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REVIEW

Glutamate pathway implication in amyotrophic lateral sclerosis: what is the signal in the noise?

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Abstract: The cause of the fatal motor neuron disease, amyotrophic lateral sclerosis (ALS), remains largely unknown. Most cases of ALS are sporadic and, for ~20% of familial ALS patients, mutations in the superoxide dismutase-1 (*SOD1*) gene have been identified. Transgenic rodents overexpressing mutant SOD1 emulate the disease and constitute the best ALS animal model so far. Several lines of evidence suggest that ALS is a multifactorial condition. In this review, we discuss the question of the involvement of the glutamate pathways in ALS-induced motor neuron death. As such, we review the data implicating glutamate metabolism alterations, glutamatergic environmental toxins, glutamate transporter/receptor defects, and Ca^{2+}-mediated glutamate toxicity in the etiopathogenesis of ALS. Given the published data, we contend that glutamate-induced neurotoxicity more likely precipitates motor neuron degeneration rather than being the initiating factor of ALS. Furthermore, we propose that glutamate-induced neurotoxicity participates in the ALS deadly molecular cascade only as an executioner to put an end to a series of molecular perturbations that have irreversibly compromised motor neuron function. This could provide an explanation for the modest effect of therapeutic strategies targeting the glutamatergic system, including the only currently FDA-approved ALS treatment, riluzole. As in diseased motor neurons, overwhelming Ca^{2+} overload may be the converging point for glutamate, endoplasmic reticulum stress, and mitochondrial dysfunctional pathways, and only therapies targeting these simultaneously or targeting the earliest alterations initiating this deleterious cascade may have a real impact on halting ALS progression.

Keywords: glutamate, excitotoxicity, amyotrophic lateral sclerosis, glutamate receptors, glutamate transporters, calcium

Overview

One of the simplest and most abundant molecules of the living, the proteinogenic L-amino acid glutamate, is a major excitatory neurotransmitter of the mammalian central nervous system (CNS). Glutamate belongs to the family of excitatory amino acids (EAAs) and is widely distributed throughout the CNS. Glutamate transmission is implicated in functions as diverse as learning, memory, sensori-motricity, brain development, and endocrine control of peripheral organs.[1–3] In addition to its role in neurotransmission, glutamate can kill neurons. The first observation of glutamate neurotoxicity was reported by Lucas and Newhouse,[4] who described that parenteral administration of monosodium L-glutamate to newborn mice triggers retinal cell degeneration. Subsequently, Olney and collaborators[5] showed that the lesion in immature rodents, caused by L-glutamate, is not restricted to the retina, but extends to central structures such as the hypothalamus, not yet protected by the blood–brain barrier (BBB). Since

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the mechanisms of Na^+-dependency of glutamate-induced neuronal depolarization started to be recognized[6] a few years before, Olney envisioned a possible relationship between the excitatory and the cytotoxic effects of glutamate, thus giving birth to the concept of "excitotoxicity" or excessive stimulation of glutamate receptors (GluRs).[7]

One can wonder whether the attribution of such powerful neurotoxicity to a ubiquitous molecule like glutamate was a regrettable error or was intended by nature. One speculation may be that, originally, it aimed at providing the brain with a cell suicide back-up plan to shut down diseased cells and to protect the integrity of the system. Whatever the answer, the discovery of excitotoxicity stressed the importance of glutamate reuptake after synaptic release not only to maintain the phasic character of neurotransmission but also to guarantee neuronal survival. Excitotoxicity became, thereafter, one of the prime suspects responsible for neurodegeneration in CNS insults. In the literature, there is now a plethora of evidence of increased extracellular glutamate levels and glutamate uptake dysfunction in numerous acute insults, such as ischemia or traumatic brain injury, and in chronic degenerative diseases, such as Huntington's disease, Parkinson's disease, Alzheimer's disease and amyotrophic lateral sclerosis (ALS),[8] although much more controversial. Even though over the years the excitotoxicity advocates built up a strong case for the involvement of excitotoxic processes in neuronal death associated with acute insults, the situation on chronic neurodegenerative diseases is still murky.[9] It certainly appears less intuitive to relate excitotoxicity to very diverse, progressive, and selective neurodegenerative processes than to acute focal waves of cell death.

In this review, we will focus on glutamate pathways in the relentless motor neuron (MN) disease, ALS, for which there is abundant literature and which has been reviewed extensively in the past. Here, we will try to give the reader a new take on the debate of glutamate/EAA dysfunction in ALS by providing a critical appraisal of the published data on the topic and by highlighting how an old hypothesis like this may be related to the newest theories on the cellular and molecular mechanisms underlying the ALS neurodegenerative process, such as mRNA defects and non-neuronal cell toxicity. After introducing glutamate/EAA-linked cytotoxic mechanisms and ALS, we will discuss the controversial history of glutamate in ALS with a particular emphasis on glutamate transport alterations, which were the first evidence to put astrocytes and noncell autonomous toxic processes at center stage in ALS. Then, we will review how GluR specificities and mRNA modifications are related to a possible peculiar vulnerability of MNs to excitotoxicity and the development of MN diseases such as ALS. Behind the mask of glutamate or other toxic processes such as oxidative stress, many believe that the real MN terminator is Ca^{2+}. We will examine how properties associated with Ca^{2+} attempt to explain the selective degeneration of MNs in ALS and how glutamate-associated Ca^{2+} overloads are related to endoplasmic reticulum (ER) stress and mitochondrial defects, two alternate pathogenic mechanisms that may account for MN demise in ALS. Our final objective is to come up with the clearest message on the level and degree of implication of glutamate dysfunction in ALS and on the perspectives that it possibly opens for therapy.

Excitatory amino acid-associated death processes

Excitotoxicity is defined as cell death resulting from excessive stimulation of GluRs, which is hypothesized to follow increased release or decreased reuptake of synaptic glutamate. However, which characteristics of GluRs may underlie this deadly capacity?

Glutamatergic receptors are classified into ion channel-coupled or ionotropic receptors (NMDA, AMPA, and kainate [KA]) and G protein-coupled or metabotropic receptors (class I, II, and III). Fast excitatory transmission is mediated by AMPA/KA receptors through the entry of Na^+ and K^+ ions triggering the instantaneous depolarization of neurons and the removal of NMDA receptor Mg^{2+} block. NMDA receptors are responsible for a slower and longer-lasting transmission and are characterized by their high permeability for Ca^{2+}, as well as Na^+ and K^+. Initially, NMDA receptors were considered as the main source of extracellular Ca^{2+} in neurons. Later, some specific forms of AMPA receptors lacking the GluR2 subunit were also identified as Ca^{2+} permeable.[10] The properties of GluR2 are generated post-transcriptionally by RNA editing at the Q/R site by the enzyme called adenosine deaminase acting on RNA 2 (ADAR2) leading to a substitution of the neutrally charged glutamine (Q) amino acid by a positively charged arginine (R).[11,12] Therefore, AMPA receptors containing an unedited GluR2 subunit are also Ca^{2+} permeable. Diversity of AMPA GluR is also obtained by alternative splicing of subunits at the flip/flop and/or C terminal sites leading to differential desensitization properties.[13,14] Finally, metabotropic receptors are responsible for a long-lasting modulation of glutamatergic transmission through G-protein-regulated enzymes producing several second messengers, such as cyclic adenosine monophosphate or Ca^{2+}.[14]

Pioneer in vitro studies, aimed at unraveling the mechanisms of excitotoxicity, have shown that GluR-mediated neurotoxicity can be separated into two components, based on differential time course and ionic dependence: an acute limited and a delayed massive toxicity, underlain by Na^+ and Ca^{2+} influx, respectively.[15,16] The acute neurotoxicity is characterized by neuronal swelling, and ultimately, lysis. This can be prevented by removal, from the culture medium, of Na^+ and Cl^- responsible for massive water entry during GluR cationic channel opening. In contrast, the delayed neurotoxicity was demonstrated to be supported by Ca^{2+} entry and its progression is not altered by prevention of the acute excitotoxicity. Among neurotransmitter receptors, permeability to Ca^{2+} being the privilege of GluRs, the lion's share of attention in excitotoxicity research has been paid to the Ca^{2+}-mediated delayed form of glutamate neurotoxicity, which can be more easily dissociated from the simple neuronal excitation. Subsequently, the necessity of prolonged intraneuronal Ca^{2+} increase to produce neuronal damage was reported.[17] It has also been documented that voltage-dependent Ca^{2+} channels, reversal of the Na^+/Ca^{2+} exchanger and group I metabotropic receptors through inositol tri-phosphate-mediated ER Ca^{2+} release may contribute to the sustained Ca^{2+} elevations during excitotoxic processes.[18] Excess free intracellular Ca^{2+} is then thought to trigger a destructive cascade via activation of proteases, phospholipases, endonucleases, and pro-oxidant enzymes leading ultimately to oxidative stress and neuronal death.[19]

Since consistent evidence of increased glutamate or other EAA levels have not been provided in chronic neurodegenerative diseases and even as physiological concentrations of glutamate become toxic during energy failure,[20] an alternative concept referred to as "secondary" or "slow-onset excitotoxicity" has developed over the years. According to this theory, in absence of any alteration of its extracellular level, glutamate may induce the death of specific neuronal populations that were hypersensitized by a genetic, endogenous or exogenous metabolic factor.[9] Impairment of energy metabolism, trophic support, oxidative stress, or alteration in GluR ionic permeability may constitute examples of such factors leading to an exacerbated neuronal vulnerability to normal glutamate levels. This hypothesis may appear tantalizing by providing some clues to why distinct neuronal populations are selectively degenerating in various chronic nervous system disorders.

Of note, the overstimulation of GluRs is not the only pathway through which increased extracellular glutamate concentrations were shown to be cytotoxic. A form of toxicity resulting from the inhibition of the xc^- cystine-glutamate antiporter by high extracellular glutamate levels has been described in vitro.[21,22] Termed "oxidative glutamate toxicity", this type of cell death is underlain by an impairment of cystine uptake, leading to glutathione depletion and oxidative stress, cystine being the limiting factor in glutathione synthesis. Interestingly, it has been suggested that oxidative glutamate toxicity may be part of the excitotoxicity cascade.[23] However, the relevance of this mechanism for mature neurons remains uncertain as this antiporter is mainly expressed by astrocytes and by immature neurons only.[24]

Amyotrophic lateral sclerosis

ALS has been described by many as one of the most dreadful diseases of the human condition. This midlife-onset paralytic disorder, inexorably lethal in 2 to 5 years after diagnosis, progresses very aggressively to lock an intellectually preserved human being in a motionless body. Only the occulomotor and continence controls are usually spared.[25] The incidence of ALS is about 1 to 2 in 100,000.[26] Pathological analyses have revealed that ALS is characterized by the progressive degeneration of the upper MNs or the lower MNs or both.[27] In the vast majority of cases (~90%), ALS is documented as a sporadic condition, ie, of unknown etiology. In the remaining ~10% of ALS cases associated with a familial history, mutations in several genes have been identified (for review see[28]). Clinically, familial and sporadic ALS are impossible to tell apart. The most commonly mutated genes in familial ALS, accounting for approximately 20% of all the cases, is the gene encoding for the enzyme superoxide dismutase-1 (SOD1).[28] To date, more than 150 mutations in this enzyme have been identified in ALS families (http://www.alsod.org) even if the pathogenicity of two of them has recently been challenged.[29] SOD1 is a ubiquitously expressed, abundant cytosolic enzyme[30] whose role is to catalyze the dismutation of superoxide into oxygen and hydrogen peroxide. Transgenic mice expressing either catalytically active SOD1 mutants[31,32] or catalytically inactive SOD1 mutants[33,34] develop a paralytic disorder emulating the clinical and neuropathological hallmarks of human ALS. In contrast, transgenic mice expressing high levels of wild-type human SOD1stay healthy,[32] even if several neuromuscular junction abnormalities were described in 24 month-old mice suggesting a premature aging of the nervous system as seen in Down's syndrome.[35] Based on these observations and others, the consensus now is that it is through a toxic gain-of-function effect that mutant SOD1 causes MN degeneration. However, despite 15 years of extensive work on this only available animal model of ALS,

the nature of the mutant SOD1 acquired deleterious effect remains elusive. To date, it has been proposed that mutant SOD1 cytotoxicity involves different mechanisms including excitotoxicity,[36] glutamate transporter failure,[37] oxidative stress,[38,39] protein aggregation,[40] aberrant protein–protein interactions,[41] decreased binding affinity for zinc,[42] mitochondrial dysfunction,[43,44] ER stress,[45] and apoptosis,[46,47] none of which are mutually exclusive. Pertinent to the possible implication of excitotoxicity in a MN disease like ALS is the fact that glutamate appears to be a key neurotransmitter in the human motor system. Indeed, MNs may receive massive glutamatergic innervation from the corticospinal tracts,[48] the spinal excitatory interneuronal pathways,[49] and the cortico-cortical association pathways.[50] On the other hand, during these last few years, an important insight made in ALS SOD1 rodents is the notion that mutant SOD1 in both MNs and non-neuronal cells, especially glial cells like astrocytes and microglia contribute to the disease process in vivo[51–55] and that in vitro mutant SOD1 expression in astrocytes is sufficient to selectively kill otherwise healthy MNs[56–58] through a soluble toxic mechanism.[57] Very recently, ALS research has turned its attention toward RNA metabolism alterations as a possible unifying cause of the degenerative process following the identification, in familial and sporadic patients, of mutations in different RNA processing proteins such as TAR DNA-binding protein 43 (TDP43)[59–61] and fused in sarcoma/translated in liposarcoma (FUS/TLS).[62,63] Mutations in the TDP-43 and FUS/TLS genes account for 5% and 4%, respectively, of familial ALS patients. It can be expected that the development of animal models of these new mutations may help ALS research leap forward in the near future.

Glutamate and ALS: history and controversies

Glutamate levels in ALS patients and animal models: the roots of the debate

The first publication found in PubMed describing an investigation related to glutamate in ALS was published in 1978.[64] This study, aimed at comparing amino acid levels in 12 ALS patients with those of 12 control patients, matched for age, sex, and severity of disability (affected by diverse paralytic disorders), reports that glutamate concentrations are unchanged in cerebrospinal fluid (CSF) and not significantly increased in serum and urine, suggesting that there is no glutamate-associated specific signature in ALS patients. However, this was challenged a decade later, when plasma[65] and CSF[66] glutamate levels of 18 to 22 ALS patients were shown to be doubled compared to healthy controls and other neurological disorders patients. From then, the controversy continued, as some groups confirmed glutamate increases in plasma[67] or CSF[68] while others did not.[69,70] Thereafter, claiming improvement in measurement techniques and in patient subclassification, several studies attempted to reconcile these divergent findings by highlighting that patients with spinal onset, but not bulbar onset, may possess higher glutamate levels in the CSF[71] and/or plasma.[72,73] Similarly, glutamate augmentations in serum were correlated to patients exhibiting severely progressive ALS versus patients with milder disease courses.[74] Even if the most recent study published finds significant elevations of glutamate in ALS patients' CSF in absence of consistent correlations to clinical presentation or duration,[75] overall, it appears reasonable to agree on the facts that: 1) glutamate elevation in patients' fluids is not a universal metabolic signature of ALS patients and hence a very unlikely factor to account for the induction of MN degeneration in ALS; 2) fluid glutamate increases are, however, a characteristic of a subset of ALS patients that needs to be further clarified to see if extracellular glutamate may be involved in modulating disease progression in these patients. Of interest, in the rodent models of ALS, the question of increased fluid glutamate levels was less investigated, or may be less reported due to negative results. In the SOD1^{G93A} mouse model, while one group has measured, by intracerebral microdialysis, a significant increase in glutamate in the cortical extracellular fluid,[76] these findings could not be confirmed by a subsequent study,[77] and another group found no difference in glutamate CSF and plasma levels among different stages of the disease,[78] providing some closure to this question.

In theory, it is not so trivial to connect glutamate elevation in patients' fluids such as plasma, serum, or even CSF, to MN death. Relevant to this issue, even if, again, some divergent data have been published,[79,80] it seems that the CSF of ALS patients is neurotoxic to cultured cortical neurons (~40% decrease in survival),[81] to spinal MNs (~60%) and, to a lesser extent, to other spinal neurons (~15%).[82] Although CSF glutamate levels have not been measured in these works, the authors have shown that the observed toxicity could be prevented by AMPA/KA GluR antagonists, but not by NMDA antagonists, supporting the involvement of excitotoxicity. In a third study that also does not report CSF glutamate concentrations, both AMPA blockade and metabotropic GluR class-I modulation showed some neuroprotective effect against ALS CSF toxicity.[83] Intriguingly, the latter study reported that the addition of glutamate at concentrations that either mimic

those found, on average, in ALS patients' CSF (ie, 5 μM), or 10 times higher (ie, 50 μM), did not recapitulate the reported ALS CSF neurotoxicity. Further, the authors showed that ALS CSF toxic activity is retained on a 5 kDa filter and is not lost after a 15 minutes of boiling, suggesting that the neurotoxic factor(s) is a macromolecule resistant to heat and, by far, higher in molecular mass than glutamate (147.13 Da). In keeping with this, a very recent study demonstrates no correlation between glutamate concentrations in human patients' CSF and in vitro neurotoxic potency.[75] Yet, in contrast to all of the previous studies, it was found that NMDA antagonists offer a complete rescue of cortical neuron survival whereas rescue is only partial with AMPA/KA antagonists. In summary, most of the studies point to the neurotoxicity of ALS patients' CSF, preferentially for MNs, recently proven to be independent of CSF glutamate levels, and possibly, mediated by an unknown macromolecular toxic factor(s) inducing a neuronal death pathway mysteriously involving ionotropic GluRs at one point. This confirms that even if elevated glutamate is not the primary insult, other neurotoxic mechanisms may lead to a secondary excitotoxicity for which MNs appear the most vulnerable. Nonetheless, without the identification of the ALS CSF macromolecular toxic factor(s), it will be difficult to explore the relevance of these in vitro findings for the in vivo pathogenic process.

In contrast to investigations of ALS patients' fluids, several groups[84–86] have found that glutamate levels in postmortem ALS patient tissues are consistently decreased, strongly supporting glutamate metabolism alterations in ALS. The analysis of the ALS patient[87] and the mutant SOD1^{G93A} mouse[88] by magnetic resonance spectroscopy revealed that, at early stages of the disease, brain glutamate content is elevated. The mouse study[88] further demonstrates that this increase is region-specific (spinal cord > medulla > sensorimotor cortex, 0 = cerebellum) and followed by a more extensive decrease at late stages of the disease, substantiating the results obtained in postmortem ALS patient tissues. Although consistent, so far the pertinence of these changes for the pathogenic processes remains murky.

Environmental excitotoxins and ALS: fame, shame, and come-back

Concomitant to the first report of increased glutamate levels in ALS patients' CSF, another early finding linking excitotoxicity to ALS had a groundbreaking impact in the field. Indeed, Spencer and colleagues showed that feeding monkeys with an environmental toxin (β-methyl-amino-L-alanine, BMAA), whose consumption is thought to be responsible for the 100 times higher ALS incidence in the western Pacific island of Guam, was causing these animals to develop an ALS/Parkinson-like MN disorder.[89] They described that the main features of neurodegeneration were in the motor cortex and ventral spinal cord and that the pathology could be alleviated by NMDA receptor antagonists. Subsequently, the hypothesis of BMAA being at the origins of Guam ALS was so widely challenged (eg, concentrations of BMAA in Guam ALS patients' brains and food that they consume are far below neurotoxic level; BMAA exhibits no consistent excitotoxic potency) that it had fallen into oblivion until a recent renewal of interest in the ALS community (for review see[90–92]). The main findings that made BMAA get back into the race are the demonstrations that: 1) it can be biomagnified in the food chain to reach neurotoxic levels; 2) human brains from ALS as well as from Alzheimer's and Parkinson's patients contain very high levels of protein-bound BMAA compared with controls, suggesting that the slow release of free BMAA from protein-bound reservoirs could last for years after remote exposures and explicate a chronic toxic effect; 3) BMAA becomes a potent excitotoxin only in the presence of bicarbonate, which is abundant in vivo. Still, recent evidence suggests that BMAA may be neurotoxic through several mechanisms other than excitotoxicity, such as inhibition of the xc^- cystine–glutamate antiporter akin to oxidative glutamate toxicity[93] and incorporation into proteins leading to protein misfolding or dysfunction.[94] This hypothesis is particularly appealing in the context of ALS, knowing that accumulation of misfolded proteins in the ER leading to chronic ER stress is one of the major current theories to account for MN death in mutant SOD1 mouse models of familial ALS.[95] If experimentally proven, BMAA-mediated protein misfolding and ER stress may provide evidence for the first common pathogenic process between familial and environment-linked sporadic ALS.

Other confirmed excitotoxins have been described as provoking MN disease-like syndromes following oral intake. For instance, the KA agonist domoic acid, biomagnified in mussels, was responsible for a food poisoning outbreak in Canada, leading initially to widespread neurologic dysfunction and later to chronic residual memory deficits and motor neuropathy or axonopathy.[96,97] More specific to MN diseases, the chronic consumption of grass pea (*Lathyrus sativus*) causes lathyrism, an upper MN disorder primarily characterized by spastic disability of the lower extremities (for review see[98]). At the neuropathological level, cortical MNs with the longest axonal projections seem to exhibit a preferential vulnerability towards these compounds. It has

been suggested that this may be due to their largest dendritic tree being covered by GluRs, potential targets for the liable grass pea excitotoxin β-N-oxalyl-L-α,β-diaminopropionic acid (β-ODAP or BOAA). Indeed, β-ODAP is a potent AMPA receptor agonist[99] which was shown to mimic several clinical features of lathyrism in monkeys following oral intake and to elicit MN death in rodent spinal cord following intrathecal injection.[98] These observations strongly support the view that MNs may be peculiarly vulnerable to excitotoxicity and exogenous excitotoxins. However, even for lathyrism, which appears to be the most salient/consensual example, the efficacy of anti-excitotoxic therapeutic strategies has not been proven in animal models or in the human disease, which opens the possibility that, like for BMAA, other properties of β-ODAP may be at the roots of MN degeneration.

Glutamate transport alterations in ALS: much ado about little

The first explanation for increased glutamate levels in ALS patient fluids emerged when the efficiency of glutamate transport in ALS patient synaptosomes was found to be selectively decreased compared with control or other neurological disease patients.[37] Further, this study showed that this transport defect was selective for glutamate and respected the regional specificity of the disease. This study finding, perfectly correlated to disease selectivity and specificity, started one of the most active controversies in the ALS literature. As glutamate transport implication in ALS was thoroughly and very impartially reviewed recently,[100] we will limit our discussion to the most significant and most recent evidence necessary to reach a fair opinion on this subject.

High affinity glutamate or excitatory amino acid transporters (EAATs) refer to a protein family of 5 members (EAATs 1–5) using the Na^+/K^+ transmembrane gradient to transport glutamate back into the cells following its synaptic release.[101] While EAAT1 (GLAST in rodents) and EAAT2 (GLT-1 in rodents) are mainly located in astrocytes, EAAT3–5 are expressed by neurons throughout the brain. Among the EAATs, astrocytic EAAT2 has a primary role in maintaining extracellular glutamate at low levels compatible with both neurotransmission and neuronal survival.[101,102] Indeed, astrocytes express much higher levels of EAAT2 than EAAT1 in vivo, and it has been demonstrated in genetic deletion studies that EAAT2 is responsible for ~95% of total glutamate transport at the synapse.[103] This gave a rationale for the avid study of EAAT2 dysfunction and expression defects to account for glutamate level alterations in ALS patients.

EAAT2 loss accounts for reduced glutamate transport in ALS

The first observation of decreased glutamate transport in ALS patients[37] was confirmed and extended to the discovery of a selective and extensive loss of EAAT2 protein in the spinal cord and motor cortex of ALS patients.[104,105] While these studies were pioneers in the field for studying the roles of glutamate transporter failure and noncell autonomous processes in ALS, they were all observations from a small number of patients, and the end-stage nature of the experimental material does not allow sorting out whether EAAT2 deficiency is a cause or a consequence of the disease process. Further, EAAT2 disappearance may not be ALS specific as this was reported for several other neurodegenerative and psychiatric diseases.[101] Along this line, despite an isolated report that identified a polymorphism (N206S) altering EAAT2 function in sporadic ALS patients,[106] and an exciting but later invalidated[107] finding of EAAT2 mRNA selective splicing defect variants in ALS affected regions,[108] no genetic linkage to the EAAT2 locus was detected and the extremely rare mutations of EAAT2 found in familial or sporadic patients do not give rise to transcript defects.[109]

Several groups also examined the function and levels of EAAT2/GLT-1 in the mutant SOD1 rodent model of ALS. In agreement with the human data, an early study demonstrated a 50% decrease in GLT-1 protein in spinal cord extracts from end-stage $SOD1^{G85R}$ mice,[33] while a second evidenced decreased glutamate uptake in synaptosomes extracted from end-stage $SOD1^{G93A}$ mice.[110] Then, addressing all of the shortcomings of these previous studies, Bendotti and collaborators demonstrated that the GLT-1 protein level reduction in $SOD1^{G93A}$ mice was specific for affected regions, selective for GLT-1 over GLAST, not associated with GLT-1 mRNA decrease, and not significant in early-symptomatic stage where MN insult signs are already present versus end-stage.[78] Altogether, these results indicate that GLT-1 decrease in $SOD1^{G93A}$ mouse spinal cord may be due to post-transcriptional alterations of the protein and, importantly, is a very unlikely primary event leading to MN degeneration. Similar findings giving further credence to the same conclusion were reported in $SOD1^{G93A}$ rats.[111,112] At best, EAAT2/GLT1 decrease may be a contributor to disease progression in advanced stages but, knowing that astrocyte expression of glutamate transporters is dependent on neuron presence and activity in vitro[113,114] and in vivo,[115] we cannot exclude that it is MN dysfunction and/or loss that is eliciting GLT-1 expression downregulation. In keeping with this, it was recently proposed that kappa B-motif binding

phosphoprotein (KBBP) regulates the activation of the GLT1/EAAT2 promoter in astrocytes as a function of presynaptic neuronal activity.[116] It was hypothesized that alteration/loss of presynaptic terminals during ALS disease process resulted in reduced astroglial KBBP expression and, ultimately, in transcriptional dysfunction of GLT-1.

A secondary role for EAAT2 dysfunctions in ALS

This notion of EAAT2 reduction playing a contributive, but not primary role, in MN degeneration in ALS is supported by several different approaches in vivo. The first strategy described, aimed at increasing EAAT2 protein through over-expression in ALS SOD1^{G93A} mice, showed that a doubled expression of EAAT2 increased glutamate uptake by 2-fold, delaying symptom onset (17%) and MN death, without, however, noticeable effects on paralysis onset, weight decline or life span.[117] Subsequently, several drugs, known to increase EAAT2 protein in astrocytes such as the β-lactame antibiotic ceftriaxone and the immunophilin ligand GPI-1046, were evidenced to both protect MN survival and to extend SOD1 ALS mouse lifespan (9% and 12% respectively).[118,119] Nevertheless, with these drugs having primarily multiple biological effects unrelated to EAAT2, we can assume that the modest improvement measured in ALS mice may be attributed to other unidentified mechanisms. To address the question from a reverse angle, Pardo et al crossed the SOD1^{G93A} mouse with a mouse heterozygous for GLT-1 ablation that displays a significant reduction in transporter protein.[120] While, as expected, they observed an earlier and an exacerbated MN loss (~30%) in mice carrying both abnormalities, they did not detect an earlier motor onset, but an accelerated decline in motor strength, accompanied by only a very modest reduction in survival (4%). Ultimately, we can conclude that these two complementary genetic studies argue for GLT-1 loss being somewhat associated with disease progression, but not a prime cause of MN degeneration.

Alternatively, it can be hypothesized that EAAT2 protein level reduction may be preceded by more discrete EAAT2 dysfunctions. These defects may be as crucial in terms of MN survival, may appear early and may be maintained long enough to pretend to play a key role in the ALS pathogenic process. Accordingly, it was established that oxidative stress results in the rapid inactivation of glutamate transporters in cultured astrocytes[121,122] or when EAAT2 is co-expressed with human mutant but not wild-type SOD1 in *Xenopus laevis* oocytes.[123] A cleavage by caspase-3 at a unique site located in the cytosolic C-terminal domain of EAAT2 was proposed to account for its oxidative stress-mediated inhibition.[124] In contrast, a study found that transfection of both mutant G93A and wild-type SOD1 in primary astrocytes results in significant GLT-1 protein reduction independent of oxidative mechanisms.[125] Dependent or not on oxidative stress, none of these studies made the link between GLT1/EAAT2 dysfunctions in astrocytes and MN death. Our group[57] and others[56,58] have evidenced that mutant SOD1-expressing astrocytes are selectively toxic to MNs in vitro. Yet, we further demonstrated that this death, while mediated by a soluble mechanism, was independent of glutamate alteration.[57] Indeed, in our hands, primary astrocytes, prepared directly from rats or mice nontransgenic or transgenic for wild-type or different SOD1 mutations, exhibit no impairment in their capacity to clear excess extracellular glutamate. Further, we found similar extracellular glutamate levels in the different astrocyte culture genotypes, no decrease in GLT-1 protein content in association with SOD1 expression (unpublished data), and no protection of MNs from astrocyte toxicity with glutamatergic receptor antagonists. This suggests that, at least in vitro, astrocytes, produced directly from a relevant animal model of familial ALS, can be detrimental to MNs independent of glutamate transport dysfunction and associated excitotoxic mechanisms.

EAAT2 is essential for the therapeutic replacement of ALS-diseased astrocytes

Supporting the idea that, in ALS, MNs reside in a hostile astrocyte neighborhood, a recent study has reported that the focal replacement of astrocytes in cervical spinal cords of mutant SOD1^{G93A} rats through the transplantation of lineage-restricted astrocyte precursors, also called glial-restricted precursors (GRPs), had significant therapeutic effect.[126] The transplanted GRPs differentiated efficiently into astrocytes in diseased tissue, reduced microgliosis, lessened MN loss (~15%), slowed motor declines (~5%) and extended animal survival (~10%) and disease duration (~25%). Interestingly, GRPs genetically deficient in GLT-1 were unable to exhibit the same neuroprotective effect, but GRPs overexpressing GLT-1 did not improve the protection offered by nonengineered GRPs. Altogether, this indicates that astrocytes that do not express mutant SOD1 exert a positive GLT1-dependent effect. However, rather than helping to cope with improbably increased extracellular glutamate levels (see previous discussion), this may mean that GLT-1 expression is essential for astrocytes to acquire neuronal supportive activities. For instance, glutamate capture into astrocytes is

known to sustain antioxidant defenses through glutathione synthesis,[127] and neuronal energy production through release of lactate.[128]

Chronic glutamate transport alteration is an unlikely culprit in MN degeneration

On the other hand, the chronic pharmacological alteration of glutamate transport in spinal cord organotypic cultures by treatment with competitive inhibitors was shown to produce a model of slow MN death, not strictly limited to MNs or to the ventral horn.[129–131] However, in vivo, the same inhibitors of glutamate transport are well known to be inefficient in causing neuronal death despite the induction of massive increases in extracellular glutamate, stressing how glutamate itself may be innocuous compared with potent neurotoxic GluR agonists.[132,133] It can be assumed that synaptic glutamate augmentations in combination with other underlying dysfunctions may be the key to triggering glutamate-associated neuronal death in vivo. These possibilities were tested, in the ALS context, by the group of Ricardo Tapia who first showed that, as in control mice, in end-stage mutant SOD1^{G93A} mice, a 2-hour perfusion of a competitive glutamate transport inhibitor caused a significant elevation in glutamate levels but no detectable associated neuronal damage, excluding collectively a potential exacerbated neuronal vulnerability to glutamate and a difference in glutamate transport capacity in these animals.[77] Then, they demonstrated that a 10-day chronic infusion of the same inhibitor in the spinal cord of nontransgenic mice was likewise harmless to MNs.[134] These two studies strongly support the view that glutamate transport deficiency is not a key factor in MN degeneration.

In conclusion, the numerous investigations on glutamate transporters in ALS have greatly increased our knowledge of the regulation of these transporters' expression and activity. Several lines of evidence point to the possibility that glutamate transport defects and the related excitotoxicity may play a role in the late stage of ALS disease progression. However, there are no consistent data to support defects in EAATs as having a primary role in the induction or the selectivity of MN degeneration taking place in ALS (see Figure 1 for summary).

Motor neuron glutamate receptors in ALS: lost in translation

Along with potential defects in extracellular glutamate levels and glutamate transport, GluR alterations have been exten-

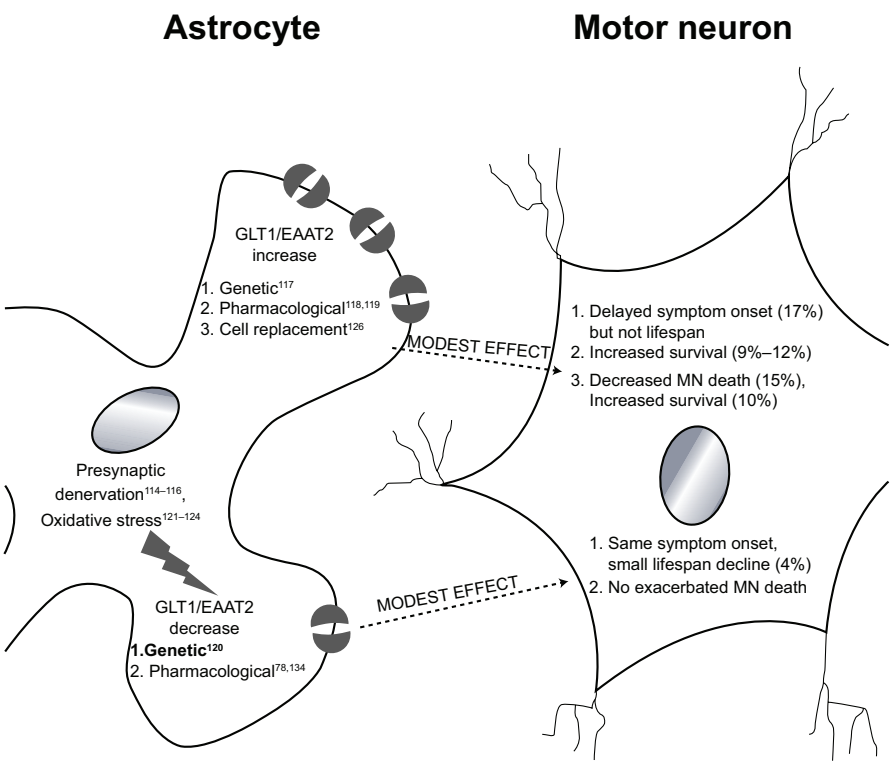

Figure 1 Modulating EAAT2/GLT1 has only modest effect in amyotrophic lateral sclerosis mice.
Note: In order to evaluate the effect of glutamate transport modulation on SOD1^{G93A} mice, EEAT2/GLT1 expression was increased or decreased via different genetic or pharmacological approaches. None of these studies revealed a major effect on motor neuron (MN) survival or animal life span.

sively investigated in ALS. It is consensually established that MNs have a high density of GluRs, express all their types,[135–137] and are more vulnerable than other neuronal subsets to excitoxicity induced by influx of Ca^{2+} through GluRs.[138–140] Most of the studies investigating the possible implication of GluRs in ALS pathogenesis are therefore particularly focused on ionotropic GluR NMDA and on GluR2 subunit expression and editing, which confer Ca^{2+} impermeability to the AMPA/KA GluR.

Differential GluR expression and vulnerability to excitotoxicity in MNs

Quantitative autoradiography studies of human tissues have determined that both NMDA and non-NMDA GluR are expressed in motor cortex, brainstem, and spinal cord with a particularly striking high density on lower MN somata.[135,141–144] Intriguingly, further, MN groups classically described as resistant in ALS, such as the oculomotor nucleus, were shown to possess fewer NMDA binding sites and much denser AMPA binding sites in comparison to vulnerable MN groups, suggesting that higher NMDA GluR expression may account for the selective vulnerability of certain MNs in ALS. However, a plethora of evidence is more consistent with the hypothesis of the non-NMDA origin of the exacerbated MN vulnerability to EAAs. For instance, the single intrathecal injection of a high concentration of NMDA, contrary to AMPA agonists, had no effect on spinal MNs.[145] Likewise, in a model of organotypic rat spinal cord culture and in vivo, larger MNs were shown to be relatively well preserved against NMDA exposure,[146] but considerably more sensitive to AMPA/KA mediated toxicity.[147–149] In addition, AMPA/KA receptor activation, through the continuous infusion of KA into the rat spinal subarachnoid space, induces a progressive and motor-selective behavioral deficit associated with a late loss of spinal MNs.[150] Further, immunohistochemistry and in situ hybridization studies have emphasized that healthy human and rodent MNs express lower[151–155] or even undetectable[156,157] GluR2 subunit mRNA and protein, suggesting that MN AMPA/KA GluR have a higher permeability to Ca^{2+}, which could account for MN hyper-susceptibility to excitotoxicity. The situation is slightly different in vitro, as in rat MN cultures[158] and spinal cord tissues,[159] GluR2-containing receptor clusters were found to be more abundant than GluR2-lacking clusters. However, the activation of micro-domains lacking GluR2 was suggested to be sufficient to greatly amplify Ca^{2+} influx locally and to participate in toxic mechanisms.[158] In agreement, another in vitro study specified that short-term exposure to KA causes a selective MN death, completely dependent on extracellular Ca^{2+}, insensitive to inhibitors of voltage-operated Ca^{2+} or Na^{+} channels, and fully inhibited by Joro spider toxin, a selective blocker of AMPA receptors lacking GluR2 subunit.[140] In contrast, cortical neuron vulnerability to excitotoxicity is not correlated to GluR2-deficient AMPA/KA receptors,[160] suggesting that Ca^{2+} influx through AMPA receptors may be especially important in MN-associated excitotoxicity. Another interesting observation is that, rather than whole-cell relative Ca^{2+} permeability of AMPA GluR, the AMPA current density, which is 2- to 3-fold higher in MNs than in other spinal neurons, may be sufficient to account for MN vulnerability to AMPA agonists.[161,162]

Of note, group I mGluRs were reported to have a dual role in excitotoxic-mediated MN degeneration,[163] as some evidence supports their neuroprotective action[164] against AMPA-mediated toxicity,[165,166] whereas the greater expression of their subtype 1a in the vulnerable MN population in rat may exacerbate excitotoxic injury by increasing intracellular Ca^{2+} levels and activating PKC.[167]

Ca^{2+}-permeable AMPA GluR are not the key to MN degeneration

To better understand the importance of AMPA GluR in MN vulnerability to EAAs, transgenic animal models targeting the AMPA GluR2 subunit were generated. Mice heterozygous for an editing incompetent GluR2 allele resulting in only 25% of Ca^{2+}-impermeable AMPA GluR die prematurely from seizures and hippocampal pathology but are not associated with any ALS-like motor phenotype or motor neuron degeneration.[168] In contrast, transgenic mice expressing a minigene for an artificial Ca^{2+}-permeable GluR2 subunit are viable, but exhibit a late decline in motor function associated with neuronal death in the spinal cord and diverse other brain areas.[169,170] As for knockout mice lacking the GluR2 subunit, they show only minor impairment in motor coordination even though the Ca^{2+} permeability of AMPA GluR was measured to be maximal in regions such as the hippocampus.[171] The conflicting phenotypic consequences observed in these different models may be partially explained by the properties of AMPA receptor trafficking and subunit incorporation. The GluR2(Q) subunit is more readily targeted to the cell surface and incorporated into AMPA receptors than is an edited GluR2(R) subunit.[172,173] We can therefore assume that, in the editing-incompetent GluR2 mice, a higher density of Ca^{2+}-permeable AMPA receptors will end up at the plasma membrane due to a more sustained assembly than in the two other models. This could ultimately lead to a greatly exacerbated vulnerability

to excitotoxicity and explicate the premature death of these mice. More recently, mice were engineered for a cholinergic neuron-selective excision of some ADAR2 exons crucial for the enzyme activity, by using a vesicular acetylcholine transporter–Cre/LoxP system.[174] These mice, referred to as AR2 mice, in which ADAR2 activity is ablated in 50% of the MNs, are relatively long lived (~81 weeks versus ~105 weeks for controls) and exhibit some motor abnormalities such as hypokinesis and decreased rotarod performance but no overt paralysis. Further, it was shown that the kinetics of the motor deficit in these mice was consistent with the degeneration of cells in the anterior horn of the spinal cord and that MNs known to be resistant in ALS such as those of the oculomotor nuclei are spared despite effective Cre/LoxP recombination. Taken together, these results indicate that GluR2-lacking or unedited GluR2-containing AMPA receptors, per se, cannot provoke severe paralytic disorders resembling ALS nor selective MN degeneration. Although the same MN subsets are observed to be more resistant to the ALS disease process and AMPA-mediated increased Ca^{2+} permeability, they may also be more resistant to any other insult.

MN GluR alteration in ALS: zoom in on GluR2 loss and editing defect

With all of these characteristics of MN GluR in mind, we may wonder whether GluRs are altered in ALS and play a key role in the pathogenic process. An opening argument for abnormal GluR permeability to Ca^{2+} in ALS is the observation of Ca^{2+} accumulation in MN terminals of ALS patients[175] and in vacuoles of degenerating MNs in $SOD1^{G93A}$ mice.[176] In ALS patients, a specific reduction of NMDA receptor binding sites and NR-1 mRNA levels was observed in the ventral horn compared with control individuals,[177,178] indicating that the MNs that were lost were expressing NMDA receptors. Reflecting earlier stages of the disease, a postsynaptic decrease of NR2A subunit expression was also recently reported in upper MNs of presymptomatic $SOD1^{G93A}$ mice.[179] Although these observations suggest that NMDA receptors could be involved in MN degeneration in ALS, intrathecal application of NMDA had no impact on MN survival in mice.[145] In contrast, memantine, a noncompetitive NMDA receptor antagonist, significantly but quite modestly prolongs the survival of ALS mice (~7%) without affecting disease onset.[180] However, because memantine is also a potent serotonin-3[180] and alpha7 nicotinic acetylcholine receptor[181] antagonist and an agonist of dopamine D2 receptors,[182] it is difficult to attribute its therapeutic effect to a selective action on NMDA receptors.

The contribution of AMPA receptors to the ALS disease process is more consistently supported by several studies. First, the survival of $SOD1^{G93A}$ ALS rodents was prolonged by ~10% after intraperitoneal injection of AMPA receptor antagonists.[183,184] Then, crossbreeding $SOD1^{G93A}$ mice with ChAT-GluR2 overexpressing mice, for which AMPA Ca^{2+}-permeability is significantly reduced in cholinergic neurons, was shown to delay disease onset (~19%) and mortality (~14%) and to alleviate SOD1 protein misfolding.[185] In contrast, crossbreeding of $SOD1^{G93A}$ mice with GluR2 knockout mice was found to accelerate disease progression and animal death (~15% for both).[186] As GluR2 mRNA levels are unchanged over the course of the disease in $SOD1^{G93A}$ mice,[187] but GluR2 protein is specifically decreased in spinal MNs before symptom onset, the beneficial effects of GluR2 overexpression may rely on both a global reduction in Ca^{2+} influx through AMPA GluR and on a compensatory GluR2 protein translation defect or instability.[184] Nevertheless, no difference in GluR2 mRNA and protein expression was observed between human ALS and control tissues,[155] or between resistant and vulnerable rat MNs.[188] Altogether these results suggest that reduced expression of GluR2 is not a crucial factor predisposing MNs to degeneration in ALS per se but that AMPA GluRs have a modulatory role worth further investigation, at least, in the mutant SOD1-linked familial form of ALS.

Besides a decrease in GluR2 expression, a deficiency in GluR2 mRNA editing at the Q/R site also leading to increased Ca^{2+} permeability of AMPA GluR was observed in the ventral spinal gray matter of ALS patients (supposedly sporadic) and in single MNs compared with controls.[189–191] This incomplete editing was region selective and was not observed in MNs from patients suffering from spinal and bulbar muscular atrophy or multiple system atrophy, suggesting that, although based on few human cases, this is specific to spinal MNs of ALS patients. In animal models crossing $SOD1^{G93A}$ mice with GluR2(N) mice that express an unedited GluR2 subunit, progeny exhibited a ~7% shorter lifespan[169] and no GluR2 editing defect was found in symptomatic mutant SOD1 rats.[192] No aberrant RNA editing was observed in the upper MN of ALS patients either,[169] indicating that this is not associated with all MNs degenerating in ALS. This does suggest that GluR2 RNA editing anomalies may be a hallmark of some form of ALS (sporadic?) but not of SOD1-associated familial ALS. However, the direct involvement of GluR2 editing defect in sporadic ALS pathogenesis remains to be determined and is not supported by animal modeling of this defect,[168,170] as discussed above. Knowing that oxidative or nitrosative stress

taking place in ALS can result in abnormal RNA editing, impaired GluR2 editing could be the effect rather than the cause of ALS, and thus be a secondary contributor to MN degeneration.[169] A single study has reported a higher proportion of flip versus flop variants of AMPA GluR subunits in spinal MN of ALS patients compared with control individuals, which suggests that the presence of slowly desensitizing AMPA receptors may also contribute to MN vulnerability in ALS.[193] The significance of this alternative defect of AMPA GluRs remains enigmatic as it has not been confirmed or further investigated.

Mutant SOD1 a potential catalyzer of MN vulnerability to excitotoxicity

Microinjection of different SOD1 mutant cDNAs into rat primary neurons has been suggested to increase MN vulnerability to normally nontoxic glutamatergic stimulation via Ca^{2+} influx through AMPA receptors and subsequent SOD1 aggregation.[194] These SOD1 aggregates are apparently forming selectively in MNs as they have not been observed in neurons resistant to ALS disease process.[40] Other groups also found a peculiar sensitivity to excitotoxicity of MNs overexpressing mutant SOD1[195,196] through AMPA/KA receptors, although others did not.[197,198] Another study suggested that exacerbated excitotoxic death of $SOD1^{G93A}$ MNs may not result directly from a greater Ca^{2+} influx through AMPA receptors but from voltage-dependent Ca^{2+} channels activated secondary to an abnormally increased AMPA-mediated depolarizing current.[199] Several differences in cell culture conditions or in mutant SOD1 overexpressing strategies may account for these discrepancies. Although there is unfortunately no direct in vivo evidence to reach a definite verdict in this case, most studies support mutant SOD1 expression as a sensitizing factor in excitotoxicity.

Astrocytes: a new piece in the excitotoxic puzzle in ALS

A very original study uncovered that spinal astrocytes from two rat strains are differently regulating GluR2 expression and their associated Ca^{2+} permeability in MNs, thereby influencing their vulnerability to excitotoxicity.[198] Importantly, astrocytes expressing mutant SOD1 lose their capacity to upregulate GluR2 expression in MNs, which could participate in the mechanisms leading to MN degeneration in ALS. Furthermore, the growth factor vascular endothelial growth factor, which is secreted by astrocytes and which stimulates GluR2 expression in MN in vivo and in vitro,[200] was found to prolong survival of ALS mice by ~18% to 30%.[201,202] Besides, a strong upregulation of mGluR5 mRNA and protein expression was observed in spinal astrocytes of ALS patients[203] and mutant SOD1 transgenic animals[204] compared with controls. This GluR5 increase was suggested to contribute to the astrocytosis observed in ALS and to constitute a "failed attempt" to stimulate astrocyte glutamate uptake, as mGluR5-mediated enhancement of glutamate transport is deficient in astrocytes derived from mutant SOD1 rats.[205]

Other putative players in the excitotoxic deadly game in ALS

Chloride influx via voltage-dependent channels is known to worsen AMPA GluR-mediated MN death through a partial repolarization that amplifies AMPA receptor conductance and toxic elevation in Ca^{2+} influx.[206] Co-administration of GABA with EAAs further enhanced this Cl^- influx and resulting cell death. This phenomenon could be particularly relevant to MNs that present a high density of AMPA receptors coupled to an important GABAergic innervation.[207]

Because Ca^{2+} can also enter the cells via voltage-sensitive Ca^{2+} channels activated by cell membrane depolarization,[208] the intrinsic MN excitability properties may play a major role in the ALS Ca^{2+}-mediated excitotoxicity. Altered electrophysiologic axonal properties such as persistent sodium and/or potassium channel conductance were actually found in ALS patients[209,210] and might explain the ectopic firing of motor units and the axonal hyperexcitability observed in presymptomatic and symptomatic patients. These abnormalities responsible for fasciculations are heterogenic and not always detected in mutant SOD1 animal models.[211,212]

D-serine, a physiological co-agonist of the NMDA receptor, was suggested to amplify ALS MN excitability by increasing the receptor's affinity for glutamate,[213,214] even though the expression of NMDA receptors is kept low in this disease.[142,177] An increase of D-serine was observed in spinal cords of mutant SOD1 mice and in familial and sporadic ALS patients.[215] Further, primary spinal cord neurons from ALS mice were shown to be more vulnerable to NMDA toxicity in a D-serine-dependent manner than those from control mice, and removal of endogenous D-serine from spinal cord cultures of SOD1 mice attenuated NMDA receptor-mediated MN death.[215]

Finally, several mechanisms related to mitochondrial dysfunction, such as oxidative stress and energy depletion, described to take place in ALS disease process, may make MNs vulnerable to "slow onset" or "secondary excitotoxicity"

(for review see[216]). These various insults can lead to the loss of membrane potential which, in turn, may release the Mg^{2+} blockade from NMDA receptors and permit excess Ca^{2+} entry into neurons. Under these conditions, levels of glutamate observed in normal neurotransmission may damage already diseased MNs.

In conclusion, data generated from several in vitro and in vivo studies suggest that alteration in GluR properties may be a secondary contributing factor but not a primary element accounting for the selective MN death in ALS (for summary see Figure 2).

Calcium: the crossroad of MN termination

Ca^{2+} is the most abundant metal by mass in many animals, of which 99% participates in the mineralization of the bones and only 1% serves a myriad of critical biological functions. Most of these physiological functions are regulated in neurons and astrocytes by transient variations of intracellular Ca^{2+} concentrations (Ca^{2+}i) via complexes exchanges across the plasma, mitochondrial and ER membranes, and interplays with Ca^{2+} buffering proteins.[217] When Ca^{2+} load exceeds the capacity of regulation of the cell, Ca^{2+}-dependent toxic pathways may be activated. Due to its crucial importance in brain functioning as both a second messenger and a cell death mediator, alterations in Ca^{2+} homeostasis have been suggested to be implicated in the onset/progression of various neurodegenerative diseases, such as Parkinson's, Alzheimer's, and Huntington's (for reviews see[218,219]).

Ca^{2+} and excitotoxicity: the never-ending story

Twenty-three years ago, using mature cortical neuronal cultures, Choi[15] was the first to demonstrate that a low glutamate exposure mediated a delayed Ca^{2+} influx via NMDA receptors leading to a gradual neuronal disintegration. As discussed in

	PRO	CON
AMPA implication in ALS pathogenic process	**• Increased survival ($SOD1^{G93A}$ mice)** • AMPA antagonist (+10%) [183,184] • GluR2 overexpression (+14%)[185] **• Accelerated death ($SOD1^{G93A}$ mice)** • GluR2 KO (+15%)[186] • unedited GluR2 (+7%)[169] **• GluR2 protein decrease (presymptomatic $SOD1^{G93A}$ MNs)[184]** **• GluR2 editing defect (ALS MNs patients)[189–191]** **• Increased flip/flop variants ratio (ALS MNs patients)[193]** **• MN loss and behavior deficit mediated by spinal KA infusion[150] or decreasing ADAR2 in MNs[174]**	**• No GluR2 mRNA/protein levels difference in:** • MNs of ALS and control patients[155] • ALS resistant and vulnerable MNs[188] **• No GluR2 editing defect in:** • symptomatic $SOD1^{G93A}$ and $SOD1^{H46R}$ rats[192] •upper MNs of ALS patients[191] **• Weakness of all the pro-arguments that are related to modest effects**
NMDA implication in ALS pathogenic process	**• Increased survival but no delayed disease onset with NMDA antagonist ($SOD1^{G93A}$, 7%)[180]** **• Decreased NMDA binding site density (upper MNs of presymptomatic $SOD1^{G93A}$ mice and ALS patient ventral horn)[177–179]**	**• Ventral horn MNs resistant to NMDA exposure[145]** **• Weakness of the few pro-arguments that are related to modest effects**

Figure 2 Evidence in favor or against the involvement of AMPA/KA and NMDA receptors in amyotrophic lateral sclerosis (ALS) pathogenic process.
Note: Many arguments support a limited but actual involvement of AMPA/KA receptors in amyotrophic ALS. Considering the weakness of all the pro-arguments, AMPA receptor defects are more likely implicated in late stage of disease progression rather than in its initiation (a). The involvement of NMDA being even less supported, its role may be further minor in ALS pathology (b).
Abbreviations: MN, motor neuron; KA, kainate.

the previous sections, initially, Ca^{2+} entered the ALS pathogenic cascade via its possible increased influx in MNs through altered Ca^{2+}-permeable GluR leading ultimately to MN excitotoxic death.[220] Interestingly, later, it was suggested that the chief determinant of glutamate-mediated Ca^{2+} neurotoxicity is not really the amount of Ca^{2+}i but the path and duration of the Ca^{2+} increase. For instance, it was suggested that focal increases in Ca^{2+} in the vicinity of GluR could be coupled to downstream neurotoxic second messengers via interactions with region-specific adaptor proteins and enzymes.[221,222] In cultures of mouse spinal and cortical neurons, it was demonstrated that the neurotoxicity induced by long-duration/low-level glutamate exposure was primarily triggered by Ca^{2+} influx through NMDA receptor channels, although the same Ca^{2+} load was mediated by NMDA, AMPA/KA, and other Ca^{2+}-permeable channels.[221,222] A similar study further demonstrated that this chronic/low glutamate exposure selectivity triggers MN death in spinal neuronal cultures in contrast to acute/high-dose glutamate exposure which is nonspecifically toxic.[223] Over the course of chronic/low glutamate exposure, spinal neurons first experience a transient increase in Ca^{2+}i, followed by a sustained and irreversible Ca^{2+}i rise, indicating imminent cell death due to the loss of Ca^{2+} homeostasis maintenance capacity.[221] Altogether, these observations are particularly relevant to both sporadic and familial ALS, knowing that Ca^{2+} accumulation was observed in MN terminals of sporadic ALS patients,[175] and that an early Ca^{2+}i increase was also reported in vulnerable spinal MNs of $SOD1^{G93A}$ mice.[176] Further, an explanation for increased Ca^{2+} in MNs is offered despite the lack of consistent evidence for increased glutamate levels in ALS patients or animal models.

One of the most consensually admitted hypotheses to explain the Ca^{2+} accumulation observed in ALS is that MN Ca^{2+} buffering capacity is impaired. Indeed, the way MNs handle Ca^{2+} overload during excitatory neurotransmission is a likely candidate for their peculiar fragility. Supporting this view, the amplitude of the Ca^{2+} signal in response to AMPA receptor or voltage-dependent Ca^{2+} channel activation was not significantly different between controls and $SOD1^{G93A}$ MNs, but the time for Ca^{2+}i recovery to basal level was significantly slower in mutant MNs after AMPA, but not voltage-dependent Ca^{2+} channel activation.[224] Interestingly, the same defect was not observed in $SOD1^{G93A}$ cortical neurons. These findings suggest that it is the capacity of Ca^{2+}i clearance following its entry through AMPA GluR which is specifically altered in ALS MNs. There is also evidence showing that vulnerable MNs, lost early in human or rodent ALS, are deficient in the Ca^{2+}-binding proteins calbindin-D28K and parvalbumin, whereas MNs damaged later or relatively spared, express high levels of these Ca^{2+}-buffering proteins.[176,225] Transgenic mice overexpressing parvalbumin in spinal MNs were interbred with mutant SOD1 mice and exhibited a ~17% delayed onset of motor defects and an ~11% prolonged survival.[226] However, the marginal survival extension in this double transgenic mouse implies that other mechanisms associated or not with intracellular Ca^{2+} are taking place in ALS pathogenesis.

Mitochondria and ER under stress

Of note, MN structures and synaptic function tightly dependent on Ca^{2+} levels are reported to be impaired in ALS. In fact, observations of ER and mitochondrial abnormalities and Ca^{2+} dysfunction have been extensively documented in MNs of sporadic ALS patients and in ALS mouse models (for review see[220]). Although several pathways may be involved in the Ca^{2+}i augmentation observed in ALS, increased Ca^{2+} permeability through GluR is very attractive in the sense that this has been shown to induce organelle-specific dysregulation in neurons. It is, however, impossible to determine, as for the chicken-and-egg mystery, which came first: the increase in Ca^{2+}i or the organelle impairment.

Under physiological conditions, mitochondrial uptake represents a major clearance mechanism for glutamate-induced Ca^{2+} loads in neurons.[227] Interestingly, a brief exposure to AMPA/KA triggers substantial mitochondrial Ca^{2+} loading in MNs but not in ALS-resistant GABAergic interneurons, although both neuronal populations express a large number of Ca^{2+} permeable AMPA/KA receptors and present a significant cytosolic Ca^{2+} increase.[139,228] Remarkably, MNs were reported to exhibit a lower mitochondrial density per volume compared with other neuronal subsets, which could contributed to their higher vulnerability to Ca^{2+} overloads.[229] Consistent with this, MN mitochondrial Ca^{2+} overload was reported even after brief glutamate/NMDA stimulation.[223] Further, repetitive short KA stimulations induce, specifically in MNs, a gradual increase in peak and baseline Ca^{2+}i due to the saturation of mitochondrial buffering capacity.[229] These findings confirm that MNs are particularly susceptible to mitochondrial Ca^{2+} overload and provide an explanation for the chronic mitochondrial Ca^{2+} overload reported in nerve terminals of ALS patients[175] and for the significant decrease in mitochondrial Ca^{2+} uptake observed in brain and spinal cord of $SOD1^{G93A}$ mice.[230] Mitochondrial Ca^{2+} overload may induce swelling, membrane potential depolarization, and finally opening of the permeability transition pore. It results in dissipation of mem-

brane potential, loss of ATP synthesis and ion homeostasis, and finally release of pro-apoptotic proteins (for review see[44]). Although the mechanisms leading to mitochondrial alteration are not clear, it is suggested that mitochondria may be damaged by their own increase in reactive oxygen species (ROS) production subsequent to GluR overstimulation and Ca^{2+} overload.[223] In MNs, excitotoxicity-mediated ROS was described as creating a vicious circle, since after freely crossing neuronal membranes, they can inflict damage to astrocytes notably through oxidation and disruption of glutamate transporters, thereby exacerbating the overstimulation of MN GluR.[231] Alternatively, elevated Ca^{2+}i was shown to promote unusual aggregation of mutant SOD1 inside of mitochondria, thereby worsening the organelle's dysfunction.[232] On the other hand, it was shown, in rat spinal cord cultures, that mitochondrial dysfunction induced by the respiratory chain inhibitors rotenone and malonate causes activation of non-NMDA and/or NMDA GluR resulting in a selective MN death.[233] This suggests that mitochondrial dysfunction and excitotoxicity may predispose MNs to each other and are tightly interrelated mechanisms. Since the rotenone- or malonate-induced MN death was blocked by glutamatergic receptor antagonists in absence of glutamate concentration elevation in the media, the authors hypothesized that these respiratory chain inhibitors may affect a portion of the membrane-incorporated GluR2 subunits or interfere with GluR2 mRNA editing. In agreement, in cortical upper MN explants, chronic mitochondrial inhibition with malonate results in MN death by excitotoxicity via both NMDA and non-NMDA receptors.[234]

Mitochondria exchanges Ca^{2+} with the ER to synchronize physiological functions. ER is another Ca^{2+} intracellular store that regulates Ca^{2+} homeostasis by taking up the ions through Na^{+}/Ca^{2+} exchanger or by fast releasing it after Ca^{2+}-induced ryanodine receptors (RyR) or inositol triphosphate receptors (IP3R) activation.[235]

Although a plethora of evidence suggests that ALS is associated with ER stress and dysfunction,[236] only a few studies imply that glutamate-mediated Ca^{2+} influx may impact ER functions in MNs. Among the few insights, it was shown that Ca^{2+} entry through AMPA receptors caused RyR-mediated Ca^{2+}-induced Ca^{2+} release from the ER in MNs.[220,237] Because some subtypes of RyR are only present in MNs, they may specifically amplify Ca^{2+}i level in MNs after GluR activation.[238] Along the same line, inhibition of ER-Ca^{2+} release with RyR and IP3R inhibitors was demonstrated to reduce cytosolic Ca^{2+} increase, mitochondrial damage, and excitotoxicity after NMDA stimulation in cultured cortical neurons and in organotypic slices.[239] Unfortunately, none of these studies has yet been confirmed or reproduced in an ALS context such as in SOD1 mutant animals.

If the reuptake of Ca^{2+} by mitochondria and ER is important in the glutamate-mediated toxic cascade, some evidence support that Ca^{2+} release from these organelles is equally crucial. Indeed, glutamate excitotoxicity was shown to be alleviated by blocking Ca^{2+} release from intracellular stores with dandrolene.[240]

In conclusion (see Figure 3 for summary), accumulating data suggest that, in ALS, MNs cannot handle large activity-induced Ca^{2+} load originating from Ca^{2+}-permeable GluR. However, abnormally high intracellar Ca^{2+} may also arise, in MNs, from defective buffering capacities due to impaired mitochondria and ER reuptake, or low endogenous Ca^{2+}-binding protein content. In ALS, Ca^{2+} homeostasis alteration may be at the center of a vicious circle between mitochondria, ER, and plasma membrane GluR precipitating MN death. If we consider how early mitochondria and ER alterations are described in ALS compared with glutamate-associated alterations, we can assume that mitochondrial and ER dysfunctions are unfortunate converging points of multiple dysfunctional pathways in the disease pathogenesis, but probably not the specific consequence of increased Ca^{2+}i after GluR activation.

Conclusion and glutamate-associated therapeutic perspectives in ALS

Based on the accumulation of evidence associating glutamate pathway alteration in ALS, we must perforce recognize that there is certainly no smoke without fire. But at which level and to what extent is glutamate crucial in the ALS disease process? The current consensus is that CSF glutamate levels are increased in 40% of sporadic ALS patients[71] and thus do not represent an universal metabolic signature for ALS. Further, the biological significance of this glutamate increase remains undetermined, as the toxicity of ALS patient CSF is independent of glutamate levels but is incongruously dependent on GluR.[75,83] Then, if the discovery of environmental EAAs predisposing to ALS or ALS-like syndrome, such as BMAA, gave a serious impetus to the investigation of excitotoxicity in ALS research, the most recent hypotheses on the origin of BMAA chronic toxicity are rather oriented toward its pathological incorporation into proteins and associated protein misfolding/aggregation/dysfunction.[94] Regarding glutamate transport and, in particular, EAAT2/GLT1 defects, it appears reasonable to agree with the fact it represents a secondary mechanism in ALS,[78,241] maybe just circumstantial to neurodegeneration,[116]

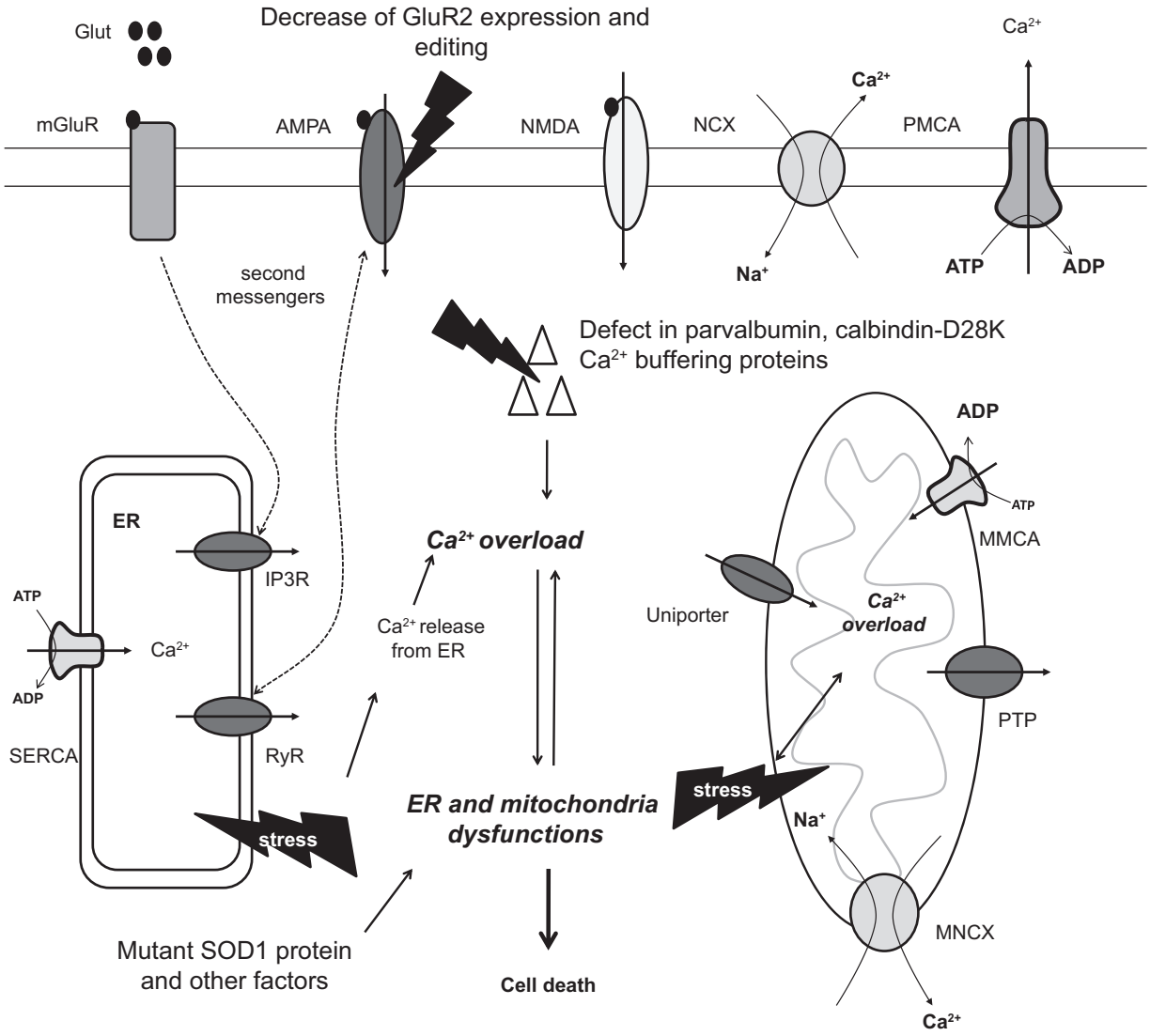

Figure 3 Converging pathological pathways leading to Ca^{2+} overload in amyotrophic lateral sclerosis (ALS) motor neurons (MNs).

Note: In ALS, glutamate initiates several mechanisms that can participate to Ca^{2+}i overload and ultimately motor neuronal death. First, the cell specific decrease of GluR2 subunit expression and editing defect may exacerbate Ca^{2+} permeability of AMPA receptors. Intracellular Ca^{2+} release may also originate from endoplasmic reticulum (ER) after indirect activation of IP3R and RyR by GluRs or from mitochondria through MNCX. The Ca^{2+}i concentration is then normally regulated by mitochondrial (MMCA, uniporter) and ER (SERCA) uptake, plasma membrane extrusion (NCX, PMCA) and by Ca^{2+} binding protein buffering. In ALS, ER, and mitochondrial Ca^{2+}-buffering capacities may be impaired by ongoing stress. Besides reduced buffering capacity due to low level of Ca^{2+}-binding proteins in ALS vulnerable MNs, evidence has been provided for mitochondrial Ca^{2+} overload. This can result in the opening of the PTP leading to membrane potential dissipation, ATP synthesis loss, and pro-apoptotic proteins release.

Abbreviations: IP3R, inositol-1, 4, 5-trisphosphate receptor; RyR, ryanodine receptor; PMCA, plasma membrane Ca^{2+} pump; NCX, Na^+/Ca^{2+} exchanger; SERCA, sarco-endoplasmic reticulum Ca^{2+} ATPase; MNCX, mitochondrial Na^+/Ca^{2+} exchanger; PTP, permeability transition pore; MMCA, mitochondrial membrane Ca^{2+} ATPase.

although it could participate in the late stage of disease progression. As for GluR receptor deficiency in ALS, most of the evidence points to a modest but preponderant role of GluR2, especially in mutant SOD1-linked familial ALS, in the late modulation but not the induction of the disease process.[183–185,187,188] Finally, if ALS MN incapacity to handle large activity-induced Ca^{2+} load appears solid,[175,176] the origin of calcium overload in ALS is more uncertain. Abnormally high influx through Ca^{2+}-permeable GluR, impaired mitochondria and ER buffering, and low endogenous Ca^{2+} binding protein content may all contribute to cytosolic Ca^{2+} accumulation. Nevertheless, knowing that mitochondrial and ER alterations are now described as the earliest detectable alterations in ALS pathogenesis (for review see[95,242]), their role may be preponderant compared to glutamate-associated Ca^{2+} alteration that may arise at a later stage.

Now, how can we translate these different glutamate-associated alterations into realistic therapeutic perspectives for ALS? First of all, we must acknowledge that the unique currently available Food and Drug Administra-

tion-approved treatment for ALS, riluzole, which prolongs patient survival by only few months, is defined as a general inhibitor of glutamatergic transmission.[243,244] Notably, riluzole inhibits voltage-dependent Na^+ channels by stabilizing their inactivate state.[245] Riluzole was also reported to enhance glutamate transport,[112,246] to inhibit glutamate release, and to prevent activation of the intracellular signaling pathway associated with GluR.[247] Despite this supposedly multilevel effect on glutamatergic transmission, riluzole efficacy is in reality very modest, which suggests that the hyperexcitability associated with glutamate or excitotoxicity may have an equally modest role in ALS pathogenesis. In agreement, in ALS SOD1 mice, riluzole did not modify disease onset and only humbly extended animal survival (11%), an effect comparable to the one observed in sporadic ALS patients.[248] Nevertheless, with most of the effects of riluzole being indirect, one can argue that more specifically targeted drugs or a combination of these could exhibit a greater therapeutic effect. When we review the previous drug-based therapeutic strategies targeting glutamate-mediated excitotoxicity in mice (AMPA antagonist,[183] EAAT2 upregulation,[118,119] and caboxypeptidase II inhibition),[249] none was found to consistently modify disease onset and all only modestly extended animal life span (6%–15%). In human clinical trials, treatment with the AMPA/KA antagonist topiramate worsened the patient's condition as it accelerated loss of muscle strength and elicited a series of adverse effects.[250] Clinical trials with the NMDA antagonists dextromethorphan or memantine (acting on other non-GluR; see GluR section) did not elicit any adverse effect or beneficial effect on motor decline, patient vitality, life quality,[251] or survival;[252] the results on life expectancy are in the process of being collected for the newest study.[251] Similarly, inhibitors of glutamate release, such as lamotrigine, were inefficient in improving human ALS.[253] The only positive outcome, other than for riluzole, ever reported for antiglutamatergic drug concerns the treatment of ALS patients with the glutamatergic modulator gabapentin;[254] however this finding was later invalidated.[255] Some have suggested that targeting glutamate transport may be a safer and more efficient strategy than targeting glutamate receptors due to the vast adverse effects of their inhibitors, which waits to be demonstrated.[256]

Altogether, these different clinical trials are not very encouraging for antiexcitotoxic-based ALS therapy. Nevertheless, the strategies targeting oxidative stress, inflammation, or energetic metabolism were no more successful (for review see[257]). So far, all of the mechanisms that have been targeted are very general cell death mechanisms involved in the final progression rather than in the initiation of the disease. The discovery of a really efficient ALS therapy will probably arise only when the earliest pathogenic mechanisms leading to MN dysfunction and death have been unraveled. In the meantime, the best hope for halting ALS propagation certainly relies on the development of multidrug therapies targeting several pathogenic mechanisms that converge on dysfunctions that overwhelm the MNs such as calcium overload.

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Disclosure

The authors declare no conflicts of interest in this work.

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