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Immuno-Pharmacological Characterization of Presynaptic GluN3A-Containing NMDA Autoreceptors: Relevance to Anti-NMDA Receptor Autoimmune Diseases

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Abstract

Mouse hippocampal glutamatergic nerve endings express presynaptic release-regulating NMDA autoreceptors (NMDARs). The presence of GluN1, GluN2A, GluN2B, and GluN3A subunits in hippocampal vesicular glutamate transporter type 1-positive synaptosomes was confirmed with confocal microscopy. GluN2C, GluN2D, and GluN3B immunopositivity was scarcely present. Incubation of synaptosomes with the anti-GluN1, the anti-GluN2A, the anti-GluN2B, or the anti-GluN3A antibody prevented the 30 μM NMDA/1 μM glycine-evoked [^{3}H]D-aspartate ([^{3}H]D-ASP) release. The NMDA/glycine-evoked [^{3}H]D-ASP release was reduced by increasing the external protons, consistent with the participation of GluN1 subunits lacking the N1 cassette to the receptor assembly. The result also excludes the involvement of GluN1/GluN3A dimers into the NMDA-evoked overflow. Complement (1:300) released [^{3}H]D-ASP in a dizocilpine-sensitive manner, suggesting the participation of a NMDAR-mediated component in the releasing activity. Accordingly, the complement-evoked glutamate overflow was reduced in anti-GluN-treated synaptosomes when compared to the control. We speculated that incubation with antibodies had favored the internalization of NMDA receptors. Indeed, a significant reduction of the GluN1 and GluN2B proteins in the plasma membranes of anti-GluN1 or anti-GluN2B antibody-treated synaptosomes emerged in biotinylation studies. Altogether, our findings confirm the existence of presynaptic GluN3A-containing release-regulating NMDARs in mouse hippocampal glutamatergic nerve endings. Furthermore, they unveil presynaptic alteration of the GluN subunit insertion in synaptosomal plasma membranes elicited by anti-GluN antibodies that might be relevant to the central alterations occurring in patients suffering from autoimmune anti-NMDA diseases.

Keywords Presynaptic NMDA autoreceptor · GluN3 · Anti-GluN antibody · Complement · NMDA internalization

Introduction

NMDA receptors are ionotropic glutamate receptors associated to voltage-dependent ionic channels permeable to divalent cations. First proposed to be exclusively postsynaptic, it is nowadays recognized that they also have a presynaptic distribution [1–3]. In particular, starting from the late 1980s, evidence support the existence of presynaptic release-regulating NMDA receptors on dopaminergic [4–9], noradrenergic [10–15], peptidergic (namely cholecystokinin and somatostatin) [16, 17], cholinergic [18], GABAergic [19, 20], and glutamatergic [21–25] terminals.

Conventional NMDA receptors consist of at least one GluN1 subunit in combination with two of four GluN2 and/or one of two GluN3 subunits. GluN1 and GluN3 subunits contain the binding domain for glycine/D-serine, while GluN2 subunits bear the glutamate binding site. The GluN1 is ubiquitously distributed in CNS and has developmental and regional variations due to the existence of eight isoforms. The GluN3 subunits exist in two different isoforms, the GluN3A and the GluN3B subunits. The GluN3A subunits have a wide distribution in neurons; they are maximally expressed postnatally and then decrease. Differently, GluN3B expression increases slowly but remains restricted to defined CNS regions.

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When present, these subunits act as dominant negative, reducing Mg^{2+} block and Ca^{2+} permeability. As to the GluN2 subunits, they exist in four isoforms (GluN2A to GluN2D). GluN2A and GluN2B are the predominant subunits, they locate synaptically, and their expression peaks postnatally; when present, they generate "high-conductance" channels that are typified by high sensitivity to Mg^{2+} block. Differently, GluN2C appears late in the development while GluN2D occurs postnatally but then drops markedly. The expression of both subunits is restricted to defined CNS regions [26–28].

Because of the large number of GluN subunits, NMDA receptors are expected to have a heterogeneous composition in subunit which should affect their pharmacological profiles and neuronal functions [1]. Deciphering the subunit composition is therefore a fundamental step to comprehend the role of these receptors in the CNS and, concomitantly, to propose therapeutics for the cure of central neurological diseases associated to NMDA receptor-mediated dysfunctions. To note, despite the unmet need in clinic of selective NMDA modulators (with the exception of memantine and derivatives, [29]), ligands acting at selected GluN subunits still represent a promising approach for the treatment of CNS disorders [30].

Our previous studies suggested that GluN1, GluN2A, GluN2B, and GluN3A subunits exist in the presynaptic component of hippocampal synaptosomal plasma membranes, where they associate to express NMDA autoreceptor (NMDAR, [24]). Accordingly, the NMDA/glycine-evoked release of glutamate was prevented by selective GluN2A and GluN2B subunit antagonists (i.e., Zn^{2+} ions and ifenprodil), as well as by ligands acting at the glycine binding site(s) (i.e., 7-Cl-kynurenate acid) [23], consistent with the presence of a receptor having a GluN1/GluN2A/GluN2B tri-heteromeric structure [24]. The NMDARs, however, also displayed insensitivity to external Mg^{2+} ions, while D-serine behaved as a partial agonist, two observations that seemed predictive of the involvement of GluN3A subunit in the NMDA receptor assembly, as already discussed in the literature [31]. The lack of selective GluN3A ligands, unfortunately, left the hypothesis unproven.

In recent years, activating or inactivating antibodies recognizing the outer side of membrane proteins emerged as appropriate tools for the characterization of the subunit composition and the functional roles of membrane receptors [31–35]. The present study aimed at improving our knowledge of the subunit composition of NMDARs by taking advantage of selective anti-GluN antibodies recognizing the NH_2 terminus of the GluN proteins. Experiments were carried out: (i) to confirm the existence of the GluN subunit, in particular the GluN3A one, in the hippocampal glutamatergic synaptosomes; and (ii) to prove its participation to the NMDA/glycine-mediated releasing activity. The results definitively demonstrate the involvement of the GluN3A subunit in the expression of the NMDARs in the hippocampus, but also unveil new unexpected events that might occur presynaptically in the brain of patients suffering from anti-NMDA autoimmune diseases.

Materials and Methods

Animals

Mice (male, strain C57BL/6J) were obtained from Charles River (Calco, Italy) and were housed in the animal facility of DIFAR, Section of Pharmacology and Toxicology, under environmentally controlled conditions (ambient temperature = 22 °C, humidity = 40%) on a 12-h light/dark cycle with food and water ad libitum. Mice were euthanized by cervical dislocation, followed by decapitation, and the hippocampi and cerebellum were rapidly removed. The experimental procedures were in accordance with the European legislation (European Communities Council Directive of 24 November 1986, 86/609/EEC) and the ARRIVE guidelines, and they were approved by the Italian Ministry of Health (DDL 26/2014 and previous legislation; protocol number no. 50/2011-B). Experiments were performed following the Guidelines for Animal Care and Use of the National Institutes of Health and in accordance with the Society's Policies on the Use of Animals and Humans in Neuroscience Research. In line with the 3Rs rules (replacement, refinement, and reduction), any effort was made to reduce the number of animals to obtain statistically reliable results.

Preparation of Synaptosomes

Mouse hippocampal purified synaptosomes were prepared as previously described [36]. Briefly, the tissue was homogenized in 10 volumes of 0.32 M sucrose, buffered to pH 7.4 with Tris-(hydroxymethyl)-amino methane (TRIS, final concentration 0.01 M) using a glass/Teflon tissue grinder (clearance 0.25 mm). The homogenate was centrifuged at 1000×*g* for 5 min to remove nuclei and debris, and the supernatant was gently stratified on a discontinuous Percoll® gradient (6%, 10%, and 20% *v*/*v* in Tris-buffered sucrose) and centrifuged at 33,500×*g* for 5 min. The layer between 10 and 20% Percoll® (synaptosomal fraction) was subsequently collected and washed by centrifugation. Synaptosomes were resuspended in a physiological solution with the following composition (mM): NaCl, 140; KCl, 3; $MgSO_4$, 1.2; $CaCl_2$, 1.2; NaH_2PO_4, 1.2; $NaHCO_3$, 5; HEPES, 10; glucose, 10; pH 7.2–7.4.

Release Experiments

Synaptosomes were incubated for 15 min at 37 °C in a rotary water bath in the presence of [^{3}H]D-aspartate ([^{3}H]D-ASP, f.c.: 50 nM). In order to study the impact of anti-GluN antibodies

on glutamate exocytosis, synaptosomes were incubated for 30 min in the presence of the following antibodies recognizing the NH_2 terminus of the GluN proteins (final concentration 1:500): mouse monoclonal anti-GluN1; mouse monoclonal anti-GluN2A; mouse monoclonal anti-GluN2B; rabbit polyclonal anti-GluN3A. As anti-GluN3B antibody recognizing an extracellular epitope of the GluN3B was not commercially available, a rabbit polyclonal anti-GluN3B antibody recognizing the intracellular epitope of the GluN3B subunit was applied. The rabbit polyclonal anti-GluN3B antibody was used as a negative control to unveil non-specific effects due to the exposure of synaptosomes to immunoglobulins. During incubation with antibodies, the radioactive tracer was added at t = 15 min of incubation to label glutamatergic synaptosomes. Identical portions of the synaptosomal suspensions were then layered on microporous filters at the bottom of parallel thermostated chambers in a Superfusion System (Ugo Basile, Comerio, Varese, Italy) [37, 38]. Synaptosomes were superfused at 0.5 ml/min for 36 min with standard physiological solution to equilibrate the system and then superfusate fractions were collected to monitor tritium release. When indicated, the pH of the superfusion medium (standard medium, pH 7.4) was adjusted to the desired value (6.60 or 8.00) with HCl or NaOH. Synaptosomes were exposed to these media starting from t = 20 min of superfusion.

In the experiments carried out to quantify the impact of NMDA/glycine on [^{3}H]D-ASP release, four consecutive 3-min fractions were collected. At t = 39 min of superfusion, synaptosomes were exposed to 30 μM NMDA/1 μM glycine for 5 min and then the agonists solution was replaced with the physiological solution until the end of the superfusion (t = 48 min).

When indicated, synaptosomes were transiently (90 s) exposed, at t = 39 min, to high KCl-containing medium (12 mM KCl, NaCl substituting for an equimolar concentration of KCl, [39] or to mouse complement (dilution 1: 300), then replaced by the standard medium until the end of the superfusion period. In these experiments, fractions were collected as follows: two 3-min fractions (basal release), one before (t = 36–39 min) and one after (t = 45–48 min) a 6-min fraction (t = 39–45 min; evoked release).

The amount of radioactivity released into each superfusate fraction was expressed as percentage of the total synaptosomal radioactivity. The NMDA/glycine-evoked release of tritium was calculated by subtracting the tritium content in the first and the fourth fractions from the neurotransmitter content in the second and the third fractions The tritium overflow elicited by exposing synaptosomes to high K^+ medium or complement was estimated by subtracting the neurotransmitter content into the first and the third fractions collected (basal release, b1 and b3) from that in the 6-min fraction collected during and after the depolarization pulse (evoked release, b2). In all the figures, the effects of the anti-NMDA antibodies or pH are expressed as percent of their respective control. Data are reported as the mean ± SEM of independent determinations obtained in different experiments run in triplicate (three superfusion chambers for each experimental condition).

Confocal Microscopy and Colocalization

Mouse hippocampal synaptosomes were fixed with 2% paraformaldehyde, permeabilized with 0.05% Triton X-100 phosphate-buffered saline (PBS), and incubated overnight at 4 °C with the following primary antibodies (diluted in 3% albumin PBS): mouse anti-GluN1 (1:500), mouse anti-GluN2A (1:500), mouse anti-GluN2B (1:500), rabbit anti-GluN3A (1:500), rabbit anti-GluN3B (1:500), and guinea pig anti-vesicular glutamate transporter type 1 (VGLUT1; 1:500). Synaptosomes were then washed in PBS and incubated for 1 h at room temperature with the following secondary antibodies: donkey anti-mouse AlexaFluor-488 and goat anti-guinea pig AlexaFluor-633 (1:1000 both, colocalization of GluN1/ GluN2A/ GluN2B receptor proteins with VGLUT1), or donkey anti-rabbit AlexaFluor-488 and goat anti-guinea pig AlexaFluor-633 (1:1000 both, colocalization of GluN3A /GluN3B receptor proteins and VGLUT1). Synaptosomes were then applied onto coverslips [40]. Fluorescence imaging (512 × 512 × 8 bit) acquisition was performed by a six-channel Leica TCS SP5 laser-scanning confocal microscope, equipped with 458, 476, 488, 514, 543 and 633-nm excitation lines, through a plan-apochromatic oil immersion objective 63X/1.4NA. Light collection configuration was optimized according to the combination of chosen fluorochromes. Sequential channel acquisition was performed to avoid spectral bleed-through artifacts. Leica "LAS AF" software package was used for image acquisition, storage, and visualization. The quantitative estimation of colocalized proteins was performed as already described [32, 36], by calculating the "-colocalization coefficients" [41]. These coefficients express the fraction of colocalizing molecular species in each component of a dual-color image and are based on the Pearson's correlation coefficient, a standard procedure for matching one image with another in pattern recognition [42]. If two molecular species are colocalized, the overlay of their spatial distributions has a correlation value higher than what would be expected by chance alone. Costes and colleagues [43] developed an automated procedure to evaluate the correlation between the green and red channels with a significance level > 95%. The same procedure automatically determines an intensity threshold for each color channel based on a linear least-square fit of the green and red intensities in the image's 2D correlation cytofluorogram. Costes' approach was carried out by macro routines integrated as plugins (WCIF Colocalization Plugins, Wright Cell Imaging Facility, Toronto Western Research Institute, Canada) in the ImageJ 1.51w software (Wayne Rasband, NIH, USA).

Immunoblot Analysis

Mouse hippocampal purified synaptosomes were lysed in modified RIPA buffer (10 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% Triton X-100, 1 mM sodium orthovanadate, and protease inhibitors) and quantified for protein content with BCA assay. Mouse cerebellum was homogenized in lysis buffer with a glass/Teflon tissue grinder, sonicated, and centrifuged at 20,000*g* for 10 min at 4 °C. Supernatant was kept for the immunoblot analysis. Samples were boiled for 5 min at 95 °C in SDS-PAGE sample buffer. Proteins (20 μg for synaptosomal hippocampal lysate and 30 μg for cerebellar homogenate) were separated by SDS-PAGE and then blotted onto PVDF membrane. Membranes were blocked for 1 h at room temperature with Tris-buffered saline-Tween (t-TBS: 20 mM Tris, pH 7.4, 150 mM NaCl, and 0.05% Tween 20) containing 5% (*w*/*v*) non-fat dried milk, and then probed with the following primary antibodies overnight at 4 °C: rabbit anti-GluN2C (1:250), rabbit anti-GluN2D (1:250), mouse anti-GluN1 (1:2000), and mouse anti-β actin (1:10000). After extensive washes in t-TBS, membranes were incubated for 1 h at room temperature with the appropriate horseradish peroxidase-linked secondary antibodies (1:20000). Immunoblots were visualized with an ECL (enhanced chemiluminescence) Western blotting detection system. Images were acquired using the Alliance LD6 images capture system (Uvitec, Cambridge, UK) and analyzed with UVI-1D software (Uvitec, Cambridge, UK).

Biotinylation Analysis

Changes in the hippocampal synaptosomal surface levels of GluN1 and GluN2B receptor proteins were evaluated by performing biotinylation and immunoblot analysis [8]. Briefly, mouse hippocampal synaptosomes were divided into three aliquots and incubated for 30 min at 37 °C under mild shaking: in physiological medium (control synaptosomes), in the presence of mouse anti-GluN1 antibody (1:500, anti-GluN1 treated synaptosomes) or in the presence of mouse anti-GluN2B antibody (1:500, anti-GluN2B treated synaptosomes). Synaptosomes (control and treated) were then labeled for 1 h at 4 °C with sulfo-NHS-SS-biotin (1.5 mg/ml) in PBS/Ca-Mg of the following composition (mM): 138 NaCl, 2.7 KCl, 1.8 KH_2PO_4, 10 Na_2HPO_4, 1.5 $MgCl_2$, 0.2 $CaCl_2$, pH 7.4. The biotinylation reaction was stopped by incubating synaptosomes with PBS/Ca-Mg with 100 mM glycine for 15 min at 4 °C. After two washes in PBS/Ca-Mg, synaptosomes were lysed in modified RIPA buffer. Samples (100 μg) were then incubated with Dynabeads MyOne Streptavidin T1 beads for 30 min at room temperature under shaking. Beads were added to the biotinylated synaptosomes to pull down the biotinylated proteins, as well as to non-biotinylated synaptosomes, to check the specificity of streptavidin pulldown. After extensive washes, samples were boiled for 5 min at 95 °C in SDS-PAGE loading buffer to isolate biotinylated proteins from the beads. Eluted fractions were analyzed through immunoblot assay (see the "Immunoblot analysis" section). The immunoreactivity of GluN1 and GluN2B receptor proteins was monitored by using mouse anti-GluN1 (1:2000) and mouse anti-GluN2B (1:2000) antibodies in the total lysate (L), in the control, anti-GluN1, or anti-GluN2B-treated biotinylated synaptosomes (C and T), as well as in the streptavidin pulldown of the non-biotinylated synaptosomal lysate (B).

Calculations and Statistical Analysis

Sigma plot 10 data analysis and graphing software package was used for data handling/statistics and for graph drawing. Analysis of variance was performed by ANOVA followed by Dunnett's multiple-comparisons test, as appropriate; direct comparisons were performed by Student's *t* test. Post hoc tests were done only if an *F* value was significant. The level of significance was set at $p < 0.05$.

Chemicals

[2,3-^{3}H]D-aspartate (specific activity 11.3 Ci/mmol) was from Perkin Elmer (Boston, MA, USA). Mouse anti-β-actin, horseradish peroxidase-conjugated anti-mouse and anti-rabbit secondary antibodies, and glycine were from Sigma (Milan, Italy). NMDA, dizocilpine, and glycine were purchased from Tocris Bioscience (Bristol, UK). Luminata Forte Western blotting detection system and guinea pig anti-vesicular glutamate transporters type 1 (VGLUT1) antibody were purchased from Millipore (Temecula, CA, USA). Donkey anti-rabbit AlexaFluor-488, goat anti guinea pig AlexaFluor-633, donkey anti-mouse AlexaFluor-488, goat anti-mouse AlexaFluor-633, and Dynabeads MyOne Streptavidin T1 were from Thermo Fisher Scientific (Waltham, MA USA). Mouse anti-GluN1 monoclonal antibody (MAB 1586), clone R1JHL, purified Ig, recognizing the fusion protein of amino acids 1-564 from rat NR1, and rabbit anti-GluN3B polyclonal antibody (no. 07-351), recognizing the 916-930 amino acid sequence of the receptor protein, were from Millipore (Temecula, CA, USA). Mouse anti-GluN2A[S327A-38] monoclonal antibody (ab 174636), purified IgG2b, recognizing the fusion protein corresponding to amino acids 75-325; mouse anti-GluN2B[S59-36] monoclonal antibody (ab 93610), purified IgG2b, recognizing the fusion protein corresponding to amino acids 20-271; and rabbit anti-GluN3A polyclonal antibody (ab 111226), recognizing a synthetic peptide from the extracellular domain, were from Abcam (Cambridge, UK). Rabbit anti-GluN2C polyclonal antibody (AGC-018), recognizing the peptide corresponding to the 365-377 amino acid sequence of the NH_2 terminus, and rabbit anti-GluN2D polyclonal

antibody (AGC-020), recognizing the peptide corresponding to the 345-359 amino acid sequence of the NH_2 terminus, were from AlomoneLabs (Jerusalem, Israel). Complement was from Gentaur (Kampenhout, Belgium).

Results

Mouse Hippocampal Synaptosomal Lysates Do Not Possess GluN2C and GluN2D Receptor Subunits

Our previous studies unveiled the presence of the GluN1, GluN2A, GluN2B, GluN3A, and GluN3B proteins in mice hippocampal synaptosomes [24, 25]. Whether these terminals also possess GluN2C and GluN2D proteins was not investigated. To fill the gap, Western blot analysis was carried out to highlight the presence of the two subunits in hippocampal synaptosomal lysates. The analysis was extended to cerebellar homogenates, because of the well documented expression of GluN2C and GluN2D proteins at this level. In both preparations, the expression of GluN1 subunits was also analyzed as a positive control of immunoblotting [44–47]. We found that the GluN2C or GluN2D immunopositivity is undetectable in hippocampal synaptosomal lysates (Fig. 1a), but clearly evident in the cerebellar preparation (Fig. 1b), while the GluN1 positivity was observed in both the hippocampal [48] and the cerebellar lysates. These observations shed doubt on the presence of GluN2C and GluN2D in hippocampal synaptosomes and on their participation to the expression of NMDARs.

Mouse Hippocampal Glutamatergic Synaptosomes Are Endowed with GluN1, GluN2A, GluN2B, and GluN3A Subunits

The finding that GluN1, GluN2A, GluN2B, and GluN3A (but not GluN3B) proteins are present in the presynaptic component of purified synaptosomes [24, 49] does not give any information on their presence in the glutamatergic nerve endings. To address the question, confocal analysis was carried to confirm the presence of GluN1, GluN2A, GluN2B, and GluN3A immunopositivity in mouse hippocampal synaptosomes that express the vesicular glutamate transporter type 1 (VGLUT1) protein, used as a selective marker of the glutamatergic synaptosomes. We identified a large immunoreactivity (44 ± 12% of colocalization, $n = 12$, Fig. 2a, right, merge, yellow) of GluN1 subunit (Fig. 2a, left, green) in VGLUT1-positive terminals (Fig. 2a, middle, red). GluN2A (Fig. 2b, left, green) and GluN2B subunit (Fig. 2c, left, green) stainings were also detected in VGLUT1 immuno-positive terminals (Fig. 2b and c, middle, red, 59 ± 9% of colocalization for GluN2A ($n = 12$) and 42 ± 3% of colocalization for GluN2B ($n = 15$), Fig. 2b and c, right, merge, yellow). Furthermore, the analysis unveiled a diffuse GluN3A staining (Fig. 2d, left, green) in VGLUT1-positive synaptosomes (Fig. 2d, middle, red) that amounted to 49 ± 8% of colocalization ($n = 12$, Fig. 2d, right, merge, yellow). Differently, GluN3B immunoreactivity (Fig. 2e, left, green) was rare (27 ± 3% of colocalization, ($n = 9$) Fig. 2e, right, merge, yellow) in VGLUT1-positive synaptosomes. This observation is largely consistent with previous findings showing the low presence of GluN3B subunits in the presynaptic component of mouse hippocampal synaptosomal preparations [24].

Immuno-Pharmacological Characterization of the Presynaptic NMDA Receptors Controlling Glutamate Release in Mouse Hippocampal Synaptosomes

Hippocampal synaptosomes were labeled with [^{3}H]D-ASP, a radioactive tracer that allows a reliable measure of the release of glutamate from nerve endings [23, 50]. The exposure of synaptosomes to low micromolar (30 μM) NMDA in the presence of saturating (1 μM) glycine elicits a dizocilpine-sensitive, Ca^{2+}-dependent release of [^{3}H]D-ASP [24]. Synaptosomes were incubated with anti-GluN antibodies (i.e., anti-GluN1, anti-GluN2A, anti-GluN2B, or anti-GluN3A antibodies) raised against the external amino acid sequence of the NMDA receptor subunits in order to assess their impact on the NMDA-induced exocytosis. Antibodies failed to alter the spontaneous release of [^{3}H]D-ASP (Table 1), but significantly reduced the tritium

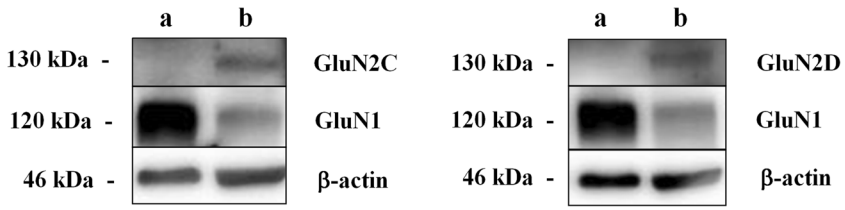
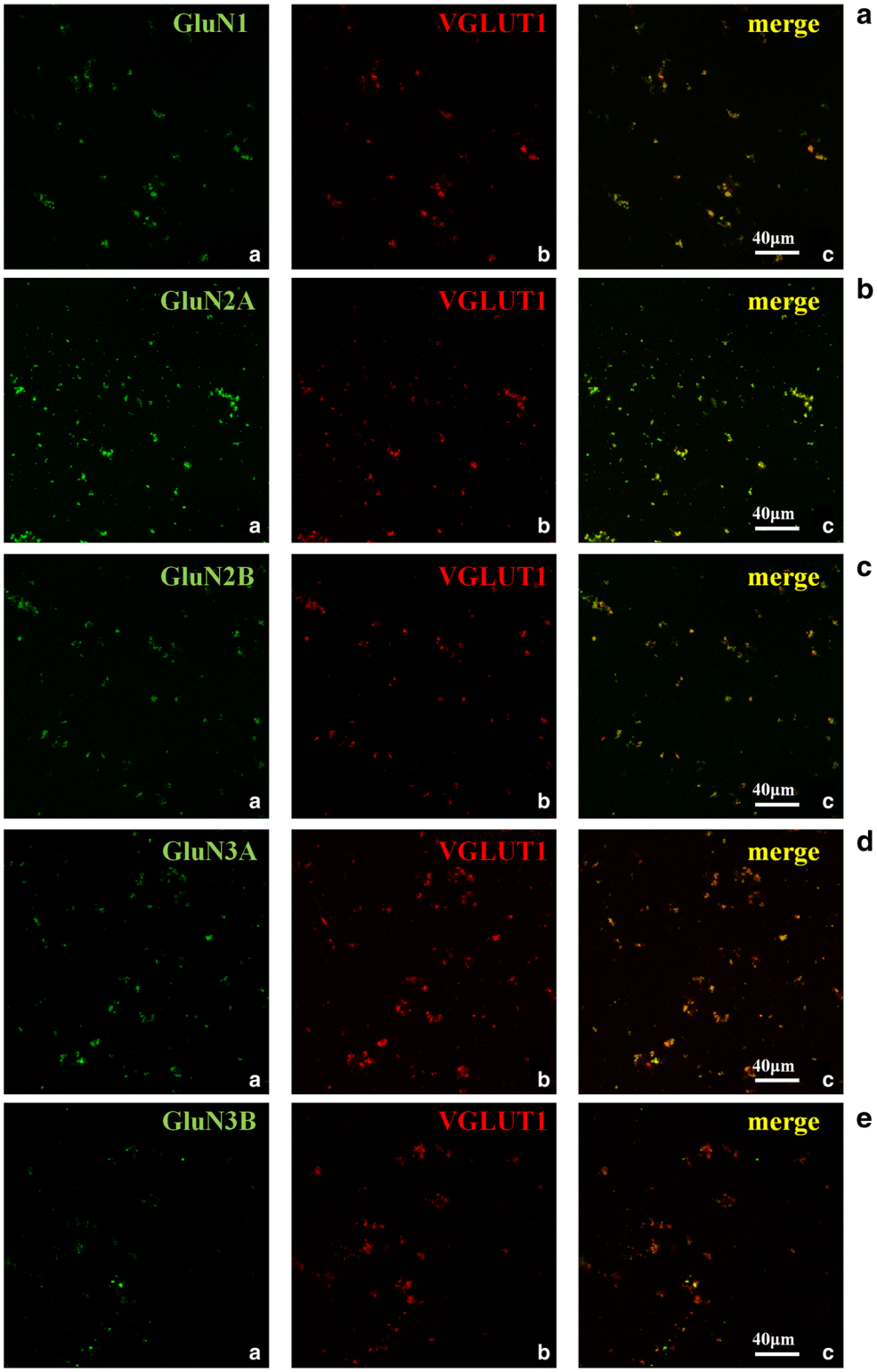


Fig. 1 Western blot analysis of GluN2C, GluN2D, and GluN1 subunit proteins in mouse hippocampal synaptosomal lysates and in mouse cerebellar homogenates. Western blot analysis unveils the presence of GluN1 receptor protein in both preparations. GluN2C (left) and GluN2D (right) immunopositivities were not detected in hippocampal synaptosomes (a) but they were evident in cerebellar homogenates (b). The figure shows a representative blot of six different analysis



overflow elicited by 30 μM NMDA/1 μM glycine (Fig. 3). We also investigated the impact of the anti-GluN3B antibody on the releasing activity. The antibody recognizes a sequence within the COOH terminus of the GluN protein and therefore should not be expected to bind the outer sequence(s) of the receptor subunit. Accordingly, Fig. 3 shows that the 30 μM NMDA/1 μM glycine-evoked [^{3}H]D-ASP release from anti-GluN3B-treated synaptosomes is comparable to that detected from the control synaptosomes.

◀ **Fig. 2** Confocal analysis of GluN subunits immunoreactivities in glutamatergic vesicular glutamate transporter type 1-positive (VGLUT1) hippocampal nerve terminals: colocalization of the receptor proteins with the glutamate transporters. (A) Confocal analysis of GluN1 subunit immunoreactivity (a, green) in VGLUT1-positive (b, red) hippocampal nerve terminals and their colocalization (c, yellow). (B) Confocal analysis of GluN2A subunit immunoreactivity (a, green) in VGLUT1-positive (b, red) hippocampal nerve terminals and their colocalization (c, yellow). (C) Confocal analysis of GluN2B subunit immunoreactivity (a, green) in VGLUT1-positive (b, red) hippocampal nerve terminals and their colocalization (c, yellow). (D) Confocal analysis of GluN3A subunit immunoreactivity (a, green) in VGLUT1-positive (b, red) hippocampal nerve terminals and their colocalization (c, yellow). (E) Confocal analysis of GluN3B subunit immunoreactivity (a, green) in VGLUT1-positive (b, red) hippocampal nerve terminals and their colocalization (c, yellow). The figure shows representative images of six independent experiments

Effects of pH Changes on the Release-Regulating NMDA Autoreceptors in Hippocampal Synaptosomes

Functional NMDA receptors are heteromultimeric assemblies of the GluN1 subunit with GluN2 and/or GluN3 subunits. Because of the alternative splicing variants in exon 5, 20 and 21, GluN1 subunits exist in at least eight different isoforms, having different expression in the CNS [51]. GluN1 subunit lacking the splice cassette of 21 amino acids (termed N1) encoded by the exon 5 in the amino-terminus domain represents a preferential partner of GluN3A proteins [52], but also makes the NMDA receptor sensible to the external concentration of protons ($[H^+]_{out}$) [7, 53]. Experiments were carried out to verify the pH sensitivity of the NMDA autoreceptors controlling glutamate exocytosis. The spontaneous release of [^{3}H]D-ASP did not significantly changed at pH 6.60 and at pH 8.00 (pH 7.4, 2.35 ± 0.18; pH 6.60, 2.55 ± 0.14; pH 8.00, 2.65 ± 0.23; data expressed as percentage of tritium released in the first fraction collected with respect to the total synaptosomal content; $n = 8$, n.s.). The 30 μM NMDA/1 μM glycine-evoked release of [^{3}H]D-ASP, on the contrary, was significantly modified when changing the pH of the superfusion medium. In particular, lowering the external pH to 6.60 significantly reduced the release of tritium elicited by 30 μM NMDA/1 μM glycine, while increasing it to 8.00 increased the tritium overflow (Fig. 4).

Table 1 Effects of incubation of hippocampal synaptosomes with anti-GluN antibodies on the spontaneous release of preloaded [^{3}H]D-aspartate

	[^{3}H]D-aspartate release in the first fraction collected	
Control	3.52 ± 0.25	
Incubation with anti-GluN1 antibody	3.39 ± 0.18	n.s.
Incubation with anti-GluN2A antibody	3.79 ± 0.33	n.s.
Incubation with anti-GluN2B antibody	3.47 ± 0.27	n.s.
Incubation with anti-GluN3A antibody	3.68 ± 0.24	n.s.
Incubation with anti-GluN3B antibody	3.56 ± 0.55	n.s.

Incubation with anti-GluN subunit antibodies did not affect the spontaneous release of [^{3}H]D-aspartate from mouse hippocampal synaptosomes. Results are expressed as percent of the total synaptosomal tritium content released in the first fraction collected. Data are means ± SEM from five experiments run in triplicate

n.s., not significative

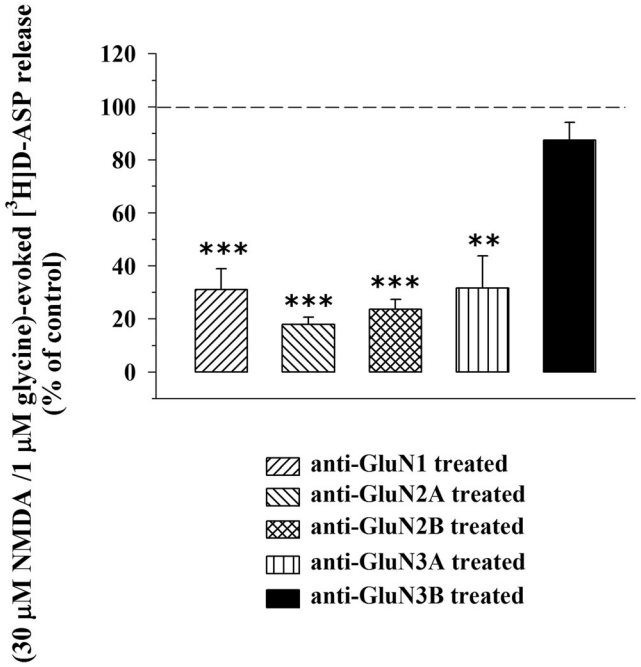


Fig. 3 Effects of the incubation of mouse hippocampal synaptosomes with anti-GluN subunit antibodies on the NMDA/glycine-evoked release of [^{3}H]D-aspartate ([^{3}H]D-ASP). Synaptosomes were incubated in the absence or in the presence of selective anti-GluN antibodies (as indicated) and then superfused to monitor the release of preloaded [^{3}H]D-ASP. Results are expressed as percentage of the NMDA/glycine-evoked release from control synaptosomes (% of control). The NMDA/glycine-evoked overflow (expressed as % of tritium over basal release) amounted to 0.85 ± 0.11. Data are expressed as means ± SEM from seven experiments run in triplicate. $^{**}p < 0.01$ versus the 30 μM NMDA/1 μM glycine-evoked tritium overflow from the control hippocampal synaptosomes; $^{***}p < 0.001$ versus the 30 μM NMDA/1 μM glycine-evoked tritium overflow from the control hippocampal synaptosomes

Incubation of Hippocampal Synaptosomes with Anti-GluN Antibodies Does Not Affect the KCl-Evoked Release of [^{3}H]D-Aspartate

Activation of NMDARs elicits the Ca^{2+}-dependent, exocytotic-like release of [^{3}H]D-ASP [23, 24]. We asked whether incubation of synaptosomes with anti-GluN antibodies could affect per se the exocytotic machinery accounting for tritium release. To answer the question, synaptosomes incubated with the anti-GluN antibodies under study were exposed in superfusion to another depolarizing stimulus (i.e., a solution containing 12 mM KCl). Figure 5 shows that the 12 mM KCl-

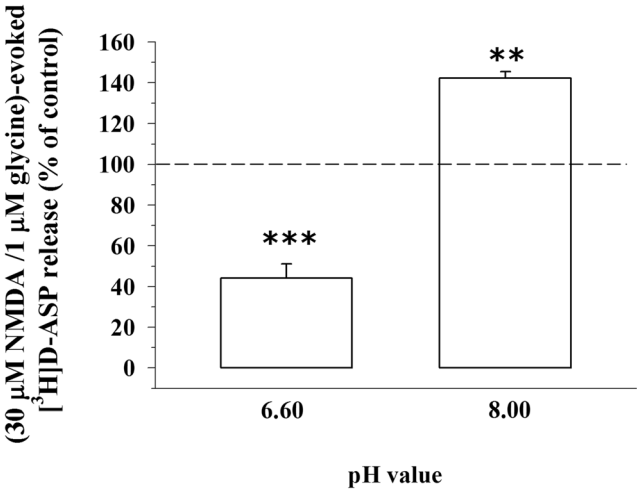


Fig. 4 Effects of external protons on the NMDA/glycine-evoked release of [^{3}H]D-aspartate ([^{3}H]D-ASP). Synaptosomes were superfused with medium at different pH to monitor the release of preloaded [^{3}H]D-ASP elicited by 30 μM NMDA/1 μM glycine. Results are expressed as percentage of the NMDA/glycine-evoked release from synaptosomes superfused at pH 7.40 (% of the control). NMDA/glycine-evoked overflow (expressed as % of tritium released over basal release), 0.92 ± 0.09. Data are expressed as means ± SEM from five experiments run in triplicate. $^{**}p < 0.01$ versus the 30 μM NMDA/1 μM glycine/pH 7.40-evoked tritium overflow; $^{***}p < 0.001$ versus the 30 μM NMDA/1 μM glycine/pH 7.40-evoked tritium overflow

evoked release of tritium from anti-GluN antibody-incubated synaptosomes did not significantly differ from that observed from the control, untreated synaptosomes, whatever the antibody under study.

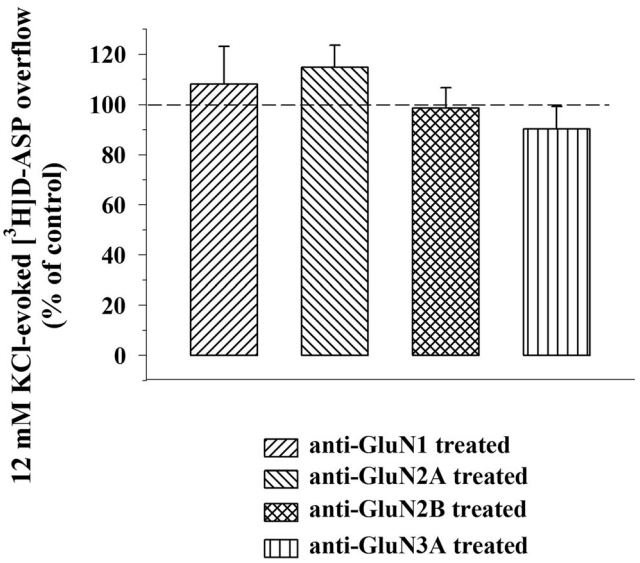


Fig. 5 Effects of the incubation of mouse hippocampal synaptosomes with anti-GluN subunit antibodies on the 12 mM KCl-evoked release of [^{3}H]D-aspartate ([^{3}H]D-ASP). Results are expressed as percentage of 12 mM KCl-evoked release from the control synaptosomes (% of control). 12 mM KCl-evoked overflow (expressed as % of tritium released over basal release), 1.53 ± 0.14. Data are means ± SEM from four experiments run in triplicate

Anti-GluN Antibodies Affect the Complement-Induced Release from Mouse Hippocampal Synaptosomes

Complement releases glutamate from nerve terminals isolated from selected CNS regions [54] and the release efficiency is amplified by the presence of an antibody/protein complex at the outer side of plasma membranes [55]. We asked whether the presence of the anti-GluN/GluN complex could affect the release of glutamate elicited by complement from mouse hippocampal synaptosomes. To this aim, untreated and antibody (anti-GluN1, anti-GluN2A, anti-GluN2B, or anti-GluN3A)-treated synaptosomes were exposed to complement (dilution 1:300), and the release of [^{3}H]D-ASP overflow from the different synaptosomal preparations was quantified. Figure 6 shows that incubation of synaptosomes with the anti-GluN1, the anti-GluN2B, or the anti-GluN3A antibody caused a significant reduction of the complement-evoked release of [^{3}H]D-ASP (Fig. 6). Differently, the release of [^{3}H]D-ASP from synaptosomes incubated with the anti-GluN2A antibody was slightly, although not significantly, diminished when compared to the control. We speculated that the complement-induced releasing activity comprises an NMDA-mediated component that is significantly reduced in synaptosomes incubated with anti-GluN antibodies. Consistent with this view, the NMDA antagonist dizocilpine (1 μM) significantly reduced the complement-evoked releasing activity (1:300

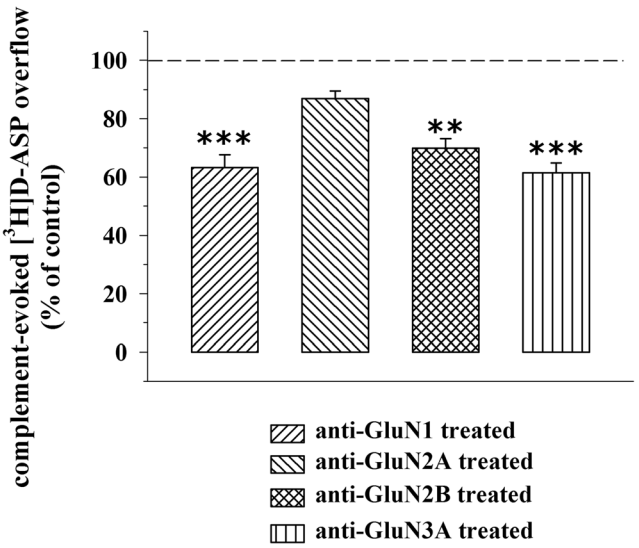


Fig. 6 Effects of the incubation of mouse hippocampal synaptosomes with anti-GluN subunit antibodies on the (1:300) complement-evoked release of [^{3}H]D-aspartate ([^{3}H]D-ASP). Results are expressed as percentage of the (1:300) complement-evoked release from control synaptosomes (% of the control). Complement-evoked overflow (expressed as % of tritium released over basal release), 3.79 ± 0.29. Data are means ± SEM from five experiments run in triplicate. $^{**}p < 0.01$ versus the (1:300) complement-evoked tritium overflow from control hippocampal synaptosomes; $^{***}p < 0.001$ versus the (1:300) complement-evoked tritium overflow from the control hippocampal synaptosomes

complement-evoked overflow = 2.43 ± 0.24; plus 1 μM dizocilpine = 1.93 ± 0.23; results expressed as induced overflow; − 20.58 ± 5.29%; $n = 5$, $p < 0.05$). At the concentration applied, the antagonist did not modify on its own the basal release of [^{3}H]D-ASP (not shown, but see [24]).

Anti-GluN1 and Anti-GluN2B Antibodies Reduce the Insertion of Presynaptic Release-Regulating NMDA Autoreceptors in Hippocampal Synaptosomes

Anti-NMDA autoantibodies were proposed to favor the internalization of NMDA receptors in cultured neurons [56–61]. We asked whether anti-GluN antibodies could promote NMDAR endocytosis also presynaptically in hippocampal glutamatergic nerve endings. To answer the question, biotinylation studies were carried out to compare the expression of GluN subunits in control nerve endings and in synaptosomes incubated with antibodies recognizing the NH_2 terminus of subunits bearing the glycine binding site (i.e., the GluN1 subunit) or the glutamate binding site (i.e., the GluN2B subunit). The data depicted in Fig. 7 clearly demonstrate that the density of the biotin-tagged GluN1 and GluN2B subunits was lower in anti-GluN1-treated (T, Fig. 7a) or in anti-GluN2B-treated synaptosomes (T, Fig. 7c) when compared to control untreated (C, Fig. 7a and c) synaptosomes. In particular, we observed a significant reduction of GluN1 and GluN2B density in hippocampal synaptosomal plasma membranes incubated with anti-GluN1 antibody (Fig. 7b). Pre-incubation of mouse hippocampal synaptosomes with anti-GluN2B antibody also caused a significant decrease of the immunoreactivity of the GluN1 and of the GluN2B subunit (Fig. 7d) in plasma membranes when compared to the control.

Discussion

The study was devoted to confirm the participation of GluN3A subunit to the presynaptic release-regulating NMDA autoreceptor in mouse hippocampal nerve endings. To this aim, we focused on two criteria that, if satisfied, support the conclusion [62, 63]: (i) the existence of the GluN subunit protein in the plasma membranes of nerve terminals and (ii) the subunit-dependent manipulation of the NMDA-mediated control of release efficiency.

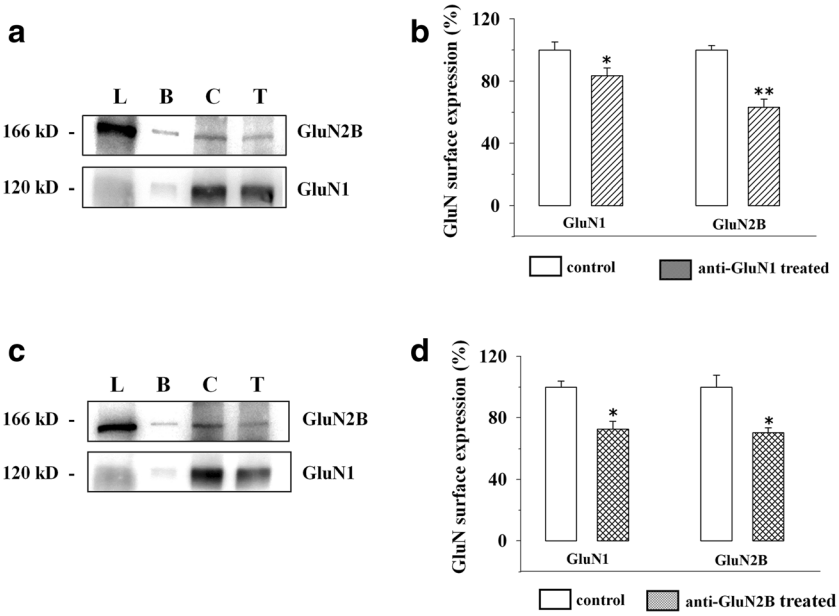


Fig. 7 Western blot analyses of GluN1 and GluN2B receptor subunits surface densities in hippocampal terminals. (a) The Western blot compares total synaptosomal lysates (lane L), synaptosomal lysates that were not treated with biotin, but were subject to streptavidin pulldown (lane B), synaptosomal lysates incubated with biotin and subject to streptavidin pulldown (lane C), and anti-GluN1 antibody-pretreated synaptosomal lysates incubated with biotin and subject to a streptavidin pulldown (lane T). Each preparation was analyzed for the presence of GluN1 and GluN2B immunoreactivities. The blot is representative of six different experiments on synaptosomal preparations from different animals. (b) Changes in GluN1 and in GluN2B subunit protein surface density in anti-GluN1 antibody-treated and in untreated synaptosomes. Results are expressed as percent of changes of the GluN surface expression. Data are means ± SEM. *$p < 0.05$ versus control; **$p < 0.01$ versus control. (c) Western blot analysis of total synaptosomal lysates (lane L), synaptosomal lysates that were not treated with biotin, but were subject to streptavidin pulldown (lane B), synaptosomal lysates incubated with biotin and subject to streptavidin pulldown (lane C), and anti-GluN2B antibody-pretreated synaptosomal lysates incubated with biotin and subject to a streptavidin pulldown (lane T). Each preparation was analyzed for the presence of GluN1 and GluN2B immunoreactivities. The blot is representative of seven different experiments carried out in different days with synaptosomal preparations from different animals. (d) Changes in GluN1 and in GluN2B subunit protein surface density in anti-GluN2B antibody-treated and in untreated synaptosomes. Results are expressed as percent of changes of the GluN surface expression. Data are means ± SEM. *$p < 0.05$ versus control

Immunochemical Evidence that Mouse Hippocampal Glutamatergic Nerve Endings Possess GluN1, GluN2A, GluN2B, and GluN3A Subunits

The presynaptic localization of selected GluN subunits, including the GluN3A protein, in the hippocampus of rodents has been matter of discussion and several studies support this view [47, 64, 65]. In particular, GluN1, GluN2A, GluN2B, and GluN3A, but not GluN3B, proteins were shown to exist in the presynaptic component of hippocampal synaptosomal plasma membranes [24, 25]. Comparable results were obtained by Henson and colleagues [49]. The authors demonstrated the presence of GluN3A subunit in mice forebrain synaptosomal membranes. Finally, the presynaptic localization of GluN3A subunit emerged on the basis of both functional and immunochemical observations [29, 64, 66].

The results from confocal microscopy described in the present study confirmed the presynaptic localization of the GluN1, GluN2A, GluN2B, and GluN3A subunits in this region, but also added new information on their synaptic distribution. Actually, GluN1, GluN2A, GluN2B, and GluN3A immunofluorescence was found to largely colocalize with the VGLUT1-staining, unveiling a diffuse expression of these proteins in the glutamatergic subpopulation of the synaptosomes. Differently, the GluN3B immunostaining in the VGLUT1-positive terminals was low, well consistent with its scarce expression in hippocampal synaptosomal lysates [24], while the almost undetectable immunopositivity of GluN2C and GluN2D subunits in synaptosomal lysates seems best interpreted by assuming that, if present [48, 64, 67], these proteins preferentially adopt a non-synaptic localization.

Thus, the first conclusion of the study was that the GluN1, GluN2A, GluN2B, and GluN3A proteins are those subunits preferentially expressed presynaptically in hippocampal glutamatergic nerve endings.

An Immuno-Pharmacological Approach to Define the NMDA Autoreceptor Subunit Composition

As to the second criterion, the low sensitivity of the NMDARs to Mg^{2+} ions together with the effects of glycinergic ligands (i.e., D-serine) described in a previous study [24] seemed predictive of the participation of the GluN3A subunit to the NMDAR assembly. The lack of selective GluN3A ligands, however, limited the possibility to verify the hypothesis. To circumvent the limitation, we turned to a new approach, we refer to as the "immuno-pharmacological" approach, which relies on the use of antibodies raised against the amino-terminal sequence of a selected receptor subunit as selective tools to highlight the participation of that protein to the receptor assembly. Basically, it is proposed that the binding of the antibody to the outer sequence of the subunit causes stereochemical modifications that emerge as changes in the receptor-mediated effects, including the receptor-mediated control of transmitter release. This approach, so far limited to the characterization of metabotropic receptors [31–35], was here extended to the ionotropic NMDA receptors, under the premise that (i) anti-GluN antibodies could affect the NMDA/glycine-evoked release efficiency by interfering with agonists at the orthosteric sites and (ii) the final outcome would strictly depend on the activating/inactivating properties of the antibodies.

Based on the previous observations obtained which suggested the involvement of the GluN1, GluN2A, and GluN2B subunits in the NMDAR assembly [24], we first investigated the impact of the anti-GluN1, anti-GluN2A, and anti-GluN2B antibodies on the NMDA/glycine-evoked glutamate exocytosis, owing to confirm our previous findings and to validate the approach. Antibodies failed to modify the spontaneous release of glutamate, but reduced significantly the NMDA/glycine-induced releasing activity, consistent with an antagonist-like activity of the antibodies at the NMDARs. Furthermore, the antibodies did not alter glutamate exocytosis elicited by other depolarizing stimuli (i.e., high K^+), confirming the specificity of the "immuno-pharmacological" approach. In a whole, these observations were largely confirmatory of our working hypothesis [32–34] and definitively prove the "immune-pharmacological" approach as a suitable technique to characterize the subunit composition of membrane receptors, including the ionotropic ones.

Based on the positive results, we turned to the anti-GluN3A antibody. Pre-treating hippocampal synaptosomes with the anti-GluN3A antibody significantly reduced the release of glutamate elicited by NMDA/glycine, leaving unchanged the [^{3}H]D-Asp overflow evoked by high KCl. Again, the results allow the conclusion that the GluN3A subunit participates to the expression of the NMDAR and influences its releasing activity.

The question arises on the subunit composition of the NMDARs under study and how GluN3A subunit participates to the receptor assembly.

It is known that GluN3A subunits can associate to GluN1 protein to form an atypical "glycine-sensitive" GluN1/GluN3A NMDA receptor, the activation of which produces desensitizing currents that are potentiated by external protons [49, 53, 68]. The NMDARs in hippocampal nerve endings, however, cannot be activated by glycine alone [24] and their releasing activity is reduced by acidifying the external medium, which allow to exclude the existence of presynaptic release-regulating GluN1/GluN3A dimers in hippocampal glutamatergic nerve endings.

Differently, the sensitivity of the NMDARs to the external pH could suggest the involvement of GluN1 subunits lacking the N1 cassette in the NMDAR assembly. This aspect deserves attention, since one of the four GluN1 splice variants lacking this cassette [51], i.e., the GluN1-1a subunit, was

shown to drive the insertion of GluN3A proteins into plasma membranes [52]. Notably, besides lacking the N1 cassette, the GluN1-1a variant also possesses two intraterminal sequences, namely the C1 and the C2 sites, that modulate NMDA receptor responses and make them sensitive to PKC-dependent phosphorylative processes, as indeed observed for the NMDARs in hippocampal synaptosomes [69]. By a speculative point of view, the pH-dependency of the NMDA-evoked glutamate exocytosis together with its sensitivity to PKC modulators would confirm the involvement of the GluN1-1a subunit (i.e., the GluN1 splicing variant able to drag the GluN3A protein in plasma membranes) in the expression of the NMDAR in hippocampal terminals. If present, this subunit would recruit GluN3A subunits to participate to the NMDAR assembly.

To note, data in the literature suggests that GluN1 subunits associate to GluN2 subunits to form tetra-heterodimeric complex (GluN1/GluN2A/GluN2B) [70], that further can evolve to multimeric association of subunit, with a high degree of complexity, which also includes GluN3 subunits [71, 72]. Accordingly, previous finding showed the physical association linking GluN3A and the GluN2B subunits in hippocampal synaptosomes [24]. Although the results so far available are insufficient to propose the exact GluN stoichiometry of the NMDARs under study, they seem best interpreted by proposing the existence of a multimeric GluN1/GluN2A/GluN2B/GluN3A NMDA complex in hippocampal glutamatergic terminals.

Anti-GluN Antibody/NMDA Receptor Complex Does Not Activate Complement, but Promotes NMDA Receptor Internalization in Nerve Terminals

In hippocampal synaptosomes, complement releases glutamate through a mechanism involving the sodium-dependent release of the amino acid [54]. Surprisingly, we here provide evidence showing that the complement-evoked release of glutamate is in part prevented by dizocilpine, compatible with the conclusion that also presynaptic release-regulating NMDARs participate to the complement-evoked releasing activity. As a matter of fact, the depolarization of the synaptosomal plasma membranes elicited by complement could have favored the opening of the NMDAR-associated voltage-dependent ionic channels in synaptosomal plasma membranes, improving the influx of cations and then amplifying transmitter exocytosis. If this was the case, NMDAR channel blockers (i.e., dizocilpine) would reduce the flowing of the positive charges through the NMDA-associated ionic channel, hampering the NMDA-mediated component of the complement-evoked overflow, as indeed observed.

Recently, we demonstrated that the presence of an antibody/receptor complex at the outer side of cortical synaptosomal plasma membranes reinforces the releasing activity of the complement by triggering an additional pathway activation of the immune complex, i.e., the classic pathway [55]. Contrary to expectation, however, the presence of anti-GluN antibody/GluN subunit complexes in hippocampal synaptosomal plasma membranes did not further increase the complement-evoked releasing activity, rather reduced it, well consistent with the antagonist-like activity of anti-GluN antibodies at the NMDA autoreceptors in hippocampal synaptosomes observed in release experiments.

As to the impact of anti-GluN autoantibodies on the NMDA receptors in CNS, the data so far available show that anti-GluN1 and anti-GluN2B antibodies, which are present in the cerebrospinal fluid of patients suffering from autoimmune neurological diseases [57 and references therein, 73], reduce NMDA receptor-mediated synaptic currents [58] and long-term potentiation at Schaffer collateral CA1 synapses [59]. These effects were proposed to rely preferentially on the increased internalization of GluN subunits in neurons [58, 60]. Starting from these observations, we asked whether incubation of synaptosomes with anti-GluN antibodies could have favored the internalization of the NMDARs instead of exerting an antagonist-like effect at the respective subunits. Biotinylation studies unveiled that the anti-GluN1 or anti-GluN2B antibody pre-treatment reduced the presence of both the GluN subunits in synaptosomal plasma membranes, which seems best interpreted by assuming the internalization of the NMDAR receptors instead of the reduced availability of a selected GluN subunit. These findings provide the first demonstration that anti-GluN antibodies trigger the internalization of the NMDA receptors also presynaptically at chemical synapsis and might account for the reduced NMDAR releasing efficiency observed in anti-GluN-treated synaptosomes when compared to the control.

Conclusion

Our results confirm the "immuno-pharmacological approach" as a useful technique to characterize pharmacologically native receptors. By using this approach, we prove the participation of GluN3A subunit to the expression of presynaptic release-regulating NMDARs in hippocampal glutamatergic nerve endings. Our study also provide evidence supporting the involvement of GluN1 subunit lacking the N1 cassette in the NMDAR assembly. The glycinergic subunit would permit the insertion of the GluN3A subunit in synaptosomal plasma membranes, favoring its association to a GluN1/GluN2A/GluN2B heterodimeric complex. Finally, we demonstrate that the anti-GluN antibodies hamper the releasing activity elicited by NMDARs by triggering the internalization of NMDA receptor complex.

Taking into account the role of anti-GluN autoantibodies in the etiopathogenesis of central neurological diseases [56, 57],

our result add new insights on the cellular events leading to disrupt synaptic plasticity in patients suffering of autoimmune anti-NMDA diseases. Since not all the antibodies targeting NMDA receptor subunits are pathogenic or result in NMDA receptor endocytosis [74], it will be of interest to analyze the impact of circulating NMDA receptor autoantibodies on the expression and the function of presynaptic release-regulating NMDA receptors in mouse hippocampal nerve endings. The results from this study will allow to verify whether anti-NMDA autoantibodies mimic anti-GluN antibodies in controlling the presynaptic release-regulating NMDA autoreceptors and how do they impact their release efficiency.

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Compliance with Ethical Standard The experimental procedures were in accordance with the European legislation (European Communities Council Directive of 24 November 1986, 86/609/EEC) and the ARRIVE guidelines, and they were approved by the Italian Ministry of Health (DDL 26/2014 and previous legislation; protocol number no. 50/2011-B).

Conflict of Interest The authors declare that they have no competing interests.

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