Phenytoin Protects Spinal Cord Axons and Preserves Axonal Conduction and Neurological Function in a Model of Neuroinflammation In Vivo

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Lo, Albert C., Carl Y. Saab, Joel A. Black, and Stephen G. Waxman. Phenytoin protects spinal cord axons and preserves axonal conduction and neurological function in a model of neuroinflammation in vivo. J Neurophysiol 90: 3566–3571, 2003. First published August 6, 2003; 10.1152/jn.00434.2003. Axonal degeneration within the spinal cord contributes substantially to neurological disability in multiple sclerosis (MS). Thus neuroprotective therapies that preserve axons, so that they maintain their integrity and continue to function, might be expected to result in improved neurological outcome. Sodium channels are known to provide a route for sodium influx that can drive calcium influx, via reverse operation of the Na+/Ca2+ exchanger, after injury to axons within the CNS, and sodium channel blockers have been shown to protect CNS axons from degeneration after experimental anoxic, traumatic, and nitric oxide (NO)-induced injury. In this study, we asked whether phenytoin, which is known to block sodium channels, can protect spinal cord axons from degeneration in mice with experimental allergic encephalomyelitis (EAE), which display substantial axonal degeneration and clinical paralysis. We demonstrate that the loss of dorsal corticospinal tract (63%) and dorsal column (cuneate fasciculus; 43%) axons in EAE is significantly ameliorated (corticospinal tract: 28%; cuneate fasciculus: 17%) by treatment with phenytoin. Spinal cord compound action potentials (CAP) were significantly attenuated in untreated EAE, whereas spinal cords from phenytoin-treated EAE had robust CAPs, similar to those from phenytoin-treated control mice. Clinical scores in phenytoin-treated EAE at 28 days were significantly improved (1.5, i.e., minor righting reflex abnormalities) compared with untreated EAE (3.8, i.e., near-complete hindlimb paralysis). Our results demonstrate that phenytoin has a protective effect in vivo on spinal cord axons, preventing their degeneration, maintaining their ability to conduct action potentials, and improving clinical status in a model of neuroinflammation.

INTRODUCTION

A large body of evidence indicates that axonal degeneration occurs in multiple sclerosis (MS) (Ferguson et al. 1997; Trapp et al. 1998), and it has been suggested that axonal degeneration contributes to the acquisition of persistent (nonremitting) clinical deficits (Davie et al. 1995; Lee et al. 2000). The loss of axons in the spinal cord, in particular, appears to contribute significantly to neurological disability in animal models of MS and in MS (Bjartmar et al. 2000; Ganter et al. 1999; Lovas et al. 2000; Wujek et al. 2002). Thus neuroprotective interventions that maintain axonal integrity and preserve conduction might be expected to slow or halt the development of neurological disability.

Although a number of studies have shown that it is possible to use immunomodulatory interventions to ameliorate the clinical course of experimental allergic encephalomyelitis (EAE), an animal model of MS in which axonal degeneration is known to occur (Korneck et al. 2000; Raine and Cross 1989; White et al. 1992), neuroprotective strategies that directly target neurons have not been studied in EAE. Evidence suggesting that voltage-gated sodium channels might be tractable targets for neuroprotection of white matter axons was provided by early studies (Stys et al. 1992a). This work demonstrated that voltage-gated sodium channels can participate in the production of calcium-mediated degeneration of white matter axons after injury by providing a route for persistent sodium current that drives reverse operation of the Na+/Ca2+ exchanger which, in turn, results in a damaging influx of calcium. As suggested by this mechanism, sodium channel blockade with tetrodotoxin (TTX) and tertiary and quarternary anesthetics has been shown to prevent the development of irreversible dysfunction of axons within the anoxic optic nerve in vitro (Stys et al. 1992a,b). In vitro studies have also demonstrated that phenytoin, a drug that blocks sodium channels and inhibits persistent sodium currents (Chao and Alzheimer 1995; Segal and Douglas 1997), has a protective effect on axons within white matter that has been subjected to anoxia (Fern et al. 1993).

Although the molecular events leading to axonal degeneration in MS have not been delineated, it has been suggested that, as a result of inflammatory damage to small blood vessels, hypoxia may contribute to neuronal damage in MS (Lassmann 2003). In addition, a role for sodium channels is suggested by the protective effects of TTX (Garthwaite et al. 2002) and of low doses of lidocaine and flecainide (Kapoor et al. 2003) on experimental axonal injury triggered by nitric oxide (NO); NO is present at elevated concentrations in MS lesions (Bo et al. 1994; Brosnan et al. 1994; Smith et al. 1999) and, like anoxia, is thought to trigger mitochondrial failure with resultant energy depletion and an increase in intra-axonal sodium (Bolanos et al. 1997; Brown et al. 1995; Kapoor et al. 2003). Reasoning that a persistent sodium current might provoke reverse Na+/Ca2+ exchange that contributes to axonal injury in inflamma-

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tory disorders of the CNS, Lo et al. (2002) recently studied the optic nerve in EAE and observed an increased number of preserved axons after treatment with phenytoin. These initial results in the optic nerve suggest that phenytoin may have a neuroprotective effect in EAE as measured by axon counts.

In the present study, we have examined the effect of phenytoin on axonal degeneration within the dorsal columns (cuneate fasciculus) and dorsal corticospinal tracts of the spinal cord. Using axonal counting, electrophysiology, and behavioral assessment after induction of EAE, we show that treatment with phenytoin results in robust protection of the integrity of spinal cord axons, preservation of action potential conduction, and amelioration of neurological deficits.

**METHODS**

**Immunocytochemistry**

We measured the number of axons within the cervical spinal cord after staining them for neurofilaments. Mice were perfused with phosphate-buffered saline (PBS) and then with 4% paraformaldehyde in 0.14 M Sorensen’s phosphate buffer. Spinal cords were carefully excised from the brain stem to the lumbar region and cryoprotected with 30% sucrose in PBS. The cervical enlargement was identified and then transected at the exact midpoint of the cervical enlargement to standardize a site along the longitudinal axis of the cord, ensuring that the same cervical spinal cord regions were analyzed for all conditions. Transverse sections were cut and incubated with antibodies against phosphorylated neurofilaments (SMI-31, 1:20,000; Sternerber Monoclonals, Lutherville, MD) and nonphosphorylated neurofilaments (SMI-32, 1:20,000) overnight at 4°C on a rotating shaker. To count all axons, including axons with both phosphorylated and nonphosphorylated neurofilaments (Lo et al. 2002; Lovas et al. 2000; Wujek et al. 2002), we incubated sections with SMI-31 and -32 antibodies in combination or individually with SMI-32, which has been used as a marker for demyelinated or damaged white matter axons (Trapp et al. 1998), and with SMI-31. (As noted in RESULTS, SMI-32 immunostained axons accounted for <5% of the total axon population in each region studied, and analysis of tissue stained for SMI-32 and SMI-31 individually did not reveal any trends that differed from those for the total axon population stained by SMI-32 and -31 in combination.) Sections reacted with SMI-31 and/or -32 were then incubated sequentially in PBS, rabbit anti-mouse IgG-biotin (1:1000, Sigma), PBS, avidin-HRP (1:1000, Sigma), PBS, and heavy metal-enhanced DAB (Pierce, Rockford, IL).

**Image acquisition and analysis**

Sections were examined with a Nikon E800 microscope equipped with a ×100 oil objective, and digital images were captured with a Spot RT color camera (Spot Diagnostic Instruments, Sterling Heights, MI). Axonal densities were determined within preselected fields (500 μm² in area) at specific sites within the dorsal column (cuneate fasciculus), lateral column, ventral column, and dorsal corticospinal tract white matter regions, using the borders of the gray matter as consistent landmarks (Fig. 1A). Neurofilament stained axons were manually counted from each 500-μm² area using IPLab software (Scanalytics, Fairfax, VA) as described previously (Lo et al. 2002). The number of mice analyzed in each group were as follows: control, 4; control + phenytoin, 5; EAE, 6; and EAE + phenytoin, 5. Statistical analyses between control and experimental groups were performed using Student’s t-test with Microsoft Excel software.

**Electrophysiological recordings**

Fourteen adult male C57/BL6 mice were used for assessing spinal cord conduction. Untreated EAE (n = 6) and phenytoin-treated EAE (n = 4) mice were studied between days 24 and 30 and age-matched phenytoin-treated mice (n = 4) were used as controls. Mice were anesthetized with halothane, and deep anesthesia was maintained with 1.1% halothane through a tracheal cannula by artificial ventilation (75–80 strokes/min, 0.7 ml/stroke) and verified by the absence of a withdrawal reflex to noxious peripheral pinch. Heartbeats (~360 beats/min) and body temperature were carefully monitored. Mice were fixed in a stereotaxic apparatus (Kopf Instruments). A midline incision was made through the skin, a laminectomy was performed, and the dura was carefully cut to expose the lower thoracic and lumbar spinal cord regions.

Silver wire electrodes (0.01-in diam, A-M Systems) insulated except at the tips were used for stimulation of dorsal column axons at L4–5 and recording of compound action potentials (CAP) at T11–12 (Fig. 3A). Using stereotaxic measurements, the electrodes were positioned 8 mm apart at the surface of the midline spinal cord. Pancuronium bromide (0.3 mg/kg, Sigma) was injected i.p. to prevent further muscular contractions throughout the experiment. The signal from the recording electrode was amplified with filters set at 300–3,000 Hz (Dam 80, WPI) and collected (CED 1401+, Cambridge Electronic Design), and a computer was used for data analysis (Spike 2 program, Cambridge Electronic Design). Single-current pulses (0.05 ms) were applied through a stimulus isolation unit (A365, WPI, and Master-8, AMPI) in increments (0.2, 0.3, 0.6, 0.8, and 1 mA). The amplitude of CAP was calculated as the value between the positive and negative peaks of the biphasic wave (Fig. 3B). The area of the CAP was calculated by rectifying the negative component (full-wave rectification using Spike 2 script software, Cambridge Electronic Design) and measuring its area. Conduction velocity was estimated by measuring the latency to the peak of the negative component of the supramaximal CAP at 8 mm, then moving the recording electrode to 9 mm and measuring latency; conduction velocities measured in this manner were within 5% of conduction velocities measured between the stimulating electrode and the recording electrode at 8 mm. At the end of each experiment, the dorsal columns and corticospinal tract were transected between stimulating and recording electrodes to confirm that a CAP could not be detected.
Induction of EAE

Experiments were carried out in accordance with National Institutes of Health guidelines for the use of laboratory animals; all animal protocols were approved by the Yale Institutional Animal Care and Use Committee. C57BL/6 mice 6–10 wk of age were injected subcutaneously with 200 μl of an emulsion of 300 μg of rat myelin-oligodendrocyte glycoprotein (MOG) 35–55 peptide (Keck Biotechnology Center, Yale University) in incomplete Freund’s adjuvant (IFA, Sigma, St. Louis, MO) supplemented with 250–500 μg of Mycobacterium tuberculosis H37Ra (Difco, Detroit, MI). The MOG injection, with mycobacterium supplemented IFA, was repeated in the opposite flank 1 wk later. The mice also received an injection of 250–500 ng pertussis toxin (Sigma) in 200 μl PBS ip immediately after the first immunization with MOG and then again 48 h later.

Immunized mice were observed daily for clinical signs and scored on a 0–6 scale, with 0.5 gradations for intermediate scores, as follows: 0, normal without clinical signs; 1, flaccid tail; 2, abnormal righting reflex; 3, partial hindlimb paralysis; 4, complete hindlimb paralysis; 5, moribund; and 6, death.

Phenytoin treatment

Beginning on day 10 after the initial MOG injection, control and EAE-induced mice were fed pelleted mice chow incorporating phenytoin (5,5 diphenylhydantoin; Sigma). A separate group of EAE mice were fed the identical standard mice chow not containing phenytoin. Serum phenytoin levels were measured by homogeneous enzyme immunoassay (Pinus and Abraham 1991) from two mice in each group on day 14 and on the day of either perfusion (days 27–28) or electrophysiological study (days 24–30). Mean serum phenytoin levels were: day 14: control + phenytoin, 18.3 μg/ml; EAE + phenytoin, 18.0 μg/ml; and day 28 (perfusion): control + phenytoin, 19.0 μg/ml, EAE + phenytoin, 20.1 μg/ml. For mice used in spinal cord electrophysiological studies, phenytoin serum levels were 16.8 μg/ml. These serum phenytoin levels are within the therapeutic range determined in the clinical setting (10–20 μg/ml).

Results

Quantification of axons

We counted the number of cervical spinal cord axons via counts within standardized 500-μm² fields at predetermined sites in the dorsal corticospinal tract, dorsal column (cuneate fasciculus), lateral column, and ventral column (Fig. 1A, Table 1). To identify all axons within these fields in the mid-cervical spinal cord, we labeled axons using a combination of antibodies directed against both phosphorylated (SMI-31) and non-phosphorylated (SMI-32) neurofilaments (Lo et al. 2002; Lovas et al. 2000). The density of axons labeled with neurofilament antibodies within the dorsal corticospinal tract was 162 ± 9/500 μm² in untreated controls and 172 ± 8/500 μm² for phenytoin-treated controls (not statistically significant; P > 0.05).

<table>
<thead>
<tr>
<th>Condition</th>
<th>n</th>
<th>CST</th>
<th>DF</th>
<th>LF</th>
<th>VF</th>
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</thead>
<tbody>
<tr>
<td>control</td>
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<td>162 ± 9</td>
<td>68 ± 8</td>
<td>69 ± 7</td>
<td>64 ± 6</td>
</tr>
<tr>
<td>control + phenytoin</td>
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<td>172 ± 8</td>
<td>74 ± 12</td>
<td>74 ± 8</td>
<td>77 ± 12</td>
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<td>42 ± 3</td>
<td>61 ± 8</td>
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<tr>
<td>EAE + phenytoin</td>
<td>5</td>
<td>125 ± 22</td>
<td>62 ± 6</td>
<td>76 ± 6</td>
<td>72 ± 8</td>
</tr>
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Values are means ± SE, n, number of mice; CST, dorsal corticospinal tract; DF, dorsal column (cuneate fasciculus); LF, lateral funiculus; VF, lateral funiculus.

Phenytoin was orally administered beginning on day 10 after the initial MOG induction of EAE. Phenytoin had a significant protective effect on dorsal corticospinal column and dorsal column (cuneate fasciculus) axons (Fig. 2A). Axon loss in the corticospinal tract of phenytoin-treated EAE was only 27.7% (125 ± 22/500 μm²) compared with 62.5% loss (P < 0.05; see preceding text) in the same region in untreated EAE. Axon loss in the dorsal column (cuneate fasciculus) was also reduced by treatment with phenytoin and was 16.7% (62 ± 6/500 μm²).
compared with a dropout of 43.1% \( (P < 0.05) \) in the same location of untreated EAE (Fig. 2B). In the lateral and ventral columns, where axonal loss was less pronounced (see preceding text), axon densities were maintained near normal in phenytoin-treated EAE \( (76 \pm 6/500 \) and \( 72 \pm 8/500 \mu m^2 \), respectively), but the protective trend in phenytoin-treated EAE compared with untreated EAE did not reach statistical significance in these regions.

Because neurofilaments exist in phosphorylated and non-phosphorylated forms, which may be individually expressed or co-expressed in axons (Sloan and Stevenson 1987), we also immunoreacted cervical spinal cord separately with SMI-32 or -31 antibodies and analyzed the axon densities in the same regions (dorsal corticospinal tract, dorsal column (cuneate fasciculus), lateral column, and ventral column) to determine whether there was a preferential effect of phenytoin on SMI-32 versus SMI-31 positive axons. This analysis did not reveal any trends that differed from those for the total axon population stained with SMI-32 and -31 in combination. The percentage of SMI-32 positive axons (i.e., SMI-32 positive/SMI-31 positive + SMI-32 positive) within the corticospinal tract in untreated EAE was 4.4%; the percentage of SMI-32 positive axons in the CST in phenytoin-treated controls was 0.5% and in phenytoin-treated EAE was 1.0%. Similarly, within the dorsal columns (cuneate fasciculus) the percentage of SMI-32 positive axons was 4.5% in untreated EAE, 0.3% in phenytoin-treated EAE, with 0.9% in phenytoin-treated controls, and 0.9% in phenytoin-treated EAE.

**Spinal cord electrophysiology**

The average compound action potential (CAP) amplitude (Fig. 3, B and C) and CAP area (Fig. 3, D and E) in the untreated EAE group were significantly smaller than in the phenytoin-treated control group \( (P < 0.01) \). In fact, a biphasic CAP could not be recorded in four of six mice in the untreated EAE group. In addition, the average threshold for evoking a CAP in two mice in the untreated EAE group, in which a CAP could be elicited, was higher \( (0.3-0.6 \) mA) than in the phenytoin-treated control group \( (0.2-0.3 \) mA; Fig. 3C). In contrast, robust CAPs with a normal positive-negative configuration were observed in phenytoin-treated EAE, with a threshold similar to that in controls (Fig. 3C), and the average CAP amplitude and area in the phenytoin-treated EAE group were not significantly different from in the phenytoin-treated control group (Fig. 3, C, D, and E). Mean \( \pm SE \) conduction velocity in the EAE group (2.28 \( \pm 1.65 \) m/s) was substantially slower than in controls \( (11.58 \pm 2.10 \) m/s) but was maintained close to normal levels \( (13.15 \pm 1.06 \) m/s; not significant, \( P > 0.05 \) compared with controls) in phenytoin-treated EAE (Fig. 3F).

**Neurological status**

We assessed the clinical score of each mouse in each experimental condition using a rating scale (see METHODS) that is sensitive to motor function, particularly in the hindlimbs. Phenytoin-treated control mice did not display any pathological clinical signs (clinical score = 0). Untreated EAE mice manifested progressive clinical impairment, with an average clinical score of 3.8 \( \pm 0.18 \) \( (n = 6) \) on day 27–28. In contrast, phenytoin-treated EAE mice exhibited a less severe clinical course than untreated mice, with an average clinical score of 1.5 \( \pm 0.26 \) on day 27–28 (Fig. 4). The clinical scores in the untreated EAE and phenytoin-treated EAE mice were significantly different \( (P < 0.05) \) at all times starting on day 21 onward.

**DISCUSSION**

The EAE model used in this study produces substantial degeneration of axons within the dorsal corticospinal tract and dorsal column (cuneate fasciculus) of the spinal cord, with the loss of 62 and 43%, respectively, of axons in these two tracts at 24–28 days of EAE. We observed substantial protection of axons in mice with EAE that were treated with phenytoin with axonal dropout reduced from 62 to 27% in the dorsal corticospinal tract and from 43 to 16% in the cuneate fasciculus. Electrophysiological assessment of axonal transmission, via
monitoring of the dorsal column CAP, demonstrated a loss of conduction along spinal cord axons in untreated EAE, but preservation of conduction along spinal cord axons in phenytoin-treated mice. Moreover, we also observed amelioration of clinical deficits in phenytoin-treated EAE (clinical score = 1.5, i.e., a minor righting reflex abnormality), compared with 3.8 (nearly complete hindlimb paralysis) in untreated EAE.

Our observations of substantial loss of axons within the spinal cord in EAE are consistent with earlier studies (Kornek et al. 2000; Raine and Cross 1989; White et al. 1992), which demonstrated axonal dropout within EAE; however, the present study is the first to document substantial loss of corticospinal tract axons. The robust nature of axonal degeneration in our EAE mice provided an animal model of loss of spinal cord axons, which is known to contribute to the progression of neurological disability in MS (Bjartmar et al. 2000; Losseff et al. 1996; Wujek et al. 2002), against which we could assess the effects of phenytoin. Consistent with the conclusion that axon loss in the spinal cord contributes to the degree of neurological disability in EAE, we found that the loss of spinal cord axons in untreated EAE is accompanied by hind-limb paralysis and that preservation of spinal cord axons in phenytoin-treated EAE is paralleled both by preservation of action potential conduction and by reduced neurological disability (i.e., reduced clinical score).

In rodents, a major component of the corticospinal tract mediating motor behavior is located adjacent to sensory axons within the dorsal columns (Brosamle and Schwab 1997, 2000). Therefore we expected spinal cord conduction to parallel clinical behavior. To functionally assess conduction in the axons of the dorsal columns and corticospinal tract, CAPs were studied in vivo. The CAPs recorded in this study were abolished by transection of the dorsal columns and the dorsal corticospinal tract at the end of each experiment, indicating that they were evoked in axons coursing through the dorsal column and the dorsal corticospinal tract and not in other (lateral or ventral column) axons. The results suggest that conduction in these axons is severely impaired in EAE and shows a protective effect of phenytoin with relatively preserved CAP amplitude and area in phenytoin-treated EAE, in agreement with the histological data and behavioral scores. We observed a substantial reduction in conduction velocity in EAE, with conduction velocities in our control and EAE spinal cords very close to those previously reported (Imaizumi et al. 1998) for control and demyelinated rodent spinal cord. Interestingly, conduction velocities were maintained at values close to normal in phenytoin-treated EAE, suggesting that treatment with phenytoin may have prevented demyelination in addition to protecting axons from degeneration.

The sodium-channel-blocking actions of phenytoin are well established (Ragsdale and Avoli 1998; Ragsdale et al. 1996) and its protective effect on axons within the anoxic optic nerve in vitro has been attributed to the block of axonal sodium channels (Fern et al. 1993; Stys et al. 1993). Imaizumi et al. (1997) demonstrated that a significant component of axonal loss, after anoxic injury to the dorsal columns in vitro, is the result of reverse operation of the sodium Na+/Ca2+ exchanger that is triggered by sodium influx via voltage-gated sodium channels. Tetrodotoxin (Agrawal and Fehlings 1996; Rosenberg et al. 1999; Teng and Wrathall 1997), procaine (Agrawal and Fehlings 1996; Rosenberg et al. 1999), and QX-314 (Agrawal and Fehlings 1997) have been shown to have a protective effect on spinal cord axons in vitro and in vivo models of traumatic injury, suggesting that sodium channels may be more generally involved in the injury-induced cascade that produces degeneration of spinal cord axons. In addition, it has been demonstrated that TTX (Garthwaite et al. 2002) and low concentrations of lidocaine and flecainide (Kapoor et al. 2003) protect axons from degeneration induced by NO, which is present at high levels in MS lesions (Bo et al. 1994; Brosnan et al. 1994; Smith et al. 1999), where it has been postulated to trigger mitochondrial failure and hypoxia-like energy depletion (Kapoor et al. 2003). Moreover, Lassmann (2003) has suggested that hypoxic injury due to inflammatory damage to small blood vessels may contribute to tissue damage in MS.

Phenytoin was originally developed as an antiepileptic medication and is also used clinically for the treatment of some pain syndromes, presumably on the basis of its sodium-channel-blocking properties. Nonetheless, phenytoin is not a specific sodium channel blocker. Phenytoin acts to stabilize membranes in excitable cells and (at concentrations higher than those required for block of sodium channels) blocks calcium channels (McLean and MacDonald 1993; Narahashi 1988) and potassium channels (Yaari et al. 1986). In the anoxic optic nerve model, phenytoin has a protective effect at concentrations of 1 μM, lower than the concentration required for block of sodium, calcium, and potassium channels; it has been suggested that the protective effect of phenytoin in the anoxic optic nerve might be due to the potentiation of sodium channel block by phenytoin by depolarization (Fern et al. 1993). Because phenytoin is not a specific sodium channel blocker, the present results do not provide conclusive evidence that sodium channels contribute to the degeneration of axons in EAE. However, consistent with this suggestion, Bechtold et al. (2002) have observed that systemically administered flecainide has a protective effect on dorsal column axons in a rat model of chronic-relapsing EAE, and TTX has been shown to protect axons so that they do not degenerate after a spectrum of insults (Agrawal and Fehlings 1996; Garthwaite et al. 2002; Stys et al. 1992a; Teng and Wrathall 1997).

In summary, the present results demonstrate that phenytoin has a protective effect on axons in vivo, reducing the degeneration of spinal cord axons in EAE. Our results also demonstrate that the preservation of axons in phenytoin-treated EAE...
is paralleled by preservation of axonal conduction within the spinal cord and by amelioration of clinical deficits. These results may provide a basis for novel therapeutic approaches that will preserve axons and axonal function in neuroinflammatory disorders such as MS.

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DISCLOSURES

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