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Assessment of Neural Cell Adhesion Molecule (NCAM) in Autistic Serum and Postmortem Brain

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Studies have identified structural abnormalities in areas of the autistic brain, with a pattern suggesting that a neurodevelopmental anomaly took place. Neural cell adhesion molecule (NCAM), which is involved in development of the central nervous system, was previously shown to be decreased in the serum of autistic individuals. In the present study, we measured NCAM protein in the sera from controls, patients with autism, siblings of autistic patients, and individuals with other neurologic disorders, but found no significant differences. We also measured NCAM protein in autistic postmortem brain samples and found the longest isoform, NCAM-180, to be significantly decreased. In addition, we investigated the mRNA expression of NCAM in these brain samples using cDNA microarrays and RT-PCR. Results show that NCAM mRNA levels are not altered in autism.

KEY WORDS: Autism; neural cell adhesion molecule; brain.

INTRODUCTION

Autism was described by Kanner (1943) over 50 years ago, however its cellular neurobiology is still unknown. Abnormalities in certain brain areas of autistic individuals have been described. These include small, tightly packed cells in the hippocampus (Raymond, Bauman, & Kemper, 1996) and decreased numbers of Purkinje cells in the cerebellum (Kemper & Bauman, 1998). Some neuroimaging studies indicate areas of the brain that are enlarged in autism, such as the lateral ventricles (reviewed in Piven, 1997; Minshew & Dombrowski, 1994). These changes have not been observed consistently and their significance is unknown, but they may be associated with delayed maturation (Piven, 1997). The pattern of neuropathological abnormalities suggests that these anomalies arise early during brain development (Kemper & Bauman, 1998).

Neural cell adhesion molecule (NCAM), a developmentally regulated protein in the brain, is a plausible candidate molecule for involvement in the pathophysiology of autism. The structure and function of NCAM have been reviewed in more detail elsewhere (Cunningham *et al.*, 1987; Doherty, Fazeli, & Walsh, 1995; Ronn, Hartz, & Bock, 1998). Briefly, NCAM is believed to be involved in the development of the nervous system by regulating axon fasciculation, neurite outgrowth, and synaptic plasticity (Baldwin, Fazeli, Doherty, & Walsh, 1996; Fields & Itoh, 1996; Mayford, Barzilai, Keller, Schacher, & Kandel, 1992; Rutishauser & Jessel, 1988). NCAM may also have a role in synaptic plasticity in the mature brain (Jorgensen, 1995) and has been associated with long-term potentiation and memory (Fields & Itoh, 1996; Luthl, Laurent, Figurov, Muller, & Schachner, 1994; Rose, 1995). NCAM is a cell-surface molecule belonging to the immunoglobulin (Ig) superfamily (Brümmendorf & Rathjen, 1995; Williams & Barclay, 1988). In brain,

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three major NCAM polypeptides of different sizes (120, 140, and 180 kDa) exist (Walsh & Dickson, 1989). In addition to these membrane-attached forms of NCAM, soluble NCAM has been identified in cerebrospinal fluid (CSF) and plasma (Nybroe, Linnemann, & Bock, 1989; Olsen, Krog, Edvardsen, Skovgaard, & Bock, 1993).

In addition to the significance of NCAM function, Plioplys, Hemmens, and Regan (1990) found that levels of a NCAM fragment were significantly decreased in autistic serum samples when compared to controls. Further, NCAM knockout mice display behavioral symptoms sometimes associated with autism such as high anxiety, decreased exploratory behavior, and abnormal stress response (Stork *et al.*, 1999). We therefore investigated NCAM protein and mRNA levels in autism. We measured NCAM protein in the sera from controls, patients with autism, siblings of autistic patients, and individuals with other neurologic disorders by Western blotting. NCAM was similarly measured in autistic and control postmortem brain samples. In addition, we used cDNA microarrays and the polymerase chain reaction with reverse transcription (RT-PCR) to investigate the mRNA expression of NCAM in these brain samples.

METHOD

Serum Samples

The 75 participants in this study constituted four clinical groups: control ($n = 25$), autism ($n = 33$), siblings of autistic patients ($n = 10$), and other neurologic disorders (OND; $n = 7$). A neurologist assigned a diagnosis of autism based on DSM-IV criteria (American Psychiatric Association [APA], 1994). The Autism Diagnostic Interview-Revised (ADI-R; Lord, Rutter, & Le Couteur, 1994) and/or Childhood Autism Rating Scale (CARS; Schopler, Reichler, DeVellis, & Daly, 1980) were also used in all cases. All autistic individuals had low-functioning autism that began after a period of apparently normal development for the first 18 to 24 months of age (Zimmerman & Gordon, 2000). Regression was defined as a loss of previously acquired language and social interaction skills and was diagnosed based on family reporting. Cognitive function in all autistic subjects was moderately to severely diminished. Seizures were not present in any of the autistic patients and all had undergone genetic and metabolic testing. From the medical records of all autistic subjects, it was determined that autism was not caused by known genetic or metabolic disorders including fragile X syndrome. The clinical data for these groups are presented in Table I. Blood was kept on ice until it was

Table I. Characteristics of Serum Samples by Group

	Clinical diagnosis			
Characteristic	Control ($n = 25$)	Sibling ($n = 10$)	Autism ($n = 33$)	OND[a] ($n = 7$)
Age (years)	35.1 ± 15.9	9.5 ± 4.3	8.9 ± 7.1	11 ± 3.8
Sex ratio (M:F)	12:13	5:5	29:4	7:0

[a] Other neurological disorder.

centrifuged at 2,500 × *g* in a Beckman G-15 centrifuge for 20 min at 4°C. A protein assay (Bradford, 1976) was then used to quantify the protein amount in the separated serum, using bovine serum albumin as a standard.

Tissue Samples

Frozen postmortem tissue samples from the cerebellar cortex of 9 autistic and 15 control individuals were obtained from the Harvard Brain Tissue Resource Center (Belmont, MA), in conjunction with the Autism Research Foundation (Boston, MA) and the University of Maryland Brain Bank (Baltimore, MD). The diagnosis of autism was assigned by a neurologist based on DSM-IV criteria (APA, 1994) and, in five out of nine cases, the ADI-R (Lord *et al.*, 1994). Additional frozen samples from the cerebellar cortex of 1 autistic and 1 control individual were obtained from the University of Miami (Miami, FL) in conjunction with the Maryland Brain and Tissue Bank. The diagnosis of autism was assigned by a neurologist based on the CARS (Schopler *et al.*, 1980). The clinical data for the autistic (A1–A10) and control (C1–C16) group are presented in Table II. Cerebellum samples (~250 mg) were prepared for protein analysis by homogenizing in SHEEP buffer (0.32 M sucrose, 4 mM Hepes, 0.1 mM EGTA, 0.1 mM EDTA, 0.3 mM PMSF). This preparation was then centrifuged at 2,000 × *g* for 10 min at 4°C. The supernatant was collected and protein concentrations were determined by a protein assay (Bradford, 1976) using bovine serum albumin as a standard. The pH of each brain sample homogenate was also measured using a Corning Chek-Mite pH-15 meter (Acton, MA).

Antibodies

MAB2122, a mouse anti-NCAM monoclonal, and MAB055-32/21, a mouse monoclonal against serum albumin, were purchased from CHEMICON International, Inc. (Temecula, CA). The albumin antibody was used at a dilution of 1:50,000, while the NCAM anti-

Table II. Clinical Characteristics of Brain Samples

ID	Age (years)	Sex	Race	PMI (hrs)[a]	Regression	Epilepsy	IQ	Cause of death
Autism								
A1	27	M	Caucasian	15	—	Yes	40–50	Accident, multiple injuries
A2	19	M	Caucasian	9.5	No	Yes	< 50	Cardiac arrest due to cardiac arrhythmia
A3	5	M	Caucasian	4.9	Yes	No	76–90	—
A4	20	M	Caucasian	15	No	Yes	< 30	Perforation of ulcer; Asphyxia second to vomiting
A5	54	M	Caucasian	4.3	No	No	50	Upper GI hemorrhage
A6	10	M	Asian	23	—	—	65	Asphyxia second to drowning
A7	21	F	Caucasian	20.6	No	No	< 20	Pneumonia/sepsis
A8	6	M	Hispanic	23.2	No	No	< 40	Asphyxia second to drowning
A9	19	M	Caucasian	15	No	No	40	Fire
A10	9	M	Caucasian	24	Yes	Yes	—	Leukemia
M	19.0 ± 14.3			14.5 ± 8.1				
Control								
C1	6	M	—	21				—
C2	16	M	African American	13				Gunshot wound to abdomen
C3	43	M	—	23				—
C4	63	M	—	23				—
C5	28	M	—	24				—
C6	24	M	African American	5				Gunshot wound to chest
C7	26	M	—	20				—
C8	19	M	African American	21				Epiglottitis
C9	20	M	—	16				—
C10	5	M	African American	19				Congenital heart disease
C11	19	M	Caucasian	17				Accident, multiple injuries
C12	53	M	—	17				—
C13	20	F	—	21				—
C14	22	M	—	12				—
C15	4	M	—	19				—
C16	5	M	—	15				—
M	23.3 ± 17.0			17.9 ± 4.9				

[a] Postmortem interval (hours).

body was used at 1:2,000. The A2066 actin antibody, a rabbit polyclonal, was purchased from Sigma (St. Louis, MO) and used at a dilution of 1:500. The NCAM antibody specifically recognized three major forms of NCAM in the brain (180, 140, and 120 kDa) as well as the major form in serum (105–115 kDa). HRP-labeled goat secondary against mouse was purchased from Jackson Immunoresearch Laboratories, Inc. (Westgrove, PA) and was used at a dilution of 1:5,000.

Western Blotting

All 75 serum samples as well as 11 control (C1–C11) and 9 autistic (A1–A9) postmortem brain samples were analyzed by Western blotting. Twenty micrograms of each serum and brain sample were loaded onto a 4–15% SDS polyacrylamide gradient gel (Bio-Rad, Hercules, CA) and electrophoresed at 60 mA for 90 min. For molecular weight determination, Bio-Rad Kaleidoscope protein markers were also electrophoresed on each gel. Separated proteins were then transferred to Protran nitrocellulose membranes (Schleicher & Schuell, Keene, NH) in Tris/glycine buffer (2.5 mM Tris, 192 mM glycine, 20% methanol) overnight at 30 mA. Nonspecific binding sites were blocked for 1 hour at room temperature in 50 mM Tris buffer containing 150 mM NaCl and 0.1% Tween 20 with 5% nonfat dry milk. Immunostaining was then performed by incubating the primary antibody for 1 hour at room temperature. The membranes were washed with PBST for 30 minutes and the membrane was incubated with the HRP-conjugated secondary for 1 hour at room temperature. After washing with PBST again for 30 minutes, an enhanced chemiluminescence detection system was used (ECL, Amersham Pharmacia Biotech, Piscataway, NJ) along with Kodak (Rochester, NY) X-OMAT AR film for detection of signal. Bands were quantified by measuring mean density using NIH image analysis

software (National Institutes of Health, Bethesda, MD). To standardize NCAM expression, the serum Western blots were reprobed with an albumin antibody, while the brain Westerns were reprobed with an actin antibody. No stripping was performed between incubations since the proteins were separated sufficiently for identification.

Western Blot Analysis

Serum Samples. To standardize each sample, the ratio of the NCAM band intensity to its albumin band intensity was calculated. A standard, which was electrophoresed on every gel, was used to normalize between gels. A natural log transformation was then used on all of the intensity values. To adjust for age and gender differences between clinical groups, a linear regression model was used to compare mean NCAM levels. In addition, a paired two-sample t test was used to compare autism patients (only the subgroup with siblings; $n = 10$) and their respective siblings.

Brain Samples. To standardize each sample, the ratio of the NCAM band intensity to its actin band intensity was calculated. A separate ratio was calculated for the 120, 140, and 180 kDa isoforms as well as for the total intensity of all three isoforms. A standard, that was electrophoresed on every gel, was used to normalize intensity values between gels. A natural log transformation was used on all of the intensity values and then four separate t tests were performed to evaluate NCAM levels (120 kDa, 140 kDa, 180 kDa, and total) between autism and control.

RNA Isolation

Total RNA from 10 autistic and 16 control brain samples (see above) was isolated using TRIZOL solution from Gibco/BRL (Gaithersburg, MD) as directed by the manufacturer. RNA yield was determined by measuring the A_{260} of a 5-μl aliquot. The A_{260}/A_{280} ratio for each sample was also confirmed to be between 1.9–2.0, the ratio pure RNA exhibits (data not shown). Integrity of RNA was also assessed by electrophoresing samples on an ethidium bromide-stained, denaturing agarose gel (data not shown).

cDNA Array Hybridization

NCAM mRNA expression was analyzed using the Atlas Human Neurobiology Array (Clontech Laboratories, Inc., Palo Alto, CA; www.clontech.com) which consists of 588 human cDNAs spotted in duplicate onto a nylon membrane. A total of 12 individual hybridizations were performed for autistic (A1, A2, A4, A5, A8, A9) and control (C1, C7–C9, C11, C12) brain samples. For each sample, poly A^+ RNA was isolated and a cDNA probe was synthesized from 15 μg total RNA using the Atlas Pure Total RNA Labeling System (Clontech) as specified by the manufacturer. The probe was separated from unincorporated ^{32}P nucleotide using Chromaspin-200 columns as described in the Atlas cDNA Expression Arrays User Manual (Clontech). The protocol for membrane prehybridization; hybridization with the probe; and washes is also detailed in this user manual. Atlas Image v.1.0 software (Clontech) was used to measure the spot intensities in the scanned images. The intensity values ($n = 588$) for each array were then imported into Partek (Partek Inc., MO) where a natural log transformation was used on all of the values. They were then globally normalized to a mean of zero and a variance of one, allowing for a comparison between arrays. Within Partek, a t test was used to evaluate the difference in NCAM expression between control and autism arrays. NCAM expression was also investigated with the UniGEM V2 microarray (Incyte Genomics, St. Louis, MO; www.incyte.com), which contains 9,374 cDNAs immobilized on a glass slide. One hundred micrograms of total RNA from six autistic brain samples (A1, A2, A4, A5, A8, A9) and total RNA from a pool of four controls (C7–C9, C11) were sent to Incyte Genomics where poly A^+ was isolated and cDNA probes synthesized with the fluorescent markers Cy3 (autistic) and Cy5 (control pool). Equal amounts of poly A^+ were used to generate probes. Six hybridizations were performed, one for each autistic sample against the control pool. Intensity values for each array, which were supplied by Incyte Genomics, were imported into Partek where a natural log transformation was used on all of the values. They were then globally normalized to a mean of zero and a variance of one. For each array, a ratio of autism intensity to control intensity was calculated. A t test was used to determine if these ratios were significantly different than 1.0 (equal expression).

RT-PCR

PCR with NCAM-specific primers was performed to verify microarray results. The samples analyzed on the microarrays as well as additional brain samples were used. First strand cDNA was synthesized from 5 μg total RNA using oligo d(T) with Superscript reverse transcriptase (Life Technologies, Gaithersburg, MD). Approximately 20 ng of cDNA were added to a 100 μL PCR mix containing 1X buffer (Qiagen, Valencia, CA). 1.5 mM $MgCl_2$, 200 μm of each nucleotide,

2.5 units of *Taq* DNA polymerase, and 0.5 μM of each gene-specific primer. Actin was used as a control to normalize NCAM expression. The primer sequences used were as follows: NCAMF (5′) CAG TCC GTC ACC CTG GTG TGC GAT GC, NCAMR (5′) CAG AGT CTG GGG TCA CCT CCA GAT AGC. ActinF (5′) CTC TTC CAG CCT TCC TTC CTG G and ActinR (5′) CTT GCT GAT CCA CAT CTG CTG G. The reaction mixtures were subjected to 24 cycles of two-step PCR (Clontech). Each cycle consisted of 20 seconds at 94°C and 1 minute at 70°C. The entire program was followed by a 5-minute extension period at 68°C. The reactions were subjected to successive, additional three cycles and viewed on an ethidium bromide-stained agarose gel to determine when the amplification of each product reached a linear phase. Bands in the linear phase were quantified by measuring mean density using NIH image analysis software (NIH, Bethesda, MD). The ratio of the NCAM density to the density of its actin band was calculated for each sample. A standard sample that was run in every RT-PCR was used to normalize between RT-PCRs. A natural log transformation was used on all the values and then a *t* test was performed to evaluate differences in NCAM expression between control and autistic brain.

RESULTS

Clinical Variables

Six *t* tests were performed to determine if there were significant differences in the mean age between the four clinical groups. The mean age of the control group was significantly higher than the mean age of the autism, sibling, and OND groups ($p < 0.0005$; $p < 2.69e^{-5}$, $p < 2.56e^{-11}$, respectively). In addition, the sex ratios of the clinical groups were different. Because of these differences, a linear model was used to evaluate NCAM differences between the groups (see below). A *t* test was also performed to determine if there were significant differences in the mean age, PMI, or pH of brain homogenate (See Table II: pH data not shown) between autism and control postmortem brain samples. No significant differences were found ($p < .51$; $p < .19$; $p < .63$). In addition, the sex ratios of the two clinical groups were similar ($p < .74$).

Serum Western Blotting

Only the major form of NCAM in serum (105–115 kDa) was recognized by MAB2122. An example of a typical Western blot of serum samples is presented in Figure 1A. A prevalent protein in serum, albumin, was used to standardize NCAM expression and was present at the expected molecular weight (68 kDa). To adjust for age and gender differences between clinical groups, a linear regression model was used to compare mean NCAM levels. We observed a trend of elevated NCAM in serum samples of patients diagnosed with autism. However, no significant differences were found in normalized NCAM protein levels between the control and autism groups ($p < .07$), between the control and sibling groups ($p < .51$), between the control and OND groups ($p < .45$), nor the autism and OND groups ($p < .68$; Fig. 1B). A paired *t* test was also performed between autistic patients and their respective siblings to determine if their relatedness was masking an effect. However, no significant difference was found ($p < .75$).

Brain Western Blotting

The NCAM antibody recognized the 120 kDa, 140 kDa, and 180 kDa isoforms of NCAM in the human brain cerebellum (Fig. 2A). A longer exposure to film was used to visualize the 180 kDa isoform in each sample. The actin A2066 antibody recognized a single band at approximately 42 kDa, the expected molecular weight. Levels of actin were similar between the samples and were used to standardize NCAM expression. After standardizing, there was no difference in the mean value of total NCAM protein between autism and the control group ($p < .48$; Fig. 2B). However, when levels of 120 kDa, 140 kDa, and 180 kDa NCAM were compared individually, levels of the largest form (180 kDa) were significantly lower in autism ($p < .04$). There was no difference in the mean value of 120 kDa or 140 kDa NCAM isoforms between autism and control groups ($p < .92$; $p < .50$).

cDNA Microarrays

The Atlas human neurobiology array and the UniGEM V2 chip were used to examine the expression of NCAM mRNA in control versus autistic postmortem brain tissue. Both arrays produced a detectable signal where the NCAM cDNA was spotted (Fig. 3A and B). Hybridization of 12 age-matched samples to the Atlas human neurobiology array showed the intensity level of NCAM expression in the six autistic samples was consistently higher than NCAM expression in the six control samples (Fig. 3A). This difference was dramatic in sample A5 versus C12. When a *t* test was performed, however, the increase was not significant ($p < .07$). Similar to the Clontech array, hybridization of the same samples to the UniGEM V2 chip (Fig. 3B) showed that NCAM mRNA expression was slightly elevated in most

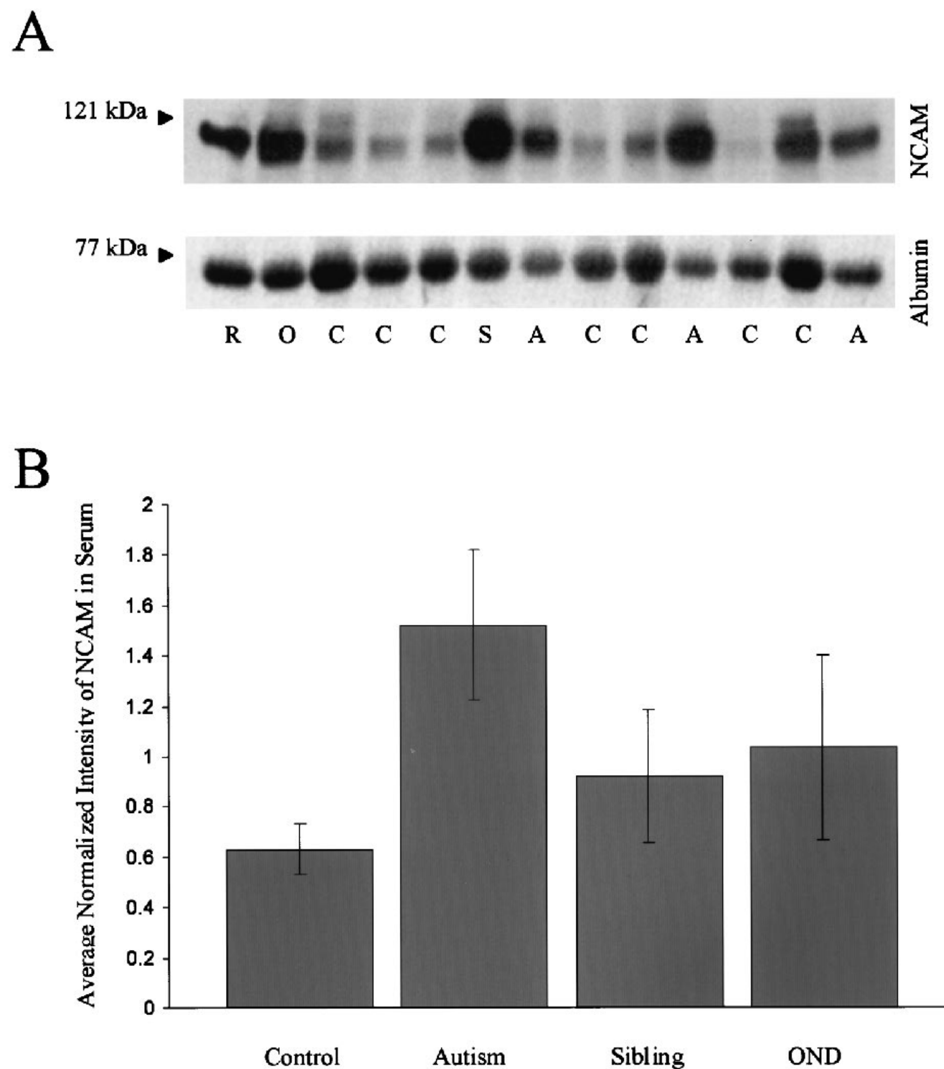
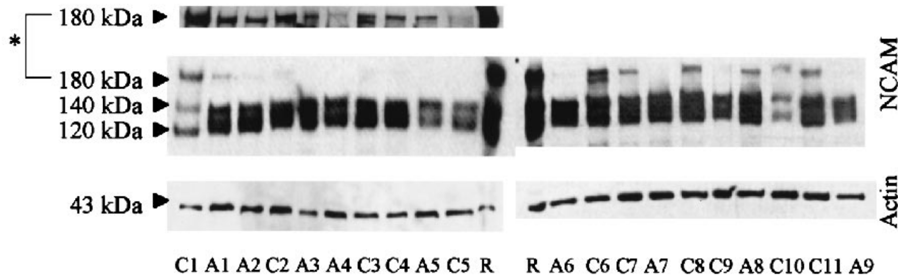


Fig. 1. Western blotting of serum samples from different clinical groups with NCAM and albumin antibodies. (A) Sample Western blot showing examples of clinical groups used in the study. The upper panel shows the major NCAM isoform in serum, and lower panel shows the albumin immunoblot used to standardize samples. R = reference; O = other neurological disorder; C = control; S = sibling of autistic; A = autism. (B) The average normalized intensity as a function of clinical group. The average normalized intensity is not significantly different between any of the clinical groups.

autism cases. When a *t* test was performed, however, the Incyte array results were consistent with those of the Clontech array in that there was no significant difference between autism and control NCAM mRNA expression ($p < .56$).

RT-PCR

RT-PCR was also used to evaluate the levels of NCAM mRNA expression in autistic versus control postmortem cerebellum samples. Samples chosen for RT-PCR were those used in the microarray experiments as well as additional samples. The PCR reactions produced single bands at the expected length for both actin (300 bp) and NCAM (700 bp; Fig. 4A). There is a large range in the level of NCAM mRNA between subjects. In some cases, there is little or no cDNA product. After additional PCR cycles are performed, however, the level of product appears to increase linearly (data not shown). Figure 4B shows the normalized intensity values of the age-matched autism and control samples. The difference in the mean normalized intensities between autistic and control groups is not significantly different when a *t* test is performed ($p < .43$; Fig. 4C).



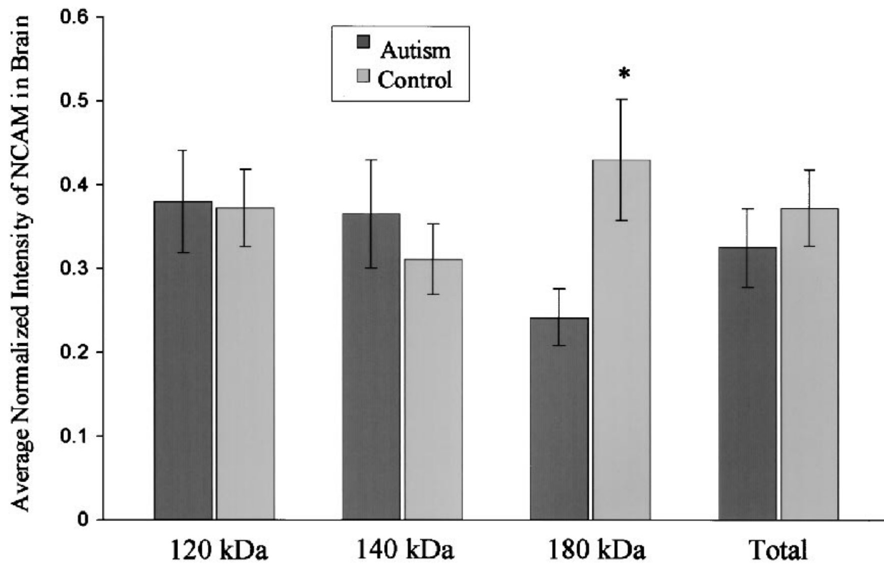


Fig. 2. Western blotting of control and autistic postmortem brain samples with NCAM and actin antibodies. (A) The upper panel shows the three major NCAM isoforms in brain (120, 140, and 180 kDa). The lower panel shows the actin immunoblot used to standardize samples. C = control; A = autism; R = reference. (*) Longer exposure is used to detect the 180 kDa NCAM in all postmortem brain samples. (B) The average normalized intensity as a function of NCAM isoform. When the 180 kDa isoform is analyzed separately, the average intensity is significantly decreased in autism as compared to controls ($p < .04$).

DISCUSSION

We have measured the levels of both NCAM protein and mRNA in autism. Our rationale for this study was based on the report of Plioplys *et al.* (1990) showing that NCAM protein levels were reduced significantly in the sera of 16 autistic individuals relative to 7 controls. That report represents one of the relatively few biological observations of significant differences between autistic and control samples. We thus sought to independently confirm the changes in NCAM.

In the present study we evaluated the levels of NCAM protein in serum and postmortem brain samples of individuals diagnosed with autism as well as various control groups (siblings, normal controls, and patients diagnosed with other neurological disorders). We found no significant differences in NCAM protein levels between these groups based on Western blot analyses. In postmortem brain samples, we detected the expected three major NCAM isoforms (120 kDa, 140 kDa, 180 kDa) and found that levels of the 180 kDa isoform were significantly reduced in brain samples

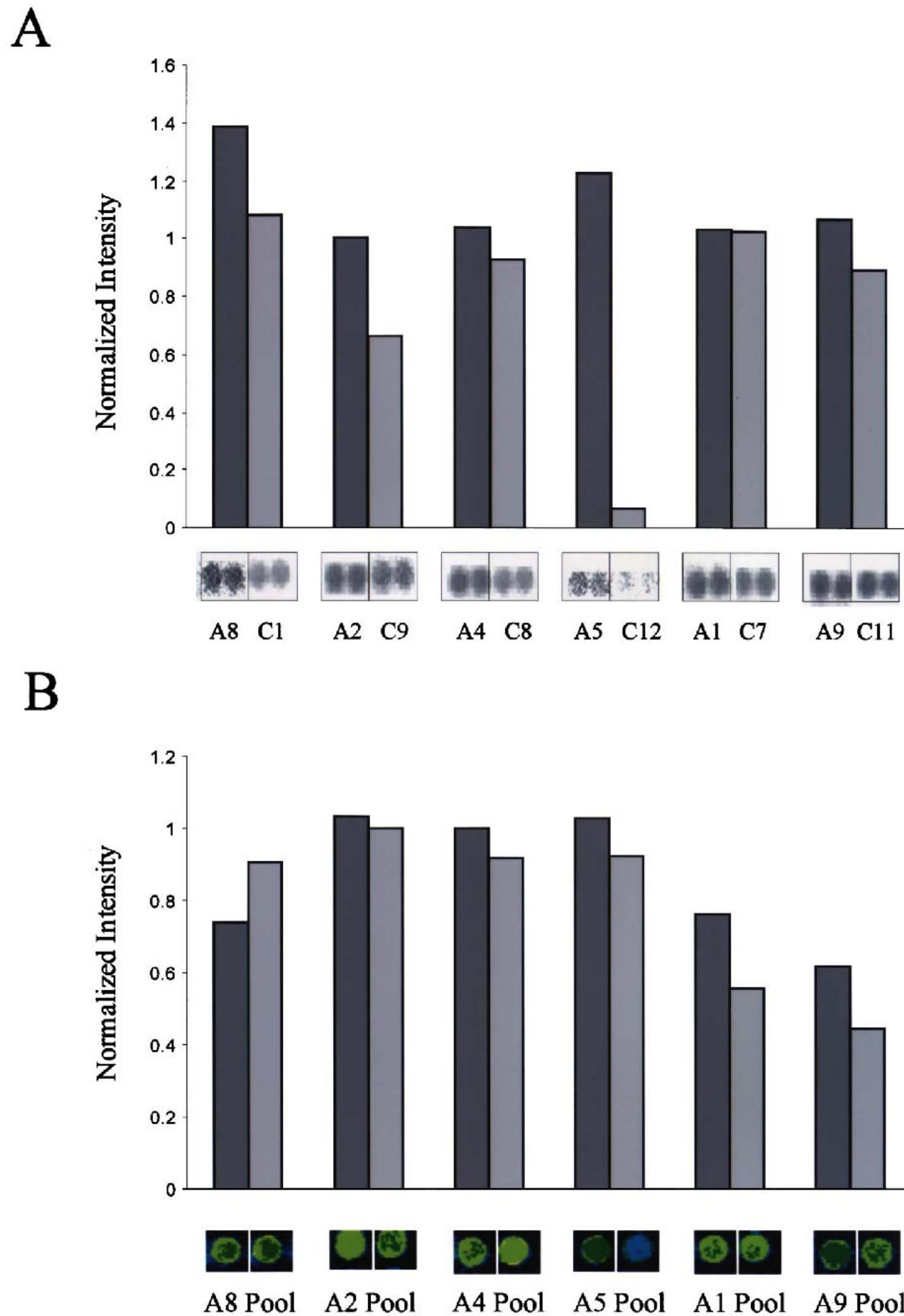


Fig. 3. cDNA microarray analyses of NCAM mRNA expression in postmortem autistic and control brains. (A) Clontech Atlas human neurobiology array. Bar graph shows normalized intensity for age-matched autism and control pairs. Below each bar is the corresponding spot on the array showing radioactively labeled probe hybridization to spotted NCAM cDNA. A = autism; C = control. (B) Incyte Genomics UniGEM V2 array. Graph shows the normalized intensity for age-matched autism and control pool pairs. Below each bar is the corresponding spot on the array showing fluorescently labeled probe hybridization to spotted NCAM cDNA. Images of each autism (cy3 channel) and control pool (cy5 channel) pair are from the same spot on one array. A = autism; Pool = control pool.

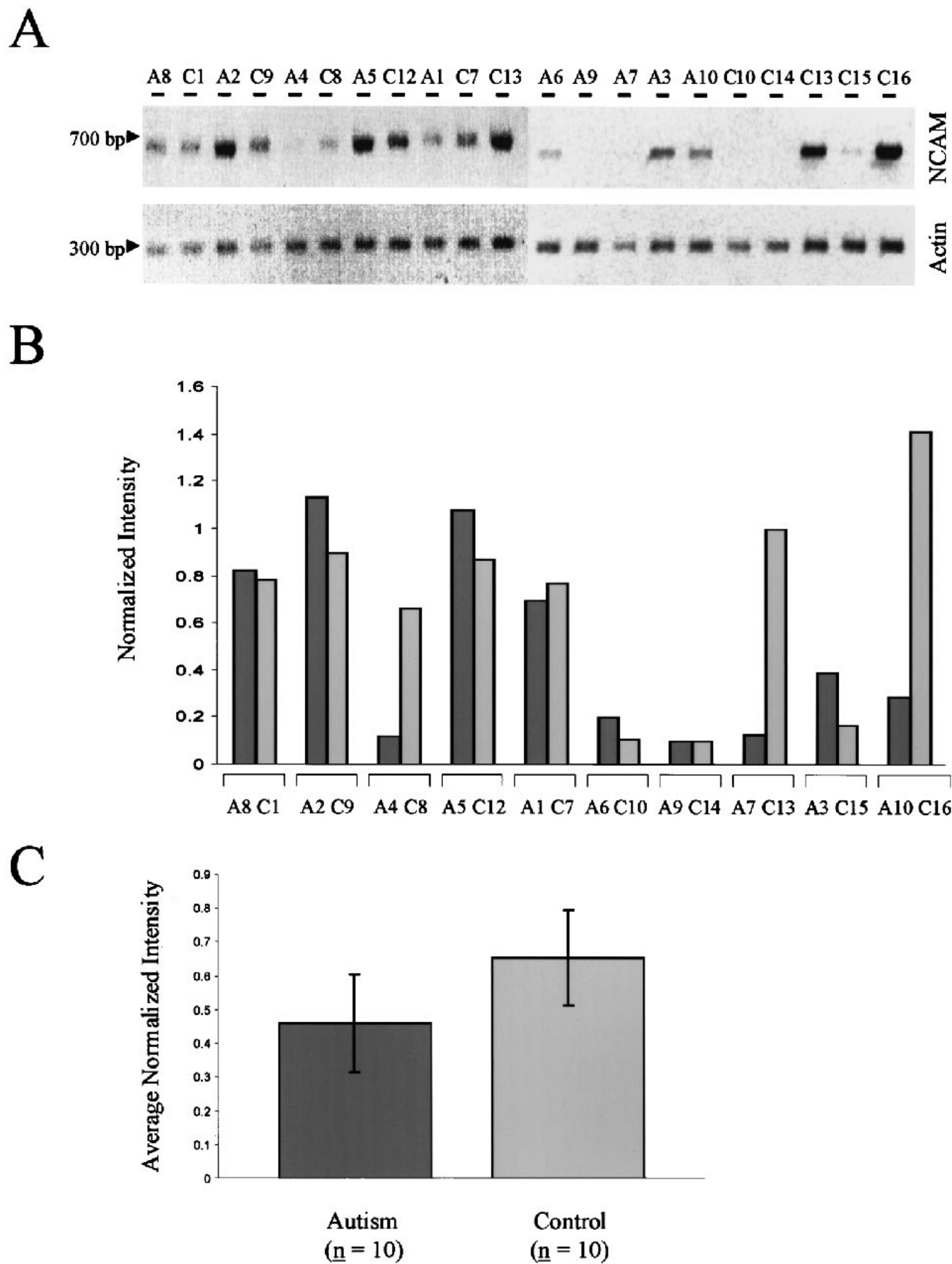


Fig. 4. Confirmation of cDNA microarray results using RT-PCR. (A) NCAM and actin products from RT-PCR of autism and control brain RNA. The upper panel shows the 700 bp cDNA NCAM product, while the lower panel shows similar actin products at 300 bp, which were used to standardize samples. (B) Normalized NCAM intensities of 10 age-matched autism and control pairs. (C) Average NCAM intensities of autism and control samples. The average normalized intensity is not significantly different between autism and control.

from individuals diagnosed with autism relative to age- and gender-matched controls. We also measured NCAM mRNA levels using cDNA microarrays from Clontech Laboratories and Incyte Genomics and found no significant changes between autistic and control post-mortem brain samples. Finally, we measured NCAM mRNA in brain by RT-PCR and also found no significant differences.

There are several explanations why our results differ from those of Plioplys *et al.* (1990). Plioplys *et al.*

used a custom-generated polyclonal antiserum that is currently unavailable (A. Plioplys, personal communication, 1999). This antiserum was shown to recognize the three major NCAM isoforms in brain, but was not tested on serum samples by Western blotting. Thus, it is unknown what isoform(s) of NCAM were measured. In our studies, we used Western blot analyses to detect NCAM. This technique is quantitative and allows us to visualize protein isoforms selectively. Western analysis revealed a single prominent band in serum samples, and the three NCAM isoforms in brain. In addition to differences in antisera used, this study examined a larger sample (n = 33 autistic serum samples, n = 25 controls), which reduces the possibility of random error. Last, it is possible that different results were obtained because the criteria used to diagnose autism differed between the studies. The autistic subjects utilized by Plioplys *et al.* were diagnosed based upon DSM-III and DSM-III-R criteria. The sera were further subdivided based upon medication status. In our study, clinical diagnoses of autism were based upon DSM-IV criteria, and in all cases CARS and/or ADI-R indices were applied. The subjects with autism were further characterized by histories of clinical regression following apparently normal development. Because of the clinical heterogeneity of autism and because of the lack of biological markers, it is possible that there were significant differences in the molecular phenotype of the autistic cases evaluated in the two studies.

We further explored the levels of NCAM protein in postmortem human brain cerebellum samples from autistic and age- and gender-matched control samples. We selected cerebellum for this study based upon availability of tissue and based upon neuropathology studies that suggest disruptions of cerebellar morphology in autism (Kemper & Bauman, 1998). Our rationale for studying brain tissue is that autism is predominantly a neurological disorder, and any changes in NCAM protein or mRNA levels would be most relevant to brain. Furthermore, the NCAM gene is expressed predominantly in brain. The rationale for studying human postmortem brain samples is further strengthened by the lack of robust animal models for autism. Biopsied tissue is rarely obtained from autistic individuals, and so postmortem brains are an important source for molecular studies.

The use of postmortem human brains for molecular studies is complicated by factors such as the postmortem interval, the agonal state, the physical state of the brain (such as pH), the manipulation of the brain (e.g., freezing and thawing), the complexity of the cells that are dissected, the difficulty of identifying the specific brain region that is dissected, and the potential lack of clinical data corresponding to each case (Colantuoni, Purcell, Bouton, & Pevsner, 2000). We have addressed each of these concerns as fully as possible. In general, the postmortem interval is not as critical a factor for obtaining brain protein and brain RNA as is the agonal state, and the pH of the brain is a good indicator of the integrity of the tissue (Harrison *et al.*, 1995). We have measured the pH of our brain samples and found that autistic and control brains have a comparable pH. The postmortem intervals were also matched and were less than 24 hours in all cases. Dissections of cerebellar cortex were performed by a qualified neuropathologist at the Harvard Brain Bank and we used comparable quantities of brain tissue (250 mg) for each case. We infer that the composition of the brain samples used in this study were not significantly different because the expression profiles of 9,000 genes expressed selectively in neurons and in glia did not differ greatly between autistic and control brains (Purcell & Pevsner, manuscript in preparation).

Autism is likely to be a neurodevelopmental disorder, and deleterious molecular changes may occur even before birth. However, we have studied both serum and brain samples from individuals of ages 4–54 years (for brain) or 3–63 years (for serum: see Tables I and II). An assumption of any studies on serum or brain samples from neurodevelopmental disorders is that changes observed at later developmental stages do occur and are relevant to the molecular phenotype of the disorder.

We extended our study of NCAM protein in brain to NCAM mRNA. We characterized the NCAM gene expression pattern by using microarrays and RT-PCR. High density cDNA microarrays allow the rapid, simultaneous measurement of the expression of up to thousands of genes on a solid support such as a nylon filter or glass slide (Duggan, Bittner, Chen, Meltzer, & Trent, 1999). We isolated mRNA from autistic and control brains and converted it to cDNA labeled with radioactivity (for Clontech arrays) or fluorescence (for Incyte arrays). Image analysis and subsequent data analysis revealed that NCAM mRNA levels were not significantly altered in autism (see Fig. 3). We also evaluated NCAM mRNA levels by performing RT-PCR and found a lack of regulation (see Fig. 4). The microarray experiments provided a detailed profile of gene expression changes for thousands of genes in autistic versus control brain samples. Some of these genes are consistently, differentially expressed in multiple autistic samples (Purcell & Pevsner, manuscript in preparation).

We also used microarrays to evaluate the levels of NCAM mRNA in brain samples from patients diagnosed with schizophrenia and Rett syndrome and in skin fibroblasts from patients diagnosed with Sturge-Weber syndrome, mucolipidosis II, Niemann Pick type C, and Chediak-Higashi syndrome (Pevsner, unpublished data). NCAM mRNA levels were not significantly regulated in any of these conditions.

In conclusion, we evaluated the levels of NCAM in autistic serum and brain samples and found no changes (in serum) or only modest changes (in one brain isoform). Thus, it is unlikely that NCAM is a useful marker for autism or that it reflects the primary pathophysiological defect(s) in autism. Further studies of genomic mutations or variants in the NCAM gene would serve to exclude NCAM in the etiology of autism. Our study may represent the first report of mRNA levels for any gene in autistic samples. In the future it may be interesting to assess the expression of NCAM or many other genes in additional brain regions. It is likely that many transcripts are consistently, abnormally regulated in autistic brain.

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