Glutamate in Amyotrophic Lateral Sclerosis: An Ageless Contestant

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6.1 Introduction

Glutamate is a crucial amino acid which serves a fundamental function in the central nervous system (CNS) and acts as a signaling substance at many excitatory synapses, ordaining on practically all central neurons. It remains in millimolar concentrations chiefly in the presynaptic terminals of excitatory neurons but obtainable throughout in the brain and spinal cord. As a closely regulated process, glutamate release and uptake are vigilantly regulated. The exposure to this neurotransmitter must be concise to neurons and at its appropriate levels it exhibits proper synaptic neurotransmission and/or neurotrophic effects. Indeed, when the extracellular concentrations of glutamate are increased and remain high for an abnormally long duration, as it happens in certain pathological conditions, glutamate acts as a toxin. In this regard, the notion of glutamatergic excitotoxicity was introduced by Olney and collaborators in the 1960s early 1970s [1–4], and studies in last decades have strongly supported the involvement of this hypothesis in neuronal death [5–7].

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6.2 Glutamate, Astrocytes, and Mitochondria

6.2.1 Glutamatergic Excitotoxicity in ALS

The case for excitotoxicity in Amyotrophic Lateral Sclerosis (ALS) began to surface thanks to the pioneer work of Andreas Plaitakis [8–10]. His work leads the way to the hypothesis that a systemic defect in the metabolism of the excitatory amino acid glutamate may lie behind the ALS-related motor neuron death, directing the attention to the role played by glutamatergic excitotoxicity in the ALS etiology. With his colleagues at the Department of Neurology at Mount Sinai School of Medicine in New York, suggested that the delivery of the glutamate between the intracellular and extracellular pools could be altered, possibly due to the outcome of a defected uptake system or release machinery(s) [11]. In the same years, Rothstein and collaborators, from the Department of Neurology at Johns Hopkins reported irregularities in excitatory amino acids in the CNS of ALS patients [12, 13]. In the 1990 study, they measured significantly higher concentrations of glutamate (by 100–200%) in the cerebrospinal fluid (CSF) from ALS patients. Although, at first, there were conflicting evidences since other groups reported a lack of raise in the glutamate concentrations of CSF and plasma of ALS patients

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[14–17], these observations set off a line of research looking at the glutamatergic system and excitotoxicity in ALS.

An additional evidence endorsing the involvement of excitotoxicity in the pathology of sporadic ALS (sALS) was provided once more by Rothstein and collaborators [18]. Using synaptosomes preparations from spinal cord or other impacted brain regions of sALS patients, they detected a functional deficiency in the uptake of high-affinity sodium-dependent glutamate, the GLT-1 glial glutamate transporter. Glutamate elimination from the extracellular space, by high-affinity and low-affinity sodium-dependent carriers expressed by astrocytes and neurons, is the primary mechanism for its inactivation [19, 20]. The low-affinity glutamate transporter sub-serves common metabolic performance. The high-affinity carrier is a constituent of the glutamate neurotransmitter scheme and is accountable for the elimination of neurotransmitter glutamate from the synaptic cleft [21]. If the extracellular concentration of glutamate remains elevated at the cleft, it becomes toxic to neurons. Hence, they examined the glutamate-transport system in brain and spinal cord tissue received from the postmortem brain of ALS patients. They found a significant alteration (decrease) in the efficiency of the glutamate transport in spinal cord and brain tissue from ALS patients, identifying excitotoxic injury as one of the noxious processes beneath motor neuron death.

These observations fuelled the interest on glutamate in ALS and encouraged studies to elucidate the underlying mechanisms.

6.2.2 Glutamate Receptors

A considerable line of research has looked at the receptors that are activated by the neurotransmitter. Physiologically when released glutamate binds to its post-synaptic receptors triggering an increase in Na+ and Ca2+ concentrations. Glutamate activates both ionotropic and metabotropic receptors, with distinct pharmacological and molecular profiles. The metabotropic receptors are members of the G-protein coupled receptor superfamily. They arbitrate synaptic neurotransmission through the activity of intracellular second messenger. Hence, they mediate slow responses.

The ionotropic are ion channels associated with the glutamate-mediated rapid responses. These are classified into three different subtypes: the *N*-methyl-D-aspartate (NMDA), the alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), and the kainate receptors. The NMDA receptors are usually associated with neuronal plasticity. Pathologically, they mediate several acute insults to the CNS, which is consistent with their predominant role during a long exposure to elevated levels of glutamate [22]. Even though slower acting, AMPA or kainate agonists are also compelling neurotoxins and may cause extensive neuronal devastation [23]. Ionotropic glutamate receptor is highly permeable to Ca2+ (with the exception of the AMPA receptors containing the edited GluR2 subunit). Intracellular Ca2+ overload is the key feature of the glutamate-mediated excitotoxicity as demonstrated by Dennis Choi in 1985 [24]. In this landmark study, glutamate excitotoxicity in neuronal cultures was enhanced in a calcium-rich extracellular solution, while a calcium-free extracellular solution noticeably decreased neurodegeneration. Then, following evaluations have established that in the glutamate-mediated injury the intracellular calcium sequestered into mitochondria plays an important role [25]. When over-activated the NMDA receptors allow the entry of excessive amounts of Ca2+ that leads to a mitochondrial calcium overload that in turn triggers mitochondrial dysfunction and activates death signals [26], leading to cell death [27]. The NMDA receptors are considered to be the primarily responsible for the glutamate-mediated Ca2+ entry [28, 29]. In ALS, however, since the 1993 work by Couratier and co-workers [30], AMPA receptors have been considered to be a major player. The authors reported that rat neuronal culture exhibited the poisonous effects when exposed to CSF obtained from ALS patients which was reversed by CNQX, an antagonist to the AMPA/kainate receptor, but not by MK-801 and AP7, two NMDA receptor antagonists. Hence, their data were a strong

indication that AMPA receptors are the main intermediaries in the glutamate-mediated motor neuron death. An additional evidence is offered by the environmental neurotoxins β-*N*-methylamino-L-alanine (BMAA), believed to be correlated to the Amyotrophic Lateral Sclerosis-Parkinsonism Dementia Complex of Guam [31, 32], that again is supposed to be toxic through the commencement of the glutamate receptors [33], mainly of the AMPA subtypes [34]. These evidences have shaped the studies that have looked at the glutamate-mediated excitotoxicity standpoint, focusing essentially on role played by the AMPA subtype of receptor in several "in vitro" [35–38] and "in vivo" [39–43] findings. Although the impact of NMDA receptors in ALS excitotoxicity shouldn't be overlooked [44–47].

These observations while helping define the role of glutamate in motor neuron death have linked glutamatergic-mediated toxicity to two additional frontrunners in the ALS-related pathophysiology: astrocytes and mitochondria.

6.2.3 Spinal Cord Astrocytes in ALS

Astrocytes are the ample populated cells in the CNS (~50% by volume). They have a virtual interaction with neurons, with a metabolism, involving energy generating pathways and amino acid homeostasis firmly coupled to that of neurons [48, 49]. When Rothstein and collaborators reported a dramatic decrease in the GLT-1/EAAT2 immunoreactive protein in motor cortex and spinal cord of patients of ALS [18], they highlighted the glial contribution to the motor neuron demeanor. Then, they punctually demonstrated, by chronically inhibit the synthesis of the glutamate transporter, employing antisense oligonucleotides, that GLT-1/EAAT2 and GLAST/EAAT1 are the main accountable for the amplified glutamate concentration extracellularly and the subsequent glutamate-mediated toxicity [50]. Beyond their precise proposition for perceptive ALS, the studies by Rothstein's group supported the view that astrocytes are directly involved in the pathological process of ALS. This hypothesis was also suggested by a study on postmortem human spinal cords, where the authors concluded that the disease mechanism in sporadic ALS may involve alterations in spinal cord astrocytes [51].

Bruijn et al. [52] reported in a study carried on in the transgenic mice overexpressing human G85R SOD1, a murine model of familial ALS (fALS), the presence of numerous inclusions in astrocytes that preceded the appearance of similar inclusions in neurons. They suggested that besides the glutamate transporter malfunction, molecular targets, present within the astrocytes, and possibly damaged by mutant SOD1, while affecting astrocytes were harming motor neurons. Additional studies confirmed that neuronal Lewy-body-like hyaline inclusion and astrocytic hyaline inclusion were morphological trademark of SOD1-linked familial ALS patients and mice expressing the human SOD1G85R mutation [53, 54]. These findings were further confirmed by Watanabe et al. [55] in two other SOD1 fALS mouse models the SOD1G93A and SOD1G37R. They reported the presence of proteinaceous accumulations in astrocytes and concluded that abnormal astroglial biology could be important in the cell death in ALS.

To clearly understand glial role in ALS genetically engineered mice with a restricted overexpression of mutant SOD1 only in astrocytes or only in neurons have been extremely valuable. Gong et al. [56] generated transgenic mice with mutant SOD1 overexpression restricted to the astrocytes to see whether these mice would extend unplanned motor neuron degeneration and astrocytic pathology. Their experiments demonstrated that when mutant SOD1 expression is limited to astrocytes it causes significant pathological changes within astrocytes but was insufficient to cause motor neuron death or motor dysfunction in vivo. Their conclusion was that astrocytosis in mutant SOD1 is the result of a combined neuronal function impairment as well as prime straight astrocytic dysfunction. Soon after Rouleau and co-workers [57] generated transgenic SOD1G37R mice driven by the neurofilament light chain promoter, to test whether motor neuron restricted expression of mutant SOD1 was adequate for disease

occurrence. They found that the neuronal cell-specific expression of mutant SOD1 does not originate noteworthy motor neuronal cell death and reported that their mice seems healthy at age of more than 18 months. On the contrary, when ubiquitously express the SOD1G37R gene causes the disease as early as 3.5 months and produces clear pathological features in motor neurons (cytoplasmic vacuoles in dendrites, proximal axons, and perikarya, including degenerating and swollen mitochondria) [58]. Another group [59] created a G85R mutant SOD1 deletion, with a confined expression to spinal motor neurons and interneurons. Their transgene generated pathological (loss of motor neurons) and immunohistochemical symbols of motor neuron degeneration (ubiquitin staining) only in the mutant SOD1-immunoreactive cells, without any clear phenotypical signs. They believed that their mice did not build up the clinical disease because the mutant SOD1 expression occur only in a few motor neurons and that a more extensive motor neuron degeneration would be necessary for the disease to become clinically apparent. Hence, they argued that their data diverged, for this reason, from earlier published studies in which mutant SOD1 focused by neuronal promoters abortive to generate either clinical or pathological verification of motor neuron degeneration [57, 60]. Whether or not this is the case, these data clearly assess that mutant SOD1 has to be overexpressed in both neurons and glia to be able to trigger the disease and show its phenotype "in vivo." An interesting manuscript is the one by Hensley et al., [61] showing that primary cultures astrocytes carrying the SOD1G93A mutation hold an altered unstable phenotype prone to produce proinflammatory substances and enter a proinflammatory state.

These observations set the tone to a new view for ALS, as a non-cell autonomous disease [62]. Classically, neurotoxicity in neurodegenerative diseases is viewed as a process where a particular neuronal population is mainly susceptible to a collective toxic load (i.e., toxic mutant proteins). The chronic damage caused by this toxicity, combined with aging, reaches a verge that crushes the neuron's protective machineries leading to its death. Initially, this process was seen as cell autonomous, self-regulating for the damage gathered within other cell types interacting with the neuronal cells. This view has now changed. Cleveland and co-workers using a Cre/loxP SOD1G37R transgenic mice have showed a contribution of diverse cell types to mutant SOD1-induced motor neuron disease. They constructed chimeric mice that incorporated combination of normal and mutant SOD1-expressing cells. Their analyses show that elevated levels of expression of mutant SOD1 in most [63] or all [64] motor neurons are insufficient for early onset of disease, thus linking disease initiation to the synthesis of mutant proteins by non-motor neurons. Then, cell type-dependent excision in mice-expressing transgenes flanked by lox sites has contributed to ascertain the identities of cells whose mutant SOD1 synthesis participates in the disease pathology. The same authors elegantly proved that the selective expression to motor neurons of a ALS-linked SOD1 mutant delayed disease onset, but the degree of disease progression did not alter after the disease onset [62, 65]. Specifically, they demonstrated that a decreased expression of SOD1G37R in microglia and activated macrophages offered slight effect on the initial phase of the disease onset, but their effect could increase with disease progression and could significantly slow down the late phase. In other words, the disease onset between this model and the one that overexpresses mutant SOD1 ubiquitously was similar, while the disease duration after the onset was significantly higher in the selective-expressing mutant.

Apart from the role played in ALS, the dependence of neurons on astrocytes for their energy metabolism and glutamate synthesis [48] is critical. Neurons need astrocytes to maintain the right levels of glutamate, behind its clearance from the cleft. They lack the enzyme pyruvate carboxylase, for this reason they rely on astrocyte cells for de novo glutamate synthesis [66–68]. Moreover, the astrocyte-derived glucose is an essential precursor for the glutamate synthesis [69], and in maintaining its optimum concentration [70].

6.3 Mitochondria and Calcium Loading in Glutamate Excitotoxicity

As discussed above, the excessive activation of the ionotropic glutamatergic receptors leads to the deficit of post-synaptic structures (i.e., dendrites) and neuronal cell bodies. In this context in a neurodegenerative disease as ALS, where neuronal cell death take place over an comprehensive time period, we can envision a condition of chronic glutamate-mediated excitotoxicity. In other words, a long repeated activation of the glutamatergic receptors determined by an increased extracellular glutamate concentration may lead to the nerve cell death.

As a proof of concept, organotypic spinal cord cultures have been utilized to investigate chronic glutamate toxicity [71]. These organotypic cultures may be asserted for up to 3 months. Using two different glutamate uptake inhibitors (threohydroxyaspartate and pyrrolidinedicarboxylic acid), the authors obtained a continual increase of glutamate in the cell culture medium that they linked to a concentration- and duration-dependent motor neuronal cell death. They also reported that the glutamate-mediated neuronal death was neutralized by non-NMDA receptor antagonists and inhibitors of glutamate synthesis or its release. Their experiments revealed that a moderate and prolonged increased of extracellular glutamate can induce toxicity.

Chronic excitotoxicity has also been linked with the Guamanian amyotrophic lateral sclerosis/Parkinson-dementia complex (ALS/PDC), BMAA toxin, from the cycad Cycas circinalis, is considered a possible cause [72]. Although the evidence of its association to the Guanamian ALS/PDC is still controversial [72], the oral dosing of BMAA to macaques causes a motor system impairment affecting both upper and lower motor neurons and also on the extrapyramidal system [73].

Mitochondria are the cellular power plant. They are highly dynamic organelles controlled by an array of physiological stimulus that change their shape through the fission/fusion cycle. Metabolic function during physiological cellular life may contribute dysfunction of mitochondria and their damage. A significant burden for their homeostasis mainly in post-mitotic tissues, such as the brain, is the oxidative damage. With age they accumulate altered proteins in their matrix (i.e., oxidized and glycoxidized), and their ATP-stimulated proteolytic activity decreases considerably [74].

They play complex, interdigitated roles in cellular physiology, have a crucial role in providing the brain with energy (ATP generation), and are central in cell death mechanisms through the activation of cellular suicide programs (i.e., apoptosis) [75]. In addition to providing the ATP necessary to maintain ionic gradients, they can also buffer cytosolic Ca2+ [76], thanks to their large electrochemical potential [77]. Mitochondria shape the Ca2+ responses in neurons by taking up large amounts of the ion [78, 79]. This has been observed in neurons stimulated with glutamate [80, 81]. Hence, there is a direct dependency between the glutamate-mediated Ca2+ response and mitochondrial homeostasis.

Mitochondrial alterations in ALS were suggested by neuropathological studies on postmortem human patients [82]. Then, the mitochondrial involvement in ALS became obvious when, thanks to the mutant SOD1 transgenes, the anatomical analyses of the affected tissues revealed the presence of numerous membrane-bound vacuoles in the G93A and G37R lines. These vacuoles were evident prior to the last phase of the disease and seem to be originated from dilated mitochondria [58, 83] and the endoplasmic reticulum [83]. Subsequent studies have confirmed that mitochondrial abnormalities are an early feature in ALS and that mitochondrial degeneration is an important early event [84, 85].

Weiss and co-workers were within the first to investigate, in spinal neurons, the downstream sequelae of Ca2+ entry by the Ca2+ permeable AMPA/Kainate ionotropic glutamate receptors [35, 86, 87]. They found that motor neurons were extremely susceptible to the chronic Ca2+-dependent mediated injury of those receptors [35, 86]. Then, they extended their analyses and focused on mitochondria and reactive oxygen

species (ROS) generation [87]. They reported that motor neurons are more susceptible, than GABAergic cortical neurons, to AMPA/kainate receptor-mediated damage essentially because their activation triggers substantial mitochondrial Ca2+ excess, mitochondrial depolarization and ROS production. They concluded that the expression of Ca2+ permeable AMPA receptor channels by motor neurons probably contributes to their extreme susceptibility in ALS.

Consistent with these data supporting the role of calcium and oxidative stress in the pathology of ALS is the work by Kruman et al. [88]. The authors further confirmed the augmented vulnerability of MOTOR NEURONs from mutant SOD1 to excitotoxicity and clarify some of the fundamental machineries. They identify elevated basal-oxidative stress and disturbed mitochondrial functions in the mutant spinal cord cultures. Moreover, excitotoxic experiments let them to conclude that mutant motor neurons were extremely vulnerable to the AMPA-mediated glutamate toxicity and that their Ca2+ homeostasis is perturbed. They also reported that antioxidant and Ca2+-reducing agents were protecting against glutamate-mediated toxicity. We have reported a differential expression of the AMPA receptor subunits in mutant SOD1G93A spinal motor neuron in culture [38]. Using the single-cell PCR technology, we were able to demonstrate that the mutant SOD1 alters the AMPA receptor isoforms and subunit composition leading to the expression of a high-gain AMPA receptor that desensitizes more slowly with a longer receptor open time. This provokes an elevated Na+ influx with a resulting extended cell depolarization and opening of voltage-sensitive Ca2+ channels, with an increase in the intracellular Ca2+ and subsequently increased excitotoxicity [38]. The mitochondrial involvement in ALS has also been demonstrated for the cortical motor neurons. Van Westerlaak and co-workers using a rat cortical explant culture model determined that the persistent mitochondrial inhibition ensued in a dose-dependent rise of cortico-spinal motor neuron death. The neuronal death was reverted by the NMDA antagonist MK-801 and the non-NMDA antagonist CNQX clearly showed the role of glutamate through both non-NMDA and NMDA receptors [89, 90].

In the work by Avossa et al. [91] using organotypic slice cultures from wild-type and SOD1G93A spinal cords, early signs of mitochondria vacuolization in the mutant ventral horns were not found. However, other works confirmed the occurrence of altered/malfunctioning mitochondria in spinal and cortical motor neurons, combined with glutamatergic excitotoxicity.

Calderò and collaborators working with an organotypic slice culture from chick embryos spinal cord examined the motor neuron response to various excitotoxins. Their results confirmed the high motor neuron sensitivity to kainate and NMDA. Moreover, their results show that motor neurons are also highly vulnerable to persistent inhibition of mitochondrial functions with malonate and 3-nitropropionic acid (3-NP), which did cause excitotoxic-like lesions. They conclude that their data reveal a positive association among excitotoxicity and mitochondrial dysfunction in MOTOR NEURONs [45]. The protective effects of pyruvate and β-hydroxybutyrate (βHB) as energy substrates in association with the antioxidants glutathione ethyl ester and ascorbate in a chronic AMPA-induced neurodegeneration were also demonstrated by Tapia and co-workers [92]. Again, more recently Tapia's group [93] showed that AMPA perfusion in the lumbar rat spinal cord causes motor neurons death and the permanent paralysis of the ipsilateral hind limb. Interestingly, they reported mitochondrial dysfunction as an early hallmark of neuronal degeneration, prevented when AMPA was perfused together with pyruvate. The authors demonstrated that the progressive motor deficits, massive death of lumbar spinal MOTOR NEURONs, and noteworthy astrogliosis in the ventral horns following "in vivo" AMPA infusion was prevented by the co-infusion of pyruvate or βHB, while the antioxidants co-infusion was ineffective. They concluded that the protection observed with pyruvate and βHB, two

well-recognized mitochondrial energy substrates, is indicative of the importance that the deficit in mitochondrial energy metabolism has in the excitotoxic AMPA-dependent motor neuron death.

Mitochondria are central in the ALS-related pathology as self-governing organelles, and as interconnected organelles in cross talk especially with the endoplasmic reticulum [94]. In this context, the endoplasmic reticulum–mitochondria–Ca2+ cycle (ERMCC) and its link to the disruption of the Ca2+ homeostasis, determined by glutamate-mediated is gaining momentum [95, 96]. Indeed, Ca2+ dysregulation, which is generally triggered by neuronal over-activation, is closely interconnected with the mitochondrial pathology Cozzolino and Carri [97].

It has been reported that glutamatergic excitotoxicity is one of the first pathological pathways related to the motor neuronal death in ALS [98]. However, this event could be submissive by other players in the ALS pathophysiology, which may lead to hyperexcitability (i.e., GABAergic interneurons impairment, Na+ channels malfunction, and altered K+ concentration at the cleft; [99]). Since the central role of glutamate in neuronal function and brain homeostasis is well accepted, it retains a noteworthy role in the disease pathology (Fig. 6.1).

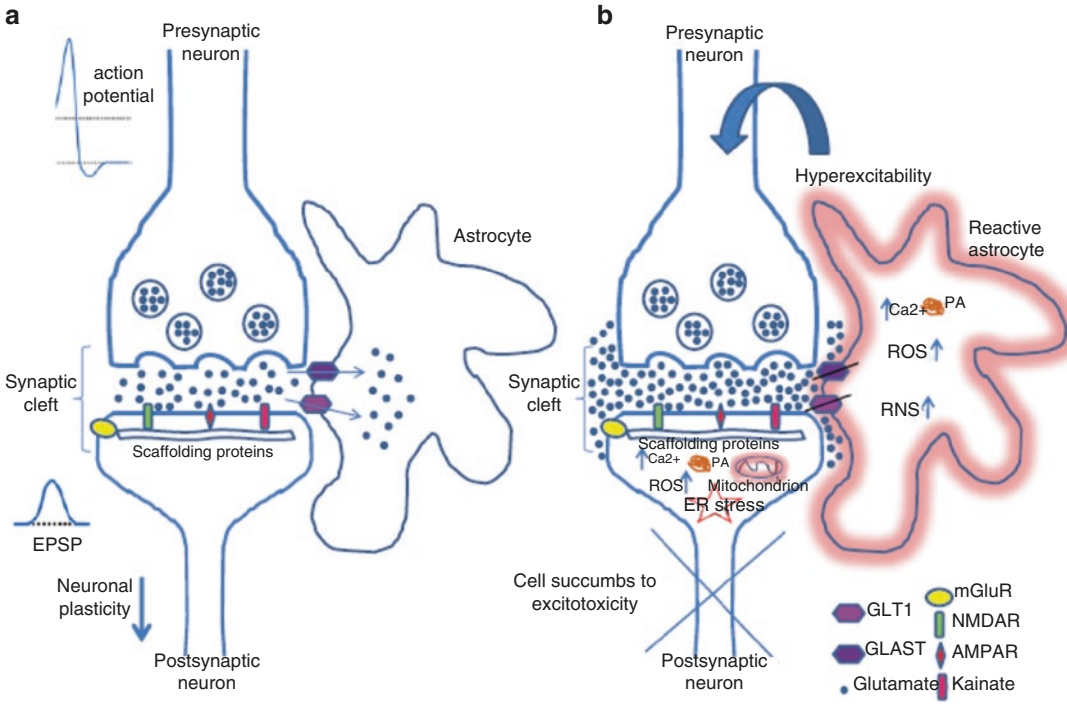

Fig. 6.1 Glutamatergic transmission in ALS pathology. (**a**) During physiological conditions, glutamate released by the presynaptic motor neuron which stimulates its receptors on the post-synaptic neuron to generate excitatory post-synaptic potentials (EPSPs) and contribute to neuronal plasticity. (**b**) In ALS presynaptic motor neuron generates excessive glutamate release. In addition, the concurrent incident of a reduced expression of the glial glutamate transporter GLAST/GLT1 ascertains a pathological rise in the extracellular levels of glutamate in the synaptic cleft. This offers an over-stimulation of the glutamate receptors on the post-synaptic neurons with a resultant cellular excitotoxicity on top of synchronized factors such as mitochondrial failure and endoplasmic reticulum (ER) stress. Moreover, both neuronal and astrocyte cells build up proteinaceous aggregates (PA), increased Ca2+ and reactive oxygen/nitrogen species (ROS/RNS) levels. The incidence of all these measures leads to cellular death. (From Spalloni et al. [47])

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