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Autoimmune receptor encephalitis in mice induced by active immunization with conformationally-stabilized holoreceptors

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Abstract

Autoimmunity to membrane proteins in the central nervous system has been increasingly recognized as a cause of neuropsychiatric disease. A key recent development was the discovery of autoantibodies to *N*-methyl-D-aspartate (NMDA) receptors in some cases of encephalitis, characterized by cognitive changes, memory loss, seizures that could lead to long-term morbidity or mortality. Treatment approaches and experimental studies have largely focused on the pathogenic role of these autoantibodies. Passive antibody transfer to mice has provided useful insights, but does not produce the full spectrum of the human disease. Here we describe a de novo autoimmune mouse model of anti-NMDA receptor encephalitis. Active immunization of immune competent mice with conformationally-stabilized, native-like NMDA receptors induced a fulminant encephalitis, consistent with the behavioral and pathologic characteristics of human cases. Our results provide evidence for neuroinflammation and immune cell infiltration as a component of the autoimmune response in mice. Use of transgenic mice indicated that mature T cells as well as antibody-producing cells were required for disease induction. This active immunization model may provide insights into disease induction as well as a platform for testing therapeutic approaches.

One Sentence Summary:

We report an active immunization mouse model of autoimmune N-methyl-D-aspartate receptor encephalitis that provides insights into the pathophysiology.

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Data and materials availability: All summary data and individual measurements are included in the paper, associated supplementary figures and tables. All materials and methods are included with supplementary materials.

Introduction

Behavioral changes, psychosis, memory impairment, and seizures have been recognized as a pattern in several forms of encephalitis (1). In some cases, this discrete clinical syndrome has been attributed to Herpes simplex virus, but the underlying cause in other cases has remained unknown. In 2007, autoantibodies targeting *N*-methyl-D-aspartate (NMDA) receptors were discovered in a subset of these patients (2, 3). NMDA receptors are ligand-gated Ca²⁺ permeable ion channels expressed postsynaptically at excitatory synapses in neurons throughout the central nervous system. These receptors play critical roles in synaptic plasticity and development, while NMDA receptor antagonists disrupt memory formation and cause schizophrenia-like symptoms (4–7). Thus loss of function of these receptors can lead to both structural and functional changes in the brain (8–10). NMDA receptors are heterotetrameric composed of two obligate GluN1 subunits and two variable GluN2 subunits (GluN2A–D) arranged around a central pore. GluN1/GluN2A/GluN2B triheteromers are the most common subunit combination in forebrain excitatory neurons (4, 11). With recognition of anti-NMDA receptor encephalitis as a clinical syndrome, diagnostic tests have revealed that the disease is relatively common across a broad range of ages (12–16). Initially considered as one of the paraneoplastic disorders, it is now clear that many affected patients do not have detectable tumors (12, 17), underscoring our lack of understanding of the disease etiology. Patient-derived serum or cerebrospinal fluid indicate a role for antibodies directed at NMDA receptor subunits in the pathogenesis, leading to treatments to reduce antibody titers with plasmapheresis or immunosuppression (15). However, recovery following standard treatments can be prolonged and incomplete (12, 18–20).

Passive transfer of antibodies from affected patients to mice have indicated that NMDA receptor antibodies can cause hypofunction in NMDA receptor-mediated synaptic transmission (17, 21–23). Yet, the initiating immunological factors as well as the potential role of immune cell infiltrates and neuroinflammation in the disease process are difficult to determine using existing models (21, 24–26). A robust animal model of the disease has the potential to address such issues.

To develop a mouse model of autoimmune encephalitis, we postulated that immunization with fully assembled receptors could be important in triggering the disease. Thus we used active immunization with intact native-like NMDA receptors composed of GluN1–GluN2B tetramers embedded in liposomes. Subcutaneous injection of these NMDA receptor-containing proteoliposomes induced fulminant encephalitis within 4 weeks in young adult mice. The mice demonstrated behavioral changes, seizures as well as histological features of neuroinflammation and immune cell infiltration that were most prominent in the hippocampus. The presence of NMDA receptor antibodies was confirmed by immunohistochemistry and western blot. CD4⁺ T cell infiltration was an early feature, and both mature T and B cells were required for disease induction.

Results

Encephalitis induced by active immunization with NMDA receptor holoprotein

We used purified GluN1/GluN2B NMDA fully-assembled tetrameric receptors (holoreceptors) embedded in liposomes (NMDA receptor proteoliposomes) to immunize C57Bl6 adult mice (Fig. 1A). The production of highly purified NMDA holoreceptors and generation of proteoliposomes is described in detail in the methods section. Proteoliposomes, like those used in the current study, are an established tool for investigating the structural and biophysical properties of membrane-bound protein complexes as well as facilitating an antigen-specific antibody response (27–35). The native-like conformation of NMDA receptors in our proteoliposome preparation was validated in a recent cryo-EM study in which the same method was used to resolve the structure of tetrameric NMDA receptors with atomic precision (34).

Using a standard immunization protocol mice received a subcutaneous injection of NMDA receptors in proteoliposomes (see methods for protein/liposome ratio and injection volume) followed by a booster two weeks later (36). Littermate control cohorts were injected only with liposomes or with saline. Overt neurological signs began to appear by four weeks, and by six weeks post-immunization nearly all of the proteoliposome-treated mice (86%) exhibited abnormal home cage behavior (Fig. 1B). The most prominent behavioral changes were hyperactivity (86%) followed by tight circling (50%), overt seizures (21%) and hunched back/lethargy (11%), (Fig. 1C & Movie S1–S5). Cumulative clinical scores (described in methods) were significantly higher in proteoliposome-treated mice (proteoliposome v. liposome $p < 0.0001$, proteoliposome v. saline $p < 0.0001$, liposome v. saline $p < 0.9999$; Kruskal-Wallis, Dunn's multiple comparisons post hoc; $n = 28$ /treatment group) (Fig. 1B). Proteoliposome-treated mice also had increased mortality by six weeks post-immunization ($n = 8/56$ proteoliposome, $n = 0/56$ controls; $p < 0.0005$; Log-rank test) (Fig. 1D). The results were not limited to the specific holoprotein as we observed a similar distribution of clinical signs in mice immunized with a rat GluN1-GluN2A proteoliposome preparation (Fig. S1A: hyperactivity (50%), circling (20%), seizure (20%), hunching/lethargy (10%); $n = 10$).

To assess behavioral phenotype, we used a battery of standardized tests. Open field testing (Fig. 2A) confirmed a hyperactive locomotor phenotype with nearly double the distance traveled in proteoliposome-treated mice (proteoliposome. 5472 ± 525.4 cm, $n = 26$; liposome = 3207 ± 111 cm, $n = 28$; saline = 3093 ± 84.9 cm, $n = 28$; proteoliposome v. liposome $p = 0.0002$; proteoliposome v. saline $p < 0.0001$; liposome v. saline $p > 0.9999$; Kruskal-Wallis test with Dunn's multiple comparisons post hoc). Proteoliposome-treated mice also showed a high degree of variability in the open field ranging from near immobility to extreme hyperactivity. Nest building, indicative of complex stereotyped behavior, was severely compromised in the proteoliposome-treated mice (Fig. 2B). At six weeks post-immunization, control mice created precise nests whereas proteoliposome-treated mice barely disturbed the nestlets as indicated by a lower nesting score: proteoliposome (24 and 48 hours) = 1 ± 0.29 and 1.48 ± 0.39 , $n = 27$; liposome = 4.60 ± 0.14 , and 4.78 ± 0.11 , $n = 28$; saline = 4.21 ± 0.14 and 4.75 ± 0.12 , $n = 28$. proteoliposome v. liposome $p < 0.0001$;

proteoliposome v. saline $p < 0.0001$; liposome v. saline $p = 0.3603$ (24 hours); proteoliposome v. liposome. $p < 0.0001$; proteoliposome v. saline $p < 0.0001$; liposome v. saline $p > 0.9999$ (48 hours); Kruskal-Wallis with Dunn's multiple comparisons post hoc). In the zero maze, often used as a measure of anxiety-like behavior, proteoliposome-treated mice spent more time in the normally aversive open-area (Fig. 2C) (% time open-area: proteoliposome = 27.90 ± 3.70 , $n = 18$; liposome = 16.08 ± 0.71 , $n = 28$; saline = 16.32 ± 0.96 , $n = 28$; proteoliposome v. liposome. $p = 0.0065$; proteoliposome v. saline. $p = 0.0081$; liposome. v. saline. $p > 0.9999$; Kruskal-Wallis with Dunn's multiple comparisons post hoc). There was no statistical difference in the total distance moved in the open or closed areas indicating that hyperactivity could not explain the observed phenotype (Fig. 2C) ([Open area: proteoliposome: 175.4 ± 26.18 cm; liposome: 118.7 ± 9.41 cm; saline: 130.4 ± 15.67 cm; proteoliposome v. liposome $p = 0.0786$; proteoliposome v. saline. $p = 0.0629$; liposome. v. saline. $p = >0.9999$]; [Closed area: proteoliposome: $572.6.4 \pm 27.29$ cm; liposome: 494.8 ± 17.83 cm; saline: 524.1 ± 22.17 cm; proteoliposome v. liposome $p = 0.1140$; proteoliposome v. saline. $p = 0.4312$; liposome. v. saline. $p = >0.9999$], Kruskal-Wallis with Dunn's multiple comparisons post hoc). These results indicated that immunization with NMDA receptor holoprotein induces a behavioral phenotype compatible with encephalitis based on the criteria used to diagnosis anti-NMDA receptor encephalitis in humans (37).

Neuroinflammation and peripheral leukocyte infiltration

The histopathology of reported cases of human anti-NMDA receptor encephalitis is heterogeneous, and can include immune cell infiltrates, neuroinflammation and occasionally neuronal loss (24–26, 38). In proteoliposome-treated mice at six weeks post-immunization, hematoxylin & eosin (H&E)-labeled perivascular cuffing was observed in multiple CNS regions including the hippocampus and neocortex of five out of six mice examined (perivascular cuffing measure: proteoliposome, 0.83 ± 0.17 ; control, 0 ± 0 ; proteoliposome v. control, $p = 0.0152$. Mann Whitney test; $n = 6/\text{group}$). Representative images of perivascular cuffing are shown in Fig. 3A, right panels. Patchy areas of cell death were observed in brain sections in one of the six mice included in the histological analysis (cell death measure: proteoliposome, 0.17 ± 0.17 ; control, 0 ± 0 ; proteoliposome v. control, $p > 0.9999$. Mann Whitney test; $n = 6/\text{group}$). Right panels in Fig. 3B shows areas of cell death in a proteoliposome-treated mouse. No evidence of inflammation or cell death were present in assessed H&E controls (Fig. 3A, B, left panels). Individual data points from the H&E assay and all subsequent experiments with an $n < 20$ are included in Table S1.

Immunolabeling with GFAP and Iba1 revealed an inflammatory response in assessed proteoliposome-treated mice at six weeks post-immunization (Fig. 4A,B, right panels) as measured by total GFAP immunoreactivity in the hippocampus (proteoliposome: $42.5 \times 10^8 \pm 8.80 \times 10^8$; control: $6.33 \times 10^8 \pm 1.64 \times 10^8$; proteoliposome v. control. $p = 0.0022$; Mann Whitney test; $n = 6/\text{group}$). Iba1 labeling revealed foci of microgliosis compared to the tiling of diffuse labeling in controls as expected for healthy mice (Fig. 4B). Total Iba1 immunoreactivity in the hippocampus of these mice was $60.9 \times 10^8 \pm 14.3 \times 10^8$ in proteoliposome-treated mice compared to $28.5 \times 10^8 \pm 1.64 \times 10^8$ in controls: $28.5 \times 10^8 \pm 1.64 \times 10^8$ ($p = 0.0022$, Mann Whitney test; $n = 6/\text{group}$).

By six weeks post-immunization there was also pronounced CNS infiltration by peripheral immune cells in the proteoliposome-treated mice assessed by immunohistochemistry (IHC). Labeling with the pan-leukocyte marker CD45R was robust in the hippocampus (Hipp), striatum (Str), thalamus (Thal), amygdala (Amyg) and neocortex (Ctx), of these mice (Fig. 5A, C). Control mice included in this assay showed sparse CD45R labeling (Fig. 5A, C; proteoliposome v. liposome: $p = 0.0022$, Mann-Whitney test; $n = 6/\text{group}$, for all anatomical regions analyzed). Peripheral immune cells including activated macrophages/microglia (Galectin3⁺), plasma cells (CD138⁺), helper T cells (CD4⁺), B cells (CD20⁺) were increased in the brains of assessed proteoliposome-treated mice as well, whereas cytotoxic T cells (CD8⁺) were sparse or absent (Fig. 5B, right panel). In contrast liposome- or saline-treated mice assessed by IHC lacked immunoreactivity to the same immune cell markers (Fig. 5B, C; proteoliposome v. liposome: $p = 0.0022$, Mann-Whitney test; $n = 6/\text{group}$, for all immune cells analyzed). Mean cell densities for control- and proteoliposome-treated mice included in the preceding analyses are summarized in Table 1, with individual values provided in Table S1.

Although mice examined at 3 weeks post-immunization did not show prominent clinical features, neuroinflammation and immune cell infiltration could be detected in some animals at this early time point (Table 1 and Fig. S2A–E). Figure S2B–E show representative images and quantification of glial markers and immune cell infiltrates. Quantification of GFAP and Iba1 immune reactivity at three weeks post-immunization showed no significant differences between treatment groups despite evidence of glial nodules in proteoliposome-treated mice (Fig. S2B, right panels; GFAP fluorescence intensity: proteoliposome, $34.74 \times 10^8 \pm 8.85 \times 10^8$; liposome, $19.12 \times 10^8 \pm 1.63 \times 10^8$; proteoliposome v. liposome: $p = 0.2857$, Mann-Whitney test; $n = 5/\text{group}$; Iba1 fluorescence intensity: proteoliposome, $40.56 \times 10^8 \pm 6.44 \times 10^8$; liposome, $22.66 \times 10^8 \pm 1.08 \times 10^8$; proteoliposome v. liposome: $p = 0.0952$, Mann-Whitney test; $n = 5/\text{group}$). Immune cell quantification at three weeks post-immunization revealed significantly increased CD45R⁺ and CD20⁺ cells in the hippocampi of assessed proteoliposome treated mice (Fig. S2C–E; Hipp CD45R⁺: proteoliposome v. liposome: $p = 0.0286$, Mann-Whitney; $n = 4/\text{group}$; Hipp CD20⁺: proteoliposome v. liposome: $p = 0.0476$, Mann-Whitney; $n = 5/\text{group}$). Table 1 and Table S1 contain mean and individual values, respectively, for all immune cell quantifications at the three week time point.

Receptor autoantibodies in serum from proteoliposome-treated mice

To assess the specificity of the IgG isolates from the serum of proteoliposome-treated mice, we used HEK293 cells to express and stain for NMDA receptor subunits. HEK cells were transfected with single subunit constructs or combinations of GluN1/GluN2A and GluN1/GluN2B. Using this HEK cell assay we tested all serum/IgG isolates from proteoliposome-treated mice included in the behavioral and IHC analyses. We observed colabeling of NMDA-receptor expressing HEK293 cells using a commercially available GluN1 antibody and IgG derived from all proteoliposome-treated mice at six weeks post-immunization with no colabeling seen in serum/IgG isolate from a randomly selected group of controls (proteoliposome, 1 ± 0 ; controls, 0 ± 0 ; proteoliposome v. controls: $p < 0.0001$; Mann-Whitney test; $n = 26/\text{group}$). Figure 6A (bottom panels) shows representative images of HEK cells co-immunolabeled with a GluN1 antibody and IgG derived from a

proteoliposome-treated mouse at six weeks post-immunization. We used the same HEK cell assay to assess the serum from a cohort of mice at three weeks post-immunization (Fig S3). At the three week time point all but one proteoliposome treated-mouse showed colabeling with a GluN1 antibody, indicating the presence of antibodies before we observed overt clinical signs (proteoliposome, 0.80 ± 0.20 ; controls, 0 ± 0 ; proteoliposome v. controls: $p = 0.0476$; Mann Whitney test; $n = 5/\text{group}$). Figure S3A shows representative HEK cell colabeling for a GluN1 antibody and IgG from a proteoliposome- and control-treated mouse at three weeks post-immunization. To ensure that the colabeling observed in our HEK cell assay was specific for the GluN1 subunit we repeated the staining in HEK cells expressing only the GluN1 subunit with a subset of serum/IgG samples from each treatment condition. Again, IgG derived from each proteoliposome-treated mice included in the assay colabeled with a GluN1 specific antibody at six weeks post-immunization (Fig. S4A, proteoliposome: 1 ± 0 ; controls, 0 ± 0 ; proteoliposome v. controls: $p = 0.0286$; Mann Whitney test; $n = 4/\text{group}$). Serum samples derived from all mice treated with rat GluN1-GluN2A proteoliposomes also showed GluN1-specific colabeling with no colabeling observed in controls at six weeks post-immunization (Fig. S1C (HEK GluN1-GluN2A), S4B (HEK GluN1-only); proteoliposome: 1 ± 0 ; liposome, 0 ± 0 ; proteoliposome v. liposome: $p < 0.0001$; Mann Whitney test; $n = 10$ proteoliposome/8 liposome). See Table S1 for individual values from all HEK293 cell assays.

In cultured mouse hippocampal neurons (DIV14–21), only the IgG isolate from proteoliposome-treated mice co-localized with a GluN1 subunit-specific antibody in IgG samples derived from proteoliposome-treated mice at both the 6 and 3 week time points (Neuron ICC: 6wk proteoliposome v. control: $p < 0.0001$, Mann-Whitney test; $n = 26/\text{group}$; 3wk proteoliposome v. liposome: $p = 0.0476$, Mann-Whitney test, $n = 5/\text{group}$). The punctate labeling we observed along dendrites and at dendritic spines follows the expected distribution of NMDA receptors at synapses (4, 8, 39, 40). Figure 6B shows colabeling of proteoliposome-derived IgG (6 weeks post-immunization) and a GluN1 antibody along a dendritic shaft. Figure S3B shows the same colabeling for proteoliposome IgG from the 3-week time point.

Hippocampal NMDA receptors are triheteromeric, composed of GluN1, GluN2A and GluN2B subunits, and are widely distributed in the neuropil (41, 42). As a demonstration of the tissue distribution of proteoliposome derived IgG labeling, we examined IgG staining pattern in naïve mouse brain sections as compared to the pattern of a GluN2A subunit-specific antibody, which was effective in these floating tissue sections (Fig. 6C, 6 weeks post-immunization). In the samples we tested in this assay, purified IgG (red) from liposome controls showed no labeling whereas proteoliposome derived IgG (red) showed the same staining pattern as the NMDA receptor antibody (green); (proteoliposome: 1 ± 0 ; controls, 0 ± 0 ; proteoliposome v. controls: $p = 0.0286$; Mann Whitney test; $n = 4/\text{group}$). Staining for mouse IgG as a proxy for the presence of autoantibodies, outlined the hippocampi of mice included in this assay (Fig. S5), consistent with the expected high levels of NMDA receptor expression in hippocampus and the IgG deposits observed in anti-NMDA receptor encephalitis (2, 21); (proteoliposome: 1 ± 0 ; controls, 0 ± 0 ; $n = 3/\text{group}$).

To confirm the NR1 labeling in the HEK293 cell assays from each mouse, we used serum from two proteoliposome-treated and two liposome-treated mice at six weeks post-immunization to examine bands on Western blots. Bands corresponding to purified recombinant rat and xenopus GluN1 subunit protein as well as xenopus GluN2B were observed. Although a putative pathogenic epitope on the GluN1 amino-terminal domain (ATD) has been identified in some human cases, immunoreactivity to GluN2A and GluN2B subunits also has been reported in a subset of cases (2, 17, 43, 44). For the mouse shown in Figure 6D, serum also labeled a *Xenopus* GluN1 subunit that lacked the ATD domain, suggesting the presence of polyclonal antibodies in at least some of the mice. Serum from control-treated mice included in Western blot did not recognize NMDA receptor subunits (Fig. 6A, middle panel); (proteoliposome: 1 ± 0 ; liposome, 0 ± 0 ; $n = 2/\text{group}$).

Serum from proteoliposome-treated mice did not acutely block NMDA receptor function, as assessed by whole-cell currents in cultured hippocampal neurons (Fig. 7A, B). NMDA ($50 \mu\text{M}$) was co-applied by local flow pipes either with serum from liposome-treated mice or serum from proteoliposome-treated mice (1:100 dilution). The NMDA-evoked current in the presence of serum from proteoliposome-treated mice was $95.9 \pm 6.8\%$ of that evoked by NMDA + serum from liposome-treated mice in the same neuron ($n = 8$; $p = 0.23$, paired t-test; Shapiro-Wilk normality test, proteoliposome serum: $p = 0.2231$; liposome serum: $p = 0.1413$). In contrast, a 24-hour incubation with serum from proteoliposome-treated mice reduced synaptically-activated NMDA receptors, which underlie the slow components of EPSCs and drive overall network activity. As shown in Figure 7C (top left), the slow components of EPSC barrages from neurons incubated in serum from liposome-treated mice were reduced by the NMDA receptor antagonist, D-AP5, as indicated by the rapid decay of the spontaneous EPSCs (Fig 7C, top right). However, after 24-hour incubation in serum from proteoliposome-treated mice, spontaneous EPSCs had reduced NMDA receptor-mediated currents and thus were less sensitive to block by D-AP5 (Fig. 7C, bottom right). For neurons incubated in serum from liposome-treated mice total charge from spontaneous EPSCs was reduced to $44.1 \pm 7.9\%$ of control charge by D-AP5 (Fig. 7D). In contrast, D-AP5 reduced total charge to only $85.6 \pm 6.0\%$ of control charge in neurons incubated with serum from proteoliposome-treated mice ($n = 8$ per group; $p < 0.005$, paired t-test; Shapiro-Wilk normality test, proteoliposome serum: $p = 0.3893$; liposome serum: $p = 0.5722$ Fig. 7D). These results demonstrate a marked reduction in NMDA receptor function after 24 hour incubation with serum from proteoliposome-treated mice. We also stained the same 24-hour treated cultures for Post-Synaptic Density-95 protein (PSD-95), a key structural component and marker of dendritic spines at excitatory synapses, and GluN1 to assess their colocalization (Fig. 7E), (45). The 24-hour incubation did not affect total synaptic puncta, but did result in a $>50\%$ decrease in GluN1 immunoreactivity (Fig. 7E), consistent with the reduction in functional measures of NMDA receptor activity ([PSD-95 puncta per μm : proteoliposome, 0.54 ± 0.019 ; liposome, 0.62 ± 0.059 ; $p = 0.4244$, Mann-Whitney test; $n = 8/\text{group}$]; NR1+PSD-95 puncta per μm : proteoliposome, 0.18 ± 0.034 ; liposome, 0.56 ± 0.064 ; $p = 0.0003$, Welch's t-test; Shapiro-Wilk normality test: proteoliposome, $p = 0.8788$; liposome, $p = 0.9482$; $n = 8/\text{group}$]). Thus antibodies generated in proteoliposome-treated mice can lead to NMDA receptor hypofunction after a 24 hour exposure.

T cells as well as B cells are necessary for proteoliposome-induced encephalitis

Studies of anti-NMDA receptor encephalitis in human cases have focused on the role of antibodies (2, 21, 22). Our results in proteoliposome-treated mice are consistent with the presence of B cell infiltrates as well as NMDA receptor autoantibodies. In human cases perivascular T cell infiltrates, primarily CD4⁺ helper T cells, have been reported however, parenchymal infiltrates appear to be infrequent (21, 24). To distinguish the roles of B and T cells in proteoliposome-treated mice, we used two well-characterized mutant mouse lines that lack either mature B cells or mature T cells (46, 47). Consistent with a role for B cells in the pathophysiology, proteoliposome treatment of MuMt⁻ mice, which lack the capacity to generate an antigen specific antibody response, showed no behavioral or histological abnormalities at 6 and 12 weeks post-immunization (Fig. S6; GFAP fluorescence intensity: proteoliposome, $51.00 \times 10^8 \pm 2.80 \times 10^8$; liposome, $60.08 \pm 3.15 \times 10^8$; proteoliposome v. liposome: $p = 0.0572$, Welch's t test, $n = 6/\text{group}$; Iba1 fluorescence intensity: proteoliposome, $24.81 \times 10^8 \pm 5.81 \times 10^8$; liposome, $25.81 \pm 5.81 \times 10^8$; proteoliposome v. liposome: $p = 0.2520$, Welch's t test, $n = 6/\text{group}$; CD45R⁺: proteoliposome v. liposome; $p > 0.9999$, Mann Whitney test, $n = 6/\text{group}$; Cell based colabeling assays: proteoliposome v. controls: $p > 0.9999$; Mann Whitney test; $n = 6/\text{group}$). To evaluate the role of mature T cells we used Tcr α ⁻ ("CD4, CD8 cell KO") mice, which lack mature helper T and cytotoxic T cells. All proteoliposome-treated Tcr α ⁻ mice survived to 12 weeks without clinical signs of disease (Fig. 8A). Likewise Tcr α ⁻ mice did not show histopathological evidence of gliosis or cell infiltrates and serum lacked detected anti-NMDA receptor antibodies (Fig. 8B–D; GFAP fluorescence intensity: proteoliposome, $60.47 \times 10^8 \pm 4.08 \times 10^8$; liposome, $59.35 \pm 4.39 \times 10^8$; proteoliposome v. liposome: $p = 0.8573$, Welch's t test, $n = 4/\text{group}$; Iba1 fluorescence intensity: proteoliposome, $21.97 \times 10^8 \pm 4.03 \times 10^8$; liposome, $20.07 \pm 3.77 \times 10^8$; proteoliposome v. liposome: $p = 0.8573$, $p = 0.5282$ Welch's t test, $n = 4/\text{group}$; CD45R⁺: proteoliposome v. liposome; $p > 0.9999$, Mann Whitney test; $n = 4/\text{group}$; Cell based colabeling assays: proteoliposome v. controls: $p > 0.9999$; Mann Whitney test; $n = 4/\text{group}$). Individual data and group statistics for all data shown in the B and T cell mutant analyses can be found in Table S1. A cohort of immune competent wild-type mice immunized in parallel with the MuMt⁻ and Tcr α ⁻ showed the expected phenotype, indicating the potency of the immunogen. These results confirm the expected role of B cells, but also indicate a requirement for mature T cells in disease pathogenesis.

Discussion

Our results demonstrate that active immunization with NMDA receptor holoproteins induces a disease state in mice that recapitulates the core features of human anti-NMDA receptor encephalitis including the presence of pathogenic anti-GluN1 autoantibodies (13). In general, active immunization to develop animal models has played an important role in the study of neurological disorders including autoimmune diseases such as myasthenia gravis and multiple sclerosis (48, 49). For example, peptide fragments from myelin have long been used to generate experimental autoimmune encephalomyelitis (EAE) with its incumbent clinical signs to test the molecular basis and therapeutic approaches in multiple sclerosis (48, 50, 51). Likewise, active immunization with neuromuscular junction proteins can cause myasthenic-like features in mice (52). Although synaptic membrane proteins have been implicated in autoimmune encephalitis, studies of these diseases have largely been limited to

passive transfer approaches (1). Our de novo autoimmune anti-NMDA receptor encephalitis model represents an additional approach to examine the pathophysiology and developing treatments for the human disease.

The etiology in some cases of encephalitis was an enigma for decades. The discovery of NMDA receptor antibodies in a subset of these patients was not only a surprise, but also provided an opportunity for a better understanding of the causes and also treatment strategies (17). The diagnostic criteria used to identify anti-NMDA receptor encephalitis (37) include a number of features that also were present in our mouse model including: behavioral changes, movement abnormalities, seizures, and the presence of antibodies against the GluN1 subunit of NMDA receptors. The clinical signs and histopathology ranged from mice with marked behavioral impairments to occasional mice that lacked clinical signs but had histological features and anti-NMDA receptor antibodies. As shown in the HEK293 cell assays, all proteoliposome-treated mice had antibodies to GluN1 at 6 weeks post-immunization, but in for those mice tested by Western blot we also saw immunoreactivity to GluN2B or to a construct that lacked the ATD of GluN1. This pattern suggests the possibility of a polyclonal response by the time fulminant symptoms were present (6 weeks post-immunization). This issue and the possibility of epitope spreading will be important to examine in the future. However, consistent with the human disease, a GluN1 epitope was predominant.

Antibodies recognizing NMDA receptor subunits have been observed in some other contexts. For example, systemic lupus erythematosus (SLE) is associated with a with a range of antibodies to nuclear antigens as well as antibodies recognizing the NMDA receptor GluN2A subunit (53, 54). However, unlike the NMDA receptor antibodies found in our mice and in human anti-NMDA receptor encephalitis patients, anti-GluN2A/B antibodies derived from SLE patients do not appear to alter synaptic responses but rather induce cell death (53, 54).

Although histopathological reports are available for only a small fraction of human cases of anti-NMDA receptor encephalitis, immune cell infiltrates and neuroinflammation have been observed in some patients (21, 24) but were less prominent in other cases (26). The presence or absence of inflammatory cell infiltrates thus likely depends on the time point at which the tissue is examined. Neuroinflammation in our mice was most prominent during the fulminant stages of the disease (6 weeks post-immunization compared to 3 weeks). In the human disease and in our mouse model, cytotoxic T cell involvement can occur, but was not a prominent feature in mice included in this assessment (24–26, 38). Also consistent with human cases, IgG infiltration of the hippocampus could be seen in proteoliposome-treated mice in which tissue slices were incubated with anti-IgG antibodies (2, 21).

Prior experimental studies of anti-NMDA receptor encephalitis used passive transfer approaches with patient-derived CSF/IgG (21–23, 55). These studies provide compelling evidence for the involvement of NMDA receptor antibodies in pathogenesis (22, 23, 44, 55). Intraventricular infusion of human NMDA receptor antibodies in mice decreased NMDA receptor density in the hippocampus (23, 56), but these mice did not exhibit movement disturbances or spontaneous seizures (23, 56–58). These observations suggest that passive

infusion of NMDA antibodies is not sufficient to fully mimic the clinical syndrome. Although no animal model is expected to perfectly mimic a human disease, our results indicate that active immunization of immune competent mice with NMDA receptor holoproteins replicates several signs of human encephalitis. The active immunization model also allows an examination of the time course of the disease process from the point of induction.

The current hypothesis for the clinical phenotype is that antibody-mediated internalization of NMDA receptors leads to hypofunction at a network level. The available evidence suggest that antibodies from human cases cause internalization of NMDA receptors in vitro and reduce NMDA responses (21, 22, 59). Thus therapies in use or proposed have been directed at removing antibodies, immunosuppression, or preventing receptor internalization (1). The hypofunction hypothesis gains further support from the behavioral side-effects observed with use of NMDA receptors antagonists, but general NMDA receptor hypofunction does not easily account for the presence of unprovoked, spontaneous seizures given the presence of NMDA receptors on both excitatory and inhibitory neurons and the complexity of the circuits involved (6, 22, 57, 60, 61). More experiments will be necessary to the relative contribution of receptor internalization to neurological signs and whether other factors contribute to the spectrum of clinical signs and histopathology.

Although prior studies of anti-NMDA receptor encephalitis have largely focused on the role of B cells and antibodies, the role of CD4⁺ T cells in autoimmune encephalitis is an area of growing interest (62). T cells could promote neuroinflammation as well as potentiate B cell- and plasma cell-mediated antibody responses. In human cases, CSF cytokine/chemokine profiles support a role for CD4⁺ T cell involvement (63–66). For example, interleukin-17, a pro-inflammatory cytokine produced by Th17 CD4⁺ T cells, is prominent in the CSF of human cases and may perform the dual role of blood-brain barrier disruption and upregulation of IL-6, a pro-B cell and plasma cell cytokine (63, 67, 68). Although cytotoxic T cells were not a prominent histological feature in our mice, the absence of disease in the TCR α [−] mice lacking mature CD4⁺ or CD8⁺ cells support an important role for at some population of T cells in disease pathogenesis. Thus therapies designed to reduce T cell-mediated inflammation, such as blocking interleukin 17 signaling, as well as those aimed at reducing B cell activation are worthy of further investigation.

The trigger for an autoimmune reaction to NMDA receptors remains unclear. Immunization using peptide fragments to produce NMDA receptor antibodies have not resulted in reports of clinical disease. In a recent study, mice immunized with NMDA receptor peptides did not show clinical signs, but the authors suggested that blood-brain barrier integrity may prevent circulating NMDA receptor antibodies from entering the CNS (69). The immunogens in our case were the tetrameric *X. laevis* GluN1/GluN2B or rat GluN1/GluN2A receptor in native-like heteromeric assembly (70). The *X. laevis* subunits had been altered to maximize protein stability, by removal of the intracellular C-termini (70), and were capable of binding glutamate or glycine, which may also have improved protein stability. The rat subunits we used had no mutations except for removal of the intracellular C-termini and thus unaltered topology in the ATD, one of the putative sites of pathogenic antibody interactions in human cases (43, 44). The use of holoprotein immunogens with intact extracellular domains likely

played a role in the high incidence of disease in our mice, and may be relevant considerations for other membrane proteins implicated as causes of encephalitis. Our results suggest that disease induction depends on conformationally restricted epitopes. This idea is consistent with prior studies in HEK293 cells, which showed an assembled NMDA receptor was necessary for reactivity of antibodies from human cases (2, 17). In human cases, the source of intact NMDA receptors to trigger the autoimmune response is unknown, but could be ectopic expression from a tumor, or membrane debris following an insult causing neuronal cell loss. For example, anti-NMDA receptor encephalitis has been reported following viral infection (12, 71). An association with viral infection was also suggested in the case report of Knut, a polar bear at the Berlin zoo (72).

Despite the prevalence of anti-NMDA receptor encephalitis, many experimental questions remain unanswered given the lack of a de novo autoimmune animal model that recapitulates the signs and symptoms. The mouse model described here provides such a platform and has already provided several new insights. For example, use of conformationally-stabilized holoproteins appears to be a critical component of immunogenicity, and our results already indicate a complex pathogenesis. Thus the initial steps in disease induction, the roles of specific immune components, and potential new therapies can now be tested.

In the future, it will be interesting to examine immunized mice at earlier time points to look for specific memory deficits, more subtle cognitive impairments as well as the evolution and atomic localization of autoantibody epitopes. We did not examine some other aspects that have been reported in the human disease such as autonomic dysfunction or the cause of death in some of our mice. Furthermore we did not investigate aberrant electrophysiological activity at the network and synaptic level in vivo or in brain slices derived from affected mice. However, these questions are all addressable using this mouse model, which offers significant advantages for further exploration of such issues.

Methods

Study Design:

We examined the effect of active immunization with NMDA receptor native-like holoproteins on normal adult mice. The aim of the study was to investigate autoimmunity to NMDA receptors in the context of anti-NMDA receptor encephalitis. Littermate controls (liposome or saline) of both sexes were used for all interventions. Results from liposome and saline controls did not differ and were thus combined for statistical analysis of “control” in some experiments as indicated. Criteria were established in advance based on pilot studies for issues including data inclusion, outliers, selection of endpoints, and sample size (see statistics section). All analyses were blinded. Observations within each animal were averaged and the value for N replicates reflects the number of animals. Detailed methods for each experimental technique and analysis are included in supplementary materials including: animal use, NMDA receptor expression and purification, NMDA receptor proteoliposome preparation and immunization, behavioral assessments, histology and immunohistochemistry, serum collection/IgG purification and western blots, in vitro assays and immunohistochemistry, electrophysiology and quantification of synaptic puncta.

Statistics:

Sample size were determined based on prior experiments of this type with an effect size of 20% and power = 0.8. Tests of normality were used to determine the appropriate test. Multiple comparisons used 1- or 2-way ANOVA or nonparametric ANOVA as indicated for each experiment. Exact p values are provided and both the number of animals and the number of observations are indicated as appropriate. Data unless other indicated are plotted as mean $\pm$ SEM. All statistical analyses were completed using Prism 7 software (GraphPad).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Clinical phenotype in proteoliposome-treated mice

(A) Timeline of treatment and behavioral testing. Adult wild-type mice received subcutaneous injections at day 0 and at day 15 with proteoliposome (purple) or control - liposome (green) or saline (blue). (B) Clinical signs were plotted from the first injection. (C) Clinical signs in proteoliposome-treated mice (D) Kaplan-Meier survival plot for proteoliposome- and control-treated mice.

Figure 2. Behavioral assessment

(A) Representative movement traces in the open field from a control mouse (field outlined in green) and two proteoliposome treated-mice (field outlined in purple) to examine hyperactivity. Total distance moved in the treatment groups are plotted at right: proteoliposome (purple), liposome (green) and saline (blue) purple. (B) Representative images of a nest created by a control mouse compared to nests in two proteoliposome treated-mice. Nests were scored at 24 and 48 hours and quantified as shown at right. (C) Mice were assessed in the zero maze for time spent in the open area (left) and for the total distance moved in the open and closed areas.

Figure 3. Histological assessment

(A) Representative Images of perivascular cuffing (black arrow, right panels) in hippocampal and neocortical tissue from a proteoliposome-treated mouse. Matched samples from liposome-treated controls are shown at left. (B) Representative tissue sections were used to examine for areas of cell loss including karyolysis (single white arrow) and pyknosis (double white arrow) in a proteoliposome- treated mouse (right), compared to control (left). Scale bar: 100µm.

Figure 4. Glial cell labeling in proteoliposome-treated mice

(A) Immunofluorescence for GFAP (white) in proteoliposome-treated mice (right) compared to controls (left). The higher magnification insets from the hippocampus show individual astrocytes. The histogram shows quantification of GFAP immunofluorescence (see methods). (B) Labeling with the microglial marker Iba1 (white) in proteoliposome-treated mice (right) compared to control (left). Higher magnification inset shows microglia in the hippocampus of proteoliposome-treated mouse compared to control. Immunofluorescence was quantified for Iba1 as for GFAP. Scale bar: 1000 μ m (inset 100 μ m).

Figure 5. Immunohistochemical labeling of CNS immune cell infiltrates

(A) Labeling with the pan-leukocyte marker, CD45R in coronal sections from a proteoliposome treated-mouse (right) and control mouse (left). Insets show labeling of individual CD45R⁺ cells (white) from the indicated region of the hippocampus. CD45R⁺ cells in controls (left). Scale bar: 1000 μm (inset 100 μm). (B) Immunohistochemical labeling for a battery of immune cell markers in brains of proteoliposome-treated mice and control mice (CD8⁺, CD4⁺, CD20⁺, CD138⁺, Gal3⁺). Proteoliposome treated-mice showed immune cell infiltrates as indicated with the insets from the hippocampal region; [Scale bar: 500 μm (inset 200 μm). (C) Left: CD45R⁺ density (cells/ μm^3) in striatum (Str), cortex (Ctx), amygdala (Amyg), hippocampus (Hipp) and thalamus (Thal) in proteoliposome-treated (black) and control mice (gray). Right: CD8⁺, CD4⁺, CD20⁺, CD138⁺, Galectin3⁺ cell densities (cells/ μm^3) in the hippocampus of proteoliposome treated (black) and control mice

(gray). Only Gal3⁺ cells were of sufficient density to be apparent above zero on the histogram in control mice.

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Figure 6. Detection of NMDA receptor subtype-specific serum antibodies and localization of IgG binding to NMDA receptors

(A) HEK293FT cells transfected with rat GluN1/2A subunits were labeled with anti-GluN1 antibody (left, top and bottom, green) or with IgG from proteoliposome-treated mice (bottom, middle, red). Merge panel shows colocalization (bottom, right, yellow). IgG derived from control mice (top middle panel, red) showed no labeling. Scale bar: 15 μ m. (B) Dendrites of cultured hippocampal neurons with the expected punctate pattern of synapses using an anti-GluN1 antibody (green, left) compared to IgG from proteoliposome-treated mice (bottom row, middle, red). The merge image is shown at bottom row, right, yellow. IgG from liposome-treated mice (top row, middle) compared to puncta observed with anti-GluN1 (top row, left and right, green). Dendritic shafts were labeled with anti-MAP2 antibody (gray). Scale bar: 5 μ m. (C) The pattern of immunoreactivity in hippocampal tissue sections for a commercial NMDA receptor antibody (GluN2A) in an untreated wildtype mouse (top)

compared to labeling using purified IgG derived from a proteoliposome-treated mouse (bottom) and purified IgG derived from a liposome-treated mouse. (Scale bar: 500µm). (D) A battery of recombinant GFP-tagged NMDA receptor subunits from *Xenopus* (XI) and Rat (R) were blotted and detected with anti-GFP antibodies (red, top panel). Incubation with serum (1:100) derived from a liposome-treated mouse (green, middle panel). Serum (1:100) derived from a proteoliposome-treated mouse showed bands corresponding to GluN1 subunit isoforms in *Xenopus* and rat (XI.GluN1–3a, R.GluN1–1a, R.GluN1–1b), *Xenopus* GluN2B as well as a *Xenopus* GluN1 lacking the ATD domain (XI.NR1–3a ATD). Blots are from representative liposome-treated control mice as well as proteoliposome-treated mice with clinical signs of disease.

Figure 7. Electrophysiological effects in neurons following acute and chronic exposure to serum from proteoliposome-treated mice

(A) Representative whole-cell currents in a neuron evoked by acute flow-pipe application of NMDA + serum from liposome-treated, or NMDA + serum from proteoliposome-treated mice, respectively. Serum dilution was 1:100. (B) Quantification of evoked whole-cell NMDA currents in neurons following acute serum application. (C, D) Spontaneous excitatory postsynaptic currents were recorded following 24-hour incubation (“chronic”) in serum from liposome or proteoliposome-treated mice. (C) Representative sEPSC traces in cells treated with serum from liposome-treated before and after D-AP5 application (top, left and right traces, respectively) and cells treated with proteoliposome-derived serum (bottom, left and right traces, respectively). (D) Quantification of D-AP5 induced reduction in sEPSCs shown in histogram. (E) Immunocytochemical labeling of dendrites, PSD-95, and GluN1 puncta in cultured hippocampal neurons following 24hr incubated in serum from liposome and proteoliposome-treated mice. PSD-95 immunoreactivity (left, top & bottom,

red). GluN1 labeled puncta from control- and proteoliposome-treated mice (middle, top and bottom, green; respectively). Right panels show overlap of GluN1 and PSD-95 immunolabeling (right, top and bottom, yellow). Dendrites are labeled with anti-MAP antibody (gray). Scale bar: 10 μ m. Quantification of PSD-95 and GluN1 positive synaptic puncta per μ m of dendrite shown in left and right histograms (liposome = gray; proteoliposome = black). Quantification of PSD-95 and GluN1 positive synaptic puncta per μ m of dendrite shown in left and right histograms (liposome = gray; proteoliposome = black).

Figure 8. Response of T cells mutant mice to proteoliposome treatment

(A) $TCR\alpha^{-/-}$ mutant mice were immunized in parallel with a cohort of wildtype mice. Graphs show clinical observations and mortality rates by 12 weeks post-immunization (liposome = green; proteoliposome = purple). (B) CD45R (top panels), GFAP (middle panels) or Iba1 (bottom panels) immunolabeling in proteoliposome- and liposome-treated $TCR\alpha^{-/-}$ mice. Scale bar: 500 μ m (C)) Immunocytochemical colabeling of GluN1 and purified IgG from control- and proteoliposome-treated $TCR\alpha^{-/-}$ mice in hippocampal neuronal cultures (top panels) and HEK cells expressing GluN1-GluN2A subunits (bottom panels). The upper two rows show GluN1 positive puncta (left, green) along a dendrite (MAP2, gray) or lack of staining for IgG from liposome-treated mice (top middle, red) or IgG from proteoliposome treated-mice (bottom middle panel, red). Scale bar = 5 μ m. The lower two rows show anti-GluN1 antibody labeling of HEK293FT cells expressing rat GluN1/2A subunits (left, green), and absent labeling for IgG from proteoliposome-treated or liposome-treated mice (bottom two rows, middle). Scale bar: 15 μ m.

Table 1.
Immune cell densities Quantification of immune cell infiltration

Mean CD45R⁺ cell densities across sampled anatomical regions in control and proteoliposome-treated mice at 6 and 3 weeks post-immunization ($\pm$ SEM). Mean immune cell subtype densities in the hippocampus of control and proteoliposome treated mice at 6 and 3 weeks post-immunization ($\pm$ SEM).

Mean CD45R cell densities by anatomical region: 6 weeks post-immunization		
Anatomical Region	Control (cells/ μm^3 ; $\times 10^{-7}$)	Proteolip. (cells/ μm^3 ; $\times 10^{-7}$)
Striatum	0.57 $\pm$ 0.05	18.3 $\pm$ 8.94 **
Cortex	1.54 $\pm$ 0.32	29.3 $\pm$ 9.92 **
Amygdala	1.32 $\pm$ 0.13	30.4 $\pm$ 9.07 **
Hippocampus	0.59 $\pm$ 0.15	32.3 $\pm$ 9.50 **
Thalamus	1.30 $\pm$ 1.89	33.9 $\pm$ 12.1 **
Mean CD45R cell densities by anatomical region: 3 weeks post-immunization		
Anatomical Region	Control (cells/ μm^3 ; $\times 10^{-7}$)	Proteolip. (cells/ μm^3 ; $\times 10^{-7}$)
Striatum	1.16 $\pm$ 0.15	108.0 $\pm$ 54.5
Cortex	2.33 $\pm$ 0.44	46.7 $\pm$ 16.9
Amygdala	0.85 $\pm$ 0.19	47.3 $\pm$ 34.4
Hippocampus	0.97 $\pm$ 0.16	88.1 $\pm$ 17.5 *
Thalamus	1.53 $\pm$ 0.36	28.9 $\pm$ 13.7
Mean Immune cell subtype densities in hippocampus: 6 weeks post-immunization		
Immune Cell Subtype	Control (cells/ μm^3 ; $\times 10^{-7}$)	Proteolip. (cells/ μm^3 ; $\times 10^{-7}$)
CD8	0.026 $\pm$ 0.014	4.74 $\pm$ 2.00 **
CD20	0 $\times 10^0 \pm 0 \times 10^0$	15.9 $\pm$ 5.33 **
CD4	0.007 $\pm$ 0.006	18.9 $\pm$ 7.69 **
CD138	0.007 $\pm$ 0.007	23.3 $\pm$ 10.7 **
Galectin3	3.21 $\pm$ 0.265	24.0 $\pm$ 4.05 **
Mean Immune cell subtype densities in hippocampus: 3 weeks post-immunization		
Immune Cell Subtype	Control (cells/ μm^3 ; $\times 10^{-7}$)	Proteolip. (cells/ μm^3 ; $\times 10^{-7}$)
CD8	0.009 $\pm$ 0.006	0.10 $\pm$ 0.03
CD20	0.03 $\pm$ 0.03	13.2 $\pm$ 9.47 *
CD4	0.38 $\pm$ 0.11	35.9 $\pm$ 16.5
CD138	0.07 $\pm$ 0.04	13.9 $\pm$ 11.4
Galectin3	2.81 $\pm$ 0.34	39.8 $\pm$ 14.0

**
p = 0.0022, Mann Whitney test; n=6 mice/group

*
p = 0.0286, Mann Whitney test; n=4 mice/group

*
p = 0.0476, Mann Whitney test; n=5 mice/group