Development/Plasticity/Repair

Polysialylated Neural Cell Adhesion Molecule Promotes Remodeling and Formation of Hippocampal Synapses

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Expression of the neural cell adhesion molecule (NCAM) has been shown to promote long-term potentiation (LTP) and stabilization of synapses during early synaptogenesis. Here, we searched for the mechanisms of synaptogenic activity of NCAM, focusing on the role of polysialic acid (PSA), an unusual carbohydrate preferentially associated with NCAM. We show that enzymatic removal of PSA with endoneuraminidase-N (endo-N) abolished preferential formation of synapses on NCAM-expressing cells in heterogenotypic cocultures of wild-type and NCAM-deficient hippocampal neurons. Transfection of NCAM-deficient neurons with either of three major NCAM isoforms (different in intracellular domains but identical in extracellular domains and carrying PSA) stimulated preferential synapse formation on NCAM isoform-expressing neurons. Enzymatic removal of heparan sulfates from cultured neurons and a mutation in the heparin-binding domain of NCAM diminished synaptogenic activity of neuronally expressed PSA-NCAM, suggesting that interaction of NCAM with heparan sulfate proteoglycans mediates this activity. PSA-NCAM-driven synaptogenesis was also blocked by antagonists to fibroblast growth factor receptor and NMDA subtype of glutamate receptors but not by blockers of non-NMDA glutamate receptors and voltage-dependent Na^+ channels. Enzymatic removal of PSA and heparan sulfates also blocked the increase in the number of perforated spine synapses associated with NMDA receptor-dependent LTP in the CA1 region of organotypic hippocampal cultures. Thus, neuronal PSA-NCAM in complex with heparan sulfate proteoglycans promotes synaptogenesis and activity-dependent remodeling of synapses.

Key words: **PSA; NCAM; adhesion; hippocampus; long-term potentiation; perforated synapses; structural plasticity; synaptogenesis**

Introduction

Dynamic changes in the structural characteristics of excitatory synapses are thought to underlie some forms of synaptic plasticity and learning and memory. These structural changes depend on rearrangements of cell-to-cell and extracellular matrix-to-cell interactions mediated by recognition molecules. The neural cell adhesion molecule (NCAM) and its putative invertebrate homologs, fasciclin II in *Drosophila* and apCAM in *Aplysia*, have been shown to be important for many processes that require cell–cell and cell–substratum interactions such as neurite growth, cell migration, and synaptic plasticity (Mayford et al., 1992; Davis et al., 1997; Schachner, 1997). Recent studies revealed that NCAM is involved in both long-term potentiation (LTP) and synaptogenesis. Constitutively and conditionally, NCAM-deficient mice exhibit impaired LTP in the CA1 and CA3 areas of the hippocampus (Muller et al., 1996, 2000; Cremer et al., 1998; Bukalo et al., 2004). The percentage of spines postsynaptically expressing the major isoform of NCAM with the longest cytoplasmic domain, NCAM180, is strongly increased after induction of LTP in the molecular layer of the dentate gyrus of adult rats (Schuster et al., 1998; Fux et al., 2003). Higher amplitudes of unitary EPSCs and higher levels of glutamate-induced potentiation and synaptic density, measured either as mean intensity of synaptophysin immunofluorescence or as the number of synaptophysin-rich spots, were observed in NCAM-expressing neurons compared with NCAM-deficient cells (Dityatev et al., 2000). Interestingly, the synaptogenic effect of NCAM was revealed only when neurons were maintained in a choice situation presented in heterogenotypic cocultures of wild-type and NCAM-deficient cells, where axons could select between wild-type and NCAM-deficient postsynaptic targets (Dityatev et al., 2000).

During early synaptogenesis at days 3 and 4 *in vitro*, NCAM is clustered in the plasma membrane, and these clusters are associated with trans-Golgi network (TGN)-derived organelles. These complexes are translocated along neurites and trapped at sites of initial neurite-to-neurite contacts, favoring accumulation of TGN organelles via heterophilic and possibly homophilic NCAM-mediated trans-interactions between contacting neurons (Sytnyk et al., 2002). Also, heterophilic interactions appear to be important for neurons maintained for 7 d *in vitro* (Dityatev et al., 2000). The interesting outcome of these two studies was that at later developmental stages, only postsynaptically localized

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NCAM is important for synaptogenesis. These observations imply that in addition to the primary stabilization of TGN organelles in the presence of NCAM either presynaptically or postsynaptically, there may be subsequent synapse selection mechanisms, which preferentially stabilize synapses expressing NCAM postsynaptically. These mechanisms and the structural determinants mediating synaptogenic activity of postsynaptic NCAM are currently unknown.

Reports indicate that an unusual carbohydrate α2,8-linked polysialic acid (PSA), which appears to be exclusively associated with NCAM, is required for correct axonal branching and fasciculation in the peripheral nervous system (Rutishauser and Landmesser, 1996), lamination, and synaptogenesis of mossy fibers (Cremer et al., 1997; Seki and Rutishauser, 1998), structural plasticity in the hypothalamic-pituitary system (Theodosis et al., 1999), migration of neural precursor cells to the olfactory bulb (Hu et al., 1996; Chazal et al., 2000), synaptic plasticity in the CA1 region of the hippocampus (Becker et al., 1996; Muller et al., 1996; Eckhardt et al., 2000), spatial learning (Cremer et al., 1994; Becker et al., 1996), and circadian rhythm (Shen et al., 1997; Prosser et al., 2003). Here, we used heterogenotypic cultures containing wild-type and NCAM-deficient neurons to study the mechanisms underlying NCAM-driven preferential synapse formation. We could show that PSA, the interaction between PSA-NCAM and heparan sulfate proteoglycans, and activation of NMDA and fibroblast growth factor (FGF) receptors are required for this process. Furthermore, both PSA and heparan sulfates are necessary for activity-dependent remodeling of synapses after induction of LTP.

Materials and Methods

DNA constructs. Rat NCAM140 and rat NCAM180/pcDNA3 were gifts from P. Maness (University of North Carolina, Chapel Hill, NC). Rat NCAM120 (gift from E. Bock, University of Copenhagen, Copenhagen, Denmark) was subcloned into the pcDNA3 vector (Invitrogen, San Diego, CA) using two *Eco*RI sites. The NCAMΔHBD (heparin-binding domain) construct with the mutated heparin-binding domain was produced as described previously (Reyes et al., 1990) with slight modifications. In brief, the Quikchange site-directed mutagenesis kit (Stratagene, La Jolla, CA) was used to mutate amino acids at the following positions: Lys 153 → Asn, Arg 155 → Leu, Lys 160, 161 → Ile. The mutant was constructed by the use of the following primers: 5′aaa cac aat gg cta gat gtc atc ctg ata ata gat gtc3′, 5′gac atc tat tat cag gaat gac atc tag gcc att gtg ttt3′. Mutations were performed on a *Hin*dIII/*Eco*RI subfragment representing the extracellular domain of NCAM180 and verified by DNA sequencing. The mutated *Hin*dIII/*Eco*RI fragment of NCAM180 was reintroduced into the pcDNA3 vectors containing the 3′ *Eco*RI/*Xho*I subfragment of NCAM180. For visualization of neurons, the enhanced green fluorescent protein (EGFP) plasmid (Clontech, Palo Alto, CA) was used.

Heterogenotypic cocultures of hippocampal neurons. Preparation of cocultures was done as described previously (Dityatev et al., 2000). *NCAM*$^{-/-}$ mice were obtained from H. Cremer (Developmental Biology Institute of Marseille, Marseille, France) (Cremer et al., 1994) and backcrossed to C57BL/6J mice for eight generations. Age-matched C57BL/6J mice served as controls. The hippocampi of 1- to 3-d-old mice were isolated, cut into small pieces, and treated with trypsin (6 mg/1.8 ml) and DNase I (1.5 mg/1.8 ml) in Ca^{2+}- and Mg^{2+}-free HBSS. After dissociation, the cells derived from wild-type and *NCAM*$^{-/-}$ mice were plated at 1:1 ratio at a density of 350 cells/mm^2 on glass (Assistent, Sondheim, Germany) coated with poly-L-lysine (100 μg/ml) and laminin (20 μg/ml). For the first 2 d, cells were maintained in culture medium (Eagle's MEM containing 5 gm/l glucose, 2 mM glutamax I, 100 μg/ml bovine transferrin, 100 μg/ml insulin, 5 μg/ml gentamycin, and specifically tested 10% horse serum). Starting from the third day, gentamycin was omitted, and the concentration of serum was reduced to 5%. The culture medium was then supplemented with 5 μM cytosine-β-arabinofuranoside and 2% B-27 supplement (Invitrogen).

Pharmacological treatment of heterogenotypic cocultures. All pharmacological treatments were done during exchange of culture medium on days 3 and 5 after plating. Neurons were analyzed after being maintained 6 d *in vitro*. Mouse PSA-NCAM-Fc, composed of the extracellular domain of NCAM and Fc portion of human IgG, was produced according to the study by Vutskits et al. (2001) using a stably transfected TE671 cell line kindly provided by G. Rougon (Laboratoire de Genetique et Physiologie du Developpement, Marseille, France). Mouse NCAM-Fc was produced using stably transfected Chinese hamster ovary cells as described previously (Chen et al., 1999). Recombinant endoneuraminidase-N (endo-N) (Gerardy-Schahn et al., 1995) was used at 0.2 μg/ml to remove PSA from cultured neurons. The efficacy of treatment was confirmed by a loss of PSA immunoreactivity in the treated cultures using the monoclonal antibody to PSA (clone 735) (Frosch et al., 1985). To digest heparan sulfates, heparinases I and III (both from Sigma, St. Louis, MO) were applied at concentrations of 0.5 U/ml (Storms and Rutishauser, 1998). Removal of chondroitin sulfate was achieved by treatment with chondroitinase ABC (chondroitin ABC lyase, EC4.2.2.4, 0.25 U/ml) from *Proteus vulgaris* (Seikagaku, Tokyo, Japan) as described previously (Bukalo et al., 2001).

Other chemicals that were applied to the cocultures are as follows: the antagonist to the NMDA subtype of glutamate receptors AP-5 (50 μM; Sigma), the antagonist to non-NMDA subtypes of glutamate receptors 2,3-dihydroxy-6-nitro-7-sulfonyl-benzo[*f*]quinoxaline (NBQX) (10 μM; BioTrend, Cologne, Germany), the blocker of voltage-gated Na^+ channels tetrodotoxin (TTX) (1 μM; Alamone Labs, Jerusalem, Israel), the inhibitor of MAPK (mitogen-activated protein kinase) kinase 1 PD98059 (2′-amino-3′-methoxyflavone) (2 μM; Calbiochem, La Jolla, CA), and the antagonist to FGF receptors PD173074 (1-tert-butyl-3-[6-(3,5-dimethoxy-phenyl)-2-(4-diethylamino-butylamino)-pyrido [2,3-d]pyrimidin-7-yl]-urea) (50 nM).

Transfection of hippocampal neurons. Hippocampal neurons were transfected 24 hr after seeding using the calcium phosphate transfection method (Xia et al., 1996). Before transfection, the cells were incubated for 1 hr in 3% CO_2 at 35°C in culture medium without serum. The DNA/calcium phosphate precipitate was prepared using the Mammalian Transfection Kit (Stratagene) following the instructions of the manufacturer. Fifty microliters of DNA/calcium phosphate precipitate was added to 1 ml of the medium without serum, mixed, and added to the cultures for 3 hr in 3% CO_2 at 35°C. The incubation was stopped by treating the cells for 1–2 min with 5% glycerol in the culture medium without serum. Cells were then washed three times and returned to 5% CO_2 and 37°C. For cotransfection, the plasmid DNAs coding for NCAM and EGFP were mixed at a ratio of 2:1.

Immunocytochemistry and measurement of synaptic density. The cultures were briefly washed with PBS, pH 7.3, fixed with 4% paraformaldehyde in PBS, and treated for 20 min with blocking solution [PBS containing 2% bovine serum albumin (BSA)]. NCAM rabbit polyclonal antibodies (30 μg/ml) (Lüthi et al., 1994) and secondary anti-rabbit IgG antibodies coupled to cyanine 3 (Cy3) (diluted 1:100; Dianova, Hamburg, Germany) were consecutively applied for 30 min at room temperature. Washing (four times for 5 min) followed each application of antibodies. After staining with secondary antibodies, the cultures were fixed with 2% paraformaldehyde in PBS for 10 min and permeabilized by PBS containing 0.2% Triton X-100 and 2% BSA. Monoclonal mouse synaptophysin antibodies (1:2; Camon, Wiesbaden, Germany) and secondary anti-mouse IgG antibodies coupled to Cy5 (1:100) were consecutively applied for 60 and 45 min, respectively. Finally, the cultures were embedded in Aqua-Poly/Mount (Polyscience, Eppelheim, Germany). Well-isolated cells with distinguishable, nonfasciculating neurites were selected for analysis without knowing the type of pharmacological treatment and the distribution of synaptophysin immunostaining. The thick tapering neurites, most likely dendrites, were outlined starting from the origin to the second and third order of branching using high-resolution (pixel size, 0.22 μm) images. The mean value of immunofluorescence within outlined areas was used for the analysis. Previously, we verified that this measure provides the same information as counting of

synaptophysin-rich spots (Dityatev et al., 2000). Identity of dendrites was confirmed in a few experiments by immunostaining with rabbit anti-microtubule-associated protein 2 polyclonal antibodies (Chemicon, Temecula, CA). Synapses on somata were not analyzed. Neurons selected for analysis had a similar local environment, as measured by the number of cells per frame that was on average 4.5–5.5 in all experiments. There was no significant difference in the numbers of somata per frame, which were counted for all experimental groups. Mean intensity of synaptophysin staining within a traced area of neurites after subtraction of background fluorescence was used to assess synaptic density. Each pharmacological treatment–transfection was repeated two to four times. For each culture preparation, ~20 images per 60 neurons were analyzed per group.

Double immunostainings for NCAM and PSA or NCAM and Fc were performed without cell permeabilization, as for single NCAM immunostaining. The monoclonal antibody 735, followed by the appropriate secondary antibody, and anti-human IgG Cy5-coupled antibody (1:100; Dianova) were used to visualize PSA and Fc, respectively.

Labeling of active synapses by FM4–64 loading. For these experiments, cells were maintained in Corning dishes (35 mm in diameter) with a glass bottom (MatTek Corporation, Ashland, MA, USA). The staining procedure was similar to the one used by Ahmari et al. (2000). Cultures were exposed to 15 μM FM4–64 *N*-(3-triethylammoniumpropyl)-4-(6-(4-(diethylamino)phenyl)hexatrienyl pyridinium dibromide in a stimulating solution containing the following (in mM): 31.5 NaCl, 90 KCl, 5 HEPES, 1 $MgCl_2$, 2 $CaCl_2$, and 30 mM glucose for 1 min in a CO_2 incubator. The washing and imaging of neurons was performed in culture medium containing 10 μM CNQX and 50 μM DL-AP-5 to block synaptic transmission and thus avoid destaining of the synapses during imaging. Dishes were sealed in the incubator with parafilm and transferred to the heating stage of the confocal laser-scanning microscope for recording at 35°C. To show that synapses are capable of active exocytosis, they were destained by incubation of cultures in stimulating solution without FM4–64 for 1 min in a CO_2 incubator. FM4–64 fluorescence was measured in the same way as synaptophysin immunofluorescence.

Analyses of perforated synapses in hippocampal slice cultures. Hippocampal organotypic slice cultures were prepared from 6- to 7-d-old rats and maintained 10–12 d in culture as described by Stoppini et al. (1991). Endo-N was purified from phage K1 (generous gift from G. Rougon) and used at a concentration of 0.8 U/ml applied as a drop directly on top of slice cultures 12–15 hr before experiments. Previous work showed that this treatment completely and specifically eliminates PSA from NCAM (Muller et al., 1996). Heparinase treatment of slice cultures (0.5 U/ml heparinase III and 0.5 U/ml heparinase I) was performed the same way.

Synaptic responses were recorded in the CA1 area and elicited using a stimulation electrode made of twisted nichrome wires placed in the CA3 area. NMDA receptor-dependent LTP was induced using theta burst stimulation consisting of five bursts at 5 Hz with each burst composed of four pulses at 100 Hz. This pattern was applied twice at 10 sec intervals and, once again, 5 min before fixation to restimulate and relabel the same synapses (Buchs and Muller, 1996; Toni et al., 1999). Thirty minutes after LTP induction, control, endo-N-treated, and heparinase-treated cultures were fixed and processed for electron microscopy as described previously (Buchs et al., 1994). Briefly, cultures were fixed overnight at 4°C in 3% glutaraldehyde, rinsed in 0.1 M phosphate buffer, pH 7.4, and postfixed 2 hr in a fresh solution of osmium tetroxide 1% (OsO_4) with 1.5% potassium chromium trisoxalate $[K_3Cr(C_2O_4)]_3$ (Sigma), pH 9.5. This technique reveals, under the form of a fine precipitate, the presence of calcium accumulated in storage organelles (Buchs et al., 1994; Buchs and Muller, 1996). After a 5 min rinse in KOH, pH 9.5, the samples were dehydrated in ethanol and embedded in Epon (Fluka, Buchs, Switzerland). Sections were cut in the middle portion of the apical arborization of CA1 pyramidal neurons (ultratome Ultracut-E; Leica, Deerfield, IL) and stained for 45 min in 0.5% uranyl acetate and 45 sec in lead citrate and analyzed on a Philips CM10 electron microscope at a magnification of 11,000–29,100×.

Synapses were defined by the presence of a clear postsynaptic density facing at least three presynaptic vesicles and perforated synapses by the presence of a discontinuity in the postsynaptic density (Geinisman et al., 1987). For morphological analyses, five to six single sections per slice culture were examined, and all labeled synapses observed in an area corresponding to the middle third of CA1 stratum radiatum and clearly identified by the presence of a postsynaptic density and precipitate were considered for statistical measurements. These single section analyses were previously shown to provide similar values as an unbiased stereological methods (Toni et al., 1999). Data from three to six cultures (194–525 synapses) per condition were obtained, and the percentage of labeled spine synapses exhibiting a perforated postsynaptic density was calculated. Data are presented as mean ± SEM.

Results

PSA-NCAM increases synaptic coverage of neurons

Because PSA carried by NCAM has been shown to mediate many of NCAM functions, we investigated whether removal of PSA impairs NCAM-dependent synaptogenic activity in heterogenotypic hippocampal cultures, that is in cocultures of NCAM-expressing and NCAM-deficient neurons (Dityatev et al., 2000). In these cocultures, cells derived from wild-type ($NCAM^{+/+}$; 50% of cells) and NCAM-deficient ($NCAM^{-/-}$; 50% of cells) mice are plated in close contact with each other. The genotype of neurons in heterogenotypic cocultures is determined by immunocytochemical labeling with NCAM antibodies. To measure the density of synaptic contacts on postsynaptic dendrites (i.e., their synaptic coverage), we immunostained for synaptophysin, a synaptic vesicle marker enriched in synaptic boutons (Fig. 1*A*).

Mean intensities of synaptophysin immunofluorescence along neurites from $NCAM^{+/+}$ neurons (174.1 ± 12.1%) were significantly higher than along neurites from $NCAM^{-/-}$ cells (100 ± 5.6%) (Fig. 1*B*), as described previously (Dityatev et al., 2000). Enzymatic treatment with endo-N during 3–6 d after plating of cells *in vitro* abolished preferential formation of synapses on $NCAM^{+/+}$ neurons: mean intensities of synaptophysin immunofluorescence were 115.1 ± 7.9% along neurites of $NCAM^{+/+}$ neurons versus 100 ± 9.4% on neurites of $NCAM^{-/-}$ neurons (Fig. 1*B*).

Expression of all major NCAM isoforms increases synaptic coverage

In addition, we asked whether transmembrane NCAM140 or NCAM180 or glycosylphosphatidylinositol (GPI)-linked NCAM120 could differentially stimulate synaptic coverage of hippocampal neurons, for instance because of different signal transduction mechanisms stimulated by these isoforms. To address this question, we transfected cells from $NCAM^{-/-}$ mice with one of the three major NCAM isoforms. Immunostaining of transfected neurons with polyclonal antibodies recognizing all major isoforms of NCAM showed similar levels of expression of NCAM120, NCAM140, and NCAM180 in the transfected neurons. NCAM was colocalized with PSA, as shown by double immunofluorescence labeling (Fig. 2).

Transfection of $NCAM^{-/-}$ neurons with the individual NCAM isoforms results in a mixture of NCAM-expressing and nonexpressing cells because of the fact that <1% of cells take up the NCAM isoform carrying vectors. Analysis of $NCAM^{-/-}$ neurons expressing NCAM120, NCAM140, or NCAM180 revealed more synaptophysin immunoreactivity associated with neurites of transfected cells (167.9 ± 11.2, 145.8 ± 8.9, and 181.8 ± 7.4%, respectively) when compared with neurites of neighboring untransfected $NCAM^{-/-}$ neurons (set to 100 ± 7.9%) (Fig. 3).

In control experiments in which $NCAM^{-/-}$ neurons were transfected to express EGFP only (Fig. 3*B*), we observed that neurites received the same synaptic coverage as neurites from nontransfected neurons (95.2 ± 3.7 vs 100 ± 4.6%), showing that

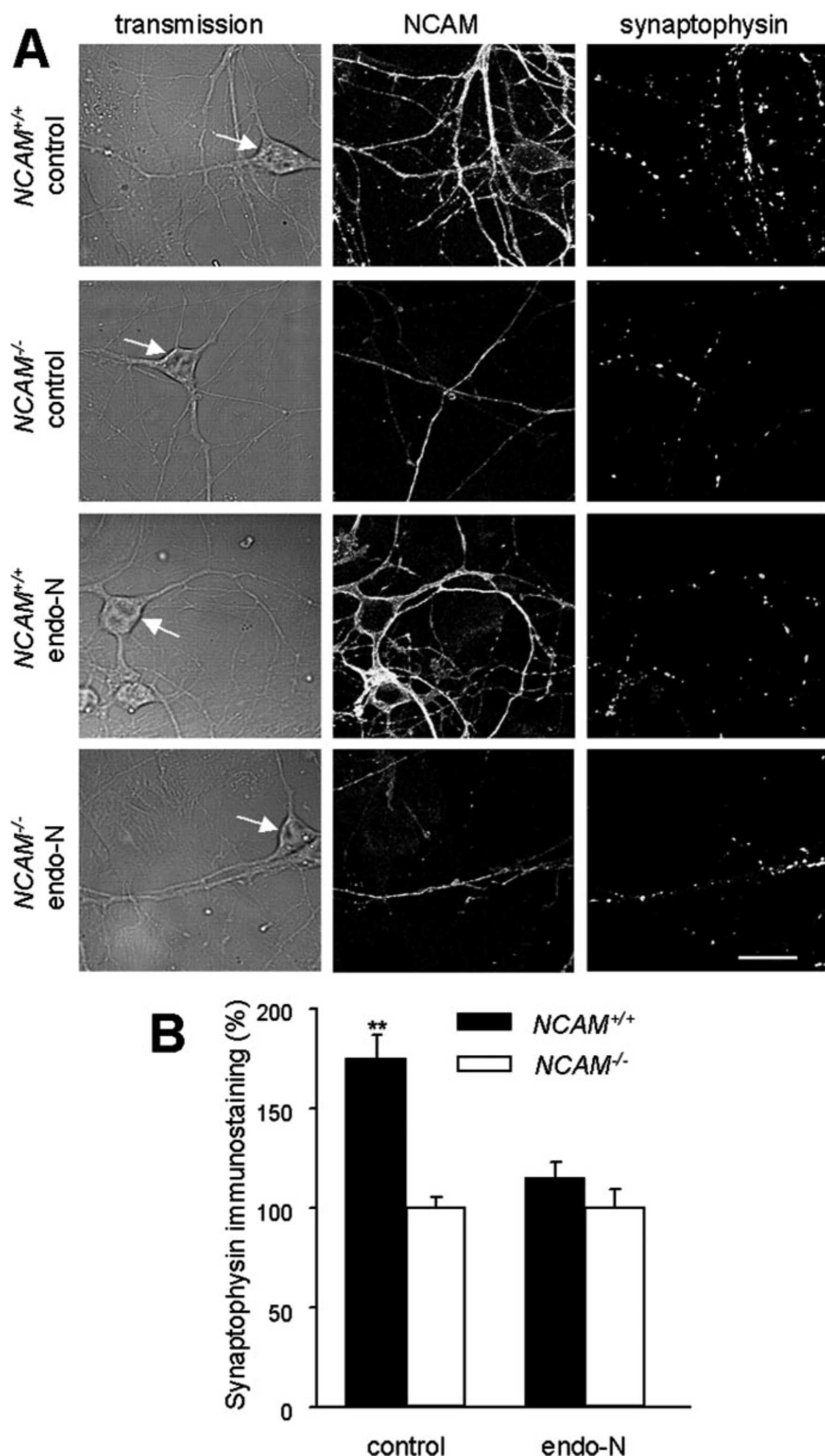

Figure 1. Removal of PSA by endo-N treatment prevents preferential formation of synapses on NCAM-expressing neurons. *A*, Synaptic coverage of $NCAM^{+/+}$ and $NCAM^{-/-}$ neurons maintained in heterogenotypic cocultures and visualized by phase-contrast imaging and coimmunolabeling with synaptophysin and NCAM antibodies. The two top rows show neurons maintained in untreated cocultures, and the two bottom rows represent neurons treated with endo-N. The arrows point to somata of pyramidal-like neurons used for analysis. Scale bar, 20 μm. *B*, Analysis of mean intensity of synaptophysin immunofluorescence within traced neurites in $NCAM^{+/+}$ and $NCAM^{-/-}$ neurons in untreated heterogenotypic cocultures and in cocultures treated with endo-N. Mean intensity of synaptophysin immunofluorescence in $NCAM^{-/-}$ neurites was used to normalize the intensities in different experiments. The asterisks indicate a difference between genotypes; $^{**}p < 0.01$, *U* test. Data are from three independent experiments.

transfection per se does not affect synaptogenesis. Thus, these data demonstrate that expression of any major NCAM isoform stimulates synaptic coverage and suggest that major synaptogenic determinants are located in the extracellular domain of NCAM. This is in agreement with our data showing all major NCAM isoforms to be polysialylated and requirement of PSA for NCAM-driven preferential synapse formation.

Postsynaptic expression of PSA-NCAM in a choice situation increased not only the total synaptic coverage measured by synaptophysin immunoreactivity but also the number of active synapses, as measured using synaptic activity-dependent uptake of fluorescent dye FM4–64 stimulated by high K^+. In these experiments, neurons were visualized in living cultures by transfection with EGFP. The levels of FM4–64 fluorescence associated with dendrites of neurons derived from $NCAM^{-/-}$ mice and cotransfected with NCAM180 and EGFP were higher than levels corresponding to neighboring untransfected $NCAM^{-/-}$ neurons or $NCAM^{-/-}$ neurons transfected with EGFP only (Fig. 4). Enzymatic treatment with endo-N abrogated the synaptogenic effects of NCAM (Fig. 4).

To interfere with interactions mediated by the extracellular domain of NCAM, we applied soluble PSA-NCAM-Fc and NCAM-Fc to heterogenotypic cocultures. Purified human Fc was used as a negative control. Immunostaining for Fc and NCAM showed binding of NCAM-Fc and PSA-NCAM-Fc, but not control Fc, to neurites of $NCAM^{+/+}$ but not of $NCAM^{-/-}$ neurons (Fig. 5*A*). These results are consistent with the notion that recombinant soluble NCAM-Fc or PSA-NCAM-Fc interact homophilically with NCAM expressed at the neuronal cell surface. Treatment with PSA-NCAM-Fc inhibited the preferential synaptic coverage of neurites from $NCAM^{+/+}$ neurons; the mean intensity of synaptophysin immunostaining associated with neurites was 99.5 $\pm$ 5.1% in treated versus 165.9 $\pm$ 9.3% in untreated cocultures (Fig. 5*B*) (100% correspond to $NCAM^{-/-}$ neurons). Exposure of neurites to NCAM-Fc, but not to Fc, also abolished preferential synaptic coverage of $NCAM^{+/+}$ neurites (Fig. 5*B*). Because both PSA-NCAM-Fc and NCAM-Fc bound to NCAM expressed endogenously on neurites and inhibited preferential synapse formation on NCAM-expressing neurons, the extracellular domain of the NCAM glycoprotein backbone appears to carry a synaptogenic deter-

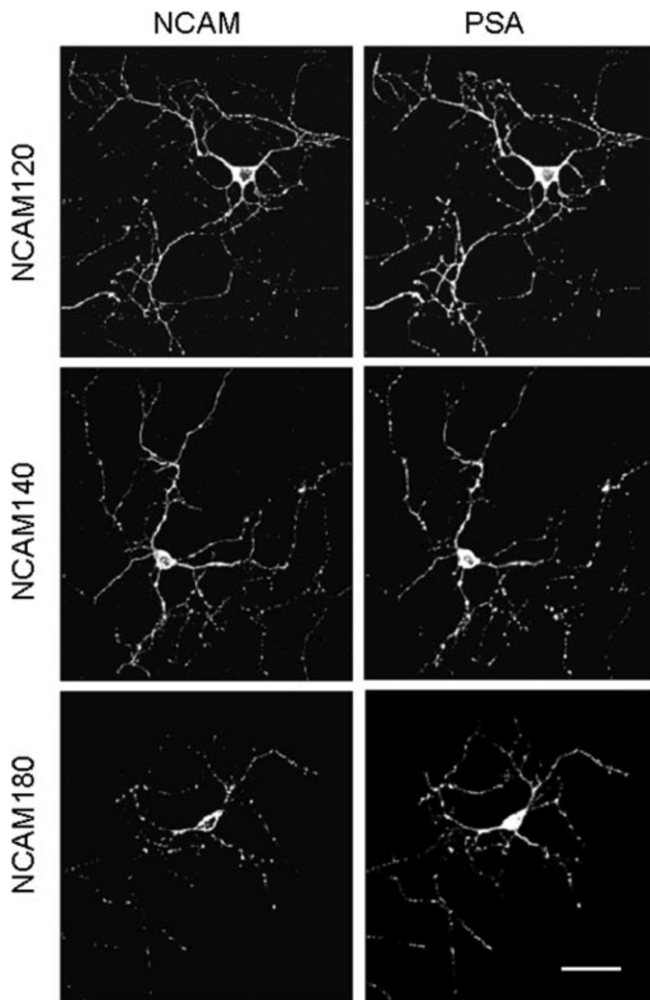

Figure 2. Expression of PSA in neurons from $NCAM^{-/-}$ mice transfected with different NCAM isoforms. Coimmunolabeling of NCAM (left) and PSA (right) in neurons derived from $NCAM^{-/-}$ mice and transfected with NCAM120, NCAM140, and NCAM180. A colocalization of PSA and NCAM is evident in dendrites, morphologically distinguishable as thick tapering neurites. Images were acquired with the same settings. Scale bar, 50 μm.

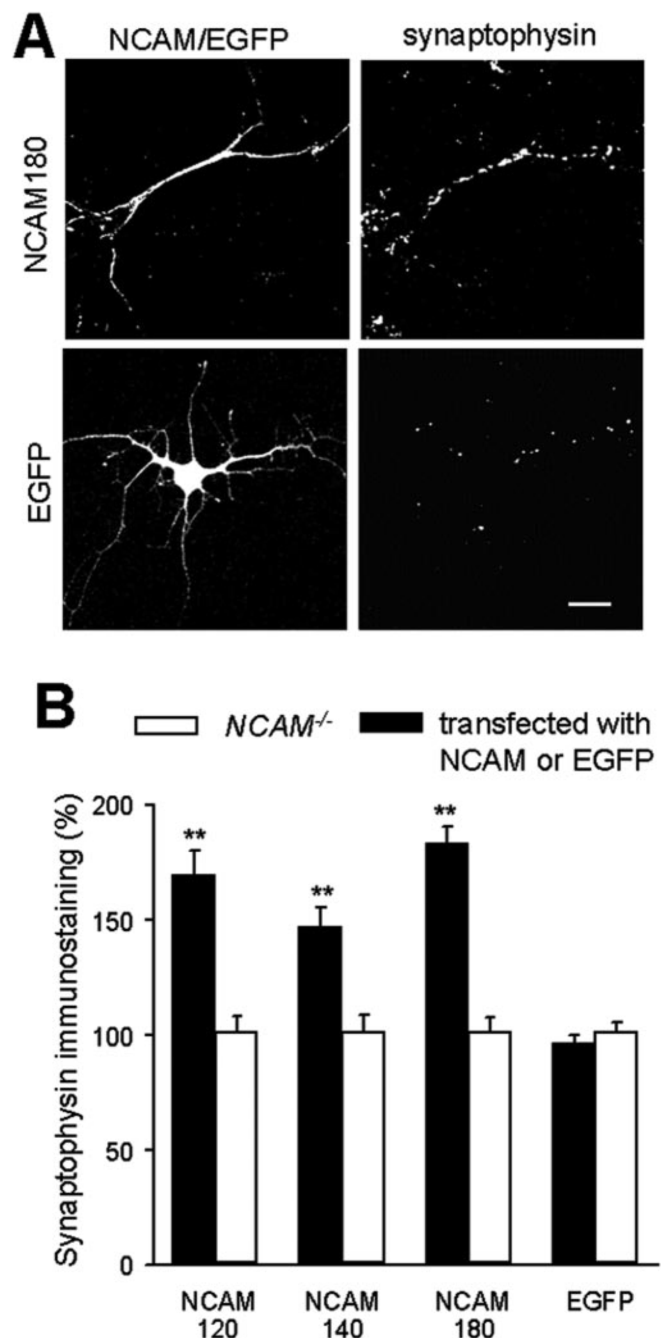

Figure 3. All NCAM isoforms promote formation of synaptic contacts. *A*, Synaptophysin immunolabeling in cultures from neurons derived from $NCAM^{-/-}$ mice and transfected with NCAM180 (top row) or EFGP (second row). NCAM180 transfected neurons were visualized by immunolabeling of NCAM. Scale bar, 20 μm. *B*, Analysis of mean intensity of synaptophysin immunofluorescence within traced neurites in $NCAM^{-/-}$ neurons transfected with NCAM120, NCAM140, NCAM180, and EGFP. Mean intensity of synaptophysin immunofluorescence in untransfected $NCAM^{-/-}$ neurons was used to normalize the intensities in the different experiments. The asterisks indicate a difference between genotypes; $^{**}p < 0.01$, *U* test. Data are from three independent experiments.

minant that can be blocked via homophilic interactions with soluble NCAM.

Interaction of NCAM with heparan sulfate proteoglycans mediates synaptogenic activity of PSA-NCAM

As a next step, we investigated the synaptogenic role of PSA-NCAM mediated interactions with heparan trans-interactions sulfate proteoglycans, the only known molecules of which transinteractions with NCAM are blocked by endo-N treatment and, thus, require the presence of PSA (Storms and Rutishauser, 1998). To disturb interactions between PSA-NCAM and heparan sulfate proteoglycans, we first removed heparin–heparan sulfates from the cell surface of live cells by heparinase treatment. This treatment resulted in the loss of preferential synapse formation on NCAM-expressing neurons in heterogenotypic cocultures (Fig. 6*A*); the mean intensity of synaptophysin immunostaining was 112.2 ± 3.4% in treated versus 178.3 ± 6.6% in untreated cocultures, compared with 100% in $NCAM^{-/-}$ neurons. As a control, enzymatic removal of chondroitin sulfate by chondroitinase ABC did not perturb preferential synapse formation on neurites from $NCAM^{+/+}$ neurons (Fig. 6*A*). Mutation of the binding site for heparin in the second immunoglobulin-like domain of NCAM (Reyes et al., 1990) of NCAM180 abolished the preferential synaptic coverage when transfected into $NCAM^{-/-}$ neurons being 108.7 ± 4.8% in comparison with 188.8 ± 8.8% observed for $NCAM^{-/-}$ neurons transfected with wild-type NCAM180 (Fig. 6*B*). Thus, interaction between the second immunoglobulin-like domain of PSA-

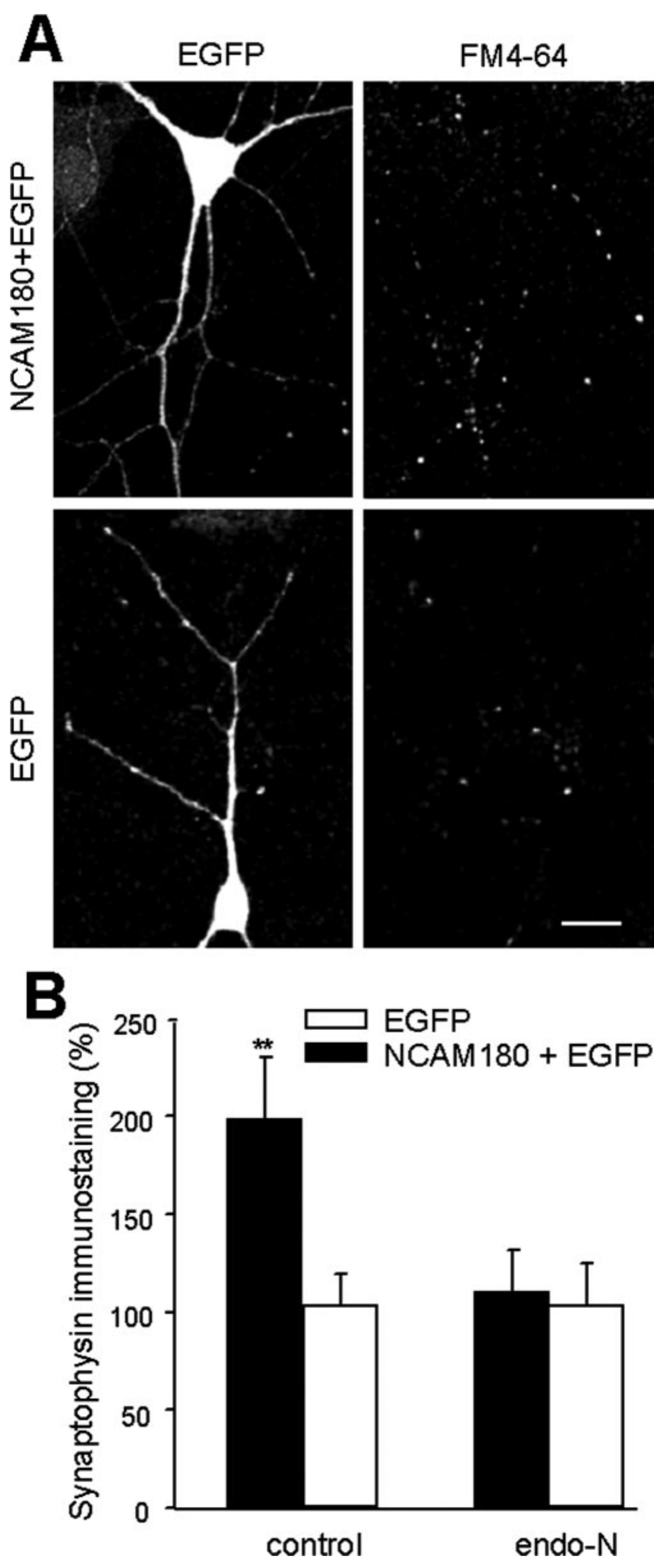

Figure 4. NCAM180 promotes formation of active synaptic contacts. *A*, FM4–64 uptake in cultures from neurons derived from $NCAM^{-/-}$ mice and transfected with NCAM180 plus EGFP (top row) or EFGP (second row). Scale bar, 20 μm. *B*, Analysis of mean intensity of FM4–64 fluorescence within traced neurites in untreated and endo-N-treated $NCAM^{-/-}$ neurons transfected with NCAM180 plus EGFP and EGFP only. Mean intensity of FM4–64 fluorescence in EGFP transfected $NCAM^{-/-}$ neurons was used to normalize intensities in different experiments. The asterisks indicate a difference between genotypes; $^{**}p < 0.01$, *U* test. Data are from two independent experiments.

NCAM and heparan sulfates appears to be important for the synaptogenic activity of PSA-NCAM.

Signaling involved in the synaptogenic activity stimulated by NCAM

To investigate several signaling pathways involved in NCAM-stimulated synaptogenesis, we performed a series of pharmacological experiments. First, we analyzed whether synaptic activity is important for the synaptogenic activity of NCAM. Preferential synaptic coverage of neurites from NCAM-expressing neurons in heterogenotypic cocultures was not affected by blocking non-NMDA glutamate receptors with NBQX or by tetrodotoxin-mediated suppression of spiking activity (Fig. 7*A*). However, preferential synaptic coverage was completely abolished by the NMDA glutamate receptor antagonist AP-5. Together, these data show that activity of NMDA receptors triggered by tetrodotoxin-resistant spontaneous glutamate release is required for the synaptogenic effect of NCAM.

In addition, we investigated the possibility that activation of FGF receptors, which are known to be expressed in hippocampal cultures (Li et al., 2002), directly interact with NCAM (Kiselyov et al., 2003), and are necessary for the neuritogenic activity of NCAM (Saffell et al., 1997; Niethammer et al., 2002), would also be important for the synaptogenic activity of NCAM. To address this issue, we used a specific antagonist of FGF receptors, PD173074 (Skaper et al., 2000; Niethammer et al., 2002). Treatment of heterogenotypic cocultures with this antagonist strongly reduced the preferential synaptic coverage of neurites originating from $NCAM^{+/+}$ neurons (115.0 ± 4.3% in treated vs 165.6 ± 8.6% in control cocultures) (Fig. 7*B*). Because FGF and NMDA receptors are known to activate MAPK cascade and inhibition of the MAPK pathway reduces NCAM-stimulated neurite outgrowth (Xia et al., 1996; Schmid et al., 1999; Kolkova et al., 2000), we also studied the effects of the inhibitor of MAPK kinase 1, PD98059. Treatment of cocultures with PD98059 produced a similar inhibition of preferential synaptogenesis on $NCAM^{+/+}$ neurons as blockers of FGF and NMDA receptors, suggesting that activities of these receptors in the presence of NCAM are necessary to activate MAPK, thus promoting NCAM-stimulated synaptogenesis.

PSA is required for formation of perforated synapses after induction of LTP

Because PSA is required for hippocampal LTP and hippocampus-dependent spatial learning and correct synaptic projection of mossy fibers (Becker et al., 1996; Muller et al., 1996; Eckhardt et al., 2000), we investigated whether PSA contributes to the morphological changes described to be associated with induction of LTP. Using a precipitation technique that reveals calcium at the electron microscopic level in subcellular structures in the form of a fine precipitate, it was possible to identify labeled synapses in the CA1 area. These labeled spine synapses were shown in previous studies to correspond to synapses loaded with Ca^{2+} and, thus, most likely reflect synapses activated by high-frequency stimulation (Buchs and Muller, 1996; Toni et al., 1999). Morphometric analysis of these labeled synapses revealed that they undergo morphological changes during the first hour after LTP induction. A major structural modification observed 30 min after stimulation was an increase in the proportion of synapses with perforated postsynaptic densities (Fig. 8*A*,*B*). The proportion of perforated synapses among labeled profiles increased from 22.3 ± 1.7% under control conditions to 45.8 ± 2.8% 30 min after stimulation. To test the role of PSA in these

Figure 5. Soluble PSA-NCAM-Fc and NCAM-Fc prevent preferential formation of synapses on NCAM-expressing neurons. *A*, Coimmunolabeling with antibodies to Fc and NCAM (first and second columns, respectively). Fc, NCAM-Fc, or PSA-NCAM-Fc were applied as indicated at the ← left. The genotypes of mice used to prepare cultures are indicated at the right. Note the bigger size of Fc-immunopositive clusters after application of PSA-NCAM-Fc compared with NCAM-Fc. *B*, Analysis of mean intensity of synaptophysin fluorescence within traced neurites in $NCAM^{+/+}$ and $NCAM^{-/-}$ neurons maintained in heterogenotypic cocultures in the presence of Fc, NCAM-Fc, or PSA-NCAM-Fc. Mean intensity of synaptophysin immunofluorescence in $NCAM^{-/-}$ neurons was used to normalize intensities in different experiments. The asterisks indicate a difference between genotypes; $^{**}p < 0.01$, *U* test. Data are from two independent experiments.

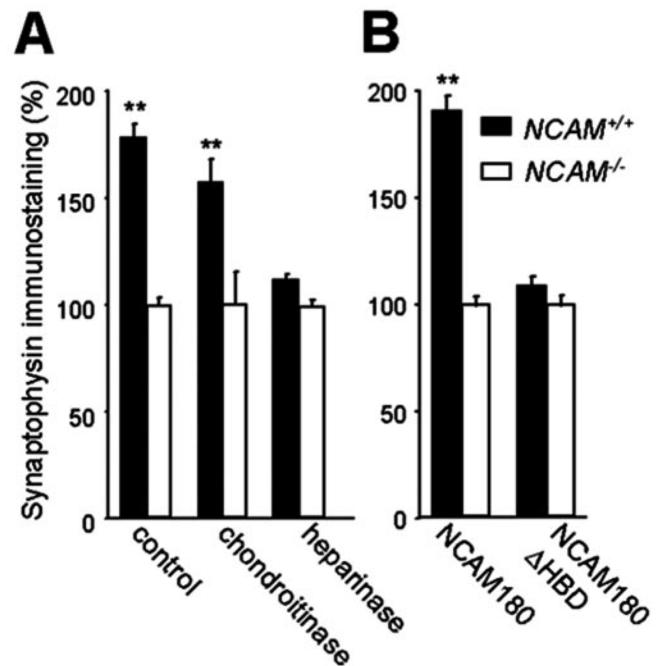

Figure 6. Synaptogenic activity of NCAM is sensitive to heparinase treatment and mutation of heparin-binding domain of NCAM. *A*, Analysis of mean intensity of synaptophysin immunofluorescence in chondroitinase ABC or heparinase-treated heterogenotypic cocultures. *B*, Analysis of mean intensity of synaptophysin immunofluorescence in $NCAM^{-/-}$ neurons transfected with wild-type NCAM180 or NCAM180 with mutation of heparin binding domain on the second immunoglobulin-like domain of NCAM, NCAM180ΔHBD. Mean intensity of synaptophysin immunofluorescence in $NCAM^{-/-}$ neurons was used to normalize intensities in different experiments. The asterisks indicate a difference between genotypes; $^{**}p < 0.01$, *U* test. Data are from two to three independent experiments.

morphological changes, we used cultures treated with endo-N, thus specifically removing α2,8-linked sialic acid. Previous work has shown that application of endo-N to a slice culture for 12 hr fully and reversibly eliminated PSA (Muller et al., 1996). High-frequency stimulation of endo-N-treated cultures failed to induce LTP and the morphological changes (Fig. 8*C*,*D*). In endo-N-treated cultures, the percentage of perforated synapses was decreased to 25.7 ± 1.9% compared with 45.8 ± 2.8% in the cultures stimulated without endo-N treatment. This effect was not caused by endo-N-induced changes in the number of activated synapses, labeling efficacy, or alterations of calcium entry into postsynaptic spines, because the same proportion of synaptic profiles was labeled when LTP was induced under control conditions and in endo-N-treated cultures (12.1 ± 0.3 and 12.4 ± 0.1%).

Because PSA-NCAM interacts with heparan sulfate proteoglycans, which also have been shown to be involved in synaptic plasticity (Lauri et al., 1999) and because this interaction appeared to be important for NCAM-stimulated synaptogenesis, we studied whether removal of heparin sulfate from heparan sul-

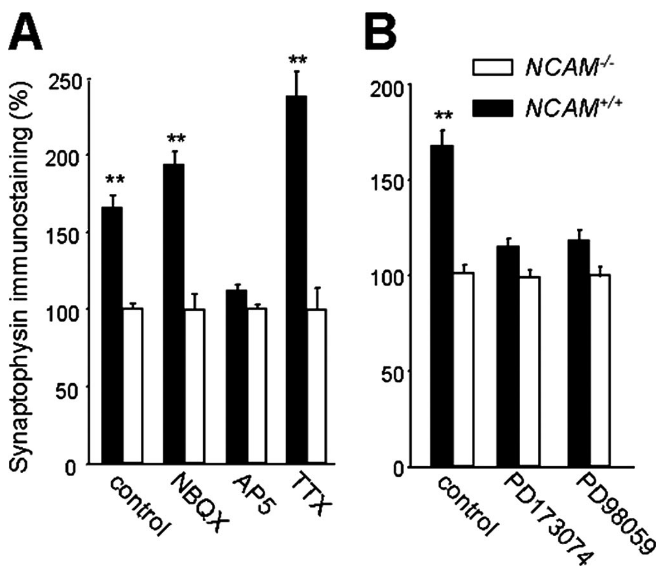

Figure 7. Synaptogenic activity of NCAM required activities of FGF and NMDA receptors. *A*, Analysis of mean intensity of synaptophysin immunofluorescence after treatment of heterogenotypic cocultures with NBQX or AP-5, as antagonists of non-NMDA and NMDA subtypes of glutamate receptors, respectively, and TTX as blocker of voltage-gated Na^+ channels. *B*, Analysis of mean intensity of synaptophysin immunofluorescence after treatment of heterogenotypic cocultures with PD173074, antagonist of FGF receptors, and PD98059 as inhibitor of MAPK kinase 1. Mean intensity of synaptophysin immunofluorescence in $NCAM^{-/-}$ neurons was used to normalize intensities in different experiments. The asterisks indicate a difference between genotypes; $^{**}p < 0.01$, *U* test. Data are from three independent experiments.

fate proteoglycans would affect structural plasticity. For this, we heparinase treated slice cultures for 12–15 hr and then assessed LTP and the proportion of perforated synapses. Figure 8, *C* and *D*, indicates that heparinase treatment resulted in very similar effects as treatment with endo-N, preventing LTP induction and increase in the proportion of perforated synapses (27.7 ± 1.8 vs 45.8 ± 2.8%; $n = 3–6$; $p < 0.05$). Together, these results indicate that PSA and an interaction between PSA-NCAM and heparan sulfate proteoglycans are likely to contribute to the morphological changes associated with induction of the NMDA receptor-dependent form of LTP (Buchs and Muller, 1996) in the CA1 region of the hippocampus.

Discussion

Our study shows that NCAM is a prominent player in synaptogenesis in that neurons expressing any one of the three major NCAM isoforms receives more synaptic contacts in a choice situation, that is, when a neurite can make contacts with either an NCAM-positive or -negative neurite. PSA and the heparin-binding domain of NCAM are necessary for the synaptogenic activity of NCAM, and FGF and NMDA receptors are involved in mediating this NCAM-dependent process.

Synaptogenic activity of PSA-NCAM

Of the two transmembrane isoforms differing in the size of their intracellular domains, only the NCAM140, but not NCAM180, activates fyn kinase (Beggs et al., 1994, 1997; Kolkova et al., 2000). Because all three major NCAM isoforms, including GPI-linked NCAM120, stimulated synaptogenesis, we think that NCAM signaling via fyn kinase is not critical for synaptogenic activity of NCAM. Because all major isoforms expressed PSA and enzymatic removal of PSA blocked preferential synaptogenesis on NCAM-expressing neurons, we conclude that the PSA-bearing extracellular domain of NCAM is required for its synaptogenic activity. This conclusion is further supported by experiments with soluble PSA-NCAM-Fc and a mutation in the heparin-binding domain of NCAM (Reyes et al., 1990), which inhibited NCAM-dependent synaptogenesis. Although the present data suggest that PSA is required for preferential synaptogenesis, they do not exclude the possibility that nonpolysialylated NCAM molecules provide additional signals, for instance via direct NCAM-FGF receptor interaction.

We have found previously that postsynaptic, but not presynaptic, NCAM increases both the number of synapses and the synaptic strength of connections generated on postsynaptically NCAM-expressing neurons in heterogenotypic cocultures (Dityatev et al., 2000). The current study provides additional support for this idea, because presynaptic $NCAM^{-/-}$ neurons preferably contact postsynaptic $NCAM^{-/-}$ neurons transfected with NCAM isoforms rather than untransfected $NCAM^{-/-}$ cells.

Previous ultrastructural analysis in the adult hypothalamic magnocellular nuclei revealed that after removal of PSA by endo-N, there was no apparent withdrawal of astrocytic processes from neurons, no increase in synaptic contacts normally induced by lactation and dehydration (Theodosis et al., 1999), and no estradiol-induced morphological synaptic plasticity (Hoyk et al., 2001). Thus, PSA has been implicated in dynamic cell interactions in that it regulates the strength of adhesivity among cells, in equilibrium between too much and too little adhesion. Molding of cell contacts as it most likely occurs in synaptogenesis and generation of perforated synapses are dynamic events that depend on the flexibility of cell interactions and, thus, on the function of a molecular structure that generates such pliancy. Additionally, because PSA enhances binding of heparan sulfates to NCAM (Storms and Rutishauser, 1998) and because heparan sulfates present FGF to its receptors (Stauber et al., 2000; Kwan et al., 2001; Loo et al., 2001), PSA may thus regulate FGF receptor activities. Because removal of PSA, heparan sulfates, or heparin-binding domain of NCAM inhibits synaptogenic activity of PSA-NCAM (similar to a block of FGF receptors), PSA-NCAM in complex with heparan sulfates and FGF may provide a positive signal for synaptic differentiation.

Heparan sulfate proteoglycans and NCAM

The interaction between NCAM and heparan sulfate was discovered many years ago (Cole et al., 1986; Cole and Akeson, 1989) and was found to depend on PSA (Storms and Rutishauser, 1998). This interaction was suggested to play an important role in cell migration (Prag et al., 2002). In the context of our study, it is striking that presynaptically secreted heparan sulfate proteoglycan agrin induces postsynaptic differentiation at the neuromuscular junction and supports NCAM-mediated heterophilic adhesion (Storms et al., 1996; Storms and Rutishauser, 1998). Heparan sulfate proteoglycans of the syndecan and glypican families may also be candidates to mediate PSA-NCAM synaptogenic activity. Syndecan-2 and -3 are the major syndecans expressed by neurons of the forebrain, differentially localized within neurons; syndecan-3 is concentrated in axons, whereas syndecan-2 accumulates in developing synapses and plays an important role in formation of spines (Ethell and Yamaguchi, 1999; Hsueh and Sheng, 1999). In the adult hippocampus, glypican-1 immunoreactivity is well detectable in projections of associational and commissural fibers, which consist of axons arising from glypican-1 mRNA-expressing hippocampal pyramidal cells (Litwack et al., 1998). Additional studies are required to identify which of the heparan sulfate proteoglycans and which heparan sulfate moi-

eties (Jenniskens et al., 2000; Dennissen et al., 2002) interact with NCAM during synaptogenesis.

Signaling via FGF receptors

Agrin binds to FGF-2, and syndecans and glypicans promote FGF-2-triggered signaling (Filla et al., 1998; Cotman et al., 1999; Matsuda et al., 2001). Several studies have shown that heparan sulfate chains serve to facilitate dimerization of FGF and FGF receptors (Stauber et al., 2000; Kwan et al., 2001; Loo et al., 2001), which activates the tyrosine kinase domain of these receptors through autophosphorylation. FGF receptors may also be activated by NCAM via direct interaction between the first two fibronectin type III domains of NCAM and the second and third immunoglobulin domains of FGF receptor (Kiselyov et al., 2003). In agreement with our present study, FGF-2 and a synthetic peptide corresponding to the binding site of NCAM with FGF receptor 1 stimulated formation of synapses in hippocampal cultures (Li et al., 2002; Cambon et al., 2004). Particularly pertinent for the many signaling mechanisms described for FGF receptor function is activation of neurite outgrowth through generation of polyunsaturated fatty acids (e.g., arachidonic acid), which activate voltage-gated Ca^{2+} channels (Williams et al., 1994; Walsh and Doherty, 1997; Archer et al., 1999). Restricted Ca^{2+} influx at the sites of FGF action may lead to phosphorylation-sensitive clustering of synaptic vesicles at these sites (Dai and Peng, 1995). Arachidonic acid may also affect synaptogenesis via activation of protein kinase C (Hama et al., 2004). These data suggest that heparin sulfate proteoglycans in association with PSA-NCAM may assist in activating axonally localized FGF receptors, thus stimulating differentiation of presynaptic specializations. Such mechanisms may explain the higher number of presynaptic contacts on postsynaptically PSA-NCAM-expressing neurons. However, it is also conceivable that NCAM and FGF receptors may interact postsynaptically and stabilize presynaptic contacts via retrograde delivery of arachidonic acid.

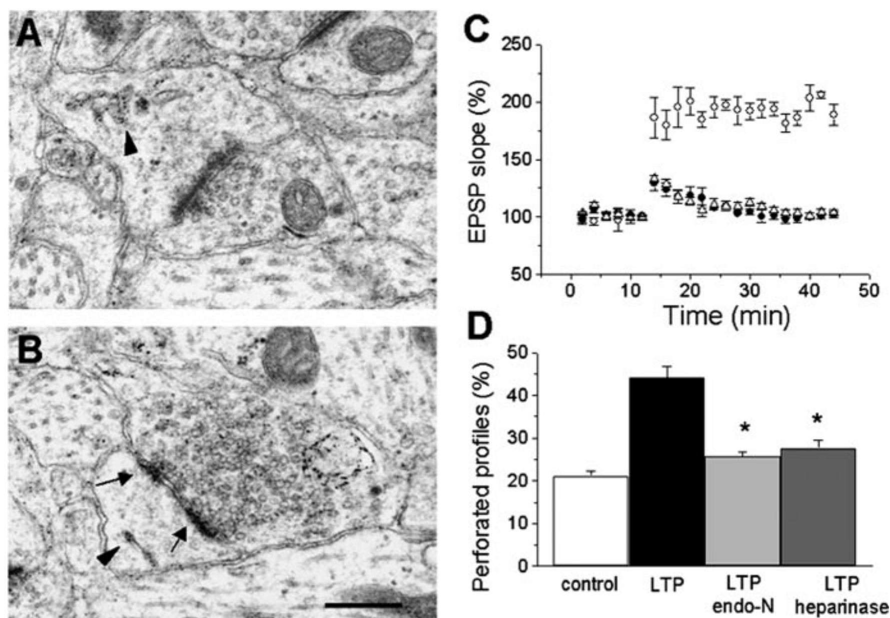

Figure 8. Enzymatic removal of PSA or heparan sulfates prevents morphological remodeling of synapses associated with induction of LTP. *A*, Illustration of a synapse with a single postsynaptic density. *B*, Illustration of a calcium precipitate-labeled synapse with a perforated postsynaptic density (arrow). Note the presence of precipitates (arrowhead) in the postsynaptic spine. Scale bar, 0.5 μm. *C*, Changes in EPSP slope observed after LTP induction using theta burst stimulation in four control (open circles), three endo-N-treated (filled circles), and six heparinase-treated (open triangles) slice cultures. *D*, Proportion of labeled synaptic profiles showing perforated postsynaptic densities observed in nonstimulated slice cultures (open column) and 30 min after LTP induction without additives (black column), with endo-N treatment (light gray column), and with heparinase treatment (dark gray column). Data are mean ± SEM of the proportion of perforated synapses observed in three to six slice cultures per condition (194–525 synapses were analyzed per condition; $^*p < 0.05$).

NCAM and NMDA receptors

Because the NMDA receptor antagonist AP-5, but not the blocker of voltage-dependent Na^+ channels tetrodotoxin and non-NMDA receptor antagonist NBQX, abolishes synaptogenic activity of NCAM, it is plausible to assume that synaptogenesis depends on activation of NMDA receptors by spontaneously released glutamate in an action potential-independent manner. Inability of tetrodotoxin to affect NCAM-mediated synaptogenesis is not surprising because we have never observed action potential-dependent activity in young cultures (our unpublished observations). Our experiments suggest that NMDA receptor activity is important for expression of PSA and NCAM and/or that NMDA receptors are involved in synaptogenesis in such a way that more synapses are stabilized on PSA-NCAM-expressing neurites. Available data support the idea that PSA-NCAM and NMDA receptors influence each other: cell surface expression of PSA-NCAM is NMDA receptor dependent (Bouzioukh et al., 2001). In synaptic membranes of nonstimulated spine synapses, NCAM180 and the NR2A subunit of NMDA receptors are accumulated in the center of postsynaptic densities and coredistribute to the edges of postsynaptic densities after induction of LTP (Fux et al., 2003). It is conceivable that NCAM and NMDA receptors are cross-linked by the membrane-cytoskeletal linker protein spectrin that binds to these two molecules (Pollerberg et al., 1986; Wechsler and Teichberg, 1998; Sytnyk et al., 2002). Our data on NCAM-mediated accumulation of the synaptic machinery in nascent synapses (Sytnyk et al., 2002) suggest that the NCAM-spectrin complex may promote the postsynaptic accumulation of NMDA receptors and/or modify their activity.

Similarity and differences in NCAM-dependent mechanisms during neurite outgrowth, synaptogenesis, and LTP

Of the NCAM isoforms, only NCAM140 stimulated neurite outgrowth as a homophilic receptor (Niethammer et al., 2002). However, all isoforms of NCAM, including GPI-linked NCAM120, are capable of promoting neurite outgrowth when presented as trans-interacting ligands (Doherty et al., 1990). Similarly, our present findings demonstrate that all major NCAM isoforms are synaptogenic. Also, NCAM-dependent neurite outgrowth and synaptogenesis are both dependent on FGF receptor activation.

Previous studies have revealed an involvement of NCAM and PSA in LTP (Lüthi et al., 1994; Ronn et al., 1995; Becker et al., 1996; Muller et al., 1996, 2000; Bukalo et al., 2004). Strikingly, NMDA and FGF receptors, and heparan sulfate proteoglycans (found here to be involved in synaptogenic activity of NCAM), have also been implicated in hippocampal LTP (Ishiyama et al., 1991; Abe et al., 1992; Collingridge and Bliss, 1995; Lauri et al., 1999). Also, formation of perforated synapses is dependent on

NMDA receptors (Jourdain et al., 2002), PSA, and heparan sulfates (our study). However, LTP is reduced in a no-choice situation in hippocampal cultures and in acute slices from NCAM-deficient mice (Muller et al., 1996, 2000; Dityatev et al., 2000; Bukalo et al., 2004), whereas effects of NCAM on synaptogenesis were evident only in a choice situation (Dityatev et al., 2000). This situation may appear artificial, but expression of NCAM is not uniform among different neurons or even subdomains of a neuron, thus providing a choice. In view of the activity-dependent regulation of postsynaptic expression of PSA and NCAM (Muller et al., 1996; Schuster et al., 1998; Fux et al., 2003), it is conceivable that upregulation of NCAM expression at distinct stimulated postsynaptic sites could accentuate a choice situation and promote synaptic remodeling and/or synapse formation at these sites.

In summary, the contribution of NCAM to synaptogenesis appears to depend on interactions with heparan sulfate proteoglycans and to require FGF and NMDA receptor activation. These observations extend and strengthen the notion that NCAM and PSA-NCAM can modulate synaptic plasticity.

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