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Neuroendocrine-Specific Protein C, a Marker of Neuronal Differentiation, Is Reduced in Brain of Patients with Down Syndrome and Alzheimer's Disease

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Neuroendocrine-specific protein C (NSP-C) is found in neural and neuroendocrine cells and associated with the endoplasmic reticulum. Its expression was found to correlate with the degree of neuronal differentiation. As the neuropathological findings in Down syndrome (DS) includes deficits of differentiation, and we detected a downregulated sequence with 100% homology with NSP-C homolog mRNA in temporal cortex of patients with DS as well as Alzheimer's disease (AD) using differential display-polymerase chain reaction (DD-PCR), we decided to examine the protein levels of NSP-C in temporal, frontal cortex and cerebellum of DS and AD. To normalize NSP-C versus neuronal density, we also determined neuron-specific enolase (NSE) levels and calculated the ratios. NSP-C was significantly reduced in DS (temporal and frontal cortex) and AD (frontal cortex) compared to controls. The significant decrease of NSP-C in DS was even more pronounced when related to NSE levels. Impaired differentiation in DS brain may well be due to absolutely and relatively decreased NSP-C levels in temporal and frontal cortex. As NSP-C was also reduced in AD frontal cortex, NSP-C deficits in these disorders may be reflecting neurodegenerative changes rather than a primary and specific finding of DS or AD pathogenesis.

Key Words: **neuroendocrine-specific protein C; neuron-specific enolase; neuronal differentiation; Down syndrome; Alzheimer's disease; neurodegenerative diseases.**

The 1.8-kb transcript neuroendocrine-specific protein C (NSP-C) is encoded by the NSP-gene, a member of the reticulon (RTN) family [13, 16] located on chromosome 14q21-q22 [10] and is translated into NSP-C protein with a molecular weight of 23 kDa [18, 24]. It is associated with membranes of the endoplasmic reticulum [24] and found in neural and neuroendocrine cells and tissues [3, 18–20]. NSP-C is expressed in peripheral nerves and in the molecular and granular layer of the cerebellar cortex, but not in Purkinje cells [23]. NSPs are constituents of the rough and the smooth endoplasmic reticulum (ER). Since ER is considered to play a major role in the rapid anterograde transport of enzymes and a variety of membrane macromolecules to be incorporated into the axolemma and synaptic vesicles, NSP proteins may well play a role in transport process or the regulation of intracellular calcium [24]. The expression of NSP-C was found to be correlating with the degree of neuronal differentiation: neuroblastoma cells that morphologically present with variable degrees of differentiation including neuritic extending processes and synapse formation with distant cell, showed NSP-C-dependent differentiation. Hens and co-workers recently showed that highly differentiated neuronal cell lines expressed NSP-C to a higher extent than cell lines showing less neuronal differentiation [9]. Moreover, induction of neuronal differentiation in undifferentiated pheochromocytoma PC12 cells induced a strong increase in NSP-C expression [25]. Performing gene hunting in Down Syndrome (DS) brain we found a downregulated sequence with high homology to NSP-C homolog mRNA and this finding along with histological data proposing impaired neuronal differentiation in DS brain made us examine NSP-C in brain of adults DS patients at the protein level. Brains of controls and of patients with Alzheimer's disease (AD) were studied as well in order to rule out that the decrease of NSP-C was simply due to neurodegeneration found in both disorders.

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TABLE 1

Autopsy Data Used in This Study

	Age (years)	Postmortem interval (h)
Temporal cortex		
Control (n = 5)	34.20 ± 11.80	31.00 ± 11.60
DS (n = 3)	57.33 ± 11.50	44.33 ± 23.97
AD (n = 5)	62.60 ± 5.27	32.00 ± 25.62
Frontal cortex		
Control (n = 7)	61.00 ± 9.11	32.57 ± 12.34
DS (n = 6)	57.83 ± 8.18	31.17 ± 23.13
AD (n = 5)	58.00 ± 5.96	25.80 ± 25.51
Cerebellum		
Control (n = 6)	63.17 ± 9.28	39.50 ± 22.35
DS (n = 5)	57.60 ± 9.29	28.80 ± 25.86
AD (n = 5)	60.80 ± 6.83	17.20 ± 8.38

MATERIALS AND METHODS

Brain samples. Postmortem human brain samples (temporal, frontal cortex, and cerebellum) were obtained from the MRC London Brain Bank for Neurodegenerative Diseases, Institute of Psychiatry (Table 1). All the DS patients were karyotyped and possessed trisomy 21. A formal cognitive assessment of dementia in DS was not performed. In all DS brains there were abundant and extensive beta-amyloid deposits, neurofibrillary tangles and neuritic plaques. The AD patients were fulfilled the National Institute of Neurological Disorders and Stroke and Alzheimer's disease and Related Disorders Association criteria for probable AD [22]. The neuropathological diagnosis of "definite AD" was confirmed using the CERAD criteria [12]. Normal control brains were obtained from individuals with no history of neurological or psychiatric illness. The major cause of death was bronchopneumonia in DS and AD and heart disease in controls. The fresh brain was dissected, and coronal slices were snap frozen and stored at −70°C until use.

RNA isolation. Temporal tissues ground using a mortar and pestle under liquid nitrogen were lysed in RNAzol B (Molecular Research Center, Newark, NJ) and added 0.2 ml of chloroform per 2.0 ml of homogenate. The homogenates were shaken vigorously for 15 s, stored on ice for 5 min and centrifuged at 12,000*g* for 15 min at 4°C. Total RNA remained in the upper aqueous phase was precipitated by mixing with an equal volume of isopropanol. The mixtures were incubated for 15 min at 4°C and centrifuged at 12,000*g* for 15 min at 4°C. Total RNA pellets were washed with 75% ethanol and dried for 10 min. Total RNA pellets were dissolved in RNase-free water. Messenger RNA was isolated using Quick Prep Micro mRNA purification kit (Pharmacia Biotech, Uppsala, Sweden) containing oligo(dT)-cellulose according to the manufacturer's instructions. The concentration and purity of total RNA and mRNA were calculated with absorbance at 260 and 280 nm using a spectrophotometer.

Differential display–polymerase chain reaction (DD-PCR). DD-PCR was performed according to the method described by Liang *et al.* [11] using the RNAimage kit (GenHunter Corp., Nashville, TN). Briefly, 100 ng of the isolated mRNA was reverse transcribed in reverse transcriptase buffer (25 mM Tris–Cl, pH 8.3, 37.6 mM KCl, 1.5 mg $MgCl_2$ and 5 mM DTT) containing 5 unit/μl of MMLV-reverse transcriptase, 20 μM dNTP mix and 0.2 μM of each one-base-anchored oligo(dT) primer (-G, -A or -C). The reverse transcription mixture was used for PCR in dilution of 1:10. Subsequent PCR (20 μl) was performed in PCR buffer (10 mM Tris–Cl, pH 8.4, 50 mM KCl, 1.5 mM $MgCl_2$ and 0.001% gelatin) containing 2 μM dNTP, 0.2 μM of one-base-anchored oligo(dT) primer, 0.2 μM of arbitrary primer, 0.2 μl of α-[^{33}P]dATP (2000 Ci/mmol) and 0.05 unit/μl of AmpliTaq DNA Polymerase (Perkin–Elmer). The thermocycler (GeneAmp PCR System 9700, Perkin–Elmer) was programmed as follows: 40 cycles at 94°C for 30 s, 40°C for 2 min, and 72°C for 30 s, and terminated with a final extension at 72°C for 5 min. ^{33}P-labeled PCR products were separated on 6% denaturing polyacrylamide gel for 3.5 h at 60 W constant power. The blotted gel on a piece of 3M paper was dried under vacuum at 80°C for 1 h. The autoradiogram oriented with the dried gel was exposed and developed. The bands of interest were cut from the dried gel, eluted by boiling in water and reamplified by PCR with the same set of primers in the same condition. The reamplified cDNA fragments were cloned in PCR-TRAP vector using PCR-TRAP cloning system (GenHunter) according to the manufacturer's instructions. DNA sequencing was performed in MWG-BIOTECH (Ebersberg, Germany). The sequence alignment was aligned in all EMBL libraries.

Western blot. The brain tissues were suspended in homogenate buffer consisting of 10 mM Tris–HCl (pH 7.5), 150 mM NaCl, 0.05% (v/v) Tween 20, 4% Chaps, 1 mM EDTA and a mixture of protease inhibitors, 1 mM PMSF and 1 μg/ml of each pepstatin A, chymostatin, leupeptin and antipain. The suspension was homogenized in a Potter-Elvehjem homogenizer at 4°C and centrifuged at 10,000*g* for 10 min. The BCA protein assay kit (Pierce, USA) determined the concentration of protein in the supernatant. Samples (10 μg) were mixed with the same volume of sample buffer (125.5 mM Tris, 70 mM SDS, 0.001% bromophenol blue, 20% glycerol, 2% 2-mercaptoethanol, pH 6.8), incubated at 95°C for 15 min and loaded onto a 12.5% homogeneous gel (Amersham Pharmacia Biotech, Sweden). The electrophoresis was performed with Multiphor II Electrophoresis System (Amersham Pharmacia Biotech, Sweden). Proteins separated on the gel were transferred onto PVDF membrane (Millipore, Bedford, MA) and the membrane was blocked in blocking buffer (10 mM Tris–HCl (pH 7.5), 150 mM NaCl, 0.1% Tween 20, 0.1% $MgCl_2$ and 1% non-fat dry milk). The membrane was incubated for 2 h at room temperature with 1:5 diluted mouse monoclonal antibody (IgG1 subtype) recognizing epitopes present in NSP-C. After 3 times washing for 15 min with blocking buffer, membranes were probed with a 1:1000 dilution of secondary antibody, anti-mouse IgG1, coupled to horseradish peroxidase (Southern Biotechnology Associates, Inc., Alabama) for 1 h. The membrane was washed 3 times for 15 min and developed with the Western blot chemiluminescence reagents (NEN Life Science Products, Inc., U.S.A.). The densities of signaling bands were measured by RFLPscan version 2.1 software program (Scanalytics, U.S.A.). Between group differences were calculated by nonparametric Mann–Whitney U test using GraphPad Instat2 program and the level of significance was considered at $P < 0.05$. All results are presented as mean ± standard deviation (SD).

RESULTS

To detect the differentially expressed genes in human brain we compared the mRNA expression pattern of temporal region from patients from DS and AD, and controls using DD-PCR. DD-PCR was performed using a total of 24 primer combinations. As shown in Fig. 1, the arrowhead-indicated cDNA band that was absent in DS and poorly expressed in AD compared to controls was excised from the gel, reamplified, subcloned and subsequently sequenced. Alignment of the nucleic acid sequence with the sequence obtained from EMBL was shown that 167-base cDNA fragment showed 100% homology with NSP-C homolog mRNA sequence (Fig. 2). Since the amino acid sequence encoded by NSP-C homolog mRNA is high homologous with that encoded by NSP-C mRNA and impaired neuronal differentia-

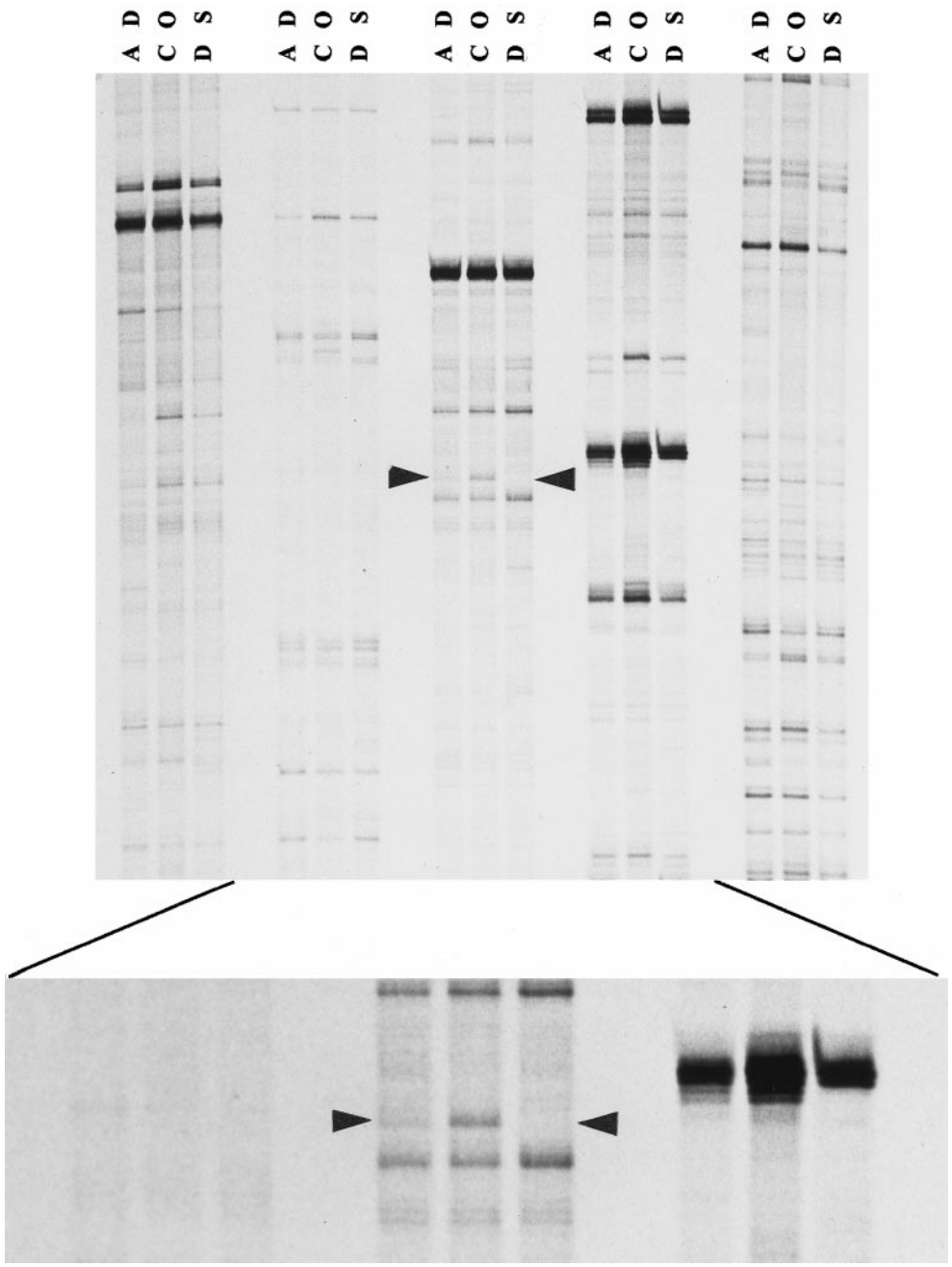

FIG. 1. Differential display to present mRNAs expressed in AD and DS compared to controls (CO). The arrowhead indicates a differentially expressed 167-base cDNA fragment that was amplified using one-base anchored oligo(dT) primer (5′-AAGCTTTTTTTTTTTC-3′) and arbitrary primer (5′-AAGCTTTGGTCAG-3′).

tion in DS brain was proposed in several studies, we investigated the protein levels of NSP-C in temporal, frontal cortex and cerebellum. The protein levels of NSP-C were significantly reduced in DS (temporal and frontal cortex) and AD (frontal cortex) (Table 2). Although there was no statistically significant difference ($P > 0.05$) in DS (cerebellum) and AD (temporal cortex and cerebellum), the protein levels of NSP-C in DS and AD showed reduced patterns compared to controls. We also determined NSE levels in order to normalize NSP-C level with neuronal density (Table 3). The significant reduction of NSP-C in DS was even more pronounced when related to NSE levels (Table 4).

DISCUSSION

We clearly show that NSP-C is expressed in cerebellum as described by Senden and co-workers [20] as well

```
                    1          11         21         31
167-base cDNA       atgtgttcat catcttaagt attgtaagct gctatgtatg
                    :::::::::: :::::::::: :::::::::: ::::::::::
NSP-C homolog mRNA  atgtgttcat catcttaagt attgtaagct gctatgtatg
                    991        1001       1011       1021

                    41         51         61         71
167-base cDNA       gatttaaacc gtaatcatat ctttttccta tctatctgag
                    :::::::::: :::::::::: :::::::::: ::::::::::
NSP-C homolog mRNA  gatttaaacc gtaatcatat ctttttccta tctatctgag
                    1031       1041       1051       1061

                    81         91         101        111
167-base cDNA       gcactggtgg aataaaaaac ctgtatattt tactttgttg
                    :::::::::: :::::::::: :::::::::: ::::::::::
NSP-C homolog mRNA  gcactggtgg aataaaaaac ctgtatattt tactttgttg
                    1071       1081       1091       1101

                    121        131        141        151
167-base cDNA       cagatagtct tgccgcatct tggcaagttg cagagatggt
                    :::::::::: :::::::::: :::::::::: ::::::::::
NSP-C homolog mRNA  cagatagtct tgccgcatct tggcaagttg cagagatggt
                    1111       1121       1131       1141

                    161
167-base cDNA       ggagcta
                    :::::::
NSP-C homolog mRNA  ggagcta
                    1151
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FIG. 2. Sequence alignment of 167-base cDNA fragment found by DD-PCR with the sequence obtained from EMBL. The sequence of 167-base cDNA fragment has 100% homology with NSP-C homolog mRNA sequence.

as in frontal and temporal cortex. It was significantly decreased in DS (temporal and frontal cortex) and AD (frontal cortex). Although there was no statistically significant difference ($P > 0.05$) in other brain regions, NSP-C showed reduced patterns compared to controls. This significant decrease was even more pronounced when related to NSE, a marker of neuronal density, in DS whereas in AD reduced levels were no longer reaching statistical significance. As NSE in DS was significantly and remarkably decreased, normalization

TABLE 2

NSP-C Protein Expression Levels in the Brain Regions of Patients with DS, AD, and Controls

	Control	DS	AD
Temporal cortex	3.54 ± 2.45	0.80 ± 0.07*	1.03 ± 0.80
Frontal cortex	4.88 ± 1.06	2.04 ± 1.95*	3.12 ± 1.50*
Cerebellum	3.22 ± 1.85	3.11 ± 3.08	2.77 ± 2.25

* $P < 0.05$.

TABLE 3

NSE Protein Expression Levels in the Brain Regions of Patients with DS, AD, and Controls

	Control	DS	AD
Temporal cortex	11.64 ± 4.23	16.53 ± 3.75	5.15 ± 3.04*
Frontal cortex	0.27 ± 0.02	0.22 ± 0.06*	0.25 ± 0.07
Cerebellum	0.75 ± 0.35	0.95 ± 0.31	0.77 ± 0.33

* $P < 0.05$.

TABLE 4

NSP-C Protein Expression Levels Normalized with NSE Protein Levels

	Control	DS	AD
Temporal cortex	0.31 ± 0.25	0.05 ± 0.02*	0.21 ± 0.13
Frontal cortex	18.18 ± 3.33	8.71 ± 6.71*	12.54 ± 5.29
Cerebellum	5.89 ± 4.45	3.01 ± 3.06	3.62 ± 2.61

* $P < 0.05$.

should have been showing even higher NSP-C levels (as related to neuronal density). The morphological correlate of the difference between DS and AD may be represented by histological findings of affected cortical layers II and IV in DS but with main pathology of cortical layers II, III and V in AD [4]. And indeed, neuronal loss is more pronounced in cortical layers of patients with DS than with AD [5, 14] reflecting NSE levels in our study. The biological meaning of absolutely and/or relatively decreased NSP-C in frontal and temporal cortex of DS may be representing deterioration of brain differentiation in this disorder rather than simply neuronal cell loss. Although there is no histological feature or pattern and histological reports are inconsistent, there is strong evidence for morphological changes in DS brain from early life [6]. Macroscopically, DS brain is different from controls. The brain weight is in the low normal range, the size of the cerebellum and brain stem is smaller compared to normals, fronto-occipital length is shortened and there is pronounced narrowing of the superior temporal gyri in at least a third of cases. Nerve cell heterotopias were described in DS and are attributed to impaired migration in fetal life. The anterior commissure is reduced in cross sectional area in adult DS patients and Golgi studies have revealed a series of architectural abnormalities.

There is now consensus that there are abnormalities of dendritic spines in infants and children with DS pointing to atrophy of the dendritic tree of the cortex. There is major evidence for defect histogenesis in DS brain including poverty of granular cells (possibly the aspinous stellate cells) throughout the cortex and in individuals ranging in an age from newborn to 14 years, there is decreased neuronal density in layers II and IV of the occipital area [17, 26]; in hypothalamic areas a diminution in the number of hypothalamic neurons has been described [27]. Again, a histologically clear picture of the wiring of the brain in DS has not emerged yet. Impaired differentiation in DS may be due to decreased NSP-C although it may also be explained by other factors involved in differentiation mechanisms as e.g., Down Syndrome cell adhesion molecule (DSCAM) or S100. DSCAM has been isolated from chromosome 21q22.2-22.3 and is considered a new class of neuronal cell adhesion molecules and is mainly expressed at times of neuronal differentiation in the neural tube [28]. Based upon the overexpression hypothesis [6], that genes encoded on chromosome 21 should be overexpressed in trisomy 21, one may propose or speculate that increase DSCAM could account for impaired differentiation in DS, although the overexpression hypothesis has been challenged recently [7, 8]. This would be also valid for another neuronal protein, encoded on chromosome 21, namely S100B, beta polypeptide, which has been found to be increase in DS hippocampus [1, 2, 15]. No systematic study on differentiation abnormalities in AD brain has been carried out so that we are left with a immunochemical finding of a decreased differentiation marker.

We conclude that a neuronal differentiation protein was significantly reduced in frontal and temporal cortex of DS and in temporal cortex of AD. The findings may express the biological meaning that differentiation is impaired in both neurodegenerative disorders, which may help to explain neuropathological findings in frontal cortex of DS [17, 21]. As we studied adult DS patients presenting with AD neuropathology—and indeed all DS patients from the fourth decade of life show AD morphological changes—we propose to assign the findings of decreased NSP-C to neurodegeneration, common to both disorders.

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