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## VASE-Containing N-CAM Isoforms Are Increased in the Hippocampus in Bipolar Disorder but Not Schizophrenia

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The neural cell adhesion molecule (N-CAM) is a cell recognition molecule that is involved in cellular migration, synaptic plasticity, and CNS development. In schizophrenia, a 105- to 115-kDa N-CAM protein is increased in CSF and in the hippocampus and prefrontal cortex. The variable alternatively spliced exon (VASE) of N-CAM is developmentally regulated and can be spliced into any of the major 120-, 140-, and 180-kDa N-CAM isoforms. We determined that the variable alternative spliced exon of N-CAM (VASE) also is increased in bipolar disorder by quantitative Western immunoblot. VASE immunoreactive proteins (triplet bands around 140 kDa and a single band around 145 kDa) were identified in soluble and membrane brain extracts and quantified in the hippocampus. Soluble VASE 140 kDa was increased in the hippocampus of patients with bipolar disorder as compared to controls, patients with schizophrenia, and suicide cases. Membrane-extracted VASE 140 and 145 kDa were unchanged in the same groups. Multiple 145-kDa VASE-immunoreactive proteins that also reacted to an N-CAM antibody were separated by isoelectric focusing and electrophoresis followed by western immunoblotting; however, the VASE 140-kDa proteins were only weakly N-CAM immunoreactive. By immunohistochemistry, VASE colocalized with GFAP-positive astrocytes in the hippocampus. VASE immunostaining was also observed in the cytoplasm of CA4 pyramidal neurons that were positive for phosphorylated high molecular weight neurofilament and synaptophysin terminals. Thus no differences in VASE were found in patients with schizophrenia, but there was a marked increase of VASE immunoreactive proteins in bipolar disorder. It is possible that abnormal regulation of N-CAM pro-

teins results in differing patterns of abnormal expression in neuropsychiatric disorders.

**Key Words:** schizophrenia; bipolar disorder; hippocampus; prefrontal cortex; neural cell adhesion molecule; variable alternative spliced exon; isoelectric focusing; immunohistochemistry; Western immunoblot; *in situ* hybridization histochemistry.

### INTRODUCTION

Schizophrenia and bipolar disorder are neuropsychiatric disorders of unknown etiology, although subtle changes in brain structure have been reported in both disorders. It has been suggested that these structural abnormalities may be developmentally derived (10, 17, 28), but also could be manifestations of an ongoing process. The possible developmental origin of these disorders suggests an involvement of proteins which mediate CNS development. The cell recognition molecule, neural cell adhesion molecule (N-CAM), is involved in CNS morphoregulatory events and is developmentally regulated. The N-CAM variable alternative spliced exon (VASE) is 30 bp in length and inserted between exons 7 and 8 (23).

Several lines of evidence suggest an association between VASE expression and decreased neural plasticity. VASE is undetectable in N-CAM from the olfactory neuroepithelium, which continues to regenerate olfactory neurons in adults (23). VASE levels in rat brain are low in the embryonic and early postnatal period; however, as maturation proceeds, VASE increases and eventually is present in up to 50% of the total N-CAM (23). In the hippocampus, VASE increases after maturation. Embryonic hippocampal neurons respond to N-CAM by increased neurite outgrowth and express low levels of VASE. Postnatal hippocampal neurons, which

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are not responsive to N-CAM, have a greater proportion of transcripts containing VASE (27). Cells transfected with N-CAM + VASE show less neurite outgrowth and adhesion compared with cells expressing N-CAM only (6, 13, 22). The adhesion mechanisms presumably involve *cis*- and *trans*-interactions of N-CAM containing VASE most likely through the binding partners N-CAM and L1 (12). The external binding of N-CAM containing VASE in cell-cell adhesion can generate intracellular signals, e.g., phosphorylation of N-CAM, p125(fak), and p59(fyn) (3, 7). Synapse stabilization (16) during plasticity probably involves primarily N-CAM transcripts that do not contain VASE (5, 23).

Previously, our group reported that concentrations of N-CAM are increased in cerebrospinal fluid (CSF) of patients with schizophrenia and affective disorders (18–20). N-CAM, corresponding to the soluble form found in CSF, was also increased in the hippocampus and prefrontal cortex of patients with schizophrenia as compared to bipolar disorder, suicides, and controls (26). VASE protein was increased in CSF of patients with schizophrenia (M. P. Vawter *et al.*, unpublished results) as compared to normal controls. In the present study we have measured VASE expression in brain from patients with neuropsychiatric disorders.

## METHODS

### *Tissue Samples*

All post-mortem brains were obtained from the NIMH Neuroscience Center at St. Elizabeths Brain Collection. Samples of bilateral hippocampus were from patients with schizophrenia ( $n = 16$ ), bipolar disorder ( $n = 6$ ), normal controls ( $n = 13$ ), and individuals who died by suicide without a concomitant diagnosis of psychosis ( $n = 7$ ). The numbers of persons using alcohol at the time of death (for hippocampal samples) were: normal controls (2), suicide (3), schizophrenia (0), and bipolar disorder (2). Samples of dorsolateral prefrontal cortex were from patients with schizophrenia ( $n = 10$ ), normal controls ( $n = 10$ ), individuals who died by suicide without a concomitant diagnosis of psychosis ( $n = 10$ ), and bipolar disorder ( $n = 10$ ). There was an overlap of 19 samples from both the hippocampus and prefrontal cortex regions for the same patient. The brains were previously screened for structural abnormalities by standard neuropathological examination (for details, cf. 26). Consecutive frozen coronal slabs were dissected from the amygdala through the posterior hippocampus. The hippocampus proper then was dissected out from the adjacent entorhinal cortex or parahippocampal gyrus on a platform cooled with dry ice. The prefrontal cortex corresponding to Brodmann's areas 5 and 46 was dissected from coronal sections. Whole hippocampi or prefrontal cortex from each brain were pulverized on dry ice prior to extraction. A published method for

homogenization and obtaining "crude membrane" and "soluble" N-CAM extracts was followed (25), with slight modifications as previously described (26). The protein content of "soluble" and "membrane" samples was measured by bicinchoninic protein assay (Pierce, Rockford, IL). Membrane extracts were measured in all hippocampus and prefrontal cortex samples. The soluble fraction was measured in all samples of hippocampus, while in the prefrontal cortex the soluble fraction was measured in 10 bipolar disorder and 3 control samples, due to technical limitations the remaining samples were not analyzed. Normal brain sections were selected randomly for immunohistochemical studies.

### *Gel Electrophoresis*

Hippocampal and prefrontal cortex (membrane and soluble) samples were diluted 1:4 in a denaturing and reducing solution consisting of 10% sodium dodecylsulfate (SDS, Bio-Rad, Hercules, CA) and 2.3% dithiothreitol (DTT, Pharmacia Biotech, Piscataway, NJ), and heated at 95°C for 4 min. The sample was allowed to cool and diluted with 2× sample buffer (Laemmli, 1970) to achieve 40 µg of loaded protein in each lane. Slab gels were cast in a Protean II multigel casting chamber (Bio-Rad), according to the manufacturer's instructions for 7.5% SDS-PAGE with piperazine di-acrylamide (Bio-Rad) as the cross-linking agent to increase the gel strength. A 3% stacking gel was overlaid on the running gel. The samples were applied to the stacking gel with a *Tris*-glycine-SDS running buffer. SDS-PAGE was carried out at 4°C, on vertical gels, 1.5 mm × 20 cm × 20 cm with separation conditions of constant current of 40 mA/gel for 5 h (9). The gels and transfer membranes (Hybond C Super supported 0.45-µm nitrocellulose membrane, Amersham Life Science, Arlington Heights, IL) were equilibrated in transfer solution consisting of Tris (24.1 mM), glycine (39 mM), and 20% v/v methanol (or 10% v/v methanol for prefrontal cortex) for 30 min. Gels were electroblotted to transfer membranes with transfer solution for 40 min using a semidry electroblotter (Bio-Rad) at 800 mA and 25 V constant setting. Silver staining with a sensitivity of 20 pg/mm<sup>2</sup> (14) of gels after electroblot transfer was used to determine protein transfer for proteins in the region of interest from 60–205 kDa. A colloidal gold stain (Amersham) of the nitrocellulose membrane was used to verify quantitative protein transfer from the gel to the membranes. High molecular weight prestained color markers (Sigma Chemical Co, St. Louis, MO) were used to estimate molecular weights, as well as efficiency of protein transfer to the membrane.

### *Two-Dimensional Electrophoresis*

High-resolution two-dimensional electrophoresis was performed as described (9), with minor revisions.

Samples were prepared for isoelectric focusing (IEF) by dilution of "crude membrane" and "soluble cytoplasmic" hippocampus samples in sample buffer to yield 100 µg of protein/60 µl of sample buffer. The 2D sample buffer contained 0.2 g SDS (Bio-Rad), 0.232 g DTT, 2 ml glycerol, 0.5 ml ampholines, pH range 4–8 (BDH, England), and 0.2 g CHAPS (Calbiochem, San Diego, CA) in 10 ml deionized water. The samples were loaded with a syringe (Hamilton, Reno, NV) on polyacrylamide tube gels at room temperature, focused overnight with a stepped voltage program of 200 V for 2 h, 500 V for 5 h, and 1200 V for 14 h using a Bio-Rad 3000xi power supply. IEF tube gels were extruded with a stream of water, rinsed with a transfer solution containing 8% SDS and 0.008% bromophenol blue in 0.02 M Tris-HCl, pH 6.8, and then transferred to a 12% SDS-PAGE slab gel (16 × 16 cm). The second dimension separation was performed on 5–10% gradient gels (or 7.5% linear gels) at 10°C, 40 mA per gel, with conditions similar to the SDS-PAGE 1D gels described above.

#### *Preparation of VASE Antisera and Fusion Proteins*

**VASE antisera.** VASE antisera (Becton-Dickinson 3854) was generated to a 19-amino acid peptide comprising the 10-amino acid VASE peptide (ASWTRPEKQE) flanked by four amino acids of human N-CAM on either side and a cysteine at the amino-terminus (Vega Biomolecules, Columbia, MD). The peptide (cseekASWTRPEKQEtldg, the underlined amino acids correspond to VASE) was coupled to keyhole limpet hemocyanin (KLH) using the cross-linking reagent *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester (Pierce Chemical, Rockford, IL) per the manufacturer's recommendation. Rabbits were immunized subcutaneously with 200 µg of peptide-KLH, initially in complete Freund's adjuvant and subsequently in incomplete Freund's adjuvant at several-week intervals.

**VASE-N-CAM fusion protein.** Synthetic oligonucleotides corresponding to nucleotides 467–487 and 1244–1264 of human N-CAM and containing *EcoRI* and *BamHI* cloning sites, respectively, were prepared. These primers were used to PCR amplify cDNA clones NIII-4 and NII-6, which lack or contain the VASE exon (8). The resulting amplification products were subcloned into plasmids, and eventually into the *EcoRI/BamHI* sites of pMAL-c2 (New England Biolabs, Inc., Beverly, MA) to generate fusion proteins with bacterial maltose-binding protein. Overnight bacterial cultures were diluted 1:50 in Leibovitz's medium and grown to mid-log phase at 37°C. Fusion proteins were induced with 0.05 mM isopropyl β-D-thiogalactopyranoside for 2.5 h. Bacteria were collected and resuspended to  $A_{660} = 20$  and boiled with an equal volume of 2× concentrated SDS-PAGE gel buffer containing DTT. Fusion protein samples were electrophoresed and probed with rabbit anti-VASE antiserum (1:500 dilution) and detected

colorimetrically with alkaline-phosphatase-conjugated anti-rabbit IgG. At this concentration of antibody, only the +VASE fusion protein was immunoreactive; at higher concentrations of antibody (1:250 and higher), immunoreactivity with both +VASE and –VASE fusion proteins was seen. The bacterial lysate expressing a fusion VASE + N-CAM protein showed immunoreactivity at 80–90 kDa.

#### *Immunoblots*

Antisera used were actin (Sigma clone AC-15), N-CAM (rabbit polyclonal Becton-Dickinson 3732), VASE antisera (3854), and monoclonal N-CAM antibody (Becton-Dickinson 18.1.3 and Becton-Dickinson 14.2). The dilutions of antibody for actin were 1:5000, polyclonal and monoclonal N-CAM antibodies were diluted 1:1000, and VASE was diluted 1:1000. Primary antibody binding to the membrane was visualized by secondary antibody (goat anti rabbit IgG or goat anti mouse IgG coupled to horseradish peroxidase was used at 1:10,000) and an enhanced chemiluminescent (ECL) reaction (Amersham). The membrane was exposed to XAR film for 1–10 min or BioMax MR film for 15 s–2 min (Kodak).

In order to sequentially probe the membrane for VASE and N-CAM each immunoblot was stripped of primary and secondary antibody with a 0.2 M glycine pH 2.7–2.9 solution for 60 min. Each membrane was neutralized with PBS, reblocked with 4% nonfat milk (Safeway, Pleasanton, CA) and 0.1% bovine serum albumin (Sigma), and reprobed with another primary antibody. The entire strip and reprobe procedure was conducted at the same time under the same conditions for each membrane with the membranes in separate trays.

We also determined whether residual primary or secondary antibody was retained after stripping and reprobing the immunoblots. Soluble hippocampi samples were run in duplicate on both halves of a membrane and probed for N-CAM and followed with secondary antibody (goat α rabbit IgG-horseradish peroxidase label; Amersham). The entire immunoblot was then stripped of antibody, the membrane separated, and one half reprobed with the secondary antibody and ECL reagent and the other half with ECL reagent only. Low molecular weight bands ~30–50 kDa and a single band at ~85 kDa developed as a result of reprobing with the secondary antibody. With application of the ECL reagent alone, no signal was detected.

#### *Immunohistochemistry*

Human prefrontal cortex and hippocampus fresh frozen sections were processed for indirect immunofluorescence. The IgG fraction from VASE antisera (3584) was preabsorbed against a detergent-extracted N-CAM-depleted human membrane preparation. The starting material for the depleted fraction was the detergent-



extracted third pellet (P3) from human prefrontal cortex membrane fraction (26). The P3 was resuspended in PBS with protease inhibitors (Sigma), anti-pain (4  $\mu\text{g/ml}$ ), pepstatin A (2  $\mu\text{g/ml}$ ), aprotinin (2  $\mu\text{g/ml}$ ), leupeptin (2  $\mu\text{g/ml}$ ), and phenyl methyl sulfonyl fluoride (0.1  $\mu\text{g/ml}$ ), and microfuged (Eppendorf, Hamburg, Germany) in a 1.5-ml tube for 1 min at 500 rpm. The low speed supernatant was removed and respun at 8000 rpm. The supernatant was discarded, and 100  $\mu\text{l}$  of anti-VASE rabbit serum was added to the pellet, vortexed, and gently mixed for 10 min. The mixture was centrifuged at 8000 rpm, and dilutions of the supernatant (1:50–1:100) were used for immunohistochemistry. Similarly, normal rabbit serum and mouse ascites fluid (Sigma) used for immunohistochemistry controls were preabsorbed with detergent-extracted membrane preparation using the same procedure. Other primary antibodies used were: mouse anti-neurofilament (SMI 32) reactive for phosphorylated heavy neurofilament (NF-H); anti-GFAP (SMI 21) monoclonal antibodies obtained from Sternberger Monoclonals Inc. (Baltimore, MD); monoclonal N-CAM antibody (Becton-Dickinson clone 18.1.3), specific for an intracellular epitope found in N-CAM 140 and N-CAM 180; and monoclonal anti-synaptophysin (Sigma, clone SVP 38).

Frozen blocks from the middle frontal gyrus and hippocampal formation were sectioned in the coronal plane at 14- $\mu\text{m}$  thickness, mounted on gelatin-coated slides, and stored at  $-70^{\circ}\text{C}$ . On the day of the experiment, the sections were thawed for 30 min, air dried, and fixed in 4% paraformaldehyde for 10 min, washed in PBS, blocked by incubation in 10% normal goat serum, and incubated at  $4^{\circ}\text{C}$  overnight with two primary antibodies diluted in 0.33% Triton X-100 in PBS. Dual-labeled sections were incubated with absorbed anti-VASE IgG (1:100) and one of four additional monoclonal antibodies: anti-N-CAM (1:400); anti-synaptophysin (1:200); anti-neurofilament specific for phosphorylated 200-kDa neurofilaments (1:2000); and anti-GFAP (1:2000). The sections were then rinsed in PBS and incubated for 1 h in biotinylated donkey anti-rabbit IgG (1:100; preabsorbed against rat, human, and mouse sera, Amersham) and sheep anti-mouse IgG conjugated to Texas Red (1:100, preabsorbed against rat, human, and rabbit sera, Amersham) at room temperature. The slides were then rinsed in PBS before a final 1-h incubation in the streptavidin-fluorescein conjugate (1:100). All slides were counterstained with 4',6-diamidino-2-phenylindole (DAPI, Sigma) at a concentration of 1  $\mu\text{g/ml}$  to show nuclei. The slides were turned on edge for 5 min and blotted to remove excess buffer before anti-fade buffer (Molecular Probes, Eugene, OR) and coverslips were applied. The three color fluorescence was visualized with a Zeiss Axiophot epifluorescence microscope. In order to z-section and view localization of VASE antibody with other immunolabels, slides

were also examined under a Zeiss Model 410 laser scanning confocal microscope.

### *Neuraminidase Treatment of Brain Samples*

Membrane samples (100  $\mu\text{g}$ ), from occipital and prefrontal cortex, and soluble hippocampus samples (100  $\mu\text{g}$ ) were treated with 2  $\mu\text{g}$  ( $\sim 0.4$  U) of neuraminidase (*C. perfringens*, Type IX, Sigma) for 2 h at  $37^{\circ}\text{C}$ . Control samples were incubated with PBS, pH 7.4. The samples were separated under denaturing or native conditions on 7.5% SDS-PAGE and proteins were detected on immunoblots with VASE and N-CAM antibody.

### *In Situ Hybridization Histochemistry*

Oligonucleotide probes were synthesized and purified on HPLC (Oligos, Etc., Wilsonville, OR). Probes were complementary to human N-CAM VASE mRNA (8), 28 bases in length (5'-CTCTTGCTTCTCTGGTC-GAGTCCACGAA-3'); and to human N-CAM, 35 bases in length (5'-CGGAACCTCCTGTGGGGTTGGCGCAT-TCTTGAACAT-3'), based upon CHB1 human N-CAM clone sequence 499–533 nt (2), and showed no stable secondary or homodimeric structures. A random 28-mer probe using the bases in VASE sequence was also synthesized (5'-AGCCTCCGATTGCCACTTAGTCTCTCGT-3'). The stem loop melting temperatures are  $46^{\circ}\text{C}$  for the VASE probe,  $10^{\circ}\text{C}$  for the random probe (Oligo Tech Software; Oligos, Etc.), and the N-CAM probe did not show any stable secondary structure. All probes were labeled, at the 3'-ends, using terminal deoxynucleotidyl transferase (New England Nuclear, Boston, MA) and deoxyadenosine  $\alpha$ - $^{35}\text{S}$ ]thio-triphosphate, (New England Nuclear) to a specific activity of  $5\text{--}10 \times 10^5$  cpm/ $\mu\text{l}$ , according to a described method (29). *In situ* hybridization histochemistry (ISHH) was carried out according to the published method (4) with slight modifications (for details see 1) with probe incubation at  $45^{\circ}\text{C}$ . Control sections were treated with the labeled random probe to evaluate the background of the VASE and N-CAM mRNA probes. The slides were exposed to Biomax film (Kodak) for 30 days along with  $^{14}\text{C}$  standards. Autoradiographs were digitized with a flat-bed optical scanner (Hewlett-Packard, Palo Alto, CA). Samples of labeling were evaluated from the following hippocampal regions: dentate gyrus, cornu ammon 1, 2, 3, 4; presubiculum, subiculum, and parahippocampal gyrus.

### *Image Analysis*

Images of immunoreactive—VASE bands were transferred from film to computer by a high resolution scanner (Hewlett Packard, ScanJet 4c/T, Palo Alto, CA). The digitized bands were quantitated by calcula-

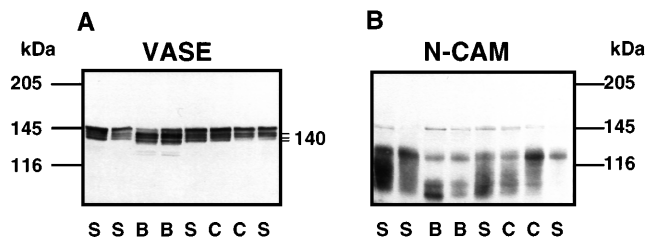
tion of the integrated mean density with Image software v 1.59 (Wayne Rasband, National Institutes of Health, Bethesda, MD) for VASE bands within the MW region of brain-derived VASE immunoreactivity. The occipital brain cortex sample was used as a reference standard. Thus, all mean density measurements were expressed as ratio values to the same concentration (40  $\mu\text{g}/\text{lane}$ ) of reference brain protein. The mean ratio values were analyzed by MANOVA (Statistica 5.0, Statsoft, Tulsa, OK) for one group factor (schizophrenia, bipolar, suicides, and controls) with VASE bands as a repeated measure. Pearson correlations for VASE and age, and post-hoc Tukey *t*-test comparisons corrected for unequal *n* and multiple comparisons, were calculated with Statistica 5.0.

## RESULTS

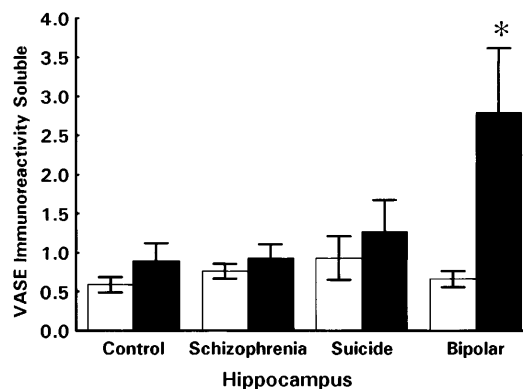
### Western Immunoblot—Hippocampus

The post-mortem interval (PMI) means for each group were previously matched (Vawter *et al.*, 1998). There was no difference for control ( $22.9 \pm 3.2$ ) (h; mean  $\pm$  SEM), schizophrenia ( $24.0 \pm 5.4$ ), suicide ( $28.1 \pm 3.7$ ) and bipolar disorder ( $29.6 \pm 3.7$ ) ( $F(3, 36) = 0.31$ ;  $P = 0.82$ ). The age at the time of death (year; mean  $\pm$  SEM) for the control ( $51.5 \pm 4.8$ ), schizophrenia ( $51.7 \pm 4.1$ ), bipolar disorder ( $45.8 \pm 4.8$ ), and suicide ( $51.3 \pm 2.7$ ) cases was not different ( $F(3, 38) = 0.24$ ;  $P = 0.86$ ). The percentage of protein extracted from each brain sample (mg protein/mg wet weight of brain tissue) did not differ between groups ( $F(3, 38) = 2.09$ ;  $P = 0.12$ ): control (mean  $\pm$  SEM,  $3.59 \pm 0.11$ ), schizophrenia ( $3.41 \pm 0.14$ ), bipolar disorder ( $3.64 \pm 0.17$ ), and suicide ( $3.94 \pm 0.15$ ).

VASE was measured in two major immunoreactive bands in the hippocampus: VASE 140 kDa which consisted of a triplet band was found in soluble and membrane brain extracts and a larger 145-kDa VASE



**FIG. 1.** Soluble hippocampal extracts immunoblotted with VASE (A) and N-CAM (B). VASE was measured in two major immunoreactive bands in the hippocampus: VASE 140 kDa which consisted of a triplet band and the larger 145-kDa VASE band, both of which were found in both soluble brain and membrane extracts. VASE immunoblots of membrane extracts (as shown in Fig. 4) appear similar to soluble fractions. The N-CAM immunoblot (B) shows reactivity in a band corresponding to the VASE 145-kDa band. Lane samples are (S) schizophrenia, (B) bipolar, and (C) control samples.



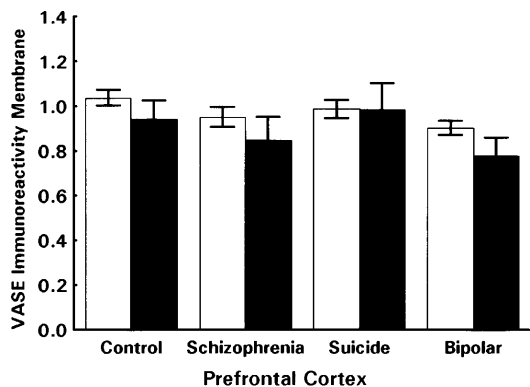
**FIG. 2.** VASE 140 kDa (dark bar) and 145 kDa (light bar) in hippocampal soluble extracts in neuropsychiatric groups. VASE immunodensity units are expressed in ratio units to a reference brain preparation in Figs. 2 and 3. VASE 140 kDa was significantly increased in bipolar disorder in the soluble fraction compared to the control ( $P = 0.0001$ ), patients with schizophrenia ( $P = 0.0001$ ), and suicide ( $P = 0.0003$ ) groups. There was no difference in VASE 145 kDa.

band which was found in both soluble brain and membrane extracts (Fig. 1). The membrane VASE 140- and 145-kDa bands were not significantly different between groups ( $F(3, 38) = 1.46$ ,  $P = 0.24$ ). However, in the soluble fractions VASE 140 kDa was significantly increased in bipolar disorder compared to two other groups (vs controls  $P = 0.0001$ ; vs schizophrenia  $P = 0.0001$ ). There was also a trend for an increase in bipolar disorder group in comparison to the suicide group ( $P < 0.07$ ). There was one outlying data point in the suicide group ( $>3$  SD above the mean of all samples). This subject was removed and the same analysis repeated (Fig. 2). For this second analysis, soluble 140-kDa VASE was significantly increased in bipolar disorder as compared to the suicide group as well ( $P = 0.0003$ ). Age, post-mortem interval (PMI), and freezer time did not correlate with VASE 140 or VASE 145 measurements in the hippocampus and thus were not included as covariants in the above analyses.

### Western Immunoblot—Prefrontal Cortex

VASE was measured in membrane fractions from the prefrontal cortex of patients with schizophrenia ( $n = 10$ ), suicides ( $n = 10$ ), bipolar disorder ( $n = 10$ ), and normal controls ( $n = 10$ ). As in hippocampus, there were no differences between groups in membrane extracted VASE 140-kDa or VASE 145-kDa proteins in the prefrontal cortex ( $F(3, 70) = 0.38$ ;  $P = 0.76$ ; Fig. 3).

Because of technical limitations, it was not possible to assay VASE in soluble extracts from all of the groups. Soluble VASE 140-kDa and VASE 145-kDa proteins were measured in extracts from the prefrontal cortex in patients with bipolar disorder ( $n = 10$ ) and controls ( $n = 4$ ). Soluble VASE 140 kDa was increased in the prefrontal cortex of bipolar patients ( $1.10 \pm 0.03$ ) as



**FIG. 3.** VASE 140 kDa (dark bar) and 145 kDa (light bar) in prefrontal cortex membrane extracts in neuropsychiatric groups. There were no differences in membrane extracted VASE 140- and 145-kDa expression in the prefrontal cortex between diagnostic groups.

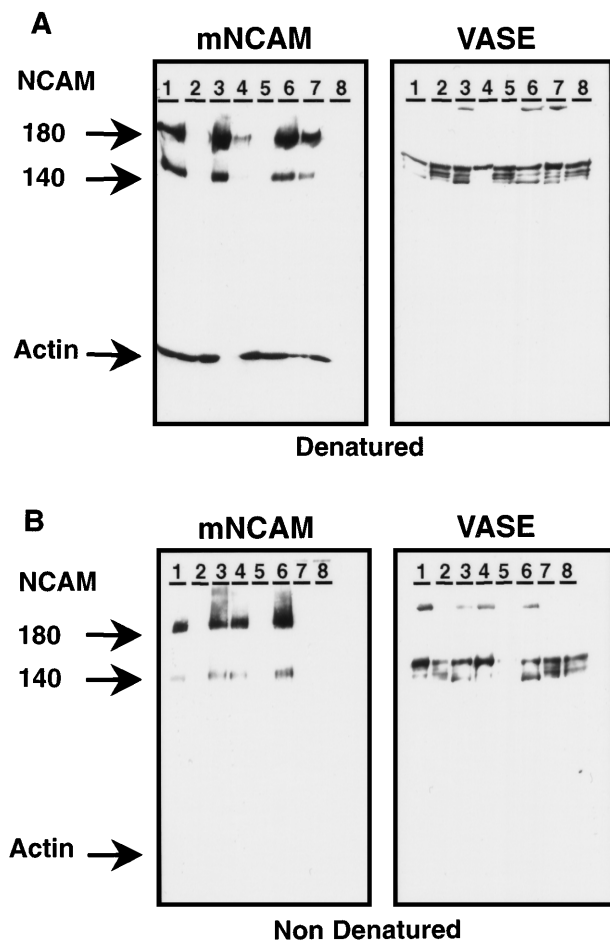
compared with the control group ( $0.28 \pm 0.0003$ ;  $P < 0.0003$ ). The soluble prefrontal cortex VASE 145-kDa expression was also increased in bipolar disorder ( $2.13 \pm 0.10$ ) as compared to the control group VASE 145 kDa ( $0.59 \pm 0.004$ ;  $P = 0.0002$ ). However, due to

the small number of controls, these results may not be representative of a larger sample.

The PMI (h; mean  $\pm$  SEM) for control ( $24.8 \pm 6.1$ ), schizophrenia ( $19.8 \pm 4.8$ ), suicide ( $35.1 \pm 8.4$ ), and bipolar cases ( $30.1 \pm 5.8$ ) were not different ( $F(3, 32) = 0.76$ ;  $P = 0.51$ ). The age at the time of death (year; mean  $\pm$  SEM) for the control ( $52.8 \pm 4.5$ ), schizophrenia ( $54.7 \pm 4.6$ ), suicide ( $58.0 \pm 0.6$ ), and bipolar cases ( $45.4 \pm 5.0$ ) was not different ( $F(3, 36) = 0.82$ ;  $P = 0.48$ ).

### VASE Characterization

We determined whether the differences in molecular weights of the four VASE bands between 140 and 145 kDa in the hippocampus and prefrontal cortex were due to differences in degree of polysialylation. Removal of polysialic acid (PSA) with neuraminidase did not alter VASE migration in SDS-PAGE (Fig. 4). Neuraminidase treatment of crude occipital cortex showed that high molecular weight N-CAM migrated faster as compared to a nonneuraminidase treated preparation under nondenaturing conditions (Fig. 4). Thus, the various 140- to 145-kDa VASE bands do not simply



**FIG. 4.** Monoclonal (m)N-CAM and VASE immunoblots of occipital, hippocampus, and prefrontal cortex soluble and crude extracts following neuraminidase treatment. The same blots were probed with a VASE antibody (right) and stripped and probed (left) with a mN-CAM antibody (18.1.3). Using denaturing (A) and nondenaturing (B) conditions, neuraminidase treatment did not alter VASE migration. In both (A) the brain samples shown are crude occipital cortex (lanes 1, 3, 6), soluble hippocampus (lanes 2, 5, 8), and crude prefrontal cortex (lanes 4 and 7). In (B), the brain samples are the same, except that lane 7 is also a soluble hippocampus sample. Soluble samples: In lanes loaded with soluble samples from hippocampus (A and B, lanes 2, 5, and 8; and B, lane 7), there is no NCAM immunoreactivity because the mN-CAM antibody was specific for the cytoplasmic epitope and recognized N-CAM 140- and 180-kDa isoforms. VASE immunoreactivity is seen in soluble samples, under both denaturing and nondenaturing conditions. Control for incubation: To examine the possibility that incubation at room temperature during neuraminidase treatment altered immunoreactivity patterns, freshly thawed samples were compared to samples incubated at room temperature without neuraminidase treatment. On each blot, lanes 3, 4, and 5 were incubated at room temperature, while lanes 6, 7, and 8 were freshly thawed. There was no apparent effect of incubation at room temperature (compare lanes 3 vs 6, 5 vs 8, or 4 vs 7 in A). Effect of neuraminidase treatment: Crude preparations of occipital cortex are shown without neuraminidase treatment (lanes 3 and 6) and after neuraminidase treatment (lane 1) in each blot. In A and B, with the mNCAM antibody, note that the high MW streaking seen in lanes 3 and 6 is removed by neuraminidase treatment in lane 1. In B, under nondenaturing conditions, the neuraminidase-treated sample (lane 1) migrated faster and without the high MW streaking, indicating removal of polysialic acid (PSA) from high molecular weight N-CAM. No apparent effect of neuraminidase treatment on VASE immunoreactivity was seen under either denaturing conditions (A, VASE, compare lanes 1 vs 3 or 6) or nondenaturing conditions (B, same comparison).

represent a single protein with differences in sialylation, as revealed by lack of mobility differences under both denaturing and nondenaturing conditions following neuraminidase treatment. A polyclonal N-CAM antibody (3732) was reactive with a band of 145 kDa on Western blots (Fig. 1). The monoclonal N-CAM antibody used for immunohistochemistry (N-CAM 18.1.3) reacts with the intracellular C-terminal epitopes of N-CAM 140 and N-CAM 180. The 140-kDa N-CAM immunoreactive band overlapped the VASE 145-kDa band and one VASE 140-kDa band identified with the polyclonal VASE antibody (Fig. 4).

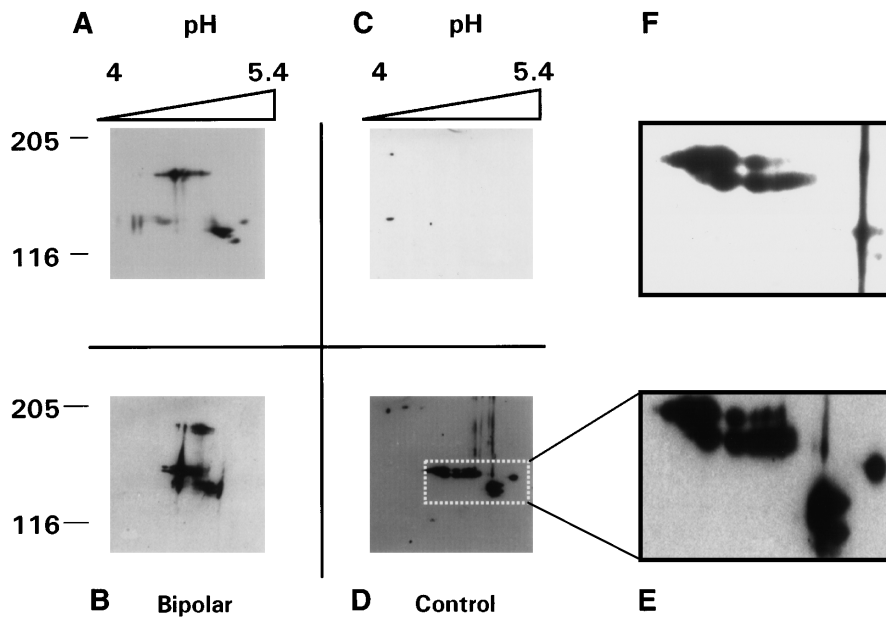
Two-dimensional electrophoresis (first dimension isoelectric focusing followed by second dimension SDS-PAGE) and Western immunoblotting resolved 16 potential VASE-containing isoforms of N-CAM. Isoelectric focusing of crude and soluble hippocampus shows individual isoelectric points for four VASE bands between pH 4.5 and 5.0 (Fig. 5) that are also N-CAM immunoreactive (Fig. 5F). This  $pK_i$  range is similar to the calculated values for human N-CAM 120 kDa and rat N-CAM 140 kDa (ExPASy Swiss Protein DataBase  $pK_i$  calculations are 4.77 and 4.81, respectively). Individual spots suggest variable glycosylation of VASE isoforms (Fig. 5E, enlargement of immunoreactive spots). Since neuraminidase treatment did not alter VASE band migration by molecular weight position in SDS-PAGE, the small spots shown after isoelectric focusing and SDS-PAGE apparently are not related to addition of polysialic acid, but may arise from other glycosylation

and/or phosphorylation events. In addition, a VASE 180-kDa band was found in normal brain.

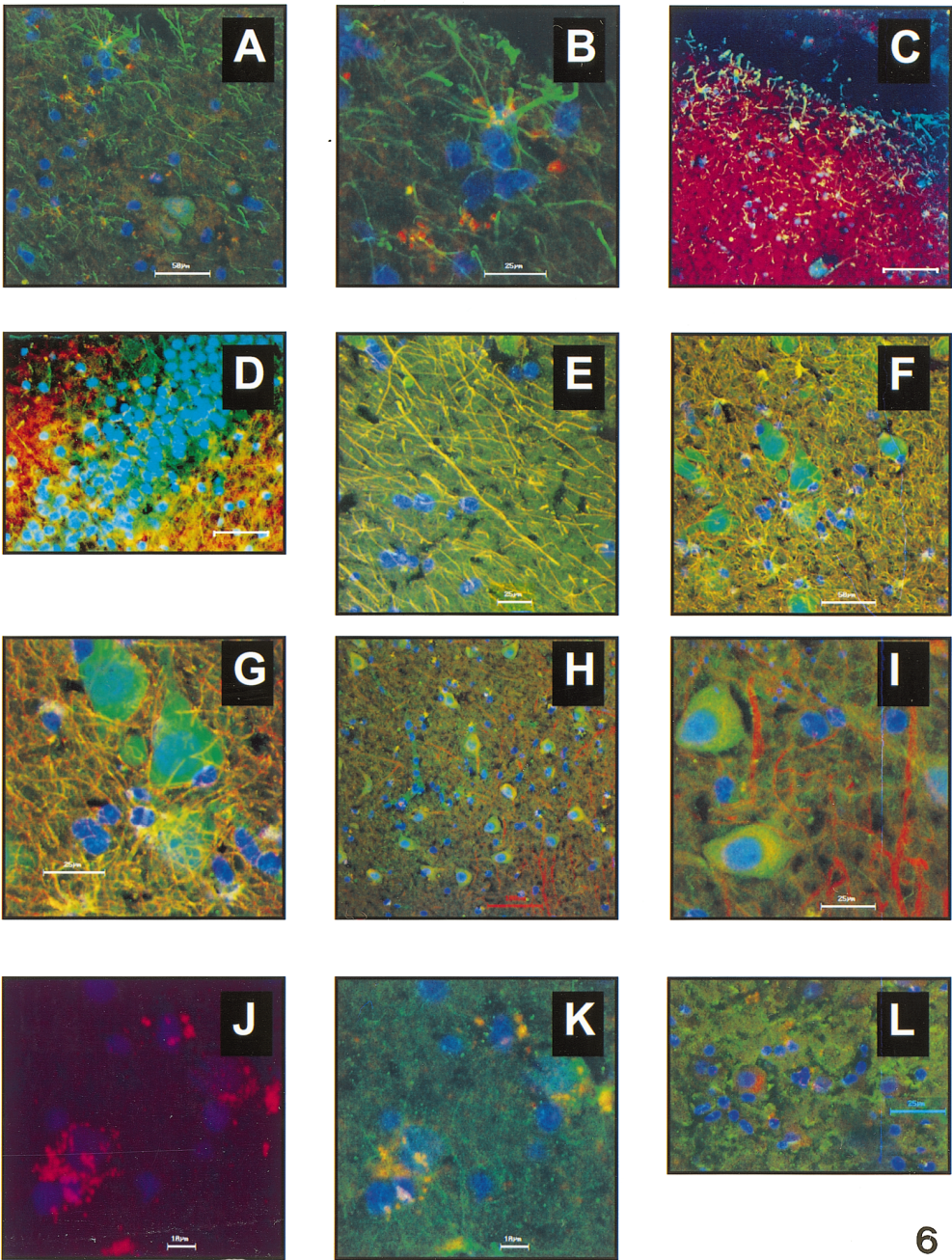
The entire proteomic data base (SwissProt) was scanned for any similarities to the VASE 10 amino acid sequence to determine whether the immunoreactive VASE bands were the result of a homologous protein. The bFGF receptor, which was the only close match (70% homology), did not cross-react with the VASE antisera (M. Vawter, J. Saffell, and P. Doherty, unpublished data).

### Immunohistochemistry

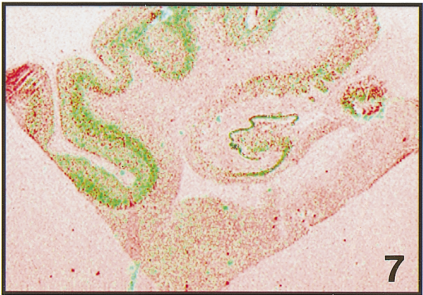
The brain sections used for immunohistochemistry controls were treated with either normal rabbit serum or mouse ascites fluid preabsorbed against a detergent-extracted membrane preparation. The control sections gave background fluorescence staining (not shown). Strong VASE immunostaining was observed in glia usually in superficial layers I/II of the entorhinal cortex and prefrontal cortex. These VASE-positive glial cells formed "foot" processes at the pial surface and appeared to be multipolar astrocytes (Figs. 6A and 6B). Strong VASE immunostaining was also localized surrounding the temporal horn of the lateral ventricle in the hippocampus (Fig. 6C). VASE positive fibers were found in the dentate gyrus (Fig. 6D), CA4 (Figs. 6E–6G), CA3, subiculum, and outer molecular layer of the dentate gyrus. Some of the VASE-positive cells were also found to be GFAP positive, and forming a subset of GFAP positive astrocytes (Fig. 6E–6G). VASE immuno-



**FIG. 5.** VASE immunoblots of the membrane (A, C) and soluble (B, D) hippocampus fractions of bipolar disorder (A, B) and control (C, D), following two-dimensional electrophoresis. The magnification of the soluble control hippocampus region (E) is indicated with a box in (D) and shows ~16 focused protein spots reactive for VASE suggesting glycosylated and phosphorylated proteins. Reprobing of the same membrane (D) with an N-CAM antibody showed strong reactivity with VASE 145 kDa and weaker reactivity of VASE 140-kDa triplet proteins (F).



6



7

**FIG. 6.** VASE immunostaining was found in the hippocampus (A–I and K) shown with a secondary FITC antibody (green). The nuclei are stained with DAPI (purple). All images are confocal microscope scans except (C) and (D), which were from a epifluorescence Zeiss microscope. Dual immunostaining with a monoclonal antibody in each section is shown with a secondary Texas Red-conjugated antibody (A–K):



staining was found in CA4 pyramidal neurons in the cytoplasm and axons (Figs. 6F and 6G) and also localized in CA4 pyramidal neurons positive for NF-H immunostaining (Figs. 6H and 6I) and synaptophysin immunostaining (Figs. 6J and 6K). VASE-positive cells were also N-CAM-positive (as shown by colocalization with monoclonal antibody to intracellular epitopes of N-CAM -140 and -180 kDa) in cell membranes in the outer molecular layer of the dentate gyrus and pyramidal cell layer of the CA4 of the hippocampus (Fig. 6L).

### *In Situ Hybridization Histochemistry*

We employed ISHH to determine the regions of hippocampus in which VASE and N-CAM mRNA are present (Fig. 7). Both antisense probes showed hybridization in the hippocampus compared with the random 28-mer probe, which showed only background signal. N-CAM and VASE probes showed a nonsignificant white matter signal. By ISHH, VASE mRNA expression was demonstrated in hippocampal subfields, CA3, CA4, dentate gyrus, subiculum, and the entorhinal cortex layers II, III, and V–VI. VASE and N-CAM mRNA signal appeared least intense in the CA1. The VASE mRNA expression coincided with N-CAM mRNA expression (Fig. 7).

### SUMMARY

In brain, VASE protein appears in both membrane extracts and soluble cytoplasmic fractions. The expression of soluble, but not membrane-bound 140-kDa VASE, was increased in bipolar patients in the hippocampus. The increase in VASE 140 kDa containing N-CAM isoforms in bipolar disorder, with no other change in N-CAM proteins (26), suggests that there are specific changes in cell adhesion molecules that are characteristic of patients with bipolar disorder and schizophrenia. Patients with schizophrenia did not show changes in VASE isoforms, but previously have been found to show an increase in soluble N-CAM 105- to 115-kDa isoform in the hippocampus and prefrontal cortex (26). However, the majority of patients with schizophrenia have received greater lifetime neuroleptic medication exposure as compared to bipolar patients. Therefore differences in neuroleptic exposure as well as lithium treatment may account partially for

differences in the specific changes in N-CAM isoforms between the two groups.

By immunohistochemistry, VASE was found in both neurons and glia cells. VASE immunostaining appeared in the cytoplasm and membranes consistent with the presence of VASE immunoreactive proteins in soluble and membrane extracts found by immunoblot. VASE immunopositive CA4 pyramidal neurons were also positive for N-CAM, phosphorylated NF-H, and synaptophysin. VASE protein expression colocalized to a subset of astrocytes positive for GFAP. VASE mRNA expression was demonstrated in hippocampal subfields, CA3, CA4, and the dentate gyrus; however, further research is required to definitively localize the origin of the increased soluble cytoplasmic VASE 140 kDa in bipolar disorder.

The degree of increase in soluble brain VASE 140 kDa in bipolar disorder as compared to controls was 284% in the hippocampus. The increase in N-CAM 100- to 120-kDa concentrations previously measured in CSF for patients with bipolar disorder (19) was 40%. Thus, there appears to be a differential alteration of N-CAM isoforms with and without VASE in bipolar disorder. Further, VASE was elevated in the bipolar group as compared to the suicide group, indicating that depression alone did not account for the findings. A study of VASE in CSF of patients with bipolar disorder and schizophrenia is currently in progress (M. Vawter *et al.*, unpublished data) and should help to clarify whether the elevated brain VASE is also reflected by changes in CSF.

N-CAM which contains VASE, in the 140- to 145-kDa MW range, does not appear to possess a significant degree of polysialylation, as compared to “embryonic N-CAM” 180–220 kDa. This result agrees in principle with prior speculation that an inverse relationship exists between VASE-containing and PSA-containing N-CAM isoforms (21).

We have also recently observed an increase of the secreted splice variant of N-CAM in the hippocampus in bipolar disorder (M. P. Vawter, unpublished data) but not in schizophrenia. Thus, in neuropsychiatric disorders the hippocampus shows abnormalities in three different N-CAM proteins, soluble N-CAM 105–115 kDa (schizophrenia), secreted N-CAM 108 and 115 kDa (bipolar disorder), and soluble N-CAM, which contains

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Synaptophysin (A, B, C, J, K); N-CAM (D); GFAP (E, F, G), and NF-H (H, I). (A, B) VASE (green), synaptophysin (red), (B) is a higher magnification of (A). (C, D) VASE (green) and N-CAM (red) in the temporal horn of the lateral ventricle (C) and in the dentate gyrus (D). (E–G) VASE (green), GFAP (red) in the CA4 region. (G) is a higher magnification of (F). (H, I) VASE (green) and NF-H (red). (I) is a higher magnification of (H). (J, K) synaptophysin (red) and VASE (green). (J) and (K) are the same section, (J) shows only synaptophysin and (K) shows VASE overlay. (L) shows VASE (red) and NCAM (green). The reference bars in micrometers are (A, 50; B, 25; C, 50; D, 50; E, 25; F, 50; G, 25; H, 100; I, 25; J, 10; K, 10; and L, 25). **FIG. 7.** Serial sections of human hippocampus were probed by in situ hybridization for VASE (green) and N-CAM (red) mRNA. The localization of VASE mRNA is clearly seen in the dentate gyrus, CA4, CA3, subiculum, and the parahippocampal gyrus. N-CAM *in situ* hybridization also confirmed the regional distribution of VASE mRNA expression in the hippocampus-entorhinal cortical regions. The two sections were overlaid and the distribution of mRNA is shown to be greater for N-CAM.

VASE (bipolar disorder). These findings might be interpreted as evidence that in neuropsychiatric disorders N-CAM protein expression is shifted among various splice variants and break-down products within the hippocampus. The hippocampus samples were collected from chronic neuropsychiatric patients; therefore, the shift in N-CAM protein expression may represent a toxic endpoint in each disorder. Further, the differences in N-CAM expression in the hippocampus may be related to volume changes in the hippocampus observed for patients with bipolar disorder (24) and schizophrenia (15). The reduction in volume of the hippocampus of patients with neuropsychiatric disorders may reflect early neurodevelopmental events or abnormalities related to ongoing differences in expression of the N-CAM proteins. On a molecular level, N-CAM proteins can alter synaptic plasticity, long-term potentiation, and cell signaling. The shifting of cell recognition molecule expression in neuropsychiatric disorders suggests the possibility that these disorders are accompanied by abnormalities at structural and functional interfaces between cells, mediated at least in part by cell recognition molecules.

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#### REFERENCES

- Bachus, S. E., T. M. Hyde, M. M. Herman, M. F. Egan, and J. E. Kleinman. 1997. Abnormal cholecystokinin mRNA levels in entorhinal cortex of schizophrenics. *J. Psychiatr. Res.* **31**(2): 233–256.
- Barton, C. H., G. Dickson, H. J. Gower, L. H. Rowett, W. Putt, V. Elsom, S. E. Moore, C. Goridis, and F. S. Walsh. 1988. Complete sequence and in vitro expression of a tissue-specific phosphatidylinositol-linked N-CAM isoform from skeletal muscle. *Development* **104**(1): 165–173.
- Beggs, H. E., S. C. Baragona, J. J. Hemperly, and P. F. Maness. 1997. NCAM140 interacts with the focal adhesion kinase p125(fak) and the SRC-related tyrosine kinase p59(fyn). *J. Biol. Chem.* **272**(13): 8310–8319.
- Chesselet, M. F., L. Weiss, C. Wuenschell, A. J. Tobin, and H. U. Affolter. 1987. Comparative distribution of mRNAs for glutamic acid decarboxylase, tyrosine hydroxylase, and tachykinins in the basal ganglia: An *in situ* hybridization study in the rodent brain. *J. Comp. Neurol.* **262**(1): 125–140.
- Doherty, P., M. S. Fazeli, and F. S. Walsh. 1995. The neural cell adhesion molecule and synaptic plasticity. *J. Neurobiol.* **26**(3): 437–446.
- Doherty, P., C. E. Moolenaar, S. V. Ashton, R. J. Michalides, and F. S. Walsh. 1992. The VASE exon downregulates the neurite growth-promoting activity of NCAM 140. *Nature* **356**(6372): 791–793.
- Gegelashvili, G., A. M. Andersson, A. Schousboe, and E. Bock. 1993. Cyclic AMP regulates NCAM expression and phosphorylation in cultured mouse astrocytes. *Eur. J. Cell Biol.* **62**(2): 343–351.
- Hemperly, J. J., J. K. DeGuglielmo, and R. A. Reid. 1990. Characterization of cDNA clones defining variant forms of human neural cell adhesion molecules N-CAM. *J. Mol. Neurosci.* **2**(2): 71–78.
- Hochstrasser, D. F., M. G. Harrington, A. C. Hochstrasser, M. J. Miller, and C. R. Merrill. 1988. Methods for increasing the resolution of two-dimensional protein electrophoresis. *Anal. Biochem.* **173**(2): 424–435.
- Kerwin, R. W., and R. M. Murray. 1992. A developmental perspective on the pathology and neurochemistry of the temporal lobe in schizophrenia. *Schizophrenic Res.* **7**(1): 1–12.
- Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**(259): 680–685.
- Lahrtz, F., R. Horstkorte, H. Cremer, M. Schachner, and D. Montag. 1997. VASE-encoded peptide modifies NCAM- and L1-mediated neurite outgrowth. *J. Neurosci. Res.* **50**(1): 62–68.
- Liu, L., S. Haines, R. Shew, and R. A. Akeson. 1993. Axon growth is enhanced by NCAM lacking the VASE exon when expressed in either the growth substrate or the growing axon. *J. Neurosci. Res.* **35**(3): 327–345.
- Merrill, C. R. 1987. Detection of proteins separated by gel electrophoresis. In *Advances in Electrophoresis* (A. Chrambach, M. Dunn, and B. Radola, Eds.), pp. 111–139. VCH Verlagsgesellschaft mbH, Weinheim, Germany.
- Nelson, M. D., A. J. Saykin, L. A. Flashman, and H. J. Riordan. 1998. Hippocampal volume reduction in schizophrenia as assessed by magnetic resonance imaging: A meta-analytic study. *Arch. Gen. Psychiatry* **55**(5): 433–440.
- Persohn, E., and M. Schachner. 1990. Immunohistological localization of the neural adhesion molecules L1 and N-CAM in the developing hippocampus of the mouse. *J. Neurocytol.* **19**(6): 807–819.
- Pilowsky, L. S., R. W. Kerwin, and R. M. Murray. 1993. Schizophrenia: A neurodevelopmental perspective. *Neuropsychopharmacology* **9**(1): 83–91.
- Poltorak, M., M. A. Frye, R. Wright, J. J. Hemperly, M. S. George, P. J. Pazzaglia, S. A. Jerrels, R. M. Post, and W. J. Freed. 1996. Increased neural cell adhesion molecule in the CSF of patients with mood disorder. *J. Neurochem.* **66**(4): 1532–1538.
- Poltorak, M., I. Khoja, J. J. Hemperly, J. R. Williams, R. el-Mallakh, and W. J. Freed. 1995. Disturbances in cell recognition molecules (N-CAM and L1 antigen) in the CSF of patients with schizophrenia. *Exp. Neurol.* **131**(2): 266–272.
- Poltorak, M., R. Wright, J. J. Hemperly, E. F. Torrey, F. Issa, R. J. Wyatt, and W. J. Freed. 1997. Monozygotic twins discordant for schizophrenia are discordant for N-CAM and L1 in CSF. *Brain Res.* **751**: 152–154.
- Rutishauser, U. 1993. Adhesion molecules of the nervous system. *Curr. Opin. Neurobiol.* **3**: 709–715.
- Saffell, J. L., F. S. Walsh, and P. Doherty. 1994. Expression of NCAM containing VASE in neurons can account for a developmental loss in their neurite outgrowth response to NCAM in a cellular substratum. *J. Cell Biol.* **125**(2): 427–436.
- Small, S. J., and R. Akeson. 1990. Expression of the unique NCAM VASE exon is independently regulated in distinct tissues during development. *J. Cell Biol.* **111**(5): 2089–2096.
- Swayze, V. W. 2d, N. C. Andreasen, R. J. Alliger, W. T. Yuh, and J. C. Ehrhardt. 1992. Subcortical and temporal structures in affective disorder and schizophrenia: A magnetic resonance imaging study. *Biol. Psychiatry* **31**(3): 221–240.
- Takamatsu, K., B. Auerbach, R. Gerardy-Schahn, M. Eckhardt,

- G. Jaques, and N. Madry. 1994. Characterization of tumor-associated neural cell adhesion molecule in human serum. *Cancer Res.* **54**(10): 2598–2603.
26. Vawter, M. P., H. E. Cannon-Spoor, J. J. Hemperly, D. Vander Putten, T. M. Hyde, J. E. Kleinman, and W. J. Freed. 1998. Abnormal expression of cell recognition molecules in schizophrenia. *Exp. Neurol.* **149**: 424–432.
  27. Walsh, F. S., J. Furness, S. E. Moore, S. Ashton, and P. Doherty. 1992. Use of the neural cell adhesion molecule VASE exon by neurons is associated with a specific down-regulation of neural cell adhesion molecule-dependent neurite outgrowth in the developing cerebellum and hippocampus. *J. Neurochem.* **59**(5): 1959–1962.
  28. Weinberger, D. R. 1987. Implications of normal brain development for the pathogenesis of schizophrenia. *Arch. Gen. Psychiatry* **44**(7): 660–669.
  29. Young, W. S., III. 1992. *In situ* hybridization with oligodeoxynucleotide probes. In *In Situ Hybridization: A Practical Approach*. (D. G. Wilkinson, Ed.), pp. 33–44. Oxford Univ. Press, New York.