Clustering of the Neural Cell Adhesion Molecule (NCAM) at the Neuronal Cell Surface Induces Caspase-8- and -3-dependent Changes of the Spectrin Meshwork Required for NCAM-mediated Neurite Outgrowth

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Changes in neuronal morphology underlying neuronal differentiation depend on rapid and sustained cytoskeleton rearrangements in the growing neurites. Whereas cell adhesion molecules are well established as regulators of neuronal differentiation, less is known about the signaling mechanisms by which they influence the cytoskeleton. Here we show that the neural cell adhesion molecule (NCAM) associates with the active form of caspase-8 and that clustering of NCAM at the neuronal cell surface leads to activation of caspase-8 and -3 followed by the cleavage of the sub-membranous brain spectrin meshwork, but not of the actin or tubulin cytoskeleton. Inhibitors of caspase-8 and -3 specifically block the NCAM-dependent spectrin cleavage and abolish NCAM-dependent neurite outgrowth. NCAM-dependent rearrangements of the membrane-associated spectrin meshwork via caspase-8-dependent caspase-3 activation are thus indispensable for NCAM-mediated neurite outgrowth.

The establishment and maintenance of neuronal morphology are essential for brain development and functioning. The neural cell adhesion molecule (NCAM)2 plays a prominent role in these processes by being involved in regulation of neuronal migration and differentiation as well as synaptogenesis in the developing nervous system and synaptic plasticity in the adult (1–4). NCAM is a member of the immunoglobulin superfamily of adhesion molecules and contains five immunoglobulin and two fibronectin-like type III domains within its extracellular part. In developing neurons, NCAM is highly expressed in two transmembrane isoforms with molecular weights of 140 kDa (NCAM140) and, to a lower extent, 180 kDa (NCAM180), which are generated via alternative splicing of the ncam1 gene. The extracellular portion of NCAM interacts with multiple binding partners on adjacent cells and in the extracellular matrix, including adhesion molecules, such as prion protein (PrP), L1, and NCAM itself (5, 6), growth factor receptors, such as FGFR and GFRα (7, 8), and other receptors, such as receptor-type protein phosphatase β or its secreted forms (9).

Clustering of NCAM at the cell surface by its ligands promotes neurite outgrowth by inducing intracellular signaling cascades, initiated by the association of the intracellular domain of NCAM with and consequent activation of kinases and phosphatases, including CaMKIIα, PKC, and RPTPα (10–12). Palmitoylation of the intracellular domain of NCAM (13) and association of NCAM with the cellular form of prion protein (PrP) (5) promote redistribution of NCAM and associated signaling molecules to lipid rafts where they activate downstream signal transducing proteins including fyn kinase and GAP43 (10, 12, 14, 15).

The cytoskeleton plays an important role in NCAM-dependent signaling, and clustering of NCAM at the cell surface induces formation of the spectrin-based cytoskeleton enriched in microdomains (3). However, little is known on how NCAM signaling is coordinated with the cytoskeleton reorganization including not only its polymerization but also local remodeling via proteolysis, which is essential for the efficient neurite outgrowth (16, 17).

Cysteinylation-dependent aspartate specific proteases, caspases, and caspase-3 in particular, are proteases, which can locally cleave spectrin and actin components of the cytoskeleton (18, 19). Caspases are a family of soluble proteins, which are expressed by all cell types, structurally consisting of a prodomain and two catalytic large and small domains, and activated by intramolecular cleavage (20, 21). Activation of the initiator caspases-8,-9, and -10 leads to the cleavage of the short prodomain-containing procaspase-3 and generation of the active effector caspase-3. Whereas caspases play a prominent role in apoptosis (20, 21), it is now well established that they are necessary for the regulation of neuronal morphology (22, 23).

In the present study, we show that NCAM associates with and regulates the activity of caspase-8 and -3 to induce the local remodeling of the spectrin cytoskeleton. We show that...
inhibitors of caspase-8 and -3 block NCAM-dependent neurite outgrowth. Thus, we reveal a novel function for a cell adhesion molecule in its function as a regulator of the neurite outgrowth-promoting remodeling of the cytoskeleton via local caspase-dependent cytoskeleton proteolysis.

EXPERIMENTAL PROCEDURES

Animals—NCAM<sup>+/−</sup> mice were provided by Harold Cremer (24) and were inbred for at least nine generations onto the C57BL/6J background. Animals for biochemical experiments were 1–3-day-old NCAM<sup>+/+</sup> and NCAM<sup>−/−</sup> littermates from heterozygous breeding pairs. To prepare cultures of hippocampal neurons, 1–3-day-old C57BL/6J mice were used.

Antibodies—Rabbit polyclonal antibodies against mouse NCAM (13) (for biochemical and immunocytochemical experiments), chicken polyclonal antibodies against mouse NCAM (Antibody Service Dr. Pineda, Berlin, Germany, for clustering of mouse NCAM in growth cones and cultured neurons), rat monoclonal antibodies H28 against mouse NCAM (for biochemical and immunocytochemical experiments, clustering of NCAM at the cell surface of cultured hippocampal neurons, (25)) react with the extracellular domain of all NCAM isoforms. Rabbit polyclonal antibodies against mouse L1 were as described (26). Mouse monoclonal antibodies against PrP were a generous gift of Dr. Man Sun Sy (Case Western Reserve University, Cleveland, OH). Rat monoclonal antibody against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was from Chemicon (Temecula, CA). Mouse monoclonal antibody against α-tubulin was from Sigma-Aldrich (Taufkirchen, Germany). Rabbit polyclonal antibodies against cleaved αII-spectrin were from CalBiochem (EMD Chemicals, Gibbstown, NJ). Goat polyclonal antibodies against contactin were from R&D Systems (Wiesbaden-Nordenstadt, Germany). Rabbit polyclonal antibodies against caspase-3, -9, -10, and cleaved caspase-3 were from Cell Signaling Technology (Danvers, MA). Chicken polyclonal antibodies against caspase-8 were from Abcam (Cambridge, UK). Rabbit polyclonal antibodies against caspase-8 and mouse monoclonal antibodies recognizing full-length and cleaved βII spectrin were from BD Biosciences (Heidelberg, Germany). Mouse monoclonal antibody against β-tubulin (clone E7) was from Developmental Studies Hybridoma Bank (The University of Iowa, Iowa City, IA). Secondary antibodies against chicken, rabbit, rat, and mouse immunoglobulins coupled to HRP, Cy2, Cy3, or Cy5, and nonspecific chicken, rabbit, rat, and mouse immunoglobulins were from Jackson ImmunoResearch Laboratories (West Grove, PA).

Inhibitors—Aprotinin and leupeptin were from Sigma-Aldrich. Caspase-3 and caspase-9 inhibitors were from Calbiochem. Caspase-8 and caspase-10 inhibitors were from BioVision (Mountain View, CA). EDTA-free complete protease inhibitor mixture was from Roche Diagnostics (Mannheim, Germany).

Preparation of Brain Tissue Homogenates—Brain homogenates of 1–3-day-old mice were prepared using a Potter homogenizer in buffer containing 1 mM MgCl₂, 1 mM CaCl₂, 5 mM Tris, pH 7.4, 0.1 mM PMSF, EDTA-free protease inhibitor mixture, and 0.32 mM sucrose (HOMO buffer).

Isolation of Cytosolic, Total Membrane, and Lipid Raft Fractions—Brain homogenates were centrifuged at 700 × g for 10 min at 4 °C to pellet nuclei and mitochondria. The resulting supernatants were centrifuged at 100,000 × g at 4 °C for 30 min. Supernatants obtained after centrifugation were used as fractions enriched in cytosolic proteins, while pellets were used as total membrane fractions. Rafts were isolated from total membrane fractions as described (12). Briefly, membranes were resuspended in ice-cold Tris-buffered saline (TBS, pH 7.5) and extracted for 20 min on ice with 4 volumes of 1% Triton X-100 in TBS. Extracted membranes were mixed with equal volume of 80% sucrose in 0.2 mM sodium carbonate, overlaid with 30% sucrose in TBS, 10% sucrose in TBS and TBS and centrifuged at 230,000 × g for 17 h at 4 °C. The lipid raft fraction was collected at the interface between 10 and 30% sucrose, pelleted by centrifugation at 100,000 × g for 1 h at 4 °C and resuspended in TBS.

Isolation of Growth Cones—Growth cones were isolated as described (27). Brain homogenates from 1–3-day-old mice were centrifuged at 1660 × g for 15 min at 4 °C. The supernatant was collected and centrifuged on a discontinuous density gradient of 0.75/1.0/2.66 mM sucrose at 242,000 × g for 30 min at 4 °C. The interface between the load and 0.75 mM sucrose, containing growth cones, was collected, resuspended in 10 ml of HOMO buffer, and pelleted by centrifugation at 100,000 × g for 40 min at 4 °C to obtain purified growth cones.

NCAM Clustering in Isolated Growth Cones—Unless otherwise indicated, all steps were performed on ice. Growth cones were mixed with 0.5 ml of 2× dilution buffer (100 mM sucrose, 20 mM glucose, 200 mM NaCl, 10 mM KCl, 2.4 mM NaH₂PO₄, 4 mM HEPES, 2.4 mM MgCl₂, pH 7.3) containing aprotinin and leupeptin to inhibit serine and cysteine proteinases, incubated for 30 min, mixed with additional 0.8 ml of the 2× dilution buffer, and incubated for 20 min. This suspension (250 μl) was added to assay tubes and preincubated with caspase-3 (1 μM) or caspase-8 inhibitors (1 μM) or vehicle for 2 h on ice. To induce clustering of NCAM at the growth cone surface, chicken polyclonal antibodies against NCAM, or pre-immune serum (IgY) as control were added to the samples, and samples were equilibrated for 15 min. Samples were then warmed up in a water bath to 37 °C for 5 min, and subsequently chilled in ice slurry for 5 min. Samples were then loaded onto 0.5-ml cushions of 0.4 mM sucrose in TBS and centrifuged at 100,000 × g at 4 °C for 1 h. Pellets were resuspended in 60 μl of Laemmli buffer and used for Western blot analysis.

Co-immunoprecipitation—For co-immunoprecipitation experiments, samples containing 1 mg of total protein were lysed for 1 h at 4 °C with lysis buffer, pH 7.5, containing 50 mM Tris–HCl, 150 mM NaCl, 1% Nonidet P-40, 1 mM Na₃PO₄, 1 mM NaF, 2 mM Na₂VO₃, 0.1 mM PMSF, and EDTA-free protease inhibitor mixture (Roche). Lysates were centrifuged for 15 min at 20,000 × g at 4 °C. Supernatants were cleared with protein A/G–agarose beads (Santa Cruz Biotechnology, Heidelberg, Germany), applied for 3 h at 4 °C, and incubated with corresponding antibodies or nonspecific
IgG applied overnight at 4 °C. Complexes were then precipitated with protein A/G-agarose beads applied for 3 h at 4 °C. Beads were washed four times with lysis buffer and three times with phosphate-buffered saline (PBS), pH 7.4, and used for Western blot analysis.

**Western Blot**—Proteins were separated by 6–16% SDS-PAGE and electroblotted to nitrocellulose transfer membranes (PROTRAN; Schleicher & Schuell, Dassel, Germany). The membranes were then incubated with appropriate primary antibodies followed by incubation with HRP-labeled secondary antibodies, which were visualized using ECL Western blotting reagents (Amersham Biosciences) or SuperSignal West Dura Extended Duration reagents (Pierce) on BIOMAX films (Sigma-Aldrich). Molecular weight markers were prestained protein standards from Bio-Rad. The chemiluminescence quantification was performed using TINA 2.09 software (University of Manchester, UK) or Scion Image for Windows (Scion Corporation, Frederick, MD).

**Cultures of Hippocampal Neurons**—Neurons were prepared in accordance with the Invitrogen protocol (Invitrogen, Carlsbad, CA) and maintained for 24 h in Neurobasal A medium (Invitrogen) supplemented with 2% B-27 (Invitrogen), glutamine (Invitrogen) and FGF-2 (2 ng/ml, R&D Systems) on glass coverslips or cell culture plates coated with poly-d-lysine (100 μg/ml). NCAM and PrP were clustered at the cell surface of live neurons as described (12). Neurite outgrowth was analyzed as described (11). Neuronal viability was analyzed by using Trypan blue exclusion test. Briefly, neurons were incubated for 2 min with 0.2% Trypan Blue in PBS, pH 7.3, washed with PBS, and fixed for 15 min in 4% formaldehyde in PBS. Cell viability was estimated by counting numbers of blue dead cells versus the total number of cells on randomly acquired images.

**Immunofluorescence Labeling**—Immunolabeling was performed as described (2). All steps were performed at room temperature and all antibodies were applied in 1% bovine serum albumin (BSA) in PBS. Neurons were fixed for 15 min in 4% formaldehyde in PBS. To label NCAM and PrP at the cell surface, fixed neurons were blocked with 1% BSA in PBS for 20 min and incubated with NCAM and PrP antibodies applied for 30 min followed by corresponding secondary antibodies applied for 30 min. Then neurons were postfixed for 5 min in 2% formaldehyde in PBS, washed with PBS, permeabilized with 0.25% Triton X-100 in PBS for 5 min, blocked with 1% BSA in PBS for 20 min, and treated with primary antibodies against intracellular proteins indicated in the text for 2 h followed by corresponding secondary antibodies applied for 45 min. Cell ELISA was performed as described (11).

**Image Acquisition and Manipulation**—Coverslips were embedded in Aqua-Poly/Mount (Polysciences, Inc., Warrington, PA). For neurite outgrowth measurements, images of neurons were acquired at room temperature using Axioskop 2 microscope (Zeiss, Jena, Germany) equipped with AxioCam HRc digital camera (Zeiss), AxioVision software (version 3.1, Zeiss) and Plan-Neofluar 40× objective (numerical aperture 0.75, Zeiss). For cell viability analysis, images were acquired at room temperature using an Nikon Eclipse TS100 microscope equipped with DS-Qi1 digital camera (Nikon), NIS-Elements Basic Research software (Nikon), and S Plan-Fluor 40× objective (numerical aperture 0.6, Nikon). Immunofluorescence images were acquired at room temperature using a confocal laser scanning microscope LSM510 (Zeiss), LSM510 software (version 3, Zeiss), and oil Plan-Apochromat 63× objective (numerical aperture 1.4, Zeiss) at 1–3× digital zoom. Contrast and brightness of the images were further adjusted in Corel Photo-Paint 9 (Corel Corporation, Ottawa, Ontario, Canada).

**Immunofluorescence Quantification**—Immunofluorescence quantification was performed essentially as described (10). To quantify levels of active caspase-3, neurons were manually outlined in the ImageJ software (National Institutes of Health, Bethesda, MD) and mean immunofluorescence intensities were measured within these outlines using ImageJ. To analyze correlation coefficients, profiles of immunofluorescence intensity along neurites were measured using ImageJ and correlation coefficients between thus obtained immunofluorescence intensity distributions were calculated in Excel (Microsoft Corp.).

**Cultures and Transfection of CHO Cells**—Chinese hamster ovary (CHO) cells were maintained in Glasgow Eagle’s medium, containing 5% of fetal calf serum. Cells were transfected using Lipofectamine and Plus reagent (Invitrogen) following the manufacturer’s instruction. When indicated, rabbit polyclonal antibodies against NCAM (10 μg/ml) or nonspecific rabbit IgG (10 μg/ml) were applied to cells in culture medium at 37 °C in a CO2 incubator.

**RESULTS**

**Caspase-3 Activation Is Reduced in NCAM−/− Brains**—We started our work by comparing levels of the apoptotic markers in NCAM−/− and NCAM+/+ mouse brains. Western blot analysis of the brain homogenates with caspase-3 antibodies recognizing both pro- and active caspase-3 showed that the levels of the 35 kDa procaspase-3, the predominant form of caspase-3 in brains of mice of both genotypes (Fig. 1), were slightly increased in NCAM−/− brain homogenates when compared with NCAM+/+ brain homogenates of young adult mice. At longer exposure times for Western blot analysis, a caspase-3 immunoreactive band at ~12 kDa, representing active caspase-3, was also detectable (Fig. 1). The level of active caspase-3 was approximately three times lower in NCAM−/− brain homogenates when compared with NCAM+/+ brain homogenates (Fig. 1).

The reason for reduced levels of active caspase-3 in NCAM−/− brains could be due to lower expression levels of caspase-3 activators, i.e. caspase-8, -9, and -10, which cleave procaspase-3, thereby producing active caspase-3 (28–31). While Western blot analysis did not reveal any statistically significant differences in levels of procaspase-8, -9, and -10 in NCAM+/+ versus NCAM−/− brain homogenates, the levels of cleaved caspase-8 were reduced in NCAM−/− brain homogenates indicating lower activation of this caspase and suggesting that NCAM is involved in regulation of caspase-3 activation via caspase-8 (Fig. 1).

**NCAM Associates with Caspase-8**—To explore possible mechanisms of NCAM-dependent caspase-8 activation, we
immunoprecipitated NCAM from NCAM$^{+/+}$ brain homogenates with NCAM antibodies and analyzed these immunoprecipitates for the presence of caspases by Western blot. Caspase-8, but not caspase-10 and -9, co-immunoprecipitated with NCAM. Interestingly, only cleaved caspase-8 but not procaspase-8 was detected in NCAM immunoprecipitates, with ~70% of all active caspase-8 molecules being in complex with NCAM (Fig. 2A).

To analyze whether the association of NCAM with caspase-8 depends on NCAM clustering at the cell surface, we used indirect immunofluorescence labeling to compare distributions of NCAM and caspase-8 in cultured hippocampal mouse neurons treated with NCAM antibodies to cluster NCAM at the cell surface or with nonspecific immunoglobulins as control. In control neurons, NCAM, and caspase-8 showed diffuse and partially overlapping distributions (Fig. 2B). Clustering of NCAM with NCAM antibodies induced pronounced aggregation of caspase-8 in NCAM clusters (Fig. 2B). The overall overlap between distributions of NCAM and caspase-8 was significantly enhanced after NCAM clustering as shown by the strongly increased correlation coefficient between distributions of these proteins (Fig. 2B). Hence, the combined observations suggest that NCAM associates with caspase-8 at the neuronal surface membrane, and clustering of NCAM induces aggregation of NCAM-associated caspase-8.

NCAM Enhances Association of Caspase-8 and -3 with Lipid Rafts—Clustering of NCAM enhances its association with lipid rafts and can direct NCAM-associated proteins to the plasma membrane and lipid rafts (10, 12, 13). To analyze whether NCAM deficiency affects the subcellular distribution of caspases, we estimated levels of caspase-3, -8, -9, and -10 in soluble cytosolic, total membrane and lipid raft fractions isolated from NCAM$^{+/+}$ and NCAM$^{-/-}$ brain homogenates.
FIGURE 2. Caspase-8 associates with NCAM. A, NCAM immunoprecipitates (IP) from NCAM^+/+^ brain lysates and 100% of input material used for IP were probed by Western blot with antibodies against NCAM and caspase-8, -10, and -9. Cleaved caspase-8 but not caspase-10 and -9 co-immunoprecipitated with NCAM. B, NCAM was clustered at the cell surface of 1-day-old cultured hippocampal neurons by NCAM antibodies. Alternatively, neurons were treated with nonspecific immunoglobulins (IgG). Neurons were then fixed and co-labeled with antibodies against caspase-8 and βIII tubulin. Images of the representative neurons (upper panels) and high magnification images of neurites outlined by dashed lines (lower panels) are shown. NCAM clustering induces aggregation of caspase-8 in NCAM clusters and enhances overlap between distributions of NCAM and caspase-8. Bar, 10 μm. The graph shows coefficients of correlation between distributions of NCAM and caspase-8 (mean ± S.E., n = 30 neurites analyzed in each group). *, p < 0.05, t test.
Western blot analysis of these fractions showed that all caspases analyzed were present at high levels in the cytosolic fraction (Fig. 3A). Caspase-8 and -3 were also enriched in the total membrane fraction, while caspase-9 and -10 showed lower preference to this fraction. Among all caspases, caspase-8 showed the highest association with lipid rafts, while only low levels of caspase-3 and -9 were detectable in lipid rafts. Caspase-10 was undetectable in lipid raft fractions (Fig. 3A). Interestingly, levels of caspase-3 and -8, but not levels of caspase-9 were reduced in the lipid raft fraction from
NCAM\textsuperscript{−/−} brains when compared with NCAM\textsuperscript{+/+} brains (Fig. 3B). Thus, it is likely that binding to NCAM enhances the association of caspase-3 and -8 with lipid rafts. In agreement with this idea, when NCAM and PrP, a marker of NCAM-containing lipid rafts, were co-clustered at the cell surface of cultured hippocampal neurons by incubating live neurons with NCAM and PrP antibodies, overlapping clusters of NCAM and PrP co-localized with caspase-3 accumulations (Fig. 3C).

Clustering of NCAM at the Cell Surface Induces Activation of Caspase-3 and -8—Aggregation of caspase-8 is known to result in its activation (32–35). To analyze whether clustering of NCAM, which is accompanied by caspase-8 aggregation in NCAM clusters (Fig. 2), results in activation of caspase-8 and consequent activation of caspase-3, we clustered NCAM at the cell surface of live cultured hippocampal neurons for 10 min and then labeled these neurons by indirect immunofluorescence with antibodies recognizing the activated form of caspase-3. These experiments showed that application of NCAM antibodies resulted in an \textasciitilde 70\% increase in levels of activated caspase-3 along neurites and in cell bodies of NCAM antibody-treated neurons when compared with neurons treated with control nonspecific immunoglobulins (Fig. 4A). The levels of activated caspase-3 in NCAM antibody-treated neurons were still much lower than in apoptotic cells occasionally observed in cultures (supplemental Fig. S1), indicating that NCAM antibodies do not induce overall apoptosis. Apoptotic cells were clearly distinguishable from non-apoptotic cells by morphological criteria, and were excluded from the analysis. Because antibodies recognizing only activated caspase-8, but not procaspase-8, are not available, immunocytochemical analysis of its activation could not be carried out. Levels of activated caspase-8 were also below Western blot detection limits when lysates of cultured neurons were analyzed. To obtain sufficient amount of material for this type of analysis, we used Chinese hamster ovary (CHO) cells which were stably transfected either with NCAM140, the major outgrowth promoting NCAM isoform (13), in a pcDNA3 expression vector or mock-transfected with this vector alone. Transfected live CHO cells were treated with control nonspecific immunoglobulins, or NCAM antibodies applied for different time periods. Lysates of these cells were then analyzed by Western blot with antibodies recognizing procaspase-8 (inactive) and cleaved activated caspase-8. This analysis showed that clustering of NCAM with NCAM antibodies resulted in a transient increase in levels of cleaved caspase-8 in NCAM140-transfected CHO cells (Fig. 4B). The peak in caspase-8 activation was observed within 5 min after NCAM antibody application, with levels of cleaved caspase-8 being \textasciitilde 2 times higher in NCAM-antibody treated CHO cells when compared with control immunoglobulin-treated CHO cells. Levels of activated caspase-8 then gradually declined reaching the baseline level at 30 min after NCAM antibody application (Fig. 4B).

Application of NCAM antibodies did not induce any changes in levels of cleaved caspase-8 in mock-transfected CHO cells (Fig. 4C), and did not have any effect on caspase-9 cleavage and activation in NCAM140-transfected or mock-transfected CHO cells (Fig. 4, B and C).

NCAM Activation Induces Proteolysis of Spectrin by Activating Caspase-3—In developing neurons, NCAM accumulates in the growth cones of neurites (Fig. 5) and regulates neurite outgrowth via modulation of the cytoskeleton and activation of the intracellular signaling cascades (10, 12). Indirect immunofluorescence labeling of cultured hippocampal neurons from NCAM\textsuperscript{+/+} mice, showed that caspase-3 was broadly distributed along growing neurites, but also accumulated in growth cones (Fig. 5, A and B). Caspase-3 distribution partially overlapped with the distributions of NCAM and its lipid raft-anchoring molecule PrP (Fig. 5, A and B).

These observations prompted us to analyze the presence and distribution of the proteolytic products generated by caspase-3, such as cleaved \textalpha II spectrin (36, 37), in cultured hippocampal neurons. Labeling of neurons with antibodies, recognizing caspase-3 cleaved \textalpha II spectrin, showed that 100\% of all neurons analyzed contained basal levels of cleaved \textalpha II spectrin (Fig. 5C). However, less than 5\% of all neurons were identified as apoptotic by labeling with antibodies against activated caspase-3. These apoptotic neurons displayed levels of active caspase-3 at least 400\% higher than levels of active caspase-3 in surrounding cells. Furthermore, cultures survived for up to 1 month, arguing against the idea that all neurons were apoptotic. Hence, the presence of cleaved \textalpha II spectrin accumulations in all neurons suggested that caspase-3 mediated \textalpha II spectrin proteolysis at low levels is a physiological process. Cleaved \textalpha II spectrin immunoreactivity was found accumulated in clusters in cell bodies, along neurites and in growth cones (Fig. 5, C and D). When NCAM was clustered at the neuronal cell surface, accumulations of the cleaved \textalpha II spectrin were also observed in NCAM clusters, suggesting that clustering of NCAM can induce proteolysis of the cytoskeleton adjacent to NCAM cluster.

To support this, first, we analyzed whether NCAM deficiency affects levels of caspase-3 substrates in growth cones isolated from brain. NCAM\textsuperscript{+/+} and NCAM\textsuperscript{−/−} growth cone fractions were analyzed by Western blot with antibodies against \textbeta II spectrin recognizing both full-length and caspase-3 generated proteolytic products (36, 37). Levels of the proteolytic products were normalized to the levels of full-length \textbeta II spectrin to estimate proteolysis of \textbeta II spectrin. Proteolysis of \textbeta II spectrin was strongly reduced in NCAM\textsuperscript{−/−} versus NCAM\textsuperscript{+/+} growth cones (Fig. 6A): \textasciitilde 13 \pm 1.3\% versus 1.3 \pm 0.8\% of the total \textbeta II spectrin protein was detectable as proteolytic fragments with molecular weights of \textasciitilde 160 and 110 kDa in NCAM\textsuperscript{+/+} and NCAM\textsuperscript{−/−} growth cones, respectively. In agreement, levels of full-length \textbeta II spectrin were \textasciitilde 20\% higher in NCAM\textsuperscript{−/−} growth cones in comparison to NCAM\textsuperscript{+/+} growth cones (Fig. 6A), again suggesting reduced proteolysis in the absence of NCAM. NCAM\textsuperscript{+/+} and NCAM\textsuperscript{−/−} growth cones contained similar levels of actin (Fig. 6A), suggesting that NCAM deficiency specifically affects spectrin proteolysis.

To verify that clustering of NCAM results in spectrin proteolysis, cultured hippocampal neurons from NCAM\textsuperscript{+/+} mice were treated for 5 min with NCAM antibodies to cluster NCAM at the surface or with control nonspecific immunoglobulins. Western blot analysis of the cell lysates with anti-
bodies against βII spectrin showed that application of NCAM antibodies resulted in an increase in levels of cleaved βII spectrin in the cultured neurons (Fig. 6B). Application of NCAM antibodies did not influence levels of actin and the cell adhesion molecule L1. NCAM-antibody induced βII spectrin cleavage was abolished in neurons pre-incubated with inhibitors of caspase-8 and caspase-3 before application of NCAM antibodies (Fig. 6B). Similar effects were also observed when isolated growth cones or cultured neurons were treated with NCAM antibodies and analyzed with antibodies against cleaved αII spectrin by Western blot or cell ELISA (supplemental Fig. S2). However changes in cleaved αII spectrin were...
smaller than those observed for βII spectrin, probably due to overall higher basal levels of cleaved αII spectrin, which may have obscured the NCAM antibody-induced effects. We conclude that clustering of NCAM at the surface of growth cones induces caspase-8 and -3-dependent proteolysis of the spectrin meshwork in growing neurites.

**NCAM-induced Neurite Outgrowth Requires Caspase-3 Activity**—Ligand-induced clustering of NCAM at the neuronal cell surface is known to result in enhanced neurite outgrowth rates (5, 10, 12). To analyze the role of caspases in NCAM-dependent neurite outgrowth, cultured NCAM+/+ hippocampal neurons maintained on substrate-coated poly-d-lysine were stimulated with the recombinant extracellular domain of mouse NCAM fused to the Fc portion of human IgG (NCAM-Fc) or NCAM antibodies. NCAM-Fc and NCAM antibodies were applied to the culture medium in the presence or absence of caspase-3 and -8 inhibitors. Incubation of neurons with NCAM-Fc or NCAM antibodies for 24 h resulted in ~70% higher neurite lengths when compared with neurons incubated with human Fc or nonspecific immunoglobulins as a negative control (Fig. 7). This effect was blocked by caspase-3 and -8 inhibitors (Fig. 7). In contrast, inhibitors of caspase-9 and -10 did not affect NCAM-dependent neurite elongation (Fig. 7). None of the treatments affected cell viability as measured by the Trypan Blue exclusion test (Fig. 7), and none of the inhibitors affected basal neurite outgrowth on poly-d-lysine alone (Fig. 7). We conclude that the activities of caspase-3 and -8 are required for NCAM-dependent neurite outgrowth.

**DISCUSSION**

The growth of neurites requires continuous changes in the structure of the cytoskeleton of the neurite and particularly in the growth cone. This cytoskeleton remodeling depends on
the assembly, stabilization but also the breakdown of cytoskeletal components (38). The idea that NCAM is involved in the regulation of the cytoskeleton was suggested by previous observations showing that NCAM associates with several cytoskeletal components including tubulin (39) and spectrin (40, 41). NCAM also induces the polymerization of the spectrin meshwork (3, 12).

Spectrin function is indispensable for axonal outgrowth and growth cone adhesion and motility (42–44). Spectrin mutations disrupt axonal outgrowth in Caenorhabditis elegans (42) and lead to defects in the architecture of the neuronal growth cone in Drosophila (45). The local proteolysis of the spectrin meshwork, presumably by calpains, is a crucial step in growth cone formation after axotomy (16, 17). How-

ever, how the local spectrin proteolysis is coordinated with the extracellular environment has remained largely unexplored. In the present study, we identify NCAM as a signal transducer that coordinates extracellular adhesion signals with spectrin proteolysis via the caspase-8/-3-dependent pathway.

Thus, our observations add new data to the growing body of evidence that caspases exert physiological functions even in the absence of cell death. Caspase-3 contributes to remodeling of the cytoskeleton as a protease in a non-apoptotic context in activated astrocytes by contributing to the reorganization of the cytoskeleton by means of vimentin cleavage (46). Caspase-3 is also implicated in the remodeling of the spectrin membrane skeleton during lens development and aging (47).

FIGURE 6. Clustering of NCAM induces caspase-3 and -8-dependent proteolytic processing of spectrin. A, NCAM+/+ and NCAM−/− growth cones were probed by Western blot with antibodies against βII spectrin and actin. Arrows indicate bands representing full-length protein and its proteolytic fragments produced by caspase-3. Note that proteolysis of βII spectrin is decreased in NCAM−/− growth cones. Actin levels are similar in both genotypes. Labeling for L1 served as loading control. Profiles of protein band densities calculated within the blot area marked with a curly bracket are shown on the right and were used to quantify differences. Graph shows quantitation of blots from several experiments with the levels of cleaved βII spectrin normalized to the total levels of βII spectrin (mean ± S.E., n = 6). *, p < 0.05, paired t test. B, NCAM+/+–cultured hippocampal neurons preincubated with inhibitors of caspase-8 or -3 or with vehicle were incubated for 5 min with chicken NCAM antibodies or nonspecific chicken immunoglobulins (Ig). Lysates of neurons were then analyzed by Western blot with antibodies against βII spectrin, actin, and L1 as loading control. Note increased levels of cleaved βII spectrin in neurons treated with NCAM antibodies in the absence of inhibitors. Profiles of protein band densities calculated within the blot area marked with a curly bracket are shown on the right and were used to quantify differences. The graph shows quantitation of blots from several experiments (mean ± S.E., n = 3) with the levels in neurons treated with Ig in the absence of inhibitors set to 100%. *, p < 0.05, paired t test.
NCAM-dependent Neurite Outgrowth Depends on Caspase-3

Inhibition of caspase-3 abrogates the formation of new growth cones of dorsal root ganglion cells and retinal cells after axotomy suggesting the role for caspase-3 in growth cone formation (22). In addition, the guidance cues netrin-1 and lysophosphatidic acid (LPA) have been shown to induce caspase-3 activation in retinal growth cones (48). Furthermore, active caspase-3 is necessary for the chemotropic responses to LPA and netrin-1, since LPA-induced growth cone collapse and netrin mediated growth cone attraction were blocked by specific caspase-3 inhibitors (48).

The association between NCAM and caspase-8 suggests that the NCAM-induced caspase-8/-3-dependent pathway is linked to the signaling network of enzymes activated by NCAM, which includes Ca$^{2+}$ influx via voltage-dependent Ca$^{2+}$ channels (11, 49). Our data showing that the levels of caspase-8 and -3 are reduced in lipid rafts isolated from NCAM$^{-/-}$ brains are in agreement with this idea. The observation that caspase-8 is enriched in lipid rafts is in agreement with observations on the association between caspase-8 and p60src protein tyrosine kinase, which accumulates in lipid rafts: In neuroblastoma cells, caspase-8 promotes the p60src mediated cell adhesion to fibronectin, an extracellular matrix glycoprotein that binds to integrins at the cell surface (50).

Caspase-8 associates with p60src via its DED domains and promotes the activation of the Erk pathway (50), which is also activated by NCAM (13). Phosphorylation of caspase-8 by p60src plays an important role in capase-8-dependent cell migration and signal transduction (51, 52). Importantly, in lipid rafts NCAM associates with fyn, another member of the Src kinase family, the activity of which is enhanced in an NCAM-dependent manner via CaMKIIα-mediated phosphorylation and activation of RPTPα which activates fyn (10, 11). Thus, it is plausible to assume that NCAM-induced caspase-8/-3 activation does not only co-exist with but also intersects with other NCAM-activated signaling cascades.

In vitro and in vivo studies show that caspase-3 contributes not only to the regulation of neuronal morphology and cell migration, but its cleavage occurs also in mature neurons during long term potentiation of synaptic activity: caspase-3 inhibitors block long term potentiation, memory and learning (53–55). Long term potentiation, memory and learning are also strongly impaired in NCAM-deficient mice (56). It remains to be investigated whether the cross-talk between NCAM and caspases is also observed in the adult brain and whether caspases are implicated in NCAM-dependent learning and memory.

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