Mitochondrial Dysfunction as a Cause of Axonal Degeneration in Multiple Sclerosis Patients

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Objective: Degeneration of chronically demyelinated axons is a major cause of irreversible neurological disability in multiple sclerosis (MS) patients. Development of neuroprotective therapies will require elucidation of the molecular mechanisms by which neurons and axons degenerate. Methods: We report ultrastructural changes that support Ca2+/H11545-mediated destruction of chronically demyelinated axons in MS patients. We compared expression levels of 33,000 characterized genes in postmortem motor cortex from six control and six MS brains matched for age, sex, and postmortem interval. As reduced energy production is a major contributor to Ca2+/H11545-mediated axonal degeneration, we focused on changes in oxidative phosphorylation and inhibitory neurotransmission. Results: Compared with controls, 488 transcripts were decreased and 67 were increased (p < 0.05, 1.5-fold) in the MS cortex. Twenty-six nuclear-encoded mitochondrial genes and the functional activities of mitochondrial respiratory chain complexes I and III were decreased in the MS motor cortex. Reduced mitochondrial gene expression was specific for neurons. In addition, pre-synaptic and postsynaptic components of GABAergic neurotransmission and the density of inhibitory interneuron processes also were decreased in the MS cortex. Interpretation: Our data supports a mechanism whereby reduced ATP production in demyelinated segments of upper motor neuron axons impacts ion homeostasis, induces Ca2+/H11545-mediated axonal degeneration, and contributes to progressive neurological disability in MS patients.

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Rapid communication between neurons requires energy and the insulation of axons by discontinuous segments of myelin. Voltage-gated Na+/H11001 channels produce nerve impulses and are concentrated at the nodes of Ranvier,1 the short unmyelinated axon segment between individual myelin internodes. The nerve impulse rapidly jumps from node to node by a process called saltatory conduction. Multiple sclerosis (MS) is an inflammatory, demyelinating disease of the central nervous system (CNS) that destroys myelin, oligodendrocytes, axons, and neurons.2 Pathologically, demyelination predominates during early stages of MS. Neurological disability associated with demyelinating lesions is initially reversible because of a variety of adaptive changes in the MS brain. As part of these changes, Na+ channels are distributed diffusely along the surface of demyelinated axons,3 resulting in slow but effective nerve communication. This also increases the energy demands of neuronal communication and renders the demyelinated axon more susceptible to hypoxic/ischemic damage (for review, see Stys4).

After an initial stage (commonly 10–15 years) of relapses and remissions (RRMS), most MS patients enter a course of irreversible and continuous neurological decline, termed secondary progressive multiple sclerosis (SPMS).5 During SPMS, new inflammatory brain lesions substantially decrease with age,5 but neurological decline continues due in part to degeneration of chronically demyelinated axons.6 To identify neuronal gene changes that may contribute to ax-
onal degeneration in chronic MS patients, we conducted an unbiased comparison of mRNA expression changes in nonlesioned motor cortex from six control and six MS patients obtained by rapid autopsy using the human U133 microarray platform (Affymetrix, Santa Clara, CA) containing more than 33,000 well-characterized genes. Previous studies have profiled gene expression in demyelinated lesions7–9 and normal-appearing white matter from MS patients.10,11 To our knowledge, this is the first study of non-lesion cerebral cortex from MS patients using the complete set of U133 arrays.

In MS cortex, we detected significant reductions in gene products specific for the mitochondrial electron transport chain and demonstrated that cortical mitochondria have a diminished capacity to exchange electrons in respiratory chain complex I and III. In addition, pre-synaptic and postsynaptic components of inhibitory neurotransmission and the density of inhibitory interneuron processes were decreased in MS motor cortex samples. We propose that these changes negatively impact on ion homeostasis in demyelinated segments of upper motor neuron axons and contribute to axonal degeneration and progressive neurological disability in MS patients.

### Patients and Methods

**Tissue**

MS brain tissues were obtained from The Cleveland Clinic. Patients or their relatives provided informed consent in the form of an advanced directive as part of an institutional review board–approved protocol. Control brain tissues were obtained from the University of Pittsburgh, Center for the Neuroscience of Mental Disorders Brain Bank and have been characterized previously by microarray analysis. MS and control donors were matched for age and sex and postmortem interval (PMI; \( p < 0.05 \); Table). MS patients were severely disabled (Expanded Disability Status Scale [EDSS] score, >7.0) at the time of death. Tissues belonging to MS and control cases used for microarray analysis, real-time polymerase chain reaction analysis was done using samples MS1–MS6 and C1–C4, C7, C8 (control C7 and C8 were included to supplement inadequate sample recovery from sample C5 and C6). Western blot analysis was performed using samples C1, C4, C5, C7, C8, M1, M3, M4, and M5. EDSS = Expanded Disability Status Scale (possible range, 1–10, with a higher score indicating a greater degree of disability); PP = primary progressive; SP = secondary progressive.

### Table. Details of Patients Examined in this Study

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<th>Postmortem Interval (hr)</th>
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<th>Duration of Disease (yr)</th>
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Samples M1 to M6 and Control C1 to C6 were used for microarray analysis. Mean age and postmortem interval (HR) for multiple sclerosis (56.3 ± 5.3; 6.5 ± 1.8) and control (57.3 ± 2.5; 11.7 ± 2.1) patients was statistically similar in samples used for the microarray analysis. Samples MS 1-10 were used for electron microscopy analysis; real-time polymerase chain reaction analysis was done using samples MS1–MS6 and C1–C4, C7, C8 (control C7 and C8 were included to supplement inadequate sample recovery from sample C5 and C6). Western blot analysis was performed using samples C1, C4, C5, C7, C8, M1, M3, M4, and M5. EDSS = Expanded Disability Status Scale (possible range, 1–10, with a higher score indicating a greater degree of disability); PP = primary progressive; SP = secondary progressive.
Electron Microscopy of Spinal Cord Axons

Spinal cords from 10 MS patients (MS1–10 in the Table) were obtained by rapid autopsy, cut into 2cm segments, and placed in 4% paraformaldehyde for 48 hours. A 5mm-thick slice then was taken from each end of the block, placed in 4% paraformaldehyde and 2.5% glutaraldehyde for 1 week, and then processed to Epon embedding (EMbed 812 kit, Electron Microscopy Sciences, Hatfield, PA) by standard procedures. Areas containing demyelinated lesions, identified in 1µm-thick sections, were sectioned for ultrastructural analysis, stained with lead citrate and uranyl acetate, and examined in a Phillips electron microscope. Equal numbers of myelinated and demyelinated axons were photographed, and the ultrastructural integrity of axoplasm was evaluated. The percentage of axons in each group was divided into those with and without intact neurofilaments, and the resulting differences were analyzed using the Student’s t-test.

Microarray Procedures and Statistical Analysis

Cortical gray matter was separated from subcortical white matter by scoring motor cortex blocks at the white matter/grey matter juncture with a scalpel prior to cutting 60µm-thick sections in a cryostat. RNA was isolated from pooled cortical sections using RNAgents Total RNA Isolation System protocol (Promega, Madison, WI). RNA integrity and 28S/18S ratios were determined with an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). Biotinylated cRNA probes (Enzo Diagnostics, Farmingdale, NY) were generated from 8µg total RNA and successfully hybridized to Test 3 arrays and then to Affymetrix Human Genome U133A/U133B arrays according to the manufacturers instructions (Affymetrix, Santa Clara, CA). Microarray data images were analyzed using Microarray Analysis Suite software 5.0 (Affymetrix). Univariate and principle component analysis determined intensity distribution and eliminated sample outliers. Microarray probe level normalization of log2-transformed data were performed using RMA and subjected to statistical significance using a two-tailed group-wise t-test assuming equal variance. In the initial microarray experiment genes were considered differentially expressed if they showed 1.5-fold change (average log2 ratio, >0.585) and p < 0.05 across replicates of the two tissue types. To detect whether selection of genes were caused by chance and not inherent differences, we performed false discovery rate (FDR) analysis using a permutation test that has been validated in previously published studies. Details of the analysis are in online Supplemental Data II. Data were visualized using Genespring version 5.0 (Silicon Genetics, Redwood City, CA). Expressed sequence tags that met the inclusion criteria were BLAST searched against public databases for sequence homology. Functional annotations of the altered transcripts were carried out using EASE (Expression Analysis Systemic Explorer; available at http://david.niaid.nih.gov/david/ease.htm) which validated occurrence of each biological category within a queried group of genes by using Fisher’s exact test.

Quantitative Real-time Polymerase Chain Reaction

RNA from the six MS samples and six control samples was DNase-treated (Qiagen, Valencia, CA) and 2µg was reverse-transcribed using random primers with Stratagene reverse transcriptase (Stratagene, La Jolla, CA). Gene-specific PCR was performed (See Supplemental Data I for primer sequences) using the SYBR Green I kit and a Roche Lightcycler (Roche Diagnostics, Indianapolis, IN) and standardized to 18S RNA (Ambion, Austin, TX). Each sample was run in triplicate. Mean fold change was calculated between control and MS RNA and compared by the Student’s t-test.

Western Blotting

Mitochondrial-enriched fractions were isolated from homogenates of total gray matter as described previously. Equal amounts of protein (10µg) were loaded on 4-12% NuPage Bis-Tris gels in MES buffer (Invitrogen, Carlsbad, CA) and blotted according to the manufacturer’s instructions. Three separate blots were performed for each protein and exposed onto biomax XAR films (Kodak, Rochester, NY). Films were scanned, and average intensity was quantitated with Scion-Image (NIH, Bethesda, MD) and compared by the Student’s t-test.

Mitochondrial Electron Transport Chain Activity

Activities of mitochondrial respiratory chain complexes I, III, and IV were measured as described. In brief, mitochondria-enriched fractions were obtained by differential centrifugation from sections from six MS blocks and six matched control blocks belonging to three sets of patients and controls. To allow free access of substrates to the complexes, freeze-thawed mitochondrial membranes were disrupted with 1% sodium cholate, and the activities of individual complexes were determined using appropriate electron donors and acceptors. Enzyme activity was corrected for nonenzymatic reduction of acceptors, using appropriate inhibitors. All measurements were performed in duplicate and standardized to protein concentration in at least three independent experiments. Data were analyzed by Student’s t-test.

Immunocytochemistry

All tissues used in this study were immunostained for proteolipid protein and MHC class II antigens as described previously. Blocks with evidence of cortical demyelination or other pathologies were excluded. For quantitative analysis of immunocytochemistry, motor cortex was embedded in paraffin and sectioned at thickness of 10µm. Multiple sections from four MS brains and two control brains were incubated with antibodies specific for parvalbumin or mitochondrial-encoded complex IV subunit I protein (cytochrome c oxidase subunit I). The percentage of cortical area occupied by parvalbumin stained processes and the area of upper motor neuron perikarya occupied by mitochondria were quantified using Photoshop 6.0 (Adobe, San Jose, CA). These data were analyzed by the Student’s t-test.

Antibodies

NADH dehydrogenase (ubiquinone) 1 alpha subcomplex 6 (NDUFA6), succinate dehydrogenase complex subunit A (SDHA), low molecular mass ubiquinone-binding protein (QPC), mitochondrial-encoded complex IV subunit I protein and porin-specific antibodies were obtained from Molecular Probes (Eugene, OR). MHC class II (HLA-DR)
antigen-specific antibody was purchased from Dako (Carpenteria, CA). Glutamic acid decarboxylase 1, parvalbumin, actin, proteolipid protein, and GABA A receptor-specific antibodies were obtained from Chemicon (Temecula, CA).

**In Situ Hybridization**
Paraffin-embedded sections from control and MS motor cortex were hybridized with riboprobes specific to NADH dehydrogenase (ubiquinone) 1α subcomplex 6 (NDUFA6, complex I), low molecular mass ubiquinone-binding protein (Qp-C, complex III), ATP synthase, H+ transporting, mitochondrial F0 complex, subunit e (ATP 51, complex V), and proteolipid protein, which was used as a control. 35S-dUTP (Perkin-Elmer, Boston, MA)–labeled riboprobes were generated using T3/T7 polymerase and the in-vitro transcription kit (Ambion, Austin, TX; primer sequences are appended in Supplemental Data I). Both the sense and antisense riboprobes containing 5 × 10⁵ cpm were placed on identical sections and hybridized overnight at 60°C. For cellular resolution of mRNA expression, sections were dipped in autoradiographic NTB2 emulsion (Kodak), stored at 4°C for 4 days, developed, counterstained with hematoxylin and eosin, and examined by dark-field and bright-field microscopy. Bright-field images were used to quantify mRNA abundance. For mitochondrial probes, grains per neuronal perikaryal area were quantified for 55 to 130 upper motor neurons in sections from both control and MS motor cortices. For proteolipid protein mRNA, total grains per oligodendrocyte perikaryal area were quantified. Statistical significance was determined by the Student’s t-test.

**Results**

**Ultrastructure of Demyelinated Axons**
Degeneration of chronically demyelinated axons has been proposed as a mechanism that drives continuous irreversible neurological disability in chronic MS patients. To elucidate possible mechanisms of this axonal degeneration, we analyzed myelinated and demyelinated axons in the spinal cords of 10 severely disabled (EDSS score, >7.0) MS patients (see Table) by electron microscopy. Specimens obtained by our rapid autopsy protocol retained significant ultrastructural integrity as indicated by preservation of myelin membranes, cytoplasmic organelles, and extracellular spaces (Fig 1A). Greater than 95% of the myelinated axons had normal-appearing axoplasm with well-preserved microtubules, mitochondria, and neurofilaments with appropriate side-arm extensions (see Fig 1B). Fifty percent of the demyelinated axons had similarly well-preserved axoplasm (see Fig 1C). Because of reduced side-arm extension, however, neurofilament spacing was less than that found in myelinated axons. The remaining 50% of demyelinated axons had abnormal axoplasm with reduced organelle content and varying degrees of neurofilament fragmentation (see Fig 1D). Because the myelinated and demyelinated axons were from the same sections, we conclude that these axoplasmic changes are inherent to 50% of the demyelinated axons and not caused by artifacts induced by autolysis or tissue processing. These observations support the possibility that half of the demyelinated axons in spinal cord lesions from chronic MS patients have activated Ca²⁺-dependent enzymes that are known to fragment neurofilaments.

**Microarray Comparisons**
Non-lesion motor cortex samples from six MS patients and six individuals without neurological disease were obtained at autopsy (see Table) and compared on Affymetrix U133A and U133B microarrays. Since our goal was to examine neuronal gene changes that may contribute to degeneration of chronically demyelinated axons, we studied tissue from MS patients with severe disability (EDSS score, >7.0). No outliers were detected in the arrays using univariate, principal component, or correlation coefficient (0.96–0.91 for controls; 0.96–0.75 for MS samples) analysis. Transcript changes separated control and MS samples into separate clusters (Fig 2A). Among the 555 significantly altered transcripts, 488 were decreased and 67 were increased in the MS samples (p < 0.05, 1.5-fold). A standardized MIAME/MGED-format description of the data set is included in Supplemental Data II. Permutation testing estimated FDR of 5.9% across the comparison of control and MS samples (see Fig 2B). This indicated that approximately 95% of the transcript alterations were caused by true biological differences between the MS and control samples and not by chance.” Altered transcripts were classified using EASE into gene ontology–based biological processes. Transcripts related to “oxidative phosphorylation” (p = 0.00049), “synaptic transmission” (p = 0.0080) “cellular transport” (p = 0.0085) “MHC related” (p = 0.000013), antigen presentation (p = 0.00029), antigen processing (p = 0.00035) and “translational initiation” (p = 0.0060) were among the major significant biological groups that were altered in MS motor cortex (complete list of classification attached as Supplemental Data II). We focus here on the analysis of biological categories of oxidative phosphorylation and synaptic transmission.

**Reduced Mitochondrial Function in Upper Motor Neurons**
Oxidative phosphorylation is catalyzed by five large multiprotein complexes that are encoded by both nuclear and mitochondrial DNA. Among the 119 nuclear-encoded mitochondrial electron transport chain genes on the arrays, 103 were called present in all samples and 26 were significantly (p < 0.05) decreased in MS samples (Fig 3A). Decreased transcripts are listed in Figure 3A and include 10 of 36 for complex I, 0 of 7 for complex II, 4 of 11 for complex III, 7 of 24 for complex IV, and 5 of 25 for complex V. Transcript
decreases were confirmed by quantitative RT PCR (see Fig 3A) and extended to protein for a subset of these genes (see Fig 3B). Levels of complex II 70kDa protein SDHA were similar in control and MS samples. The decrease in mitochondrial electron transport gene expression appear to be functionally relevant as the activ-

Fig 1. Ultrastructure of myelinated and demyelinated axons from spinal cords of chronic multiple sclerosis patients. The electron micrograph in panel A contains four axons (Ax1–Ax4) at the edge of a demyelinated lesion; each displays varying degree of ultrastructural integrity. The myelinated axon (Ax1) has normal-appearing axoplasm (B) with intact and appropriately oriented neurofilaments. Axoplasm of demyelinated Ax2 (C) is also intact, but neurofilament spacing is significantly reduced. Neurofilaments in demyelinated Ax 3 (D) are fragmented and barely detectable in demyelinated Ax4. Less than 5% of the myelinated axons and 50% of demyelinated axons analyzed had abnormal axoplasm (E). Scale bars; A = 2μm, B–D = 200nm. *p < 0.00000001.
The activities of respiratory chain complex I and III were significantly decreased by 61% and 40%, respectively (compared with control), in mitochondria-enriched fractions isolated from postmortem MS cortex (see Fig 3C). Complex IV activity was not changed significantly. Mitochondrial density was similar in upper motor neuronal cell bodies in MS and control tissue as quantified by staining for the mitochondrial encoded complex IV subunit 1 protein (see Fig 3D), levels of porin (see Fig 3B), and normal mRNA levels for 75%
Mitochondrial electron transport chain gene transcripts are decreased in multiple sclerosis motor cortex. Twenty-six electron transport chain transcripts are decreased significantly ($p < 0.05$) in multiple sclerosis motor cortex (A). These include NDUFB7 (NADH dehydrogenase ubiquinone 1 beta subcomplex), NDUFB8 (NAD dehydrogenase ubiquinone 1 beta subcomplex 8), NDUFB2 (NADH dehydrogenase ubiquinone 2 beta), NDUFS2L (NADH-ubiquinone oxidoreductase 39kDa protein), NDUFV2 (NADH dehydrogenase ubiquinone flavoprotein 2), NDUFS2 (NAD dehydrogenase ubiquinone Fe-S protein 2), NDUFA6 (NADH dehydrogenase ubiquinone 1 beta subcomplex 6), NDUFS4 (NADH dehydrogenase ubiquinone Fe-S protein 4), NDUFB1 (NADH dehydrogenase ubiquinone 1B subcomplex 1), QP-C (low molecular mass ubiquinone binding protein), UQRC2 (ubiquinol-cytochrome c reductase core protein II), UQBP (ubiquinone binding protein), UQCR (ubiquinol-cytochrome c reductase), COX5B (cytochrome c oxidase subunit 5B), COX7B (cytochrome c oxidase subunit 7B), COX7C (cytochrome c oxidase subunit 7C), COX17 (cytochrome c oxidase assembly protein 17), COX5A (cytochrome c oxidase subunit 5A), COX11 (cytochrome c oxidase assembly protein), ATP5I (ATP synthase, $H^+$ transporting, F0 complex subunit e), ATP5 (ATP synthase, $H^+$ transporting, F0 complex subunit b), ATP5J (ATP synthase, $H^+$ transporting, F0 complex subunit F6), ATP5G3 (ATP synthase, $H^+$ transporting, F0 complex subunit c). Expression levels of NDUFS4, QP-C, COX5A, and ATP5G3 mRNA decreased when measured by real-time polymerase chain reaction. Mean fold change $\pm$ SEM, $^*$ $p < 0.05$. Western blots of mitochondrial proteins NDUFA6 (complex I) and QP-C (complex III) also show significant decrease in multiple sclerosis motor cortex, whereas complex II protein, SDHA II, was unchanged (B). Protein levels were normalized to porin. Fold changes represent the average of three separate blots. $^*p < 0.01$. Activity of electron transport complexes I and III are decreased in mitochondrial-enriched fractions from motor cortex of multiple sclerosis patients (C). Complex I function, as assayed as NADH-ferricyanide reductase activity, was decreased in multiple sclerosis motor cortex by 61%. Complex III function, as assayed by decylubiquinol-ferricytochrome c oxidoreductase activity, was decreased by 40%. Complex IV function was similar. (black bars) control; (gray bars) multiple sclerosis motor cortex. $^*p < 0.006$. Confocal images of upper motor neurons immunostained with antibodies specific for complex IV/subunit 1 depict similar percentage of neuronal cell body area occupied by mitochondrial in control (left side panel D) and multiple sclerosis (right side panel D). Scale bars = 10$\mu$m.
of the nuclear-encoded mitochondrial genes. Our data therefore support mitochondrial dysfunction in the motor cortex of MS patients due to dysfunction of specific genes and not due to a deficiency in the number of mitochondria themselves.

To investigate whether mitochondrial transcripts were reduced in upper motor neurons, we performed quantitative in situ hybridization for several mitochondrial transcripts (Fig 4). Localization of proteolipid protein mRNA served as control. When viewed by dark-field microscopy, mitochondrial mRNAs were highly enriched in upper motor neurons in both control (see Fig 4A, C) and MS (see Fig 4B, D) sections, whereas proteolipid protein mRNAs were enriched in glial cells (see Fig 4E, F). The densities of silver grains representing mitochondrial mRNA were significantly reduced in upper motor neurons in MS tissue sections while proteolipid protein mRNA densities were similar (see Fig 4G). Thus, the mitochondrial mRNA transcript reductions detected in microarrays and by RT PCR occur predominately in neurons. Based on silver grain densities, mitochondrial mRNA reductions appeared to occur in most upper motor neurons analyzed. Thus, reduced mitochondrial gene expression is unlikely to occur only in neurons that may have transected axons. Only 6 of the 26 nuclear-encoded mitochondrial genes (see Fig 3A) were significantly altered in white matter lesions (a tissue with axonopathy) from MS patients (Peterson JW, Supplemental Data I). Furthermore, none of these mitochondrial genes were significantly decreased in microarray comparisons of normal-appearing white matter representing primarily the glial cell population (Supplemental Data I,11). These data suggest mitochondrial gene expression therefore is not globally altered in brains from MS patients. Collectively, our data support reduced nuclear-encoded mitochondrial genes expression in upper motor neurons in the motor cortex of patients with MS.

**Decreased Inhibitory Neurotransmission**

Approximately 25% of the neurons in the cerebral cortex utilize the inhibitory neurotransmitter GABA to modulate the firing of motor and other neurons.23 The majority of GABA receptors on the surface of cortical neurons consist of subunits α1, β3, and γ2.24,25 mRNA encoding GABA A α1 (−1.84, p = 0.041) and β3 (−1.52, p = 0.003) receptor subunits were significantly decreased in microarrays from MS cortex (Fig 5A). The γ2 subunit was also reduced on the microarrays but did not reach statistical significance (−1.46, p = 0.073). mRNA encoding GABA A receptor associated protein (GABRAP), which modulates

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**Fig 4. Mitochondrial transcripts are decreased in neurons from the motor cortex of multiple sclerosis patients.** Distribution of mitochondrial complex I (NDUFA6) mRNA in sections from control (A, C) and multiple sclerosis motor cortex (B, D). Mitochondrial transcripts were highly enriched in neurons. Silver grain densities were determined for at least fifty upper motor neurons (layers V–VI) in sections from three multiple sclerosis and two control blocks and expressed as grains/μm² soma area (G). Levels of mRNA encoding proteins specific for respiratory chain complex I (NADH dehydrogenase (ubiquinone) 1 alpha subcomplex 6), III (low molecular mass ubiquinone-binding protein), and V (ATP synthase, H⁺ transporting, mitochondrial F0 complex subunit e) were significantly decreased in multiple sclerosis motor neurons. Dark-field images of proteolipid protein mRNA in sections from control (E) and multiple sclerosis (F) motor cortex. Proteolipid protein mRNA densities were similar in oligodendrocytes in multiple sclerosis and control cerebral cortex sections (G). Scale bars, A, B, E, F = 25μm; C, D = 10μm. p < 0.0002, *p < 0.00002.
GABA receptor function was decreased 1.45-fold (p = 0.022). Reductions in select transcripts were confirmed by quantitative RT PCR using the RNA from the same six MS and six control samples (see Fig 4A). All changes as measured by RT PCR were slightly larger than changes observed by microarray comparisons and mRNA encoding GABA A receptor subunit 3 was significantly reduced (see Fig 5A). GABA A receptor protein was decreased 3.8-fold (p = 0.032) in Western blot analysis (see Fig 5B).

Four transcripts related to presynaptic inhibitory interneuron function were significantly decreased in microarrays of MS motor cortices. mRNA encoding GAD67, the enzyme that synthesizes GABA, was decreased 1.9-fold (p = 0.029) in microarrays and 4.4-fold (p < 0.05) by RT PCR (see Fig 4A). GAD67 protein was decreased 4.4-fold (p < 0.005) by Western blot (see Fig 5B). Parvalbumin, a Ca2+-binding protein, expressed by a subpopulation of inhibitory interneurons was decreased 2.7-fold by microarrays and 4-fold by RT PCR. We quantified the percentage of cortical area stained for parvalbumin in tissue sections from control (see Fig 5C) and MS (see Fig 3D) motor cortex and found 30% reduction in parvalbumin-stained processes in MS motor cortex (see Fig 5E).

Two other inhibitory interneuron transcripts, cholecystokinin (CCK) (−1.57, p = 0.018) and tachykinin (TAC1; −1.76, p = 0.023) were also decreased in MS samples (see Fig 5A). Neurotransmitter-related changes appear to be specific for inhibitory neurotransmission as levels of glutamate receptor subunit mRNAs were similar in control and MS samples (see Fig 5A). Collectively, our data suggest that inhibitory neurotransmission is reduced at both pre-synaptic and postsynaptic levels in the motor cortex of chronic MS patients.

**Discussion**

Degeneration of chronically demyelinated axons is a major cause of the continuous, irreversible neurological disability that occurs in the chronic stages of MS. In a cohort of MS patients similar to that investigated here, axonal loss in chronically demyelinated spinal cord lesions averaged 68%. We show here that approximately 50% of the remaining demyelinated axon profiles in chronic lesions of MS have pathological changes that suggest Ca2+-activated protease activity including fragmented neurofilaments, depolymerized microtubules, and decreased organelle content. We con-
include, based on global transcript profiles, biochemical analysis, and morphological studies of motor cortex samples, that motor neurons in chronic MS patients have significantly impaired mitochondrial function and decreased inhibitory innervation. These data support increased excitability of upper motor neurons that have a reduced capacity to produce ATP.

The objective of microarray comparisons is to decipher inherent biological differences keeping technical variations (noise, artifacts, false-positives) to a minimum. To maximize statistical validity of our microarray data, we performed rapid autopsies, 6 by 6 comparisons, FDRs, and RT PCR (for select transcripts). Being more sensitive, RT PCR almost always produced greater differences than microarray comparisons. The biological relevance of the transcript changes were extended to protein expression levels as determined by western blots and cell specificity by immunohistochemistry and in situ hybridization. Our data demonstrate the value of an unbiased search of the whole genome as changes in neuronal mitochondrial gene expression and inhibitory neurotransmission have not been reported in previous studies of MS pathogenesis.

Our data are consistent with the hypothesis that a mismatch between energy demand and reduced supply of ATP causes degeneration of chronically demyelinated axons in MS patients. The energy demand of nerve conduction is increased by the diffuse distribution of voltage-gated Na⁺ channels along the demyelinated axolemma and possibly by increased firing of upper motor neurons due to decreased inhibitory input. Both of these changes increase Na⁺ influx into the axon, which is normally exchanged for extracellular K⁺ by the Na⁺/K⁺ ATPase in a rapid and energy-dependent manner. We speculate that this exchange is impaired in chronically demyelinated axons because the upper motor neuron supplies it with dysfunctional mitochondria. Increased axoplasmic Na⁺ concentrations will reverse the energy-independent Na⁺/Ca²⁺ exchanger and exchange axoplasmic Na⁺ for extracellular Ca²⁺. Chronic increases in axoplasmic Ca²⁺ concentrations depolymerize microtubules and activate proteases that fragment neurofilaments, as observed in the electron microscopic studies reported here. Cytoskeletal pathology and reduced ATP production decrease axonal transport, reduce axoplasmic organelle density, and eventually cause axonal degeneration.

The reduction in respiratory chain complex I (61%) and III (40%) activities in mitochondrial preparations from the motor cortex of MS patients (see Fig 3C) indicates significant neuronal mitochondrial dysfunction and by inference decreased ATP production in demyelinated axons. Because the Na⁺/K⁺ ATPase utilizes approximately 50% of available CNS energy, it is likely that its function is impaired in chronically de-myelinated axons in chronic MS brains. The ionic cascade described above has been unequivocally documented as the cause of myelinated axon degeneration in experimental models of CNS white matter hypoxia and ischemia (for review see Stys). Because myelinated axons in chronic MS spinal cord displayed little evidence of Ca²⁺-mediated pathology, demyelination appears to be a dominant factor in producing the axonal pathology documented in Figure 1.

Previous studies have reported reduced mitochondrial respiratory chain complex I activity in chronic active lesions from MS patients and attributed it to oxidative damage of mitochondrial DNA by nitric oxide. When applied to myelinated axons in vitro, nitric oxide causes conduction block and these axons degenerate when they are activated. In our microarray comparisons, levels of nitric oxide (NO)–synthesizing enzymes were similar between control and MS cortex. Because neuronal nitric oxide synthase (nNOS) activity and NO production is Ca²⁺ dependent, it is possible that NO and reduced mitochondrial gene expression may act in concert to reduce energy production in chronically demyelinated axons.

We demonstrated both reduced GABA-related gene transcripts and density of inhibitory interneuron processes in the motor cortex samples from MS patients. We have shown previously that activated microglia in the cerebral cortex of MS patients physically associate with neuronal perikarya and proximal dendrites. In rodents, similar targeting of neurons by activated microglia can be initiated by nerve transection, demyelination, delayed-type hypersensitivity, and systemic lipopolysaccharide administration. Furthermore, these microglia separate pre-synaptic and postsynaptic specializations and thereby remove what is predominately inhibitory interneuron input. It is possible that this microglial response is initially neuroprotective as reducing inhibitory innervation of motor neurons increases firing of synaptic NMDA (N-methyl-D-aspartate) receptors and inhibits cell death pathways by triggering cAMP-response element binding protein (CREB) activity and brain-derived neurotrophic factor (BDNF) expression. In the context of chronic demyelination, however, decreased inhibitory innervation of cortical neurons may cause axonal degeneration by increasing the firing of demyelinated axons that have higher than normal energy demands and an impaired ability to produce ATP.

Although little is known about the molecular regulation of mitochondrial genes in neurons, neuronal mitochondrial gene expression can be regulated by neuronal functional activity. Functional magnetic resonance imaging (MRI) studies of MS patients have described reduced cortical energy consumption after inflammatory white matter lesions at sites that contain their axonal projections. Experimental inhibition of sensory

Dutta et al: Mitochondrial Dysfunction in MS 487
input to the human cortex rapidly reduces cortical GABA but not glutamate levels as detected by magnetic resonance spectroscopy (MRS).46 Because the hallmark of MS disease progression is altered neuronal function, it is reasonable to suggest that decreased neuronal mitochondrial gene expression is a downstream event of demyelination and possibly reduced inhibitory innervation. Coordinated regulation of genes involved in similar functions occurs by shared transcriptional regulatory motifs or RNA stability sequences.47,48 Understanding the mechanisms that regulate nuclear-encoded mitochondrial genes in upper motor neurons may lead to therapeutics that increase demyelinated axonal ATP production. A variety of ionotrophic and metabotropic receptors can be activated by reverse operation of the Na+ transporters leading to further overload of cellular Ca2+.49 The Na+ channel blockers phenytoin and flecainide have been shown to reduce axonal degeneration and neurological disability in rodent models of MS.50,51 Our data hence provide further rationale for modifiers of neurotransmitter system Na+ channel and/or Na+/Ca2+ exchanger blockers as candidates for neuroprotective therapeutics in chronic stages of MS.

Additional Information

Primers used for RT PCR and in situ hybridization studies and fold changes of mitochondrial transcripts compared between control, normal-appearing white matter, and white matter lesions are included in Supplemental Data I. Details of the microarray experimentation and analysis and biological classification of altered transcripts are enclosed as Supplemental Data II.

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References


