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RESEARCH ARTICLE

N-CAM Dysfunction and Unexpected Accumulation of PSA-NCAM in Brain of Adult-Onset Autosomal-Dominant Leukodystrophy

Marco Piccinini[1]; Barbara Buccinnà[1]; Giovanni De Marco[1,2]; Elisa Lupino[1]; Cristina Ramondetti[1]; Silvia Grifoni[2]; Barbara Votta[2]; Maria Teresa Giordana[2]; Maria Teresa Rinaudo[1]

[1] Department of Medicine and Experimental Oncology, Section of Biochemistry and [2] Department of Neuroscience, University of Turin, Torino, Italy.

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ADLD, cerebral white matter, N-CAM, PSA-NCAM, PST, STX.

Corresponding author:
Maria Teresa Rinaudo, PhD, Department of Medicine and Experimental Oncology, Section of Biochemistry, Via Michelangelo n 27/b-10126, Torino, Italy (E-mail: *mariateresa.rinaudo@unito.it*)

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Abstract

Previously, myelin from cerebral white matter (CWM) of two subjects of a family with orthochromatic adult-onset autosomal-dominant leukodystrophy (ADLD) was disclosed to exhibit defective large isoform of myelin-associated glycoprotein (L-MAG) and patchy distribution only in the elder subject. L-MAG and neural cell adhesion molecule (N-CAM) (N-CAM 180, 140, and 120) are structurally related and concur to myelin/axon interaction. In early developmental stages, in neurons and glia N-CAM is converted into polysialylated (PSA)-NCAM by two sialyltransferases sialyltransferase-X (STX) and polysialyltransferase-1 (PST). Notably, PSA-NCAM disrupts N-CAM adhesive properties and is nearly absent in the adult brain.

Here, CWM extracts and myelin of the two subjects were searched for the expression pattern of the N-CAM isoforms and PSA-NCAM, and their CWM was evaluated for N-CAM, STX and PST gene copy number and gene expression as mRNA. Biochemically, we disclosed that in CWM extracts and myelin from both subjects, PSA-NCAM accumulates, N-CAM 180 considerably increases, N-CAM 140 is modestly modified and N-CAM 120 remarkably decreases; duplication of genes encoding N-CAM, STX and PST was not revealed, whereas PST mRNA was clearly increased. Immunohistochemically, in CWM of both subjects, we found an unusually diffuse accumulation of PSA-NCAM without inflammation markers. PSA-NCAM persistence, up-regulated PST mRNA and previously uncovered defective L-MAG may be early pathogenetic events in this ADLD form.

INTRODUCTION

Adult-onset autosomal-dominant leukodystrophy (ADLD) is a rare disorder affecting white matter in the central nervous system (CNS) and characterized by autonomic dysregulation, pyramidal signs and cerebellar dysfunctions. The main feature of the disease consists of symmetrical primary myelin loss in CNS visible on magnetic resonance images or computed tomographic scans (5, 6, 16, 33). The pathogenetic mechanisms underlying ADLD are not extensively documented; a limited number of biochemical studies performed on affected individuals have produced inconsistent results concerning myelin lipids (21, 31, 44). Even less is known about the profile of the myelin proteins in the CNS of these individuals; in this regard, a duplication of the gene encoding the nuclear laminar protein lamin B1 (*LMNB1*) has been reported in brain of symptomatic members of Irish American, Japanese and French Canadian families affected by ADLD, and in one Italian individual with familial ADLD (12, 27, 33); in brain tissue of some affected individuals, the genomic duplication resulted in an increased gene dosage at the mRNA and protein levels (33). However, additional studies are required to elucidate the involvement of lamin B1 overexpression in myelin damage in ADLD.

This study is part of an ongoing investigation carried on two members belonging to an Italian family affected by orthochromatic ADLD (19). One member (49 years old at death) was clearly symptomatic with appreciable myelin loss and slight axonal damage, the other (27 years old at death) was apparently asymptomatic.

In the CNS myelin of both subjects, the large isoform of myelin-associated glycoprotein (L-MAG) lacks a short peptide at the C-terminus region; this alteration is associated with quantitatively deficient myelin basic protein (MBP) along with unaltered proteolipid protein (PLP) and cyclic nucleotide phosphodiesterase (CNP) (19). All these proteins are selectively generated by oligodendrocytes, that in the CNS are specifically involved in the production of myelin in which L-MAG is prevalently distributed in the periaxonal region (29, 42).

The neural cell adhesion molecule (N-CAM) family (CD56) shares a number of structural and functional properties with MAG. Like MAG, N-CAM is constitutively and extensively glycosylated at its extracellular N-terminal domain (9), belongs to the

immunoglobulin superfamily of which MAG is the major component (17, 49, 50) and contributes to the complex interaction between myelinating glia cells and neurons, which results in the formation of a myelin-axolemmal complex consisting of axonal and oligodendroglial plasma membranes (28, 37). All these features largely rely on the structural properties of N-CAM, as well as on its wide distribution in the CNS, which partly overlaps that of L-MAG (4, 18, 25, 26, 28, 36).

Many N-CAM isoforms result from differences in splicing of the precursor mRNA (17, 43). These can be categorized into three main species, defined by their molecular mass as N-CAM 180, N-CAM 140 and N-CAM 120; the former two are integral membrane proteins, whereas the latter is linked to the membrane via a glycosylphosphatidylinositol anchor (26, 36). N-CAM 180 is prevalent in neurons, where it is localized in postsynaptic densities (22); N-CAM 140 is also distributed in neurons, preferentially in migratory growth cones, as well as in astroglia (22, 49); N-CAM 120 is predominantly expressed in oligodendrocytes and thus in myelin sheaths, and also in glia (astrocytes) (7, 18, 20, 26, 34, 36). Notably, N-CAM 120 is the most adhesive of the N-CAM isoforms (51).

Early in development, N-CAM is converted into its highly polysialylated (PSA) form, PSA-NCAM, through attachment of α2-8 linked polysialic polymers to carbohydrate moieties on specific glycosylation sites that are distributed on the N-terminal domain; these sialic acid polymers (about 150 units) comprise approximately 30% of the protein molecular mass (9, 15). N-CAM polysialylation is catalyzed by two closely related Golgi-resident sialyltransferases, sialyltransferase-X (ST8Sia II/STX) and polysialyltransferase-1 (ST8Sia IV/PST) (23, 32, 35, 48). By raising the molecular mass of N-CAM isoforms, this extensive polysialylation increases the hydrodynamic radius of their extracellular regions, with a consequent widening of the intermembrane space and disruption of the adhesive properties of N-CAM and other adhesion molecules. In this regard, PSA-NCAM is considered a global inhibitor of cell adhesion and is involved in cell migration, axonal growth, path finding and synaptic plasticity (13, 30, 48). Of relevance to this study, PSA-NCAM expression in the adult brain is circumscribed to restricted regions harboring stem cells, or exhibiting plasticity (2, 23, 30, 48).

Keeping in mind the previously discovered MAG alteration affecting the cerebral white matter (CWM) of the two subjects, the signs of demyelination restricted to the elder subject, the structural and functional similarities of L-MAG and N-CAM, and the duplication of the gene encoding lamin B1; in this study, the expression pattern of N-CAM and PSA-NCAM was investigated in CWM from postmortem specimens of the two subjects; in parallel, our attention focused on the copy number evaluation of the genes coding for N-CAM, ST8Sia II, ST8Sia IV and lamin B1 and on their expression in terms of mRNA (N-CAM, ST8Sia II, ST8Sia IV) and protein (lamin B1).

MATERIALS AND METHODS

Subjects

Clinical data of the ADLD affected family and clinical and pathological data of the two family members were previously detailed (19). Briefly, transmission was autosomal dominant, with a mean age at onset of 46.6 years. The clinical features included spastic paraparesis, action tremor and memory impairment. A linkage to a region on chromosome 5q, in which the ADLD gene is localized (16, 27, 33) was demonstrated in this family; the disease-linked haplotype was found in both patients (unpublished data).

The first subject died after a 4-year history of neurological impairment; patchy demyelination was a feature of its CWM. The second subject died at a presymptomatic age with apparently normal myelin distribution. Both subjects underwent sudden death due to causes unrelated to the disease. The neuropathological diagnosis was orthochromatic leukodystrophy (19).

In the present study, the biochemical analyses were carried out on tissue extracts, total membrane preparations and purified myelin isolated from frozen postmortem specimens of CWM. Postmortem interval was 24 h. The immunohistochemical analyses were performed on formalin-fixed, paraffin-embedded (FFPE) tissue sections. The investigations were performed upon request of the family. For the biochemical assays, CWM specimens of three adult individuals, 30, 42 and 50 years old, who died without neurological disorders, were used as controls.

Biochemical investigations

Reagents

Antibodies to glial fibrillary acidic protein (GFAP), neurofilament protein (NF-L AB11604), N-CAM (AB5032), PSA-NCAM (MAB 5324), PLP (MAB 388) and CNP (MAB 326) were from Chemicon (Temecula, CA, USA); monoclonal mouse antilamin B1 was from Zymed Laboratories (San Francisco, CA, USA); bacterial neuraminidase (N8271), selective for the release of α-2→3, α-2→6, α-2→8 and α-2→9 linked sialic acids from complex glycans, antibody to β actin and protease inhibitor cocktail were from Sigma-Aldrich (Milan, Italy); secondary antibodies conjugated to horseradish peroxidase (HRP) were from Santa-Cruz (Heidelberg, Germany); enhanced chemiluminescence detection reagent (ECL-Plus), hyperfilm and polyvinylidene difluoride (PVDF) membranes were from GE Healthcare (Milan, Italy); nucleotides, recombinant Taq polymerase and Platinum® SYBR® Green qPCR SuperMix-UDG were from Invitrogen (Milan, Italy); ArrayGrade FFPE RNA Isolation Kit and Reaction Ready First Strand cDNA Synthesis Kit were from SuperArray Bioscience Corporation (Frederick, MD, USA); molecular markers for protein (161-0373), molecular biology grade agarose and RC DC Protein Assay Kit I were from BioRad (Milan, Italy); molecular markers for DNA electrophoresis were from Fermentas (Milan, Italy). All the other chemicals were analytical grade from Sigma-Aldrich.

CWM extract preparation

CWM specimens were washed with ice-cold phosphate-buffered saline (PBS) and homogenized in 10 volumes of urea buffer A, which is comprised of 40 mM Tris-HCl pH 7.4, 4 M urea, 5% (w/v) sodium dodecyl sulfate (SDS) and 2% (v/v) β-mercaptoethanol (45). The homogenized tissue was centrifuged at 100 000 *g* for 30 minutes at 20°C, and the clear supernatant was aliquoted and stored at −70°C.

Total membrane and myelin preparation

Total membranes were isolated from CWM specimens by homogenization in ice-cold buffer containing 20 mM Tris pH 7.2, 5 mM ethylenediaminetetraacetic acid (EDTA), 10% sucrose and 10 μL/mL of protease inhibitor cocktail and centrifuged at 100 000 *g* for 2 h at 4°C; the pellet was suspended in PBS pH 6, aliquoted and stored at −70°C.

Myelin was isolated from CWM specimens on a discontinuous 10%–30% (w/v) sucrose gradient as previously reported (19), aliquoted and stored at −70°C.

CWM extracts, total membrane and myelin preparations were then evaluated for protein content using the RC DC protein assay. In addition, the degree of purity of the myelin preparations was assessed by western immunoblotting. The myelin preparations showed high immunoreactivity to antibodies against the myelin selective proteins MBP, PLP and CNP, whereas their immunoreactivity toward antibodies against the astrocyte-specific protein GFAP and neuron-specific protein NF-L was at the detection limit.

Western immunoblotting

All frozen samples were combined with urea buffer B, which is comprised of 50 mM Tris-HCl pH 6.8, 2% (v/v) glycerol, 5% (w/v) SDS, 4 M deionized urea, 40 mM dithiothreitol, 0.05% (w/v) bromophenol blue (45), incubated for 2 h at room temperature, centrifuged at 18 000 *g* at 20°C, and then subjected to SDS-PolyAcrylamide Gel Electrophoresis (SDS-PAGE) on 7.5% polyacrylamide gels and western blotting on PVDF membranes (8). In addition to the above-mentioned antibodies, blotted proteins were immunostained with selective antibodies to N-CAM (1:1000), PSA-NCAM (1:1000) and lamin B1 (1:1000). Antibodies to β actin (1:20000) were used as a control for equal protein loading. Immunostained bands were detected by ECL and subjected to densitometric analysis using a G : BOX Chemi XT16 imaging system (Syngene, Cambridge, UK).

Finally, experiments were run in which aliquots of the total membrane preparation were combined with a volume of the reaction buffer Sigma Product No R-0266 pH 6, supplemented with or without neuraminidase (2 μL of the commercial preparation for 15 μL of the reaction mixture, according to the manufacturer). After 24 h at 37°C, all samples were combined with an equal volume of urea buffer B, processed as described above, and then immunostained with antibodies to PSA-NCAM or N-CAM.

DNA extraction and real-time polymerase chain reaction (PCR) analysis

High molecular weight genomic DNA was extracted from CWM specimens of the two family members and controls, according to previously described procedures (41). Real-time PCR analysis was used to check the copy number of the genes coding for lamin B1, N-CAM, ST8Sia II/STX and ST8Sia IV/PST. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control to normalize the amount of genomic DNA that was subjected to amplification. Primers were designed using Primer3 software (web-based program, http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) and synthesized by MWG (Martinsried, Germany). Primers were specific for: (i) *LMNB1* (GeneID: 4001) exon 4, positions 8–157 (forward 5′-ACGAGAC CAGAAGGAAGCA-3′ and reverse 5′-TCTGCTCCAGCTCC TCCTTA-3′); (ii) *NCAM1* (GeneID: 4684) exon 10 positions 2–179 (forward 5′-GGCAGGAGATGCCAAAGATA-3′ and reverse 5′-ATCCTCGCCTGTAACCACAC-3′); (iii) *ST8Sia II* (GeneID: 8128) exon 7, positions 1274–1435 (forward 5′-GAGCTTGAAAGGGTGCTGTC-3′ and reverse 5′-CAGAA TGCAGAAGGCAAACA-3′); (iv) *ST8Sia IV* (GeneID: 7903) exon 6, positions 26–192 (forward 5′-ATCAAAAGACC CAGCACAGG-3′ and reverse 5′-GGGCTTGCATTGGAAAA GTA-3′); and (v) *GAPDH* (GeneID: 2597) exon 6 positions 7–201 (forward 5′-CAACGAATTTGGCTACAGCA-3′ and reverse 5′-AGGGGTCTACATGGCAACTG-3′).

Real-time PCR analysis of gene copy number was performed in the presence of 50 ng of DNA and 200 nM specific forward and reverse primers for each gene, in a final volume of 25 μl, using Platinum SYBR Green qPCR Super Mix. The amplification profile was as follows: activation of DNA polymerase at 95°C for 3 minutes, followed by 50 amplification cycles of denaturation at 95°C for 15 s and annealing/extension at 60°C for 30 s, in a BioRad Mini Opticon thermal cycler. Product identity was confirmed by ethidium bromide-stained agarose gels and melting point analysis. Samples were run in triplicate, and the mean C_T values were used for calculation. Because the different primer sets had similar amplification kinetics, it was reasonable to use the comparative threshold cycle number (C_T) method to detect the relative gene amount. Using this method, the $2^{-\Delta\Delta C_T}$ formula can be applied to calculate the gene level in affected sample relative to control sample, where $\Delta\Delta C_T = [C_T$ GAPDH (control sample) $- C_T$ LMNB1 or other genes (control sample)] − [C_T GAPDH (affected sample) − or other genes (affected sample)] (24).

The values in Table 1 were obtained using a variation of the Livak method, in which the dosage of each gene is calculated by the $2^{(C_T \text{ GAPDH} - C_T \text{ checked gene})}$ formula. In the control, this value is used as a term of calibration (C); in the affected samples, these values are a measure of alteration (A). Consequently, the relative gene dosage in controls is expressed by the ratio C/C and thus is equal to 1; in affected subjects, the gene dosage is expressed by A/C.

Table 1. Evaluation of relative gene copy number.

Genes	Control (*C/C)	Family subjects (†A/C)
NCAM1	1	0.95 ± 0.22
ST8Sia II	1	0.92 ± 0.19
ST8Sia IV	1	0.89 ± 0.23
LMNB1	1	0.86 ± 0.25

The results are the mean ± standard deviation values of experiments in triplicate carried out on at least three different DNA preparations.
The assay was performed on BWM specimens from control and the two family subjects. The table refers only the values relative to the elder subject because they mirrored those of the younger.
Real-time polymerase chain reaction analysis was applied to check for gene copy number using the ΔC_T method, a variation of the Livak method (24).
*$C = 2^{C_T \text{GAPDH (control sample)} - C_T \text{checked gene (control sample)}}$
†$A = 2^{C_T \text{GAPDH (affected sample)} - C_T \text{checked gene (affected sample)}}$

RNA isolation, reverse transcription and real-time PCR analysis

Total DNA-free RNA was extracted from FFPE CWM sections using the FFPE RNA Isolation Kit. Purified RNA was quantified spectrophotometrically and stored in aliquots at −70°C. cDNA synthesis reactions were performed using 0.3 μg RNA and the Reaction Ready First Strand cDNA Synthesis Kit, consistent with the manufacturer's instructions.

PCR primers used were designed using the Primer 3 server (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) and synthesized by MWG. To amplify *NCAM1* mRNA (NM_181351) between positions 1025 and 1147, which is shared by the N-CAM 180, N-CAM 140 and N-CAM 120 splice forms, the following primers were used: forward 5′-CTTCTACCCGGAACATCAGC-3′ and reverse 5′-TGTACTCTCCGGCATCAGTG-3′. To amplify *ST8Sia II* (NM_6011) and *ST8Sia IV* (NM_5668) mRNAs between positions 80 and 179 and 30 and 130, respectively, the following primers were used: forward 5′-GGGAGGCAGAGGTACAA TCA-3′ and reverse 5′-AACAACAGCTGGTGATGAGG-3′ and forward 5′-AGAACTGAGGAGCACCAGGA-3′ and reverse 5′-CCAGCCTTTCGAATGATTTT-3, respectively. The primers reported at point 5 were used to amplify *GAPDH* mRNA (NM_2046).

Real-time PCR analysis was performed in the presence of 5 ng of reverse transcribed total RNA as described in point 5.

Neuropathological investigations

The following antibodies were used on 5-μm-thick paraffin sections: mouse anti-PSA-NCAM (1:250, MAb 5324, Chemicon), specificity: PSA moiety; rabbit anti-NCAM (1:200, AB5032, Chemicon), specificity: NCAM; goat anti-NCAM (C-20) (1:250, sc-1507, Santa Cruz, CA), specificity: C-terminal of human NCAM. The presence of inflammatory lymphocytes was determined using an antibody against the CD3 component of the T Cell Receptor (Dako, Carpintera, CA, 1:50). Immune reactions were visualized with the Streptavidin-ABC Vectastain kit using diaminobenzidine as the chromogen. Sections were counterstained with hematoxylin.

Statistical analysis

Data were analyzed using the Student's *t*-test. Values given are the mean ± standard deviation from three independent experiments, each carried out using a CWM extract or myelin preparation from specimens of a single control. In each experiment, a different CWM extract and a different myelin preparation from specimens of the two family subjects were used. Significant differences were set at $P < 0.05$.

RESULTS

Biochemical observations

Immunodetection of N-CAM in CWM extracts and myelin preparations

Western blots of CWM extracts (Figure 1A,C) and myelin preparations (Figure 1B,D) stained with an antibody to N-CAM revealed a number of protein bands ranging in size from approximately 170–200 kDa to 130–110 kDa. In both blots, the band around 180 kDa was much more intensively immunostained in the two subjects compared to the controls; this band was broad in the former but sharp in the latter. In CWM blots, the immunoreactivity of the band around 140 kDa was approximately comparable in all samples, as opposed to the immunoreactivity of the band around 120 kDa, which was at the detection limit in the two subjects but easily appreciable in the controls. In myelin blots, the immunoreactivity of the band around 140 kDa was slightly more intense in the two subjects with respect to the controls, as opposed to that of the 120 kDa band that was also weaker in the two subjects in comparison to the controls, particularly the elder subject. These differences were not attributable to a discrepancy in the amount of protein loaded on the gel, as revealed by β actin signal intensity.

Immunodetection of PSA-NCAM in CWM extracts and myelin

Blots of CWM extracts and myelin preparations stained with an antibody directed to the polysialyl moiety of PSA-NCAM revealed a strongly immunoreactive and widely smeared protein band in the molecular mass range 160–250 kDa in the two subject; contrastingly, this band was barely appreciable in the controls (Figure 2A,B).

Immunodetection of PSA-NCAM and N-CAM in CWM total membrane preparations exposed to a neuraminidase that cleaves α-2→8 glycosidic bonds on polysialic polymers

Blots carried out on preparations of total membranes isolated from CWM specimens of the elder (Figure 3A) and the younger (Figure 3B) subjects and then exposed to neuraminidase (+neurN) were minimally stained by an antibody to PSA; the immunoreactivity was instead preserved in the blots carried out on the membrane preparation not exposed to neuraminidase (−neurN). By contrast, blots from +neurN, which were evaluated for immunoreactivity to N-CAM, revealed a pattern of protein bands sized around 180, 140 and 120 kDa, considerably different with respect to that shown in a blot from −neurN. Specifically, the 180 kDa band was smeared in −neurN but minimally so in +neurN; the 120 kDa band was darker in +neurN than in −neurN in which it was very faint; the 140 kDa band was more intensively stained in +neurN than in −neurN in the case of the elder subject; this difference was not appreciable in the case of the younger subject.

NCAM1, ST8Sia II/STX, ST8Sia IV/PST and LMNB1 gene copy number evaluation

In an attempt to shed light on the above-described accumulation of N-CAM and PSA-NCAM, the genes coding for N-CAM, ST8Sia II/STX and ST8Sia IV/PST were searched for duplication in CWM specimens from the two individuals and the controls. The assay did not reveal appreciable differences between the two subjects and the controls for all genes considered (Table 1).

The evaluation of *LMNB1* gene copy number and lamin B1 protein failed to uncover differences between the two subjects and

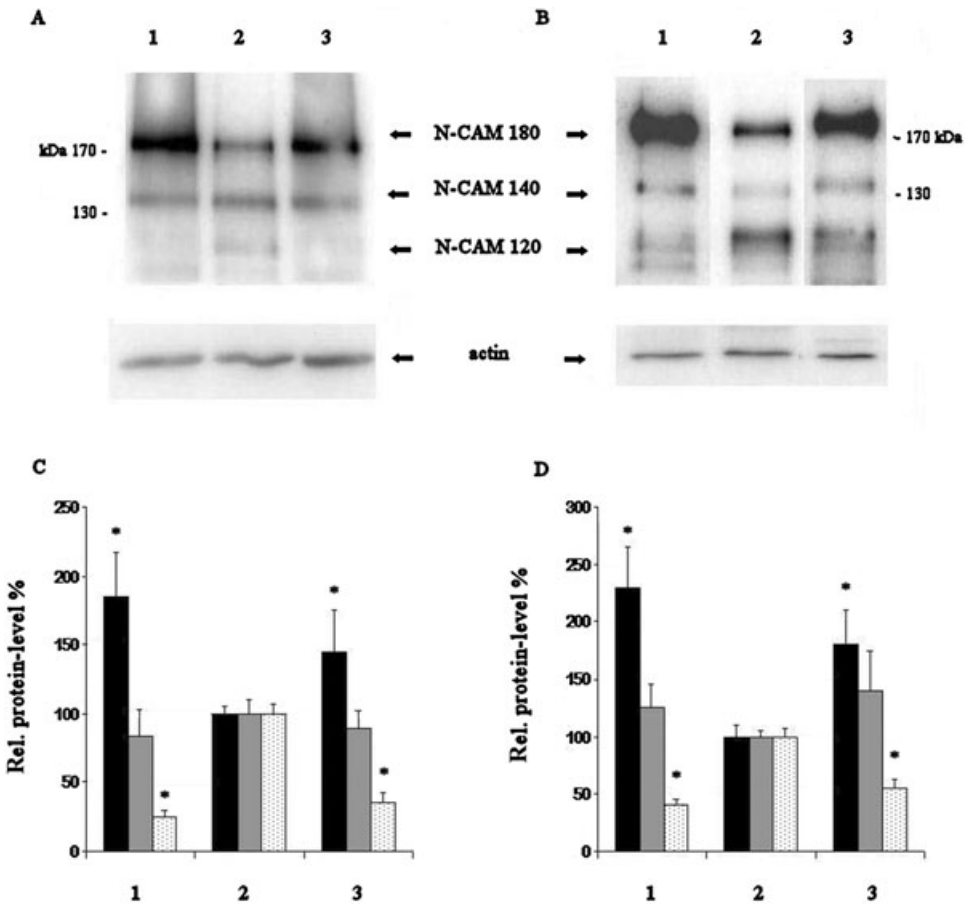

Figure 1. Immunoblotting of neural cell adhesion molecule (N-CAM) isoforms in cerebral white matter extracts (**A**) and myelin preparations (**B**) from postmortem specimens of two subjects with familial orthochromatic adult-onset autosomal-dominant leukodystrophy. Lane 1: the elder subject; lane 2: a neurologically unaffected subject (control); lane 3: the younger subject. Blots were stripped and reprobed with an anti-β actin antibody to verify equal protein loading. The molecular mass markers included proteins in the range 250–10 kDa. The figure illustrates the outcome of one experiment, which was reproduced in two additional independent experiments, each carried out using a different control specimen. The bar charts report the mean ± standard deviation values of the optical density of three independent experiments performed on CWM (**C**) and myelin (**D**). Results were displayed as relative N-CAM amounts, with the value obtained for controls set to 100%. $^*P < 0.01$. ■ N-CAM 180; ▭ N-CAM 140; ▨ N-CAM 120.

controls whether in terms of DNA (Table 1) and protein (not reported).

NCAM1, ST8Sia II/STX and ST8Sia IV/PST mRNA expression

The amount of mRNA transcript coding for N-CAM and ST8Sia II/STX was similar in the elder and the younger subjects and was comparable to that of the controls (not shown). However, the mRNA coding for ST8Sia IV/PST was significantly up-regulated in both subjects as compared to the controls (Figure 4).

Neuropathological and immunohistochemical observations

The neuropathology of the disease consisted of patchy myelin loss in the CWM of the elder subject, as opposed to the younger subject in whom myelin was grossly preserved (19); these findings were confirmed in the present study (Figure 5A,B). Mild gliosis, occasional axonal damage, moderate microglial reaction and absence of inflammatory lymphocytes were previous additional outcomes (19) that were confirmed in this study and exemplified by only isolated CD-3 immunoreactive lymphocytes detected in the CWM of the elder subject (Figure 5E). A novel result of this study concerns the CWM of the elder subject that was diffusely immunopositive with the PSA-NCAM-specific antibody (Figure 5C); this feature was reproduced in the CWM of the younger subject (not shown) but not so in the CWM of an age-matched control that was negative as expected (Figure 5D).

DISCUSSION

This study evaluated the expression pattern of N-CAM and its polysialylated form, PSA-NCAM, in CWM autopsy specimens

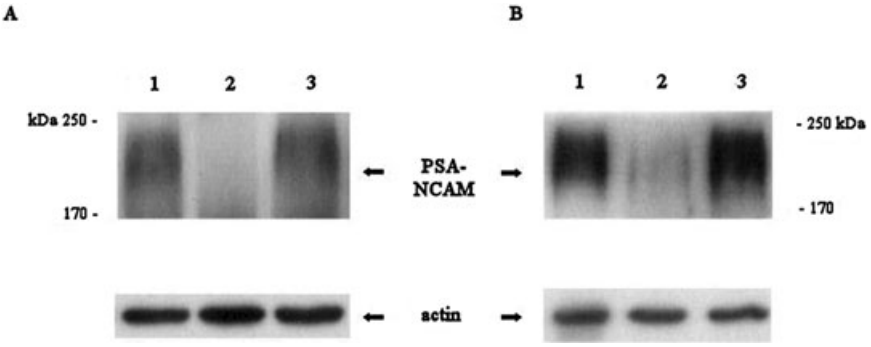

Figure 2. Immunoblotting of polysialylated neural cell adhesion molecule (PSA-NCAM) in cerebral white matter extracts (**A**) and myelin preparations (**B**) from postmortem specimens of two subjects with familial orthochromatic adult-onset autosomal-dominant leukodystrophy. Lane 1: the elder subject; lane 2: a neurologically unaffected subject (control); lane 3: the younger subject. Blots were stripped and reprobed with an anti-β actin antibody to verify equal protein loading. The figure illustrates the outcome of one experiment, which was reproduced in two other independent experiments, each carried out using a different control specimen.

from two subjects who belonged to a large family that has been affected by orthochromatic ADLD over eight generations (19).

An accumulation of PSA-NCAM was found in CWM extracts, total membrane preparations, and purified myelin from the two subjects, paralleling the increased and decreased levels of N-CAM 180 and N-CAM 120, respectively. The presence of PSA-NCAM in both subjects, together with its considerable accumulation, is puzzling if one considers the normally restricted time interval of PSA-NCAM expression during development and the age of the two subjects at death. These findings can thus be taken as a clear indication of a remarkable molecular dysfunction that affects the two subjects.

PSA-NCAM expression is relevant during embryogenesis, peaks in the perinatal phase before the onset of myelination and then progressively decreases, in a tissue-related manner, during development (39, 47). In the developing mammalian brain, PSA-NCAM is mainly found in neurons, although its synthesis proceeds also in other cell types including oligodendrocytes precursors cells (OPC).

However, N-CAM polysialylation is down-regulated in OPCs as soon as they reach their final destination and start to differentiate into mature myelinating oligodendrocytes; in neurons, PSA-NCAM expression begins to be down-regulated when their axons become myelinated (18). In mature nervous tissue, PSA-NCAM is normally absent except in very limited zones of the cerebral cortex (1), the hippocampus mossy fibers (2, 30) and also in those restricted areas of the brain that exhibit neurogenesis and/or plasticity, such as the dentate gyrus, olfactory bulb and hypothalamus-hypophyseal system (23, 48). With the exception of these areas, the presence of PSA-NCAM in the adult human brain is associated with a pathologic status, such as schizophrenia (3), pituitary tumors (46) and multiple sclerosis (MS) (14). In all these cases, PSA-NCAM is localized in specific regions (demyelinated axons in MS

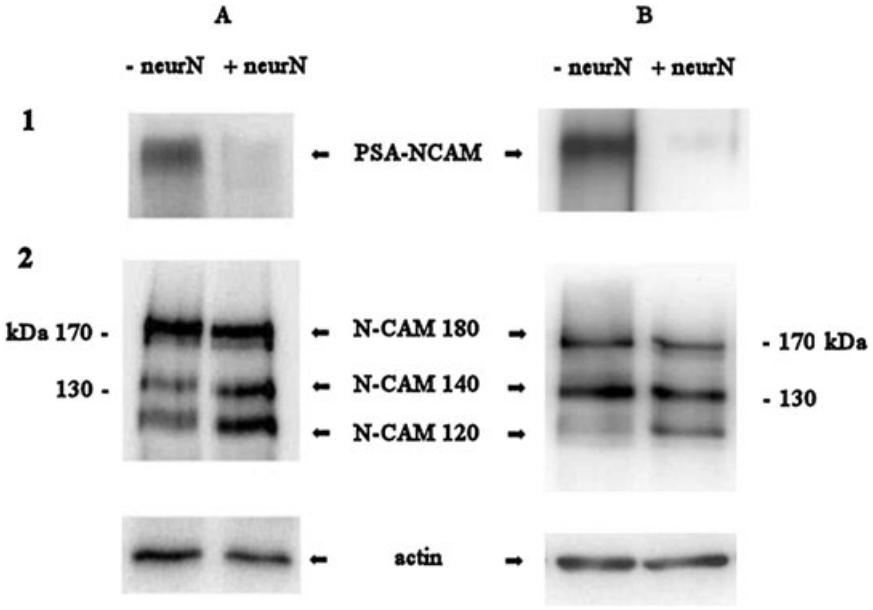

Figure 3. Immunoblotting of neural cell adhesion molecule (N-CAM) and polysialylated (PSA)-NCAM in membrane preparations that were isolated from cerebral white matter of the elder subject (**A**) and the younger subject (**B**) and then exposed to exogenous neuraminidase to disrupt α2-8 glycosidic bonds between polysialic polymers and N-CAM molecules. Blots were stained with anti-: (i) PSA-NCAM; and (ii) N-CAM antibodies from membranes not exposed (–neurN) or exposed (+neurN) to exogenous neuraminidase. Blots were stripped and reprobed with an anti-β actin antibody to verify equal protein loading.

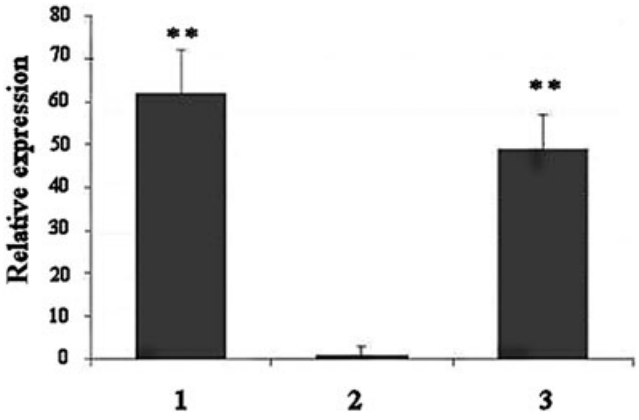

Figure 4. Real-time polymerase chain reaction evaluation of the mRNA expression of polysialyltransferase-1 (ST8Sia IV/PST) extracted from the cerebral white matter of the two subjects with familial orthochromatic adult-onset autosomal-dominant leukodystrophy. The assays were performed on three different total RNA preparations from the two subjects and controls. Duplicate determinations were performed for each RNA preparation, and each assay was carried out in duplicate. The mean ± standard deviation values are reported in the bar graph. $^{**}P < 0.001$.

plaques or neurogenic areas in the other cases, including response to damage). By contrast, PSA-NCAM immunoreactivity is diffusely distributed in the CWM of the two family subjects and absent, as expected, in healthy individuals as revealed by the immunohistochemical analysis.

Therefore, it is likely that the observed accumulation of PSA-NCAM is an anomalous trait affecting broad areas of the CWM of the two subjects, so constituting a novelty with regard to the distribution of PSA-NCAM in pathologic conditions. This concept is supported by the biochemical analysis, which revealed a considerable PSA-NCAM accumulation in CWM extracts, total membrane preparations and purified myelin samples from the two subjects, and all preparations were obtained starting from relatively large tissue fragments. In addition, this analysis revealed that the abnormal accumulation of PSA-NCAM is paralleled, in terms of protein, by increased N-CAM 180 and N-CAM 140, mostly the former and modestly the latter, but decreased N-CAM 120. However, following experimentally induced disruption of polysialic acid polymers (exposure to neuroaminidase), the levels of N-CAM 140 and N-CAM 120 recovered, consistently the latter, while the level of N-CAM 180 remained almost unvaried. It is thus reasonable to believe that up-regulated PSA-NCAM expression in the CWM of the two subjects is due to recruitment of N-CAM 120 and, to a lesser extent, N-CAM 140 (although restricted to the elder subject) to serve as substrates for extensive polysialylation. If this hypothesis is correct, at least some of the N-CAM 120 molecules, and even less so as concerns N-CAM 140, are shifted toward higher mass values through polysialylation. Because the various N-CAM isoforms are distributed differently in the CNS, with N-CAM 120 predominant in oligodendrocytes, N-CAM 180 predominant in neurons, and N-CAM 140 distributed between neurons and glia (astrocytes), and because N-CAM 180 is apparently minimally or even not implicated in the observed PSA-NCAM enhancement in the two subjects, we conclude that the abnormal preservation of PSA-NCAM in this form of ADLD results from metabolic dysfunctions of oligodendrocytes rather than neurons. This feature constitutes an additional novelty of this study because neurons are considered the main sites of PSA-NCAM synthesis during development and under physiological and pathological conditions in the adult brain (see above).

Although minimally or not involved in the abnormal PSA-NCAM accumulation, N-CAM 180 is undoubtedly increased in CWM extracts, myelin and total membrane preparations in the two

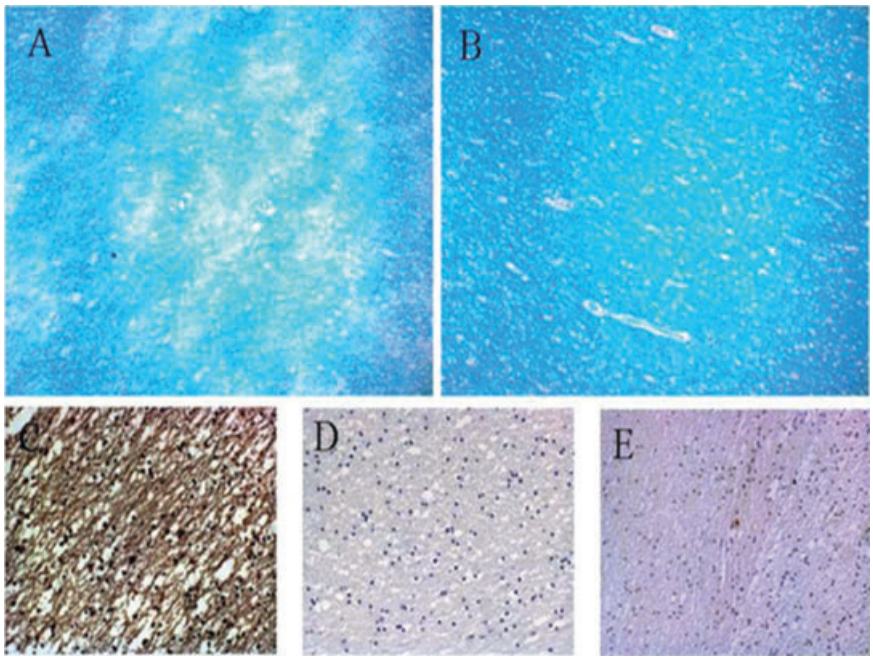

Figure 5. (**A**) Patchy myelin loss in the cerebral white matter of the clinically affected elder subject (Luxol Fast Blue B, ×40); (**B**) Normal myelin staining in the younger subject (Luxol Fast Blue B, ×40); (**C**) cerebral white matter (CWM) of either subject was diffusely immunopositive to a polysialylated neural cell adhesion molecule (PSA-NCAM) antibody, as exemplified by the immunoreactivity shown on the blot of the elder subject (DAB, ×100); (**D**) CWM matter from a control case is negative to a PSA-NCAM antibody (DAB, ×100); (**E**) Isolated T-lymphocytes (CD3-DAB, ×100) were found in the demyelinated white matter of each subject, as exemplified by the immunoreactivity in the blot from the elder subject.

subjects, irrespective of treatment with neuraminidase. Without extra copies of the N-CAM gene or modification of its mRNA expression (Table 1), the question arises as to the mechanisms underlying the increased expression of N-CAM 180 as a protein. In this context, post-transductional and/or post-translational events that lead to protein stabilization, possibly relying on delayed protein degradation, may be at work. Whatever the case, the observed N-CAM 180 up-regulation in the two subjects should stand for an effort by neurons to counter a generalized blockage of cell–cell adhesion caused by the accumulation of anti-adhesive PSA-NCAM, remarkable in olygodendrocyte membranes, and the decreased contribution to cell–cell adhesion relying on the drastically reduced expression of N-CAM 120 paralleled by its considerable conversion into its anti-adhesive polysialylated form. The aforesaid appears reasonable considering the role played by N-CAM in favoring myelin compaction by strengthening myelinating glia cell and neuron interaction, and the localization at the axonal membrane of N-CAM 180. The effort would be successful in the younger subject, in whom myelin was not overtly affected, but unsuccessful in the elder one on the basis of the observed patchy myelin loss.

In addition, or as an alternative, the N-CAM 180 accumulation could represent an attempt to compensate for any potential changes in cell adhesion resulting from the structural alteration of L-MAG, previously described as a trait of both subjects (19). In this respect, it is of note that, following interaction with external ligands, L-MAG transduces signals that lead to up-regulation of the gene encoding MBP, a role that is confined to its C-terminal domain (19). This domain is precisely the site where the L-MAG defect was localized. In both subjects, MBP was quantitatively deficient; of note, this alteration concerns the MBP isoform that stabilizes the myelin sheaths [references in (19).

The conversion of N-CAM into PSA-NCAM is carried out by two specific polysialyl transferases, STX and PST. The regulation of PST and STX is suggested to occur at the transcriptional level and can be modulated by post-translational events (10, 11, 15, 32, 38, 40) The observed accumulation of PSA-NCAM in the CWM of the two individuals apparently does not rely on extra copies of the genes encoding the two transferases, while the up-regulation solely of ST8*Sia IV* at the mRNA level can have a part. This constitutes a still not debated aspect in the ADLD. Furthermore, our results, by disclosing that PTX mRNA expression continues to be wrongly up-regulated in the brain of the two subjects, also revealed that this expression is not circumscribed to defined brain areas. This is, in our view, an additional novel dysfunction characterizing at least this ADLD form being the expression of the mRNA of the two sialyltransferases high in fetal brain, and that of PST the only one preserved in the adult brain and only in well-defined areas that harbor permanent neurogenesis and neural plasticity (15).

To conclude, we suggest that the alterations in the neural adhesion proteins highlighted in this particular form of ADLD point to cross-talk between the axonal membrane and myelin as an early target of an unknown pathogenic mechanism underlying the disease. The alterations disclosed in the younger subject indicate that structurally defective L-MAG, together with decreased amounts of MBP and N-CAM 120, increased amounts of PSA-NCAM and N-CAM 180, and up-regulated PST mRNA expression, are the manifestation of pathogenic events underlying the disease that originate long before the onset of symptoms, rather than subsequent steps of a myelin impairment.

These events are not associated with an extra copy of the gene encoding the nuclear laminar protein lamin B1 (Table 1) discovered in a number of ADLD affected families ending into up-regulated protein expression in some of them (12, 27, 33). More likely, they are related to impaired signaling in oligodendrocytes by the structurally truncated L-MAG at its C-terminus, resulting in the previously described down-regulation of MBP and in the up-regulation of PST, accumulation of PSA-NCAM and subtraction of N-CAM 120 from its role as adhesive molecule here revealed. This complex sequence of derailments in oligodendrocytes is suspected to be at the heart of impaired signaling toward neurons, causing delayed N-CAM 180 degradation and consequent accumulation, and dysfunctional cell-to-cell adhesion finally ending into myelin degradation. In this respect, all these defects are novel additional unsuspected factors that may contribute to ADLD pathology, thus underscoring the complexity of the causative events at the core of this group of neurological disorders.

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