REVIEW ARTICLE

PROGRESS IN CLINICAL NEUROSCIENCES: The Evidence for ALS as a Multisystems Disorder of Limited Phenotypic Expression

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ABSTRACT: Traditionally, amyotrophic lateral sclerosis (ALS) is considered to be a unique neurodegeneration disorder in which motor neurons are selectively vulnerable to a single disease process. Our current understanding of ALS, however, suggests that this is far too limited an approach. While motor neuron degeneration remains the central component to this process, there is considerable phenotypic variability including broad ranges in survivorship and the presence or absence of cognitive impairment. The number of familial variants of ALS for which unique genetic linkage has been identified is increasing, attesting further to the biological heterogeneity of the disorder. At the cellular level, derangements in cytoskeletal protein and glutamate metabolism, mitochondrial function, and in glial interactions are clearly evident. When considered in this fashion, ALS can be justifiably considered a disorder of multiple biological processes sharing in common the degeneration of motor neurons.

RÉSUMÉ: Observations indiquant que la SLA est une maladie multisystémique à expression phénotypique limitée. Traditionnellement, la SLA était considérée comme une maladie neurodégénérative dans laquelle les motoneurones sont vulnérables de façon sélective à un processus pathologique unique. Notre compréhension actuelle de la SLA suggère cependant que cette approche est beaucoup trop étroite. Bien que la dégénérescence des motoneurones demeure l'élément central de ce processus, il existe une variabilité phénotypique considérable particulièrement quant à la survie et à la présence ou à l'absence de déficit cognitif. Le nombre de variantes familiales de la SLA pour lesquelles une liaison génétique a été identifiée augmente, attestant de l'hétérogénéité biologique de la maladie. Au niveau cellulaire, il existe des perturbations de la protéine cytosquelettique et du métabolisme du glutamate, de la fonction mitochondriale et des interactions gliales. Quand on regarde la SLA sous cet aspect, on peut à juste titre la considérer comme une maladie due à des processus biologiques multiples ayant en commun la dégénérescence de motoneurones.

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In spite of the passage of over 100 years since the initial clinicopathological description of amyotrophic lateral sclerosis (ALS), our ability to treat this devastating disease remains limited.[1] However, significant gains in our understanding of the pathogenesis of ALS have been achieved, leading to a renewed optimism that efficacious therapies will become available. This article will review our current understanding of the clinical and neurobiological features of ALS, how this relates to a potential understanding of its pathogenesis, and illustrate how this new knowledge has led to the concept of ALS as a clinical and biological disorder affecting multiple aspects of the central nervous system. Approaching ALS in this fashion, not as a unique disease process but rather as the limited phenotypic reflection of a broad spectrum of biological processes, has become integral to our understanding of its potential pharmacotherapy.

NEW CONCEPTS IN CLINICAL PHENOMENOLOGY

Increasing age-related mortality rates

With increasing incidence rates with age, ALS is amongst the three major neurodegenerative diseases of our aging population. Alzheimer's disease and Parkinson's disease complete the triad. Although juvenile and early adult onset cases are recognized, these are either uncommon or restricted to specific geographic

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foci (e.g., juvenile onset ALS in Tunisia).[2] In North America, as with most developed countries, the fact that the majority of ALS cases will arise from the older aged segment of the population is of economic significance given the aging of the "baby boomers", of which 75 million were born between 1945 and 1964. For instance, in 1996, 12.7% of the American population was greater than 65 years in age. By 2020, this is anticipated to be 18.0%. Of these patients, the "old old" will rise to 7.0 million from 3.7 million.[3] Because of this effect of aging of the population, increasing numbers of ALS patients can be anticipated.

However, age-adjusted mortality rates are even now increasing at a rate disproportionate to that predicted on the basis of societal aging alone.[4,5] Age-adjusted mortality rates for ALS in the USA significantly increased in the aged population in the interval from 1977 to 1986, an interval too short to be accounted for solely on the basis of the aging of the population.[6] Similar findings of an increased incidence of ALS, particularly amongst the elderly, are evident in Sweden,[7,8] Israel,[9] the United Kingdom[10] and Canada.[11] The most parsimonious interpretation of these epidemiological observations is that an environmental factor, acting cumulatively, must contribute to the pathogenesis of ALS.[12] Although such a concept must seem intuitive, identifying such factors has proven difficult.

The strongest evidence for an environmental trigger in the induction of motor neuron degeneration has been that of the previously hyper-endemic focus of ALS in the Western Pacific. In this geographically unique region, westernization of the diet of the native peoples is held to have been sufficient to correct chronic nutritional deficiencies of calcium and magnesium, reversing the chronic exposure to a readily bioavailable form of aluminum.[13,14] When exposed to a similar calcium-deficient, aluminum supplemented diet, nonhuman primates were found to develop a motor neuron disorder bearing many of the features of ALS.[15]

For the more common sporadic variants of ALS, only geographic (rural or farming exposure) and industrial (antecedent electrical injury or plastics exposure) factors are associated with disease. While we tend to think of ALS as being in part related to industrialization, farmers and shepherds in Sardinia are amongst those with the highest incidence rates.[16]

Variability survivorship

Survival curves in ALS are skewed with a broad survival range from months to decades of survival following symptom onset. Long-term survival is not as uncommon as originally thought, with the age at symptom onset and gender being amongst the most important predictors of prolonged survival, irrespective of whether the variant of ALS under consideration is sporadic or familial.[17-19] The magnitude of this effect can be readily seen in the patient population of southwestern Ontario in whom symptom onset at less than the age of 45 is associated with a median survival of 54.8 months (40.5, 66.2 months; 25th and 75th percentile, respectively) in contrast to a median survival of 25.4 months with symptom onset after age 45 (9.9, 37.8 months; 25th and 75th percentile, respectively) ($p < 0.001$). This age-dependency effect is most clearly evident in males, who enjoy a significantly better survivorship associated with a younger age of symptom onset. The gender discrepancy between males and females is lost with increasing age. Although less robust, the site of symptom onset also predicts survival patterns in that amongst all groups, limb onset survival exceeds that of bulbar onsetting disease. On the whole, young males with hand onset of symptoms are amongst the most likely to enjoy long-term survivorship. Whether each of these factors (age and site of symptom onset; gender) simply influences the phenotypic expression of a common disease process, or whether truly biologically discrete processes give rise to each clinical phenotype remains a critical biological question in ALS. It is hard to imagine, however, that the fundamental disease process of a young woman with a malignant disease course is biologically identical to that of a similarly aged male who will likely enjoy a prolonged survival.

Cognitive dysfunction in ALS

Amongst the most convincing arguments in support of ALS as a multisystems disorder has been the recognition of cognitive dysfunction as an integral component of the disease process. The occurrence of cognitive impairment or dementia has been previously considered to be either rare or extremely uncommon in ALS.[20] Although the exact prevalence is not known, cognitive impairment will be evident in approximately a third of all patients when carefully assessed.[21] Deficits are primarily those of frontal and temporal functions, including mental flexibility, verbal and nonverbal fluency, abstract reasoning and in memory for both verbal and visual material.[22-26] Although instances of dementia antedating the onset of amyotrophy are well-recognized,[27] and can even manifest as the Klüver Bucy syndrome,[28] more often the findings are subtle.[29] We observed that individuals with bulbar onsetting disease were more likely to demonstrate cognitive impairment.[30] In addition to the features described above, we also found deficits in working memory and problem solving ability – consistent with a frontal temporal lobar degeneration.

Although not evident in all cognitively impaired ALS patients, both static and dynamic neuroimaging studies support the clinical impression of frontal and temporal lobar degeneration, including atrophy on CT scanning and increased T2 signal on MR imaging in both frontal and temporal white matter.[22,31,32] These findings are complementary to observations of reduced blood flow in both frontal and temporal neocortices using functional imaging modalities such as SPECT employing either ^{123}I-Imp (^{123}I-N-isopropyl-*p*-iodoamphetamine) or [^{99m}Tc]-*d,l*-HMPAO.[33-36] In the presence of cognitive impairment, reduced rCBF in the anterior cerebral hemispheres and the anterior cingulate gyrus is evident with PET scanning.[22,37,38] Defining this further, Abrahams and colleagues[39,40] have utilized verbal fluency/word generation tasks for functional PET and observed reduced metabolism in the right dorsolateral prefrontal cortex and left middle and superior temporal gyrus. We observed a significant reduction in the NAA/Cr ratio with MR ^{1}H spectroscopy (consistent with neuronal loss) of the left anterior cingulate gyrus at the earliest time interval studied in those patients developing cognitive impairment.[30]

The neuropathological correlates of this process include frontal lobar atrophy (Figure 1A), a marked neuronal loss accompanied by spongiform changes in the 2nd and 3rd cortical layers of the frontal lobes and precentral gyrus (Figure 1 B & C) with intraneuronal inclusions in a number of neuronal

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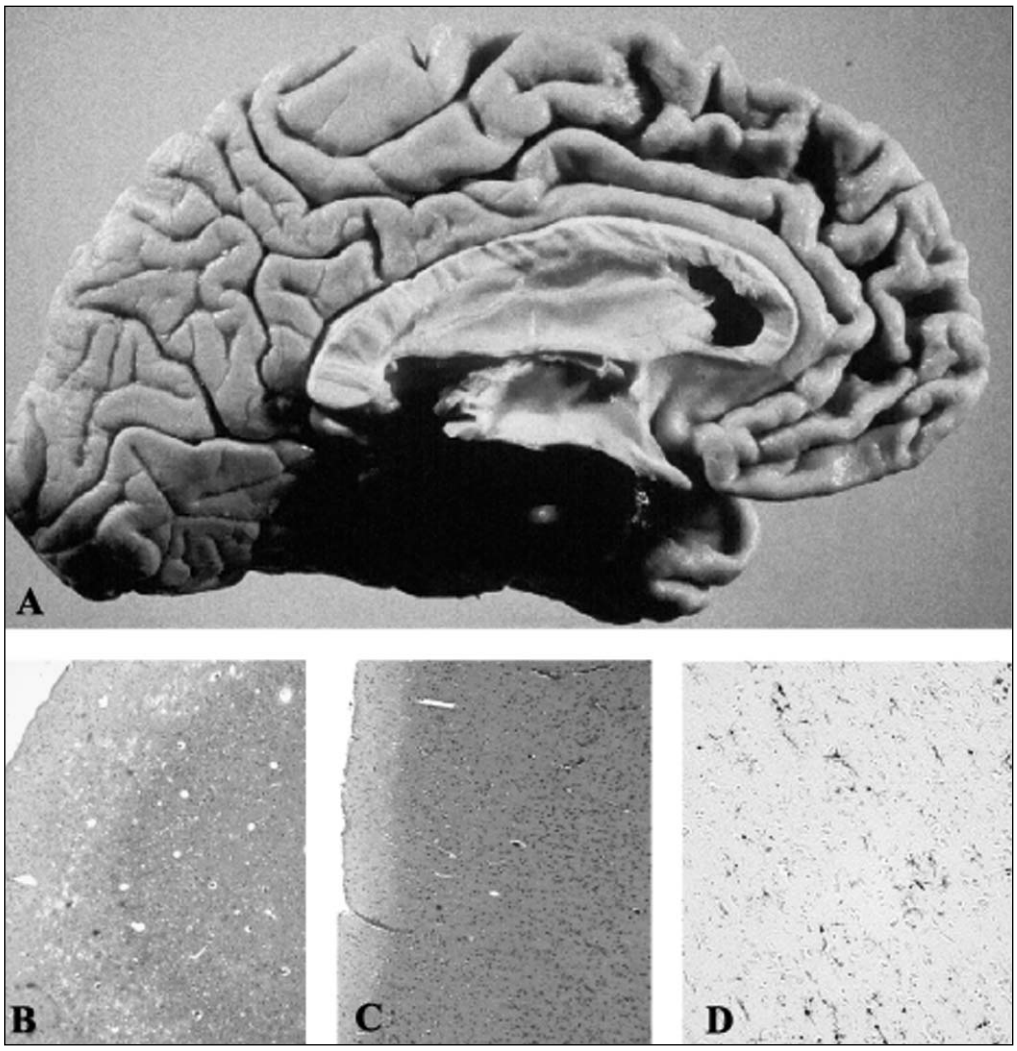

Figure 1: *Neuropathological features of cognitive impairment in sporadic ALS. Consistent with the imaging findings, prominent frontal atrophy can be observed in cognitively impaired patients with ALS. In Figure A, striking atrophy is evident throughout the frontal lobe, including the anterior cingulate gyrus (photograph courtesy of Dr. David Munoz). The only histological feature identified as a consistent feature of cognitive impairment is the presence of superficial linear spongiosis (B, 10x mag.) not observed in cognitively intact patients (C, 10x mag.). In the majority of patients, this was also accompanied by a transcortical microglia activation (D, 20x mag.; HLA-DR immunostaining of microglia with the antigen:antibody conjugate detected using 3,4-diaminobenzidine, giving rise to a brown coloration.). The later finding was not, however, restricted to the first and second cortical layers, those that are most affected by the superficial linear spongiosus.*

populations not traditionally thought to be involved in ALS. Using immunohistochemical markers, these inclusions are seen to be unique to ALS and are ubiquitin immunoreactive intraneuronal aggregates assuming either a discrete Lewy body-like morphology or a more amorphous perinuclear arcuate shape. Unlike the aggregates of nonALS patients with a frontotemporal dementia, these inclusions are not immunoreactive for either the microtubule-associated protein *tau* or for α-synuclein.[41-44]

It does not appear, however, that the presence of cognitive impairment is an all or nothing phenomenon in ALS. Rather than a strict correlation with the presence or absence of neuropathological changes described above, we have observed that cognitive impairment in ALS appears to best correlate with the extent and load of ubiquitin-immunoreactive intraneuronal aggregates and dystrophic neurites in the frontal and temporal lobes.[45] Our findings suggest that there is a continuum of neuropathological change in which neuronal ubiquitin positive aggregates are present in both cognitively intact and cognitively impaired ALS patients but with a greater total load in the latter. Only the presence or absence of superficial linear spongiosus affecting the layers I and II of the frontal cortex clearly discriminated between the two (Figure 1 B & D). This finding of superficial linear spongiosus is a frequently observed finding of the frontal temporal lobar degenerations.[46-48]

The concept of a continuum of nonmotor neuronal involvement in ALS is also supported by the neuropathological

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Table 1: Familial ALS and related disorders – known inheritance patterns

Inheritance Pattern	Chromosomal linkage	Unique features	Reference
Autosomal dominant			
ALS 1	21q22.1	Cu/Zn superoxide dismutase mutations	55
ALS X	Xp11 – q12	Adult onset	206
ALS 3		Adult onset; absence of linkage to Cu/Zn superoxide dismutase	207
ALS 4	9q34	Juvenile onset, complete penetrance; very slow progression; distal limb amyotrophy with pyramidal signs	208,209
ALS 6	9q21 – q22	Frontotemporal dementia associated	53
ALS with bulbar onset	unknown	Japanese family; juvenile onset with prominent early onset bulbar dysfunction; slow progression; dementia	210
NFH	22q12.1 – q22	Mutations in KSP repeats (not observed in *f*ALS; only found in sporadic ALS)	115-118
Autosomal recessive			
ALS 2	2q33 – q35	Spastic pseudobulbar syndrome with spastic paraplegia; childhood onset; slow progression,	2,211
ALS 5	15q15.1 – q21.1	Not pseudobulbar; distal amyotrophy; minor spasticity; long-term survival	212
Brown-Vialetto-van Laere syndrome		Progressive bulbar paralysis; childhood onset; progressive deafness; pyramidal signs;	213
X-linked			
Kennedy's syndrome	Xq12	Progressive muscle atrophy; gynecomastia; reduced fertility; Androgen receptor gene mutation (trinucleotide (CAG) repeat)	214-217
Miscellaneous			
Hexosaminidase A and B	15q23 – q24	Late onset GM2 gangliosidosis	218-221
Disinhibition-dementia-parkinsonism-amyotrophy syndrome	Chr 17	Allelic with frontotemporal dementia and parkinsonism	54

analysis of ventilator dependant ALS patients in whom long-term survival is attained, and in whom neuronal loss and spongiform degeneration of layer II of the frontal cortex is observed.[49-52]

The above findings are of particular interest in that they provide convincing evidence that ALS is not a disease purely of motor neurons. There is clearly a subset of nonmotor neurons that can become integrally involved in the disease process. The recent observation of genetic linkage of cognitive impairment in familial ALS (*f*ALS) to chromosome 9q21-22 suggests that such a process may also be under the control of specific modifier genes.[53] This latter process is distinct from the uncommon chromosome 17 linked disinhibition-parkinsonism-amyotrophy syndrome.[54]

Familial variants of ALS (*f*ALS)

Although accounting for <10% of ALS cases, advances in our understanding of *f*ALS have provided significant insights into the complexity of the pathogenesis of ALS. While the majority of pedigrees are inherited in an autosomal dominant fashion, autosomal recessive forms are recognized, as are X-linked variants (Table 1). Many inherited variants of motor neuron disease might be best considered as true spinal muscular atrophies, as highlighted by the X-linked spinobulbar atrophy (Kennedy's syndrome) in which corticospinal tract degeneration does not occur. However, others are more clearly variants of ALS in which the triad of bulbar, lower motor neuron and corticospinal tract involvement is evident but with divergent rates of progression or severity.

To highlight the complexity of understanding the genetics of *f*ALS, one need only to examine the striking clinical heterogeneity associated with the most common mutation in *f*ALS. Mutations in the copper/zinc superoxide dismutase (SOD1) gene on chromosome 21 are associated with approximately 15% of the dominantly inherited cases of *f*ALS.[55] In spite of extensive studies, the exact mechanism by which alterations in SOD1 directly induce the process of motor neuron degeneration in ALS is still unknown. It has been suggested that these mutations confer a gain of aberrant activity to the SOD1 enzyme, increasing the accessibility of peroxynitrite ($ONOO^-$) to the Cu/Zn binding site and leading to increased rates of reactive nitrating species formation.[56,57] Another theory suggests that enhanced rates of hydroxyl radical formation would be catalyzed leading to DNA and membrane damage.[58-60] However, the

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striking variability in both the clinical and neuropathological characteristics of both human pedigrees and transgenic mice harboring mutations in the SOD1 enzyme suggest that the pathogenesis of ALS cannot be attributed solely to aberrant activity of the SOD1 enzyme.[61]

First, beyond the significant clinical variability associated with the various SOD1 mutations (Table 2), there is significant variability in the neuropathological manifestations amongst the cases of *f*ALS in which SOD1 mutations have been identified. For example, cases expressing the E100G mutation in exon 4 demonstrate features typical of *f*ALS with posterior column involvement but with the additional involvement of both ascending sensory and efferent cerebellar pathways.[62] Aggregates of phosphorylated neurofilament are not a significant feature. In contrast, the expression of the I113T mutation in exon 4 is associated with profound neurofilamentous aggregate formation with little ubiquitin immunoreactivity, the absence of posterior column pathology, and the striking finding of *tau* immunoreactive neurofibrillary tangles in multiple brain stem nuclei.[63] The same mutation in another pedigree is associated with marked neurofilamentous aggregate formation restricted to the lower motor neurons.[64] *f*ALS cases harboring the A4V mutation manifest with neuropathological features of ALS with posterior column degeneration but, in addition, the unique presence of intracytoplasmic inclusions with intense SOD1 immunoreactivity.[65] The A4T mutation in exon 1 is associated with ALS with posterior column involvement without the inclusion formation.[66] The most striking example of the extent of phenotypic variability that can occur in a single mutation is observed in families harboring the D90A SOD1 mutation. In these, such divergent manifestations as classical ALS, segmental spinal muscular atrophy, spinal muscular atrophy, or variants of Charcot-Marie-Tooth disease have been observed.[67,68] Hence, there is a sufficiently high degree of variability in the neuropathological manifestations of human pedigrees bearing *f*ALS SOD1 mutations to question whether the mutated enzyme, acting alone, is sufficient to induce the disease process.

These human observations are paralleled in transgenic mice expressing SOD1 mutations in which the neuropathological and clinical manifestations vary markedly with the specific SOD1 mutation. Although the initial G93A constructs developed motor dysfunction accompanied by pronounced vacuolar degeneration within motor neurons in the absence of neurofilamentous inclusion formation, the subsequent generation of the G93A mutants developed cytoskeletal pathology reminiscent of ALS.[69,70] G85R constructs developed a profound astrocytic pathology consisting of SOD1 and ubiquitin immunoreactive inclusions.[71] It is likely, therefore, that specific modifying genes, as yet unknown, are of importance to the ultimate disease phentoype.

Absolute changes in the level of SOD1 activity also cannot explain the induction of motor neuron pathology. While increased levels of SOD1 mRNA have been reported in motor neurons of sporadic ALS (*s*ALS),[72] reduced red blood cell SOD1 activity has been documented in heterozygotes for the SOD1 mutation.[73] Also, while the down-regulation of SOD1 activity in PC12 cells is associated with apoptotic cell death,[74] both the A4V and G37R mutants, when transfected into yeast lacking SOD1, are associated with increased rates of apoptosis.[75]

Table 2: Familial ALS 1 - general syndrome features associated with specific point mutations in Cu/Zn superoxide dismutase[222]

Clinical Phenotype	Cu/Zn SOD mutation
Lower motor neuron predominant	A4V; L84V; D101N
Slow progression	G37R (18y); G41D (11y); G93C; L144S; L144F
Rapid progression	A4T (1.5y); N86S (homozygous, 5 months); L106V (1.2y); V148G (2y)
Late onset	G85R; H46R
Early onset	G37R; L38V
Female predominant	G41D
Bulbar onset	V148I
Low penetrance	D90A; I113T

Moreover, SOD1 knockout mice fail to develop motor neuron disease.[76]

Hence, if alterations in the expression of SOD1 are integral to the development of motor system degeneration, this cannot be the only determinant of the disease expression. This concept is supported by the studies of Cleveland and colleagues in which $SOD1^{G85R}$ mice mated with either wild-type SOD1 knockouts or transgenics expressing 6-fold elevated levels of SOD1 failed to modify the extent of clinical or neuropathological disease progression.[77] Recalling also that ALS is a chronic neurodegenerative disease with age-dependant incidence rates, little is known of chronic low-level SOD1 mediated neurotoxicity or the effect of age-dependant oxidative damage to the SOD1 enzyme itself.[78] In beginning to address this, Cleveland and colleagues have recently observed chronic caspase 1 activation associated with mutant SOD1 expression *in vitro*, culminating ultimately in apoptotic cell death heralded by caspase 3.[79] This novel observation suggests a possible mechanism of the induction of apoptosis in a chronic disease state by the sequential activation of caspases and has been subsequently confirmed in an elegant study utilizing a small peptide caspase inhibitor (zVAD-fmk) in the G93A SOD1 transgenic mice to induce a significant increase in survival.[80] It is worth recognizing, however, that whether cell death in ALS is apoptotic remains to be ascertained with certainty.[81,82]

ALS AS A MULTIFACTORIAL DISEASE PROCESS

There is little doubt that at the cellular level, ALS can be attributed to a number of discrete biological processes. In the preceding discussion, this is most clearly highlighted by the similarities of ALS phenotype between the *f*ALS and *s*ALS cases, in spite of clearly differing genetic compositions. ALS also affects a number of neuronal metabolic pathways, including such diverse processes as oxidative injury, excitotoxicity, altered cytoskeletal protein homeostasis, a failure of calcium homeostasis and alterations in mitochondrial function. Whether these are truely discrete processes, each of which can serve as an etiological trigger for the disease, or whether they represent

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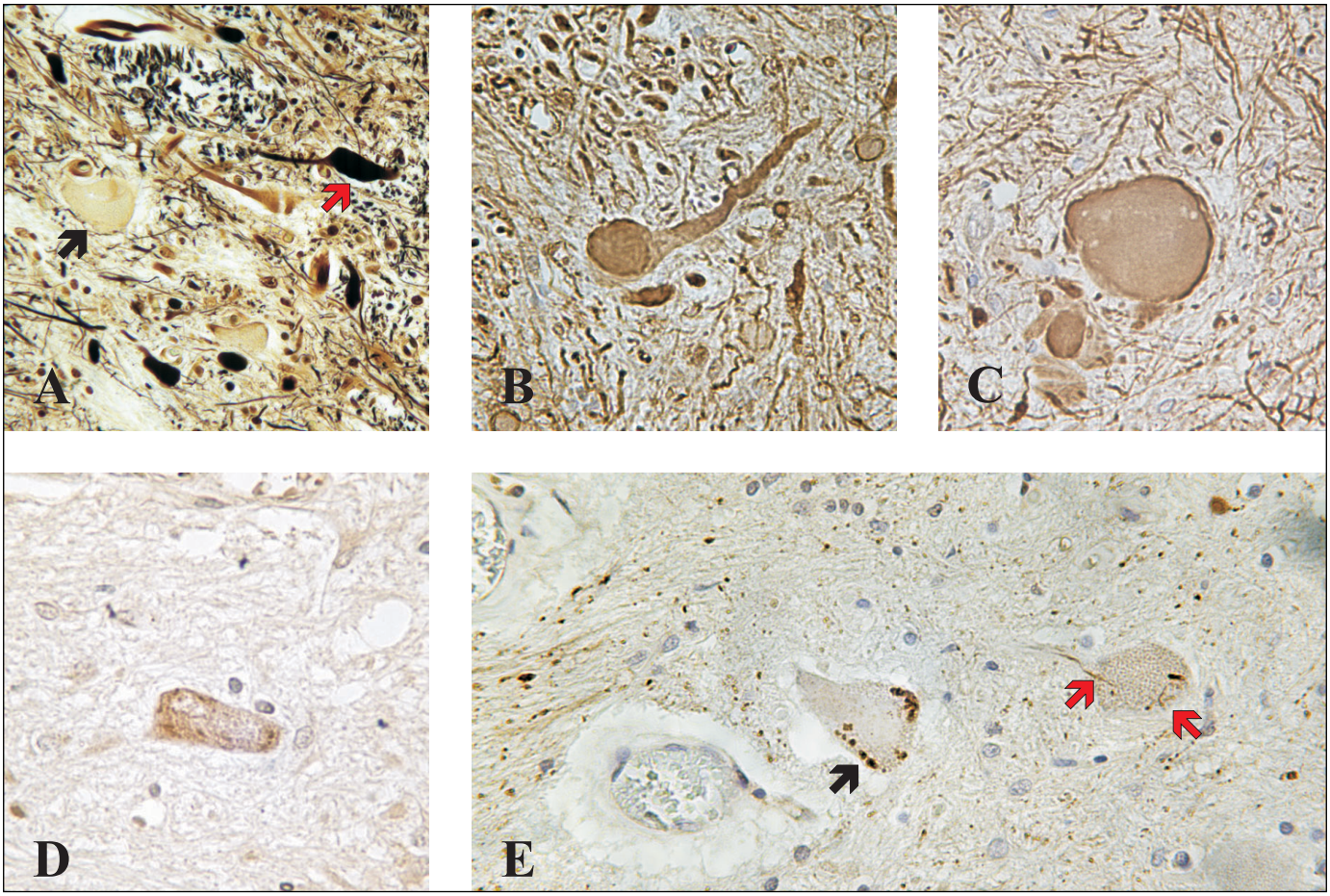

Figure 2: *The spectrum of intraneuronal inclusions observed in ALS. By routine silver staining techniques, motor neurons are observed to contain dense, argentophilic material in the form of neuroaxonal spheroids (A; arrow) or a hyalinized cytoplasmic clearing (red arrow) (10x mag.). Using monoclonal antibodies against phosphorylated neurofilaments (SMI 31), dense intracytoplasmic neurofilamentous aggregates (B; 40x mag.) or neuroaxonal spheroids are evident (C, 40x mag.). Both cortical and spinal cord motor neurons are immunoreactive to nitrotyrosine (D, 40x mag). Ubiquinated inclusions within spinal motor neurons can assume a punctate appearance (E, arrow) or a skein-like morphology (E, red arrow) (40x mag.).*

integrally related processes as a part of a cascade of neuronal degeneration ultimately culminating in cell death, remains to be determined. Intuitively, the latter would seem the more likely process. While considerable interest has arisen with regards to the nonneuronal biological effects in ALS (e.g., alterations in skin glycosaminoglycans[83]), this review will focus on the neurobiological aspects of the pathogenesis of ALS.

Neurofilaments

The neuropathological diagnosis of ALS is established by the finding of widespread motor neuron selective degeneration in a topographically specific pattern that includes a loss of specific motor neuron pools with chromatolytic neurons, degeneration of descending innervation pathways and atrophy of ventral spinal roots.[84] In familial variants of the illness, pallor of the spinocerebellar tracts and posterior columns with degeneration of Clarke's nucleus may also be observed.[85] As discussed above, this concept at minimum must now be modified to incorporate an understanding of the nonmotor neuronal degeneration that forms the basis of cognitive impairment in a population of ALS patients.

The ultrastructural hallmark of ALS is an accumulation of neurofilamentous material within degenerating neuronal perikaryal and axonal processes, the deposition of ubiquitin-conjugated material, and the immunohistochemical evidence of oxidative damage[86-89] (Figure 2). The neurofilamentous aggregates consist of masses of interwoven skeins of neurofilamentous material, appearing either as Lewy body-like inclusions, or amorphous aggregates infiltrating the perikaryon and extending into neuritic structures. Such aggregates are also immunoreactive to antibodies recognizing α internexin, a related intermediate filament.[90] In contrast, neuroaxonal aggregates localized to the neuritic process and consisting primarily of neurofilamentous material are immunoreactive for peripherin.[91] This suggests that the composition of such aggregates is dependant to some degree on the somatotopic localization of the aggregate within the neuron and the relative contribution of the cytoskeleton to the normal cellular structure at that point.

Immunohistochemical and molecular studies of ALS have provided us with some degree of understanding of the generation of neurofilamentous aggregates in ALS. Amongst neuronal

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populations, motor neurons possess the greatest axonal lengths and complexity relative to perikaryal size and thus giving rise to the necessity of a high content of neurofilament (NF) proteins.[92] These proteins, members of the highly conserved intermediate family of cytoskeletal proteins, are three separate but closely related proteins consisting of a highly conserved α-helical core domain, a N-terminus domain that is integral to the initial assembly process, and a C-terminus domain in which the size is based largely upon the number of multiphosphorylation repeats (KSVP sequences).[93] Based on this latter property, NFs are defined on the basis of molecular weight as low molecular weight (NFL), intermediate molecular weight (NFM) or high molecular weight (NFH). Disruption of the assembly of the NF triplet protein, a heteropolymer composed of the initial homopolymerization of the NFL proteins followed by the layering on of the NFM or NFH proteins, results in a motor neuron degeneration.[94-97] Altering the stoichiometry of NF expression in transgenic models also results in the formation of neurofilamentous aggregates and a motor neuron degeneration.[98-100] Altering the NFL rod domain through a point mutation will sufficiently disrupt NFL homopolymerization to inhibit the triplet protein assembly. As demonstrated by Julien and colleagues,[101] double transgenic mice containing a NFL deletion and over expression of peripherin develop a motor neuron degenerative state containing a striking number of parallels to ALS.

Perhaps the most convincing evidence to date that alterations in NF biochemistry can be integral to ALS include the demonstration by Bergeron and colleagues of selective suppressions of NFL mRNA steady state levels in degenerating ALS motor neurons[102] and our demonstration that this alters the stoichiometry of NFL, NFM and NFH steady state mRNA levels in a fashion consistent with transgenic mouse models of motor neuron disease.[90]

Secondly, motor neurons are rich sources of free oxygen radicals, nitric oxide synthase and SOD1. The presence of free oxygen radicals and nitric oxide leads to the formation of peroxynitrite which, in the presence of SOD1, can be catalyzed to form reactive nitrating species. Reactive nitrating species will preferentially modify phenolic residues (e.g., tyrosine residues), of which NFL is an abundant source. The nitration of NFL as the end result of SOD1 mediated catalysis of peroxynitrite has thus been proposed as a mechanism in the pathogenesis of ALS, and indeed, when NFL is nitrated and then added to an otherwise intact NF isolate containing NFL, NFM and NFH, the NF triplet assembly is inhibited.[103] All the key constituents necessary to drive this reaction have been observed in ALS motor neurons, including the constitutive expression of the neuronal isoform of NOS,[104] the byproducts of nitric oxide synthesis,[105] an abundance of both SOD1 mRNA[106] and enzyme[107,108] and intense nitrotyrosine immunoreactivity co-localizing to intracellular aggregates.[109] 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 high performance liquid chromatography, are elevated compared to controls.[110] Such elevations are reminiscent of those observed in transgenic mice expressing the human $SOD1^{G93A}$ mutation.[111] These observations suggest that elevated levels of reactive nitrating species are present in ALS. Further, SOD1 mutations observed in *f*ALS alter the activity of SOD1 to preferentially generate reactive nitrating species and increase nitration of NFL.[112]

The hypothesis that NFL nitration will give rise to the motor neuron degeneration of ALS thus becomes rather appealing. Unfortunately, neither the role of altered SOD1 activity or of reactive nitrating species in the pathogenesis of ALS is fully understood. When NF isolates were examined from both ALS and age-matched control cases, we found no evidence for a significant alteration in the extent of nitration in ALS NFL.[113] Rather, these have led to our proposal that, distinct from its role in NF triplet assembly, NFL may function as a biological sink for reactive nitrating species. In support of this, we have observed *in vitro* that spinal motor neurons derived from NFL -/- transgenics are more sensitive to the toxicity of reactive nitrating species than are either control or *h*NFL +/+ cultures.[114]

The third piece of evidence that alterations in NF homeostasis are of significance in ALS relates to the findings of mutations in the NFH C-terminus domains in a number of *s*ALS cases.[115-118] Not every study has replicated these findings.[119] These mutations, for the most part, are localized to the MPR regions and would be predicted to alter the phosphorylation state of NFH. Although this represents an absolute minority of ALS cases, these observations serve to highlight that the phosphorylation domains of the NF proteins may be critical to the genesis of ALS. While our recent observation that there are no significant differences in the physicochemical properties of ALS NFH when contrasted to control NFH isolates would seem to argue against this, to date, we know little of the dynamics of NFH phosphorylation and how this is altered in ALS.[120]

Mitochondrial dysfunction and oxidative stress in ALS

There is considerable evidence of mitochondrial dysfunction in ALS. At the ultrastructural level, this includes the observations of abnormal mitochondrial morphology in motor nerve terminals,[121] liver[122,123] and muscle.[124] Metabolic studies of the central nervous system have found significant reductions in cytochrome oxidase activity,[125] increases in either complex I activity alone,[126] or both complex I and II.[127]

Nonneuronal tissue derived from ALS patients also demonstrates abnormalities in mitochondrial function, including ALS derived platelets.[128] In ALS derived lymphocytes, increased cytosolic calcium concentration and impaired responses to uncouplers of oxidative phosphorylation suggest impaired mitochondrial function.[129]

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 neurofilaments (potential of enhanced cross-linking) and for further damage to the mitochondrial energy transfer site with a resultant increase in mitochondrial proton loss and cell death. Mitochondrial damage will 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 ability to buffer calcium, has been suggested to be a determinant of the motor neuron sensitivity observed in

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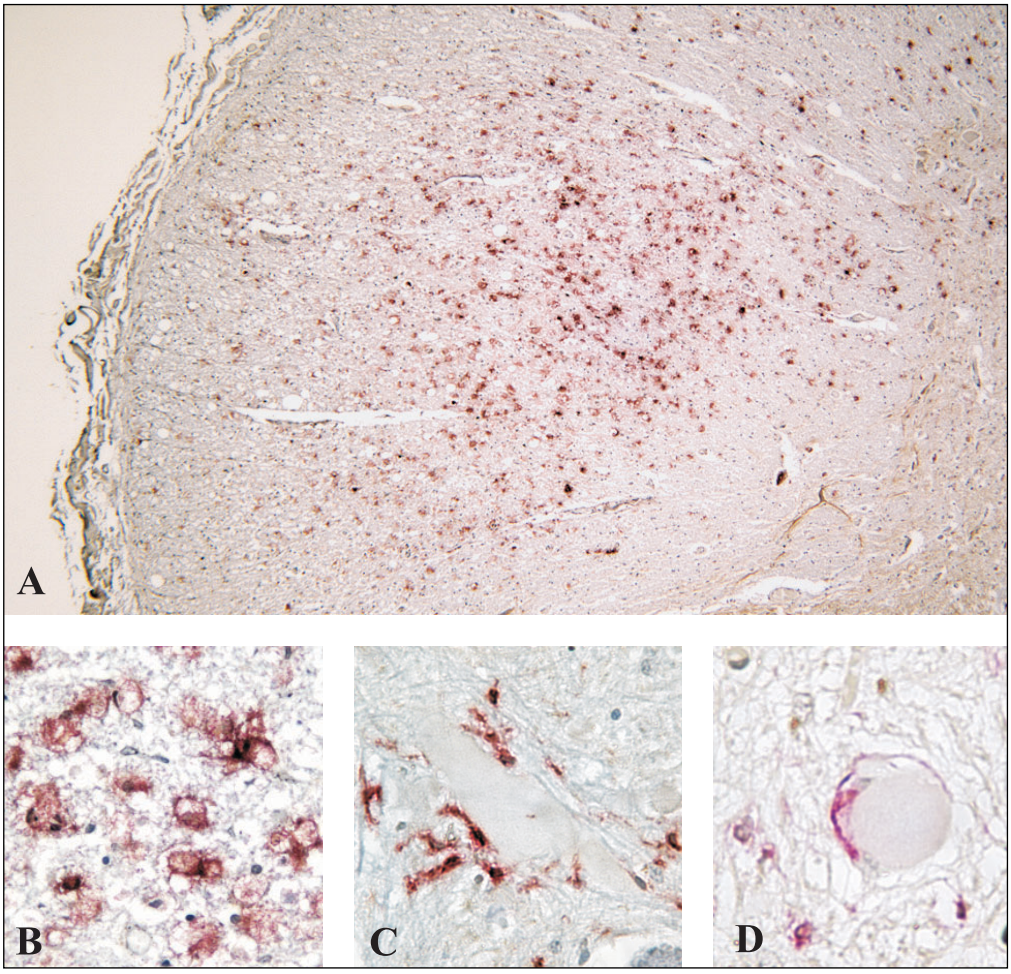

Figure 3: *Microglia activation in ALS assumes two morphological characteristics, including a robust activation and proliferation in the degenerating corticospinal tracts (A; 4x mag.) in which the microglia assume plump, phagocytic morphologies (B; 40x mag.). In the vicinity of degenerating neurons, the microglia are observed in direct opposition to the motor neurons (C; 40x mag.) or surrounding neuroaxonal spheroids (D, 40x mag.). Microglia immunostained with a monoclonal antibody directed to HLA DR with the antigen:antibody conjugate detected using alkaline phosphatase development, yielding a reddish/brown coloration.*

ALS.[92,130-135] Hence, while there is no evidence that ALS is a mitochondrial cytopathy, there is considerable evidence that mitochondrial dysfunction is of significance.

The net effect of the increased generation of reactive oxygenating species, fueled by abnormalities of the electron transport chain and an increased leakage of oxygen radicals from the damaged mitochondria is compounded by a deficiency of key free radical scavenging enzymes.[136,137] As a consequence, increased protein carbonyl formation in both the motor cortex and in the spinal cord has also been observed.[59,126,138,139]

Excitotoxicity in ALS

Although there remains controversy surrounding the potential mechanisms by which glutamate-mediated excitotoxicity might occur in ALS, there is considerable evidence in both the clinical and experimental literature to implicate glutamate-mediated motor neuron toxicity in ALS.[140,141] Glutamate is released into the synaptic cleft when the pre-synaptic terminal is depolarized. It then diffuses across the synaptic cleft to activate the post-synaptic neuron by interacting with either ionotropic (e.g., NMDA, AMPA, or kianate) or metabotropic (G-protein coupled) receptors. Excitatory synaptic transmission is terminated by the rapid uptake of glutamate. Five glutamate transporters have been cloned, including the asctrocyte-specific variant EAAT-2 (GLT-1). Following uptake of glutamate, it is either transaminated to form glutamine or metabolized to α-ketoglutarate; both of which serve as neuronal precursors to glutamate synthesis. Excesses of extracellular glutamate induce neurotoxicity by either increasing neuronal sodium and chloride influx during depolarization, or by an excess of calcium influx. The latter has direct consequences in the activation of a number of calcium-dependant enzymes (i.e., phospholipases, xanthine oxidase, neuronal nitric oxide synthase, etc.) and in inducing DNA damage, lipid peroxidation, and mitochondrial dysfunction.

It was of some interest to then find that a reduction in the glial glutamate transporter GLT-1 and an alteration in the mRNA

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encoding the astrocytic glutamate transporter, EAAT-2 existed in the majority of *s*ALS cases.[142,143] Initially predicted to affect upwards of 80% of *s*ALS patients, this alteration in RNA processing was postulated to give rise to excessive extracellular levels of glutamate, thereby leading to glutamate-mediated cytotoxicity. In subsequent studies, however, it has become less clear that these alterations in RNA processing are specific to ALS.[144-146]

Regardless, astrocytic proliferation is a key neuropathological feature of ALS and, while likely a response to the induction of motor neuron degeneration, it cannot be ignored. Transgenic mice models expressing *f*ALS mutations have been associated with the initial formation of SOD-immunoreactive aggregates in astrocytes, suggesting that, at least in these models, astrocytic pathology may be a harbinger of subsequent neuronal damage. It is also relevant that the *in vivo* activation of the AMPA/kainate receptor decreases the expression of NF mRNA and NF phosphorylation,[147] both of which are key considerations discussed earlier.

Microglial activation in ALS

Although it has been generally held that the immune system plays little, if any, role in the pathogenesis of ALS, microglial (CNS resident macrophages) proliferation and activation is a prominent feature of ALS.[148-151] In the ventral and lateral funiculi of the spinal cord, microglia assume a phagocytic morphology (foamy macrophages) suggesting a not unexpected response to the corticospinal tract degeneration (Figure 3 A&B). In contrast, activated microglia of the ventral horns are in close approximation to otherwise healthy-appearing motor neurons and do not demonstrate the morphology of phagocytic microglia (Figure 3 C&D). In cognitively impaired ALS patients, microglial activation is a prominent feature accompanying superficial linear spongiosis (Figure 1 C&D). The central question remains as to whether such microglial activation participates directly in the pathogenesis of ALS.

The inter-relationship between injured neurons and microglia is complex. When present in the "resting state", microglia have finely branched processes that extend in multiple directions. In response to a variety of pathological insults, microglia rapidly activate and their processes retract and hypertrophy, resulting in a phagocytic morphology. In concert with this activation, microglia upregulate the expression of a number of cell surface antigens and become active secretory cells. The observation of a prominent perineuronal microglial proliferation and migration within 24 hours of a neuronal injury suggests that injured neurons possess the inherent capacity to induce a microglial response.[152,153] Inhibition of this response, for instance in the model of optic nerve transection with inhibition of the intraretinal microglial response with a macrophage inhibitory peptide, is associated with an enhanced rate of optic nerve axon survival and a greater degree of axonal regeneration.[154,155] In contrast, induction of the post-axotomy microglial response with a macrophage stimulating factor at the time of axotomy induces a faster rate of ganglion cell degeneration. Similarly, the *in vivo* inhibition of microglial activation will attenuate neuronal degeneration induced by either ischemia[156] or by the excitatory neurotoxin ibotenic acid.[157]

Microglial neurotoxicity can be mediated through a number of cytotoxic pathways or by phagocytosis. This includes the synthesis of glutamate and other NMDA receptor agonists,[158,159] and of toxic superoxide radicals,[160] the expression of an inducible form of nitric oxide synthase (iNOS) that renders them a potent source of the nitric oxide, relevant to the earlier discussion of oxidative injury, and the secretion of a number of proteolytic enzymes, active lysosomal proteases and arachidonic acid metabolites – all of which are cytotoxic.[161] Microglia can also be neuroprotective, and can inhibit NO-donor (sodium nitroprusside) induced neuronal apoptosis *in vitro* through a TNF-α dependant mechanism.[162]

Hence, the critical issue remains the extent to which microglia participate directly in the pathogenesis of ALS. To address this, we have examined the role of microglia in an experimental model of motor neuron degeneration in which clinical and neuropathological recovery is possible and determined that the absence of a microglial response was permissive to recovery.[163,164] We have subsequently demonstrated that injured motor neurons release soluble factor(s) that induce microglial activation, and that following activation, these microglia are able to stimulate nitric oxide generation in otherwise healthy motor neurons. These findings suggest that microglial cells can in fact be direct participants in the neurodegenerative process of ALS. In this light, the recent observation of increased interleukin-6 levels in CSF of ALS patients is thus of considerable interest, although earlier studies had failed to observe this.[165,166]

Lessons from pharmacotherapeutics

Given the complexity of the biology of ALS described above, it should not be surprising that pharmacologically modifying the course of ALS has been fraught with failure, in spite of the utilization of individual agents with strong theoretical potential to be effective. These include agents potentially designed to inhibit or prevent cell damage (antiglutamatergic or neurotrophic agents, antioxidants, antiviral agents), to enhance neuronal repair (gangliosides), to inhibit immune-mediated damage (immuno-modulatory agents) or to enhance neuromuscular function (monoamines or cholinergic agents). With the sole exception of the antiglutamatergic and neurotrophic therapies, there is no evidence of efficacy for the remaining classes of therapy.[167]

Antiglutamatergic agents

The only antiglutamatergic agent for which a suggestion of efficacy is available is rilutek (Riluzole). Riluzole appears to improve survival but the degree of improvement is small. In the pivotal phase III study, NNT values ranged from 20 to 14 with broad 95% confidence intervals (approaching infinity).[168] Riluzole did not appear to slow the rate of decline of patient functional assessments in either of the pivotal studies, although a subsequent retrospective analysis suggested a prolongation of time spent within a less severely affected stage of the illness.[169] More recent evidence utilizing proton density magnetic resonance spectroscopy has, however, suggested that patients receiving riluzole demonstrate less neuronal loss in the motor strip and may, in fact, demonstrate an arrest of neuronal loss.[170]

A number of other antiglutamatergic therapies have been used without success in ALS. These include L-threonine,[171] branched chain amino acids,[172,173] dextromethorphan,[174] gabapentin,[175] lamotrigine[176] and verapamil.[177]

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Neurotrophic therapies

There is little evidence that the use of neurotrophic factors has had a significant impact on the rate of progression of ALS, with recombinant human insulin-like growth factor (rhIGF-1) amongst the most promising. However, only one of two valid random controlled trials of rhIGF-I revealed results favouring improvement in mortality, rate of clinical decline, and quality of life in ALS. In the North American trial, NNT to progress less than 20 points on A-ALS scale over nine months using 0.1 mg/day rhIGF-1 sc was six (95%CI = 3-25), and to survive 30 months was eight (95%CI = 4-∞).[178] Ackerman and colleagues concluded that rhIGF-1 was most effective in patients at an earlier stage of disease, or if they possessed a more rapid disease course.[179] However, only 53% of patients completed the North American study protocol. In contrast, the European protocol failed to show a significant difference in either measure.[180]

The list of failed neurotrophic factor therapies in ALS is daunting, and includes ciliary neurotrophic factor,[181,182] growth hormone,[183] thyrotropin releasing hormone[184-187] and, most recently, either subcutaneous or intrathecally administered brain-derived neurotrophic factor (BDNF).

Immunomodulatory therapy

In spite of the apparent role of microglia in the disease process, immunomodulatory therapies have been largely unsuccessful. These have included cyclophosphamide,[188-190] cyclophosphamide combined with IvIg[191] or with prednisone,[190] plasmapheresis alone[192] or with azathioprine,[193] total lymph node irradiation,[194] and cyclosporine.[195]

Others

A number of failed clinical trials, while limited in scope, have examined a variety of other treatment modalities in ALS. These have included studies of monoamine therapies utilizing deprenyl[196-198] or L-dopa.[199] Direct attempts at enhancing cholinergic function have also failed, including physostigmine alone[200] or in combination with neostigmine,[201] 3,4-diaminopyridine[202] and tetrahydroaminoacridine.[203] Antiviral therapies, in spite of the recent interest surrounding polymerase chain reaction evidence of viral DNA fragments in ALS motor neurons, have been ineffective.[204,205]

Summary

Can some semblance of cohesion be brought forward from the above? Clearly, the clinical, neuropathological and neurochemical evidence mandates that ALS no longer be considered to be a discrete disorder of the motor neurons, but rather one in which the manifestation of neuromuscular dysfunction is one of a heightened threshold for the development of dysfunction in motor neurons, but not a selectivity. Understanding this propensity for degeneration within selective populations of motor neurons has thus become paramount in understanding the pathogenesis of ALS, and by corollary, its treatment.

Previously thought to be the sole domain of ALS patients whose survival was artificially prolonged through aggressive respiratory support, alterations in cognition are an integral component of ALS within a defined subpopulation of patients. The lessons from *f*ALS would suggest that the development of cognitive impairment in ALS may be under the control of modifier genes, similar in many ways to the determinants of phenotypic variation or risk in Alzheimer's disease. In *s*ALS, the manifestation of cognitive impairment is not an all or nothing phenomenon, but rather a reflection of the total burden of neuropathological damage. Determining whether this is a ubiquitous phenomenon in *s*ALS will require careful longitudinal cliniconeuropathological studies.

The striking diversity of genetic defects observed in both juvenile and adult variants of *f*ALS also attests to the pathogenetic heterogeneity of ALS, yielding clinical syndromes with little clinical variability. The most poignant argument, however, rests with the mutations in the SOD1 gene in which a single enzyme, mutated by a wide variety of point mutations, yields divergent neuropathological and clinical phenotypes.

Integrating the neuropathological and neurochemical features of ALS is somewhat more challenging, but again, the disorder must be considered to be multifactorial and multisystem. In many senses, it may not be relevant for the majority of individuals whether the nature of the initial triggering event is known. Whatever the trigger, motor neurons appear to be placed at a greater risk for disease, based on their large size and extensive axonal processes requiring an abundance of NF and mitochondria. This is coupled with a lack of key calcium binding proteins (e.g., calbindin D-28K and parvalbumin), a lack of the GluR2 AMPA receptor subunit (enhancing its risk for calcium mediated neurotoxicity) and the high expression of the SOD1 enzyme. By the time the illness is clinically evident, a lethal cascade has been established with the involvement of not only multiple biological intracellular processes (including, but not exclusive to, NF aggregate formation with potential axostasis, mitochondrial damage with increased cytosolic calcium and activation of caspase 1, oxidative injury with DNA damage) but clearly involvement of the adjacent glial cells.

Although the proposed deficiency of glutamate transporter EAAT2 remains to be confirmed, there is considerable evidence to suggest that CSF and tissue glutamate levels are increased in ALS and that this will have a deleterious effect on motor neuron survival. This will be further augmented by the nature of the interaction between microglial cells and motor neurons. Upon injury, motor neurons signal to microglia to induce proliferation, upregulation of activity, and migration. Stimulated microglia are amongst the most potent generators of glutamate in the central nervous system. Failure of glutamate uptake by astrocytes defective in EAAT2 would leave the already vulnerable motor neuron open to excitotoxic injury. The increased influx of calcium induced by such glutamate mediated activation of NMDA receptors will not have a single effect, but rather a cascade of effects that would enhance cellular injury. This includes upregulating nNOS expression and activation, with a consequent increase in nitric oxide generation and the formation of reactive metabolites. We have shown that, in the presence of neurofilament aggregates induced by alterations in NF stoichiometry, motor neurons are at a higher risk for the development of a neurotoxic cell death.[114]

Finally, the virtually total failure of pharmacotherapeutic agents to impact on ALS progression, in spite of multiple potential sites of effect, strongly suggest that the biological process of ALS is far more complex than anticipated. A

parsimonious view of ALS thus should include the intimate nature of the interactions between all of these cell types and the suggestion that once induced, this triumvirate of cells (motor neuron, astrocyte and microglia) is largely responsible for the manifestations of ALS as we recognize them. Pharmacotherapy should thus reflect a similar approach to the triumvirate.

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