Imaging Correlates of Decreased Axonal Na^+/K^+ ATPase in Chronic Multiple Sclerosis Lesions

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Objective: Degeneration of chronically demyelinated axons is a major cause of irreversible neurological decline in the human central nervous system disease, multiple sclerosis (MS). Although the molecular mechanisms responsible for this axonal degeneration remain to be elucidated, dysfunction of axonal Na^+/K^+ ATPase is thought to be central. To date, however, the distribution of Na^+/K^+ ATPase has not been studied in MS lesions.

Methods: The percentage of axons with detectable Na^+/K^+ ATPase was determined in 3 acute and 36 chronically demyelinated lesions from 13 MS brains. In addition, we investigated whether postmortem magnetic resonance imaging profiles could predict Na^+/K^+ ATPase immunostaining in a subset (20) of the chronic lesions.

Results: Na^+/K^+ ATPase subunits $\alpha 1$, $\alpha 3$, and $\beta 1$ were detected in the internodal axolemma of myelinated fibers in both control and MS brains. In acutely demyelinated lesions, Na^+/K^+ ATPase was detectable on demyelinated axolemma. In contrast, 21 of the 36 chronic lesions (58%) contained less than 50% Na^+/K^+ ATPase-positive demyelinated axons. In addition, magnetic resonance imaging-pathology correlations of 20 chronic lesions identified a linear decrease in the percentage of Na^+/K^+ ATPase-positive axons and magnetization transfer ratios ($p < 0.0001$) and T1 contrast ratios ($p < 0.0006$).

Interpretation: Chronically demyelinated axons that lack Na^+/K^+ ATPase cannot exchange axoplasmic Na^+ for K^+ and are incapable of nerve transmission. Loss of axonal Na^+/K^+ ATPase is likely to be a major contributor to continuous neurological decline in chronic stages of MS, and quantitative magnetization transfer ratios and T1 contrast ratios may provide a noninvasive surrogate marker for monitoring this loss in MS patients.

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Multiple sclerosis (MS) is a demyelinating disease of the central nervous system that leads to irreversible neurological decline. MS afflicts more than 1.5 million people in North America and Europe, where it is the major cause of nontraumatic neurological disability in young adults.[1] Degeneration of chronically demyelinated axons is now considered to be a major contributor to the permanent neurological disability that most MS patients eventually endure.[2,3] The preservation of axons within chronic lesions of MS presents a unique therapeutic challenge. Although we are beginning to elucidate potential mechanisms for this axonal degeneration,[4] we have no means by which to measure this axonal pathology in a clinical setting.

Myelin is a tightly compacted membrane spiral that surrounds axons in the central and peripheral nervous systems. During formation of the myelin sheath, voltage-gated sodium (Na_v) channels are concentrated at the nodes of Ranvier, small, unmyelinated axonal segments that separate adjacent myelin internodes. Because axonal membrane depolarization occurs only at the nodes, conduction velocities of myelinated axons are approximately 100 times faster than those of unmyelinated axons where Na_v channels are diffusely distributed.[5] After each depolarization, the Na^+/K^+ ATPase rapidly exchanges axonal Na^+ for extracellular K^+ in an energy-dependent manner. Rapid repolarization permits rapid and repetitive axonal firing, a necessary event for proper neuronal function. When axons are demyelinated, the Na_v channels diffuse away from the nodes.[6,7] This Na_v channel redistribution increases both Na^+ influx during impulse conductance and the demand for ATP during repolarization of the axolemma. Reduced ATP production and Na^+/K^+ AT-

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Pase dysfunction are thought to initiate a cascade of ionic imbalances that lead to degeneration of chronically demyelinated axons.[8] Specifically, as axonal Na^+ levels increase, the Na^+/Ca^{++} exchanger operates in reverse and exchanges axoplasmic Na^+ for extracellular Ca^{++}.[9–11] Increased axonal Ca^{++} will activate proteolytic enzymes and eventually lead to degeneration of chronically demyelinated axons, and by extension, to progressive neurological decline during the latter stages of MS. Despite the potentially important role of axonal Na^+/K^+ ATPase during axonal degeneration in MS, and a report detailing a loss of Na^+/K^+ ATPase enzymatic activity in chronic MS lesions,[12] axonal Na^+/K^+ ATPase distribution has not been compared in myelinated and demyelinated axons.

Currently, magnetic resonance imaging (MRI) is the most commonly used tool for diagnosis and monitoring of MS. Because of its high sensitivity, MRI is an invaluable method for following the subclinical progression of the disease. Unfortunately, conventional MRI is limited by its low pathological specificity. Postmortem imaging studies have shown that classification of MS lesions, based on multiple imaging modalities, can improve correlations between MRI and specific aspects of pathology.[13–15] For example, hypointensity on T1-weighted images correlates with axonal loss,[16] and decreased magnetization transfer ratio (MTR) is correlated with demyelination.[17] Recently, we have utilized a composite of T2, T1 contrast ratio, and MTR intensity characteristics to identify chronic lesions of MS with axonal loss and increased demyelinated axon diameter.[15] Techniques such as diffusion tensor imaging and magnetic resonance spectroscopy also provide valuable information on underlying pathology,[18] particularly for axonal pathology, but these studies are more difficult to perform in a typical clinical setting. As yet, there are no reliable MRI markers that are both specific for axonal pathology and feasible to measure on a routine basis.

It is well established that much of the disease process in MS patients is clinically silent. As such, brain imaging has become the major surrogate marker of disease activity and the best predictor of disease progression. In this study, we determined the distribution of Na^+/K^+ ATPase subunits in normal human brain and in demyelinated lesions from brains of patients with MS. Subunits $\alpha1$, $\alpha3$, and $\beta1$ were detected on the internodal axolemma and absent from the nodal axolemma of myelinated fibers. In acutely demyelinated brain tissue, all three subunits were retained on demyelinated axolemmas. In contrast, 58% of chronically demyelinated lesions of MS contain less than 50% Na^+/K^+ ATPase-positive axons. We then compared Na^+/K^+ ATPase measurements and postmortem MRI in a subset of chronic MS lesions, and found a linear correlation between the percentages of demyelinated axons without Na^+/K^+ ATPase and decreased MTR and T1 contrast ratios.

Subjects and Methods

Tissue and Lesions

Brains were obtained from 13 patients with MS (Table). Postmortem, in situ MRI was collected on 11 brains, as described previously.[15] Brains were removed and placed in fixative after an average postmortem interval of 5.9 hours. Left and right hemispheres were separated and processed differently. In brains with postmortem MRI, one hemisphere was fixed in 4% paraformaldehyde for 4 weeks, and then rescanned and sliced to ensure coregistration of lesion location. Lesions with MRI data came from this hemisphere (n = 7 hemispheres). The remaining brains or hemispheres were cut into 1cm-thick slices and placed in either 4% paraformaldehyde or rapidly frozen. Lesions without MRI data came from this hemisphere (n = 8 hemispheres). Lesions were identified macroscopically in fixed slices, blocked, cryoprotected, frozen, and sectioned (30μm-thick) on a freezing-sliding microtome. Sections were stained for myelin proteins and major histocompatability complex class II, and classified as acute, chronic active or chronic inactive, as described previously.[19] Three acute and 36 chronic lesions were identified. Control brains (n = 4) were collected and processed as described earlier, but no MRI was obtained.

Magnetic Resonance Imaging and Image-Guided Tissue Sampling

Details of the methods used for MRI-pathology correlations and image-to-tissue coregistration have been described previously.[15] In brief, MRIs were obtained postmortem, using a standardized protocol that included a T2-weighted fluid-attenuated inversion recovery image, a T1-weighted spin-echo image, a pair of images to calculate MTR, and a high-resolution magnetization prepared rapid gradient echo (MPRAGE) image for coregistration purposes. The images were analyzed to segment T2 lesions (hyperintense on fluid-attenuated inversion recovery), and each T2 lesion was further classified based on T1 and MTR characteristics. T1 and MTR contrast ratios were calculated for each region as the mean intensity within the region divided by the mean intensity of the normal-appearing (nonlesional) white matter in the same slice. Based on MTR and T1 contrast ratios, 20 demyelinated lesions from 7 brains were selected for Na^+/K^+-ATPase immunostaining.

Immunocytochemistry

Fixed tissue was cryoprotected, frozen, and sectioned with a sliding microtome (30μm-thick). Sections were rinsed in phosphate-buffered saline, microwaved in 10mM citric acid buffer (pH 6.0), and incubated in 3% hydrogen peroxide and 1% Triton X-100 (Sigma, St. Louis, MO) in phosphate-buffered saline (pH 7.4) for 30 minutes. Double- and triple-labeled sections for confocal analysis were pretreated as described earlier (but without hydrogen peroxide), immunostained for 3 to 5 days at 4°C, and then incubated with fluorescently conjugated secondary antibodies, as described

previously.[19] Sections were mounted and coverslipped with Vectashield (Vector Labs, Burlingame, CA).

Antibodies

The antibodies used in this study are well-characterized and include rabbit anti–myelin basic protein (1:500; Dako, Glostrup, Denmark); mouse anti-human major histocompatability complex class II (1:250 dilution; Dako); mouse anti-Na^+/K^+ ATPase α1 (clone α6F; 1:50 dilution; Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA); goat anti-Na^+/K^+ ATPase α3 (clone C-16; 1:100 dilution; Santa Cruz Biotechnology, Santa Cruz CA); mouse anti-Na^+/K^+ ATPase β1 (clone M17-P5-F11; 1:100 dilution; Affinity BioReagents, Golden, CO); rabbit anti-neurofilament light, medium, and heavy chain (1:2,000 dilution for light and medium, and 1:1,000 dilution for heavy; Serotec, Raleigh, NC); and rabbit anti-Caspr (1:250 dilution; generous gift from James S. Trimmer, University of California, Davis, CA).

Confocal Microscopy

Fluorescently labeled sections were scanned with a Leica SP5 confocal microscope (Leica, Heidelberg, Germany). Laser intensities were adjusted to eliminate channel cross talk. Single optical slices were used for quantitation (Figs 1D–G, 2, and 4; Figs 1A–C represent projected z-stacks of three to five slices covering 1–3μm in depth).

Quantification of Pathology

Percentages of Na^+/K^+ ATPase-labeled neurofilament-positive axons were quantified in control brain, acute and chronic demyelinated lesions, and normal-appearing white matter from individuals with MS from images obtained by confocal microscopy. Six fields (one single optical section was obtained using a 100× NA 1.3 objective lens and 1.96X zoom, resulting in an area of 79.4μm^2) were counted for each lesion or area of normal-appearing white matter (an average of approximately 108 axons for each field), and data

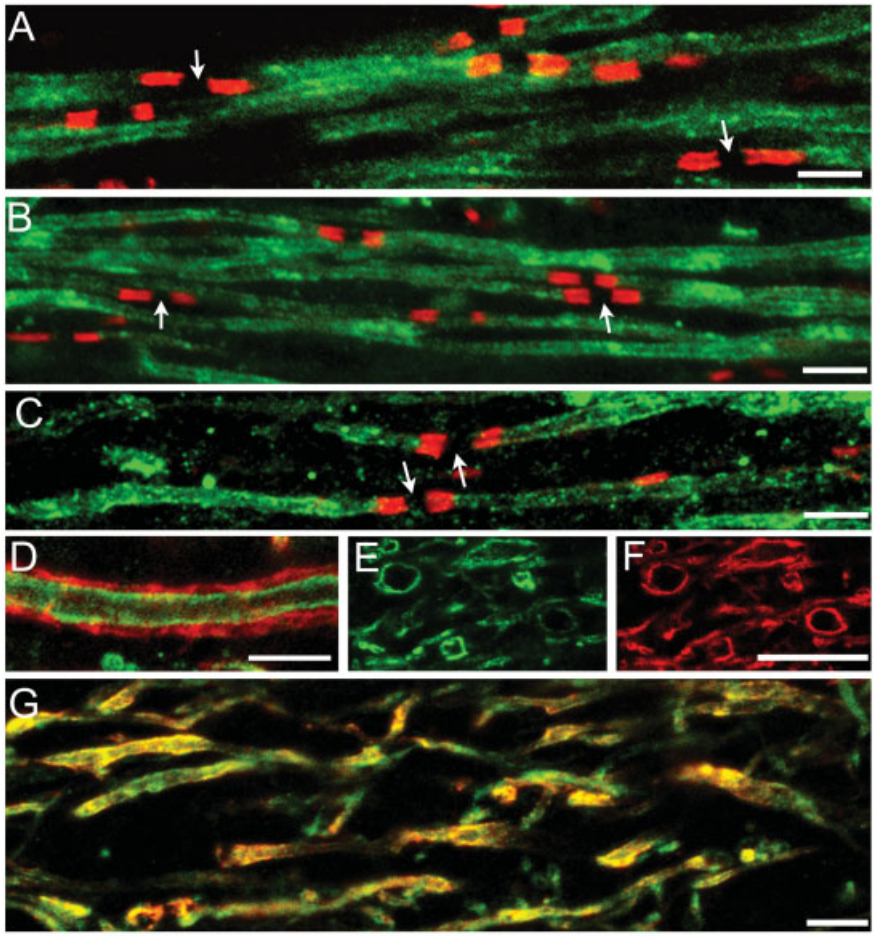

Fig 1. Na^+/K^+ ATPase is enriched in the internodal axolemma of myelinated axons in the adult human brain. Na^+/K^+ ATPase subunits α1 (A, green), α3 (B, green), and β1 (C, green) are located at the axolemma of myelinated axons. As demarcated by the paranodal marker Caspr (A–C, red), the Na^+/K^+ ATPase is enriched in the internodal axon and not detected in paranodal regions or nodal axolemma (A–C, arrows*). Na^+/K^+ ATPase (D, green) was clearly expressed along the axolemma and below myelin sheaths stained with myelin basic protein antibodies (D, red). The α subunits displayed differential labeling in some axons (E, α1, green; F, α3, red). In the majority of axons, the α1 (red) and α3 (green) subunits colocalized (G). Scale bars = 5μm.*

Table. **Characteristics of Patients and Number of Lesions Studied**

Patient No.	Age (yr)/ Sex	Clinical Status	Disease Duration (yr)	EDSS Score	Postmortem Interval (hr)	Total Lesions Stained for Na^+/K^+ ATPase (n = 39)	Subset of Lesions with MRI- Na^+/K^+ ATPase Correlation (n = 20)
1	53/M	SP	15	9	17	4	0
2	43/M	RR	1	6	2.8	1	0
3	61/F	SP	35	9.5	10	8	4
4	46/M	SP	23	8	3	8	4
5	57/F	PP	15	6.5	5.5	1	0
6	48/F	SP	27	9	4.7	1	0
7	52/M	SP	25	9.5	4.5	1	0
8	56/F	SP	27	8.5	4.5	3	0
9	53/F	SP	19	6.5	5.9	3	3
10	77/F	SP	46	8	5	2	2
11	56/M	SP	33	9.5	3	3	3
12	52/M	SP	30	8.5	6.7	3	3
13	65/M	SP	46	8.5	4.5	1	1
14	47/F	C[a]	0	0	15	NWM	—
15	52/F	C[b]	0	0	13.5	NWM	—
16	89/M	C[c]	0	0	13	NWM	—
17	65/M	C[d]	0	0	8.5	NWM	—

Control subject (C) causes of death: [a]necrotizing pancreatitis; [b]unknown; [c]fatal hemorrhage; [d]cardiac arrest.
EDSS = Expanded Disability Status Scale; MRI = magnetic resonance imaging; SP = secondary progressive; RR = relapsing remitting; PP = primary progressive; NWM = normal white matter.

are presented as the mean of 6 fields (see Supplementary Table 1). An axon was considered positive for Na^+/K^+ ATPase if it had a neurofilament-positive core and an Na^+/K^+ ATPase-positive region surrounding the core. An axon was considered negative if it had a neurofilament-positive core and no detectable (signal above background level) Na^+/K^+ ATPase labeling. Differences between groups were determined using a Kruskall–Wallis one-way analysis of variance with Dunn's multiple-comparisons posttest. Standard deviation from mean was calculated for each lesion. Fields were scored by two individuals blind to the lesion stage and corresponding MRI characteristics. Images were taken from lesion core, an area equidistant from all myelinated borders.

To assess the relation between Na^+/K^+ ATPase and MTR or T1 contrast ratios, we used a linear mixed model. The model also accounted for correlated data, to allow for multiple regions from a single brain to be grouped.

Results

Na^+/K^+ ATPase Subunits α1, α3, and β1 Are Located in Internodal Axolemma

To determine the normal distribution of Na^+/K^+ ATPases, we colocalized subunits α1 and α3 with Caspr, a protein enriched in paranodal axolemma. As described previously,[20,21] Caspr antibodies intensely stained paranodal regions of central nervous system myelinated fibers (see Figs 1A–C, red). Both α1 and α3 subunit antibodies displayed specific and relatively uniform labeling of the internodal axolemma, with little or no detectable immunoreactivity at the nodal (arrows) or paranodal axolemma (see Figs 1A, B, green). Na^+/K^+ ATPase internodal immunoreactivity did not appear to differ between large or small axons. In sections double-labeled with α1 or α3 Na^+/K^+ ATPase and myelin basic protein antibodies, ATPase immunoreactivity was associated with the internodal axolemma and did not appear to be present in the surrounding myelin sheath (see Fig 1D). When sections were double-labeled with α1 and α3 antibodies, most axons contained both α1 and α3 immunoreactivity, although the relative α1 and α3 axolemmal staining intensity appeared to vary between some axons (see Figs 1E–G; Fig 1E, α1; Fig 1F, α3; Figs 1E, F, arrowheads). The Na^+/K^+ ATPase β1 subunit had a similar distribution to α1 and α3 subunits labeling the internodal but not the nodal or paranodal axolemma (see Fig 1C).

Distribution of Na^+/K^+ ATPase Subunits in Multiple Sclerosis Lesions

The distribution of Na^+/K^+ ATPase α1 and α3 subunits was determined in 39 MS lesions (3 acute, 36 chronic). Lesion stage was based on the distribution and density of major histocompatability complex class II–positive cells, as described previously.[4] Sections were stained with Na^+/K^+ ATPase, myelin basic protein, and neurofilament antibodies, and the percentage of Na^+/K^+ ATPase-positive demyelinated axons were quantified in sections double-labeled for Na^+/K^+ ATPase (a cocktail of both α1 and α3 antibodies) and neurofilament. In the 3 acute and 15 of the 36 chronic MS lesions (42%), Na^+/K^+ ATPase α1 and α3 antibodies stained demyelinated axolemmas with a similar distribution and intensity to that found in myelinated axons in brain sections from control and MS patients (see Figs 2A, B). In contrast, axons in 21 of the 36 chronic MS lesions (58%) had less than 50% Na^+/K^+ ATPase α1- and α3-positive axons (see Fig 2C). In lesions containing ATPase-negative axons, axonal ATPase staining was present in myelinated fibers at the lesion edge, and the transition between ATPase-positive and -negative staining correlated with the presence and absence of myelin (see Fig 2C). These patterns of labeling were consistent in both diaminobenzidine- and fluorescence-labeled sections. In one brain slice, one chronic lesion contained intense Na^+/K^+ ATPase labeling, whereas another chronic lesion had little to no labeling (as demonstrated in Figs 4C, D). Na^+/K^+ ATPase immunoreactivity was either consistently present (see Fig 2D, higher magnification of Fig 2A core) or consistently absent (see Fig 2E, higher magnification of Fig 2C core) along the length of demyelinated axons within the lesion core. The percentage of Na^+/K^+ ATPase-positive axons was similar between control white matter, normal-appearing white matter from MS brains, and acute demyelinated lesions from MS brains (Fig 3). In contrast, in chronic MS lesions, the proportion of Na^+/K^+ ATPase-positive axons ranged from 100 to 0% (see Fig 3). Both control ($p < 0.0001$) and normal-appearing white matter ($p < 0.001$) groups were significantly different from the chronic group. The significance of the difference between acute and chronic groups could not be tested because of the small sample number of acute lesions. The average standard deviation (6 fields/lesion) for the 39 lesions studied was 4.8. The bimodal distribution of Na^+/K^+ ATPase-positive axon percentage in chronic lesions did not correlate with their immunohistochemical classification (ie, lesions with greater percent Na^+/K^+ ATPase-positive axons were not necessarily chronic active, lesions with lower percent Na^+/K^+ ATPase-positive axons were not necessarily chronic in-

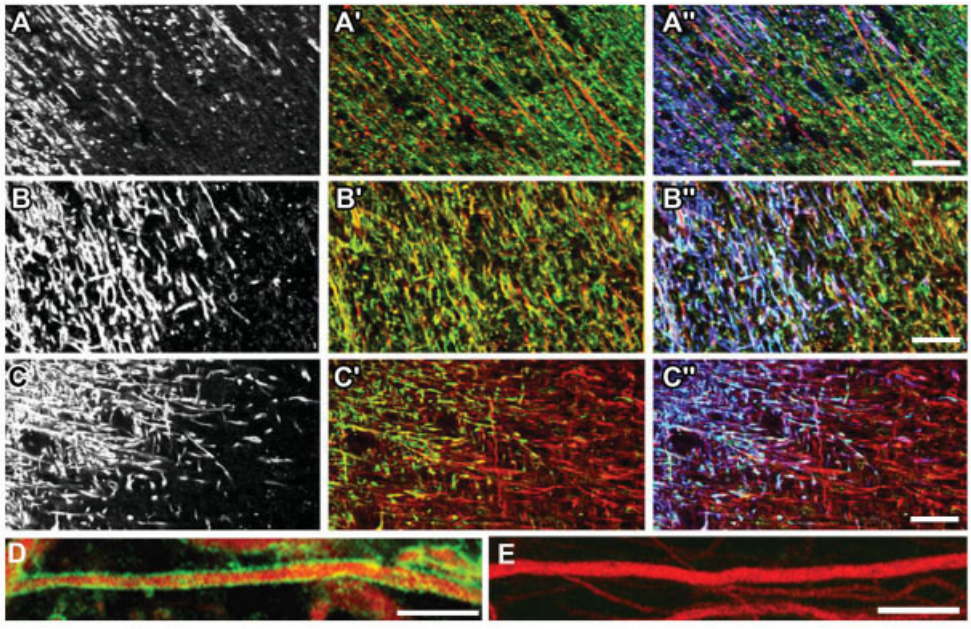

Fig 2. Demyelinated axons in some chronic multiple sclerosis (MS) lesions lack Na^+/K^+ ATPase. Myelin basic protein (MBP) immunoreactivity (white in A–C; blue in A″–C″) identifies myelinated axons and the lesion border. Neurofilament (NF) staining (red) labels myelinated and demyelinated axons. Na^+/K^+ ATPase (green, A–E) distribution is continuous along demyelinated axons in acute lesions (A′, A″) and a subset of chronic lesions (B′, B″). In contrast, Na^+/K^+ ATPase immunostaining is absent from other chronic lesions (C′, C″) but present along myelinated axons at the border of the lesion (C′, C″). Staining for Na^+/K^+ ATPase was consistently present (D, from lesion in A) or absent (E, from lesion in C) along individual axons within the core of a demyelinated lesion. Scale bars = 40μm (A–C); 5μm (D, E).

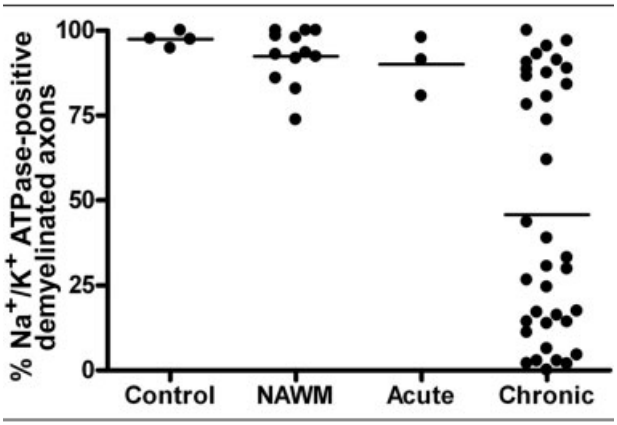

Fig 3. Quantification of Na^+/K^+ ATPase-positive axons in control human brain and multiple sclerosis (MS) lesions. The percentages of Na^+/K^+ ATPase-positive axons in control, normal-appearing white matter (NAWM), and acute MS lesions were closely grouped, all containing high percentages of Na^+/K^+ ATPase-positive axons. In contrast, in chronic MS lesions, the percentage of Na^+/K^+ ATPase-positive axons varied, with 58% (21/36) having Na^+/K^+ ATPase-positive axon percentages of less than 50%. Statistical analysis (analysis of variance with Dunn's multiple-comparisons posttest) confirmed a significant difference between both the control ($p < 0.0001$) and NAWM ($p < 0.001$) groups and the chronic lesion group. Statistical analysis of the difference between the acute lesion group and the chronic lesion group was impossible due to small "acute lesion" group size. Horizontal lines *indicate the mean value for each group. Control tissue, n = 4; NAWM, n = 7; acute lesion, n = 3; chronic lesion, n = 16.*

active). Concordance between measurements made by two observers was high (0.993), indicating excellent reproducibility.

Lesions were also double-labeled with α1 or α3 Na^+/K^+ ATPase and neurofilament antibodies to determine whether demyelinated axons preferentially express either subunit. Demyelinated axons expressed both or neither subunit (see Figs 1E–G), with rare exceptions (not shown). In all stained sections included in this study, normal-appearing white matter served as an internal control for Na^+/K^+ ATPase labeling. Thus, the differential pattern of Na^+/K^+ ATPase labeling in lesion subtypes appears to be a valid observation and not because of staining artifact or tissue processing. Because only a small proportion of a brain's total lesion load was studied, it was not possible to correlate patient sex, duration of disease, or Expanded Disability Status Scale score with percentage of Na^+/K^+ ATPase-negative demyelinated lesions.

Imaging Correlates of Axonal Na^+/K^+ ATPase

One possible explanation of the data described earlier is that chronic demyelination leads to loss of axonal Na^+/K^+ ATPase. We recently correlated axonal loss and increased demyelinated axon diameter with reduced T1 contrast ratios and reduced MTR in postmortem MS brains.[15] In this study, we investigated whether postmortem MRI measurements, selected to represent opposite ends of a scale of pathological severity, could identify MS lesions with (Fig 4A) and without (see Fig 4B) axonal Na^+/K^+ ATPase. Twenty of the 39 demyelinated lesions had postmortem MRI data available. We compared T1 and MTR contrast ratios with the percentage of axons without Na^+/K^+ ATPase. As demonstrated in Figure 4C, demyelinated lesions with MTR contrast ratios greater than 0.8 had normal Na^+/K^+ ATPase distribution and lesions with ratios less than 0.7 had less than 20% of axons with Na^+/K^+ ATPase. Likewise, demyelinated lesions with T1 contrast ratios greater than 0.85 had normal Na^+/K^+ ATPase distribution and lesions with T1 contrast ratios less than 0.75 had less than 20% of axons with Na^+/K^+ ATPase (see Fig 4D). To ensure that lesions were not clustered with other same-brain lesions, we identified lesions from each brain by a unique color symbol in Figures 4C and D. There was a statistically significant linear relationship between percentage axons with Na^+/K^+ ATPase and both MTR ($p < 0.0001$) and T1 contrast ratio ($p < 0.0006$). These observations are the first to correlate MRI characteristics with molecular properties of axons.

Discussion

These data impact our understanding of the pathogenesis of permanent neurological disability during chronic stages of the disease MS in two ways. More importantly, axons in 58% of the 36 chronically demyelinated lesions of MS examined in this study contained less than 50% Na^+/K^+ ATPase-positive axons. Second, quantitative MRI of a subset of these lesions (20) was able to differentiate chronic lesions with and without axonal Na^+/K^+ ATPase. Axons lacking Na^+/K^+ ATPase cannot efficiently transmit action potentials. Reduced exchange of axonal Na^+ for extracellular K^+ will also increase axonal Na^+ concentrations, which will, in turn, reverse the Na^+/Ca^{++} exchanger. Although this will increase axonal Ca^{++} and contribute to Ca^{++}-mediated axonal degeneration, our data support the concept that many chronically demyelinated axons are nonfunctional before degeneration. We propose that loss of axonal Na^+/K^+ ATPase is a contributor to the progressive neurological decline that most MS patients eventually face, and that quantitative MRI may provide a valuable predictor of this process in longitudinal studies of MS patients.

We used well-characterized antibodies to localize the neuronal Na^+/K^+ ATPase subunits to internodal axolemma. The internodal distribution of Na^+/K^+ ATPase spatially uncouples Na^+ influx at nodes from Na^+ efflux at internodes and identifies the periaxonal

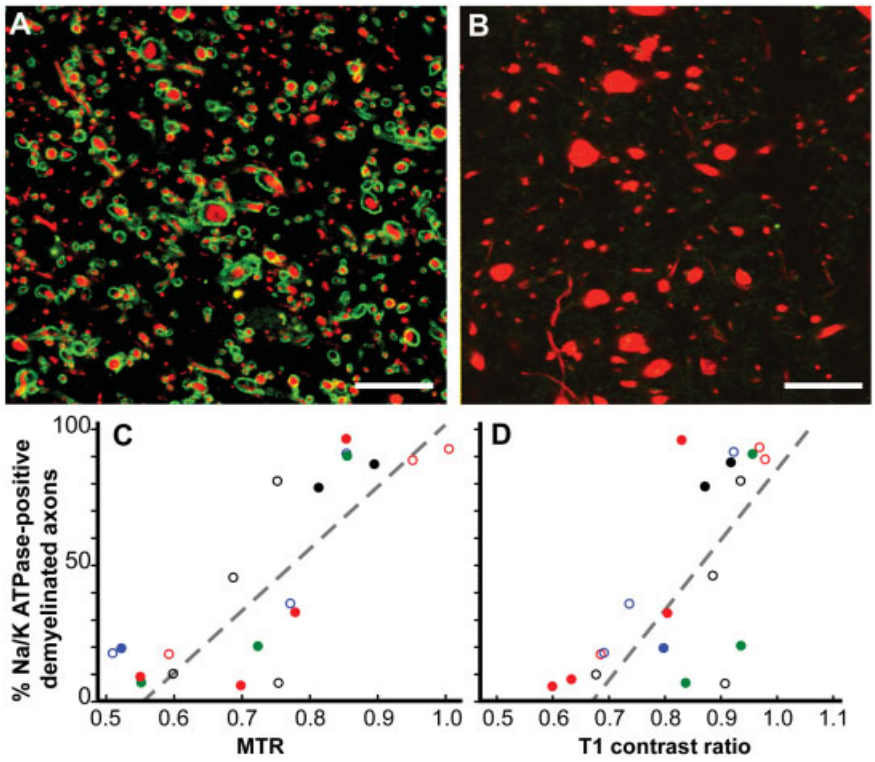

Fig 4. Magnetization transfer ratios (MTRs) and T1 contrast ratios linearly correlate with the percentage of Na^+/K^+ ATPase-positive axons in chronic MS lesions. The percentage of Na^+/K^+ ATPase-positive axons in chronically demyelinated MS lesions were correlated with quantitative postmortem MTR and T1 contrast ratios. (A, B) Chronically demyelinated lesions stained for Na^+/K^+ ATPase (green) and neurofilament (red). The percentage of Na^+/K^+ ATPase-positive axons varied from near 100 (A; MTR = 0.9) to 0% (B; MTR = 0.5). Many axons without Na^+/K^+ ATPase had increased diameters (B). A comparison of the percentage of Na^+/K^+ ATPase-positive axons in chronically demyelinated MS lesions were correlated with quantitative postmortem MTR ($p < 0.0001$; C) and T1 contrast ratios ($p < 0.0006$; D). Dashed lines denote a fit curve to the regression coefficient. Each data point is from a single lesion and each unique color-symbol combination denotes one of the brains studied. Scales bars = 5μm.

space as an important player in regulating membrane potentials during nerve conduction. The periaxonal space is a cloistered, 12- to 14nm-wide, extracellular space restricted by the axolemma, periaxonal membrane of the myelin internode, and the septate junctions, which tether the paranodal loops to the axon.[21] Na^+ and K^+ exchange through the periaxonal space implicates the myelin/oligodendrocyte unit as an active player in nerve conduction and not just a passive insulating participant that renders the internodal axolemma inert to the dynamics of ion exchange and nerve transmission. To date, such ion channels or pumps have not been localized to the periaxonal membrane of the central nervous system myelin internode. If present, they will likely be enriched in the membranes of the inner tongue process, the small cytoplasm-containing tube that extends the length of the myelin internode and is contiguous with the cytoplasm of the paranodal loops. The inner tongue process occupies less than 20% of the periaxonal surface in mature central nervous system internodes; the remainder of the periaxonal membrane is "fused" with the cytoplasmic surface of the first compact myelin lamella.

The percentage of axons without Na^+/K^+ ATPase varied from lesion to lesion within and between postmortem brains (see Figs 2 and 3). Most demyelinated axons in acute MS lesions contained Na^+/K^+ ATPase distribution similar to myelinated areas, whereas a subset of chronic lesions contained a large percentage of axons without Na^+/K^+ ATPase. Pathologically, chronic lesions are categorized as active or inactive based on the presence or absence of activated immune cells at the lesion border.[22] In this study, the loss of Na^+/K^+ ATPase was not preferentially associated with either chronic active or chronic inactive lesions. Based on postmortem MRI analysis, we recently classified pathological characteristics of MS lesions.[15] Compared with T2-only demyelinated lesions, lesions that were abnormal by T2, T1, and MTR ("T2T1MTR lesions") contained fewer axons, and these axons were swollen (increased axonal diameter). We show here that the percentage of Na^+/K^+ ATPase-positive axons in demyelinated T2-only lesions (see Supplemental Fig 1) was similar to that of myelinated axons. In addition, quantitative MTR and T1 contrast ratios delineated chronic MS lesions with and without detectable axonal

Na^+/K^+ ATPase, and those axons without Na^+/K^+ ATPase were swollen (compare axons in Figs 4A, B). A decrease in the MTR or T1 contrast ratio characteristics, therefore, is indicative of the presence of swollen axons and a loss of axonal Na^+/K^+ ATPase. Chronic loss of Na^+/K^+ ATPase, therefore, is likely to contribute to a persistent ion imbalance across the axolemma, which eventually leads to water influx, axoplasmic swelling, and possibly, degeneration.

Although the acute symptoms of MS are not likely to be directly correlated with a depletion of axonal Na^+/K^+ ATPase, loss of the pump on chronically demyelinated axons will contribute to the continuous neurological decline experienced by most MS patients. Based on a dystrophic[19] and/or swollen[23] appearance and reduced Na_v channels,[24] it has been proposed that many demyelinated axons in chronic MS lesions may be functionally compromised before degeneration. The loss of Na^+/K^+ ATPase on chronically demyelinated and swollen axons described here provides additional support to this hypothesis. In addition, the ability of quantitative MTR and T1 contrast ratios to identify MS lesions with little or no axonal Na^+/K^+ ATPase raises the possibility that noninvasive brain imaging techniques may monitor and predict neurological decline and efficacy of neuroprotective therapies in patients with MS.

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References

1. Noseworthy JH, Lucchinetti C, Rodriguez M, et al. Multiple sclerosis. N Engl J Med 2000;343:938–952.
2. Waxman SG. Axonal conduction and injury in multiple sclerosis: the role of sodium channels. Nat Rev Neurosci 2006; 7:932–941.
3. Bjartmar C, Kidd G, Mork S, et al. Neurological disability correlates with spinal cord axonal loss and reduce *N*-acetyl aspartate in chronic multiple sclerosis patients. Ann Neurol 2000;48: 893–901.
4. Trapp BD, Peterson J, Ransohoff RM, et al. Axonal transection in the lesions of multiple sclerosis. N Engl J Med 1998;338: 278–285.
5. Waxman SG. Conduction in myelinated, unmyelinated, and demyelinated fibers. Arch Neurol 1977;34:585–589.
6. Bostock H, Sears TA, Sherratt RM. The spatial distribution of excitability and membrane current in normal and demyelinated mammalian nerve fibres. J Physiol 1983;341:41–58.
7. Waxman SG, Craner MJ, Black JA. Na+ channel expression along axons in multiple sclerosis and its models. Trends Pharmacol Sci 2004;25:584–591.
8. Dutta R, McDonough J, Yin X, et al. Mitochondrial Dysfunction as a cause of anxonal degeneration in multiple sclerosis patients. Ann Neurol 2006;59:478–489.
9. Baker PF, McNaughton PA. Kinetics and energetics of calcium efflux from intact squid giant axons. J Physiol 1976;259: 103–144.
10. Stys PK, Waxman SG, Ransom BR. Ionic mechanisms of anoxic injury in mammalian CNS white matter: role of Na+ channels and Na(+)-Ca2+ exchanger. J Neurosci 1992;12: 430–439.
11. Stys PK, Sontheimer H, Ransom BR, et al. Noninactivating, tetrodotoxin-sensitive Na^+ conductance in rat optic nerve axons. Proc Natl Acad Sci USA 1993;90:6976–6980.
12. Hirsch HE, Parks ME. Na+- and K+-dependent adenosine triphosphatase changes in multiple sclerosis plaques. Ann Neurol 1983;13:658–663.
13. Bruck W, Bitsch A, Kolenda H, et al. Inflammatory central nervous system demyelination: correlation of magnetic resonance imaging findings with lesion pathology. Ann Neurol 1997;42:783–793.
14. Schmierer K, Scaravilli F, Altmann DR, et al. Magnetization transfer ratio and myelin in postmortem multiple sclerosis brain. Ann Neurol 2004;56:407–415.
15. Fisher E, Chang A, Fox R, et al. Imaging correlates of axonal swelling in chronic multiple sclerosis brains. Ann Neurol 2007; 62:219–228.
16. van Walderveen MA, Kamphorst W, Scheltens P, et al. Histopathologic correlate of hypointense lesions on T1-weighted spin-echo MRI in multiple sclerosis. Neurology 1998;50: 1282–1288.
17. van Waesberghe JH, Kamphorst W, De Groot CJ, et al. Axonal loss in multiple sclerosis lesions: magnetic resonance imaging insights into substrates of disability. Ann Neurol 1999;46: 747–754.
18. Bammer R, Augustin M, Strasser-Fuchs S, et al. Magnetic resonance diffusion tensor imaging for characterizing diffuse and focal white matter abnormalities in multiple sclerosis. Magn Reson Med 2000;44:583–591.
19. Chang A, Tourtellotte WW, Rudick R, et al. Premyelinating oligodendrocytes in chronic lesions of multiple sclerosis. N Engl J Med 2002;346:165–173.
20. Einheber S, Zanazzi G, Ching W, et al. The axonal membrane protein Caspr, a homologue of neurexin IV, is a component of the septate-like paranodal junctions that assemble during myelination. J Cell Biol 1997;139:1495–1506.
21. Rios JC, Melendez-Vasquez CV, Einheber S, et al. Contactin-associated protein (Caspr) and contactin form a complex that is targeted to the paranodal junctions during myelination. J Neurosci 2000;20:8354–8364.
22. Lassmann H, Raine CS, Antel J, et al. Immunopathology of multiple sclerosis: report on an international meeting held at the Institute of Neurology of the University of Vienna. J Neuroimmunol 1998;86:213–217.
23. Yin X, Kidd GJ, Pioro EP, et al. Dysmyelinated lower motor neurons retract and regenerate dysfunctional synaptic terminals. J Neurosci 2004;24:3890–3898.
24. Black JA, Newcombe J, Trapp BD, et al. Sodium channel expression within chronic multiple sclerosis plaques. J Neuropathol Exp Neurol 2007;66:828–837.