T-Cell-Mediated Disruption of the Neuronal Microtubule Network

Correlation with Early Reversible Axonal Dysfunction in Acute Experimental Autoimmune Encephalomyelitis

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During the course of the central nervous system autoimmune disease multiple sclerosis (MS), damage to myelin leads to neurological deficits attributable to demyelination and conduction failure. However, accumulating evidence has indicated that axonal injury is also a predictor of MS clinical disease. Using the animal model of MS, experimental autoimmune encephalomyelitis (EAE), we examined whether axonal dysfunction occurred early in disease and correlated with disease symptoms. We tracked axons during EAE by using transgenic mice that express yellow fluorescent protein (YFP) in neurons. At the onset of disease, we observed a loss of YFP fluorescence in the spinal cord in areas that coincided with immune cell infiltration, before prominent demyelination. These inflammatory lesions also exhibited evidence of axonal injury but not axonal loss. During the recovery phase of EAE, the return of YFP fluorescence occurred in parallel with the resolution of inflammation. Using *in vitro* cultured neurons expressing YFP, we demonstrated that encephalitogenic T cells alone directed the destabilization of microtubules within neurites, resulting in a change in the pattern of YFP fluorescence. This study provides evidence that encephalitogenic T cells directly cause reversible axonal dysfunction at the onset of neurological deficits during an acute central nervous system inflammatory attack. (*Am J Pathol* 2006, 169:999–1011; DOI: 10.2353/ajpath.2006.050791)

etiology. It is the leading cause of neurological disability in young adults and affects more than 1.1 million individuals worldwide.^{1,2} Lesions in the CNS of MS patients are characterized by an inflammatory infiltrate consisting primarily of T cells and macrophages surrounding demyelinated plaques.³ Loss of conduction along demyelinated nerve fibers is believed to play a role in the pathogenesis of MS; however, recent studies have renewed interest in axonal pathology as a mediator of clinical symptoms.^{4,5} In brain biopsies taken from newly diagnosed MS patients, axonal injury, detected by the presence of amyloid precursor protein (APP) aggregates, was observed in the early stages of disease in both active demyelinating plaques as well as in normal-appearing white matter.^{3,6} These studies suggested that the myelination status of the neuron is not the sole predictor of vulnerability to injury in diseases such as MS. The relationship between inflammation, oligodendrocyte damage, and neuronal dysfunction is complex, and the mechanisms that lead to tissue damage and clinical symptoms are not well understood. Furthermore, recent evidence from magnetic resonance imaging studies has shown that current treatments such as β -interferon do not stop the accumulation of axonal injury throughout time.⁷ This study and others suggest that current therapies may be inadequate at treating the degenerative component of MS.

Experimental autoimmune encephalomyelitis (EAE), the animal model for MS, has been used to study the mechanisms of axonal damage during CNS inflammation. In this model, immunization of mice with myelin proteins in adjuvant or the transfer of activated myelin-specific T cells induces an ascending paralytic disease

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Multiple sclerosis (MS) is an autoimmune inflammatory disease of the central nervous system (CNS) of unknown

that mimics some MS symptoms. CNS lesions in EAE are also composed of inflammatory cells accompanied by demyelination that primarily occurs in the white matter tracts of the spinal cord. During EAE, axonal damage has been observed in both early and late active demyelinating plaques.³ However, in the Theiler's murine encephalomyelitis virus model of viral-induced demyelination, damaged axons, detected by an increase in nonphosphorylated neurofilament-H, were present before the onset of demyelination.⁸ Therefore, it is unclear the extent to which neuronal dysfunction precedes myelin degeneration in EAE and MS.

The ability to track axonal degeneration or functional loss during disease has been difficult because of lack of reagents that label both neuronal cell bodies and processes. Fluorescent tracers are useful, but consistent labeling is technically difficult in mice. Previous studies have demonstrated that axonal injury can be visualized using two antibodies, APP⁹ and SMI-32.¹⁰ These antibodies detect two different parameters of degeneration. SMI-32, which detects nonphosphorylated neurofilament-H, has previously been correlated with transected neurites or axonal swelling in MS lesions,¹⁰ whereas APP-positive axons are believed to occur when axonal transport is disrupted leading to an accumulation of APP that reacts with the antibody.^{11,12} Both SMI-32 and APP have previously been shown to correlate with axonal injury, neuritic swelling, and axonal transport defects during disease conditions in the CNS such as viral infections, Alzheimer's disease, and traumatic brain injury.^{4,13}

The integrity of the neuronal transmission is dependent on proper delivery to the axon terminal of synaptic vesicles and proteins that are synthesized in the cell body. In addition, viability is maintained by retrograde transport of neurotrophic factors from the synapse to the cell body.¹⁴ Because the axon terminal can be a significant distance from the cell body, delivery of critical factors occurs by the procession of molecular motors carrying cargo on a network of microtubules. Microtubules consist of α - and β -tubulin that form a tubular protofilament. Motor proteins such as dynein and kinesin associate with these filaments and deliver vesicles and proteins in both the anterograde and retrograde direction.^{15,16} The stability of microtubules in the neuritic processes is maintained by microtubule-associated proteins. The disruption of microtubule integrity that has been observed in Huntington's chorea and the early stages of Wallerian degeneration was associated with axonal transport defects resulting in the accumulation of cytosolic proteins in discrete domains along the axon, termed a "beads on a string" pattern.^{12,17} Thus, dysregulation of microtubule stability is an early event during neurodegeneration.

In MS, neither the temporal relationship nor the interrelatedness of CNS pathologies—including immune cell infiltration, neuronal damage/dysfunction, and demyelination—are well understood or characterized. The understanding of how these pathological events are connected is important for the development of therapeutic modalities for the treatment of MS. One difficulty in tracking CNS events is the lack of markers for neuronal damage or dysfunction. The expression of green fluorescent protein

(GFP) or its derivatives has revolutionized the tracking of cells and molecules. With the use of cell-specific promoters, GFP labeling can be used to delineate specific cell types during various experimental conditions. Therefore, to track neuronal health during EAE, we used the Thy1-yellow fluorescent protein (YFP) transgenic mouse, which expresses YFP in the cell body and dendritic and axonal processes in both motor and sensory neurons, providing a vital marker for neurons under various experimental conditions.¹⁸ Using an acute model of EAE associated with spontaneous recovery, we found that YFP fluorescence was lost from the white matter regions of the spinal cord at the onset of clinical symptoms, before evidence of demyelination. Areas of the spinal cord that lacked YFP fluorescence also contained inflammatory infiltrates. As EAE clinical disease progressed, demyelination was observed along with an increased loss of YFP fluorescence and the presence of damaged axons. Axonal damage was indicated by the detection of nonphosphorylated neurofilament-H and the accumulation of APP. On recovery from EAE clinical disease, the inflammatory lesions resolved and were accompanied by remyelination and the return of YFP fluorescence. Using electron microscopy (EM), we found that there was no overt loss of axons during the acute disease course. Furthermore, we showed that encephalitogenic T cells disrupt microtubule integrity in cultured neurons.

Therefore, the loss of YFP fluorescence in inflammatory lesions is a good indicator of EAE CNS pathology and demonstrates that functional changes occur in neurons during inflammation in the CNS, which are likely mediated by T cells. Thus, neuronal dysfunction likely contributes to many of the clinical symptoms in MS and EAE, and identifying early events in neuronal degeneration may help to develop therapies that delay the progression of disease and promote remission of clinical symptoms.

Materials and Methods

Mice

B10.PL (H-2^d), B6.Cg-Tg(Thy1-YFP)16Jrs/J (Thy1-YFP), FVB, and Tg(TcrHEL3A9)Mmd/J (HEL-TCR) mice were purchased from the Jackson Laboratory (Bar Harbor, ME).¹⁸ The Thy1-YFP founder mice were on the C57BL/6 background and were backcrossed to B10.PL for five generations. (Thy1-YFP $\times$ FVB) F_1 mice were generated in our colony. The myelin basic protein (MBP)-T-cell receptor (TCR) transgenic mice expressing a TCR transgene specific for the acetylated NH₂-terminal peptide of MBP (Ac1-11) were generated as previously described.¹⁹

Peptides and Antibodies

The Ac1-11 (Ac-ASQKRPSQRSK) and HEL 46-61 (NT-DGSTDYGILQINSR) peptides were synthesized and high performance liquid chromatography purified by the Peptide Core Laboratory at BloodCenter of Wisconsin, Blood Research Institute. The anti-mouse antibodies for CD11b, the β chain of the TCR, IgG₁, and IgG_{2b} were purchased

from eBioscience (San Diego, CA). The SMI-99 and SMI-32 monoclonal antibodies that detect MBP and a nonphosphorylated epitope of neurofilament-H, respectively, were purchased from Sternberger Monoclonals (Lutherville, MD). Anti- β tubulin was purchased from Sigma-Aldrich (St. Louis, MO) or Chemicon (Temecula, CA).

Cells

Total splenocytes from MBP-TCR (I-A^u restricted) and HEL-TCR (I-A^k restricted) transgenic mice were activated, as described,¹⁹ in the presence of 5 μ g/ml Ac1-11 or the HEL 46-61, respectively. Naïve splenocytes were isolated from B10.PL mice and, for some experiments, were cultured overnight in 2.5 μ g/ml concanavalin A.

EAE Induction

EAE was induced by the adoptive transfer of MBP-specific encephalitogenic T cells generated as described.¹⁹ Briefly, 1×10^6 activated MBP-TCR T cells were intravenously injected into irradiated (360 rads) 5- to 8-week-old B10.PL or Thy1-YFP recipients. Animals were assessed daily for clinical symptoms and scored using a scale from 1 to 5 as follows: 0, no disease; 1, limp tail and/or hind limb ataxia; 2, hind limb paresis; 3, hind limb paralysis; 4, hind and forelimb paralysis; and 5, death. On days 10, 15, and 30 after induction of EAE, three mice were deeply anesthetized and perfused intracardially with 0.1 mol/L phosphate buffer (PB), followed by a paraformaldehyde-lysine-periodate fixative. Brains and spinal cords were harvested and fixed overnight at 4°C in paraformaldehyde-lysine-periodate fixative. After fixation, the tissue was washed in 0.1 mol/L PB and cryoprotected in solutions of 10 and 20% sucrose. The tissue was snap-frozen at -80°C in Tissue-Tek OCT (Sakura, Torrance, CA). Transverse sections of spinal cord at 10- μ m thickness were used for immunofluorescence.

Statistical Evaluation

Images of lumbar spinal cord were taken using MetaMorph software (Universal Imaging Corp., Downingtown, PA) from three individual mice at each time point (untreated, and days 10, 15, 22, and 35). The average fluorescence intensity in the columns was measured and expressed as mean $\pm$ SD. Statistical significance was determined using a one-way analysis of variance for multiple comparisons. Significance thresholds were $P < 0.05$.

Immunofluorescence

Sections of spinal cord were rehydrated in 0.1 mol/L PB with 0.01% Triton for 10 minutes. If a biotinylated primary antibody was used, sections were blocked with an avidin/biotin blocking kit (Vector Laboratories, Burlingame, CA) followed by a 1-hour incubation in 100% fetal calf serum.

The primary antibodies anti-CD11b-biotin (1:75), anti-TCR β -phycoerythrin (1:75), and SMI-99 (1:100) were diluted in 30% fetal calf serum/0.1 mol/L PB and incubated for 2 hours. After washing in 0.1 mol/L PB, sections with anti-CD11b-biotin were incubated with streptavidin-Alexa Fluor 350 (1:250) (Molecular Probes, Eugene, OR) for 1 hour. After incubation with SMI-99, the slides were washed in 0.1 mol/L PB and rat anti-mouse IgG_{2b}-Texas Red (1:200) was applied to sections for 1 hour. The slides were washed in 0.1 mol/L PB followed by a wash in distilled water and then coverslipped with Aquamount (Biomedex, Foster City, CA) and imaged using a Zeiss Axiostop microscope and Sensys camera with MetaMorph imaging software (Universal Imaging Corp.). Images represent spinal cords taken from three separate experiments. For neuronal cultures, 4% paraformaldehyde for 10 minutes was used for fixation and treated as for frozen sections, staining with anti- β -tubulin for 2 hours followed by a 1-hour incubation with anti-mouse IgG₁-Texas Red.

Electron Microscopy

Control, irradiation-only mice, and mice with EAE on days 10, 15, and 30 were deeply anesthetized and then perfused intracardially with PB followed by a solution of ice-cold 2.5% glutaraldehyde in 0.1 mol/L cacodylate buffer. Spinal cords were dissected and fixed overnight in 2.5% glutaraldehyde. Ultrathin sections were prepared and toluidine blue staining and EM were performed by the EM facility at the Medical College of Wisconsin (Milwaukee, WI). Three mice per group were analyzed at each time point.

Embryonic Neuronal Cultures

Embryonic neurons were cultured using an adapted protocol from Ransom and colleagues.²⁰ Brains and spinal cords were isolated on embryonic day 15 from Thy1-YFP or (Thy1-YFPxFVB)F₁ mice and titrated in DISGH buffer (135 mmol/L NaCl, 5 mmol/L KCl, 0.3 mmol/L Na₂HPO₄, 0.2 mmol/L KH₂PO₄, 16.5 mmol/L glucose, 22 mmol/L sucrose, and 9.86 mmol/L HEPES), followed by digestion in 0.67 mg/ml of papain at 37°C for 30 minutes. The tissue was then titrated in 40 μ g of DNase diluted in minimal essential medium (Mediatech, Herndon, VA), and the cells were pelleted by centrifugation and resuspended in minimal essential medium containing 10% horse serum and 10% fetal calf serum. Neurons were plated on polyethylenimine-coated plates, and after 8 hours the medium was replaced with neurobasal media containing N2 supplement (Invitrogen, Carlsbad, CA), 2 mmol/L glutamine, 100 μ g/ml gentamicin, and 2.5 μ g of fungizone. Three days later, the cultures were treated with 0.054 mmol/L fluorodeoxyuridine (Sigma-Aldrich) and 0.014 mmol/L uridine (Sigma-Aldrich) to inhibit proliferation of astrocytes. Fresh medium was added to the culture every 2 days, and cultures were used ~10 days after seeding. T cells and splenocytes were diluted in neurobasal medium before addition to the neuronal cultures. Live cell

Figure 1. EAE clinical disease and loss of YFP fluorescence during EAE in Thy1-YFP mice. EAE was induced by the adoptive transfer of MBP-specific encephalitogenic T cells. **A:** The average daily EAE clinical disease score of five Thy1-YFP mice is shown. **B–F:** Transverse sections from the lumbar spinal cord of Thy1-YFP mice were generated from either untreated (**B**) mice or mice with EAE on days 10 (**C**), 15 (**D**), 22 (**E**), or 35 (**F**) and analyzed by immunofluorescence for the expression of YFP. Images shown are representative of three mice examined at each time point from three separate experiments. **G:** Quantitation of average intensity of fluorescence in white matter of lumbar spinal cord of three mice expressed as mean $\pm$ SD. Original magnifications, $\times 100$ (**B–F**).

imaging was conducted using a Nikon fluorescent microscope with a Spot camera and Meta Morph Imaging Software (Universal Imaging Corp.). In experiments using taxol (paclitaxol, Sigma-Aldrich), neurons were pre-treated for 1 hour with 10 $\mu\text{mol/L}$ taxol and washed in neurobasal media, and then encephalitogenic T cells were added to the cultures. Colchicine (Sigma-Aldrich) (200 $\mu\text{mol/L}$) was added to Thy1-YFP cultures for 1 hour before live cell imaging. For some experiments, neurons were labeled with 10 $\mu\text{mol/L}$ carboxy SNARF-1 acetate (Molecular Probes) for 30 minutes at 37°C and washed twice before addition of T cells.

Results

Thy1-YFP Mice Exhibit a Monophasic EAE Disease Course

Because we backcrossed the Thy1-YFP mice onto the B10.PL background, we first determined the nature of the EAE disease course in the Thy1-YFP mice. As we previously reported for wild-type B10.PL mice,^{19,21} the Thy1-YFP mice exhibited a monophasic acute disease course (Figure 1A). The mice first showed signs of clinical disease 7 to 10 days after the adoptive transfer of the MBP-specific T cells, presenting with a weak tail or hind

limb ataxia. The severity of disease increased until the peak of disease on days 13 to 16, at which time the mice exhibited hind limb paresis or paralysis. The mice then underwent spontaneous recovery completely resolving clinical symptoms by day 35 (Figure 1A).

To determine the distribution of YFP fluorescence in the Thy1-YFP B10.PL mice, we examined spinal cords from untreated mice. Continuous YFP fluorescence was evident in the ventral, lateral, and dorsal white matter columns as well as in cell bodies of neurons in both dorsal and ventral horns (Figure 1B and data not shown). We next determined whether changes in YFP fluorescence could be detected in areas of the lumbar spinal cord where we have previously shown inflammatory lesions to reside in EAE in B10.PL mice.²² At the onset of EAE clinical symptoms (day 10), small areas exhibiting loss of YFP fluorescence were observable in the white matter (Figure 1C). As EAE clinical disease progressed, the loss of YFP fluorescence became more prominent and was spread throughout the ventral, dorsal, and lateral columns by the peak of disease (Figure 1D). The YFP fluorescence that remained, surrounding areas of loss, exhibited a distinct punctate pattern (Figure 1D). Loss of YFP in these areas correlated well with the symptoms of sensory and motor loss in the lower extremities displayed by the mice. YFP fluorescence returned once the mice began to recover (Figure 1E); however, the punctate

Figure 2. Loss of YFP fluorescence occurs in lesions containing immune cell infiltration. **A–W:** Transverse frozen sections of spinal cords were generated from unmanipulated Thy1-YFP mice (**A, F, K, P, U**) or from mice with EAE on the day of onset (**B, G, L, Q, V**), at the peak of disease (**C, H, M, R, W**), during the recovering phase (**D, I, N, S**), and once fully recovered (**E, J, O, T**). The sections were stained with monoclonal antibody specific for TCRβ and CD11b and analyzed by immunofluorescence for the expression of YFP (green) alone (**A–E**), TCRβ (red) alone (**F–J**), CD11b (blue) alone (**K–O, U**), merged YFP/TCRβ/CD11b (**P–T**), or merged YFP/CD11b (**V, W**). **Arrows** indicate a typical lesion in the lateral column. Data shown are representative of three mice examined at each time point. Original magnifications: $\times 100$ (**A–T**); $\times 400$ (**U–W**).

fluorescence pattern did not resolve until the mice showed signs of full recovery (Figure 1F). Changes in YFP fluorescence were quantified, and total YFP fluorescence decreased steadily until the peak of disease at which time the loss was statistically significant (Figure 1G). As observed in the histological sections, YFP fluorescence returned once the mice showed signs of clinical disease recovery (Figure 1G). These data indicate that the status of YFP fluorescence in neurons correlates with EAE clinical disease.

Loss of YFP in the Spinal Cord Is Associated with Immune Cell Infiltration

To determine the relationship between the distribution of YFP fluorescence in the Thy1-YFP B10.PL mice and the presence of inflammatory cells, we examined spinal cords from untreated and mice at days 10, 15, 22, and 30 during EAE for the presence of T cells and macrophages.

Figure 3. Loss of YFP precedes demyelination in EAE. Transverse frozen sections from the lumbar spinal cord were generated from unmanipulated Thy1-YFP mice (**A, F, K**), and from mice with EAE at onset (**B, G, L, P**), peak of disease (**C, H, M, Q**), during recovery (**D, I, N**), and in recovered mice (**E, J, O**). The sections were stained with monoclonal antibody specific for MBP and analyzed by immunofluorescence for the expression of YFP (green) alone (**A–E**), MBP (red) alone (**F–J**), or merged YFP/MBP (**K–Q**). **Arrows** indicate a typical lesion in the ventral column. Data shown are representative of three mice examined at each time point. Original magnifications: $\times 100$ (**A–O**); $\times 400$ (**P–Q**).

In control mice without EAE, continuous YFP fluorescence was observed (Figure 2A), whereas T cells bearing the TCR β chain were absent (Figure 2F), and the CNS resident microglial cells exhibited dull CD11b staining (Figure 2, K and U). At both the onset and peak of disease, areas exhibiting loss of YFP fluorescence (Figure 2, B and C) also contained T cells (Figure 2, G and H) and macrophages (Figure 2, L and M). By overlaying YFP fluorescence with T cell and macrophage staining (Figure 2, Q and R), it is evident that loss of YFP fluorescence occurs only in the areas containing inflammatory infiltrates. This is particularly evident at high magnification, where an overlay of YFP fluorescence with CD11b staining at both the onset (Figure 2V) and peak of disease (Figure 2W) shows that areas with a complete loss of YFP fluorescence co-localized with macrophage accumulation. As the mice entered the recovery phase and were fully recovered, the inflammatory infiltrates resolved (Figure 2, I, J, N, O, S, and T) and YFP fluorescence returned (Figure 2, D and E). These data show that the loss of YFP fluorescence in axons can be used as an additional biological marker of EAE clinical disease and that it correlates with the presence of inflammatory infiltrates.

Demyelination in the Spinal Cord Follows Loss of YFP Fluorescence

Because the loss of YFP correlated with clinical disease and the presence of inflammatory infiltrates, we next determined whether YFP loss occurred in parallel with demyelination. In Thy1-YFP mice with no EAE, YFP fluorescence (Figure 3A) and the presence of myelin, as detected by staining for MBP (Figure 3F), were co-localized throughout the lumbar spinal cord (Figure 3K). On the day of EAE onset (day 10), when loss of YFP fluorescence was evident (Figure 3B), we were unable to detect signs of demyelination by staining for MBP (Figure 3G). Intact myelin was present in the lesions where loss of YFP was evident, as shown by a lack of yellow fluorescence in the overlay of YFP and MBP fluorescence (Figure 3L). A high-power magnification image, shown in Figure 3P, clearly demonstrates the presence of myelin in the absence of YFP fluorescence. These data suggest that loss of YFP fluorescence and demyelination are not temporally related. During peak of disease, areas of diminished YFP fluorescence were more prominent (Figure 3C), and

these same regions exhibited loss of myelin (Figure 3H and overlay Figure 3M). Figure 3Q is a high-power image of the lesion at the ventral horn of Figure M showing that neurons that exhibit a punctate YFP fluorescence pattern are both myelinated (large arrow) and demyelinated (small arrow). As the clinical symptoms subsided in the recovery phase, a return of both YFP fluorescence in the axons along with the return of myelin was evident (Figure 3, D, I, and N). In recovered mice, both YFP fluorescence (Figure 3E) and the presence of MBP (Figure 3J) completely co-localized (Figure 3O). These data show that loss of YFP fluorescence is an earlier marker of clinical disease than demyelination, and that the two pathologies are likely independent events.

Loss of YFP Fluorescence Correlates with Markers of Neuronal Dysfunction but Not Overt Neuronal Loss during EAE

Because the loss of YFP appeared before the detection of demyelination, we examined whether these areas also showed early evidence of neuronal dysfunction. Two antibodies, SMI-32 and anti-APP, have previously been shown to correlate with axonal damage.⁴ Antibodies specific for APP detect early axonal dysfunction in MS lesions,⁶ which is thought to be because of disruption of axonal transport leading to an accumulation of APP, which can be detected with antibodies. In Thy1-YFP mice with no EAE (Figure 4A), APP staining is not detected (Figure 4D). However, in mice with EAE clinical symptoms, APP accumulation was detectable (Figure 4E) in regions where YFP fluorescence is absent (Figure 4B and overlay Figure 4H). In mice that have recovered from EAE and in which YFP fluorescence has returned (Figure 4C), APP accumulation is no longer detectable (Figure 4F and overlay Figure 4I).

SMI-32 specifically stains an epitope expressed on nonphosphorylated neurofilament-H. Demyelination of axons as well as neuritic swelling confers SMI-32 reactivity.¹⁰ In control Thy1-YFP mice without EAE (Figure 4J), no SMI-32 immune reactivity is detectable (Figure 4M and overlay Figure 4P). However, during clinical disease, areas of YFP loss in the white matter columns (Figure 4K) showed evidence of positive SMI-32 staining (Figure 4N and overlay Figure 4Q). The inset in Figure 4Q shows a higher power image of SMI-32 reactivity in an area in which YFP fluorescence is absent. As with APP, SMI-32 immunoreactivity was lost on clinical recovery (Figure 4, L, O, and R). These data suggest that YFP loss correlates with a disruption in axonal function that can be detected at the very early stages of disease in Thy1-YFP mice and that there is a reversal of this dysfunction when inflammation is resolved and the mice recover clinically.

Because clinical recovery is observed in Thy1-YFP mice along with the return of continuous YFP fluorescence, we examined whether this recovery of YFP was attributable to preservation of axons during the inflammatory episode. We examined this via EM by examining myelin and axonal integrity during the EAE clinical disease course. First, we performed toluidine blue staining

on ultrathin sections prepared for EM from mice at EAE onset (Figure 5B), at peak of disease (Figure 5C), and in recovered mice (Figure 5D). During all three phases of EAE disease, healthy myelinated axons were present throughout the spinal cord, similar to control animals (Figure 5A). At both onset (Figure 5B) and peak of disease (Figure 5C), inflammatory lesions were detectable in the toluidine blue-stained sections in the white matter, as indicated by the arrows. EM was subsequently performed on these spinal cord sections to determine whether alterations in the axons or surrounding myelin occurred that were not detectable at the gross microscopic level. Because EAE is induced in irradiated mice and EM can detect very minor disturbances in the integrity of axons or myelin, we first determined that irradiated mice had normal compacted myelin with intact axolemma (Figure 5, A and E). In addition, there were no detectable alterations in the organization or structure of cytoskeletal components or organelles (Figure 5E). During the onset of disease, the majority of axons appeared healthy with minimal affect on myelin compaction, even when adjacent to cells resembling T lymphocytes (Figure 5F). However, in some areas of the spinal cord, loss of myelin compaction was evident (Figure 5G). Despite this disruption in myelin structure, axons continued to exhibit normal organelle and cytoskeletal structure. No demyelination was detected in the roots (Figure 5H). Therefore, clinical symptoms were likely dependent on changes observed in the spinal column.

Demyelination of axons became evident at peak of disease (Figure 5I, boxed area), consistent with the results obtained with immunofluorescence (Figure 3, M and Q). Macrophages that contained phagocytosed myelin were found in proximity with demyelinated axons (Figure 5I, arrows). Axons that were completely demyelinated retained a normal morphology with intact axolemma and no evidence of disrupted mitochondria or degeneration (Figure 5, I and J). During recovery, axons in the white matter continued to show structurally normal mitochondria and cytoskeleton along with restoration of myelin compaction (Figure 5K). The width of the myelin was reduced in the remyelinated axons (Figure 5K), as compared to mice without EAE (Figure 5E). This finding is consistent with previous studies in MS.²³ A minority of axons did exhibit evidence of degeneration; however, these were surrounded by normal-appearing axons in most instances (Figure 5K, arrow). These data indicate that the majority of axons maintained their integrity throughout the acute inflammatory episode. The small number of degenerating axons that we observed in the spinal cord could not account for large areas of YFP loss that occur in the spinal cord during acute EAE.

Loss of YFP Fluorescence Is Associated with Disruption of β -Tubulin in Axons

To examine whether encephalitogenic T cells could directly mediate the loss of YFP fluorescence in neurons during EAE, we cultured embryonic neurons from Thy1-YFP mice and co-cultured them with MBP-specific en-

Figure 5. Axonal integrity is maintained throughout EAE clinical disease in Thy1-YFP mice. Ultrathin sections of the lumbar spinal cord were prepared from irradiated mice (360 rads) 24 hours later (**A, E**) or from mice with EAE at disease onset (**B, F, G, H**), peak of disease (**C, I, J**), or from recovered mice (**D, K**). Toluidine blue staining was performed to examine tissue integrity and to localize inflammatory lesions (**A–D**, arrows). **H:** At the peak of EAE disease a root is shown surrounded by a Schwann cell. In **F**, **T** refers to the nuclei of cells resembling T lymphocytes. In **I**, the boxed area surrounds three demyelinated axons and the arrows indicate myelin debris inside a macrophage. The arrow in **K** indicates a degenerating axon. Scale bars: 1 μm (**E–G**; **J, K**); 2 μm (**H**).

cephalitogenic T cells used for EAE induction. Neurons expressing the YFP transgene exhibited continuous YFP fluorescence in both the cell body and neuritic processes (Figure 6, A and B). However, when MBP-specific CD4⁺ encephalitogenic T cells were added to the neuronal cultures, there was a redistribution of the cytosolic YFP protein into aggregated beads contained within the neurites (Figure 6, C and D; arrows). A higher magnification of the YFP redistribution along a neurite is shown in the inset in Figure 6C. The accumulation of YFP at distinct points along the neurite initiated at the distal process and was evident along the entire process within 30 minutes. Neither naïve (Figure 6E) nor ConA-activated (Figure 6F) B10.PL splenocytes induced changes in YFP distribution, suggesting that the T cells needed to be activated effectors. To examine this further, we activated splenic HEL-specific T cells *in vitro* in an identical manner as MBP-specific T cells¹⁹ and on addition to YFP neurons, they also induced YFP redistribution (Figure 6G), demonstrating that neither antigen nor MHC specificity is required.

Because the redistribution of YFP resembled a “beads on a string” pattern often seen when axonal transport is disrupted,²⁴ we next examined whether the integrity of

microtubules in the axons was disrupted by staining for β -tubulin. As with YFP fluorescence, control Thy1-YFP neurons exhibited continuous β -tubulin staining along the length of the neurite as well as in the cell body (Figure 6H). After co-culture with either MBP- or HEL-specific T cells, the β -tubulin staining was discontinuous, indicating a disruption in the axonal microtubule structure (Figure 6, I and J, respectively; arrows). To confirm that microtubules were disrupted by co-culture with T cells, we pre-treated neurons with taxol, a drug that stabilizes the microtubule network by binding to tubulin subunits and preventing their dissociation. After taxol treatment, the encephalitogenic T cells were unable to induce changes in YFP fluorescence (Figure 6K) or disruption of microtubule integrity (Figure 6L). To further demonstrate that microtubule disruption leads to altered YFP fluorescence, neuronal cultures were treated with colchicine, a known microtubule destabilizer (Figure 6M). Finally, we examined whether the change in the pattern of YFP fluorescence and microtubule structure was because of loss of membrane integrity. We accomplished this by labeling cultured neurons with the pH-dependent vital dye SNARF-1 acetate before the addition of encephalitogenic

Figure 4. Loss of YFP fluorescence in Thy1-YFP mice correlates with two markers of neuronal dysfunction. Transverse frozen sections from the lumbar spinal cord were generated from unmanipulated Thy1-YFP mice (**A, D, G, J, M, P**), from mice at EAE onset (**B, E, H**), from mice at peak of disease (**K, N, Q**), or from mice that had recovered from EAE (**C, F, I, L, O, R**). The sections were stained with monoclonal antibody specific for APP (**D–F**) or nonphosphorylated neurofilament-H (SMI-32) (**M–O**) and analyzed by immunofluorescence for the expression of YFP (green) (**A–C, J–L**), APP (**D–F**), merged YFP/APP (**G–I**), nonphosphorylated neurofilament-H (red) (**M–O**), or merged YFP/nonphosphorylated neurofilament-H (**P–R**). The arrow indicates a typical lesion in the ventral column. Data shown are representative of three mice examined at each time point. Original magnifications: $\times 100$ (**A–R**); $\times 400$ (**Q**, inset).

Figure 6. Co-culture of Thy1-YFP neurons with activated MBP-specific T cells results in disassembly of microtubules but not neuronal death. Embryonic neurons were cultured from Thy1-YFP mice and live cell fluorescence imaging was performed on cells alone (**A**, **B**, **H**) or in the presence of 1×10^6 MBP-specific encephalitogenic CD4 T cells (**C**, **D**, **I**), naïve splenocytes (**E**), ConA-activated splenocytes (**F**), or HEL-specific CD4 T cells (**G**, **J**) for 1 to 2 hours. **H–J**, and **L**: After co-culture with T cells or taxol, the neurons were fixed and stained for β -tubulin. **K** and **L**: Neurons were pretreated with 10 μ mol/L taxol before the addition of T cells. **M**: Neurons were treated with 200 μ mol/L colchicine for 1 hour before live cell imaging. **N** and **O**: Neurons were labeled with SNARF-1 acetate before the addition of T cells and imaged by bright field (**N**) and immunofluorescence (**O**). Original magnifications: $\times 400$ (**A–O**); $\times 600$ (**C**, inset).

T cells, such that the loss of membrane integrity would result in the loss of the dye and diminished fluorescence. In the presence of T cells, shown by the arrow in a bright-field image (Figure 6N), no loss of SNARF-1 acetate fluorescence was observed (Figure 6O), indicating that the T cells did not result in neuronal cell death. Further evidence to support this conclusion is the observation that the redistribution of YFP fluorescence is reversible on removal of the T cells (data not shown). These data indicate that T cells are capable of directly affecting the function of neurons by disrupting the integrity of β -tubulin.

Discussion

Axonal injury is thought to play a key role in the progression of disease in patients with MS. The goal of this study was to examine neuronal integrity during an acute inflammatory episode of EAE using the Thy1-YFP mice. In these mice, loss of YFP fluorescence was observed in the white matter columns of the spinal cord at the onset of clinical symptoms in regions containing inflammatory infiltrates (Figure 2), and preceded detectable demyelination (Figure 3). Areas of YFP loss were also associated with markers of neuronal dysfunction (Figure 4), in the absence of axonal loss (Figure 5). On clinical recovery, YFP fluorescence returned to control levels and signs of neuronal injury dissipated. In addition, using *in vitro* cultured Thy1-YFP neurons, we showed that encephalitogenic T cells are sufficient to induce structural changes in β -tubulin resulting in the accumulation of YFP in discrete domains along the axon (Figure 6). These data demonstrate that neuronal injury, detected by a loss of YFP fluorescence, is likely a T-cell-mediated event that occurs early in the EAE clinical disease and likely contributes to disease symptoms not associated with demyelination.

In addition to our study, the Thy1-YFP mice have also been used to study neuronal integrity in other model systems. In the myelin oligodendrocyte glycoprotein model of chronic EAE, YFP fluorescence was used to detect abnormalities in motor neuron dendrites during disease,²⁵ whereas in a transgenic model of Alzheimer's disease, neuritic dystrophy produced an accumulation of YFP at distinct points in the axon in areas of amyloid β accumulation.²⁶ In addition, after nerve crush injury, YFP fluorescence was lost in the peripheral nerve, and its return was used to monitor nerve regeneration.²⁷ In these models as well as ours, expression of YFP served as a marker of neuronal integrity without the use of antibodies.

The importance of neuronal pathology as a predictor of clinical symptoms in MS and EAE has gained increasing attention. In MS, co-expression of the sodium channel $\text{Na}_v1.6$ with the $\text{Na}^+/\text{Ca}^{2+}$ exchanger was shown to be associated with axonal degeneration in MS,²⁸ indicating that functional changes in conductance occur during inflammation in the CNS. The disruption of neuronal function was also detected in several models of EAE. This was observed at the molecular level by gene expression analysis, which showed that genes involved in transmission and neuronal function were dysregulated, even at early

time points in EAE.²⁹ At the histological level, neuronal injury detected by dendritic beading was observed in Lewis rats immunized with MBP.³⁰ Finally, at the electrophysiological level, motor and sensory transmission defects in SJ/L mice corresponded with the appearance of neurological deficits and occurred in both the inflammatory period of the acute attack as well as in the chronic demyelinating stages.³¹ This study indicated that defects in synaptic function, and not solely demyelination, resulted in clinical symptoms. Our study showing that loss of YFP fluorescence parallels the onset of clinical disease and inflammation are in accordance with the above studies, which collectively demonstrate that altered neuronal function correlates with the appearance of neurological disease.

Although the importance of neuronal dysfunction in MS has recently become an area of intense interest, the presence and consequences of demyelination in MS plaques has been an area of study for decades. It is thought that demyelination of neurons has a dramatic negative affect on the rate of conductance of nerve impulses, which is thought to contribute to motor defects in MS patients.³² It is also known that interactions between oligodendrocytes and neurons influence the activity and health of neurons.³³ Studies have suggested that neurons become vulnerable to cell death in the absence of oligodendrocytes.^{34,35} However, the interrelationship between neuronal dysfunction and demyelination are not completely understood. In our study, we detected neuronal injury at the onset of clinical disease before detectable demyelination, suggesting that early EAE symptoms that include hind limb tremors³⁶ and hind limb ataxia, but not loss of motor function, are because of neuronal dysfunction. In addition to the spinal cord, spinal roots have been shown to undergo demyelination during EAE in the Lewis rat.³⁷ We did not observe demyelination in the roots by EM, indicating that demyelination at this anatomical location is likely not involved in early disease manifestations in our EAE model.

Similar to our observation, a study using Theiler's murine encephalomyelitis virus as a model of demyelination and inflammation detected nonphosphorylated neurofilament-H in the spinal cord white matter before overt demyelination.⁸ We also saw the accumulation of APP aggregates in areas of YFP loss at EAE onset before demyelination, suggesting that demyelination alone cannot account for early neurological dysfunction. Studies in human MS tissue have also determined that the amount of demyelination does not always predict disease course.³⁸ Further evidence that neuronal dysfunction and demyelination are independent events, is the presence of both myelinated and demyelinated axons containing what appears to be YFP aggregation during the peak of disease (Figure 3R). As with APP,^{11,12} YFP aggregation is likely attributable to a transport defect along the axon, and because it occurs in inflammatory lesions, is likely an immune-mediated event. Our *in vitro* data showing that encephalitogenic T cells were sufficient to disrupt β -tubulin integrity in neuronal axons, thereby disrupting axonal transport, support this hypothesis.

Although models of EAE are characterized by infiltrating immune cells and demyelination similar to those seen in MS, the neurodegeneration exhibited in these models may not recapitulate the primary description of the pathology of MS demyelination with relative preservation of axons.³⁹ In our model of EAE, the disease course is acute, allowing the study of the recovery phase of the inflammatory episode. This acute disease may more reliably model early symptoms seen in MS patients who often show complete remission from disease and only later develop irreversible accumulation of disability. We found that mice in the recovery period exhibited resolution of inflammation and regained myelin and YFP expression. Furthermore, no loss of axons was detected by EM. This suggests that a reversible dysfunction is occurring in neurons early in the EAE disease course and that remyelination occurred because axons were not lost during disease. In MS, similar cycles of demyelination and remyelination have been reported in which remyelination has been found to occur after inflammation has resolved forming shadow plaques.³ Most studies examining neurodegeneration in MS examine postmortem tissue. In these samples, axonal transection and degeneration are prominent features of the disease; however, because of the advanced stage of disease in samples, it was not possible to study early changes in neurons that influence the disease course. In the Thy1-YFP mice, we show that early disease symptoms correlate with disruption of YFP, making these mice an ideal model to study the mechanism of early axonal dysfunction that cannot be studied in MS patients.

The mechanism of tissue injury in MS and EAE is still not clear. It has been proposed that T cells may directly injure neurons by either transecting them or producing soluble molecules that result in degeneration.^{8,40–42} Because immune cells are present in the CNS in areas of neuronal dysfunction early in EAE disease (Figure 2), it is likely that the immune cells directly alter neuronal functions resulting in clinical symptoms. In a recent study, TRAIL, a soluble member of the tumor necrosis factor family, has been shown to induce neuronal cell death.⁴³ However, in our model, YFP fluorescence returned to control levels as the mice recovered clinically, suggesting death of neurons is not the mechanism for YFP loss. Mechanisms such as nitric oxide secretion by macrophages/microglia may also play a role in neuronal injury. *In vitro*, it has been shown that nitric oxide release by microglia co-cultured with neurons resulted in a decrease in the transport of synaptic vesicle precursors.⁴⁴ Inhibition of neurotransmitter release would not only result in loss of signal conduction between neuronal cells, but also may decrease release of growth factors necessary for the viability of synapsing neurons. Thus early damage to neurons mediated by infiltrating immune cells may then predispose oligodendrocytes, which are associated with damaged neurons, to further attack by inflammatory cells or mediators and result in later demyelination. Our studies showing the localization of YFP loss to areas of immune cell infiltration before demyelination support this model.

Protection of neurons during episodes of CNS inflammation is critical to prevent the accumulation of permanent neurological disability. The early, reversible axonal dysfunction seen in the Thy1-YFP mice is an ideal target for neuroprotective therapies. Because clinical symptoms correlated well with YFP loss, prevention of the underlying mechanism leading to this dysfunction may alleviate MS symptoms and prevent subsequent demyelination.

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