CMLS, Cell. Mol. Life Sci. 57 (2000) 1810–1816
1420-682X/00/121810-07 $ 1.50 + 0.20/0

CMLS Cellular and Molecular Life Sciences

Research Article

Decreased levels of complex III core protein 1 and complex V β chain in brains from patients with Alzheimer's disease and Down syndrome

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Received 14 June 2000; received after revision 14 August 2000; accepted 4 October 2000

Abstract. Ubiquinol:cytochrome c oxidoreductase (complex III) and ATP synthase (complex V) are important enzymes in the mitochondrial electron transport chain. Defects in mitochondrial respiratory enzymes have been reported for several neurodegenerative diseases. In this study, we applied the proteomic approach to investigate protein levels of complex III core protein 1 and complex V β chain in brain regions of Alzheimer's disease (AD) and Down syndrome (DS) patients. Complex III core protein 1 was significantly reduced in the temporal cortex of AD patients. Complex V β chain was significantly reduced in the frontal cortex of DS patients. We conclude that decreased mitochondrial respiratory enzymes could contribute to the impairment of energy metabolism observed in DS. These decreases could also cause the generation of reactive oxygen species and neuronal cell death (apoptosis) in DS as well as AD.

Key words. Ubiquinol:cytochrome c oxidoreductase; ATP synthase; mitochondrial electron transport chain; Alzheimer's disease; Down syndrome; neuronal cell death; neurodegenerative disease.

Defects in the mitochondrial electron transport chain are known to be integrally related to programmed cell death (or apoptosis), and this is characteristic of several neurodegenerative diseases such as Alzheimer's disease (AD), Down syndrome (DS), Parkinson disease (PD), and Huntington disease (HD) [1–6].

Ubiquinol:cytochrome c oxidoreductase (complex III) and ATP synthase (complex V) are important enzymes of the mitochondrial electron transport chain for energy metabolism [7]. Complex III catalyzes electron transfer from ubiquinol to cytochrome c and consists of nine to ten polypeptides. Complex V as the terminal enzyme of the respiratory chain is responsible for ATP synthesis from ADP and inorganic phosphate at the expense of protonic energy derived from the operation of respiratory complex I, III, and IV. Complex V is composed of two principle sectors, a catalytic core (F_1) and a membrane proton channel (F_0). F_1 has five subunits (α, β, γ, δ, and ε) and F_0 has three subunits (A, B, and C).

Recently, defects of mitochondrial electron transport chain enzymes were found repeatedly in various cells and tissues of patients with neurodegenerative diseases. Biochemical findings demonstrated a generalized depression of activity of electron transport chain complexes (complex I, II, III, and IV) in AD brain mitochondria using assays for electron transport chain activities [8]. Significantly decreased activity of complex IV was reported in frontal, temporal, parietal, and

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occipital cortex from AD patients compared to age-matched controls [9]. Reduction in complex V was screened in the hippocampus of brains from AD patients by blue native polyacrylamide gel electrophoresis, but complex III and complex IV were present at almost normal concentrations [10]. In PD, complex I activity was significantly and selectively reduced in the substantia nigra in the absence of mitochondrial damage or reduction in polypeptide composition [11]. Deficiencies in complex I subunits (30-, 25-, and 24-kDa subunits) were reported in the striata of patients with PD using immunoblot analysis [12], and low mean activity of complex III was reported in skeletal muscle from patients with PD [13].

Here, we applied the proteomic approach to study protein levels of complex III core protein 1 and complex V β chain in several brain regions of AD and DS. Two-dimensional (2D) gel electrophoresis with subsequent matrix-assisted laser desorption ionization mass spectroscopy (MALDI-MS) identification and specific software for quantification of proteins was used. We also investigated the protein level of aconitate hydratase to show the specificity of the reduction of mitochondrial electron transport chain enzymes rather than simply mitochondrial loss. Moreover, we investigated protein levels of vacuolar ATP synthase (V-ATP synthase, B and F subunits) to identify whether another ATP synthase from a different compartment is also reduced in neurodegenerative diseases.

Deranged levels of complex III and V proteins do not classify DS or AD as mitochondriopathies, but contribute a puzzle piece to the open controversy surrounding mitochondrial enzyme expression and activation as well as mitochondrial dysfunction.

Materials and methods

Brain samples. Postmortem brain samples were obtained from the MRC London Brain Bank for Neurodegenerative Diseases, Institute of Psychiatry (table 1). All 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 β-amyloid deposits, neurofibrillary tangles, and neuritic plaques. The AD patients were selected prospectively and examined clinically, and fulfilled the National Institute of Neurological Disorders and Stroke and Alzheimer's Disease and Related Disorders Association criteria for probable AD [14]. The neuropathological diagnosis of 'definite AD' was confirmed using the CERAD criteria [15]. Normal control brains were obtained from individuals with no history of neurological or psychiatric illness. The fresh brain was dissected, coronal slices snap frozen and stored at -70°C until required. Seven brain regions (cerebellum, frontal, temporal, occipital, parietal cortices, thalamus, and caudate nucleus) of patients with AD, DS, and controls were used for the studies at the protein level. The postmortem interval had no influence upon protein expression (data not shown) and the freezer storage time showed no effect.

2D gel electrophoresis. Brain tissue was suspended in 0.5 ml of sample buffer consisting of 40 mM Tris, 5 M urea (Merck, Darmstadt, Germany), 2 M thiourea (Sigma, St. Louis, Mo.), 4% CHAPS (Sigma), 10 mM 1,4-dithioerythritol (Merck), 1 mM EDTA (Merck) and a mixture of protease inhibitors, 1 mM PMSF and 1 µg each of pepstatin A, chymostatin, leupeptin, and antipain. The suspension was sonicated for approximately 30 s and centrifuged at 10,000*g* for 10 min and the supernatant was centrifuged further at 150,000*g* for 45 min. The protein concentration of the supernatant was determined by the Coomassie blue method [16]. 2D gel electrophoresis was performed essentially as reported elsewhere [17]. Samples of approximately 1.5 mg were applied on immobilized pH 3–10 nonlinear gradient strips. The proteins were focused at 300 V for 1 h, after which the voltage was gradually increased to 3500 V within 6 h. Focusing was continued at 5000 V for 24 h. The second-dimensional separation was performed on 9–16% linear gradient polyacrylamide gels. After protein fixation with 40% methanol containing 5% phosphoric acid for 12 h, the gels were stained with colloidal Coomassie blue (Novex, San Diego, Calif.) for 48 h. The molecular mass was determined by running standard protein markers at the right side of selected gels. The size markers (Gibco, Basel, Switzerland) covered the range 10–200 kDa. pI values were used as given by the supplier of the immobilized pH 3–10 nonlinear gradient strips (IPG, Pharmacia Biotechnology, Uppsala, Sweden). The gels were destained with H_2O and scanned in a Molecular Dynamics Personal

Table 1. Autopsy data used in this study.

	Control	AD	DS
n	18	13	9
Age (years)	50.00 ± 16.94	58.54 ± 7.57	55.67 ± 7.48
Postmortem interval (h)	38.78 ± 18.84	30.31 ± 26.17	31.44 ± 19.56
Major cause of death	heart disease	bronchopneumonia	bronchopneumonia

densitometer. The images were processed using PhotoShop (Adobe) and PowerPoint (Microsoft) software. In partial 2D gel images including either complex III core protein 1 or complex V β chain and the neighboring proteins, the percentage of the volume of the spot representing either complex III core protein 1 or complex V β chain was quantified. This was then compared to the total proteins present, using the ImageMaster 2D Elite software (Amersham Pharmacia Biotechnology) referring to the previously constructed 2D map of human brain proteins [18].

Matrix-assisted laser desorption ionization mass spectroscopy. MALDI-MS analysis was performed as described [19] with minor modification. Briefly, spots were excised, destained with 50% acetonitrile in 0.1 M ammonium bicarbonate and dried in a speedvac evaporator. The dried gel pieces were reswollen with 3 µl of 3 mM Tris-HCl, pH 8.8, containing 50 ng trypsin (Promega, Madison, Wis.). After 15 min, 3 µl of H_2O was added and 1 µl was applied to the dried matrix spot. The matrix consisted of 15 mg nitrocellulose (Bio-Rad) and 20 mg α-cyano-4-hydroxycinnamic acid (Sigma) dissolved in 1 ml acetone:isopropanol (1:1, v/v). A 0.5-µl portion of matrix solution was applied on the sample target. The digest mixtures were analyzed in a time-of-flight PerSeptive Biosystems mass spectrometer (Voyager Elite, Cambridge, Mass.) equipped with a reflectron. An accelerating voltage of 20 kV was used. Calibration was internal to the samples. The peptide masses were matched with the theoretical peptide masses of all proteins from all species of the SWISS-PROT database. For protein search, monoisotopic masses were used and a mass tolerance of 0.0075% was allowed.

Statistics. Results are expressed as means $\pm$ standard deviation (SD). Between-group differences were investigated by a nonparametric Mann-Whitney U test. Significance was set at $P < 0.05$. All statistical analyses were performed by GraphPad Instat2 software, version 2.05.

Results

Identification of human brain proteins. Identification of human brain proteins based upon molecular weight, pI value, and peptide matches was done in previous work by 2D gel electrophoresis and MALDI-MS [18].

Protein levels of complex III core protein 1 and complex V β chain. In this study, we used 2D gels with proteins from seven brain regions of controls and patients with AD and DS to identify differences and any regional variation at the protein level of complex III core protein 1 and complex V β chain. We observed reduced protein levels of both in brain regions of patients with AD and DS compared to controls. As shown in table 2, the complex III core protein 1 level was significantly reduced in temporal cortex from patients with AD. The complex V β chain level was significantly reduced in frontal cortex from patients with DS (table 3). It was also decreased in temporal and occipital cortices as shown for complex III core protein 1, without reaching statistical significance. The 2D gel protein spots representing complex III core protein 1 and complex V β chain are illustrated in figures 1 and 2. The intensity of the protein spot from the region with the significant reduction in AD or DS was relatively weak compared to that in controls. To identify whether reduction of mitochondrial electron transport chain enzymes was a specific finding rather than due simply to mitochondrial loss, we determined protein levels of mitochondrial aconitate hydratase: as shown in table 4, there were no statistically significant differences in the protein levels of mitochondrial aconitate hydratase.

Table 2. Complex III core protein 1 levels in brain regions of patients with AD and DS and in controls.

	Control	AD	DS
Cerebellum	2.11 ± 0.95 (n = 11)	2.10 ± 1.21 (n = 4)	2.04 ± 0.56 (n = 5)
Frontal cortex	2.12 ± 1.00 (n = 8)	1.67 ± 0.53 (n = 4)	1.33 ± 0.69 (n = 6)
Temporal cortex	2.47 ± 1.31 (n = 12)	1.31 ± 0.39** (n = 6)	1.65 ± 0.39 (n = 3)
Occipital cortex	2.07 ± 0.80 (n = 9)	1.46 ± 0.55 (n = 7)	1.46 ± 0.60 (n = 7)
Parietal cortex	2.11 ± 0.93 (n = 7)	2.45 ± 1.56 (n = 3)	1.50 ± 0.24 (n = 6)
Thalamus	1.35 ± 0.67 (n = 8)	2.16 ± 1.02 (n = 5)	1.71 ± 0.79 (n = 3)
Caudate nucleus	1.57 ± 0.42 (n = 7)	1.27 ± 0.33 (n = 3)	1.40 ± 0.47 (n = 2)

Proteins from seven brain regions of patients with DS and AD, and the control group, were separated on 2D gels and visualized following staining with colloidal Coomassie blue. In partial gel images, including complex III core protein 1 and the neighboring proteins, intensities of spots representing complex III core protein 1 were quantified as a percentage of the total proteins present using ImageMaster 2D software. Statistical analyses were performed by Graphpad Instat2 software. Results are expressed as means $\pm$ SD. ** $P < 0.01$.

Protein levels of V-ATP synthase B and F subunits. We measured protein levels of V-ATP synthase B and F subunits to identify whether other ATP synthase protein levels were also decreased in AD and DS. As shown in table 5. The V-ATP synthase B subunit was significantly reduced in cerebellum from patients with DS, and V-ATP synthase F subunit was significantly reduced in frontal cortex from patients with DS (fig. 3). For V-ATP synthase B subunit, we measured the right spot because the left spot was not well separated.

Table 3. Complex V β chain levels in brain regions of patients with AD and DS and in controls.

	Control	AD	DS
Cerebellum	3.84 ± 1.08 (n = 7)	3.86 ± 1.46 (n = 4)	3.64 ± 0.76 (n = 5)
Frontal cortex	4.73 ± 1.24 (n = 4)	2.85 (n = 1)	2.71 ± 1.37* (n = 6)
Temporal cortex	3.64 ± 1.21 (n = 7)	3.05 ± 1.61 (n = 8)	3.14 ± 1.65 (n = 7)
Occipital cortex	3.37 ± 0.68 (n = 7)	3.18 ± 0.82 (n = 8)	2.35 ± 1.67 (n = 6)
Parietal cortex	3.99 ± 1.45 (n = 5)	5.25 ± 1.04 (n = 2)	3.05 ± 1.19 (n = 5)
Thalamus	3.73 ± 0.59 (n = 3)	2.04 (n = 1)	0.75 (n = 1)
Caudate nucleus	2.69 (n = 1)	1.34 (n = 1)	3.74 ± 2.36 (n = 2)

* $P < 0.05$.
Intensities of the spots representing complex V β chain were quantified and analyzed as described in table 2.

Discussion

The mitochondrial electron transport and oxidative phosphorylation system is composed of five protein-lipid complexes [7], and deficiencies in mitochondrial respiratory chain enzymes have been suggested to contribute to the progression of the pathology of neurodegenerative diseases. In AD brain, determination of electron transport chain activities revealed that defective activity was most marked for complex IV (cytochrome c oxidase or COX) activity [8]. In mitochondria from platelets of AD patients, complex IV activity was deficient and confirmed by analysis of postmortem brain tissue [20–22]. Complex IV activity was also significantly reduced in all hippocampal areas of AD patients but the protein levels of subunits II and IV did not differ between controls and AD [23]. An ATP synthase deficiency was reported in the hippocampus of brains from AD patients by blue native gel electrophoresis [10]. Activities of complex I, II, and IV were decreased in skeletal muscles biopsies of PD [24], and in HD, studies of platelet mitochondria also showed decreased complex I activity [25]. Deterioration of individual mitochondrial enzymes of energy metabolism could lead to deficient mitochondrial function and structure and may contribute to the abnormality of energy metabolism.

A consequence of defective mitochondrial energy production is the increased generation of free radicals (such as superoxide and hydroxyl radicals), which are normally produced as by-products of oxidative metabolism. These free radicals are able to oxidize lipids, proteins, nucleic acids, and other molecules, altering their structure and function. Oxidative stress due to increased free radicals is a common feature of neurodegenerative disease such as AD and DS [26, 27]. Mitochondria are themselves susceptible to damage by reactive oxygen species (ROS) [28]. Several studies have demonstrated an increase in oxidative DNA damage in AD and impairment in the ability to repair oxidative damage to mitochondrial DNA in DS [29–31]. DS neurons showed increased free radical production preceding neuronal death compared with controls [32].

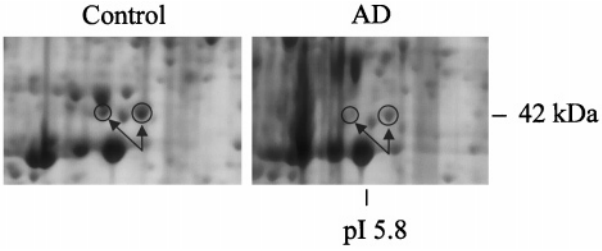

Figure 1. 2D gel images of complex III core protein 1 in temporal cortex. The human brain proteins were extracted and separated on an immobilized pH 3–10 nonlinear gradient strip, followed by a 9–16% linear gradient polyacrylamide gel. The gel was stained with Coomassie blue and the spots analyzed by MALDI-MS. Partial 2D gel images are shown; protein spots corresponding complex III core protein 1 are circled and arrowed.

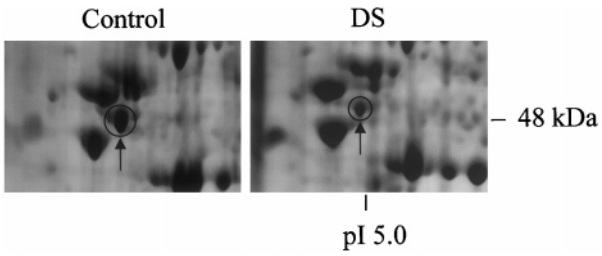

Figure 2. 2D gel images of complex V β chain in frontal cortex. Gels were prepared and the protein spots analyzed as described in the legend to figure 1. Partial 2D gel images are shown; a protein spot corresponding to complex V β chain is circled and arrowed.

Table 4. Mitochondrial aconitate hydratase levels in brain regions of patients with AD and DS and in controls.

	Control	AD	DS
Cerebellum	13.27 ± 4.28 (n = 8)	11.65 ± 2.46 (n = 6)	16.72 ± 6.00 (n = 5)
Frontal cortex	10.89 ± 3.51 (n = 6)	11.11 ± 2.12 (n = 6)	11.00 ± 2.60 (n = 6)
Temporal cortex	11.87 ± 5.40 (n = 9)	13.03 ± 3.86 (n = 10)	10.91 ± 3.45 (n = 7)
Occipital cortex	11.05 ± 3.02 (n = 8)	10.95 ± 2.87 (n = 9)	9.07 ± 3.01 (n = 8)
Parietal cortex	10.53 ± 3.12 (n = 6)	9.27 ± 2.92 (n = 4)	12.27 ± 3.87 (n = 6)
Thalamus	12.82 ± 2.85 (n = 8)	12.38 ± 2.43 (n = 7)	10.55 ± 2.77 (n = 6)
Caudate nucleus	11.62 ± 6.34 (n = 5)	8.82 ± 2.84 (n = 7)	9.65 ± 1.51 (n = 5)

Intensities of spots representing mitochondrial aconitate hydratase were quantified and analyzed as described in table 2.

Table 5. V-ATP synthase B and F subunit levels in brain regions of patients with AD and DS and in controls.

	Control	AD	DS
V-ATP synthase B subunit			
Cerebellum	2.62 ± 1.65 (n = 9)	1.61 ± 0.63 (n = 8)	1.08 ± 0.43** (n = 6)
Frontal cortex	2.71 ± 1.22 (n = 9)	1.99 ± 1.15 (n = 7)	2.99 ± 1.31 (n = 8)
Temporal cortex	2.00 ± 1.45 (n = 13)	1.67 ± 0.90 (n = 9)	2.41 ± 1.91 (n = 9)
Occipital cortex	2.02 ± 1.25 (n = 11)	2.42 ± 1.03 (n = 10)	2.20 ± 0.74 (n = 8)
Parietal cortex	2.98 ± 1.40 (n = 7)	3.75 ± 2.07 (n = 5)	2.64 ± 1.47 (n = 7)
Thalamus	2.04 ± 1.27 (n = 10)	2.35 ± 1.15 (n = 7)	2.36 ± 1.40 (n = 7)
Caudate nucleus	2.40 ± 1.29 (n = 9)	2.73 ± 1.58 (n = 7)	2.37 ± 1.03 (n = 8)
V-ATP synthase F subunit			
Cerebellum	1.99 ± 0.61 (n = 4)	1.90 ± 0.76 (n = 9)	1.52 ± 0.79 (n = 6)
Frontal cortex	4.14 ± 1.21 (n = 8)	3.38 ± 0.71 (n = 5)	2.30 ± 1.31* (n = 4)
Temporal cortex	4.15 ± 1.69 (n = 9)	6.32 ± 4.61 (n = 8)	4.53 ± 1.30 (n = 8)
Occipital cortex	3.73 ± 1.70 (n = 13)	3.12 ± 1.28 (n = 9)	4.46 ± 2.32 (n = 8)
Parietal cortex	3.10 ± 2.82 (n = 6)	2.34 ± 1.47 (n = 6)	3.15 ± 2.08 (n = 5)
Thalamus	2.99 ± 1.21 (n = 8)	3.31 ± 0.83 (n = 7)	3.67 ± 2.05 (n = 7)
Caudate nucleus	3.02 ± 1.45 (n = 7)	3.44 ± 1.91 (n = 7)	3.65 ± 1.74 (n = 7)

Intensities of spots representing V-ATP synthase B and F subunits were quantified and analyzed as described in table 2. ** $P<0.01$; * $P<0.05$.

Direct evidence for increased oxidative stress in DS was provided by the finding of significantly elevated levels of 8-hydroxy-2′-deoxyguanosine, a biomarker of oxidative damage to DNA, and malondialdehyde, a biomarker of lipid peroxidation, evaluated by the chemical analyses of urine samples [33]. Increased lipid peroxidation has also repeatedly been shown in brain of AD patients [34–36]. Although up-regulating antioxidant defense enzymes such as superoxide dismutase, catalase, and glutathione reductase can detoxify free radicals and products of oxidative metabolism, they are not completely effective in preventing the increasing oxidative damage that can induce programmed neuronal cell death (apoptosis) [37, 38]. A defect in energy production could also result in an increase in neuron vulnerability to excitatory amino acids, leading to neuronal degeneration by a slow excitotoxic process [5].

Recent research using specific mitochondrial respiratory chain inhibitors has provided additional clues for mitochondrial deficiencies and apoptosis. The mitochondrial inhibitors rotenone (complex I), antimycin A (complex III), and oligomycin (complex V) induced an apoptotic suicide response in cultured human lymphoblastoid and other mammalian cells, but apoptosis induced by respiratory chain inhibitors was not inhibited by the presence of Bcl-2 [39]. The complex I inhibitor, 1-methyl-4-phenyl-pyridinium (MPP^+), could induce apoptosis in PC12 cells and cerebellar granule cells [40, 41]. In PD cytoplasmic hybrids (cybrids), MPP^+ could also induce a decrement in complex I activity, increased oxygen radical production, and apoptotic cell death [42]. The physiological consequences of partial complex I inhibition at the cellular level were studied using the cybrid technique [43]. When complex I was inhibited, cells were depleted of ATP, and ROS production was enhanced, damaging mitochondrial membranes to result in a slight membrane depolarization and permeability transition. These mitochondrial alterations are the starting point of a cascade of events promoting apoptosis.

In previous work, we reported deteriorated mRNA levels of NADH3 (complex I) in cerebellum of patients with DS using differential display polymerase chain reaction [44]. We have also identified significantly reduced protein levels of complex I 24- and 75-kDa sub-

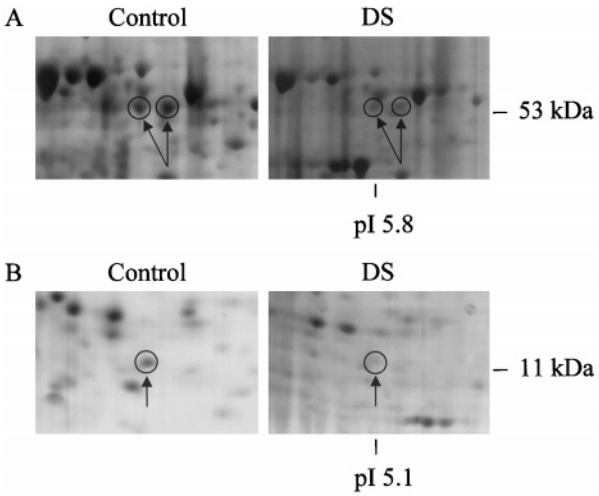

Figure 3. 2D gel images of V-ATP synthase B and F subunits in significant brain regions. Gels were prepared and protein spots analyzed as described in the legend to figure 1. (*A*) The protein spot corresponding to V-ATP synthase B subunit in the cerebellum is indicated by circles and arrows. (*B*) The protein spot corresponding V-ATP synthase F subunit in frontal cortex is indicated by a circle and arrow.

units in human brain of patients with DS and AD compared to controls, using the proteomic approach (unpublished data). Here, we report significantly decreased protein levels of complex III core protein 1 in AD (temporal cortex) and of complex V β chain in DS (frontal cortex). In other regions, there was no significant difference between controls and either DS or AD in the levels of these proteins (table 3). In this study, reduction of mitochondrial electron transport chain enzymes rather than simply mitochondrial loss is proposed because protein levels of mitochondrial aconitate hydratase were unchanged (table 4).

Oxidative stress by mitochondrial damage has been implicated in the degeneration of both synapses and neurons in neurodegenerative diseases. In previous work, we observed decreased levels of synaptosomal associated protein 25 (snap-25) in the brain of patients with AD and DS [45]. In addition, in a comparable cohort, protein levels of synaptosomal proteins β-soluble N-ethylmaleimide-sensitive factor attachment protein in temporal cortex of AD and DS, and synaptotagmin I in temporal cortex of AD were decreased [unpublished data], i.e., in brain regions where complex III or V defects were found.

In addition, we also identified significantly decreased protein levels of the V-ATP synthase B subunit in cerebellum of DS patient and of V-ATP synthase F subunit in frontal cortex (where numerous degenerative changes and decreased protein levels of complex V β chain were observed) of DS patients. A recent study showed that the specific inhibitors of V-ATP synthases, concanamycin A and bafilomycin A1, could induce apoptotic cell death [46]. Concanamycin A triggered apoptotic cell death of osteoclasts and induced DNA fragmentation in B cell hybridoma HS-72 cells [47, 48]. Bafilomycin A1 could also induce apoptosis in the human pancreatic cancer cell line, Capan-1 [49]. Based on the literature and our results, we suggest that a functional impairment due to reduction in the V-ATP synthase subunit might be associated with neuronal cell death.

In conclusion, deficient mitochondrial respiratory enzymes may contribute to mitochondrial dysfunction in neurodegeneration, which may in turn induce apoptotic neuronal cell death in DS as well as AD [5, 50]. Furthermore, from a clinical perspective, decreased subunit levels of complex V and V-ATP synthase may reflect the abnormal energy metabolism and reduced resting metabolic rate observed in DS [51, 52].

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