Quantitative Ultrastructural Analysis of a Single Spinal Cord Demyelinated Lesion Predicts Total Lesion Load, Axonal Loss, and Neurological Dysfunction in a Murine Model of Multiple Sclerosis

Sith Sathornsumetee,* Dorian B. McGavern,[‡]
Daren R. Ure,* and Moses Rodriguez*^{†‡}

From the Departments of Neurology* and Immunology,[‡] and the
Molecular Neuroscience Program,[‡] Mayo Clinic and Foundation,
Rochester, Minnesota

Infection of susceptible mice with Theiler's murine encephalomyelitis virus results in neurological dysfunction from progressive central nervous system demyelination that is pathologically similar to the human disease, multiple sclerosis. We hypothesized that the development of neuropathology proceeds down a final common pathway that can be accurately quantified within a single spinal cord lesion. To test this hypothesis, we conducted quantitative ultrastructural analyses of individual demyelinated spinal cord lesions from chronically infected mice to determine whether pathological variables assessed within a single lesion accurately predicted global assessments of morphological and functional disease course. Within lesions we assessed by electron microscopy the frequencies of normally myelinated, remyelinated, and demyelinated axons, as well as degenerating axons and intra-axonal mitochondria. The frequency of medium and large remyelinated fibers within a single lesion served as a powerful indicator of axonal preservation and correlated with preserved neurological function. The number of degenerating axons and increased intra-axonal mitochondria also correlated strongly with global measures of disease course, such as total lesion load, spinal cord atrophy, and neurological function. This is the first study to demonstrate that functional severity of disease course is evident within a single demyelinated lesion analyzed morphometrically at the ultrastructural level. (*Am J Pathol* 2000, 157:1365-1376)

The role of axonal injury in the development of permanent neurological dysfunction is an important focus of research studying central nervous system (CNS) demyelinating diseases in humans. Multiple sclerosis (MS) is the most common CNS demyelinating disease in humans,¹ and recent studies describing the extent of axonal injury

in MS lesions have stimulated new interest in the role of axonal loss in long-term neurological disability during the course of this disease.^{2,3} Despite the numerous reports of axonal pathology in MS patients, axonal damage after chronic demyelination is not completely understood. Therefore, the study of axonal loss in animal models of demyelinating disease may aid in the understanding of disease pathogenesis and the development of potential treatment strategies.

Theiler's murine encephalomyelitis virus (TMEV) causes a chronic progressive demyelinating disease in susceptible strains of mice that is pathologically indistinguishable from MS.⁴⁻⁹ Intracerebral injection of TMEV into mice of the prototypic susceptible background (SJL/J) results in a biphasic disease characterized by an acute neuronal polioencephalitis followed by chronic white matter demyelination and neurological deficits.¹⁰ Twenty-one days after postinfection, viral persistence is observed in oligodendrocytes, not neurons, and therefore, should not contribute directly to progressive neuronal disruption.^{11,12} During the chronic phase of disease, multifocal demyelinating lesions are primarily localized to the white matter of the brain stem and spinal cord, and the demyelinating process is at least in part immune mediated.¹³ Small, focal demyelinating lesions are detectable by 15 days postinfection,⁴ and the demyelinating phase of the disease plateaus by 100 days postinfection.¹⁴ After this time point, susceptible SJL/J mice continue to decline in neurological function, and this decline is associated with significant increases in spinal cord atrophy and a reduction in myelinated axon frequencies.

Supported by the National Institutes of Health (Grants RO1 NS24180 and RO1 NS32129), and the generous contributions of Mr. and Mrs. Eugene Applebaum and Ms. Kathryn Peterson. S. S. is supported by the Faculty of Medicine, Siriraj Hospital, Mahidol University, Thailand. D. B. M. is supported by a predoctoral NRSA from the National Institute of Mental Health (Grant 1F31ME12120). We also appreciate Ms. Dyana Saenz's contribution to the project.

Accepted for publication July 10, 2000.

S. S. and D. B. M. contributed equally to the manuscript.

Address reprint requests to Moses Rodriguez, M.D., Mayo Clinic, 200 First St., SW, Rochester, MN 55905. E-mail: rodriguez.moses@mayo.edu.

Disease course after TMEV infection of susceptible SJL/J mice is heterogeneous. Mice studied during the chronic phase of disease vary in lesion load, axonal loss, and neurological function. The goal of this study was to determine whether pathological variables assessed from a single demyelinated lesion serve as an indicator of neurological dysfunction and the severity of disease course. This hypothesis was based on recent studies in MS indicating that mechanisms of immunopathogenesis within individual patients may serve as an indicator of disease severity.^{15,16} We tested this hypothesis by conducting quantitative ultrastructural analyses of individual demyelinated spinal cord lesions from SJL/J mice infected for 192 days. We previously demonstrated significant total spinal cord demyelination (a measure of lesion load), spinal cord atrophy, myelinated axon loss in the normal-appearing white matter, and neurological dysfunction in the chronically infected SJL/J mice used in this study.¹⁴ This provided the unique opportunity to conduct an independent, blinded study to determine whether correlative relationships existed between variables assessed within a single demyelinated lesion and global assessments of spinal cord pathology and neurological function.

Materials and Methods

Mice

At 8 weeks of age, SJL/J mice ($n = 7$) (Jackson Laboratories, Bar Harbor, ME) used in these studies were anesthetized and injected intracranially with 2×10^6 pfu of the Daniel's strain of TMEV in a 10 μ l volume. Six age-matched SJL/J mice were sham-infected intracranially with 10 μ l of phosphate-buffered saline and used as controls. Care and handling of mice conformed to the guidelines of both the National Institutes of Health and the Mayo Clinic Animal Care and Use Committee.

Tissue Processing

Mice were anesthetized intraperitoneally with 10 mg of pentobarbital at 192 days postinfection and perfused via intracardiac puncture with 50 ml of Trump's fixative (phosphate-buffered 4% formaldehyde with 1% glutaraldehyde, pH 7.2). The spinal cords were sectioned transversely into 1-mm blocks, postfixed with osmium tetroxide, and embedded in Araldite (Polysciences; Warrington, PA). Cross-sections (1- μ m thick) were cut from every other 1-mm block to obtain a complete representation of all cord regions. These 1- μ m cross-sections were stained with 4% paraphenylenediamine to label myelin. An average of 15 sections per mouse was analyzed by light microscopy to determine percentages of total spinal cord demyelination and C7 combined lateral and anterior column areas. For quantitative ultrastructural analyses using electron microscopy, a single mid-thoracic spinal cord lesion was selected by light microscopy from the anterolateral columns (Figure 1) using the 1- μ m paraphenylenediamine-stained cross-sections for all in-

fected and sham-infected SJL/J mice. The lesion was selected from the anterolateral columns of the thoracic spinal cord because this is the most common location to observe lesions.¹⁷ Thin sections were cut at 0.1 μ m and counterstained with uranyl acetate and lead citrate.

Quantitative Ultrastructural Analysis

All ultrastructural analyses were performed in a blinded and nonbiased manner on $\times 3,000$ photographs captured using an electron microscope (JEOL 1200; JEOL, Peabody, MA). For all mice infected with TMEV, nonoverlapping photographs were taken of an entire demyelinated lesion from the anterolateral columns (mean lesion area, 18,355 μ m²) (see example in Figure 1B). Comparable areas (mean area, 25,606 μ m²) were photographed from the normal white matter in the anterolateral columns of sham-infected mice to serve as a control (example in Figure 1A). An average of 36 photographs was analyzed per mouse. The boundaries of lesions were identified on the electron microscope by the presence of the surrounding normal-appearing white matter.

Calculation of Axonal Areas

Cross-sectional axons were traced on the electron microscope photographs by placing a transparency (3 mol/L; Austin, TX) over the photograph and then outlining the axolemma of each axon in the field. Longitudinal axons and astrocytic processes were excluded from the analysis. An average of 3,756 axons was traced for each mouse. The total number of axons traced for sham-infected and infected mice was 32,377 and 16,450, respectively. Axons traced in the demyelinated lesions of infected mice were divided into three separate categories: demyelinated, remyelinated, and normally myelinated. A demyelinated axon was defined as one that completely lacked a surrounding myelin sheath (examples in Figure 1D). Oligodendrocyte-mediated remyelination was identified by the appearance of abnormally thin myelinated axons relative to axonal diameter when compared to normally myelinated axons (examples in Figure 1E).¹⁸ To further ensure that remyelinated axons were categorized separately from normally myelinated axons, we calculated the ratio of myelin thickness to axonal diameter in remyelinated axons using a $\times 10$ magnifier that contained a micrometer. We reported previously that remyelinated axons have a myelin thickness to axonal diameter ratio of < 0.14 (mean, 0.08).¹⁹ Axons remyelinated by infiltrating Schwann cells were identified by thicker myelin sheaths, a surrounding basement membrane, and a one-to-one relationship between the Schwann cell body and the axon (Figure 1F). After tracing all of the axons for each of the photographs, transparencies were digitized using a Hewlett Packard scanner. Axonal areas for normally myelinated, remyelinated, and demyelinated fibers were then quantified using a program written for the KS400 image analysis software (Kontron Elektronik GmbH; Munich, Germany). The program calculated the number and area of axons from each

Figure 1. Ultrastructural analysis of the spinal cord white matter from sham-infected and 192-day-infected SJL/J mice. A representative mid-thoracic spinal cord cross-section is shown for a sham-infected (A) and a 192-day-infected (B) SJL/J mouse. White matter was trimmed from the anterolateral columns (black outline) and prepared for electron microscopy. Representative electron micrographs are shown for sham-infected (C) and 192-day-infected (D–F) SJL/J mice. Areas of normally myelinated axons were quantified from sham-infected mice (C). Note the intact axons and thick myelin sheaths. Areas of normally myelinated, demyelinated, and remyelinated axons were quantified from the lesions of 192-day-infected mice (D–F). Demyelinated axons have an intact axolemma but no myelin sheath. Representative examples are denoted with asterisks (D). Examples of normally myelinated axons in D are denoted with arrows. Axons were remyelinated by either oligodendrocytes (O) or infiltrating Schwann cells (S). The majority of the axons in E are remyelinated by oligodendrocytes (examples denoted with asterisks). Note the thin myelin sheaths when compared to the normally myelinated axons in C. Less than 1% of the remyelinated axons were remyelinated by Schwann cells. F illustrates Schwann cell remyelination (examples denoted with asterisks). Note the thicker myelin sheaths and increased space between individual axons.

transparency. Axonal data for each mouse were represented as axonal frequency distributions. Axonal frequency distributions were calculated by dividing the number of axons with areas ranging from 0 μm^2 to 85

μm^2 (using 0.5- μm^2 intervals as bins) by the total number of axons sampled. Relative frequencies were calculated for all fibers sampled and for individual fiber types (normally myelinated, remyelinated, and demyelinated). For

example, axonal frequency distributions for myelinated axons were calculated by dividing the number of normally myelinated axons in each $0.5\text{-}\mu\text{m}^2$ bin by all of the normally myelinated axons sampled. Frequency histograms were further divided into three different size categories to facilitate comparisons: 0 to $4\text{ }\mu\text{m}^2$ (small fibers); $4\text{ }\mu\text{m}^2$ to $10\text{ }\mu\text{m}^2$ (medium fibers); and $>10\text{ }\mu\text{m}^2$ (large fibers).

Quantification of Mitochondria

Intraaxonal mitochondria were counted in all normally myelinated, remyelinated, and demyelinated axons sampled. The data were represented as the number of mitochondria per axonal area (in μm^2).

Quantification of Degenerating Axons

Degenerating axons were identified as axons containing an axoplasmic mass filled with numerous floccular dense bodies, swelling mitochondria, and granular disintegration of neurofilaments and vesicles (see examples in Figure 3, A and B).²⁰ Myelin ovoids devoid of underlying axolemma or axoplasm were also included as degenerating axons. Ovoids within macrophages were not included as degenerating axons. Data were represented as the total number of degenerating axons counted.

Total Spinal Cord Demyelination

The percentage of total spinal cord demyelination (or total lesion load) is a measure of all lesions in the spinal cord white matter and was assessed as described.¹⁴ Briefly, a Zeiss interactive digital analysis system and camera lucida attached to a Zeiss photomicroscope (Carl Zeiss Inc., Thornwood, NY) were used to first determine the total white matter area from 10 to 12 paraffin-phenylenediamine-stained spinal cord sections per mouse by tracing all of the white matter. The area of total spinal cord demyelination was determined by tracing each of the demyelinated lesions. Data were represented as percent total spinal cord demyelination per mouse by dividing the total area of demyelination by the total area of white matter sampled and then multiplying by 100.

Spinal Cord Atrophy

We measured the C7 combined lateral and anterior column area as described.¹⁴ There is atrophy in both cervical and thoracic spinal cord of 192-day-infected SJL/J mice. C7 was selected because this spinal cord level was reproducibly obtained for all mice used in the study and allowed for assessment of both ascending and descending fiber tracts. Briefly, an Olympus Provis AX70 microscope fitted with a SPOT color digital camera and a $\times 1.25$ objective was used to digitize the spinal cord cross-section corresponding to C7 for each mouse. A program written for the KS400 image analysis software was then used to calculate the anterior and lateral white

matter area (which included the anterior, lateral, and anterolateral columns) from each section after manually outlining the region. Data were represented as the C7 anterior and lateral column area (in mm^2) for each mouse.

Rotarod Analysis

The Rotamex rotarod (Columbus Instruments, Columbus, OH) measures motor coordination/balance and was used as described.¹⁴ Briefly, sham-infected and infected SJL/J mice were trained at 191 days after injection using a constant speed rotarod assay (speed, 5 rpm; time, 3 minutes; trials, 3) to familiarize them with the rotarod. This was followed by analysis of all mice at 192 days using an accelerated (7 rpm/minutes) rotarod assay (start speed, 5 rpm; end speed, 40 rpm; time, 5 minutes; trials, 3). The amount of time on the rotarod (seconds) was recorded for each of the three trials per mouse and then averaged.

Statistical Analyses

Sham-infected and infected relative axonal frequencies were compared using an unpaired Student's *t*-test ($P < 0.05$). Mitochondria per axonal area for sham-infected and infected mice were statistically compared using a one-way analysis of variance. Pairwise comparisons were made using the Student-Newman-Keuls Method ($P < 0.05$). All correlation coefficients were calculated using a Pearson product moment correlation ($P < 0.05$).

Results

Ultrastructural Pathology Observed in the Demyelinated Lesions of 192-Day-Infected SJL/J mice

We used electron microscopy to conduct a detailed analysis of individual lesions from SJL/J mice infected with TMEV for 192 days. Comparable areas from the anterolateral columns were selected from sham-infected, age-matched control mice (Figure 1, A and B, black boxes). Individual spinal cord lesions sampled from TMEV-infected mice ranged in size from 7,296 to 35,000 μm^2 (Table 1). These lesions contained varying degrees of pathology that included macrophage infiltration, astrocytic scars, collagen deposition, denuded axons, axonal degeneration, and remyelinated axons (Table 1). Using electron micrographs, we accurately classified axons into three different categories based on myelin sheath thickness: normally myelinated, remyelinated, and demyelinated. Normally myelinated axons have thick myelin sheaths relative to the axon diameter, and were observed in the white matter of sham-infected mice (Figure 1C) and in the lesions of 192-day-infected mice (Figure 1D, arrows). An average of 45% of the axons sampled from the lesions of infected mice were normally myelinated (Table 1; range, –11 to 73%). Demyelinated axons represented 25% of the axons sampled in infected mice (Table 1;

Table 1. Lesion Summaries of Individual SJL/J Mice Infected for 192 Days

Mouse	Lesion area (μm^2)	Axons (n)	Lesion fiber types (%)			Macrophages (n)	Degenerating axons (n)
			NM	RM	DM		
1	13,000	911	73	9	19	26	147
2	35,000	3,910	53	25	22	49	469
3	8,190	1,133	52	41	7	7	94
4	7,296	1,002	64	24	12	5	171
5	20,000	4,111	46	28	26	18	547
6	32,000	4,283	11	56	33	44	324
7	13,000	1,100	20	26	54	38	158

Abbreviations: NM, normally myelinated; RM, remyelinated; DM, demyelinated.

range, -7 to 54%) and were identified as axons with axolemma but no myelin sheaths (Figure 1D, asterisks).

In chronically infected SJL/J mice spontaneous remyelination is incomplete and occurs only late in disease when gliosis is also present.⁴ Axons can be remyelinated by oligodendrocytes (Figure 1E) or infiltrating Schwann cells (Figure 1F). Oligodendrocyte-mediated remyelination was identified by a thin myelin sheath relative to axon diameter (Figure 1E, asterisks). Oligodendrocyte-mediated remyelination was responsible for $>99\%$ of the total remyelination observed in the lesions of infected SJL/J mice. Schwann cell remyelination accounted for $<1\%$ of the total remyelination quantified (Figure 1F, asterisks). An average of 30% of the axons sampled from the lesions of infected mice were remyelinated (Table 1; range, -9 to 56%).

Reductions in Large Axonal Frequencies Are Observed in Demyelinated Lesions

We determined the severity of axonal loss from individual demyelinated lesions. All axons (normally myelinated, remyelinated, and demyelinated) were measured from the anterolateral columns of sham-infected and 192-day-in-

fectured mice and plotted as axonal frequency distributions (Figure 2, A–C). We represented the data as axonal frequency distributions rather than axonal densities (axons per unit area) because axonal frequencies are not influenced by factors in the lesions that increase extracellular space, such as edema, inflammatory cells, and gliosis. When axonal frequencies were compared between sham-infected and infected SJL/J mice, no reductions were observed in small axons (Figure 2A). A 9% decrease was observed in the medium axons (Figure 2B) but did not reach statistical significance. The most significant decrease (63%) was observed in the large axons (Figure 2C) of 192-day-infected mice, which is consistent with the 62% decrease in large myelinated fibers estimated previously from the normal-appearing white matter of these same mice.¹⁴ This supports the hypothesis that axonal loss quantified in the normal-appearing white matter results from axons that traverse inflammatory lesions.

Reductions in the frequencies of medium and large axons may result from a combination of axonal atrophy and loss. Axonal atrophy could have resulted from the transection of axons secondary to inflammatory demyelination and/or the loss of trophic support²¹ as a consequence of oligodendrocyte injury or death during the

Figure 2. Axonal frequency distributions calculated from the anterolateral columns. Axonal frequency distributions (A–C) were calculated for all axons sampled from sham-infected and 192-day-infected SJL/J mice. No significant differences in axon frequency distributions for small (A) and medium (B) fibers were observed in 192-day-infected mice when compared to sham-infected controls. The frequency of large fibers (C) was decreased in 192-day-infected mice by 63% . Scale bars represent averages $\pm$ SEM. Asterisk denotes statistical significance by an unpaired Student's *t*-test ($P < 0.05$). Percent decreases from sham-infected controls are shown when statistical significance was reached.

Figure 3. Degenerating axons quantified in the lesions of 192-day-infected SJL/J mice. (**A** and **B**) Some degenerating axons contain an axoplasmic mass filled with numerous floccular dense bodies, swelling mitochondria, and granular disintegration of neurofilaments and vesicles (**single arrows**). Other degenerating axons have a dark amorphous axoplasm (**B**, **double arrow**). Myelin ovoids devoid of axoplasm were occasionally engulfed by macrophages, however, these were not included as degenerating axons (**B**, **asterisks**). Oligodendrocytes are denoted with an O. **C:** A strong negative correlation was found between the combined C7 lateral and anterior column area and the number of degenerating axons quantified in the lesions. **D:** A strong negative correlation was also found between rotarod performance and the number of degenerating axons. Correlation coefficients were calculated using a Pearson product moment correlation ($P < 0.05$).

course of demyelinating disease.²² However, we hypothesize that axonal loss rather than atrophy best explains the reductions in medium and large fibers. We determined the average cross-sectional area reduction in the thoracic combined lateral and anterior columns of 192-day-infected SJL/J mice as compared to age-matched, sham-infected controls to be $326,036 \mu\text{m}^2$. We calculated that if a single axon was not lost during the course of disease, axonal atrophy alone could only account for $<10\%$ of actual spinal cord area reduction observed (calculated reduction because of axonal atrophy, $27,703 \mu\text{m}^2$). This calculation was made using the mean axolemma area of all small, medium, and large fibers sampled previously¹⁴ from sham-infected (mean, $1.94 \mu\text{m}^2$) and 192-day-infected (mean, $1.80 \mu\text{m}^2$) and the estimated number of total fibers (192,943) in the combined lateral and anterior columns of sham-infected mice. A similar result was obtained if the mean axolemma areas obtained from this study were used in the calculation.

The Number of Degenerating Axons in a Lesion Correlates with Spinal Cord Atrophy and Neurological Deficits

Normally myelinated, remyelinated, and demyelinated degenerating axons were frequently observed in the lesions of 192-day-infected SJL/J mice (Table 1 and Figure 3, A and B). Axons were identified as degenerating if their axoplasm contained numerous floccular dense bodies, swelling mitochondria, and granular disintegration of neurofilaments and vesicles (Figure 3, A and B, single arrows). Some degenerating axons also had a dark amorphous axoplasm (Figure 3B, double arrows). Myelin ovoids devoid of axoplasm were occasionally engulfed by macrophages (Figure 3B, asterisks). The number of degenerating axons counted in the lesions of infected mice ranged from 94 to 469 (Table 1).

To determine whether degenerating axons correlated with other pathological or functional variables, we assessed the relationships between the number of degenerating axons and total spinal cord demyelination, atrophy, and rotarod performance. No correlation was observed between the percentage of total spinal cord demyelination and the number of degenerating axons sampled from a single demyelinated lesion (plot not shown). The C7 combined lateral and anterior column area has been shown to correlate almost perfectly with the frequency of medium and large myelinated axons ($\geq 4 \mu\text{m}^2$) measured in the normal-appearing spinal cord white matter, suggesting that this measure of spinal cord atrophy is a strong indicator of axonal loss.¹⁴ In the present study, a near perfect negative correlation was found between the C7 combined lateral and anterior column area and the number of degenerating axons within a single lesion (Figure 3C) ($r = -0.90$, $P = 0.014$). These data suggest that axonal degeneration results in reduced spinal cord areas and are consistent with the hypothesis that the C7 combined lateral and anterior column area is a measure of spinal cord atrophy and axonal loss.

The rotarod is a sensitive measure of motor coordination and balance and has been shown to correlate with measures of spinal atrophy and axonal loss in 192-day-infected SJL/J mice.¹⁴ Interestingly, the number of degenerating axons within a single demyelinated lesion correlated almost perfectly with rotarod performance (Figure 3D) ($r = -0.91$, $P = 0.005$), demonstrating that quantification of the number of degenerating axons within one lesion can predict the overall neurological function in a 192-day-infected mouse. The number of degenerating axons quantified in a lesion likely reflects both axonal loss and lesion size, as a positive linear correlation approaching statistical significance was obtained between lesion size (Table 1) and the number of degenerating axons (plot not shown) ($r = 0.73$, $P = 0.063$).

Analysis of Medium and Large Axons within a Single Lesion Correlates with Total Lesion Load, Spinal Cord Atrophy, and Neurological Dysfunction

We hypothesized that the frequency of medium and large axons ($\geq 4 \mu\text{m}^2$) in a lesion would serve as the strongest indicator of other global pathological and functional variables, because axonal fibers in this size category were preferentially affected in the normal-appearing white matter of 192-day-infected mice.¹⁴ Lesion axonal frequency distributions were calculated for all fibers and normally myelinated, remyelinated, and demyelinated fibers, separately (see Materials and Methods). The percentage of total spinal cord demyelination serves as a measure of total lesion load. A strong negative correlation was obtained between lesion load and the frequency of all medium and large ($\geq 4 \mu\text{m}^2$) axons (Figure 4A) ($r = -0.84$, $P = 0.020$). To determine which fibers were responsible for this correlation, we plotted the percentage of total

spinal cord demyelination *versus* the frequency of medium and large normally myelinated, remyelinated, and demyelinated fibers, separately. Each was expressed as a function of the total number of normally myelinated, remyelinated, and demyelinated fibers sampled, respectively. For example, the frequency of medium and large normally myelinated fibers was calculated by dividing the number of normally myelinated fibers $>4 \mu\text{m}^2$ by the total number of normally myelinated fibers sampled. Thus, the frequency of medium and large normally myelinated fibers only reflects normally myelinated fibers, but not demyelinated or remyelinated fibers. The strongest negative correlation was obtained between percentage of total spinal cord demyelination and the frequency of medium and large ($\geq 4 \mu\text{m}^2$) normally myelinated axons (Figure 4B) ($r = -0.91$, $P = 0.005$). Correlation coefficients did not reach statistical significance for frequencies of medium and large remyelinated ($r = -0.67$, $P = 0.103$) or demyelinated ($r = -0.37$, $P = 0.415$) fibers (plots not shown). These data suggest that the frequency of medium and large normally myelinated fibers measured within a single lesion is a powerful indicator of total lesion load in a 192-day-infected mouse, and that involving more of the spinal cord white matter in the demyelinating process results in greater reductions of medium and large normally myelinated fibers.

Reductions in the C7 combined lateral and anterior column area reflect axonal loss. A near perfect positive correlation was found between the C7 combined lateral and anterior column area and the frequency of all medium and large ($\geq 4 \mu\text{m}^2$) axons assessed within a single lesion (Figure 4C) ($r = 0.93$, $P = 0.007$). To determine which fibers were responsible for this correlation, we plotted C7 combined lateral and anterior column areas *versus* the frequency of medium and large normally myelinated, remyelinated, and demyelinated fibers, separately. A near perfect positive correlation was obtained between C7 combined lateral and anterior column areas and the frequency of medium and large remyelinated fibers (Figure 4D) ($r = 0.95$, $P = 0.003$). Correlation coefficients did not reach statistical significance for the frequency of medium and large normally myelinated ($r = 0.58$, $P = 0.226$) or demyelinated ($r = 0.72$, $P = 0.106$) fibers (plots not shown). No significant linear relationships were obtained if the small fibers ($\leq 4 \mu\text{m}^2$) were used in any correlation. These data indicate that frequency of medium and large remyelinated fibers measured from a single thoracic demyelinated lesion reflects the degree of atrophy measured at a different level of the spinal cord.

We hypothesized that the frequency of medium and large remyelinated fibers within a lesion serves as a surrogate marker for the preservation of medium and large axons in the entire cord. In other words, higher frequencies of medium and large axon remyelinated fibers within a lesion may signify less axonal loss in this size category during the course of disease, and thus, less spinal cord atrophy. Because spinal cord atrophy correlates with rotarod performance (a measure of motor coordination), we tested this hypothesis by plotting rotarod performance *versus* the frequency of medium and large

Figure 4. Linear relationships between the frequency of medium and large fibers ($>4 \mu\text{m}^2$) in the lesions of 192-day-infected mice and other pathological/neurological variables. Axonal frequency distributions for the lesions of individual 192-day-infected mice were plotted against the percentage of total spinal cord demyelination (a measure of lesion load) (**A** and **B**), the C7 lateral and anterior column area (a measure of spinal cord atrophy and axon loss) (**C** and **D**), and rotarod performance (a measure of motor coordination and balance) (**E** and **F**). The frequencies of all medium and large fibers (normally myelinated, remyelinated, and demyelinated) are plotted on the x axis for panels **A**, **C**, and **E**. The frequencies of medium and large individual fiber types that resulted in the strongest correlation coefficients were plotted on the x axis for panels **B**, **D**, and **F**. **A** and **B**: The frequency of all medium and large fibers within the lesion correlated negatively with the percentage of total spinal cord demyelination. Of the individual fiber types, the frequency of medium and large normally myelinated fibers showed the strongest correlation with the percentage of total spinal cord demyelination. **C** and **D**: The frequency of all medium and large fibers within the lesion correlated positively with the C7 lateral and anterior column area. The frequency of medium and large remyelinated fibers showed the strongest correlation with this measure of spinal cord atrophy. **E** and **F**: The frequency of all medium and large fibers within the lesion correlated positively with rotarod performance. The frequency of medium and large remyelinated fibers showed the strongest correlation with this measure of motor coordination. Correlation coefficients were calculated using a Pearson product moment correlation ($P < 0.05$).

axons in the lesion. Interestingly, a positive correlation approaching statistical significance was obtained between rotarod performance and the frequency of all medium and large fibers in the lesion (Figure 4E) ($r = 0.75$,

$P = 0.055$). The strongest correlation was found between rotarod performance and the frequency of medium and large remyelinated fibers (Figure 4F) ($r = 0.79$, $P = 0.033$). Correlation coefficients did not reach statistical

significance for the frequencies of medium and large normally myelinated ($r = 0.44$, $P = 0.318$) or demyelinated ($r = 0.53$, $P = 0.198$) fibers (plots not shown). These data are consistent with the hypothesis that the frequency of medium and large remyelinated axons within a single lesion is an indicator of axonal loss, spinal cord atrophy, and thus, neurological function. In further support of this hypothesis, a strong negative correlation (plot not shown) ($r = -0.84$, $P = 0.018$) was obtained between the frequency of medium and large remyelinated axons and the number of degenerating axons (Table 1) within a lesion. This indicates that increased frequencies of medium and large remyelinated fibers are associated with less degenerating axons.

Alternatively, it is possible that the frequency of medium and large remyelinated axons within a lesion represents functionally repaired axons that aid in the preservation or maintenance of neurological function. To test this possibility, we asked if any relationships existed between the actual percentage of remyelinated axons in a lesion (expressed as a function of the total number of fibers sampled) (Table 1) and rotarod performance. No correlation was obtained between the percentage of remyelinated axons within a lesion and rotarod performance (plot not shown). Furthermore, the percentage of remyelinated medium and large fibers (expressed as function of the total number of medium and large fibers sampled) did not correlate with rotarod performance (plot not shown). In concert, these data are consistent with the former hypothesis that the frequency of medium to large remyelinated fibers within a lesion (expressed as a function of the total number of remyelinated fibers sampled) is a surrogate marker for the survival of all medium to large fibers in the combined lateral and anterior column area. These fibers seem to be required for the maintenance of neurological function assessed by the rotarod.

Intraaxonal Mitochondria Are Increased in Demyelinated Lesions

Increases in intra-axonal mitochondria have been observed previously in demyelinated lesions and could reflect CNS injury or repair.^{20,23-27} We quantified the number of intra-axonal mitochondria for normally myelinated, remyelinated, and demyelinated axons in infected mice to gauge the overall health of axons within a demyelinated lesion (Figure 5A). Mitochondria quantified in the myelinated axons of sham-infected mice were used as controls. Intra-axonal mitochondria were significantly increased in normally myelinated (mean, 0.66 ± 0.03), remyelinated (mean, 0.81 ± 0.05), and demyelinated (mean, 0.97 ± 0.06) fibers of 192-day-infected mice when compared to the myelinated (mean, 0.39 ± 0.03) fibers of sham-infected controls (Figure 5A). Furthermore, within the demyelinated lesions, increases in intra-axonal mitochondria were associated with decreases in myelin surrounding the axons. For example, the highest intra-axonal mitochondria were observed in the demyelinated fibers followed by lower numbers of mitochondria in remyelinated and normally myelinated fibers, respectively.

Because the role of increased intra-axonal mitochondria is not completely understood, we analyzed whether there was any relationship between increased mitochondria and other pathological variables in 192-day-infected mice, such as the percentage of total spinal cord demyelination (a measure of lesion load) and spinal cord atrophy. Interestingly, a strong positive correlation was obtained between the percentage of total spinal cord demyelination and intra-axonal mitochondria for normally myelinated (Figure 5B) ($r = 0.89$, $P = 0.007$) and demyelinated (plot not shown) ($r = 0.78$, $P = 0.037$) axons. These data suggest that involving more of the total spinal cord white matter area in the demyelinating process results in increased intra-axonal mitochondria quantified within a single lesion. A strong negative correlation was also obtained between the C7 combined lateral and anterior column area and the intra-axonal mitochondria for demyelinated axons (Figure 5C) ($r = -0.86$, $P = 0.029$). These data suggest that the relative increases in mitochondria within demyelinated axons of a single lesion are an accurate reflection of the severity of spinal cord atrophy.

Discussion

This study demonstrated that quantitative ultrastructural analysis of pathological variables within a single demyelinated lesion from anterolateral columns of the spinal cord accurately reflects the percentage of total spinal cord demyelination, spinal cord atrophy measured at C7, and neurological dysfunction assessed by the rotarod. Our findings indicate that measures of axonal preservation or health serve as the strongest indicators of neurological function during the course of TMEV-induced demyelinating disease. More importantly, the present study demonstrated that the severity of disease course is evident within a single spinal cord lesion located in the anterolateral columns. This may be relevant to the study of MS and other demyelinating diseases.

The results from this study support the hypothesis that the assessment of axonal loss within the normal-appearing white matter represents the secondary effects of axons that projected through inflammatory lesions. This concept has also been described recently in a study of three patients with large, solitary brain demyelinating lesions of the type seen in early MS.²⁸ One month after observing the lesion in the patients, reductions in NAA (a measure of neuronal integrity)²⁹ were observed in the normal-appearing white matter of the contralateral hemisphere that were homologous to those observed in the lesions. In the present study, we observed a reduction in the frequency of large fibers ($\geq 10 \mu\text{m}^2$) in the lesions of the anterolateral columns of 192-day-infected mice when compared to sham-infected controls. Moreover, the reduction in the frequency of large fibers observed in individual demyelinated lesions was comparable to the reduction observed previously¹⁴ in the normal-appearing white matter (63% versus 62%, respectively). A small reduction (9%) in the frequency of medium fibers ($4 \mu\text{m}^2$ to $10 \mu\text{m}^2$) was observed that did not reach statistical significance.

Figure 5. Quantification of intra-axonal mitochondria. **A:** Intra-axonal mitochondria were calculated for the myelinated axons of sham-infected mice, and the normally myelinated (NM), remyelinated (RM), and demyelinated (DM) axons of 192-day-infected mice. Intra-axonal mitochondria were increased for all fibers assessed in the 192-day-infected mice when compared to the normally myelinated fibers of sham-infected controls. **B:** The mitochondria quantified in the normally myelinated axons of 192-day-infected mice correlated positively with the percentage of total spinal cord demyelination (a measure of lesion load) calculated from the entire spinal cord white matter ($r = 0.89$, $P = 0.007$). **C:** The mitochondria quantified in the demyelinated axons of 192-day-infected mice correlated negatively with the combined C7 lateral and anterior column area (a measure of spinal cord atrophy and axon loss) ($r = -0.86$, $P = 0.029$). Scale bars in **A** represent average $\pm$ SEM. Asterisks denote statistical significance using a one-way analysis of variance ($P < 0.05$). Correlation coefficients in **B** and **C** were calculated using a Pearson product moment correlation ($P < 0.05$).

One of the most interesting findings from this study was the fact that although TMEV-induced demyelinating disease is heterogeneous, the frequencies of medium and large fibers calculated within a single demyelinated lesion served as a powerful indicator of disease course in individual 192-day-infected mice. By analyzing individual lesions located in the anterolateral columns, we could accurately estimate total lesion load, spinal cord atrophy, and neurological function. A strong negative correlation ($r = -0.91$, $P = 0.005$) was obtained between the percentage of total spinal cord demyelination and the frequency of medium and large myelinated axons within the

lesion. The results suggest that the animals with the most total spinal cord demyelination had the greatest medium and large normally myelinated fiber loss during the course of disease. A near perfect positive correlation ($r = 0.95$, $P = 0.003$) was obtained between the C7 combined lateral and anterior column area and the frequency of medium and large remyelinated axons within the lesion. Interestingly, the number of degenerating axons within a single lesion also correlated negatively with C7 combined lateral and anterior column area ($r = -0.90$, $P = 0.014$). This confirms that the C7 combined lateral and anterior column area is a measure of spinal cord atrophy, and

thus, axonal loss. These data are also in agreement with magnetic resonance imaging literature that use brain and spinal cord atrophy as a surrogate marker for axonal loss in MS.^{30,31}

In the TMEV model of progressive demyelination, we hypothesize that the frequency of medium and large remyelinated axons remaining in a lesion serves as a strong indicator of the medium and large axon loss that occurred during the course of disease. We propose the following as an explanation. During the course of TMEV-induced demyelinating disease, some axons in all size categories are demyelinated. Based on our studies, the small axons are relatively preserved, whereas the medium and large demyelinated axons will either remain demyelinated, degenerate, or begin the process of repair (ie, remyelination). Degeneration will eventually result in decreased medium and large normally myelinated fibers assessed in the normal-appearing white matter. The medium and large axons that survive the demyelinating process and begin the process of repair will become remyelinated. Thus, we hypothesize that if greater frequencies of medium and large remyelinated axons are observed in the lesion, then less medium and large axons were lost during the course of disease. In support of this hypothesis, a strong negative correlation ($r = -0.84$, $P = 0.018$) was obtained between the frequency of medium and large remyelinated axons and the number of degenerating axons within a lesion, indicating that less axonal degeneration is associated with greater preservation of medium and large axons. Furthermore, it is also important to note that the frequency of medium and large remyelinated fibers correlated with a measure of motor coordination ($r = 0.79$, $P = 0.033$), demonstrating the role of these medium and large fibers in the maintenance of neurological function.

The mechanism(s) that results in the preferential loss of medium to large fibers is not completely understood. We propose two hypotheses to explain this preferential loss. In murine/human amyotrophic lateral sclerosis only the largest caliber, neurofilament-rich axons are lost.^{32,33} Abnormal accumulations of neurofilaments are observed in the motor neurons that give rise to these axons.^{34,35} Deficits in slow³⁶ and fast³⁷ axonal transport precede motor neuron degeneration and clinical disease in two murine models of amyotrophic lateral sclerosis. Based on these studies, it has been suggested that axonal transport may be especially crucial to the long, large caliber axons and disruptions may result in axonal strangulation of these large, neurofilament-rich axons.³⁸ Therefore, disruptions in axonal transport resulting from demyelination and alterations in neurofilaments could explain the preferential loss of medium and large fibers in the TMEV model.

The second hypothesis for the preferential loss of medium and large fibers in the TMEV model involves intra-axonal calcium levels. Electron probe X-ray microanalysis has demonstrated that large CNS and peripheral nervous system axons have threefold to fourfold more intra-axonal calcium than small fibers.³⁹ *In vitro* models of white matter injury have demonstrated that axonal damage can result from increases in intra-axonal calci-

um.^{40–44} Because large axons have a pre-existing high concentration of Ca^{2+} , a threshold may be reached more rapidly, resulting in the activation of effector proteins⁴⁵ that have the potential to cause axonal injury and degeneration. The role of this mechanism of axonal damage/loss has been demonstrated recently for spinal cord injury⁴⁶ in which large fibers are preferentially lost.^{47–50}

Mitochondria are essential for oxidative processes and energy utilization in axons, and their function may be disrupted by increases in intra-axonal calcium.⁵¹ Increases in intra-axonal mitochondria have frequently been observed by electron microscopy in injured axons.^{20,23–27} Interestingly, we found increased intra-axonal mitochondria in normally myelinated, remyelinated, and demyelinated axons of 192-day-infected mice when compared to the myelinated axons of sham-infected controls. The reason for increased mitochondria in injured axons is not completely understood, but may reflect oxidative stress,⁵² impaired axonal transport,²⁵ attempts at restoring conduction,²⁷ and/or axonal regeneration.⁵³ Strong correlation coefficients were obtained between the percentage of total spinal cord demyelination and intra-axonal mitochondria for normally myelinated and demyelinated axons. This may signify that more stress is being placed on individual axons at multiple levels of the spinal cord. Furthermore, strong correlation coefficients were also obtained between the C7 combined lateral and anterior column area, the number of degenerating axons, and intra-axonal mitochondria for demyelinated axons. These data suggest that increases in intra-axonal mitochondria are at least in part an indicator of axonal injury, although alternative explanations are also plausible.

In summary, this is the first study to demonstrate in a model of progressive spinal cord demyelination that quantifying pathological variables at the ultrastructural level within a single demyelinated lesion can serve as a strong indicator of functional severity. A number of factors may induce axonal loss during the course of a progressive demyelinating disease. Future studies will investigate potential mechanisms that lead to axonal loss after progressive CNS demyelination and will use therapeutic interventions in an attempt to reduce this axonal loss and the subsequent irreversible neurological dysfunction.

References

1. Noseworthy JH: Progress in determining the causes and treatment of multiple sclerosis. *Nature* 1999, 399:A40–A47
2. Ferguson B, Matyszak MK, Esiri MM, Perry VH: Axonal damage in acute multiple sclerosis lesions. *Brain* 1997, 120:393–399
3. Trapp BD, Peterson J, Ransohoff RM, Rudick R, Mork S, Bo L: Axonal transection in the lesions of multiple sclerosis. *N Engl J Med* 1998, 338:278–285
4. Dal Canto MC, Lipton HL: Primary demyelination in Theiler's virus infection. *Lab Invest* 1975, 33:626–637
5. Lipton HL, Dal Canto MC: Chronic neurologic disease in Theiler's virus infection of SJL/J mice. *J Neurol Sci* 1976, 30:201–207
6. Leirich JR, Arnason BGW, Hochberg FH: Demyelinative myelopathy in mice induced by the DA virus. *J Neurol Sci* 1976, 29:149–160
7. Dal Canto MC, Lipton HL: Multiple sclerosis. Animal model: Theiler's virus infection in mice. *Am J Pathol* 1977, 88:497–500
8. Dal Canto MC, Lipton HL: Recurrent demyelination in chronic central

- nervous system infection produced by Theiler's murine encephalomyelitis virus. *J Neurol Sci* 1979, 42:391–405
9. Rodriguez M, Oleszak E, Leibowitz J: Theiler's murine encephalomyelitis: a model of demyelination and persistence of virus. *Crit Rev Immunol* 1987, 7:325–365
10. Lipton HL: Theiler's virus infection in mice: an unusual biphasic disease leading to demyelination. *Infect Immun* 1975, 11:1147–1155
11. Dal Canto MC, Lipton HL: Ultrastructural immunohistochemical localization of virus in acute and chronic demyelinating Theiler's virus infection. *Am J Pathol* 1982, 106:20–29
12. Rodriguez M, Leibowitz J, Lampert PW: Persistent infection of oligodendrocytes in Theiler's virus-induced encephalomyelitis. *Ann Neurol* 1983, 13:426–433
13. Lipton HL, Dal Canto MC: Theiler's virus-induced demyelination: prevention by immunosuppression. *Science* 1976, 192:62–64
14. McGavern DB, Murray PD, Rivera-Quinones C, Schmelzer JD, Low PA, Rodriguez M: Axonal loss results in spinal cord atrophy, electrophysiologic abnormalities, and neurologic deficits following demyelination in a chronic inflammatory model of multiple sclerosis. *Brain* 2000, 123:519–531
15. Lucchinetti CF, Bruck W, Rodriguez M, Lassman H: Distinct patterns of multiple sclerosis pathology indicates heterogeneity in pathogenesis. *Brain Pathol* 1996, 6:259–274
16. Lucchinetti CF, Bruck W, Parisi JE, Scheithauer B, Rodriguez M, Lassman H: A quantitative analysis of oligodendrocytes in multiple sclerosis lesions—a study of 113 cases. *Brain* 1999, 122:2279–2295
17. Rivera-Quinones C, McGavern D, Schmelzer JD, Hunter SF, Low PA, Rodriguez M: Absence of neurological deficits following extensive demyelination in a class I-deficient murine model of multiple sclerosis. *Nat Med* 1998, 4:187–193
18. Ludwin SK: Remyelination in the central nervous system and the peripheral nervous system. *Adv Neurol* 1988, 47:215–254
19. Miller DJ, Rodriguez M: Spontaneous and induced remyelination in multiple sclerosis and the Theiler's virus model of central nervous system demyelination. *Microsc Res Tech* 1995, 32:230–245
20. Lampert PW: A comparative electron microscopic study of reactive, degenerating, regenerating, and dystrophic axons. *J Neuropathol Exp Neurol* 1967, 26:345–366
21. Bernstein M, Lichtman JW: Axonal atrophy: the retraction reaction. *Curr Opin Neurobiol* 1999, 9:364–370
22. Tsunado I, Kurtz CI, Fujinami RS: Apoptosis in acute and chronic central nervous system disease induced by Theiler's murine encephalomyelitis virus. *Virology* 1997, 228:388–393
23. Perier O, Gregoire A: Electron microscopic features of multiple sclerosis lesions. *Brain* 1965, 88:937–952
24. Perier O: Electron microscopic observations on human demyelinating diseases. *Int Arch Allergy* 1969, 36:452–462
25. Friede RL, Ho KC: The relation of axonal transport of mitochondria with microtubules and other axoplasmic organelles. *J Physiol* 1977, 265:507–519
26. Prineas JW, Connell F: The fine structure of chronically active multiple sclerosis plaques. *Neurology* 1978, 28:68–75
27. Mutsaers SE, Carroll WM: Focal accumulation of intra-axonal mitochondria in demyelination of the cat optic nerve. *Acta Neuropathol* 1998, 96:139–143
28. De Stefano N, Narayanan S, Matthews PM, Francis GS, Antel JP, Arnold DL: *In vivo* evidence for axonal dysfunction remote from focal cerebral demyelination of the type seen in multiple sclerosis. *Brain* 1999, 122:1933–1939
29. Urenjak J, Williams SR, Gadian DG, Noble M: Proton nuclear magnetic resonance spectroscopy unambiguously identifies different neural cell types. *J Neurosci* 1993, 13:981–989
30. Simon JH, Jacobs LD, Campion MK, Rudick RA, Cookfair DL, Herndon RM, Richert JR, Salazar AM, Fischer JS, Goodkin DE, Simonian N, Lajaunie M, Miller DE, Wende K, Martens-Davidson A, Kinkel RP, Munschauer FE, Brownschidle CM: A longitudinal study of brain atrophy in relapsing multiple sclerosis. *Neurology* 1999, 53:139–148
31. Trapp BD, Ransohoff RM, Rudick R: Axonal pathology in multiple sclerosis: relationship to neurologic disability. *Curr Opin Neurol* 1999, 12:295–302
32. Kawamura Y, Dyck PJ, Shimono M, Okazaki H, Tateishi J, Doi H: Morphometric comparison of the vulnerability of peripheral motor and sensory neurons in amyotrophic lateral sclerosis. *J Neuropathol Exp Neurol* 1981, 40:667–675
33. Bruijn LI, Becher MW, Lee MK, Anderson KL, Jenkins NA, Copeland NG, Sisodia SS, Rothstein JD, Borchelt DR, Price DL, Cleveland DW: ALS-linked SOD1 mutant G85R mediates damage to astrocytes and promotes rapidly progressive disease with SOD1-containing inclusions. *Neuron* 1997, 18:327–338
34. Carpenter S: Proximal enlargement in motor neuron diseases. *Neurology* 1968, 18:841–851
35. Hirano A, Donnenfeld H, Sasaki S, Nakano I: Fine structural observations of neurofilamentous changes in amyotrophic lateral sclerosis. *J Neuropathol Exp Neurol* 1984, 43:461–470
36. Williamson TL, Cleveland DW: Slowing of axonal transport is a very early event in the toxicity of ALS-linked SOD1 mutants to motor neurons. *Nat Neurosci* 1999, 2:50–56
37. Collard J, Cote F, Julien J: Defective axonal transport in a transgenic mouse model of amyotrophic lateral sclerosis. *Nature* 1995, 375:61–64
38. Cleveland DW: From Charcot to SOD1: mechanisms of selective motor neuron death in ALS. *Neuron* 1999, 24:515–520
39. Stys PK, Lehning E, Saubermann AJ, LoPachin RM: Intracellular concentrations of major ions in rat myelinated axons and glia: calculations based on electron probe x-ray microanalyses. *J Neurochem* 1997, 68:1920–1928
40. Ransom BR, Stys PK, Waxman SG: The pathophysiology of anoxic injury in central nervous system white matter. *Stroke* 1990, 21:III52–III57
41. Stys PK, Ransom BR, Waxman SG, Davis PK: Role of extracellular calcium in axonic injury of mammalian central white matter. *Proc Natl Acad Sci USA* 1990, 87:4212–4216
42. Stys PK, Sontheimer H, Ransom BR, Waxman SG: Noninactivating, tetrodotoxin-sensitive Na^+ conductance in rat optic nerve axons. *Proc Natl Acad Sci USA* 1993, 90:6976–6980
43. Waxman SG, Black JA, Ransom BR, Stys PK: Axonic injury of rat optic nerve: ultrastructural evidence for coupling between Na^+ influx and Ca^{2+} -mediated injury in myelinated CNS axons. *Brain Res* 1994, 644:197–204
44. Imaizumi T, Kocsis JD, Waxman SG: Anoxic injury in the rat spinal cord: pharmacological evidence for multiple steps in Ca^{2+} -dependent injury of the dorsal columns. *J Neurotrauma* 1997, 14:299–311
45. Shields DC, Schaefer KE, Saido TC, Banik NL: A putative mechanism of demyelination in multiple sclerosis by a proteolytic enzyme, calpain. *Proc Natl Acad Sci USA* 1999, 96:11486–11491
46. Rosenberg LJ, Teng YD, Wrathall JR: Effects of the sodium channel blocker tetrodotoxin on acute white matter pathology after experimental contusive spinal cord injury. *J Neurosci* 1999, 19:6122–6133
47. Blight AR, Decrescito V: Morphometric analysis of experimental spinal cord injury in the cat: the relation of injury intensity to survival of myelinated axons. *Neuroscience* 1986, 19:321–341
48. Fehlings MG, Tator CH: The relationships among the severity of spinal cord injury, residual neurological function, axon counts, and counts of retrogradely labeled neurons after experimental spinal cord injury. *Exp Neurol* 1995, 132:220–228
49. Rosenberg LJ, Wrathall JR: Quantitative analysis of acute axonal pathology in experimental spinal cord contusion. *J Neurotrauma* 1997, 14:823–838
50. Rosenberg LJ, Teng YD, Wrathall JR: 2,3-Dihydroxy-6-nitro-7-sulfamoyl-benzo(f)quinoxaline reduces glial loss and acute white matter pathology after experimental spinal cord contusion. *J Neurosci* 1999, 19:464–475
51. Carafoli E: Calcium homeostasis in excitable cells. *Biochem Soc Trans* 1988, 16:519–520
52. Mecocci P, MacGarvey U, Beal MF: Oxidative damage to mitochondrial DNA is increased in Alzheimer's disease. *Ann Neurol* 1994, 36:747–751
53. Dimova RN, Markov DV: Changes in the mitochondria in the initial part of the axon during regeneration. *Acta Neuropathol* 1976, 36:235–242