

## Characterization of Human Cleaved N-CAM and Association with Schizophrenia

Marquis P. Vawter,<sup>\*,†</sup> Nsima Usen,<sup>\*</sup> Linn Thatcher,<sup>\*</sup> Bruce Ladenheim,<sup>\*</sup> Peisu Zhang,<sup>\*</sup> Dale M. VanderPutten,<sup>‡</sup> Katherine Conant,<sup>§</sup> Mary M. Herman,<sup>||</sup> Daniel P. van Kammen,<sup>¶</sup> Goran Sedvall,<sup>\*\*</sup> David L. Garver,<sup>††</sup> and William J. Freed<sup>\*,1</sup>

<sup>\*</sup>Development and Plasticity Section, Cellular Neurobiology Research Branch, National Institute on Drug Abuse, 5500 Nathan Shock Drive, Baltimore, Maryland 21224; <sup>‡</sup>DirectGene, 2661 Riva Road, Annapolis, Maryland 21401; <sup>§</sup>Department of Neurology, Johns Hopkins University, Baltimore, Maryland 21287; <sup>||</sup>Clinical Brain Disorders Branch, NIMH-IRP, 36 Convent Drive, Bethesda, Maryland 20892;

<sup>¶</sup>The Robert Wood Johnson Pharmaceutical Research Institute, 920 Route 202, Raritan, New Jersey 08869; <sup>\*\*</sup>Department of Clinical Neuroscience, Psychiatry Section, Karolinska Institute & Hospital, Stockholm, Sweden S-171 76; <sup>††</sup>University of Louisville

School of Medicine, Department of Psychiatry and Behavioral Sciences, Louisville, Kentucky 40222; and

<sup>†</sup>University of California, Irvine, Psychiatry & Human Behavior, Irvine, California 92697-1675

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The neural cell adhesion molecule (N-CAM) is a cell recognition molecule involved in cellular migration, synaptic plasticity, and CNS development. A 105- to 115-kDa isoform of N-CAM (cleaved N-CAM or cN-CAM) is increased in schizophrenia in hippocampus, prefrontal cortex, and CSF. We purified and partially characterized cN-CAM, a putative novel isoform, and confirmed that the first 9 amino acids were identical to exon 1 of N-CAM, without the signal sequence. Analysis of trypsin-digested cN-CAM fragments by matrix-assisted laser desorption ionization on a time-of-flight mass spectrometer (MALDI-TOF) yielded peptides that could be identified as being derived from the first 548 amino acid residues of the expected N-CAM amino acid sequence. Immunological identification with four specific N-CAM antisera directed toward cytoplasmic, secreted, variable alternative spliced exon, or GPI epitopes failed to indicate other known splice variants. Neuraminidase treatment of cN-CAM produced a minor alteration resulting in a faster migrating immunoreactive band, indicating partial glycosylation of cN-CAM. Membranous particles from cytosolic brain extract containing cN-CAM were obtained by ultracentrifugation; however, CSF contained few such particles. cN-CAM and synaptophysin were colocalized on these particles. Both cN-CAM and N-CAM 180 were present in synaptosomal preparations of human brain. Following incubation of synaptosomes or brain tissue without protease inhibitors, N-CAM 180 was degraded and cN-CAM was increased. A cN-CAM-like band was present in human fetal neuronal cultures, but not in fetal astrocyte cultures. Thus, cN-CAM represents a protease- and neuraminidase-susceptible fragment

possibly derived by proteolytic cleavage of N-CAM 180. An enlargement in ventricular volume in a group of adult patients with schizophrenia over a 2-year interval was found to be correlated with CSF cN-CAM levels as measured at the time of the initial MRI scan ( $r = 0.53$ ,  $P = 0.01$ ). cN-CAM is associated with ventricular enlargement; thus, the release of N-CAM fragments may be part of the pathogenic mechanism of schizophrenia in vulnerable brain regions such as the hippocampus and prefrontal cortex. Alternatively, the increases in cN-CAM in schizophrenia may be a reflection of a more general abnormality in the regulation of proteolysis or of extracellular matrix stability.

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**Key Words:** synaptosome; MALDI-TOF; protein sequence; ventricular enlargement; protease; N-CAM; cell adhesion molecule; cell recognition molecule; schizophrenia, proteolysis.

### INTRODUCTION

The neural cell adhesion molecule (N-CAM) is a cell recognition molecule involved in cellular migration, synaptic plasticity, and CNS development. N-CAM plays important roles in many neurodevelopmental processes including axonal guidance, synapse stabilization, and cell migration (16, 25, 47). The membrane N-CAM isoforms in human brain have molecular weights by SDS-PAGE of 120, 140, and 180 kDa, which correspond to 761-, 858-, and 1115-amino acid lengths. Additionally, a 105- to 115-kDa isoform previously termed cytosolic N-CAM and now termed "cleaved N-CAM" (cN-CAM) was identified in human brain on the basis of immunoreactivity to N-CAM antibodies (42);

<sup>1</sup> To whom correspondence should be addressed. Fax: 410-550-1621. E-mail: wfreed@intra.nida.nih.gov.

however, the amino acid identity of cN-CAM is unknown.

N-CAM alterations in schizophrenia were initially proposed as an explanation for disorientation of cells in the hippocampus (6). N-CAM may play a role in several features of schizophrenia such as ventricular enlargement, cognitive dysfunction, and in the putative neurodevelopmental etiology of schizophrenia (41). There are several reports of N-CAM alterations in schizophrenia, including decreased polysialylated N-CAM in the hippocampus (1), increased cN-CAM in the prefrontal cortex and hippocampus (20, 42), and increased N-CAM in the CSF (32, 33, 40). Alterations in the variable alternative spliced exon (VASE) and secreted (SEC) N-CAM isoforms were found in patients with bipolar disorder, but not schizophrenia (45, 46). Total N-CAM was similar in schizophrenia and control brains (1, 3).

The enlargement of ventricular volume in patients with schizophrenia is regarded as a hallmark of brain imaging studies [for review (24, 49)]. We hypothesized a relationship between CSF N-CAM and ventricular enlargement based on several lines of evidence. N-CAM 180 knock-out mice show ventriculomegaly (48). Ventriculomegaly in schizophrenia is speculated to result from decreased neuropil and neuronal size (15, 49). Further, soluble N-CAM may be developmentally toxic, as indicated by a gene targeting study in which removing all cytoplasmic domains of N-CAM was lethal to mice (34). The relationship between changes in ventricle size and N-CAM was examined in patients with schizophrenia who underwent consecutive MRI scans for a previous study (13). The increase in ventricle size over time was correlated with concentrations of N-CAM in the CSF, which were also measured in a separate prior study (44). In order to elucidate the nature of this novel N-CAM isoform, we partially purified and characterized cN-CAM from human postmortem brain tissue, compared this isoform to other splice variants of brain N-CAM, addressed the subcellular localization of cN-CAM, and examined the role of proteolysis and neuraminidase in the generation of cN-CAM in brain.

## METHODS

### *Antibodies*

Polyclonal antibodies that detect splice variants in N-CAM isoforms were directed toward the following: SEC (46), the intracellular domain of the 180/140 N-CAM exons (Becton Dickinson, 14.1.2), VASE (Becton Dickinson 3854) (45), and the GPI-linked N-CAM (GPI) epitopes. An antibody to an extracellular epitope of N-CAM (Becton Dickinson Polyclonal 3732, raised against purified human N-CAM) was used for detection of cN-CAM 105- to 115-kDa. Monoclonal antibodies

against actin (Sigma Clone AC-15) and synaptophysin (Sigma Clone SVP38) were used. Dilutions were 1:2500 for actin, 1:1000 for synaptophysin, and 1:1000 for all polyclonal N-CAM antibodies.

### *Western Immunoblot Analyses*

The procedure used previously for detection of N-CAM, synaptophysin, and actin was as described previously (42). Briefly, the cytosolic and membrane extracts were diluted (1 vol sample:1 vol loading buffer, SeptraSol, Integrated Separation Systems, Natick, MA) and denatured at 95°C and 40–80 µg of protein was separated by 7.5% SDS-PAGE (17). The immunoblots were probed with N-CAM, actin, or synaptophysin antisera. Primary antibody binding to the blotted membrane was visualized with a secondary goat anti-rabbit IgG horseradish peroxidase conjugate (Sigma, St. Louis, MO) diluted 1:5000 using an enhanced chemiluminescent reaction (Amersham Pharmacia Biotech, Inc., Piscataway, NJ). The membranes were exposed to BioMax MR film (Kodak) for 60–300 s. The films were developed and images transferred by a flatbed scanner with 600 dpi resolution (HPScanJet) to a computer. The optical density for each band was measured with NIH Image software (v 1.59b, Wayne Rasband, NIH).

### *Brain Tissue Extract*

Control human postmortem brain tissue was obtained from the NIMH Clinical Brain Disorders Brain Collection. A gross and microscopic neuropathological examination of each brain was conducted to rule out abnormalities, such as neuritic pathology hemorrhage, ischemia, neuronal loss, active infection, or tumor.

"Membrane extracts" and "cytosolic" tissue extracts of human occipital cortex were prepared as previously described (42). Note that the "cytosolic" extract would also contain soluble extracellular material. Frozen pulverized occipital pole (Brodmann Area 17) ~50 g was suspended in 1000 ml of cold 0.05 M Tris-buffered saline (TBS, pH 7.4) with protease inhibitor cocktail (PIC): antipain (4 µg/ml), pepstatin A (2 µg/ml), aprotinin (2 µg/ml), leupeptin (2 µg/ml), and phenylmethylsulfonyl fluoride (0.1 µg/ml). The solution was homogenized (Tissumizer; Tekmar, Cincinnati, OH) for 5 × 10-s pulses in a 4°C ice bath and with a 30-s cooling interval between pulses. The homogenate was centrifuged for 30 min at 42,000g at 4°C. The clear supernatant with visible lipid removed was the cytosolic fraction. The pellet was resuspended in cold TBS-protease inhibitor cocktail and recentrifuged at 42,000g for 30 min at 4°C, and the supernatant was discarded. The pellet was extracted by stirring at 4°C for 30 min in cold TBS + protease inhibitor + 1% NP-40 detergent. The resulting preparation was then centrifuged at 42,000g for 30 min, and the supernatant was the membrane extract. Protein content of the cytosolic and

membrane extract samples was measured by the micro bicinchoninic acid method (Pierce, IL). Extracts of rodent brain (Hsd: Sprague Dawley rats, Harlan, Indianapolis, IN; Swiss Webster mice, Taconic Farms, Germantown, NY) and occipital cortex obtained from squirrel monkey (NIDA, Dr. Ira Baum) were made using the same procedure described for human brain (42).

#### *Neuraminidase and Protease Inhibitor Treatment of Cytosolic and Membrane Brain Extracts*

Neuraminidase from vibrio cholerae (~2.49 units/ml, Fluka) in pH 5.5, 0.15 M NaCl, 4 mM CaCl<sub>2</sub> was diluted with 50 mM sodium acetate, 4 mM CaCl<sub>2</sub>, and 0.2 mM EDTA (pH 5.0; Buffer A) to 1.25 U/ml. Aliquots of cytosolic or membrane extracts of brain (100  $\mu$ l) were mixed with 100  $\mu$ l of diluted neuraminidase and incubated for 18 h at 37°C. Control aliquots of cytosolic and membrane extracts were treated with 100  $\mu$ l of Buffer A. Additional controls were refrozen for 18 h. Samples were later analyzed by immunoblotting N-CAM. Membrane and cytosolic fractions from human occipital cortex were incubated in the presence of protease inhibitors (10  $\mu$ M) for varying lengths of time at RT. An aliquot was analyzed by gel electrophoresis and immunoblotted with N-CAM 3732 antibody.

Protease inhibitors that were tested individually were: aprotinin, AEBSF, antipain, bestatin, amastatin, elastatinal, AMPSF, leupeptin, pepstatin A, and *trans*-epoxysuccinyl. Serine protease inhibitors are aprotinin, AEBSF, leupeptin (acetyl-leucyl-leucyl-arginal), elastatinal (Leu-(Cap)-Gln-Ala-al, *N*-[(*S*)-1-carboxyisopentyl]-carbamoyl- $\alpha$ -(2-imino-hexahydro-4(*S*)-pyrimidyl)-L-glycyl-L-glutaminy-L-alaninal), antipain ([(*S*)-1-carboxy-2-phenyl]-carbamoyl-Arg-Val-arginal), APMSF ((4-amidino-phenyl)-methane-sulfonyl fluoride), and PMSF. Cysteine protease inhibitors are leupeptin, *L-trans*-epoxysuccinyl-leucylamide-(4-guanido)-butane. Aminopeptidase inhibitors are bestatin ([(*2S,2R*)-3-amino-2-hydroxy-4-phenylbutanoyl]-L-leucine) and amastatin ([(*2S,2R*)-3-amino-2-hydroxy-5-methylhexanoyl]-Val-Val-Asp-OH). All protease inhibitors were obtained from Sigma (St. Louis, MO).

#### *Fetal Cell Cultures*

Human fetal astrocyte and neuronal cell cultures were compared to examine the origin of cytosolic N-CAM. Brain tissue from 9- to 12-week-old human fetuses was obtained in accordance with National Institutes of Health guidelines (<http://ohrp.osophs.dhhs.gov/humansubjects/guidance/publiclaw103-43.htm>) and (<http://www.nih.gov/niams/grants/notice/not93-235.html>). Excess tissue that was not needed for pathology was provided without patient identifiers, and no one profited from the transfer of the tissue. The preparation of astrocyte cultures was according to a previously

published method (5, 8). The neuronal cultures were prepared by washing tissue in PBS, mechanically disrupting the tissue by aspiration through a 19-gauge needle, and resuspension in minimal essential medium (MEM; GIBCO, Gaithersburg, MD) supplemented with 2 mM L-glutamine, 5  $\mu$ g/ml gentamicin, and 10% fetal calf serum. The disrupted cells were then distributed into poly-D-lysine-coated tissue culture plates. After 72 h, the medium was changed to serum-free MEM and tissue culture flasks were placed on an orbital incubator shaker at 225 rpm for 4–6 h at 37°C. The nonadherent cells were then placed into poly-D-lysine-coated tissue culture plates and maintained for a minimum of 10 days in Neurobasal medium (GIBCO) containing 1  $\times$  B27 and N2 supplements (GIBCO).

The membrane and cytosolic extracts of neuronal and astrocyte cultures were prepared by washing cells quickly in 4°C calcium-free PBS buffer. The cells were scraped, centrifuged (14,000 rpm in a microfuge for 15 s) to pellet the cells, and then resuspended in homogenization buffer (25 mM Tris-HCl, 4 mM EGTA, 2 mM EDTA, 250 mM glucose, 5 mM DTT, and 1 mM PMSF, pH 7.5). The pellet was homogenized (10 strokes with a Dounce homogenizer) and then centrifuged at 100,000g for 30 min. The supernatant was saved as the cytosolic fraction. Homogenization buffer containing 0.5% Triton X-100 was then added to the pellet. Following resuspension by sonication, the suspension was centrifuged at 100,000g for 30 min. The supernatant was then saved as the detergent-extracted membrane particulate fraction.

#### *Psychotropic Drug Treatment of Rats*

Four groups of rats (Sprague Dawley SD, Harlan Sprague Dawley, Inc., Indianapolis, IN) were injected daily for 30 days with either 2 mg/kg haloperidol (McNeil Labs, PA; *n* = 8), haldol vehicle (1.8 mg methylparaben/kg and 0.2 mg/kg propylparaben, Sigma; *n* = 7), lithium carbonate (2 mEq/kg/day, Sigma; *n* = 7), or physiological saline ip (*n* = 8). The animals were maintained in accordance with the NIH Guide for the Care and Use of Animals. The animals showed sedation following neuroleptic administration for the first 3 days and resumed normal activity throughout the remainder of the experiment. Two animals in the lithium group died, and the lithium dosage was decreased by one-half on Day 10. On Day 31, the animals were euthanized under CO<sub>2</sub>, and the hippocampus was dissected and stored at -78°C for Western immunoblot assay. In a separate experiment, mice (Swiss-Webster, Taconic Farms) were treated with methamphetamine (*n* = 10; 4 sc injections, 10 mg/kg every 2 h) or saline (*n* = 10). The mouse hippocampus, prefrontal cortex, and striatum were analyzed for N-CAM immunoreactivity. Mice were used for methamphetamine experi-

ments because of previous experience using this model by our group [e.g., (4)].

#### *Purification of Cytosolic N-CAM from Human Brain*

Cytosolic N-CAM 105- to 115-kDa was prepared by first passing a cytosolic extract (135 ml at 4.47 mg/ml) over a 50-ml DEAE ion-exchange column (MacroPrep, Biorad, Hercules, CA). The column was washed with 450 ml 0.1 M Tris-HCl, pH 6.8, until no protein eluted into the absorbance detector. The absorbed protein was eluted with a linear gradient composed of 200 ml 0.1 M Tris-HCl, pH 6.8, and 200 ml of 0.1 M Tris-HCl, pH 6.8, 1 M NaCl. The resulting fractions were analyzed by SDS-PAGE and Western immunoblotting. The DEAE ion-exchange fractions with the highest N-CAM immunoreactivity were dialyzed and lyophilized. The DEAE fractions containing N-CAM were then partially purified by preparative SDS-PAGE. The bands from the preparative SDS-PAGE gel were visualized by zinc staining and Western immunoblot. The resulting bands with the highest N-CAM immunoreactivity/protein ratio were electroeluted from the preparative SDS-PAGE gel and dialyzed against distilled water, and the protein content was assayed by a NanoOrange fluorescence assay (Molecular Probes, Wilson, OR). In brief, the protein samples were added to NanoOrange reagent (1:500), and the mixtures heated at 95°C for 10 min and cooled at room temperature. Then the fluorescence emissions were measured directly on a fluorometer (TD-700 Fluorometer, Turner Designs, Sunnyvale, CA) with 486-nm excitation and 510- to 700-nm emission using bovine serum albumin standards (Pierce). Purity of the protein (0.05–10  $\mu$ g) at this stage was determined by SDS-PAGE and silver staining procedure (19) to detect impurities and by Western immunoblotting. The final purification was accomplished by SDS-PAGE, zinc staining, destaining in-gel, and transfer to PVDF membrane. Bands were visualized with amido black stain and Western immunoblotting. The band containing N-CAM immunoreactivity and amido black staining was excised from the PVDF membrane. Approximately 10 pmol of purified cN-CAM was sequenced by N-terminal Edman degradation. A duplicate PVDF membrane sample of purified cN-CAM was also subjected to enzymatic digestion with trypsin. The peptide fragments were characterized by matrix-assisted laser desorption ionization on a time-of-flight mass spectrometer (MALDI-TOF) (11).

#### *Ultracentrifugation of Brain and CSF*

A sample of cytosolic extract (see Brain Tissue Extract) was ultracentrifuged for 120 min at 38,000 rpm (266,000g) in a Beckman L8-M ultracentrifuge using a SW40Ti rotor at 4°C through a 0.5, 1.0, and 1.2 M sucrose density step gradient. The fractions and pellet were analyzed for N-CAM and synaptophysin by West-

ern immunoblot. The pellet was resuspended and centrifuged for 60 min at 38,000 rpm at 4°C in the SW40Ti rotor. The overlay of PBS was removed except approximately 0.5 ml of PBS, and the resulting pellet was resuspended by pipetting up and down. A 100-ml aliquot was treated with 1.9 ml of NP-40 detergent (5% solution in 1  $\times$  PBS and PIC) or control treatment consisting of 1.9 ml of PBS with PIC without NP-40. The tubes were then incubated at 4°C on a rocker for 30 min. Each treated aliquot was overlaid on a step sucrose density gradient (4, 3, 2, 1.5, 1 M sucrose in PBS and PIC). The tubes were topped off with 0.3 M sucrose and centrifuged at 30,000 rpm (159,600g) at 4°C for 60 min. One-milliliter fractions from each tube were removed and the absorbance at 280 nm was measured against a 1 M sucrose blank. Each sucrose fraction was run on a gel (70  $\mu$ l sample and 30  $\mu$ l 2 $\times$  denaturing solution) and analyzed for N-CAM and synaptophysin.

A 100-ml sample of discarded CSF was obtained from a patient with hydrocephalus, and PIC and sodium azide (0.01%) were added (CSF pool A). A sucrose density step gradient (6-ml size) was formed by layering 1 ml of each sucrose solution (4, 3, 2, 1.5, 1.0, and 0.5 M sucrose in PBS and PIC) in a polyallomer 14  $\times$  89-mm ultracentrifuge tube (Beckman, Fullerton, CA). A CSF layer of 6 ml on the top of the sucrose gradient was centrifuged at 30,000 rpm (159,600g), 4°C, for 60 min. Upon completion of centrifugation, the absorbance at 280 nm and weight of each sucrose layer were measured and the samples analyzed by Western immunoblot for N-CAM. A 50-ml aliquot of the same CSF pool (CSF Pool B) was precipitated with 50 ml of 4.1 M ammonium sulfate, pH to 5.0. The acidified CSF was precipitated on ice for 1 h and centrifuged at 42,000g, and the precipitate was resuspended in 500  $\mu$ l of 0.32 M sucrose in PBS, pH 7.4, with PIC. The precipitate did not resuspend and was dialyzed against 100 ml of sucrose PBS and PIC, pH 7.4, overnight at 4°C. A 400- $\mu$ l aliquot of the dialyzed precipitate was loaded on the top of a sucrose step gradient (3, 2.5, 2.0, 1.5, 1.0, 0.5 M) using 2-ml sucrose layers and centrifuged at 30,000 rpm (159,600g), 4°C, for 60 min.

#### *Immunostaining*

The high-density particles identified by ultracentrifugation of cytosolic brain extract were immunostained in chamber slides with antibodies to N-CAM and synaptophysin. All steps were performed at room temperature in an eight-well chamber slide precoated with 0.1% BSA for 15 min, washed, and allowed to air dry in a dust-free environment. An aliquot of the N-CAM-enriched sucrose gradient sample was diluted 1:10 in PBS and 2  $\mu$ l was spotted into the center of each chamber. The slide was fixed with 20  $\mu$ l of 5% paraformaldehyde in PBS, pH 7.4, for 10 min and

washed with 200  $\mu$ l of PBS three times. N-CAM and synaptophysin primary antibodies (1:1000) were added for 45 min followed by three PBS washes and a final incubation with 100  $\mu$ l of secondary antibody (1:250 anti-rabbit IgG FITC and anti-mouse rhodamine, Boehringer Mannheim Corp.) for 40 min followed by three PBS washes. The chambers were removed and an anti-fade solution (Molecular Probes, OR) was added prior to coverslipping. The immunostained particles were imaged by confocal laser scanning microscopy (Zeiss Model 410, Germany).

### *Electron Microscopy*

A suspension of particles from the pellet obtained by sucrose density gradient ultracentrifugation of brain cytosolic extract was fixed with 2% glutaraldehyde in PBS on ice for 20 min. The particles were pelleted at 10,000g for 1 h and the pellet was resuspended in 25  $\mu$ l PBS and then mixed with 25  $\mu$ l of 3% agarose at 60°C. A 1- $\mu$ l droplet of agarose-particle mixture was solidified on parafilm and cut into 1- to 2-mm cubes. The cubes were rinsed 3  $\times$  15 min in PBS, fixed with 1% osmium tetroxide in PBS for 1 h, and rinsed 3  $\times$  10 min in PBS. The cubes were en block stained with 2% uranyl acetate for 1 h and dehydrated through a series of 30, 50, 70, 80, 95, and 100% ethanol solutions for 5 min each. The dehydrated cubes were infiltrated overnight at 55°C with unaccelerated Durcupan ACM resin (Electron Microscopy Sciences, Fort Washington, PA), embedded into a flat mold with accelerated Durcupan resin, and polymerized at 55°C for 72 h. Ultrathin sections of 90 nm from two different cubes were prepared on an Ultracut E ultramicrotome. The sections were stained with uranyl acetate and lead citrate, and viewed with Zeiss 600 EM10A transmission electron microscope at 60 kV.

### *Subcellular Fractionation*

A protocol for synaptosomal localization of N-CAM (29) was used with several modifications. A sample of right human occipital cortex (49.4 g) was obtained 12 h postmortem and stored overnight at 4°C, diluted 20% w/v in solution A (0.32 M sucrose solution with 1 mM NaHCO<sub>3</sub>, 1 mM MgCl<sub>2</sub>, and 0.05 mM CaCl<sub>2</sub> with protease inhibitors) and homogenized by 12 strokes with a glass-Teflon homogenizer. The homogenate was filtered with a nylon mesh screen sieve and centrifuged at 1475g. The supernatant was saved and the resultant pellet (P1) was resuspended with solution A and centrifuged at 755g. This supernatant was combined with the first supernatant, and the combined supernatants (S1A) were centrifuged at 17,500g. The resultant pellet (P2) containing synaptosomes and mitochondria was resuspended in solution B (0.32 M sucrose solution with 1 mM NaHCO<sub>3</sub>), loaded into a sucrose density gradient of 0.85, 1.0, 1.2 M, and centrifuged for 2 h at

100,000g. The band between 1.0 and 1.2 M sucrose, which contains synaptosomes (S2) was saved as well as the other sucrose fractions S3 (top), S4 (0.85 M), S5 (1.0 M), and S6 (1.2 M). The synaptosome band (S2) was combined with 4 vol of solution B and centrifuged at 48,200g for 20 min.

The pellet was resuspended in solution C (0.16 M sucrose with 6 mM Tris-HCl containing 0.5% Triton X-100) and centrifuged at 48,200g. The supernatant was saved (S7) and the pellet was resuspended in solution B (S8), loaded on another sucrose gradient density with density steps of 1.0 (S10), 1.5 (S11), and 2.0 M (S12), and centrifuged for 2 h at 275,000g. The band between 1.5 and 2.0 M (S11) contains the postsynaptic densities, and the floating band at the top contains the synaptosomal membranes (S9). The fractions (S3-S12) were filtered through Amicon filters (MW cutoff 3000). Each fraction was analyzed by Western immunoblot pre- and postfiltration.

### *CSF N-CAM and Ventricular Volume of Patients with Schizophrenia*

Serial volumetric magnetic resonance images of the cerebral ventricles of 20 patients with schizophrenia ( $n = 18$ ) or schizoaffective disorder ( $n = 2$ ) were obtained using repeated blind measurements of total ventricular volume. Diagnoses were made according to DSM-IV criteria. The patients age was  $33.4 \pm 9.3$  years (means  $\pm$  SD). Freezer storage was  $32.9 \pm 4.0$  months, and illness duration was  $7.1 \pm 8.0$  years. Sixteen were male and 4 were female. The first scan was conducted in a drug-free state and the second scan after a 2-year period in which the patient was taking medications (27). CSF cN-CAM was previously determined for each patient (44). The CSF cN-CAM measurements were correlated with ratings on the Scale for the Assessment of Positive Symptoms and ventricular enlargement (mm<sup>3</sup>) occurring during a 2-year period.

### *CSF N-CAM in Neuroleptic-Naive and Neuroleptic-Free Patients*

CSF samples ( $n = 36$ ) were obtained from the Karolinska Institute. The samples were assayed for cN-CAM as previously described (46). There were three groups with similar numbers of female and male subjects (F/M): controls (8/7), neuroleptic-naive (4/6), and neuroleptic-free patients (5/6). The neuroleptic-naive patients had never been treated, while neuroleptic-free patients had received treatment but had been withdrawn. The average age ( $\pm$  SD) in each group was matched in controls ( $29.0 \pm 6.8$ ), neuroleptic-naive ( $26.8 \pm 4.0$ ), and neuroleptic-free patients ( $32.6 \pm 6.6$ ). Freezer time (years  $\pm$  SD) for the samples was significantly shorter for the neuroleptic-free patients ( $13.5 \pm 2.6$ ) compared to neuroleptic-native ( $16.5 \pm 2.5$ ) and

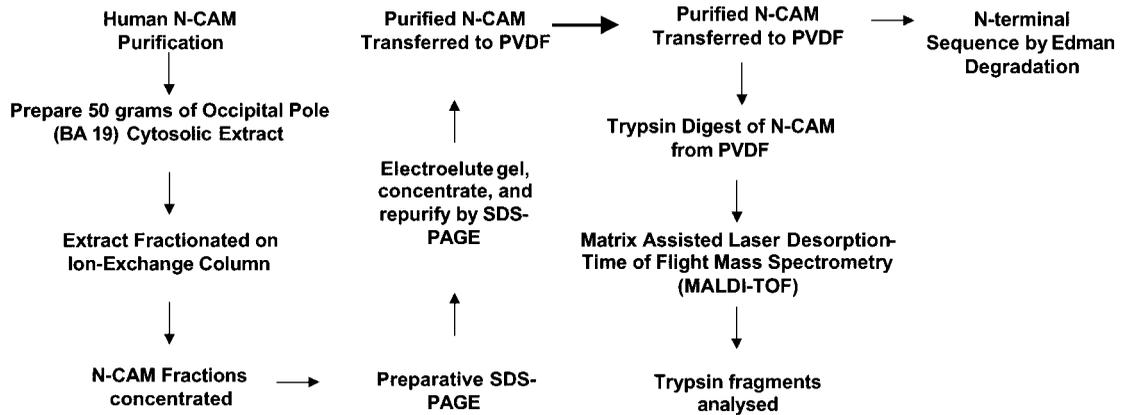


FIG. 1. Overview of the scheme for purification and characterization of cN-CAM.

control ( $15.6 \pm 1.1$ ) groups ( $P < 0.02$  for both comparisons).

## RESULTS

### Isolation of cN-CAM

An overview of the characterization steps for cN-CAM is shown in Fig. 1. The initial processing of 50 g of brain tissue into a cytosolic extract yielded 135 ml at 4.47 mg protein/ml. The cytosolic extract was passed over a DEAE ion-exchange column and fractions with

the highest N-CAM immunoreactivity were identified by immunostaining (Fig. 2A). The fractions with highest N-CAM immunoreactivity were dialyzed, lyophilized, and then partially purified by SDS-PAGE. The bands in the 105- to 115-kDa range visualized by zinc staining (Fig. 2B) with the highest N-CAM immunoreactivity (Fig. 2C, bands 4 and 5) were electroeluted from the gel from each preparative lane.

After electroelution, bands 4 and 5 were dialyzed against distilled water and a sample was repurified by

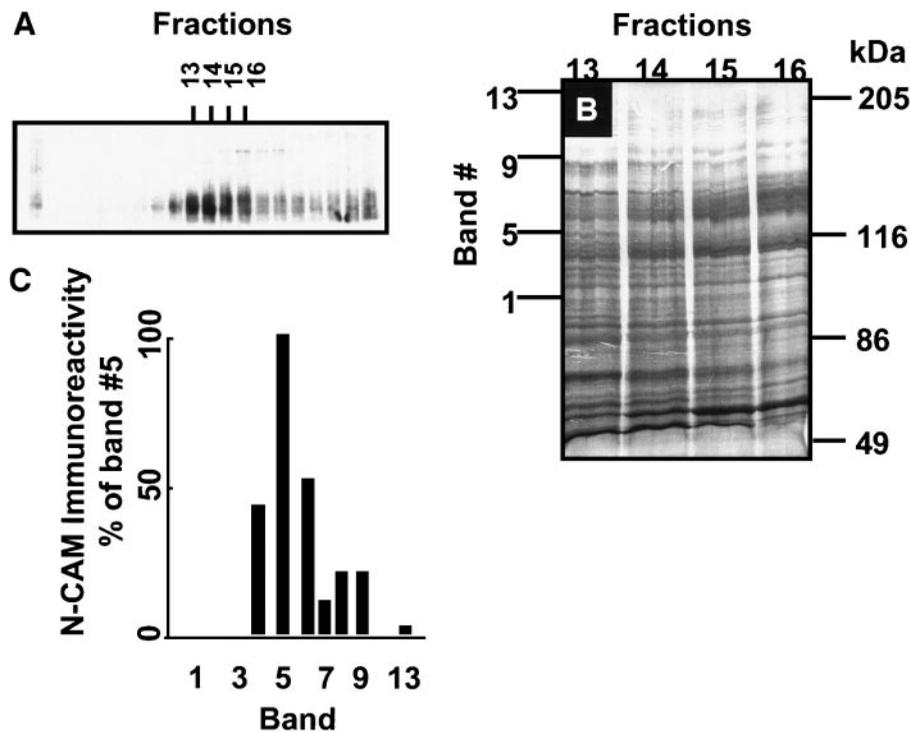
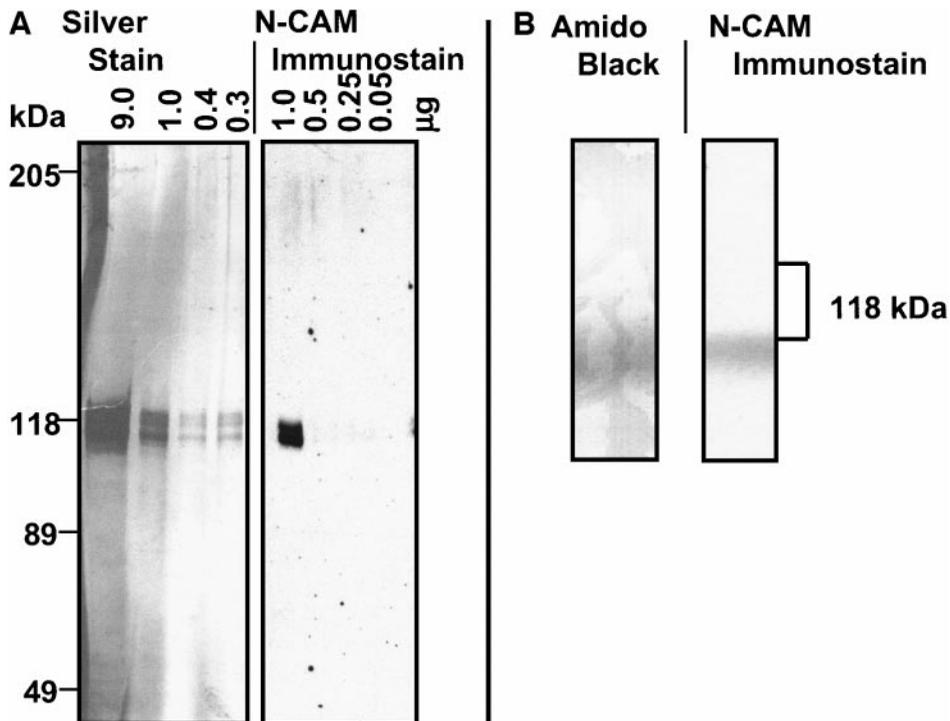


FIG. 2. A cytosolic brain extract was passed over a DEAE ion-exchange column and fractions with the highest N-CAM immunoreactivity were determined by immunostaining (A). The fractions with highest N-CAM immunoreactivity (Nos. 13–16) were dialyzed, lyophilized, and then partially purified by SDS-PAGE. The gel bands were visualized by zinc staining (B). Bands 4 and 5 were in the 105- to 115-kDa range and showed the highest N-CAM immunoreactivity (C). These two bands were electroeluted and repurified by SDS-PAGE (see Fig. 3).

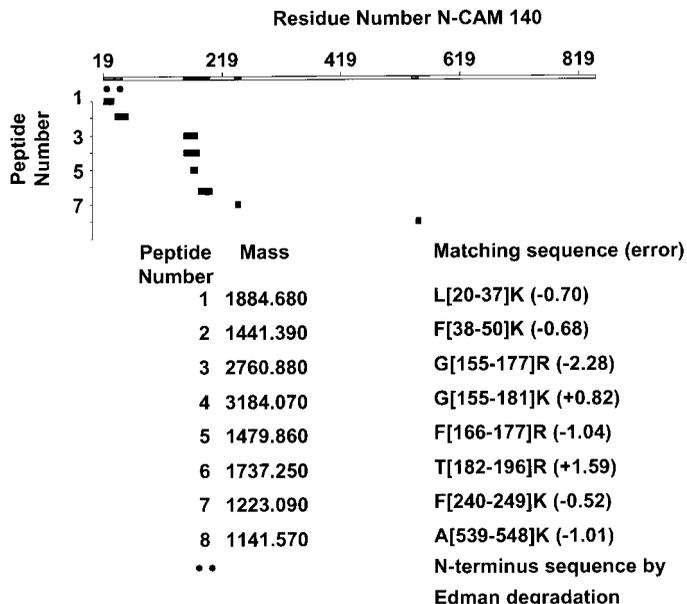


**FIG. 3.** Cleaved N-CAM final purification by SDS-PAGE. The band in the 105- to 115-kDa range with the highest N-CAM immunoreactivity (Fig. 2C, band 5) was repurified by SDS-PAGE and analyzed by silver staining and immunoblotting to detect impurities. Three protein bands were detected by silver staining and two distinct bands were seen by immunoblotting (A). The final preparative SDS-PAGE purification gel was stained with zinc, destained, and transferred to PVDF membrane, and the membrane stained with amido black and immunoblot (B) for location of N-CAM. The band corresponding to N-CAM immunoreactivity and amido black staining was excised and sequenced.

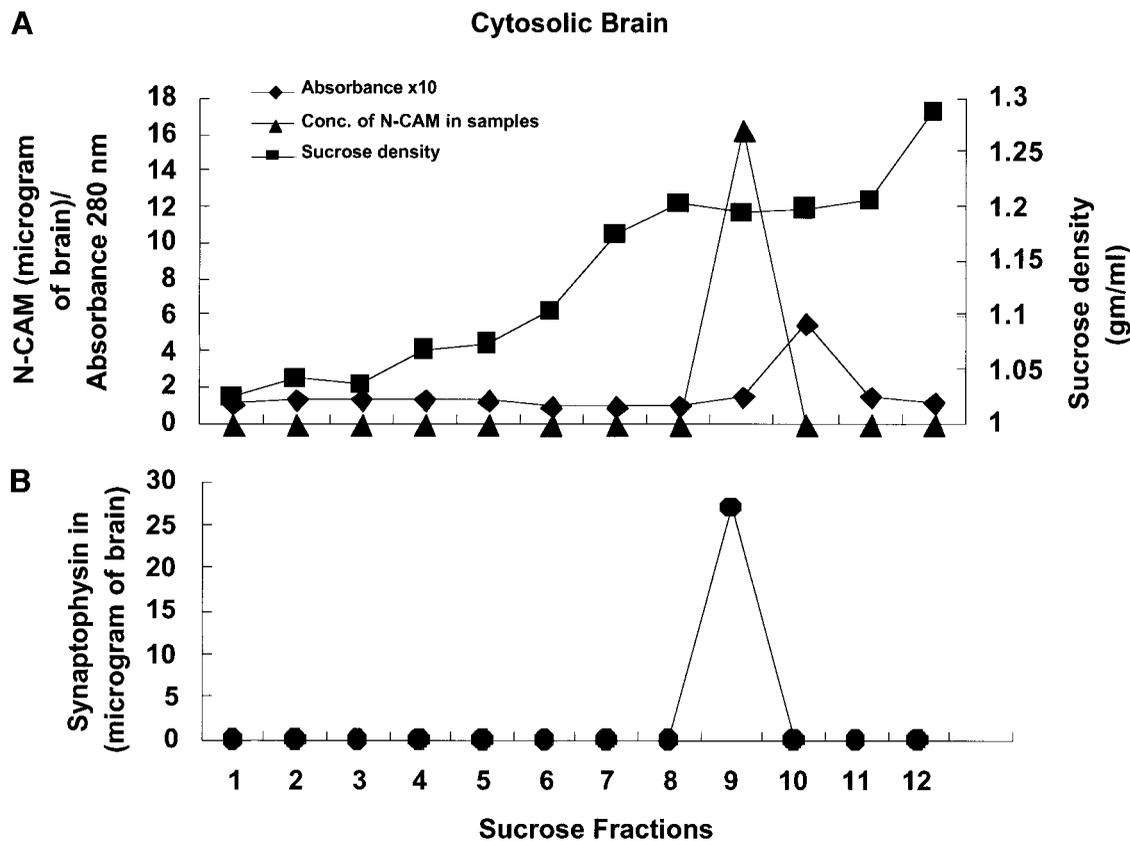
SDS-PAGE and analyzed by silver staining and immunoblotting to detect impurities. Three protein bands were detected by silver stain and two bands were seen by immunoblot (Fig. 3A). The final preparative SDS-PAGE purification gel was stained with zinc, destained, and transferred to PVDF membrane, and the membrane stained with amido black and immunoblot (Fig. 3B) for location of N-CAM. The band corresponding to N-CAM immunoreactivity and amido black staining was excised and sequenced.

*N-Terminal Sequence and MALDI-TOF*

A PVDF membrane with approximately 10 pmol of purified cN-CAM was sequenced by N-terminal Edman degradation. The resulting amino acid sequence X-Q-V-D-I-V-P-S-Q confirmed the first 8 amino acids to be exon 1 of N-CAM, without the signal sequence. This sequence is found in all of the higher MW N-CAM isoforms (180, 140, and 120 kDa), which verifies the common amino terminus identity of the N-CAM 105- to 115-kDa protein. A duplicate PVDF membrane sample of purified N-CAM 105–115 kDa was characterized by MALDI-TOF following trypsin digestion *in situ* to identify the masses of the peptide cleavage fragments (Fig. 4). Eight peptide fragments were found to match the



**FIG. 4.** A PVDF membrane sample of purified cN-CAM was characterized by MALDI-TOF following trypsin digestion to measure the masses of the peptide cleavage fragments. Matches within 2 mass units (*m/z*) corresponding to theoretical masses of the digested peptides were seen for eight peptide fragments.



**FIG. 5.** High-density particles from sucrose density ultracentrifugation of cytosolic brain obtained at a sucrose density of 1.194 g/L. The graphs (left side) show N-CAM concentrations in arbitrary density units (triangles), absorbance at 280 nm (diamonds), and sucrose density (g/L), as a function of sucrose fraction numbers. (B, D) Synaptophysin for the same sucrose fractions depicted in (A) and (C), respectively. (A) N-CAM immunoreactivity. Synaptophysin immunoreactivity comigrated with cN-CAM (B). Pretreatment of the brain cytosol with NP-40 prior to ultracentrifugation altered the migration of N-CAM, so that approximately 50% stayed at the top of the gradient and the remainder sedimented to the 1.237 g/L density level (C). NP-40 pretreatment disrupted the migration of synaptophysin (D), so that synaptophysin was seen in low concentration in all sucrose fractions.

theoretical masses of the digested peptides within 2 mass units ( $m/z$ ). These peptide fragments each comprise sequences found within the first 548 amino acids in N-CAM 120, 140, and 180 isoforms, of which all lie within the extracellular domain.

#### *Enriched Particles from Brain Containing cN-CAM*

High-density particles from sucrose density ultracentrifugation of cytosolic brain were obtained at a sucrose density of 1.194 g/L (Fig. 5A) and showed N-CAM immunoreactivity. The step gradient also showed synaptophysin immunoreactivity that was present in the same fraction as cN-CAM (Fig. 5B). Pretreatment of the brain cytosol with NP-40 prior to ultracentrifugation altered the migration of N-CAM so that approximately 50% stayed at the top of the gradient and the remainder sedimented to the 1.237 g/L density layer (Fig. 5C). The NP-40 pretreatment disrupted the migration of synaptophysin that was distributed throughout all sucrose fractions (Fig. 5D), presumably due to membrane solubilization.

The control fraction from the brain sucrose density preparations (without NP-40) was visualized by fluorescent immunocytochemistry and confocal microscopy, showing that a single class of particles was immunoreactive for both N-CAM (Fig. 6A) and synaptophysin (Fig. 6B). Electron microscopy showed irregular clusters of densely packed aggregated spheres and membranous particles (Fig. 6C).

High-density particles containing cN-CAM were isolated from CSF following the method outlined for brain. CSF showed cN-CAM predominantly in the lower density range fractions (1.03–1.07 g/L) at the beginning of the gradient (not shown). Approximately 15% of total CSF N-CAM was pelleted at a density of 1.28 g/L, which is near the same density value obtained for brain particles (1.19–1.23 g/L).

#### *Antibody Epitopes in cN-CAM*

Western blotting was performed with antibodies to epitopes that are present in various other N-CAM isoforms. Antibodies to the secreted exon, cytoplasmic

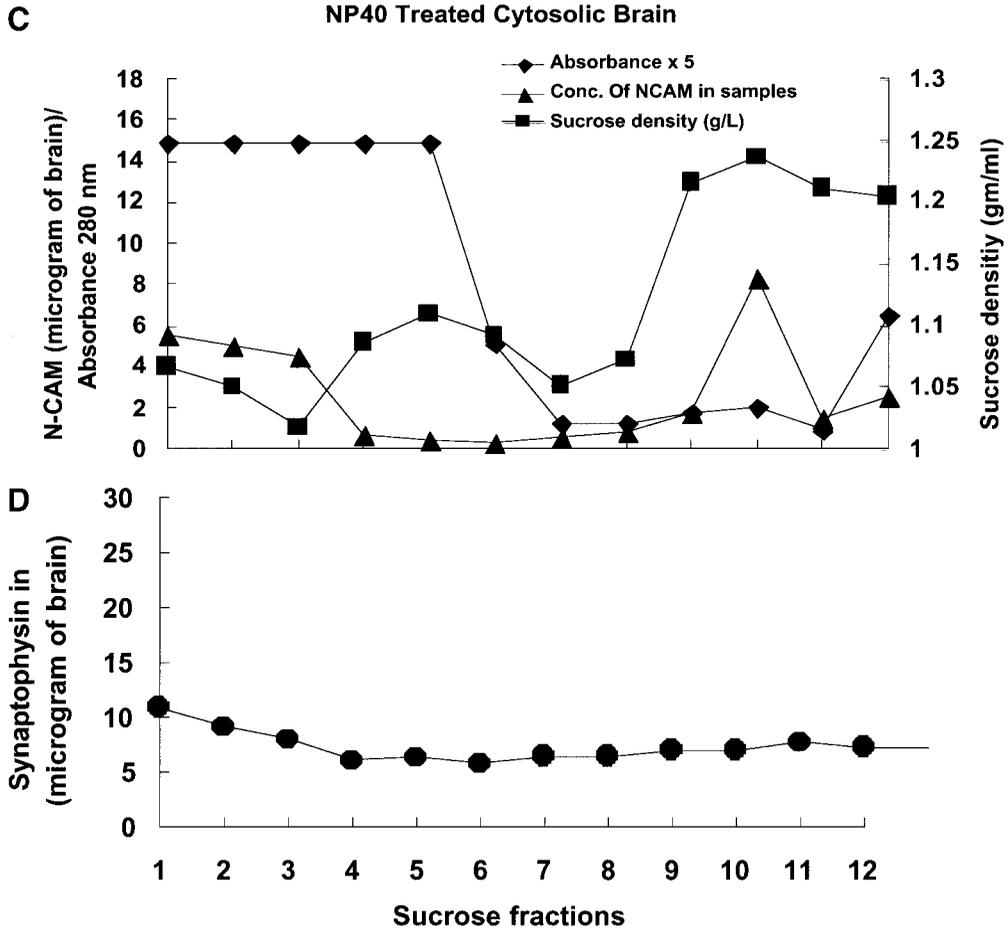


FIG. 5—Continued

exon (180/140), variable alternative spliced exon, and glycoposphatidylinositol exon did not react with cN-CAM 105- to 115-kDa (data not shown).

*In Vitro cN-CAM*

cN-CAM was prominent in adult fetal neuron cultures, but not in fetal astrocyte preparations (Fig. 7). The N-CAM immunoblot shows human adult brain cytosolic and membrane fractions (Fig. 7, lanes 1, 2). No N-CAM was present in the media supernatant from 15- to 17-week fetal cultures (Fig. 7, lanes 3, 4). The cytosolic cell extracts of 17 week neuronal cultures (Fig. 7, lane 5) show a doublet N-CAM band. The lower of the two bands corresponds to the cN-CAM band in the brain cytosolic fraction (Fig. 7, lane 1). The fetal neuronal membrane extracts show the high-molecular-weight embryonic N-CAM band with a molecular weight greater than 180 kDa, as well as a small amount of cN-CAM (Fig. 7, lane 6). Whole-cell extracts of astrocyte cultures (cytosolic and membrane fraction combined, Fig. 7, lane 7) and astrocyte media supernatant (Fig. 7, lane 8) are negative for N-CAM immunoreactivity.

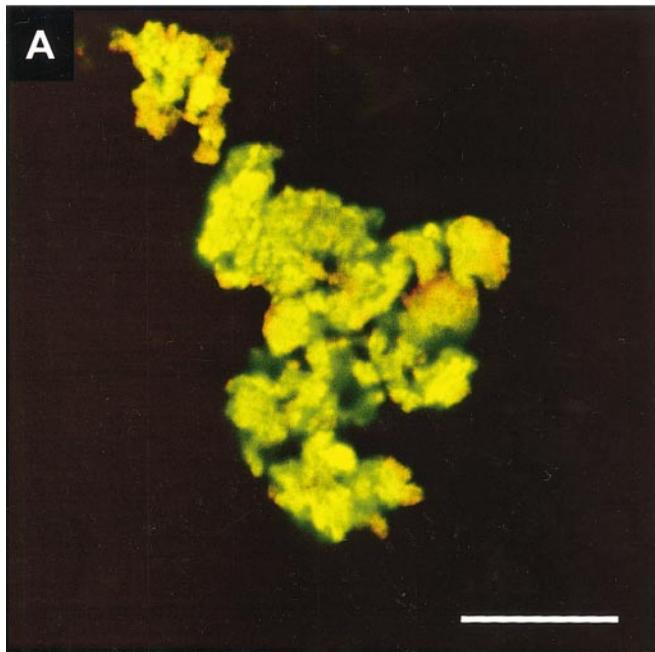
*Synaptosomal Preparation*

The subcellular fractionation of human occipital cortex showed N-CAM 180 to be present in synaptosome fractions that contain PIC (Fig. 8, preconcentrate, lanes 3–6). The presence of the synaptic vesicle marker synaptophysin also indicated that N-CAM 180 colocalized in the fractions that were expected to contain synaptosomes (Fig. 8, lanes 4–6). The postsynaptic density fraction (Fig. 8, lane 3) was negative for synaptophysin, but positive for cN-CAM. After removal of the PIC through filtration (Fig. 8, concentrate) the N-CAM 180 band was lost and a cN-CAM-like band was predominant (Fig. 8, concentrate lanes 3–6). Fractions S9–S12 did not contain detectable N-CAM. Band density for the same data was quantified and is shown in Fig. 8B.

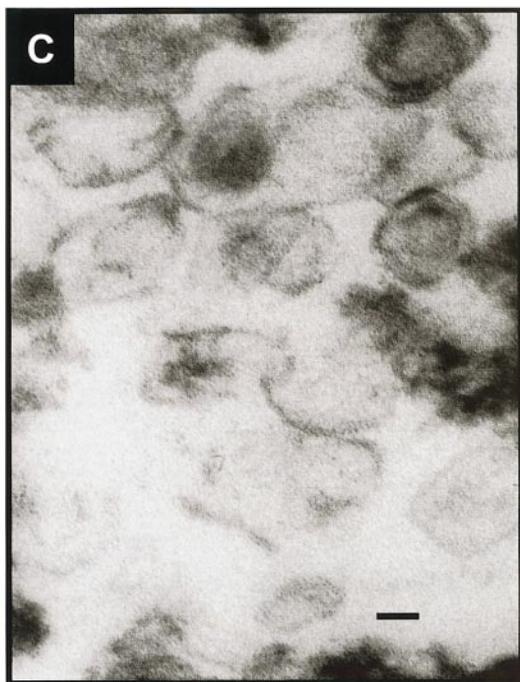
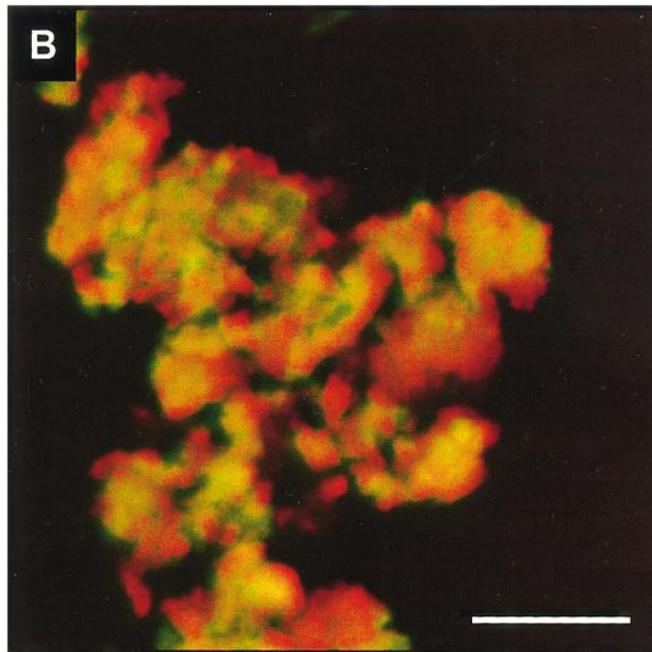
*Human Brain Proteolysis*

The pulverized adult occipital cortex samples incubated at RT showed a decrease in N-CAM 180 at 4–48 h (Fig. 9A). These cytosolic samples showed

## N-CAM (FITC)



## Synaptophysin (Texas Red)

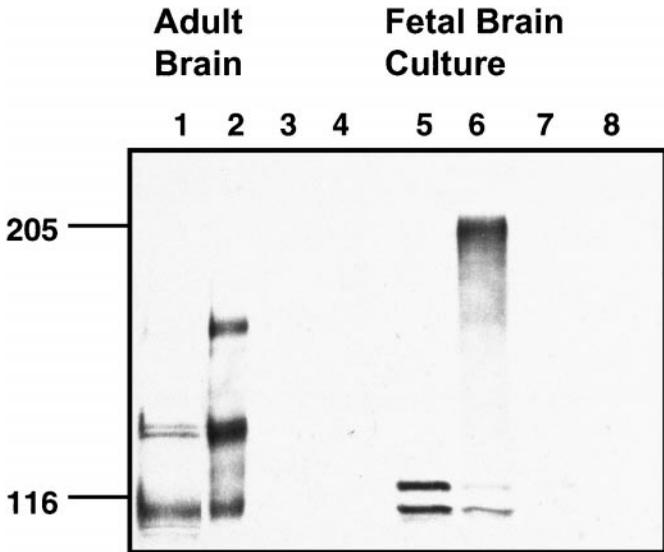


0.1  $\mu\text{m}$  —

**FIG. 6.** The control fraction (without detergent treatment) from the brain sucrose density preparations was visualized by fluorescent immunocytochemistry for N-CAM (A) and synaptophysin (B). A single class of particles was positive for both antigens. Electron micrographs showed irregular clusters of densely packed aggregated spheres and membranous particles (C). Bars, 10  $\mu\text{M}$  (A); 5  $\mu\text{M}$  (B).

an accumulation of cN-CAM with PIC present (Fig. 9A, lane 3+). When no PIC was present, cN-CAM was partially degraded in the cytosolic frac-

tion after 48 h incubation (Fig. 9A, lane 3-), but N-CAM 180 was essentially not detectable (Fig. 9A, lane 3).



**FIG. 7.** cN-CAM was prominent in fetal neuron preparations relative to fetal astrocyte preparations. The N-CAM immunoblot shows human adult brain cytosolic (lane 1) and membrane fractions (lane 2). N-CAM is absent in media supernatants from 15- to 17-week fetal cell cultures (lanes 3, 4). The cytosolic cell extracts from 17-week neuronal cultures (lane 5) show a doublet N-CAM band, the lower band corresponding to the cN-CAM band in the adult brain cytosolic fraction (lane 1). The fetal neuron membrane extracts also show a high-molecular-weight N-CAM, embryonic N-CAM with a molecular weight greater than 180 kDa, corresponding to embryonic N-CAM (lane 6). Whole-cell extracts of astrocyte cultures (cytosolic and membrane fractions combined; lane 7) and astrocyte media supernatant (lane 8) do not show N-CAM immunoreactivity.

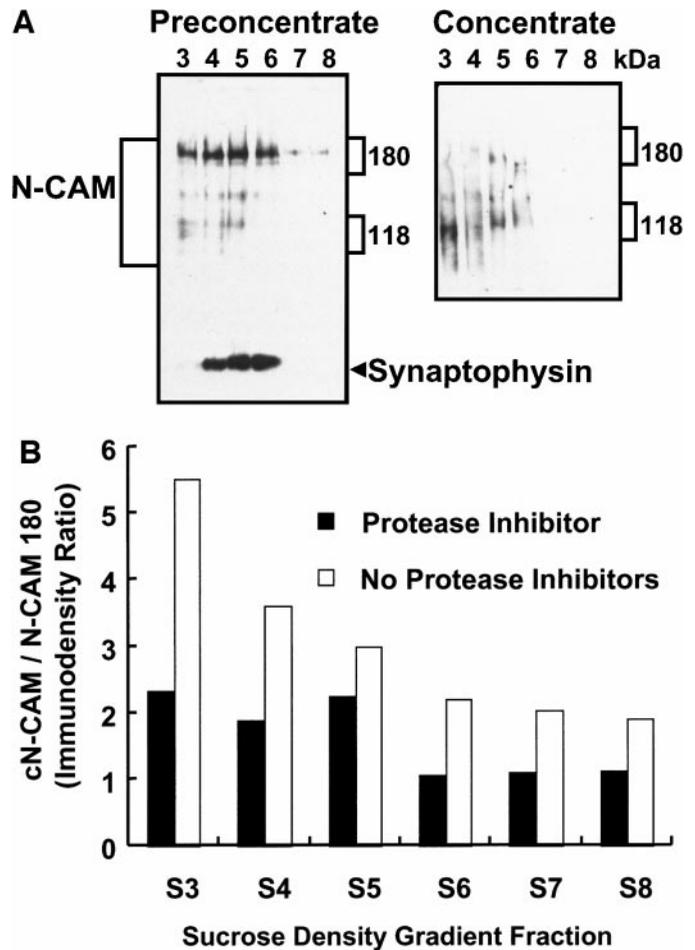
*Protease Inhibitors and N-CAM*

Protease inhibitors were tested at 10  $\mu$ M concentrations over 48 h since N-CAM 180 was shown to be lost completely by 48 h in the absence of protease inhibitor cocktail. The most effective protease inhibitors in blocking loss of N-CAM 180 were leupeptin (Fig. 10B) and antipain (Fig. 10C) compared to the standard control PIC (Fig. 10A). Inhibition of serine/cysteine proteases with leupeptin and antipain also prevented a buildup of cN-CAM apparently by preventing a degradation of N-CAM 180 to cN-CAM. Amastatin, bestatin, elastatinal, and *trans*-epoxysuccinyl were also effective to varying degrees (data not shown). Aprotinin, AEBSF, AMPSF, and pepstatin A were ineffective or produced complex effects that were difficult to interpret (data not shown).

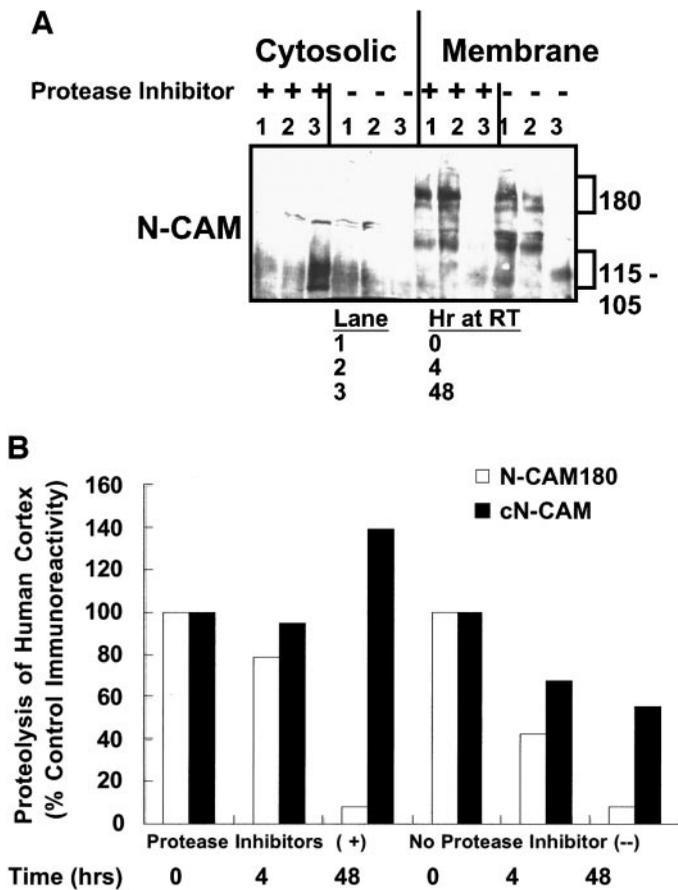
*Neuraminidase Treatment of Human Brain Extracts*

After neuraminidase enzyme treatment of membranes to remove polysialic acid, N-CAM 180 immunoreactivity completely disappeared and a faint cN-CAM band was seen (Fig. 11A, membrane +). Neuraminidase enzyme treatment of the cytosolic fraction produced a light doublet band resembling cN-CAM (Fig. 11A, cytosolic, +). The loss of immunoreactivity N-

CAM 180 following neuraminidase treatment was associated with the appearance of a strong N-CAM immunoreactive band at 50–60 kDa (degraded N-CAM or dN-CAM, Fig. 11A, membrane, +), which was not seen in any other control lanes (Fig. 11A, membrane, –). Figure 11B shows CSF from six separate individuals, showing N-CAM bands at 105- to 115-kDa corresponding to cN-CAM and a separate band at 75 kDa. The loss of N-CAM 180 and appearance of cN-CAM and dN-CAM (Fig. 11C) following removal of polysialic acid suggest that polysialic acid plays a role in protecting N-CAM 180 from degradation.



**FIG. 8.** The effect of protease inhibitors on cortical synaptosome preparation from human brain. (A) The synaptosome fractions from sucrose density ultracentrifugation were identified by synaptophysin immunoreactivity and also analyzed for N-CAM in the preconcentrated fractions. The protease inhibitor was removed from the synaptosomal preparation in the concentrated fractions. The results show a decrease in N-CAM 180 and increase in cN-CAM in the concentrated fractions, without protease inhibitors. The lane numbers refer to the sucrose density gradient fraction numbers. (B) Quantitation of the immunodensity shows that protease inhibitors decreased the amount of cN-CAM relative to N-CAM 180 in each sucrose density brain fraction, compared to removal of protease inhibitors.



**FIG. 9.** The effect of protease inhibitors on proteolysis of N-CAM 180 in human cortex. (A) Cytosolic extracts of cortical samples were left at room temperature for 4 or 48 h and treated with protease inhibitors (+) or no protease inhibitor (-). Membrane extracts were similarly treated. The results show that membrane N-CAM 180 is predominantly converted to a band similar to cytosolic N-CAM at 48 h. Without protease inhibitors the proteolysis is not halted and all N-CAM isoforms are degraded by proteolysis. (B) Quantitation of band density shows that accounts of cN-CAM and N-CAM 180 are similar with or without protease inhibitors at zero h; however, as proteolysis proceeds at 4 and 48 h there is a progressive loss of N-CAM 180 and a relative accumulation of cytosolic N-CAM (cN-CAM). By 48 h, N-CAM 180 is essentially lost under both conditions, whereas cN-CAM is relatively resistant to proteolysis.

### Drug Treatment and N-CAM

N-CAM 180, cN-CAM, and synaptophysin were measured in rats following lithium or haloperidol and mice following methamphetamine treatments. There were no changes in cN-CAM, N-CAM 180, or synaptophysin in the striatum, hippocampus, or prefrontal cortex in any of the drug-treated groups of animals compared to controls (data not shown).

### Ventricular Volume and CSF cN-CAM

The rate of ventricular expansion was  $2.2 \pm 1.6$  mm<sup>3</sup>/year (mean  $\pm$  SD) in the patients with schizophrenia and  $0.7 \pm 0.6$  mm<sup>3</sup>/year in controls as previ-

ously reported (13, 27). CSF cN-CAM was previously reported for this patient population (44). There was a significant correlation between CSF cN-CAM and ventricular change ( $r = 0.52$ ,  $P = 0.016$ ; Fig. 12A) in patients with schizophrenia. The correlation between CSF cN-CAM and the Scale for the Assessment of Positive Symptoms barely failed to reach significance ( $r = 0.442$ ,  $P = 0.051$ ; Fig. 12B).

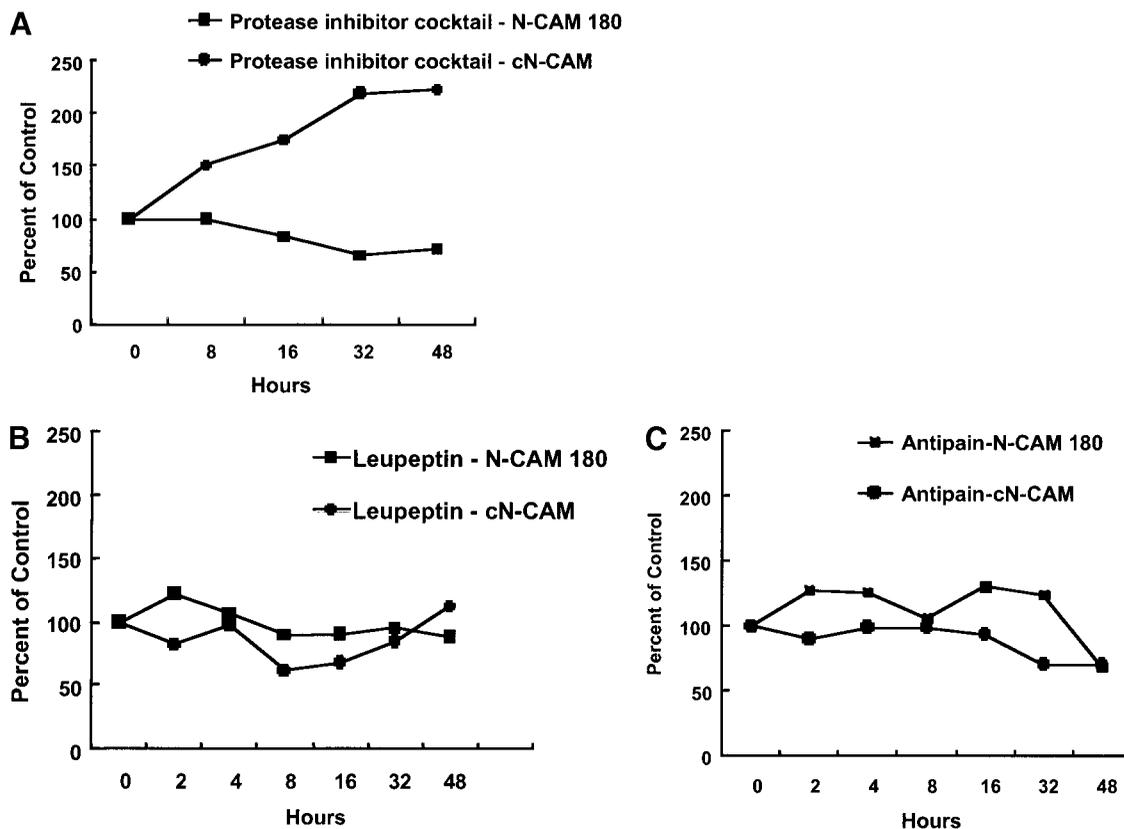
### CSF cN-CAM in Neuroleptic-Naive and Neuroleptic-Free Patients

CSF cN-CAM was not significantly different between groups (Fig. 13;  $F(2, 30) = 0.28$ ,  $P = 0.75$ ). The gender factor was significant ( $F(1, 30) = 4.46$ ,  $P = 0.043$ ). The chronic male patients that were neuroleptic-free at the time of CSF withdrawal had a significantly higher CSF cN-CAM concentration than female neuroleptic-free patients ( $P = 0.017$ ). Freezer-time was also entered as a covariable into an ANCOVA since it was shorter in the neuroleptic-free patients. The results were not changed for gender factor ( $F(1, 29) = 4.28$ ,  $P = 0.047$ ). Freezer time was not significantly correlated with CSF cN-CAM ( $r = -0.14$ ).

## DISCUSSION

The basic conclusion of the present study is that cN-CAM is a fragment of the 180-kDa N-CAM isoform, which is generated by proteolysis. Since cN-CAM is increased in schizophrenia, this could be a reflection of an aberration of proteolysis in schizophrenia, or of a more specific abnormality in the regulation of N-CAM stability and degradation. We characterized cN-CAM 105- to 115-kDa, as this isoform is increased in the brain and CSF of patients with schizophrenia. The N-terminal sequence (X-Q-V-D-I-V-P-S-Q; X refers to an undetermined first residue) corresponds to the N-terminal sequence for mature human N-CAM 180, 140, and 120. The first 19 predicted amino acids, which represent the signal sequence, were not present. This suggests that we did not isolate cN-CAM prior to signal peptide cleavage that presumably occurs during translocation through the Golgi trans-membrane apparatus to the cell surface. The results from trypsin digestion and MALDI-TOF analysis of purified cN-CAM showed peptides with sequences extending to the 548-amino acid region of the N-CAM protein. Therefore, cN-CAM 105- to 115-kDa shares a common N terminal with all known N-CAM isoforms. At this time, the C terminus of cN-CAM 105- to 115-kDa is unknown. cN-CAM does not share epitopes characteristic of any known splice variants for larger N-CAM isoforms: VASE, SEC, or the intracellular domain of N-CAM found on N-CAM 180 or N-CAM 140 kDa.

Localization of cN-CAM to particles in brain was shown by isolation of the particles and immunostain-

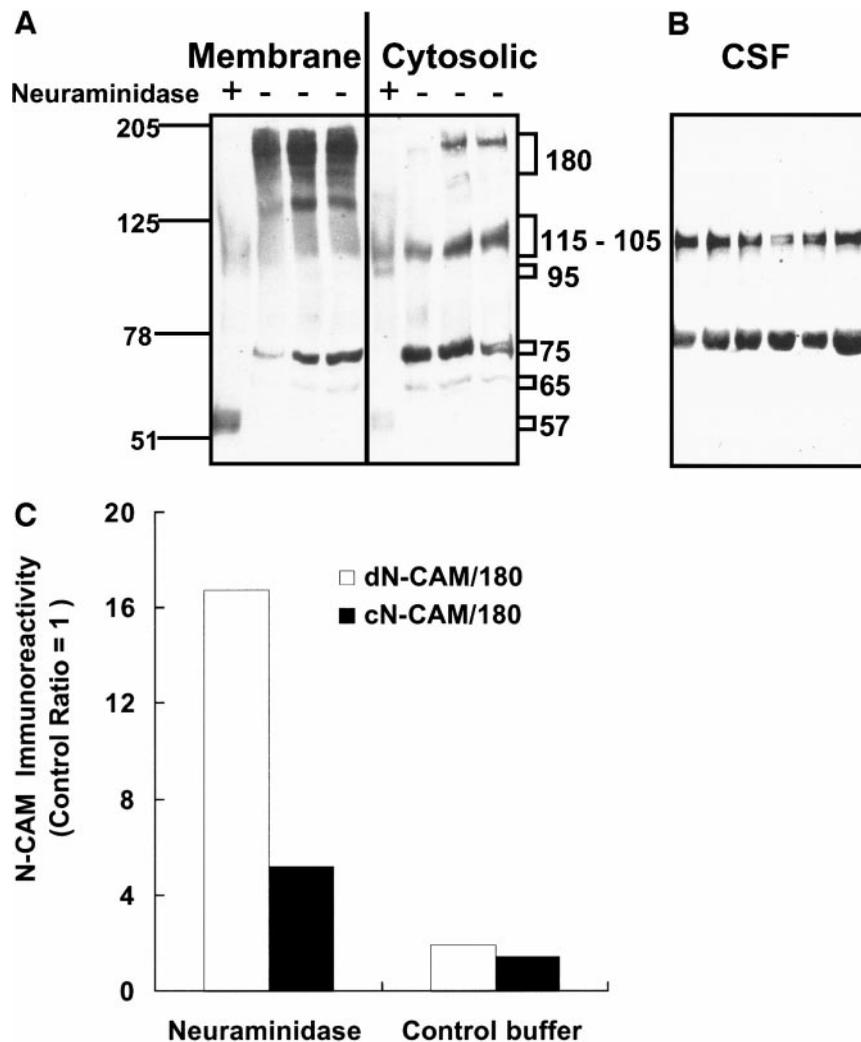


**FIG. 10.** Protease inhibitor effect upon N-CAM 180 and cN-CAM. (A) The protease inhibitor cocktail allows an accumulation of cN-CAM and loss N-CAM 180 over a 48-h incubation period at room temperature. Incubation with leupeptin (B) or antipain (C) at higher concentrations reduces the loss of N-CAM 180 and the accumulation of cN-CAM.

ing. Electron microscopy revealed membranous particles, some vesicular in shape. Detergent treatment of brain cytosol eluted ~50% of N-CAM from the particles. Isolation of cN-CAM from CSF showed that less than 15% was attached to particles based upon sedimentation. Since we observed that synaptophysin was also localized to these particles, we prepared synaptosomal fractions from fresh adult human brain. About 50% of cN-CAM was found to comigrate with synaptosomes. Our evidence of synaptosomal localization of cN-CAM is consistent with an observation that the three major isoforms of N-CAM localize in synaptosomes (30).

cN-CAM was found in fetal human neuron cultures, but not astrocyte cultures. Other cell culture systems (e.g., tumor cells and cell lines) were negative for cN-CAM (unpublished data). One possibility is that cN-CAM is a nonfunctional molecule, which is generated as a byproduct of an abnormality in the regulation of extracellular proteolysis. Alternatively, cN-CAM may play a role in N-CAM-related functions. It seems that cN-CAM is released from neuronal membranes through an enzymatic process, which is supported by the observation that membrane-attached 180-kDa N-CAM appeared to convert to cN-CAM upon incubation

at room temperature. Although this process can continue slowly in the presence of a mixture of protease inhibitors, the protease inhibitors leupeptin and antipain were potent in preventing the breakdown of N-CAM 180 and accumulation of cN-CAM. This result is consistent with previous observations that the serine protease tissue plasminogen activator when added to a soluble fraction of hippocampus also produces a 75-kDa CAM fragment that is reactive with N-CAM antibodies (18, 19). This extracellular proteolysis can occur after NMDA receptor activation, presumably by a release of tissue plasminogen activator from depolarized hippocampus (14), and subsequent degradation of N-CAM 180 (9, 10). There are also data suggesting an intracellular degradation route for N-CAM via a calpain-sensitive site (38). Thus, proteolytic degradation of N-CAM 180 and the resulting appearance of fragmented N-CAM isoforms such as cN-CAM (105- to 115-kDa), dN-CAM (~50 kDa), and the 75-kDa fragment might provide a mechanism for regulation of synaptic adhesiveness. Alternatively, however, the possibility that cN-CAM is generated as a byproduct of a proteolytic process which is primarily directed at other targets cannot currently be dismissed.

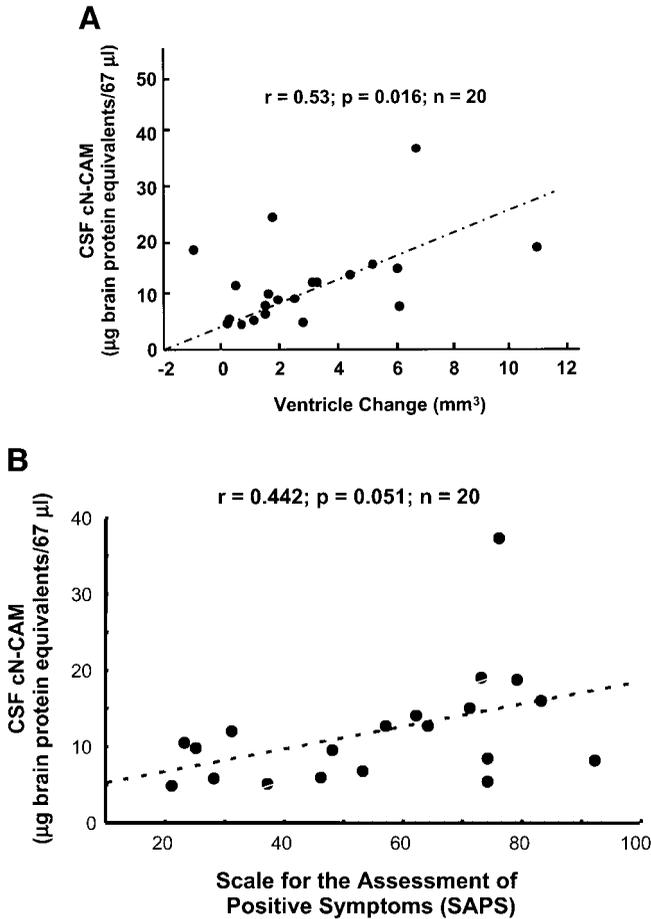


**FIG. 11.** The effect of neuraminidase on conversion of N-CAM 180 to cN-CAM. (A) Neuraminidase enzyme treatment (+) of membrane and cytosolic human brain extracts is shown to be permissive for conversion of N-CAM 180 to a low-molecular-weight N-CAM fragment (dN-CAM). (B) The CSF N-CAM immunoblot also shows cN-CAM and N-CAM 75 kDa similar to brain tissue. Lanes represent CSF from six separate individuals. (C) Ratio of dN-CAM to N-CAM 180 kDa (open bars) or of cN-CAM to N-CAM 180 kDa (solid bars) under two different conditions, either treatment with neuroaminidase or the control buffer. These ratios were arbitrarily set to "1" for the original sample (shown in lane 4, panel A). Lane 2 in (A) was treated with the control buffer and lane 3 was untreated. Lane 1 was treated with neuroaminidase. In the neuroaminidase-treated samples (A, lane 1) an increase in cN-CAM and dN-CAM can be seen (A, lane 1 and C). Thus, neuraminidase treatment to deglycosylate N-CAM 180 permits proteolytic processing of N-CAM 180 to cN-CAM and dN-CAM isoforms.

Increased cN-CAM in schizophrenia could result from an increased susceptibility of membrane N-CAM to protease and/or neuraminidase activity. Our present results suggest that cN-CAM can be generated from N-CAM 180 after removal of polysialic acid. The cleavage site appears to be extracellular, thus removing cytoplasmic epitopes. Known extracellular epitopes such as GPI, VASE, or SEC were not present in cN-CAM. Extracellular proteases such as tissue plasminogen activator and the protease inhibitor neuroserpin are regulated by neuronal activity (2, 9, 10, 18, 19, 23), and could be involved in the generation of cN-CAM. At the present we can predict that cN-CAM is derived from extracellular cleavage approximately between

amino acids 594 and 700, based upon an observed molecular weight of 105- to 115-kDa and an intact N terminus. Inspection of this region of the protein sequence reveals a consensus serine protease sequence site at residues 699–700, which is just upstream of the spliced variants of GPI and SEC exons.

The cN-CAM particles found in brain might be generated by membrane endocytosis. Both the N-CAM 180-kDa isoform and cN-CAM appear to be ubiquitous in synaptosomal preparations, and we further observed that disappearance of N-CAM 180 and appearance of cN-CAM occurred concurrently. This process was accelerated by the absence of protease inhibitors. cN-CAM-containing particles were immunoreactive for



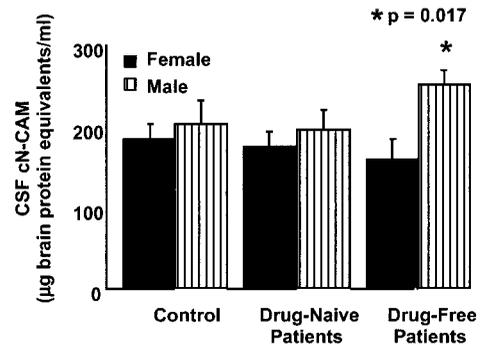
**FIG. 12.** Scatterplot showing the relationship between cN-CAM in CSF and changes in ventricular volume as measured by repeat MRI scans at a 2-year interval (A) and the Scale for the Assessment of Positive Symptoms (SAPS) (B). (A) CSF N-CAM and ventricle expansion show a statistically significant correlation. Patients with higher initial CSF cN-CAM concentrations showed greater ventricular enlargement. (B) CSF, cN-CAM, and SAPS ratings are modestly correlated in patients with schizophrenia. CSF cN-CAM is expressed as micrograms of brain equivalents/67  $\mu$ l of CSF. That is, a standard curve was run with varying amounts of protein from control reference brain cytosolic extract (44). The data are expressed as amounts of cN-CAM in units of micrograms brain protein equivalents present in the sample volume that was assayed (67  $\mu$ l).

synaptophysin, suggesting that the particles might contain some presynaptic membranes. Thus, the synaptosome and brain particle experiments agree in colocalizing N-CAM 180, cN-CAM, and synaptophysin to the synapse.

Several roles, although speculative, are suggested for N-CAM 180 degradation in humans. N-CAM can regulate and stabilize synaptic contacts as previously suggested in learning studies (26, 37). Further when peptide ligands of N-CAM that prevent N-CAM internalization are administered, passive avoidance learning is disrupted (12). We can speculate that our prior observations of increased cN-CAM in the CSF and brain of patients with schizophrenia (32, 33, 40, 42, 44)

are reflective of a dysfunction in the regulation of synaptic adhesion. The present data suggest the possibility of treating schizophrenia, and perhaps other neuropsychiatric disorders, with protease inhibitors and possibly neuraminidase inhibitors to prevent the breakdown of N-CAM 180 to cN-CAM and other breakdown fragments, e.g., dN-CAM and the 75-kDA fragment. In a previous study, we identified a substantial decrease in expression of the neuroserpin gene in the prefrontal cortex (43). Neuroserpin protein is an endogenous serine protease inhibitor (21), which is secreted from axons (28) and is transcriptionally regulated in concert with neuronal depolarization (2). The decrease in neuroserpin expression is therefore consistent with the hypothesis that an increase in protease inhibition might be of benefit in schizophrenia. This decrease in neuroserpin mRNA is also theoretically consistent with the observations of increased cN-CAM in schizophrenia. Neuroserpin might therefore be a potential target for manipulation in the treatment of schizophrenia. Interestingly, there is a familial dementia which is caused by a neuroserpin mutation (7).

There is an additional possible interpretation of the present results. It is possible that protease degradation of N-CAM 180, which results in the formation of cN-CAM, is a regulated process. The level of resistance of N-CAM 180 to degradation might be regulated by neuraminidase and by the activity of protease inhibitors such as neuroserpin. In schizophrenia, inhibition of N-CAM proteolysis might be deficient because of abnormalities in one or both of these processes. For N-cadherin, the resistance to protease degradation is regulated by dimerization, which occurs following depolarization (39). Our work suggests that removal of polysialic acid by neuraminidase has the capacity to decrease N-CAM protease resistance. It is therefore tempting to speculate that following synaptic modifications N-CAM 180 loses PSA residues, and thereby



**FIG. 13.** CSF cN-CAM in samples from the Karolinska Institute in a group of controls ( $n = 15$ ), never-treated patients ( $n = 10$ ), and a group of chronic patients that had been treated with neuroleptics and withdrawn ( $n = 11$ ). cN-CAM was significantly increased only in the male chronic patients. CSF cN-CAM is expressed as micrograms of brain cytosolic protein equivalents per milliliter of CSF.

becomes less resistant to serine proteases such as tissue plasminogen activator. It has been reported that polysialylated N-CAM is reduced in the hippocampus of patients with schizophrenia (1). Thus, if N-CAM protease resistance is regulated in synapses by PSA or by protease inhibitors such as neuroserpin, reduced neuroserpin gene expression in schizophrenia or an unidentified abnormality in PSA processing could also lead to increased cN-CAM.

An association of CSF N-CAM with ventricular enlargement was found in first-episode patients with schizophrenia that were followed for 2 years. It is possible that cN-CAM is related to the underlying pathogenetic mechanisms of ventricular enlargement. Certainly, N-CAM has multiple roles in neural plasticity, brain size, ventricular size, and learning (35, 36). Further, patients with schizophrenia show alterations in synaptic density, neuropil volume, neuronal size, ventricular enlargement, and learning. Since cN-CAM is not a significant component of serum (22, 31, 32), it is presumed that the brain and CSF expression abnormalities reflect abnormal function in the brain.

The possibility that concentrations of cN-CAM are elevated in brain parenchyma of first-episode patients cannot presently be dismissed. This possibility is not, however, feasible to study directly. It is proposed that as the schizophrenia illness progresses, cN-CAM in the brain parenchyma is gradually increased. Eventually, enough cN-CAM is released into CSF from the extracellular space so that an elevation occurs in the CSF in chronically ill patients. From previous experiments, we believed that since first-episode patients did not show marked elevations of CSF N-CAM, the elevations of CSF N-CAM seen in chronic patients were caused by a very long-term neuroleptic effect. However, we have utilized rodent models in which haloperidol, lithium, and methamphetamine were administered and found no apparent changes in N-CAM 180 or cN-CAM. These experiments do not rule out the possibility that changes in cN-CAM might be produced by acute drug administration. In first-episode patients that were given <20 mg of haloperidol prior to CSF N-CAM measurement, there was a significant decrease in CSF cN-CAM (44). Thus, it is possible that haloperidol tends to normalize CSF cN-CAM, as in first-episode patients. However, we should emphasize that the evidence from our animal studies of haloperidol are short-term, and involved rodents only. Therefore, we cannot exclude long-term medication as the cause of cN-CAM alterations in schizophrenia. The alternate explanation, based on the relationship of cN-CAM to ventricular enlargement and SAPS scores, is that the cN-CAM in CSF is related to the severity or progression of the illness.

The current data suggest that cN-CAM accumulation is related to abnormal proteolysis in schizophrenia, perhaps brought about by an imbalance in synap-

tic protease and protease inhibitor functions. It is possible that the accumulation of cN-CAM is itself related to a deteriorative or degenerative process in schizophrenia. Alternatively, it is also possible that abnormal regulation of proteolysis, and consequent instability of extracellular matrix and cell surface proteins, is related to the progression of schizophrenia. Accumulation of cN-CAM might then be a byproduct of a more general abnormality of proteolysis in schizophrenia.

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