Review Article

The Pathobiology of Amyotrophic Lateral Sclerosis: A Proteinopathy?

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Abstract

Amyotrophic lateral sclerosis (ALS) is increasingly considered to be a disorder of multiple etiologies that have in common progressive degeneration of both upper and lower motor neurons, ultimately giving rise to a relentless loss of muscle function. This progressive degeneration is associated with heightened levels of oxidative injury, excitotoxicity, and mitochondrial dysfunction—all occurring concurrently. In this article, we review the evidence that suggests, in common with other age-dependent neurodegenerative disorders, that ALS can be considered a disorder of protein aggregation. Morphologically, this is evident as Bunina bodies, ubiquitin-immunoreactive fibrils or aggregates, neurofilamentous aggregates, mutant copper/zinc superoxide dismutase (SOD1) aggregates in familial ALS variants harboring mutations in SOD1, peripherin-immunoreactive aggregates within spinal motor neurons and as neuroaxonal spheroids, and in an increasingly greater population of patients with ALS with cognitive impairment, both intra- and extraneuronal tau aggregates. We review the evidence that somatotopically specific patterns of altered kinase and phosphatase activity are associated with alterations in the phosphorylation state of these proteins, altering either solubility or assembly characteristics. The role of nonneuronal cells in mediating motor neuronal injury is discussed in the context of alterations in tyrosine kinase activity and enhanced protein phosphorylation.

Key Words: Cdk5, Neurofilament, Protein aggregation, Regulation/deregulation, SOD1, Topographic phosphorylation.

INTRODUCTION

Despite significant advances in our understanding of the biology of motor neuron degeneration, amyotrophic lateral sclerosis (ALS) remains one of the most catastrophic diseases of our aging population. There are as yet no pharmacotherapies that will significantly alter the disease course, and the natural history remains one of relentless progression of muscle atrophy and weakness culminating in death for the majority within 5 years of symptom onset. The societal impact of ALS is further compounded by the shift in the demographics that will occur as the “baby boomers” of the 20th century become the aged population of the 21st century (1).

At the core of ALS neuropathology is a selective degeneration of both the upper and lower motor neurons with degeneration of the cortical motor neurons (Betz cells) and Wallerian degeneration of the descending corticospinal tracts. Degeneration of the lower motor neurons, including brainstem and spinal motor neurons, ultimately gives rise to muscle atrophy. Among the key neuropathologic features of ALS are an extensive array of protein aggregates, including Bunina bodies, ubiquitinated inclusions, and neurofilament-rich “hyaline conglomerate inclusions” (2), and increasingly, protein aggregation in nonneuronal cells. In this review, we discuss the pathobiology of these inclusions and their potential roles in the induction of motor neuron degeneration in ALS.

NEUROPATHOLOGIC FEATURES OF AMYOTROPHIC LATERAL SCLEROSIS

Core Neuropathologic Features

ALS is first and foremost a disease of the upper and lower motor neurons. Accompanying this degeneration is a prominent disruption of the neuronal cytoskeleton. A number of intraneuronal aggregates have been associated with ALS, including Bunina bodies, ubiquitinated inclusions, and neurofilament-rich “hyaline conglomerate inclusions” (Fig. 1A, B). Of these, Bunina bodies appear to be unique. These inclusions are dense, refractile eosinophilic inclusions of lysosomal origin that are immunoreactive for the lysosomal cysteine protease inhibitor cystatin C (3). Although the substrate associated with the abnormal aggregation of ubiquitin in ALS is not known, both skeins and aggregates of ubiquitin-immunoreactive material are commonly observed in degenerating neurons in ALS (Fig. 1F, G). Many are, however, also immunoreactive to a range of neuronal-intermediate filaments (for example, neurofilament [NF], peripherin, α-internexin), suggesting that an abnormal degradative processing of these proteins is associated with ALS (4–6). The observation of serpin–serine protease complexes within NF aggregates further suggests an inhibition of the degradation of oxidatively modified NF (7).
FIGURE 1. Cytoskeletal protein aggregation in spinal motor neurons in amyotrophic lateral sclerosis. Using silver staining (Bielschowsky staining), a number of coexistent pathologies can be observed (A, B). (A) Intensely argentophilic, well-circumscribed neuroaxonal spheroids (white arrow) are observed along with less intensely staining, more amorphous aggregates (red arrow). Magnification: 20× before reproduction. Within motor neurons, areas of cytoplasmic clearing corresponding to hyalinized intracellular conglomerates, are present (yellow arrow), whereas adjacent motor neurons appear normal. Intensely argentophilic, punctate aggregates corresponding to Bunina bodies observed on routine stains are also readily apparent (B, arrow, 40× magnification). With immunostaining using a monoclonal antibody recognizing highly phosphorylated NF-H ([C], 40× magnification), neuroaxonal spheroids are intensely staining (black arrow), whereas perikaryal neurofilament aggregates are more amorphous and heterogeneous (red arrow). Peripherin immunoreactivity colocalizes to dense perikaryal aggregates ([D], arrow) and prominently to neuroaxonal spheroids (E). Ubiquitin-immunoreactive threads and aggregates (F, G) are invariably observed as early markers of aberrant protein aggregate in otherwise healthy appearing motor neurons. In cognitively impaired patients with amyotrophic lateral sclerosis, argentophilic intraneuronal aggregates are evident in cortical frontal neurons (H), as are argentophilic astrocytic aggregates (J) (Gallyas Brack silver stain, 40× magnification and 20× magnification, respectively). The latter are most readily observed at the interface of cortical layers II and III. (I, K) Both neuronal and astrocytic frontal cortical aggregates are immunoreactive to monoclonal antibodies recognizing tau protein (40× before magnification) and tau-immunoreactive neuropil threads are evident.
These aggregates are also immunoreactive with antibodies recognizing SOD1, dorfin, and nitric oxide (8, 9). The presence of galectin-1 within NF aggregates suggests that this protein, involved in axonal regeneration, cell growth, and differentiation, has also been mislocalized within the neuron or entrapped in the process giving rise to the NF aggregate (10). As discussed, a key research issue remains the extent to which these varied proteins are “innocent bystanders” entrapped with proteinaceous aggregates or primary participants in aggregate formation.

**Frontotemporal Lobar Degeneration in Amyotrophic Lateral Sclerosis**

Although the occurrence of an overt dementia is uncommon in ALS, more subtle deficits in frontal and temporal lobe function are observed in as many as 50% of patients with ALS (11). ALS with cognitive impairment is a subtle disorder marked by a frontal dysexecutive syndrome for which impairments in verbal praxis and fluency are hallmarks. There is overlap with a frontal behavioral syndrome in which emotional lability is prominent. At the core of these syndromes is a frontotemporal lobar degeneration (FTLD) sharing many of the neuropathologic features of more commonly recognized FTLDs. This includes superficial linear spongiosis, astrocytic proliferation (predominantly cortical layers II and III), and a microglial proliferative response (12). Both intra- and extraneuronal tau-immunoreactive aggregates are observed (Fig. 1H–K). These include neuropil thread-like structures, argyrophilic granules, or dense, rounded aggregates with irregular fibrillary margins (13). Nonneuronal tau aggregation can be observed in glial cells (as coiled bodies) within the hippocampus, parahippocampal gyrus, and amygdala (14). The appearance of astrocytic tau aggregates within the frontal neocortex is unique to ALS with cognitive impairment and is disproportionate to that observed with normal aging (15). Of note, similar neuropathologic features can be observed in patients with ALS in whom no evidence of deficits in cognition was observed antemortem, suggesting that there may exist a spectrum in which ALS with motor system degeneration alone overlaps, or is a continuum with, an FTLD. We have recently shown that the deposition of tau observed histopathologically is associated with an alteration in tau phosphorylation and solubility.

Finding an alteration in tau metabolism in sporadic ALS, although unexpected, is not without precedent. Tau aggregation is a hallmark of the Western Pacific variant of ALS in which ALS, parkinsonism, and dementia coassociate. In this variant, tau-immunoreactive neuronal inclusions with the morphology of neurofibrillary tangles (NFTs) are found throughout frontal cortical layers II and III (16). Similar to ALS with cognitive impairment, tau isolated from this variant of ALS is also highly insoluble and hyperphosphorylated (17). Additional evidence of a potential disturbance in tau metabolism in ALS is garnered from the finding of mutations in the tau gene on chromosome 17 (FTDP-17) in association with a syndrome characterized by behavioral changes, psychosis, loss of executive functioning, and a progressive loss of speech output (18). Corticospinal tract disturbances, muscle wasting, and fasciculations typical of ALS have been found in a small proportion of these families.

Although the exact prevalence of tau aggregation in sporadic ALS remains to be determined, as does its relationship to frontotemporal dementia, there is sufficient evidence to now consider that alterations in tau metabolism coexist with motor neuron degeneration in a subgroup of patients with ALS.

**Nonneuronal Cell Involvement**

Both microglial and astrocytic proliferation is observed in ALS, regardless of the variant (Fig. 2). A number of neurochemical markers of microglial activation have been observed in ALS, including an increased expression of the receptor for macrophage-colony stimulating factor in the precentral gyrus, and both TNF-α and the soluble extracellular domains of its receptors TNFRI and TNFRII in ALS serum (19, 20). An increased expression of proinflammatory cytokines, COX-2, and of microglia-mediated protein oxidative pathology is also observed in ALS (21–23).

It is less clear whether the microglia are activated as a reflection of a primary microglial problem in ALS and thus initiate the disease process or whether microglial activation is a consequence of motor neurons degenerating and then, once activated, contributing directly to the propagation of neuronal degeneration. Experimentally, there is considerable evidence that microglial activation is temporally linked to neuronal degeneration in a number of murine models of motor neuron disease. In the wobbler mouse (wr/wr), motor neuron dysfunction is preceded by the ensheathment of otherwise healthy appearing motor neurons by microglia (24). In the mnd mouse in which the intraneuronal accumulation of a lipofuscin-like material results in a late-onset motor neuron disease, brain and spinal cord TNF-α levels are elevated coincident with the onset of motor neuron dysfunction (25). In presymptomatic mnd mice, a dramatic upregulation of the TNF receptor TNFRI is preceded by an increase in the expression of CD11b-immunoreactive microglia in the spinal cord ventral horns (26). The development of motor neuron dysfunction in SOD1G93A transgenic mice is also preceded by an early alteration in the expression and upregulation of proinflammatory factors in the presymptomatic phase. A prominent and sustained microglial response is observed throughout the active phase of the disease progression (27, 28). Specifically, both TGF-β1 and macrophage-colony stimulating factor expression are upregulated in presymptomatic mice, with TNF-α expression being increased by month 4, well in advance of the appearance of motor deficits (29). By month 6, a significant increase in microglia numbers is observed. This process is associated with an increased level of COX-2 mRNA and protein, and an increase in PGE2 content limited to the regions associated with motor neuron pathology, further confirming a role for microglial activation (23). Support for the role of microglia in motor neuron degeneration is also gained from the observation that the administration of minocycline, a tetracycline derivative, prolongs the symptom-free interval before the onset of motor dysfunction in SOD1G93A transgenic mice and reduces the...
extent of motor neuron microvacuolar degeneration at 120
days (30, 31).

Although these studies suggest that microglial activation
may precede the development of motor neuron degeneration,
the most critical experimental observation is that nonneuronal
cells that do not express mutant SOD1 significantly impact on
the survival of mutant SOD1 expressing motor neurons in a
chimeric model of ALS (32). This suggests that microglia play
a crucial role in modulating the disease process rather than
actually triggering it in this specific model of familial ALS. We
have been examining this relationship using transgenic mice
(NFL−/− or hNFL+/+) that develop NF aggregates. In these
models, NF aggregate formation precedes morphologic evi-
dence of microglial proliferation, but once present, microglial
proliferation is associated with a loss of NF aggregate-bearing
neurons (32a).

Astrocytic proliferation, long held to be secondary to the
neuronal loss, is of increasing importance given the specific
loss of the glial glutamate transporter GLT-1 (EAAT2) in
sporadic ALS and a hypothesized potentiation of glutamate-
mediated neurotoxicity that may be critical to the pathogenesis
of ALS (33).

**GENETICS OF AMYOTROPHIC LATERAL SCLEROSIS**

Although it is uncommon for ALS to be inherited, when
present (approximately 5% of patients), inheritance in the
majority is in an autosomal-dominant fashion (Table 1). Both
recessive and X-linked variants also occur. The most common
gene defect in familial ALS is a mutation in the copper/zinc
superoxide dismutase gene (SOD1) at 21q22 (ALS1) (34).
Among these families, significant intra- and interfamilial vari-
ability exists in the age at symptom onset, rate of progression,
and degree of clinical deficit (35). Although most SOD1 muta-
tions are autosomal-dominant, an autosomal-recessive variant
(SOD1D90A) with incomplete penetrance is observed (36).

Additional inherited variants of ALS include an
autosomal-dominant variant with onset at a young age and
with slow progression linked to mutations in the senataxin
gene on 9q34 (ALS4) (37), a frontotemporal dementia with parkinsonism and amyotrophy linked to the microtubule-associated tau protein at 17q21-22 (38, 39), and a recessively inherited juvenile-onset variant that has been linked to mutations in ALSin at 2q33. This region encodes for a 184 kDa protein that may function as a putative guanine exchange factor and play a critical role in signaling cascades, membrane transport, and organization of the cytoskeleton (40, 41).

A number of susceptibility genes have been identified for sporadic ALS, including codon deletions or insertions in

### TABLE 1. Genetics of Amyotrophic Lateral Sclerosis

| Inheritance Pattern* | Linkage | Gene Defect | Unique Features | Age at Onset | Clinical Features |
|-----------------------|---------|-------------|-----------------|--------------|-------------------|
| ALS 1 [105400]†       | AD      | 21q22.1     | Copper/zinc superoxide dismutase (SOD1) [147450] | Adult-predominant | Classic ALS phenotype |
| ALS 2 [205100]        | AR      | 2q33-q35    | ALSin [606352]  | Juvenile     | First or second decade of life; spastic pseudobulbar syndrome with spastic paraplegia; distal amyotrophy; slow progression; first identified among Tunisian kindred with spasticity of limb and facial muscles; mutations also observed in AR-HSP and AR-PLS |
| ALS 3 [606640]        | AD      | 18q21       | Unknown         | Adult        | Classic ALS phenotype; non-SOD1-linked |
| ALS 4 [602433]        | AD      | 9q34        | Senataxin [608465] | Juvenile     | Onset typically in second decade, slow progression with normal lifespan; distal weakness and amyotrophy; severe loss of motor neurons in brainstem and spinal cord |
| ALS 5 [602099]        | AR      | 15q15.1-q21.1 | Unknown      | Juvenile     | Absence of pseudobulbar features; distal amyotrophy; minor spasticity; long-term survival |
| ALS 6 [608030]        | AD      | 16q12.1-q12.2 | Unknown       | Adult        | Majority with short duration; rarely FTD |
| ALS 7 [608031]        | AD      | 20ptel      | Unknown        | Adult        | Linkage in only 2 family members; similar phenotype to chromosome 16 linked |
| ALS 8 [608627]        | AD      | 20q13.33    | Unknown        | Adult        | Slow progression, Brazilian family |
| FTD-MND [105550]      | AD      | 9q21-q22    | Unknown        | Adult        | ALS with dementia with features of frontotemporal degeneration (socially inappropriate, impulsive behavior, deterioration in activities of daily living) |
| FTDP-17 [157140]      | AD      | 17q21       | Microtubular associated protein tau | Adult        | Disinhibition–dementia–parkinsonism–amyotrophy syndrome |
| ALS-FTD “San Francisco family A” | AD | 17 | Not linked to tau | Adult | Prominent tau positive and α-synuclein-positive inclusions |
| ALS with bulbar onset | Unknown          | Unknown     | Juvenile     | Adult | Japanese family; prominent early-onset bulbar dysfunction; slow progression; dementia |
| Miscellaneous MND syndromes |  |  |  |  |  |
| Brown-Vialetto-van Laere syndrome [211530]‡ | AD (?AR variants) | Unknown | Unknown | Juvenile | Progressive bulbar paralysis; childhood onset; progressive deafness; pyramidal signs are infrequent |
| Kennedy disease [313200] | X-linked | Xq11-12 | Androgen receptor gene mutation (trinucleotide [CAG] repeat) | Adult-predominant | Progressive muscle atrophy with proximal greater than distal involvement; bulbar involvement; absence of upper motor neuron features; slowly progressive; gynecomastia, testicular atrophy, oligospermia, and erectile dysfunction |

* Inheritance patterns: AD, autosomal-dominant; AR, autosomal-recessive.
†, [Online Mendelian Inheritance in Man reference number] (http://www.ncbi.nlm.gov/omim/).
‡, Also known as pontobulbar palsy with deafness.
§, Although the failure to observe signs of upper motor neuron dysfunction distinguishes Kennedy syndrome from typical ALS, ALS rarely presents with only bulbar and spinal lower motor neuron dysfunction; prominent perioral fasciculations favors a diagnosis of Kennedy disease.

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the lysine-serine/proline (KSP) repeat domain of NF-H, mitochondrial DNA microdeletions encoding for cytochrome c oxidase, RNA processing errors in the glutamate transporter EAAT2, an abnormal copy number of the survival motor neuron gene (SMN), and gene deletions of the chromosome 5q13-linked neuronal apoptosis inhibitory protein (NAIP) gene. Neurotoxic splice variants of peripherin (per61) have been identified in sporadic ALS and may confer a heightened risk of apoptotic motor neuron death (42, 43). There is an increased frequency of the cytochrome P450 debrisoquine hydroxylase CYP2D6(B) allele associated with a “poor drug metabolizer” phenotype. The presence of the ε3/ε4 genotype is associated with bulbar symptom onset, whereas the ε2/ε3 genotype is associated with a limb-onset variant. Expression of the ε4 allele is also associated with an earlier onset.

**NEUROCHEMISTRY**

Although this review largely focuses on the role of abnormal protein aggregation in the pathogenesis of ALS, it is clear that the final biologic expression of ALS is the cumulative result of the interaction of a number of deranged biochemical processes, none of which alone can be considered pathonamic of ALS. These include features of excitotoxicity, deranged mitochondrial function and oxidative injury, and neuroinflammation. Each of these will impact on the biology of the motor neuron and, in turn, the metabolism of cytoskeletal proteins.

**Excitotoxicity**

There is considerable evidence for deranged glutamate metabolism in ALS (44, 45). Increased glutamate-mediated calcium influx is thought to be a key aspect of this process, augmented by a reduced astrocytic glutamate transporter (EAAT-2). Although initial estimates suggested that approximately 80% of patients with sporadic ALS have a deficiency in EAAT-2 resulting from RNA missplicing (33, 46), the specificity of alterations in EAAT-2 RNA processing to ALS has been challenged (47). Nonetheless, when applied to organotypic motor neuron cultures, glutamate induces a selective loss of motor neurons through a non-NMDA-mediated pathway. In addition, spinal motor neurons in ALS appear to be at a heightened risk for direct glutamatergic neurotoxicity as a result of a relative deficiency of calcium-binding proteins. Of note, in vivo activation of the AMPA/kainate receptor decreases the expression of NF mRNA and alters NF phosphorylation—a process that may be relevant to NF aggregate formation (48, 49).

**Oxidative Injury**

SOD1-Mediated

Of the inherited variants of ALS, approximately 15% are linked to one of over 100 different mutations in the antioxidant enzyme copper/zinc superoxide dismutase (SOD1) (for an updated listing of mutations, see http://www.alsod.org). SOD1, a highly conserved 32 kDa homodimer with one copper- and one zinc-binding site, exists in its highest concentration within motor neurons (50). There is no evidence that either the loss of SOD1 activity or increased activity explains the toxicity of mutant SOD1. Currently, the neurotoxicity of mutant SOD1 is believed to be conferred by a toxic gain of function directly resulting from the mutation with 2 scenarios being proposed. In the first, mutant SOD1 is associated with oxidative injury, whereas in the second, either intracellular misfolding or aggregation leads to toxicity.

The oxidative hypothesis holds that the toxic gain of function is mediated through the enhanced generation of peroxynitrite in association with increased rates of lipid peroxidation, sulfhydryl oxidation, and the generation of hydroxyl radicals. By interacting with the copper of the active site of the SOD1 enzyme, peroxynitrite is catalyzed to a reactive nitrating species, leading in turn to increased rates of nitrotyrosine formation. Although concentrations of free 3-nitrotyrosine (a specific marker of reactive nitrating species formation) and its metabolite, 3-nitro-4-hydroxyphenol acetic acid as measured by HPLC chromatography, are elevated in patients with ALS compared with control subjects (51), the extent of protein nitration, the predicted end result of this process, is not significantly different between ALS and age-matched control subjects (52). Modifications in SOD1 activity through alterations in the copper/zinc-binding domain similarly do not account for the neurotoxicity of mutant SOD1. Zinc-depleted SOD1, as a direct effect of the failure of zinc binding to the mutant SOD1 copper/zinc-binding domain or by a competitive inhibition of zinc access to the SOD1-binding domain, could potentially result in altered SOD1 activity either through the reduced ability to catalyze the dismutase of superoxide or by catalyzing the reverse reaction of oxygen to superoxide and then combining with NO to generate peroxynitrite (53). Against this is the failure of nNOS-deficient mice to alter SOD1G93A mouse survival (54). The toxicity of mutant SOD1 is also equivalent in the presence or absence of CCS, a specific copper chaperone (55).

Mutant SOD1 does, however, have a significant potential to form aggregates both in vitro and in vivo (56, 57). Ubiquitin-immunoreactive intraneuronal and astrocytic mutant SOD1 protein aggregates are observed in both transgenic mice expressing mutant SOD1 protein and in familial patients with ALS harboring a SOD1 mutation. Mutant SOD1 aggregates contain dorfin, a RING finger-type E3 ubiquitin ligase, important for targeting for proteosomal degradation. SOD1 aggregates have been proposed to induce neurotoxicity either by interfering with normal proteosomal function or by altering chaperone interactions (58, 59). Regarding the latter, mutant SOD1 and not wild-type forms detergent-insoluble aggregates that associate with Hsp70 and Hsp 40. The critical role of Hsp 70 in preventing protein folding and aggregation has been demonstrated by the observation that increased Hsp 70 expression in cultured primary motor neurons harboring mutant SOD1 reduces the extent of SOD1 aggregate formation and prolongs survival (60). The failure to express HSF1 in motor neurons, required for upregulation of Hsp 70 expression, may place motor neurons at a heightened risk for SOD1 aggregate formation (61). The trigger for aggregate formation is as yet undefined, although an enhanced susceptibility of zinc-deficient mutant SOD1 to oxidation-induced aggregation has been demonstrated as has a propensity for mutant SOD1 to form...
monomeric SOD1 and thus a greater rate of fibril formation (62, 63).

A novel mechanism by which mutant SOD1 may lead to aggregate formation relates to the recent description of mutant SOD1 as an NFL mRNA-binding protein capable of destabilizing NFL mRNA. Both NFL and NFM steady-state mRNA levels were suppressed when a motor neuron hybridoma cell was transfected with the SOD1G93A mutant (64). When the same cell line was cotransfected with wrSOD1, SOD1G93A, or SOD1G41S and the human NFL, only those cells transfected with either mutant SOD1G93A or SOD1G41S demonstrated significant reductions in human NFL mRNA levels (65). Mutant SOD1 proteins, but not wrSOD1, bound directly to the human NFL mRNA in binding elements located within the 3' UTR, destabilizing the mRNA. In doing so, mutant SOD1 potentially alters NF stoichiometry, leading to aggregate formation.

A direct interaction between mutant SOD1 and mitochondria has also been recently observed (66). The close association of mutant SOD1 to the inner mitochondrial membrane suggests a preferential import of mutant, but not wild-type, SOD1 into the mitochondria. This suggests a potential pathway for the induction of selective mitochondrial damage in mutant SOD1 harboring familial ALS, where mitochondria-associated mutant SOD1 has also been shown to sequester the antiapoptotic Bcl-2 protein (67). As discussed subsequently, both neuronal mitochondrial dysfunction and apoptosis are critical biochemical events in the pathogenesis of ALS.

### Reactive Oxygenating Species Toxicity

In animal models (principally of the familial ALS type 1) and in sporadic ALS, there is considerable evidence of oxidative injury. This includes the finding of increased protein carbonyl content, lipid peroxidation, 4-hydroxyxenonanal protein conjugates, CML-protein adducts, oxidative DNA damage, and the observation of ROS activity and nitrotyrosine formation within both neurofilamentous and hyaline aggregates. Although the major intracellular source of reactive oxygenating species (ROS) is mitochondrial, with “leakage” of free oxygen radicals generated by the electron transport chain, other neuronal sources of ROS include free transition metals catalyzing the formation of hydroxyl radicals, xanthine and monoamine oxidase production of superoxide, and ROS liberated by lipid metabolism and proteolysis. Extraneuronal sources of ROS are also critical to this process and include microglia as a major contributor to both superoxide and nitric oxide. The generation of oxidative injury can thus be seen as a combination of factors resulting from both an increased generation of ROS from a number of differing sources as well as a loss or reduction in endogenous antioxidant activity. Oxidative injury can lead to a wide range of intracellular effects, including damage to nucleic acids, protein modifications, and lipid modification. Of particular interest in ALS has been the observation of oxidative protein modifications. Although a virtual plethora of modifications are possible, those of keen interest include protein carboxylation (for example, glycation, glycoxidation, and lipid peroxidation) and nitration. Once formed, glycation products can be further modified to advanced glycation end products. The net effect of this process can vary but in general includes either a failure of the oxidatively modified proteins to function normally or that the proteins participate in the further generation of ROS, thereby amplifying the process.

Motor neurons appear to be at an increased risk for oxidative injury as a result of a combination of factors, including, but not limited to, a high metabolic activity associated with neurotransmission, a high availability of unsaturated lipids in the neuronal membrane, high levels of glutamatergic excitatory input (discussed previously) accompanied with an increase in intracellular calcium, deficiencies in cytosolic calcium-binding proteins (for example, calbindin and parvalbumin), and low levels of reduced glutathione (68, 69), a free radical scavenger.

### Mitochondrial Dysfunction

Although traditionally, mitochondria have not been considered to be a primary pathogenic target in ALS, mutant SOD1 binds to, and aggregates with, Bcl-2 in spinal cord mitochondria, suggesting a potential role in familial ALS (66, 67). In sporadic ALS, abnormal mitochondrial morphology is observed in motor nerve terminals, liver, and muscle. Metabolic studies have found significant reductions in cytochrome oxidase activity, increases in either complex I activity alone, or both complex I and II. Increased protein carbonyl formation in both the motor cortex and in the spinal cord has also been found.

The implications of mitochondrial dysfunction in ALS relate specifically to the consequent increased extent of oxidative damage, including oxidative damage to SOD1 (reduced activity), to NF (potential of enhanced crosslinking), and for further damage to the mitochondrial energy transfer site with a resultant increase in mitochondrial proton loss and cell death. Mitochondrial damage can also lead to altered calcium homeostasis and, through cytochrome C release, increased rates of apoptosis. In concert with mitochondrial damage, the lack of expression of calcium-binding proteins (calbindin D-28K, parvalbumin) within specific populations of motor neurons, and hence the loss of ability to buffer calcium, has been suggested to be a determinant of the motor neuron sensitivity observed in ALS (70–72).

### Alterations in Intermediate Filament Metabolism

#### Altered Neurofilament Expression

As discussed earlier, a neuropathologic hallmark of ALS is the formation of protein aggregates, with neurofilamentous aggregates among the most prominent. Neurofilaments are neuron-specific intermediate filaments. The individual proteins are defined on the basis of molecular mass as determined by SDS.PAGE (200 kDa: high-molecular-weight NF [NF-H]; 160 kDa: intermediate-molecular-weight NF [NF-M]; 68 kDa: low-molecular-weight NF [NF-L]). All neurofilaments are comprised of a central alpha helical coil-coiled domain flanked by a globular aminoterminal head domain. In the case of NF-H and NF-M, they contain a hypervariable carboxyterminal tail domain that is absent in NF-L. The central rod domain is involved in coil-coiled formation of the filamentous structure, whereas the globular aminoterminal head domain is involved...
in neurofilament assembly. Assembly into the triplet protein is triggered by the dimerization of NF-L either alone, or as heteropolymers with related intermediate filaments, followed by assembly of the dimers in a staggered, antiparallel array in which NF-M and NF-H subunits associate. Elongation of the protein occurs by head to tail assembly of NF-L by a process regulated by the N-terminus domain. The carboxyterminal domain forms sidearms that extend from the filament and appear to form links between adjacent neurofilaments and between neurofilaments and adjacent structures. The most striking feature of the sidearms in NF-H and NF-M are the KSP repeat motifs that are phosphorylated by Cdk5 and other proline-directed kinases. These repeats are extensively phosphorylated in axons in vivo and as a result of these motifs, NF-H is one of the most extensively phosphorylated neuronal proteins. Mass spectroscopic analyses of NF-H in humans, rats, canines, and squid have shown that most of the KSP repeats are phosphorylated in vivo. Together with microtubules, microtubule-associated proteins (MAPs), and actin, NFs make up the dynamic axonal cytoskeleton. Developmentally, the neuronal cytoskeleton has to accommodate the morphologic and behavioral transitions from migrating target-seeking axons to the stability of the mature neurons. This is achieved by axonal phosphorylation of the NFs.

Disturbances in the assembly of the NF triplet impacts on neuronal function. For instance, N-terminal deletions of the NF-L gene yields assembly-incompetent NF-L polypeptides (74); cleavage of the NF-L N-terminus inhibits assembly of NF-L into 10-nm filaments (75); and a single missense mutation in the rod domain (Leu394Pro) of NF-L (76) leads to a profound accumulation of NF within motor neurons resulting in motor neuron death and atrophy of muscle fibers. In the latter transgenic mouse, the mutation disrupts the binding of a ribonucleoprotein complex to the 3' UTR of NF-L mRNA and thereby alters mRNA stability (77).

The latter is of relevance to the formation of NF aggregates in ALS in that alterations in the stoichiometry of NF mRNA consistent with that which is associated experimentally with the induction of NF aggregate formation (Table 2) have been observed in ALS. Specifically, NF-L steady-state mRNA levels are selectively reduced in degenerating spinal motor neurons in ALS (5, 64, 78). The potential for alteration in mRNA stability underlie this process is highlighted by the finding that the stability of murine NF-L mRNA could be regulated, in part, through the binding of unique transacting mRNA stability determinants (79), and that these transacting-binding proteins that regulate the stability of human NF-L mRNA are differentially expressed in ALS compared with neurologically normal, age-matched, control spinal cord homogenates (80). We have recently shown that mutant but not wild-type SOD1 is one such transacting-binding protein capable of destabilizing NF-L mRNA (65), thus providing a potential linkage between the expression of a familial ALS-associated mutation and the genesis of an alteration in NF stoichiometry.

Peripherin Splice Variants in Amyotrophic Lateral Sclerosis

Neurofilamentous aggregates intensely colocalize peripherin, a related intermediate filament protein (Fig. 1D, E). Although encoded by a single gene located on chromosome 12, 3 alternative splice variants of peripherin have been identified with molecular weights of 56, 58, and 61 kDa. The 58-kDa variant is the most common, whereas the 61-kDa variant carries and addition 96 basepair insertion within the α-helical rod domain. Peripherin can coassemble with the NF subunits in vitro and has been observed to coaggregate with NF aggregates in ALS. Either the overexpression of the most abundant isoforms (per58) or the expression of the 61-kDa isoform is associated with an enhanced rate of motor neuron death (43). Of note, peripherin expression can be driven by TNF-α, highlighting the close interrelationship between neuronal and nonneuronal cells in ALS (42).

Functional Consequences of Neurofilament Aggregate Formation

Although NF aggregate formation is a hallmark of ALS, alterations in NF expression are not always neurotoxic. In doubly transgenic mice expressing the SOD1G93A mutation, altering the stoichiometry of NF expression with an increased level of either NF-M or NF-H expression results in a slowing of disease progression, approaching 5 months with increased expression of NF-H. Although direct proof is elusive, it is tantalizing to suggest that the formation of intraneuronal aggregates associated with alterations in NF expression results in the sequestration, or buffering, of key cellular proteins or oxidative species, thus reducing their impact on neuronal function. One such example is the presence of cyclin-dependent kinase 5 (Cdk5) colocalizing to NF aggregates in SOD1G37R mice (81). An alternate hypothesis would hold that reduced levels of axonal NF expression results in reduced axonal transport of mutant SOD1, thereby modulating its extent of damage (82).

Once formed, NF aggregates can also function dynamically to disturb neuronal homeostasis by acting as intraneuronal sinks. For example, NF aggregate-bearing motor neurons in vitro demonstrate a heightened NMDA-mediated calcium influx in response to a glutamatergic stimulus, a process that we have postulated is the result of the shunting of neuronal nitric oxide synthase (nNOS) to the NF aggregate. In doing so, this inhibits nNOS translocation to the cell surface membrane where it would normally downregulate the NMDA receptor and inhibit calcium influx (49).

SIGNAL TRANSDUCTION AND KINASE CASCADE REGULATION AND DeregULATION: A ROLE IN PHYSIOLOGY AND PATHOLOGY

Key to the understanding of aggregate formation in ALS is understanding the role of altered protein phosphorylation. The phosphorylation of cytoskeletal proteins is tightly regulated in the nervous system under physiological states. Cytoskeletal proteins, particularly neurofilaments and microtubule-associated proteins such MAP-2 and tau, are extensively phosphorylated (83). Most of this phosphorylation takes place on proline-directed serine and threonine residues of the proteins in a topographically regulated manner where the cell body of normal neurons contains little or no phosphorylation...
### TABLE 2. Murine Transgenic Models of Targeted Disruption of Neuronal-Intermediate Filament Gene Expression

| Age* (months) | Mouse Strain | Neurpathologic Effect | Axonal Loss | Effects on IF Content Phenotype | Phenotype |
|---------------|--------------|-----------------------|-------------|---------------------------------|-----------|
| 2–3           | C57BL/6      | ≥50% reduction in ventral root axonal caliber | ~20% loss | Reduction in both NF-M and NF-H protein levels in sciatic nerves and brain homogenates; significant loss of NF in myelinated axons | Normal development, no overt phenotype; reduced rate of regeneration post-axotomy |
| 6             | B6AF1J       | Massive perikaryal aggregates of neurofilament; axonal NF aggregates | None | Increased NF density, no alteration in NF-L or NF-H expression levels | Variable motor phenotype; cataract formation |
| 4             | 129 Sv/J bred to C57BL/6 | ≥50% reduction in ventral root axonal caliber | ~20% | Normal development, no overt phenotype | Normal development, no overt phenotype |
| 4             | P70 (adult)  | None | None stated | None | No overt phenotype |
| 4             | 129 Sv/J bred to C57BL/6 | ≥50% reduction in ventral root axonal caliber; loss of large diameter myelinated axons | ~10% loss | Significant reduction in NF-L, protein levels in NF-M −/− mice; concomitant increase in NF-H protein levels in brain but not spinal cord | No overt phenotype |
| 3–12          | C57BL6/DBA2J | Prominent perikaryal NF-L immunostaining | Reduced axonal NF | Increased levels of NF-L expression; reduced phosphorylation of NF-H; increased axonal NF density | Age-dependent deficits on reference and memory tasks |
| 3             | C57BL6 or 129 Sv/J bred to C57BL/6 | ≥50% reduction in ventral root axonal caliber; enhanced NF-L immunoreactivity in motor neuron perikarya | 25% | Significant loss of intermediate filaments within axonal processes; most axons devoid of NFs; loss of NF-L | No overt phenotype |

NF-M

| Age* (months) | Mouse Strain | Neurpathologic Effect | Axonal Loss | Effects on IF Content Phenotype | Phenotype |
|---------------|--------------|-----------------------|-------------|---------------------------------|-----------|
| 4             | 129 Sv/J bred to C57BL/6 | Significant reduction in the development of large-diameter myelinated axons with concomitant increase in smaller-diameter fibers | No | Approximately 10%–25% reduction in NF-L protein level, no effect on NF-M protein levels | Normal development; no overt phenotype |
| 4, 9, and 1 year | C57BL6/C3 | NF-H dose-dependent induction of severe axonal and perikaryal NF aggregate formation in spinal cord, reduction in axonal caliber | Reduced large-diameter axons if perikaryal aggregates formed | Reduced NF-L and NF-M molar ratios | Normal phenotype; no evidence of neurogenic atrophy (even at 2 years) |
| 4             | C57BL6/C3H | Prominent perikaryal NF aggregate formation; dying back axonopathy | Severe axonopathy | Elevated level of NF-H expression, no alteration in NF-L or NF-H levels | Progressive motor neuronopathy with death by 1 year |

(continued on next page)
of these proteins, whereas extensive phosphorylation exists in the axonal compartment (84–87).

Proline-directed S/T kinases (for example, Cdk5, MAPKs, and GSKs) are emerging as strong contributors to the pathogenesis of ALS. Cdk5 is a member of the cyclin-dependent kinases (Cdks) that are involved in the regulation of the proliferative cell cycle. As their name suggests, Cdks require the association with a cyclin for their activity. Cdk5 is unique, however, among these kinases because it is not activated by a cyclin, although it has the ability to bind to cyclin D/E (84). Instead, Cdk5 associates with its activators p35 and p39. Although Cdk5 is widely expressed in a number of tissues, its activity is restricted primarily to postmitotic neurons as a result of the neuronal-specific distribution of its activators (88, 89). To date, the best-characterized activator of Cdk5 is p35. Deregulation of Cdk5 by its truncated coactivators, p25 and p29, contributes to neurodegeneration by altering the phosphorylation state of cytosolic and cytoskeletal proteins and, possibly, through the deregulated activity of Cdk5. p35, the major regulator of this kinase, is anchored to the membrane through its myristoylation residue present in its N-terminus domain. Various neuronal insults result in elevated levels of intracellular calcium and results in the activation of calpains, the calcium-activated proteases. This induces the cleavage of membrane-associated p35 to p25 that is present in the cytosolic as well as nuclear compartments of the neurons. Thus, the regulation of Cdk5 is deregulated under pathologic conditions and induces aberrant phosphorylation of the cytoskeletal proteins in various neurodegenerative disorders, including ALS. The role of Cdk5 and MAPKs in ALS is discussed later.

### Phosphorylation of Neurofilament Proteins

It has been shown that the regulated topographic phosphorylation induces compartmentally restricted phosphorylation by proline-directed kinases (MAPKs, Cdk5, and GSKs) (90). The signals in particular cell types are summarized in Table 3. An EGF stimulus activates ERK1/2 to increase the phosphorylation of NF-M. In PC-12 cells, a similar result is achieved through membrane depolarization leading to calcium influx. In SH-SY5Y neuroblastoma cells, the activation of the integrin receptor, through its ligand laminin, activates Cdk5 to increase NF-H/M phosphorylation. A similar signal in motor neurons activates ERK1/2 to increase NF-M phosphorylation. As a balancing factor, the inhibition of MEK1/2 in hippocampal neurons inhibits ERK1/2 activity to decrease the phosphorylation of NF-M, NF-H, tau, and microtubule-associated proteins. These studies suggest that activation of the proline-directed kinases phosphorylate the carboxyterminal

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**TABLE 2. (continued) Murine Transgenic Models of Targeted Disruption of Neuronal-Intermediate Filament Gene Expression**

| Mice | Age* (months) | Mouse Strain | Neurpathologic Effect | Axonal Loss | Effects on IF Content | Phenotype |
|------|---------------|--------------|-----------------------|-------------|-----------------------|-----------|
| Aminoterminal deletion, NF-H | 9 weeks | C57BL/6J | ~20% reduction in both large and small motor neuron axonal area | No | No alteration in NF-L; approximately 2-fold increase in NF-M levels | No phenotype |
| Peripherin | | | | | | |
| Knockout Murine | 6–10 | C57BL/C3H | Diffuse, increased peripherin immunoreactivity in perikaryal and neurites; peripherin-immunoreactive aggregates in aged mice | ~35% motor axonal loss, age-dependent | Normal | Late-onset (>2 years) motor dysfunction |
| Knockout Murine | 6–8 | C57BL/C3H | Significant (~64%) loss of large motor neurons, increasing with age; increased peripherin staining in motor neurons with peripherin aggregates | ~46% loss of ventral root motor axons | Not stated | Progressive loss of hindlimb mobility beginning at 6–8 months |
| α-Internexin | | | | | | |
| Murine (−/−) | 3 | C57BL/6/129 Sv | Normal | No | Normal | No developmental delay; no overt phenotype |
| Murine (+/+) | 12–18 | B6CBA F1/J | Enhanced α-internexin immunostaining in cerebellum, neocortex, and thalamus | None | Normal | Reduced motor coordination and balance on rotorod |

*Age at which neuropathologic analysis undertaken.

For more detailed referencing for this table, please contact the author.
TABLE 3. Regulation of Cytoskeletal Protein Phosphorylation by Activation and Inhibition of Kinase Cascades

| Cell Type          | Signal                              | Kinase     | Cytoskeletal Proteins |
|--------------------|-------------------------------------|------------|-----------------------|
| NIH 3T3            | EGF                                 | Erk 1/2 ↑  | ↑ NF-Mp               |
| PC12               | Ca²⁺/membrane depolarization        | Erk 1/2 ↑  | ↑ NF-Mp               |
| Hippocampal neurons| MEK inhibition                       | Erk 1/2 ↓  | ↑ NF-M/NF-Mp, tau and MAPs phosphorylation |
| SH-SY5Y (human neuroblastoma) | Laminin (integrin)               | Cdk5 ↑     | ↑ NF-Hp               |
| Motor neurons      | Laminin (integrin)                  | Erk 1/2 ↑  | ↑ NF-Mp               |

A variety of cell culture systems (neuronal and nonneuronal) were used to investigate the phosphorylation of the proteins. Examples were the activation NIH 3T3 cells by epidermal growth factor (EGF), PC12 cells by membrane depolarization through the elevation of potassium levels in the extracellular medium, SH-SY5Y human neuroblastoma cells, and motor neurons through laminin. In addition, primary hippocampal neuron cultures were treated by the MEK (MAPKK) inhibitor PD98059, which inhibits Erk1/2 activity. The studies summarized in this table conclude that the activation of Erk1/2 and Cdk5 resulted in the phosphorylation of neurofilament (NF-M and NF-H), tau, and high-molecular-weight microtubule-associated proteins. These studies established that signal transduction processes are involved in cytoskeletal protein phosphorylation.

Myelin-Associated Glycoprotein Regulates Cytoskeletal Protein Phosphorylation by the Activation of Cdk5 and ERK1/2

Phosphorylation of NF proteins occurs in the axonal compartment in close proximity to myelin sheaths, suggesting that myelination may be an activating signal (91). It is also possible that a signal from Schwann cells or oligodendrocytes may activate Cdk5 and other proline-directed kinases such as ERK1/2. Increased phosphorylation of MAPs is also caused by myelination (92), and increases in axonal caliber and phosphorylation of NFs is also affected by myelination. Although the specific molecules in myelinizing cells, and the mechanisms that regulate signaling cascades affecting the expression and phosphorylation of cytoskeletal elements, have not been adequately identified, myelin-associated glycoprotein (MAG) is a potential neuronal ligand capable of modulating glial as well as axonal events (93). Although MAG is not essential for myelin formation, MAG-null mice display subtle abnormalities, including disruption of the periaxonal junction, delayed myelination, and formation of dystrophic oligodendrocytes (94). MAG-null mice show decreased NF phosphorylation in association with decreased Cdk5 and ERK1/2 activity. In vitro experiments have been used to determine MAG interactions with neurons and whether MAG directly influences the expression and/or phosphorylation of cytoskeletal proteins and their associated kinases. When COS-7 cells stably transfected with MAG were cocultured with primary dorsal root ganglion neurons, total amounts of NF-M, microtubule-associated protein 1B (MAP1B), MAP 2, and tau were upregulated significantly in dorsal root ganglion neurons, accompanied by an increased expression of phosphorylated NF-H, NF-M, and MAP1B. Similarly, total and phosphorylated NF-M levels were increased significantly in PC12 neurons cocultured with MAG-expressing COS cells or when treated with a soluble MAG Fc-chimera. This increased expression of phosphorylated cytoskeletal proteins in the presence of MAG in vitro was associated with increased activities of Cdk5 and ERK 1/2. These in vitro and in vivo findings have led to the proposal that the interaction of MAG with an axonal receptor(s) induces a signal transduction cascade that regulates expression of cytoskeletal proteins and their phosphorylation by these proline-directed protein kinases reinforcing the hypothesis that MAG itself is a component of the signaling system that affects the neuronal cytoskeleton (95) (Fig. 3).

A balance between kinase and phosphatase activities in the neuron tightly regulates the phosphorylation of cytoskeletal proteins. The signal transduction pathways currently known to be involved in cytoskeletal protein phosphorylation are shown in Figure 4. The influx of calcium ions into the neuron through membrane depolarization under normal conditions may lead to the activation of Ras and the subsequent activation of c-Raf. In turn, c-Raf activates MEK1/2, which phosphorylates ERK1/2. The activation of ERK1/2 leads to cytoskeletal protein phosphorylation, including NF, tau, and MAPs. Another pathway involves the binding of the matrix to the integrin receptor. The intracellular signal that results from this leads to p35/Cdk5 as well as MAPK phosphorylation of cytoskeletal proteins. Not shown in the figure, however, are the recent findings that Cdk5 activity regulates the activity of ERK1/2 through direct phosphorylation or indirectly by upstream Rac activation (96). Thus, the intricate pathways of regulating Cdk5 and ERK1/2 activities are critical in maintenance of “normal” cytoskeletal protein phosphorylation, and when this regulation is lost in neurodegenerative diseases, the activities of the kinases are deregulated, leading to aberrant cytoskeletal protein hyperphosphorylation.

The involvement of glia–axon interactions in the topographic phosphorylation processes as described previously is also responsible for these regulated kinase cascades. In normal topographic regulation of phosphorylation, there are higher levels of tyrosine phosphorytases in the cell body and lower levels in the axon (97). This results in lower levels of phosphorylated cytoskeletal proteins in the cell body and elevated levels along the axon. A hypothetical model of topographic regulation of cytoskeletal protein phosphorylation is
shown in Figure 5. Under physiological conditions, the receptor kinases are downregulated in the cell body compartment as a result of higher expression and activity of protein tyrosine phosphatases. This will reduce the activity of proline-directed kinases in the cell body compartment. Although there is lower expression and activity of the similar protein phosphatases in the axonal compartment, this results in higher receptor activation. In addition, the glial/axonal interaction activates proline-directed kinases in the axon. These factors are responsible for constitutive activation of the kinases responsible for stably phosphorylated cytoskeletal proteins, including NFM/H.

We propose that various neuronal insults induce the deregulation of the tightly regulated topographic phosphorylation as a result of aberrant activation of the kinase cascades

FIGURE 3. A model of neuronal cytoskeletal protein phosphorylation in myelinated axonal compartment. Studies were conducted on the myelin-associated glycoprotein (MAG) in wild-type and MAG knockout mice. It is proposed that MAG is one of the factor(s) responsible for axonal cytoskeletal protein phosphorylation. There are always constitutively active kinases present in the axonal compartment. This may be the result of glial axonal interaction and activation of proline-directed kinases induced by MAG. This results in higher constitutively active receptor kinases and active proline-directed kinases resulting in higher phosphorylation of cytoskeletal proteins such as NF-H and NF-M in the axonal compartments.

FIGURE 4. The involvement of signal transduction pathways in neuronal cytoskeletal protein phosphorylation. The various signal transduction pathways involved in the activation of proline-directed kinases such as Erk1/2 and Cdk5 and the resultant phosphorylation of the cytoskeletal proteins are summarized. These include calcium ion influx through membrane depolarization, binding of growth factors and the matrix to their receptors, as well as glial/axonal cell–cell interaction. These pathways lead to cytoskeletal protein phosphorylation.

FIGURE 5. Normal topographic regulation of cytoskeletal protein phosphorylation in neurons. From data collected from a large number of studies, this figure represents a hypothesis on topographic regulation of cytoskeletal protein phosphorylation in the neuron under normal conditions. The topographic regulation of protein phosphorylation is tightly regulated as a result of an appropriate balance between kinase (TyK) and phosphatase (TyP) activities in different compartments in the neuron. The lower activity of the receptor kinases (RTyK) in the cell body may be the result of higher protein tyrosine phosphatase activity in this compartment compared with the axonal compartment.

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The aberrant phosphorylation of cytoskeletal proteins observed in many neurologic diseases can be attributed to aberrant activation of signal transduction pathways and kinase cascades, which results in deregulation of compartment-specific regulated phosphorylation. This hypothesis is consistent with the studies described by Strong et al (98) in which they found the extensive phosphorylation of NF-H in human spinal cervical motor neurons of ALS but not in control subjects. However, the phosphomass spectroscopic analysis of NF-H from ALS showed no new phosphorylation sites in ALS compared with control subjects (40). These data, as well as studies from other laboratories on other neurodegenerative disease such as Alzheimer disease and Parkinson disease, led us to propose the deregulation of the topographic cytoskeletal protein phosphorylation model associated with neurodegenerative disease pathology. In this model, tyrosine kinase activity in the cell body is upregulated, leading to abnormally activated kinase cascades and increased cytoskeletal protein phosphorylation.

FIGURE 6. Topographic deregulation of neuronal cytoskeletal protein phosphorylation. This figure depicts a hypothetical model to explain the aberrant activation of kinases and deregulation of topographic cytoskeletal protein phosphorylation. This proposal is based on studies conducted by Strong et al (98) and Green et al (99). The phospho-specific mass spectroscopic analysis of NF-H isolated from normal and ALS samples carried out did not uncover any new phosphosites in ALS compared with normal control subjects (40). In addition, the residues phosphorylated in hereditary canine spinal muscular atrophy (HCSMA) were identical to sites phosphorylated in NF-H isolated from control samples. In both HCSMA and ALS, there was 4 or 5 times more phosphorylated NF-H (41). These data, as well as studies from other laboratories on other neurodegenerative disease such as Alzheimer disease and Parkinson disease, led us to propose the deregulation of the topographic phosphorylation model associated with neurodegenerative disease pathology. In this model, tyrosine kinase activity in the cell body is upregulated, leading to abnormally activated kinase cascades and increased cytoskeletal protein phosphorylation.

MECHANISMS OF CELL DEATH

Although a detailed discussion of the mechanisms of cell death in ALS is beyond the scope of this review, ultimately the motor neurons die. This process appears to be apoptotic. In ALS, a cytosol-to-membrane and membrane-to-cytosol redistribution of cell death proteins (Bax and Bak) and caspase-3 activation has been observed, as has an increase in p53 expression (100). Other markers of apoptosis have also been identified in ALS tissue, including expression of Le antigen and of Par-4, reduced levels of Bcl-2 mRNA and elevated levels of bax mRNA in ALS lumbar spinal cord, and the upregulation of the proapoptotic “BH-3 only” peptide human protein Hrk.

The induction of apoptosis is also regulated through the expression of heat shock proteins (HSPs). These highly conserved proteins are expressed following a number of stresses and function to protect the cell against further damage, in part by preventing protein aggregation or promoting protein disaggregation. They also play a key role in regulating apoptosis at the level of the formation of the apoptosome. After release into the cytosol, cytochrome c binds to Apaf-1, inducing an ATP-dependent oligomerization of Apaf-1 and resulting in the recruitment of procaspase-9, which then undergoes an autoproteolytic cleavage to an active caspase. It then, in turn, activates the effector caspases 3 and 7. HSP 90 inhibits this process by inhibiting Apaf-1 oligomerization, HSP 70 interacts directly with Apaf-1 to prevent activation but does not prevent oligomerization, whereas HSP 27 binds and sequesters cytochrome c away from Apaf-1. There is then a delicate balance between the induction of apoptosis and the response of the cell to oxidative injury through the generation of HSPs.

CONCLUSIONS

Among the multiple theories for the pathogenesis of ALS, none has gained supremacy as being clearly linked to the triggering of the disease process. In part, this is almost certainly the result of the tight integration among the many disturbances in neuronal metabolism in ALS. The exact role that alterations in cytoskeletal protein metabolism, in particular NF, peripherin, and tau, play in the disease process remains to be fully defined. However, if ALS is viewed as a disorder of protein aggregation, then neuronal cytoskeletal proteins must be considered a primary target for the disease process, with the deregulation of topographic phosphorylation resulting from aberrant activation of various kinase cascades playing an important role. ALS, like with many neurodegenerative sporadic disorders, demonstrates a slow progression and the disease onset is late. The accumulation of various neuronal insults...
potentially results in a substantial imbalance between kinase and phosphatase activities such that the system can no longer regulate itself. This is the result of aberrant activation of kinases and deregulation of the tightly regulated processes inducing pathology.

Additionally, there is little doubt that altering intermediate filament expression stoichiometry, and specifically that of the individual NF proteins, induces motor neuron degeneration in those neurons in which NFs are the most abundant protein. The pathology observed in transgenic mice in which NF stoichiometry is altered is variable in terms of severity and in clinical phenotype, but in 2 in which the human disease state is most closely recapitulated (NF-L [−] with or without peripherin overexpression), there is a very good parallel to ALS. Moreover, the former shows a striking age dependency in the development of a phenotype, and the temporal correlation between NF aggregate formation and motor neuron death with astrocytic and microglial activation is striking in animal models.

A hypothetical model has been proposed to explain the phosphoimmunocytotoxicity and the absence of the phosphoepitopes of the cytoskeletal proteins and their transport and is left behind causing the deposits of the phosphoepitopes of the cytoskeletal proteins and pathology.

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