Neurodegeneration in multiple sclerosis

Defining the problem

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ABSTRACT It is increasingly apparent that neurodegeneration in multiple sclerosis (MS) begins earlier in the course of the disease than was previously believed. The loss of axons, which results in permanent deficits, is distributed beyond regions of abnormal-appearing CNS white matter, and a constant background of neuron loss continues to take place while clinical symptoms wax and wane. It is therefore important to precisely define the scope of neurodegeneration so that experimental and clinical approaches to providing neuroprotective therapies can be devised that will halt and reverse neuron damage. Some of the complexities involved in cellular interactions in the normal-appearing and obviously affected CNS are reviewed here as a starting point for the consideration of approaches to neuroprotective strategies. **NEUROLOGY 2007;68 (Suppl 3):S5–S12**

MS IS NOT MERELY AN INFLAMMATORY DEMYELINATING DISEASE OF WHITE MATTER Although multiple sclerosis (MS) is classically defined as a T-helper cell (Th1)-mediated autoimmune disease, with clear evidence of B-cell/antibody involvement in its pathogenesis, we now know that the consequences are not simply inflammation and demyelination in the white matter. The physical sites and the timing of the causal insults are being redefined as technologies used to visualize cellular and molecular morphology are modified and improved. In some cases these new technologies have allowed rediscovery of findings made years ago with much less sophisticated techniques. A challenge to the concept that the initial lesion in MS results from autoimmune activity comes from studies demonstrating that inflammation may not be the original trigger of lesion formation. Extensive apoptosis of oligodendrocytes (OGCs) and activation of microglia have been described in MS specimens that did not yet show evidence of lesions with lymphocytic infiltration or myelin-positive phagocytes.[1] Microglial activation without perivascular infiltration of T and/or B cells appears to be the initial lesion in normal-appearing white matter (NAWM).[2,3] This suggests the presence of a type 3 lesion[4,5] or, conceivably, a type 4 lesion as the precursor to the classic (and later-developing) types characterized by the presence of inflammatory cells and macrophages (types 1 and 2). Histologic and proton magnetic resonance spectroscopy (MRS) methods have also shown that the changes to axon integrity, as measured by neuron-specific *N*-acetyl aspartate (NAA) levels, occur in patients with MS who have no clinically significant disability or changes in total brain volume.[6–9] Even patients early in the disease (<3 years) have a reduced NAA:creatine (Cr) ratio but low T2-weighted MRI lesion loads, suggesting that axon injury occurs very early during development of MS and might initially occur, in some but not necessarily all areas, independently of myelin damage. Previous MRS studies showed that the NAA:Cr ratio was decreased in patients with relapsing–remitting MS (RRMS) and those with secondary-progressive MS (SPMS) but that total lesion volume in the patients with SPMS was lower than that in the patients with RRMS.[10] Admittedly, the latter may represent an effect of atrophy. The metabolic information provided by MRS analysis of NAA:Cr also proved to be a better predictor of Expanded Disability Status Scale (EDSS) score levels for patients with RRMS than did T2-weighted lesion volumes.[11,12] MRS can also be used to measure levels of choline, which is derived from myelin membrane lipids. Choline levels are higher in areas of NAWM that go on to develop MRI lesions 6 to 12 months later than in NAWM that does not develop lesions.[13] This,

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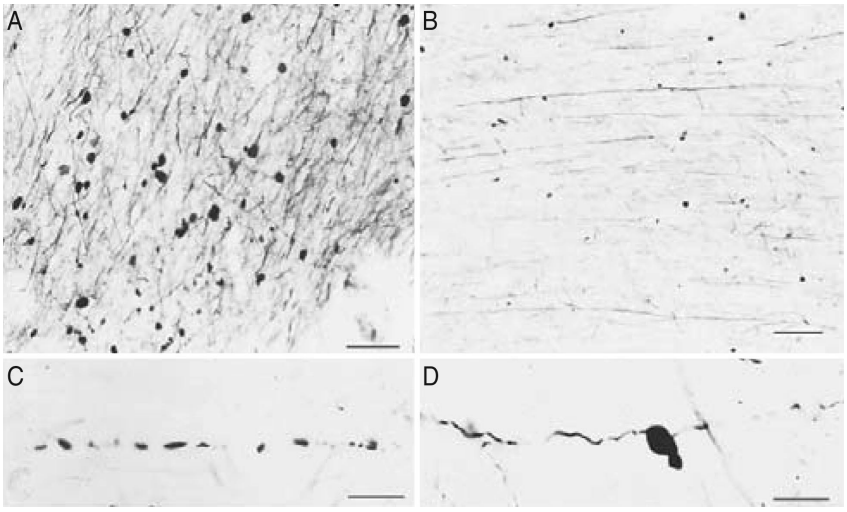
Figure 1 Patterns of pathologic axon changes in MS lesions



In active lesions (A), SMI-32-positive ovoids and axons are abundant. At the edges of chronic active lesions (B), nonphosphorylated neurofilament-positive axons and ovoids are less abundant and the ovoids are smaller. Most nonphosphorylated neurofilament-positive axons in MS lesions have a normal appearance (A,B). Some have discontinuous staining for nonphosphorylated neurofilaments (C), which is characteristic of axon degeneration. Other axons have constrictions, dilatations, or large swellings (D). Scale bars 60 μm (A,B), 52 μm (C,D). From Trapp et al.,[64] *with permission.*

again, suggests that myelin membrane damage occurs early, at least up to a year before development of MRI-detectable lesions, i.e., before the establishment of an obvious inflammatory lesion with changes in the blood–brain barrier (BBB) or plaques. Of course, one cannot exclude concomitant lower-grade inflammation in a more diffuse fashion, as has been suggested in more progressive phases of MS.[14]

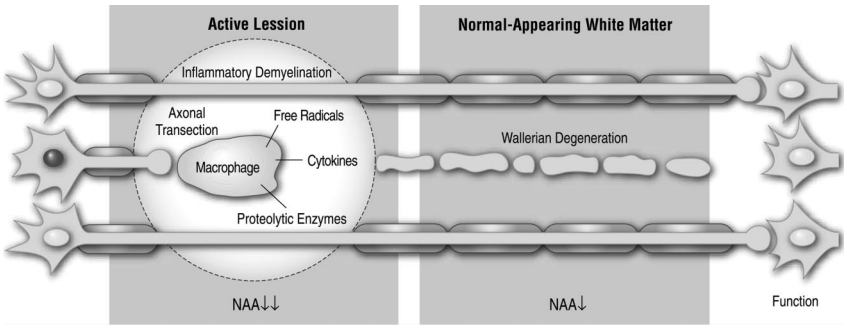
This and other new (and in some cases rediscovered) knowledge of early axon and neuron abnormalities has changed the study of MS in recent years. The progressive loss of neurologic function associated with MS, which continues in spite of therapies that reduce immune activity, implies the loss of CNS axons. Axon transections, which manifest as terminal ovoids that do not stain for myelin-associated proteolipid protein (PLP) but do stain for nonphosphorylated neurofilaments (with SMI-32 antibody), are found in high density in both active and chronic active lesions of brain specimens, with greatest densities within active lesions or on the edges of chronic active lesions (figure 1).[15] SMI-32–staining ovoids and axon tracts in the lesion sites are also associated with major histocompatibility complex (MHC) II-expressing cells, indicative of activated macrophages and microglial cells at these sites. Axon loss is not limited to the brain but is also found in spinal cord lesions in MS. An average of 68% of spinal cord lesions in samples from paralyzed patients with MS (EDSS score >7.5) contained axon transections, and NAA levels were reduced by 50% in cross-sections of spinal cord that contained lesions.[16] Based on axon volume, NAA levels were reduced 42% in demyelinated axons but were also reduced by 30% in axons of NAWM from patients with MS. In other words, NAA levels are reduced even in still-myelinated axons, and axon pathology is not limited to the lesion sites but extends into NAWM (figure 2). Because the reduced NAA levels, like the increased choline levels,[13] are detectable early in MS, at least some of the axon degeneration revealed by these altered compound levels is believed to remain clinically silent initially, perhaps due to compensatory mechanisms that are based in the cortex.[17]

In addition to the white matter lesions typically associated with MS, gray matter regions also exhibit demyelination that probably contributes to cognitive dysfunction (reviewed by Kutzelnigg et al.[14]) and other symptoms. Conventional MRI methods cannot easily distinguish low-contrast differences in gray matter, but diffusion tensor MRI, functional MRI, and MRS techniques have been used to quantify and chemically analyze gray matter lesions. Lesions in these areas also present with sharply defined demyelinated edges in cerebral cortex, basal ganglia, and gray matter areas of brainstem and spinal cord (see articles by Zivadinov and Arnold in this supplement). Unlike the WM lesions, the gray matter lesions are associated with 13-fold fewer $CD3^+$ lymphocytes and sixfold fewer $CD68^+$ macrophages/microglia. Microglia were found in these regions associated with apical dendrites, neuronal perikarya, and dendrites, but not with the ends of transected neurons, and it is still not clear whether or not microglia contribute to cortical lesion formation.[18]

Anatomic areas remote from the sites of inflammation and obvious myelin lesions also contain transected axons. DeLuca et al.[19] examined postmortem samples from brain, brainstem, and spinal cord of patients with MS and found axon loss in all three regions. However, there was little correlation between plaque load and axon loss, suggesting that demyelination may not be the primary determinant of spinal cord axon loss. Furthermore, there was an apparent selective loss of axons in corticospinal and posterior column tracts, implying increased sensitivity of these axons to MS-associated damage mechanisms. "Sick" OGCs and Schwann cells (in the peripheral nervous system [PNS]) may not provide appropriate signals, adhesion molecules, and/or trophic factors to axons, even when there is no inflammatory attack on the myelinating cell.[20–25]

RETHINKING MS AS A BIPHASIC DISEASE Although some investigators now consider MS to have two distinctly separate phases that reflect distinct pathogenetic mechanisms, inflammatory and noninflammatory, such an absolute division of phases is now also being challenged. Gadolinium (Gd)-

Figure 2 Axon damage caused by inflammatory demyelination in an active lesion



Substances produced by activated immune and glial cells may mediate tissue injury, including axon transection. Levels of the neuronal marker *N*-acetyl aspartate (NAA) are markedly decreased within lesions. NAA becomes reduced as a consequence of Wallerian degeneration in normal-appearing white matter distal to the lesion. Denervation of target neurons causes functional loss and possible downstream effects. From Bjartmar et al.,[16] with permission.

enhancing lesions are five- to tenfold more sensitive than clinical findings for the assessment of disease activity, but patients show deficit progression as well as progressive atrophy in the absence of Gd-enhancing lesions or new T2 lesions. T1-weighted, Gd-negative hypointense lesions[26,27] and overall CNS atrophy[28–32] are perhaps better ways to visualize tissue alterations that contribute to irreversible neurologic damage. Significant CNS atrophy begins during the earliest stages of disease, and there is a strong association between brain atrophy and disability.[33] Contributions of relapses to the accrual of disability have been described,[34] although others have not found this effect.[35] Disability accumulating in the absence of relapses, or during treatments that reduce relapse rate, challenges the division of the disease into distinct, mutually exclusive relapsing–remitting and progressive phases.[36] For example, interferon (IFN)-β causes a 30% decrease in relapse rate and a >50% reduction in MRI activity, but the effect of this drug on disability and brain atrophy is minimal (see Arnold article in this supplement). Support for an ongoing accumulation of subclinical neuron damage in the background of intermittent inflammation-related relapses stems from the recognition of the age-dependence of clinical phenotypes, regardless of whether the disease presents as RRMS or primary-progressive MS (PPMS).[37] According to this view, disability milestones that reflect irreversible neuron damage are reached on a predictable schedule that is not influenced by relapses. However, these studies have some limitations, in that patients were not entirely without any therapeutic intervention and the effect of some of these interventions could not be reliably predicted. In this view, MS is therefore a single disease but with either relapsing–remitting or progressive types of onset. Consistent with this view is the lack of correlation between clinical status and any of the four types of immunopathogenetic patterns described by the MS Lesion Project[38] and the evidence that distinct types of demyelinated lesions do correlate with the types of MS onset. Specifically, active focal demyelinated plaques are found primarily in patients with RRMS, whereas cortical demyelination or diffuse WM injury is mostly found in patients with SPMS and PPMS.[39] However, there is evidence that some changes in cortical gray matter and deep nuclei occur early in the disease.[8,18,40–47]

There may not be a complete dissociation of inflammatory lesions from axon and neuron changes, depending on the sensitivity of the imaging method being used. Triple-dose (TD) Gd-MRI is more sensitive than standard-dose (SD) Gd-MRI for quantification of lesion sites, although both show a correlation between lesion count and volumes of total and new enhancing lesions,[48] and there are probably additional insights to be gleaned from using magnets with higher field strengths. Tortorella et al.[15] used dual-echo and T1-weighted MRI with TD Gd-MRI to show that breakdown of the BBB is an obligatory event in the early phase of lesion formation. However, with the use of SD Gd-MRI and frequent scanning, new T2 lesions may appear without a preceding or concomitant Gd^+ enhancement. However, Gd provides information only about the extent of BBB disruption to humoral factors and not about the degree of cellular infiltration or inflammation already ongoing on the parenchymal side of the BBB. In fact, in glatiramer acetate (GA)-treated patients with RRMS, GA significantly reduced the number of Gd-enhancing lesions on SD and TD scans, but there was no correlation between Gd dose (in terms of number of total and new enhancing lesions) and treatment efficacy.[49] In other words, the Gd dose used was not important, and the effect of GA therefore did not depend on the severity of BBB disruption, at least as defined by Gd enhancement.

The emerging consensus is that the accumulated damage to neuronal structures best correlates with dysfunction in MS, which places increasing emphasis on the study of damage to axons and neurons. This is predicated on increasing evidence for the correlation of permanent deficits with loss of axons and neurons, and permanent disability with atrophy as assessed by MRI methods. It follows that the most effective treatments would be those that control or restore self-control to the inflammatory system in the many affected regions of the CNS, and that also provide, bolster, or reinitiate those types of mechanisms that fall within a definition of direct or indirect neuroprotective or neuroregenerative processes.

THE CONCEPT OF "NEUROPROTECTION" FOR MS AND OTHER CNS DISORDERS. How narrowly or how widely we define neuroprotection re-

flects, in turn, how well we understand the direct etiologies of the damage and what is perceived to be in need of restoration. Neuroprotection should relate not just to neurons and axons but also to OGCs, myelin, astrocytes, and microglia, and to the molecules they use for intercellular communication, all of which are vital components of normal CNS function. The definition of neuroprotection may include injury prevention, as in the preemption of neural cell damage. For example, polio and rabies vaccines are the ultimate form of neuroprotection in the sense that the potential damage from the causative agents is interdicted. In the case of polio vaccine, the anterior horn cells remain unaffected because the peripheral immune system prevents the polio virus from attacking the cells. In MS, if immunomodulatory drugs were perfect and/or there were no primary noninflammatory process affecting OGCs or neurons, the situation would be similar to that of the polio vaccine. However, the therapies are not perfect, and we do not as yet know if there might be a pathogenetic process independent of the effects of infiltrating inflammatory cells.

Neuroprotection is perhaps easier to think of as protection against further neuron/axon damage or damage to adjacent neurons and axons once the injury has started. The scope of neuroprotection may also include the regeneration of those cells of the CNS that have the capacity to regenerate. In the case of MS, this would include endogenous OGCs, OGC precursors (OPCs) that are able to lead to remyelination of damaged axons, local precursor cells that develop into mature neurons or OPCs or OGCs, or precursor cells that migrate into an area of damage and then proliferate and mature. It may also mean similar mechanisms for neurons and neuron precursors.[50]

Axon and neuron recovery in MS requires more than just protection/regeneration of neurons or OGCs in isolation, because the CNS comprises multiple cell types that interact with immune system cells and with each other. Alterations of normal features of CNS cells may influence the extent of immunopathology that contributes to neuron damage. For example, the extent and specificity of local inflammatory processes may cause larger-scale bystander cell damage. In the rat model of experimental autoimmune encephalomyelitis (EAE), rats that transgenically overexpress PLP have greater levels of myelin degradation than normal rats when both types are injected with activated T cells specific for myelin OGC glycoprotein (MOG) or PLP. This increased demyelination is associated with greater numbers of activated microglial cells, increased numbers of reactive T cells, and with increased sensitivity of OGCs and myelin to antimyelin antibodies.[51] Moreover, overexpression of PLP itself causes damage.[52,53]

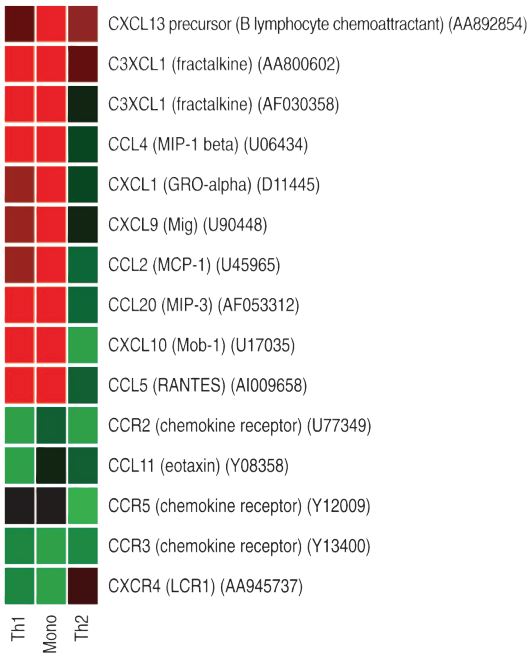
Interactions between normal cells of the CNS are also bidirectional, with initiation and feedback signals between neurons/axons and glial cells. These latter cell types not only are the cause of some CNS dysfunction (such as activated microglia) but also contribute to potential neuron repair and maintenance mechanisms. For example, recent studies indicate that astrocytes are involved in the regulation of synaptogenesis and maintenance of synaptic health. During ontogeny, astrocytes develop before the majority of synaptic junctions are formed, suggesting that synapse formation is astrocyte-dependent. Studied in isolation, retinal ganglion cells form synapses, but the number of synapses is increased and the activity of these synapses is greater when glial cells are present (reviewed in Slezak et al.[54]). Soluble factors, including cholesterol, are released by astrocytes and OGCs, stimulating synapse formation by an unknown mechanism. Still other factors appear to regulate aspects of synapse maturation, including receptor density and subunit composition. Once synapses are formed, astrocytes can also functionally regulate synaptic transmission (reviewed by Newman[55]). The intimate physical interactions of astroglial cells with pre- and postsynaptic nerve cell terminals allow their concomitant activation after presynaptic release of compounds that bind to postsynaptic receptors. The activated glial cells, in turn, release transmitters that bind to the postsynaptic membrane and feed back to the presynaptic membrane to alter further release of neurotransmitters. In addition to these roles in synapse formation and maintenance, astrocytes also have several protective functions in the CNS and interact with microglial, perivascular (pericytes), and vascular cells to help form the BBB.

SOME KEY QUESTIONS TO BE ANSWERED IN MS

In view of the above findings, a more complete understanding of how to develop, apply, and assess the efficacy of neuroprotective therapies requires that we first answer some basic questions, some of which have been considered elsewhere.[56,57]

Why don't OGCs naturally repair or remyelinate damaged neurons? It is possible that exhaustion or elimination of OGCs and/or OPCs or other precursor cells is part of the process that results in incompletely remyelinated lesions. Chang et al. have shown that OPCs do exist in mature healthy brain tissue, and both OGCs and OPCs are seen in some MS lesions.[4,58–60] The chondroitin sulfate-

Figure 3 Changes in chemokine gene expression induced by cytokine mixtures



For Th1 cytokines, expression increased 358-fold for CCL5 (RANTES) and 35-fold for CXCL10 (Mob-1); expressions of CCL11 (eotaxin), CCR2, and CCR3 all decreased about 20-fold. From Lisak et al.,[65] *with permission.*

containing protein NG2, which is found in OPCs, is distributed in both WM and gray matter in normal healthy adult brain.[61] $NG2^+$ stellate/elongate cells, which are distinct from astrocytes and microglial cells, are also found in chronic MS lesions. A possible reason why OGCs do not repair or remyelinate may be that damage to OGCs and/or OPCs is just as permanent and irreversible as damage to neurons. Antibodies to components of OPCs have been reported as well.[62] The persistence of cytokines, effector molecules, damaging or inhibitory molecules, and complement factors, all released by immune cells, might have effects on OGCs. On the other hand, glial cells (OGCs or OPCs) may be capable of remyelination if the appropriate environment is provided. However, if certain stimulatory factors are absent or are not present in the correct sequence, or if they persist when they are no longer required for the next step of maturation, then the required series of signaling events would not be properly provided. This might account for what appears to be completion of all but the ultimate step in remyelination of axons by OGCs in MS. Premyelinating OGCs in the periphery of chronic active lesions extend processes toward and make contact with demyelinated axons. They also synthesize detectable amounts of the myelin components PLP and MOG but for unknown reasons do not remyelinate the contacted axons.[63] Perhaps such axons do not provide the proper signal to the OGCs telling them to myelinate.[4,64]

As a result of the infiltration of immune cells and activation of local microglial cells, cells of the MS lesion site are very probably influenced by growth factors, neurotrophins, cytokines, and chemokines secreted by the immune cells. Perhaps just as important, if not more important, is the effect on receptors for these molecules. Distinct types of immune cell populations, including Th1, Th2, and monocytes/macrophages (M/Ms), release distinct mixtures of compounds and might thus elicit different responses, including protective responses, from CNS glial cells. We have started to test the response of glial cells to exposure to mixtures of cytokines by examining changes in gene expression in mixed glial cell cultures exposed to mixtures of cytokines typical of Th1 and Th2 lymphocytes and M/Ms.[65] Very many changes were seen in the expression of genes encoding proteins involved in immune effector and surveillance functions, confirming that resident glial cells are highly responsive to the cellular makeup and secretion product repertoire of the lesion site (figure 3). Of possible relevance to the lack of remyelination by OGCs mentioned previously[63] is the fact that, in response to Th1 and M/M cytokine mixtures, the cultures expressed increased amounts of chemokine (C-X-C motif) ligand (CXCL)1. CXCL1 in the presence of platelet-derived growth factor induces OPCs to remain in place and continue to proliferate as nonmyelinating precursors.[66,67] In addition, molecular mechanisms for myelination and remyelination are probably not identical.[68–70] Extension of these studies to neuron cultures and co-cultures in which remyelination is occurring should begin to reveal which combination(s) of factors dictates the direction—either damage or repair—that a MS lesion site takes.

What are the important aspects of neuron and axon development that have neuroprotective implications? The differentiation of the CNS is a complex process that depends on precise timings and sequential migration and on influx of precursor cells that interact physically and via secreted factors. There are differences in neuron development in different areas of the brain and spinal cord. In the mature CNS there are still other regional differences in regenerative potential. For example, the hippocampus has a very active capacity to regenerate neurons, whereas other areas do not.[50] There is heterogeneity of neurons even within individual regions of the CNS, and different factors are important in the development and maintenance of the different cell types. Synaptogenesis, which includes complex interactions of pre- and postsynaptic proteins, trophic development factors, effects of contact, and neurotransmitters and receptors for development and

maintenance of synapses, may be critical for regeneration of neurons and formation of new synapses. The mature neuron pool needs to be maintained, and for this neurons and axons require various growth factors, interaction with glial cells, binding of transmitters, and expression of ion channels. It is not yet clear which of these features of CNS development and maintenance are most critical for restoration of function in patients with MS. As noted earlier, if the myelin-forming glial cells are dysfunctional, axons may become abnormal and, by extension, may not be able to respond to signals for repair.

Why don't neurons/axons survive or regenerate in MS lesions? Not all MS pathology is associated with irreversible neuron death via cytotoxic and/or apoptotic mechanisms. This is indicated by pathologic studies and by recovery of patients from relapses. However, the continued accumulation of neurologic dysfunction indicates that eventually there is net axon and/or neuron loss, also confirmed by pathologic and imaging studies. As noted previously with OGC development, this might result from inappropriate expression of growth factors, neurotrophins, cytokines, chemokines, change in receptors for these molecules, and other factors which, during normal CNS development, must all work in the correct sequence and combinations, and with programmed times of persistence, to recapitulate the necessary stages of CNS development that allow regeneration. Once again, as with OGCs, there are likely to be some differences in molecular mechanisms between development and regeneration of damaged neurons. Cytokines, chemokines, and growth factors often have dual natures, promoting degeneration if proinflammatory and promoting protection and regeneration if noninflammatory. INF-γ, interleukin (IL)-6, and transforming growth factor (TGF)-β each have distinct effects on neurons, as well as on astrocytes, OGCs, and microglia (reviewed in Benveniste[71]). IL-1 may be proinflammatory or harmful in some situations but is essential for regeneration in others, such as the sciatic nerve crush model. IL-2, a classic Th1 cytokine, can stimulate low levels of OGC proliferation that inhibits early OGC development[72] but, more importantly, can also help OGCs to mature,[73] which is essential for their interaction and for maintenance of neurons. IL-3 may be protective, but with overexpression may lead to motor neuron degeneration.[74–76]

SUMMARY Neurodegeneration in MS and desired subsequent neuroprotection are highly complex processes. It is therefore necessary to carefully define neuroprotection in the context of MS and to appropriately design experiments to study various potential methods of neuroprotection and neuroregeneration. Implicit in all neuroprotective endeavors is the understanding that the biological players often serve dual roles—as mediators of inflammation in some contexts and of regeneration in others. Neuron generation, protection, and remyelination probably occur through a very specific chain of events, including feedback loops, with specific timing and in an optimal environment. In addition, interactions among cells of the CNS, as well as the nature of the cells in a particular area of the CNS involved in a disease, may also affect outcome. Identifying which of these conditions are causally affected in MS, and how to remedy them, are the current research challenges.

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