not cause gross brain damages.

LTP

Unlike the LTP studies in *in vitro* models, the *in vivo* models allow the assessment of the chronic effect of the transferred patient antibodies. In mice infused with patient CSF, LTP in Schaffer collateral-CA1 synapses from acute hippocampal slices was impaired, accompanied by a decrease in the density of NMDARs and EphB2 at the synapses. Infusion of the soluble ephrin-B2, the cognate ligand and activator of EphB2, prevented the effect of patient antibodies on the density of cell-surface and synaptic NMDARs and EphB2, and on the long-term synaptic plasticity [24]. The impairment of LTP in hippocampal CA1 was also observed in female Wistar rats that were infused with patient CSF [37]. In the hippocampal CA3, there are two types of LTP: the NMDAR-dependent LTP at the associational-commissural (A/C) fiber-CA3 synapse, and the NMDAR-independent LTP at the mossy fiber (MF)-CA3 synapse. Infusion of patient CSF (containing NMDAR antibodies) in female Wistar rats did not affect the LTP at the mossy fiber (MF)-CA3 synapse, but did impair the LTP at the associational-commissural (A/C) fiber-CA3 synapse even though the degree of impairment varied among CSF samples [38]. Furthermore, LTP in dentate gyrus of adult female rats that were infused with patient CSF was significantly reduced [39].

Memory and behavioral defects

Several paradigms of behavioral tests are available in rodent models, and the selection of each paradigm is based on its scope of measurement and its possible correlation to human cognitive deficits. Morris water maze is used to test the spatial memory, novel object recognition (NOR) is utilized to measure the short term/working memory, sucrose intake is an indicator of anhedonic behaviors, and depression-like behaviors are tested using tail-suspension and forced swimming tests. Elevated platform tests and light-dark box tests are conducted to examine anxiety-like behaviors.

Spatial memory measured in the Morris water maze task was compromised when mouse brains were infused with patient antibodies [40]. A number of studies used the NOR to measure the effect of patient antibodies on the short-term/working memory in mice [23, 24, 27, 41] and in rats [37]. The principle behind the NOR test is that rodents tend to spend more time exploring novel objects, and the novel object discrimination index indicates such a preference. A higher index reflects greater memory retention for the object at the familiar location [23]. However, if the short-term memory is compromised, then the novel object discrimination index will be reduced. It was reported that patient NMDAR antibodies con-

sistently reduced the novel object discrimination index in mice and rats after passive transfer of patient antibodies, suggesting a decrease in short-term/working memory [23, 24, 27, 37, 41]. The time course was followed: when the infusion stopped at day 10, the discrimination index reached the bottom level at day 18, and recovered at day 25 [23, 24]. However, there was one conflicting report that the infusion of patient antibodies did not compromise NOR [42]. In addition, animals that received passive transfer of patient antibodies demonstrated a decrease in the preference of sucrose compared to control animals.

The sucrose intake was designed to test whether the animals experience some symptoms of anhedonia, which is common in depression [23, 24]. Researchers also investigated the depression-like behaviors in mice using tail-suspension and forced swimming tests [23, 24]. In these tests, immobility time is an indication that the animals have given up their escape effort. In a report using tail-suspension tests, animals infused with patients' CSF had significantly longer periods of immobility compared with those infused with control CSF on day 12. On day 20, the experimental animals did not show a significant increase in immobility time compared to control animals in the forced-swimming tests [23, 24]. Since these two behavioral tests were performed on different days after the infusion and recovery, it was not possible to compare them directly.

The deficits induced by patient antibodies, i.e, impairment in novel object recognition and depression-like behaviors, were all prevented by inclusion of ephrin-B2, the cognate ligand of EphB2 [24]. Besides depression, the investigators performed anxiety tests on the animals treated with patient or control CSF [23, 24]. Using the elevated platform test and the light-dark box test, Planagumà and co-workers found no difference between the animal groups [23]. The lack of anxiety-like behaviors in animals infused with patient antibodies was also reported in a different scheme using the open field test in rats [37]. Furthermore, the effect of passive transfer of patient antibodies on animal locomotor activity was studied. While one team reported that the patient antibodies increased the locomotor activity in the running-wheel test [43], other studies so far have found no evidence of change in locomotor activity in passive transfer models [23, 37, 41]. While the reason for the discrepancy is unknown, the differences in animal species/strains, infusion methods, variety among the patient antibodies, test paradigms, and even the time courses after the start/stop of the infusion, could have contributed to the differences. Finally, a new addition to the passive transfer model studies is the psychotic-like behavior test. Pre-pulse inhibition of the acoustic startle reflex (PPI), a classic method to measure alterations in sensorimotor gating, was recently employed to examine psychotic-like changes in mice after patient antibody infusion [27]. Patient CSF caused significant decreases in the PPI on day 11 and day 19, but recovered to almost control level at day 25 [27]. In summary, a host of cognitive and behavioral tests have been used to demonstrate that patient antibodies cause deficits in learning, memory, and behaviors.

Seizures

Seizures develop in 75% of patients with anti-NMDAR encephalitis, and represent a major cause of morbidity, and, in case of refractory status epilepticus, mortality [18]. In a stereotaxic injection experiment, one time injection of patient antibodies into mice did not induce seizures; however, intra-peritoneal injection of sub-threshold level of chemo-convulsant pentylenetetrazol (PTZ) 48 hours after the patient antibody injection produced increased numbers of observed convulsive seizures in test mice [44]. A more recent study reported a mouse model of seizures in anti-NMDAR encephalitis through passive transfer [42]. Patient CSF, or purified IgGs from CSF, was chronically infused through a mini-pump. Seventy five to 93 percent (75-93%) of test mice developed seizures [42], a number close to clinical data [18]. No motor signs or only subtle signs accompanied the majority of the electrographic seizures detected in the study, presumably because the site of seizures did not reach the motor control regions of the brain [42]. It's not immediately clear why and how hypofunction of an excitatory neurotransmitter receptor GluN1 would tip the balance toward more excitation in the brain, but a cell-type specific inhibition of the NMDARs in the GABAergic neurons would provide an explanation for how the patient anti-

bodies might contribute to the removal of inhibitory tone [18]. The creation of a seizure model in anti-NMDAR encephalitis opens new avenues for discovery in mechanistic studies.

In summary, the experimental results obtained from the aforementioned passive transfer models have provided new insights into the mechanisms of pathogenesis of anti-NMDAR encephalitis. Studies using these models generated affirmative evidence that chronic transfer of patient antibodies decreased endogenous NMDARs in synapses, reduced NMDAR-mediated currents and calcium-entry, impaired NMDAR-dependent LTP, compromised spatial memory and novel object recognition, caused depression-like behaviors, and produced seizures. Most of the deficits were recoverable after the infusion stopped. The transfer failed to produce anxiety-like behaviors or change locomotor activity in most studies. Moreover, the passive transfer models in general exhibited no gross anatomical changes, or salient neuronal loss, milder inflammatory infiltrates, more frequent B cell or plasma cell infiltrates than T cells, and lack of activation of the complement system [23, 24].

Given the nature of the passive transfer, the approach of infusing or injecting patient antibodies into animals has an inherent limitation: it is hard to explain how the patient antibodies against GluN1 are achieved. In addition, it is difficult to address the potential role of immune cell infiltrates and neuroinflammation in the disease processes. Therefore, an active immunization approach is warranted.

In vivo active immunization models

In the active immunization approach, experimental animals are injected with the immunogens and adjuvants, and the animals are induced to produce autoantibodies against the antigens. Three active immunization models of anti-NMDAR encephalitis have been developed so far. One of them is an indirect approach by immunizing with herpes simplex virus (HSV). Another two are direct approaches: one uses synthetic peptide, the other uses native-like holoproteins in the form of proteoliposomes [45-47].

HSV infection

Herpes simplex encephalitis (HSE) is a strong risk factor for anti-NMDAR encephalitis, and a quarter of HSE patients develop autoimmune encephalitis, frequently associated with NMDAR antibodies [15]. As an indirect approach, in a pilot study, six female mice were intranasally inoculated with HSV-1 [45]. Four out of the six mice developed NMDAR antibodies at various time points post-inoculation. Immunofluorescence and Western blot analysis showed that these mice had decreased synaptic NMDARs in the hippocampus. This proof of principle study paved the way for behavioral tests and further studies can address how the autoimmune antibodies against NMDARs induce pathogenesis in HSE animals [45]. The herpes simplex virus infection models successfully recapitulated the clinical observation that herpes simplex encephalitis can trigger antibodies against NMDARs and with further studies may explain neurologic symptoms that occur after the onset of herpes simplex encephalitis.

Holoprotein immunization

Since the NMDAR antibodies are known to recognize the native state of the NMDARs [29] but not denatured NMDARs, it is a technical challenge in the preparation of antigens in proper forms. That is why one novel approach was to immunize with fully assembled receptors [46]. The key innovation lies in assembling native-like GluN1-GluN2B heterotetramers in liposomes, so-called proteoliposomes. The conformationally intact and stabilized holoprotein receptors are required to avoid producing a population of antibodies against the denatured proteins. Using the new approach, Jones and colleagues [46] reported a *de novo* immunized mouse model of anti-NMDAR encephalitis. In that model, there was clear evidence of production of GluN1 antibodies, and the antibodies could bind to GluN1 co-expressed with GluN2A on the HEK293 cells, and to NMDARs on the hippocampal neurons as well [46]. The antibodies also decreased the NMDAR-component of spontaneous EPSCs from cultured hippocampal neurons after 24-hour incubation [46]. The experimental animals showed hyperactive locomotor phenotype, severely compromised nest-building activity, and anxiety-like behaviors in the O maze [46]. The noticeable behavioral changes included hyperactivity, tight circling, overt seizures, and hunched back/lethargy. The report also provided evidence of neuroinflammation and immune cell filtrations

in the disease process. Both B cells and T cells were found in the filtrates. The role played by T cells was deemed essential because immunization of transgenic mice that were T cell negative did not cause the encephalitis phenotype [46].

Synthetic peptide immunization

In a recent report of the attempt to generate antibodies against GluN1, a group immunized mice with a mixture of four synthetic peptides from different regions of GluN1 (all located on the extracellular side, including one peptide encompassing the ATD/NTD N368/G369 region). The immunized mice did generate GluN1 antibodies but showed no signs of inflammation in the brain or any significant changes in behavioral tests [48]. The synthetic peptide immunization approach should not be automatically dismissed, as we learn from a more recent report [47]. Two synthetic peptides from the GluN1 were designed: the GluN1 (168-187) control peptide and the GluN1 (359-378) experimental peptide, which encompasses the ATD/NTD N368/G369 key epitope site revealed by previous studies [20]. The GluN1 (359-378) is merely two more amino acids more than the peptide AA361-376 on each side, a peptide previously used by Pan and colleagues [48]. The GluN1 (359-378), but not the control peptide, produced antibodies that recognize the GluN1/GluN2B expressed on the HEK293 cells. The animals immunized with GluN1 (359-378) peptide, but not control peptide, demonstrated spatial memory impairment and anxiety- and depression-like behaviors. General locomotor activity was not affected by either peptides [47]. In this peptide immunization model, there was no overt T-cell filtration, but strong B-cell filtration was observed with the B cells originated from the peripheral lymphoid organs. Furthermore, blocking the B cell responses decreased the symptoms in mice with encephalitis. And finally, mice immunized with GluN1 (359-378) were more sensitive to subthreshold injection of PTZ to generate seizures [47].

It is apparent that both active immunization models using synthetic peptide and holoprotein shared both common ground and striking differences. Both immunization models generated NMDAR antibodies that reacted with GluN1/GluN2 expressed on the HEK293 cells, induced inflammation of the brain mimicking the encephalitis involving lymphocyte infiltration, showed memory and behavioral deficits, and increased the risk for seizures. The two immunization models also showed striking differences. While the holoprotein immunization model showed hyperactive locomotor phenotypes, the synthetic peptide model did not; whereas the holoprotein immunization model was more dependent on the T-cell involvement, the peptide model relied more on B cells. One key difference is that due to the small size of the peptide, the variability and diversity of antibodies generated could be small compared to that of antibodies against the holoproteins. Indeed, as reported, the antisera in holoprotein immunization model not only contained antibodies against native GluN1 protein, but also antibodies against denatured GluN1 (as evidenced by Western Blot), and antibodies against GluN2B, the effect of which is unknown [46].

Regardless of the differences, the *de novo* active immunization models have enhanced our understanding of the anti-NMDAR encephalitis and may have potential to provide further answers for some critical questions. How are the native NMDARs changed in the mouse brains? Can the super-resolution imaging techniques be used to study the NMDAR dynamics *in vivo* and *ex vivo*? How is LTP affected in the hippocampal regions in slices from the animal models? How does the immunization affect the synaptic activities at network level? What about more subtle cognitive functions like Morris water maze, the NOR, depression-like symptoms, or the Pre-Pulse Inhibition? Most importantly, we anticipate that the animal models could be used for the studies searching for new therapeutics. It is worth pointing out that no single active immunization model is expected to recapitulate all the pathophysiological features of the disease. Multiple immunization models will be required to address specific questions [45-47, 49, 50].

Conclusion and unresolved questions

In a little more than a decade since the discovery of anti-NMDAR encephalitis, the research towards understanding its pathogenic mechanisms has advanced from the *in vitro* approach to the *in vivo* passive transfer model, and to *in vivo* active immunization models. There is

general agreement that the NMDAR autoimmune antibodies are the culprit behind the encephalitis, as the NMDAR antibodies cross-link the NMDARs, disrupt NMDAR-EphB2 interactions, and cause NMDAR internalization. The internalization results in hypofunction of NMDARs, which is believed to be responsible for the impaired long-term potentiation and observed memory and behavioral deficits, and increased susceptibility to seizures. As we strive to better understand the disease, there are some critical unanswered questions: How is the autoimmune response against NMDARs initiated? How do the NMDAR antibodies affect triheteromers such as GluN1/GluN2A/GluN2B? Do the patient antibodies affect synaptic vs extra-synaptic receptors in the same way? What is the exact mechanism of antibody cross-linking and subsequent internalization? What is the relationship between lateraldiffusionobservedinquantum-dotsandnano-object size examined under the super-resolution microscopy? How does the hypofunction of NMDARs at the cellular level lead to memory deficit, behavioral changes, and seizures? A multi-pronged approach complementing aspects of *in vitro* and *in vivo* models may provide us answers and open new avenues for the development of therapeutic strategies.

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None.

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