Conference report

Neurodegeneration and neuroprotection in multiple sclerosis and other neurodegenerative diseases

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Abstract

Multiple sclerosis is considered a disease of myelin destruction; Parkinson's disease (PD), one of dopaminergic neuron depletion; ALS, a disease of motor neuron death; and Alzheimer's, a disease of plaques and tangles. Although these disorders differ in important ways, they also have common pathogenic features, including inflammation, genetic mutations, inappropriate protein aggregates (e.g., Lewy bodies, amyloid plaques), and biochemical defects leading to apoptosis, such as oxidative stress and mitochondrial dysfunction. In most disorders, it remains uncertain whether inflammation and protein aggregation are neurotoxic or neuroprotective. Elucidating the mechanisms that orchestrate neuronal diseases should facilitate development of neuroprotective and neurorestorative strategies.

Keywords: Neurodegeneration; Neuroprotection; Multiple sclerosis; Parkinson's disease; ALS; Alzheimer's disease; Stroke; Caspase-1; Glatiramer acetate

1. Introduction

This paper highlights a series of lectures presented by the authors at a symposium, *Neuroprotective Strategies in Multiple Sclerosis*, held May 21, 2005 in Princeton, NJ. Conference goals were to discuss the mechanisms of neuronal damage in multiple sclerosis and in other neurological disorders, to identify mechanistic commonalities among neurodegenerative diseases, and to share ideas regarding therapeutic approaches to neuroprotection.

2. Multiple sclerosis: the inflammatory paradigm

The prevailing paradigm in multiple sclerosis (MS) pathogenesis holds that MS is an immunogenic disease that leads to immune attack on the central nervous system (CNS). Although the initiating event is a matter of debate,

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the long held belief is that the disease begins with inflammation, orchestrated by autoreactive T lymphocytes (Fig. 1) (Dhib-Jalbut, 2002). An unidentified antigen, likely an autoantigen, virus, or bacterium, is recognized by an antigen presenting cell (APC). The APC presents the antigen to a CD4+ precursor cell, which subsequently differentiates into a T helper type 1 (Th1) or type 2 (Th2) cell. Both T cell subtypes are present in MS, though Th1 cells, which are thought to be more important to the pathogenic mechanisms in MS, produce cytokines generally considered to be pro-inflammatory. Adhesion molecule (e.g., ICAM, VCAM) expression is increased on the surface of Th1 cells, and cytokines produced by Th1 cells upregulate receptors for these adhesion molecules on the endothelium of the blood–brain barrier (BBB). Th1 cells also secrete matrix metallo-proteinases (MMP), which are proteolytic enzymes that compromise the integrity of the BBB matrix membrane. MMP9 is believed to be important in the extravasation of Th1 cells into the CNS.

Upon entry to the CNS compartment, activated Th1 cells must be restimulated, otherwise they die or leave the CNS. Restimulation, perhaps via an autoantigen or a microbial antigen presented on microglia, causes clonal expansion of the Th1 cells. Th1 cells release an array of pro-inflammatory cytokines, including interleukin 1 (IL-1), interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α), leading to monocyte activation. Activated monocytes and macrophages can also release a variety

of neurotoxic elements, including nitric oxide, oxygen free-radicals, and MMP, all of which contribute to the destruction of the myelin sheath, and perhaps, axons (Dhib-Jalbut, 2002).

Glutamate receptors are upregulated within MS lesions, suggesting microglia-produced glutamate is a factor in MS pathology. Other components of the immune response in MS include mast cells, γδ-T cells, and CD8+ cytotoxic T cells. Mast cells release several molecules that can damage myelin and neurons, and can contribute to the opening of the BBB, facilitating the influx of immunocytes into the CNS. The γδ-T cells appear to recognize a heat shock protein that can destroy oligodendrocytes, and CD8+ cytotoxic T cells may also destroy oligodendrocytes, probably through a Fas-ligand-mediated mechanism (Sospedra and Martin, 2005; Ziemssen, 2005).

Th2 cells may have a dual role in MS. They produce cytokines that activate B cells to produce immunoglobulins as part of the humoral immune system, which, along with complement, can cause myelin destruction. On the other hand, experimental models indicate anti-inflammatory cytokines released by Th2 cells can down-regulate the immune response (Dhib-Jalbut, 2002). Similarly, the role of astrocytes in MS pathology is not clear but they, too, can have contradictory immunogenic effects. Like Th2 cells, astrocytes promote the release of anti-inflammatory cyto-kines, such as transforming growth factor β (TGF-β) and IL-10, giving them a beneficial regulatory role. Conversely,
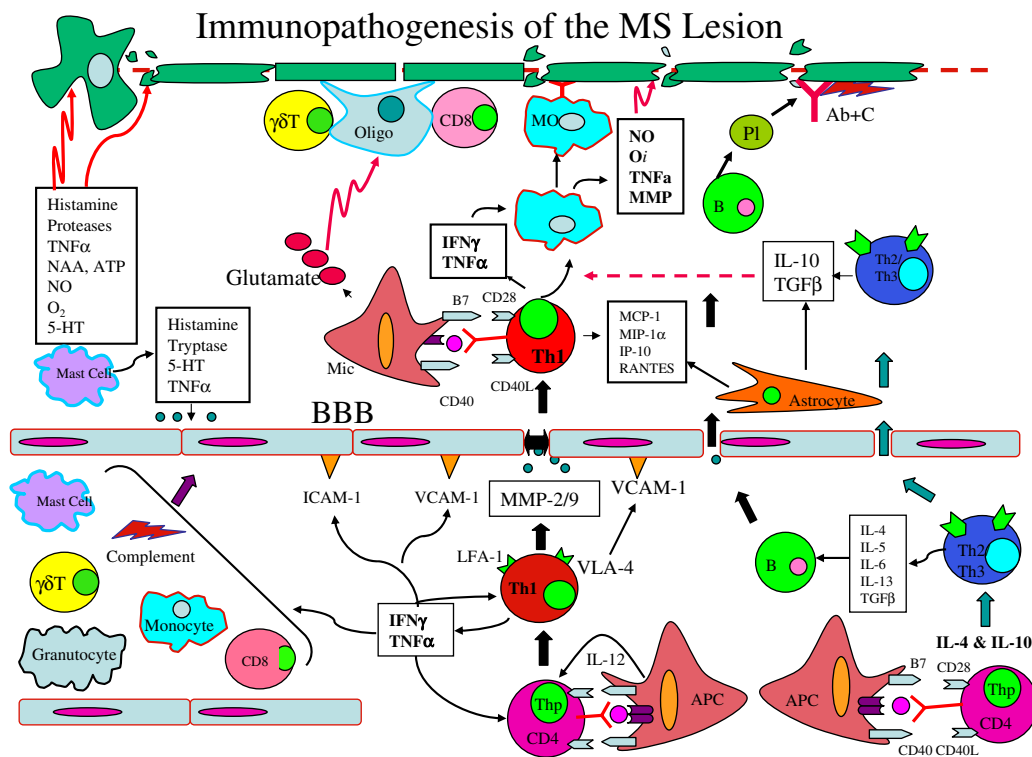

Fig. 1. Immunopathogenesis of the MS lesion (APC=antigen presenting cell; IFN=interferon; IL=interleukin; Mic=microglia; MMP=matrix metalloproteinase; MO=monocyte; NAA=nacetylaspartate; NO=nitric oxide; Pl=plasma; VCAM=vascular cell adhesion molecule).

they can release chemokines that help recruit and retain autoreactive Th1 cells within the CNS compartment.

The concept that inflammation subsequent to autoimmunity precedes and causes neurodegeneration has prevailed for almost 50 years. However, there are data to support the argument that MS is a primary disease of axons, neurons, or oligodendrocytes, and that the immune response is secondary to neurodegeneration (Bo et al., 2003a). Some acute brain lesions display microglial activation and oligodendrocyte death with no evidence of T-cell infiltration (Barnett and Prineas, 2004). What seems certain is that the triggering event and the final effector mechanisms causing myelin destruction and axonal damage in MS are multifactorial.

3. Neuroinflammation and neuroprotection in MS

A preponderance of evidence suggests inflammation is a key contributor to axonal injury and neuronal cytotoxicity in MS. Histological data from the spinal cord of mice afflicted with experimental autoimmune encephalomyelitis (EAE), an MS-like disease, and autopsy samples of MS lesions show a strong correlation, in both the presence and the degree, of inflammation and axonal injury. Active MS lesions in normal appearing white matter (NAWM) of MS patients demonstrate perivascular lymphocyte infiltration that spreads into the parenchyma (Giuliani et al., 2003). Activated Th1 cells accumulating in the CNS have at least the potential to disrupt axons and kill neurons. In vitro, polyclonally activated Th1 cells align along axons and soma of cultured human neurons causing substantial neuronal death (Giuliani et al., 2003). In this system, CNS cell types other than neurons (e.g., oligodendrocytes, astrocytes) are not killed by the activated T cells.

Inflammatory processes are not always detrimental. Myelin-reactive T cells can also be neuroprotective, an example of what has become known as "protective autoimmunity" (Schwartz and Kipnis, 2001). Untreated crush injury to the spinal cord of experimental animals results in primary and secondary neurodegeneration (Hauben et al., 2000). However, secondary degeneration and the loss of axons and projection neurons are attenuated when animals are treated with myelin-reactive T cells after injury (Yong, 2004). The mechanism underlying protective autoimmunity is not clear; however, it may be related to production of neurotrophic factors by activated T cells. The perivascular infiltrate in human MS brain lesions contains brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and nerve growth factor (NGF) (Yong, 2004). Despite the capacity to produce neurotrophic factors, the potential danger of using autoreactive T cells as therapies outweighs their potential benefit.

Currently, the best therapeutic options for neuroprotection in MS are the immunomodulatory agents, glatiramer acetate (GA) and the interferon beta (IFNβ) drugs. The neuroprotective effects of IFNβ on CNS inflammation are "passive," in that IFNβ activity remains outside the BBB (Fig. 2). These drugs interact with receptors on T cells to decrease antigen presentation and activation of Th1 cells (and T cell activity in general), reduce T-cell production of MMP, and decrease expression of adhesion molecule by T cells and endothelial cells. As a result, they impede influx of T cells into the CNS (Dhib-Jalbut, 2002). The IFNβ protein itself is not thought to infiltrate the CNS.

In contrast, glatiramer acetate (GA) plays an active role against inflammation within the CNS. GA polarizes T cells toward a Th2 bias. GA-reactive Th2 cells cross the BBB,
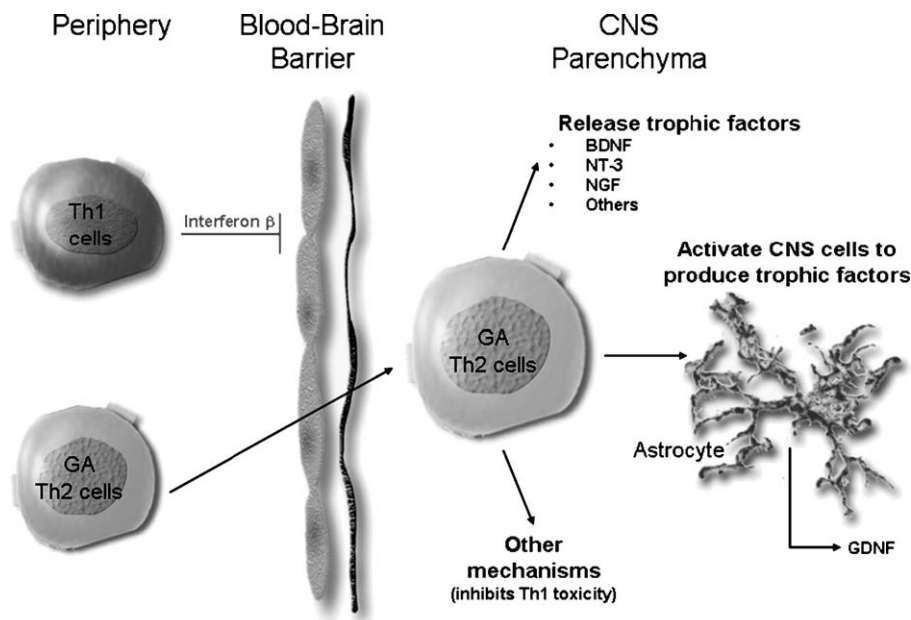

Fig. 2. Possible neuroprotective activities in the CNS of current MS immunomodulators (BDNF = brain derived neurotrophic factor; GA = glatiramer acetate; GDNF = glial cell line derived neurotrophic factor; NGF = nerve growth factor; NT3 = neurotrophin-3).

where they are reactivated by myelin antigens and then release Th2 cytokines. Once in the CNS, GA-reactive Th2 cells may also confer neuroprotection, as demonstrated in animal models of ocular glutamate excitotoxicity, optic nerve crush, facial nerve resection, parkinsonism, amyotrophic lateral sclerosis (ALS), and EAE (Schori et al., 2001; Angelov et al., 2003; Gilgun-Sherki et al., 2003; Kipnis et al., 2000; Benner et al., 2004). The neuroprotective activity of GA may be related to the stimulation of anti-inflammatory Th2 cytokine production and/or to promotion of neurotrophic factors, as has been demonstrated in vitro and in vivo (Aharoni et al., 2003). GA may also promote neurogenesis, as shown in a murine EAE model (Aharoni et al., 2005). Data in humans showing preservation of *N*-acetylaspartate (NAA), a marker of neuronal integrity, suggest GA may confer neuroprotection in MS patients as well (Khan et al., 2005).

Minocycline, a tetracycline antibiotic under evaluation for use in neurodegenerative diseases, has shown remarkable benefit in animal models of several neurologic disorders, among them, MS, Parkinson's disease, Huntington's disease, ALS, stroke, and spinal cord injury (Yong et al., 2001, 2004). Minocycline reduces neuroinflammation through a number of mechanisms, including blocking microglial activation, reducing inflammatory cytokine production, attenuating T cell proliferation and migration, inhibiting MMP, decreasing T cell-microglial interactions, antagonizing glutamate excitotoxicity, and attenuating cell death through mechanisms involving apoptosis (Fig. 3) (Yong et al., 2004a). Combination therapy with GA and minocycline provides synergistic neuroprotective effects in an EAE model (Giuliani et al., 2005).

Preliminary data suggest minocycline has clinical benefit in MS patients. Data from an open-label 36-month trial of oral minocycline 100 mg twice daily in 10 RRMS patients will soon be available. MRI and clinical data for these patients at 24 months are encouraging (Yong et al., 2004a).

4. The "Neurodegeneration First" hypothesis for MS

MS is often regarded as a disease of white matter, however, myelinated axons are not restricted to white matter. Indeed, the extent of demyelination in the cerebral cortex may exceed that in the white matter in some patients. Results of immunocytochemical studies show significant neuronal damage, axonal transection, dendritic transection, and neuronal death by apoptosis in cortical lesions with little or no hematogenously derived immune cells (Bo et al., 2003a). Increasing evidence suggests MS is a disease with heterogenous pathogenic mechanisms, and the hypothesis that MS is a primary neurodegenerative disease that in some instances has secondary inflammatory demyelination merits consideration.

Four immunopathological patterns of demyelination have been described in MS (Lucchinetti et al., 2000). In two types, pathogenic oligodendrocyte death rather than autoimmunity appears to be the primary event, with secondary inflammation present to phagocytosize myelin debris. A third type is characterized by antibody/complement-associated demyelination and the fourth type exhibits inflammatory demyelination, which the researchers believe to be the primary event.

In a postmortem study of 50 MS brains, Peterson et al. (2001) identified three types of cortical lesions. Type 1 lesions are contiguous with subcortical white matter lesions; Type 2 lesions are small, confined to the cortex, and often contain a centrally located vessel; and Type 3 lesions indicate a wave of demyelination extending from the pial surface into
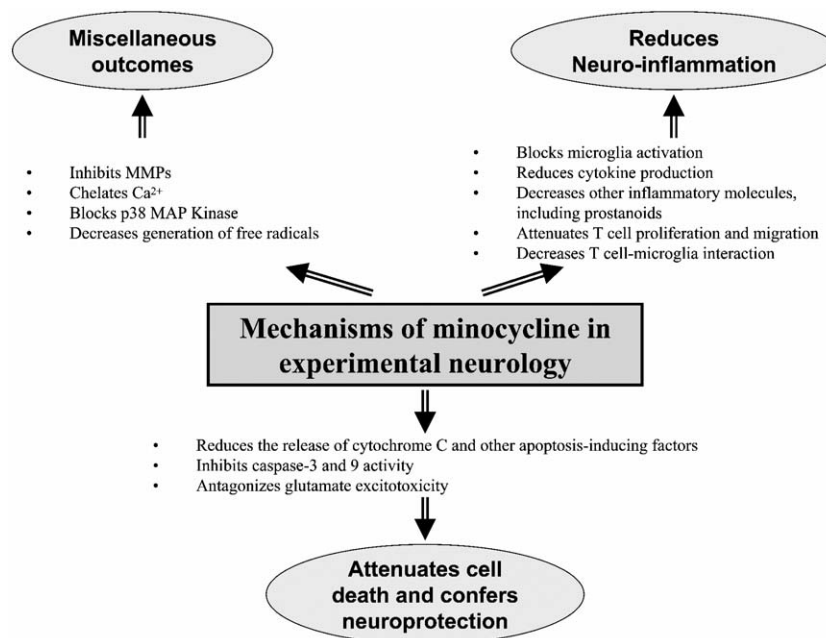

Fig. 3. Mechanisms of minocycline in neuroprotection (MAP=mitogen activated protein).

the cortex, often stopping at layer 3 or 4 and comprising multiple gyri. There was no evidence that Type 3 lesions entered the white matter. Type 1 and Type 3 lesions constituted approximately 98% of the cortical lesion load in these samples. These cortical lesions had vastly reduced inflammatory cell content compared with white matter lesions. Little is known about the dynamics of these lesions; because they are not inflamed, they elude detection on MRI.

In another postmortem study, Bo and colleagues (2003b) examined 20 MS brains to determine the extent and pattern of demyelination within the cerebral cortex. The autopsy cohort was typical; ages at death ranged from 37 to 77 years, and disability level ranged from very minor to quadriplegia. Disease duration ranged from 4 to 37 years. Tissue blocks from four predetermined areas (cingulate gyrus and frontal, parietal, and temporal lobes) were studied, irrespective of macroscopically evident MS plaques and all tissue blocks contained cerebral cortex and periventricular and/or subcortical white matter. Immunocytochemical staining with antibodies against the major histocompatibility complex (MCH) class II and myelin proteins indicated the mean percentage of demyelinated area in these samples was significantly higher in the cerebral cortex (26.5%) than in the white matter (6.5%). The percentage of cortical demyelination did not correlate with the duration of disease, nor was EDSS predictive of demyelination. For example, two patients, with disease durations of 4 and 8 years and EDSS scores of 4.5 and 8.5, respectively, both had percent demyelination close to the mean. Moreover, the two patients with the lowest EDSS scores (EDSS 2 and 3 with approximately equal disease durations) exhibited demyelination of 25% and 2%, respectively. EDSS scores may be an adequate measure of motor function but cognitive deficits or psychiatric symptoms related to lesions in the cerebral cortex may not be reflected by them.

The lack of inflammatory infiltrate or evidence of inflammatory cell recruitment from the periphery, and increases in resident microglia cells in Type 3 intracortical lesions suggest infiltration of immune cells from the periphery into the MS lesion is a secondary effect of the degeneration of myelin, recruited when the myelin load is too great for the microglia to remove. Furthermore, the lack of lymphocyte infiltration suggests there is no breakdown of the BBB, perhaps explaining the difficulty of detecting these lesions using classical MRI, which is based on changes in water content. In Type 1 lesions, there is extensive breakdown of the BBB in the white matter but not in the gray matter. Similarly, IgG was detectable in the white matter portion of Type 1 lesions but not in the gray matter.

Natural history data support a "neurodegeneration first" argument. A study by Confavreux et al. (2000) of the association between disease duration and neurological disability in a large cohort (N=1844) of MS patients in Lyons, France, showed that though the time between clinical disease onset and EDSS 4 could vary widely among relapsing–remitting (RRMS) and primary-progressive

(PPMS) patients, once patients with either phenotype reached a level of irreversible disability (EDSS 4), they all progressed at the same rate. This was true whether it took 1 year or 31 years to reach EDSS 4, suggesting some mechanism may occur early in the disease that causes "preprogramed" neurodegeneration later.

Epidemiological studies suggest earlier disease onset is associated with a more positive prognosis in terms of long-term disability (Weinshenker, 1989); however, measures of disease duration are subjective and the chronological age of the patient may be a more meaningful clinical factor. In a large cohort (N=1463) of MS patients in Italy, Liguori et al. (2000) evaluated the relative associations between age at disease onset and current age with EDSS scores. Current age was a significant predictor of neurological disability, whereas there was no association between clinical disability and patient age at disease onset.

These data raise the possibility that halting inflammation in white matter lesions may not prevent chronic irreversible neurological disability in MS patients. Like ALS and Alzheimer's disease, MS may be a classic neurodegenerative disease with a silent stage of neuronal loss preceding symptoms or detection of lesions by MRI. Early neurodegeneration is silent largely due to the brain's remarkable ability to compensate for neuronal loss through various mechanisms. Thus, the primacy of neurodegeneration in MS is plausible and MS may present the disease of choice for development of neuroprotective therapies because the inflammatory lesions identify the patient before significant neuronal loss or axonal transection occurs.

5. Imaging neurodegeneration and neuroprotection in MS

Although MRI cannot establish the mechanisms of neurodegeneration and neuroprotection, increasingly sophisticated imaging techniques are making it possible to determine when these processes occur. Quantitation of myelin content, imaging of neuronal and axonal integrity, and assessment of cerebral atrophy signal beneficial and deleterious pathologic changes within the CNS.

In MS, if the axon is not transected by inflammatory segmental demyelination, insertion of new sodium channels can re-establish conduction across the demyelinated segment and improve function. However, these channels do not have the same electrical characteristics as the original ones, and may even be responsible for some MS symptoms. The viability of the focally demyelinated axon is reduced, predisposing it to Wallerian degeneration. This process is thought to be independent of current inflammation but is not necessarily independent of inflammation that had occurred previously.

Measuring myelin protection and repair can be accomplished in several ways, some more specific than others. The least specific method is measuring the evolution of black holes, which are hypointense lesions on T1-weighted

images. Not all T1-hypointensities become persistent, chronic black holes, which are intrinsically more destructive and are associated with loss of both myelin and axons (van Walderveen et al., 1999).

A more specific way to determine neuroprotection is to quantify the amount of myelin in the lesions and in the normal appearing white matter (NAWM). Currently, there are three approaches. The first involves imaging the short (10–50 ms) component of the T2-weighted relaxation distribution that originates from water in myelin sheaths. In principle, quantitation of this peak is equivalent to quantitation of water trapped within myelin (Moore et al., 2000). A second method is to quantify magnetization transfer. This technique quantifies the semi-solid pool of protons bound to macromolecules that are not visible on conventional MRI. This is accomplished by saturating the magnetization of these bound protons and observing the effect of the transfer of this saturation to the visible water pool, which reduces the signal from the visible water pool. It is then possible to indirectly estimate the size of the semisolid pool. Changes in magnetization transfer in cerebral white matter are dominated by changes in myelin density. Thus, changes in MTR are relatively specific for changes in myelin density. Full quantitation of magnetization transfer requires sophisticated acquisition techniques and mathematical modeling of the phenomenon, which are not widely available. However, semi-quantitative measurement of magnetization transfer can be relatively easily accomplished by performing two images, one with, and one without, a pulse that saturates the semi-solid proton pool, and then calculating the magnetization transfer ratio (MTR) image. MTR imaging is feasible to perform on most modern MRI scanners. The images are intrinsically semi-quantitative and can be used to follow changes in myelin content. Sophisticated image post-processing techniques can be combined with MTR imaging to enable global, regional and even voxel-based quantification of demyelination and remyelination in vivo (Chen et al., 2005).

Ongoing MTR studies in MS patients who have undergone immunoablation followed by autologous stem cell transplantation are providing information regarding the evolution of demyelination and remyelination in MS lesions. Theoretically, patients who undergo immunoablation have all myelin-reactive T cells deleted. Indeed, in these patients, T1-weighted measures of gadolinium-enhancing lesions drop from pretreatment values to zero. However, MTR images in these patients are illuminating: for years after immunoablation and stem cell transplant, both demyelination and remyelination are detected, in the absence of overt inflammation (i.e., new Gadolinium enhancement). The amount of demyelination is slightly higher than the amount of remyelination resulting in a small preponderance of demyelination over remyelination in the lesion and a progressive demyelination within the existing lesion for up to 5 years after lesion formation. Changes in MTR are also present in "pre-lesional" regions (i.e., regions of NAWM that will subsequently become lesion) for years prior to lesion appearance on conventional MRI {Pike Radiology (2000)}. This could reflect very low grade inflammation brewing before lesion development, or it could be indicative of focal neurodegeneration. The MTR of the NAWM also indicates slowly evolving demyelination that could reflect either low grade inflammation or degeneration.

There are two main ways to measure axonal protection. The first is MR spectroscopy (MRS), the specificity of which is based on the ability to quantitate the concentration of *N*-acetylaspartate (NAA) in the tissue. NAA is an amino acid localized almost exclusively in nerve cells and their axons; therefore, NAA concentration is an indicator of neuronal, axonal, and dendritic density (Miller, 2004). In acute MS lesions, NAA may be reduced by 70–80% and the reduction is only partially reversible when the lesion has resolved. The NAWM also shows decreasing NAA concentration early in the course of MS; in fact, the rate of decrease is slightly faster in early MS than it is later in the disease.

Measures of brain atrophy are important to obtain a complete picture of neurodegeneration and neuroprotection. Atrophy of the white matter or gray matter likely reflects axonal and neuronal loss (Miller, 2004). Loss of brain volume has been shown to be attenuated by the immuno-modulatory therapies used in MS (Jacobs et al., 2000a,b; Sormani et al., 2004).

Although indicative of pathologic changes, current imaging techniques cannot resolve the "inflammation or neurodegeneration first" debate. That anti-inflammatory therapy can attenuate atrophy early but not later in the progressive phase of MS supports the contention that inflammation precedes, at least a component of, neurodegeneration. However, the assessment of the relation of "lesions" to degeneration and atrophy is confounded by the fact that lesions in gray matter are not directly visible on MRI using current technology (Arnold, 2005).

6. Lessons from other neurodegenerative diseases

6.1. Parkinson's disease: molecular genetics and new therapeutic targets

Parkinson's disease (PD) is a multigenic neurodegenerative disorder affecting 1% of the population over age 65. The main pathology is loss of dopaminergic neurons in the substantia nigra. PD has a heterogeneous clinical presentation: age of onset, rate of progression, and predominant symptoms vary among different individuals. Some present with tremor while others present with only rigidity and bradykinesia. Further, a subset of patients has cognitive and psychiatric manifestations in addition to their motor impairment (Mouradian, 2002). At least 20% of Parkinson's cases are familial; others have no clear family history. To date, five genes for PD have been identified: α-synuclein and dardarin/LRRK2 are associated with dominantly inherited

disease, while parkin, DJ-1 and PINK1 usually cause recessively inherited PD (Cookson et al., 2005). Molecular studies focusing on the cellular mechanisms by which these gene products contribute to PD pathology are uncovering new and important signaling cascades in the life and death of dopaminergic neurons.

Genetic studies indicate that point mutations in, as well as duplication or triplication of, the α-synuclein gene lead to the clinical phenotype of PD (Polymeropoulos et al., 1997; Singleton et al., 2003; Ibanez et al., 2004). α-Synuclein protein readily aggregates and is a major fibrillar component of Lewy bodies (Spillantini et al., 1997). Over-expression of α-synuclein in several animal models results in protein aggregation and various degrees of neuronal impairment (Masliah et al., 2000; Feany and Bender, 2000; Lakso et al., 2003). Additionally, cultured cells over-expressing α-synuclein generate reactive oxygen species (ROS) (Junn and Mouradian, 2002). α-Synuclein may also influence the dopamine transporter, promoting dopamine release into cells (Lee et al., 2001). Thus, excess α-synuclein appears to be deleterious to dopaminergic neurons.

Enzymatic cross-linking with tissue transglutaminase (tTG) is one of the mechanisms by which α-synuclein aggregation can be enhanced (Junn et al., 2003). tTG belongs to a family of enzymes that catalyze a transamidation reaction to form γ-glutamyl-ε-lysine (GGEL) bonds between proteins, resulting in oligomerization and aggregation. tTG is a ubiquitous enzyme present in neurons primarily in the cytoplasm but can also be found in the nucleus. In the presence of tTG, α-synuclein forms insoluble aggregates and cytoplasmic inclusions. In addition, the halo of Lewy bodies in the brains of patients with PD or dementia with Lewy bodies stains positively for an antibody that recognizes isodipeptide bonds (a marker of tTG cross-linking), suggesting that α-synuclein is cross-linked within Lewy bodies (Junn et al., 2003).

tTG expression and activity are highly regulated. Calcium is an important activator of TG2 while guanosine triphosphate (GTP) is an important inhibitor. These two modulators act in a competitive manner to control tTG activity (Gentile and Cooper, 2004). The mitochondrial complex I impairment in PD and the associated increase in intracellular calcium levels could activate tTG resulting in α-synuclein aggregation. Similarly, mitochondrial complex II inhibition is associated with decreased GTP and ATP content, thereby increasing tTG activity. Moreover, proteins that are oxidatively damaged or nitrated tend to be better substrates for tTG. Since such proteins are not uncommon in the parkinsonian brain, tTG activity is believed to be elevated in the substantia nigra based on limited studies. tTG can also cross-link and aggregate pathogenic proteins in other neurodegenerative diseases, including amyloid precursor protein (APP), huntingtin, and tau (Bailey et al., 2005).

Phosphorylation promotes α-synuclein aggregation as well. In postmortem studies of PD and other α-synucleinopathies, α-synuclein in Lewy bodies is hyperphosphorylated (Fujiwara et al., 2004). Interestingly, co-expression of α-synuclein and its interacting protein synphylin-1 results in the formation of cytoplasmic eosinophilic inclusions (Engelender et al., 1999; Lee et al., 2004). The interaction between these two proteins is regulated by phosphorylation; when phosphorylation is blocked inclusion formation is hampered (Lee et al., 2004).

Several mutations in *DJ-1* have been described in PD including point mutations and a large exonic deletion (Bonifati et al., 2003). Wild-type DJ-1 is a dimer but the disease-associated loss-of-function mutation, L166P, occurs at the dimer interface, preventing dimerization. This suggests that dimerization is important for normal DJ-1 function (Wilson et al., 2003). DJ-1 is expressed ubiquitously, found in most tissues, including nigral dopamine neurons and astrocytes. Wild type DJ-1 localizes in the cytoplasm and the nucleus, whereas the mutant form might localize in mitochondria and the nucleus. It should be noted that these subcellular localization studies have yielded inconsistent results.

DJ-1 quenches ROS in a variety of models. In vitro, as concentrations of hydrogen peroxide increase, cell death is aggravated. But, in the presence of over-expressed wild-type DJ-1, cells are protected (Junn et al., 2005). The L166P mutant is incapable of protecting cells against oxidative stress. However, the ROS-quenching potential of DJ-1 does not sufficiently explain its protective effect against cell death, implying additional mechanism(s) through which DJ-1 acts as a cellpreserving factor. In fact, DJ-1 interacts with Daxx, a death-associated protein important in the regulation of apoptosis (Junn et al., 2005). Daxx is known to interact with and activate apoptosis signal-regulating kinase 1 (ASK1) (Chang et al., 1998). Activated ASK1, in turn, phosphorylates JNK and p38, triggering the apoptotic cascade. ASK1 is also activated by other insults, including ROS, endoplasmic reticulum stress, and TNF-α. Deletion of ASK1 renders cells resistant to ROS (Matsuzawa et al., 2002).

Wild-type DJ-1 and Daxx coexist within the nucleus of an unstressed cell while ASK1 is normally present in the cytoplasm. When the cell is stressed, Daxx translocates from the nucleus to the cytoplasm to interact with its effector kinase, ASK1 (Song and Lee, 2003; Charette et al., 2000). Wildtype DJ-1 sequesters Daxx in the nucleus and prevents its translocation to the cytoplasm and, therefore, interferes with the ASK1-mediated apoptotic cascade. In contrast, L166P mutant DJ-1 cannot block Daxx translocation to the cytoplasm and, hence, cannot block ASK1 activation and cell death (Junn et al., 2005).

Recently, the role of ASK1 in neurodegenerative diseases is beginning to be recognized. The cytoplasmic domain of APP dimerizes and forms a complex with ASK1 via JIP-1b, the JNK interacting protein, causing sustained ASK1/JNK mediated neurotoxic signal (Hashimoto et al., 2003). Dominant negative mutant ASK1 reportedly blocks this form of cell death. *ASK1*$^{-/-}$ embryonic fibroblasts are resistant to the E2 ubiquitin conjugating enzyme E2-25K/Hip-2 which mediates amyloid-β neurotoxicity (Song et al.,

2003). In addition, Fas-triggered death of normal embryonic motoneurons requires transcriptional upregulation of neuronal nitric oxide synthase (NOS) and involves Daxx, ASK1, and p38 together with the classical FADD/caspase-8 cascade. Motor neurons from transgenic mice over-expressing ALS-linked superoxide dismutase (SOD1) mutants display increased susceptibility to activation of this pathway (Raoul et al., 2002).

Multiple different mutations in the parkin gene have been identified in PD patients including point mutations and exonic deletions (Kitada et al., 1998; Moore et al., 2005). Parkin is believed to be an E3 ubiquitin ligase, adding ubiquitin chains onto substrate proteins. Once a substrate is polyubiquitinated, it is recognized by the proteasome for degradation. When parkin is overexpressed in cellular models, small inclusions can be detected in the cytoplasm of unstressed cells. If cells are stressed with a proteasome inhibitor, the small aggregates coalesce into a single large inclusion next to the nucleus, called an aggresome. These inclusions attract molecular chaperones and many other proteins, including α-synuclein and its interacting partner, synphilin-1 (Junn et al., 2002). A number of similarities are recognized between these inclusions and Lewy bodies, both morphologically and biochemically, leading to the hypothesis that a Lewy body may be a type of aggresome in neurons (Junn et al., 2002; Olanow et al., 2004).

Whether these inclusions are neurotoxic or protective has been debated. Recent cell biologic studies have provided the tools to address this question experimentally. When a cell is treated with a drug that blocks apoptosis, the percentage of cells with apoptotic nuclei decreases but the percentage of cells with inclusions does not change (Tanaka et al., 2004). Additionally, when cells are stressed by over-expressing α-synuclein, apoptosis increases as expected. But examination of the apoptotic pool of cells reveals that only a small minority contains inclusions, whereas the majority lacks inclusions (Tanaka et al., 2004). These observations suggest that inclusions are likely a survival strategy mounted by neurons to clear out misfolded or otherwise damaged proteins from the cytoplasm.

Based on the current knowledge about the molecular mechanisms of α-synuclein, DJ-1, and parkin described above, Fig. 4 proposes a model scenario of the fate of dopaminergic neurons in PD. Monomeric α-synuclein is prone to oligomerize and form early aggregates, termed protofibrils. These intermediate aggregates are believed to be toxic, while the subsequent fibrillization of α-synuclein into Lewy bodies is presumed to be a detoxification step. Thus, early aggregates of α-synuclein in the soluble fraction of the cytoplasm constitute a major stressor. Packaging these aggregates in Lewy bodies is an attempt by the cell to limit the concentrations of toxic species of α-synuclein. In addition to point mutations or having multiple copies of the α-synuclein gene, aggregation is accelerated by many other insults, including oxidative stress and mitochondrial impairment. Further, α-synuclein protofibrils can increase the release of dopamine from vesicles, and can impact the plasma membrane dopamine transporter, rendering it more leaky. Heavy metals and post-translational modifications such as phosphorylation also accelerate the aggregation process (Uversky et al., 2001; Fujiwara et al., 2004). The E3 ligase function of parkin is thought to protect cells by clearing aggregated or misfolded proteins. DJ-1 can protect cells through minimizing oxidative stress as well as by blocking ASK1 mediated apoptosis at a downstream site (Junn et al., 2005).
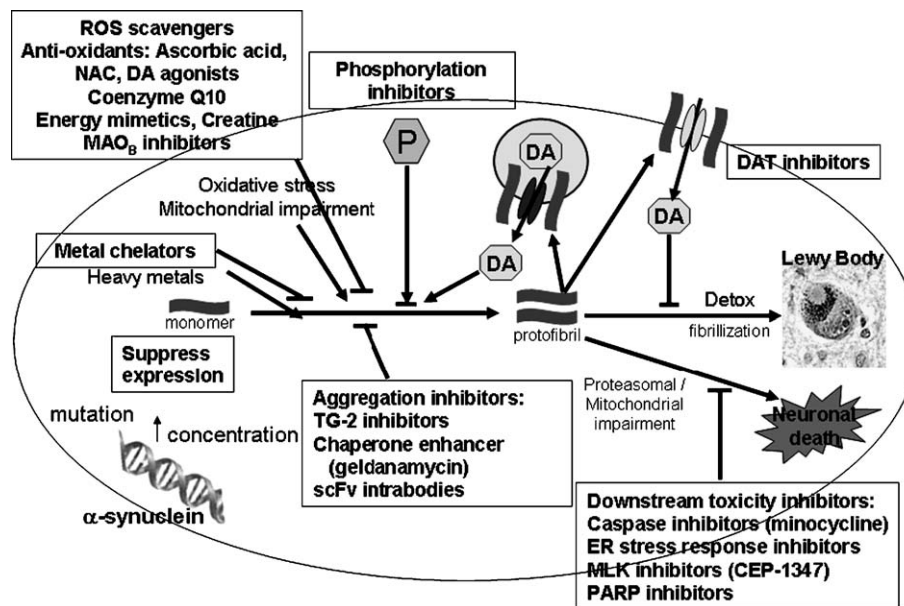

Fig. 4. Putative pathogenetic events in Parkinson's disease and potential neuroprotective targets against α-synuclein toxicity (ROS=reactive oxygen species; MAO=monoamine oxidase; DA=dopamine; DAT=dopamine transporter; P=phosphorylation; TG-2=tissue transglutaminase; MLK=mixed lineage kinase; PARP=poly(ADP-ribose) polymerase; NAC=n-acetylcysteine; ER=endoplasmic reticulum; scFv=single-chain variable fragment).

This model highlights potential therapeutic targets for slowing neuronal degeneration. These targets include early steps such as mitigation of oxidative stress, minimizing dopamine reentry through the transporter, measures to reduce protein aggregation, and interference with downstream sites to block cell death.

6.2. Neurodegeneration and inflammation in Parkinson disease

Neurotoxin-based models have been instrumental in elucidating the molecular cascade of cell death in PD (Dauer and Przedborski, 2003). The loss of dopaminergic neurons in the substantia nigra is often associated with nonneuronal pathology, for example, with activated microglial cells and reactive astrocytes (Przedborski and Goldman, 2004). The most frequently used neurotoxic model in PD, the 1-methyl-4-phenyl-1,2,3,6 tetrahydropyridine (MPTP) model, has been utilized to determine whether neuronal death is cell autonomous; i.e., whether processes within neurons are sufficient to explain their demise, or whether the environment in which neurons are imbedded plays a significant role in the fate of these cells. Processes taking place outside the dopaminergic neurons include excitotoxicity mediated by glutamatergic projections to, and microglial activation in, the substantia nigra.

In animals, MPTP causes a dramatic loss of cell bodies in the substantia nigra and even more profound loss of fibers in the striatum, with activation of microglia and astrocytes. The loss of cell fibers in the striatum and cell bodies in the substantia nigra of MPTP-treated mice is mitigated by minocycline (Wu et al., 2002). This may reflect reduced apoptosis or minocycline may prevent microglial activation by inhibiting p38 mitogen-activated protein (MAP) kinase. In this model, minocycline decreased the production of inflammatory cytokines, including interleukin (IL)-1β, inducible nitric oxide synthase (iNOS, the main producer of nitric oxide in inflammation), and nicotinamide adenosine dinucleotide phosphate (NADPH)-oxidase (an enzyme that produces ROS), compared with levels in untreated animals (Wu et al., 2002). Observations of minocycline-related attenuation of these factors prompted investigation of their role in the cytotoxic response. Much work has been done on IL-1β and association with caspase-induced cell death but less is known about the relative contributions of iNOS and NADPH-oxidase to neurodegeneration.

There is no evidence of iNOS expression in the brain of healthy animals (Liberatore et al., 1999). Within 24 h of MPTP administration, iNOS-positive cells are present and the morphology of these cells within the nigra resembles macrophages. When iNOS-knockout mice are given MPTP, the loss of dopaminergic neurons at 7 days is lower than that in the wild type, though the effect is mild Liberatore et al., 1999).

Normally, very few microglia are detected in the vicinity of dopaminergic neurons, and when present, they appear to be resting with fine, long processes. After MPTP administration, these microglial cells are more numerous and have short processes, suggesting they are activated, and they can now be found in the proximity of remaining dopaminergic neurons. NADPH-oxidase is present within these activated microglia (Wu et al., 2003). Minocycline attenuates NADPH-oxidase associated production of ROS, though, as with iNOS, the effect is mild (Liberatore et al., 1999; Wu et al., 2003).

MPTP also causes increased expression of cyclooxygenase-2 (COX-2) in dopaminergic neurons (Teismann et al., 2003). In this model, COX-2 behaves as marker of cellular distress rather than as an inflammatory element. COX-2 expression is not an artifact of the MPTP model; in postmortem human brain samples, normal brains have no COX-2, whereas PD brains have relatively significant amounts in the dopaminergic neurons and in the Lewy bodies (Teismann et al., 2003). It is a matter of speculation what the role of COX-2 in dopaminergic neurons is, perhaps it contributes to the formation of α-synuclein protofibrils. Corroborating this view is the demonstration that COX-2 stimulates the demise of dopaminergic neurons and the formation of dopamine protein adducts in the MPTP model (Teismann et al., 2003).

A method of determining the role of activated microglia in PD is to modify their behavior. As noted above, the immunomodulator, glatiramer acetate (GA), promotes a Th2 generally antiinflammatory phenotype of T-cells. Two days after administration of MPTP to mice, T cells accumulate at diseased substantia nigra close to microglial cells. After establishing MPTP-induced lesions, passive transfer of T cells in splenocytes from animals vaccinated with GA attenuated neurotoxicity (Fig. 5) (Benner et al., 2004). The control (passively transferred T cells reactive against ovalbumin) had no effect on MPTP-induced neurodegeneration. Animals vaccinated with GA-reactive T cells showed a dramatic reduction in microglial activation and increased levels of glial cell line-derived neurotrophic factor (GDNF). Confocal microscopy indicated co-localization of GDNF and GFAP (a marker of astrocytes), suggesting GA-reactive T cells accumulating in the diseased area stimulate astrocytes to produce GDNF (Benner et al., 2004).

Results of neurotoxin models in PD reinforce findings from transgenic animal models: dopaminergic neuronal degeneration is the result of multiple pathogenic factors occurring both within and outside of the cell.

6.3. Alzheimer's disease

Alzheimer's disease is the most common cause of dementia in people ages 65 years and older. Approximately 4.5 million people in the US have the disease and incidence is increasing as the population ages. Chronic impairment of cognitive function and memory typify Alzheimer's disease and there are currently no effective treatments available. Approximately 95% of all Alzheimer's disease cases are
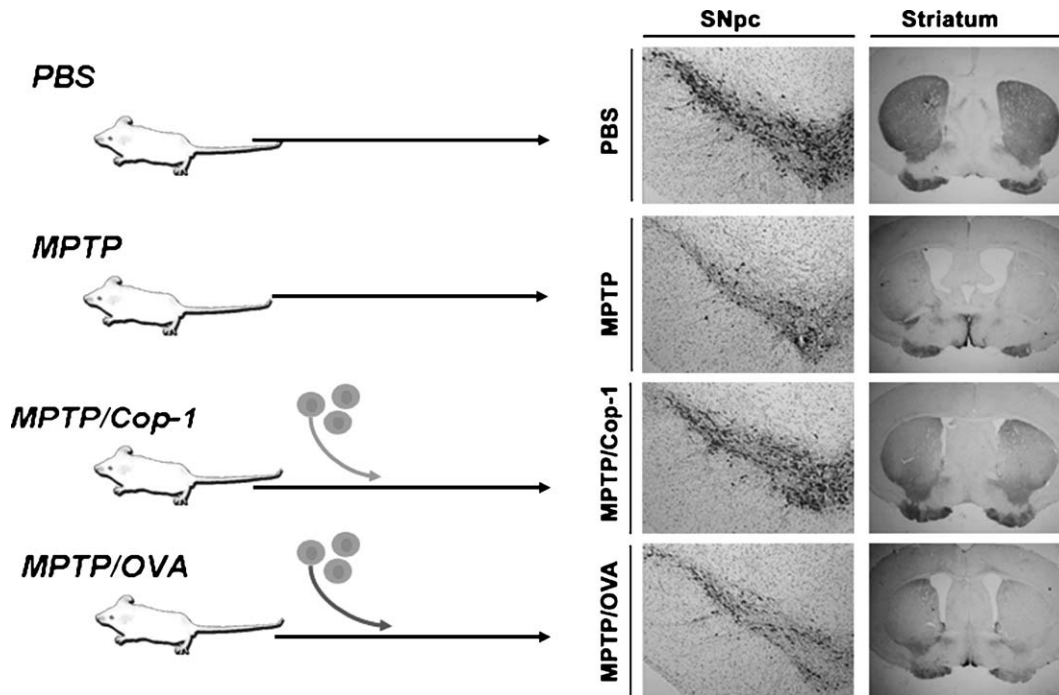
Fig. 5. Glatiramer acetate splenocytes protect against MPTP (MPTP=methyl-4-phenyl-1,2,3,6 tetrahydropyridine; Cop-1=glatiramer acetate [formerly copolymer-1]; SNpc=substantia nigra pars compacta).

sporadic while the rest are autosomal dominant, but both forms share the same pathological hallmarks: amyloid plaques and neurofibrillary tangles. Amyloid plaques are extracellular protein assemblies found in the brain parenchyma and in blood vessels (amyloid angiopathy). Neurofibrillary tangles consist of intraneuronal tau protein aggregates and occur mainly in large pyramidal neurons. Other prominent features of Alzheimer's disease are astrocytosis, microgliosis, and neuronal cell loss.

Amyloid plaques, which are insoluble aggregates of amyloid-β (Aβ) peptide, are associated with dystrophic neurites but it is now widely believed that soluble Aβ aggregates (i.e., oligomers) cause neurotoxicity. Aβ peptide is produced from amyloid precursor protein (APP) via two secretase cleavages and mutations found in genetic forms of Alzheimer's disease occur within close proximity of the two cleavage sites. Data strongly point to Aβ as key in the pathogenesis of Alzheimer's.

Neurofibrillary tangles occur throughout regions in the hippocampus and the cortex, as indicated by immunostaining for tau protein. Tau resides preferentially in neurons and is involved in the assembly of microtubules. Tau activity is regulated by phosphorylation and, as was described in PD, when tau is abnormally phosphorylated it begins to aggregate. The cause of tau hyperphosphorylation remains unknown.

Inflammatory processes significantly modulate the pathogenesis of Alzheimer's disease. Transforming growth factor-β (TGF-β) can be produced by any cell type and every cell has receptors for it (Buckwalter and Wyss-Coray, 2004). Transgenic mice expressing TGF-β1 (the most abundant and well-studied TGF-β isoform) exhibit massive activation of microglial cells compared with wild types. These mice are largely protected from excitotoxic injury and when crossed with a mouse model for Alzheimer's disease, they develop less amyloid deposition (Wyss-Coray et al., 2002; Buckwalter and Wyss-Coray, 2004). Thus, TGF-β1 has a protective effect even though it is associated with prominent microglial activation, suggesting activated microglia need not be detrimental. Indeed, the concept of "microglial activation" may be too simplistic. Microglial cell types have varying functions. Resting microglia cells can be very plastic and may differentiate into varying functions, all of which are "activated." These functions can be phagocytic, neurotoxic, neurotrophic, or antigen-presenting. Furthermore, these functions are not mutually exclusive and the cells may switch roles back and forth.

The complement system is also implicated in Alzheimer's disease (Wyss-Coray et al., 2002; Wyss-Coray and Mucke, 2002). The complement system consists of about 30 different proteins, the most abundant of which is C3. Activation of C3 via the classical pathway or the alternative pathway leads to the formation of C3 convertases that cleave C3 into C3a and C3b (Fig. 6). The classical pathway is typically activated by antibodies but can also be activated by fibrillar proteins. The alternative pathway is activated by repetitive structures and bacterial cell surfaces, and possibly also by dead cells.

C3a is implicated in chemotaxis and vasodilatation. C3b can tag or opsonize structures for phagocytosis, or it can further activate complement in the C5b-9 or "lytic" pathway. The lytic pathway leads to the formation of a membrane
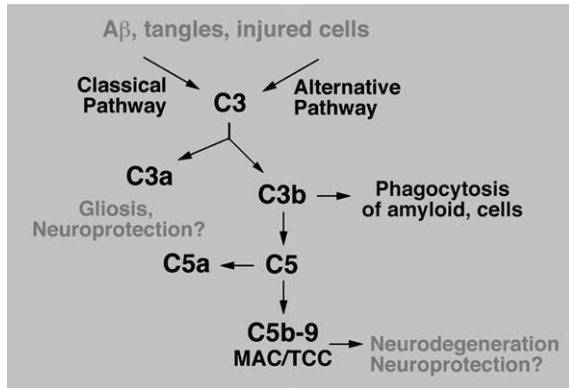
Fig. 6. Possible roles of complement in Alzheimer's disease (MAC/TCC = membrane attack complex/terminal complement complex).

attack complex, which is inserted into a target cell membrane and forms a pore, causing cell lysis. The membrane attack complex could be implicated in neurodegeneration by promoting cell death but there is also evidence that a "sublytic" concentration of the complex activates kinase-Akt and PI3K pathways, which are anti-apoptotic and may be cytoprotective (Wyss-Coray et al., 2002).

In Alzheimer's disease, complement can be activated by Aβ deposits and possibly neurofibrillary tangles. Moreover, apoptotic cells may activate complement and be labeled for phagocytosis via C3b (Wyss-Coray et al., 2002). To elucidate the role of complement in Alzheimer's disease, mice that express a complement inhibitor called sCrry have been used. sCrry inhibits the conversion of C3 into C3a and C3b, effectively inhibiting the activation of downstream complement mechanisms. Human amyloid precursor protein (hAPP) transgenic mice overproduce APP and Aβ and develop amyloid plaques. When hAPP mice were crossed with sCrry mice, complement inhibition led to increased formation and accumulation of Aβ deposits (Wyss-Coray et al., 2002). Aβ accumulation in the hAPP/sCrry mice with reduced complement activation was 2–3 times that in the hAPP single transgenic mice. Furthermore, electromicroscopy indicated increases in degenerating cells in the tissue. These results suggest complement plays a beneficial role in Alzheimer's disease by clearing extracellular Aβ deposits; as Aβ fibrils are formed they are tagged by complement and cleared via complement receptors on microglial cells (Wyss-Coray et al., 2002).

To investigate the relationship between complement activation and neurofibrils, a mouse model is used that over expresses a mutant form of tau (AT8 phospho-epitope). This tau epitope is also associated with aggregation of hyperphosphorylated tau into tangle-like structures in neurons. Surprisingly, inhibition of complement in the double transgenic (tau/sCrry) mice almost completely prevented the formation of these aggregates. This was also true in single transgenic sCrry mice and in non-transgenic mice. Subsequent data from cell culture studies suggest that activation of complement, specifically formation of the membrane attack complex pore, is detrimental because it promotes tau hyperphosphorylation.

Therefore, while activation of complement may have beneficial effects on Aβ clearance, the effect of complement activation on neurons may actually promote pathology. This may reflect difference in pathologic mechanisms controlling extracellular (amyloid) vs. intracellular (tau) aggregates.

6.4. Amyotrophic lateral sclerosis

Amyotrophic lateral sclerosis (ALS; Lou Gehrig's disease) is a chronic, progressive disease marked by gradual degeneration of motor neurons. Most incidences are sporadic, with approximately 10% of cases inherited in an autosomal-dominant manner (Bruijn et al., 2004). The cause of ALS is unknown; however, in 2% of all cases (20% of familial disease), the primary cause is a mutation of the Cu/Zn superoxide dismutase gene (SOD1). SOD1 is quite abundant, comprising about 1% of total brain protein, and is ubiquitous, expressed in every cell type and at relatively high levels. The function of SOD1 is to convert superoxide (oxygen with an extra electron) to hydrogen peroxide or molecular oxygen in two asymmetric steps, each of which requires catalytic copper.

There have been many hypotheses about how dominant mutation in this enzyme leads to the selective killing of motor neurons. Approximately 117 mutations in the SOD1 enzyme have been identified; they occur on almost every surface of the protein and all yield dominantly inherited disease.

Loss of SOD1 activity is not the primary event that gives rise to ALS. Some mutations retain full dismutase activity yet cause disease, while others have no detectible dismutase activity. In mice, complete deletion of SOD1 does not cause motor neuron disease and does not affect lifespan. Mice express a variety of different SOD1 mutations; in some cases, mutations that raise overall dismutase activity cause progressive paralysis and death while other mutations can cause disease without affecting endogenous dismutase activity (Gurney et al., 1994; Wong et al., 1995; Bruijn et al., 1997). Since all 117 SOD1 mutants can cause ALS, they must share one or more toxic properties, however, the least important appears to be dismutase activity.

It was initially speculated that SOD1-related disease may be related to oxidative damage caused by superoxide. SOD1 is a metalloprotein that is loaded in vivo by the action of an intracellular chaperone, called the copper chaperone for SOD (CCS), which accepts copper from the membrane-bound copper transporter and translocates it to its only known substrate, SOD1 (Culotta et al., 1997). To test the oxidative chemistry performed by SOD1, copper binding was eliminated by removing the CCS in transgenic mice. Mating the CCS-deletion mouse to a SOD1-mutation mouse showed that removing the copper catalyst did not attenuate toxicity but rather modestly accelerated it (Subramaniam et al., 2002). Similarly, transgenic mice in which all four copper-

coordinating residues of SOD1 are mutated (preventing binding of catalytic copper at all) still acquire progressive heritable motor neuron disease. Thus, catalytic copper is not required for cytotoxicity.

As in PD and Alzheimer's disease, intracellular and extracellular aggregates are present in ALS; this is true in all of models of SOD1-mediated disease in mice. These protein aggregates are intensely immunoreactive with antibodies to SOD1, supporting the theory that mutant SOD1 is toxic because it co-aggregates with other essential proteins. As in the other neurodegenerative diseases, the question regarding whether these protein aggregates are neurotoxic or neuro-protective remains to be answered.

Mitochondria act as "gate-keepers" of cell death, stimulating production and release of cytochrome *c* that triggers downstream apoptotic process, including caspase activation (Fig. 7). The stimulus for mitochondrial-initiated cell death was investigated. Study of transgenic mice that express a SOD1 mutation showed mutant protein aggregates onto mitochondria but only in cells within the spinal cord (Liu et al., 2004). The mutant aggregation initiated before the disease was symptomatic and continued. Importantly, this protein aggregation onto mitochondria occurred only in the tissues at risk in ALS, while mutant SOD1 did not associate with mitochondria in muscle, liver, and brain (Liu et al., 2004). Thus, mutant protein aggregates appear to associate only with mitochondria in tissues affected in ALS, consistent with known differences in mitochondria of different cell types. This observation may help explain the selectivity of SOD1 mutations to affect motor neurons.

To examine whether mechanisms of motor neuron destruction in ALS are cell autonomous, a series of chimeric mouse models was created to determine which cells develop mutant SOD1 mediated damage that contributes substantial-ly to motor neuron death (Clement et al., 2003). Are mutant motor neurons damaged by mutant action acting within them and can normal neighbors help them to survive? Mice which had a high percentage of mutant motor neurons but variable levels of normal non-neuronal cells, did not develop disease (Clement et al., 2003), while mice that express mutant SOD1 ubiquitously are dead at half the age of the surviving animals. Examination of motor axons (in the L5 root bundle of axons—about 1000 axons in normal animals) showed no sign of axonal degeneration, despite the fact that 35% of the axons expressed the SOD1 mutant. Thus, mutant expression in motor neurons was insufficient to kill any of those neurons, much less cause disease. This finding suggests ALS is not cell autonomous and may have implications for treatment. Replacing motor neurons would be a daunting task, whereas improving the microenvironment by replacing non-neuronal cells could be beneficial.

Which non-neuronal cells in the previous experiment may have prevented neurotoxicity is unclear. Robust microgliosis occurs during ALS disease progression and, as in other neurodegenerative diseases, it is unknown whether microglia play a beneficial or a damaging role, and whether mutant SOD1 expression within microglia affect their role. Micro-glia come from a cell progenitor that ultimately gives rise to the macrophage and microglial lineages. Selectively remov-ing a SOD1 mutant from the macrophage/microglial lineage indicated that mice with the SOD1 mutant removed from their microglial lineage live longer than those with mutant SOD1 in the microglia.

Astrocytes with mutant SOD1 activity exhibit impaired glutamate transporter function and may accelerate disease development. The only known glutamate transporter in the spinal cord is EAAT2, which is present only in astrocytes. Blocking glutamate transporters leads to excessive glutamate
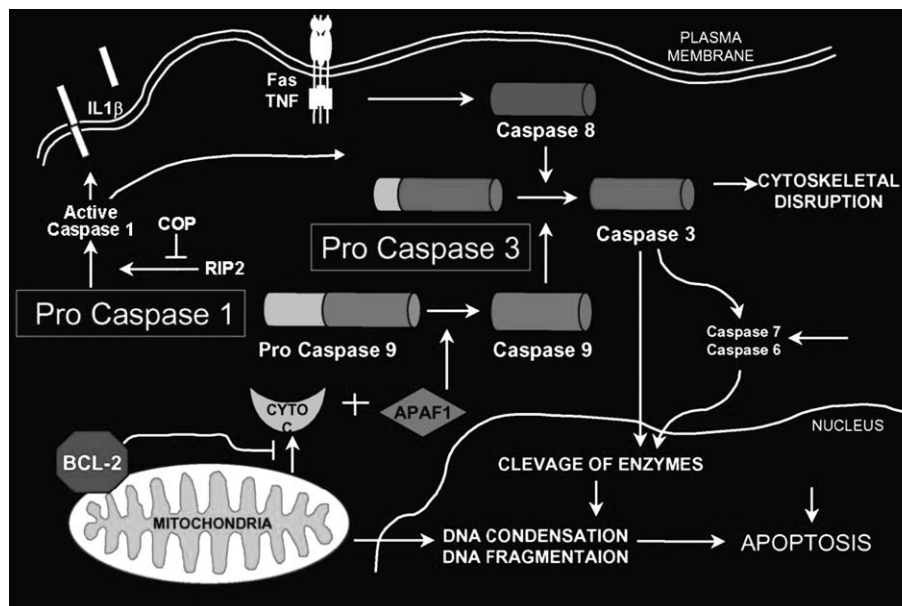

Fig. 7. Abbreviated cascade of apoptotic events.

pools in the synaptic cleft, causing repetitive firing, calcium entry, and ultimately, motor neuron death. In normal rats, glutamate transporters are present in the ventral area of the spinal cord where the cell bodies of the motor neurons reside. But in rats expressing a SOD1 mutant and about to develop disease, the glutamate transporter is selectively lost in the ventral horn just prior to disease initiation (Howland et al., 2002).

Theories to explain the selective death of motor neurons in ALS include oxidative stress, aggregation, mitochondrial damage, glutamate, and microglial cell mediated damage. It is why all of these likely that all of these theories are correct to some extent and each of these processes could be an appropriate therapeutic target.

6.5. Neuroprotection in acute stroke

Ischemic injury is complex and, like neurodegenerative disorders, multiple pathways may be involved. An important difference regarding neurodegeneration in stroke (i.e., due to ischemia) is the time period for salvage, which is minutes and hours as opposed to days, weeks, months, or years. There is a "therapeutic time window" in which ischemic brain evolves from potentially salvageable to irreversibly damaged, related to the extent of cerebral blood flow decline. Tissue plasminogen activator (tPA) was approved for use in acute stroke within a 3-h window because half of the patients in the pivotal clinical trial were treated within 90 min of stroke onset (National Institute of Neurological Disorders and Stroke rt-PA Stroke Study Group, 1995). However, the percentage of ischemic tissue that is potentially salvageable varies over time and among individual patients. The odds ratio (OR) for successful outcomes is high if the patient is treated within 90 min; unfortunately, the odds of seeing and treating a patient within 90 min are not good. Between 90 and 180 min, the OR with tPA is 1.5, which translates to an absolute benefit of 6% to 7%. However, the benefit of tPA actually extends to approximately 4.5 h, when the absolute benefit is about 5%.

The most important therapeutic target in the ischemic brain is functionally impaired but surviving brain tissue—the ischemic penumbra. Different approaches have been tried to extend the survival time of the penumbra until vessels can be reopened with intravenous thrombolysis. For example, animal data show lowering brain temperature extends survival of penumbral tissue for many hours (Colbourne et al., 1997). Hypothermia alone, however, is not effective because when stopped, lesions begin to evolve again. Administration of normobaric hyperoxia extends the therapeutic time window in rats subjected to focal cerebral ischemia (Kim et al., 2005). A desired approach is to use a variety of neuroprotective agents to help stabilize the penumbra before lytic therapy is initiated; however, no such agent is currently available and a long list of neuroprotective drugs have fared poorly in phase 3 clinical trials (Table 1). Results of current studies of neuroprotective

Table 1
Neuroprotection drug failures

NMDA antagonists	Cerestat
	MK-801
	Dextrorphan
	Selfotel
Other NMDA drugs	Eliprodil
	GV150526
	ACEA 1021
	AMPA Antag
Presynaptic effects	Fosphenytoin
	Lubeluzole
	BW619C89
Others	Nimodipine
	Tirilazad
	bFGF
	Cervene
	Enlimomab
	ICOS
	Maxipost
	Clomethiazole

agents in stroke are mixed. Preliminary data for NXY 059, a spin trapping agent that acts as a free radical scavenger, are promising (Lees et al., 2005). ReoPro, a GPIIb/IIIa antagonist that likely has an effect on the microcirculation, had shown promising results in phase II studies but a phase III study was recently (October, 2005) halted due to a higher-than-expected rate of brain hemorrhaging (Society for Vascular Surgery, 2005). Several drugs are in phase II studies.

Among the reasons for poor outcomes in clinical trials is inappropriate study design. Before initiating a study, a reasonable animal testing paradigm should be employed. Embolic models are probably the closest mimicker of human stroke. The suture-occlusion model involves leaving a monofilament suture in the animal until it is sacrificed. The occlusion is permanent but the suture can be withdrawn at any time so a degree of reperfusion is possible. Multiple outcomes should be assessed in animal stroke models. The primary outcome should be histological, i.e., measuring infarct volume, but it is also important to assess biochemical markers and behavioral outcomes.

Enrolling the right patients is also crucial; therapy trials must include patients still able to respond to therapy. Moreover, therapeutic plasma levels are important; a number of the drugs that were protective in animals failed in humans because they were too toxic at the same plasma concentrations. Finally, the mechanism of drug action must be considered in the trial design. For example, there are clear differences between the mechanisms of ischemic injury in gray matter compared with those in white matter during acute ischemia. Many animal models use infarct size as the main endpoint, which occurs primarily in gray matter in rats; however, human trials often include patients with lacunar stroke, which is a white matter injury.

An area of high interest in animal–and recently human–studies is the evaluation of ischemic injury in vivo using diffusion/perfusion MRI. Diffusion-weighted imaging is

used to localize the ischemic injury, follow its evolution, and track effects of therapy in vivo. Perfusion imaging allows assessment of the adequacy of the vascular occlusion in experimental models and also allows measurement of the reperfusion effects of thrombolysis. Combining the two techniques and detecting the "mismatch" between areas of diffusion and perfusion provides an approximation of the location and size of ischemic penumbra; that is, the area that is hypoperfused but does not have an acute lesion. Use of this technique in animal experiments has provided a tool for assessing optimal duration of treatment, onset of treatment response, and overall response to treatment in vivo. This approach is now being used in human clinical trials and even (infrequently) in clinical practice (Ribo et al., 2005).

Use of diffusion/perfusion MRI allowed a serendipitous discovery of the surprising neuroprotective effect of dimethyl sulfoxide (DMSO). While experimenting with another molecule for which DMSO was the solvent, the control sample (DMSO alone) showed a large degree of neuroprotection. Animals with a permanent occlusion were infused with 30% DMSO for 3 h beginning 4 h after infarct. Results showed no increase in the volume of the diffusion lesion, even at 24 h (Bardutzky et al., 2005). DMSO treatment effectively "froze" the penumbra and it never evolved subsequent to initiation of the therapy. DMSO is a free-radical scavenger; however, these results suggest it may have additional neuroprotective mechanisms.

7. Caspase-1: a common pathway to neurodegeneration

Caspase-mediated cell death appears to be a commonality in several, if not all, neurologic diseases. Caspases are proteinases implicated in apoptosis. Caspase-1, also known as interleukin (IL)-1β converting enzyme (ICE), is involved in the production of the inflammatory mediator, IL-1β. Caspase-1 is the only enzyme required for activation of pre-IL-1β; therefore, the presence of mature IL-1β indicates caspase-1 activation. When caspase-1 function is blocked, neurologic injury in many different disease models is decreased (Zhang et al., 2003). Thus, determining what activates this enzyme, and by what mechanism it promotes neuronal death may provide targets for neuroprotective therapy.

The R6/2 mouse is frequently used animal model of Huntington's disease. By 4 weeks of age, these mice develop ubiquinated neuronal inclusions and neuroreceptor loss and their brains atrophy. The R6/2 mouse loses weight by 7 weeks, develops progressive neurologic dysfunction, has tremors and seizures, and dies, usually by 14 weeks. There is an approximate 2.5-fold increase in the amount of mature IL-1β in the R6/2 mice compared with wild type mice. (Interestingly, the brains of patients who died of advanced HD have an approximate 2-fold increase in capsase-1 compared with control brains.)

A number of experiments in different cell lines expressing a caspase-1 dominant negative protein (M17) confirm that blocking caspase-1 function prevents cell death. A transgenic mouse was created using the neuron-specific enolase (NSE) promoter to target expression of M17 specifically in neurons. The transgenic caspase-1 dominant negative mouse was developmentally normal in all ways, including its brain. The R6/2 mice were crossed with the caspase-1 dominant negative mice to create a double-transgenic mouse (R6/2-M17Z) to determine the effect of inhibiting caspase-1 activation in the R6/2 mice (Ona et al., 1999). Rotorod tests of motor strength and coordination in the R6/2 mice at 9 weeks of age indicative of early stage disease; these mice could not complete 10 min on the treadmill, whereas the double transgenic R6/2-M17Z mice could complete 10 min at the highest speed. Thus, there was a delay in disease onset associated with caspase-1 inhibition. At 12 weeks, the R6/2 mice had advanced disease and the R6/2-M17Z mice began to show rotor rod deficits, indicating disease progression was not stopped but was delayed. Mortality was also delayed by approximately 20% when capsase-1 was inhibited. Direct delivery of a peptide caspase inhibitor into the brain also resulted in a delay of disease progression and extended survival in the R6/2 mice (Ona et al., 1999).

In ischemia and hypoxia models, primary cerebrocortical neurons exposed to oxygen/glucose deprivation (OGD) die in a time-dependent manner, providing a model to investigate caspase-1-instigated cell death. In vitro, neurons from wild-type mice exposed to OGD die as expected, whereas neurons from M17Z (caspase-1 knockout) mice exposed to OGD demonstrate almost complete inhibition of OGD-mediated cell death. Thus, knocking out caspase-1 alone improves neuronal survival (Zhang et al., 2003).

How caspase-1 is activated and regulated remains unknown though it appears mitochondria are involved in the activation of caspase-dependent pathways (Fig. 7). Any kind of stress, such as exposure to ROS or increased intracellular calcium can induce a change in mitochondrial membrane permeability, which leads to mitochondrial swelling, rupture of the outer membrane, and a significant decline in mitochondrial membrane potential. Changes in membrane potential lead to release of apoptotic factors (e.g., cytochrome *c*; caspase-9, caspase-3), and eventually, to cell death. In wild type mice exposed to OGD, membrane potential dissipates but in caspase-1 knockout mice, membrane potential is preserved during OGD, suggesting caspase-1 is activated early in the cell-death pathway Zhang et al., 2003).

The protein, Rip-2 (or CARDIAK), has been shown to activate caspase-1 under inflammatory conditions, though until recently its role in cell death was unknown. Wild type mouse primary neurons transfected with Rip-2 undergo substantial cell death. In contrast, neurons from caspase-1 knockout mouse transfected with Rip-2 do not die, suggesting Rip-2 is an activator of caspase-1 (Zhang et al., 2003). An endogenous inhibitor of Rip-2 has been identified, called the CARD only protein (COP); the protein has a high degree of a sequence homology to caspase-1 and appears to act as a decoy in Rip-2-mediated caspase-1 activation.

Adding COP to cells incubated with Rip-2 and caspase-1 inhibited cell death. In vivo, an increase in Rip-2 in neurons of R6/2 mice is detected early in disease development. As the disease progresses, Rip-2 migrates into the nucleus (Wang et al., 2005).

Evidence suggests caspase-1 is an early trigger of neuronal cell death, involved in the early orchestration of activities between the filamentary pathways and the cell death pathway. Rip-2 is a stress-induced activator of caspase-1 and requires caspase-1 to promote cell death. COP appears to be an important inhibitor of Rip-2, and decreased COP, may, in part, be responsible for caspase-1 activation. Once active, caspase-1 mediates mitochondrial collapse. As neurologic disease progresses, caspase-1 transcription is increased. As the cell becomes more ill, increases in IL-1β, TNFα, and free radicals affect neighboring cells. Reactive microglia and reactive astrocytes further mediate inflammatory and cell-death pathways.

8. Conclusion

There is a great need for the discovery of biomarkers of neurodegenerative diseases because by the time the patient is diagnosed, extensive neuronal damage has usually already occurred. Biomarkers could allow earlier diagnosis and intervention. Additionally, commonalities among neurodegenerative diseases have implications for disease prevention and development of effective therapies. Genetic mutations (SOD1, parkin, huntingtin), protein misfolding and aggregation (Lewy bodies, Amyloidβ, neurofibrillary tangles), mitochondrial dysfunction, and caspase-mediated apoptosis, are present in seemingly very different neurologic disorders. In most cases, no single dysfunction has been implicated in causing the disorder and it is unlikely that a single drug or targeting a single mechanism will be sufficient to halt neurodegenerative processes. As has become common in other fields such as cancer or HIV, combination therapies may be most effective for these disorders.

Acknowledgements

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