Dendritic and Synaptic Pathology in Experimental Autoimmune Encephalomyelitis

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Evidence has shown that excitotoxicity may contribute to the loss of central nervous system axons and oligodendrocytes in multiple sclerosis and experimental autoimmune encephalomyelitis (EAE). Because dendrites and synapses are vulnerable to excitotoxicity, we examined these structures in acute and chronic models of EAE. Immunostaining for microtubule-associated protein-2 showed that extensive dendritic beading occurred in the white matter of the lumbosacral spinal cord (LSSC) during acute EAE episodes and EAE relapses. Retrograde labeling confirmed that most motoneuron dendrites were beaded in the white matter of the LSSC in acute EAE. In contrast, only mild swelling was observed in the gray matter of the LSSC. Dendritic beading showed marked recovery during EAE remission and after EAE recovery. In addition, synaptophysin, synapsin I, and PSD-95 immunoreactivities were significantly reduced in both the gray and white matter of the LSSC during acute EAE episodes and EAE relapses, but showed partial recovery during EAE remission and after EAE recovery. Pathologically, both dendritic beading and the reduction in synaptic protein immunoreactivity were well correlated with inflammatory cell infiltration in the LSSC at different EAE stages. We propose that dendritic and synaptic damage in the spinal cord may contribute to the neurological deficits in EAE. (*Am J Pathol* 2003, 162:1639–1650)

Central nervous system (CNS) inflammation and neurodegeneration are two major pathological processes in multiple sclerosis (MS). Understanding the pathology and mechanisms of CNS degeneration in MS is essential for protecting neural structures and functions in MS patients. In addition to the demyelination and the loss of oligodendrocytes, axonal degeneration in MS has received substantial attention. It has been realized that axonal loss starts early in the disease course,^{1,2} and that the accumulation of axonal damage parallels in general the progressive neurological dysfunction in MS patients.³ In experimental autoimmune encephalomyelitis (EAE), an

animal model with features of CNS autoimmune inflammation, similar neurodegenerative changes have been described.^{4,5}

Recent studies suggest that excitotoxicity may play an important role in the pathogenesis of both MS and EAE. Increased levels of glutamate were observed in the cerebrospinal fluid from MS patients,⁶ and a correlation was shown between the cerebrospinal fluid glutamate and the disease activity and severity.⁷ CNS-infiltrating macrophages and activated microglia are probably the major sources because these cells release large quantities of glutamate after activation *in vitro*.^{8,9} Moreover, both glutamate degradation enzymes and glutamate transporters were markedly reduced in MS and EAE lesions.^{10,11} Importantly, treatment with NBQX, an AMPA receptor antagonist, significantly reduced EAE severity, oligodendrocyte loss, and axonal degeneration without mitigating CNS inflammation.^{12,13} Consistent with these findings, oligodendrocytes are known to be exquisitely vulnerable to glutamate excitotoxicity both *in vitro*¹⁴ and *in vivo*.¹⁵

Neuronal dendrites receive most of the synaptic inputs and are very sensitive to excitotoxicity. A 10-minute exposure of cultured cortical neurons to sublethal concentrations of N-methyl-D-aspartate (NMDA 30 $\mu\text{mol/L}$) or kainate (KA, 100 $\mu\text{mol/L}$) resulted in dramatic dendritic beading.^{16,17} Hippocampal slice cultures treated with glutamate receptor agonists showed similar dendritic changes.¹⁸ Dendritic beading has also been observed in the cochlea after traumatic noise exposure¹⁹ and in models of cerebral ischemia,²⁰ epilepsy,²¹ and CNS trauma.²² Excitotoxicity is believed to be an important pathogenic factor in these situations. Emerging evidence suggests that more severe excitotoxic insults may damage synaptic structures as well. NMDA treatment (50 $\mu\text{mol/L}$ for 3 hours) in organotypic hippocampal cultures resulted in postsynaptic calpain activation and PSD-95 degradation.²³ In Alzheimer's disease, the loss of synaptophysin in the midfrontal cortex is associated with the local reduction in glutamate uptake.²⁴

Spinal motoneurons have especially large dendritic trees, which constitute ~97% of the surface area and

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75% of the volume of these neurons.²⁵ Within the dendritic tree, ~75% of the surface area and 55% of the volume were located more than 300 μm away from the soma.²⁵ Motoneuron dendrites not only form an intricate network within the spinal cord gray matter, but also extend widely into ventrolateral white matter.^{26,27} The pattern of synaptophysin immunoreactivity in the white matter is close to the distribution of dendrites.²⁸ We hypothesized that there might be damage to the dendrites and the synapses in inflamed spinal cord during EAE attacks, and examined these structures in both acute and chronic EAE models.

Materials and Methods

EAE Induction and Observation

All reagents except those specifically mentioned were obtained from Sigma-Aldrich (St. Louis, MO). Male Lewis rats and female DA rats (~200 g of body weight), as well as female guinea pigs (~350 g of body weight), were obtained from Charles River Canada (St. Constant, Quebec, Canada). The protocols for animal experiments were approved by the Animal Care Center, University of British Columbia. To induce acute EAE, each Lewis rat was immunized subcutaneously close to the inguinal lymph nodes with 100 μL of myelin basic protein (MBP)/complete Freund's adjuvant emulsion, which contained 50 μg of guinea pig MBP and 500 μg of heat-inactivated *Mycobacterium tuberculosis* H37RA (Difco Laboratories, Detroit, MI). To induce chronic EAE, each DA rat was immunized subcutaneously at the tail base with 200 μL of guinea pig spinal cord/compleat Freund's adjuvant emulsion, which contained 50 mg of guinea pig spinal cord homogenate and 2 mg of heat-inactivated *M. tuberculosis* (Difco). The degrees of EAE severity were scored as follows: 0^o, no clinical signs; 0.5^o, incomplete tail paralysis; 1^o, complete tail paralysis; 2^o, unsteady gait or incomplete paraplegia; 3^o, complete paraplegia.

Tissue Collection and Processing for Histology

In most MBP-immunized Lewis rats, acute EAE had an onset at 10 days post-immunization (dpi), a peak at 12 dpi, and was completely recovered by 18 dpi. To compare the dendritic and synaptic pathology at different EAE stages, we collected lumbosacral spinal cord (LSSC) tissue from four groups of Lewis rats: normal animals, animals at EAE onset (EAE score, 0.5^o to 1^o, 10 dpi), animals at EAE peak (3^o, 12 dpi), and animals fully recovered from EAE (0^o, 20 dpi). In our study, guinea pig spinal cord-induced chronic EAE in DA rats only had a single relapse episode after a complete remission. In most animals, the first EAE episode was from 9 dpi to 14 dpi, and the EAE relapse was from 18 dpi to 31 dpi. We collected LSSC tissue from five groups of DA rats: normal animals, animals at the peak of first EAE episode (3^o, 11 dpi), animals at EAE remission (0^o, 16 dpi), animals at the peak of EAE relapse, (2.5 to 3^o, 21 dpi), and animals fully

recovered from EAE relapse (0^o, 43 dpi). Three to four animals were included in each group.

Animals were sacrificed at different EAE stages by intraperitoneal Euthanyl injection, and then transcardially perfused with 150 ml of 0.1 mol/L phosphate-buffered saline (PBS) and 150 ml of 4% paraformaldehyde in PBS. The LSSC was dissected out, postfixed in 4% paraformaldehyde overnight and then cryoprotected with 10 to 30% sucrose. The LSSC was then cut into nine segments approximately corresponding to L1 to S3 segments. These segments were immersed in TissueTek in a cryomold and were oriented with the rostral ends toward the bottom. They were snap-frozen in 2-methylbutane cooled with liquid nitrogen, and 10- μm transverse spinal cord sections were prepared.

Immunohistochemistry

Tissue sections were incubated with 10% normal goat serum and 1% bovine serum albumin for 30 minutes to block the background staining, and then incubated with primary antibodies and 0.5% Triton-100 at 4°C overnight. Monoclonal anti-microtubule-associated protein 2 (MAP2), anti-synaptophysin, and anti-synapsin I antibodies were used at 1:1000, 1:200, and 1:150 dilutions, respectively. Monoclonal anti-PSD-95 antibody (BD Sciences, Mississauga, Ontario, Canada) was used at 1:100 concentration. After rinsing, the sections were incubated with 0.3% H₂O₂ in PBS for 15 minutes to block the endogenous peroxidase activity, and then incubated with biotinylated secondary antibody (Jackson ImmunoResearch, West Grove, PA) at 1:800 for 1 hour. After rinsing, the sections were incubated with avidin-biotin-peroxidase complex (Vector, Burlingame, CA) at 1:500 for 1 hour. Immunoreactivity was visualized by incubating in 0.5 mg/ml of 3,3'-diaminobenzidine solution and 0.03% H₂O₂ for 3 to 5 minutes. The sections were then washed in distilled water, dehydrated in a graded series of alcohols, cleared in xylenes, and finally coverslipped. Tissue sections collected from different EAE stages of either EAE model were stained simultaneously with the same procedures to facilitate later image analysis.

Retrograde Labeling of Spinal Motoneurons and Their Dendrites

Lyophilized cholera toxin B subunit (CTB; List Biological Laboratories, Campbell, CA) was dissolved in saline and injected into the middle part of the gastrocnemius muscles on both sides of the animal (100 μg CTB/muscle). Three animals were included in each of the normal, acute EAE peak, and EAE recovery groups. To label dendrites at EAE peak and after EAE recovery, CTB was injected on 9 dpi and 25 dpi, respectively. These animals were sacrificed 3 days later, and LSSCs were obtained and processed as described above, except that tissue sections were 40 μm in thickness. The sections were then immu-

nostained with an anti-CTB antibody (List Biological Laboratories) at 1:5000.

Image Analysis

To quantify immunostaining results, images were captured under microscope ($\times 200$) using the RS Image software and a CoolSnap camera (both from Roper Scientific, Trenton, NJ). Same light intensity and exposure time (58 ms) were applied to all photographs. The linear property of the camera at this setting was confirmed by analyzing the mean gray values of images obtained from one to six stops of neutral density filters. To minimize the variability, all images were captured from L3 or L4 spinal cord sections, and were acquired separately from the gray and white matter of LSSC. To take gray matter pictures, the microscope was focused on the middle region of the ventral horn, and to take white matter pictures, the microscope was focused on the area lateral to the ventral horn. Six gray matter and six white matter pictures were collected from the two sides of L3/L4 sections from three animals at a specific EAE stage. All images were converted to grayscale and then analyzed with ImageJ v1.29 (the Windows version of NIH Image, downloaded from <http://rsb.info.nih.gov/ij/>). A fixed threshold range of 0 to 160 was chosen to well highlight the staining signals in normal spinal cord sections, and the total area within this range was measured, averaged, and compared among different EAE stages. The numbers of neurons in the ventral gray matter of LSSC, which were positively stained for synaptic protein immunohistochemistry (IHC), were also quantitated. These neurons were counted separately from the two sides of the nine-level LSSC sections, and results from three animals were averaged, and then compared between different EAE stages. One-way analysis of variance and Bonferroni post hoc tests were applied to examine the statistical significance of differences in mean values.

Immunoblotting

The entire LSSC segments from individual animals were obtained at different stages of acute EAE. Protein samples were prepared by homogenizing the LSSC tissue in a lysis buffer (50 mmol/L Tris-Cl, pH 8.0, 150 mmol/L NaCl, 0.02% sodium azide, 0.1% sodium dodecyl sulfate, 100 μ g/ml phenylmethyl sulfonyl fluoride, 1 μ g/ml aprotinin, 1% Nonidet P-40, 0.5% sodium deoxycholate). Twenty μ g of proteins from each sample were loaded in each lane, and then separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Proteins were transferred onto a polyvinylidene difluoride membrane, and the blot was blocked in 5% fat-free instant milk at 4°C overnight. Immunodetection was performed by incubating the blot for 1 hour with the primary antibody. The blot was then incubated for 1 hour with horseradish peroxidase-labeled goat anti-mouse IgG1 (1:5000 dilution), and developed with an ECL kit (Amersham Biosciences, Piscataway, NJ).

Results

Dendritic Pathology in Acute and Chronic EAE

Hematoxylin and eosin (H&E) staining showed that at the peak of acute EAE in Lewis rats, severe perivascular and parenchymal inflammation developed in the gray and white matter of LSSC in similar degrees (Figure 1, compare B with A). The clinical recovery from acute EAE was correlated with the resolution of pathological inflammation in LSSC, and very little inflammation could be detected at 20 dpi (Figure 1C).

It is well established that MAP2 is expressed in neuronal somata and dendrites, but is not present in the axons or glial cells.²⁹ In this study, MAP2 IHC strongly stained large motoneurons and the intricate dendritic network in the gray matter of LSSC (Figure 1, D and G). Most cross-sectioned dendrites were stained as solid dots. At the edge of the gray matter, MAP2-positive dendrites were organized into transverse bundles and extended heavily into the ventral and lateral white matter (Figure 1, D and J). As they penetrated more deeply into the white matter, the dendrites showed branch-like patterns. Many dendrites extend nearly as far as the peripheral edge of the white matter, and no dendritic beading was observed in normal spinal cord sections. When EAE reached the 3rd peak at 12 dpi, mild swelling occurred in some neuronal nuclei and dendrites in the gray matter (Figure 1, E and H). Some cross-sectioned gray matter dendrites contained MAP2-negative vacuoles at the center. However, the majority of gray matter dendrites had relatively normal morphology, and no patterns of alternating swelling and constriction were observed. In contrast, most dendrites in the white matter of LSSC manifested a beaded pattern at EAE peak, and very thin intersegment between the beads could be seen (Figure 1, E and K). In the cervical spinal cord, where no significant inflammation was present, MAP2 staining showed primarily normal dendritic morphology (data not shown), suggesting that local inflammation is necessary for generating dendritic beading in EAE. When EAE recovered clinically (20 dpi), most dendrites in the LSSC recovered normal morphology (Figure 1; F, I, and L). Moreover, we observed that both inflammation and dendritic beading were less severe in LSSC at EAE onset (0.5 to 1st) than at EAE peak (data not shown).

To more specifically examine spinal motoneuron dendrites, we performed CTB retrograde labeling in the acute EAE model. Approximately 8 to 12 large motoneurons with their dendrites were labeled on each side of L2-L4 spinal cord sections. No dendritic beading was observed in the gray matter of LSSC from either normal or EAE animals (Figure 2; A to C). Many CTB-positive dendrites extended deeply into the ventral and lateral white matter in a similar pattern as we observed with MAP2 immunostaining (Figure 2; D to F). In the normal spinal cord, labeled white matter dendrites were stained as smooth lines, which tapered very little along their length (Figure 2D). However, most motoneuron dendrites showed severe beading in the white matter at EAE peak (Figure 2E). The swellings were ~ 10 to 20 μ m in diame-

Figure 1. Pathological inflammation and dendritic beading in Lewis rat LSSC in acute EAE. **A** to **C** and **D** to **F** show H&E staining and MAP2 IHC in LSSC transverse sections obtained from normal, 12-dpi EAE peak, and 20-dpi EAE recovered animals, respectively. The lateral part of the ventral horn is shown on the **left** of each photograph, and the white matter lateral to the ventral horn is shown on the **right**. **G** to **I** and **J** to **L** show MAP2 staining in the gray matter (GM) and the white matter (WM) separately under higher magnifications. In normal LSSC sections, H&E staining shows a few large motoneurons and some other neural cells (**A**). MAP2 immunostaining strongly stained motoneuron perikarya and the intricate dendritic network in gray matter (**D** and **G**). The dendrites form transverse bundles at the gray and white matter junction area, and extensively penetrate into the lateral funiculus of the white matter (**D** and **J**). At the EAE peak, H&E staining (**B**) shows severe perivascular and parenchymal inflammation in LSSC. In the gray matter, MAP2 staining (**E** and **H**) shows that some proximal dendrites have mild swelling, but there is no dendritic beading. In contrast, most dendrites are beaded in the white matter (**E** and **K**). When EAE is recovered, inflammation (**C**) and dendritic changes (**F**, **I**, and **L**) resolve in both the gray and white matter, although some residual dendritic beading is still present in the white matter (**L**). Scale bars: 50 μ m (**A-F**); 25 μ m (**G-L**).

ter, and the intersegments were thinner than normal dendrites. When animals had recovered from clinical EAE, most white matter dendrites regained their normal morphology (Figure 2F). Consistent with previous reports,²⁵ we did not observe dendritic spines in spinal motoneurons with either MAP2 staining or CTB retrograde labeling.

To further examine the correlation between spinal cord inflammation and dendritic pathology, we studied spinal cord dendrites in a chronic relapsing-remitting EAE model (Figure 3). H&E staining showed that severe in-

flammatory cell infiltration developed in the gray and white matter of LSSC during the first EAE episode and EAE relapse (Figure 3, B and D), but largely resolved during EAE remission (Figure 3C) and after EAE recovery (Figure 3E). Consistent with the findings in acute EAE, MAP2 immunostaining showed no dendritic beading in the gray matter of LSSC during EAE attacks (Figure 3; F to J). In the white matter, marked dendritic beading was observed during the first EAE episode (Figure 3L) and at EAE relapse (Figure 3N), but showed substantial recov-

Figure 2. CTB retrograde labeling of L2-L4 spinal motoneurons in the acute EAE model. **A, B,** and **C** show gray matter labeling in normal, 12-dpi EAE peak and 28-dpi EAE recovered animals. The morphology of motoneurons and the gray matter dendrites do not show marked differences, and there was no dendritic beading at the EAE peak. **D, E,** and **F** show white matter dendritic labeling in normal, EAE peak, and recovered animals. White matter dendrites in normal spinal cord (**D**, arrowheads) are labeled as fine lines with fairly constant thickness. At the EAE peak (**E**), most white matter dendrites show a typical beading pattern, and the majority of swellings (arrowheads) are 10 to 20 μm in diameter. The intersegments, although continuous between beads, are thinner than normal dendrites. These changes resolve in most white matter dendrites (**F**) after clinical EAE recovery. Scale bars, 25 μm .

ery during EAE remission (Figure 3M) and after EAE recovery (Figure 3O).

To examine whether there were overall changes in MAP2 signals in the gray and white matter of LSSC at different EAE stages, we quantitated MAP2 staining with the NIH Image software (Figure 4). Statistical analysis was focused on comparing whether the MAP2 level at each EAE stage was significantly different from its immediate previous stage, and on comparing whether the MAP2 levels after full EAE recovery had significant differences with those in the normal tissue. In the gray matter of LSSC, MAP2 signals were moderately but significantly reduced at the peak of acute EAE (14%) and in the first episode of chronic EAE (12%). After full EAE recovery, the gray matter MAP2 level was not significantly different from the normal level in either EAE model. In the white matter, MAP2 signals were more markedly reduced in acute EAE (32%), in the first episode (52%) and in the relapse phase (21% reduction from the remission level) of chronic EAE. There was significant recovery of white matter MAP2 signals during EAE recovery and remission. However, after 12 days in full recovery from chronic EAE, MAP2 signals were still significantly lower than normal levels. Immunoblot analysis with samples collected from Lewis rats at various stages of the acute EAE confirmed that MAP2 protein level was markedly reduced in LSSC during EAE, but was largely recovered after clinical EAE recovery (Figure 5).

We conclude from these experiments that: 1) dendritic damage in inflamed LSSC is more severe in the white matter during EAE attacks; 2) dendritic beading and reduced MAP2 signals in the white matter of LSSC are correlated with clinical EAE signs and pathological inflammation in LSSC; 3) there is significant recovery in

both dendritic beading and MAP2 signals in the white matter of LSSC during EAE remission and after EAE recovery; 4) in the chronic EAE model, the recovery of MAP2 signals in the white matter of LSSC is less satisfactory after full EAE recovery.

Synaptic Pathology in Acute and Chronic EAE

Because most synapses are present on neuronal dendrites, we further examined whether synaptic structure in LSSC was damaged in EAE. Synaptophysin and synapsin I are presynaptic proteins. Synaptophysin is localized on the membrane of synaptic vesicles, and synapsin I is a cytoplasmic protein linking synaptic vesicles to actin filaments and other cytoskeleton proteins.³⁰ In addition, PSD-95 is an important postsynaptic density protein, which is associated with NMDA receptors at excitatory synapses.³¹

In the gray matter of LSSC sections from normal Lewis rats, immunostaining for these synaptic proteins yielded punctuated signals, which collectively encircled large spinal motoneurons (Figure 6; A to C). PSD-95 IHC stained the cytoplasm of motoneurons as well. The thicker proximal dendrites in the gray matter were stained along their membrane, so that a thin tube-like pattern could be observed. Remaining punctuated signals may represent the synapses on the thinner dendrites. At the peak of acute EAE, the positive staining around neuronal somata and along proximal dendrites was greatly reduced for all three proteins (Figure 6; D to F). The overall staining intensity also became much paler. Consistent with previous reports,^{32,33} PSD-95 IHC stained some infiltrating inflammatory cells (Figure 6F). Nevertheless,

Figure 3. Pathological inflammation and dendritic beading in DA rat LSSC during chronic EAE. Please note that in this figure various EAE stages are arranged in columns while different staining is in rows. H&E staining (**A–E**) shows that perivascular and parenchymal inflammation are evident in both the gray and white matter of LSSC at the first EAE peak (**B**) and EAE relapse (**D**), but resolve at EAE remission (**C**) and after EAE recovery (**E**). Throughout the entire chronic EAE course, MAP2 IHC shows little morphological changes in the gray matter dendrites (**F–J**). In contrast, extensive dendritic beading occurs in the white matter at the first EAE peak (**L**) and EAE relapse (**N**), but is substantially recovered at EAE remission (**M**) and after clinical EAE recovery (**O**). Scale bars: 50 μ m (**A–E**); 25 μ m (**F–O**).

substantial recovery in these stainings was observed after clinical EAE recovery (Figure 6; G to I).

In the white matter of normal LSSC sections from Lewis rats, IHC for these synaptic proteins showed branch-like

patterns that closely resemble the dendritic distribution shown in MAP2 IHC (Figure 7; A to C). At the EAE peak, positive staining for all three markers was greatly reduced (Figure 7; D to F). Instead, inflammatory cells were

Figure 4. Quantitation of MAP2 IHC at different stages of acute EAE (A) and chronic EAE (B). Analysis of variance tests showed significant differences ($P < 0.01$) in all four groups of data, and post hoc tests were applied to examine the significance of differences between individual subgroups. **Asterisks** are shown above the bars when the mean values are significantly different ($P < 0.05$) from their immediate previous ones. **Asterisks** are also shown when the differences between the normal and recovery levels are statistically significant ($P < 0.05$). Results show that in the gray matter of LSSC, MAP2 signals are significantly reduced in acute EAE and in the first episode of chronic EAE. After EAE recovery, there are no significant differences in the gray matter in either EAE model. In white matter, signals of MAP2 IHC are markedly reduced in acute EAE, in the first episode and relapse phase of chronic EAE. There is significant recovery of white matter MAP2 signals during EAE recovery and remission. In the chronic but not acute EAE model, white matter MAP2 signals are significantly lower than normal after clinical EAE recovery.

strongly labeled with the anti-PSD-95 antibody. Similar to the gray matter, there was marked recovery of staining signals after EAE fully recovered (Figure 7; G to I).

Quantitation showed that at the acute EAE peak, positively stained areas in synaptophysin, synapsin I, and PSD-95 IHC were reduced by 29%, 15%, and 44% in gray matter (Figure 8A) and 55%, 47%, and 53% in the white matter on average (Figure 8C). The numbers of positively stained neurons in the ventral gray matter of LSSCs were reduced by 47%, 66%, and 83% in synaptophysin, synapsin I, and PSD-95 IHC at the EAE peak (Figure 8B), suggesting that in the gray matter there is more dramatic reduction of synaptic proteins around neuronal somata. Although there was significant recovery of staining signals after EAE recovery, statistical analysis showed that except for the positive areas of synaptophysin and synapsin I IHC in gray matter, all other measurements at 20 dpi were still significantly lower than normal levels (Figure 8; A to C).

To confirm the marked reduction of synaptic proteins in acute EAE, immunoblot for these synaptic proteins was performed in LSSC protein samples obtained at different

EAE stages (Figure 9). The data show that immunoreactivity for all three proteins was markedly reduced at EAE peak. Although the reduction of synaptophysin and synapsin I immunoreactivity correlated with EAE severity, the reduction of PSD-95 immunoreactivity was very strong even at the EAE onset. There was marked recovery in immunoreactivity of these synaptic proteins after full EAE recovery at 20 dpi. Because of the small sample sizes, no further quantitation was performed.

Synaptic protein IHC in chronic EAE showed quite similar results. Quantitation showed that during the first EAE episode, the areas positively stained with synaptophysin, synapsin I, and PSD-95 IHC were reduced by 28%, 22%, and 44% in the gray matter (Figure 10A), and 31%, 48%, and 42% in the white matter (Figure 10C) on average. In addition, the numbers of positively stained neurons in ventral gray matter were reduced by 50%, 36%, and 66% in synaptophysin, synapsin I, and PSD-95 IHC (Figure 10B). Marked reductions in these measurements were also observed in EAE relapses, and significant recovery was observed during EAE remission and after EAE recovery. However, after 12 days in full EAE recovery, only partial recovery in synaptic protein IHC was reached, except for the synaptophysin-positive and synapsin I-positive areas in the gray matter.

We conclude from these experiments that: 1) presynaptic and postsynaptic structures were damaged in both the gray and white matter of LSSC during EAE attacks; 2) in the gray matter of LSSC, more severe synaptic damage was observed around the neuronal somata; 3) substantial recovery of synaptic structure occurs during EAE remission and after EAE recovery; 4) at the time point of EAE recovery we examined, the recovery of synaptic structures in most measurements is incomplete. This is especially apparent in the white matter of LSSC.

Discussion

Our data show that there is extensive dendritic beading in the white matter of LSSC during clinical EAE, and this change is associated with local inflammatory cell infiltration. Dendritic damage appears to be separable from CNS demyelination, because it is present in the acute EAE in a similar degree.

Dendritic beading signifies that dendrites lose their smooth shape and appear as alternating swellings and constrictions.³⁴ Electron microscopy studies found that microtubules were greatly reduced and became short fragments in swollen regions.³⁵ In contrast, tightly packed microtubules were found in the thin intersegments. Dendritic beading occurs most frequently in excitotoxic conditions. The morphology of beaded dendrites in EAE as shown by our CTB retrograde labeling is very similar to that described in cortical neuron culture treated with glutamate receptor agonists.¹⁷ Studies have shown that excessive Na^+ influx through glutamate receptor channels is both necessary and sufficient to induce dendritic beading in excitotoxic conditions, whereas Ca^{2+} influx is unnecessary.³⁶ Our data also showed that MAP2 signals were decreased in both the

Figure 5. Immunoblot analysis of MAP2 levels in LSSC at different stages of acute EAE [0.5°, 1°, 2°, 3°, and recovery (R)] compared with those in normal (N) LSSC. Different lanes represent protein samples from different animals. The amount of loaded protein was shown with β -actin immunoblot. The data show that MAP2 protein level in LSSC was markedly reduced during EAE, but was almost completely recovered after clinical EAE recovery.

Figure 6. The changes of synaptic protein immunostaining in the gray matter of LSSC in acute EAE. In the normal spinal cord (**A–C**), large motoneurons and their proximal dendrites in the ventral horns are positively stained in synaptophysin, synapsin I, and PSD-95 IHC. Punctate staining in thinner dendrites is also evident. At the EAE peak (**D–F**), the positive staining around neuronal somata as well as the overall gray matter staining is reduced. The synaptic protein signals are substantially recovered after clinical EAE recovery (**G–I**). Scale bars, 25 μ m.

white and gray matter of LSSC during EAE attacks. MAP2 loss may also be induced by excitotoxicity, because treatment with either a glutamate release inhibitor riluzole or calpain inhibitors reduced MAP2 loss in spinal cord trauma models.³⁷ However, it remains to be determined whether exposure to other inflammatory factors, such as free radicals, inflammatory cytokines, or proteases may also result in dendritic beading or MAP2 loss.

Although similar levels of inflammation were present in the gray matter and white matter of LSSC during EAE, dendritic damage and MAP2 loss were much milder in the gray matter than in the white matter. Several possibilities may be considered. First, in spinal motoneurons, the conductance of excitatory synapses on distal dendrites may be many times higher than that on proximal dendrites.^{38,39} Therefore, similar levels of excitotoxic conditions may more severely damage distal dendrites. Secondly, the gray matter may have higher capacity to clear the extracellular glutamate because the gray matter contains much more synapses than does the white matter. Thirdly, the glutamate uptake system may be preferentially damaged in the spinal cord white matter. It was recently reported that GLT-1 expression in oligodendrocytes was markedly reduced in the white matter regions adjacent to MS lesions.¹¹ A similar reduction in EAE may

result in more pronounced dendritic damage in the white matter of LSSC.

We found profound reductions in synaptophysin, synapsin I, and PSD-95 immunoreactivity in both the white and gray matter of LSSC during acute and relapsing EAE episodes. Because there is marked gray matter involvement, dendritic beading is not the direct cause for synaptic damage in EAE. PSD-95 is associated with NMDA receptors, and is a substrate for calpain.²³ Because calpain is also present in the postsynaptic density,⁴⁰ the loss of PSD-95 can be associated with excessive NMDA receptor activation under excitotoxic conditions.^{23,41} It has also been reported that marked loss of presynaptic proteins, such as synaptophysin and synapsin I, which is characteristic for lesions in Alzheimer's disease,⁴² is correlated with localized loss of glutamate transporter activity.²⁴ Recent studies in transient cerebral ischemia models, in which excitotoxicity may play an important pathogenic role, showed significant loss of many presynaptic proteins, including synaptophysin and synapsin I, after 1 to 3 days of reperfusion.^{43,44} Although both dendritic beading and loss of synaptic proteins may involve excitotoxicity, their different distribution in EAE spinal cord may reflect different receptor-based mechanisms. NMDA receptors are uniformly distributed or even more

Figure 7. The changes of synaptic protein immunostaining in the white matter of LSSC in acute EAE. Synaptophysin, synapsin I, and PSD-95 IHC (A–C) all show branch-like patterns in the normal white matter, which are similar to the MAP2 staining pattern. At the EAE peak, signals for all three synaptic proteins are markedly reduced (D–F), while some inflammatory cells are positive for PSD-95 (F). Synaptic protein signals show partial recovery at 20 dpi when clinical EAE is fully recovered (G–I). Scale bars, 25 μ m.

enriched in proximal dendrites, whereas AMPA/KA receptors are expressed more highly in distal dendrites.⁴⁵ It is possible that Na^+ influx through the AMPA/KA or NMDA receptors is mainly responsible for dendritic beading, whereas Ca^{2+} influx through NMDA receptors is mainly responsible for the loss of synaptic proteins in our EAE models.

Other possible mechanisms for synaptic damage in EAE include inflammation and synapse-directed autoimmunity. It has been shown that presynaptic protein loss and inflammation are co-localized in Alzheimer's lesions, but both are absent in amyloid-deposited brain areas in nondemented seniors.⁴⁶ Consistent with this, dendritic and synaptic damage have been reported in HIV en-

Figure 8. Quantitation of synaptic protein IHC in acute EAE. Both the area of positive staining (A) and the number of stained neurons (B) were quantitated from the ventral gray matter. The area of positive staining in the white matter was also quantitated (C). Analysis of variance tests showed that significant differences existed within all nine groups ($P < 0.01$), and post hoc tests were performed to examine the significance of differences between individual subgroups. Asterisks were shown in the same way as described in Figure 4. The results show that positive signals for all three markers were significantly reduced in both the gray and white matter of LSSC at the acute EAE peak. Significant recovery in these synaptic protein signals was found after clinical EAE recovery. However, except for the gray matter area positive in synaptophysin and synapsin I IHC, other measurements only show partial recovery at 20 dpi.

Figure 9. Immunoblot analysis of synaptophysin, synapsin I, and PSD-95 levels in LSSC at different EAE stages [0.5°, 1°, 2°, 3°, and recovery (R)] compared with those in normal (N) LSSC. Different lanes represent protein samples from different animals. The amount of loaded protein was shown with β -actin immunoblot. The two bands in synapsin I immunoblot represent the 80 kD of synapsin Ia and the 77 kD of synapsin Ib. The marked reduction in synaptophysin and synapsin I immunoreactivity was well correlated with EAE severity. The reduction in PSD-95 level was strong even at the EAE onset. There was marked recovery of immunoreactivity for these synaptic proteins on clinical EAE recovery at 20 dpi.

cephalitis⁴⁷ and murine retroviral encephalitis.⁴⁸ Because inflammatory cells can release large amounts of glutamate,⁴⁹ and can down-regulate the expression of glutamate transporters,⁹ inflammation and excitotoxicity are closely related. Less is known about whether other inflammatory mediators, such as free radicals and various proteases, may also contribute to the synaptic damage.⁵⁰ On the other hand, it has been reported that both anti-MBP antibody and MBP-specific T cells can cross-react with synapsin I,^{51,52} and the presence of synapsin I-crossreacting T cells correlated with clinical EAE signs.⁵³ Therefore, autoimmunity is another possibility for the presynaptic structure damage in EAE. However, this mechanism seems less relevant to the loss of postsynaptic PSD-95.

The recovery of synaptic proteins, especially synaptophysin and synapsin I, has been reported in models of cerebral ischemia^{54,55} and spinal cord trauma.⁵⁶ However, whether the recovery in EAE reflects the replenishment of synaptic proteins or a plastic process of neosynaptogenesis needs to be determined. This recovery is a relatively rapid process, because it is evident during a 3- to 5-day period of remission. Nevertheless, even 12 days after full clinical recovery from chronic EAE, the signals of all three synaptic proteins were still significantly lower

than those in normal LSSCs, especially in the white matter. It is possible that frequent episodes of CNS autoimmune inflammatory attacks or chronic inflammatory processes may result in cumulative synaptic damage.

We propose that marked dendritic beading and synaptic protein loss in LSSC may contribute to the neurological dysfunction in EAE. In rat hippocampal slices that were treated with NMDA, dendritic beading was accompanied by AMPA receptor internalization and a rapidly occurring and long-lasting depression in synaptic transmission.¹⁸ Kainate-induced dendritic beading in cochlear spiral ganglion neurons is also associated with depressed auditory evoked potentials and reduced auditory sensitivity to sound.⁵⁷ Studies have suggested that the extensive white matter dendritic branches from spinal motoneurons have important physiological functions. In rats, spinal motoneurons receive cortex-generated voluntary movement commands indirectly through propriospinal fibers, and receive neuromodulatory innervation through bulbospinal fibers.⁵⁸ In the transverse plane of all levels of the spinal cord, the centrifugal dendritic branches in the white matter run parallel with the centripetal propriospinal and bulbospinal fibers, and serial contact and synapses are formed between the dendrites and the axons.²⁷ This powerful anatomical organization is further supported by the distribution of high-conductance excitatory synapses in distal dendrites of motoneurons,^{37,38} and the extensive 5-HT receptor expression along the entire dendritic tree.⁵⁹ Therefore, the transmission failure in white matter dendrites could possibly contribute to the movement dysfunction in EAE. More importantly, both presynaptic and postsynaptic proteins are markedly reduced in EAE spinal cord. Because spinal motoneurons depend on synaptic transmission to receive information from propriospinal and supraspinal fibers, synaptic protein loss is also likely to contribute to the motor dysfunction. 4-aminopyridine (4-AP), a potassium channel blocker, has been used to temporarily relieve the neurological symptoms in MS patients at a dose 1000 times lower than that required to restore electrical transmission in demyelinated fibers.⁶⁰ Evidence has shown that low-dose 4-AP treatment enhances synaptic transmission by increasing synaptic vesicle release.⁶¹ On the other hand, both dendritic beading and the loss of syn-

Figure 10. Quantitation of synaptic protein IHC in chronic EAE. Analysis of variance tests showed that significant differences existed within all nine groups ($P < 0.01$), and the pairwise data are presented in the same way as in Figure 7. The results show that the reduction of synaptic protein signals was significant in the first EAE episode and during EAE relapse, and that the recovery of staining signals was significant in EAE remission and EAE recovery. The only exception is that there is no significant recovery of synapsin I-positive neuron numbers at EAE remission. Except for the gray matter area positive for synaptophysin and synapsin I, other measurements only show partial recovery 12 days after full clinical EAE recovery.

aptic proteins in EAE may be self-protective mechanisms of spinal motoneurons.

Further study is required to demonstrate the dendritic and synaptic changes in EAE at the ultrastructural level. Electrophysiological study is needed to examine whether spinal motoneurons actually fail to receive propriospinal and bulbospinal information during EAE attacks. EAE models with more relapses and longer EAE course may be applied to determine whether dendritic and synaptic damage might accumulate after multiple relapses. It will also be interesting to examine whether similar dendritic and synaptic damage occurs in MS lesions, especially in spinal cord lesions and cortical lesions.

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