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Review

Molecular and Cellular Mechanisms Affected in ALS

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Abstract: Amyotrophic lateral sclerosis (ALS) is a terminal late-onset condition characterized by the loss of upper and lower motor neurons. Mutations in more than 30 genes are associated to the disease, but these explain only ~20% of cases. The molecular functions of these genes implicate a wide range of cellular processes in ALS pathology, a cohesive understanding of which may provide clues to common molecular mechanisms across both familial (inherited) and sporadic cases and could be key to the development of effective therapeutic approaches. Here, the different pathways that have been investigated in ALS are summarized, discussing in detail: mitochondrial dysfunction, oxidative stress, axonal transport dysregulation, glutamate excitotoxicity, endosomal and vesicular transport impairment, impaired protein homeostasis, and aberrant RNA metabolism. This review considers the mechanistic roles of ALS-associated genes in pathology, viewed through the prism of shared molecular pathways.

Keywords: oxidative stress; mitochondria dysfunction; axonal transport; autophagy; endocytosis; secretion; excitotoxicity; RNA metabolism; MND

1. Introduction

Amyotrophic lateral sclerosis (ALS) is the most frequent motor neuron disease (MND), with an estimated ~223,000 patients being affected globally in 2015 [1]. The pathology affects both upper motor neurons (UMN) in the cortex and lower motor neurons (LMN) in the brainstem and spinal cord [2]. Paralysis and death usually occur between three to four years after symptom onset [3], and there are currently no effective treatments to slow disease progression [4]. Approximately 90% of ALS cases are sporadic, while 10% are familial, defined by the occurrence of ALS in more than one family member [5]. Around 30 different genes are linked with ALS [5,6], explaining ~20% of all ALS cases and associated with different molecular functions and disease phenotypes [7], so that the task of understanding the relationships between affected pathways is complex.

To investigate the different molecular pathways affected in ALS, various in vivo models, including drosophila [8–11], C-elegans [12], zebrafish [13–16], and rodents [17], as well as in vitro cell models such as patient lymphoblastoid cell lines [18] and hybrid [19,20] or primary murine cell lines, [21] have been developed. Most of these models investigate the pathological effects of mutations to ALS genes, including *Fused in Sarcoma* (*FUS*), *Superoxide dismutase* (*SOD1*), *TAR DNA-binding protein 43* (*TDP-43*), and *Chromosome 9 open reading frame 72* (*C9orf72*) [22,23]. Their study has resulted in numerous cellular and molecular mechanisms being proposed to explain motor neuron death. Mechanisms frequently implicated include: reactive oxygen species (ROS)-associated oxidative stress [24–27], mitochondrial dysfunction [24], axonal

and vesicular trafficking dysregulation [28,29], glutamate-mediated excitotoxicity [30–33], proteostatic impairments [34–38], and altered RNA metabolism and/or processing [39–42].

Alteration to one or more of these cellular processes may be present, not only in the motor neurons themselves but, also, in neighboring cell populations, such as glial cells, peripheral inflammatory cells, and muscles, as ALS is increasingly considered a multisystemic disease that culminates in motor neuron death [6,24]. For example, astrocytes and microglia have been implicated in the release of proinflammatory mediators that lead to chronic neuroinflammation and motor neuron toxicity [43]. In addition, the selective overexpression of mutant SOD1 in skeletal muscle was shown to cause mitochondrial abnormalities, induce microglial activation in the central nervous system (CNS), and result in severe muscle atrophy in mice [44].

Consensus is yet to be reached regarding the causal mechanisms involved in the onset and propagation of ALS. The aim of this review is to identify and summarize the different molecular mechanisms implicated in various forms of the disease, including sporadic and familial cases. In doing so, it is hoped that new insights may be gained regarding the role of different pathways across different forms of the disease.

2. Oxidative Stress

Oxidative stress results from an imbalance between the production and elimination of reactive oxygen species (ROS) [45], as well as an impaired ability to repair ROS-mediated toxicity [46], and has been of particular interest in ALS pathogenesis ([47] and Figure 1) since the discovery of *SOD1* mutations in familial forms of ALS [48]. Increased levels of oxidized proteins, RNA, DNA, and lipids have been observed in post-mortem tissue from both sporadic and *SOD1* ALS cases [27,49,50], as well as in the cerebrospinal fluid (CSF), serum, and urine of sporadic ALS patients [26].

SOD1 is a major antioxidant enzyme that is ubiquitously expressed and catalyzes radical superoxide anions into molecular oxygen and hydrogen peroxide [51]. Approximately 80 of the 160 *SOD1* mutations reported in ALS are missense mutations that fail to cause a loss of SOD1 activity [52], and many SOD1 mouse models show a progressive, late-onset motor phenotype with concomitant astrogliosis and motor neuron pathology when mutated forms of human SOD1 are overexpressed [17]. Evidence from human samples have shown that there is a 42% reduction in overall SOD1 activity in familial SOD1 patients [53], potentially leading to an imbalance between ROS production and degradation (Figure 1). This imbalance might be exacerbated by the disruption of the NRF2-ARE (Nuclear erythroid 2-Related Factor—antioxidant response element) signaling pathway that is observed in *SOD1* ALS [54], thus affecting the expression of antioxidant proteins [55] (Figure 1). Supporting these hypotheses, oxidative damage such as protein glycoxidation and lipid peroxidation were observed in the motor neurons of the anterior horn from *SOD1* familial ALS (fALS) patients [56] and $SOD1^{G93A}$ mice [57,58].

The generation of ROS could result from the activity of NADPH oxidase in the lipid raft membrane compartment. Interestingly, the *ATXN2* gene encodes the ataxin-2 polyglutamine (PolyQ) protein, and intermediate-length PolyQ expansions (27–33 Qs), which are known to be a significant risk for ALS [59–61], can interact with NADPH oxidase and may lead to an increase in ROS production, DNA damage, and mitochondrial distress [62] (Figure 1).

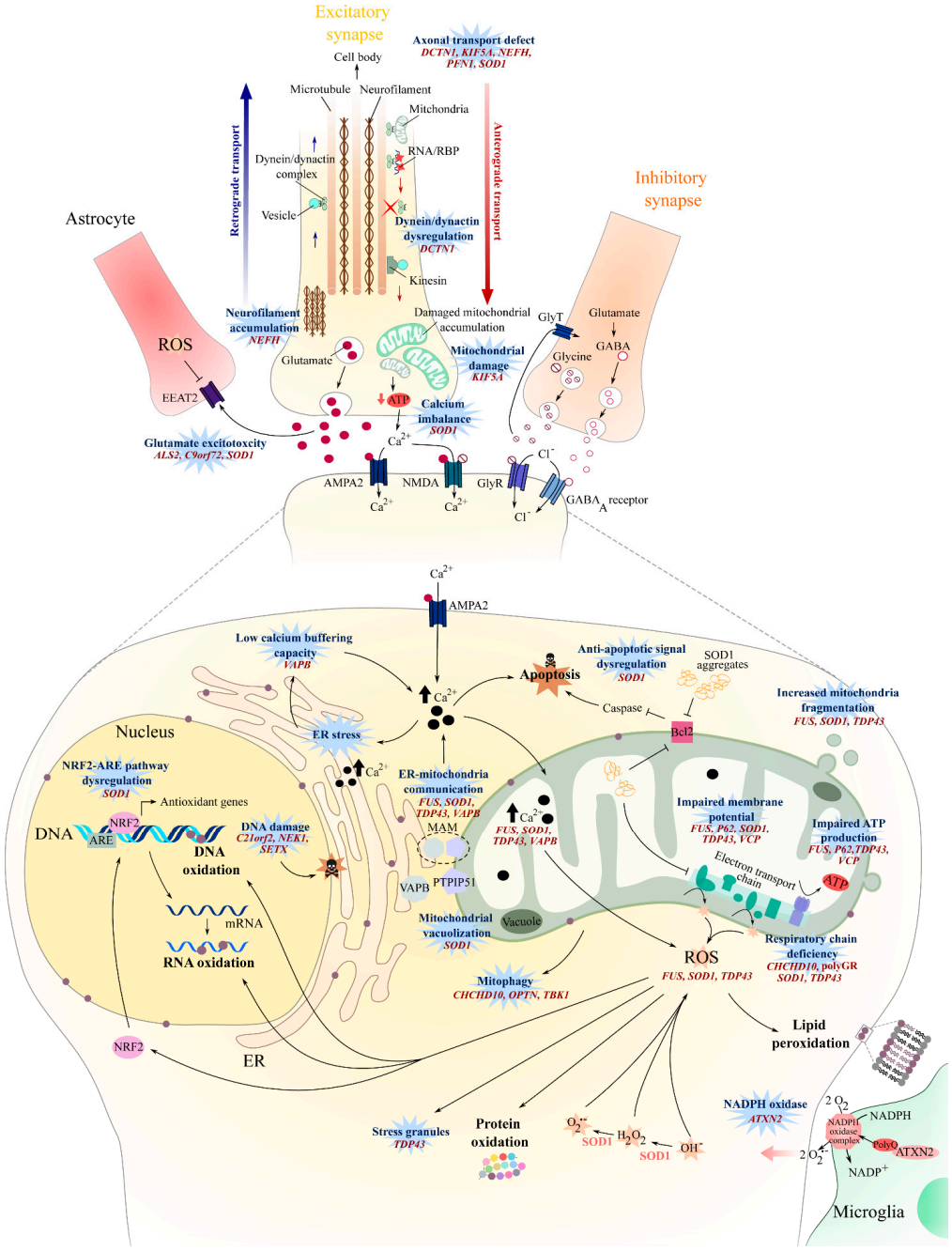

Figure 1. Oxidative stress, mitochondrial dysfunction, axonal transport, and glutamate excitotoxicity in amyotrophic lateral sclerosis (ALS). An increase in oxidative stress can result from defects in detoxifying pathways. Such defects include the loss of SOD1 function, aberrant DNA damage repair machinery, or a decrease in expression of antioxidant genes affecting the NRF2-ARE pathway. Oxidative stress can also be increased by the stimulation of ROS production via increased NADPH oxidase activity or from disrupted mitchondrial respiratory chain activity. Mitochondrial activity can be affected by several ALS mutations, such as those leading to the accumulation of protein aggregates, or to decreased mitochondrial biogenesis and transport, or to increased cytosolic Ca^{2+} (as observed when glutamate receptor activity is stimulated or when the Ca^{2+}-buffering capacity is decreased). Consequently a disruption of the mitochondrial respiratory chain will lead to an increase in ROS production and, thus, to an accumulation of oxidized proteins, lipids, DNA, and RNA. Oxidative damage occurring over time may then stimulate apoptotosis and, thus, cell death. Defective axonal transport affects not only the mitochondria but, also, the transport of other proteins and RNA, with consequences on the axon structure and function being accompanied by neurofilament accumulation.

Defective glutamate uptake by astrocytes, and/or a defect in glutamate receptor clearance or in AMPA or GABA receptors, can lead to increased Ca^{2+} permeability and can impact the post-synaptic hyperexcitability and mitochondrial function. ARE: antioxidant response element, AMPA2: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor 2, ATXN2: ataxin 2, Bcl2: B-cell lymphoma 2, C9orf72: Chromosome 9 open reading frame 72, C21orf2: Chromosome 21 open reading frame 7, CHCHD10: coiled-coil helix coiled-coil helix domain-containing 10, DCTN1: Dynactin 1, EEAT2: Excitatory amino acid transporter, ER: endoplasmic reticulum, FUS: Fused in Sarcoma, GABA: gamma-Aminobutyric acid, GlyR: glycine receptor, GlyT: glycine transporter, KIF5A: Kinesin heavy-chain isoform 5A, MAM: Mitochondria-associated ER membranes, NEFH: heavy-weighted neurofilaments, NEK1: (NIMA)-related kinase 1, NMDA: N-methyl-D-aspartate receptor, NRF2: Nuclear erythroid 2-Related Factor, PFN1: profilin-I, PTPIP51: Protein tyrosine phosphatase-interacting protein 51, SETX: senataxin, SOD: Superoxide dismutase 1, SPG11: Spatacsin, TDP-43: TAR DNA-binding protein 43, VAPB: vesicle-associated membrane protein-associated protein B, VCP: valosin-containing protein, and ROS: reactive oxygen species.

Recurrent oxidative stress and/or mitochondrial dysfunction occurring throughout the life of the cell can lead to DNA damage—damage that can be fixed by activating the DNA damage repair machinery. Several genes known to encode for proteins involved in DNA damage repair [63–65] are also associated with ALS: *NEK1* [66], *C21orf2* [67], and *SETX* [68]. These encode for the proteins never in mitosis-A (NIMA)-related kinase 1 (NEK1), cilia and flagella-associated protein 410, and the DNA/RNA helicase senataxin, respectively. Mutations in these genes may therefore increase the susceptibility to ALS as a result of dysregulated DNA damage repair machinery [67,69,70], leading to an impaired ability of motor neurons to cope with oxidative stress, consequently leading to cell death [64,70] (Figure 1). For example, induced pluripotent stem cell (iPSC) motor neurons derived from $NEK1^{c.2434A>T}$-mutated ALS patients exhibit an increased level of DNA damage, as well as a failure to repair DNA double-strand breaks [70]. Primary motor neurons from $SETX^{R2136H}$ and $SETX^{L389S}$ murine models were unable to cope with induced oxidative stress and showed an increased stress granule formation [71].

Altogether, these studies suggest that oxidative stress might be increased in sporadic and familial ALS patients. Increased oxidative stress may affect mitochondrial function [72], exacerbate endoplasmic reticulum stress [73], and impact protein homeostasis mechanisms [74], ultimately leading to cell damage and neuronal loss.

3. Mitochondrial Dysfunction

Mitochondria are key organelles for ATP generation, calcium buffering, and apoptosis regulation [75], and their dysfunction in the dorsal root ganglion cells of sporadic ALS patients has been described previously [76]. Several mechanisms can trigger mitochondrial dysfunction in ALS (Figure 1).

The maintenance of mitochondrial cristae organization is crucial to ensure respiratory chain function [77] and requires cardiolipin, the ATP synthase dimer, and large protein complexes such as the mitochondrial contact site complex (MICOS) and dynamin-like Opa1/Mgm1 [78,79]. The coiled-coil helix coiled-coil helix domain-containing protein 10 (CHCHD10), known to be associated with ALS [23], is suspected to be either part of [80,81] or interact with MICOS [82]. Consequently, mutations in *CHCHD10* result in the loss of mitochondria cristae [80], mitochondria fragmentation [81], and defective mitochondrial repair [80,83] (Figure 1).

Mitochondrial biogenesis can also be directly affected, as observed in *FUS*-mutated conditions [84,85]. While *FUS* encodes for a DNA/RNA-binding protein [86] predominantly localized to the nucleus, mutated forms of FUS can accumulate in the cytosol and possibly become toxic [87,88] and affect the mitochondrial function. For example, the mutated FUS^{P525L} can interact with mitochondrial chaperone proteins and induce mitochondria fragmentation and elevated ROS production [84,85].

Aberrant swollen mitochondria morphology has also been observed in neuronal, non-neuronal cells, and muscle tissue of other fALS cases, such as *SOD1* [24,89] and *C9orf72* [25,89]-mutated ALS patients but, also, in both $SOD1^{G93A}$ and $TDP\text{-}43^{A315T}$ murine models [44,90,91]. The aberrant morphology may result from a cascade of events involving the mutated protein aggregates. For example, insoluble mutant SOD1 can aggregate in mitochondria in the spinal cord of $SOD1^{G93A}$ mice [92], causing the formation of vacuoles in the outer- and inter-mitochondrial membrane [93], affecting mitochondrial respiration, energy production, and ultimately, increasing the level of oxidative stress [94] (Figure 1). ALS patients with the $SOD1^{A4V}$ mutation show significant increases in complex I and III activity of mitochondria in the motor cortex [95,96]. The overactivation of complexes I and III increased the production of mitochondrial ROS [97] and may explain the high level of oxidative stress observed in SOD1 mice and patients.

The G4C2 hexanucleotide repeat expansion mutation (HREM) in the *C9orf72* gene explains 40–50% of familial ALS cases and 5–10% of sporadic cases [98–101]. There are several hypotheses regarding the mechanisms by which this leads to toxicity, and evidence exists for both loss and gain-of-function-mediated toxicity. One hypothesis suggests that the repeat-associated non-AUG (RAN) translation of G4C2 repeats is causal in the expression of toxic dipeptide repeat (DPR) proteins. RAN translation can occur in both sense and antisense reading frames [41], resulting in the production of five different DPRs: glycine-alanine (GA), glycine-arginine (GR), proline-arginine (PR), proline-alanine (PA), and glycine-proline (GP) [38]. Interestingly, the expression of poly-GR results in early abnormalities in the mitochondiral respiratory chain by interacting with ATP5A1, a complex V protein, and leads to its ubiquitination and degradation in *C9orf72* ALS-FTD patients [102]. Mitochondrial dysfunction [103] and an increased oxidative stress [104] are reported in fibroblasts and iPSC-derived astrocytes obtained from *C9orf72* ALS patients (Figure 1).

Nonfunctional and damaged mitochondria can be targeted by NIP3-like protein X (NIX) or PTEN-induced putative kinase protein 1 (PINK1)-E3 ubiquitin ligase parkin, then sequestered into isolation membranes and degraded after fusion with the autophagosome or lysosome [105]. Optineurin (OPTN) and TANK-binding kinase 1 (TBK1) are key actors for mitochondria engulfment [106]. Consequently, ALS mutation in *OPTN* [107] and *TBK1* [23] will affect the mitophagic flux and may lead to an accumulation of nonfunctional mitochondria over time and result in motor neuron death (see [108] for review). Taken together, except for *CHCHD10*, these studies point toward mitochondria dysfunction and damage being a downstream effect of ALS gene mutations that lead to protein aggregations and/or proteostasis dysfunction (see Section 7: Impaired Protein Homeostasis).

In addition, damage to mitochondria and alterations in their functions can disrupt calcium homeostasis, increasing the sensitivity of neurons to glutamate excitotoxicity and the risk of motor neuron damage ([109], Figure 1). Mitochondrial dysfunction can also activate proapoptotic signals [93], such as the caspase-dependent [110] or bcl-2-dependant pathways [93], and might lead to motor neuron degeneration.

4. Axonal Transport

Motor neurons have exceptionally long axons, up to 1 m in length, placing extreme demands on cellular physiological functions that rely on the axonal transport of organelles such as mitochondria or of molecules including proteins, lipids, and RNA to and from the synapse [111]. Axonal transport, as well as the conduction of electrical impulses and the maintenance of the axon structure, are heavily regulated processes linked with control of the neurofilament structure [112,113]. In both sporadic ALS (sALS) and fALS patients, the disorganization of neurofilament networks has been reported [38].

Neurofilaments are neuron-specific intermediate filaments that are stretch-resistant and are major cytoskeleton proteins [114]. They form parallel coiled-coiled heterotetramers composed of light, medium, and heavy-weighted neurofilaments (NF-L, NF-M, and NF-H, respectively) and α-internexin or peripherin [112,114]. Eight heterotetramers form cylindrical structures known as unit-length filaments (ULFs) with the tail domains sticking out [112,114]. A series of ULFs form a filament that

matures into neurofilament after a radial compaction of the cylindrical structure [112]. Consequently, variants in the *NEFH* gene affecting the crosslinking properties of the NF-H protein may result in abnormal neurofilament accumulations and in axonal transport defects [115].

Neurofilaments form cross-bridges not only with each other but, also, with actin filaments, actin rings, and microtubules [114], constituing a protein network that might participate to the maintenance of the axon structure [114,116]. Actin polymerization requires the small actin-binding proteins profilin I and II and phosphoinositide islands localized at the membrane [117]. Mutations in the *PFN1* gene encoding profilin-I are associated with ALS [118], and the expression of mutant hPFN1^{G118V} in a murine model resulted in dysregulated actin polymerization [119]. Consequently, the attachement of actin to the microtubules might be affected, probably impacting anterograde and retrograde transport and, thus, leading to an accumulation of fragmented mitochondria and, ultimately, to upper and lower motor neuron death ([119], Section 3 and Figure 1).

Microtubules and motor proteins such as the dynein-dynactin complex [28,120,121] and the kinesins [120,122,123] are involved in the long-distance transport of cellular cargo. Microtubules are composed of dimers of α- and β-tubulin. The alpha tubulin subtype TUBA4A is an ALS-associated protein [124], and ALS-associated mutations of *TUBA4A* lead to microtubule polymerization defects and network destabilization [124].

The dynein-dynactin complex [28,120,121], along with the kinesins [120,122,123], are key drivers of the anterograde and retrograde movements of diverse cargoes along the microtubule cytoskeleton, including organelles, vesicles, neurofilaments, AMPA and GABA receptors, and RNAs. Interestingly, mutations in *dynactin subunit 1 (DCTN1)* affecting the tertiary structure of the dynactin protein and its capacity to bind to microtubules can cause ALS [125]. When the interaction between dynein-dynactin is interrupted by the overexpression of dynamitin, axonal transport is impaired, and mice develop a late-onset motor pathology that recapitulates late-onset progressive ALS [126]. Kinesins form a superfamily of molecular motors that can be divided into three groups [120]. KIF5, a member of kinesin 1-group, is a tetramer with two kinesin heavy chains (KHCs) that contains a motor domain and two kinesin light chains (KLCs) that facilitate connections with cargo. There are three KIF5 isoforms—KIF5A, KIF5B, and KIF5C—all three isoforms being associated with the neuronal function and anterograde transport of proteins and organelles [127]. Mutations in the C-terminal of KIF5A, leading to a loss of function, are associated with ALS [128] and are suspected to disrupt the axonal transport (Figure 1). This hypothesis is supported by the defective axonal transport of mitochondria, the local accumulation of neurofilament, and the reduced axonal growth and survival observed in the primary culture of motor neurons from KIF5A$^{-/-}$ mice [129].

Distal axonal transport is also affected in SOD1^{G93A} mice at an early stage, with an early decrease in kinesin expression in asymptomatic mice, followed by a decrease in dynein expression in older presymptomatic mice [130]. Defective axonal transport may contribute to the accumulation of impaired mitochondria at distal sites ([93], Figure 1), resulting in decreased ATP production and disrupted calcium homeostasisis at the neuromuscular junction, consequently leading to a distal axonopathy in SOD1^{G93A} mice [109,131,132] and *SOD1* patients [24,28]. Kinesin-dynein machineries have been described to be affected in sporadic ALS, where KIF1Bβ and KIF3Aβ, two kinesin-related proteins, were found to be downregulated in motor cortex samples of sporadic patients [133]. However, the expression level of another kinesin-related protein, KIFAP3, is inversely correlated with sporadic ALS patient survival [134].

In conclusion, different mutations associated to ALS can directly alter the architecture and dynamics of the cytoskeleton, affecting the axonal transport machinery. Interestingly, aberrant axonal transport has also been observed in sALS patients and in fALS patients harboring mutations in non-cytoskeletal-related genes. Disrupted transport mechanisms can then affect the mitochondrial metabolism and degeneration (Section 3), as well as protein degradation (Section 7) and RNA transport (Section 8), ultimately leading to motor neuron death.

5. Glutamate Excitotoxicity

Glutamate is the most abundant neurotransmitter in the CNS and is released from presynaptic neurons into the synaptic cleft, resulting in the activation of NMDA and AMPA receptors that mediate calcium and sodium influxes in postsynaptic neurons. Excess glutamate may result in the abnormal activation of glutamate receptors, causing an excessive influx of Ca^{2+} in the postsynaptic neuron (Figure 1), which leads to extreme neuronal firing [135], resulting in excitotoxicity, which is potentially implicated in a number of pathological conditions, including multiple sclerosis [136], Parkinson's diasease [137], and ALS [138,139]. Glutamate excitotoxicity is thought to occur as a result of defective glutamate uptake and transport mechanisms that lead to excessive neuronal Ca^{2+} intake, aberrant Ca^{2+} homeostasis, downstream mitochondrial dysfunction, and increased ROS production [140,141] (Figure 1). Glutamate-gated AMPA receptors are abundant in human and animal motor neurons [142,143] and are made up of four subunits, GluA1 to GluA4 (also GluR1–4) [144]. The overactivation of AMPA receptors has been shown to result in hindlimb paralysis and motor neuron degeneration in wild-type rats, highlighting the susceptibility of motor neurons to Ca^{2+} dysregulation [145]. The Ca^{2+} permeability of AMPA receptors is mediated by the presence of the GluA2 subunit, the absence of which, in addition to impaired transcriptional editing at the Q/R site, confers increased AMPA Ca^{2+} permeability [146]. Interestingly, spinal motor neurons have been reported to display a reduced expression of GluA2 relative to dorsal horn neurons from the same region, providing some explanation for the selective susceptibiltiy of motor neurons in ALS [147], and GluA2 transcriptional editing has been found to be impaired in motor neurons of sporadic ALS patients relative to controls [148]. Furthermore, evidence suggests that GluA2 editing is also impaired in ALS oculomotor neurons, despite their spared function in disease. However, spared functionality has been hypothesized to be the result of increased Ca^{2+}-binding proteins and, in particular, parvalbumin, which is highly abundant in oculomotor neurons and present at low levels in spinal motor neurons [149]. GluA2 transcriptional editing into the Ca^{2+} impermeable subunit is mediated by adenosine deaminase acting on RNA 2 (ADAR2) activity [150,151]. A reduced ADAR2 expression has been reported in sporadic ALS patients and has been shown to result in an increased aggregation of TDP-43 in spinal motor neurons [152]. Taken together, the evidence suggests that a decreased GluA2 expression and impaired transcriptional editing in spinal motor neuron AMPA receptors is a contributing factor to the increased uptake of Ca^{2+} and the downstream susceptibility to excitotoxicity in ALS (Figure 1). Moreover, the finding that AMPA receptor dysfunction can result in the aggregation of misfolded TDP-43 is an important finding for linking ALS pathology with the glutamate excitotoxicity hypothesis.

In addition to dysregulated GluA2 subunit function, research has reported dysfunctional glutamate transport mechanisms in ALS. Under normal physiological conditions, glutamate at the synaptic cleft is cleared by the excitatory amino acid transporter (EAAT2), which functions to maintain low levels of extracellular glutamate and, thus, prevent excessive increases in intracellular Na^{+} and Ca^{2+} levels [89,153]. EAAT2 is found primarely on the synaptic processes of astrocytes, and the loss of EAAT2 has been reported to induce increased extracellular levels of glutamate and cause motor neuron toxicity and muscle paralysis in animal models [154], whilst the pharmalogical stimulation of EAAT2 was found to rescue motor neuron degeneration and delay paralysis in $SOD1^{G93A}$ mice [155,156]. Abnormalaties in EAAT2 have been suggested to occur post-translationally after a post-mortem research highlighted no differences in EAAT2 mRNA expressions between sporadic ALS patients and controls, despite a 95% decrease in protein levels in sALS subjects [157]. Further support of EAAT2 loss and its implications in ALS were reported by a separate group who demonstrated a reduced EAAT2 immunoreactivity in anterior horn cells of sporadic ALS and lower motor neuron disease patients relative to healthy controls [158]. Together, these studies highlight the role of dysfunctional glutamate uptake and transport mechanisms in sporadic cases of ALS (Figure 1).

Excitotoxicity has also been associated with genetic forms of the disease. *SOD1* mutations have been implicated in the glutamate excitotoxicity hypothesis, and research has demonstrated an increased glutamate release [159], as well as motor neuron and inter-neuron hyperexcitability two to three months

prior to motor neuron degeneration and phenotype onset in SOD1^{G93A} mice [160]. *SOD1* mutations have also been shown to reduce the expression of astrocytic GluA2 in vitro and in vivo, thereby diminishing their ability to protect against motor neuron excitotoxicity [21]. In patients, the deterioration of neuronal dendrites was observed in sporadic and familial ALS cases, but not in healthy or Alzheimer's disease controls, leading to the suggestion that ALS is a synaptopathy [161], which is perhaps attributable to the excessive levels of glutamate observed in the CSF of patients [138,139]. Indeed, metabolomic analyses suggest that ALS patients show elevated serum levels of glutamate [32], and there is evidence that sporadic and familial ALS cases show heightened levels of cortical excitablity, which can be detected even in the presymptomatic stages in familial *SOD1* mutation carriers [162]. However, other studies have failed to find evidence for elevated glutamate levels in ALS patients [163–165]. *C9orf72* has also been implicated in the glutamate excitotoxicity hypothesis after iPSC motorneurons from ALS patients were found to have impaired autophagosome formation and aberrant accumulations of glutamate receptors [166–168]. This has been supported in vivo with *C9orf72* knockout mice showing GluR1 upregulation in the hippocampus and a greater susceptibility to excitotoxicity compared to controls [166]. In addition, *C9orf72* knockout mice demonstrated a complete loss of SMCR8 [169], a protein that functions in a complex with C9orf72 and WD40 repeat domain 41 (WDR41) to regulate membrane trafficking and autophagy [170]. The concomitant abnormalities in autophagy and aberrant accumulations of GluR1 has led to the hypothesis that *C9orf72* loss-of-function leads to an impaired clearance of excess glutamate receptors, which, in turn, results in a greater glutamate uptake and increased susceptibility to excitotoxicity (Figure 1). *C9orf72* patients have also been reported to demonstrate elevated glutamate levels in their cerebropsinal fluid (CSF), which has been hypothesized to occur as a result of DPR-mediated splicing defects to EAAT2 and subsequent impairments in glutamate clearance [168]. Research has also implicated ALS2 in the glutamate exicitotoxicity hypothesis by virtue of its interaction with Rab5 and the endosomal pathway. ALS2$^{-/-}$ knockout mice have been reported to show significant increases in glutamate receptor degradation, including GluA2 [171], the loss of which is believed to contribute to excitotoxicity and motor neuron degeneration. Similarly, *ALS2*$^{-/-}$ spinal motor neurons were found to be more susceptible to glutamate excitotoxicity as a result of reduced GluA2 at the synapses of neurons, which was attributed to an altered glutamate receptor interacting protein 1 (GRIP1) function, caused by the genetic loss of *ALS2* [171].

Although there is evidence to suggest the presence of glutamate transport and uptake defects in both sporadic and familial cases of ALS, it is unclear how these defects lead to the specific deterioration of motor neurons in disease. Furthermore, it has been 25 years since the approval of the antiglutamatergic drug riluzole for the treatment of ALS, yet there is no understanding as to why it, as well as other antiglutamatergics, including gabapentin, memantine, and ceftriaxone, fail to delay symptom progressions in ALS by more than ~three months [172,173].

6. Endosomal Pathway and Vesicle Secretion

Extracellular vesicles encompass different types such as apoptotic vesicles, microvesicles, and exosomes, all of which can affect the functionality of the recipient cells [174–177]. The last decade has seen several investigations into the relevance of exosomes to ALS, either in propagating the disease or as biomarkers [6,178–181]. In the ALS context, exosomes secreted by astrocytes, neurons, or microglia are suspected to carry neurotoxic elements such as mutated SOD1 or C9orf72-derived DPR and to be responsible for motor neuron death [178,179,182]. Interestingly, sporadic muscle cells present an accumulation of vacuole and multivesicular bodies in their cytosol, suggesting that vesicle trafficking is disrupted in these cells ([183] and personal data) and that extracellular vesicle secretion might have an important role in ALS.

Exosome biogenesis requires the formation of inward buds in the multivesicular body (MVB), followed by their fission and release as vesicles into the MVB lumen. The generation of intraluminal vesicles can be either Endosomal Sorting Complex Required for Transport (ESCRT)-dependent or ESCRT-independent. The ESCRT is composed of four complexes—ESCRT-0, ESCRT-I, ESCRT-II,

and ESCRT-III—each complex acting one after the other to form intraluminal vesicles. Interestingly, charged multivesicular protein 2B (CHMP2B) is a component of ESCRT-III involved in the processing of cargo into intraluminal vesicles and is associated with ALS [184]. The dysfunction of ESCRT-III may lead to abnormal and dysmorphic endosomes [185] (Figure 2).

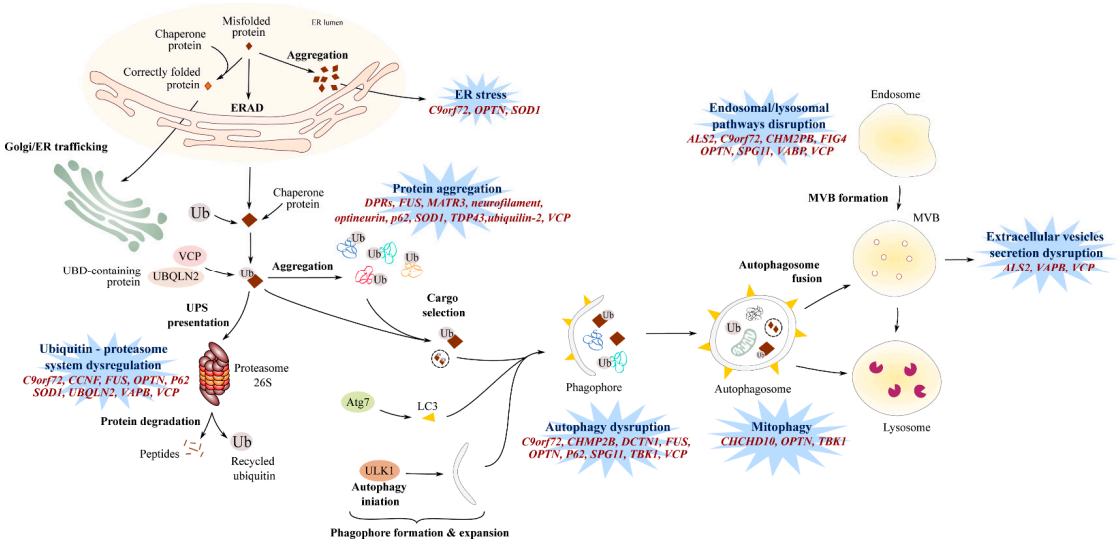

Figure 2. Protein homeostasis dysregulation. Dysregulated protein homeostasis is mediated by multiple pathways encompassing defects in autophagy, the dysregulated ubiquitin-proteasome system (UPS), endo-lysosomal pathway disruptions, or endoplasmic reticulum (ER) stress. The presence of misfolded proteins activates endoplasmic reticulum-associated protein degradation (ERAD), leading to proteasome-mediated degradation to avoid misfolded protein accumulations in the ER lumen and subsequent ER stress. Several ALS-associated gene mutations induce proteasome-mediated toxicity via the sequestration of ubiquilin and chaperone proteins involved in the UPS pathway. The proteolytic activity of the proteasome has also been demonstrated to be targeted by gene mutations in ALS. The autophagic pathway involves the formation and maturation of phagophores that engulf selected transported-cargo and form autophagosomes. Fusion with the lysosome enables the degradation of autophagosome contents. Defects in autophagy initiation and expansion, dysregulated phagophore formation, and/or impaired cargo transport are observed in ALS patients. Mutations in ALS-associated genes also cause defects in mitophagy, a specific form of autophagy. Defects in the endolysosomal have been associated with ALS gene mutations, including defective endolysosomal trafficking and altered lysosomal hoemostasis and degradation. Defects in the autophagy/lysosomal pathway may affect vesicle secretion. Genes implicated in dysregulated protein homeostasis are indicated in red. ER: endoplasmic reticulum, ERAD: endoplasmic reticulum-associated protein degradation, ALS2: Alsin, C9orf72: Chromosome 9 open reading frame 72, CCNF: Cyclin F, CHCHD10: coiled-coil helix coiled-coil helix domain-containing 10, CHMP2B: chromatin-modifying protein 2B, DCTN1: Dynactin 1, FIG4: Phosphoinositide 5-phosphatase, FUS: Fused in Sarcoma, MATR3: matrin 3, MVB: Multivesicular bodies, OPTN: Optineurin, SOD1: Superoxide dismutase 1, SPG11: Spatacsin, TDP-43: TAR DNA-binding protein 43, TBK1: TANK-binding kinase-1, UBQLN2: Ubiquilin-2, VAPB: vesicle-associated membrane protein-associated protein B, and VCP: valosin-containing protein.

Endocytosis and vesicle trafficking from one cellular compartment to another are regulated by small Rab GTPases [186]. For example, Rab5 is associated with the formation of early and late endosomes, while Rabs 11, 35, and 27 have direct roles in exosome biogenesis and secretion. Alsin is an ALS-associated protein [187] and is a guanine nucleotide-exchange factor involved in endosome motility and fusion with the lysosome [171]. Consequently, an absence of alsin expression in hippocampal neurons leads to an accumulation of Rab5-positive vesicles and an enhanced lysosome-mediated

degradation, suggesting an enhanced degradation of endosomal vesicles [171], thus probably affecting the production and secretion of exosomal vesicles.

The C9orf72 protein structure presents some similarities with the Differentially Expressed Normal versus Neoplastic (DENN) guanine nucleotide exchange factor and, thus, may activate Rab proteins [101] such as RAB8A and RAB39B [188]; Rab1a [34]; or Rabs 1, 7, and 11 [189], which are associated with autophagy and vesicle-trafficking processes [190–192], as well as exosome biogenesis and secretion [186]. In *C9orf72* knockdown cell lines [189], a *C9orf72* knockout murine model [193], and in ALS patient fibroblasts and iPSC-derived motor neurons [194], transgolgi and endosomal trafficking were reduced, a defective autophagy pathway was observed [34,194], and exosomal secretion was affected [194].

Vesicle-associated membrane protein-associated protein B (VAPB) is involved in vesicle trafficking between the endoplasmic reticulum and the golgi apparatus [195,196]. Interestingly, VAPB has been described to interact with RAB7 and colocalize with CD63 [197], two proteins involved in late-endosome formation and exosome biogenesis [186]. However, the impact of ALS-associated VAPB mutation in exosome biogenesis and secretion still needs to be investigated.

Multivesicular body formation is at a crossroad between the autophagy (Figure 2) and secretion pathways, and an autophagic failure may lead to cell secretion [198]. The *VCP* gene is associated with ALS [199] and encodes for a valosin-containing protein, an ubiquitous AAA+ ATPase that interacts with clathrin to form early endosomes but, also, with the autophagy pathways [200]. In this context, a mutation in a valosin-containing protein (VCP) may affect the endosomal pathway, and one can hypothesize that it has an impact on the formation and secretion of exosomes. Other gene mutations associated to ALS, such as protein polyphosphoinositide 5-phosphatase (FIG4) [201,202] or spastacsin (Spg11) [203,204], are associated with the blockade of lysosomal clearance (see Section 7)—the blockade of which could potentially lead to vesicle secretion [198]. However, futher investigations related to exosome pathways in ALS in vivo are needed.

7. Impaired Protein Homeostasis

Protein aggregates positive for TDP-43 [36,205], neurofilament [41], FUS [87], or SOD1 [206] are observed in the vast majority of ALS patients, with TDP-43 being present in as many as 98% of sporadic and familial cases [207], meaning that the presence of such aggregates is widely regarded as a hallmark feature of ALS pathology. These deposits can occur in the cytoplasm of neurons [208] and skeletal muscle [99,209], and their presence is highly suggestive of an imbalance between protein synthesis and degradation pathways (Figure 2).

7.1. Proteasome and Autophagic Degradation Pathways

In the late 1960s and early 1970s, the presence of protein inclusions in the anterior horn cells of sporadic and familial ALS patients was described [210–212]. Later, these inclusions were found to be ubiquitin positive [35], and SOD1 was the first ALS-associated protein found to be immunoreactive within the inclusions of familial patients [213]. Subsequently, ubiquinated inclusions have often been found to be immunoreactive for the ubiquitin-binding protein p62 [214], and up to 98% of sALS and fALS cases show inclusions that are TDP-43-positive [215], with the exception being *SOD1* [216] and *FUS* [216] patients who do not demonstrate TDP-43 inclusions but do demonstrate SOD1 and FUS immunoreactive inclusions. Other ALS proteins that have been implicated in the formation of cytoplasmic inclusions include optineurin (OPTN) [107], ubiquilin 2 (UBQLN2) [217], dynactin 1 (DNCT1) [218], valosin-containing protein (VCP) [219], and matrin 3 (MATR3) [220]. Studying the structure of the main proteins SOD1, FUS, and TDP-43 helped to unravel potential mechanisms involved in protein misfolding and self-propagation within the cells and in surrounding cells. SOD1 is a stable homodimer, thanks to the intrasubunit disulfide bond and its ability to bind zinc and copper. However, a reducing and metal-poor intracellular environment or mutations [221–227] can abolish these features and destabilize SOD1, leading to the formation of aggregates and amyloid fibril

structures [228–231] that can self-propagate in vitro [229,230]. FUS and TDP-43 proteins possess a low complexity domain that presents similarities with yeast prions [209] and can form large aggregates and amyloid fibril structures [209,229,232,233]. Interestingly, mutated forms of FUS and TDP-43 can induce the misfolding of wild type forms of FUS and TDP-43, respectively [229], and have also been shown to induce the misfolding of wild type forms of SOD1 in vitro [234]. Altogether, these studies suggest a potential mechanism for the self-propagation of misfolded proteins in vitro—misfolded proteins that can potentially be transferred from cell to cell via secreted vesicles, thus propagating the misfolding mechanism to neighboring cells (see Section 6, [178,229,235]). The presence of these protein aggregates has been suggested to impair the proteasome and autophagic degradation pathways and could be key mediators in ALS pathogenesis [38,236,237] (Figure 2).

Dysregulation of the Ubiquitin–Proteasome System (UPS) in ALS patients was first suspected following the identification of mutations in genes encoding ubiquilin 2 [238] and VCP [199], two proteins involved in protein clearance via the ubiquitin-proteasome pathway [239]. Mutations in *OPTN* [240] and *SQSTM1/P62* [241] were then identified, and following this, *SOD1* [236], *VABP* [242], *C9orf72* [25,208], and *CCNF* (cyclin F) [243] mutations were all reported to reduce UPS activation. Ubiquitin-positive inclusions were observed in post-mortem neuronal and muscular tissues of fALS and sALS patients [35] and, more specifically, in *C9orf72* patients [244]. Similarly, SOD1 [245], FUS [87], ubiquilin 2 [246], and C9orf72-derived DPR proteins [247] can generate toxic aggregates positive for some proteasome components [248]. These ubiquitin-positive inclusions can also contain and "trap" nonmutated forms of SOD1 [48], TDP-43 [205], optineurin [107], and ubiquilin 2 [249], thus exacerbating the already disrupted cellular homeostasis in ALS.

The degradation of ubiquitinated proteins through the autophagy/lysosomal pathway occurs in four steps: (1) the initiation and extension of the bilayer vacuole into phagophores; (2) the transport of selective cargoes (including ubiquitinated proteins, dysfunctional mitochondria, and protein aggregates); (3) the maturation into autophagosomes; and (4) fusion with low pH lysosomes to form autolysosomes where degradation of the cargoes can proceed [250].

The fusion of the endosome with the lysosome for degradation is an tightly regulated event [251] involving the protein polyphosphoinositide 5-phosphatase, FIG4 [252]. Deleterious mutations in *FIG4* in ALS leads to abnormal lysosomal storage [201,202]. Spastacsin is also involved in lysosomal clearance, and the absence of *Spg11* expression impaired the lysosomal-autophagy pathway and is accompanied by an accumulation of lipid within the lysosomes [203,204]. Nonfunctional TBK1 [253] and p62 [254] inhibit the transport of targeted cargoes toward the autophagosome. Interestingly, impaired autophagosome maturation was also observed in ALS cells mutated for *FUS* [255], *VCP* [89], *CHMP2B* [184], and *OPTN* [256]. The importance of autophagy can be observed in studies that stimulate autophagic activation in the presence of ALS mutations. For instance, the stimulation of autophagy in murine and human iPSC-derived neurons expressing *TARDBP* mutations demonstrated a greater clearance of TDP-43 aggregates relative to nonstimulated cells and resulted in improved motor neuron survival [257].

C9orf72 mutations can interfere with the autophagy pathway at several levels. When *C9orf72* expression is abolished or decreased as suggested by the haploinsufficiency hypothesis [258,259], autophagy is inhibited [34,189,193,194], leading to simultaneous increases in the number of cytoplasmic inclusions immunoreactive for ubiquitin, p62, and TDP-43 [34,101,260]. The impairment of autophagy in *C9orf72* cells may also result in the accumulation of cytotoxic DPR proteins encoded by the G4C2 HREM and, ultimately, lead to neuronal loss [261]. Similarly to cells expressing *TARDBP* mutations [257], the stimulation of autophagy abolished the accumulation of poly-DPR proteins and neuronal toxicity [261].

Altogether, these studies illustrate the importance of autophagy for the efficient clearance of misfolded and aggregated proteins and is indicative of the underlying impairments in proteostatic mechanisms that mediate ALS physiopathology (Figure 2).

7.2. Endoplasmic Reticulum Stress

During the formation of misfolded proteins, the unfolded-protein response (UPR) may be initiated to transport defective proteins to the endoplasmic reticulum (ER), where ER-resident chaperones will properly fold the protein [262]. The accumulation of misfolded proteins in the ER activates the ER stress response pathway, also known as the endoplasmic reticulum-associated protein degradation (ERAD) pathway. The ERAD pathway involves the translocation of misfolded proteins from the ER lumen to the cytosol, where they undergo ubiquitination and degradation through the ubiquitin-proteasome pathway [262]. In ALS patient cells, mutated SOD1 agregates were observed in ER and colocalized with UPR markers, leading to an increase in ER stress [263] by interacting with ER stress response proteins and inhibiting their function in the ERAD response [236]. The presence of poly (GA) aggregates, observed in neuronal post-mortem *C9orf72* ALS patients, can inhibit proteasome activity and induce ER stress, which can be abolished when using ER stress inhibitors such as salubrinal and tauroursodeoxycholic acid (TUDCA) [208]. Concordantly, the cerebropsinal fluid (CSF) of sporadic ALS patients displays an accumulation of ER stress markers [263], and when healthy neurons were exposed to patient CSF, the ER became fragmentated and caspase-dependent apoptosis was activated, suggesting an increase in ER stress [264].

Vesicle-associated membrane protein-associated protein B (VAPB) is localized in the endoplasmic reticulum membrane and has a key role in vesicle trafficking between the endoplasmic reticulum, golgi apparatus, and the nuclear envelope [195–197]. The $VAPB^{P56S}$ mutation associated with ALS leads to a misfolded protein that accumulates in the ER [265] and can cause a defect in nuclear envelope protein transport, leading to an aberrant nuclear envelope structure [266]. Interestingly, the accumulation of VAPB has also been observed in the endoplasmic reticulum of peripheral blood mononuclear cells of sporadic ALS [267].

Optineurin is a TBK1 partner and is involved in mitophagy (Section 3). When the association of optineurin with myosin VI is disrupted, as osberved in fALS cases associated with *OPTN* mutations, optineurin is diffused in the cytosol of neuronal cells and results in ER stress and Golgi apparatus fragmentation, as well as an inhibition of the autophagy pathway ([256], Figures 1 and 2).

Altogether, these studies suggest that protein degradation could be directly and indirectly affected in ALS, causing protein aggregation that leads, in turn, to the disruption of the function of organelles such as nuclei (Section 7.2) and mitochondria (Section 3) or to the blockage of lysosomal activity that can potentially affect cell-cell communication (Section 6).

8. Aberrant RNA Metabolism

FUS [86,268] and TDP-43 [269] are RNA-binding proteins involved in multiples steps of RNA metabolism. In ALS patients, mutations in both genes give rise to the translation of proteins frequently mislocalized to the cytoplasm [87,88,270] and, subsequently, result in downstream complications that affect RNA-processing mechanisms. Dysregulated RNA metabolism is another key feature of ALS pathogenesis and includes transcription defects, alternate splicing changes, miRNA biogenesis, stress granule formation, and RNA nucleocytoplasmic transport (Figure 3).

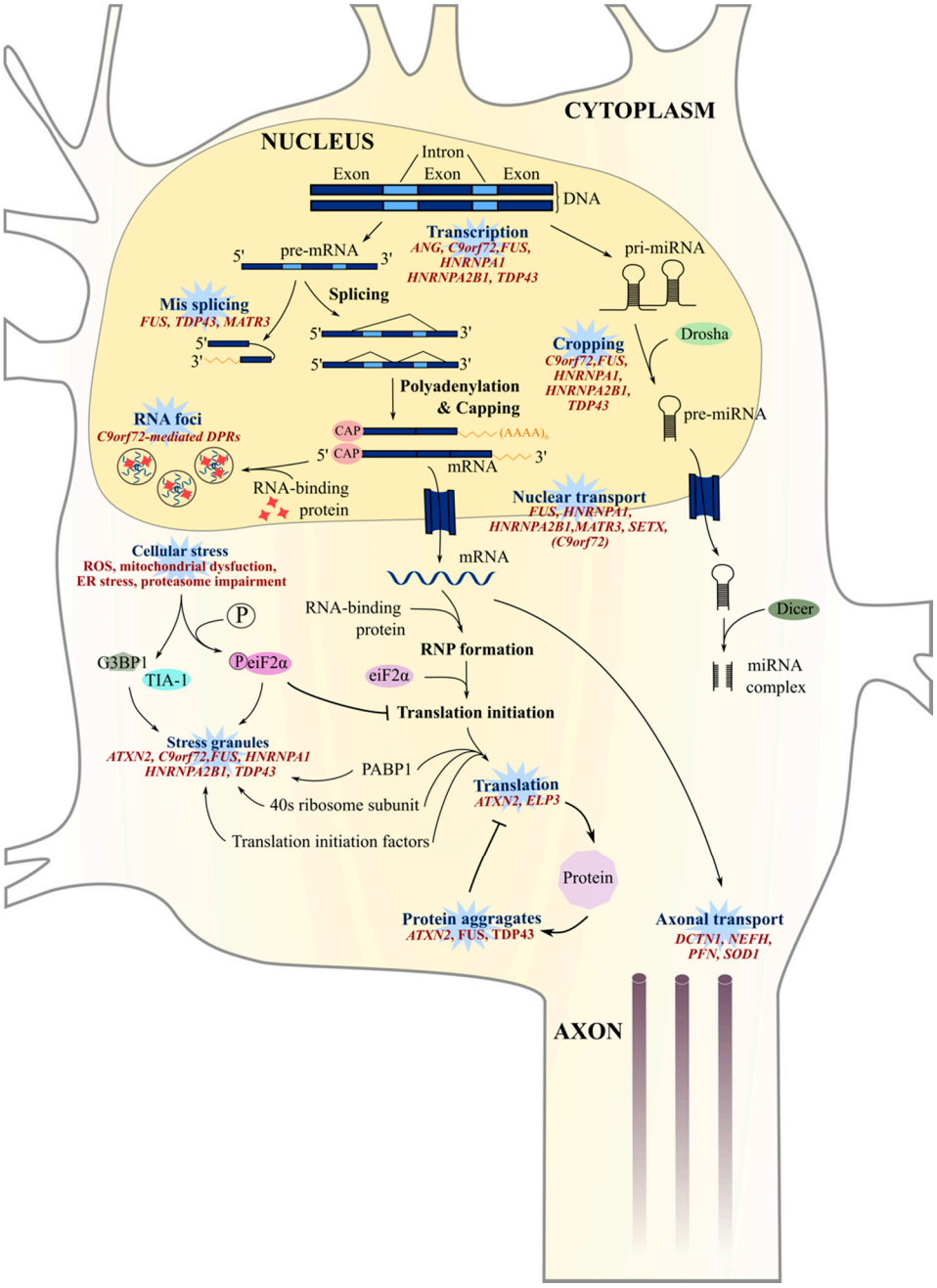

Figure 3. RNA and miRNA biogenesis defects in ALS. Many processes in RNA and miRNA pathways are disrupted in ALS patients, including transcription defects, alternate splicing events, miRNA biogenesis, and nucleus-cytosol transport impairment. RNA metabolism defects are particularly relevant in ALS pathogenesis, since TDP-43 and FUS are both well-known ALS-associated genes involved in RNA processing. Both FUS and TDP-43-mutated proteins mislocalize to the cytoplasm of ALS motor neurons, leading to a probable loss and/or toxic gain-of-function of these proteins. ANG: Angiogenin, ATXN2: Ataxin-2, C9orf72: Chromosome 9 open reading frame 72, DCTN1: Dynactin 1, eIF2α: Eukaryotic translation initiation factor 2A, ELP3: Elongator protein 3, FUS: Fused in Sarcoma, G3BP1: Ras GTPase-activating protein-binding protein 1, hNRNPA1: Heterogeneous nuclear ribonucleoprotein A1, hnRNPA2B1: Heterogeneous nuclear ribonucleoprotein A2B1, MATR3: matrin 3, NEFH: Neurofilament heavy subunit, PABP1: Polyadenylate-binding protein 1, PFN1: Profilin, SETX: Senataxin, SOD1: Superoxide dismutase 1, TDP-43: TAR DNA-binding protein 43, and TIA-1: TIA1 Cytotoxic Granule-Associated RNA-Binding Protein.

8.1. RNA Splicing and Translation

Given the large number of possible protein-protein interactions between *FUS* or *TDP-43* and their partners, it is easy to expect alterations to important RNA-processing mechanisms in ALS patients [89]. *FUS* and *TDP-43* also regulate the expression of multiple proteins involved in neuronal physiology, including components of the synaptic plasticity pathways [39,271,272] and dendritic branching processes [272–274]. In addition, the HREM in *C9orf72* generates repeat RNA and RNA foci, which repress the gene expression of RNA metabolism regulators (such as *hnRNPA3*) [275] or sequester TDP-43 [275,276] and FUS [275] proteins and, thus, indirectly inhibits the transcription of RNA metabolism-associated genes. Similarly to *C9orf72*-mediated RNA-processing defects, *FUS* mutations have been associated with major transcriptional defects [268].

Ataxin-2 is a polyglutamine (polyQ) protein that is involved in mRNA translation, and it interacts with RNA-binding proteins such as TDP-43 and FUS [277]. In ALS spinal cords, ataxin 2 exhibited significant cytoplasmic accumulation and enhanced the toxicity of TDP-43 in Drosophila via RNA binding [59].

TDP-43, *FUS*, *hnRNPA1*, *hnRNPA2B1*, and *MATR3* are associated with ALS and involved in pre-mRNA processing [220,278–281]. Consequently, *TDP-43* knockdown in murine tissues results in the alternate splicing dysregulation of numerous mRNA transcripts [42,282], and the loss-of-function of *FUS* also induces many splicing defects [39], suggesting important alternate splicing events in ALS patients (Figure 3). These downstream complications are not surprising given the ability of the protein FUS to sequester numerous components of the splicing process, such as key splicing factors [283] U1 snRNP and U11/U12 snRNPs [274,284], which are involved in minor intronic splicing. Alternative splicing changes have been identified in neuronal genes involved in cytoskeleton organization, axonal growth, and guidance in *FUS*-mutated ALS patients [285,286], and, interestingly, axonopathy and axon retraction occur in the early stages of ALS [287].

ALS-linked MATR3^{S85C} and MATR3^{P154S} mutations were observed to affect Matrin 3 interactions with the TRanscription and EXport (TREX) protein complex, altering the global nuclear export of mRNA [288]. As a result, mRNA is sequestered within the nucleus, causing export defects of TDP-43 and FUS mRNA [288], which may affect mRNA splicing directly [289] and indirectly [278] (Figure 3). Consequently, as observed in the *MATR3*^{S85C} murine model, dysfunctional MATR3 may lead to astrocyte and microglia activation and result in spinal motor neuron degeneration [290].

The *ELP3* gene encodes for elongator protein 3, a histone acetyltransferase subunit of the RNA polymerase II elongator complex responsible for RNA translation (Figure 3). Mutations in the *ELP3* gene are associated with ALS [291,292] and result in the shortening and abnormal branching of motor neurons, as observed in *ELP3* knockdown in zebrafish embryos [291], and altered tRNA modification, triggering proteome impairment and the subsequent aggregation of susceptible proteins [292].

Angiogenin, encoded by the hypoxia-inducible gene *ANG*, is a member of the pancreatic ribonuclease superfamily [293] and, as well as angiogenesis, is also involved in ribosomal biogenesis [294,295]. Defects in this protein are associated with the impairment of its nuclear localization and diminished ribonucleolytic activity [295] (Figure 3), both of which are essential for normal ANG functioning and motor neuron viability.

Together, these findings suggest that RNA processing is a key pathway affected in ALS, either due to mutations directly affecting proteins involved in RNA processing or as a consequence of protein aggregations.

8.2. RNA Foci

Sense and antisense RNA generated from the bidirectional transcription of G4C2 repeats have been proposed to induce a toxic gain-of-function in ALS *C9orf72* patient cells by forming RNA foci that may sequester RNA-binding proteins, thus disrupting RNA metabolism and processing in cells [101] (Figure 3) widely throughout the central nervous system [296,297]. Both sense and antisense RNA foci are frequently observed in nucleoli, with antisense RNA foci being denser [296]. In addition, the antisense RNA foci correlate with TDP-43 aggregation in the cytosol of *C9orf72* motor

neurons [296,297]. An in situ hybridization of post-mortem *C9orf72* ALS tissue revealed that 78.7% of the neurons and 24.9% of the glial cells in the motor brain and spinal cord regions were positive for antisense RNA foci [297]. Interestingly, extra-motor brain regions also show a high percentage of cells positive for antisense RNA foci, with 89.4% of neurons and 46.1% of glia being positive [297].

8.3. Epigenetic Modulation

Epigenetic mechanisms such as microRNA regulation maintain cell type and tissue identity and may be involved in the onset and progression of neurodegenerative diseases, including ALS. The decreased expression of miRNAs, including miRNAs let-7e, miR-148b-5p, miR-577, miR-133b, and miR-140-3p, were observed in post-mortem spinal cords of sporadic ALS patients [298], suggestive of impairment in the genes and pathways associated with miRNA biogenesis, neuroinflammation, and apoptosis.

Interestingly, the class II ribonuclease, Drosha, interacts with TDP-43, FUS, and *C9orf72*-mediated DPRs [299–301], while the Dicer enzyme interacts with TDP-43 protein [301] and FUS can interact with pri-miRNA [302] (Figure 3). Consequently, mutated *TDP-43* may impair the post-transcriptional regulation of miRNAs and lead to an altered expression of miR-132-3p and miR-132-5p (involved in the regulation of neuronal outgrowth [301]), miR-143-3p and miR-143-5p (involved in myoblast cell differentiation [303]), miR-558-3p (involved in neurofilament stability [304]), and miR-574-3p (associated with stroke [305]) [301]. Similarly, the downregulation of FUS in a neuroblastoma cell line had a considerable impact on the biogenesis of miRNAs, with an altered expression of miR-9, miR-125b, and miR-132 implicated in neuronal differentiation, activity, and function [302], while mutated *FUS* affected the expression levels of miR125 and miR192 [302], which are involved in early neural conversion [306] or senescence [307].

Altogether, these findings are consistent with defective miRNA processing in ALS patients, which may affect downstream pathways with an impact on motor neuron survival.

8.4. Stress Granules and Nucleocytoplasmic Transport

In response to stressful conditions, RNA granules, also known as stress granules, are generated and can recruit FUS and TDP-43 [41,308]. Mutations in *FUS* and *TDP-43* can increase the persistence of stress granules in the cytoplasm, resulting in a possible toxic gain-of-function [237] by inhibiting mRNA translation and, thus, contributing to the progression of ALS pathology (Figure 3). The heterogeneous nuclear ribonucleoprotein particle proteins hnRNPA1 and hnRNPA2B1 are RNA-binding proteins and binding partners of TDP-43 and are involved in RNA processing, including miRNA maturation, the nucleocytoplasmic transport of mRNA, and RNA metabolism [300,309]. Mutations in the prion-like domains of hnRNPA2/B1 and hnRNPA1 increase fibril formation and aggregation potential, as well their hyperassembly into stress granules [310,311]. Stress granules are then targeted to the lysosome by the autophagic machinery involving VCP. Indeed, the pharmacological inhibition or RNAi knockdown of VCP is accompanied by reduced stress granule clearance, while Hela cells expressing VCP^{A232E} and VCP^{R155H} mutations showed a constitutive appearance and accumulation of stress granules containing TDP-43 [312]. Concordantly, the ALS-VCP mutation is accompanied by an increase in stress granules [313].

In *C9orf72* patients, stress granules are also involved in the sequestration of proteins required for effective nucleoplasmic transport, such as RAN GAP [40], or importing and exporting proteins [40,41]. The impairment of the nucleoplasmic transport of molecules in *C9orf72* ALS cells is controverisal. Indeed, while some studies observed that newly formed DPRs such as poly-PR can bind to nuclear pore transporters, thereby impairing the subsequent translocation of molecules [314], other studies did not observe any disruption in the nucleocytoplasmic transport with poly-GR or poly-PR [314]. However, with the expression of poly-GA, defects were observed both in import and in export in a SH-SY5Y cell line and in iPSC-derived motor neurons, respectively [101].

9. Concluding Remarks

Over 150 years have passed since ALS was first reported by Charcot, and still, the etiology of the disease remains elusive. Although research is progressing and genetic studies continue to identify novel gene mutations in familial cases of ALS [315], many questions remain surrounding the pathological mechanisms associated with already established mutations, their roles in the disease phenotype, and the as-yet-undiscovered mechanisms that underly sporadic onset. The most investigated mechanisms revolve around neurocentric deficits in dysfunctional mitochondria and oxidative stress, axonal transport, glutamate excitotoxity, protein homeostasis, and RNA processing (Figure 4).

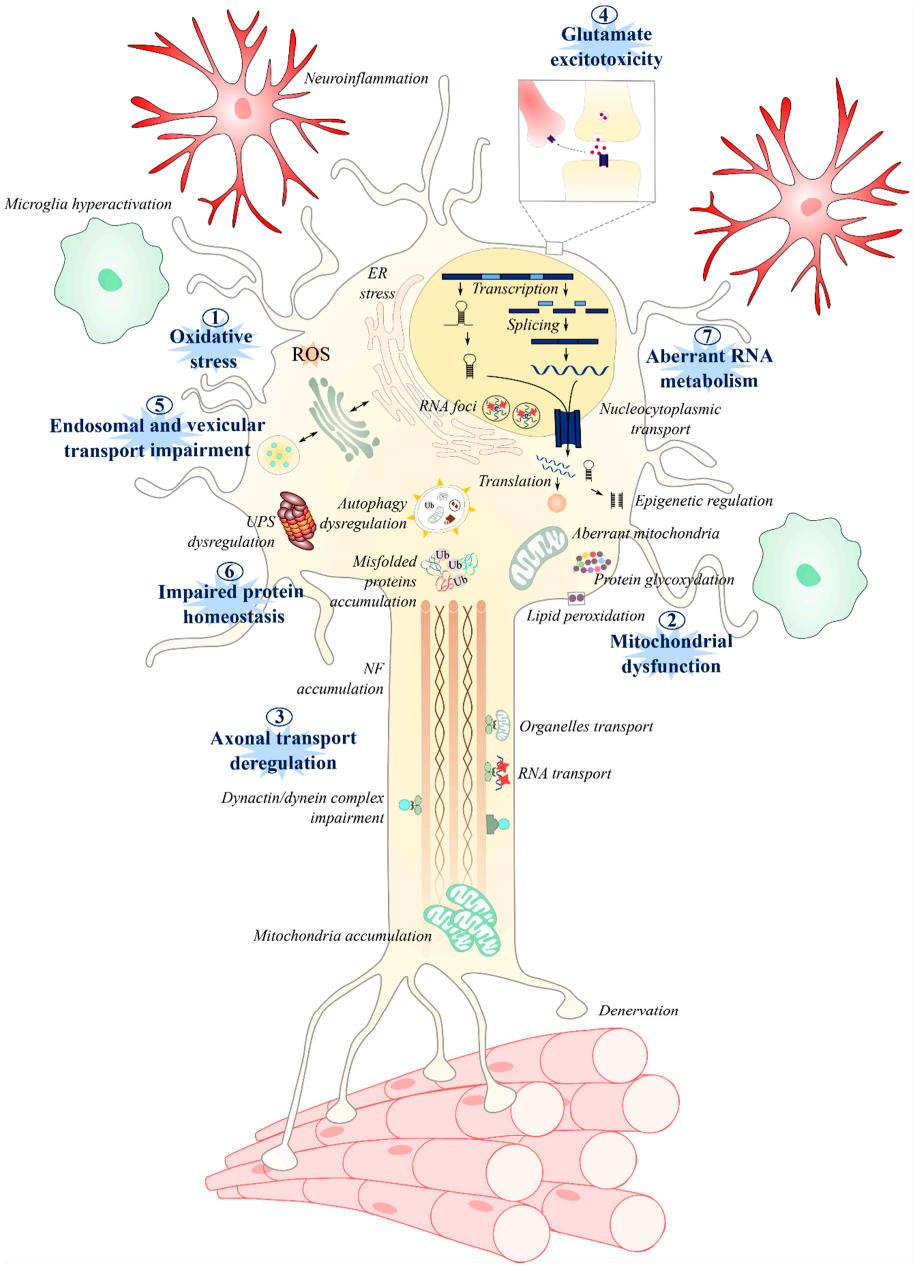

Figure 4. Summary of the different molecular and cellular mechanisms involved in ALS pathogenesis.

Among the most studied and well-established pathways are: oxidative stress, mitochondrial dysfunction, axonal transport, glutamate excitotoxicity, endosomal and vesicle secretions, protein homeostasis, and RNA metabolism. One pathway may lead to another, exacerbating the disruption of cellular homeostasis. The disruption of these pathways can lead to microglia activation, neuroinflamation, astrocytosis, and, ultimately, to motor neuron death and muscle denervation.

By detailing, as has been done in this review, the molecular events of the various pathways that are implicated in ALS, it becomes clear that these pathways can be linked to each other—in some cases, with one leading to another. For example, disrupted axonal transport can lead to an accumulation of nonfuctional mitochondria, while ATP deficiency and increased oxidative stress may damage proteins and DNA, which, in turn, could exacerbate the disruption of cellular homeostasis, leading to motor neuron death (Figure 4). These pathways are disrupted not only in motor neurons [24] but, also, in astrocytes [316,317], microglia [318,319], peripheral blood cells [43,320], and muscle [37,321–323], suggesting multisystemic [6] involvement in motor neuron death. Thus, by considering ALS from the perspective of shared molecular pathways [6,324], a cohesive understanding may yet emerge of the cellular mechanisms driving this pathology. It may be that different molecular pathways correspond to sub-strata of patients, such as among those with known genetic forms of ALS, as suggested in [6]. However, the identification of these strata may prove to be extremely challenging in non-monogenic forms of the disease [324].

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