J Neuropathol Exp Neurol
Vol. 75, No. 7, July 2016, pp. 663–672
doi: 10.1093/jnen/nlw037

OXFORD

ORIGINAL ARTICLE

Decreased Coenzyme Q10 Levels in Multiple System Atrophy Cerebellum

Emanuele Barca, MD, Giulio Kleiner, PhD, Guomei Tang, PhD, Marcello Ziosi, PhD, Saba Tadesse, MS, Eliezer Masliah, MD, Elan D. Louis, MD, MS, Phyllis Faust, MD, PhD, Un J Kang, MD, Jose Torres, MD, Etty P. Cortes, MD, Jean-Paul G. Vonsattel, MD, Sheng-Han Kuo, MD, and Catarina M. Quinzii, MD

Abstract

In familial and sporadic multiple system atrophy (MSA) patients, deficiency of coenzyme Q_{10} (CoQ_{10}) has been associated with mutations in *COQ2*, which encodes the second enzyme in the CoQ_{10} biosynthetic pathway. Cerebellar ataxia is the most common presentation of CoQ_{10} deficiency, suggesting that the cerebellum might be selectively vulnerable to low levels of CoQ_{10}. To investigate whether CoQ_{10} deficiency represents a common feature in the brains of MSA patients independent of the presence of *COQ2* mutations, we studied CoQ_{10} levels in postmortem brains of 12 MSA, 9 Parkinson disease (PD), 9 essential tremor (ET) patients, and 12 controls. We also assessed mitochondrial respiratory chain enzyme activities, oxidative stress, mitochondrial mass, and levels of enzymes involved in CoQ biosynthesis. Our studies revealed CoQ_{10} deficiency in MSA cerebellum, which was associated with impaired CoQ biosynthesis and increased oxidative stress in the absence of *COQ2* mutations. The levels of CoQ_{10} in the cerebella of ET and PD patients were comparable or higher than in controls. These findings suggest that CoQ_{10} deficiency may contribute to the pathogenesis of MSA. Because no disease modifying therapies are currently available, increasing CoQ_{10} levels by supplementation or upregulation of its biosynthesis may represent a novel treatment strategy for MSA patients.

Key Words: Cerebellar ataxia, Coenzyme Q10, Multiple system atrophy, Oxidative stress.

From the Department of Neurology, College of Physicians and Surgeons, Columbia University, New York (EB, GK, GT, MZ, ST, UJK, S-HK, CMQ); UOC of Neurology and Neuromuscular Disorders, Department of Clinical and Experimental Medicine, University of Messina, Messina, Italy (EB); Department of Neuroscience and Pathology, University of California, San Diego, California (EM); Department of Neurology, Yale School of Medicine, Yale University, New Haven, Connecticut (EDL); Department of Chronic Disease Epidemiology, Yale School of Public Health, Yale University, New Haven, Connecticut (EDL); Center for Neuroepidemiology and Clinical Neurological Research, Yale School of Medicine, Yale University, New Haven, Connecticut (EDL); Department of Pathology and Cell Biology, Columbia University Medical Center and the New York Presbyterian Hospital, New York (PF, JT, EPC, J-PGV); and Taub Institute for Research on Alzheimer's Disease and the Aging Brain, Department of Pathology and Cell Biology, College of Physicians and Surgeons, Columbia University, New York (JT, EPC, J-PGV).

Send correspondence to: Sheng-Han Kuo, MD, Department of Neurology, College of Physicians and Surgeons, Columbia University, New York, NY 10032; E-mail: sk3295@columbia.edu

Authors Emanuele Barca, Giulio Kleiner, Sheng-Han Kuo and Catarina M. Quinzii contributed equally to this work.

Guomei Tang has received research support from the National Institutes of Health: NIMH K01 MH096956. Elan D. Louis has received research support from the National Institutes of Health: NINDS R01 NS042859 (principal investigator), NINDS R01 NS39422 (principal investigator), NINDS R01 NS086736 (principal investigator), NINDS R01 NS073872 (principal investigator), NINDS R01 NS085136 (principal investigator), and NINDS R01 NS088257 (principal investigator). Sheng-Han Kuo is supported by American Brain Foundation Research Training Fellowship, Parkinson Disease Foundation, NINDS K08 NS083738, Louis V. Gerstner Jr. Scholarship, American Parkinson's Disease Association, International Essential Tremor Foundation. Catarina M Quinzii is supported by NIH P01 HD080642-01, Muscle Dystrophy Association (MDA), and Department of Defense (DOD).

The authors have no duality or conflicts of interest to declare.

Supplementary Data can be found at http://www.jnen.oxfordjournals.org.

INTRODUCTION

Multiple system atrophy (MSA) is a late-onset, sporadic neurodegenerative disorder clinically characterized by autonomic failure, parkinsonism, and cerebellar ataxia (1). Recently, *COQ2* mutations have been described in patients with familial and sporadic MSA in Japan (2). *COQ2* encodes 4-para-hydroxybenzoate: polyprenyl transferase, the second enzyme in the biosynthetic pathway of Coenzyme Q_{10} (CoQ_{10}), a potent antioxidant and electron carrier in the mitochondrial respiratory chain. CoQ_{10} deficiency can lead to various clinical manifestations, with cerebellar ataxia being the most common presentation. This suggests that the cerebellum might be selectively vulnerable to low levels of CoQ_{10} (3). The association between *COQ2* and MSA has been independently confirmed in a Taiwanese cohort (MSA-C) (4). Moreover, Ogaki et al (6) identified 1 heterozygous carrier of a known loss of function p.S146N substitution in a severe MSA-C pathologically confirmed patient, and Schottlaender et al (7) reported 1 heterozygous p.R197H carrier in 300 pathologically confirmed MSA cases. However, several other groups did not find the association between MSA and *COQ2* variants. This may be due to differences in ethnic backgrounds of the study populations because MSA-C is the predominant type in

© 2016 American Association of Neuropathologists, Inc. All rights reserved.

Downloaded from https://academic.oup.com/jnen/article/75/7/663/2579780 by guest on 25 April 2024

East Asia but not in other geographical areas (4–11). Whether CoQ_{10} deficiency is present in MSA brains, independent of the presence of *COQ2* mutations, and whether it plays a role in the pathogenesis of the disease, has not been investigated. Cerebellar ataxia associated with CoQ_{10} deficiency can be primary, if it is associated with mutations in genes encoding enzymes involved in the biosynthesis of CoQ or its regulation, or secondary to molecular defects in genes encoding proteins unrelated to CoQ biosynthesis and/or regulation (12, 13). Therefore, we hypothesize that MSA is part of a broad clinical spectrum associated with CoQ_{10} deficiency. Here, we provide evidence of CoQ_{10} deficiency and impaired CoQ biosynthetic pathway in postmortem cerebellar samples from pathologically confirmed MSA cases without *COQ2* mutations.

MATERIALS AND METHODS

Samples

We conducted a case-control study to compare CoQ_{10} levels in postmortem MSA brains versus age-matched control brains. We collected 12 pathologically confirmed MSA cases from the New York Brain Bank, available at Columbia University Medical Center (14). All MSA cases had characteristic glial cytoplasmic inclusions (GCIs) (15). Of the 12 MSA cases, 7 were pathologically diagnosed with olivopontocerebellar atrophy, 3 with striatonigral degeneration, and 2 with mixed olivopontocerebellar atrophy and striatonigral degeneration pathology (14). We collected 12 age-matched controls from the New York Brain Bank. The control brains were from individuals followed at the Alzheimer's disease Research Center, and the Washington Heights Inwood Columbia Aging Project (WHICAP) at Columbia University. Those individuals were followed prospectively with serial neurological examinations, and showed no clinical signs of Alzheimer disease, essential tremor (ET), Parkinson disease (PD), Lewy body dementia, MSA, or progressive supranuclear palsy. From the same brain bank we also collected 9 PD cases and 9 ET cases as disease controls. The diagnosis of PD was made by the movement disorders specialists at Columbia University during life and was pathologically confirmed by the presence of Lewy bodies in the substantia nigra (16). The diagnosis of ET was made by the treating neurologist during life and confirmed by an Essential Tremor Centralized Brain Repository (ETCBR) study neurologist (Elan D. Louis) using medical records, detailed videotaped neurological examinations, and ETCBR diagnostic criteria (17, 18). All disease and control brains were processed under the same protocol in the New York Brain Bank with standardized neuropathological assessment (19). Demographic data for the samples used in specific assays are shown in Tables 1–3.

CoQ_{10} Measurement

CoQ_{10} was extracted from brain homogenate by mixing tissue extracts with 1 − propanol. After 2-minute vortex, the suspension was centrifuged at 15 700 rcf for 5 minutes. The resultant supernatant containing the lipid extract was injected in a high-performance liquid chromatography system and the results were expressed in ng CoQ_{10}/mg protein (20).

Mitochondrial Respiratory Chain Enzymes Activities

CoQ_{10}-dependent respiratory chain activities (NADH cytochrome c reductase and succinate cytochrome c reductase [complexes I + III and II + III]) were measured spectrophotometrically in tissue homogenates (21). All following reactions were measured at 30°C. Complex I + III activity was measured in the presence of 10 mM potassium cyanide, 2 mM NADH, and 1 mM cytochrome c, as the rotenone-sensitive reduction of cytochrome c assessed at 550 nm. The results were expressed in nmol reduced cyt c/min/mg protein. Complex II + III activity was measured in the presence of 10 mM KCN and 30 mM succinate in KH_2PO_4 buffer (pH 7.5). The reaction was initiated by addition of 1 mM cytochrome c and the decrease in absorbance was monitored at 550 nm. The results were expressed in nmol reduced cyt c/min/mg protein. Cytochrome c oxidase (COX or complex IV) activity was measured following the oxidation of cyt c catalyzed by COX. Samples were incubated in medium containing 0.01 mM KH_2PO_4 (pH7.0) and 1% reduced cyt c, and the reaction followed for 3 minutes at 550 nm. The results were expressed in nmol oxidized cyt c/min/mg protein. Citrate synthase activity was measured following the reduction of 1 mM 5,5′-dithiobis (2-nitrobenzoic acid) in 1M Tris–HCl (pH 8.1) at 412 nm for 2 minutes in the presence of 10 mM acetyl-CoA, and 10 mM oxalacetic acid. Citrate synthase activity was expressed as nmol/min/mg protein.

TABLE 1. Demographic Data and Cerebellar Coenzyme Q_{10} Levels

	Controls	MSA Cases	PD Cases	ET Cases	p value
N	12	12	9	9	
Gender (Male/Female)	7/5	5/7	2/7	4/5	0.18[a]
Age (years)	65.4 ± 2.8	63.8 ± 2.3	76.7 ± 1.2	82.6 ± 1.7	< 0.001[b]
Postmortem interval (hours)	7.9 ± 6.8	3.9 ± 3.4	3.6 ± 3.5	2.4 ± 1.5	0.039[b]
CoQ_{10} levels	113.1 ± 7.16	68.1 ± 10.03	132.2 ± 8.47	176.3 ± 22.92	< 0.0001[b]

MSA, multiple system atrophy; PD, Parkinson disease; ET, essential tremor.
Mean ± SE and frequency (%) are reported.
[a]Chi-square test.
[b]Analysis of variance.

Downloaded from https://academic.oup.com/jnen/article/75/7/663/2579780 by guest on 25 April 2024

Oxidative Stress Analysis

Oxidative damage was assessed in cerebellum and striatum from MSA patients and age-matched controls (6 vs. 4 and 5 vs. 4, respectively, Table 3) by immunohistochemistry using 2 assays specific for lipid peroxidation: 4-hydroxynonenal (4-HNE) and DNA oxidative damage 8-hydroxy-2′-deoxyguanosine (8-OHdG), respectively. Briefly, 7-μm-thick paraffin-embedded cerebellar tissues slides were deparaffinized, rehydrated, rinsed in PBS, and blocked with 3% hydrogen peroxide in methanol. For antigen retrieval, slides for lipid peroxidation staining were placed in 0.1 M sodium citrate buffer (pH 6.0) and heated in a microwave oven for 15 minutes; slides for DNA damage staining were treated with 10 μg/ml proteinase K in PBS at 37°C for 40 minutes, according to the manufacturer's instructions. All sections were then incubated with donkey serum (D9663; Sigma-Aldrich, Saint Louis, MO) for 1 hour at room temperature, followed by incubation with anti-4 hydroxynonenal antiserum (1:1000, HNE11-S; Alpha Diagnostic International Inc., San Antonio, TX) or anti-8-hydroxy-2′-deoxyguanosine (1:200, AB5830; Millipore, Billerica, MA) overnight at 4°C. Sections were subsequently rinsed in PBS, and incubated with species-specific, biotinylated secondary antibody (1:100, GE Healthcare Life Sciences, Pittsburgh, PA) for 1 hour at room temperature. Immunoreactivity was detected by avidin-biotin complex with 3,3′-diaminobenzidine (DAB) substrates (Vectastain ABC Kit, Vector Laboratories, Burlingame, CA). All slides for 4-HNE and 8-OHdG immunohistochemistry were stained and developed at the same time. Controls consisting of omission of the primary antibodies were performed for the antibodies employed. Slides were examined by light microscopy using an Olympus BX51 microscope (Olympus, Tokyo, Japan) and images were captured with a QImaging Retiga EXi digital camera, using QCapture software version 2.68.6. Images were acquired at 20× or 40× magnification and processed by NIH ImageJ 1.50e software. All morphological analyses were done using a blinded code, which did not allow distinguishing between MSA cases and controls.

The numbers of positive dark stained Purkinje cells or striatal neurons were determined from analyzing 5 randomly chosen different areas per sample with a 40× lens. The function "color deconvolution, H DAB" was used to unmix the RGB image into separate channels. On the new panel corresponding to DAB color, we measured the mean gray level of background and region of interest (ROI). The intensity numbers were then converted to optical density (OD) with the following formula: OD = log (max intensity/mean intensity), where max intensity = 255 in an 8-bit image. The values of the ROIs were referred as percentage of background, considered as 100%. Cells with ROIs ≥140% were considered positive. OD readings were significantly below the ROI OD readings used as criteria accepted as "dark neurons". The numbers of positive Purkinje cells or striatal neurons were normalized to the total number of the respective cells in 5 randomly chosen fields (22).

To assess whether 4-HNE staining colocalizes with α-synuclein aggregates, we performed double anti-4-HNE/α-synuclein staining of representative MSA and control cerebellum samples. Slides were deparaffinized, rehydrated, and rinsed in PBS. For antigen retrieval, slides were placed in 0.1 M sodium citrate buffer (pH 6.0) and heated in a microwave oven for 15 minutes. Slides for DNA damage staining were

TABLE 2. Demographic Data and Striatal and Occipital Cortical Coenzyme Q_{10} Levels

	Striatum			Occipital Cortex		
	MSA Cases	Controls	p value	MSA Cases	Controls	p value
N	7	7		9	10	
Gender (Male/Female)	4/3	6/1	0.56[a]	2/7	4/5	0.31[a]
Age (years)	70.3 ± 2.03	70.1 ± 2.2	0.96 [b]	69.7 ± 1.65	69.6 ± 2.72	0.98 [b]
Postmortem interval (hours)	9.4 ± 4.0 [median= 295]	8.0 ± 5.9	0.24 [c]	8.6 ± 4.4	7.2 ± 5.3 [median=5.2]	0.22 [c]
CoQ_{10} levels	244.2 ± 27.2	230.8 ± 28.6	0.74 [b]	277.1 ± 29.7	267.3 ± 21.8	0.79 [b]

MSA, multiple system atrophy.
Mean ± SE, [median], and frequency (%) are reported.
[a]Fisher exact test;
[b]Independent t-test;.
[c]Mann-Whitney test.

TABLE 3. Demographic Data of Controls and Multiple System Atrophy Cases Used for Analyses of Oxidative Stress Markers

	Cerebellum			Striatum		
	Controls	MSA Cases	p value	Controls	MSA Cases	p value
N	4	6		6	6	
Gender (Male/Female)	2/2	2/4	1.00[a]	4/2	3/3	1.00[a]
Age (years)	65.5 ± 6.6	68.2 ± 1.5	0.96[b]	67.8 ± 13.8	69.3 ± 8.3	0.82[b]
Postmortem interval (hours ±)	5.8 ± 8.8	4.4 ± 3.2	0.74[b]	3.1 ± 4.1	2.6 ± 2.4	0.88[b]

MSA, multiple system atrophy.
[a]Fisher exact test;
[b]Independent t-test.

Downloaded from https://academic.oup.com/jnen/article/75/7/663/2579780 by guest on 25 April 2024

subsequently treated with 10 μg/ml proteinase K in PBS at room temperature. All slides were blocked with horse serum for 25 minutes followed by a 1.5-hour incubation at room temperature with primary antibody anti α-synuclein (1:100, 610786; BD Bioscience, San Jose, CA), and then incubated with horse anti-mouse (1:200, BA-2000; Vector Laboratories) and with streptavidin Alexa Fluor 488 (1:300, S32354; Thermo Fisher Scientific, Waltham, MA). Half of the slides were blocked for 25 minutes with donkey serum, incubated overnight at 4°C with anti 4-HNE (1:100) and with donkey anti rabbit Alexa Fluor 594 (1:4000, A-11037; Thermo Fisher Scientific) for 30 minutes at room temperature. The other half were blocked for 25 minutes with rabbit serum, incubated overnight at 4°C with anti 8-OHdG (1:400), with biotinylated rabbit anti goat (1:200, BA-5000; Vector Laboratories), and with streptavidin Alexa Fluor 594 (1:4000, S11227; Thermo Fisher Scientific) for 30 minutes at room temperature. Slides were then covered with DAPI (H-1200; Vector Laboratories). Single Z stacks were acquired with Leica SP5 confocal microscope and entire images were elaborated with ImageJ public software (NIH) and Photoshop CS5 (Adobe).

COQ2 Molecular Analysis

DNA was extracted from cerebellar tissues of 12 MSA patients and 12 controls. Mutational analysis was performed by PCR followed by direct Sanger sequencing of coding exons and flanking intronic regions of human *COQ2* (NM_015697.7). PCR products were purified on Montage PCR96 Cleanup Plates (Millipore, Billerica, MA) and used in sequencing reaction with ABI BigDye Terminator Kit (Applied Biosystems, Foster City, CA). Primers and PCR conditions have been described by Emmanuele et al (3).

Western Blot Analyses of COQ Biosynthetic Proteins and TOM20

To assess steady state levels of proteins involved in CoQ_{10} biosynthetic pathway, approximately 40 μg of frozen cerebellar tissues from 12 MSA cases and 12 controls were homogenized on ice in 10 mM Tris-HCl buffer (pH 7.4) containing sucrose. The homogenates were centrifuged at 1000 rcf for 10 minutes at 4°C and the supernatant was collected and centrifuged at 17 000 rcf for 45 minutes. Thirty micrograms of protein were mixed with 4× LDS sample buffer and 25% DDT. After denaturation at 55°C for 5 minutes, samples were loaded in 10%–20% SDS-PAGE gels. Proteins were transferred on a methanol activated PDVF membrane and blocked with 5% nonfat milk at room temperature for 1 hour. Membranes were incubated at 4°C overnight with primary antibodies, anti-PDSS1 (1:100, AV46195, Aviva, San Diego, CA), anti-COQ2 (1:200, ab89706, Abcam, Cambridge, MA), anti-COQ7 (1:200, PA5-25774, Thermo Scientific Fisher), anti-COQ5 (1:200, PA5-26327, Thermo Scientific Fisher), anti-ADCK3 (1:100, H00056997-M04A, Abnova, Taipei City, Taiwan), and anti-TOM20 (1:500, sc-11415, Santa Cruz Biotechnology Inc., Santa Cruz, CA), a mitochondrial outer membrane protein, used as index of mitochondrial mass, and anti-β actin (1:1000, A1978, Sigma-Aldrich), used as loading control. Protein–antibody interaction was detected by peroxidase-conjugated antibodies, using ECL Prime Western Blotting detection reagents (GE Healthcare Life Sciences). Quantification of proteins was carried out using NIH ImageJ 1.50e software. Average gray value was calculated within selected areas as the sum of the gray values of all the pixels in the selection divided by the number of pixels. No postprocessing modifications of the gels were made prior to quantification.

Statistical Analyses

Demographic and clinical characteristics, and cerebellar CoQ_{10} levels in controls, MSA, PD, and ET cases were all normally distributed (Kolmogorov-Smirnov test) and thus were analyzed using analysis of variance (ANOVA) followed by Tukey *post hoc* analysis. We additionally constructed a linear regression model for the cerebellar CoQ_{10} levels in different diagnostic groups and adjusted for age, gender, and postmortem interval (PMI). To compare striatal and occipital cortical CoQ_{10} levels, CoQ biosynthetic pathway proteins levels, and TOM20, between MSA and controls, we used independent t-test. The results of oxidative stress markers from immunohistochemical assays in MSA cases and controls were not normally distributed; therefore, we used nonparametric Mann-Whitney test. Correlation analyses were performed using Pearson's correlation coefficient. Statistical significance was assumed for $p < 0.05$. Data are expressed as mean ± SE.

RESULTS

CoQ_{10} Measurements

Cerebellar CoQ_{10} levels were measured in: 12 MSA cases, 9 PD cases, and 9 ET cases, and 12 controls. These 4 groups showed no differences in gender ($p = 0.18$) but differ in age of death ($p < 0.001$) and Post-mortem interval (PMI) ($p = 0.039$). Age of death was similar between controls and MSA cases, whereas PD cases and ET cases were significantly older than the other 2 groups (Tukey *post hoc* analysis, $p < 0.01$). In addition, PMI was not significantly different between controls and MSA cases or PD cases but significantly higher in controls when compared to ET cases (Tukey *post hoc* analysis, $p = 0.042$) (Table 1). Cerebellar CoQ_{10} levels in the control group did not correlate with either age of death or with PMI (For age of death: Pearson's $r = 0.14$, $p = 0.67$; for PMI: $r = 0.006$, $p = 0.99$).

CoQ_{10} levels were significantly different among 4 groups (ANOVA, $p < 0.0001$). MSA cerebellum had a 40% decrease in CoQ_{10} levels versus control cerebellum (MSA = 68.1 ± 10.03 nmol CoQ_{10}/mg protein, control = 113.1 ± 7.16, $p = 0.047$). Levels of CoQ_{10} in PD cerebellum (132.2 ± 8.47 nmol CoQ_{10}/mg protein, $p = 0.71$) were not significantly different from levels in control cerebellum; however, ET cerebellum had significantly greater CoQ_{10} levels (176.3 ± 22.92 nmol CoQ_{10}/mg protein, $p = 0.006$) versus controls (Fig. 1a, b). Cerebellar CoQ_{10} levels still remained significant between MSA cases and controls in a linear regression model adjusting for age, gender, and PMI ($\beta = -47.53$ nmol CoQ_{10}/mg protein, $p = 0.016$). Within MSA cases we did not observe

Downloaded from https://academic.oup.com/jnen/article/75/7/663/2579780 by guest on 25 April 2024

significant differences in CoQ_{10} levels between patients with MSA-olivopontocerebellar atrophy, MSA-striatonigral degeneration, or MSA-olivopontocerebellar atrophy and striatonigral degeneration pathology (data not shown).

Striatum is another brain region that is histologically affected whereas the occipital cortex is relative spared in MSA (5, 15). Therefore, we investigated whether these brain areas had altered CoQ_{10} levels in MSA cases. We analyzed striatum of 7 MSA cases and 7 age-matched controls, and occipital cortex of 10 MSA cases and 9 age-matched controls (Table 2). CoQ_{10} levels in both striatal and the occipital cortical samples were similar in MSA cases and controls (occipital cortical CoQ_{10} levels: 277.1 ± 29.73 nmol CoQ_{10}/mg protein in MSA cases vs. 267.3 ± 21.88 in controls, $p = 0.79$; striatal CoQ10 levels: 244.2 ± 27.16 nmol CoQ10/mg protein in MSA cases vs.230.8 ± 28.62 in controls, $p = 0.74$) (Fig. 1c, d).

Mitochondrial Respiratory Chain Enzymes Activities and Mitochondrial Mass

To assess the effects of CoQ_{10} deficiency on the mitochondrial bioenergetics, we measured activities of CoQ-dependent respiratory enzymes, complexes I + II and II + III, which showed no differences between MSA cases and control cerebellar samples (Fig. 2a). To exclude the possibility that low CoQ_{10} in MSA cerebellum was due to the marked neuronal loss and degenerative changes relative to controls, we measured the activity of citrate synthase (a mitochondrial matrix enzyme), and the level of TOM20 (a mitochondrial outer membrane protein), indices of mitochondrial mass. We also assessed COX activity, a non-CoQ_{10}-dependent mitochondrial respiratory enzyme. Citrate synthase and COX activities as well as TOM20 levels were comparable in MSA cases and controls (Fig. 2b). Therefore, CoQ_{10} levels in MSA cerebellum were significantly reduced compared with controls also when normalized to citrate synthase (17.26 ± 2.87 vs. 36.66 ± 7.46; $p = 0.030$). These data indicated that CoQ_{10} reduction was not a consequence of mitochondrial mass reduction in MSA cerebellum.

We assessed mitochondrial respiratory chain and mitochondrial mass also in the striatum. As expected, complexes I + III and II + III activity, and citrate synthase were comparable between MSA patients and controls (Supplementary Data Fig. 1a); however, we observed a slight but significant increase in

Downloaded from https://academic.oup.com/jnen/article/75/7/663/2579780 by guest on 25 April 2024

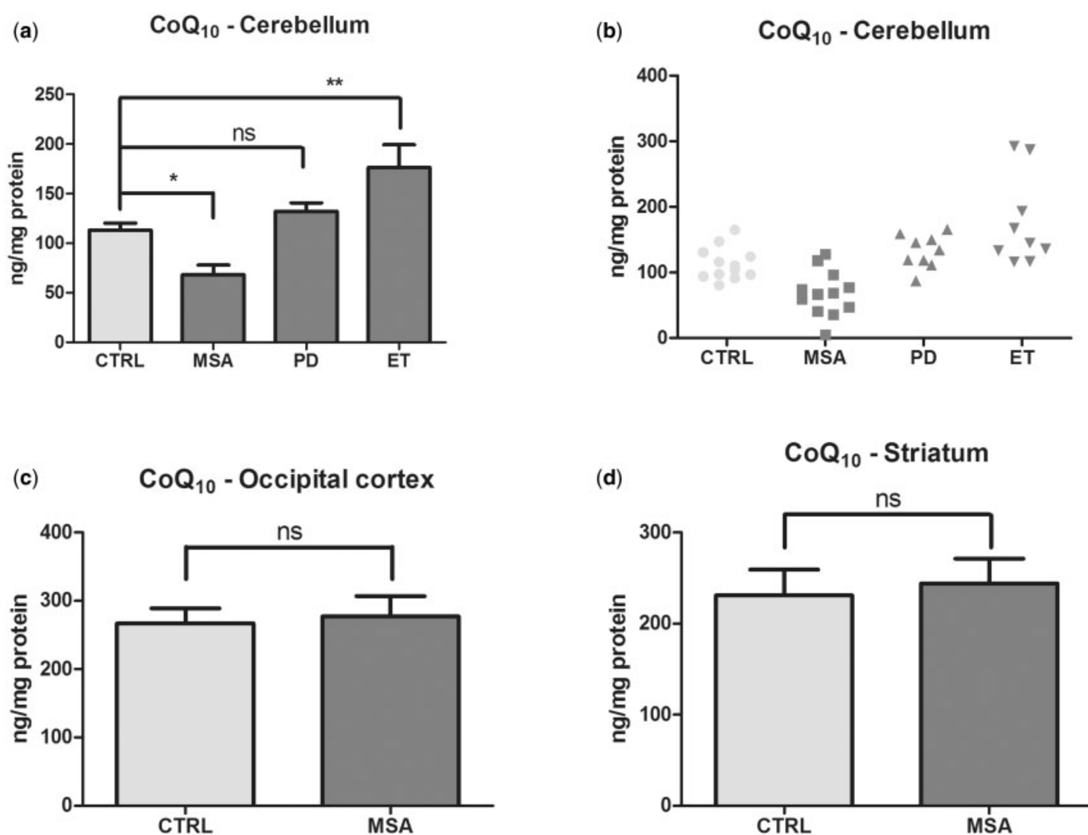

FIGURE 1. CoQ_{10} levels in different brain regions. **(a, b)** CoQ_{10} is significantly reduced in the cerebellum of multiple system atrophy ([MSA] n = 12) versus control ([CTRL] n = 12) patients, but not in Parkinson disease ([PD] n = 9) patients. Essential tremor ([ET] n = 9) patient samples had increased CoQ_{10} levels versus controls. **(c, d)** CoQ_{10} levels in MSA occipital cortex (n = 9) **(c)** and striatum (n = 7) **(d)** are comparable to those in control occipital cortex (n = 10) and striatum (n = 7). *$p < 0.05$; **$p < 0.01$; ns, not significant.

COX activity of MSA patients. On the contrary, TOM20 was reduced in MSA striatal samples (Supplementary Data Fig. 1b).

Oxidative Stress Analyses

Because CoQ_{10} acts as an antioxidant, we assessed oxidative stress as a possible consequence of CoQ_{10} deficiency in MSA samples. Specific staining against oxidized membrane lipids revealed that a subset of Purkinje cells had increased signals for 4-HNE (Fig. 3a). We quantified the percentage of Purkinje cells positive for 4-HNE staining and found that in MSA cases the percentage of 4-HNE-positive Purkinje cells was almost 4-fold higher than in controls (88 ± 9.15% vs. 22.9 ± 13.8%; $p = 0.049$) (Fig. 3b). Also, the proportion of Purkinje cells positive for 8-OHdG was higher in MSA cases than controls although the difference was not significant

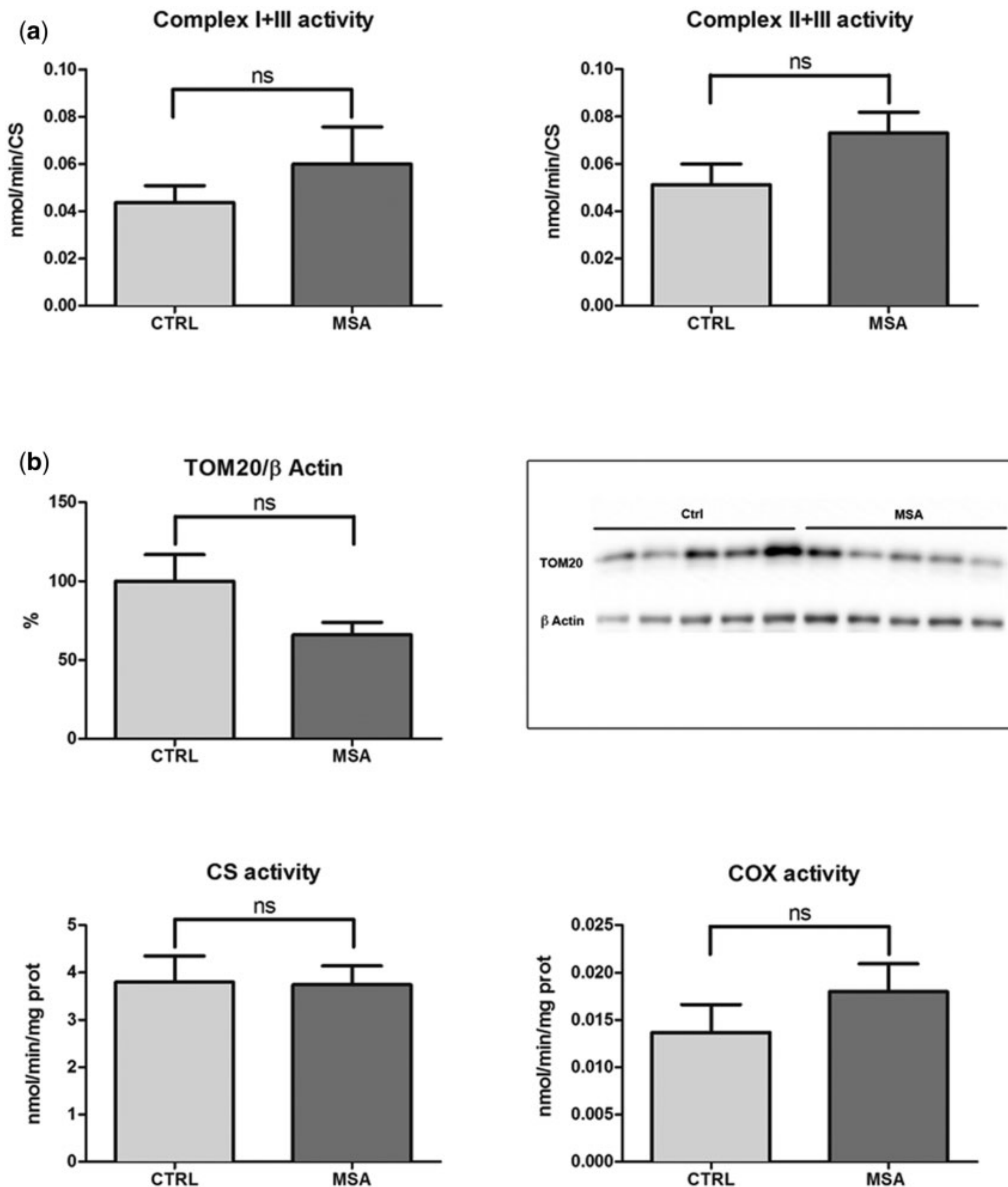

FIGURE 2. Cerebellum. **(a)** Activities of CoQ-dependent respiratory chain enzymes, NADH cytochrome c reductase and succinate cytochrome c reductase (complexes I + III and II + III), in samples from MSA patients are not different from those in control samples. **(b)** Mitochondrial mass: measurements of TOM20 levels, citrate synthase (CS) activity, indices of mitochondrial mass, and cytochrome c oxidase (COX, complex IV) activity showed no difference between MSA brain and control brain samples. A representative Western blot with 5 controls and 5 MSA cases is shown. CTRL, controls, n = 12; MSA, multiple system atrophy, n = 12; ns, not significant.

Downloaded from https://academic.oup.com/jnen/article/75/7/663/2579780 by guest on 25 April 2024

(17.1 ± 8.98% vs. 2.08 ± 2.08%, p = 0.33) (Fig. 3b). We did not detect any significant staining in the omitted primary antibody control samples (Supplementary Data Fig. 2). Oxidative stress was assessed also in striatum, a region affected by MSA but with normal CoQ10 levels. Both 4-HNE and 8-OHdG immunostaining were not different in MSA versus control samples (Supplementary Data Fig. 3).

To verify that lipid oxidation was independent of α-synuclein inclusions in Purkinje cells, double immunofluorescence staining was performed. Immunofluorescence confirmed that 4-HNE signal is increased in Purkinje cells of MSA patients (Fig. 4B, B'''), and that α-synuclein accumulated in the white matter of MSA patient samples but not in Purkinje cells of controls (Fig.4A, A'') or of MSA patients (Fig. 4B, B'').

COQ Biosynthetic Enzymes Analysis

By direct sequencing of DNA from cerebellar samples, we did not find any *COQ2* sequence variants in the patients of the cohort tested, including the, p. S146N, p. R197H, or p. V393A mutations previously identified in MSA

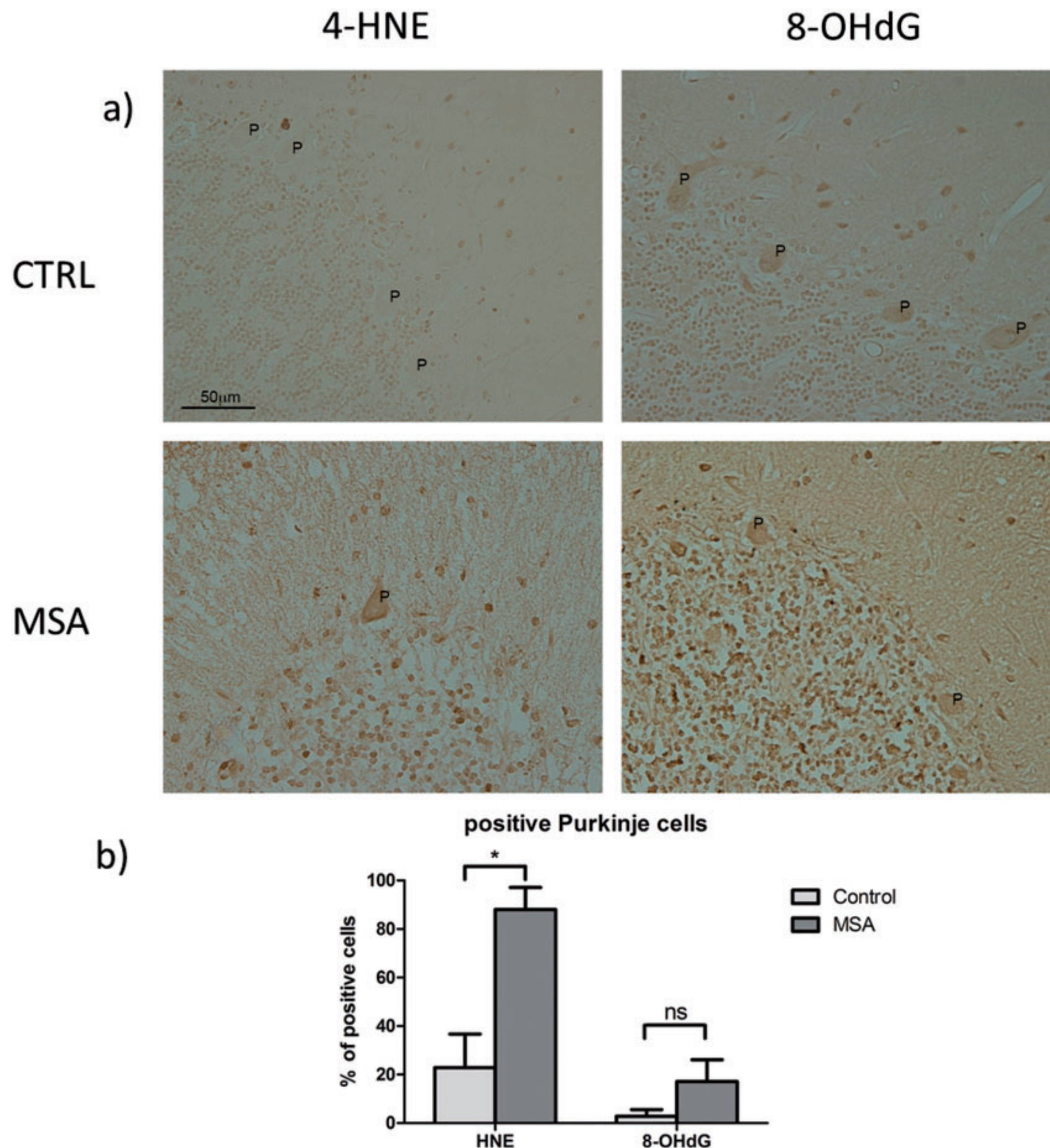

FIGURE 3. Oxidative stress in cerebellum. **(a)** Representative images of immunohistochemistry with anti-4-HNE and anti-8-OHdG (indices of lipid and DNA oxidation, respectively) in samples from controls (CTRL) (n = 4 per staining) and multiple system atrophy (MSA) patients (n = 6 per staining). Figures are representative of 5 different areas per slide. Scale bar: 50 μm. **(b)** Image J analysis showed increased signals in brains of MSA cases versus controls for HNE but not for 8-OHdG. 4-HNE, 4-hydroxynonenal; 8-OHdG, 8-hydroxy-2′-deoxyguanosine; P, Purkinje cells. *$p < 0.05$.

Downloaded from https://academic.oup.com/jnen/article/75/7/663/2579780 by guest on 25 April 2024

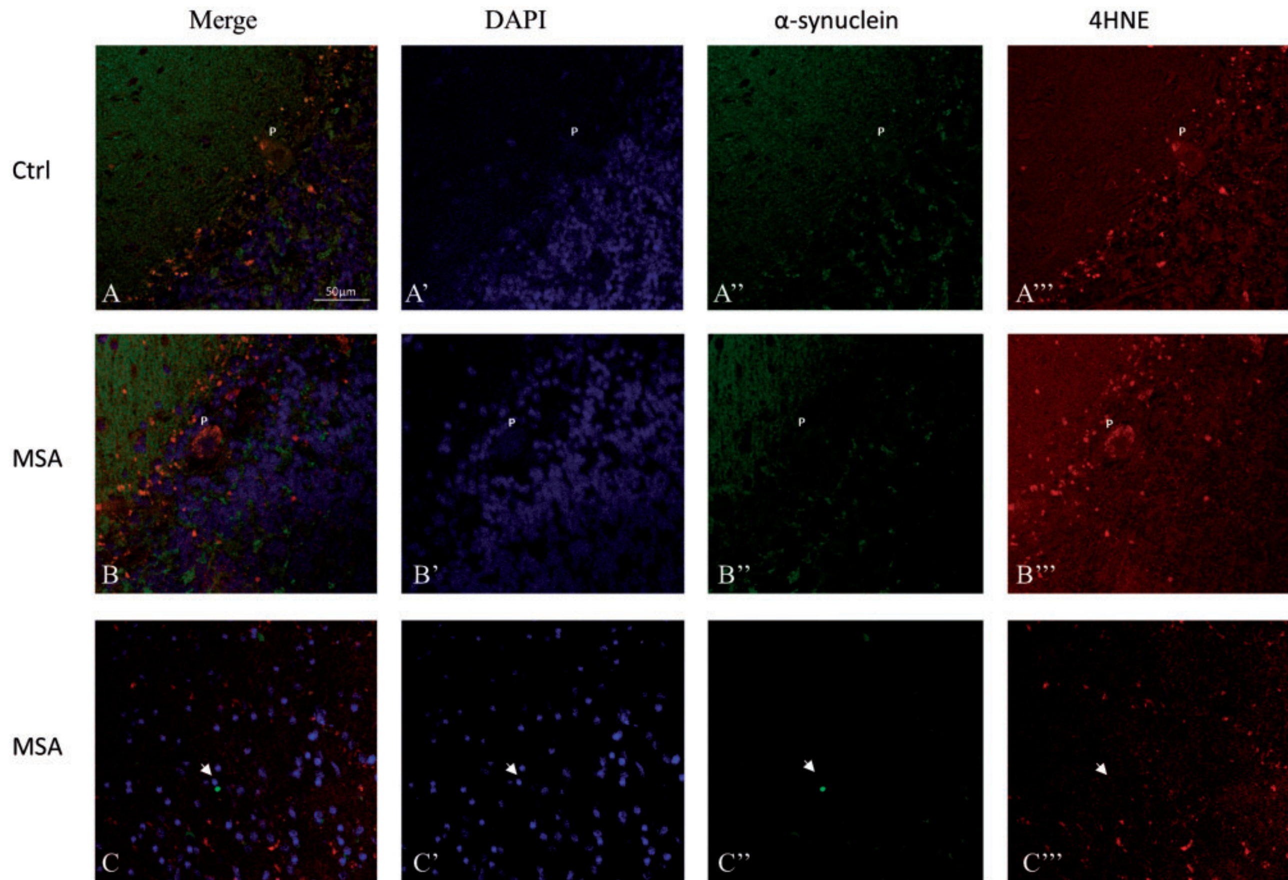

FIGURE 4. Anti-α-synuclein and anti-4-HNE double immunofluorescence. **(A)** Control Purkinje cell. **(B)** MSA sample Purkinje cell. **(C)** α-synuclein accumulation in the white matter of a sample from an MSA patient (arrows). 4-HNE, 4-hydroxynonenal.

patients (2). To assess whether CoQ_{10} biosynthesis was impaired, we measured steady state levels of proteins involved in CoQ_{10} synthesis and its regulation (PDSS1, COQ2, COQ5, COQ7, ADCK3). Levels of PDSS1 and COQ5, but not of other CoQ_{10} biosynthetic proteins, were significantly reduced in MSA compared with control samples (Fig. 5a, b).

DISCUSSION

We found decreased CoQ_{10} levels in MSA cerebellum even in samples from patients without *COQ2* mutations. Although the relatively mild reduction of CoQ_{10} observed in this study was insufficient to impair mitochondrial respiratory chain electron flux, we noted signs of increased oxidative stress, which has been described in MSA (23), and frequently observed with CoQ_{10} deficiency *in vitro* (24) and *in vivo* (25). Consistent with the fact that CoQ_{10} inhibits lipid peroxidation directly and indirectly (via recycling of vitamin E), we observed significantly increased 4-HNE staining indicating enhanced lipid peroxidation in MSA cerebellum. We excluded the possibility that CoQ_{10} deficiency was caused by nonspecific degenerative processes leading to the loss of mitochondria in MSA cerebellum compared to control tissue because we did not observe any significant decrease in the total mitochondrial mass in MSA cases.

Furthermore, we observed that PD cerebellum had normal CoQ_{10} levels whereas ET cerebellum had increased CoQ_{10} levels. The increased CoQ_{10} in ET is intriguing because ET also shows cerebellar degenerative changes, although milder than those in MSA cases (18, 26). Because dysfunction of autophagy and increased mitochondrial accumulation have been observed in ET cerebellum (27), the increased CoQ_{10} levels in ET cases may reflect increased mitochondrial mass.

Whether oxidative stress is increased in MSA brains remains controversial (28, 29). Shibata et al (28) described 4-HNE immunoreactivity in α-synuclein-containing GCIs and neuronal cytoplasmic inclusions (NCIs) in pontine neurons, and it is possible that increased 4-HNE staining could be due to the presence of GCIs and NCIs in MSA cases. Therefore, we analyzed oxidative stress in Purkinje cells, which have very few α-synuclein-containing NCIs in MSA (29). We found that 88% of Purkinje cells had dark 4-HNE staining and that Purkinje cells in the MSA cases did not have α-synuclein-containing NCIs. Therefore, our observation of increased 4-HNE staining in MSA Purkinje cells cannot be attributed to NCIs. Furthermore, in Purkinje cells of MSA cases, we observed 4-HNE in a diffuse cytoplasmic distribution rather than in localized cytoplasmic inclusions, further supporting our hypothesis that increased 4-HNE

Downloaded from https://academic.oup.com/jnen/article/75/7/663/2579780 by guest on 25 April 2024

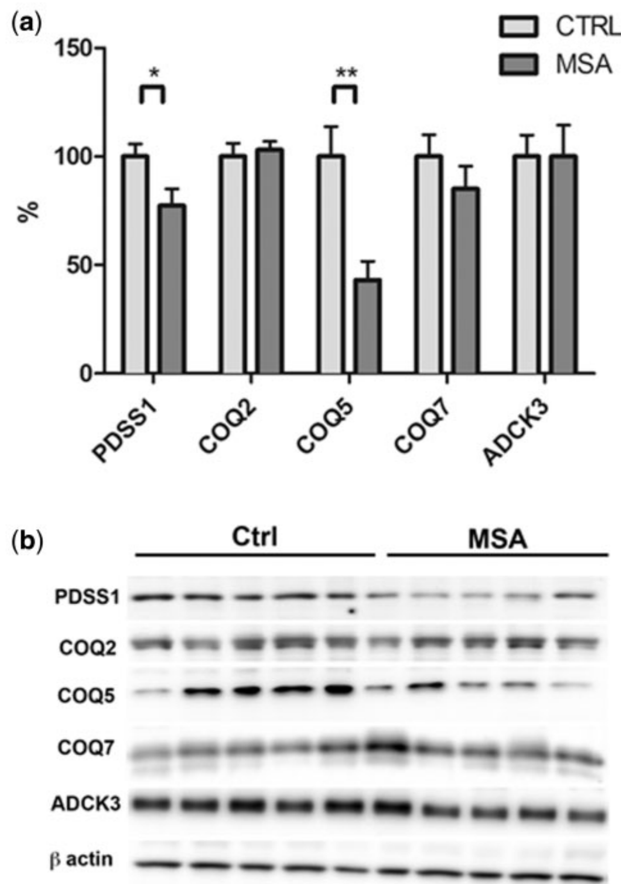

FIGURE 5. CoQ_{10} biosynthetic pathway. **(a)** Measurements of protein steady-state levels of enzymes involved in CoQ biosynthesis and its regulation in MSA and control brains showed significantly decreased levels of PDSS1 and COQ5. **(b)** Representative Western blot showing the expected sizes bands in 5 MSA and 5 controls. Ctrl, controls, n = 12; MSA, multiple system atrophy, n = 12; *$p < 0.05$; **$p < 0.01$.

staining is not secondary to neuronal inclusions. In addition, we observed colocalization of 4-HNE and α-synuclein-containing GCIs, consistent with the previous report (28). Nevertheless, we cannot exclude the possibility that lipid peroxidation in MSA is due to the combination of excessive α-synuclein and deficiency of CoQ_{10}.

Importantly, we noted that CoQ_{10} deficiency is limited to MSA cerebellum whereas the striatum, another affected area in MSA, is spared. The cerebellum is frequently affected in CoQ_{10} deficiency, suggesting specific vulnerability of this brain region. The low levels of CoQ_{10} in normal cerebellum compared with the other brain regions observed in this and previous studies suggest that a threshold is necessary for CoQ_{10} biological function. In MSA, CoQ_{10} may be below a physiological threshold necessary for normal cerebellar function (30). However, we observed reduced TOM20 and increased COX activity in MSA striatum samples. Therefore, other pathogenetic mechanisms involving mitochondria and independent of CoQ_{10} deficiency may account for the disease in this area. This notion is further supported by our observation that there was a nonsignificant trend of increased 4-HNE and 8-OHdG staining in striatal neurons. A study of a larger sample size is needed.

Interestingly, low CoQ_{10} levels in MSA brains were associated with low levels of 2 CoQ_{10} biosynthetic proteins, PDSS1 and COQ5, which may contribute to the CoQ_{10} deficiency in MSA cerebellum. PDSS1 is one of the 2 subunits of decaprenyl diphosphate synthase, the first enzyme of the CoQ_{10} biosynthetic pathway, which generates the decaprenyl side chain of CoQ_{10}. COQ5 is the C-methyltransferase of the CoQ_{10} biosynthetic pathway (31). MSA patients without *COQ2* mutations might have CoQ_{10} deficiency because of the decrease of these 2 critical enzymes in the cerebellum.

Our results are consistent with those in a recent report of Schottlaender et al (32), and further indicate that deficiency of CoQ_{10} and ensuing oxidative stress could play a role in the pathophysiology of MSA. Because disease-modifying therapies for this disorder are not currently available, increasing CoQ_{10} levels by supplementation or upregulation of the biosynthetic pathway might represent a novel treatment strategy for MSA patients.

REFERENCES

1. Lin DJ, Hermann KL, Schmahmann JD. Multiple system atrophy of the cerebellar type: Clinical state of the art. Mov Disord 2014;29:294–304
2. Multiple-System Atrophy Research Collaboration. Mutations in *COQ2* in familial and sporadic multiple-system atrophy. New Eng J Med 2013: 369;233–44
3. Emmanuele V, Lopez LC, Berardo A, et al. Heterogeneity of coenzyme Q10 deficiency: Patient study and literature review. Arch Neurol 2012; 69:978–83
4. Lin CH, Tan EK, Yang CC, et al. *COQ2* gene variants associate with cerebellar subtype of multiple system atrophy in Chinese. Mov Disord 2015;30:436–7
5. Chen YP, Zhao B, Cao B, et al. Mutation scanning of the *COQ2* gene in ethnic Chinese patients with multiple-system atrophy. Neurobiol Aging 2015;36:1222.e7–11
6. Ogaki K, Fujioka S, Heckman MG, et al. Analysis of *COQ2* gene in multiple system atrophy. Mol Neurodegen 2014;9:44
7. Schottlaender LV, Houlden H, Multiple-System Atrophy Brain Bank Collaboration. Mutant COQ2 in multiple-system atrophy. N Eng J Med 2014;371:81
8. Sharma M, Wenning G, Kruger R. European Multiple-System Atrophy Study Group (EMSA-SG). Mutant COQ2 in multiple-system atrophy. N Engl J Med 2014;371:80–1
9. Yang X, Xi J, An R, et al. Lack of evidence for an association between the V393A variant of *COQ2* and amyotrophic lateral sclerosis in a Han Chinese population. Neurol Sci 2015;36:1211–5
10. Jeon BS, Farrer MJ, Bortnick SF. Korean Canadian Alliance on Parkinson's disease and related disorders. Mutant COQ2 in multiple-system atrophy. N Eng J Med 2014;371:80
11. Fanciulli A, Wenning GK. Multiple-system atrophy. N Engl J Med 2015; 372:249–63
12. Quinzii CM, Hirano M, DiMauro S. Mutant COQ2 in multiple-system atrophy. N Engl J Med 2014;371:81–2
13. Mitsui J, Tsuji S. Mutant COQ2 in multiple-system atrophy. N Engl J Med 2014;371:82–3
14. Ramirez EP, Vonsattel JP. Neuropathologic changes of multiple system atrophy and diffuse Lewy body disease. Sem Neurol 2014;34:210–6
15. Burn DJ, Jaros E. Multiple system atrophy: Cellular and molecular pathology. Mol Pathol 2001;54:419–26
16. Virmani T, Moskowitz CB, Vonsattel JP, et al. Clinicopathological characteristics of freezing of gait in autopsy-confirmed Parkinson's disease. Mov Disord 2015;30:1874–84
17. Louis ED, Borden S, Moskowitz CB. Essential tremor centralized brain repository: Diagnostic validity and clinical characteristics of a

Downloaded from https://academic.oup.com/jnen/article/75/7/663/2579780 by guest on 25 April 2024

highly selected group of essential tremor cases. Mov Disord 2005;20: 1361–5
18. Babij R, Lee M, Cortes E, et al. Purkinje cell axonal anatomy: Quantifying morphometric changes in essential tremor versus control brains. Brain 2013;136:3051–61
19. Vonsattel JP, Del Amaya MP, Keller CE. Twenty-first century brain banking. Processing brains for research: The Columbia University methods. Acta Neuropathol 2008;115:509–32
20. Lopez LC, Schuelke M, Quinzii CM, et al. Leigh syndrome with nephropathy and CoQ10 deficiency due to decaprenyl diphosphate synthase subunit 2 (PDSS2) mutations. Am J Hum Genet 2006;79:1125–9
21. Kirby DM, Thorburn DR, Turnbull DM, et al. Biochemical assays of respiratory chain complex activity. Methods Cell Biol 2007;80:93–119
22. Ruifrok AC, Johnston DA. Quantification of histochemical staining by color deconvolution. Anal Quant Cytol Histol 2001;23:291–9
23. Yamashita T, Ando Y, Obayashi K, et al. Oxidative injury is present in Purkinje cells in patients with olivopontocerebellar atrophy. J Neurol Sci 2000;175:107–10
24. Quinzii CM, Lopez LC, Gilkerson RW, et al. Reactive oxygen species, oxidative stress, and cell death correlate with level of CoQ10 deficiency. FASEB J 2010;24:3733–43
25. Luna-Sanchez M, Diaz-Casado E, Barca E, et al. The clinical heterogeneity of coenzyme Q10 deficiency results from genotypic differences in the Coq9 gene. EMBO Mol Med 2015;7:670–87
26. Kuo SH, Tang G, Louis ED, et al. Lingo-1 expression is increased in essential tremor cerebellum and is present in the basket cell pinceau. Acta Neuropathol 2013;125:879–89
27. Kuo SH, Tang G, Ma K, et al. Macroautophagy abnormality in essential tremor. PLoS One 2012;7:e53040
28. Shibata N, Inose Y, Toi S, et al. Involvement of 4-hydroxy-2-nonenal accumulation in multiple system atrophy. Acta Histochem Cytochem 2010; 43:69–75
29. Mori F, Piao YS, Hayashi S, et al. Alpha-synuclein accumulates in Purkinje cells in Lewy body disease but not in multiple system atrophy. J Neuropathol Exp Neurol 2003;62:812–9
30. Naini A, Lewis VJ, Hirano M, et al. Primary coenzyme Q10 deficiency and the brain. BioFactors 2003;18:145–52
31. Kawamukai M. Biosynthesis of coenzyme Q in eukaryotes. Biosci Biotechnol Biochem 2015;80:23–33
32. Schottlaender LV, Bettencourt C, Kiely AP, et al. Coenzyme Q10 levels are decreased in the cerebellum of multiple system atrophy patients. PLoS One 2016;11:e0149557

Downloaded from https://academic.oup.com/jnen/article/75/7/663/2579780 by guest on 25 April 2024