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Alterations of NMDAR Subunits in the Cerebrospinal Fluid Across Neurodegenerative and Immunological Disorders

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ABSTRACT

N-methyl-D-aspartate receptors (NMDARs) are glutamate-binding calcium channels that play a key role in brain function and have been linked to many neurological disorders. NMDARs are multi-pass membrane heterotetrameric complexes composed of two compulsory GluN1 subunits and two GluN2 (A-D) or GluN3 (A-B) subunits, from which GluN1, GluN2B, GluN2A, and GluN3A are widely expressed in the adult brain. This study assesses the presence of GluN1, GluN2B, GluN2A, and GluN3A in the cerebrospinal fluid (CSF) from healthy individuals, viral and autoimmune encephalitis, Huntington's disease (HD) and Alzheimer's disease (AD) patients. Samples were run in SDS-PAGE under reducing conditions and resolved with different anti-ectodomain and anti-C-terminal antibodies that combined with immunoprecipitation analyses, served to demonstrate the presence of full-length GluN1, GluN2A, GluN2B, and GluN3A in CSF. These NMDAR subunit complexes are not associated with extracellular vesicles. As a proof of concept of the identity of NMDAR subunits in the CSF, we demonstrated reduced levels of

Abbreviations: A β , β -amyloid peptide; AD, Alzheimer's disease; CSF, cerebrospinal fluid; EVs, extracellular vesicles; H0, asymptomatic phase of Huntington's disease; H1, symptomatic phase of Huntington's disease; HD, huntington's disease; KO, knock out; NMDARs, N-methyl-D-aspartate receptors; P10K, pellet obtained from centrifuging CSF that contains apoptotic bodies and large extracellular vesicles; P100K, pellet obtained from centrifuging CSF that contains mainly small extracellular vesicles (<200 nm in diameter); P-tau, Phospho-tau; SynF, synaptic fraction.

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GluN1 in the CSF from patients with autoimmune encephalitis caused by anti-GluN1 antibodies compared with other causes of encephalitis; and showed a depletion of CSF GluN3A in a *Grin3a* knockout mouse model. Moreover, we observed higher GluN3A levels in CSF in both asymptomatic and symptomatic HD patients; while GluN2A levels were lower in CSF from AD patients. In conclusion, here we demonstrate the presence of NMDAR full-length subunits in CSF and that changes in NMDAR subunits balance could serve to identify alterations related to pathological conditions.

1 | Introduction

N-methyl-D-aspartate receptors (NMDARs) are cation channels gated by the neurotransmitter glutamate that play a key role in brain development and synaptic transmission, crucial in synaptic plasticity events (Gardoni and Di Luca 2021; Paoletti et al. 2013). As a consequence, dysfunctions in NMDAR physiology contribute to the pathogenesis of many neurological disorders (Dupuis et al. 2023). NMDAR dysfunction may arise from various alterations, including changes in receptor expression levels, subunit composition, or in the balance of synaptic versus extrasynaptic localization within the plasma membrane (Hardingham and Bading 2010).

NMDARs are multipass membrane proteins and heterotetrameric, giving rise to multiple subtypes. NMDARs are composed of the mandatory subunit GluN1, with the remaining subunits comprising either GluN2 (A-D) or GluN3 (A-B) (Paoletti 2011; Sanz-Clemente et al. 2013). GluN2A and GluN2B subunits have been well studied as they are widely expressed in the cerebral cortex of adult humans and mice (Paoletti et al. 2013; Pegasiou et al. 2020). The GluN3A subunit, whose role in development is well documented (González-González et al. 2023), also plays a role in the adult brain, but its functions remain less studied (Crawley et al. 2022).

Among the NMDAR-related diseases, one relatively common and potentially fatal is the anti-NMDAR autoimmune encephalitis, mediated by antibodies against GluN1 (Gleichman et al. 2012). Active immunization against the GluN1 ectodomain has been demonstrated to cause encephalitis in a mouse model (Ding et al. 2021).

In Huntington's disease (HD), GluN3A plays a crucial role (Parsons and Raymond 2014). HD is a fully penetrant neurodegenerative disorder caused by a dominant genetic mutation consisting of a CAG trinucleotide repeat expansion in the *Huntingtin* gene (McColgan and Tabrizi 2018). GluN3A is ordinarily downregulated during postnatal development but less so in HD patients and mouse models. This is probably because mutant huntingtin interferes with the selective endocytic removal of NMDARs that contain GluN3A (Marco et al. 2013; Pérez-Otaño et al. 2006). Accordingly, increased levels of GluN3A have been found in the striatum of human HD patients; furthermore, the induction of aberrant GluN3A expression leads to symptomatology even when huntingtin is not mutated (Marco et al. 2013) and, in the absence of GluN3A, mouse models carrying mutant huntingtin do not develop symptoms associated with HD (Marco et al. 2018).

GluN2A and GluN2B levels appeared altered in Alzheimer's disease (AD) (Danysz and Parsons 2012; Jacob et al. 2007;

Zhang et al. 2016), the most common form of dementia, which is associated with β -amyloid ($A\beta$) toxicity and hyperphosphorylation of the tau protein. GluN2A and GluN2B play a key role in neuronal death mechanisms and neurodegeneration. Cell death observed in AD, as well as in HD, may be partially attributable to an imbalance between synaptic and extrasynaptic NMDAR signaling. This imbalance could result from synapse loss or the redistribution of NMDARs from synaptic to extrasynaptic sites (Hardingham and Bading 2010). By isolating the synaptic and extrasynaptic fractions from human cortex, we found recently reduced levels of GluN2B and GluN2A in the synaptic fraction of individuals with AD, compared to control samples (Escamilla, Badillos, et al. 2024). Studies of NMDAR expression in human samples are few compared to those in mouse models (reviewed in Escamilla, Sáez-Valero, and Cuchillo-Ibáñez 2024).

The presence of NMDAR subunits in the human cerebrospinal fluid (CSF) could provide a readout of synaptic alterations in the brain of individuals affected by neurological disorders. However, to the best of our knowledge, this has not been investigated before. Here, we provide the first characterization of NMDAR subunits in human CSF. As a proof of concept, we demonstrate a strong reduction of GluN1 in the CSF in cases of anti-NMDAR encephalitis and a depletion of GluN3A in a *Grin3a* knockout mouse model (Das et al. 1998). We have also found high GluN3A levels in HD CSF from both asymptomatic and symptomatic phases and low GluN2A levels in AD CSF.

2 | Material and Methods

2.1 | Human CSF Samples

This study was exploratory and not preregistered. A generic consent was obtained from all subjects involved in the study. This study was approved by Universidad Miguel Hernández ethics committee (IN.JSV.04.20). The study was carried out in accordance with the 1964 Declaration of Helsinki and its later amendments. Stroke and hemorrhages were listed as exclusion criteria. No sample size was calculated a priori; the sample size was estimated according to our previous experience (Escamilla, Badillos, et al. 2024; Escamilla, Sáez-Valero, and Cuchillo-Ibáñez 2024; Lennol et al. 2025) and limitations of samples particularly for encephalitis in which lumbar puncture is not a habitual procedure.

For the collection of CSF samples from patients with encephalitis, samples and data were collected in the setting of a multicentre, hospital-based, longitudinal cohort study on encephalitis during the COVID-19 pandemic (ENCOVID-study; ethical approval# NP 4067; Pilotto et al. 2021; Lennol

et al. 2025). All clinical analyses were performed at the Neurology Unit, University of Brescia following the ENCOVID research protocol, as described in previous studies (Pilotto et al. 2021; Lennol et al. 2025). In this study, we only analyzed samples from patients with encephalitis without SARS-CoV-2 infection. Each subject underwent brain magnetic resonance imaging, standard electroencephalography, thyroid function, and antibodies (anti-thyroglobulin and anti-thyroid peroxidase), IgM, and IgG for *Borrelia burgdorferi*. CSF viral screening included herpes simplex virus [HSV-1, HSV-2, HSV-6, HSV-8, CMV, Epstein-Barr virus (EBV), varicella zoster virus (VZV)], adenovirus, and enterovirus; and standard immunological screening, which included antibodies against NMDARs, LGI1, CASPR2, GABAbR, AMPARs, DPPX, Ri, Yo, Ma2, CV2, Hu, amphiphysin, titin (Euroline and Mosaic kit, Euroimmun, Luebeck) and MOG (live cell-based assay). In our analysis, samples were subgrouped between anti-NMDARs encephalitis ($n=4$; 1 females/3 male) and other causes of encephalitis ($n=8$; 4 females/4 male) including 4 autoimmune (3 IgLo1, one anti-Yo), 4 viral (2 HSV-1, one EBV, and one VZV). The average age of anti-NMDAR encephalitis subjects is 49 ± 11 years, and of subjects with other causes of encephalitis is 62 ± 21 years.

CSF samples from a cross-sectional cohort of HD mutation carriers were obtained from the Movement Disorders Unit at Hospital de la Santa Creu i Sant Pau (Barcelona, Spain; ethical approval# 18/067). Subjects were diagnosed and categorized according to CAG repeats of the smaller and larger allele, manifestation of the disease (H0 asymptomatic and H1 symptomatic), and disease burden or CAG score (CAG repeats $\times$ age) (Zhang et al. 2011; Schobel et al. 2017). Accordingly, the cohort was composed of control individuals with no signs of dementia [$n=9$; 5 males/4 females; 40 ± 15 (means $\pm$ SEM) years], H0 ($n=10$; 5 males/5 females; 40 ± 8 years) and H1 patients ($n=9$; 4 males/5 females, 53 ± 12 years). In H0 patients, the number of CAG repeats in the smaller allele was 18.9 ± 2.8 , and the number of CAG repeats in the larger allele was 42.5 ± 2.0 , with a CAG score, the parameter that reflects disease severity, of 349 ± 72 in H0 patients. In H1 patients, the number of CAG repeats in the smaller allele was 21 ± 7 , the number of CAG repeats in the larger allele was 44 ± 3 , and the CAG score was 542 ± 226 . See Table S1 for details.

The CSF collection for AD study was obtained from the Clinical Neurochemistry Laboratory (Mölndal, Sweden). The CSF samples used were deidentified left-over aliquots from clinical routine analyses, following procedures approved by the Ethics Committee at the University of Gothenburg. Patients who were designated as AD or non-AD controls had typical core CSF biomarker levels using cutoffs that are $>90\%$ specific for AD (Hansson et al. 2006). AD patients ($n=15$; 9 males/6 females; 73 ± 8 years) have a CSF biomarker profile indicative of AD, including increased total tau (T-tau: 728 ± 234 pg/mL) and phosphorylated tau (P-tau: 111 ± 30 pg/mL) together with low A β 1-42 (A β 42: 539 ± 44 pg/mL) concentrations in CSF. Non-AD controls ($n=20$; 12 males/8 females; 74 ± 8 years) have normal levels of all three CSF biomarkers (T-tau: 268 ± 58 pg/mL, P-tau: 32 ± 8 pg/mL, A β 42: 904 ± 299 pg/mL). See Table S2 for details.

2.2 | Knock-Out Mice and CSF Extraction

The *Grin3a* knockout strain was generated by back-crossing F1 hybrids 8 into a C57Bl6/J background for more than 12 generations (Das et al. 1998). Animals were generously provided by Prof. I. Pérez-Otaño (Instituto de Neurociencias, CSIC-Universidad Miguel Hernández, Spain). Housing conditions consisted of a Makrolon Type three cage, which besides food (Kliba Nafag) and tap water ad libitum, contained deeper bedding and nesting material (Inotiv) and a tunnel (MCViso). The number of companions in each cage was 3–4. Wild-type and *Grin3a* KO animals were anesthetized with a concentration of 5% isoflurane administered in an induction chamber with a flow rate of 1.0–1.5 L/min and then maintained at 2.5%–3% with a mask to rapidly achieve a surgical plane of anesthesia. Oxygen for induction and maintenance of anesthesia was obtained through a surgical O₂ concentrator. For euthanasia, the thoracic cavity was opened, and blood was withdrawn by cardiac puncture with heparinized needles and syringes. Then, CSF was obtained by cisternal puncture with a needle (BD Micro-Fine™+Demi 0.3 mL) inserted in the suboccipital region through the atlanto-occipital membrane, with a single incision of the subarachnoid space as previously described (Sáez-Valero, de Gracia, and Lockridge 2005; García-Ayllón et al. 2013). Around 4 μ L were extracted from each animal. CSF samples were centrifuged at $1000 \times g$ for 10 min at 4°C and stored at -80°C until further analysis. For Western blot analysis, we pooled the CSF from 3 *Grin3a* KO [~ 3 months old; 2 males (weight 29–32 g), 1 female (weight ~ 24 g)] and 4 wild-type (~ 3 months old; 2 males, 2 female; similar weights) animals, and the same volume (12 μ L, a minimal volume demonstrated in preliminary experiments to be sufficient for the determination of NMDAR subunits in CSF) was loaded.

2.3 | Subcellular Fractionation Protocol

Synaptic fractions from human frozen cerebral cortex were obtained as previously reported (Escamilla, Badillos, et al. 2024). In brief, human frozen frontal cortices were cut into pieces (100 mg), trying to avoid white matter and excess vascular tissue. Each piece was homogenized in 100 μ L of homogenization buffer (ice-cold sucrose buffer containing 0.32 M sucrose, 10 mM Tris-HCl (pH 7.4), 1 mM Na₃VO₄, 1 mM NaF, 1 mM HEPES, 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM EGTA, protease, and phosphatase inhibitors) in a 1.5 mL eppendorf using a Heidolf homogenizer (10 strokes). The subsequent centrifugations were all performed at 4°C.

Cortical homogenates were centrifuged at $1000 \times g$ for 10 min to obtain a nuclear-free supernatant and a pellet containing the nucleus. Further centrifugation of the nuclear-free supernatant at $10000 \times g$ for 15 min resulted in a supernatant that contained cell cytosol and microsomes and a pellet of plasma membranes. The plasma membrane fraction was incubated with homogenization buffer containing 1% TX-100 (w/v) for 20 min in rotation at 4°C and then centrifuged at $32000 \times g$ for 20 min. The pellet fraction, containing the insoluble fraction, was solubilized in RIPA buffer. This pellet was mainly composed of postsynaptic densities and, therefore, synaptic membranes of synaptic fraction (SynF).

2.4 | Over-Expression of NMDAR Subunits in HEK Cells

HEK293 cells were grown in 6 well-plates in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich Co) supplemented with 10% fetal bovine serum (FBS; Gibco) and 100 µg/mL penicillin/streptomycin (Gibco). After 24 h, cells were transfected using Lipofectamine 3000 (Invitrogen) following the manufacturer's instructions. For expression of GluN1, GluN2A, and GluN2B, we used constructs encoding rat *GRIN1* (Regalado et al. 2001) kindly provided by Prof. Juan Lerma (Instituto de Neurociencias de Alicante, CSIC-Universidad Miguel Hernández, Spain), and *GRIN2A* and *GRIN2B* (Woodward 2002) kindly provided by Prof. John J. Woodward (University of South Carolina, South Carolina, USA). After 48 h of transfection, cells were resuspended in cold lysis buffer (Tris-HCl (50 mM, pH 7.4), NaCl (150 mM), Triton X-100 (0.5%), Nonidet P-40 (0.5%) and a cocktail of protease inhibitors), centrifuged for 10 min (10 000 × rpm, 4°C) and the supernatant fraction (cell extracts) was kept.

2.5 | Western Blot

NMDAR subunits were detected by fluorescent-based imaging after sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting. CSF samples (30 µL for human CSF and 12 µL for mouse CSF) were run on SDS-PAGE (7.5% Tris-glycine) after boiling at 98°C for 5 min in 6 × Laemmli sample buffer (Thermo Scientific). Electrophoreses were run at 4°C, 120 V, and 120 min. Transference was performed into 0.45 µm nitrocellulose membranes for 2.5 h at 300 mA in the cold room. Then, the membrane was blocked with Odyssey Blocking Buffer for 1 h. Primary antibodies were used against GluN2B C-terminal (mouse, 1:800 antibody dilution, Invitrogen MA1-2014), GluN2B N-terminal (rabbit, 1:800, Alomone AGC-003), GluN2B N-terminal (mouse, 1:200, NeuroMab Clone N59/36 Cat# 75-101), GluN2A C-terminal (rabbit, 1:800, Invitrogen A6473), GluN2A C-terminal (rabbit, 1:800, Millipore 07-632), GluN1 N-terminal (guinea pig, 1:1000, Alomone AGP-046), GluN1 C-terminal (mouse, 1:800, Millipore 05-432), GluN1 N-terminal (mouse, 1:200, Hybridoma bank Clone N308/48), GluN3A C-terminal (rabbit, 1:1000, Millipore 07-356), GluN3A N-terminal (rabbit, 1:800, Alomone AGC-030), and Alix (mouse, 1:1000, Cell Signaling #2171). Primary antibody binding was visualized with fluorescent secondary antibodies (IRDye, 1:10000, Bioss). Primary antibodies were incubated overnight at 4°C. All primary and secondary antibodies were prepared in Odyssey Blocking Buffer. After primary antibody incubation, membranes were washed 3 times for 10 min in PBS. After secondary antibody incubation, membranes were washed 2 times for 10 min in PBS-Tween 0.1% (v/v) and 2 washes for 10 min in PBS. Images were acquired using an Odyssey CLx Infrared Imaging system (LI-COR Biosciences GmbH). Band intensities were analyzed using LI-COR software (ImageQuant Studio). The raw full blots selected as representatives for each figures, are shown as Figures S1–S6.

All experiments were conducted and analyzed in a blind fashion (experimental group assignment and blot design were performed by a different person than the experimenter). All samples were analyzed at least in duplicate. Total protein staining by No-Stain

Protein Labeling Reagent (ThermoFischer Scientific) served to monitor potential loading inaccuracies in individual blots. In the study of the HD and AD cohorts, immunoreactive signal for NMDAR subunits GluN2A, GluN2B, and GluN3A was normalized to the immunoreactivity of the compulsory subunit, GluN1, detected in the same blot. A reference CSF sample (previously aliquoted to avoid freezing–thawing cycles) was included in each blot to serve as a loading reference and for normalizing the immunoreactivity signal between blots.

2.6 | Immunoprecipitation Assays

CSF (400 µL) was incubated on a roller overnight at 4°C with magnetic beads (PureProteome NHS FlexiBind LSKMAGN04) coupled to antibodies against GluN1 N-terminal (guinea pig, 20 µL, Alomone AGP-046), GluN2A C-terminal (rabbit, 20 µL, Millipore 07-632), GluN2B N-terminal (rabbit, 1:800, Alomone, catalog number AGC-003), and GluN3A N-terminal (rabbit, 1:400, Alomone AGC-030). The same amount of beads without coupled antibodies was used as a negative control. The input, bound, and unbound fractions were analyzed by western blotting using antibodies against GluN2B C-terminal (mouse, 1:800, Invitrogen, catalog number MA1-2014), GluN2A C-terminal (rabbit, 1:800, Invitrogen REF A6473), GluN1 C-terminal (mouse, 1:800, Millipore 05-432), and GluN3A C-terminal (rabbit, 1:400, Millipore, catalog number 07-356).

2.7 | Extracellular Vesicle (EV) Isolation

CSF samples were processed by differential ultracentrifugation as described earlier (Stuendl et al. 2016). Briefly, CSF aliquots were thawed and subjected to subsequent centrifugation steps: 3500 × g for 10 min, two times 4500 × g for 10 min, 10 000 × g for 35 min, and finally at 100 000 × g for 70 min to pellet EVs. The supernatant was saved, and the EV pellet was washed in 1 mL of 1 × PBS and centrifuged again at 100 000 × g for 70 min. The EV pellet, as well as the pellet obtained after the 12 000 × g centrifugation and an aliquot of the kept supernatant, were prepared in loading buffer 1 × for Western blot analysis.

2.8 | Statistical Analysis

No sample size was calculated a priori. The sample size was estimated based on our previous experience and practical limitations associated with human sample availability. For the animal experiments, the number of individuals was further constrained by the availability of mice. The distribution of data was tested for normality using a D'Agostino-Pearson test. ANOVA was used for parametric variables and the Kruskal–Wallis test for nonparametric variables for comparison between groups. A two-tailed Student's *t*-test for parametric variables and a Mann–Whitney U test for nonparametric variables were employed for comparison between two groups and for determining *p* values. Correlations were performed by Pearson correlation coefficients since all distributions analyzed were parametric. Outliers were screened using the ROUT method. When found, outliers were left out of the analysis (indicated in figure legends). The results are presented as

the means $\pm$ SEM, and all the analyses were performed using GraphPad Prism (version 7; GraphPad Software Inc). p value < 0.05 was considered significant.

3 | Results

3.1 | The NMDAR Subunits GluN1, GluN2A, GluN2B, and GluN3A Are Present in the CSF

To determine the presence of NMDAR subunits in CSF, we first examined CSF samples in parallel to brain extracts by western blotting. GluN1, GluN2A, and GluN2B immunoreactive species of similar molecular mass were identified in CSF and synaptic membranes (Figure 1A). Due to the lack of reports about NMDAR in the CSF, we focused on the immunoreactive bands whose molecular weight matched that of the full-length species, as reported for the human brain (Escamilla, Badillos, et al. 2024). We also verified the specificity of GluN3A immunoreactivity by using the CSF from *GluN3a* KO mice and wild-type. We observed that GluN3A was only found in the wild-type, and it was absent in the KO (Figure 1B). In this study, we served of the same antibodies used in the characterization of GluN1, GluN2A, GluN2B, and GluN3A in synaptic and extrasynaptic

membranes from the human cortex (Escamilla, Badillos, et al. 2024). Moreover, the specificity of the staining for GluN1, GluN2A, and GluN2B was confirmed in HEK293 cells transfected with expression vectors encoding the GluN1 and GluN2A subunits, or the GluN1 and GluN2B subunits (Figure S1).

The use of alternative antibodies confirmed the specificity of the NMDAR subunits in CSF samples. We used a combination of different antibodies against the N-terminal ectodomain and the C-terminal domains of specific NMDAR subunits (Figure 1C). To corroborate this, we also performed immunoprecipitation assays that were resolved by Western blots with alternative antibodies (Figure 1D). Overall, we detected immunoreactivity for GluN1, GluN2A, GluN2B, and GluN3A in human CSF compatible with the full-length species.

Since NMDARs are heteromers, we wondered whether the subunits still maintain interactions between them in the CSF. By co-immunoprecipitations, we observed that the full-length GluN2B and GluN2A interact with GluN1 (Figure 2A), suggesting that at least some subunit interactions are present.

In this scenario, a possible mechanism for NMDAR subunits to be present in the CSF could be their association with

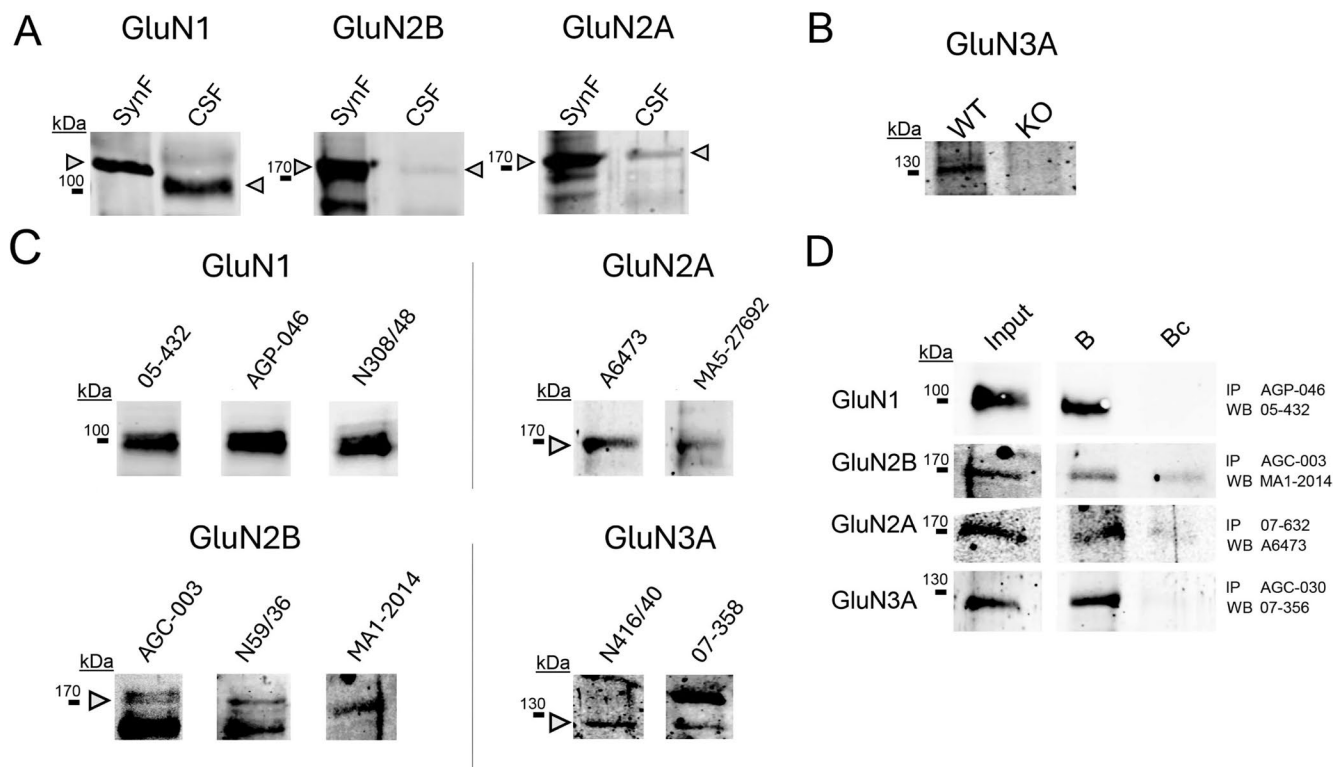


FIGURE 1 | N-methyl-D-aspartate receptor (NMDAR) subunits GluN2A, GluN2B, GluN1, and GluN3A are present in human cerebrospinal fluid (CSF). (A) Western blot of human synaptic membranes and CSF for GluN1 (antibody AGP-046), GluN2B (antibody AGC-003), and GluN2A (antibody A6473). (B) Western blot of CSF [pooling CSF obtained from 3 to 4 animals, and loading same volume (12 μ L)] from wild-type mouse (WT), and a mouse KO for *GluN3a* (GluN3A subunit, antibody 07-356). (C) Combination of different antibodies detecting the same subunit, either GluN1 (C-terminal: 05-432; N-terminal: AGP-046 and N308/48), GluN2B (N-terminal: AGC-003 and N59/36; C-terminal: MA1-2014), GluN2A (C-terminal: A6473 and MA5-27692), or GluN3A (C-terminal: 07-356; N-terminal: AGC-030). (D) Immunoprecipitation (IP) of control CSF against the four different subunits using alternative antibodies to reveal the western blot (WB). Together with the input and the bound fraction (B), a control IP (Bc) was also revealed, corresponding to the incubation of the sample performed with an irrelevant IgG of the same animal species as the specific anti-NMDAR antibody. Arrowheads point the immunoreactive band considered for further analysis. The inputs samples were resolved in the same gels but are shown separately to optimize contrast for defining discrete bands. The uncropped blots are available as Figure S2.

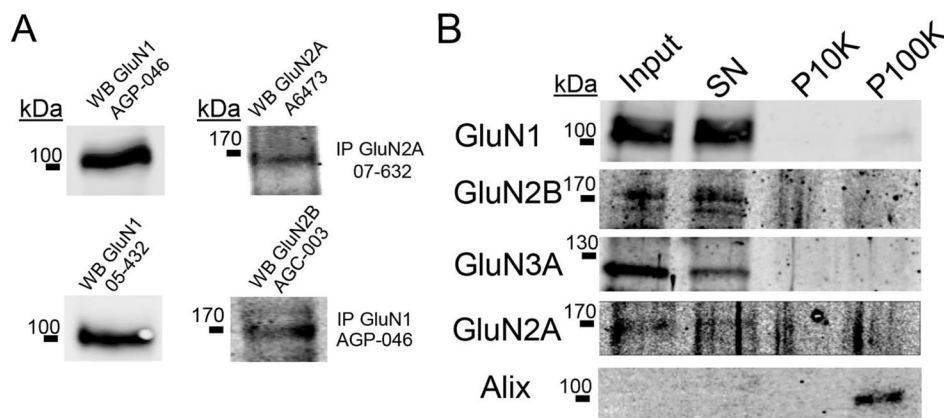


FIGURE 2 | Co-immunoprecipitation of N-methyl-D-aspartate receptor (NMDAR) subunits and lack of the NMDAR immunoreactivities in extracellular vesicles. (A) The specific NMDAR subunit was immunoprecipitated (IP) from human cerebrospinal fluid (CSF) with the indicated antibody and assayed in immunoblots (WB) probed with an alternative antibody against the same subunit and also with an additional antibody against a different subunit, as indicated. (B) Western blot against the four different subunits using the following antibodies: GluN1 AGP-046, GluN2B AGC-003, GluN2A A6473, and GluN3A 06-356. We used CSF as input and three different fractions of the extracellular vesicles (EVs) fractionation protocol (see Section 2 for details). Supernatant (SN) did not contain any EVs. Pellet P10K was obtained after centrifugation of 10000×g and contained apoptotic bodies and larger EVs. Pellet P100K was obtained after an ultracentrifugation of 100000×g and contained small EVs, as demonstrated by the presence of Alix, a canonical EV marker. The uncropped blot are available as Figure S3.

brain-derived extracellular vesicles (EVs), as reported for other plasma membrane proteins (Fauré et al. 2006; Solana-Balaguer et al. 2023). To examine this, EVs were isolated from CSF following a protocol based on differential ultracentrifugation (Stuendl et al. 2016) that generated different fractions: a supernatant depleted of EVs, a first pellet (P10K) that contains apoptotic bodies and large EVs, and a final pellet (P100K) that contains small EVs (<200 nm in diameter), including exosomes (Welsh et al. 2024). However, we did not find any of the subunits studied, GluN1, GluN2A, GluN2B, or GluN3A, in the P10K or P100K, indicating that NMDAR subunits are not part of CSF EVs; instead, the four subunits were present in the supernatant fraction (Figure 2B).

Lastly, as a proof of concept for evaluating the identity of NMDAR subunits in the CSF, we assessed GluN1 levels in the CSF from patients with autoimmune encephalitis. Anti-NMDAR encephalitis is diagnosed by the presence of antibodies against GluN1 in the CSF (Beaurain et al. 2024). This could somehow affect the amount of NMDAR subunits in this fluid, particularly GluN1. We determined GluN1 levels in the CSF from individuals with anti-NMDAR encephalitis and compared them with those from encephalitis by other causes (not related to NMDARs). The CSF from individuals with anti-NMDAR encephalitis exhibited significantly lower levels of GluN1 (~79% reduction; $t=2.362$, $p=0.045$) in comparison with CSF from subjects with encephalitis by other causes (Figure 3A,B).

3.2 | GluN3A Subunit Levels Are Higher in Huntington's Disease Than in Control CSF Samples

Since high GluN3A levels are observed in the brain of HD patients and HD mouse models (Marco et al. 2013), we considered the possibility that changes could be detected in the CSF from HD patients. In this disease, two stages have been described, the asymptomatic (H0) and the symptomatic phase (H1) (Schobel

et al. 2017). Accordingly, GluN3A and GluN1 levels were determined by western blot in H0, H1, and in control samples (see Table S1). When GluN3A values were normalized with respect to GluN1 levels (Figure 4A), we found that GluN3A levels were higher in H0 (118% increase; $t=3.912$, $p=0.001$) and H1 (56% increase; $t=2.720$, $p=0.015$) compared to those from control samples (Figure 4B). We found a significant correlation between GluN3A levels and disease burden (defined as CAG repeats × age, see Methods) only within the H0 group (Pearson's correlation, $p=0.009$; $R^2=0.5870$). No statistically significant correlations were found between GluN3A levels and age, CAG repetitions (Pearson correlation), or gender (Mann-Whitney test). These data indicate that the HD-associated brain changes that occur in GluN3A can be detected early in the CSF, before symptoms appear.

3.3 | GluN2A Subunit Levels Are Lower in Alzheimer's Disease Than in Non-AD Control CSF Samples

We also explored whether changes in NMDAR levels reported in AD brain are reflected in the CSF. GluN1, GluN2A, and GluN2B levels were determined in samples from a cohort of AD and non-AD controls (Table S2) by Western blots (Figure 5A). GluN2A and GluN2B values were normalized with respect to GluN1. After analysis, normalized GluN2A levels were lower in AD with respect to controls (47% reduction; $t=3.231$, $p=0.002$; Figure 5B), whereas GluN2B levels did not show differences (Figure 5C). No statistically significant correlations were found between GluN2A or GluN2B levels and P-tau, Aβ42 levels or age (Pearson correlation), or gender (Mann-Whitney test). No significant correlation was found between tau and GluN2B, nor for GluN2A when the AD and control groups were considered independently. Only a significant inverse correlation was observed between GluN2A and tau levels when all cases were considered together (Pearson's correlation, $p=0.025$; $R^2=0.1989$).

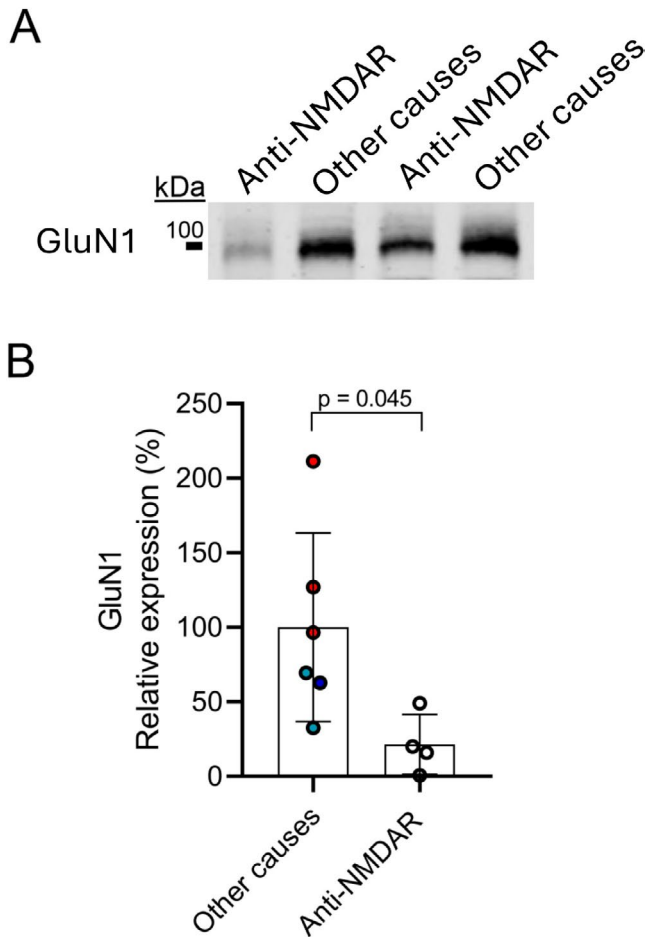


FIGURE 3 | N-methyl-D-aspartate receptor (NMDAR) subunit GluN1 levels in the cerebrospinal fluid (CSF) of anti-NMDAR encephalitis patients. (A) Representative Western blots of GluN1 (antibody AGP-046) subunit in samples from patients anti-NMDAR ($n=4$) encephalitis and encephalitis by other causes ($n=6$; red is viral encephalitis, light blue is LG1 encephalitis and dark blue is anti-Yo encephalitis). (B) GluN1 subunit was quantified. Data are expressed as percentages with respect to values from viral encephalitis samples. ROUT method identified no outliers. Exact P value obtained by t -test is shown. Error bars represent SEM. The uncropped blot are available as Figure S4.

4 | Discussion

It has been proposed that changes in synaptic proteins precede neurodegeneration (Lleó et al. 2019), and several synaptic proteins, such as neurogranin, have demonstrated potential as AD CSF biomarkers (Blennow and Zetterberg 2018). Here, we describe the presence of NMDAR subunits in CSF and demonstrate that their levels are associated with specific neuropathological conditions.

In previous studies from our laboratory, we reported the presence in the CSF of several full-length transmembrane proteins that preserved their intracellular domains. We demonstrated the existence of AD-related proteins such as APP (Cuchillo-Ibañez et al. 2015) and C-terminal fragments of APP containing the transmembrane and intracellular domains (García-Ayllón et al. 2017), BACE1 (Lopez-Font, Boix, et al. 2019), ADAM10 (Sogorb-Estève et al. 2018), and presenilin-1 as well as other

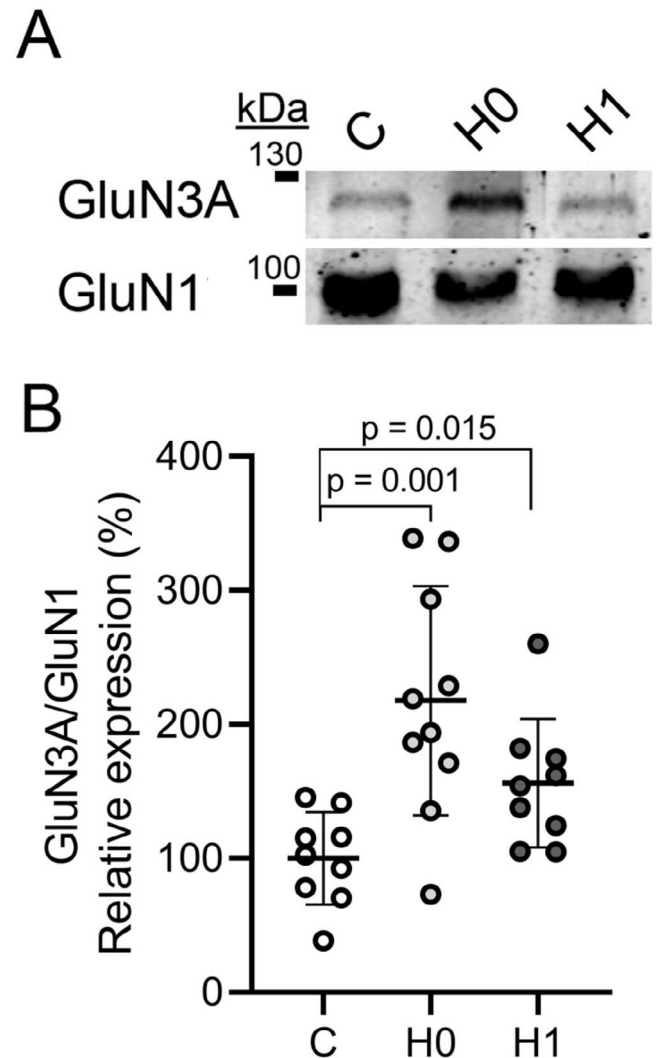


FIGURE 4 | N-methyl-D-aspartate receptor (NMDAR) subunit GluN3A levels in the cerebrospinal fluid (CSF) of Huntington's disease (HD) mutation carriers. (A) Representative Western blots of GluN3A (antibody 07-356) and GluN1 (antibody AGP-046) subunits in cases control ($n=9$), cases of HD in the asymptomatic phase (H0; $n=10$), and cases in the symptomatic phase (H1; $n=9$). (B) Quantification of GluN3A subunit levels was normalized to the GluN1 subunit. An internal sample was used to normalize the values among different membranes. ROUT method identified 1 outlier from the control group and one from the H1 group, which were removed from the analysis. Data are expressed as percentages with respect to controls. Error bars represent SEM. Exact p values obtained by t -test are shown. Given the exploratory nature of this study, we performed pairwise comparisons using independent t -tests to specifically assess differences between C and each disease group (H0 and H1). The uncropped blot are available as Figure S5.

subunits of the multi-pass γ -secretase complex (García-Ayllón et al. 2013).

Here, our data shows that the full-length GluN1 subunit interacts with GluN2B and GluN2A subunits in the CSF, which suggests that they are still able to maintain heteromeric complexes in this fluid. In the brain, this interaction occurs mainly through the N-terminal and ligand-binding domains of NMDAR subunits (Lee et al. 2014). These complexes in the

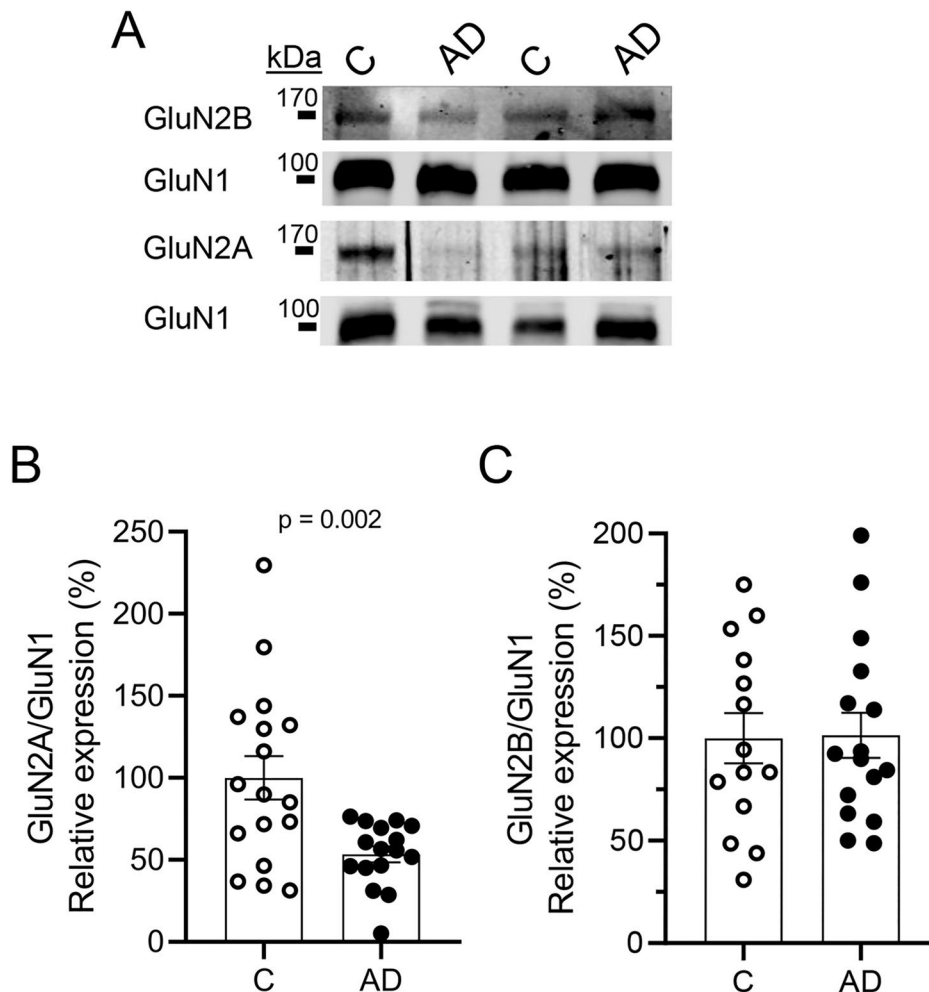


FIGURE 5 | N-methyl-D-aspartate receptor (NMDAR) subunits GluN2A and GluN2B levels in the cerebrospinal fluid (CSF) of patients with Alzheimer's disease (AD). (A) Representative western blots of GluN2A (antibody A6473), GluN2B (antibody AGC-003), and GluN1 (antibody AGP-046) subunits in human CSF from AD ($n = 16$) and non-AD control cases ($n = 17$). An internal control sample was used to normalize among different membranes. (B) Quantification of GluN2A and (C) GluN2B subunit levels were normalized to the GluN1 subunit. ROUT method identified 3 outliers from the control group when measuring GluN2B, which were removed from the analysis. Data are expressed as percentages with respect to controls. Error bars represent SEM. Exact p value obtained by t -test is shown. The uncropped blots are available as Figure S6.

CSF should come from brain neuronal membranes; although most likely, they are nonspecific aggregations of the NMDAR subunits. However, the resolution of this question deserves further investigation.

The mechanisms by which these membrane-bound proteins reach the CSF are unknown (discussed in Lopez-Font et al. 2015). The possibility of their association with brain-derived EVs was tested since they carry APP and APP C-terminal fragments, among other transmembrane proteins (Pérez-González et al. 2020). However, we did not find evidence pointing to this. It is also plausible that the neuronal damage and blood-brain barrier dysfunction that occurs in neurodegeneration lead to an abnormal release of cellular components into the CSF (Nation et al. 2019; Preis et al. 2024), including the NMDAR subunits. Nonetheless, our data showed that, compared to non-disease CSF samples, NMDAR subunit levels are higher in HD and lower in AD, reflecting brain changes related to pathology but not in an indiscriminate manner.

The relationship between changes in NMDAR subunit levels and disease severity remains unclear. In this context, we observed elevated GluN3A levels in the CSF of HD subjects not only during the symptomatic phase (H1) but also in the asymptomatic phase (H0). Notably, a significant correlation between GluN3A levels and disease burden was found only within the H0 group. Whether GluN3A levels increase during the presymptomatic stage and then reach a plateau at symptom onset warrants further investigation. For AD and non-AD cases included in this study, all CSF samples were obtained from individuals seeking medical evaluation for cognitive impairment. Diagnosis was established based on core AD biomarkers, and no additional measures of disease severity were available. Within each group, we did not detect significant correlations between GluN2A levels and AD core biomarkers. In our previous study on GluN2A expression in AD brain tissue, GluN2A levels were significantly reduced in Braak stages III–IV and exhibited a decreasing trend in the earlier Braak stages (Escamilla, Badillos, et al. 2024). These findings suggest that GluN2A alterations may also be associated with the onset of clinical symptoms. Further studies

are warranted to evaluate the clinical and neuropathological underpinnings of glutamatergic alterations in HD and AD and their potential association with specific biological subtypes of the disease.

Detection and quantification of NMDAR subunits in CSF are challenging due to their low abundance and the limitations of the Western blot technique. Thus, we have focused our analysis on full-length species that were characterized by combinations of alternative antibodies. We have observed that GluN1 levels are depleted in anti-NMDAR encephalitis, GluN3A appeared increased in the CSF of HD patients, even at asymptomatic stages, and GluN2A levels decreased in AD subjects with respect to controls. In the last cases, we referred the changes to the GluN1 levels, the compulsory subunit present in all NMDAR complexes.

It is possible that NMDAR subunit fragments also reach the CSF, although we did not focus on them in this work. Proteolytic processing has been described for GluN2B (Ng et al. 2012; Wu et al. 2007) and GluN2B fragments have been found in the human brain as ~150 kDa and ~130 kDa proteolytic fragments, which levels increase during post-mortem degradation (Bayés et al. 2014; Wang et al. 2000). Fragments of transmembrane receptors, such as ErbB4 (Lopez-Font, Sogorb-Esteve, et al. 2019) and ApoER2 (Lopez-Font, Iborra-Lazaro, et al. 2019) have been identified in CSF; all probably corresponding to ectodomains originated by proteolytic processing. In the future, the characterization of NMDAR subunit fragments in CSF should be addressed. Moreover, we identified distinct glycoforms of GluN2B and GluN2A in extrasynaptic membranes of the human brain (Escamilla, Badillos, et al. 2024), which may contribute to the diversity of NMDAR subunit species present in the CSF.

Although it is commonly assumed that NMDARs are mainly located in neurons, they are also present in other cell types, including glial cells (Kirchhoff 2017; Verkhatsky and Kirchhoff 2007); thus, inflammation may also contribute to the release of NMDAR subunits into the CSF. Identifying the brain cell origin of the NMDAR subunits in CSF would be relevant as a readout of cell-specific alterations occurring in the brain. In this regard, post-translational modifications such as glycosylation and phosphorylation can serve to characterize NMDAR subunits located at subcellular localization (discussed in Escamilla, Badillos, et al. 2024; Escamilla, Sáez-Valero, and Cuchillo-Ibáñez 2024), but also with different cellular origins.

Lastly, elucidating whether the NMDAR subunits in the CSF belong to synaptic or extrasynaptic receptors would be highly informative and warrant further investigation. GluN3A subunits are preferentially located at extrasynaptic sites in human (Escamilla, Badillos, et al. 2024) and mouse brains (Pérez-Otaño et al. 2006); therefore, we presume that the higher levels of GluN3A in HD CSF are probably reflecting extrasynaptic NMDAR alterations. In the same manner, GluN2A subunits are more abundant in synaptic than in extrasynaptic membranes (Escamilla, Badillos, et al. 2024); therefore, the lower levels of GluN2A in AD CSF could reflect alterations in synaptic NMDARs. Hence, altered CSF NMDAR levels could mirror

brain changes related to synapsis but also with extrasynaptic dysfunction.

In conclusion, here we demonstrate the existence of NMDAR full-length subunits in the CSF forming complexes not associated with EVs. In this study, we have focused our analysis on full-length species and on the quotients between the compulsory subunit, GluN1, and other subunits related to the pathologic process. Knowledge of the mechanism by which NMDARs reach the CSF in healthy and disease conditions, as well as the complete characterization of the complexes, is needed to advance in its clinical significance. Overall, the analysis suggests that detecting NMDAR subunits in CSF can be a valuable readout of brain changes and may constitute a future tool for exploring the glutamatergic synaptic involvement across different neurological conditions.

Author Contributions

Sergio Escamilla: investigation, methodology, writing – original draft, formal analysis. **Rocío Pérez-González:** investigation, methodology, writing – original draft, formal analysis. **Carmen Márquez-Marco:** investigation, formal analysis. **Elena Camporesi:** investigation, methodology. **Gunnar Brinkmalm:** investigation, methodology. **Alessandro Padovani:** conceptualization, funding acquisition, supervision. **Andrea Pilotto:** funding acquisition, writing – review and editing, formal analysis, supervision. **Chiara Tolassi:** investigation, methodology. **Davide Arici:** investigation, methodology. **Irene Volonghi:** investigation, methodology. **Henrik Zetterberg:** supervision, formal analysis, writing – review and editing, conceptualization, funding acquisition. **Kaj Blennow:** conceptualization, funding acquisition, writing – review and editing, formal analysis, supervision. **Jesús Pérez-Pérez:** investigation, methodology, formal analysis. **Jaime Kulisevsky:** conceptualization, methodology, formal analysis, writing – review and editing. **Inmaculada Cuchillo-Ibáñez:** conceptualization, investigation, methodology, writing – review and editing, formal analysis. **Javier Sáez-Valero:** conceptualization, investigation, funding acquisition, writing – original draft, methodology, writing – review and editing, supervision.

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Disclosure

The authors have nothing to report.

Conflicts of Interest

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Data Availability Statement

All clinical and CSF analyses data are available from authors upon reasonable request.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Appendix S1:** jnc70192-sup-0001-AppendixS1.pdf.