OXIDATIVE STRESS AND THE PATHOGENESIS OF NEURODEGENERATIVE DISORDERS

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Microglia-derived inflammatory neurotoxins play a principal role in the pathogenesis of neurodegenerative disorders including Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, and HIV-associated dementia; chief among these is reactive oxygen species. The detrimental effects of oxidative stress in the brain and nervous system are primarily a result of the diminished capacity of the central nervous system to prevent ongoing oxidative damage. A spectrum of environmental cues, mitochondrial dysfunction, accumulation of aberrant misfolded proteins, inflammation, and defects in protein clearance are known to evolve and form as a result of disease progression. These factors likely affect glial function serving to accelerate the tempo of disease. Understanding the relationships between disease progression, free radical formation, neuroinflammation, and neurotoxicity is critical to elucidating disease mechanisms and the development of therapeutic modalities to combat disease processes. In an era where populations continue to age, the prevalence and incidence of age-related neurodegenerative diseases are on the rise; therefore, the need for novel therapeutic strategies that attenuate neuroinflammation and protect neurons against oxidative stress is ever more immediate.

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I. Introduction: Free Radicals, Immunity, and the Nervous System

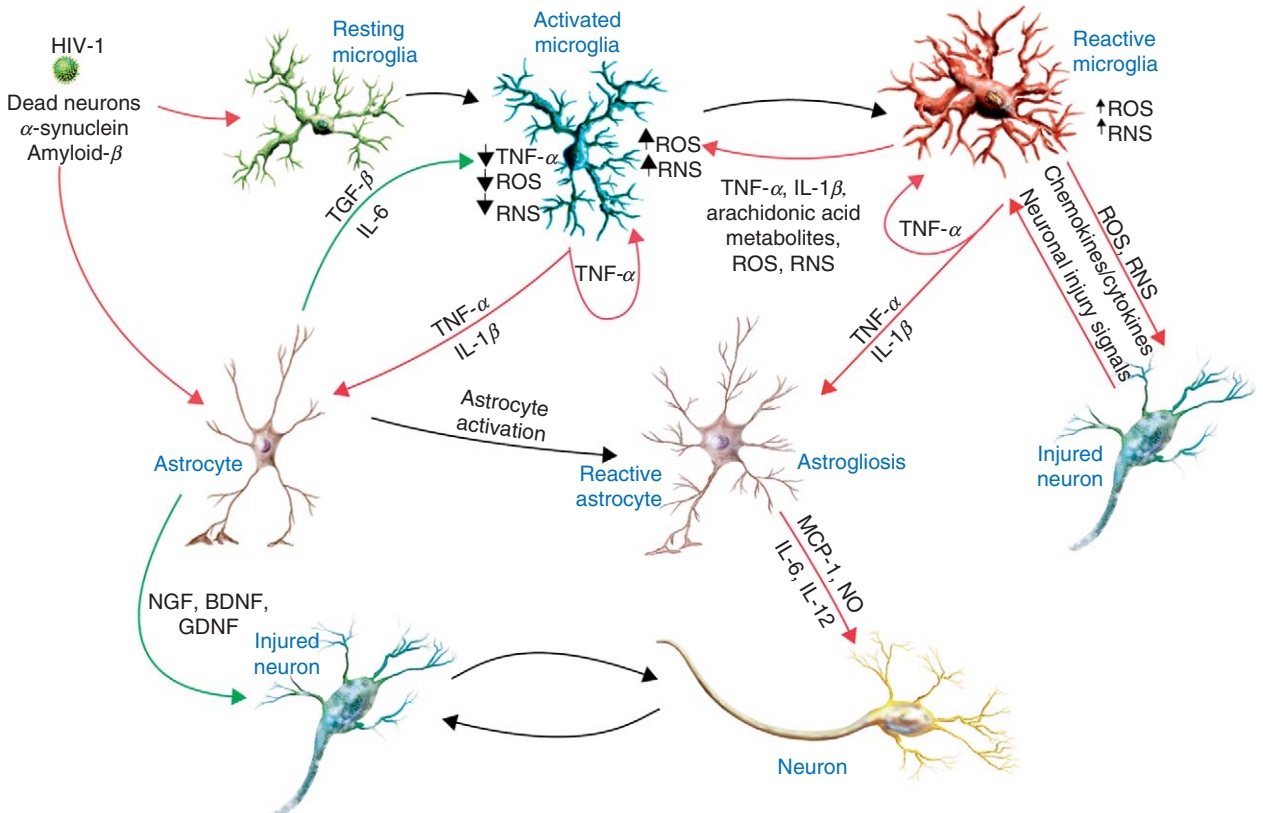
Cells within the brain, notably neurons, are highly vulnerable to the detrimental effects of reactive oxygen species (ROS). This occurs because of their high metabolic rate, rich composition of fatty acids prone to peroxidation, high intracellular concentrations of transition metals capable of catalyzing the formation of reactive hydroxyl radicals, low levels of antioxidants, and reduced capability to regenerate. Glial cells (microglia and astrocytes) are the key support cells of the nervous system. However, during neuroinflammatory processes, they produce free radicals early and often, and serve as key pathogenic elements in neurodegenerative diseases [Alzheimer's and Parkinson's disease (AD and PD), amyotrophic lateral sclerosis (ALS), and HIV-1-associated dementia (HAD)]. Free radicals have the capacity to attack proteins, polysaccharides, lipid bilayers, and DNA, causing cellular oxidative damage. Nucleic acid oxidation occurs in neurons during disease and is detected as elevated levels of 8-hydroxyl-2-dexoyguanosine in DNA and 8-hydroxyguanosine in RNA. Hydroxyl radical-mediated DNA damage often results in strand breaks, DNA-protein cross-linking, and base modifications (Nunomura *et al.*, 1999). All of these events can lead to neuronal injury. Oxygen free radicals are also found in brain regions affected by neurodegenerative diseases. These include the hippocampus in AD patients, the substantia nigra and caudate putamen in PD patients, and spinal fluids in ALS and HAD patients. Therapeutic strategies that inhibit free radical formation and prevent downstream formation of hydroxyl radicals can slow disease and remain an active area of investigation.

The innate and adaptive immune responses modulate much of the free radical formation within the central nervous system (CNS) in both health and disease (Carlson *et al.*, 2002). As to the former, innate immunity is defined as a nonspecific defense response that is activated after an antigen (microbe, aberrant protein) emerges. Although nonspecific mechanisms also include physical barriers in the CNS, innate immunity is best characterized by the production and release of a plethora of secretory products by antigen-exposed immunocytes that are affected or regulated by redox pathways. The principal immune cells in the nervous system that react in this manner include microglia and astrocytes. Microglia are highly mobile cells with numerous roles in protecting the nervous system (acting as scavengers and chemical secretors, and to present antigen to induce an immune response). They also affect the pathobiology of neurodegenerative disorders by eliciting inflammatory reactions as a consequence of infection or disease. Activated microglia participate in inflammatory processes linked to neurodegeneration by producing neurotoxic factors including quinolinic acid, superoxide anions, matrix metalloproteinases, nitric oxide (NO), arachidonic acid and its metabolites, chemokines, proinflammatory cytokines, and excitotoxins including glutamate. Astrocytes are a major brain cell

that provide physical support to neurons as well as assisting the microglia during cleanup of debris. Astrocytes also provide direct support to neurons by secreting growth factors required for proper function of the nervous system and control of fluid composition surrounding neurons. Neurons also play a role in innate immune function, but to a significantly lesser degree than microglia or astrocytes. The cellular machinery of microglia, astrocytes, and neurons and their production and regulation of toxic free radicals are illustrated in Figs. 1 and 2. Briefly, nervous system cells contain the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex, which when assembled and activated, produces free radicals in abundance that can lead to tissue damage (McGeer and McGeer, 2002a). Another source of these destructive oxidants is credited to mitochondrial metabolism, and is estimated that up to 1% of the mitochondrial electron flow leads to formation of superoxide radicals (Lass *et al.*, 1997). Albeit the production of free radicals and ROS facilitates the function of the innate immune system in host defense against invading organisms, the secondary consequence of such reactions, if not properly controlled (as occurs in neurodegenerative diseases), is tissue injury.

Adaptive immunity refers to antigen-specific immune responses, and is significantly more complex. Antigen first must be processed and recognized by antigen-presenting cells such as dendritic cells, macrophages, and microglia. Once an antigen has been recognized, the adaptive immune system expands large numbers of immune cells (commonly T cells) specifically designed to attack and destroy the foreign antigen. The role of free radicals produced and regulated by T cells in the context of neurodegenerative diseases is incompletely understood. However, for neurodegenerative disorders, the presence within the brain of major T cell subsets in ratios exceeding those typically found in the periphery suggests a more profound role in disease than merely performing a surveillance function. How these T cells are activated, whether they are antigen-specific, or migrating in response to microglial inflammation has yet to be determined.

Oxidative abnormalities and damage are a major pathogenic feature of AD, PD, ALS, and HAD (Andersen, 2004; Tabner *et al.*, 2001). Importantly, compensatory mechanisms are operative and neurons have the capacity to upregulate antioxidant defenses, which suggest a balance for oxidant damage in disease (Carri *et al.*, 2003). However, when pro-oxidants surpass the endogenous controls or antioxidants, a formula for oxidative stress arises and affects disease. Oxidative stress has been linked to protein misfolding and aggregation, and ultimately to microglial activation, thus ascribing a pathogenic trigger to the chronic inflammatory response and neurodegeneration. The processes involved in oxidative stress and protein aggregation are not mutually exclusive. The exact mechanisms by which protein aggregates formed in AD, PD, and ALS mediate neuronal cell death remain incomplete, has been linked to ROS generation, either directly or through microglial activation that ultimately affects cell demise.

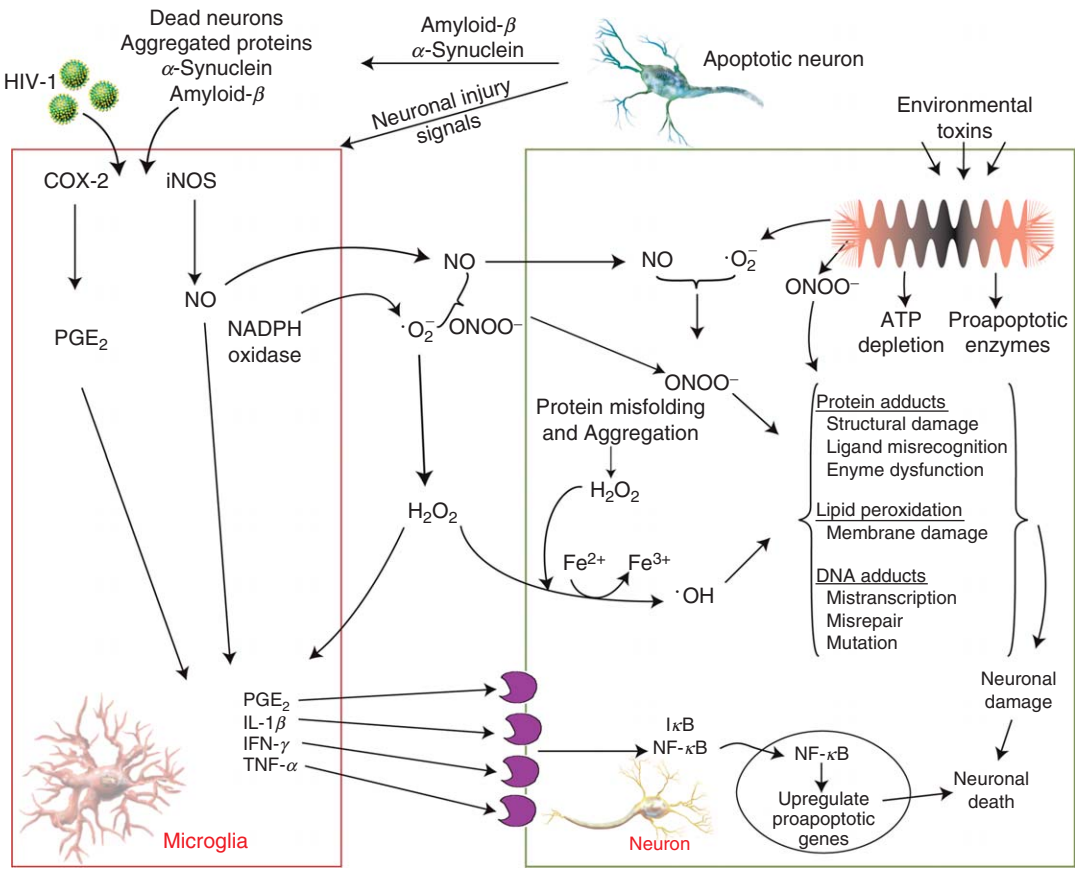

HIV-1
Dead neurons
α-synuclein
Amyloid-β
Resting microglia
Activated microglia
Reactive microglia
↑ROS
↑RNS
↓TNF-α
↓ROS
↓RNS
↑ROS
↑RNS
TGF-β
IL-6
TNF-α, IL-1β, arachidonic acid metabolites, ROS, RNS
TNF-α
ROS, RNS
Chemokines/cytokines
Neuronal injury signals
TNF-α
TNF-α
IL-1β
TNF-α
IL-1β
Astrocyte activation
Astrocyte
Reactive astrocyte
Astrogliosis
Injured neuron
MCP-1, NO
IL-6, IL-12
NGF, BDNF, GDNF
Injured neuron
Neuron

A novel mechanism that may initiate the production of ROS is the effect of hydrogen peroxide on protein aggregation, which in the presence of transition metals, such as iron, transforms into hydroxyl radicals (Fig. 2) and initiates oxidative damage before antioxidant defenses can intervene. Genetic mutations found within AD, PD, and ALS disorders lead to irregular processing of misfolded proteins, promoting the deposition of protein aggregates. This occurs in the cytosol, extracellular spaces, and/or nucleus, leading to CNS amyloidosis. In this scenario, soluble proteins are altered into insoluble, filamentous polymers that begin to develop cross-linked β-pleated sheet structures (Sipe and Cohen, 2000). β-Sheets accumulate, form fibrillar amyloid deposits, and aid in the establishment of neurodegeneration (Forman *et al.*, 2004; Zhang *et al.*, 2005). Amyloid-β (Aβ), α-synuclein, and superoxide dismutase 1 (SOD1) are the primary proteins found within the protein deposits in tissues of patients with AD, PD, and ALS, respectively. Senile plaques along with neuronal and glial inclusions are typical of protein aggregates found in histological preparations from AD patients, while Lewy bodies and hyaline inclusions represent protein aggregates typically found in PD and ALS (Tabner *et al.*, 2005). Protein aggregates may also bind and attach to cellular components, or be phagocytosed by resident microglia and incite ROS production leading to oxidative damage (Thomas *et al.*, 2007; Zhang *et al.*, 2005). Microglial activation as a result of interactions with aggregated proteins encompasses a common mechanism by which aggregated proteins can facilitate ROS production and perpetuate the neurodegenerative process.

II. Neuropathogenesis of Neurodegeneration

AD is the most common neurodegenerative disease. Sporadic AD is extremely rare in individuals younger than 60 years of age (representing less than 5% of all reported cases), but the incidence of AD dramatically increases to 40% after the age of 85 (Forman *et al.*, 2004). Neuronal cell loss in the hippocampus and amygdala, the brain subregions responsible for learning and memory, underlies

FIG. 1. Neuroinflammatory interactions between microglia, astrocytes, and neurons during neurodegenerative diseases. Microglia and astrocytes communicate through numerous cell-signaling pathways, resulting in reactive cell phenotypes and hastening the neuroinflammatory process. Activation of resting microglia results in amplification of secretions and the production of ROS, RNS, chemokines, and proinflammatory cytokines initiating a generalized inflammatory response and ultimately leading to neuronal injury or death. Astrocyte activation (astrogliosis) develops from reactive microglia that alter the astrocytic phenotype from neurotrophic to neurotoxic. (Green arrow = protective response, red arrow = destructive response, and black arrow = spontaneous.)


Dead neurons
Aggregated proteins
α-Synuclein
Amyloid-β
HIV-1
Amyloid-β
α-Synuclein
Neuronal injury signals
Apoptotic neuron
Environmental toxins
COX-2
iNOS
NO
NO
·O2−
ONOO−
ATP depletion
Proapoptotic enzymes
PGE2
NADPH oxidase
·O2−
ONOO−
ONOO−
Protein misfolding and Aggregation
Protein adducts
Structural damage
Ligand misrecognition
Enyme dysfunction
Lipid peroxidation
Membrane damage
DNA adducts
Mistranscription
Misrepair
Mutation
H2O2
H2O2
Fe2+
Fe3+
·OH
Neuronal damage
PGE2
IL-1β
IFN-γ
TNF-α
IκB
NF-κB
NF-κB
Upregulate proapoptotic genes
Neuronal death
Microglia
Neuron

AD pathobiology (Markesbery and Mira, 1996). Two types of lesions characterize AD neuropathology, senile plaques and neurofibrillary tangles (NFT) composed of Aβ. Aβ is a cleavage product of the amyloid precursor protein (APP), which is phosphorylated tau functioning as a microtubule stabilizer. When tau is hyperphosphorylated, it enhances the formation of fibrillar aggregates that leads to NFT. Although plaques and fibrils appear to be the most prevailing features of AD pathology, they alone are not sufficient to generate the significant and profound neuronal loss that is characteristic of disease. Neuronal damage caused by neurotoxic factors initiated from inflammatory responses by immune-activated glial cells appears to be the best link to cognitive deterioration. Aβ plaques prime microglia, and an inflammatory cascade is created and supported by secondary factors including proinflammatory cytokines, chemokines, or by T cells trafficking in and out of the nervous system (reviewed in Carlson *et al.*, 2002). These factors can also contribute to the breakdown of the blood–brain barrier (BBB), allowing leukocytes entry into the brain and propagating the CNS inflammatory cascade. As a consequence of BBB breakdown and local immune activation, the brain's resident macrophages or microglia release a plethora of neurotoxins such as proteases, glutamate, arachidonic acid and its metabolites, including platelet-activating factor and ROS; of which all affect synaptic transmission and can lead to neuronal damage. Epidemiological studies for AD have shown that nonsteroidal anti-inflammatory drugs (NSAIDs), which are known to reduce microglia responses, also reduce the risk of AD (McGeer and McGeer, 2002b).

PD is the second most common age-related neurodegenerative disease and is grouped among motor system disorders. PD is predominantly defined by the loss of dopaminergic neurons (dopamine-producing neurons) of the substantia nigra pars compacta (SNpc) and their axons projecting to the caudate-putamen or striatum. The etiology of PD remains unknown; however, patterns of familial inheritance and several animal models suggest a possible connection involving abnormal protein processing and accumulation. Indeed, a pathological feature of PD is the formation of inclusion bodies or Lewy bodies that are primarily composed of ubiquitin and α-synuclein aggregates. The majority of PD cases are sporadic and may be due, in part, to mitochondrial defects at complex I (Dauer and Przedborski, 2003). Complex I inhibitors, such as 1-methyl-4-phenylpyridine and rotenone, can recapitulate pathological features of PD in humans and animal

FIG. 2. Oxidative stress and neuronal damage. ROS can arise in several distinct ways such as glial cell activation, mitochondrial dysfunction, and protein aggregation. When ROS tips the balance outweighing antioxidants, oxidative stress is generated and neuronal cell injury or death ensues. Oxidative stress destroys several organic structures of the cell, including proteins, lipids, and DNA, causing irreversible and detrimental damage. This figure was adapted from F. Gao and colleagues (Du *et al.*, 2001).

models of disease (Dauer and Przedborski, 2003; Dawson and Dawson, 2003). Reactive microglial responses, thought to play a central role in dopaminergic neuronal death, predominate the site of dopaminergic neuronal injury, and may be amplified through paracrine and autocrine processes (Dawson and Dawson, 2003). In PD, a clear connection exists between inflammation and neurodegeneration, even more so than aberrant protein processing and accumulation. There is an increase in activated microglia in the SNpc and striatum of patients with idiopathic PD (Kohutnicka *et al.*, 1998; Kurkowska-Jastrzebska *et al.*, 1999; Wu *et al.*, 2002). Indeed, attenuation of microglial activation in PD models can protect greater than 90% of the dopaminergic neurons otherwise destined to die (Choi *et al.*, 2005; Du *et al.*, 2001; Kurkowska-Jastrzebska *et al.*, 1999; Teismann and Ferger, 2001; Teismann *et al.*, 2003; Vijitruth *et al.*, 2006; Wu *et al.*, 2002). Furthermore, epidemiological data has shown that the use of NSAIDs decreases the risk for the PD as it does for AD (Chen *et al.*, 2003, 2005).

HAD is another neurodegenerative disorder that is secondary to HIV-1 infection. A common disturbance associated with HIV infection is the development of neurological disorders. Approximately 60% of HIV-1-infected patients display some form of neurological dysfunction, most likely due to early entry of HIV-1 into the CNS. The virus infects the brain through CD4+ T lymphocytes (Haase, 1999) and mononuclear phagocytes (MPs: dendritic cells, monocytes, and macrophages) (Tardieu and Boutet, 2002). The brain lacks sufficient viral control methods and adaptive immunity; therefore, active viral replication is virtually unrestricted and the most detrimental form of HIV-1-associated tissue damage can occur within the CNS. Antiretroviral therapy (ART) was introduced to help extend the amount of time a patient might have before the development of CNS disease and its associated immune suppression takeover (d'Arminio Monforte *et al.*, 2000; Yong *et al.*, 2001). Patients that responded poorly to therapy, or those that did not receive ART, display signs of damage to their immune systems and a more rapid progression of the disease (Ho *et al.*, 1995; Krishnakumar, 2005; Wei *et al.*, 1995). Even though HAD can be observed in up to 40% of infected individuals, ART has considerably reduced both disease incidence and its destruction. HIV-1-associated cognitive impairment is composed of a wide spectrum of conditions from the mild HIV-1 motor cognitive-motor disorder to the severe and debilitating HAD. The progression of HAD is extremely variable and may depend on several issues including genetic factors of the infected host, systemic and brain HIV burden, BBB integrity, the speed of CD4+ T-lymphocyte decline, and the genotype of the viral strains that gain access to the brain or induce neurovirulent activities (Anderson *et al.*, 2002). The pathology of HAD revolves around the formation of multinucleated giant cells of the MP lineage, which are also the main reservoirs for the virus in the brain. MPs can secrete neurotoxins and induce neuronal injury leading to neurocognitive

impairments and ultimately to HAD (Aquaro *et al.*, 2000; Elbim *et al.*, 2001; Kaul *et al.*, 2001; Luo *et al.*, 2003; Xiong *et al.*, 2000). Under normal conditions, MPs secrete neurotrophins and eradicate foreign material to maintain CNS homeostasis (Gras *et al.*, 2003). However, during HAD, MP-mediated control over the neurotrophic factors becomes altered and infected MPs aberrantly affect the development of a metabolic encephalopathy. In addition, infected MPs secrete proinflammatory cytokines, chemokines, eicosanoids, excitatory amino acids, TNF-related apoptosis inducing ligand, viral proteins, reactive nitrogen species (RNS) and ROS (Ensoli *et al.*, 2000; Floyd *et al.*, 1999; Gendelman *et al.*, 1998; Raber *et al.*, 1998). The actual severity of the dementia is strongly associated with the numbers of activated macrophages and microglia rather than the actual viral load in the CNS (Adle-Biasette, 1999). Therefore, neuronal damage observed during HAD is linked more to macrophage activation than to HIV-1 itself.

ALS, also known as Lou Gehrig's disease, is a devastating motorneuron disorder. Muscle weakness is the hallmark sign of ALS, occurring in approximately 60% of patients. Unlike the previous diseases, progression of ALS is rapid. Individuals with ALS lose function of motorneurons within 3–5 years (Weydt and Moller, 2005) terminating with complete neuromuscular failure and death (typically caused by compromised respiratory function). ALS affects about 5–7 in 100,000 adults throughout the world (Rowland and Shneider, 2001). The majority of ALS cases are sporadic (90–95%) (Cleveland and Rothstein, 2001), while the remaining cases are attributable to familial etiology (Cleveland and Rothstein, 2001; Valentine, 2002). The pathology of ALS is characterized by neuronal degeneration and atrophy confined almost entirely to the upper and lower motorneurons (Weydt and Moller, 2005). The hypothesized mechanisms that lead to neurodegeneration, such as glutamate toxicity, exogenous factors, neurofilament accumulation, neuroinflammation, and oxidative stress (Bruijn *et al.*, 2004; Rowland and Shneider, 2001; Strong, 2003), could be independent factors or could cooperate to cause motorneuron loss. A key discovery was the identification of missense mutations in the gene on chromosome 21 encoding for a Cu/Zn-binding protein called SOD1 (Weydt *et al.*, 2002). SOD1 is a key antioxidant in the front line of defense against oxidative stress; it detoxifies oxidative agents by converting superoxide to hydrogen peroxide and dioxygen, thus decreasing levels of superoxide within its proximity. This enzyme is located predominately in the nucleus, cytosol, and mitochondrial intermembrane space (Lyons *et al.*, 1999). Presently, over 100 SOD1 mutations have been identified in familial ALS patients (Andersen, 2001; Gaudette *et al.*, 2000; Guegan and Przedborski, 2003). The implication of SOD1 mutations is still under intense investigation; however, it is now hypothesized that the ALS phenotype is caused by a gain of a novel, unknown toxic property of the SOD1 mutant enzyme rather than by diminished SOD1 activity (Valentine, 2002).

III. Free Radicals and Neurodegenerative Disorders

Inflammatory responses induced by reactive microglia, macrophages, and proinflammatory T cells provide a primary source of free radicals, ROS (O_2^-, H_2O_2, -OH, HOCl, ferryl, peroxyl, and alkoxyl) and RNS [NO, peroxynitrite ($ONOO^-$), and peroxynitrous acid (ONOOH)] with the capacity to modify proteins, lipids, and nucleic acids. Copious amounts of ROS production, known as respiratory burst, have detrimental effects on delicate neuronal networks in the CNS. ROS include superoxide, hydrogen peroxide, and hydroxyl free radicals as well as nitrogen intermediates (NO and peroxynitrite) and can cause damage to neurons if produced in excess as occurs during prolonged neuroinflammatory processes. Microglial-derived ROS such as superoxide cannot efficiently traverse cellular membranes, and therefore unlikely to gain access to neurons and trigger intraneuronal toxic events (Beckman and Crow, 1993). However, superoxide can rapidly react with NO in the extracellular space to form peroxynitrite (Beckman and Koppenol, 1996), which can readily cross cell membranes and damage intracellular components. NO is produced in many tissues including neurons, astrocytes, and microglia, and can be secreted by glial cells on activation with proinflammatory cytokines including, but not limited to IL-1β and TNF-α (Mollace and Nistico, 1995). NO alone has a relatively low neurotoxic capacity; however, in conjunction with superoxide, it additively contributes to neuronal destruction. Nitrated species have been associated with the disruption of mitochondrial electron transport chain, lipid peroxidation, DNA damage, and protein nitration (Beckman, 1996). Therefore, superoxide production by microglia, by contributing to peroxynitrite formation, provides a significant contribution to the pathogenesis of neurodegenerative disorders.

The notion that oxidative stress is involved in AD stems from the free radical hypothesis of aging, which simply states that, as one grows older, accumulation of more ROS, coupled with diminished antioxidant capacity, results in more destruction to major cellular components (Beal, 1995; Pratico and Delanty, 2000; Reiter, 1995). ROS in AD is caused by several factors, including active microglia, redox-active metals, advanced glycation, and Aβ peptide. Near neuritic plaques and throughout the entire brain, activated microglia are heavily populated, releasing high levels of ROS. Both the NFT and Aβ deposits contain the redox-active transition metal iron (Smith *et al.*, 1997). Within affected neurons, iron is found in the cytoplasm and within lipofuscin granules. Additionally, alterations of iron homeostasis occur in AD and are supported by finding of elevated serum levels of the iron-binding protein p97 in diseased patients. This coupled with the presence of redox-available iron in AD brain regions and activation of the receptor for advanced glycation end products leading to oxygen radicals and cell injury supports the importance of oxidative stress in AD pathogenesis.

Free radicals that are considered to cause neuronal loss in AD are believed to be produced as a result of the deposition of aggregated Aβ peptide. However, whether Aβ functions as a producer of ROS or a modulator of redox reactions is not clear. Accumulated evidence of surrogate markers for oxidant injury from postmortem tissues indicates a strong association between oxidative stress and the pathology of AD. These markers show increased lipid peroxidation, protein oxidation, 8-OHdG levels, and a marked decline in oxidative-sensitive enzymes (Castellani *et al.*, 2001; Markesbery and Carney, 1999; Nunomura *et al.*, 1999, 2000, 2001; Zhu *et al.*, 2004).

Oxidative stress contributes to the cascade leading to dopaminergic neurodegeneration in PD (Tabner *et al.*, 2001). Brain regions that are rich in catecholamines, such as, adrenaline, noradrenaline, and dopamine, are exceptionally vulnerable to free radical generation. Catecholamines can spontaneously break down to free radicals, or be metabolized to free radicals by endogenous enzymes such as monoamine oxidases. Activated microglia also contribute to the degeneration of dopaminergic neurons by releasing neurotoxic factors such as NADPH oxidase-derived superoxide and cytokines. Peroxynitrite production contributes to mitochondrial dysfunction and nitration of tyrosine residues in cellular proteins and enzymes such as α-synuclein. Indeed, soluble nitrated α-synuclein is able to activate microglia to produce copious amounts of ROS through modulation of specific ion channels (Thomas *et al.*, 2007). Nitration of α-synuclein can significantly enhance fibril formation *in vitro*, similar to the biophysical properties of α-synuclein isolated form PD brains (Norris *et al.*, 2003). Aberrant protein conformations of modified α-synuclein can also potentially overload the cellular proteasome, and by doing so, may increase cellular stress associated with the accumulation of misfolded proteins in affected neurons (Vila and Przedborski, 2004). Hydrogen peroxide alone or with help from downstream ROS products can also facilitate toxic events in dopaminergic neurons by either intensifying other cytotoxic factors or by elevating the generation of neurotoxic factors in microglia (Andersen, 2004).

Oxidative stress plays a critical role in the neuropathogenesis of HIV-1. As stated earlier, the process of HIV infection leads to the generation of inflammatory products, thus in turn giving rise to an excessive amount of ROS. In HAD, production of excess-free radicals accompanied by dysregulation of antioxidants (e.g., SOD, glutathione, and catalase) diminishes the protective potential to establish a pro-oxidative stress environment (Mollace *et al.*, 2001). Superoxide anions, peroxynitrite, and NO are produced in this scenario. Peroxynitrite is a potent oxidant that can nitrate tyrosine residues of structural proteins. Neurofilament, a structural protein that provides stability to neurons, is one of the target proteins for peroxynitrite (Beckman, 1996). Besides affecting CNS injury, free radicals produced during HAD including ceramide, sphingomyelin, and 4-hydroxynonenal can also adversely affect the disease course including the

permeability of the BBB (Mollace *et al.*, 2001), which may increase the tempo of HIV-associated nervous system dysfunction and lead to progressive cognitive deficits and death.

A primary role for oxidative stress in ALS is evident. The discovery of mutations in the *SOD1* gene encoding the Cu/Zn SOD in familial ALS patients was responsible for associating this disease to ROS metabolism (Deng *et al.*, 1993; Olanow, 1993; Rosen *et al.*, 1993). Mutant forms of SOD proteins have altered enzyme activity and can result in increased superoxide radicals such that the formation of peroxynitrite from superoxide and NO would be favored. A decline in the activity of SOD1 measured in patient's tissues (Deng *et al.*, 1993) suggests that higher levels of superoxide activity may form and increase the potential of developing hydroxyl radicals and eventually neuronal damage (de Belleroche *et al.*, 1996). Interestingly, knockout SOD1 mice show limited motor symptoms by 6 months of age (Shefner *et al.*, 1999). These mice do not develop an ALS phenotype, whereas mutants of the Cu/Zn SOD1 proteins are inevitably lethal. The human mutant (G93A) *SOD1* gene, introduced to a murine model, resulted in an ALS phenotype characterized by elevated levels of lipid peroxidation, protein oxidation, and DNA oxidation. This human G93A SOD1 mutation confers a toxic gain of function that destroys motorneurons (Cudkowicz *et al.*, 1997). This toxic gain of function was shown to come from the ability of familial ALS-mutant Cu/Zn SOD1 proteins to catalyze oxidation reactions of hydrogen peroxide or peroxynitrite to hydroxyl radicals (Valentine, 2002). These results support the notion that expressed mutations in the *SOD1* gene lead to ALS pathogenesis by increasing the amount of hydroxyl radicals and generating vast amounts of oxidative stress capable of inducing motorneuron degeneration (Barzilai *et al.*, 2002).

IV. Glutathione System, Glutamate–Glutamine Cycle, and the CNS

Production of ROS and NO in neurons is buffered primarily by the glutathione system. Glutathione is the major thiol present in brain tissue, and the most important redox buffer in cells. This antioxidant molecule cycles between reduced glutathione (GSH) and oxidized glutathione disulfide (GSSG), and serves as a vital sink for control of ROS levels in cells. GSH reacts with oxygen- and nitrogen-free radicals resulting in the reduction of peroxides (Dringen *et al.*, 2000). Although varying in different regions of the brain, all GSH levels diminish by about 30% in the elderly (Chen *et al.*, 1989), suggesting a possible link with the age-associated risk factor of AD and PD. Depletion of GSH may render cells more sensitive to toxic effects of oxidative stress and potentiate the toxic effects of reactive microglia (Dringen *et al.*, 2000; Sian *et al.*, 1994; Winterbourn and Metodiewa, 1994).

A common mechanism for neuronal demise may underlie most neurodegenerative diseases. The excitotoxicity metabolic pathway that modulates neuronal injury has remained a focus of neuroscience research efforts for the past three decades. In excitotoxicity, glutamate release overstimulates neuronal ionotropic glutamate receptors resulting in significant calcium influx and neuronal cell death stroke and neurodegenerative diseases. Indeed in this pathway, the major excitatory amino acid neurotransmitter is glutamate as well as aspartate, while γ-aminobutyric acid, glycine, and taurine are inhibitory. Glutamate is, without question, the principal excitatory neurotransmitter and is the key factor implicated in affecting neuronal death. Increased extracellular glutamate also leads to oxidative stress and oxidative glutamate toxicity. During steady state, neuronal glutamate acts as a neurotransmitter. Its extracellular concentration is kept to very low levels through transporters that are present in glia and primarily in astrocytes. Moreover, extracellular concentrations of glutamate are modulated through the glutamate–cystine antiporter that serves to regulate excitotoxicity and neuronal injury. Increased levels of extracellular glutamate deplete cells of cystine by blocking the gradient-driven glutamate–cystine antiporter, a dimeric transport system composed of a specific subunit, xCT, and a 4F2 heavy chain. Cystine is required for the synthesis of GSH, thus excess depletion renders cells incapable of removing ROS. Due to the involvement of increased oxidative stress in the pathogenesis of PD, it is thought that diminished levels of GSH in the substantia nigra precede neuronal degeneration. Indeed, when GSH levels are diminished, the uptake of cystine is induced leading to increased glutamate efflux and neurotoxic outcomes (Jiang *et al.*, 2001; Simantov, 1989) (Fig. 3).

Both *in vivo* and *in vitro* studies have demonstrated that activated microglia express the transporters and enzymes of the glutamate–glutamine cycle (reviewed in Gras *et al.*, 2006), suggesting that these cells may exhibit neuroprotective properties in addition to their neurotoxic properties and can partially compensate for the deleterious reactive state within the context of neurodegeneration. This is underscored by the observation that, unlike astrocytes, microglia do not constitutively express glutamate transporters, but are only expressed on activation. Therefore, the microglia constitute a local, inducible cell population that can mediate glutamate clearance and metabolism. This property may be critical to neuronal survival and disease outcome in CNS inflammation.

V. Modulators of Microglial Activation

Critical to understanding the role of oxidative stress and inflammation in neurodegeneration is an understanding of the disease process in and of itself. A common pathological feature to most neurodegenerative disorders is the

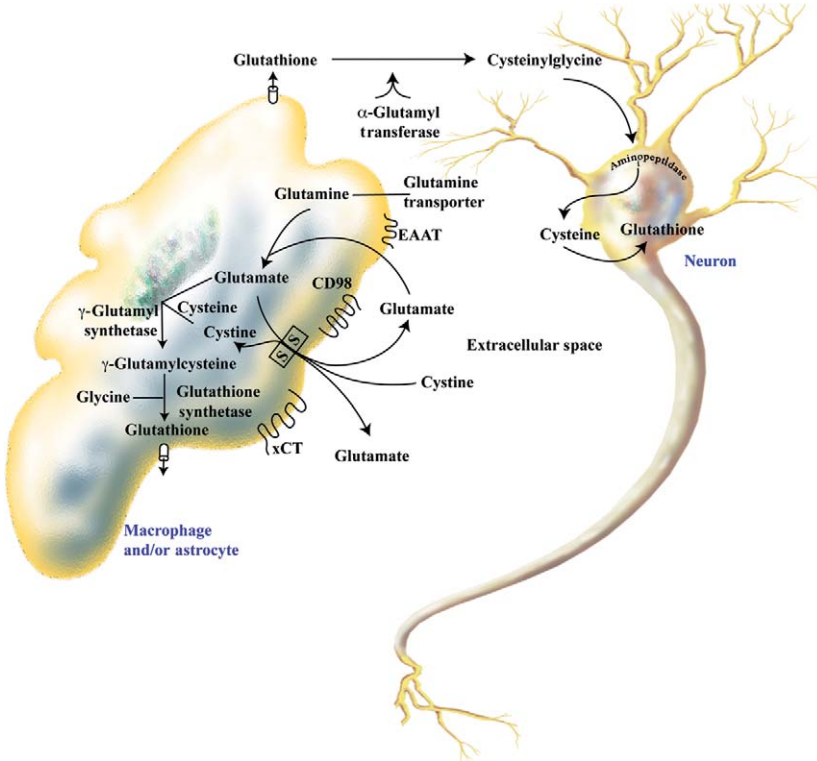


FIG. 3. Glutamate–glutamine transporters for glutamate, cysteine, and cystine in microglia (or astrocytes) and neurons. Communication links for redox pathways in the nervous system are outlined. A family of excitatory amino acid transporters (EAAT) and other transporter proteins (receptors) regulate extracellular concentrations of glutamate. When intracellular glutamate is higher than the plasma concentration, transport of glutamate across the luminal membrane occurs. The transporters on the ablumenal membrane surface of astrocytes or microglia provide a mechanism to increase intracellular glutamate concentration and removal of glutamate from cells. In microglia (or macrophages), cystine is taken up through the CD98/xCT cystine–glutamate antiporter. The cystine–glutamate antiporter then exchanges extracellular cystine for intracellular glutamate.

activated microglial cell. However, mechanisms underlying microglia activation to incite a neuroinflammatory cascade in disease are incompletely understood.

The pathogenic process in AD involves deposition of insoluble aggregates of Aβ, oxidative stress, and activation of inflammatory cytokine cascades involving microglia. Activated microglia congregate in and around amyloid plaques, where they can produce cytokines, ROS, and excitotoxins that can kill or injure neurons. Oxidative stress in AD is more attributed to the free radical generation by affected neurons more so than activated microglia. This can occur as a result of impaired

mitochondrial oxidative metabolism (Mattson *et al.*, 1997). Alternatively, a major source of free radicals in the AD brain is likely to include microglia, which have the potential to produce large amounts of ROS. Free radical generation by activated microglia has been demonstrated on direct interaction with Aβ peptides and activation of the NADPH oxidase complex to produce superoxide radicals (Bianca *et al.*, 1999; Klegeris *et al.*, 1997a,b; Van Muiswinkel *et al.*, 1999). Moreover, Aβ can potentiate the production of free radicals by phagocytic cells treated with other stimulatory agents such as IFN-γ, lipopolysaccharide (LPS), and TNF-α (Goodwin *et al.*, 1995; McDonald *et al.*, 1997; Meda *et al.*, 1996). In addition to oxygen-free radicals, large amounts of NO can be produced by activated microglia through induction of iNOS (Goodwin *et al.*, 1995). Activated microglia also release the excitotoxins glutamate (Piani *et al.*, 1992) and quinolinic acid, and on activation by amyloid plaques also evoke fulminant excitotoxicity activity (Giulian *et al.*, 1995). All of which can contribute to inflammatory mechanisms.

In PD, reactive microglia predominate within the SNpc of PD brains at autopsy (Croisier *et al.*, 2005; McGeer *et al.*, 1988; Yamada *et al.*, 1992). A significant increase in the number of reactive microglia shown phagocytosing dopaminergic neurons (McGeer *et al.*, 1988) correlated with the deposition of α-synuclein (Croisier *et al.*, 2005). In addition, microglia in the vicinity of dopaminergic neurons in disease appear to have an upregulated capacity for ROS production due to increased expression of NADPH oxidase. How microglia are activated in PD and affect disease are incompletely understood, but the release of aggregated and nitrated α-synuclein from dying or damaged dopaminergic neurons in the SN is thought to contribute, in part, to their activation (Thomas *et al.*, 2007; Zhang *et al.*, 2005). Several lines of evidence support this contention. First, evidence of α-synuclein linkage to familial PD is derived from the discovery of three missense mutations (A53T, A30P, and E46K) in the gene encoding α-synuclein (Kruger *et al.*, 1998; Polymeropoulos *et al.*, 1997; Spira *et al.*, 2001; Zarranz *et al.*, 2004) as well as duplication and triplication of the gene (Singleton *et al.*, 2003). Second, oxidation of α-synuclein leads to formation of aggregates and filaments found to be a major component of LB (Giasson *et al.*, 2000; Souza *et al.*, 2000). Third, α-synuclein itself can activate microglia, causing release of ROS and neurotoxicity (Thomas *et al.*, 2007; Zhang *et al.*, 2005). Fourth, oxidized and aggregated α-synuclein, when released from dying neurons, may stimulate scavenger receptors on microglia resulting in their sustained activation and subsequent dopaminergic neurodegeneration (Croisier *et al.*, 2005; Wersinger and Sidhu, 2006; Zhang *et al.*, 2005). Taken together, several lines of research provide corroborating evidence that implicate aggregation and oxidative modification of α-synuclein as key components that facilitate much of the neuroinflammation and degeneration in sporadic and some familial forms of PD.

Activated MPs are the primary perpetrators of neuronal injury in HIV-1-associated CNS disease. It is widely accepted that these MPs act to induce neuronal injury primarily through indirect mechanisms. These indirect mechanisms are alterations in secretory function of chemokines, cytokines, arachidonic acid derivatives, and platelet-activating factor, as well as NO, free radicals, and excitatory amino acids (Kadiu *et al.*, 2005). Direct mechanisms also bring about neurotoxicity, but probably play a lesser role. These mechanisms consist of soluble viral proteins and glycoproteins that work through neuronal receptors (D'Aversa *et al.*, 2005). MP activation during HIV-1 infection may occur by several mechanisms. Proinflammatory cytokines, such as IFN-γ and TNF-α, are potent MP activators. TNF-α allows astrocytes and microglia to amplify immune activation, resulting in coactivation of other microglia. Axonal injury also results in activation of MPs in the CNS. Chemokines are yet another method of macrophage activation. Macrophage inflammatory protein (MIP)-1β and RANTES (regulated on activation, normal T cell expressed and secreted) act through CCR5 (Cocchi *et al.*, 1995) on the cell surface, and stromal cell–derived factor (SDF)-1α through CXCR4 (Oberlin *et al.*, 1996). Fractalkine, a brain chemokine expressed by neurons, astrocytes, and endothelial cells, binds CX3CR1 to mediate macrophage recruitment and activation (Tong *et al.*, 2000). T cells also activate MPs by cytokines as well as direct contact. Activated T cells will enter the blast phase and the CNS. As they migrate through the parenchyma, they secrete the cytokines that serve as a source for microglia activation (Diesing *et al.*, 2002; Lawrence and Major, 2002). As these T cells die in the brain, the debris is removed by the brain macrophages, also contributing to macrophage activation. Viral proteins such as gp120 (Brenneman *et al.*, 1988), gp41 (Adamson *et al.*, 1996), and the nonstructural proteins Tat (New *et al.*, 1997; Price *et al.*, 2005), Nef, Vpr, and Rev secreted by infected MP can (Price *et al.*, 2006) directly disrupt glial and neuronal function through alteration of calcium homeostasis (Lannuzel *et al.*, 1995), induction of ROS and RNS (Mollace *et al.*, 1993), induction of apoptosis, or enhanced secretion of proinflammatory cytokines such as TNF-α and IL-1β as well as arachidonic acid metabolites that are implicated in HIV-1 neuropathogenesis (Jana and Pahan, 2004; Nath, 2002; Song *et al.*, 2003).

The interplay between motorneurons and glia cells is important in the pathological progression of motorneuron diseases, and release of ROS and RNS or cytokines form microglia could contribute to the demise of motorneurons as seen in ALS (reviewed in Agar and Durham, 2003). The deposition of specific proteins into intracellular hyaline inclusions in motorneurons and astrocytes is a characteristic neuropathological finding in familial ALS as well as in sporadic ALS (Kato *et al.*, 2000; Shibata *et al.*, 2000). These inclusions are strongly immunopositive for both mutant and wild-type SOD1 in patients and transgenic mice expressing mutated forms of human SOD1. Interestingly, mutant SOD1 is more susceptible to oxidation-induced aggregation *in vitro* than the wild-type

enzyme (Rakhit *et al.*, 2002). Furthermore, studies on mice-expressing mutated human SOD1 revealed that elevated markers for activated microglia are present at times of neuronal loss suggesting that activated microglia may contribute to the oxidative insult. In addition to exogenous activators of microglia, microglia in ALS may have intrinsic cytotoxic potential as a result of a defect in SOD1 function that becomes apparent following activation (Weydt *et al.*, 2004). Therefore, in this context, mutant SOD1 renders microglia more susceptible to other toxic agents that generate ROS (Liu *et al.*, 1998).

VI. Growth Factors, Antioxidants, and Anti-Inflammatory Drug Therapies

Many diverse mechanisms, factors, and pathways are involved in neurodegenerative disorders; thus, several different therapeutic methods have been developed to target a specific factor or a whole intricate pathway with the intent of ameliorating, preventing, or reversing neuronal cell damage (Tabner *et al.*, 2001). Inflammation and oxidative stress are both time and site specific, thus in order to treat the effects of these components on neurodegenerative disorders, knowing the temporal and spatial activities involved in those processes is critical for development of efficacious therapeutics. Because inflammation and oxidative stress are linked, attenuation of inflammatory responses could slow the neurodegenerative process. Therapeutic paths could include neurotrophic factor enhancement (brain-derived neurotrophic factor, glia cell line-derived neurotrophic factor, and nerve growth factor), upregulation of anti-inflammatory cytokines (IL-4, IL-10, and TGF-β), inhibition of enzymatic activities that encourage neurotoxicity (GSK-3β, γ-secretase), Ca^{2+} and glutamate excitotoxicity blockers that inhibit NMDA receptor function, suppression of neuronal cytotoxicity (memantine, lithium, sodium valproate), attenuation of inflammation by NSAIDs, minocycline, dextromethorphan, and sequestering misfolded proteins with antibodies (Fig. 4A). Due in part to late diagnosis of the disease, treatment is initiated well after neurodegeneration has started and its detrimental effects become symptomatic. The failure in late- or end-stage clinical trials of promising therapeutic modalities emphasizes the need for presymptomatic treatment.

Growing evidence suggests that, in disease, microglia may contribute to redox stress by producing ROS during phagocytosis of debris from dying and degenerating neurons (Green *et al.*, 2001; Qin *et al.*, 2004; Zhang *et al.*, 2005). As compensatory ionic fluxes are required to sustain ROS generation, characterization of the relative roles of plasma membrane ion currents in the generation of ROS in response to pathological stimuli is important, with the long-term goal of abrogating neuronal damage by modulating these fluxes during disease-associated microglial activation. Depending on the neuroinflammatory context,

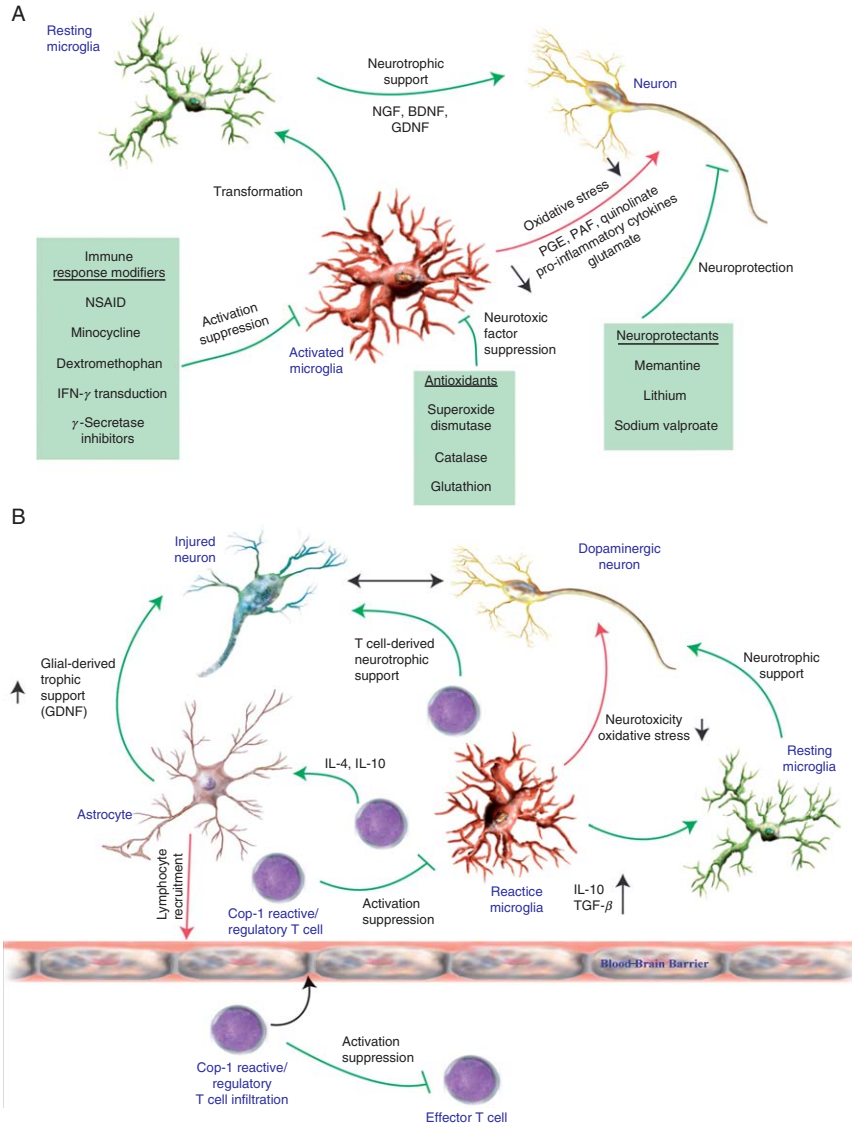


FIG. 4. Putative therapeutic anti-inflammatory and antioxidant interventions for neurodegenerative diseases. (A) Induced neuronal inflammation can be attenuated by reduction or alterations in neurodegenerative environmental cues (Aβ, α-synuclein, HIV, SOD-1) and from deactivation of activated microglia via anti-inflammatory agents and reduction in neurotoxic ROS activities through endogenous enzymes and exogenous vitamins responsible for stimulating antioxidants. (B) Immunomodulation using T cell–mediated therapies, such as immunization with Cop-1, has proven efficacious in a variety of neurological disorders. Whether Cop-1-mediated effects are through modulation of Th1/Th2 and regulatory T cell responses, or innate immunity to confer neuroprotection, the underlying etiology of disease may dictate the cellular response.

different ionic conductances may play divergent roles that depend on unique signaling pathways. Indeed, while direct activation of microglial NADPH oxidase is mediated primarily through chloride currents, ROS production following microglial activation with aggregated α-synuclein was predominately mediated through voltage-activated K^+ currents or proton currents (Thomas *et al.*, 2007). Therefore, identification and blockade of specific ion channels may attenuate redox-related stress and may slow disease progression in a variety of neuroinflammatory conditions.

VII. Therapeutic Immunomodulation

T cell-mediated immune responses are another potential therapeutic avenue for neurodegeneration. While naive T cells are precluded from CNS entry, neuroinflammation aggressively recruits activated components of the adaptive immune system to sites of active neurodegeneration. Investigations provide evidence that a well-controlled response of activated T cells is neuroprotective (Angelov *et al.*, 2003; Benner *et al.*, 2004; Butovsky *et al.*, 2006b; Kipnis and Schwartz, 2002; Kipnis *et al.*, 2000). A generalized efficacy for immunization with Copolymer-1 (Cop-1, glatiramer acetate) in divergent models of human neurological disorders including multiple sclerosis, spinal cord injury, glaucoma, PD, AD, HAD, and ALS substantiates this observation (Arnon and Sela, 2003; Benner *et al.*, 2004; Butovsky *et al.*, 2006a; Haenggeli *et al.*, 2006; Kipnis *et al.*, 2000; Laurie *et al.*, 2007). To date, the exact mechanism of action of Cop-1 is unknown. Possible mechanisms include preferential induction of a Th2 response, competition between Cop-1 and myelin basic protein for binding sites on MHC class II molecules, or bystander suppression by a yet undefined mechanism. Cop-1 is a potent inducer of Th2 regulatory cells, which secrete IL-4, IL-5, IL-10, and TGF-β through induction of a cytokine shift (Aharoni *et al.*, 2000). Cop-1-induced Th2 adaptive immune responses can affect microglial responses and lead to neuroprotection in models of metabolic and traumatic disorders. T cells reactive to Cop-1 could also be a source of brain-derived neurotrophic factor and other neurotrophic factors (Arnon and Sela, 2003) or can induce production of neurotrophins by microglia or astrocytes (Benner *et al.*, 2004). Investigations in our laboratory and others have extended these observations in deciphering mechanisms of Cop-1-mediated neuroprotection in animal models of PD, AD, HAD, and ALS (Fig. 4B) (Benner *et al.*, 2004; Butovsky *et al.*, 2006a; Haenggeli *et al.*, 2006; Laurie *et al.*, 2007). Parallel investigations suggest that Cop-1 may induce the conversion of CD4+CD25− effector T cells to CD4+ CD25+ regulatory T cells (Hong *et al.*, 2005). Indeed, an increased regulatory T cell

population in Cop-1-immunized animals demonstrates a possible role for regulatory T cells in neuroprotection.

Recent data generated in our laboratories demonstrated that regulatory T cells can suppress microglial-mediated ROS production to near prestimulatory levels in response to inflammatory activities including proinflammatory cytokines (e.g., TNF-α and LPS) (Fig. 5). These observations also support the notion that therapeutic strategies that induce regulatory T cell responses can be used to attenuate inflammation and promote neuronal survival for neurodegenerative disorders. A therapeutic vaccine approach using Cop-1 in conjunction with specific adjuvants that elicit specific regulatory T cell responses represents a potential interdictory modality for slowing the progression of neuroinflammation

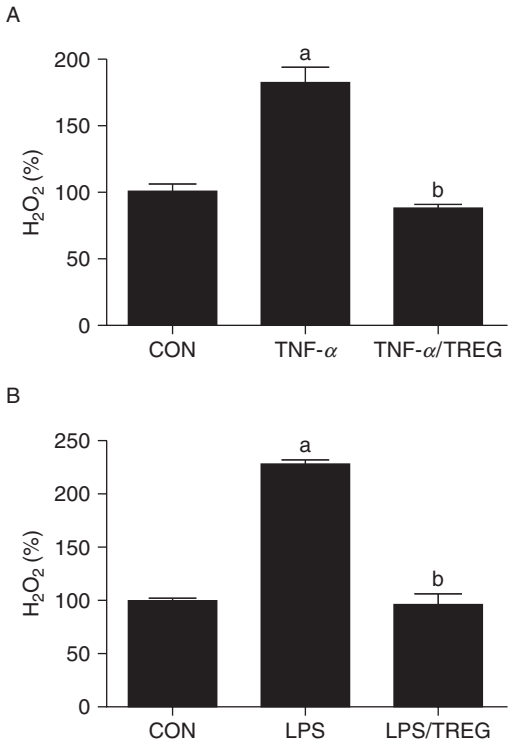


FIG. 5. CD4+CD25+ regulatory T cells attenuate microglial ROS production. ROS production by microglia in response to inflammatory stimuli was measured with Amplex Red (Molecular Probes, Carlsbad, CA). The total levels of hydrogen peroxide (H_2O_2) were determined based on the conversion of superoxide to H_2O_2 by horseradish peroxidase. Microglia produce copious amounts of ROS in response to inflammatory stimuli such as TNF-α (A) and LPS (B) ($p < 0.01$). Cocultivation of microglia with regulatory T cells with inflammatory stimuli reduced the ROS response.

and secondary neurodegeneration. This may be considered in conjunction with other anti-inflammatory or antioxidant therapies as part of a broad based therapeutic approach.

VIII. Summary

Thus, neurodegenerative disorders are strongly associated with inflammation and oxidative stress. Ironically, although essential for life, ubiquitous oxygen also is, by its products of metabolism, toxic to cells. Consequently, a strong association exists between production of free radicals, aging, and neurodegenerative diseases. Oxidative stress has been correlated with the development of cellular injuries leading to neuropathology in these various disease states. However, whether oxidative stress is the primary force driving neurodegenerative disorders is still unclear. A complete comprehension of the cellular and molecular mechanisms, and of the specificities of oxidative damage in these neurological disorders, may lead to the development of therapeutic strategies to prevent or slow the progression of disease.

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