Motor neuron pathology in experimental autoimmune encephalomyelitis: studies in THY1-YFP transgenic mice

P. G. Bannerman,1 A. Hahn,1 S. Ramirez,1 M. Morley,1 C. Bönnemann,1 S. Yu,2 G.-X. Zhang,2 A. Rostami2 and D. Pleasure1

1Neurology Research, Abramson Pediatric Research Center, Children’s Hospital of Philadelphia and 2Department of Neurology, Thomas Jefferson Hospital, Philadelphia, Pennsylvania, USA

Correspondence to: David Pleasure MD, Room 516H Abramson Research Building, The Children’s Hospital of Philadelphia, 34th and Civic Center Blvd, Philadelphia, PA, USA
E-mail: pleasure@email.chop.edu

Using adult male C57BL/6 mice that express a yellow fluorescent protein transgene in their motor neurons, we induced experimental autoimmune encephalomyelitis (EAE) by immunization with myelin oligodendrocyte glycoprotein peptide 35–55 (MOG peptide) in complete Freund’s adjuvant (CFA). Control mice of the same transgenic strain received CFA without MOG peptide. Early in the course of their illness, the EAE mice showed lumbosacral spinal cord inflammation, demyelination and axonal fragmentation. By 14 weeks post-MOG peptide, these abnormalities were much less prominent, but the mice remained weak and, as in patients with progressive multiple sclerosis, spinal cord atrophy had developed. There was no significant loss of lumbar spinal cord motor neurons in the MOG peptide-EAE mice. However, early in the course of the illness, motor neuron dendrites were disrupted and motor neuron expression of hypophosphorylated neurofilament-H (hypoP-NF-H) immunoreactivity was diminished. By 14 weeks post-MOG peptide, hypoP-NF-H expression had returned to normal, but motor neuron dendritic abnormalities persisted and motor neuron perikaryal atrophy had appeared. We hypothesize that these motor neuron abnormalities contribute to weakness in this form of EAE and speculate that similar motor neuron abnormalities are present in patients with progressive multiple sclerosis.

Keywords: motor neuron; experimental autoimmune encephalomyelitis (EAE); dendrite; axonal degeneration; multiple sclerosis

Abbreviations: CFA = complete Freund’s adjuvant; EAE = experimental autoimmune encephalomyelitis; hypoP-NF-H = hypophosphorylated neurofilament heavy; MAP2a = microtubule-associated protein 2a; MBP = myelin basic protein; MOG = myelin oligodendrocyte glycoprotein; PBS = phosphate-buffered saline; YFP = yellow fluorescent protein

Received February 11, 2005. Revised April 4, 2005. Accepted April 21, 2005

Introduction

Multiple sclerosis is an immune-mediated disease characterized clinically by relapsing-remitting or progressive neurological deficits, and pathologically by multiple plaques of CNS inflammation and demyelination. While multiple sclerosis was for many years considered to be primarily a myelin sheath disorder, it is now clear that, as Charcot (1877) first observed, axonal fragmentation is an important component of the disease (Trapp et al., 1998; Perry and Anthony, 1999; Bjartmar et al., 2000, 2003). In vivo neuroimaging supports the concept that loss of CNS axons contributes substantially to progressive multiple sclerosis disability (De Stefano et al., 1998, 2001; Edwards et al., 1999; Miller et al., 2002; Lin et al., 2003, 2004). Occasionally, neuronal perikarya are also lost in multiple sclerosis (Peterson et al., 2001).

Experimental autoimmune encephalomyelitis (EAE) is elicited in susceptible animal strains by immunization with various myelin antigens. As in multiple sclerosis, pathological features of EAE include foci of CNS inflammation, demyelination, and axonal blebbing, tortuosity and fragmentation (Slavin et al., 1998; Pitt et al., 2000; Kornek et al., 2001;
Onuki et al., 2001). Reversible motor neuron dendritic beading has also been observed during the acute phase of myelin basic protein (MBP)-induced EAE in Lewis rats (Zhu et al., 2003). Death of neurons has been documented in greater detail in EAE than in multiple sclerosis. For example, approximately one quarter of spinal cord ventral horn neurons are lost in Lewis rats with MBP-induced EAE (Smith et al., 2000) and neuronal apoptosis occurs in the CNS of mice and rats with MBP- or myelin oligodendrocyte glycoprotein (MOG) peptide-induced EAE (Meyer et al., 2002; Zhang et al., 2003). MOG-EAE and control thy1-YFP transgenic mice were sacrificed shortly after the onset of deficits (14 days post-immunization), at peak mean clinical severity (21 days post-immunization) and late in the course of their illness (98 days post-immunization).

**Spinal cord tissue fixation and processing**

MOG peptide-EAE and control thy1-YFP mice were anaesthetized with ketamine/xylazine, then perfused via the heart with saline followed by phosphate-buffered 4% (w/v) paraformaldehyde. Spinal cords were removed, post-fixed at 25°C with phosphate-buffered 4% paraformaldehyde for 1 h, then transected in the mid-lumbar region and processed for paraffin and cryostat sectioning. The paraffin sections corresponded to spinal cord levels L5 and L6, whereas the cryostat sections corresponded to spinal cord levels L5 and L4.

To prepare paraffin sections for fluorescence microscopy, spinal cord was washed three times with phosphate-buffered saline (PBS) post-fixation, dehydrated through ascending ethanol, cleared in xylene, and infiltrated with Paraplast wax (Surgipath, Richmond, IL, USA). Six μm transverse sections were collected on Superfrost/Plus slides (Fisher Scientific Morris Plains, NJ, USA) and melted at 56°C for 20 min, then deparaffinized in xylene and rehydrated through descending alcohols.

For cryostat sections, lumbar spinal cord was cryoprotected by overnight immersion in 30% sucrose at 4°C prior to embedding in OCT cryostat mounting medium (Miles Inc, Kankakee, IL, USA). After freezing in 2-methylbutane cooled with liquid nitrogen, transverse 10 μm cryostat sections were mounted on poly-l-lysine coated slides.

**Spinal cord immunohistology**

The primary antibodies used in this study are listed in Table 1. Paraffin sections were deparaffinized and treated with 10% (v/v) Triton X-100 in PBS for 30 min, then immunostained with mouse monoclonal antibodies (mAbs) using a mouse-on-mouse kit (Vector Labs Burlingame, CA, USA) as per the manufacturer’s instructions. Binding of the biotinylated secondary layer to the primary antibody was detected with rhodamine-conjugated streptavidin (diluted 1 : 100, Jackson Immunoresearch Labs, West Grove, PA, USA). For combined detection of CD11b+ microglia/macrophages or CD40+ lymphocytes and TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling) histochemistry for intranuclear DNA nicking (Gavrieli et al., 1992; Ray et al., 2000), frozen sections were incubated overnight with rat anti-CD11b or goat anti-CD40 in block A [minimum essential medium containing 15 mM HEPES buffer, 10% foetal bovine serum (FBS) and 0.05% sodium azide].

Detection of primary antibody binding was performed using rhodamine-conjugated secondary antibodies. Then we applied a minor modification of the TUNEL procedure (Gavrieli et al., 1992), substituting Pacific blue-conjugated streptavidin for streptavidin peroxidase (Yasuda et al., 1995). Following immunolabelling with primary and secondary antibodies, all sections, both paraffin and cryostat, were post-fixed with −20°C methanol, counterstained (with the exception of the slides used for TUNEL) with the nuclear dye.
Table 1 Antibodies used in this study

<table>
<thead>
<tr>
<th>Antibody specificity</th>
<th>Name</th>
<th>Titre used</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hypophosphorylated NF-H</td>
<td>SMI-32 ascites 1 : 800</td>
<td></td>
<td>Sternberger Monoclonals Inc., Lutherville, MD, USA</td>
</tr>
<tr>
<td>Hypophosphorylated NF-H</td>
<td>RMDO9.5 ascites 1 : 100</td>
<td></td>
<td>V.M.-Y. Lee PhD, Pathology, University of Pennsylvania, PA, USA</td>
</tr>
<tr>
<td>Myelin basic protein</td>
<td>SMI-99 ascites 1 : 1000</td>
<td></td>
<td>Sternberger Monoclonals Inc.</td>
</tr>
<tr>
<td>CD11b antigen</td>
<td>M1/70 10 µg/ml</td>
<td></td>
<td>Serotec, Raleigh, NC, USA</td>
</tr>
<tr>
<td>CD40 antigen</td>
<td>sc-975 10 µg/ml</td>
<td></td>
<td>Santa Cruz Biotechnology, Inc., Santa Cruz, California, USA</td>
</tr>
<tr>
<td>Microtubule-associated</td>
<td>AP14 ascites 1 : 200</td>
<td></td>
<td>V.M.-Y. Lee PhD, Pathology, University of Pennsylvania (2005) Page 4 of 10</td>
</tr>
<tr>
<td>Protein 2a (MAP2a)</td>
<td>AP5B3 1 : 750</td>
<td></td>
<td>Dr A. Beggs</td>
</tr>
<tr>
<td>Sarcospan</td>
<td>81864 1 : 2500</td>
<td></td>
<td>Dr L. Kunkel</td>
</tr>
</tbody>
</table>

Skeletal muscle immunohistology
Cryostat sections (10 µm) of tibialis anterior obtained from anaesthetized mice prior to perfusion were fixed in 100% methanol for 20 s, pre-incubated in vehicle (10% FBS, 1x PBS, 0.1% Triton X-100) for 1 h, and then incubated with primary antibodies diluted in vehicle for 1 h, all at room temperature. Antibodies used were rabbit polyclonal AP5B3 (1 : 750) raised against alpha-actinin 3, which specifically stains type 2B skeletal muscle fibres, and rabbit polyclonal 81864 raised against sarcospan to outline the sarcolemma of individual muscle fibres (1 : 2500). The slides were then washed 3x for 20 min each with 1x PBS, and incubated with an Alexa fluor-labelled secondary antibody at room temperature for 1 h in the dark. After three additional washes, the slides were mounted in Vectashield.

Imaging and image analysis
Fluorescent images were captured with a Hamamatsu Orca-ER digital camera mounted on a Leica DMR upright microscope or by confocal microscopy with a Leica DM IRE2 HC fluo TCS 1-B-UV microscope coupled to a Leica TCS SP2 spectral confocal system. To evaluate numbers and sizes of motor neurons in EAE versus control animals, laser scanning confocal microscopic scans were performed on paraffin sections corresponding to spinal cord levels L5 through L6. The areas of ventral horn YFP positive cell bodies were measured using a Leica software program developed specifically for the confocal system (Leica Microsystems, Heidelberg, Germany).

The area of ventral horn investigated mapped to motor neurons innervating the following muscle groups: iliopsoas/sartorius, quadriceps, adductors, hamstrings, gluteal and other proximal muscles of the thigh, posterior compartment of the distal hind limb (Vanderhorst and Holstege, 1997), i.e. the major muscle groups controlling hind limb mobility. To standardize the measurements, only perikarya in which the nucleus and nucleolus were in the plane of the section were counted and measured (McPhail et al., 2004).

We devised a semi-quantitative scale to score the extent of motor neuron dendritic pathology, employing microtubule-associated protein 2a (MAP2a) immunostaining with the AP14 antibody. The scorer was not provided with information as to whether a specimen was from an EAE or a control mouse; nor, in the EAE mice, as to the duration and severity of EAE. A score of 0 (normal) was assigned when most or all motor neuron dendrites were of normal thickness and length. A score of + was assigned when the majority of motor neuron dendrites were thinner than normal, a score of ++ when the majority of motor neuron dendrites were shortened or fragmented, and a score of +++ when the majority of motor neuron dendrites were lost.

Results
Clinical features of MOG peptide-EAE
Control thy1-YFP transgenic mice (n = 20) did not demonstrate weight loss or neurological deficits at any point following CFA injection. Thirty-four mice were immunized with MOG peptide in CFA. Of these, four did not show weight loss or neurological deficits at any point during the next 98 days; these four were excluded from further analysis. In the remaining 30 MOG peptide-injected mice, neurological deficits first appeared 12–14 days post-immunization, preceded by 1 or 2 days of weight loss which averaged 10% of pre-symptomatic body weight.

Five EAE mice and four CFA controls were sacrificed on day 14 post-immunization. Clinical severity in the remaining EAE mice peaked between days 19 and 21 post-immunization (Fig. 1). Fifteen EAE mice and eight CFA controls were sacrificed on day 21. In the remaining 10 EAE mice, clinical deficits then subsided to a plateau level. However, in three of these 10, a relapse occurred between day 21 and day 98 post-immunization, defined as an increase in severity of neurological deficits of one grade or more that persisted for ≥3 days. Two of the 10 EAE mice had become entirely asymptomatic by day 98 post-MOG peptide. These remaining 10 EAE mice and eight CFA controls were sacrificed on day 98.

Lumbosacral spinal cord inflammation, demyelination and axonal fragmentation in MOG peptide-EAE
Accumulations of inflammatory cells in spinal cord were prominent on day 14 after administration of MOG peptide in CFA, but were less apparent by days 21 and 98, and were not present in CFA control mice. Combined immunofluorescence/TUNEL histochemistry demonstrated frequent CD11b+ or CD40+ cells undergoing apoptosis on day 14 post-MOG peptide, as has previously been reported by Ray et al. (2000). Demyelination, as visualized by MBP immunoreactivity, was most evident in mice sacrificed...
21 days post-MOG peptide. Substantial remyelination had occurred by day 98 post-MOG peptide. Disrupted and balloononed axons, visualized by hypophosphorylated neurofilament heavy (hypoP-NF-H) immunohistology (Sternberger and Sternberger, 1983; Lee et al., 1987), were present in spinal cord white matter on day 14 post-MOG peptide, and had become more prominent by day 21 (data not shown).

Spinal cord grey and white matter atrophy occur in MOG peptide-EAE
Spinal cord atrophy has been well documented in multiple sclerosis (Edwards et al., 1999; Lin et al., 2003, 2004; Ukkonen et al., 2003). To determine whether spinal cord atrophy also occurs in MOG peptide-EAE, we compared the areas of grey and white matter in paraffin-embedded cross sections of L5,6 spinal cord from 98-day post-MOG peptide and 98-day post-CFA control mice. There were ~20% smaller cross-sectional areas of both grey matter (EAE = 0.948 ± 0.137 mm²; control = 1.230 ± 0.204 mm², mean ± SD, P < 0.01) and white matter (EAE = 0.891 ± 0.178 mm²; control = 1.106 ± 0.206 mm², mean ± SD; P < 0.05) in the MOG peptide-EAE mice.

Motor neurons survive in MOG peptide-EAE
Loss of spinal cord ventral horn neurons has previously been noted in rats with MBP-induced EAE (Smith et al., 2000). Motor neurons of the thy1-YFP transgenic mice expressed both cytoplasmic and nuclear YFP, with most intense fluorescence in the nucleus (Fig. 2). This intrinsic YFP fluorescence facilitated visualization of motor neurons in these mice.

Three lines of evidence indicated that substantial numbers of motor neurons were not lost as a consequence of MOG peptide-EAE in these mice. First, we saw no TUNEL+ YFP+ neurons in any of the mice we examined on day 14, 21, or 98 post-MOG peptide (data not shown). Secondly, there were no significant differences between densities of YFP+ motor neurons in cross sections of L5,6 spinal cords of MOG peptide-EAE and CFA control mice at day 98 post-immunization (MOG peptide-EAE mice = 114 ± 24; YFP control mice = 132 ± 20; mean ± SD, n = 8; P > 0.1). Thirdly, examination of L5,6-innervated skeletal muscles using polyclonal anti-sarcospan antibodies, which outline skeletal muscle sarcolemma, showed neither atrophic nor angulated skeletal muscle fibres in the day 98 post-MOG peptide EAE animals; and, using polyclonal anti-alpha-actinin 3 antibodies which specifically label type 2b skeletal muscle fibres, there was no fibre type-specific grouping (data not shown). These immunohistological results indicated that skeletal muscle fibres were neither denervated nor reinnervated by axonal collateral sprouting at this late time-point in these mice.

Motor neuron phenotype is altered in MOG peptide-EAE
We documented alterations in motor neuron perikaryal size, perikaryal cytoskeletal phosphorylation and dendrites in the
MOG peptide-EAE mice. First, while the mean L5,6 spinal cord motor neuron perikaryal cross-sectional area in these mice was not significantly different from that in the CFA control mice, the average transverse area of L5,6 motor neuron perikarya in the 98-day post-MOG peptide mice was 15% smaller than that in simultaneous CFA controls (427 ± 75 versus 502 ± 48 μm², respectively, mean ± SD, P < 0.02).

Secondly, we noted a widespread, but reversible, alteration in motor neuron perikaryal neurofilament phosphorylation in the EAE mice at day 21 post-MOG peptide immunization. As previously reported (Carriedo et al., 1996; Tsang et al., 2000), motor neurons of CFA control mice expressed abundant perikaryal hypoP-NF-H immunoreactivity. Whereas >80% of lumbar spinal cord motor neuron perikarya

Fig. 2 Motor neuron perikaryal hypoP-NF-H immunoreactivity is depleted in MOG peptide-EAE. In these transversely oriented sections through L5,6 spinal cord anterior horns, Hoechst nuclear staining is shown in blue, hypoP-NF-H (SMI-32 mAb) immunostaining in red, and YFP fluorescence in green. Panels A, C and E are identical fields from a 21-day post-CFA control mouse. Panels B, D and F are identical fields from a 21-day post-MOG peptide EAE mouse with a clinical disability score of 3.5. Note that YFP fluorescence was most intense in motor neuron nuclei in both control and EAE mice (A, B). Also note the co-localization of YFP and hypoP-NF-H in motor neuron perikarya of the CFA control (E), resulting in the yellow colour and the paucity of hypoP-NF-H in the YFP+ motor neuron perikarya in the MOG peptide-EAE mouse (F). Magnifications are the same in the six panels; the size bar denotes 40 μm.
expressed abundant hypoP-NF-H immunoreactivity in the 21-day post-CFA control mice (n = 8), this proportion had fallen to <70% in 21-day post-MOG peptide EAE mice with mild to moderate clinical deficits (deficit score 2.5 or lower, n = 6), and to <60% in 21-day post-MOG peptide EAE mice with severe clinical deficits (deficit score 3 or higher, n = 9) (P < 0.01, χ² test).

An example of the depletion of hypoP-NF-H immunostaining in a mouse with grade 4 clinical deficits is shown in Fig. 2. In the fields illustrated in this figure, average motor neuron perikaryal size was larger in the CFA control mouse than the MOG peptide-EAE mouse, but this was not a consistent finding at this time-point. Even though clinical deficits persisted beyond 21 days in most mice, motor neuron perikaryal hypoP-NF-H immunoreactivity had returned to normal by 98-day post-MOG peptide immunization (data not shown).

The third abnormality we observed in motor neurons of the MOG peptide-EAE mice was dendritic thinning, shortening and fragmentation. This was evident from both YFP fluorescence and MAP2a immunofluorescence microscopy (Papandrikopoulou et al., 1989; Riederer et al., 1995) (Fig. 3). Note the paucity of motor neuron dendrites in the 14-day post-MOG peptide EAE mouse (clinical deficit score 2.5) in Fig. 3C and D compared with those of the 14-day CFA control mouse shown in Fig. 3A and B. MAP2a immunostaining was particularly useful in highlighting this dendritic abnormality.

To permit semi-quantitation of dendritic alterations in the 30 MOG peptide-EAE mice and 20 CFA control mice that we autopsied, we devised a semi-quantitative score that reflected the aggregate severities of motor neuron dendritic shortening, fragmentation and thinning, assigning grades varying between normal (0) and very marked dendritic pathology (+++) (see Methods). An example of each of these scores is shown in Fig. 4; each panel is a Z-stack of 12 sequential 500-nm confocal optical slices through an L5,6 lumbar anterior horn. An observer without knowledge of the history of individual mice then scored the motor neuron dendritic pathology in L5,6 ventral horns in each of the 50 mice, again using Z-stacks of 12 sequential 500-nm confocal optical sections. Only the red (MAP2a) channel was viewed in order to avoid potential bias introduced by the presence or absence of inflammatory cells in the field. Results are summarized in Fig. 5.

Nineteen of the 20 CFA control mice, but only four of the 30 MOG peptide-EAE mice, received a score of 0. Moderate to severe dendritic pathology (++) or (+++) was scored for 17 of the 30 MOG peptide-EAE mice, but was not seen in any of the CFA control mice. Dendritic abnormalities were observed in the majority of the MOG peptide-EAE mice both early (day 14 post-immunization) and late (day 98 post-immunization) in their illness. Interestingly, the two EAE mice with

![Fig. 3](image-url) Motor neuron dendrites are disrupted in MOG peptide-EAE. In these transverse paraffin sections through L5,6 spinal cord, nuclear Hoechst staining is shown in blue, MAP2a (AP14) immunostaining in red, and YFP fluorescence in green. Co-localization of YFP (green) and MAP2a (red) in the cytoplasm of motor neurons in (A) and (C) results in the yellow coloration of portions of the motor neuron perikarya and dendrites. (A) and (B) are identical fields from a 14-day post-CFA control mouse, while (C) and (D) are identical fields from a 14-day post-MOG peptide-EAE mouse with a clinical score of 2.5. Note the depletion of AP14+ dendrites in (C) and (D). Magnifications are the same in the four panels; the size bar denotes 50 μm.
grade 0 (normal) L5,6 motor neuron dendrites on day 98 post-MOG peptide (see Fig. 5) were the only two in the group of 10 EAE mice examined at that time-point which had shown full clinical recovery from their clinical neurological deficits.

**Discussion**

Until 1998, most clinicians attributed all neurological deficits in multiple sclerosis to demyelination, with permanent deficits presumed to result from incomplete remyelination and nerve action potential conduction block. But the rediscovery of multiple sclerosis-associated axonal disruption by confocal microscopy (Trapp et al., 1998), followed by longitudinal in vivo imaging and magnetic resonance spectroscopic evidences of axonal loss in patients with multiple sclerosis (De Stefano et al., 1998, 2001; Edwards et al., 1999; Filippi et al., 2003; Lin et al., 2003, 2004) have led to recognition that axonopathy is a major cause of progressive and permanent neurological disability in patients with multiple sclerosis.
(Bjartmar et al., 2000, 2003). The causes of axonopathy in multiple sclerosis and EAE have still not been fully established (Kornek et al., 2001; Craner et al., 2004; Stys, 2004). However, axonal disruption is most prevalent within plaques of demyelination (Trapp et al., 1998; Bjartmar et al., 2003), suggesting that inflammatory processes centred in or bordering plaques target axons as well as myelin or, alternatively, that a primary loss of myelin within plaques causes secondary loss of axonal integrity.

Most of the surviving mice with EAE in the present study still demonstrated clinical neurological deficits 98 days after receiving MOG peptide. At this late time-point, spinal cord white matter atrophy had developed, presumably as a consequence of depletion of myelinated axons. While we are not aware of prior reports of spinal cord white matter atrophy in EAE, autopsy and in vivo MRI studies have demonstrated white matter atrophy in patients with progressive multiple sclerosis (Charcot, 1877; Edwards et al., 1999; Lin et al., 2003, 2004; Ukkonen et al., 2003).

Neuronal apoptosis occurs in the spinal cord and retina in EAE (Ahmed et al., 2002; Diem et al., 2003; Hobom et al., 2004) and in multiple sclerosis plaques that involve cerebral cortical grey matter (Peterson et al., 2001). The observation that a substantial number of ventral horn neurons are lost within 2 weeks after immunization of Lewis rats with myelin basic protein (Smith et al., 2000), together with reports of spinal cord motor neuron dysfunction, focal amyotrophy and generalized weakness and fatigue in some patients with multiple sclerosis (Petajan, 1982; Fisher et al., 1983; Shefner et al., 1992; de Haan et al., 2000; Chaudhuri and Behan, 2004), motivated us to evaluate whether neuronal perikarya are targeted in MOG peptide-EAE. Based on the report by Smith et al. (2000), we anticipated that YFP fluorescence imaging might reveal substantial depletion of motor neurons in the MOG peptide-EAE mice. In fact, however, these mice did not show a statistically significant loss of motor neurons.

In accord with this result, TUNEL histochemistry did not demonstrate motor neuron apoptosis in the MOG peptide-EAE mice and, 98 days after MOG peptide administration, these mice did not show immunohistological evidences of skeletal muscle fibre denervation or re-innervation. The discrepancy between the observations of Smith et al. (2000) and our own may reflect the divergent species and immunogens used in the two studies, but differences in the neuron labelling protocols may also have contributed. Smith et al. (2000) counted all ventral horn neurons, including interneurons, labelled by cresyl violet, a Nissl stain, whereas we determined the density in L5,6 spinal cord of YFP+ motor neurons. Thus, it is possible that either ventral horn interneurons are selectively susceptible to death in EAE or the use by Smith et al. (2000) of cresyl violet resulted in substantial motor neuron undercounts in their EAE rats, as has been reported when this stain has been used in other circumstances where there is motor neuron perikaryal atrophy (McPhail et al., 2004).

Late in the course of EAE in the thy1-YFP transgenic mice, the mean cross-sectional area of lumbar spinal cord motor neuron perikarya was 15% below that in CFA control mice of the same strain and age. Motor neuron atrophy has not previously been documented in EAE or multiple sclerosis, nor has atrophy of this magnitude been reported in other neuronal subsets in these disorders (Miller et al., 2002), though a slight diminution in the size of parvocellular neurons in the lateral geniculate body was noted in patients with multiple sclerosis (Evangelou et al., 2001). Motor neuron perikaryal atrophy in the MOG peptide-EAE mice is unlikely to have been a consequence of motor neuron axotomy (McPhail et al., 2004), since there were no evidences in these mice of skeletal muscle denervation or re-innervation. Other possible causes for motor neuron shrinkage in these mice include a loss of necessary trophic input owing to EAE-induced motor neuron dendritic pathology (Zhu et al., 2003, and the present study) or a deleterious effect of chronic exposure to pro-inflammatory cytokines (Villarroya et al., 1997).

Much of the NF-H in normal motor neuron perikarya is hypophosphorylated, and becomes hyperphosphorylated only after transport into the axon (Carriedo et al., 1996; Sun et al., 1996; Veeranna et al., 1998; Brownlee et al., 2000; Tsang et al., 2000; Ackerley et al., 2004). In the MOG peptide-EAE mice, the proportion of motor neurons that displayed perikaryal hypophosphorylated NF-H-P immunoactivity had fallen substantially by day 21 post-MOG peptide. This perikaryal cytoskeletal phosphorylation alteration was most widespread in mice with the most severe clinical deficits. Our studies do not shed light on the cause of this temporary alteration in perikaryal neurofilament phosphorylation. However, prior studies have shown that increased phosphorylation of neuronal perikaryal NF-H can be elicited by raising the extracellular concentration of glutamate (Ackerley et al., 2000) and perturbed CNS extracellular glutamate homeostasis has been documented in EAE and multiple sclerosis (Hardin-Pouzet et al., 1997; Matute et al., 2001; Werner et al., 2001).

Using both YFP fluorescence and MAP2a immunofluorescence, we observed persistent alterations in the architecture of motor neuron dendrites in the MOG peptide-EAE mice, including proximal dendritic thinning, shortening and fragmentation. Even by stacking multiple confocal optical slices, we could not visualize all of the proximal dendrites of individual motor neurons accurately; this would best be achieved using in vivo cholera toxin or post-mortem Dil retrograde tracing techniques (Snider and Palavali, 1990; Ritz et al., 1992; Zhu et al., 2003). However, using a semi-quantitative scoring system to grade abnormalities visualized by MAP2a immunostaining, we were able to document motor neuron dendritic abnormalities throughout the course of MOG peptide-EAE. In addition, while the number of mice with EAE we examined at day 98 was small (n = 10), our observations suggested a correlation between full remission of clinical illness and restoration of normal dendritic architecture.
Motor neurons in EAE

We are not aware of prior reports of persistent dendritic abnormalities in motor neurons in multiple sclerosis or EAE. However, acute reversible beading of distal motor neuron dendrites in spinal cord white matter has been documented by both MAP2 immunostaining and in vivo cholera toxin retrograde labelling techniques in Lewis rats with MBP-induced EAE (Zhu et al., 2003).

In summary, we have documented perikaryal atrophy and disruption of dendritic architecture in lumbarosacral motor neurons of mice with MOG peptide-induced EAE. Morphometric studies will be required to determine the relative extents to which motor neuron perikaryal atrophy and dendritic disruption contribute to late spinal cord grey matter atrophy in these mice. Further investigation is also needed to evaluate whether similar motor neuron abnormalities occur in multiple sclerosis.

Acknowledgements

We wish to thank J. Golden, R. Kalb and J. Grinspan for their critical reading of this manuscript. This work was supported by the National Multiple Sclerosis Society, Muscular Dystrophy Association, and NIH grant NS25044.

References


