Neural Cell Adhesion Molecule-associated Polysialic Acid Inhibits NR2B-containing N-Methyl-D-aspartate Receptors and Prevents Glutamate-induced Cell Death*

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The neural cell adhesion molecule (NCAM) and its associated glycan polysialic acid play important roles in the development of the nervous system and N-methyl-D-aspartate (NMDA) receptor-dependent synaptic plasticity in the adult. Here, we investigated the influence of polysialic acid on NMDA receptor activity. We found that glutamate-elicited NMDA receptor currents in cultured hippocampal neurons were reduced by ∼30% with the application of polysialic acid or polysialylated NCAM but not by the sialic acid monomer, chondroitin sulfate, or non-polysialylated NCAM. Polysialic acid inhibited NMDA receptor currents elicited by 3 μM glutamate but not by 30 μM glutamate, suggesting that polysialic acid acts as a competitive antagonist, possibly at the glutamate binding site. The polysialic acid induced effects were mimicked and fully occluded by the NR2B subunit specific antagonist, ifenprodil. Recordings from single synaptosomal NMDA receptors reconstituted in lipid bilayers revealed that polysialic acid reduced open probability but not the conductance of NR2B-containing NMDA receptors in a polysialic acid and glutamate concentration-dependent manner. The activity of single NR2B-lacking synaptosomal NMDA receptors was not affected by polysialic acid. Application of polysialic acid to hippocampal cultures reduced excitotoxic cell death induced by low micromolar concentration of glutamate via activation of NR2B-containing NMDA receptors, whereas enzymatic removal of polysialic acid resulted in increased cell death that occluded glutamate-induced excitotoxicity. These observations indicate that the cell adhesion molecule-associated glycan polysialic acid is able to prevent excitotoxicity via inhibition of NR2B subunit-containing NMDA receptors.

Cell interactions play important roles during development and in maintenance and modification of synaptic functions in the adult. The question is whether recognition molecules that specify and modulate contacts between neural cells may influence other parameters essential for nervous system function, such as neurotransmitter release and receptor activity. Among the recognition molecules with widespread functions is the neural cell adhesion molecule (NCAM)4 that starts to be expressed at the time of neural tube closure and remains detectable at lower levels in the adult. Its importance in shaping synaptic functions in the adult has long been recognized (1–2). NCAM is unique among recognition molecules in that its adhesive and concomitant signal transduction functions are modified by an unusual glycan, polysialic acid, a highly negatively charged and voluminous carbohydrate, which is regulated by its attachment to the protein backbone (3). PSA is a polymer of α2,8-linked sialic acid residues with chain lengths of up to 200 residues. It has not been detected on other recognition molecules. PSA is synthesized by two sialyltransferases, ST8Sia-II and ST8Sia-IV, and attached to the fifth immunoglobulin-like domain of NCAM (4). Even more so than the protein backbone of NCAM, expression of PSA is developmentally regulated, with high expression during embryonic stages and gradual reduction as development proceeds. However, it remains expressed in some areas of the brain during adulthood, including the hippocampus, which undergoes functional changes underlying synaptic plasticity. In particular, PSA-NCAM is required for NMDAR-dependent long-term potentiation and spatial learning (5–9).

The NMDARs are a subtype of ionotropic glutamate receptors that are found widely throughout the brain. NMDARs are heteromers assembled from the NMDAR subunits NR1 and at least one type of NR2 subunit (10). CA1 pyramidal cells, for instance, express mostly two different NR2 subunits, NR2A and NR2B, and perinatally, NR2D (11). It is believed that the different NR2 subunits confer distinct gating and pharmacological

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4 The abbreviations used are: NCAM, neural cell adhesion molecule; CA, colo- minic acid; endo-N, endo neuraminidase-N; NMDAR, N-methyl-D-aspartate (NMDA) subtype of glutamate receptors; PSA, polysialic acid; PSA-NCAM, polysialylated NCAM; SA, sialic acid; MOPS, 3-(N-morpholinopropanesulfonic acid; CV, coefficient of variation; APV, 2-amino-5-phosphonopentanoic acid; AMPA, α-amino-3-hydroxy-5-methylisoxazole-4-propionate subtype of glutamate.
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properties to NMDARs (12) and couple them to distinct intracellular signaling mechanisms (13), which may shape their characteristic roles in synaptic plasticity (14–16). NMDARs are expressed both synaptically and extra-synaptically. The subunit composition at these locations is not uniform; synaptic NMDARs predominantly contain NR2A, whereas extra-synaptic NMDARs contain mostly the NR2B subunit (17), although recent data in mature cultured hippocampal neurons support a view that both subtypes can be located in either synaptic or extrasynaptic compartments (18). Importantly, synaptic and extra-synaptic NMDARs may activate opposing pathways to prevent or induce glutamate-induced excitotoxic cell death, respectively (19).

Several lines of evidence indicate that there are intriguing links between PSA-NCAM and glutamate receptors. NMDARs are co-redistributed with NCAM after induction of long-term potentiation (20), and NCAM associates with the postsynaptic spectrin-based scaffold cross-linking NCAM with the NMDAR and calmodulin kinase IIα (21). Activity of NMDARs is required for PSA-NCAM-stimulated synaptogenesis (22), and PSA directly increases the probability of the open state of α-amino-3-hydroxy-5-methylisoxazole-4-propionate subtype of glutamate (AMPA) receptors (23). To address the question of whether PSA may also modulate NMDARs, we examined the effects of PSA on glutamate-evoked NMDAR currents in cultured hippocampal neurons and synaptosomal NMDARs incorporated in artificial lipid bilayers.

EXPERIMENTAL PROCEDURES

Chemicals—Polymers containing 25–50 sialic acid residues were purified from colominic acid (Fluka, Buchs, Switzerland) using anion exchange chromatography on a Hamilton PRPX column dissolved in 20 mM Tris, pH 7.4, with 0–500 mM NaCl and detected at 214 nm (24). The concentration was determined using a colorimetric resorcinol method adapted to microtitre plate format (25).

Mouse PSA-NCAM-Fc, containing the extracellular domain of NCAM and the Fc portion of human IgG, was produced using the value recorded in the presence of glutamate at +60 mV. Statistical comparisons of the current/voltage curves were done in Statistica 5.0 (StatSoft Inc., Tulsa, OK) using analysis of variance with “voltage” and “treatment” as repeated measures. To evaluate whether PSA induces changes in the conductance or gating of NMDAR channels in cultures, the fluctuation analysis of glutamate-activated NMDAR currents was performed (31, 32). The coefficient of variation was calculated for glutamate-evoked NMDAR currents at −60 mV as the following ratio: $CV = \frac{\text{variance (glutamate response)}}{\text{mean (base line)}}$. This measure is useful in light of the following considerations. The mean and variance of current generated by $n$ independent channels, which are open with a probability $p$ and have a conductance $q$, are described by a binomial law and equal to $qnp$ and $q^2np(1 - p)$, respectively. Then $CV^{-2} = np(1 - p)$ is a measure of variability that does not depend on $q$ and changes only when the number of channels or their open probability is changed. Any changes in $q$ alone would not affect this measure. Using these equations one can derive that the ratio between mean currents before and after inhibition of channel activity ($x$) would be proportional to the ratio between corresponding
The hippocampi were isolated from 7–10 days old Sprague-Pared as described elsewhere (33) with minor modifications. PSA-NCAM-Fc (10 μg/ml) was added to the buffer to minimize proteolysis. The homogenate was diluted with 1.60 ml of additional Krebs buffer after being homogenized with 5 turns of a handheld pestle. The mixture loaded into the 1-cc tuberculin syringe was forced through three layers of nylon (Tetko, 100-μm pore size) pre-wet with 150 μl of Krebs buffer and collected in an Eppendorf tube. After another filtering with a pre-wet Millex filter (5-μm pore size polyvinylidene difluoride Millipore filter), the filtrate was centrifuged at 1000 x g for 15 min in a microcentrifuge at 4 °C. After removing the supernatant, the pellet, which contained the synaptosomes, was resuspended in 100 μl of Krebs buffer for electrophysiological recordings.

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**Incorporation of synaptosomal NMDARs in artificial lipid bilayers**—Incorporation of synaptosomal NMDARs in artificial lipid bilayers was carried out using the “tip-dip” method (34). The phospholipid bilayer was formed at the tip of a polished glass pipette (World Precision Instruments Inc., Sarasota, FL). The P-2000 laser micropipette puller (Sutter Instrument Co., Novato, CA) was used to pull pipettes with 100-megohm resistance. The synthetic phospholipids were prepared by dissolving 1,2-diphytanoyl-sn-glycero-3-phosphocholine (Avanti Polar-Lipids Inc., Alabaster, AL) in hexane (Sigma-Aldrich) to obtain a concentration of 1 mg/ml. About 3–5 μl of this phospholipid preparation was delivered into 300 μl of bath solution. The bilayer formation was initiated by successive transfer of two monolayers onto the tip of the patch pipette in an asymmetric saline condition with “outside-out” configuration. The bath solution contained pseudoextracellular fluid composed of 125 mM NaCl, 5 mM KCl, 1.25 mM NaH2PO4, and 5 mM Tris HCl. The pseudoextracellular fluid consisting of 110 mM KCl, 4 mM NaCl, 2 mM NaHCO3, 1 mM MgCl2, 0.1 mM CaCl2, and 2 mM MOPS was used as the pipette solution. After forming a stable membrane, a 3–5-μl suspension of synaptosomes was transferred to the pseudoextracellular fluid. Gentle stirring facilitated fusion of synaptosomal fragments into the bilayer.

**Single Channel Recording and Analysis**—Single synaptosomal NMDA receptor channel currents were elicited by application of glutamate (Sigma-Aldrich) and 1 μM glycine (Sigma-Aldrich) in the presence 1 μM picrotoxin (Sigma-Aldrich), 2 mM tetrodotoxin (Tocris), which block AMPA, kainate, and glycine receptors, and potassium and sodium channels, respectively. PSA (colomnic acid) was delivered to the pseudoextracellular fluid in increasing concentrations ranging from 1 to 3 μg/ml. Chondroitin sulfate

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**FIGURE 1.** NMDAR currents in dissociated hippocampal neurons are inhibited by PSA and PSA-NCAM-Fc. A, voltage clamp protocol used to record the NMDAR currents (Vh = −80 mV with voltage steps from −100 to +60 mV; upper trace) and representative recordings showing glutamate evoked NMDAR currents before, during, and after application of colomnic acid (CA). Glutamate induced currents (second trace) are reduced by CA (third trace) in a fully reversible manner (fourth trace shows currents in 5 s after washout of CA). The horizontal bar indicates the application of glutamate. B, current/voltage relationship of NMDAR currents evoked by glutamate (3 μM, filled squares). NMDAR currents are inhibited by ~30% by colomnic acid (40 μg/ml, open squares) at all voltages tested. Data represent the mean ± S.E., n = 40, F0.05 = 60.34, p < 0.000001. C, current/voltage relationship of NMDAR currents evoked by glutamate (3 μM, filled squares). NMDAR currents are not inhibited by endo-N-digested colomnic acid (40 μg/ml, open squares). Data represent the mean ± S.E., n = 40, F0.05 = 60.34; *****, p < 0.000001), high performance liquid chromatography-purified colominic acid (purified CA, 10 μg/ml, n = 4, F0.05 = 4.28; ***, p < 0.005), sialic acid (40 μg/ml, n = 4, F0.05 = 2.12, p > 0.05), chondroitin sulfate (CS, 40 μg/ml, n = 7, F0.05 = 0.33, p > 0.05), endo-digested colominic acid (Endo-N/CA, 40 μg/ml, n = 8, F0.05 = 0.98, p > 0.05), PSA-NCAM-Fc (10 μg/ml, n = 7, F0.05 = 19.38, ****, p < 0.000001), and NCAM-Fc (10 μg/ml, n = 3, F0.05 = 2.41, p > 0.05).
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A (Sigma-Aldrich) was used as a control at a concentration of 10 μg/ml. The single channel currents were amplified (Axopatch 200B, Molecular Devices, Sunnyvale, CA), digitized using a PCM interface (VR-10B Digital Data Recorder, Instrutech Corp., Elmont, NY), and stored on VHS tape. For off-line analysis, the data were filtered at 2 kHz and digitized between 5 and 25 kHz with PClamp6 software. The channel currents, which were sensitive to NR2B specific antagonist 1 μM Ro (Ro 25-6981 maleate, Tocris), were included in the analysis. The data processing of single channel recordings from synaptosomal receptors was described elsewhere (34).

Only the data exhibiting long stretches of single channel current transition without base-line drifts were chosen for quantitative analysis. All point current amplitude histograms were constructed and fitted with Gaussian curves to identify the individual conductances. The single channel conductance of NMDA receptors were obtained by plotting current as a function of membrane voltage, and the conductance was determined according to the equation $g = 1/(V - V_o)$, where $I$ is the single channel current, $V$ is the voltage, and $V_o$ is the reversal potential. The single channel open probability was estimated as $P_o = R_o/(R_o + R_c)$, where $R_o$ and $R_c$ stand for the areas under the current-amplitude histogram corresponding to close and open states, respectively.

Excitotoxicity Assay—Cell survival assays were performed using dissociated hippocampal cultures maintained in vitro for 7 days (in glass-bottomed 96-well plates, which are well suited for pharmacological experiments) or for 12 days (in cloning cylinders on glass coverslips, which are superior in comparison to plates in terms of cell survival after 9 days in vitro). The conditioned culture medium (70 μl/well) was removed from wells and saved for the later use (see below). Compounds used during the induction of excitotoxicity were applied in Lockes buffer (154 mM NaCl, 5.6 mM KCl, 10 mM CaCl$_2$, 3.6 mM NaHCO$_3$, 5 mM HEPES, and 5.5 mM glucose, pH 7.4. Lockes buffer (70 μl) alone or Lockes buffer containing the compounds of interest was then added to and immediately removed from the cells twice. A further 70 μl of the respective solutions was then added, and the cells were returned to the incubator for 10 min. For washing, solutions were exchanged 3 times by replacing 70% of the total volume at each step to avoid drying out the cells during the washing procedures and to obtain a >95% solution exchange after the last step. After this, the conditioned medium (saved as culture supernatant) was returned to the cells. Cell survival was measured 20–24 h after treatment using the live cell marker, calcein (0.5 μM, Molecular Probes, Eugene, OR), and the dead cell marker, propidium iodide (3 μM, Sigma-Aldrich). Three visual fields were counted per well, and there were three wells per treatment group per culture preparation. Excitotoxicity was calculated as the ratio of dead cells to total cells. The t test was used to determine the statistical differences between the experimental groups.

RESULTS

Reduction of NMDAR Currents by PSA in Cultures of Dissociated Hippocampal Neurons—Whole-cell voltage clamp recordings were used to measure the NMDAR currents in pyramidal-like neurons from 12–20-day-old cultures (Fig. 1A). The currents were evoked by the application of glutamate (3 μM) and recorded at different membrane potentials from −100 to +60 mV. Because previous experiments showed an effect of 40 μg/ml bacterially produced PSA, colicin acid, on AMPA receptor currents in hippocampal neurons (23), we tested whether this concentration of colicin acid would affect NMDAR activity. Co-application of colicin acid with glutamate reduced the NMDAR currents by ~30% ($n = 40$, $F_{1.312} = 60.34, p < 0.000001$) at all tested membrane potentials (Fig. 1B). After wash-out of colicin acid, the NMDAR currents in seconds returned to control levels (Fig. 1A).

To verify that the reduction of NMDAR currents was specific for colicin acid, a series of control experiments was performed. First, to show that the effect was due to PSA and not
impurities in the sample. Polymers of sialic acid with 25–50 residues were purified. Co-application of the purified PSA (10 μg/ml) with glutamate produced a reduction of NMDAR currents of ~20% (n = 4, F_{8,24} = 4.28, p < 0.005) in comparison to glutamate alone. This effect was not different from the effect of unpurified colominic acid (Fig. 1D).

Because PSA is a polymer of sialic acid, there was the possibility that the effect was not specific for the polymeric and highly negatively charged PSA but could also be induced by equally overall negatively charged monomers. To investigate this possibility, sialic acid monomers (40 equiv overall negatively charged monomers) were co-applied with glutamate. This, however, did not produce any significant change in the glutamate-induced NMDAR currents compared with glutamate alone (n = 4, F_{8,24} = 2.12, p > 0.05, Fig. 1D). As another control, colominic acid was treated with the enzyme endo-N, which is known to specifically cleave α2,8-linked PSA. Endo-N-treated colominic acid did not change NCAM-Fc (10 g/ml), containing the extracellular domain of NCAM in fusion with the Fc portion of human IgG, reduced NMDAR currents that were not different from those induced by glutamate alone (n = 6, F_{8,56} = 0.98, p > 0.05, Fig. 1D). Thus, the influence of colominic acid on NMDARs is specific to the unique PSA polymer of sialic acid.

Because these experiments were performed using a bacterial-produced PSA, we next addressed the question of whether mammalian PSA associated with NCAM would affect NMDARs. Co-application of glutamate with mouse PSA-NCAM-Fc (10 μg/ml), containing the extracellular domain of NCAM in fusion with the Fc portion of human IgG, reduced NMDAR currents in the same manner as colominic acid (n = 7, F_{8,48} = 19.38, p < 0.000001). Application of non-polysialylated NCAM-Fc (10 μg/ml) did not affect NMDAR currents (n = 3, F_{8,16} = 2.41, p > 0.05). Thus, PSA carried by NCAM, but not the NCAM protein backbone that carries glycans chains not related to PSA, inhibits NMDAR currents (Fig. 1D).

Pharmacological and Fluctuation Analyses of the Effects of PSA on NMDARs—Because divalent cations forming a complex with PSA may modify properties of the PSA chains (35, 36) and regulate the activity of NMDARs (12, 37), we tested whether modulation of NMDAR currents by colominic acid depends on the extracellular concentrations of either Ca^{2+} or Mg^{2+}. Therefore, the effects of colominic acid on NMDAR currents were compared in the same cells for two Ca^{2+} concentrations, 0 or 2 mM, or for two Mg^{2+} concentrations, 0.5 or 1.5 mM (Fig. 2A). There was no difference in modulation of NMDAR currents by colominic acid under these conditions. These experiments suggest that association of the negatively charged PSA with positively charged divalent cations is not critical for the effects of PSA on NMDARs. Also, the addition of the NMDAR agonist spermidine (100 μM) or omitting 2 mM glycine normally included in the perfusion solution did not alter the inhibition of NMDAR currents by colominic acid (data not shown). However, increasing the concentration of glutamate from 3 to 30 μM abolished the inhibition of NMDAR currents by colominic acid (Fig. 2A), suggesting that PSA acts as a competitive antagonist, possibly at the glutamate binding site.

To investigate whether PSA reduces the conductance of NMDARs or the number of functional channels and probability of NMDAR channel opening, a fluctuation analysis was performed. Only recordings with a low level of background noise (S.D. < 2.5 pA) were selected for this analysis to provide a reliable estimate of the coefficient of current variation. Fig. 2B presents a summary of experiments performed at the two concentrations of colominic acid, 0.01 and 40 μg/ml, and 10 μg/ml PSA-NCAM-Fc, showing the relationship between changes in glutamate-induced current (I, recorded at −60 mV), and CV^2, where CV is the coefficient of current variation (Fig. 2B). Obvi-

FIGURE 3. Inhibition of NR2B-containing NMDAR by application of PSA but not by cell surface-associated PSA. A, NMDAR currents evoked by glutamate (3 μM, n = 6, filled squares) are inhibited by colominic acid (40 μg/ml, F_{8,48} = 7.94, p < 0.00001, open squares) and ifenprodil (10 μM, F_{4,48} = 11.42, p < 0.000001, filled circle). Furthermore, ifenprodil (ifen) occludes the inhibition by colominic acid (F_{8,48} = 1.49, p > 0.05, open triangles). Data represent the mean ± S.E. Insets, representative recordings showing glutamate-evoked NMDAR currents in neurons (glu). Co-application of colominic acid (glu + CA), ifenprodil (glu + ifen), and both ifenprodil and colominic acid (glu + ifen + CA) inhibits the NMDAR currents. The horizontal bar indicates application of glutamate. B, current densities for total NMDAR currents, non-NR2B NMDAR currents, and NR2B NMDAR currents in control, wild type (NCAM+/+, n = 14), endo-N-treated NCAM−/− (n = 12, F_{8,192} = 0.26, p < 0.05, F_{9,192} = 0.07, p > 0.05, F_{9,192} = 0.25, p > 0.05), and NCAM-deficient (NCAM−/−) neurons (n = 8, F_{8,160} = 6.26; ***p < 0.00001, F_{1,165} = 2.47; **p < 0.05, F_{9,165} = 4.24; ***p < 0.0001). NMDAR currents are normal in endo-N-treated cultures but are increased in NCAM−/− neurons. n.P, picofarads. C, similar levels of inhibition of NMDA receptor currents at +60 mV in NCAM+/+, endo-N-treated NCAM−/−, and NCAM−/− neurons by colominic acid (40 μg/ml, F_{1,165} = 6.25, p > 0.05) and Ro 25-6981 (0.5 μM, F_{1,20} = 0.16, p > 0.05).
viously, the ratio between amplitudes of currents recorded before and after application of 0.01 μg/ml colominic acid is close to 1, i.e. there is no effect of the glycan, in contrast to a strong reduction of current amplitude elicited by 40 μg/ml PSA-NCAM-Fc. Because values of parameters are grouped around the line of identity \( y = x \), one can conclude that reduction in the current amplitude is mostly mediated by a decrease in number of functional channels.

The NR2B Subunit Is Necessary for Inhibition of NMDA Receptors by PSA—The results derived by the fluctuation analysis and the fact that PSA only partially inhibited NMDAR currents suggest that PSA inhibits a fraction of NMDARs at the cell surface. To test whether NMDARs containing NR2B subunit are affected by PSA, the NR2B specific antagonist, ifenprodil (10 μM), was applied to cultures of hippocampal neurons. Ifenprodil reduced NMDAR currents by \( \approx 40\% \) \((n = 6, F_{8.40} = 11.42, p < 0.000001)\), which is a level of inhibition similar to that generated by PSA when applied to these cells (Fig. 3A). Furthermore, ifenprodil fully occluded the inhibition of NMDAR currents elicited by colominic acid (Fig. 3A).

Similarly, another potent antagonist of NR2B-containing NMDARs, Ro 25-6981 (0.5 μM), mimicked the effects of colominic acid (Fig. 3C).

Data described so far provide evidence that exogenous soluble PSA and PSA-NCAM inhibit NMDARs. Next, we asked whether endogenous PSA-NCAM expressed by hippocampal neural cells may regulate the activity of NMDARs. We used endo-N as the most specific treatment to remove PSA. As a complementary approach, we used neurons derived from NCAM-deficient mice (38). We, thus, treated wild type neurons with endo-N or used neurons derived from NCAM-deficient mice to estimate the total, ifenprodil-sensitive (NR2B subunit-mediated) and insensitive (NR2A subunit-mediated) NMDAR current components in neurons deficient in PSA or both polysialylated and non-polysialylated forms of NCAM. There was no difference between control and endo-N-treated neurons in the amplitude of the total, ifenprodil-sensitive and insensitive components of NMDAR currents (Fig. 3B), showing that removal of PSA does not affect NMDAR currents. However, the magnitudes of the

![Sample traces show currents from NMDA receptors reconstituted in lipid bilayers, activated by 2 μM glutamate and voltage-clamped at +104 mV. Channel activity is evident by upward transitions of the current representing the open state. Respective amplitude histograms, with peaks corresponding to the closed and open states are shown to the left of each sample trace.](image)

**FIGURE 4.** PSA reduces open probability of NR2B-containing synaptosomal NMDA receptors. Sample traces show currents from NMDA receptors reconstituted in lipid bilayers, activated by 2 μM glutamate and voltage-clamped at +104 mV. Channel activity is evident by upward transitions of the current representing the open state. Respective amplitude histograms, with peaks corresponding to the closed and open states are shown to the left of each sample trace. A, sampled recordings of single channel activity in the presence of 2 μM glutamate. B–D, decline in single channel open probability in the presence of 1, 2, and 3 μg/ml colominic acid, respectively. E, full blockade of residual activity of the NMDAR channel by the NR2B subunit-specific antagonist Ro 25-6981. F, sampled recordings of single channel activity in the presence of 2 μM glutamate. G, neither single channel open probability nor conductance of NR2B subunit containing NMDA receptors was significantly altered in the presence of 10 μg/ml chondroitin sulfate (CS). H, Ro 25-6981 completely blocked the NMDAR currents that were not affected by chondroitin sulfate, confirming that the recorded channel contained the NR2B subunit.
channel conductance (see Fig. 6, A and B). When colominic acid was applied to NMDARs at 0.01 and 0.1 μg/ml, it produced no significant effects (see Fig. 6, A and B). Also chondroitin sulfate (even at 10 μg/ml) did not affect activity of NMDARs, confirming the specificity of PSA action (see Fig. 6, A and B).

Because experiments with hippocampal neurons revealed that PSA inhibits only NMDARs activated by low glutamate concentrations, we investigated this aspect also using single-channel recordings. Application of glutamate at increasing concentrations (2, 4, and 6 μM) to a single channel in the presence of colominic acid (3 μg/ml) resulted in restoration of channel openings (see Fig. 6C). The single channel conductance was not affected at any tested concentration of glutamate (see Fig. 6D). In summary, these data demonstrate that PSA affects gating of NR2B-containing NMDARs at low glutamate concentrations.

To verify whether effects of PSA are specific for NR2B-containing NMDARs, we performed analysis of single channel activity in the presence of Ro 25-6981 (Fig. 5A). There were no significant effects of colominic acid on opening and conductance of Ro 25-6981-insensitive channels (Figs. 5B and 6, E and F). Because these channels were blocked by dl-2-amino-5-phosphonopentanoic acid (APV) but not Ro 25-6981, they are NMDARs that lack the NR2B subunit. Thus, PSA specifically inhibits NR2B-containing NMDARs.

PSA Reduces Excitotoxic Cell Death Induced by Low Micromolar Concentrations of Glutamate—To measure the proportion of live cells in cultures, dead cells were labeled by propidium iodide and live cells were labeled by calcein, and the ratio between the number of live cells to the total number was calculated. Exposure of hippocampal cultures to 5 or 30 μM glutamate for 10 min induced a 20 and 40% increase, respectively, in cell death within 24 h (Fig. 7, A–C). We chose these concentrations of glutamate since colomicin acid significantly inhibited NMDAR currents activated by 5 μM glutamate but failed to inhibit NMDAR currents activated by 30 μM glutamate (Fig. 2A). Application of colomicin acid, the general NMDAR antagonist APV, or the antagonist of NR2B-containing NMDARs, Ro 25-6981, abolished the excitotoxic effect of 5 μM glutamate (Fig. 7B). Although both APV and Ro 25-6981 also abolished the excitotoxic effect of 30 μM glutamate, colomicin acid failed to do so (Fig. 7C). These results show that glutamate-induced excitotoxic cell death depends on NMDARs containing the NR2B subunit and that colomicin acid is able to prevent this effect at low glutamate concentrations. The failure of colomicin acid to inhibit the excitotoxicity induced by a higher concentration of glutamate is in accordance with the electrophysi-
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![Graphs](image)

**FIGURE 6. Inhibitory effects of PSA on open probability of NR2B-containing synaptosomal NMDA receptors are PSA- and glutamate concentration-dependent.** A, concentration-dependent effect of CA on open probability ($P_o$) of NR2B-containing synaptosomal NMDARs in the presence of 2 μM glutamate. Bars represent the mean ± S.E. CA significantly reduces $P_o$ at concentrations ≥1 μg/ml (**, $p < 0.0001; n = 10$). There is no change in $P_o$ in the presence of lower concentrations of CA ($p > 0.1, n = 5$) or chondroitin sulfate (CS: $p > 0.1; n = 5$). B, CA and chondroitin sulfate do not significantly alter the single channel conductance of NR2B subunit-containing NMDARs ($p > 0.1; n = 10$ for CA and $n = 5$ for chondroitin sulfate). C, inhibitory effect of CA on $P_o$ of NR2B-containing synaptosomal NMDARs depends on glutamate concentration; the reduction of $P_o$ is many times smaller in the presence of 6 μM glutamate than at the presence of 2 μM glutamate (**, $p < 0.001$; ***, $p < 0.0001$, $n = 5$). D, CA does not alter single channel conductance of NR2B subunit-containing NMDARs in the presence of 2, 4, and 6 μM glutamate ($p > 0.1; n = 5$). E, CA did not affect the open probability of NR2B-lacking synaptosomal NMDARs activated by 2 μM glutamate in the presence of 1 μM Ro 25-6981 ($p > 0.1; n = 5$). F, CA does not alter single channel conductance of NR2B-lacking NMDA receptors ($p > 0.1; n = 5$).

In vitro induced cell death that could be prevented by colominic acid, Ro 25-6981 or APV (Fig. 7D).

To investigate the role of endogenous PSA in cell survival, hippocampal cultures were pretreated with endo-N for 3 h before exposure to glutamate. Endo-N pretreatment by itself increased cell death to a level similar to that observed after application of 5 μM glutamate (Fig. 7E). Pretreatment of cultures with endo-N occluded the induction of further cell death by 5 μM glutamate and its block by co-application of colominic acid (Fig. 7E). To investigate if survival-promoting effects of endogenous PSA are mediated by inhibition of NR2B-containing NMDARs, we tested whether co-application of Ro 25-6981 would reverse the effect of endo-N. In accordance with this notion, co-application of neurons with endo-N and Ro 25-6981 restored neuronal cell survival to control levels, which was the survival seen in the absence of additives (Fig. 7E).

**DISCUSSION**

Our study shows that both bacterially produced PSA (colominic acid) and eukaryotically produced PSA-NCAM specifically inhibit the NR2B subunit-containing NMDARs of cultured hippocampal neurons. Furthermore, PSA inhibits the activity of single synaptosomal NR2B-containing NMDARs reconstituted in lipid bilayers. Because 10 μM ifenprodil occluded effects of PSA in hippocampal neurons, it is likely that PSA acts on NR1/NR2B receptors rather than on triheteromeric NR1/NR2A/NR2B receptors that are more resistant to ifenprodil (39). Effects of PSA are prominent at the rather low concentration of 1 μg/ml that corresponds to 44 nM, assuming a chain length of 73 sialic acid residues that is the estimated average size of colominic acid used in this study. Because the inhibition of NMDARs by PSA is immediate and similar in hippocampal neurons and lipid bilayers, we take this as an indication that PSA may act directly on NMDARs. The same concentrations of PSA as used in the present study have been previously shown to potentiate purified AMPA receptors reconstituted in lipid bilayers and AMPA receptor-mediated currents in cultured astrocytes and immature hippocampal neurons (23). The fact that PSA inhibited NMDAR currents at lower but not at higher concentrations of glutamate suggests that PSA competes with glutamate in binding to positively charged amino acids in the S1 and S2 extracellular domains of NR2 subunits, which form the glutamate binding site of NMDARs (40). Computer modeling predicts that there are only 4–6 NR2 subunit-specific amino acid residues exposed to the glutamate binding pocket of NR2 subunits (41). These residues are located at the edge of the glutamate binding pocket, indicating that only larger sized antagonists may provide subtype-specific inhibition of NMDARs. This notion is in line with our observation that only polymers but not monomers of sialic acid inhibited NR2B-containing NMDARs. Because inhibition of NMDARs by exogenous PSA was observed in endo-N-treated and NCAM-deficient neurons, it is not mediated by neuronal cell surface-expressed PSA-NCAM or NCAM. Although the data obtained, particularly in artificial lipid bilayers, suggest that PSA directly interferes with binding of glutamate to NMDARs, we cannot exclude the possibility that this influence is indirect, for instance, via interaction with a lipid membrane.
FIGURE 7. Application of PSA and endogenous PSA inhibits excitotoxicity. A, representative images showing live cells marked by calcein (upper row) and dead cells identified by propidium iodide (lower row) under non-treated conditions (Control), after treatment with glutamate (5 μM), and after co-treatment with 5 μM glutamate and 40 μg/ml colominic acid. Images in one column were taken at the same position. B, neural cell survival on the 7th day in vitro under control conditions (control, 0.74 ± 0.03, number of culture preparations n = 11) and after treatments with 5 μM glutamate (glu, 0.52 ± 0.03, n = 8; ****, p < 0.0001 as compared with control, here and everywhere below), 5 μM glutamate, and 40 μg/ml colominic acid (glu + CA, 0.69 ± 0.05, n = 5, p > 0.1), 5 μM glutamate, and 0.5 μM Ro 25-6981 (glu + Ro, 0.71 ± 0.04, n = 4, p > 0.1), and 5 μM glutamate and 50 μM APV (0.77 ± 0.06, n = 3, p > 0.1). Note that colominic acid, APV, or Ro 25-6981 rescued neural cell survival. C, neural cell survival on the 7th day in vitro under control conditions (Control, 0.74 ± 0.03, n = 11) and after treatments with 5 μM glutamate (glu, 0.32 ± 0.03, n = 9; ****, p < 0.0001), 30 μM glutamate, and 40 μg/ml colominic acid (glu + CA, 0.39 ± 0.04, n = 5, p < 0.0001), 30 μM glutamate and 0.5 μM Ro 25-6981 (glu + Ro, 0.62 ± 0.09 n = 3, p > 0.1), and 30 μM glutamate and 50 μM DL-2-amino-5-phosphonovaleric acid (glu + APV, 0.66 ± 0.05, n = 3, p > 0.1). Colominic acid failed to rescue cell survival at this concentration of glutamate, unlike APV or Ro 25-6981 rescued neural cell survival. D, neural cell survival on the 12th day in vitro under control conditions (Control, 0.80 ± 0.03, number of cultures n = 4) and after treatments with 5 μM glutamate (glu, 0.64 ± 0.04, n = 4; ****, p < 0.0005), 5 μM glutamate, and 40 μg/ml colominic acid (glu + CA, 0.81 ± 0.03, n = 4, p > 0.1), 5 μM glutamate and 0.5 μM Ro 25-6981 (glu + Ro, 0.81 ± 0.03, n = 4, p > 0.1), and 5 μM glutamate and 50 μM APV (APV, 0.85 ± 0.02, n = 4, p > 0.1). Note that colominic acid, APV, or Ro 25-6981 rescued neural cells. E, neural cell survival on the 7th day in vitro under control conditions (Control, 0.74 ± 0.03, n = 11) and after pretreatment with endo-N (Endo, 0.59 ± 0.04, n = 6; **, p < 0.01), endo-N and 0.5 μM Ro 25-6981 (Endo + Ro, 0.70 ± 0.03, n = 4, p > 0.1), endo-N followed by 5 μM glutamate (Endo/glu, 0.57 ± 0.05, n = 5; ***, p < 0.01), and endo-N followed by 5 μM glutamate plus μg/ml colominic acid (Endo/glu + CA, 0.58 ± 0.07, n = 5; *, p < 0.05). Note that Ro 25-6981 rescued neural cell survival.
near the receptor that could modify its configuration and, thus, indirectly affect ligand binding.

An increase in total, ifenprodil-sensitive and -insensitive NMDAR currents in NCAM-deficient neurons as compared with wild type neurons suggests that NCAM is involved in regulation of NR2B- and NR2A-containing NMDAR activity. Because endo-N pretreated neurons have normal NMDAR currents, this regulation appears to be PSA-independent but NCAM glycoprotein backbone-dependent. These results are supported by biochemical analyses showing that NR1 levels are increased in NCAM−/− brain homogenates, indicating that the overall expression of NMDA receptors in NCAM−/− brains is increased (21).

The observation that pretreatment of neurons with endo-N does not lead to changes in NMDAR currents demonstrates that only PSA added to neurons in the form of soluble PSA, as colominic acid or as recombinant PSA-NCAM, has access to NMDARs. Although we did not observe any influence of endo-N treatment on currents mediated by NR2B subunits in electrophysiological recordings, endo-N treatment promoted NR2B-mediated excitotoxicity. This difference between the electrophysiological and excitotoxicity assays can be accounted for by the following argument; soluble molecules are likely to be washed out in the perfusion chamber in which electrophysiological recordings are performed, whereas soluble NCAM remains present in the neural culture supernatant (42) and, thus, may interfere with glutamate-induced cell death in the closed volumes of small wells used for the excitotoxicity assay. Indeed, we were able to detect PSA by Western blotting using PSA-specific antibodies in the medium of cultures maintained for 7 days in vitro.5 This observation is noteworthy because soluble PSA-NCAM has also been detected in brain tissue and cerebrospinal fluid (43, 44). Furthermore, it is interesting in the context of the biological significance of our present observations that expression and cleavage of the extracellular domain of NCAM/PSA-NCAM is regulated by metallocproteinase activity (45) and activation of NMDARs and the plasmin/tissue plasminogen activator system (43, 46). Thus, inhibition of NMDAR by PSA-NCAM released by neuronal activity from the cell surface may provide feedback to reduce NMDAR currents and excitotoxic damage of the activated neurons.

Our analysis of glutamate-induced excitotoxicity revealed that application of PSA prevents cell death, whereas removal of neuronal cell surface-expressed PSA promotes cell death, occluding the excitotoxic effects of glutamate. These results suggest that it is the same population of neural cells that is susceptible to 5 μM glutamate and endo-N-induced cell death. It is noteworthy in this respect that removal of PSA with endo-N has previously been shown to reduce survival of cultured cortical neurons due to reduced brain-derived neurotrophic factor-mediated signaling (26). Supplementation of culture medium with an excess of brain-derived neurotrophic factor in these experiments was able to rescue survival of endo-N treated cortical neurons. Under our experimental conditions, however, the addition of brain-derived neurotrophic factor to endo-N-treated hippocampal neurons did not affect endo-N-induced cell death,5 suggesting that other mechanisms are operant.

Excitotoxicity contributes to neuronal degeneration in many traumatic insults to the nervous system, such as ischemia, amyotrophic lateral sclerosis, and epilepsy (47). Excitotoxic activity of NR2B-containing NMDARs is likely to mediate selective neurodegeneration of striatal medium-sized spiny projection neurons in Huntington disease (48), neurotoxicity associated with alcohol-withdrawal (49), and neuronal cell death after transient cerebral ischemia (50). A recent study highlights the particular vulnerability of immature (1-week-old neurons, as in the present study) neurons to NMDA-induced toxicity, which is caused by activation of NR2B- but not NR2A-containing NMDARs (51). Because PSA inhibits NR2B-containing NMDARs and is highly expressed during early development and up-regulated during synaptic activity (3, 52), our present results suggest that PSA is well in place to prevent excitotoxic neuronal cell death during development and under pathological conditions, resulting in glutamate release, at least in cases when glutamate is accumulated in the extracellular space at low micromolar concentrations. These concentrations are physiological and found in normal and epileptic brains but may be far exceeded during transient cerebral ischemia (53–56).

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