1 General Information

1.1 Identification of the study

Title: **Antibody-mediated NMDA receptor encephalitis: symptoms, biomarkers, and mechanisms of the prolonged recovery stage**
Títol: **Encefalitis del receptor NMDA mediada per anticossos: símptomes, biomarcadors i mecanismes de l'etapa de recuperació prolongada**

Código o número de identificación del protocolo: HR22-00221
Versión y fecha: V2; 07/12/2022

1.2 Identification of the promoter

Josep Dalmau, as principal investigator
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1.3 Identification of principal investigators in our center

Josep Dalmau: IDIBAPS, Cellex 3A, Ext: 1738
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1.4 Identification of principal investigators in other participating centers

Thais Armangué: Hospital de Sant Joan de Déu, Barcelona

2 Justification

The discovery of anti-NMDA receptor encephalitis (NMDARe) in 2007 changed the landscape of neurology and psychiatry and initiated an era of investigations identifying similar disorders and underlying pathogenic mechanisms.[1] Since then we and others have identified 15 additional diseases mediated by antibodies to synaptic proteins, with NMDARe representing the groundwork for many of these investigations.[2] After identifying NMDARe, we characterized the syndrome and triggers, developed a specific diagnostic test, provided guidelines for the differential diagnosis and treatment, and demonstrated the antibody pathogenicity in cellular and animal models.[3-6] Similar studies by other investigators have contributed to a better knowledge of the disease.[7, 8] Despite these efforts, NMDARe remains an intriguing disorder with many unanswered questions. The disease evolves in stages, each with different clinical manifestations and treatment requirements that involve different specialists along the clinical course.[3] In a common scenario, a patient may initially present with acute psychosis indistinguishable from a psychiatric disease, followed a few days later by the onset of seizures, orofacial or limb dyskinesias, tachycardia or bradycardia (which may require a temporary pacemaker), and decrease of the level of consciousness or

hypoventilation, requiring immunotherapy, multiple symptomatic treatments, and appropriate specialists. Some patients may undergo surgery for tumor removal (e.g., ovarian teratoma is a known trigger of the disease). If diagnosed and treated promptly, many symptoms improve while others persist or emerge, including behavioral and psychiatric alterations, and deficits of memory and other cognitive domains that interfere with familial, social, school, and work activities.[9, 10] To date, most studies have focused on the acute phase of the disease, and less frequently on the long-term outcome;[11, 12] thus, many reports represent snapshots of a specific time point in the clinical course, with a remarkable lack of prospective longitudinal studies that include the post-acute stage. Nonetheless, clinical, EEG, and neuroimaging studies suggest that patients often have protracted or permanent deficits that are poorly understood.[11-14] Several reasons explain this limited knowledge: 1) NMDARe is a rare disease with an annual incidence of ~1.5/million persons (likely an underestimate), 2) symptom severity in the acute phase complicates the transfer of patients to referral centers, 3) the long post-acute stage (or "recovery phase") is often perceived as a "non-active phase" and has been much less investigated than the acute phase, 4) there are no centers specialized in this stage of the disease and patients, families or caregivers often have to confront most of the indicated problems alone.[9] Lastly, there are no clinical or biological biomarkers for this disease stage or that accurately predict outcome; a few reports on B cell biology or CSF cytokines were focused on the acute phase without systematic follow-up. The physiopathology of this stage is also unknown. For example, there are no animal models examining the underlying inflammatory and synaptic alterations in the post-acute stage. Thus, it is unclear whether the neuropsychiatric deficits result from a persistent presence of antibody-producing cells (leading to synaptic dysfunction), from the associated inflammatory mediators (e.g., microglia, proinflammatory cytokines), or from a slow/incomplete recovery of NMDAR-dependent circuits.

The following gaps of knowledge will be addressed in this proposal and for which we have preliminary data:

a) Characterizing the post-acute stage. There is very limited knowledge of the post-acute stage of NMDARe; it is even unclear if immunotherapy or only symptomatic treatment is needed (antibody titers are not useful for these decisions). In a pilot prospective study we found an array of previously under-recognized symptoms (sleep and psychiatric alterations, inappropriate behavior, memory and cognitive deficits) [9]. Some patients were re-admitted to psychiatric facilities and we are aware of patients prosecuted for inappropriate social behavior. In this study, which also included a group of persons with schizophrenia (SCZ) and a group of healthy participants, we found that the cognitive and psychiatric symptoms of the post-acute stage of NMDARe resembled those of patients with stabilized SCZ. Despite sustained improvement in psychiatric scales, longitudinal follow-up showed that the cognitive deficits of NMDARe, but not those of SCZ, improved initially but stagnated at ~6 months. This represents an opportunity for targeted treatment that we will address in this proposal. The mechanisms underlying these alterations are unknown. Psychophysical testing showed that patients with NMDARe or SCZ had intact precision in a working memory (WM) task but a strong reduction of serial dependence (which is the influence of previous experiences in performing the WM task [17]). In animal models serial dependence of similar WM task relates to synaptic plasticity [18], suggesting a role for NMDAR-dependent short-term synaptic potentiation in NMDARe.[17] In longitudinal studies, the WM alteration of serial dependence along with other symptoms improved gradually in NMDARe towards the value of healthy controls. Additionally, in the same pilot study, we identified that patients with NMDARe had changes in the slope of individual slow-waves during deep sleep (postulated to reflect synaptic strength).

b) Exploring the immunopathology. In CSF or autopsy studies of NMDARe we found predominance of B cell mechanisms (e.g., more abundant B cells/plasma cells compared with T cells; increase of CXCL13; microglial activation, and mild/absent neuronal loss).[19-21] In another study we showed that serum and CSF neurofilament light chain (NfL) levels in the acute stage correlated with symptom severity but not with long-term outcome; we and others postulate that NfL in the post-acute stage may correlate better with outcome.[22] However, it is unclear whether these or other features (B cell activation signature; specific interleukins/cytokines) are affected in the post-acute stage or have clinical/prognostic correlates that could guide treatment (e.g., bortezomib; anti-IL6).

c) Modeling mechanisms and treatment. We previously reported the pathogenicity of NMDAR antibodies using a mouse model of cerebroventricular transfer of patients' antibodies [6] and showed that a positive NMDAR allosteric modulator (SGE-301) reversed antibody pathogenicity [23]. This experience led us to develop a mouse model of active NMDAR immunization that associates with brain inflammation, chronic (beyond 70 days) systemic and intrathecal synthesis of NMDAR antibodies, reduction of NMDAR synaptic clusters, and impairment of long-term potentiation (LTP), memory and behavior. In preliminary studies, the clinical and synaptic alterations of this model resemble those of patients with NMDARe, but other inflammatory-immune alterations (as those listed in the previous paragraph) were not assessed. In the current project we will compare side by side the patients' blood and CSF inflammatory-immune alterations with those of the mouse model (using blood, CSF, and also brain tissue). This model offers the opportunity to test different treatment strategies during the acute and post-acute stages of NMDARe, which are goals of the current project.

To address these questions, we have assembled a multidisciplinary group of clinical and basic investigators with expertise on NMDARe ranging from patients' bedside assessment to animal models. Patients with NMDARe from our adult and pediatric centers (Hospital Clínic de Barcelona [HCB]/IDIBAPS; Hospital Sant Joan de Déu [SJD]), given the high prevalence of the disease in adolescents and young adults, will be studied during their post-acute stage of NMDARe. They will undergo biological sampling (the most invasive test will be a lumbar puncture), EEG and MRI investigations, and they will benefit from a cognitive rehabilitation program. Findings from this study will help to understand at the synaptic and network levels how specific neuronal antibodies alter memory and cognition, and will assist to treat patients with diseases in which the same or other synaptic proteins are altered by other autoantibodies (e.g., AMPA, GABA receptors).

2.1 Relevant bibliography

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3 Hypotheses of the study

The hypotheses of this proposal are:

(1) A better knowledge of the post-acute stage of NMDARe will improve treatment and long-term functional outcome of patients

(2) At the post-acute stage of NMDARe, when able to collaborate, patients will benefit from a remote cognitive rehabilitation program

(3) Development of psychophysical and biological markers will facilitate monitoring of the prolonged post-acute stage of the disease and treatment decisions

(4) Modeling the post-acute stage in mice will help to understand the physiopathology and improve treatment

4 Objectives and aims of the study

The study has 3 objectives:

1. Clinically characterize the post-acute stage of NMDARe, provide tools to remotely follow cognitive, behavioral and psychiatric deficits, and assess the impact of a cognitive rehabilitation program.

We will perform comprehensive neurologic, cognitive and psychiatric evaluations, as well as polysomnography (PSG), MRI and EEG studies on patients with NMDARe at the post-acute stage of the disease. We will validate remote working memory and sleep/EEG psychophysical studies as biomarkers of cognitive outcome. We will assess the efficacy of a remote cognitive rehabilitation program.

2. Identify biomarkers of autoimmune, inflammatory, and neuronal injury as signatures of the acute and post-acute stages of NMDARe.

We will perform blood/CSF immunophenotyping (cell sorting) and transcriptomic analysis

(RNA/NanoString) of targeted gene expression specifically related to immunological/cytokine pathways (44 genes profiling activation/function of B cells, T cells, microglia, several interleukin/chemokine signaling), and serum/CSF NfL levels (SiMoA). Findings from the post-acute stage will be compared with those of the acute stage assessing the need of immunotherapy change or maintenance.

3. Determine in an animal model the pathogenic mechanisms (including the contribution of NMDAR antibodies, inflammation, and delayed recovery of NMDAR function) and treatment (whether immunotherapy and NMDAR allosteric modulation ameliorate those alterations) of the post-acute stage of NMDARe.

In an active immunization mouse model of NMDARe, we will examine the memory/behavioral alterations, brain inflammatory infiltrates, presence of antibodies, changes in structure/function of NMDAR, NMDAR-dependent long and short-term potentiation, and the effect of immunosuppression (rituximab) and NMDAR allosteric modulation (SGE-301) on reversibility of chronic symptoms and synaptic alterations. Brain RNA/NanoString analysis targeting the same genes as in patients will be done with mouse-specific primers; findings from the acute (day 42) and post-acute (day 71) stages will be compared.

4.1 Primary and secondary variables

Description of primary and secondary variables by specific objectives:

1. Clinically characterize the post-acute stage of NMDARe, provide tools to remotely follow cognitive, behavioral and psychiatric deficits, and assess the impact of a cognitive rehabilitation program.

1.1 General clinical data that will be obtained at each visit from all patients include (all primary variables): age, gender, vision condition, handedness, general medical history, allergies, description and duration of symptoms related to NMDARe, treatments, functional status according to modified Rankin Scale (mRS).

1.2 Presential neurocognitive assessment (primary variables): general neurological examination and a battery of 16 tests (with standardized versions adapted for adults and children) encompassing 8 cognitive domains: intelligence quotient, working memory, learning and memory, psychomotor speed, executive functions, selective and sustained attention, language, visuospatial perception.

1.3 Presential psychiatric assessment (primary variables): Structured clinical interview to assess the presence of psychiatric disorders following DSM-IV-TR guidelines (SCID-I for adults and K-SADS-PL for school-age children, psychotic symptoms (Positive and Negative Syndrome Scale, symptoms of depression (Hamilton Depression Rating Scale), symptoms of mania (Young Mania Rating Scale), Global Assessment of Functioning Scale or Children's Global Assessment Scale.

1.4 Remote cognitive assessment (secondary variables): customized panel of 8 tests for 6 cognitive domains covering working memory, learning and memory, psychomotor speed, executive functions, selective and sustained attention. It will be performed using one of the most validated cognitive research software available (CANTAB: https://www.cambridgecognition.com/cantab/). Our group already has experience working

with it, and our feedback from patients is usually satisfactory. During presential visits, patients will be trained to use the online platform, and once at home they will run the exercises on an iPad that they will borrow from us and return to us after the tests (we will provide a courier service, and the iPad will have all the necessary software installed, ready to use). An experienced neuropsychologist from our team will guide them through the process and supervise the timings.

1.5 Remote neuropsychiatric assessment (secondary variables): all indicated in point 1.3 above except the structured clinical interview, and a functional neurological scale (mRS). It will be performed with the patient and preferably a close relative through an online videoconference (either by phone or computer) with an experienced neuropsychologist from our team.

1.6 Psychophysical testing of working memory (WM) (secondary variables): patients will use a remote device to acquire behavioral and EEG data during sleep and during a cognitive task that will provide quantitative estimates of (1) spatial WM precision, (2) systematic biases in spatial WM, and (3) serial biases in spatial WM, based on the behavioral responses in a computerized task. This testing is carried out using a validated visual stimulation package from PsychoPy software (pyschopy.org) as reported [17]. A working memory (WM) task, in which participants report the location of a previously presented brief visual cue, is run in a laptop that records behavioral responses (mouse clicks). Briefly, the subject maintains fixation on a central cross on the computer screen. An eye-tracker is used to confirm that the subject is looking at the cross. A brief (1 sec) visual stimulus appears on the screen, consisting of 1 coloured dot randomly located on an invisible circle. After a delay of 1-3 sec, subjects report with the mouse the remembered location of the dot. After response, the fixation cross reappears on the screen to signal a new trial. The task runs in sessions of 45-min, composed of 5-min blocks, each block with 30 trials. Each block is followed by a 1-2 min pause, with feedback on performance. Sessions start with a minimum of 10 training trials; 80% success leads to proceed with the main task.
A kit (the same iPad tablet as per variable 1.4, portable cap/EEG) will be shipped to patients for remote psychophysical tests during 1 week. The WM task will be implemented in the iPad tablet [26] and mildly gamified to make it engaging; it will be synchronized with a portable EEG (Dreem 3 for Research, https://dreem.com). Patients will be instructed to use the kit every day (1 week) to do the task with EEG and for nocturnal EEG recording. Acquired behavioral and EEG data will be transferred to our servers for off-line analysis. These studies will provide a follow-up of WM effects and sleep microstructure parameters, stability, evolution and correlation with clinical features.

1.7 MRI studies (secondary variables): will be conducted on a 3 Tesla Prisma scanner using a 32-channel head coil. Scanning takes ~50 min including 3D T1-weighted in sagittal plane; T2*axial EPI; axial diffusion weighted EPI; 3D sagittal FLAIR; resting state functional MRI and glutamate and H2O univoxel spectroscopy in dorsolateral prefrontal cortex and hippocampus. There is no contrast used for the MRI scans.

1.8 Polysomnography (PSG) (primary variables): will be adapted to patient's sleep habits (~23:00 to 07:30) using a digital polygraph (Deltamed). This includes EEG in 43 scalp channels + 11 channels for electrooculography, electrocardiography, electromyography, and audiovisual recording (sampling rate 256 Hz). Sleep stages will be scored manually (AASM criteria) using 30-s epochs, with modifications depending on sleep alterations, as reported.[9] Data will be analyzed off-line in Python.

1.9 Electroencephalogram (EEG): it will include standard clinical EEG protocol (43 channels, 512 Hz18) (primary variables), and EEG reactivations of memories prior to new trials

(secondary variables) while participants perform WM tasks, which will be synchronized with the task software in a laptop. We will decode the memory content from alpha power across electrodes as done previously,[18] and relate the decoding accuracy in different task periods to disease treatment and recovery and to behavioral parameters (WM precision, serial biases).

2. Identify biomarkers of autoimmune, inflammatory, and neuronal injury as signatures of the acute and post-acute stages of NMDARe

2.1 Cell immunophenotyping (secondary variables): whole blood/CSF obtained in a EDTA tube will be analyzed in a FACS Canto II cytometer to assess the proportion of CD4, CD8, CD8+CD45RA+, CD8+CD45RO+, iNKT, CD19, B naive (IgM+IgD+CD27-), B memory (IgD-), regulatory B cells, plasmablasts, and NK cells.

2.2 Immune/inflammatory signaling-target gene expression pathways (secondary variables): Whole blood/CSF will be collected using PAXgene® Blood RNA Tubes (Qiagen) shipped to the centers. Blood from healthy subjects and CSF from patients with normal pressure hydrocephalus will serve as controls. Total RNA will be extracted using PAXgene® Blood RNA Kit (Qiagen). RNA samples are quantified using Qubit 2.0 Fluorometer (Life Technologies) and RNA integrity is determined with Agilent 2100 Bioanalyzer (Agilent Technologies). Expression levels of 44 genes related to immune/inflammatory (or cytokine) pathways (Table 2) will be measured with the nCounter® Digital Analyzer (NanoString). The raw copy number of mRNA transcripts of each gene will be standardized (stdGene) using the geometric mean of the 4 housekeeping genes for each subject. Then, a Z score for each gene will be calculated using the mean and the standard deviation (SD) of a group of 30 healthy subjects: Z score Gene = stdGene - mean stdGene control group/SD stdGene control group. A signature for each immune/inflammatory pathway in each subject will be calculated using the median of the Z scores of the genes involved in that pathway. The signature will be considered positive (e.g., activated) if the value is ≥1.96 (>98centile) (one tail analysis is used since only positive signatures are expected). We reported similar methods for the interferon pathway in another disorder.[27] It will include genes of B cell activation/function (CCL19, CxCL13, BAFF, APRIL, SYK, TACI, BAFFR, BCMA, CD40), T ell activation/function (CLDN1, IL23A, CTLA4, ICOS, ICOSLG, PDCD1, PDCD1LG2, CD40LG), TH17 signaling (IL17A, IL17RA), IL1 signaling (IL1RN, IL1B, IL1R1), IL2 signaling (IL21, IL21R), IL6 signaling (IL6, IL6R), other cytokine signaling (CXCL3, CXCL8, CX3CL1, IL12B, TNFAIP3), type 1 interferon signaling (IFI27, IFI44, IFI44L, ISG15, RSAD2, SIGLEC1), housekeeping genes (ALAS1, HPRT1, TBP, TUBB).

2.3 Neurofilament light chain (NfL) levels (secondary variables): will be determined in serum and CSF using the SiMoA Quanterix technique. These studies will provide a signature of molecular biomarkers in the acute and post-acute stages. We expect they will correlate with symptoms and psychophysical biomarkers (Aim1) and will assist in treatment decisions.

3. Determine in an animal model the pathogenic mechanisms and treatment of the post-acute stage of NMDARe.

3.1 Memory and behavioral tests for mice (primary variables)

3.2 Surface clusters of NMDARs on sections of cortex/hippocampus (primary variables)

3.3 Hippocampal long-term (LTP) and short-term potentiation (STP) (secondary variables)

3.4 Immune/inflammatory signaling-target gene expression pathways (secondary variables): will include genes of B cell activation/function (CCL19, CxCL13, BAFF, APRIL, SYK, TACI, BAFFR, BCMA, CD40), T ell activation/function (CLDN1, IL23A, CTLA4, ICOS, ICOSLG, PDCD1, PDCD1LG2, CD40LG), TH17 signaling (IL17A, IL17RA), IL1 signaling (IL1RN, IL1B, IL1R1), IL2 signaling (IL21, IL21R), IL6 signaling (IL6, IL6R), other cytokine signaling (CXCL3, CXCL8, CX3CL1, IL12B, TNFAIP3), type 1 interferon signaling (IFI27, IFI44, IFI44L, ISG15, RSAD2, SIGLEC1), housekeeping genes (ALAS1, HPRT1, TBP, TUBB).

5 Design of the study

Human studies

This is a prospective single center, single arm, national study of 3 years duration.

The study will be conducted following the protocol that we developed for a previous prospective longitudinal observational study that was performed between 2017 and 2021 with patients with anti-NMDAR encephalitis during the post-acute stage, and that was approved by the CEIm of HCB (**Study title: "Immune-mediated diseases of the synapse: symptoms, brain networks, and the link to human memory"; PI: Josep Dalmau; Code: HCB/2016/0596**). We will perform the exact same protocol with 3 main novelties: (1) we include five remote online study visits (clinical assessments and tasks using portable devices); (2) patients will undergo a cognitive rehabilitation program, and (3) they will have two CSF studies, as detailed below.

Left over samples of blood and CSF that were obtained and stored during the acute stage of the disease (by the time of symptom onset and local hospital admission) will be obtained from the core laboratory (CBD) for patients seen at our center or will be sent to our center from the referring doctors. The diagnosis of NMDARe requires demonstration of antibodies in CSF (serum can be negative); thus, initial CSF studies form part of standard of care in this disease. For the current study, the left-over routine care serum and CSF samples from the 20 recruited patients will be used after their informed consent. For patients followed at our institution, we will take advantage of a regular follow-up visit to inform about the study and obtain their consent. For patients cared for at other hospitals, the referring doctors will explain the possibility of participating in the current study and, if the patients consent, we will contact them to provide further explanations and in such case obtain the informed consent. These acute samples will be tested for the indicated (see below) biomarkers of autoimmunity, inflammation, and neuronal injury. We are a referral center for the diagnosis and study of this disease, and therefore we have ample experience in receiving and handling the samples.

During the post-acute stage (by the time that patients will come to Hospital Clínic de Barcelona), blood and CSF samples will be obtained for identical studies as those performed in the acute stage serum and CSF samples. Two lumbar punctures will be performed, after informed consented, one at the first presential visit (V1) and the other at the last presential visit (V3). Patients will be given the option to opt out of the two spinal taps. Based on the recently published pilot study and extensive experience with the disease, all patients at V1 have cognitive and psychiatric alterations and most still have residual deficits at V3; thus, the CSF studies are important for investigations on biomarkers of disease severity, outcome and prognosis, and for better optimization of therapeutic options.

In addition to human studies, the current project includes an experimental animal model of

NMDARe using active immunization with the NMDA receptor. Thus, human biological samples will not be used in this experimental animal model (detailed later).

6. Selection of participants

Study group

20 newly diagnosed NMDARe patients (age ≥12 years) will be recruited. Families and patients will be approached during the acute phase of the disease and invited to participate in the study after hospital discharge. Informed consent and general clinical information through a structured questionnaire will be obtained from participants/legal guardians. Identification of patients will be done in (1) our hospitals, (2) through a network of Spanish hospitals, both adults and pediatrics (already in place, specified in the Annex), and (3) the Neuroimmunology lab at HCB/IDIBAPS (referral center for NMDARe). Because NMDARe predominates in women (8:2), it is expected that there will be a female predominance.

Inclusion criteria

Patients ≥12 years old with NMDARe in the post-acute stage of the disease, ≤6 months from hospital discharge (acute phase), will be eligible to participate in the study.

Exclusion criteria

They include: inability to obtain informed consent or inability to travel to our center.

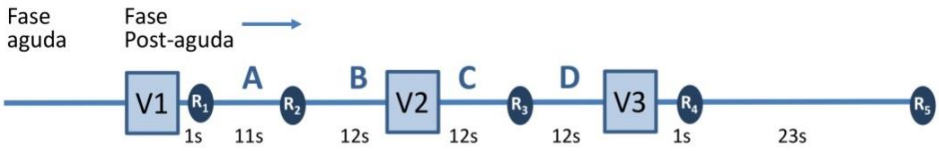
External control group

Patients from a pilot study (n=20; follow-up ≥ 2 years; **HCB/2016/0596**) who underwent similar presential visits (without cognitive rehabilitation or remote assessments/tests) will be contacted to extend their follow-up for an additional year. For patients followed up at our institution, we will take advantage of a regular follow-up visit to inform them about the study and obtain their consent. For patients cared for at other hospitals, the referring doctors will explain the possibility of participating in the current study and, if the patients consent, we will contact them to provide further explanations and in such case obtain the informed consent. They will have 2 additional presential visits (as above) 1 year apart, and blood tests. This group will serve as control and will be compared with newly recruited patients (with cognitive rehabilitation/remote tests). They will provide a long-term follow-up (≥ 3 years) without remote cognitive rehabilitation or tests and will help to assess the impact of these interventions in outcome, and whether immunologic/ inflammatory signatures or signs of neuronal injury (NfL) are chronically present in blood.

7. Treatment and calendar of the study

The general outline of the study including presential and remote visits and cognitive training is summarized in **Figure 1;** and the evaluations and tests during each of the three presential visits (except CSF studies that will be performed only in the first and last visit) are shown in **Figure 2**. A more detailed description of tests, interventions and calendar of the studies is provided following both figures.

Figure 1: Outline of presential and remote visits and cognitive training



V1, V2, V3= visitas presenciales; **V1**= 1st visita de estudio; **V2**= seguimiento a los 6 meses; **V3**= seguimiento al 1 año
A→D= entrenamiento cognitivo; **A,B**= 2 sesiones/semana; **C**= 1 sesión/semana; **D**= 1 sesión/2 semanas
R_1, R_4, R_5 = evaluación cognitiva remota (estudio comparativo con **V1, V2** y validación de estudio remoto)
R_2, R_3, R_5 = evaluación psiquiátrica remota **R_2, R_3**= dispositivos portátiles (Memoria, EEG/sueño); s= semanas

Figure 2. Schedule for each presential visit

Presential visits

This study includes a total of 3 presential visits during 1 year. After hospital discharge, patients will come to our center ~2-5 months after disease onset for the **first (baseline) presential study visit (V1)** and for **follow-up visits 6 months (V2)** and **12 months (V3)** after V1. Each visit will consist of 2 days and 1 night admission at HCB (children, adults) during which they will undergo clinical examinations and tests according to a pilot study, including: neuropsychiatric and cognitive assessments, psychophysical studies, MRI, v-PSG, EEG. At V1 and V3, patients will undergo blood and CSF studies (variables 2.1, 2.2 and 2.3 will be analyzed in these paired blood/CSF samples and will be compared with those from the acute stage).

- Presential standard neuropsychiatric examinations and cognitive assessments will be conducted as per routine clinical practice and with a reported panel of neurological functional, cognitive and psychiatric tests (variables 1.1, 1.2 and 1.3). Total time ~4h spread over 3 sessions (the exact timetable can be adapted to patients' preferences and the length of the interviews will vary according to patients' clinical status; the times are indicative and are overestimated to be sure). These clinical evaluations will take place in the same room where the patients will sleep, and patients will have enough pauses in between to rest, eat/drink and go for a walk outside the hospital with their relatives.
- Psychophysical testing of working memory: The duration of this task depends on the

performance of the patients; maximum ~1:30h. It will be done in the same room.
- MRI studies (variables 1.7). It is a passive examination, patients will not need substantial mental or physical efforts. Total time ~45min.
- PSG (variables 1.8): It is a passive examination, patients will not need substantial mental or physical efforts. Patients will sleep in the same room where the other clinical examinations and psychophysical testing will be carried out, so they will get used to and adjust to the environment during the day. Total time: night sleep.
- EEG (variables 1.9): It is a passive examination, patients will not need substantial mental or physical efforts. Total time ~45min.
- Patients' blood, serum, and CSF will be examined at the time of diagnosis (sent from local hospitals to our center via courier) and at the post-acute stage: V1 (1st visit in our center) and V3 (1 year follow-up). It will be performed in the same room; total time ~30min

Remote visits

This study includes a total of 5 online visits that will take place in between presential study visits during 1.5 years. See Fig. 1 for details about what tests are applied in each visit R1-R5. Briefly:

- R1: remote cognitive assessment (variable 1.4). 1 week after V1; total time ~40min.
- R2: remote neuropsychiatric assessment (variable 1.5) and remote psychophysical tests (working memory via iPad for variables 1.6; portable EEG/sleep for variables 1.8 and 1.9). 12 weeks after V1. Total time: ~15min once for the neuropsychiatric assessment, ~10min daily during 1 week for psychophysical testing, and night sleep while passively being recorded during 1 week.
- R3: remote neuropsychiatric assessment (variable 1.5) and remote psychophysical tests (working memory via iPad for variables 1.6; portable EEG/sleep for variables 1.8 and 1.9). 12 weeks after V2; total time ~15 min once, ~10-15min daily during 1 week, and night sleep while passively being recorded during 1 week.
- R4: remote cognitive assessment (variable 1.4). 1 week after V3; total time ~40min.
- R5: remote cognitive and neuropsychiatric assessments (variables 1.4 and 1.5). 6 months after V3; total time ~1h.

Remote cognitive rehabilitation program will be performed through an online validated platform (Guttmann Neuropersonal Trainer [GNPT]: https://gnpt.es/) run by our team. This is a Sanitary Product with CE certification (Producto Sanitario RPS/430/2014; International Patent [PCT/ES2008/00677]) and here will be used within its approved indications. The rehabilitation program will increase in difficulty and decrease in frequency during the first year of follow-up (V1-V3).

Patients/families will be instructed to perform remote cognitive rehabilitation during the presential visits. Patients will be able to use their personal computer and access via an available link and a user code the GNPT platform. In case that they do not have easy access to adequate equipment to access the platform, we will provide the iPad tablet with all the necessary software installed and ready to use to support their training. The indicated frequency of cognitive training is based on results of a recently published pilot study in which patients were followed with only presential visits (no remote visits or cognitive training was provided). The study showed that a slow progressive cognitive improvement occurred in most patients during the 1 year follow-up. However, the improvement was more marked during the first 6 months compared with the last 6 months. This finding suggested that intensive cognitive training (particularly during the first 6 months) could

accelerate recovery and improve outcome. Accordingly, for the current study patients will undergo cognitive rehabilitation twice per week for the first 6 months and once per week for the last 6 months. Similar programs are currently used by our team for patients with multiple sclerosis (along with collaborations with Institute Guttmann, Barcelona). Remote cognitive sessions will take ~45-60min.

Experimental mouse model of NMDARe

These studies are separate from the human studies. The animal study protocol is currently under revision by the CEEA-UB with registration number 420.22, already pre-approved. As indicated, no human biological samples will be used for the experimental animal model. Of note, although the animal studies are independent of human studies, the running in parallel of both sets of investigations will inform on the mechanisms of the disease and may have future treatment and prognostic implications.

Mice C57BL/6J, 8-10 weeks old will be used. Three experimental groups will be established according to 3 treatment approaches. All mice will be immunized twice (days 0 and 28) with a GluN1 peptide (amino acids 356-385) that contains the main epitope region of NMDARe. As adjuvant, we will use Addavax+Pertussis toxin that primes B cell and antibody responses with better tolerability than Freund's adjuvant. This approach results in very high titers of NMDAR antibodies with reactivity similar to those of patients (Fig 6 a,b). The corresponding 3 control groups will receive only Addavax+Pertussis toxin without peptide.

B.1. Behavioral studies

From baseline (pre-immunization) until day 71 (euthanized), animals will be regularly tested with memory/behavioral paradigms, as reported in our model of passive transfer of antibodies.[6]

B.2. Effects on NMDAR

The antibody effects will be determined in subsets of mice euthanized at day 42 (acute stage) and the rest of mice on day 71 (post-acute stage) (Fig 8). Sections of cortex/hippocampus will be examined using (1) confocal microscopy and IMARIS software to quantify the surface clusters of NMDARs, and (2) electrophysiology to assess hippocampal long-term (LTP) and short-term potentiation (STP). These techniques were described in detail in our passive immunization model [6,23] and STP in [8].

B.3. Treatment approaches to the post-acute stage

After immunization, 3 subsets of mice (and corresponding controls) will be established: not treated; treated with a mouse anti-CD20 [29] (Biogen Idec; analogous to rituximab); or treated with the anti-CD20 and a NMDAR positive allosteric modulator (SGE-301) [23]. All groups will be regularly tested with the indicated behavioral panels; on day 71 animals will be euthanized for brain confocal microscopy, LTP/STP, and studies listed below (3.5).

B.4. Immune cells and immune-inflammatory pathways

From tissue samples obtained on days 42 and 71, immune/inflammatory cells (B, T, activated microglia, etc.) will be examined by immunohistochemistry.[4,6] RNA will be extracted from brain for RNA/NanoString analysis targeting the same genes as described in patients, with mouse-RNA primers. Findings from the acute (day 42) and post-acute (day 71) stages will be compared.

8. Statistics

8.1. Sample size

20 newly recruited NMDARe patients and 20 patients from a previous pilot study for extended follow-up. Given that NMDARe is a rare disease, we are not able to perform a sample size estimation. We propose this sample size based on the feasibility according to our previous experience on the number of patients that we annually diagnosed in our centre and based on the results from the pilot study.

8.2. Statistical analyses

For human studies, we will analyze differences in cognitive and psychiatric variables between and within groups (newly recruited NMDARe patients and those from the pilot study) in the longitudinal follow-up using multilevel linear mixed-effect models, adjusting for group, age, sex, and socioeconomic status to control for possible confounds. Lme4 R package will be used to fit LME models, and residual plots will be used to validate them. Comparisons will be done with emmeans library and Tukey method for post-hoc correction for multiple testing. The global tendency of recovery of cognitive deficits in NMDARe (proportion of patients compared to the general population in each domain) will be assessed with the Cochran-Armitage test for trend; other comparisons within NMDARe will be done with Wilcoxon signed-rank and McNemar's tests. The patient behavioral, PSG and EEG data will be analyzed with linear regression using multiple regressors (task parameters, fraction of NREM N3, disease condition, with/without remote training, etc.). All analyses will be addressed considering a two-tailed type I error of 5% with statistical significance set at p-value <0.05, using SPSS (IBM Corp, Armonk, NY) and R package (Vienna).

For animal behavior studies with multiple determinations in time (e.g., new object recognition) we will use the double variance analysis (ANOVA). For behavior studies with a single determination in time (e.g., depressive behavior; "tail suspension") we will use the simple ANOVA. Electrophysiological studies (long-term potentiation) will be analyzed with double ANOVA. In all experiments, the post-hoc analysis will use the Bonferroni correction for multiple tests. A value of $p < 0.05$ will be considered significant. The alpha-error will be set to 0.05. The analyses will be done using GraphPad Prism. Animal sample size: To detect a difference between the two groups with a 95% CI, assuming an estimated standard deviation of 20% for behavioral tests and a 20% procedural mortality, we will need 12 mice per group.

9. Risks and benefits for participants in the study

It is possible that patients will not benefit from participating in this study. However, based on the results of previous similar studies (“Immune-mediated diseases of the synapse: symptoms, brain networks, and the link to human memory”, IP: Josep Dalmau, Code: HCB/2016/0596; “A Translational Model of Antibody-mediated Synaptic Disease: Symptoms, Neuronal Circuits, and the Mechanisms of Memory Loss and Recovery”, IP: Josep Dalmau, Code: HCB/2018/0561) we expect that patients will directly and immediately benefit from their participation in the carefully monitored cognitive training program and the close clinical follow up in an experienced unit.

Our previous studies with this same load of tests in presential visits were successful and

patients with anti-NMDAR and anti-LGI encephalitides returned for the most part in successive retest sessions after 3, 6, 12 and even 24 months (Guasp et al. Lancet Neurology 2022; Ariño et al. Neurology 2020; Stein et al. Nat Commun 2020). This demonstrates that the load of tasks and frequency of visits was not unbearable and patients were happy to maintain participation in the project for an extended period of time and repeat sessions with identical task loads. We specifically gauged participant satisfaction in a gathering of participating families at Hospital Clínic in 2018, where we presented the objectives and initial results of the study and allowed participants to ask questions and express their concerns. Participants were very satisfied with the study and appreciated the opportunity to be monitored in the reference center for this disease in Europe, and to get in touch with other families afflicted by the same rare disease. We plan to repeat this experience in the framework of the present project in order to detect possible dissatisfactions with our experimental protocols and adapt them for the comfort of the participants.

Nonetheless, the detailed risks for each of the procedures in the current study are as follows:

a) Risks of presential Neurologic, Psychiatric and Cognitive assessments: These evaluations are innocuous and do not represent any substantial risk for participants. The main risk of these evaluations is fatigue, and they may be demanding in terms of execution time, but patients will be able to take breaks during the testing to alleviate it. Moreover, they will be guided by a member of our team to ensure that it does not cause any major discomfort to the patient.

b) Risks of remote Neurologic, Psychiatric and Cognitive assessments: These will be run either by telephone or via a video-conference (online platform link provided by us) at various time points during the participant monitoring (Fig. 1). The main risk is fatigue, but a member of our team will ensure that it does not cause any major discomfort to the patient.

c) Risks of Magnetic Resonance Imaging (MRI): The known risks associated with MRI studies are minimal. The procedure uses radio waves and a magnetic field to take pictures. Some individuals may feel claustrophobic (uncomfortable in small areas) during the MRI scan or may be disturbed by the sounds of the machine (which are loud and repetitive). Patients can be given music to listen to during the scan or can hear the voice of the technician. These have been shown to help patients relax during the scan. If the subject experiences these feelings and is uncomfortable, they can always discontinue the scan

d) Risks of presential EEG studies: EEG is considered a safe procedure and causes no discomfort. During the recording, subjects can relax. Some people may develop a mild rash where the electrodes were attached that resolves without treatment.

e) Risks of presential Sleep studies: The same for EEG studies, as well as possible discomfort before falling asleep and reduced night rest. However, the cabling system is optimized to facilitate the free movement of patients at night, and the room is soundproofed.

f) Risks of Psychophysical testing and Brain recordings (both presential and remote): These evaluations are innocuous and do not represent any substantial risk for participants. The main risk of these evaluations is fatigue, reduced night rest, and they may be demanding in terms of execution time. However, they will only be performed in the course of two weeks separated by 6 months, for each participant. The computerized task will have a game component to make it engaging, as well as the device is specifically designed to be comfortable during sleep for nocturnal recordings. In the first day of the week of recordings, a member of our team will make a video-conference with the participant (including family members if they are minors) to assist with the setup of the device for

testing and recording. Of note, the remote EEG device does not require complex setup or extensive preparations and it is easy to wear, as it consists of a cap with electrodes already attached that communicates wireless with the iPad tablet. Thus, there is no risk of mild rash. The cap may feel uncomfortable similar to that of a bathing cap due to its tightness; patients can acclimate to this by wearing the cap for short periods of time in the days before the testing. There are no cables to connect or technical adjustments to make. Because the task to be run in the iPad tablet will have a game component, we anticipate that younger participants will be particularly happy to engage in this part of the protocol. This will allow us to determine whether a remote device is useful to assess patient evolution. This knowledge will not provide an immediate benefit to the participants of this study, but may represent an important improvement for the follow-up of anti-NMDARe patient recovery in the future, minimizing presential follow-up visits.

g) Risks of Blood Tests: Risks associated with blood drawing include infection, bleeding, bruising, fainting and discomfort.

h) Risks of Lumbar Puncture: Although lumbar puncture (spinal tap) is generally recognized as safe, it does carry some risks. These include post-lumbar puncture headache that can last from a few hours to a week or more. Lying flat and drinking fluids helps to relieve this headache. Patient may experience temporary back discomfort or pain, and mild bleeding that may occur near the puncture site. These side effects resolve without treatment. It is possible that patients will not benefit from the lumbar puncture, but for those patients with unfavourable clinical evolution or relapse, NMDAR antibody testing in CSF will help guide therapeutic decisions (antibody titters in CSF, but not in serum, have clinical correlation).

9. Ethics and legal aspects

The study will be carried out in compliance with the Helsinki Declaration (current version, Fortaleza, Brazil, October 2013) and in accordance with the protocol and with the relevant legal requirements, which in this case is Biomedical Research Law 14/2007 of July 3, and Regulation 2017/745 on Medical Products. Informed consent will be obtained for all patients before their inclusion in the study, and their samples stored in the Neuroimmunology Tissue Collection.

10. Data management

Clinical data from patients will be coded. Each patient will be assigned a code. In another separate database, the IP will store the relationship between each code and the patient's medical record number. In all data management softwares used in this project (PsychoPy, CANTAB, GNPT, Dreem 3 for Research) participants will be indexed by their code and never by their personal identification data.

Coded information from patients will be stored in online Redcap database from HCB (redcap.clinic.cat).

Ipads and wearables will be stored at Cellex 3A, Neuroimmunology laboratory.

11. Treatment of data and archiving of registers. Confidentiality of the data

The treatment, communication and transfer of personal data of all participants will comply with EU Regulation 2016/679 of the European Parliament and of the Council of April 27, 2016 regarding the protection of natural persons in terms of to the processing of personal data and the free circulation of data, being mandatory as of May 25, 2018. The legal basis that justifies the processing of your data is the consent given in this act, in accordance with the provisions of the Article 9 of EU Regulation 2016/679.

The data collected for these studies will be collected identified only by a code, so no information will be included that would allow the participants to be identified. Only the study doctor and his collaborators with the right of access to the source data (clinical history), may relate the data collected in the study with the patient's medical history. In the future, the data collected and properly coded may be used also to address other questions relevant for the understanding of NMDARe, and we will request specific consent for this potential prospective use of the data.

The identity of the participants will not be available to any other person except for a medical emergency or legal requirement.

The health authorities, the Research Ethics Committee and personnel authorized by the study promoter may have access to the identified personal information, when necessary to verify data and study procedures, but always maintaining confidentiality in accordance with current legislation. .

Only coded data will be transferred to third parties and other countries, which in no case will contain information that can directly identify the participant (such as name and surname, initials, address, social security number, etc.). In the event that this transfer occurs, it would be for the same purpose of the study described and guaranteeing confidentiality.

If a transfer of encrypted data takes place outside the EU, either in entities related to the hospital center where the patient participates, to service providers or researchers who collaborate with us, the data of the participants will be protected by safeguards such as contracts or other mechanisms established by the data protection authorities.

As promoters of the project, we undertake to process the data in accordance with EU Regulation 2016/679 and, therefore, to keep a record of the treatment activities that we carry out and to carry out a risk assessment of the treatments we carry out, to know what measures we will have to apply and how to do it.

In addition to the rights that the previous legislation already contemplated (access, modification, opposition and cancellation of data, deletion in the new Regulation) now participants can also limit the processing of data collected for the project that are incorrect, request a copy or that moved to a third party (portability). To exercise these rights, they should contact the main researcher of the study or the Data Protection Delegate of the Hospital Clínic de Barcelona through protecciodades@clinic.cat. They also have the right to contact the Data Protection Agency if they are not satisfied.

The data cannot be deleted even if a patient leaves the study, to ensure the validity of the research and to comply with legal duties and drug authorization requirements.

The Researcher and the Sponsor are obliged to keep the data collected for the study at least until 5 years after its completion. Subsequently, personal information will only be kept by the

city center or their health and by the promoter for other purposes of scientific research if the patient has given their consent to do so, and if this is permitted by law and applicable ethical requirements.

12. Management of biological samples

The serum and CSF of the patients will be stored in the Neuroimmunology Tissue Collection (registered in the ISCII with number C0000051 and in the Biobank of IDIBAPS with the number R091217-012). All patients consent to their inclusion in this collection. The samples are coded so that the samples can be related to the clinical information but not to the donor patient.

13 Funding

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14 Publication policy

We commit to making public the results of the study, whether they are positive or negative.