Immune-mediated injury of motoneurons in ALS

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Introduction

Although our understanding of the etiology of sporadic neurodegenerative disease such as ALS is limited, there is no shortage of models of pathogenesis and selective vulnerability of cell injury in these disorders. Our own studies document that calcium is increased in motor nerve terminals in human ALS [1], and that increased calcium within motoneurons can be reproduced by the passive transfer of ALS IgG to mice [2]. Ionized calcium (Ca$^{2+}$) is the most common signal transduction element in both nonexcitable and excitable cells such as neurons and is tightly regulated through numerous binding and specialized exclusion proteins [3]. Prolonged high intracellular calcium levels have been associated with cell death in numerous systems [4] and appear to be a key event in motoneuron degeneration in sporadic amyotrophic lateral sclerosis.

In the discussion which follows, we delineate the evidence for the hypothesis that calcium influx and altered calcium homeostasis may play a pivotal role in motoneuron injury and neurodegeneration in amyotrophic lateral sclerosis. We also suggest that the triggering mechanism may be an autoimmune process, which enhances the entry of extracellular calcium, activating several signal transduction elements as well as the release of neurotransmitters, possibly including glutamate. The relative absence of calcium binding proteins in motoneurons [5], the down-regulation of glial glutamate transport [6], and the presence of inflammatory cells [7,8] could aggravate the initial motoneuron injury leading to cell death.

Altered calcium in motor nerve terminals in sporadic ALS

To determine whether increases in intracellular calcium and altered morphology are present in motor nerve terminals of ALS patients in vivo, muscle biopsies were obtained from ALS patients, nondenervating disease controls, and patients

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with denervating neuropathies. Rapid fixation with oxalate-pyroantimonate was employed to preserve in situ calcium distribution [2,9], and the specimens were analyzed with ultrastructural techniques. The calcium content of precipitates were confirmed by electron spectroscopic imaging. In presynaptic terminals from disease control samples, calcium-containing precipitates were present primarily in synaptic vesicles and were rarely seen in mitochondria or other membrane enclosed structures [1]. In presynaptic terminals from ALS patients, the calcium present in mitochondria was increased but the calcium precipitates in synaptic vesicles were similar to controls (Table 1). In addition, the volume of mitochondrial calcium precipitates, normalized either to mitochondrial or terminal volume, demonstrated increased calcium accumulation in the motor nerve terminals from the ALS patients compared either to non-denervating disease controls or denervating neuropathies. Furthermore, synaptic vesicle density in terminal cross-sectional profiles as well as within active zones were significantly higher in ALS than in either of the control groups. The increased mitochondrial calcium precipitates and synaptic vesicle density did not appear to be related either to denervation, reinnervation, or sprouting. In denervating neuropathies, Schwann cell envelopment was increased and also differentiated these specimens from ALS specimens. Furthermore, the average cross-sectional area of axon terminals, and the ratio of postsynaptic to presynaptic surface membrane was the same in ALS and control motor terminals. These data provide the first direct evidence for the presence of increased neuronal calcium in amyotrophic lateral sclerosis, and also suggest the importance of the presynaptic motor terminal as a targeted site in the disease.

**ALS IgG selectively increase intracellular calcium in motoneurons in vivo**

To determine whether the increased intracellular calcium and ultrastructural alteration in ALS patients in vivo can be triggered by immune mechanisms, IgG fractions from six ALS patients were injected intraperitoneally into mice. Following fixation with the oxalate-pyroantimonate technique, motoneurons, sensory neurons, and Purkinje
cells were assayed by ultrastructural techniques for calcium content [2]. After 24 h, all six ALS IgG (40 mg/animal) increased vesicle number in spinal motoneuron axon terminals, and in boutons synapsing on spinal motoneurons. These ALS IgG also produced a dose-dependent increase in calcium in axon terminal synaptic vesicles and mitochondria. ALS IgG with the highest titer of calcium channel antibodies also demonstrated increased calcium in rough endoplasmic reticulum, mitochondria, and Golgi complex of spinal motoneurons and frontal cortex pyramidal cells. These changes in calcium precipitates as well as in mitochondria and synaptic vesicles were restricted to motoneurons, and were not noted in spinal cord sensory neurons or in Purkinje cells. Furthermore, IgG from a normal control, a patient with Guillain-Barré, with myasthenia gravis, with Lambert-Eaton myasthenic syndrome, with peripheral neuropathy, and with Alzheimer's disease, did not alter either the calcium content or the motoneuron ultrastructure.

Our laboratory had previously demonstrated that the passive transfer of ALS IgG to mice could alter motoneuron function in vivo, producing a selective increase in acetylcholine release from motor nerve presynaptic terminals, and an increase in postsynaptically measured miniature endplate potential frequency [10]. We postulated that these ALS IgG-mediated increases in acetylcholine release had resulted from increased intraterminal calcium [10,11]. Our ultrastructural study confirmed the presence of increased calcium-containing precipitates in synaptic terminals and provided a plausible explanation for the enhanced neurotransmitter release. However, although increased intraterminal calcium could enhance acetylcholine release [12,13], the latency prior to fixation in our ultrastructural study prevented us from attributing specific physiologic relevance to the increased calcium demonstrated at the axon terminals. Nevertheless, it is of interest that reductions in quantal number with concomitant increased probability of quantal release have been documented at neuromuscular junctions of patients with ALS [14].

The dilatation of the Golgi system following the administration of ALS IgG may reflect early changes which could subsequently lead to degeneration. Gonatas et al. [15] demonstrated Golgi system fragmentation as an early sign of cell damage in ALS spinal motoneurons. However, the presence of small amounts of calcium in the Golgi complex of normal motoneurons from untreated animals with no alterations in morphology, and the presence of calcium precipitates in the Golgi system of oligodendroglia with no morphologic changes, may suggest that the entry of calcium into the Golgi system may be a secondary consequence of damage to the Golgi system rather than the cause of such damage. Nevertheless, it is of considerable interest that the passive transfer of ALS IgG to mice can reproduce, at least to a limited extent, the early involvement of the Golgi apparatus which might lead to the fragmentation described by Gonatas.

The ability of ALS IgG to enhance calcium precipitates in synaptic boutons on ventral horn spinal motoneurons is of particular interest since many of the boutons synapsing on motoneurons are glutamatergic [16]. Although our techniques did not identify specific neurotransmitters, it is possible that the increased calcium precipitates as well as increased vesicle density could be associated with increased transmitter release such as glutamate, just as the increased calcium precipitates are associated with increased acetylcholine release at the neuromuscular junction.
For the present discussion, the main point is that ALS IgG administered to mice produce the changes in increased axon terminal calcium and increased vesicle density noted in ALS patients in vivo. Such data suggest the importance of ALS IgG in triggering the ultrastructural changes, and also call attention to the motor axon terminal as a target for some of the earliest changes in motoneurons since all our reported changes were observed within 24 h after injection of IgG. Since glutamate receptors have not been described to play a role in enhancing neurotransmitter release at the mammalian neuromuscular junction, it appears unlikely that excitotoxic mechanisms trigger the ultrastructural and physiological changes. However, the demonstrated alterations (increased calcium precipitates and vesicle density) of synaptic boutons on the cell body of the motoneuron suggest that enhanced release of glutamate could occur, and could aggravate the initial motoneuron changes. Our current efforts are in following such ultrastructural changes over more prolonged periods to determine whether motoneuron degeneration can occur. Nevertheless, even within 24 h, ALS IgG can increase intracellular calcium in motor axon terminals and cell bodies of upper and lower motoneurons.

Cytotoxic effects of ALS IgG on a hybrid motoneuron cell line

To define the cellular and molecular mechanisms underlying the effects of ALS IgG on cell survival, a motoneuron–neuroblastoma hybrid cell line (VSC 4.1) was developed with techniques previously employed to develop substantia nigra hybrid cells [17]. Like mammalian motoneurons, these motoneuron hybrid cells contain specific antagonist binding sites for L-type voltage-gated calcium channels (VGCC) (dihydropyridine), N-type VGCCs (ω-conotoxin), and P-type VGCCs (agatoxin IVa). These cells also possess many other properties similar to both rat motoneurons and other motoneuron hybrids previously reported in other laboratories [18,19]. Using either direct viable cell counts, or quantitation of propidium iodide and fluorescein diacetate-labeled cells, or lactate dehydrogenase release to assess cell survival, we documented that ALS IgG killed 40–70% of cyclic AMP differentiated VSC 4.1 cells within two days [20]. The sporadic ALS IgG-mediated cytotoxicity could be prevented by 1,000-fold reduction of extracellular Ca\(^{2+}\) with EGTA, by preincubating immunoglobulins with purified intact L-type VGCCs or with VGCC \(\alpha_1\)-subunit (but not with other VGCC subunits), or by preincubating cells with inhibitors of N-type or P-type VGCCs (but not with inhibitors of dihydropyridine-sensitive L-type VGCCs).

In physiological experiments using whole cell patch-clamp of cyclic adenosine monophosphate (cAMP)-differentiated VSC 4.1 cells, sporadic ALS IgG significantly increased high-threshold VGCC currents. These effects were reproducible using immunoglobulins purified from sera of five of six patients with sporadic ALS, but were not noted with any of eight disease control IgG preparations [21]. The calcium currents were blocked by the polyamine funnel-web spider toxin, FTX, which has been previously shown to block Ca\(^{2+}\) currents and evoke transmitter release at mammalian motoneuron terminals. Such data suggested that antibody-induced
increases in calcium entry through voltage-gated calcium channels may occur in motoneurons (21).

Using laser scanning confocal microscopy and the Ca$^{2+}$-sensitive dye fluo-3, we also demonstrated that sporadic ALS IgG-mediated Ca$^{2+}$ entry into VSC 4.1 cells indeed increased intracellular Ca$^{2+}$ [22]. A fast transient increase was observed within 15–120 s of IgG addition, and a second slower progressive increase was found within 4 h of the initial transient. The fast transient appeared to correlate with cytotoxicity as monitored in parallel experiments.

Furthermore, the observed cell death was apoptotic, as DNA fragmentation and laddering were observed within 12 h of sporadic ALS IgG addition [23]. Cell blebbing and fragmentation began shortly thereafter. Sporadic ALS IgG-induced apoptosis could be prevented by preincubation of cells with the endonuclease inhibitor aurintricarboxylate or with the protein synthesis inhibitor cycloheximide but not by inhibitors of glutamate receptors. In differentiated VSC 4.1 cells, glutamate alone did not cause cytotoxicity nor did it enhance the cytotoxic effects of added sporadic ALS IgG. Nevertheless, since adult mammalian motoneurons are known to have several different glutamate receptors, the absence of any effects of glutamate in our motoneuron cell line does not rule out a potential role for glutamate in motoneuron degeneration in vivo.

Selective vulnerability of motoneurons

Selective vulnerability of motoneurons is unlikely to be explained by interaction of ALS IgG with unique calcium channels in motoneurons, since such channels are present in the plasma membranes of most neurons, and antibodies to VGCC should therefore affect most neurons. Purkinje cells, for example, have an abundance of P-type calcium channels, and ALS IgG can be documented to enhance calcium current through P-type calcium channels [24]. However, Purkinje cells are not compromised in ALS. Furthermore, in our passive transfer experiments, increased calcium was not detectable within Purkinje cells, suggesting that regulation of calcium homeostasis may be more active or more finely adjusted in Purkinje cells than in motoneurons. Thus, a susceptible calcium channel, per se, cannot explain the pattern of selective vulnerability, and other factors may also influence the ability of neurons to cope with an increased calcium load.

Among the factors which are increasingly being recognized to play a role in calcium homeostasis are the calcium binding proteins, calbindin-D$_{28K}$ and parvalbumin [25]. These proteins are elevated in Purkinje cells, and significantly reduced or practically absent in adult motoneurons. In fact, the regional lack of calbindin-D$_{28K}$ and/or parvalbumin immunohistochemical reactivity in motoneurons parallels the known selective vulnerability of motoneurons in ALS (where motoneurons affected early in the disease lack immunoreactivity for these calcium binding proteins), while motoneurons controlling eye muscles (cranial nerves III, IV, VI) as well as Onuf's nucleus motoneurons controlling bladder muscles, are relatively spared and have high levels of calcium binding proteins [5,26]. In addition, in our motoneuron cell line, VSC 4.1, only differentiated cells are injured by ALS IgG, and such differentiated
cells lack calbindin-D_{28k} and parvalbumin [5]. Undifferentiated VSC 4.1 possess ample calbindin-D_{28k} and parvalbumin and are relatively resistant to cytotoxic effects of ALS IgG. To determine the specificity of these findings for the hybrid motoneuron cell line, we examined two other cell lines, the parent neuroblastoma, N18TG2 cells, as well as a substantia nigra-neuroblastoma hybrid, MES23.5 cells [17]. Both of these cell lines retained immunoreactive calcium binding proteins following differentiation, and demonstrated no significant cytotoxic effects of ALS IgG.

To provide additional evidence for the role of calcium binding proteins in motoneuron vulnerability, VSC 4.1 cells were infected with a retrovirus carrying calbindin-D_{28k} cDNA under the control of the promoter of the phosphoglycerate kinase (PGK) gene. Differentiated calbindin-D_{28k} cDNA infected cells expressed high levels of calbindin-D_{28k}, and increased resistance to ALS IgG-mediated toxicity. Further, treatment with calbindin-D_{28k} antisense oligodeoxynucleotides, which significantly decrease calbindin-D_{28k} expression, rendered these cells vulnerable again to ALS IgG toxicity [27].

The function of these calcium binding proteins is still unclear, but they appear to enhance calcium homeostasis and may alter calcium entry through VGCCs as well as promote calcium extrusion and compartmentalization [28].

**ALS pathophysiology: converging mechanisms**

Our ultrastructural studies of human ALS motor nerve terminals demonstrate increased intracellular calcium, altered mitochondrial volume and synaptic vesicle density. Similar changes can be demonstrated by the passive transfer of ALS IgG to mice. Furthermore, in vitro, ALS IgG can be demonstrated to enhance calcium entry through voltage-gated calcium channels leading to apoptotic cell death. The cytotoxicity appears to be triggered by transient Ca^{2+} entry, suggesting that Ca^{2+} may initiate a signal transduction cascade leading to cell injury. An important question is how to relate an immune-mediated mechanism manifested by increased intracellular Ca^{2+} leading to motoneuron injury with the finding that glutamate can injure motoneurons in vitro by activating neuronal AMPA/kainate receptors [29]. Excitotoxic mechanisms could also explain why synaptosomes from ALS spinal cord have decreased glutamate uptake [30], and why the glial glutamate transporter is downregulated in ALS tissue [6]. However, it is unclear what triggers the changes in glutamate metabolism. At present, there is no evidence for a genetic defect responsible for the glutamate alterations, and thus it is likely that such changes are secondary to another event. For example, motoneuron loss is associated with decreased glutamate transport in MND mice [31], and many different events including IgG enhanced glutamate release could also downregulate the glial glutamate transporter. Proof for such a concept is lacking, but it is of interest that calcium precipitates are noted in synaptic boutons on spinal motoneurons following passive transfer of ALS IgG; and such increased calcium could be associated with increased release of constituents such as glutamate and could add to the motoneuron damage caused by the entry of Ca^{2+} through VGCCs.
Free radicals could also derive from increased intracellular Ca\(^{2+}\) by enhancing mitochondrial activity [32] as well as oxidative injury [33,34]. Ca\(^{2+}\) chelators can prevent H\(_2\)O\(_2\)-induced DNA breakdown in cytotoxicity [35]; and increased intracellular Ca\(^{2+}\) may be derived from intracellular stores and induce DNA fragmentation [36].

The mechanism by which mutations in SOD1 in familial amyotrophic lateral sclerosis can lead to motoneuron cell death is unknown [37,38]. Suggestions that a decrease in the enzymatic activity of Cu/Zn SOD may be responsible for cell death have been questioned because SOD activity is not decreased, but is, in fact, increased in transgenic mice which express the G93A mutation and demonstrate motoneuron degeneration [39]. The role of both increased and decreased SOD enzyme activity have been questioned, since mutations leading to motoneuron degeneration appear to involve amino acid residues associated with enzyme dimerization rather than amino acid sequences corresponding to the active site [37,38,40,41]. Of great interest are the recent studies of Rabizadeh et al. [42] in which expression of the SOD1 mutants A4V and G37R in immortalized mammalian neural cells increased SOD1 activity, but more significantly, enhanced apoptosis. Such findings dissociate the mutant SOD1 enzymatic activity from an effect on neural cell death, suggesting another yet unknown mechanism by which SOD1 mutation may bring about neural cell death. Since the mechanism of cell death could be apoptotic it is possible that increased intracellular calcium may play a key role, making motoneurons especially vulnerable in SOD1 mutations.

Thus, regardless of whether the insult to motoneurons is mediated by enhanced apoptosis in SOD1 mutations, or by antibodies enhancing the entry of calcium through neuronal channels and/or excitotoxic mechanisms, the presence of increased intracellular Ca\(^{2+}\) and the relative absence of calbindin-D\(_{28K}\) and parvalbumin appear to be major factors in the selective vulnerability of motoneurons to cell death. Clearly, further studies are necessary to define the mechanisms by which increased intracellular calcium can lead to motoneuron cell death, and the multiple sites which may be available for therapeutic intervention.

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