PROTEIN MISFOLDING AND PROTEOSTASIS IMPAIRMENT IN AGING AND NEURODEGENERATION: FROM SPREADING STUDIES TO THERAPEUTIC APPROACHES

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EDITORIAL
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Editorial: Protein Misfolding and Proteostasis Impairment in Aging and Neurodegeneration: From Spreading Studies to Therapeutic Approaches

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Editorial on the Research Topic

Protein Misfolding and Proteostasis Impairment in Aging and Neurodegeneration: From Spreading Studies to Therapeutic Approaches

Misfolding, aggregation, and deposition of proteins in the nervous system are common features of several neurodegenerative diseases such as Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), frontotemporal dementia (FTD), amyotrophic lateral sclerosis (ALS), and prion diseases, among others. Aging is the most relevant risk factor for all these diseases and recent evidence suggests that the buffering capacity of the proteostasis network decreases with aging and contributes to the etiology of these neuropathologies (Martinez et al., 2017). In this Research Topic, we have compiled a list of interesting manuscripts which describe protein misfolding and proteostasis impairment in aging and neurodegeneration.

AD is the most iconic disease associated with protein misfolding. This disease is the most common form of dementia in the elderly population, affecting about 10% of individuals over the age of 65, with its frequency increasing to nearly 40% by the age of 85. Pathologically, the most prominent features of AD are the extracellular deposition of amyloid-β (Aβ) peptides and the intracellular accumulation of hyper-phosphorylated tau (p-tau) proteins in the brain. These features have been associated with a series of detrimental events, including exacerbated brain inflammation and oxidative stress, leading to synaptic dysfunction and neuronal death. Recent data has emerged suggesting that the progressive axonal degeneration in early stages of AD is associated with Aβ and tau accumulation, which has been discussed by Salvadores et al.

Another pathological property of misfolded proteins involves their ability to spread or propagate within different anatomical structures of the nervous system through prion-like mechanisms (Gomez-Gutierrez and Morales, 2020). This has been particularly well-studied for Aβ and tau proteins involved in AD. Despite extensive research conducted in the last decade, the mechanisms dictating the organized spread and neurodegeneration associated with these disease-associated

proteins is not completely understood. In this line, d'Errico and Meyer-Luehmann clearly summarized the current literature involving the spreading mechanisms of Aβ and tau pathology in AD. Their article focuses on evidence supporting the prion-like model for Aβ and tau spreading and how those proteins can be secreted and internalized by cells. They also highlight the need for better understanding of these disease pathways and the design of accurate and efficient intervention methods based on these mechanisms (d'Errico and Meyer-Luehmann).

A comprehensive review by McAlary et al. further describes evidence for the prion-like propagation of other proteins prone to misfold. Their article specifically focuses on amyotrophic lateral sclerosis (ALS), a fatal neurodegenerative disease affecting motoneurons and leading to death due to respiratory failure (Taylor et al., 2016). The authors cover literature involving biochemical and seeding properties of major ALS-linked proteins including superoxide dismutase 1 (SOD1), transactive response DNA-binding protein 43 (TDP-43) and fused in sarcoma (FUS) in different systems including invertebrate and mammalian models. TDP-43-positive inclusions are detected in over 90% of ALS patients and recent work has demonstrated its seeding potential. TDP-43 and FUS contain prion-like low complexity domains that can drive liquid-liquid phase separation forming membraneless organelles such as stress granules. These structures may link cellular stress responses to age-related proteostasis collapse, driving neurodegeneration. Collectively, the literature reviewed in this article supports the relevance of prion-like propagation of toxic protein species to ALS pathogenesis.

Knowledge collected on infectious prions has been used to explain relevant mechanisms associated with several neurodegenerative diseases. Importantly, prion diseases are unique among other neurodegenerative disorders due to their confirmed infectious etiology. Most mammals are susceptible to prion diseases either naturally or under experimental conditions. However, some animal species such as dogs, horses and rabbits have proven to be resistant to a diverse array of prion isolates. Several *in vitro* and *in vivo* studies have been conducted to identify the elements in the prion protein sequence that provides infection-resistance. These include the comparison of prion protein structures from animals susceptible and resistant to infection, *in vitro* prion conversion assays, and bioassays using actual animals and transgenic systems (mice and flies). Considering all these studies, few aminoacids in the prion protein (PrP) sequence have been identified as key to provide resistance to templated-conversion. This experimental evidence is conscientiously summarized and discussed by Myers et al. who hypothesize that these protective amino acids generate more compact and stable structures in the C-terminal domain of the prion protein, making it more resistant to acquire disease-associated isoforms. Research in this area is key to understanding the probability of certain animal species to get naturally infected by infectious prions and may help to develop drugs stabilizing specific domains involved in prion conversion.

Regarding mechanisms associated with ALS pathogenesis, the review by Jagaraj et al. systematically describes alterations of redox pathways in this disease. The authors present major pathways controlling redox balance, including sources of reactive oxygen species such as NADPH oxidases and defense antioxidant systems. The paper highlights the protein disulfide isomerases (PDIs), a class of oxidoreductases that promote disulfide bond formation in the endoplasmic reticulum (ER), as key components of the proteostasis network dysregulated in ALS. According to the evidence discussed, redox modifications such as S-nitrosylation and ALS-linked mutations in PDIs impair their redox activity, resulting in protein misfolding and disulfide-dependent aggregation. It will be of great importance determining whether such disulfide-crosslinked proteins can serve as templates to propagate toxic conformations along the central nervous system (CNS). The failure of previous clinical trials using redox-active compounds indicate that targeting redox mechanisms in ALS depends on more specific targets.

The study by Charif et al. uncovers important evidence involving TDP-43 toxicity. Under normal conditions, TDP-43 is localized in the nucleus. In ALS and other neurodegenerative diseases, however, TDP-43 is mislocalized to the cytoplasm where it may gain a toxic function (Taylor et al., 2016). Employing cell culture and transgenic mouse models, the authors show that cytoplasmic TDP-43 impairs the proteostasis network by inhibiting translation, which was monitored by puromycin labeling, in addition to preparation of mRNA-ribosome complexes from brain tissue. According to the authors, multiple mechanisms may be associated to suppression of protein synthesis by TDP-43 that deserve further investigation, including disruption of mRNA recruitment by ribosomes, sequestration of ribosome components into protein inclusions, aberrant formation of stress granules, ER stress and activation of the unfolded protein response, among others. Determination of the underlying pathogenic mechanisms triggered by cytoplasmic TDP-43 can lead to the design of novel therapeutics to restore translation rates in ALS.

As mentioned, a reduction in the capacity of homeostatic mechanisms during aging may increase the accumulation of misfolded proteins leading to age-related neurodegenerative diseases. One of the key pathways that mediates cell adaptation is the integrated stress response (ISR), which promotes translational arrest and induction of selected adaptive elements through the phosphorylation of the eukaryotic translation initiation factor 2 alpha (eIF2α). However, under chronic activation, ISR can also induce cell death, being considered an important regulator of neural physio(patho)logical conditions. With this focus, Martinez et al. (2021) reviewed evidence linking protein kinase R (PKR), an ISR sensor, to physiological conditions and neurodegenerative processes contributing to age-related pathologies. The PKR-eIF2α complex has been involved in both long-term potentiation (LTP) and long-term depression (LTD) in the hippocampus, physiological mechanisms that have been proposed to correlate with performance of mouse models in tasks of cognitive memory. Martinez et al. also described the role of the PKR signaling pathway in pathological conditions, including AD. Increased PKR levels have been identified in mouse and human samples, correlating with the severity of cognitive impairment. PKR involvement in other neurodegenerative diseases including PD

and HD has also been widely documented (Martinez et al., 2021).

An emerging model explaining the impairment of cellular homeostasis during aging leading to subsequent development of neurodegenerative diseases involves mitochondrial dysfunction. The mitochondrial unfolded protein response (UPR^{MT}), which involves the transcriptional activation of mitochondrial chaperones, proteases and antioxidant enzymes, is a mechanism to repair defective mitochondria relevant for neurological disorders. Muñoz-Carvajal and Sanhueza summarized several lines of investigation supporting this hypothesis, highlighting the factors that influence mitochondrial homeostasis during normal and pathological aging.

Similarly to ALS, spinal and bulbar muscular atrophy (SBMA) is classified as a motor neuron disorder (MND), with both diseases sharing common clinical manifestations and pathological features marked by accumulation of misfolded proteins in different areas of the nervous system involving multiple anatomical regions and cell types. On one hand, the different etiology of MNDs make the study of pathogenic mechanisms and search for therapeutic approaches challenging. On the other hand, both ALS and SBMA share a common pathological feature: the imbalance of the protein quality control system. The article by Cristofani et al. describe the pathological mechanisms involved in ALS and SBMA, specifically focused on the elements of the protein quality control system that are altered in these diseases. These include chaperones, protein degradation systems (the ubiquitin proteasome system and the autophagy-lysosomal pathway), the unfolded protein response, and the release of disease-associated proteins through extracellular vesicles. Along this line, the authors discuss how these different elements of the protein quality control system may be manipulated for therapeutic purposes.

Similarly, the review article by Troncoso-Escudero et al. discusses the different therapeutic approaches under development to treat the two most common movement disorders associated with misfolded proteins: PD and HD. Here, the authors focus on both preclinical approaches and clinical trials involving pharmacological, cellular replacement and genetic manipulation strategies, in addition to the use of growth factors and electrical modulation therapies. Pros and cons of each treatment avenue are thoroughly discussed, serving as a reference for clinicians and basic investigators.

Chaperones are relevant players in maintaining proper protein folding, facilitating that proteins acquire their final conformation. They are known as heat shock proteins (Hsp) as they are crucial in stress response. The activity of the molecular chaperones is compromised in chronic cellular stress and age-related neurodegenerative diseases, including AD, PD, and HD (Winklhofer et al., 2008). In fact, the expression of some Hsp is decreased in AD (Winklhofer et al., 2008). Overexpression of chaperones can decrease neurodegeneration, whereas their reduction may accelerate disease rate (Park et al., 2017). Tittelmeier et al. review the dual role of molecular chaperones in neurodegenerative diseases. On one hand, chaperones are necessary in maintaining cellular proteostasis by folding proteins and assisting degradation of those that are not properly folded. On the other hand, they can also mediate in the progression of several protein misfolding disorders, interfering in the aggregation and spreading of different disease-related amyloids. In addition, non-natively folded proteins can be degraded in lysosomes by chaperone-mediated autophagy (CMA). Chaperones associated with this specialized protein degradation machinery can become dysfunctional during brain aging, neurodegenerative disorders and even brain tumors. Auzmendi-Iriarte and Matheu (2021) review alterations observed in this autophagy mechanism in healthy and disease conditions and propose the development of CMA modulators to successfully intervene in different brain disorders. Chaperones not only assist in (re)folding of intracellular proteins, but they can also be secreted to the extracellular space. Alterations in secreted chaperones from neurons and glial cells have been lately associated with neurodegenerative disorders, as evaluated by Chaplot et al. This pathological mechanism may be key in amyloid spreading and a therapeutic target for prion-like disorders.

Overall, all the reports building this special issue further our understanding on the mechanisms contributing to protein misfolding processes, proteostasis impairment and neuronal dysfunction in aging and neurodegeneration. Considering normal and pathological aging as a multifactorial condition, a deep understanding of the cross-talk between protein misfolding, proteostasis and neurodegeneration could be useful to establish promising multi levels therapies.

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REVIEW
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A Crucial Role for the Protein Quality Control System in Motor Neuron Diseases

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Motor neuron diseases (MNDs) are fatal diseases characterized by loss of motor neurons in the brain cortex, in the bulbar region, and/or in the anterior horns of the spinal cord. While generally sporadic, inherited forms linked to mutant genes encoding altered RNA/protein products have also been described. Several different mechanisms have been found altered or dysfunctional in MNDs, like the protein quality control (PQC) system. In this review, we will discuss how the PQC system is affected in two MNDs—spinal and bulbar muscular atrophy (SBMA) and amyotrophic lateral sclerosis (ALS)—and how this affects the clearance of aberrantly folded proteins, which accumulate in motor neurons, inducing dysfunctions and their death. In addition, we will discuss how the PQC system can be targeted to restore proper cell function, enhancing the survival of affected cells in MNDs.

Keywords: motor neuron, protein quality control, CASA complex, HSPB8, BAG3, BAG1

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INTRODUCTION

Motor neuron diseases (MNDs) are neurodegenerative diseases (NDs) characterized by the loss of motor neurons in the brain cortex, in the bulbar region, and/or in the anterior horns of the spinal cord; the consequence of motor neuron death is the lack of control on the skeletal muscle fibers. While motor neurons are considered the primary target in MNDs, muscle and glial cells may also be directly involved, and this affects motor neuron survival. MNDs are generally fatal diseases, clinically characterized by severe loss of voluntary movements, muscle weakness, spasticity, and atrophy. MNDs appear as sporadic or inherited forms, which have been extensively studied in the last 30 years. The inherited forms are associated with gene mutations that result in the production of altered RNA or proteins with reduced [loss-of-function (LOF)] or aberrant neurotoxic [gain-of-function (GOF)] functions. Mixed LOF and GOF are also possible. In LOF, the RNA or the protein affected are generally essential for motor neuron viability; thus, their reduced activity often causes motor neuron death [e.g., in spinal muscular atrophy (SMA); Lefebvre et al., 1995]. In these cases, the therapeutic intervention is aimed to restore the proper activity of the missed/altered RNA or protein (Poletti and Fischbeck, 2020), and successful therapies have been recently approved worldwide from regulatory agencies (Finkel et al., 2017; Mendell et al., 2017; Mercuri et al., 2018). In GOF, different neurotoxic mechanisms

have been reported to take place in a given mutant RNA or protein. Unfortunately, this makes difficult to identify a common therapeutic target for MNDs. Therefore, these approaches must be specifically designed for each MND's form. However, it is now clear that many familial MND forms are characterized by alterations of common intracellular pathways, which are often also altered in sporadic MNDs. Thus, these pathways might serve as potential therapeutic targets to reduce motor neuron death. In this review, we will focus on one of the most common pathways affected in MNDs, the protein quality control (PQC) system. In fact, in several MNDs, which include spinal and bulbar muscular atrophy (SBMA) and amyotrophic lateral sclerosis (ALS), the PQC system becomes unable to correctly handle misfolded proteins (mainly produced by the mutant gene), letting them become harmful to motor neurons and/or to glial and skeletal muscle cells.

MISFOLDED PROTEINS ASSOCIATED WITH MOTOR NEURON DISEASES

Spinal and Bulbar Muscular Atrophy

SBMA is the first MND for which a specific gene mutation has been linked to the disease as the cause of neuronal cell death (La Spada et al., 1991). SBMA, initially defined as a pure MND, is presently also classified as a neuromuscular disease. In fact, in SBMA, the primarily affected cell populations are lower motor neurons localized in the bulbar region of the brain (brain stem containing motor neurons of the lower cranial nerves) or in the anterior horn of the spinal cord (Sobue et al., 1989; La Spada et al., 1991; Brooks and Fischbeck, 1995; Li et al., 1995; Brooks et al., 1997). Dorsal root ganglia (DRG) neurons may also be affected in SBMA (Chua and Lieberman, 2013) and the combination of motor and DRG neurons loss is responsible for the clinical signs which include muscle fasciculations, weakness, and subsequent atrophy, including dysphagia and dysarthria with atrophy of the bulbar, facial, and limb muscles, as well as sensory disturbances at distal extremities (Sobue et al., 1989). So far, there is no evidence for the involvement of other brain cell types (e.g., glial cells or microglia). In addition to neuronal cells, skeletal muscle cells are also directly affected in SBMA (Chua and Lieberman, 2013; Cortes et al., 2014a; Lieberman et al., 2014; Rinaldi et al., 2014; Rusmini et al., 2015; Cicardi et al., 2019). This specific cell susceptibility is because the gene responsible for SBMA encodes for the androgen receptor (AR), and this gene is highly expressed in all the cell types described above (Poletti, 2004; Marron et al., 2005). The same cells express high levels of androgen-activating enzymes (Poletti et al., 1994, 1997, 2001; Pozzi et al., 2003). SBMA patients show mild endocrine alterations, like hypogonadism, possibly due to modification of the gonadal-hypothalamic axis or gynecomastia (Sobue et al., 1989; Kazemi-Esfarjani et al., 1995; Polo et al., 1996; Belsham et al., 1998; Piccioni et al., 2001). These alterations are often associated with reduced AR function.

Since the AR gene locus is on the X-chromosome, SBMA exists only as X-linked inherited form, but only males are affected (La Spada et al., 1991). Notably, the mutated AR protein is inactive in the absence of androgens [testosterone or its derivative 5α-dihydrotestosterone (DHT)], while it acquires toxic properties upon agonist binding (Katsuno et al., 2002, 2003), and the presence of androgens is thus mandatory for symptoms appearance and disease manifestation. This is possible since the AR mutation found in SBMA is radically different from those responsible for partial or complete androgen insensitivity syndrome (PAIS or CAIS) or tumors like prostate cancer (Brinkmann, 2001). In SBMA, the mutant AR gene is characterized by an expansion of a CAG (cytosine, adenine, guanine) tandem repeat (La Spada et al., 1991). The CAG sequence is expressed in exon 1 of the mRNA and then translated into a polyglutamine tract in the AR N-terminus (ARpolyQ). In normal individuals, the polyQ length of AR is highly polymorphic, ranging from 15 to 35 Qs (Edwards et al., 1992; Kuhlenbäumer et al., 2001); in SBMA patients the polyQ size becomes longer than 37 Qs (to a maximum of 72; Fischbeck, 1997; Kuhlenbäumer et al., 2001; Grunseich et al., 2014; Madeira et al., 2018). CAG repeat expansions coding for elongated polyQ tracts have been found in other eight genes, which are unrelated to AR; the mutant protein products of these genes cause other similar NDs (Ross, 2002). The ARpolyQ retains approximately 30% of its transcriptional functions, which explains the endocrine signs present in SBMA, but acquires a novel toxic function that impacts neuronal and muscle cell viability. As mentioned above, this toxic function of ARpolyQ appears after its activation by androgens. These AR ligands (testosterone or DHT) may induce aberrant protein conformations to ARpolyQ (protein misfolding), which becomes highly prone to aggregation (Stenoien et al., 1999; Simeoni et al., 2000; Piccioni et al., 2002). Details of this pathological mechanism are provided below.

Amyotrophic Lateral Sclerosis

ALS is a typical MND characterized by the loss of both the cerebral motor cortex or brainstem (upper) motor neurons and the cranial nerves and ventral horns of the spinal cord (lower) motor neurons. Neurons located in the frontotemporal cortex may be involved in some specific forms of ALS (Robberecht and Philips, 2013), which may clinically manifest in a pure MND form or be associated with a different extension to frontotemporal dementia (ALS-FTD). Differently from SBMA, the surrounding non-neuronal glial cells [astrocytes (Trotti et al., 1999; Boillee et al., 2006; Nagai et al., 2007), oligodendrocytes (Philips et al., 2013), and Schwann cells (Lobsiger et al., 2009; Manjaly et al., 2010)] are indirectly or directly affected in ALS. Reactive microglia are also present in ALS-affected tissues, but not in SBMA (Philips and Robberecht, 2011), proving that neuroinflammation and oxidative stress may play a significant role in ALS (Ferraiuolo et al., 2011). As in SBMA, the striatal skeletal muscle target cells can also be directly affected in ALS (Dobrowolny et al., 2008; Onesto et al., 2011; Cicardi et al., 2018; Meroni et al., 2019). Ninety percent of ALS cases appear as sporadic (sALS) forms, and only 10% of cases are caused by inherited mutations linked to familial (fALS) forms. The two types of ALS are clinically indistinguishable. Up to now, more than 30 genes have been found altered in fALS (Robberecht and

Philips, 2013; Cook and Petrucelli, 2019; Mathis et al., 2019; Mejzini et al., 2019), and each of these accounts for disease, which mainly occurs as monogenic disease, even if disease modifier genes might exist. It is noteworthy that several of the gene products that cause a specific fALS have been reported to acquire an aberrant behavior of their wild-type (wt) forms in sALS. This suggests the existence of common pathways that lead to motor neuronal death in both fALS and in sALS (Neumann et al., 2006; Daoud et al., 2009; Bosco and Landers, 2010).

ALS has a very high variability in terms of both age of onset and disease progression, and it seems to occur earlier in males compared to females (Vegeto et al., 2020), with a male/female ratio of 1–3 in the geographic region and population evaluated in the study (Kurtzke, 1982; Haverkamp et al., 1995; Manjaly et al., 2010). The two sexes also show different symptomatology, since in males the disease predominantly begins in the lumbar tract of the spinal cord, while in females ALS mainly begins in the bulbar region (see Blasco et al., 2012 for an extensive review). It is likely that hormonal sex steroids may influence the neurotoxicity of factors involved in the pathogenesis of ALS (see Vegeto et al., 2020 for an extensive review).

Historically, the *superoxide dismutase 1 (SOD1)* gene is the first gene associated with fALS. However, this mutation only accounts for 15% of all fALS cases. *SOD1* encodes a ubiquitously-expressed antioxidant enzyme that acts as a free radical scavenger enzyme (Bendotti et al., 2012). The most frequent fALS form (almost 50% of all fALS) is due to a mutation in the *C9orf72* (chromosome 9 open reading frame 72) gene; in particular, the mutation consists of an expansion of a hexanucleotide (G_4C_2) repeat located in the 5′-untranslated region of the *C9orf72* gene. Surprisingly, despite its location in an intronic sequence, the G_4C_2 expansion (which is transcribed in both directions) is utilized by ribosomes as a starting point for translation; this results in the production of five different dipeptides (DPRs; Ash et al., 2013; Gendron et al., 2013; Lashley et al., 2013; Mori et al., 2013). The process has been identified as an unconventional translation and named "repeat-associated non-ATG (RAN) translation" (Zu et al., 2011). The five DPRs do not have a physiological role, but they only exert toxicity in the expressing cells of affected individuals. Other mutant genes are less frequently represented in fALS: examples are the genes encoding TAR DNA-binding protein 43 (TDP-43), the ALS-linked fused in sarcoma/translocated in liposarcoma (FUS/TLS), the ubiquilin-2, the optineurin, the valosin-containing protein/p97 (VCP/p97), and others. These alterations occur in a few fALS families, but the same proteins (even if in the wt form) can be dysregulated in sALS, suggesting that their functions are crucial to maintain neuronal homeostasis (a list of the most common gene mutations identified so far in fALS is reported in **Table 1**). In particular, TDP-43 is considered a hallmark for sALS since it mislocalizes from nucleus to cytoplasm, where it aggregates in inclusions. These inclusions are enriched by TDP-43 caspase-3-cleaved fragments containing the C-terminal unstructured domain (Ratti and Buratti, 2016).

A careful analysis of the gene products identified so far suggests that several of their coded proteins have functions that cluster in specific intracellular processes. One of the most represented pathways is the PQC system (**Table 1**). In fact, different ALS-associated proteins are directly involved in the PQC system and others indirectly affect the PQC system due to their mutation. Indeed, when mutated, they become unable to properly reach the folded conformation and misfold. Misfolded proteins must be cleared from cells, and with this mechanism they may overwhelm the PQC system capability to handle proteotoxic stresses. As in the case of ARpolyQ and in all other elongated polyQ-containing proteins, which cause adult-onset MNDs, the misfolded ALS proteins tend to segregate from the nuclear or cytoplasmic compartments *via* a liquid-liquid phase partitioning (Molliex et al., 2015; Patel et al., 2015; Ganassi et al., 2016; Lee et al., 2016; Alberti et al., 2017; Boeynaems et al., 2017; Freibaum and Taylor, 2017; Mackenzie et al., 2017). This leads to an initial seed of aggregates with well-defined physical-chemical properties, which then mature into aggresomes and insoluble inclusions (Davies et al., 1997; DiFiglia et al., 1997; Li et al., 1998; Lieberman et al., 1998; Kopito, 2000; Mediani et al., 2019). The accumulating proteins may thus damage the PQC system by saturating its functional capabilities or by clogging the pathways devoted to protein clearance. For these reasons, by forming aggregates, misfolded ARpolyQ or ALS-associated proteins may perturb not only the PQC system, but also a series of pathways that depend on the proper functioning of the PQC system to maintain the correct cellular homeostasis.

THE PROTEIN QUALITY CONTROL SYSTEM

Most cell types affected in MNDs are post-mitotic or generally characterized by a poor mitotic index. This means that these cells might accumulate aberrant proteins that cannot be diluted by cell self-renewal or by simple partitioning into duplicated intracellular compartments generated as a result of cell division. Thus, these cells must develop a very sophisticated system to maintain their proper protein homeostasis. Therefore, post-mitotic non-dividing cells like neurons, motor neurons, or skeletal muscle cells, as well as poorly replicating cells, like glial cells, are highly prone to respond to misfolded protein species. Misfolded species may be produced in response to different cell stresses or as a consequence of gene mutations. These cells are able to respond to these stresses in a very powerful way: the overexpression of specific chaperones and co-chaperones, paralleled by the potentiation of the degradative pathways. All these factors are extremely well-coordinated to protect against proteotoxicity, and their synergic activities constitute the PQC system mentioned above. The PQC system thus acts as the first line of defense and because of its protective action, its selective modulation represents a valuable target for therapeutic intervention in all protein misfolding diseases, including MNDs like SBMA and ALS.

The PQC system is composed of a very large number of factors clustered in specific families of proteins that work together to define the fate of every single protein starting from its proper folding after synthesis or denaturation, and it routes proteins to degradation in case the folding fails.

TABLE 1 | Gene mutations reported in familial amyotrophic lateral sclerosis (fALS).

Name	Gene symbol	Protein function	Aggregation prone	PQC function	Sporadic (s) / familial (f)
Spinal and bulbar muscular atrophy (SBMA)					
-	AR	Steroid hormone receptor for androgens	ooo		
Amyotrophic lateral sclerosis (ALS)					
ALS1	SOD1	Superoxide dismutase	ooo		s/f
ALS2	ALS2	Rho Guanine Nucleotide Exchange Factor			f
ALS3	ALS3	-	-	-	-
ALS4	SETX	DNA/RNA helicase			s
ALS5	SPG11	Maintenance of cytoskeleton stability/regulation of synaptic vesicle transport		o	f
ALS6	ALS7	-	-	-	-
ALS6	FUS	RNA-binding protein	o		s/f
ALS8	VAPB	ER-membrane protein	o	o	f
ALS9	ANG	Actin-binding protein; ribonuclease			s/f
ALS10	TARDBP	RNA-binding protein	ooo		s/f
ALS11	FIG4	Regulates synthesis and turnover of phosphatidylinositol 3,5-bisphosphate			s/f
ALS12	OPTN	Autophagy adaptor	oo	ooo	s/f
ALS13	ATXN2	Endocytosis/RNA metabolism	o		s/f
ALS14	VCP	Ubiquitin segregase		ooo	s/f
ALS15	UBQLN2	Protein degradation	ooo	ooo	s/f
ALS16	SIGMAR1	Lipid transport from the endoplasmic reticulum		oo	f
ALS17	CHMP2B	Component of the endosomal sorting required for transport complex III (ESCRT-III); involved in sorting of endosomal cargo proteins.		oo	f
ALS18	PFN1	Actin-binding protein	oo		s/f
ALS19	ERBB4	Member of the epidermal growth factor (EGF) receptor			s/f
ALS20	HNRNPA1	RNA-binding protein	oo		s/f
ALS21	MATR3	RNA-binding protein	oo		s/f
ALS22	TUBA4A	Microtubules subunit	oo		s/f
ALS23	ANXA11	Vesicle trafficking, apoptosis, exocytosis, and cytokinesis	oo	oo	s/f
FTD-	C9ORF72	Guanine nucleotide exchange factor—involved in autophagy	ooo	oo	s/f
FTD-	CHCHD10	Mitochondrial protein			s/f
FTD-	SQSTM1	Autophagy adaptor	ooo	ooo	s/f
FTD-ALS4	TBK1	Innate immune response, autophagy, inflammation and cell proliferation		oo	s/f
LAHCDA	GLE1	Required for the export of mRNAs from the nucleus to the cytoplasm			s/f
-	DAO	Regulates the level of the neuromodulator D-serine in the brain			f
-	ELP3	RNA polymerase II component			s
-	EWSR1	RNA/DNA binding protein	o		s
-	HNRNPA2/B1	RNA-binding protein	o		s
-	KIF5A	Microtubule-based motor protein			s
-	CCNF	Catalyzes ubiquitin transfer to substrates for UPS		ooo	s/f
-	CFAP410	Regulation of cell morphology and cytoskeletal organization			s/f
-	DCTN1	Component of dynein motor complex			s/f
-	NEFH	Cytoskeletal component	oo		s/f
-	NEK1	Cytoskeletal dynamics			s/f
-	TAF15	RNA-binding protein	oo		s/f
-	TIA1	RNA-binding protein	ooo		s/f

*The table shows the list of the most common mutated genes identified in fALS. The columns describe ALS name, gene symbol, protein function, aggregation propensity, involvement in PQC and sporadic vs. familial form (***o** *= low;* **oo** *= mid;* **ooo** *= high). Aggregation prone proteins are highlighted in brown, proteins involved in PQC system in orange, and those that show both conditions in yellow.*

The Chaperones

The family of intracellular chaperones and their co-chaperones is composed of more than 180 different proteins, some of which share a high degree of homology. These chaperones generally act in a specific subcellular compartment: for example, some chaperones localize exclusively in the endoplasmic reticulum, in the mitochondria, in the lysosomes, and/or in the cytoplasm, where they mainly exert their protective activities. Most chaperones are also expressed in a cell- and tissue-specific manner, with some chaperones localized exclusively in one tissue (e.g., in the testis), while others are ubiquitously expressed. In addition, chaperones may be regulated in response to cell stresses. Indeed, chaperones have been discovered as proteins induced by heat shock, and found to protect cells against thermal damages. Because of this, they have been named "heat shock proteins" or HSPs (DiDomenico et al., 1982). This name still stands for many chaperones, even if they have been demonstrated to possess much wider activities against a spectrum of variables capable of damaging intracellular proteins (e.g., oxidative stress, hypoxia, DNA damage, aberrant translation, etc.). Based on their structure and functions, these factors have been classified in subfamilies of chaperones. Originally, chaperones were grouped based on their apparent molecular weight after their biochemical identification in SDS-PAGE (small HSPs, HSP40s, HSP60s, HSP70s, HSP90s, and HSP100), but this classification now reflects their functions in the folding processes. Based on HUGO Gene Nomenclature Committee, a new nomenclature has been adopted for the human HSP families: HSPB (small HSP), DNAJ (HSP40), HSPD (HSP60), HSPA (HSP70), HSPC (HSP90), and HSPH (HSP110; Kampinga et al., 2009; see also Kampinga and Craig, 2010) for an extensive review). Chaperones often require the assistance of co-chaperones, which serve as nucleotide exchange factors (NEFs), like the members of the BCL2-associated athanogene (BAG) protein family (Takayama and Reed, 2001; **Figure 1**).

The Degradative Systems

Cells, including post-mitotic cells like neurons and skeletal muscle cells, utilize two major degradative systems to enzymatically destroy aberrant proteinaceous materials and recycle their components for other proteins production. This process is assisted by chaperones (and their co-chaperones), which route aberrant proteins to degradative systems.

Proteins undergoing this degradation are damaged proteins or regulatory proteins that ended their functions. The two degradative systems are: (a) the ubiquitin proteasome system (UPS); and (b) the autophago-lysosomal pathway (ALP). Of note, UPS acts both in the cytosolic and nuclear compartment, while ALP acts only in the cell cytoplasm.

(a) The UPS is a highly specific and very selective proteolytic system mainly devoted to the clearance of short-lived proteins. The UPS inactivates proteins controlling cell cycle progression, apoptosis, transcription, and cell differentiation. Moreover, the UPS mediates the immune response and it is responsible for the clearance of damaged monomeric proteins. UPS is based on two subsequent steps: the protein is labeled by a covalent binding to ubiquitin (a small protein of 76 amino acids), which is itself ubiquitinated forming a poly-ubiquitin chain of several molecules of ubiquitin (Pickart, 2001a,b); and this poly-ubiquitinated protein is degraded by the 26S proteasome. The recognition of the protein to be degraded is mediated by different chaperones of the

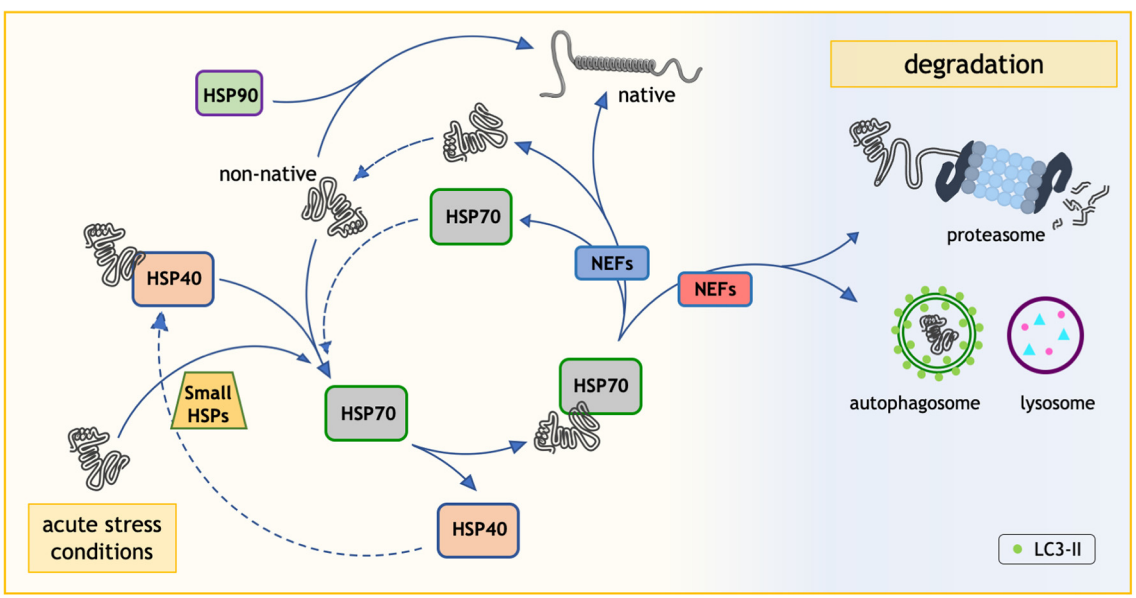

FIGURE 1 | The role of heat shock proteins (HSP70) in the protein quality control (PQC) system. HSP70 plays an essential role in the protein folding process. Through its interaction with HSP40, HSP70 is able to fold the proteins in non-native conformations. HSP70 and HSP40 are not the only HSPs involved. In fact, the HSP90 system can assist protein folding in an independent way and the small HSPs respond to acute stress conditions. Notably, when necessary nucleotide exchange factors (NEFs/BAGs) route HSP70 client proteins to degradative pathways (Ubiquitin-proteasome system and/or autophagy).

HSP70/HSP40 families in a complex (**Figure 1**). HSP70 has the ability to interact with specific E3-ubiquitin ligases (such as the C-terminus HSP70 interacting protein, CHIP), which selectively ubiquitinate misfolded proteins (Ciechanover, 1994; Ciechanover and Brundin, 2003; Ciechanover and Kwon, 2015). The ubiquitination cascade is rather complex. Ubiquitination initially requires the activation of E1 enzymes that activate ubiquitin; next, the activated ubiquitin is transferred to E2 enzymes, which in concert with the E3-ubiquitin ligases bind ubiquitin to a lysine residue of the substrate protein. E3-ubiquitin ligases have slightly different functions (Jackson et al., 2000; Joazeiro and Weissman, 2000). In addition, deubiquitinating enzymes (DUBs) are involved in this process (Amerik and Hochstrasser, 2004); DUBs maintain the cellular pool of free ubiquitin by processing ubiquitin precursors and recycling ubiquitin from poly-ubiquitinated substrates. Once polyubiquitinated, the substrate protein is recognized by the SQSTM1/p62, and other proteins of this class (Klionsky et al., 2016) and routed to the proteasome for degradation. The 26S proteasome has a typical barrel shape constituted by a large, multi-subunit protease complex: a 20S core complex with catalytic activity and a 19S regulatory complex, the cap. The cap receives the polyubiquitinated substrate, removes the poly-ubiquitin chain and induces its translocation into the 20S complex. Here, the substrate protein must enter the narrow central 20S cavity for the enzymatic degradation to small peptides. To this aim, folded proteins must be unfolded by the 19S subunit to reach a "linear" conformation. Thus, globular or aggregated proteins are not processed by the proteasome (Ciechanover and Brundin, 2003), and may even clog its catalytic core. Molecular chaperones and co-chaperones cooperating with the proteasomal-mediated degradation of ubiquitinated substrates include the already mentioned HSP70/HSP40 (now identified as HSPAs/DNAJs) and the HSP70/BAG1 complexes (**Figure 1**; Demand et al., 2001; Alberti et al., 2002; Kampinga and Craig, 2010; Kampinga and Bergink, 2016; Cristofani et al., 2017; Cicardi et al., 2018, 2019). In the latter case, the HSP70/CHIP complex, initially described as required for the substrate ubiquitination, can associate to BAG1, and together with SQSTM1/p62 it drives the ubiquitinated misfolded protein to proteasomal degradation.

(b) The lysosomal-mediated system collects proteins from various origins. The system is typically divided into microautophagy, chaperone-mediated autophagy (CMA), and macroautophagy (normally identified as autophagy). These systems are evolutionarily well-conserved processes required for the degradation of proteins or large cytosolic components *via* the lysosome (Mizushima et al., 2008). In the case of microautophagy, the cytosolic components are directly engulfed into lysosomes *via* an invagination of its membrane (Sahu et al., 2011). In CMA, only a specific subset of proteins can be processed: those containing a pentapeptide lysosome-targeting motif KFERQ or related consensus motifs (also generated by specific post-translational modifications; Orenstein et al., 2013; Kaushik and Cuervo, 2018; Kirchner et al., 2019); the sequence allows the direct translocation of cargo into lysosome. CMA requires the docking to the lysosomal receptor lysosome-associated membrane protein 2A (LAMP2A), as well as the protein unfolding by a chaperone complex containing HSC70, BAG1, HSC70-interacting protein (HIP), Hsp-organizing protein (HOP), and HSP40 (DNAJB1; Kampinga et al., 2009; Kampinga and Craig, 2010; Kampinga and Bergink, 2016). Instead, in macroautophagy (which the general term "autophagy" usually refers to), the cytosolic components are engulfed into the autophagosome, a double-membrane vesicle that then fuses with the lysosome, in order to deliver its content to the lysosome for degradation (Xie and Klionsky, 2007). Initially considered as a sort of non-specific degradation for long-lived proteins, organelles, or protein aggregates, it is now clear that autophagy is tightly regulated by several pro-autophagic factors (Mizushima et al., 2008; Sardiello et al., 2009). In this latter form of autophagy, it is also possible to distinguish between "in bulk" autophagy and selective autophagy. While "in bulk" autophagy is characterized by a very high clearance capability but is rather non-specific since it entraps large portion of cytoplasm, selective autophagy is highly specific and involves specific molecular regulators (Kaushik and Cuervo, 2018). Selective autophagy includes chaperone-assisted selective autophagy (CASA; Arndt et al., 2010; Kettern et al., 2011; Sarparanta et al., 2012; Ulbricht et al., 2013, 2015; Ghaoui et al., 2016; Sandell et al., 2016; Cicardi et al., 2019; Cristofani et al., 2019; Rusmini et al., 2019), organelles-specific types of autophagy (mitophagy, lysophagy, ribophagy, granulophagy, etc.), or processes aimed at removing large protein aggregates (aggrephagy; Nivon et al., 2012; Stürner and Behl, 2017; Aparicio et al., 2020).

CASA has attracted large attention in the field of NDs, specifically in MNDs, since this highly selective autophagy is based on the recognition of misfolded substrates by a heteromeric complex composed of a small HSP, the HSPB8, with its co-chaperone BAG3. Once the misfolded protein is bound to HSPB8/BAG3, the HSP70/CHIP dimer (already seen in the UPS pathway) can be recruited. Here, the misfolded protein is rapidly ubiquitinated by CHIP, allowing recognition by the autophagy receptor SQSTM1/p62 (and related proteins) and forming the CASA complex. Some studies include HSP40 or DNAJ proteins in this complex (Sarparanta et al., 2012; Sandell et al., 2016). In this context, the role of SQSTM1/p62 is different from that exerted in association with BAG1/HSP70/CHIP, which allows the use of the UPS pathway. When acting with HSPB8/BAG3/HSP70/CHIP, the SQSTM1/p62 protein interacts with the ubiquitinated misfolded proteins (or other cargoes) and the lipidated form of the microtubule-associated proteins 1A/1B light chain 3B (LC3-II) anchored to the autophagosome membrane. To allow SQSTM1/p62 and LC3-II-action, the CASA complex takes advantage of a dynein binding motif present in the BAG3 sequence. The CASA complex bound to dynein is transported along microtubules to the microtubule organizing center (MTOC). Ubiquitinated and SQSTM1/p62-positive misfolded proteins are concentrated at MTOC to form the aggresomes. Meanwhile, LC3-II decorated-autophagosomes are generated, allowing aggresome insertion into a nascent autophagosome. The autophagosome containing the CASA

complex and the misfolded proteins fuses with the lysosome to allow the degradation of the engulfed material following the canonical autophagic pathway.

Selective autophagy is also involved in the degradation of damaged organelles like mitochondria and lysosomes. In mitophagy, the damaged mitochondria stabilize PINK1 on its outer membrane. PINK1 recruits E3-ubiquitin ligases, like Parkin, which amplify the ubiquitination of proteins in the outer membrane mediating recruitment of the autophagic receptors that interact with LC3-II present on the forming autophagosome membrane (Youle and Narendra, 2011). Some of the mitochondrial membrane proteins, like mitofusin, are polyubiquinated with K48 ubiquitin chains. These proteins are substrates of VCP/p97, an AAA^+ ATPase, that segregates these proteins from the mitochondria membrane and promotes their degradation *via* UPS. The removal of these proteins is necessary for mitochondria degradation (Tanaka et al., 2010; Tanaka, 2010; Kimura et al., 2013). In lysophagy, ruptured lysosomes expose galectins (Gal-3, Gal-8) as damage signals. Gal-8 is directly recognized by autophagy receptors, while Gal-3 recruits and binds TRIM16. Gal-3/TRIM16 complex promotes ubiquitination of lysosomal proteins and recruits autophagy initiation factors to trigger local phagophore formation (Thurston et al., 2012; Chauhan et al., 2016). Moreover, K63-ubiquitinated proteins recruit autophagy receptors, while K48-ubiquitinated proteins are targeted by VCP/p97 to UPS degradation. VCP/p97 recruitment to lysosome membranes and functioning are mediated by its cofactors and adaptors YOD1, UBXD1, and PLAA. The removal of K48 polyubiquitinated proteins is a critical step to promote lysosome degradation (Fujita et al., 2013; Akutsu et al., 2016; Papadopoulos et al., 2017).

The Unfolded Protein Response (UPR) and the Endoplasmic Reticulum-Associated Degradation (ERAD)

UPR and ERAD are two other key pathways devoted to the PQC in cells. UPR is typically activated in the presence of an abnormal excess of misfolded proteins, while ERAD mediates their degradation by taking advantage of the cytosolic proteasome mentioned above. In fact, the accumulation of misfolded proteins in the endoplasmic reticulum (ER) activates the UPR. This action is mediated by three different "sensors"—inositol requiring enzyme 1 (IRE1α), PKR-like endoplasmic reticulum kinase (PERK), and activating transcription factor 6 (ATF6; Hetz, 2012)—that signal to dedicated pathways to stimulate either protein folding or protein degradation. During this process, ribosomes are forced to attenuate protein translation. ERAD has a specific function in PQC system since the ER is a major site for protein folding. When aberrant ER-resident proteins are processed by ERAD, they are released into the cytosol for proteasomal (when these are still soluble) or for autophagic clearance (when they are in an aggregated form; Hetz, 2012). Even in the case of ERAD, the proteins are ubiquitinated by specific E3-ubiquitin ligases, like HRD1 in the SEL1L-HRD1 protein complex (where SEL1L acts as a cofactor). Ubiquitinated misfolded proteins can be "retro-translocated" or "dislocated" (extracted) from the ER membrane and transported to the cytosol mainly by the activity of VCP/p97. VCP/p97 in complex with UFD1-NPL4 first binds HRD1 and the ubiquitinated proteins, then addresses substrates to the proteasome *via* shuttle cofactors (Ye et al., 2005; Senft and Ronai, 2015). Even in the case of the UPR-ERAD, a central role is played by an HSP70, BiP (or HSPA5 or GRP-78), which has low intrinsic ATPase activity, enhanced by co-chaperones of the DNAJ-proteins (the same class of the HSP40, like ERdj4 or DNAJB9). In addition to protein folding, the ER controls the Ca^{2+} homeostasis, being the major intracellular Ca^{2+} reservoir (Hetz and Mollereau, 2014). When misfolded proteins accumulate in the ER, the depletion of ER Ca^{2+} impacts on cell activity and enhances stress. Store-operated Ca^{2+} influx is activated in these conditions to assure the replenishment of Ca^{2+} levels (Szegezdi et al., 2006). If ER stress is prolonged, the ability of the UPR to restore ER homeostasis is reduced and this may cause ER stress-induced apoptosis by activation of caspase 12 (Yoneda et al., 2001). Once activated, the UPR-ERAD converges on the proteasome or to autophagy; therefore, in this review we will only focus on the proper degradative pathways. Details on UPR-ERAD can be found elsewhere (for an extensive review see Hwang and Qi, 2018).

Release Mediated by Extracellular Vesicles

Emerging data strongly suggest that the extracellular secretion may also play an important role in the maintenance of intracellular protein homeostasis by cooperating with or even being a part of the PQC system (Desdín-Micó and Mittelbrunn, 2017; Xu et al., 2018; Guix, 2020). In fact, it has been found that several NDs-related proteins are secreted in double membrane spherical particles known as extracellular vesicles. This is the case for the amyloid-beta peptide and tau/phosphorylated tau for Alzheimer's disease (Pérez et al., 2019), alpha-synuclein for Parkinson's disease (Longoni et al., 2019), misfolded/mutant SOD1, TDP-43 and its pathological-related C-terminal fragments (of 35 kDa and 25 kDa) and FUS for ALS (Basso and Bonetto, 2016; Iguchi et al., 2016; Hanspal et al., 2017; Sproviero et al., 2018), and progranulin, TDP-43, and C9orf72 DPRs for FTD and ALS-FTD (Benussi et al., 2016; Iguchi et al., 2016; Westergard et al., 2016). The extracellular vesicles are heterogeneous in size and are mainly classified into three different types: exosomes, microvesicles, and apoptotic bodies. These vesicles differ for size, proteins, and lipids composition and intracellular origin. In fact, exosomes are secreted membrane vesicles (approximately 30–120 nm in diameter) formed intracellularly and released from exocytosis of multivesicular bodies, whereas apoptotic bodies (approximately 1,000–4,000 nm in diameter) are released by dying/apoptotic cells. Microvesicles (approximately 200–1,000 nm in diameter) are shed from cells by an outward protrusion (or budding) of the plasma membrane followed by fission of their membrane stalk (for a detailed review see Akers et al., 2013; Colombo et al., 2014; van Niel et al., 2018). A tight connection between PQC and extracellular vesicles is particularly true for exosomes (Xu et al., 2018). As stated above, exosomes are intraluminal vesicles of the endosomal compartment that maturate into a structure

called the multivesicular body after a very dynamic process. The multivesicular body may release its content into the lysosome for degradation or, under certain conditions, it may fuse with the plasma membrane and secrete its intraluminal vesicles, the exosomes. Interestingly, components of the CASA complex may also affect/take part in extracellular vesicles pathway: for example, STUB1/CHIP deficiency resulted in an increased secretion of small extracellular vesicles that are enriched in ubiquitinated and/or undegraded proteins and protein oligomers (Ferreira et al., 2019), and BAG3 is found to be involved in the exosome secretion of mutant Huntingtin upon proteasome blockade (Diaz-Hidalgo et al., 2016). These evidences suggest that extracellular vesicles have to be considered as new actors in the proteostasis scenario, together with chaperones and the degradative systems.

HOW THE PROTEIN QUALITY CONTROL SYSTEM PROTECTS AGAINST MISFOLDED PROTEIN TOXICITY IN SBMA AND ALS

Data collected over the last 30 years suggest that ARpolyQ and several ALS-associated proteins (listed in **Table 1**) may lead to PQC system alterations (Kabashi and Durham, 2006; Voisine et al., 2010; Rusmini et al., 2016, 2017; Cristofani et al., 2018, 2019). At the same time, the boost of key proteins involved in PQC system regulation is protective in SBMA and ALS (Waza et al., 2006; Yu et al., 2011; Giorgetti et al., 2015; Crippa et al., 2016b; Rusmini et al., 2016, 2017, 2019, 2020; Cristofani et al., 2018, 2019; Mandrioli et al., 2019).

Figure 2 summarizes how all the PQC system components work synergistically to prevent misfolded protein accumulation in these diseases.

Folding Process

The first line of PQC system intervention on the misfolded protein is an attempt to restore the proper protein folding. Even if the folding process is well-understood, many questions still remain open in the case of disease-associated misfolded proteins; in particular, to what extent the refolding of a protein may occur after the first aggregation steps. The main actors in the folding process are the HSP70s (also named HSPAs), which are similar to nanomachines capable of switching conformation using hydrolysis of ATP (**Figure 1**). This allows HSP70 to change conformation in order to assist protein folding, disaggregation, and degradation (see Kampinga and Craig, 2010; Kampinga and Bergink, 2016 for an extensive review). HSP70 is a hub that requires the assistance of HSP40s (or DNAJ proteins) in order to recognize the protein to be folded, and of NEFs, like the BAGs, which exchange ADP/ATP during the hydrolytic process (Sondermann et al., 2001; Rauch and Gestwicki, 2014).

Misfolded proteins responsible for SBMA and ALS are able to alter this finely-tuned process. These misfolded proteins escape the correct folding and expose unstructured domains highly prone to aggregate. Such domains are present in the

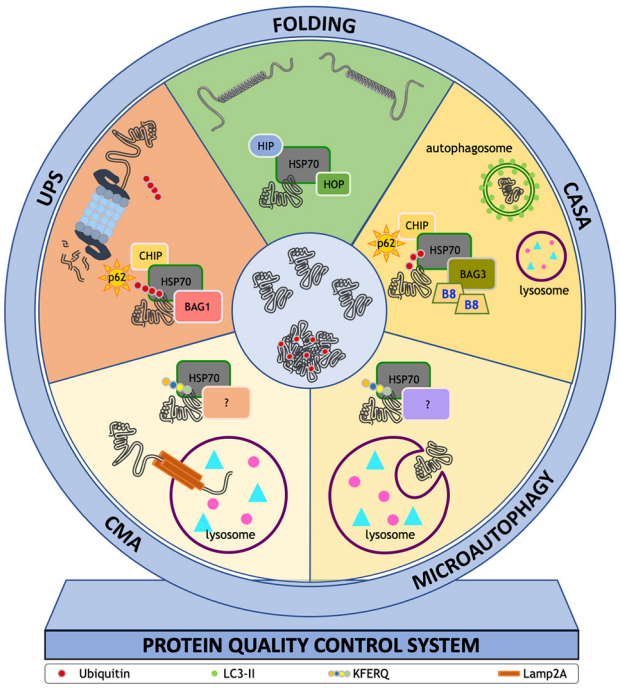

FIGURE 2 | The PQC system. The fate of misfolded proteins is finely tuned by the PQC system. This system is centered on a group of chaperones and co-chaperones assisting proteins to reach their correct conformation or directing proteins to degradative systems. Each pathway needs specific proteins that assist the action of HSP70: (i) HSC70 interacting protein 1 (HIP) and HSP70-HSP90 organizing protein (HOP) in the folding process; (ii) sequestosome 1 (SQSTM1/p62), E3-ubiquitin ligase C-terminus HSP70 interacting protein (CHIP), BAG family molecular chaperone regulator 3 (BAG3) and heat shock protein B8 (HSPB8, B8 in figure) in chaperone assisted selective autophagy (CASA); (iii) Lysosome-associated membrane glycoprotein 2 (Lamp2A) in chaperone mediated autophagy (CMA); and (iv) SQSTM1/p62, CHIP, BAG family molecular chaperone regulator 1 (BAG1) in ubiquitin proteasome system (UPS). The HSP70 interactors at lysosome membrane remain to be determined [indicated in figure as (?)] even if coimmunoprecipitation and colocalization studies identified HSP90, HSP40, HOP, HIP, and BAG1. Their role in CMA and microautophagy remains to be determined.

ARpolyQ in its poorly structured N-terminus containing the polyQ stretch, in the prion-like domains of TDP-43 and FUS proteins, and maybe also in the five DPRs derived from the C9orf72 mRNA, which do not possess tertiary structures. These unstructured domains may clamp together in a liquid-liquid partitioning of phases, forming membraneless organelles attracting other compatible molecules (e.g., RNAs or proteins which normally interact with these unstructured proteins). The mutations in these proteins greatly enhance their capability to generate liquid-liquid intracellular compartments, which soon after their formation may mature into aggresomes, stable aggregates, and even insoluble inclusions trapping specific intracellular factors (Molliex et al., 2015; Patel et al., 2015; Ganassi et al., 2016; Alberti et al., 2017; Boeynaems et al., 2017; Mackenzie et al., 2017; Mateju et al., 2017; Marrone et al., 2018). Specific proteins are known to accelerate the conversion of the aggregates formed after phase separation into stable

insoluble aggregates. Conversely, chaperones and co-chaperones may prevent this conversion, delaying the maturation into stable structures and facilitating the disassembling of the newly formed membraneless organelles. This activity of chaperones and co-chaperones should permit the refolding process of a misfolded protein even after its entrapping in the aggresomes when they are still dynamic (Jaru-Ampornpan et al., 2013; Mattoo and Goloubinoff, 2014; O'Driscoll et al., 2015; Zaarur et al., 2015; Mathew and Stirling, 2017; Kitamura et al., 2018; Alexandrov et al., 2019).

Alterations of UPS in SBMA and ALS

Evidence suggests that ARpolyQ, SOD1, TDP-43, and its ALS associated fragments, as well as other ALS-proteins, including at least one out of five DPRs of C9orf72, are processed *via* the proteasome (Rusmini et al., 2007, 2010, 2011, 2013, 2019; Sau et al., 2007; Crippa et al., 2010b, 2016a; Onesto et al., 2011; Cristofani et al., 2017, 2018; Cicardi et al., 2018, 2019). However, the large amounts of misfolded proteins formed when specific gene mutations occur may overwhelm the UPS capability to degrade them efficiently. This process is accentuated in aged cells in which the chaperone and UPS activities are reduced (Ciechanover and Brundin, 2003; Terry et al., 2004, 2006; Wang K. et al., 2018; Hegde et al., 2019). It is also possible, as in the case of the elongated polyQ tract of the AR, that the proteasome proteolytic capability is unable to digest the long polyQ sequence since no consensus cleavage sites for its enzymatic activity are present between the Qs; thus, long uninterrupted polyQ size might block the narrow catalytic site, where only a single protein can enter and be degraded. We showed that while the wtAR with a 23Q stretch can be cleared *via* the proteasome even in presence of androgens (Rusmini et al., 2007), the mutant ARpolyQ may impair the UPS function; in fact, by expressing the ARpolyQ in basal condition (absence of androgens), we noted an accumulation of the proteasome activity reporter GFP-CL1 (GFPu) as an indication that the elongated polyQ is poorly processed by the UPS and interferes with the activity of this degradative pathway. Interestingly, the inactivated ARpolyQ does not play toxicity in all cell models tested. Surprisingly, when the ARpolyQ is activated by androgens (which bound at the AR C-terminus), the protein is thought to acquire toxic conformations (Stenoien et al., 1999; Simeoni et al., 2000; Piccioni et al., 2002; Poletti, 2004), but the UPS is fully functional (Rusmini et al., 2007) since the GFPu reporter is fully degraded by the UPS. An explanation for this unexpected phenomenon is that by inducing the ARpolyQ toxic conformation, androgens also induce its misfolding (possibly *via* a phase partitioning phenomenon; Eftekharzadeh et al., 2019; Escobedo et al., 2019) and sequestration into subcellular compartments (the aggregates), protecting the cell from this dangerous protein conformation (Rusmini et al., 2007, 2010). The formation of aggregates acts as a sink that permits UPS desaturation from the excess of "free" polyQ to be processed. Meanwhile, aggregates might stimulate autophagy for ARpolyQ clearance (see below). It is thus expected that also autophagy alterations might contribute to the accumulation of stable insoluble ARpolyQ aggregates in cells (Rusmini et al., 2013; Giorgetti et al., 2015; Cristofani et al., 2017; Cicardi et al., 2019). As will be discussed below, the potentiation of CASA restores normal ARpolyQ clearance. A similar UPS role has been found involved in the clearance of ALS-misfolded proteins. Mutant SOD1 is mainly cleared by the UPS, and its pharmacological inhibition with MG132 results in an accumulation of ubiquitin-positive SOD1 aggregates in cells (Crippa et al., 2010a,b, 2016a; Cicardi et al., 2018). This aggregated SOD1 is poorly removed by autophagy but, as seen for ARpolyQ, the induction of CASA restores complete clearance of aggregating mutant SOD1 (Crippa et al., 2010a,b, see below). TDP-43 and its 35 kDa and 25 kDa TDP-43 fragments follow the same route of degradation identified for inactive ARpolyQ and mutant SOD1 (Crippa et al., 2016a; Cicardi et al., 2018, 2019). Even in this case, UPS inhibition results in an accumulation and mislocalization of TDP-43 and fragments, aside from the 25 kDa TDP-43 fragment. CASA induction reverts also this phenotype (Crippa et al., 2016a; Cicardi et al., 2018). It is unclear whether autophagy defects play a major role in the accumulation of these TDP-43-related aberrant species and this may underline differences in the type of toxicity exerted by these MNDs proteins. Recent works have shown that TDP-43 inclusions and TDP-43 hyperphosphorylation (typical hallmarks of ALS-motor neurons) are also present in muscles in sALS patients. This discovery raised a question: whether TDP-43 misfolded species could accumulate and exert toxicity in muscle cells. We found that the insoluble TDP-43 fragments also accumulate in muscle C2C12 cells, but their aggregation is reverted by tuning the expression of key components of the CASA complex. Whether the accumulation of these fragments in muscle tissue is causative of muscle atrophy is yet to be elucidated (Cicardi et al., 2018).

Also, the C9orf72 DPRs degradation is mediated by UPS and autophagy, even with different behavior of the five DPRs, since only the polyGP is efficiently degraded by the UPS (Cristofani et al., 2018). PolyGP is also degraded *via* autophagy that is able to efficiently remove polyPA, polyGR, and polyGA (Cristofani et al., 2018). Conversely, only the polyPR seems to be resistant to both degradative systems in basal condition. The reasons for these differences are still unclear, but CASA activation prevents the accumulation of all five DPRs (Cristofani et al., 2018), as will be described below.

Collectively, these data suggest that several MND-associated misfolded proteins can be cleared by the UPS system, possibly in a monomeric state. It is expected that UPS overwhelming will result in an accumulation of these species that, once concentrated in specific subcellular compartments (liquid-liquid, aggresomes, etc.), may reversibly aggregate. This mechanism might protect from misfolded protein toxicity since these species are sequestered, limiting their potential toxicity. If the accumulation persists, the aggregates may mature to more stable and potentially toxic species, and thus must be removed using alternative strategies by the cells.

Alteration of Autophagy and CASA in SBMA and ALS

Alteration of autophagy has been reported in animal and cell models of SBMA (Montie and Merry, 2009; Yu et al., 2011; Doi et al., 2013; Rusmini et al., 2013, 2015, 2019; Chua et al.,

2014; Cortes et al., 2014b; Thellung et al., 2018; Cicardi et al., 2019) and ALS (Kabuta et al., 2006; Morimoto et al., 2007; Li et al., 2008; Wang et al., 2012; Crippa et al., 2013; Xiao et al., 2015; Evans and Holzbaur, 2019; Nguyen et al., 2019). Despite this, the complexity of the autophagic pathway makes it difficult to fully understand which level of this multistep process is affected by the presence of misfolded proteins. It is evident from experimental data that autophagy activation has a beneficial role in disease since pharmacological or genetic induction of autophagy ameliorates disease phenotype (e.g., delaying disease onset, slowing down its progression or ameliorating motor behavior; Montie et al., 2009; Wang et al., 2012; Castillo et al., 2013; Kim et al., 2013; Tohnai et al., 2014; Zhang et al., 2014; Giorgetti et al., 2015; Li et al., 2015; Perera et al., 2018; Wang Y. et al., 2018; Rusmini et al., 2019). Unfortunately, not all studies agree with these observations (Zhang et al., 2011). By focusing on CASA, which has already been mentioned above, it must be noted that HSP70 chaperones and others require the assistance of co-chaperones, like the member of the NEF family (Kampinga and Craig, 2010), which includes the BAGs (Takayama and Reed, 2001). Cells utilize different BAGs to route misfolded proteins either to the UPS or to autophagy (**Figure 1**). BAG1 associates to HSP70 and CHIP to route misfolded proteins to the UPS, while BAG3, in association with HSPB8, interacts with HSP70/CHIP to route misfolded proteins to autophagy (**Figure 2**). This allows to select which pathway has to be followed by misfolded proteins to be efficiently cleared from cells; the perturbation of this equilibrium may result in misfolded proteins accumulation (Cristofani et al., 2017, 2019; Rusmini et al., 2017). The importance of the CASA complex in cell protection against proteotoxicity is underlined by the fact that mutations in the genes coding for almost all components of the CASA complex have been associated with human diseases. Indeed, mutations in HSPB8 cause diseases of motoneurons and/or muscle cells [Charcot-Marie-Tooth (CMT) type 2L disease, hereditary distal motor neuropathy type II (dHMN-II), or distal myopathy; Fontaine et al., 2006; Irobi et al., 2010; Ghaoui et al., 2016; Al-Tahan et al., 2019]. Mutations in BAG3 are causative of dilated cardiomyopathy (Arimura et al., 2011), muscular dystrophy (Selcen et al., 2009), giant axonal neuropathy, and late-onset axonal CMT neuropathy (Jaffer et al., 2012; Shy et al., 2018). Interestingly, three BAG3 mutations involve the Pro209 residue (Pro209Leu, Pro209Ser, Pro209Glu), which falls in one of the two HSPB8-interacting Ile-Pro-Val (IPV) motifs. These Pro209 mutants still retain the ability to bind to all CASA members but they impair HSP70 client processing, and they accumulate at the aggresome preventing target protein degradation and sequestering CASA members (Meister-Broekema et al., 2018; Adriaenssens et al., 2020). Mutation in STUB1/CHIP have been found in Gordon Holmes syndrome (multisystemic neurodegeneration; Hayer et al., 2017) and more recently in SCA48 (Genis et al., 2018), and a destabilized CHIP (linked to six different variants) is present in SCA16 (Pakdaman et al., 2017; Kanack et al., 2018); also, a missense mutation in the CHIP-ubiquitin ligase domain was reported as the cause of a form of spinocerebellar autosomal recessive 16 (SCAR16; Shi et al., 2013, 2018). Mutations of the SQSTM1/p62, which recognizes the CHIP-ubiquitinated cargo inside the CASA complex (some authors include it as a member of this complex), are responsible for fALS (Fecto et al., 2011; Teyssou et al., 2013). Of note, it has been suggested that in skeletal muscle, DNAJB6 of the DNAJ/Hsp40 family (HSP70 co-chaperones) suppresses aggregation of misfolded proteins involved in NDs (Hageman et al., 2010) and participates to the formation of the CASA complex (Sarparanta et al., 2012). Interestingly, a mutation in *DNAJB6* causes Limb-girdle muscular dystrophies (LGMDs), characterized by aggregates of DNAJB6 sequestering CASA complex proteins (Sandell et al., 2016).

The CASA complex is involved in mutant SOD1-associated fALS (Crippa et al., 2013). Indeed, mutant SOD1 induces a robust autophagic response both in the spinal cord and in muscle. BAG1, BAG3, HSPB8, LC3, and SQSTM1/p62 are significantly upregulated in mutant SOD1 transgenic ALS mice at the symptomatic stage (16 weeks). Notably, the autophagic response is much higher in muscle than in spinal cord, supporting the absence of high molecular weight insoluble species of mutant SOD1 in muscle; this also suggests that the toxicity exerted by mutant SOD1 in muscle cells is probably not related to the classical mechanism of intracellular protein aggregation (Galbiati et al., 2012, 2014). Interestingly, an analysis performed in SBMA knock-in mouse model revealed that the CASA complex is highly upregulated in skeletal muscle after disease onset, while no variations were observed in the spinal cord. In fact, HSPB8 and BAG3 mRNA and protein levels are increased in SBMA mice at the symptomatic stage compared to control, as well as the co-chaperone BAG1, involved in routing misfolded proteins to UPS. The increased BAG3 to BAG1 ratio suggested that autophagy is the main proteolytic pathway activated in muscle tissue during SBMA progression and CASA complex is involved in reducing ARpolyQ toxicity in skeletal muscle, which is a primary site of SBMA pathogenesis (Rusmini et al., 2015). HSPB8 seems to be a limiting factor for the CASA complex (Crippa et al., 2010a,b). HSPB8 overexpression rescues from protein accumulation and aggregation of mutant SOD1 and TDP-43 in cell models of ALS (Crippa et al., 2010a,b), while its silencing has opposite effects favoring misfolded proteins accumulation in motor neurons (Crippa et al., 2010a,b). Overlapping data were obtained with other misfolded proteins implicated in Alzheimer's disease, Parkinson's disease, a form of spinal cerebellar ataxia, (SCA3), SBMA, fALS, and FTD. In fact, HSPB8 enhances the autophagy clearance of beta-amyloid, alpha-synuclein (α-syn), the polyQ proteins huntingtin, ataxin-3, and ARpolyQ, as well as all five DPRs from the *C9orf72* mRNA (Chávez Zobel et al., 2003; Wilhelmus et al., 2006; Carra et al., 2008a,b; Crippa et al., 2010b, 2016a; Bruinsma et al., 2011; Seidel et al., 2012; Rusmini et al., 2013, 2016; Giorgetti et al., 2015; Cicardi et al., 2018), while HSPB8 downregulation has the opposite effects (Crippa et al., 2010b, 2016a,b; Rusmini et al., 2013; Cristofani et al., 2017).

Since HSPB8 may be a limiting factor of CASA complex activity and its overexpression is sufficient to restore autophagy, it is clear that this protein represents a valid

therapeutic target for these NDs. It has been demonstrated that HSPB8 expression is induced by estrogens and other selective estrogen receptor modulators (SERMs; Sun et al., 2007; Piccolella et al., 2014, 2017; Meister-Broekema et al., 2018), and this could help to explain why gender differences occur in the appearance of several NDs (Vegeto et al., 2020). Recently, we set up a high throughput screening (HTS) using a reporter luciferase gene under the transcriptional control of the human HSPB8 promoter. With this system, we found that colchicine [a Food and Drug Administration (FDA)- and European Medicine Agency (EMA)-approved drug] stimulates HSPB8 expression and enhances the autophagy clearance of the insoluble TDP-43 species (Crippa et al., 2016a) in models of ALS. The drug is presently in phase II clinical trial for ALS (Mandrioli et al., 2019). Other HSPB8 inducers are some disaccharides, like trehalose, melibiose, or lactulose (Rusmini et al., 2013, 2019; Giorgetti et al., 2015). Trehalose has been tested in mouse models of Huntington's disease, ALS, Parkinson's disease, Alzheimer's disease, succinate semialdehyde dehydrogenase deficiency, and oculopharyngeal muscular dystrophy, and found to be capable of ameliorating disease course and symptomatology (Tanaka et al., 2004; Davies et al., 2006; Rodriguez-Navarro et al., 2010; Perucho et al., 2012; Schaeffer and Goedert, 2012; Castillo et al., 2013; Du et al., 2013; Sarkar et al., 2014; Zhang et al., 2014; He et al., 2016). The mechanism of action of trehalose and its analogs, melibiose and lactulose, was recently uncovered. These disaccharides induce transient lysosomal permeabilization and possibly calcium release from lysosomes. These events trigger the Transcription Factor EB (TFEB) pathway, mediated by the calcium-dependent phosphatase PPP3/calcineurin, which dephosphorylates TFEB. Trehalose-activated TFEB migrates into the nucleus where it acts on CLEAR responsive elements to enhance the expression of genes controlling autophagy and lysosomal biogenesis. With this mechanism trehalose/TFEB-mediated activation of autophagy promotes the clearance of damaged lysosomes through lysophagy, but in parallel exerts neuroprotection by promoting the degradation of mutant and misfolded proteins from neurons (Rusmini et al., 2019).

Both colchicine and trehalose also induce BAG3 expression (Lei et al., 2015; Crippa et al., 2016b), indicating that these compounds may act *via* a general potentiation of CASA. Other drugs have been found able to stimulate BAG3 expression (e.g., proteasome inhibitors, TNF-related apoptosis-inducing ligand, fludarabine, cytosine arabinoside, and etoposide) but unfortunately, these drugs are used in chemotherapy with relevant side effects, and are thus not suitable for NDs (Romano et al., 2003; Chiappetta et al., 2007; Rapino et al., 2014). However, they might serve as molecule templates for the development of safer and better tolerated derivatives.

Alteration of CMA in SBMA and ALS

Nothing is known so far about the involvement of CMA in the degradation of ARpolyQ in SBMA. Instead, recent data suggest that CMA may play a role in NDs, including ALS (Ormeño et al., 2020). Indeed, CMA is essential in Parkinson's disease where its dysregulation modifies the onset or progression of the disease (Arias and Cuervo, 2011; Cuervo, 2011; Alfaro et al., 2018; Kaushik and Cuervo, 2018). Alpha-synuclein protein, leucine-rich repeat kinase 2 (LRRK2), Parkinson disease protein 7 (PARK7), and DJ-1, as well as myocyte-specific enhancer factor 2D protein (MEF2D), which are dysregulated or mutated in Parkinson's disease, are CMA substrates (Vogiatzi et al., 2008; Yang et al., 2009; Arias and Cuervo, 2011; Cuervo, 2011; Orenstein et al., 2013; Murphy et al., 2015; Alfaro et al., 2018; Kaushik and Cuervo, 2018). Alzheimer's disease is also associated with CMA since the beta-amyloid peptide (Aβ), the microtubule-associated protein Tau or the Regulator of calcineurin 1 (RCAN1) are involved in Alzheimer's disease and are dysregulated when CMA is altered (Liu et al., 2009; Wang et al., 2009, 2010; Park et al., 2016). CMA also plays a role in Huntington's disease (Koga et al., 2011; Qi et al., 2012), and mutant huntingtin can sequester LAMP2A and HSC70, two major players of CMA (Alfaro et al., 2018).

In ALS, CMA has been involved in TDP-43 metabolism (Huang et al., 2014). These data were recently corroborated by a study of the group of Budini et al., who pointed out that also TDP-43 can be a CMA substrate (Ormeño et al., 2020). This study started from the observation that TDP-43 contains a KFERQ-like domain, the consensus sequence that allows the interaction with HSC70 (Huang et al., 2014); mutation in this domain blocks the ubiquitin-dependent binding of TDP-43 with HSC70. Other authors have shown that LAMP2A downregulation induces the intracellular accumulation of the ALS-associated TDP-43 fragments of 35 and 25 kDa (Huang et al., 2014), and TDP-43 can also be forced to be degraded *via* CMA (Tamaki et al., 2018). Ormeño et al. (2020) showed *in vitro* that a recombinant form of TDP-43 is processed by isolated rat liver lysosomes, a process that can be reduced by competition with the GAPDH protein, a typical CMA substrate. Endogenous TDP-43 accumulates in CMA^+ lysosomes of the brain (Ormeño et al., 2020). By using an artificial TDP-43 aggregate-prone protein, Ormeño et al. (2020) demonstrated its interaction with HSC70 and LAMP2A, which causes an upregulation of CMA activity and lysosomal damage. These data open up the question of how CMA is involved not only in the few fALS forms associated with mutations of TDP-43, but also in the vast majority of sALS forms characterized by an intense mislocalization and accumulation of TDP-43 in affected neuronal and motor neuronal cells of ALS patients. By analyzing the two CMA regulators (LAMP2A and HSC70) in peripheral blood mononuclear cells (PBMCs) of ALS patients, it was found that the levels of the lysosome receptor LAMP2A were similar in control and ALS PBMCs, while the expression of the cytosolic chaperone HSC70 was found reduced, but the total amount of insoluble TDP-43 protein was found increased and accompanied by aberrant intracellular localization (Arosio et al., 2020). In parallel, HSC70 downregulation in human neuroblastoma cells correlates with the increased accumulation of the TDP-43 protein (Arosio et al., 2020). These data are in line with experimental observation showing that HSC70 is reduced in motor neurons of TDP-43-based ALS fly models, as well as in iPSCs C9orf72 models differentiated

to motor neurons (Coyne et al., 2017). In addition to these observations, in ALS-PBMCs, the ratio of the expression levels and protein of BAG1 and BAG3, which determines the equilibrium between proteasome and autophagy (including CASA), was also found altered (Arosio et al., 2020). Thus, even if CMA is not directly affected in ALS-PBMCs, the reduction of the CMA regulator HSC70 may be involved in ALS pathogenesis.

Alteration of UPR-ERAD in SBMA and ALS

As mentioned above, proteasome and autophagy work together in response to proteotoxic stimuli. Both pathways are also involved downstream in the UPR occurring in the ER. The UPR, activated in the ER lumen, generates a transient translational inhibition along with the induction of chaperones and the stimulation of the degradative pathways. Misfolding proteins here are identified by BiP/GRP78 (an HSP70), which assists the ERAD, also activating PERK and IRE1. The PERK receptor attenuates translation in response to UPR involving oligomerization and autophosphorylation of PERK with eIF2alpha phosphorylation. In parallel, the transcription factor XBP1 activated by alternative splicing induces UPR stress genes, while cleaved activated ATF6 exits the ER and moves to the nucleus to stimulate other UPR genes. Collectively, this restores ER activities: in SBMA, an ARpolyQ N-terminal fragment activates ER stress-inducible promoter *via* ATF6, IRE1, and PERK. Indeed, ARpolyQ toxicity is enhanced by ATF6 blockage and reverted by ATF6 overexpression. Also, stimulation of PERK increases ARpolyQ toxicity (Thomas et al., 2005). Thus, ARpolyQ induces UPR, while UPR stimulation is protective in SBMA (Rusmini et al., 2016). In a SBMA knock-in mouse model, the downregulation of transcription factor C/EBP homologous protein (CHOP), involved in UPR-ERAD, worsened muscle atrophy (Yu et al., 2011). In parallel, in mouse embryonic stem cells (ESCs), ARpolyQ inclusions sequester CHIP and BiP/GRP78, inducing ER stress and apoptosis. UPR was found with the induction of the ER chaperones BiP/GRP78 and GRP94 and the stress markers ATF6, phosphorylated PERK, GADD153/CHOP, and spliced XBP-1. Notably, BiP/GRP78 overexpression reverted this phenotype, while BiP/GRP78 downregulation had the opposite effect (Yang et al., 2013). As mentioned above, ER stress and Ca^{2+} homeostasis are tightly connected. In mouse model of SBMA (Sopher et al., 2004; Malik et al., 2011; Montague et al., 2014) alteration of Ca^{2+} homeostasis has been reported in embryonic motor neurons in response to ER stress causing ER-stress-induced apoptosis (Montague et al., 2014). ARpolyQ specifically depleted ER Ca^{2+} levels and the store-operated Ca^{2+} influx (Hetz and Mollereau, 2014; Tadic et al., 2014), possibly *via* the reduction of the sarcoendoplasmic reticulum Ca^{2+} ATPases (SERCA) 2b pump activity. This pump allows ER Ca^{2+} re-uptake (Foradori and Handa, 2008), and its dysregulation activates caspase 12 (Montague et al., 2014). Thus, ER stress is also involved in SBMA pathogenesis and may represent an additional therapeutic target for this disease.

ER morphology alterations occur both in ALS patients and ALS mouse models (Dal Canto and Gurney, 1995; Dal Canto, 1995; Oyanagi et al., 2008; Lautenschlaeger et al., 2012), possibly because of protein accumulation in ER causing ER stress (Sasaki, 2010). Also, the Golgi apparatus is affected in ALS (Fujita et al., 2000; Stieber et al., 2000). Mutant SOD1 inclusions in ER are positive for BiP/GRP78 and calnexin (Wate et al., 2005; Kikuchi et al., 2006), while some ER chaperones are upregulated in ALS patients and mice (Atkin et al., 2006). Notably, mutant SOD1 specifically binds Derlin-1, which controls the ERAD machinery, and triggers ER stress-induced apoptosis (Nishitoh et al., 2008). ER stress in ALS may also result from altered ER calcium homeostasis (Grosskreutz et al., 2010) or by ER-mitochondria calcium cycle unbalance (Damiano et al., 2006; Grosskreutz et al., 2010; Jaronen et al., 2014). In addition, ATF6, phospho-PERK, and phospho-eIF2α are elevated in ALS mice and cell models (Atkin et al., 2006, 2008; Saxena et al., 2009). In the spinal cord of ALS patients and mice, IRE1 is increased (Atkin et al., 2006, 2008) and its phosphorylated form correlated with spliced XBP1 in ALS mice (Kikuchi et al., 2006). Notably, autophagy is induced in double knockout/transgenic mice with mutant G86R-SOD1 and XBP1 blockage (Hetz et al., 2008; Hetz, 2012; Hetz and Mollereau, 2014), suggesting that autophagy may serve to protect when UPR/ERAD fails. A recent study performed by the group of de Belleroche suggests that at least 40 different target genes, associated with ERAD and regulated by XBP1 or ATF6, are altered in spinal cord specimens from ALS patients; this is paralleled by severe alterations and activation of the IRE1α-XBP1 and ATF6 pathways (Montibeller and de Belleroche, 2018). Among these genes, co-chaperones of the DNAJ family (*DNAJB9* and *DNAJC10*) modulating HSPA5 (BiP/Grp78, which is the only HSP70 in the ER; Kampinga and Bergink, 2016) were increased in this dataset. Both DNAJB9 and DNAJC10 are involved in ERAD (Behnke et al., 2015) and may suppress cell death induced by ER stress (Kurisu et al., 2003). As occurs in SBMA, misfolded proteins also impact ERAD-UPR in ALS, suggesting that similar strategies based on the reinforcement of this pathway can contribute to restore protein homeostasis in affected cells.

CONCLUSIONS

In conclusion, data accumulated over the past 30 years have suggested that specific proteins cause MNDs by triggering aberrant responses in neurons and other cells involved in this group of diseases. The alteration of the PQC system is presently thought to be one of the major factors responsible for both the onset and progression rate of the disease. PQC systems failure could be directly associated with a mutant protein involved in one of the PQC pathways, or indirectly associated with effects caused by the overproduction of misfolded proteins that saturate or impair the PQC system activity. This leads to a reduced PQC potential to maintain the proper cellular homeostasis, especially during cell stresses. Notably, this system is presently considered a potential druggable target, since it provides huge numbers of players with activity that can be pharmacologically or genetically enhanced or modulated. Indeed, several of the

cooperative factors playing a role in the PQC system can be specifically induced or downregulated, allowing the potentiation of a single arm of this defense mechanism. In many cases, the restoration of the proper function of one PQC arm has positive effects on the other arms of the system; they together provide a redundant mechanism capable of efficiently clearing most of the aberrant aggregating proteins, thus reducing cell death. Different approaches aimed to potentiate one or more arms of the PQC system have already been preclinically tested and are under investigation in clinical trials. Hopefully, these approaches will identify new treatments to counteract neurodegeneration in MNDs.

AUTHOR CONTRIBUTIONS

RC, MG, VC, PR, and AP designed and wrote the manuscript and critically discussed all sections of the minireview. In addition, RC prepared the figures. MEC, VF, BT, EC, MC, EM, and MP critically revised the manuscript and the figures. All authors have provided final approval of the version to be published.

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REVIEW
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Insight From Animals Resistant to Prion Diseases: Deciphering the Genotype – Morphotype – Phenotype Code for the Prion Protein

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Prion diseases are a group of neurodegenerative diseases endemic in humans and several ruminants caused by the misfolding of native prion protein (PrP) into pathological conformations. Experimental work and the mad-cow epidemic of the 1980s exposed a wide spectrum of animal susceptibility to prion diseases, including a few highly resistant animals: horses, rabbits, pigs, and dogs/canids. The variable susceptibility to disease offers a unique opportunity to uncover the mechanisms governing PrP misfolding, neurotoxicity, and transmission. Previous work indicates that PrP-intrinsic differences (sequence) are the main contributors to disease susceptibility. Several residues have been cited as critical for encoding PrP conformational stability in prion-resistant animals, including D/E159 in dog, S167 in horse, and S174 in rabbit and pig PrP (all according to human numbering). These amino acids alter PrP properties in a variety of assays, but we still do not clearly understand the structural correlates of PrP toxicity. Additional insight can be extracted from comparative structural studies, followed by molecular dynamics simulations of selected mutations, and testing in manipulable animal models. Our working hypothesis is that protective amino acids generate more compact and stable structures in a C-terminal subdomain of the PrP globular domain. We will explore this idea in this review and identify subdomains within the globular domain that may hold the key to unravel how conformational stability and disease susceptibility are encoded in PrP.

Keywords: prion disease, prion protein, disease susceptibility, animal models, protein structure, structure–function, amino acid substitution

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INTRODUCTION

The prion protein (PrP) is a 230 amino acid-long secreted glycoprotein anchored to the extracellular aspect of the membrane by a C-terminal glycosylphosphatidylinositol (GPI) anchor. PrP is highly expressed in brain neurons, but mice devoid of PrP (*Prnp0/0*) are viable and only show mild behavioral perturbations (Bueler et al., 1992; Tobler et al., 1996; Schmitz et al., 2014). PrP plays a central role in prion diseases in humans, a heterogeneous class of neurodegenerative disorders with cognitive, movement, or sleep manifestations (Zlotnik and Rennie, 1965; Mathiason, 2017). Prion diseases or transmissible spongiform encephalopathies (TSE) are fairly unique because they

can present with sporadic, genetic, and infectious etiologies. The transmissible agent is proposed to be a proteinaceous molecule highly resistant to denaturing agents that contains misfolded conformations of PrP (resistant PrP [PrP^{res}] or scrapie PrP [PrP^{Sc}]) and other factors (Prusiner, 1998). Although rare, these are devastating diseases with an aggressive course of a few months from clinical manifestation and no effective treatments.

Another unique feature of these disorders is that they have direct pathological correlates in other animals, but are limited to some mammals. The common pathological features of human and animal TSEs are vacuolar (spongiform) degeneration of the brain and accumulation of misfolded, aggregated PrP conformations (Colby and Prusiner, 2011; Kraus et al., 2013; Scheckel and Aguzzi, 2018). Other than humans, some ruminants are the only mammals known to develop endemic prion diseases: scrapie in sheep and goat, and chronic wasting disease (CWD) in deer and moose (Mathiason, 2017). Several mammals proved susceptible to TSE in the laboratory in early transmission experiments following the discovery of kuru in the 1950s: chimpanzee, mouse, hamsters, bank vole (Chandler and Fisher, 1963; Zlotnik and Rennie, 1963, 1965; Chandler, 1971). Interestingly, one lab animal proved resistant to prions: the rabbit (Gibbs and Gajdusek, 1973; Barlow and Rennie, 1976). Decades later, a large-scale unintended experiment resulted in the zoonotic transmission of prions to cattle, which developed a new disease, bovine spongiform encephalopathy (BSE), or mad-cow disease, that was traced back to the consumption of scrapie-contaminated bone meal (Wells et al., 1987; Wilesmith, 1988; Winter et al., 1989). Shortly thereafter many domestic and zoo animals exposed to BSE-contaminated prions - felines, mustelids, and others – developed new prion diseases, expanding the TSE universe (Kirkwood and Cunningham, 1994; Sigurdson and Miller, 2003). Remarkably, a few animals exposed to the same contaminated feed seemed to be resistant to prion diseases: horse, domestic dog and other canids (wolf, coyote), and pigs (Kirkwood and Cunningham, 1994). The unfortunate spread of TSEs revealed a heterogeneous landscape of susceptibility to prion diseases, with some animals suffering endemic disease, others easily infected in the lab, and others showing a relatively high or complete resistance to infection. This scenario presents a unique opportunity to uncover the molecular mechanisms mediating disease transmission and neurodegeneration.

ANIMALS RESISTANT TO PRION DISEASE: INTRINSIC vs. EXTRINSIC FACTORS

Prion diseases affect humans and other mammals, but not birds or other vertebrates. The fact that distant mammals like humans and ungulates develop sporadic and infectious forms of TSEs may erroneously suggest that all mammals are equally susceptible to TSEs. Early studies on TSEs assumed that these conditions were caused by some type of small virus. The susceptibility to these new infectious agents was tested by inoculating brain homogenates from affected humans and sheep into several animals, including apes, New- and Old-World monkeys, rats, guinea pigs, cats, and rabbits (Gibbs and Gajdusek, 1973; Barlow and Rennie, 1976). These animals received intracerebral injections from kuru or Creutzfeldt-Jacob disease (CJD) human extracts or with ME7 scrapie from sheep. This intracerebral route accelerated the disease course and shortened the incubation time, maximizing the possibility of identifying positives by clinical or pathological analysis. The first set of experiments showed that human prions can be transmitted to apes, monkeys, and cats, but were unsuccessful in rabbits (Gibbs and Gajdusek, 1973). A few years later, ME7 scrapie was inoculated into the brains of rats, guinea pigs, and rabbits. Whereas rats demonstrated a pattern of disease progression similar to that seen in mice, guinea pigs and rabbits showed no disease, although guinea pigs showed low level prion replication (Barlow and Rennie, 1976). These two studies showed that prions did not replicate in rabbits and the infectious agent was quickly disposed of in rabbits.

Now that TSEs are well-characterized pathologically and molecularly, including the key role of PrP as the disease-causing agent, it is clear that few mammals suffer prion diseases under natural conditions, suggesting underlying differences in their susceptibility to TSE. The most significant animals lacking TSE are rabbits, horses, dogs, and pigs. Of these four, only one is a laboratory animal, the rabbit, and the rest are large and have long lifespans, making them unsuitable for experimental work. At this time, we only have positive or negative evidence for animals directly exposed to BSE during the mad-cow epidemics in the United Kingdom. Thus, animals not present in zoos nor fed the same contaminated bone meal could theoretically be susceptible or resistant within the known spectrum. Why is it important to understand the risk of TSE transmission for other animals? Because many domestic and wild animals are part of the human food chain and even those not eaten by humans may shed prions in the environment that could be transmitted to other animals. Additionally, studying animals naturally susceptible or resistant to TSE can contribute to decipher the molecular mechanisms governing the pathogenesis of TSEs. Despite the clear challenges of studying non-model animals, modern technologies provide the ability to study the structure and biological properties of PrP from many animals. These experiments can help better understand the mechanisms responsible for the spectrum of TSE susceptibility among mammals. Lastly, studying variations in a protein for many animals allows us to infer the evolutionary processes shaping PrP: natural selection or neutral genetic drift with unintended consequences in post-reproductive age.

The different animal susceptibility to TSEs led to two hypotheses to explain its underlying mechanisms: intrinsic factors (sequence-structure) vs. extrinsic factors (cellular milieu, cofactors) regulate TSE susceptibility. These two mechanisms cannot be separated when highly resistant animals (rabbits) are infected with prions. But PrP from these animals can be studied *in vitro*, *ex vivo*, and *in vivo* in the cellular context of susceptible animals and, vice versa, susceptible PrP can be studied in the cell context of resistant animals. Rabbit epithelial RK13 cells with low or undetectable levels of endogenous PrP transfected with ovine PrP (Rov9) result in high susceptibility to infection by sheep prions (Vilette et al., 2001). Moreover, transgenic rabbit expressing ovine

PrP are susceptible to disease (Sarradin et al., 2015). This is evidence, along with other persuasive experiments, that rabbit cells do not express co-factors that inhibit prion replication, further supporting the idea that PrP conversion is mainly encoded by intrinsic factors. Thus, natural variations in the PrP sequence affecting its conformational dynamics is the likely mechanism underlying disease susceptibility. Hence, identifying the key residues conferring conformational stability/instability to the globular domain of PrP will contribute to uncover the molecular mechanisms mediating PrP neurotoxicity and disease susceptibility. However important, sequence is not the only intrinsic determinant of PrP aggregation dynamics and toxicity. Recent studies reveal a key contribution of post-translational modifications, particularly glycosylation, on the efficiency of PrP aggregation, fidelity of strain replication, neurotropism, and toxicity (reviewed in Baskakov et al., 2018). PrP contains two facultative *N*-glycosylation sites leading to three co-existing isoforms. Changes in PrP sequence can modulate the accessibility to the glycosylation sites whereas the ratio of the three resulting isoforms can restrict their possible quaternary assemblies due to the steric limitations imposed by the large glycans. Although glycosylation is a critical determinant of several properties of prions, we will focus this review on the impact of sequence variations in PrP conformational dynamics and toxicity.

PrP 3D STRUCTURE: NMR AND X-RAY CRYSTALLOGRAPHY

The interest on PrP as an infectious agent responsible for incurable neurodegenerative disorders led to significant work to uncover its structure. The classic method for resolving the 3D structure of biomolecules is X-ray crystallography due to its high spatial resolution, but the limiting step is the crystallization of the purified molecule. The first resolution of the PrP structure was obtained by NMR (nuclear magnetic resonance) using solution full-length and C-terminal domain from both mouse and Syrian hamster PrP (Riek et al., 1996, 1997; James et al., 1997; Liu et al., 1999). These studies revealed an unstructured N-terminal fragment (23–124) and a C-terminal globular domain (125–228) containing three α-helices and a short antiparallel β-sheet between helices 1 and 2. Throughout the paper we identify amino acids based on the human PrP sequence to avoid confusion (**Figure 1A**). The structure of the globular domain is highly conserved between mice and hamsters, and later structures for human, sheep, bovine PrP and others showed that this basic organization is highly conserved (**Figure 1B**) (Zahn et al., 2000; Knaus et al., 2001). Contemporaneous studies established that disease transmission and neurodegeneration were associated with a loss of helical content and an increase of β-sheet content (β-state) (Telling et al., 1995). These studies assigned a key role to a 3D domain in the C-terminal region consisting of the β2-α2 loop and distal helix 3, the C-terminal 3D (CT3D) domain (**Figure 1B**). Remarkably, this is a region of high sequence variability (**Figure 1A**) and the proposed binding site of a hypothetical protein (Protein-X) necessary for PrP conversion (Telling et al., 1995; Kaneko et al., 1997).

The structure of PrP from several resistant animals (dog, horse, rabbit, pig) were resolved during the 2010s to uncover how PrP toxicity and replication ability are encoded (Lysek et al., 2005; Khan et al., 2010; Perez et al., 2010; Wen et al., 2010). Essentially, these studies showed that the basic structure of PrP from these animals is very similar to that of animals susceptible to TSEs (**Figure 1C**). The globular domains contain three helices, but the β-sheet seems shorter in dog, horse, and rabbit PrP (**Figure 1D**). Detailed analysis of these structures identified a significant surface charge change in dog PrP due to the presence of D159 instead of the common N159 (Lysek et al., 2005) and increased organization of the β2-α2 loop in both horse and rabbit PrP (**Figure 1E**) (Khan et al., 2010; Perez et al., 2010; Wen et al., 2010). The stability of the β2-α2 loop is accompanied by increased contacts with the distal portion of helix 3 in horse and rabbit PrP, resulting in more stabilizing interactions within the CT3D domain (Khan et al., 2010; Perez et al., 2010; Wen et al., 2010). The X-ray crystal of rabbit PrP revealed a new feature not observed by NMR: a helix-capping domain at the start of helix 2 created by a double hydrogen bond (H-bond) between N171 and S174 (Khan et al., 2010). This structure is not observed in rabbit PrP-S174N, supporting the relevance of this finding. Interestingly, pig PrP carries the same S174 residue, whereas most mammals carry N174, suggesting a shared stabilizing domain with rabbit PrP. However, the NMR structure for pig PrP does not show the helix-capping domain (Lysek et al., 2005), making this domain uncertain. The new structural features in rabbit PrP offer a unique opportunity to examine genotype – morphotype – phenotype correlations, but the structural and phenotypic impact of other rabbit-specific substitutions needs to be considered as well. Intriguingly, compared to rabbit PrP, the reported structural changes in dog and horse PrP are subtle, suggesting that either small changes are sufficient or several subtle changes cooperate to stabilize PrP^C and delay or prevent disease.

CONFORMATIONAL DYNAMICS OF PrP PROBED BY MOLECULAR MODELING

Thanks to its relatively small size and to the abundance of experimental structures, the globular domain of PrP has been the subject of profuse computational studies. The Daggett group showed that at low pH, the β-strand structures extend beyond the short domain to include the N-terminus and almost the entire β2-α2 loop (Alonso et al., 2001). Later, the same group (DeMarco and Daggett, 2004) built a protofibril model consistent with experimental data in which the extended β-sheet formed the interface between PrP monomers. Simulations performed by the Thirumalai group (Dima and Thirumalai, 2004) identified two main regions of instability in the protein: the second half of helix 2 and the C-terminus of helix 3 (residues 213–223). Other works focused instead on the fibril-forming capabilities of shorter peptide sequences of PrP (Kuwata et al., 2003; Collu et al., 2018; Zheng et al., 2018b) and on the stability of individual secondary structure domains (Camilloni et al., 2012). Simulations of the mouse PrP showed that the pathogenic mutation D178N associated with inherited

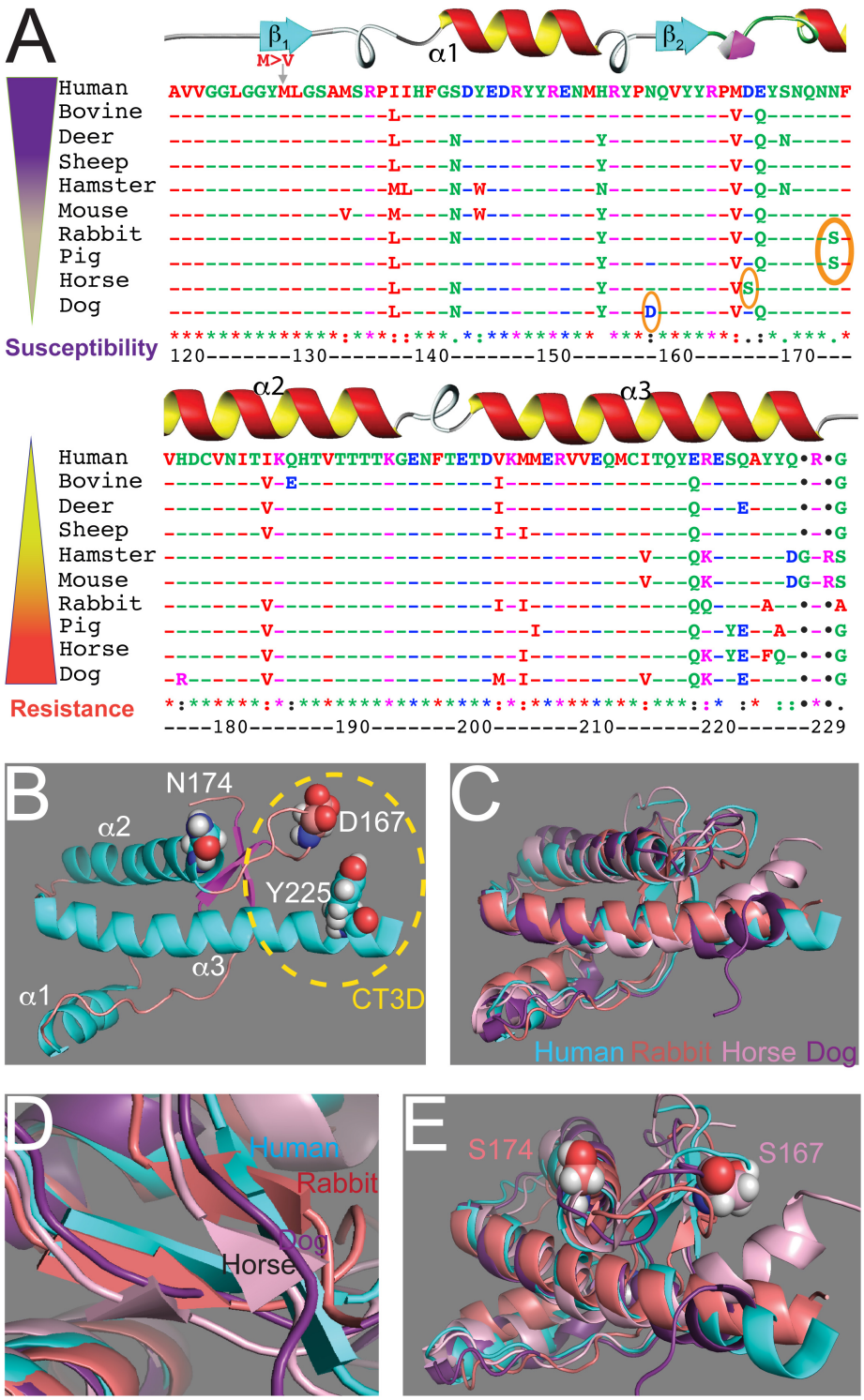

FIGURE 1 | Sequence and structural alignments for the globular domains of mammalian PrP. **(A)** Sequence alignment for the structured domain of human, bovine, deer, sheep, Syrian hamster, mouse, rabbit, horse, dog, and pig PrP. *Amino acid numbering refers to human PrP*. The secondary structure is overlaid on top. The three key residues considered to be protective are circled. On the left, two inverse gradients indicate disease susceptibility and resistance. **(B)** Structure of the globular domain of human PrP (1qm2). Three amino acids are highlighted: D167, N174, and Y225. **(C–E)** Alignment of the globular domain of human (cyan), rabbit (salmon, 2fj3), horse (pink, 2ku4), and dog (purple, 1xyk) PrP. **(C)** Notice the overall similarity and the small differences around the CT3D domain. **(D)** The β-sheet content is smaller in rabbit and horse PrP and it disappears in dog PrP. **(E)** Detail of the CT3D domain showing the position of D167 in horse PrP and S174 in rabbit PrP. This figure contains published materials collated for illustrative purposes.

CJD or fatal familial insomnia lowered the stability of the β-sheet (Barducci et al., 2005) and another group attempted to map the unfolding of the entire structured domain of PrP (Chamachi and Chakrabarty, 2017; Singh et al., 2017). Collectively, these contributions indicate that the PrP domain encompassing the N-terminus, the β-sheet, the β2-α2 loop, and the α-helix 3 C-terminus are regions of instability that may be prone to unfolding and to protein aggregation. The Caflisch group (Huang and Caflisch, 2015a,b) underlined the critical role of Y169, a highly conserved residue in mammalian PrP, in stabilizing the 3_{10} helical turn involving residues 165–168 within the β2-α2 loop. These findings were confirmed by Parrinello (Caldarulo et al., 2017) employing advanced sampling techniques.

Although most works focused on the protein, others pointed out the relevance of the water structure and dynamics in the stability of PrP (De Simone et al., 2005) and in the formation of oligomers (Thirumalai et al., 2012). Other research has shown that the correct modeling of electrostatic interactions (Zuegg and Gready, 1999) is important to correctly describe the protein's stability, which is also affected by histidine protonation states (Langella et al., 2004) and by pH (Campos et al., 2010). An endeavor to systematically characterize the differences in the secondary structure and in the flexibility of the protein for a large number of PrP species through molecular dynamics simulations was attempted by Zhang (2018). These studies identified a salt bridge between R164 and D178 (Zhang and Wang, 2016) as important for the β2-α2 loop stability.

PURIFIED AND *IN VITRO* MODELS OF RESISTANT PRP MISFOLDING

Until the early 2000s, the evidence accumulated from highly resistant animals consisted of laboratory experiments with rabbits and negative epidemiological data for non-model animals: horses, dogs and other canids, and pigs. Modern biological and biochemical techniques enable the study of the intrinsic properties of PrP from these animals: *in vitro*, *ex vivo*, and transgenic animals. At the most basic level (sequence), the key question is determining which amino acid changes are responsible for altering PrP biological properties. At a deeper level, the idea is to understand how specific amino acid changes impact PrP conformation and dynamics by either enhancing or suppressing PrP misfolding, propagation, and toxicity. This key insight will shed light on the elusive genotype – morphotype – phenotype correlation.

The focus on rabbit PrP brought forth a number of studies to uncover the mechanisms mediating resistance to TSE. Though over a decade apart, two studies emerged as highly similar in goals (Vorberg et al., 2003; Eraña et al., 2017). These studies identified 22 amino acid differences between full-length rabbit and mouse PrP, and set out to determine which residues impact prion transmissibility by complementary approaches. These studies offer a unique opportunity to analyze the impact of complementary amino acid substitutions in either the mouse or rabbit PrP backbones. First, the Priola group used a scrapie-infected mouse neuroblastoma (Sc^+-MNB) cell model persistently infected with a mouse-adapted scrapie prion (RML) (Vorberg et al., 2003). The Sc^+-MNB cells express WT and recombinant mouse PrP (recPrP) carrying rabbit PrP-specific amino acid changes. The assay consisted on determining which substitutions inhibited the ability of recPrP from replicating prions. More recently, the Castilla group used recombinant rabbit PrP carrying mouse-specific changes (Eraña et al., 2017). Their approach was to use the powerful cell-free PMCA (protein misfolding cyclic amplification) technique (Saborio et al., 2001) to determine which mouse-specific substitutions enabled conversion of the naturally resistant rabbit PrP.

Of the 22 differences between mouse and rabbit PrP (**Figure 2**), six reside in the unstructured N-terminal domain. Previous studies have shown that residues 1–94 do not play any significant roles in TSE resistance (Rogers et al., 1993; Fischer et al., 1996; Lawson et al., 2001). Of the 16 remaining, Priola introduced seven substitutions into mouse PrP, whereas Castilla introduced the same seven plus an additional four located in the C-terminal region into rabbit PrP (**Figure 2**) (Vorberg et al., 2003; Eraña et al., 2017). Of notice, both studies skipped the five amino acid differences at the end of the C-terminal (**Figure 2**) in part because their proximity to the GPI anchor makes them less likely to contribute to the mechanism of misfolding. For Priola, four of the seven residue replacements inhibited mouse PrP^{res}. For Castilla, 8 of the 11 replacements enabled conversion of rabbit PrP. These complementary studies agreed on two effective replacements: N/G100 and L/M109. The studies describe conflicting results for five residues, where the changes affected one assay but not the other: N/S108, M/L138, Y/W145, N/S174 and I/V215 (Vorberg et al., 2003; Eraña et al., 2017). The Castilla group further tested the eight protective residues in rabbit PrP with a new prion strain, but only three permitted conversion this time: N/S108, M/L109, and V/I203. Moreover, this group validated their observations by introducing 11 amino acid replacements from rabbit on mouse PrP. Unexpectedly, all 11 changes decreased the propagation activity below WT, with N100G completely inhibiting propagation (Eraña et al., 2017). Interestingly, both groups created the corresponding double mutants N/S108 and M/L109 in the mouse and rabbit backbones. Both experiments were negative, reversing the positive effect of the single mutants. These studies highlight the asymmetric impact of the reverse amino acid substitutions on rabbit and mouse PrP, and lack of cooperativity of effective substitutions, underscoring our limited understanding of the rules governing PrP conformational dynamics.

Since the discovery that TSEs are caused by the conversion of endogenous PrP from a primarily α-helical state into a mostly β-sheet state (β-state) (Swietnicki et al., 1997; Baskakov et al., 2001), extensive work has been invested to study the misfolding propensity of PrP from different animals. Chakrabartty's group developed a method to test the structural state of recombinant PrP using circular dichroism to measure the propensity of PrP from various organisms to populate the β-state under favorable conditions for PrP misfolding like low pH (Khan et al., 2010). This group found that rabbit PrP had a much lower tendency to

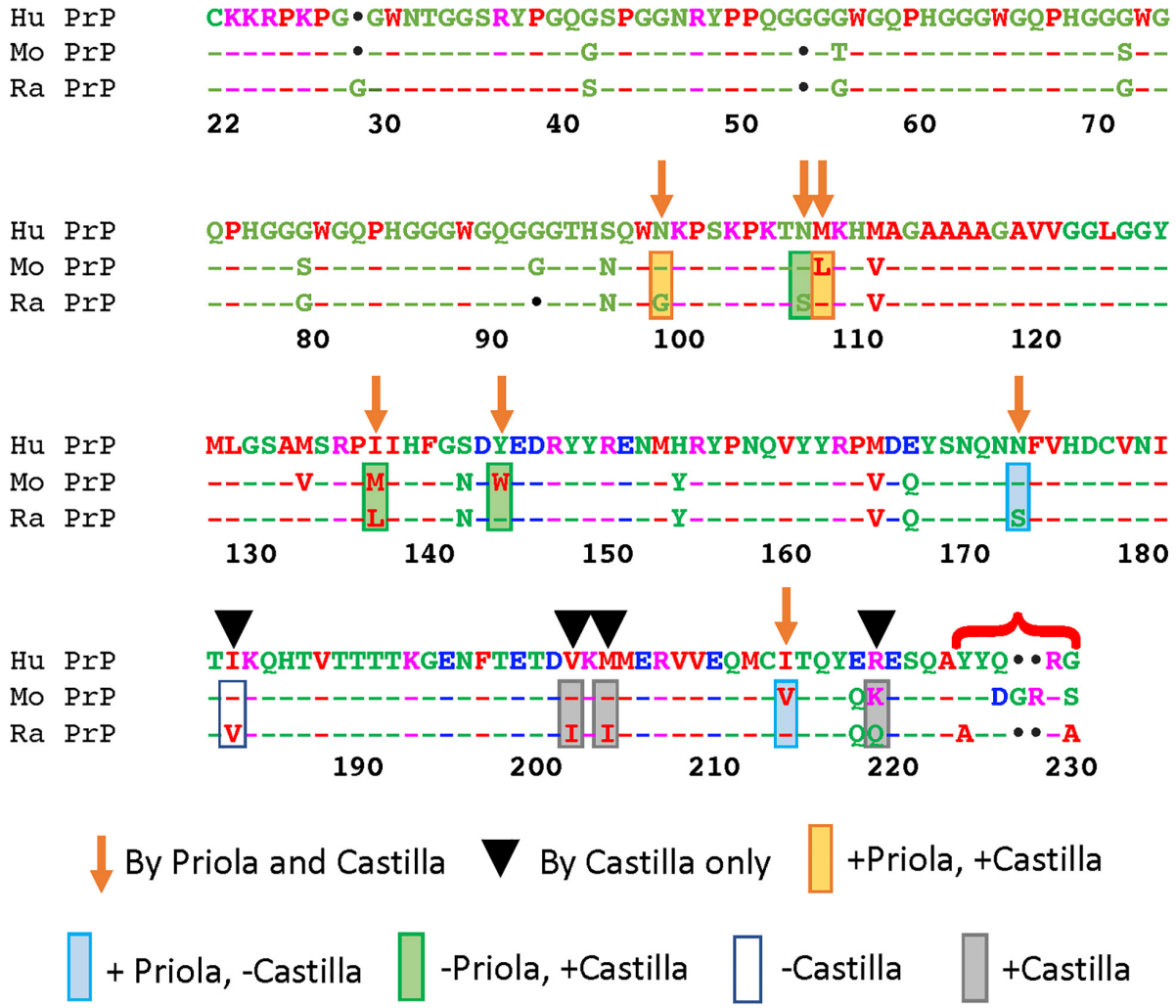

FIGURE 2 | Functional consequences of complementary amino substitutions in mouse and rabbit PrP. Sequence alignment of human, mouse, and rabbit PrP highlighting the amino acids that were mutated in mouse PrP (arrows) (Vorberg et al., 2003) and those introduced in rabbit (arrows and arrowheads) (Eraña et al., 2017). The bracket indicates differences in the C-terminal that were not modified in these studies. Orange boxes: amino acid changes that worked in both assays. Blue boxes: amino acid changes that worked in mouse but not in rabbit. Green boxes: amino acid changes that worked in rabbit but not in mouse. Gray boxes: amino acid changes that worked in rabbit. Blank box: amino acid change that did not work in rabbit. This figure contains published materials collated for illustrative purposes.

occupy the β-state than did hamster and mice PrP. Horse and dog PrP were even less likely to occupy the β-state than rabbit PrP, with dog PrP being the most resistant (Khan et al., 2010). This resistance changed for rabbit PrP-S174N, which correlates with the structural studies pointing to the key role of S174 in the formation of the helix-capping domain. These results add evidence to the stability of PrP from these animals and their resistance to misfolding critical for prion diseases.

HIGHLY-RESISTANT TO PRION DISEASE, NOT IMPERVIOUS

With the use of PMCA, researchers have explored the limits of the resistance of rabbit, horse, and dog PrP. Recent experiments showed that rabbit PrP can replicate *in vitro* and that rabbits are susceptible to TSEs under highly favorable conditions. Using PMCA with various prion strains as seeds, they showed that rabbit PrP can be converted into PrP^{res} (Chianini et al., 2012). Even unseeded rabbit PrP^{C} was able to generate PK-resistant PrP through several rounds of PMCA, indicative of spontaneous conversion. It took at least three rounds of seeded PMCA to accomplish conversion of rabbit PrP; in the case of unseeded PrP, it took 13 rounds. When the novel rabbit PrP^{res} was inoculated into three rabbits expressing WT PrP, one of the rabbits developed prion disease. Upon second passage from this positive animal, two out of 10 rabbits developed disease and accumulated PrP^{res} (Chianini et al., 2012). These experiments demonstrate that rabbits are not absolutely resistant to prions, but they also underscore the difficulty of transmitting prions

under highly favorable experimental conditions that are unlikely to be found in nature. Overall, compared to other animals, rabbits demonstrate a high degree of resistance to prion disease, a quality that is mainly encoded in its unique sequence and structure.

Pigs have received less attention than other resistant animals for two main reasons: they are large animals and no unique structural features were identified in PrP by NMR despite sharing the S174 substitution with rabbit PrP. Prior to the spread of the mad cow epidemic to other animals, experimental study of pigs was of little concern. Still, when they were challenged with a strain of Kuru, they remained resistant (Dawson et al., 1994; Jahns et al., 2006). When BSE came onto the scene, the concerns for transmission grew because pigs are not only a human food source but are also routinely fed scraps, including scrapie-infected scraps at that time. Parenteral inoculations of pigs with BSE successfully infected pigs, though they remained resistant to the oral route of infection (Dawson et al., 1990; Ryder et al., 2000; Wells et al., 2003; Konold et al., 2009). Despite confirmed experimental transmission of TSE, there remains no documented natural cases. Yet, because pigs for consumption are typically slaughtered at around 6 months of age, development of clinical TSE in pigs is unlikely because of the long-incubation times. However, some pigs are aged and used for breeding and still there are no observations of natural occurrence in that population, supporting a natural resistance to prions.

TRANSGENIC ANIMALS EXPRESSING HIGHLY-RESISTANT PrP

The *in vitro* and *in vivo* studies reviewed above support the hypothesis that PrP conversion and TSE susceptibility are primarily or solely dependent on intrinsic factors encoding the conformational stability of PrP. However informative, *in vitro* studies indirectly infer the pathological consequences of the substitutions introduced on PrP. The next level is to study PrP from TSE-resistant animals and their protective residues in flexible animal models. The two genetic models used in the study of PrP are fruit flies (*Drosophila melanogaster*) and mice. Fruit flies are a powerful research tool that lacks endogenous PrP, which is not conserved in invertebrates, providing an ideal environment to study PrP behavior in a naïve system. Moreover, generating transgenic flies is economic and fast, enabling the generation of multiple transgenes (Pfeiffer et al., 2010; Mohr et al., 2014). Flies have a small but complex tripartite brain homologous to the mammalian brain (Reichert, 2005) that contains 10^5 neurons, an estimated 10^6 synapses, and well characterized centers that control sophisticated behaviors, providing a robust system for studying neurodegenerative diseases (Simpson, 2009; Bellen et al., 2010; Zheng et al., 2018a). Mice are more time-consuming and expensive but offer the best context for observing the disease process in a mammal. Additionally, mice lacking PrP (*Prnp0/0*) provide an empty genetic background to express heterologous PrP from other animals (Bueler et al., 1992).

Transgenic flies expressing PrP from Syrian hamster, mouse, or sheep show progressive neurodegenerative changes accompanied by PrP misfolding into relevant toxic conformations (Gavin et al., 2006; Fernandez-Funez et al., 2009; Thackray et al., 2012). Additionally, flies demonstrate high sensitivity to natural PrP sequence, resulting in a gradient of toxicity: hamster > mouse > rabbit (Fernandez-Funez et al., 2010). In support of this observation, we described a similar gradient in PrP misfolding and aggregation as demonstrated by sucrose gradient. In a follow-up study, we generated transgenic flies expressing WT rabbit, dog, and horse PrP. Neither PrP caused neurodegeneration confirming the hypothesized conformational stability of these disease-resistant PrPs (Sanchez-Garcia and Fernandez-Funez, 2018). On the other extreme of this continuum of PrP toxicity, expression of human PrP in flies leads to extremely high toxicity, including a new eye phenotype (Fernandez-Funez et al., 2017). Overall, these experiments support that the spectrum of PrP toxicity is due to changes in PrP sequence.

The Castilla group first used PMCA to produce PrP^{res} from rabbit and dog PrP with BSE as seed, resulting in "adapted" prion strains after several rounds: BSE-rabbit PrP^{res} and BSE-dog PrP^{res}. These strains were then inoculated into bovine-PrP mice. First passage showed similar incubation time as cattle BSE-inoculated mice. Upon second passage, BSE-dog PrP^{res} and BSE-rabbit PrP^{res} had significantly reduced incubation times. In a different test against mice expressing human PrP, only BSE-rabbit established infection upon first passage (Vidal et al., 2013a). Often when a prion infects a new species, the incubation period is long and infectivity is low (Vidal et al., 2013b). However, once adapted to the new host, the incubation period is reduced and infectivity increases. This concern was addressed above by incorporating a second passage. The Castilla group further tested the susceptibility of rabbit PrP in transgenic mice. Intracerebral inoculation of transgenic mice overexpressing rabbit PrP with misfolded PrP seeds achieved 100% transmission (Vidal et al., 2015), a more efficient result than in wild type rabbits likely due to the overexpression of rabbit PrP. In an *in vitro* assay against several prion strains to demonstrate strain-specific susceptibility, rabbit PrP converted to PrP^{Sc} in all cases (Vidal et al., 2015). The story was different, though, in an *in vivo* test of those same strains against rabbit PrP in transgenic mice. Some of the strains induced infection to varying degrees, but others did not, including CWD and SSBP/1 (Vidal et al., 2015).

Another group generated transgenic mice expressing WT horse PrP (tgEq) at twice the levels that it is expressed in the horse brain (Bian et al., 2017). These mice were intracerebrally inoculated with various prion strains and observed for development of prion disease. Out of ten different strains, only strain SSBP/1 caused disease in tgEq PrP mice, albeit in only two of the six mice. Interestingly, upon second passage with brain homogenates from the infected mice into tgEq PrP mice, there was neither disease nor accumulation of PrP^{Sc} (Bian et al., 2017). However, transgenic mice expressing ovine PrP did develop symptoms when inoculated with the same homogenate from SSBP/1-tgEq PrP mice. In another part of this study, PMCA was used to convert horse PrP^{C} into PrP^{Sc}, but when it was inoculated into tgEq PrP mice, the mice did not develop pathology. This report strongly supports the stability of horse PrP and high resistance to misfolding and

TSE infection, although a small number of cases were positive. Considering that these tgEq PrP are inoculated by a non-natural route (intracerebral) that directly exposes PrP^C to the inoculum, horse PrP demonstrates high resistance to conversion described in *in vitro* (Khan et al., 2010) and *Drosophila* models (Sanchez-Garcia and Fernandez-Funez, 2018).

As with horse and rabbit PrP, researchers forced dog PrP^C to convert to PrP^{Sc}. To test the stability of dog PrP, they once again utilized the powerful PMCA to induce the misfolding of dog PrP in a cell-free system (Fernandez-Borges et al., 2017). Dog PrP proved to be incredibly resistant, but after several rounds of PMCA, they were able to generate PrP^{Sc} only with two of the six prion strains used as seeds: classical BSE and sheep BSE.

Pigs are critical in the human food chain and understanding the risk of contracting prion diseases from other animals is a key economic issue. Generation of transgenic mice expressing pig PrP allowed for quicker experimental studies of pig PrP outside of its normal host. The Torres group introduced pig PrP into *Prnp0/0* mice (poTg001) in which pig PrP was expressed fourfold in the mouse brain than in the pig brain (Castilla et al., 2004). The poTg001 model was used in successive studies challenging the pig PrP with an array of prion strains (Castilla et al., 2004; Espinosa et al., 2009, 2020). Only classic BSE and some BSE-derived strains successfully infected the mice, but with a low attack rate. The studies report conflicting results on the infectivity of one scrapie strain: in one study it caused conversion – at a low attack rate – while in a later study it did not cause PrP conversion. Similar results were observed in *in vivo* experiments using PMCA with the panel of prion strains (Espinosa et al., 2020).

PROTECTIVE ACTIVITY OF UNIQUE RESIDUES FROM RESISTANT ANIMALS

After reviewing the evidence supporting the different susceptibility of animals to TSEs, the main hypothesis is that amino acid changes on PrP encode its conformational dynamics and propensity to cause disease. The next step is to determine *how* specific amino acids induce conformational changes that result in high vs. low toxicity in animal models. Fortunately, many PrP sequences and structures are available, providing unparalleled resources for addressing this critical question. The most N-terminal domain (residues 1–94) does not appear to drive PrP conversion (Rogers et al., 1993; Fischer et al., 1996; Lawson et al., 2001). Therefore, we will focus mainly on the globular domain. Although the overall PrP sequence and structure are highly conserved in mammals, several changes are evident (**Figures 1A,C**). Sequence alignments identify 10–15 amino acid changes between human and other animals only in the globular domain. Of these, many are conservative changes not expected to largely impact the 3D conformation. The alignment shows relatively high variation in the β2-α2 loop (residues 166–170) and in the C-terminus of helix 3 (residues 219–229). Interestingly, these two regions are spatially close and several contacts are confirmed by structural studies, indicating that the β2-α2 loop and the C-terminus of helix 3 form a 3D domain. The variability in the distal helix 3 has been traditionally assumed to have less impact on the globular domain because of its proximity to the GPI anchor, which may underestimate the role of these variants. Combining sequence and structural data revealed three prominent amino acid changes likely to encode PrP conformational stability: D159 in dog, S167 in horse, and S174 in rabbit and pig. Next, we will review the work done in transgenic animals to examine the consequences of altering these residues.

DOG PrP – D159

Most animals, including humans, have an asparagine (N) at position 159, but dogs and other members of the Canidae family (wolf, fox, coyote) have either an aspartic acid (D) or a glutamic acid (E) at this position. Two mustelids, the wolverine and the marten, also share this acidic residue at 159 (Stewart et al., 2012; Fernandez-Borges et al., 2017). The NMR structure of dog PrP shows a conserved globular domain with subtle changes. The short β-sheet seems to be gone by NMR and the surface charge is more negative around D159 due to its negative charge and the increased solvent exposure (Lysek et al., 2005). This change in the surface charge may affect the interactions with other proteins like chaperones. Utilizing the versatile fruit fly, we generated transgenic flies expressing mouse PrP with the N159D substitution (Sanchez-Garcia et al., 2016). Expression of mouse PrP-N159D showed improved locomotor performance compared to flies expressing mouse PrP-WT, a change that correlated with lower levels of pathogenic conformations of PrP (Sanchez-Garcia et al., 2016). We recently conducted the reverse experiment by introducing the D159N substitution in dog PrP in flies. Flies expressing dog PrP-WT show no toxicity in behavioral and anatomical assays. However, flies expressing dog PrP-D159N exhibit progressive locomotor disfunction and degeneration of brain neurons (Sanchez-Garcia and Fernandez-Funez, 2018). The consistent results in the reverse substitutions in mouse and dog PrP strongly support the critical role of D159 in encoding higher PrP stability.

In continuation of their *in vitro* studies, the Castilla group generated transgenic mice expressing mouse PrP carrying the N159D substitution (Fernandez-Borges et al., 2017). When these mice were inoculated with prions, they showed no clinical signs nor accumulated PrP^{Sc}. In a follow-up study, they found that co-expression of mouse PrP-N159D with mouse PrP-WT significantly increased survival, indicating that N159D has a dominant-negative effect on the ability of PrP-WT to misfold and induce disease (Otero et al., 2018). They next generated mice expressing bank vole PrP carrying the same N159D substitution with a polymorphism at residue 109 (I109) that further increases the propensity of bank vole PrP to spontaneously misfold (Otero et al., 2019). Challenging the mice expressing bank vole PrP-N159D with two prion strains resulted in a 100% attack rate, but the disease onset was significantly delayed compared to mice expressing a control bank vole PrP construct. A more recent study compared transgenic mice expressing dog PrP-WT and -D159N (Vidal et al., 2020). Of note, dog PrP-WT carried the E159 polymorphism instead of the typical D159. Mice expressing dog

PrP-WT[E159] and -D159N were challenged with various prion strains: no prion strain propagated in dog PrP-WT[E159], but did so in the D159N model. Overall, experiments conducted in transgenic animals confirmed the protective activity of D/E159 in the context of dog, mouse, or bank vole PrP.

HORSE PrP – S167

The NMR structure of horse PrP revealed increased structural definition of the β2-α2 loop compared to mouse PrP (Perez et al., 2010). The NMR structure of mouse PrP carrying the horse substitutions D167S, Q168E, and N173K, along with double mutants, showed that D167S conferred the β2-α2 loop a well-defined structure and increased the long-distance interactions between the loop and helix 3, similar to those observed in horse PrP (Perez et al., 2010). Transgenic mice expressing high levels of mouse PrP-D167S developed spontaneous spongiform pathology, neurologic disease, and PrP^{Sc} deposits, whereas a control line overexpressing mouse PrP-WT (tga20) did not (Sigurdson et al., 2011). In contrast, mice expressing moderate levels of mouse PrP-D167S were similar to control mice, except for a lower fraction of insoluble PrP. The reverse experiment was conducted in transgenic flies expressing horse PrP-S167D. In contrast to flies expressing horse PrP-WT, expression of horse PrP-S167D showed aggressive locomotor dysfunction and degeneration of brain neurons (Sanchez-Garcia and Fernandez-Funez, 2018). Remarkably, horse PrP-S167D induced a form of neurodegeneration not seen before with other PrP in which the cell bodies swelled up causing a significant enlargement of the neuronal clusters (Sanchez-Garcia and Fernandez-Funez, 2018). The same cellular phenotype was described in flies expressing Aβ42 and linked to aberrant autophagy (Ling et al., 2009). So far, these limited studies show conflicting results regarding the protective activity of S167.

RABBIT PrP – S174

S174 has been proposed as a key residue mediating the stability of rabbit PrP based on structural, biochemical, cell culture, and cell-free evidence (Vorberg et al., 2003; Khan et al., 2010; Wen et al., 2010). The S174N substitution in rabbit PrP disrupted stability, changed overall surface charge, and made the β2-α2 loop less rigid and more flexible. Following on these studies, we generated transgenic flies expressing rabbit PrP-S174N expecting to find an increase in toxicity. However, flies expressing rabbit PrP-S174N in brain neurons exhibited no changes in locomotion nor in brain architecture (Sanchez-Garcia and Fernandez-Funez, 2018). These experiments were conducted in parallel with the D159N and S167D mutants in dog and horse PrP, respectively, that increased PrP toxicity. This puzzling result suggests that this single amino acid change has a limited impact on PrP structural dynamics *in vivo* inconsistent impact of S/N174 on *in vitro* PrP replication were reviewed above (**Figure 2**) (Vorberg et al., 2003; Eraña et al., 2017). Taken together, these results suggest that multiple amino acids contribute to the high stability of rabbit PrP and that no single amino acid is sufficient to induce dramatic changes on PrP. These observations still leave open the question about how is the conformational stability of rabbit PrP encoded in its sequence and structure. It is likely that multiple amino acids in the β2-α2 loop and helix 3 cooperate to increase the stability of the CT3D domain, complicating the experimental demonstration.

LESSONS LEARNED: HOW IS DISEASE SUSCEPTIBILITY ENCODED IN PrP STRUCTURE?

The evidence discussed so far agrees that rabbit, horse, dog, and pig PrP are comparatively more resistant to conversion and less toxic than PrP from naturally susceptible animals. However, under the right experimental conditions, rabbit, horse, dog, and pig PrP can convert into PrP^{Sc} and cause disease *in vitro* and *in vivo*. *In vitro* systems like PMCA provide a highly flexible environment that accelerates conversion by exploring high energy states perhaps facilitated by the inhibition of protective proteostasis mechanisms in cell-free systems. Conversion of rabbit PrP in PMCA resulted in prions that infected wild type rabbits, albeit with low efficiency, demonstrating the power of PMCA to lower the species and strain barriers (Fernandez-Borges et al., 2012). Mice expressing horse PrP seem to be permissive for spontaneous prionopathy, whereas mice expressing horse, rabbit, dog, and pig PrP were permissive to transmission of some prion strains. However, this experimental work does not mean that rabbits, dogs, horses, and pigs are naturally susceptible to TSE: these animals do not develop spontaneous disease. In fact, the experiments showed that these four PrPs are harder to convert than PrP from naturally susceptible animals like mouse or bank vole. Generating misfolded rabbit PrP required several rounds of PMCA (Vidal et al., 2015); horse PrP needed 14 rounds of PMCA (Bian et al., 2017) and dog PrP required a modified protocol for PMCA because 10 rounds of standard PMCA drew negative results (Fernandez-Borges et al., 2017). A previous report described that using more than one round of PMCA exceeds a natural test of the transmission barrier (Fernandez-Borges et al., 2009). Furthermore, the transmission studies were conducted by intracerebral inoculation, an unnatural and favorable mode of infection that skips the less efficient peripheral replication. It must be noted that transgenic mouse models overexpress rabbit, horse, and pig PrP^{C} multiple times the levels of endogenous PrP (Vidal et al., 2015), a condition that may further increase the likelihood of the mice developing spontaneous TSE. Overall, these studies demonstrate four important things. (1) The PrP from rabbits, dogs, horses, and pigs exhibit a remarkable resistance to conversion. (2) PMCA is a powerful tool to overcome the conversion-barrier. (3) PrP from these resistant animals can indeed misfold, in other words, they are not impervious to conversion. Thus, it is unlikely that any animal is completely resistant to forming PrP^{Sc} given the evolutionary constraints on PrP. (4) Although each of these four highly resistant PrPs show low susceptibility to misfolding, horse and dog PrP seem to be more resistant than rabbit and

pig PrP under similar conditions, identifying two targets for further research.

The impact of D/E159, S167, and S174 on PrP structure has been tested in multiple systems (**Table 1**); of these three, S174 is the only proposed to generate a new structural feature. Unfortunately, the evidence for the protective activity of S174 is mixed: in some experiments, S/N174 modifications had the expected effect and in others, it did not (Vorberg et al., 2003; Khan et al., 2010; Eraña et al., 2017; Sanchez-Garcia and Fernandez-Funez, 2018). In contrast, D/E159 and S167 are associated with subtle structural changes and the experimental evidence is generally supportive for D/E159 being protective but is mixed for S167. The contradictory results observed in different assays add some confusion to the role of these three residues but are not completely surprising. The hypothesis is that amino acids with a determining protective role on PrP structure will show opposite effects when eliminated from a resistant PrP or introduced into a susceptible PrP. The lack of consistent results for the S/N174 manipulations suggest a relatively modest role, possibly in combination with other rabbit-specific amino acids. The strong effects observed on structural/biochemistry experiments are consistent with the ability to modify protein structures when the environment is controlled chemically (pH, urea). The most comparable experiments altering multiple amino acids in mouse and rabbit PrP are indeed quite different, one using cell culture and the other a cell-free system (PMCA) (Vorberg et al., 2003; Eraña et al., 2017). The cell-free system is expected to provide more flexibility by being subject to incubations and sonications, whereas living cells provide a more restrictive environment regulated by homeostatic mechanism. Rabbit PrP-S174N expressed in flies behaves the same as WT, supporting the buffering effect of the cellular environment (extrinsic factors) in response to mild structural changes. Overall, these results hint at a gradient of effects on PrP stability, with S174 having mild effects, followed by S167, and D/E159 having the most drastic protective effects despite the lack of novel structural perturbations.

To further test the *in vivo* protective activity of D159, S167, and S174, we recently generated transgenic flies expressing human PrP carrying these three variants: N159D, D167S, and N174S alone and in combinations. Human PrP is highly toxic in flies, making it an ideal model for testing putative protective residues (Fernandez-Funez et al., 2017). These studies will provide further evidence for the ability of these three key amino acids to suppress the toxicity of the highly toxic

TABLE 1 | Summary of experimental manipulations of candidate protective residues 159, 167, and 174.

Residue	Assay/model	Result	Source
D/E/N159	NMR/X-ray of dog PrP	Change in area surface charge – more negative	Lysek et al., 2005
D/E/N159	Fruit flies expressing mouse PrP-N159D	N159D reduced the amount of PrPSc-like conformations	Sanchez-Garcia et al., 2016
D/E/N159	Fruit flies expressing dog PrP-D159N	D159N caused degeneration of brain neurons	Sanchez-Garcia and Fernandez-Funez, 2018
D/E/N159	Mice expressing mouse PrP-N159D	N159D was protective against TSE	Fernandez-Borges et al., 2017
D/E/N159	Mice co-expressing mouse PrP-WT and mouse PrP-N159D	Co-expression increased survival	Otero et al., 2018
D/E/N159	Mice expressing bank vole PrP-N159D	Disease onset was delayed	Otero et al., 2019
D/E/N159	Mice expressing dog PrP-D159N vs. D159E (WT polymorphism)	Prion strains were able to propagate in dog PrP-D159N, but not in dog PrP-WT	Vidal et al., 2020
S/D167	NMR on mouse PrP-D167S	β2-α2 loop more organized than mouse PrP-WT	Perez et al., 2010
S/D167	Fruit flies expressing horse PrP-S167D	S167D caused degeneration of brain neurons	Sanchez-Garcia and Fernandez-Funez, 2018
S/D167	Mice expressing mouse PrP-D167S	High expression increased susceptibility to TSE. Moderate expression did not	Sigurdson et al., 2011
S/N174	X-ray crystallography on rabbit PrP-WT vs. rabbit PrP-S174N	Helix-capping domain form interactions of residues N171 and S174, eliminated by S174N	Khan et al., 2010
S/N174	Rabbit PrP-WT vs. rabbit PrP-S174N treated with urea	Rabbit PrP-S174N populated the beta-state, but rabbit PrP-WT did not	Khan et al., 2010
S/N174	Cell culture of mouse PrP-N174S	N174S was protective against TSE	Vorberg et al., 2003
S/N174	PMCA with recombinant rabbit PrP-S174N	S174S did not protect against TSE	Eraña et al., 2017
S/N174	PMCA with recombinant mouse PrP-N174S	N174S was protective against TSE	Eraña et al., 2017
S/N174	Fruit flies expressing rabbit PrP-S174N	S174N did not cause neurodegeneration	Sanchez-Garcia and Fernandez-Funez, 2018

human PrP. As we wait for those experiments, it is important to consider the possibility that, based on the above results, single amino acid changes are unlikely to introduce substantial conformational changes in human PrP. Thus, it is likely that even subtle changes in the size of the side chains can impact the ability of the β2-α2 loop to interact closely with helix 3 and stabilize the CT3D domain. Therefore, double or triple mutations should be introduced in coordinated combinations, i.e., they need to come from the same animal to add the cooperative effect of multiple small changes. Alternatively, it is possible that we are still missing the key residues encoding PrP conformation and toxicity, particularly those encoding the high toxicity of human PrP. There are several areas of sequence and structure divergence between human PrP and rabbit, dog, and horse PrP in the CT3D domain (**Figure 1A**). It is likely that substitutions in the β2-α2 loop and helix 3 work cooperatively to increase the stability of this domain, but the distal helix 3 has received little attention in experimental studies thus far. For instance, the tammar wallaby carries A225,A226 in helix 3 instead of the common Y225,Y226 in human PrP and other animals, two substitutions that replace bulky tyrosines with the smaller alanines, possibly promoting closer contacts between helix 3 and the β2-α2 loop (Christen et al., 2009). However, little is known at this time about the susceptibility of wallabies to TSEs. Interestingly, rabbit, horse, and pig PrP carry relevant substitutions in these two positions that could contribute to the stability of the entire CT3D domain in combination with S167 and S174. We are currently examining the protective effect of introducing Y225A in distal helix 3 into human PrP, a first look at the role of distal helix 3 in the conformation of human PrP.

DISCUSSION

The natural variability in susceptibility to TSE and the exceptional resources to analyze PrP sequence and structure provide unique opportunities to decipher the code governing PrP misfolding, toxicity, and infectivity. The basic hypothesis is that amino acid changes between susceptible and resistant animals are responsible for the different conformational stability of PrP. Sequence alignments and structural studies identified three residues proposed to mediate the stability of dog (D159), horse (S167), and rabbit and pig (S174) PrP. The studies reviewed here show partial support for the protective activity of these three residues. Although differences between assays should be taken into consideration, the results discussed so far suggest that D159, S167, and S174 alone are insufficient to explain the different structural properties of highly resistant PrPs. Beyond these three residues, two systematic studies demonstrated the contribution of other resides to the differences between mouse and rabbit PrP while disagreeing on the role of S174 (Vorberg et al., 2003; Eraña et al., 2017). Thus, cracking the code of PrP toxicity requires expanding our focus to subtle amino acid differences *in combination* with D159, S167, and S174. Structural studies suggest that PrP stability is partially encoded in a rigid β2-α2 loop and in strong interactions between the loop and distal helix 3, resulting in a compact and stable CT3D domain.

The next efforts should be focused on defining the combinations of amino acids that achieve these structural goals. Generating double, triple, and multiple mutants is time consuming, particularly testing them in animal models. The structural data is crucial to define the best candidates to cooperatively stabilize the CT3D domain, which can be followed by *in silico* predictions of the impact of these combinations in molecular dynamics simulations. Remarkably, the main fold of the globular domain is not affected by point mutations, which instead appear to change the structure and dynamics of protein subdomains. This observation, combined with the small size of PrP, makes molecular dynamics simulations an ideal tool to systematically probe the effects of mutations on the conformational dynamics of PrP (van der Kamp and Daggett, 2011). The work by Daggett (Alonso et al., 2001; DeMarco and Daggett, 2004), Caflisch (Huang and Caflisch, 2015a,b), Parrinello (Caldarulo et al., 2017), and others, has shown that computer simulations can enrich experimental structural information by providing a description of the conformational landscape accessible to a mutant. In addition, provided appropriate sampling of the phase space, simulations can determine the thermodynamic stability of local conformations, the kinetics of their transitions, and validate these data against experimental observables (Caldarulo et al., 2017). Importantly, molecular dynamics simulations provide an atomistic description of the interactions that stabilize or destabilize a certain conformation, which allows to make predictions about the effect of mutations, and to rapidly test these predictions *in silico* (Huang and Caflisch, 2015a,b). Combinations with a significant impact on simulations can then be tested in transgenic animals, starting with fruit flies due to the fast and economic process of determining the toxicity of the mutants.

We still have extensive work to do to crack the complex code regulating PrP toxicity, which seems to involve subtle effects from multiple residues in the globular domain that, combined, result in vastly different morphotypes and phenotypes. Moreover, recent work focused on PrP glycosylation revealed two mechanisms for glycans to impact PrP aggregation, replication, and toxicity (Katorcha et al., 2014; Callender et al., 2020; Makarava et al., 2020). One is steric limitations due to the large size of the glycans. The second one can be even more important: glycans can be terminally sialylated, which adds a significant negative charge that prevents direct stacking of monomers but is compatible with a rotation (Baskakov et al., 2018). Thus, post-translational modifications add significant restrictions to the quaternary structures that misfolded PrP can explore. These restrictions are imposed on top of the tertiary conformations accessible based on the internal dynamics of several sub-domains. Overall, variations in both sequence and glycosylation limit and direct the generation of unique prion strains, which are defined by biochemical properties and phenotype (neurotropism and clinical symptoms). These considerations also help explain how different phenotypes can originate from the same sequence. Whereas there is clear

experimental evidence that the PrP ordered domain adopts a single well-defined structure, the structure of the pathogenic misfolded monomers *and* aggregates may vary (Bessen and Marsh, 1994; Caughey et al., 1998; Safar et al., 1998; Peretz et al., 2002; Cobb and Surewicz, 2009). In other words, different strains can be associated to different structures of the aggregates. Different glycosylation patterns have also been associated to different phenotypes (Cobb and Surewicz, 2009), which have been proposed to arise from intermolecular contacts involving glycans in PrP^{Sc}. In addition, it is plausible that sequences showing high conformational dynamics of the β2-α2 loop and high exposure of hydrophobic residues may be more likely to generate multiple structures of the aggregates and, in turn, multiple strains. In the nucleation-polymerization model (Jarrett and Lansbury, 1993), a PrP sequence characterized by a dynamic loop adopting many conformations may be more prone to provide the right template structure for a variety of aggregates' structures (and strains), whereas a sequence with limited conformational polymorphism may be able to provide the template only for one aggregate's structure, and thus originate only one strain. Ultimately, understanding the code mediating the phenotype – morphotype – phenotype relationship for PrP may guide the design of compounds that stabilize PrP and prevents disease progression. Ultimately, similar rules may apply to prion-like proteins (tau, α-synuclein, Amyloid-β42) responsible for highly prevalent neurodegenerative disorders with a significant impact in society: Alzheimer's and Parkinson's diseases.

AUTHOR CONTRIBUTIONS

PF-F and RM conceived the study. PF-F, RM, and AC conducted the research and wrote and revised the manuscript. All authors contributed to the article and approved the submitted version.

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Secreted Chaperones in Neurodegeneration

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Protein homeostasis, or proteostasis, is a combination of cellular processes that govern protein quality control, namely, protein translation, folding, processing, and degradation. Disruptions in these processes can lead to protein misfolding and aggregation. Proteostatic disruption can lead to cellular changes such as endoplasmic reticulum or oxidative stress; organelle dysfunction; and, if continued, to cell death. A majority of neurodegenerative diseases involve the pathologic aggregation of proteins that subverts normal neuronal function. While prior reviews of neuronal proteostasis in neurodegenerative processes have focused on cytoplasmic chaperones, there is increasing evidence that chaperones secreted both by neurons and other brain cells in the extracellular – including transsynaptic – space play important roles in neuronal proteostasis. In this review, we will introduce various secreted chaperones involved in neurodegeneration. We begin with clusterin and discuss its identification in various protein aggregates, and the use of increased cerebrospinal fluid (CSF) clusterin as a potential biomarker and as a potential therapeutic. Our next secreted chaperone is progranulin; polymorphisms in this gene represent a known genetic risk factor for frontotemporal lobar degeneration, and progranulin overexpression has been found to be effective in reducing Alzheimer's- and Parkinson's-like neurodegenerative phenotypes in mouse models. We move on to BRICHOS domain-containing proteins, a family of proteins containing highly potent anti-amyloidogenic activity; we summarize studies describing the biochemical mechanisms by which recombinant BRICHOS protein might serve as a therapeutic agent. The next section of the review is devoted to the secreted chaperones 7B2 and proSAAS, small neuronal proteins which are packaged together with neuropeptides and released during synaptic activity. Since proteins can be secreted by both classical secretory and non-classical mechanisms, we also review the small heat shock proteins (sHsps) that can be secreted from the cytoplasm to the extracellular environment and provide evidence for their involvement in extracellular proteostasis and neuroprotection. Our goal in this review focusing on extracellular chaperones in neurodegenerative disease is to summarize the most recent literature relating to neurodegeneration for each secreted chaperone; to identify any common mechanisms; and to point out areas of similarity as well as differences between the secreted chaperones identified to date.

Keywords: proteostasis, neurodegeneration, sHsp, clusterin, BRICHOS, 7B2, proSAAS, progranulin

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INTRODUCTION

Protein homeostasis, or proteostasis, is a combination of events that govern protein quality control, namely, protein translation, folding, processing and degradation. Disruptions in these events can lead to protein misfolding and aggregation. Proteostatic disruptions can arise normally as a consequence of normal aging or can be due to genetic mutations and environmental stressors such as heat shock, altered energy demands, or pH changes, and can lead to cellular changes such as ER and/or oxidative stress; organelle dysfunction; and even cell death. Protein misfolding and aggregation are central to several proteinopathies such as neurodegenerative disease, various amyloidosis, cystic fibrosis and sickle cell anemia, among others. Chaperones, ubiquitous proteins dedicated to the management of protein homeostasis, reside in every cellular compartment. For example, a well-studied class of abundant chaperones known as HSPs, are mostly cytoplasmic, are ATP-dependent, and often work in a protein network or in a sequential pathway (reviewed in Hartl et al., 2011; Klaips et al., 2018). Chaperones are responsible for binding nascent protein chains, preventing them from aggregating, and for promoting the formation of secondary and tertiary structures to obtain the stable conformations required for proper function. Chaperones also play a role in regulating active and inactive functional protein states, as well as in downstream protein processing events such as proteolytic cleavage and post-translational modification. Finally, chaperones are involved in unfolding and delivering proteins, working closely with ubiquitin ligase complexes and proteases, to degradative pathways.

Players in Secretory Pathway Proteostasis

Typically, proteins destined for secretion pass through the membrane-bound secretory pathway of the cell. Upon or during translation, the pre-proteins are channeled into the ER with the help of the signal sequence. Following chaperone-assisted folding, proteins are then subject to further processing which includes posttranslational modifications, and/or proteolytic maturation cleavages. Protein modification occurs throughout the secretory pathway, across the ER, ER-Golgi intermediate compartment, Golgi complex, *trans*-Golgi network and finally, secretory vesicles. Once secreted into the extracellular matrix, proteins can be taken up by the neighboring cells through endocytosis and eventually subjected to lysosomal degradation. Throughout this process, chaperones resident within these various compartments aid in maintaining client protein stability and localization.

The extremely high protein concentrations present in neurons and endocrine cells, which possess both regulated (stimulus-dependent) and constitutive (basal) secretory pathways, are conducive to homo- and heterotypic aggregation, and ER-resident chaperones, which assist the retro-translocation of aggregated proteins to the cytosol, play an important role in preventing misfolded proteins from causing a bottleneck in the secretory pathway during stress. An example of an ER stress-related chaperone is the ER-localized protein BiP, a member of the HSP70 family. BiP chaperones ER-based proteins under normal conditions; however, in the presence of ER stress, it binds and retro-translocates misfolded proteins from the ER to the cytoplasm for proteasomal degradation as a member of the ERAD machinery (Pobre et al., 2019). In another example, evidence has emerged linking the UPR to the regulation of extracellular proteostasis. In response to ER stress, the chaperone HSP40 (a.k.a ERdj3) is co-secreted from the cell with unstable protein clients, which may assist in prevention of cytotoxic aggregation (Genereux and Wiseman, 2015b; Genereux et al., 2015a).

Cytoplasmic Versus Secreted Chaperones: The Challenge of the Extracellular Space

The challenges faced by chaperones secreted from the cell are markedly different than those of cytoplasmic chaperones. While cytoplasmic chaperones deal with initial folding of nascent chains of secretory proteins (and unfolding during degradation), additional chaperone assistance is required during transport through the secretory pathway – not only for stabilizing protein folds, but also for proper localization of proteins in the secretory pathway compartments, as well as aiding their secretion into the extracellular matrix.

The extracellular space presents unique conditions such as low pH, low protein density and low ATP availability that affects client-chaperone interaction. The low pH of the secretory pathway and the extracellular space directly affect protein stability, necessitating chaperone action even under normal conditions. While the concentration of chaperones such as clusterin in the CSF is 30–50 nM [(Přikrylová Vranová et al., 2016); see section "Clusterin" below], the CSF concentration of other chaperones such as 7B2 is even lower, in the low nanomolar range (Iguchi et al., 1987a), rendering client interaction even more challenging. It may be speculated that the release of neuronally synthesized chaperones directly into the synaptic space alleviates this scarcity. Another challenge relates to the fact that cytoplasmic chaperones are mostly ATP-dependent, enabling them to bind hydrophobic regions of nascent proteins, and release folded proteins with ADP conversion. Owing to low extracellular ATP, secreted chaperones appear to act more as "holdases" rather than "foldases," binding to exposed hydrophobic regions of misfolded proteins,

Abbreviations: Abeta, amyloid beta; ACD, alpha crystallin domain; ACTH, adrenocorticotropic hormone; AD, Alzheimer's disease; ALS, amyotrophic lateral sclerosis; APP, amyloid precursor protein; BiP, binding immunoglobulin protein; CAA, cerebral amyloid angiopathy; CRED, chaperone/receptor-mediated extracellular protein degradation; CSF, cerebrospinal fluid; ER, endoplasmic reticulum; ERAD, ER-associated degradation; FAD, familial Alzheimer's disease; FBD, familial British dementia; FDD, familial Danish dementia; FTD, frontotemporal dementia; GRN, granulin; GWAS, genome-wide association studies; HSPGs, heparan-sulfated proteoglycans; HSPs, heat shock proteins; IDR, intrinsically disordered regions; NRBP1, nuclear receptor binding protein 1; PD, Parkinson's disease; PP1, protein phosphatase 1; proPC2, pro-prohormone convertase 2; proSP-C, prosurfactant protein C; sHSP, small heat shock proteins; SNP, single nucleotide polymorphism; SPARC, secreted protein acidic and rich in cysteine; TDP-43, TAR DNA-binding protein 43; TFEB, transcription factor EB; UPR, unfolded protein response.

thereby preventing protein self-assemblies and aggregation (reviewed in Wyatt et al., 2013) (Hartl et al., 2011; Klaips et al., 2018). Secreted chaperones may also deliver proteins to cellular membrane receptors that aid in their internalization and subsequent lysosomal degradation (Yerbury et al., 2005; Wyatt et al., 2013).

Proteostasis in Neurodegeneration

A majority of neurodegenerative diseases involve the pathologic aggregation of proteins that subverts normal neuronal function (Soto and Pritzkow, 2018). These protein aggregates, which include plaques composed of beta amyloid peptides, Lewy bodies containing synuclein, tangles containing tau proteins, and cytoplasmic aggregates of triple-repeat proteins, represent physical hallmarks of neurodegenerative processes, and reflect aberrant protein handling mechanisms that are predominantly focused in neural tissues. These protein deposits arise from aggregation-prone proteins that often possess intrinsically disordered domains, allowing them to form self-replicating structures that tend to oligomerize and form fibrillary or non-fibrillary aggregates. The oligomeric species are most often found to be the toxic form that interferes with normal cellular function. Both extracellular and intracellular proteins are at risk of aberrant aggregation, and new data suggest that there is cross-talk between different disease processes such that "seeds" from one aggregating species can cause aggregate deposition of entirely different proteins (reviewed in Peng et al., 2020). The demonstration of transsynaptic propagation of a variety of misfolded proteins during neurodegenerative disease progression suggests that extracellular processes are critical to the process of neurodegeneration (Peng et al., 2020). The significant co-morbidity of diabetes with AD suggests a generalized risk to secretory tissues in neurodegenerative disease, potentially from common metabolic factors with increased susceptibility to errors in normal protein handling (Shieh et al., 2020). While most reviews of neuronal proteostasis in neurodegenerative processes have focused on cytoplasmic chaperones (Hartl et al., 2011; Kim Y.E. et al., 2013; Balchin et al., 2016), there is increasing evidence that chaperones secreted by neurons and other brain cells play important roles in neuronal proteostasis (reviewed in Wyatt et al., 2013).

In this review, we will summarize various secreted chaperones and describe the evidence for their involvement in neurodegenerative processes. We have necessarily omitted many secreted proteins which may in future turn out to contribute to proteostasis in neurodegenerative disease; examples include SPARC/osteonectin, which exhibits extracellular chaperone activity (Chlenski et al., 2011) and has repeatedly been linked to neurodegeneration (reviewed in Chen S. et al., 2020; Pedrero-Prieto et al., 2020). However, its general chaperone activity against neurodegeneration-related aggregates has not yet been documented. A variety of personal chaperones, for example, PCSK9 (proprotein convertase subtilisin/kexin 9), RAP (receptor-associated protein) and MESD (mesoderm development), all of which accompany LDLR family members through the secretory pathway, are not discussed, nor are the many other personal chaperones that accompany other secretory proteins to specific subcellular destinations, including COSMC (core 1 β3GalT specific molecular chaperone), PPCA (protective protein/cathepsin A), Praf/STMC6 (prenylated Rab acceptor family members), and the iRhom (inactive rhomboid) proteins. However, as found with the personal chaperones 7B2 and proSAAS, it is possible that future work will reveal that other secretory pathway personal chaperones have a wider client base than previously suspected.

We have also not discussed Hsps and other chaperone proteins resident within the secretory pathway which can under specialized stress circumstances be secreted, such as ERDj3 (Genereux et al., 2015a); other ER-resident chaperones (Trychta et al., 2018); cyclophilins (Hoffmann and Schiene-Fischer, 2014); or ERAD-associated proteins such as Stch/HSPA13 (Chen et al., 2009). While these proteins clearly function as chaperones within the secretory pathway, strong evidence for their secretion during neurodegeneration is still lacking (for example documented presence within extracellular aggregates and/or altered CSF levels associated with disease). Finally, in the interest of brevity, we do not discuss well-known secreted chaperones such as α-2-macroglobulin and transthyretin which, although abundant in serum and able to cross the blood–brain barrier, are not highly expressed by the brain, and have also been recently reviewed in the context of neurodegeneration (Buxbaum and Johansson, 2017; Cater et al., 2019; Giao et al., 2020).

The secreted chaperones described in this review have all been associated with neurodegenerative disease based on specific features which include, among others, their physical presence in protein deposits; and proteomics and transcriptomics studies from human as well as animal models that highlight altered levels in neurodegenerative disease. Many of these features are summarized in **Table 1**. The presence of a given chaperone within protein deposits is consistent with, but does not prove, its propensity to sequester aggregation-prone protein clients; it may, for example, simply be an easily aggregated bystander. In addition, altered extracellular or intracellular levels seen in disease might represent a disease-related response to combat excessive misfolding, but also might point to a generally dysregulated secretory pathway. However, the additional ability of the secreted chaperones discussed here to profoundly reduce the rate of protein oligomerization *in vitro* supports a likely role in regulating disease pathogenesis.

In the following sections, select chaperones will be described that have been clearly identified as causative agents in neurodegenerative diseases: clusterin, progranulin, and BRICHOS domain-containing proteins. Next, the neural and endocrine-specific proteins, 7B2 and proSAAS, are discussed with respect to their potent anti-aggregant activity and association with neurodegenerative disease. We will then focus on extracellular actions of certain secreted small Hsps (sHsps); while sHsps have been extensively reviewed recently, most reviews in neurodegeneration have focused on their cytoplasmic actions (Muranova et al., 2019; Webster et al., 2019). Finally, we will elaborate on possible biochemical and physiological mechanisms of extracellular chaperones and discuss their therapeutic potential in treating neurodegenerative disease.

TABLE 1 | Extracellular chaperones implicated in neurodegenerative diseases - Human studies.

Name(s)	Gene name(s)		Disease link	Location	Method	Citations
Clusterin, Apo-J, SP40/40, CLI	*CLU*	AD –	Associated with Abeta40 plaques	C	IF/IHC	Howlett et al. (2013)
			Genetic risk factor		GWAS	Harold et al. (2009), Lambert et al. (2009)
			Increased protein levels	CSF	MS	Reviewed in Pedrero-Prieto et al. (2020)
		PD –	Associated with Lewy bodies	C	IHC	Sasaki et al. (2002)
		CJD –	Associated with protein deposits	CB	IHC/IP	Freixes et al. (2004)
			Increased expression	C/CB	DNA micro-array	Freixes et al. (2004)
		ALS –	Associated with TDP43 inclusions	SC	IF/IHC	Gregory et al. (2017)
			Increased protein levels	Serum	MS	Xu et al. (2018)
Progranulin	*GRN*	AD –	Associated with Abeta plaques	HC	IF/IHC	Gliebus et al. (2009), Gowrishankar et al. (2015), Mendsaikhan et al. (2019a)
			Genetic risk factor		GWAS	Chen et al. (2015)
			High protein levels	CSF	ELISA	Suárez-Calvet et al. (2018)
		FTD –	Null mutations		Linkage analysis	Baker et al. (2006)
			Decreased protein levels	CSF	WB	Wilke et al. (2017)
Bri2, Bri3	*ITM2B, ITM2C*	AD –	Associated with Abeta40 and Abeta42 plaques	HC	IF/IHC	Del Campo et al. (2014), Dolfe et al. (2018)
		FBD and FDD –	Read-through mutations causing protein deposits	C	IF/IHC	Reviewed in Rostagno et al. (2005)
ProSAAS	*PCSK1N*	AD –	Associated with Abeta plaques	HC	IF/IHC	Hoshino et al. (2014)
			Decreased protein levels	CSF	MS	Abdi et al. (2006), Finehout et al. (2007), Jahn et al. (2011), Choi et al. (2013), Holtta et al. (2015), Spellman et al. (2015)
			Increased mRNA levels	C	RNA-seq	Mathys et al. (2019)
		PD –	Associated with Lewy bodies	HC	IF	Jarvela et al. (2016)
			Decreased protein levels	CSF	MS	Rotunno et al. (2020)
		FTD –	Decreased protein levels	CSF	MS	Davidsson et al. (2002)
		DLB –	Decreased protein levels	CSF	MS	Van Steenoven et al. (2020)
7B2	*SCG5*	AD –	Associated with plaques	HC	IF/IHC	Helwig et al. (2013)
			Slightly increased protein levels	C	WB	Iguchi et al. (1987a)
		PD –	Associated with Lewy bodies		IF/IHC	Helwig et al. (2013)
		ALS –	Increased protein levels	CSF	MS	Ranganathan et al. (2005)
		FTD –	Increased protein levels	CSF	MS	Mattsson et al. (2008)
HspBl	*HSPB1*	CMT –	Causative mutations		GWAS, NGS	Muranova et al. (2019), Vendredy et al. (2020)
		AD –	Associated with plaques	HC	IF/IHC	Wilhelmus et al. (2006), Ojha et al. (2011)
		PD –	Increased protein levels	C	IF/IHC	Renkawek et al. (1999)
HspB3	*HSPB3*	CMT –	Causative mutations		GWAS, NGS	Muranova et al. (2019), Vendredy et al. (2020)
		AD –	Associated with plaques	HC	IF/IHC	Wilhelmus et al. (2006), Ojha et al. (2011)

(Continued)

TABLE 1 | Continued

Name(s)	Gene name(s)		Disease link	Location	Method	Citations
			Increased protein levels	HC, C, CG	MS	Koopman and Rüdiger (2020)
		MS –	Increased protein levels	Serum	WB	Ce et al. (2011)
HspB5	*HSPB5*	AD –	Increased protein levels	HC, C, CG	MS	Koopman and Rüdiger (2020)
HspB6	*HSPB6*	AD –	Increased protein levels	HC, C, CG	MS	Koopman and Rüdiger (2020)
HspB8	*HSPB8*	CMT –	Causative mutations		GWAS, NGS	Muranova et al. (2019), Vendredy et al. (2020)
		AD –	Associated with plaques	HC	IF/IHC	Wilhelmus et al. (2006), Ojha et al. (2011)
			Increased protein levels	HC, C, CG	MS	Koopman and Rüdiger (2020)
		CAA –	Associated with amyloid plaques	C	IF/IHC	Wilhelmus et al. (2009)

AD, Alzheimer's disease; ALS, amyotrophic lateral sclerosis; CAA, cerebral amyloid angiopathy; CMT, Charcot-Marie-Tooth; DLB, dementia with Lewy bodies; FBD, familial British dementia; FDD, familial Danish dementia; FTD, frontotemporal dementia; CJD, Creutzfeldt–Jakob disease; PD, Parkinson's disease; C, cortex; CB, cerebellum; CG, cingulate gyrus; CSF, cerebrospinal fluid; HC, hippocampus; SC, spinal cord; ELISA, enzyme-linked immunosorbent assay; GWAS, genome wide association study; IHC, immunohistochemistry; IF, immunofluorescence; IP, immunoprecipitation; NGS, next generation sequencing; MS, mass spectrometry; RNA-seq, RNA sequencing; WB, Western blot.

CLUSTERIN

Clusterin is a heavily glycosylated ∼60 kDa heterodimeric protein (Kapron et al., 1997) derived from a single gene, known as *CLU*, which represents both a potent as well as a highly abundant extracellular ATP-independent chaperone. It is able to block Abeta aggregation (Matsubara et al., 1996) as well as amyloid formation from a variety of amyloidogenic substrates (Yerbury et al., 2007) and has the ability to prevent stress-induced precipitation of a wide range of protein substrates (Humphreys et al., 1999; Poon et al., 2000). This versatile chaperone plays many roles in homeostatic processes throughout the body, including lipid transport (reviewed in Park et al., 2014), tissue remodeling (Gobé et al., 1995), cytoprotection at fluid/tissue interfaces (reviewed in Fini et al., 2016), and endocytosis-mediated clearance of extracellular debris (Bartl et al., 2001). Additionally, increased clusterin expression is linked to cancer progression and treatment resistance (reviewed in Peng et al., 2019). This versatility is thought to derive from clusterin's ability to interact with a variety of different client misfolded proteins. As discussed below, clusterin accumulation is found in many different neurodegenerative diseases.

Structure and Expression

Clusterin is expressed in many cell types throughout the body, most notably in specialized secretory cells and epithelial cells (Aronow et al., 1993). Within the brain, clusterin is ubiquitously expressed in neurons and glia, and is especially abundant in astrocytes, while being absent from microglia (Yao et al., 2020)[1]. Circulating clusterin levels are very high in serum, predominantly derived from the liver (Seo et al., 2020), and approximate 100 μg/ml (about 1.6 μM). Although at much lower levels than in plasma, clusterin is also abundant in the CSF, with normal levels between 2–9 μg/ml (30–150 nM) (Sihlbom et al., 2008; Přikrylová Vranová et al., 2016).

Within the cell, as a signal-bearing protein, clusterin exists primarily within the secretory pathway. Under various types of stress conditions, cellular clusterin levels are increased (Viard et al., 1999), and a portion of this increase may be within the cytoplasm and/or nucleus (Nizard et al., 2007; Prochnow et al., 2013). However, the origins of cytoplasmic and nuclear clusterin are still unclear (reviewed in Rohne et al., 2016) since current hypotheses to explain these subcellular locations are discordant. Cytoplasmic clusterin may arise from *CLU* transcripts translated directly into the cytosol; from secretory clusterin that has prematurely exited the secretory pathway; or from reuptake of secreted mature clusterin (discussed in Foster et al., 2019). Alternatively, intracellular and nuclear clusterin may be produced from rare distinct mRNA transcripts that arise from alternative splicing and different in-frame start sites (reviewed in Garcia-Aranda et al., 2018). However, these transcripts make up less than 0.34% of total clusterin mRNA (Prochnow et al., 2013), suggesting that the vast majority of clusterin protein is produced from the primary transcript. *CLU* expression is affected by histone acetylation, DNA methylation, and a variety of transcription factors and signaling molecules (Garcia-Aranda et al., 2018). Additionally, clusterin expression responds to oxidative and proteotoxic stress, through heat shock transcription factor-1 and activator protein-1 elements in the *CLU* promoter (reviewed in Trougakos, 2013).

For secreted clusterin, intracellular cleavage of the clusterin precursor results in the production of α- and β-subunits arranged in an anti-parallel fashion, linked by five disulfide bridges. Heavy N-linked glycosylation accounts for ∼30% of its apparent mass (reviewed in Wilson and Easterbrook-Smith, 2000). Clusterin contains five conserved putative amphipathic helices, with a large percentage of intrinsically disordered structure (Bailey et al., 2001). The anti-parallel organization of the two chains creates a polarized order-to-disorder motif within the entire protein (Bailey et al., 2001) similar to that of a partially folded, molten globule-like domain capable of interacting with a wide variety of ligands. Clusterin has been

[1]https://celltypes.brain-map.org/rnaseq/human_m1_10x

shown to exist in various pH-dependent multimeric states (Hochgrebe et al., 2000).

Function and Mechanism

Of the three major functions that protein chaperones carry out (assisting folding/refolding, preventing precipitation/aggregation, and preparation for degradation), clusterin has been implicated in the latter two. In 1999, clusterin was shown to be a proficient solubilizing chaperone for heat-stressed glutathione-*S*-transferase by the Wilson group, exceeding the client:chaperone molar ratios of sHSPs (Humphreys et al., 1999). In the last two decades, clusterin has been found to bind and solubilize a variety of heat-stressed proteins (Poon et al., 2002), as well as to prevent the aggregation and fibrillation of disease-associated aggregating proteins, including: Abeta (Yerbury et al., 2007), α-synuclein (Yerbury et al., 2007), TAR DNA-binding protein 43 (TDP-43) (Gregory et al., 2017), and transthyretin (Lee et al., 2009; Magalhães and Saraiva, 2011). Single-molecule characterization has shown that clusterin can bind small Abeta40 oligomers at equimolar ratios (Narayan et al., 2011); small α-synuclein oligomers at equimolar ratios; and large oligomers at sub-stoichiometric ratios (Whiten et al., 2018). Clusterin binding also appears to inhibit oligomer interaction with lipid membranes and the production of reactive oxygen species (Whiten et al., 2018). However, at low molar ratios of clusterin to Abeta, clusterin promotes precipitation and increases fibrillation (Yerbury et al., 2007). *In vitro*, clusterin can sequester Aβ oligomers of various sizes (Narayan et al., 2011). Taken together, these data show that clusterin is capable of sequestering small oligomers, preventing both assembly into fibrils as well as blocking downstream toxic events.

In vivo, clusterin promotes transcytosis of Abeta across the blood–brain barrier through [lipoprotein-related protein 2 (LRP2)-mediated endocytosis] (Zlokovic, 1996; Wojtas et al., 2017). More broadly, clusterin has been shown to promote internalization and degradation of cellular debris and misfolded proteins in non-professional phagocytic cells through LRP2 (Bartl et al., 2001) and through HSPGs (Itakura et al., 2020). Itakura et al. (2020) demonstrated that clusterin binds HSPGs through an electrostatic interaction to promote co-degradation of clusterin and client proteins. HSPGs are broadly expressed and have been previously linked to the endocytosis of a variety of ligands (reviewed in Sarrazin et al., 2011), including Abeta (Kanekiyo et al., 2011), among others. Whether this endocytic pathway represents a major functional contribution of clusterin in neurodegenerative diseases remains to be established.

Disease Relevance and Therapeutic Potential

Clusterin immunoreactivity has been identified within a variety of proteopathic aggregates, including amyloid plaques (Howlett et al., 2013; Craggs et al., 2016), perivascular amyloid deposits (Craggs et al., 2016), cortical Lewy bodies (Sasaki et al., 2002), and protease-resistant prion protein deposits in Creutzfeldt-Jakob disease (Freixes et al., 2004). Clusterin shows a preference for colocalization with Abeta40 over Abeta42 (Howlett et al., 2013; Craggs et al., 2016). Clusterin levels are increased at synapses in human post-mortem AD brains, where it colocalizes with Abeta at presynapses near plaques (<10 μm). This increase is greater in *APOE4* carriers (Jackson et al., 2019), and may be related to the increase in intracellular clusterin levels observed after Abeta-induced stress (Killick et al., 2014).

Considerable genetic evidence connects clusterin to neurodegenerative disease. After *APOE* and *BIN1*, *CLU* is the third largest genetic risk factor for late onset AD. Multiple GWAS (Harold et al., 2009; Lambert et al., 2009) and meta-analyses (Liu et al., 2014; Zhu et al., 2018) have confirmed a strong correlation of the rs11136000T allele with decreased AD risk in Caucasian populations; however, the evidence for this genetic link is weaker in other ethnic groups (Han et al., 2018). This protective allele is associated with increased clusterin expression (Ling et al., 2012). Additional rare polymorphisms have been identified in patients suffering from late onset AD, in which secreted clusterin levels are reduced due to folding abnormalities (Bettens et al., 2015). Six independent proteomics studies show that CSF levels of clusterin are significantly increased in AD patients (see meta-analysis in Pedrero-Prieto et al., 2020); plasma levels also rise in ALS (Xu et al., 2018). Additionally, while brain clusterin levels increase within the brain during AD progression, the levels of Abeta increase to a greater extent, resulting in a declining molar ratio between clusterin and Abeta specifically within regions of the brain with high Abeta deposition (Miners et al., 2017). Thus, while assessing clusterin levels in CSF may eventually prove to be a powerful diagnostic tool, these data suggest that brain clusterin levels will need to be increased to a great extent in order to provide a therapeutic benefit.

Animal studies of clusterin expression in neurodegenerative disease have yielded paradoxical results. When crossed with clusterin null mice, PDAPP AD model mice (which express the human APP mutant V717F at ten times the level of endogenous APP) show reduced levels of neuritic dystrophy and fibrillary amyloid plaques within the brain, without an overall change in levels of Abeta (DeMattos et al., 2002). Thus, this phenotype exhibits less neuritic toxicity than in APP model mice expressing clusterin. Another group who performed the same clusterin null cross with a different APP mouse model (APP/PS1; PS1 = presenilin1), which expresses lower levels of a mouse/human chimeric *APP* transgene found a reduction in fibrillar Abeta plaques concomitant with a reduced Abeta load (Wojtas et al., 2017). In this mouse model, Abeta deposits are found as increased CAA deposits, and overall Abeta load is decreased within the brain (Wojtas et al., 2017). These paradoxical results were corroborated by another group who showed that clusterin expression is required for Abeta toxicity in human induced pluripotent stem cell (iPSC)-derived neurons (Robbins et al., 2018). In agreement, in primary neurons, siRNA silencing of clusterin expression provides protection against Abeta toxicity (Killick et al., 2014). However, as shown by Robbins et al. (2018), the loss of clusterin expression results in a change in the expression of a variety of genes; thus, it is currently unclear if the paradoxical effect is directly related to clusterin loss or is due to pleiotropic effects. Adding to this complexity, in the 5xFAD (familial Alzheimer's disease) model mouse background, clusterin null mice showed fewer Abeta plaques and cognitive

performance deficits compared to clusterin-expressing mice at 5 months of age, though the differences disappeared by 10 months, suggesting that clusterin expression is only required for early toxicity (Oh et al., 2019). We speculate that the variation in results when using different mouse models may be due at least in part to differences in the clusterin: Abeta ratio inherent in the different experimental models. Alternative explanations for the positive effects of clusterin null mutations on neurodegenerative pathology include altered expression of other genes, as observed in Robbins et al. (2018), and thus this paradox remains unexplained.

Despite these controversial mouse studies, substantial biochemical evidence supports the positive effects of clusterin expression on Abeta load within the brain. Thus, several groups have directly examined the effects of increased brain clusterin on amyloid deposition in various mouse models of AD. Peripheral administration of recombinant human clusterin, either in complex with HDL or lipid-free, was shown to reduce the levels of insoluble Abeta as well as CAA in AD model mice (de Retana et al., 2019). Neuronal loss in the hippocampus was also decreased; whether this positive effect was due to the reduced damage from Abeta accumulation, or to clusterin's known anti-apoptotic effects (Koch-Brandt and Morgans, 1996) is unclear. One caveat to this study is that recombinant clusterin was not detected within the brain, though it was found in the intrameningeal ventricle lumen. This is consistent with studies that show that while clusterin can cross the blood–brain barrier through interaction with LRP2 (Zlokovic, 1996), plasma levels of endogenous clusterin are sufficient to saturate the transporter (Shayo et al., 1997). These data suggest that the protective effects provided by recombinant clusterin are greater than simple blockade of Abeta accumulation and may possibly occur via upregulation of microglial Abeta phagocytosis (Yeh et al., 2016). Intraperitoneal (Montoliu-Gaya et al., 2018) or intraventricular (Qi et al., 2018) administration of a short clusterin-derived peptide can decrease Abeta deposition in mouse models, as well as ameliorate cognitive defects (Qi et al., 2018). This peptide corresponds to a D-amino acid version of one of clusterin's predicted amphipathic helices (Bailey et al., 2001) and may play a role in recognizing Abeta (Wyatt et al., 2009) or in stimulating phagocytic activity, perhaps by upregulation of LRP2 (Qi et al., 2018) and thereby impacting clearance of Aβ across the blood–brain barrier.

In ALS patient tissues, immunoreactive clusterin is localized within cytoplasmic inclusions of TDP-43 in motor neurons, whereas in control tissues, clusterin staining is primarily found in the ER (Gregory et al., 2017). Further, in ALS mismatch cases – those patients with high TDP-43 burden but without cognitive deficits – clusterin expression was increased in gray matter neurons, compared to controls and ALS cases with cognitive decline. Additionally, glial clusterin expression was higher in cases of ALS with cognitive decline than in controls or mismatch cases (Gregory et al., 2020). Importantly, this effect could not be ascribed to general proteostatic differences, as the levels of the cytoplasmic chaperone HSPB8 were unchanged between controls and either disease case. These data suggest that the specific increase in neuronal expression of clusterin may be protective in an autologous fashion. It remains to be determined if elevations in clusterin are a consequence of a distinct disease progression that results in reduced cognitive decline, or instead represent a homeostatic means to provide neurological protection. It will also be important to assess whether similar proteostatic pathway changes are found in mismatch cases in other neurodegenerative diseases, such as AD, in which some diagnosed patients are known to have low levels of CSF Abeta, but no Abeta accumulation is visible in positron emission tomography (Mattsson et al., 2015).

Single nucleotide polymorphisms in clusterin have also been linked to early cognitive decline in PD (Gao et al., 2011; Sampedro et al., 2020). Clusterin has also been investigated as a potential therapeutic for PD. The preincubation of α-synuclein oligomers with clusterin prevented cell death and the production of reactive oxygen species, as well as TLR4 (toll-like receptor 4) activation (Hughes et al., 2019).

Summary

It is clear that clusterin plays a variety of different roles throughout the brain (Wilson and Zoubeidi, 2017). While we do not yet fully understand the many mechanisms by which the clusterin chaperone operates to provide such a strong genetic link to AD, several common themes have emerged from research during the past two decades. With a client ratio of 1:10 for blocking Abeta amyloid formation (Yerbury et al., 2007), clusterin clearly represents a potent chaperone, and increasing evidence indicates that its extracellular chaperone action may directly impact the course of neurodegenerative disease. In addition, extracellular clusterin appears to act as a positive force in the endocytosis of (potentially toxic) aggregates and oligomers. Lastly, clusterin expression influences clearance of Abeta from the brain. Further research will be required to determine whether a specific mechanism represents a dominant phenotype, and whether other genes work together with clusterin to provide neuroprotection.

PROGRANULIN

Progranulin (gene name: *GRN*), a secreted, cysteine-rich glycoprotein, is highly expressed in cells of myeloid lineage such as macrophages and microglia; in epithelial cells; in a subset of neurons in the cortex, hippocampus, and cerebellum; and in motor neurons. Unlike other chaperones discussed in this review, progranulin functions as a personal chaperone for lysosomal proteins (reviewed in Bateman et al., 2018); however, it is secreted, suggesting extracellular action. Mammalian progranulin contains seven and a half granulin (GRN) domains, interspersed with variable length linkers. These GRN domains contain repeats of a 12-cysteine motif which can fold into tight beta-sheets linked by disulfide bonds, forming a "beads-on-a-string" arrangement. Progranulin can be cleaved into individual GRNs which can have functions independent of progranulin.

Structure and Expression

The GRNs are referred to alphabetically by order of discovery, or numerically by order within progranulin, with "P" referring to the partial GRN in each case. From the amino-terminus, the order of

the GRNs is P-G(1)-F(2)-B(3)-A(4)-C(5)-D(6)-E(7). Progranulin is secreted as a homodimer (Nguyen et al., 2013). Computational algorithms to determine protein disorder predict a high-low pattern where the more disordered GRN-G, GRN-B, GRN-C, and GRN-E are separated by the more ordered GRN-F, GRN-A, and GRN-D (Ghag et al., 2017). Further, recombinant GRN-B, while predicted to be disordered, forms a highly thermodynamically stable monomer, though when disulfide bonds are reduced, it becomes completely disordered (Ghag et al., 2017). This interspersing of order with disorder maybe be crucial to the chaperone activity of progranulin.

In the brain, expression of the progranulin gene is strong in microglia (Daniel et al., 2000; Baker et al., 2006; Ryan et al., 2009). *GRN* expression is also high in motor neurons in the spinal cord (Ryan et al., 2009). Progranulin is secreted in an activity-dependent manner at the synapse, with increasing neuronal activity correlating with increased progranulin levels within axons (Petoukhov et al., 2013). Thus, progranulin is appropriately expressed in extracellular locations where aggregating proteins and peptides are known to be released. At the transcriptional level, expression of progranulin is regulated by the master lysosomal biogenesis regulator transcription factor EB (TFEB) (reviewed in Kao et al., 2017), allowing for increased expression when increased degradative capacity is required.

Progranulin is found both in the lysosome and extracellular space. It reaches the lysosome either directly from the *trans*-Golgi, or after secretion and endocytosis via sortilin (Hu et al., 2010). In neurodegenerative diseases, progranulin and/or GRNs are detected within amyloid deposits (Gliebus et al., 2009; Gowrishankar et al., 2015; Mendsaikhan et al., 2019b), suggesting that progranulin may have additional extracellular roles in the formation of neurodegeneration-related protein aggregates.

Function and Mechanism

Progranulin is known to exert a protective chaperone function on certain lysosomal enzymes [cathepsin D and beta-glucocerebrosidase (GCase)]. *In vitro,* progranulin added to recombinant cathepsin D protects this protein from high temperature-induced denaturation/degradation (Beel et al., 2017). Progranulin stabilizes the propeptide of cathepsin D, promoting autocatalysis at the active site (Butler et al., 2019). In the absence of progranulin, levels of cathepsin D (both pro and mature) accumulate, although enzyme activity decreases, a key indicator of lysosomal dysfunction (Götzl et al., 2018). The final 98 amino acids of progranulin, corresponding to GRN-E plus a linker sequence, are required for binding GCase, and also function to recruit non-canonically translocated HSP70 in a ternary interaction (Jian et al., 2016). We speculate that the intrinsic disorder predicted in both GRN-E and the C-terminal tail is important for recognizing multiple client proteins and for chaperone function.

A variety of *in vitro* and *in vivo* evidence supports a chaperone role for secreted GRNs. Recombinant GRN-B potently increases the fibrillation of Abeta *in vitro,* while reducing toxic oligomer formation (Bhopatkar et al., 2019). Abeta incubated under oligomerizing conditions with GRN-B at equimolar concentrations exhibits reduced caspase activation compared to Abeta incubated alone. However, GRN-B also seems to promote the formation of insoluble TDP-43 inclusions, exacerbating TDP-43 cytotoxicity (Bhopatkar et al., 2020); this is consistent with effects observed in *C. elegans* during GRN-B overexpression (Salazar et al., 2015). This aggregate promotion effect is increased following reduction of GRN-B; Bhopatkar et al. (2020) have speculated that the instability of reduced GRN-B can better disrupt proper TDP-43 folding, thus promoting its aggregation. Interestingly, known human mutations in GRN-B which disrupt the predicted beta-hairpin stack structure are linked to FTD, suggesting that the stacked structure is important for chaperone activity (van der Zee et al., 2007). Similar studies with GRN-C and TDP-43 show that GRN-C reduces thioflavin T-positive fibrillation of TDP-43 and promotes TDP-43 liquid-liquid phase separation *in vitro* (Bhopatkar et al., 2020). Taken together, these results suggest that certain individual secreted GRNs may function as sequestrase chaperones to remove aggregating proteins from solution and prevent the accumulation of small soluble toxic oligomers. The remaining GRNs and full-length progranulin remain to be mechanistically studied for *in vitro* chaperone activity.

Disease Relevance and Therapeutic Potential

A variety of mutations in the progranulin gene are now known to result in FTD; these diminish levels of secreted progranulin either through reduced translation or via improper folding (Baker et al., 2006; Cruts et al., 2006; Chen et al., 2015). In FTD patients who lack *GRN* mutations (the more common form of FTD), CSF progranulin levels are still reduced as compared to healthy controls, suggesting that indirect changes in progranulin levels might contribute to these forms of FTD (Wilke et al., 2017). Loss of progranulin results in degeneration of the frontal and temporal lobes, causing dementia and finally death. Studies of rare individuals with two faulty copies of *GRN*, and of mouse progranulin knockout models, both support a severe impact of progranulin loss on lysosomal function; in humans, the complete loss of progranulin results in lysosomal storage disease (Ahmed et al., 2010; Smith et al., 2012).

In brain samples from patients with AD, immunoreactive progranulin colocalizes with Abeta deposits (Gowrishankar et al., 2015). Progranulin immunoreactivity is interspersed within most Abeta plaques in low pathology AD brains with fewer and smaller plaques (Mendsaikhan et al., 2019a), suggesting extracellular chaperone action. In a mouse model with mild amyloid formation, reduced progranulin levels increase amyloid deposition in the brain, while in the 5xFAD mouse model with high amyloid formation, overexpression of progranulin decreases Abeta plaque load (Minami et al., 2014). Genetic evidence also links progranulin to AD; the rs5848 polymorphism is linked to a 1.36-fold increased risk of late onset AD (Chen et al., 2015). This same polymorphism has been linked to increased risk of hippocampal sclerosis, as well as to increased CSF levels of tau (Fardo et al., 2017). As AD progresses, CSF levels of progranulin increase in the same time frame as

neurodegeneration and neurofibrillary tangle formation occur (Suárez-Calvet et al., 2018).

Given the genetic risks associated with low progranulin levels in FTD and AD, increasing brain levels of progranulin via recombinant protein, viral delivery, or small molecule regulators, have all been proposed as potential disease treatments (reviewed in Gass et al., 2012). Determining the appropriate dosage will be critical, since adeno-associated virus- (AAV)-mediated overexpression of progranulin can cause hippocampal toxicity and neuronal degeneration via increased infiltration of T cells into the brain (Amado et al., 2019). Increasing progranulin expression can also induce ER stress in a variety of cell types (Li et al., 2015), likely due in part to the large number of disulfide bonds and complex folding. Nevertheless, a variety of studies are currently ongoing which involve therapeutic modulation of progranulin (recently reviewed in Cui et al., 2019). Of these, lentivirus-mediated overexpression of progranulin has likely been the most successful, and has been shown to reduce plaque burden and synapse loss in a mouse model of AD (Van Kampen and Kay, 2017). This same group had earlier shown that viral progranulin delivery into the substantia nigra protects dopaminergic neuronal health in a mouse model of PD (Van Kampen et al., 2014). In a null GRN mouse background, AAV-expressed progranulin rescues lysosomal function and reduces lipofuscinosis (Arrant et al., 2018). Oral administration of the disaccharide trehalose, an upregulator of autophagy, also increases progranulin expression in a haploinsufficient mouse model (Holler et al., 2016). Taken together, these data suggest that upregulation of progranulin expression, while potentially challenging to accomplish, may represent a promising treatment for FTD, AD, and PD.

Summary

Modulating progranulin levels as a therapy in neurodegenerative disease shows great promise. However, achieving efficacious progranulin upregulation will require therapeutic discrimination between its various pro-growth, inflammatory, and chaperone activities. To better understand the role of progranulin as a general chaperone, *in vitro* assays of the effects of full-length progranulin will be required to complement the current studies of the individual domains on the aggregation and fibrillation of a variety of toxic proteins. Experiments defining the interactions between progranulin and/or individual GRNs with aggregating proteins are only now being attempted with mechanistic scrutiny (Bhopatkar et al., 2020).

THE BRICHOS DOMAIN

The BRICHOS domain was originally found in, and named after, a set of chaperone proteins – Bri2, chondromodulin-1 and proSP-C – which demonstrate anti-amyloidogenic properties. Since its discovery in 2002, the BRICHOS domain has been found in 12 different protein families, with expression in various tissues. BRICHOS-domain proteins are ER membrane proteins and contain the BRICHOS domain in the C-terminal region; this domain is cleaved off by furin and other enzymes in the secretory pathway lumen and can then be secreted out of the cell. Interestingly, mutations in the genes encoding these proteins act as causative disease agents, for example Bri2 in dementias, chondromodulin-1 in cancer, and proSP-C in lung fibrosis. Cleaved products of Bri2 and proSP-C are also prone to form amyloid deposits. Abeta has been well studied as a client of the BRICHOS chaperone domain (reviewed in Willander et al., 2011; Buxbaum and Johansson, 2017).

Structure and Localization

BRICHOS-domain containing proteins are generally ER-based type-II transmembrane proteins which contain a BRICHOS domain connected to the N-terminal transmembrane domain via a linker region. A 17-amino acid C-terminal luminal end is cleaved off by proteases, such as furin, forming the mature protein (Kim et al., 1999; Wickham et al., 2005). The luminal domain of Bri2 is further cleaved by ADAM10, thereby releasing the roughly 100-amino acid long BRICHOS domain into the secretory pathway (Martin et al., 2008). The crystal structure of recombinant human proSP-C BRICHOS domain shows beta sheets flanked by two alpha helices, with a tendency to form dimers and oligomers (Willander et al., 2012). All BRICHOS domains contain three conserved amino acids, two cysteines and one aspartic acid. The proteins also contain regions with the non−polar residues valine, isoleucine, phenylalanine and cysteine, which are prone to form beta-sheets with a tendency to aggregate (reviewed in Buxbaum and Johansson, 2017). While proSP-C (encoded by *SFTPC*) is expressed in the lungs, Bri2 (encoded by *ITM2B*) is expressed ubiquitously, and Bri3 (encoded by *ITM2C*) is brain-specific; both Bri2 and Bri3 are highly expressed in the hippocampus, cerebellum and cerebral cortex (Akiyama et al., 2004). In neuronal cell lines, Bri2 and Bri3 have been localized to the ER and Golgi complex, as well as within neurites (Martins et al., 2016). Localization of Bri2, but not its proteolytic cleavage product, to the plasma membrane appears to be regulated by glycosylation (Tsachaki et al., 2011).

Function and Mechanism

Bri2 physically interacts with several proteins involved in membrane trafficking and the cytoskeleton (Martins et al., 2018). Several different BRICHOS-containing proteins were identified within the post-synaptic compartment in a mass spectrometry study and implicated in vesicle recycling, neurite growth and plasticity, neuronal differentiation, and signaling (Martins et al., 2018). In particular, Bri2 and Bri3 have been found to be physically complexed with, and be phosphorylated by, PP1, which regulates its functions in neurite growth and neuronal differentiation, as well as its proteolytic processing (Martins et al., 2016, 2017). Bri2 and Bri3 bind to APP during biosynthesis, shielding it from the secretases involved in APP cleavage, and reducing the production of the aggregation-prone peptide products Abeta40 and Abeta42 (Matsuda et al., 2005, 2011). These studies indicate a role for Bri2 and Bri3 in the functional regulation of APP processing under normal conditions.

Different *in vitro* studies point to a variety of possible mechanisms proposed to explain how recombinant human BRICHOS might modulate Abeta oligomerization, namely

via blocking secondary nucleation and/or by blocking fibril formation and elongation. *In vitro,* the various recombinant BRICHOS domains of Bri2, Bri3 and proSP-C have all been shown to reduce toxic Abeta oligomerization and fibrillation (Biverstal et al., 2015; Dolfe et al., 2016). The assembly state of the BRICHOS domain appears to dictate its specific effect on amyloid oligomerization and fibrillation. Recombinant BRICHOS monomers adopt different quaternary structures; they also form dimers, which act as the subunits for oligomerization (Chen et al., 2017). The monomeric and dimeric states have been shown to be more potent at reducing Abeta fibril formation as compared to oligomeric states (Cohen et al., 2015; Chen et al., 2017). Dimers also appear to be more potent than monomers in reducing Abeta fibril elongation and secondary nucleation by binding to Abeta fibrils (Cohen et al., 2015; Chen et al., 2017).

The BRICHOS domain mutations R221E and S95R, which promote a stable monomeric state, were found to significantly reduce secondary nucleation and to delay fibrillation *in vitro*, respectively, as compared to mutant oligomeric states (Biverstal et al., 2015; Chen G. et al., 2020). Immunogold electron microscopy showed that formation of Abeta fibrils *in vitro* was drastically delayed in the presence of recombinant Bri2 and proSP-C BRICHOS domains, likely due to the fact that BRICHOS domain binds to Abeta fibrils, shielding it from further surface nucleation (Willander et al., 2012). More recently, cryo-EM studies showed that the process of Abeta fibrillation consisted of both formation of free floating protofibrils as well as fibrils with surface nucleation; in the presence of the BRICHOS domain, a higher number of free-floating Abeta protofibrils were found, while in its absence, secondary nucleation was favored (Tornquist et al., 2020). This supports the idea that the possible mechanism of action is by reducing secondary nucleation rather than prevention of *de novo* fibril formation. Interestingly, BRICHOS domain oligomers are also able to reduce non-fibrillar aggregation of Abeta (Chen et al., 2017). Taken together, these biochemical studies strongly support a role for the BRICHOS domain to act as an anti-aggregant for Abeta, and additionally identify monomeric variants that could be tested in cell culture and animal models to provide effective neuroprotection against Abeta toxicity.

Disease Relevance and Therapeutic Applications

Genetic evidence supports the association of BRICHOS domain-containing proteins with neurodegenerative disease. Read-through mutations in Bri2, leading to the expression and cleavage of an additional 11 amino acids in the C-terminal peptide product, result in the formation of amyloid protein deposits in the brain, and are responsible for the familial British and Danish dementias (FBD and FDD, respectively) (Rostagno et al., 2005). Bri2 and Bri3 BRICHOS domains have been found to be colocalized with amyloid plaques in AD patients (Del Campo et al., 2014; Dolfe et al., 2018). Bri2 and Bri3 were also shown to be able to bind Abeta and/or APP in the CA1 region of the hippocampus in transgenic APP mice (Dolfe et al., 2018). However, while levels of Bri2 levels were increased in AD patient brains, levels of Bri3 were reduced (Dolfe et al., 2018). In cell culture, Bri2, but not Bri3, has been detected in the medium of overexpressing cell lines (Dolfe et al., 2018). The dissimilarities between Bri2 and Bri3 levels and localization support differences in mechanism of action between the two chaperones. While the Bri2 BRICHOS-domain probably interacts with Abeta extracellularly and/or upon reuptake, it is likely that Bri3 BRICHOS-domain binds Abeta only within the secretory pathway.

In vivo and *ex vivo* studies highlight the importance of BRICHOS-containing proteins in combating Abeta toxicity. Although *in vitro*, recombinant BRICHOS dimers exhibit higher activity against Abeta fibrillation than monomers, physiologically, it is the BRICHOS monomers rather than dimers that reduce Abeta-induced damage to neuronal networks in mouse hippocampal slices treated with Abeta. However, these studies involve the use of BRICHOS domain protein at the relatively high chaperone: client molar ratio of 1:1 (Chen et al., 2017). Recombinant Bri2 BRICHOS domain and its monomeric variant, R221E, were also able to reduce cytotoxic effects induced by exposure of hippocampal slices to Abeta42 monomers, and also partially to preformed Abeta fibrils (Poska et al., 2016; Chen G. et al., 2020). In a *Drosophila* model of AD, coexpression of Bri2 with Abeta42 reduced Abeta aggregation in adult brains as well as Abeta-induced retinal degeneration; overexpressed Bri2 also rescued lifespan and motor defects (Hermansson et al., 2014). In the presence of Bri2, Abeta42 became diffusely colocalized with Bri2 in the mushroom bodies, the seat of cognition and learning in the adult *Drosophila* brain, instead of exhibiting the punctate morphology observed in the absence of Bri2 (Poska et al., 2016), providing evidence that Bri2 can prevent Abeta42 deposition *in vivo*. In a mass spectrometry screen, the ubiquitin ligase NRBP1 was recently found to be a substrate receptor for Bri2 and Bri3, recruiting these proteins during ERAD for ubiquitination by the Cullin-RING ligase complex and thereby targeting them for proteasomal degradation (Yasukawa et al., 2020). Transgenic mice expressing fused Bri2-Abeta40/42 exhibited delayed formation of Abeta plaques, and were devoid of cognitive or behavioral decline, supporting the sequestering activity of Bri2 as a likely mechanism to prevent the spread of toxic oligomers and reduce neuronal death (Kim J. et al., 2013). Reduced cognitive decline in Bri2-Abeta40/42 expressing transgenic mice compared to controls suggests that this specific mechanism normally serves to prevent or delay processes that much later result in the development of AD (Kim J. et al., 2013). A similar effect has not been identified in AAV-mediated expression of Bri-Abeta40/42 in rats, which developed pathological symptoms of AD (Lawlor et al., 2007). Efforts to increase Bri2 and Bri3 expression could prove to be therapeutic in AD by regulating events from Abeta processing to fibril formation.

Summary

The data obtained to date indicate that BRICHOS domain-containing proteins–while themselves susceptible to amyloid formation–can effectively combat Abeta-mediated cytotoxicity at various stages: by preventing APP cleavage and Abeta toxic

peptide production, as well as by interacting with Abeta to reduce fibrillation both within the secretory pathway and the extracellular milieu.

7B2 AND PROSAAS

While all other secreted chaperones discussed in this review are expressed ubiquitously in the body, there are two small chaperone proteins, 7B2 and proSAAS, whose expression is mainly restricted to cells containing a regulated secretory pathway, namely neurons, neuroendocrine, and endocrine cells. Both proteins are similar in size, around 250 amino acids, contain functionally similar core segments, and are cleaved at least once by the proprotein convertase furin, releasing about-20 kDa N-terminal domains with chaperone functionality. However, 7B2 and proSAAS have no amino acid homology. Interestingly, 7B2 is an ancient protein, found in organisms as primitive as flatworms and rotifers (see PFAM PF05281) while the proSAAS protein is much more recent, first appearing only in vertebrates (see PFAM PF07259).

Structure and Localization

The neuroendocrine chaperone 7B2 (gene name *SCG5*) was first identified in neuroendocrine tissues by direct peptide sequencing over 28 years ago (Hsi et al., 1982); it is predominantly expressed in pituitary, all areas of the brain, pancreas, and adrenal (Iguchi et al., 1984, 1985). Within the cell, 7B2 is concentrated within regulated secretory granules, from which it is released following stimulation (Iguchi et al., 1987b); see also review (Mbikay et al., 2001). CSF concentrations approximate 3 ng/ml or 0.14 nM (Iguchi et al., 1987a) and decline with age (Natori et al., 1987). In a specific substrain of mice, loss of 7B2 results in a lethal phenotype between 5 and 8 weeks of age owing to hypersecretion of ACTH from the pituitary (Westphal et al., 1999; Laurent et al., 2002). These data indicate a possible role for 7B2 in peptide hormone storage. One early study indicates that the C-terminal peptide can depolarize vasopressin- and oxytocin-containing neurons in hypothalamic explants (Senatorov et al., 1993). However, no other studies have shown neuropeptide-like actions for 7B2, and no receptors for 7B2-derived peptides have been identified.

Full-length 27 kDa 7B2 is cleaved by Golgi-resident furin, releasing a 21 kDa product (Ayoubi et al., 1990). Both 27 kDa and 21 kDa 7B2 contain a central IDR, as indicated both by the PONDR prediction algorithm and a lack of secondary structure detected by circular dichroism, with the 27 kDa form being more compact than 21 kDa form (Dasgupta et al., 2012).

ProSAAS (gene name *PCSK1N*) is an abundantly expressed brain protein discovered 20 years ago using mass spectrometric techniques applied to brain peptide extracts (Fricker et al., 2000). Like 7B2, it is predominantly expressed within the brain as well as in endocrine and neuroendocrine tissues (Lanoue and Day, 2001; Sayah et al., 2001; Morgan et al., 2005), where, like 7B2, it is stored within secretory granules (Wardman et al., 2011; Wardman and Fricker, 2014). While the 7B2-encoding gene contains a heat shock-responsive element (Martens, 1988; Mbikay et al., 2001), the mouse or human proSAAS-encoding genes do not. Interestingly, heat shock does increase the quantity of cellular proSAAS in cell culture (Shakya et al., 2020). Expression of the *Pcsk1n* gene in differentiating neural tube neurons was observed in developing rat embryos as early as 12 days of gestation, while proSAAS processing begins in mid-gestation (Morgan et al., 2005). Due to the lack of an adequately sensitive radioimmunoassay, the concentration of proSAAS in CSF and in plasma is not yet known, but brain concentrations have been estimated to be between 10 and 500 nM depending on region (Jarvela et al., 2016).

Within the secretory pathway, basic residue pairs within the amino- and carboxy-terminal portions of 27 kDa proSAAS are cleaved by proprotein convertases and carboxypeptidase E to produce various secreted peptide products (Fricker et al., 2000; Mzhavia et al., 2001, 2002; Sayah et al., 2001; Wardman et al., 2011), and specific proSAAS-derived peptides are thought to have biological functions (Hatcher et al., 2008). The 21 kDa N-terminal domain of proSAAS, separated from the carboxy-terminal domain by a furin consensus sequence, harbors an internal predicted coiled coil region as well as a predicted IDR (Kudo et al., 2009), through which it potentially interacts with client proteins.

Function and Mechanism

Thirteen years following its discovery, 7B2 was identified as an anti-aggregant chaperone for prohormone convertase 2 (proPC2) (Zhu and Lindberg, 1995; Lee and Lindberg, 2008), functioning through an evolutionarily conserved PPNPCP motif within a 36-residue region in the middle of the protein (Zhu et al., 1996; Muller et al., 1999). The chaperone activity of this anti-aggregant region is reminiscent of the α-crystallin-like domain within soluble sHSPs (see below), although the two types of proteins bear no sequence similarity. Like sHsps, 7B2 demonstrates tendencies to both dimerize and to exhibit concentration-dependent polydispersity (Dasgupta et al., 2012); we speculate that as in sHsps, the presence of IDRs as well as the formation of oligomers may be important for binding aggregation-prone proteins such as proPC2 (Lee and Lindberg, 2008), insulin-like growth factor (Chaudhuri et al., 1995), and islet amyloid polypeptide (Peinado et al., 2013).

Like 7B2, proSAAS is also capable of reducing the fibrillation of aggregating proteins. To date known proSAAS clients include Abeta (Hoshino et al., 2014); α-synuclein (Jarvela et al., 2016); and islet amyloid polypeptide (Peinado et al., 2013). Exciting new results indicate that cytoplasmic expression of proSAAS results in the formation of phase-separated proSAAS spheres which are able to trap the aggregating protein TDP-$43^{214-414}$ within their cores (Peinado et al., 2020). Further structure-function analysis should permit us to determine the self-associating domains of proSAAS as well as the residues lining the sphere interior, which clearly favor aggregate binding.

Disease Relevance and Therapeutic Applications

Evidence of extracellular action for 7B2 is its colocalization with Abeta plaques; immunoreactive 7B2 is also found in Lewy

bodies in PD patient samples (Helwig et al., 2013). While levels of 7B2 in the CSF were shown to decrease during normal aging (Natori et al., 1987), three studies reported increased CSF 7B2 levels in FTD (Mattsson et al., 2008) and/or ALS patients (Ranganathan et al., 2005; Jahn et al., 2011). However, among AD patients, contradicting studies have reported either a slight increase (Winsky-Sommerer et al., 2003) or no change (Iguchi et al., 1987a) in 7B2 levels. APP transgenic mouse brains also do not show alterations in 7B2 levels, indicating that 7B2 is not upregulated during the course of disease (Jarvela et al., 2018). Majerova et al. (2017) showed increased levels of CSF 7B2 in proteomic studies of a tauopathy transgenic rat model of AD. While no genetic evidence directly implicates 7B2 in disease processes, the potent chaperone action of 7B2, and the presence of 7B2 in a variety of extracellular protein deposits supports the idea that brain 7B2 levels may be relevant to proteostatic processes in neurodegeneration.

Immunofluorescence studies have similarly shown that proSAAS co-localizes with aggregated proteins involved in neurodegenerative disease, namely tau tangles in dementia (Kikuchi et al., 2003); Abeta plaques in AD (Hoshino et al., 2014); and Lewy bodies in PD (Helwig et al., 2013). Seven independent proteomics studies have shown that the level of proSAAS in CSF taken from AD and/or FTD patients is reduced as compared to controls, suggesting possible cellular retention within the brain (Davidsson et al., 2002; Abdi et al., 2006; Finehout et al., 2007; Jahn et al., 2011; Choi et al., 2013; Holtta et al., 2015; Spellman et al., 2015; reviewed in Pedrero-Prieto et al., 2020). Two very recent CSF studies support this reduction (Rotunno et al., 2020; Van Steenoven et al., 2020).

The idea that proSAAS plays a role in neurodegenerative proteostasis is further supported by human transcriptomics studies, which indicate increased proSAAS expression during AD progression (Mathys et al., 2019). Increased levels of proSAAS have also been found in brain tissues of patients with CAA (Inoue et al., 2017), as well as in models of neurodegenerative diseases including horses (McGorum et al., 2016) and rats (Chatterji et al., 2014). Lastly, recent data from our laboratory indicate that cellular proSAAS levels are upregulated following ER and even heat stress (Shakya et al., 2020); interestingly, in parallel experiments, similar upregulation was not observed for 7B2. Lastly, in an experiment to determine which endogenous CSF proteins bind to the amyloid fold, proSAAS was identified (Juhl et al., 2019). Collectively, these data strongly support the involvement of proSAAS in proteostatic mechanisms of neurodegenerative disease.

Biochemical studies indicate possible similar mechanisms of action for 7B2 and proSAAS with respect to their ability to block aggregative processes in neurodegeneration. For example, *in vitro* fibrillation studies demonstrate that both chaperones potently reduce the oligomerization of aggregation-prone proteins (Helwig et al., 2013; Hoshino et al., 2014; Jarvela et al., 2016). Structure-function studies have revealed that for both proteins, a conserved internal domain of about 100 residues is responsible for anti-fibrillation chaperone activity (Helwig et al., 2013; Jarvela et al., 2016). Both proteins act at sub-stoichiometric client ratios; while both proSAAS and 7B2 are able to reduce the fibrillation of Abeta at a 1:10 chaperone: client molar ratio, proSAAS is able to efficiently diminish α-synuclein fibrillation at a molar ratio of 1:70. While both chaperones reduce Abeta and α-synuclein fibrillation, neither is able to disaggregate preformed fibrils, and the addition of ATP and/or HSP70 has no effect on their activity (Helwig et al., 2013; Jarvela et al., 2016). These results support the idea that these chaperones act alone rather than in concert with other chaperones or disaggregases. How these two chaperones are able to become trapped within aggregates is unclear, but if lessons from small cytoplasmic Hsps apply, then perhaps initial anti-aggregant activity is transformed into a sequestrase function as client levels progressively overwhelm chaperone levels (Mogk et al., 2019).

Limited studies in cell and animal models also support the idea of extracellular anti-aggregant action for proSAAS and 7B2. Both chaperones are cytoprotective in cell culture as well as in rodent models of AD and PD (Helwig et al., 2013; Hoshino et al., 2014; Jarvela et al., 2016). Application of recombinant 21 kDa proSAAS was found to reduce cytotoxicity in α-synuclein-expressing SH-SY5Y cells and in Abeta oligomer-treated Neuro2A cells, indicating effective extracellular chaperone function against these two aggregating proteins (Hoshino et al., 2014; Jarvela et al., 2016). Lentiviral expression of proSAAS increased the number of tyrosine hydroxylase-positive cells in rat primary nigral cell cultures expressing AAV-encoded α-synuclein (Jarvela et al., 2016). Similarly, external application of recombinant 21 kDa 7B2, as well as AAV-mediated overexpression of intact 7B2 in Neuro2A cells, were both cytoprotective against toxic Abeta oligomers (Helwig et al., 2013).

Paradoxically, APP model mice lacking 7B2 expression (by crossing with 7B2 knockout mice) exhibit a reduction rather than an increase in Abeta plaques (Jarvela et al., 2018). 7B2 null APP model mice also do not exhibit alterations in soluble Abeta, cognition, or memory compared to similar mice expressing 7B2 (Jarvela et al., 2018). These results are reminiscent of similar paradoxical results obtained in various crosses of clusterin knockout mice with APP model mice (DeMattos et al., 2002; Wojtas et al., 2017; Oh et al., 2019) which were attributed to the dominance of clearance effects rather than aggregate formation (Wojtas et al., 2017). We speculate that for both clusterin and 7B2, chaperone loss may directly result in lower extracellular aggregate sequestration through some as-yet undefined mechanism. However, as with clusterin, it is also possible that the loss of 7B2 impacts the expression of other genes, which indirectly causes the observed reduction in plaques. Whether the expression of either proSAAS or 7B2 impacts brain Abeta clearance is not yet known. Similar studies in proSAAS knockout mice (Morgan et al., 2010) have not yet been performed.

Summary

Since proSAAS and 7B2 are secreted from neurons, are associated with protein deposits extracellularly, and (in the case of proSAAS), exhibit increased brain expression during the development of neurodegenerative disease, it is feasible to speculate that these chaperones act extracellularly to perform a protective proteostatic function. Thus, overexpression of these

chaperones represents a potential approach for slowing the progression of AD and PD, and indeed our studies using a rat model of PD support the idea that manipulation of brain proSAAS levels is beneficial to disease outcome (Lindberg et al., 2018). Additional *in vivo* studies to decipher the physiological mechanisms involved in cytoprotection are needed to identify the precise biochemical contribution of these chaperones in disease processes. Similarly, additional *in vitro* structure-function experiments will shed light on the precise regions within each protein that contribute to anti-aggregant function.

SMALL HEAT SHOCK PROTEINS (sHsps)

The sHsp family of chaperones has been frequently reviewed within recent years, even specifically within the context of neurodegeneration (Kourtis and Tavernarakis, 2018; Muranova et al., 2019; Webster et al., 2019; Vendredy et al., 2020). These reviews have amply covered the clearly protective roles of intracellular sHSPs. For the purpose of this review, we will focus on studies relating to secreted sHsps in the context of neurodegenerative disease. Studies implicating extracellular mechanisms of sHsps in neurodegenerative disease fall into the following three categories: disease-associated analyses of biological fluids, such as CSF and plasma; demonstration of immunohistochemical association with extracellular protein aggregates; and direct cellular secretion experiments.

Structure and Localization

All ten members of this family of chaperones (HspBs 1–10) are small proteins of less than 45 kDa and all possess a conserved α-crystallin domain ("ACD") which is both required for chaperone action and responsible for the self-association phenomenon that creates molecular mass polydispersity. This domain is almost always flanked by two other domains; the N-terminal domain is about 50 residues, while the C-terminal domain may be quite short in some family members.

sHSPs are expressed in every tissue, but three family members are especially abundant in brain: HspB1, HspB5 and HspB8; HspB2, HspB3, HspB6, and HspB7 are also detected in brain but at much lower levels (Quraishe et al., 2008; Kirbach and Golenhofen, 2011). Interestingly, these abundant sHsps are predominantly expressed by non-neuronal cells such as glia, rather than by neurons (reviewed in Golenhofen and Bartelt-Kirbach, 2016), where they are found associated with intracellular aggregates in various tauopathies.

Within the cell sHSPs are predominantly located within the cytoplasm but may under certain circumstances be released from cells through various unconventional means that include exosomal and/or endolysosomal secretion, other mechanisms such as tunneling nanotubes, and even direct secretion (recently reviewed in Reddy et al., 2018; Webster et al., 2019). Astrocytes are able to release HspB1 via exosomes (Nafar et al., 2016), and retinal pigment epithelium cells also use exosomes to secrete HspB5 (Sreekumar et al., 2010); indeed, the latter chaperone may be required for exosome synthesis (Gangalum et al., 2016). In contrast, unconventional secretion of this chaperone from COS cells requires the autophagic pathway and is controlled by phosphorylation (D'Agostino et al., 2019). A dynamic relationship between sHsp secretion and extracellular proteostasis has not yet been established.

Function and Mechanism

sHsps are important ATP-independent holdase chaperones that interact with monomers of aggregation-prone proteins to stabilize them in preparation for refolding or disposal. Most sHsps are found in homo-oligomers of 10–20 subunits, composed of dimer subunits interacting via the ACD (Kim et al., 1998). Through partial unfolding, sometimes in response to environmental stressors (Kirbach and Golenhofen, 2011; Alderson et al., 2020), they are able to interact with a variety of client proteins (reviewed in Webster et al., 2019). Supportive of an extracellular role in proteostasis, HspB5, also known as alpha crystallin, αBC, and CRYAB, has long been known as a potent anti-aggregant *in vitro* against a variety of fibrillating proteins; these studies span the last two decades [recently reviewed in Boelens (2020); see also Selig et al. (2020) and Bendifallah et al. (2020) for recent results concerning Abeta and synuclein, respectively].

Considerable evidence implicates sHsp family members in extracellular proteostasis. sHsp chaperones are frequently found associated with both intracellular as well as extracellular protein deposits (reviewed in Hilton et al., 2013; Reddy et al., 2018). Phosphorylation induces structural changes resulting in oligomer dissociation, which can be associated with reduced chaperone capacity (D'Agostino and Diano, 2010). The HspB1 chaperone (also known as Hsp27 in humans and Hsp25 in rodents) is a stress-responsive chaperone which both facilitates folding and acts as an antioxidant; the secretion of this chaperone under various cellular conditions – the majority of which are cancer-related – has been nicely summarized in Reddy et al. (2018). A large number of studies have demonstrated the presence of HspB5 within extracellular brain aggregates, supporting its secretion during proteostatic failure (recently comprehensively reviewed in Muranova et al., 2019; Webster et al., 2019; Vendredy et al., 2020). Extracellular chaperone action may occur through facilitation of the sequestration of Abeta-related species rather than by refolding attempts (Ojha et al., 2011). This phenomenon illustrates the apparent paradox of chaperone trapping within insoluble aggregates. Intracellularly, during stress when proteins become unstable, sHsps form an outer shell composed of dimer subunits that sequester early unfolded intermediates of these proteins in order to preserve their partially folded structure, thus preventing their interaction with one another (Mogk et al., 2019). Intracellular sHsps commonly require ATP-dependent chaperones to resolubilize unfolded "held" proteins; since these same ATP-dependent chaperones are apparently not secreted in sufficient quantities, secreted sHsps may operate mainly to assist extracellular sequestration events rather than assisting unfolding. The exact mechanism for extracellular sequestration is as yet unclear but could be similar to intracellular sequestration. However, client: chaperone ratios likely differ inside and outside the cell, which could impact chaperone shell formation and subsequent core sequestration. A high client: chaperone ratio

may underlie chaperone trapping within protein deposits, whereas a low ratio might result in trapping of toxic oligomers inside the chaperone shell to prevent further aggregation.

In non-neuronal systems, secreted sHSPs appear to exert other extracellular roles, for example as signaling peptides or as peptides involved in immunity and inflammation (Arac et al., 2011; discussed in Reddy et al., 2018); whether this also occurs in brain, and how these other roles might interact with chaperone functions has not yet been established.

Disease Relevance and Therapeutic Applications

Evidence of the involvement of sHSPs in neurodegenerative disease comes from studies of human mutations; for example, mutations in the three family members most abundant in brain, HspB1, HspB3, and HspB8, have been implicated in certain forms of Charcot-Marie-Tooth neuropathy and/or distal hereditary motor neuropathy (Muranova et al., 2019; Vendredy et al., 2020). Renkawek et al. (1999) found increased expression of HspB1 in PD; interestingly, this same group showed that HspB8, also known as Hsp22, is upregulated in brains from AD model mice (Wei, 2020). This chaperone colocalizes with extracellular amyloid deposits found in CAA, a common comorbid condition in AD (Wilhelmus et al., 2009). A recent meta-analysis of protein quality control pathways in the AD brain clearly shows upregulation of sHSPs (Koopman and Rüdiger, 2020). Multiple immunohistochemical studies have shown that sHsps are present within extracellular amyloid plaques (Wilhelmus et al., 2006; Ojha et al., 2011; see Reddy et al., 2018 for review). Coupled with multiple reports of upregulation of sHsp expression in AD and other neurodegenerative diseases (discussed further in Webster et al., 2019) these findings support the idea that sHsps may operate extracellularly within the brain to reduce Abeta toxicity in AD.

With regard to other neurodegenerative diseases, elevated serum levels of HspB1 have been reported during attacks in multiple sclerosis (Ce et al., 2011). However, in a large proteomics meta-analysis of Alzheimer's CSF biomarkers, no sHSPs were identified as differentially expressed in any of the over 40 collated studies (Pedrero-Prieto et al., 2020) indicating that AD progression does not involve alterations in the secretion of sHsps into the CSF. The extracellular (blood) presence of HspB5 has been demonstrated by inference in the form of autoantibodies found in the sera of AD and PD patients (Papuc et al., 2016).

Many studies have shown that overexpression of HspB5 is neuroprotective in a variety of cell and animal model systems (reviewed in Kourtis and Tavernarakis, 2018; Muranova et al., 2019; Webster et al., 2019; Vendredy et al., 2020).

With regard to therapeutic applications, the large number of interacting client proteins renders the notion of specific drug-induced enhancement of a specific aggregating target problematic. However, Rothbard et al. (2019) have recently demonstrated that administration of HspB5 was therapeutic in animal models of multiple sclerosis, retinal and cardiac ischemia, and stroke.

Summary

The sHsps are a group of small chaperones with no energetic requirements for client binding which efficiently bind to a large number of aggregated proteins involved in neurodegenerative disease. While they are clearly secreted (mostly from glia) under certain circumstances, we are only now beginning to understand the non-canonical secretion mechanisms which might allow these abundant cellular proteins to assist in extracellular proteostasis. We clearly also need a more complete understanding of the biochemical mechanisms underlying the extracellular sequestration of aggregating proteins by sHSPs.

GENERAL DISCUSSION

In this review, we have attempted to summarize the available information on select secreted chaperones associated with neurodegenerative disease, using biochemical and genetic evidence to focus on those proteins with the strongest evidence for both proteostatic as well as extracellular actions in neurodegeneration.

Common Mechanisms of Action?

A common theme in many of the chaperones discussed above is the presence of a specific domain functionally similar to the α-crystallin domain which is required for chaperone activity. In sHsps, this is the ACD itself; for clusterin, this may involve residues 286–343, which exhibit 25% similarity with a canonical ACD (Wilson and Easterbrook-Smith, 2000). In BRICHOS domain-containing proteins, and in 7B2 and proSAAS, an interior segment of about 100 residues, with no sequence similarity to α-crystallin, is required for chaperone activity. A similar functional segment has not yet been identified in progranulin. In sHsps, these same sequences also function to promote self-association (Kim et al., 1998; Wyatt et al., 2009). Clusterin, 7B2 and proSAAS also form polydisperse assemblies; we speculate that similarly to sHsps, polydispersity results in the exposure of a range of different intrinsically disordered surfaces, permitting these chaperones to bind diverse clients.

None of the chaperones discussed in this review work together with other ATP-dependent chaperones to refold proteins, but instead appear to act as holdases to bind unstable protein monomers and small oligomers, initially to prevent aggregation/fibrillation, with evidence suggesting both extracellular and intracellular scavenging action (**Figure 1**, left panel). Presumably, when overwhelmed with client, these chaperones act as sequestrases to bind and sequester toxic oligomers, potentially leading to the formation of insoluble protein deposits such as extracellular plaques and intracellular Lewy bodies (**Figure 1**, right panel). These chaperones, like their client counterparts, contain IDRs of varying degrees that essentially enable the holdase and sequestrase activities, and allows them to form functional multimers that can bind to toxic protein species, preventing cellular damage. Support for this mechanism has been presented for clusterin, BRICHOS proteins, and cytoplasmic sHsps (Rohne et al., 2016; Chen et al., 2017; Mogk et al., 2019) and recent work shows similar properties

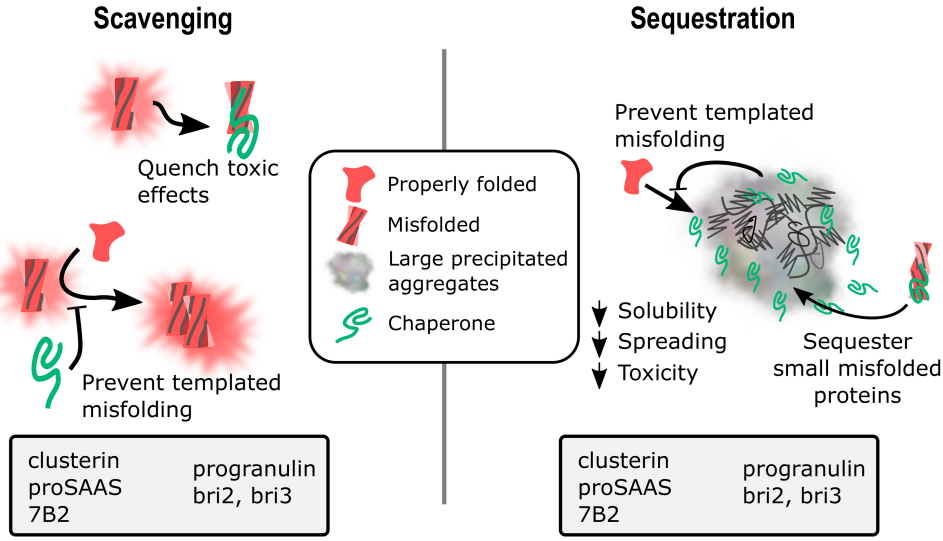

FIGURE 1 | Potential mechanisms for extracellular chaperone action in neurodegenerative disease: Scavenging and sequestration. A schematic diagram showing two proposed mechanisms of action for extracellular chaperone activity on misfolded and aggregating substrates. (1) Scavenging. Chaperones can bind soluble monomers and low molecular weight oligomers to prevent toxic effects from the misfolded protein as well as block the template misfolding/aggregation of natively folded monomers. Chaperones with data showing the ability to block aggregation of misfolded proteins are listed in the gray box. (2) Sequestration. Chaperones can interact with soluble misfolded proteins and drive their recruitment into insoluble aggregates, reducing toxic species solubility, spreading ability, and toxicity. Chaperones can also serve as a buffer to block templated misfolding of natively folded proteins, and may also interact with insoluble aggregates. Chaperones with data showing colocalization with amyloid plaques, neurofibrillary tangles, Lewy bodies, and/or intracellular inclusions are listed in the gray box.

for GRN-C (Bhopatkar et al., 2020) and for proSAAS (Peinado et al., 2020). In addition, the finding of reduced plaque number in AD model mice lacking 7B2 expression (Jarvela et al., 2018) or clusterin (DeMattos et al., 2002; Wojtas et al., 2017) suggests possible roles for 7B2 and clusterin in sequestration events (**Figure 1**, right panel).

Possible mechanisms of chaperone action downstream of misfolded protein sequestration have been identified for clusterin and could serve as plausible pathways for other chaperones. Clusterin mediates the clearance from the plasma (via the liver and kidney) and CSF (via transcytosis across the blood–brain barrier (BBB) of a variety of client proteins (**Figure 2**, panel 1). Clusterin also promotes endocytosis and degradation via non-professional phagocytic cells, through binding cell surface HSPGs (Itakura et al., 2020) (**Figure 2**, panel 2) and via microglial cells by binding the surface receptor TREM2 (Triggering Receptor Expressed on Myeloid Cells 2) (Yeh et al., 2016) (**Figure 2**, panel 3). Similar membrane receptors in brain cells promote extracellular clearance of client-chaperone complexes. These processes would essentially prevent the likely spread of toxic oligomeric species across the blood–brain barrier (**Figure 2**, panel 1) and between neurons (**Figure 2**, panel 4). Of note, transsynaptic transmission of misfolded proteins is increasingly recognized as a major source of spread of pathogenesis across brain regions (Peng et al., 2020).

Remaining Questions

Much additional *in vivo* work is required to document the protective role of each of these chaperones *in vivo*. For example, while there is evidence that intravenous administration of recombinant clusterin in mice can reduce insoluble Abeta levels and diminish hippocampal neuronal loss, clusterin-deficient mice models expressing human APP/presenilin-1 have decreased rather than increased numbers of hippocampal Abeta plaques; similar results were found with 7B2-deficient mice. While these paradoxical results may be partially explained by predominant effects on Abeta clearance, no direct evidence supports this idea; and overexpression of other chaperones, including sHsps, has been shown to be beneficial in animal models of neurodegenerative disease (reviewed in Kourtis and Tavernarakis, 2018; Webster et al., 2019). The cleaved BRICHOS domain has been shown to be anti-amyloidogenic *in vitro*, and cytoprotective *ex vivo* in hippocampal slices and *in vivo* in *Drosophila* models of AD and likely also in transgenic mice expressing fused Bri2-Abeta40/42. However, *in vivo* data from deficient or transgenic mice crossed with mice modeling neurodegenerative disease are lacking for many of the other chaperones discussed here, including proSAAS, BRICHOS domain-containing proteins and progranulin.

Recent studies have identified the transmission of pathological protein aggregates between cells as an important mechanism underlying the progression of a variety of neurodegenerative diseases (reviewed in Peng et al., 2020). Thus, the role of the chaperones discussed here should be examined using experimental paradigms that can discern cell-to-cell transmission *in vivo* (**Figure 2**, panel 4), which have now been published for α-synuclein, Abeta, tau, and TDP-43 (reviewed in Peng et al., 2020). Experiments involving supplementation or depletion of individual chaperones using these spread paradigms

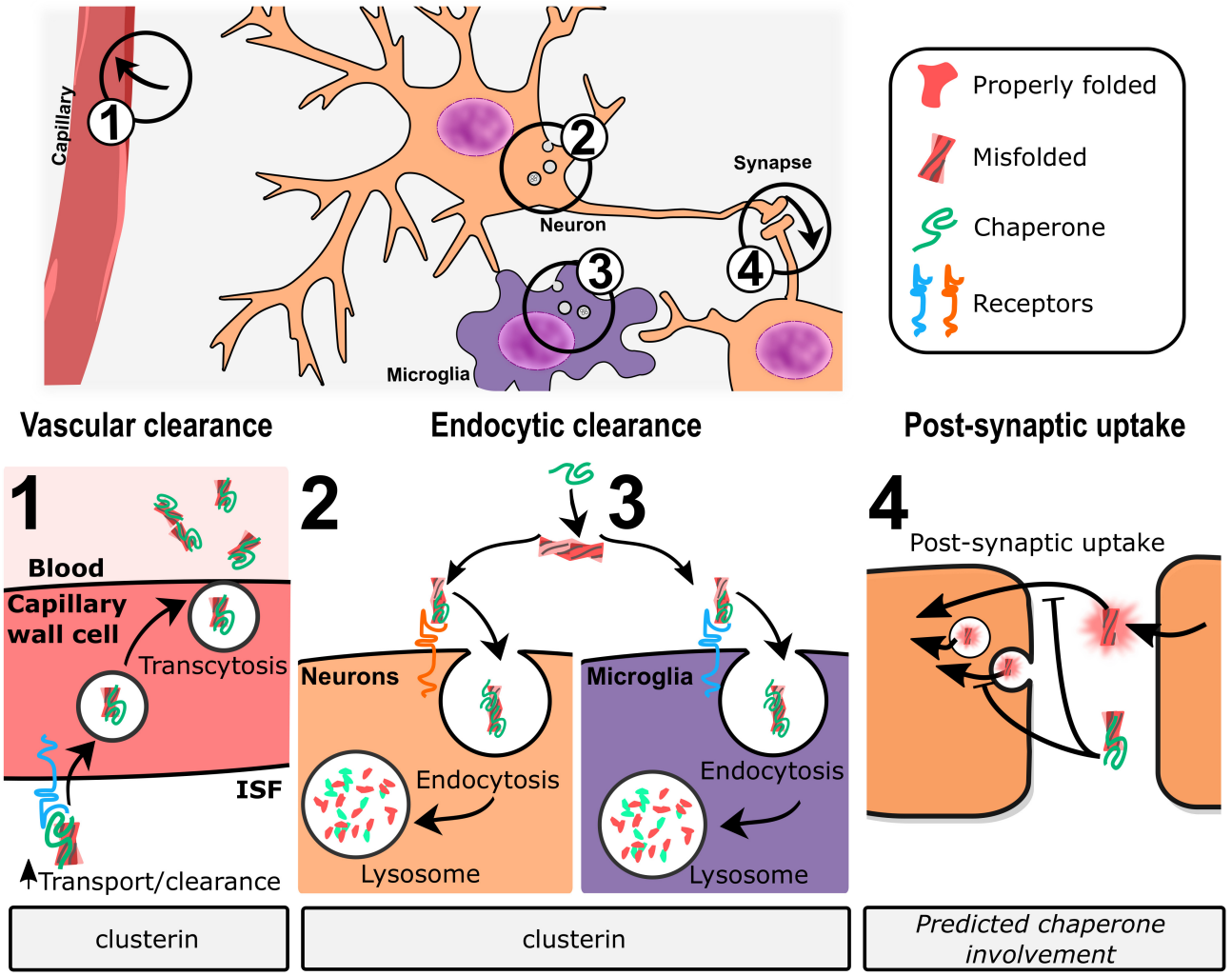

FIGURE 2 | Potential mechanisms for extracellular chaperone action in neurodegenerative disease: Clearance and transmission. A schematic diagram of proposed mechanisms of action of extracellular chaperones activity on transmission and clearance of misfolded and aggregating substrates. (1) Vascular clearance. Chaperone–client complexes are recognized by cell surface receptors on the capillary endothelium for transcytosis from the interstitial fluid (ISF) to the blood for removal. Chaperones that promote clearance across the blood–brain barrier are listed in the gray box. Endocytic clearance by (2) neurons or (3) microglia. Chaperone–client complexes interact with cell surface receptors on to promote the endocytosis and lysosomal degradation of chaperone–client complexes. Chaperones that show increased endocytosis and degradation of proteopathic seeds are listed in the gray box. (4) Post-synaptic uptake. Chaperones bind to secreted proteopathic seeds at the synapse to prevent their uptake (through either direct membrane penetration or endocytosis) by the post-synaptic neuron to reduce cell-to-cell transmission of disease pathology. (Note that no chaperone has yet been directly implicated in this pathway).

should provide new mechanistic insights of extracellular chaperone function.

Another interesting question is the contribution of secreted chaperones to neuronal health under normal conditions. The lack of animal models for many of these chaperones represents a gap in our understanding of their mechanisms of action under healthy conditions. Alternative mechanisms of how these chaperones protect against disease can be obtained by elucidating their normal chaperone functions *in vivo*. Bri2 and Bri3, for example, are known to suppress the cleavage of APP in order to prevent formation of toxic peptides, potentially representing an effective mechanism to delay AD pathology; this may or may not occur during the normal lifespan. For progranulin, its many other bioactivities (pro-growth and anti-inflammatory) are not clearly related to its chaperone roles, which provides an additional complication; however, attempts should be made to tease out its specific chaperone contributions to neuroprotection.

Clear evidence of cytoprotective activity in cell culture and animal disease models warrants the pursuit of many of these chaperones as therapeutics, and indeed several of the chaperones discussed are already being exploited as pharmacologic agents. While different modes of administration, using either gene therapy or peptide therapy (including intravenous or intraperitoneal injections of viral vectors; more targeted routes such as intracerebral inoculations; and nasal sprays) have all been suggested, at present it is not clear which

route might be most effective. In addition to directly increasing chaperone levels, pharmacological agents that indirectly impact either chaperone levels or activity could also be effective, albeit with possible off-target effects.

In summary, while the last decade has resulted in an explosion of information on the mechanism of action of many cellular chaperones, we are only now beginning to understand the biochemical mechanisms involved in extracellular proteostasis. Future work in this area will provide us with a complete appreciation of the likely many redundant mechanisms brain cells employ to carry out extracellular proteostasis over the lifetime.

AUTHOR CONTRIBUTIONS

KC and TJ planned, edited, and wrote the majority of the manuscript. KC and TJ composed the figures and table. IL wrote, supervised, edited, and reviewed the manuscript. All authors contributed to the article and approved the submitted version.

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Mechanisms of Pathogenic Tau and Aβ Protein Spreading in Alzheimer's Disease

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Alzheimer's disease (AD) is pathologically defined by extracellular accumulation of amyloid-β (Aβ) peptides generated by the cleavage of amyloid precursor protein (APP), strings of hyperphosphorylated Tau proteins accumulating inside neurons known as neurofibrillary tangles (NFTs) and neuronal loss. The association between the two hallmarks and cognitive decline has been known since the beginning of the 20th century when the first description of the disease was carried out by Alois Alzheimer. Today, more than 40 million people worldwide are affected by AD that represents the most common cause of dementia and there is still no effective treatment available to cure the disease. In general, the aggregation of Aβ is considered an essential trigger in AD pathogenesis that gives rise to NFTs, neuronal dysfunction and dementia. During the process leading to AD, tau and Aβ first misfold and form aggregates in one brain region, from where they spread to interconnected areas of the brain thereby inducing its gradual morphological and functional deterioration. In this mini-review article, we present an overview of the current literature on the spreading mechanisms of Aβ and tau pathology in AD since a more profound understanding is necessary to design therapeutic approaches aimed at preventing or halting disease progression.

Keywords: Alzheimer's disease, amyloid-β, tau, propagation, spreading

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THE SPREAD OF TAU

The tau protein is a phosphoprotein that is codified by alternative splicing of the microtubule-associate protein tau (MAPT) gene (Goedert et al., 1988, 1989; Andreadis et al., 1992; Andreadis, 2005; Pittman et al., 2006) and is enriched in axons of mature neurons where it regulates microtubule stability to ensure proper cytoskeletal organization and trafficking (Aamodt and Williams, 1984; Aronov et al., 2001; González-Billault et al., 2002; Zhang et al., 2004; Bertrand et al., 2013). Of all different post-translational modifications that tau can undergo, the phosphorylation is of particular interest because of its involvement in a group of neurodegenerative disorders known as tauopathies (Goedert and Spillantini, 2011; Arendt et al., 2016), including

Alzheimer's disease (AD). Indeed, whereas phosphorylation is fundamental for tau function under physiological conditions, the affinity of tau for tubulin decreases under pathological conditions, and the protein starts to accumulate in the cytosol of the somatodendritic compartments where insoluble structures are built. Those neurofibrillary tangles (NFTs) disturb the microtubule network and alter the normal axoplasmatic flow, which in turn compromises the functions and viability of neurons (Brion et al., 1985; Wood and Zinsmeister, 1989; Gastard et al., 2003). In AD, the development of NFTs evolves in the brain with a predictable and hierarchical distribution pattern that starts from layer II of the entorhinal cortex, spreads through the limbic and associations areas to finally reach the hippocampus and neocortex (Braak and Braak, 1991). Pathological tau can distribute from one cell to another thereby propagating the pathology from affected to interconnected healthy areas of the brain, implicating similar mechanisms than in prion diseases (Liu et al., 2012; Jucker and Walker, 2013). A large number of pieces of evidence support a prion-like model for tau spreading, consisting of abnormal proteins with the capacity to convert normal proteins into a pathological form. The inoculation of brain extracts from mice or humans with tauopathy into the brain of wild-type animals induced tau pathology in recipient animals and its propagation from the site of injection along neuronal connections (Clavaguera et al., 2009, 2013; Lasagna-Reeves et al., 2012; Ahmed et al., 2014; Guo et al., 2016; Gibbons et al., 2017; Narasimhan et al., 2017; Smolek et al., 2019a,b). Similar results were obtained when synthetic tau fibrils were injected into young mice overexpressing mutant human tau (P301S) which resulted as well in the formation of NFT-like inclusions that propagated from the injected sites to connected brain regions in a time-dependent manner (Iba et al., 2013). Experiments of human tau viral induction in cortical neurons in young *vs* old mice showed age- and brain region-dependent misfolding and spreading of tau (Wegmann et al., 2019). Furthermore, *in vitro* studies showed, that extracellular aggregates of tau can be internalized by naïve cells promoting fibrillization of intracellular tau that can be transferred between co-cultured cells (Frost et al., 2009; Guo and Lee, 2011, 2013) also *via* synaptic contacts between neurons that facilitate pathology propagation (Calafate et al., 2015). Tau propagation was extensively studied and different mechanisms involved in trans-cellular diffusion were described. The prion-like propagation implies an active and regulated passage of tau in the extracellular space (secretion) and a mechanism of tau uptake by an adjacent recipient cell, although the passive release of tau from dying cells cannot be excluded as an alternative scenario.

Tau filaments can exist in the brain as a variety of distinct conformational strains associated with various tauopathy phenotypes and different rates of network propagation (Sanders et al., 2014; Guo et al., 2016; Kaufman et al., 2016; He et al., 2020). Although the distribution of tau with different conformations can be suggested as cause also of AD heterogeneity, high-resolution analysis of tau structures by using cryo-electron microscopy recently revealed no significant variation in tau filament structures within and between

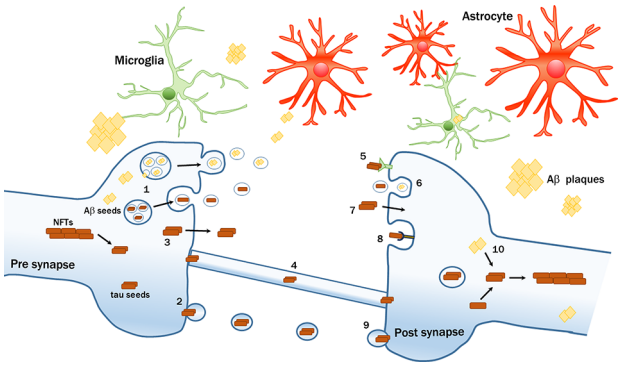

FIGURE 1 | Intercellular transmission of pathological amyloid-β (Aβ) and tau proteins. Seeds of pathological proteins can be released at the presynaptic level: (1) in exosomes after the fusion of Multivesicular bodies (MVBs) with plasma membrane (PM); (2) in larger vesicles called ectosomes; (3) as naked protein freely crossing the PM; or (4) can be transferred *via* tunneling nanotubes. Mechanisms of up-take by recipient neurons include; (5) receptors-mediated endocytosis; (6) bulk-endocytosis; (7) fluid-phase translocation; (8) macropinocytosis mediated by heparan sulfate proteoglycans (HSPGs); and (9) fusion of large tau-containing large vescicles with PM. (10) Intraneuronal Aβ seeds can trigger or enhance the formation of tau pathological aggregates. The transmission process can be modulated by multiple factors, including glial cells.

the brains of individuals with AD (Fitzpatrick et al., 2017; Falcon et al., 2018).

Tau can be actively secreted by neurons following three main routes (**Figure 1**):

First, at the presynaptic level, tau can be packed into microvesicles and further released by a process (Simón et al., 2012; Fontaine et al., 2016), that is modulated by neuronal electrical activity (Lachenal et al., 2011; Pooler et al., 2013; Yamada et al., 2014; Sokolow et al., 2015; Wang et al., 2017). Following this route, phosphorylated tau is internalized by cytoplasmic exosomes, so-called intraluminal vesicles formed in multivesicular bodies (MVBs) that are finally released into the extracellular space after MVBs fusion with the plasma membrane (PM; Saman et al., 2012). Alternatively, tau can also be internalized in ectosomes, larger vesicles (100–500 nm in diameter) that are formed by evaginations of the PM incorporating tau (Dujardin et al., 2014). These routes are unconventional secretion pathways since they do not involve signal peptides and exclude the endoplasmic reticulum (ER)-Golgi system. Extracellular vesicles containing phospho-tau were found in the brains of transgenic mice (Baker et al., 2016; Polanco et al., 2016) and in peripheral fluids such as blood or CSF of AD patients (Saman et al., 2012; Fiandaca et al., 2015; Winston et al., 2016).

Second, the majority of tau is found extracellularly as a membrane-free form. Soluble hyperphosphorylated tau can translocate directly across the PM (Plouffe et al., 2012; Pooler et al., 2012) upon interaction with phosphatidylinositol 4,5 phosphate PI(4,5)P_2 cholesterol and sphingolipids. The penetration and release are facilitated by the binding with heparan sulfate proteoglycans (HSPG) on the cell surface (Katsinelos et al., 2018; Mari et al., 2018; Merezhko et al., 2018).

Other studies suggested that tau is secreted *via* the fusion of vesicles from ER or Golgi with the PM (Ponnambalam and Baldwin, 2003). Tau was also shown to form pore-like structures in the PM that operate like channels for the passage of pathogenic proteins, a feature that can be regulated either by pathological mutations, by tau oligomerization (Lasagna-Reeves et al., 2014; Patel et al., 2015; Merezhko et al., 2018), or by specific sequences of human tau that act as binding motifs to facilitate the secretion of pathological tau (Sayas et al., 2019).

Third, another mechanism that has also been proposed involves the passage through tunneling nanotubes, filamentous actin-containing channels that connect adjacent cells, and transport proteins intercellularly (Abounit et al., 2016; Tardivel et al., 2016).

Once released, tau can be internalized by recipient cells (**Figure 1**). Intracranial or peripheral administration of pathological tau (Clavaguera et al., 2014; Mudher et al., 2017) together with *in vitro* experiments have shown that tau is mainly internalized by active endocytic processes (Frost et al., 2009; Wu et al., 2013). In particular, three kinds of endocytosis were described:

Bulk-endocytosis represents the first one, where a large portion of presynaptic PM is internalized in a dynamin-dependent manner in form of vacuoles or endosomes from which multiple synaptic vesicles can subsequently be generated (Takei et al., 1996; Wu et al., 2013).

The second, actin-dependent macropinocytosis is mediated by HSPGs on the cell surface (Holmes et al., 2013; Rauch et al., 2018; Weisová et al., 2019). Recently, the silencing of the low-density lipoprotein receptor-related protein-1 (LRP1), which works in conjunction with HSPGs, was shown to block the uptake of tau oligomers *in vitro* and reduced *in vivo* the propagation of tau between neurons (Rauch et al., 2020). Other receptors can also be involved in the uptake of pathogenic tau, such as the extracellular portion of amyloid precursor protein (APP; Takahashi et al., 2015) and muscarinic receptors (Morozova et al., 2019). Recently, the cellular prion protein was also shown to act as a receptor that facilitates the uptake of tau aggregates by cultured cells (De Cecco et al., 2020).

Finally, clathrin-mediated endocytosis was also proposed as mechanism (Evans et al., 2018), but is still under debate because the use of specific clathrin inhibitors or its silencing resulted in continued tau aggregate uptake (Calafate et al., 2016).

In general, the different mechanisms of secretion and internalization depend largely on the cell types, the size, and the different tau species involved (Dujardin et al., 2018; Evans et al., 2018). Once tau is internalized, it can escape the endosomal vesicles inducing their rupture (Calafate et al., 2016; Flavin et al., 2017) and accumulates in the cytoplasm where it becomes a potential template for the misfolding of tau (Clavaguera et al., 2009; Guo et al., 2016; **Figure 1**). Although the biochemical mechanisms driving the conversion of normal tau into the pathological form are still unclear, different models of tau seeding were proposed (Congdon et al., 2008; Mirbaha et al., 2018).

Glial cells such as astrocytes (Martini-Stoica et al., 2018; Perea et al., 2019), oligodendrocytes (Narasimhan et al., 2017) and in particular microglia were implicated in tau spreading (Asai et al., 2015; Maphis et al., 2015; Hopp et al., 2018). Recently, a study demonstrated that microglia isolated from AD cases and mouse models of tauopathy contain tau seeds that can be released into the medium (Hopp et al., 2018), proposing that microglia can uptake tau but not to completely digest it, thus representing a possible source for tau spreading. The ability of microglia to engulf tau aggregates was already documented by different *in vitro* and *in vivo* studies (Luo et al., 2015; Bolós et al., 2016, 2017). Moreover, microglia depletion was shown to prevent tau propagation. Microglia phagocytosed and released tau-containing exosomes whereas inhibiting exosome synthesis significantly reduced tau propagation *in vitro* and *in vivo* (Asai et al., 2015). Finally, increased microglial activation has been reported not only to accelerate tau pathology and behavioral abnormalities in the human Tau mouse model of tauopathy (Bhaskar et al., 2010; Bemiller et al., 2017; Ising et al., 2019), but also its propagation in the brain (Maphis et al., 2015). Together, these studies highlight the involvement of microglia in spreading tau pathology.

THE SPREAD OF Aβ

Aβ is a proteolytic product of APP, that is highly expressed in neurons and physiologically involved in many functions such as regulation of neurite outgrowth and axonal guidance, regulation of synaptic functions and plasticity, involvement in early nervous system development and in neuroprotection (Van den Heuvel et al., 1999; Leyssen et al., 2005; Priller et al., 2006; Young-Pearse et al., 2008; Mueller et al., 2018). APP can be processed in two different ways: in the non-amyloidogenic pathway, APP is cleaved first by α- followed by γ-secretase that cuts the protein within the Aβ domain. In the amyloidogenic pathway, APP is consecutively cut by β- and γ-secretase to be finally released extracellularly as Aβ fragments of different lengths, but mainly consist of 40 (Aβ1–40) or 42 (Aβ1–42) amino acids (Haass et al., 2012). Once released, monomeric Aβ can aggregate into different assemblies giving origin to oligomers, protofibrils, and amyloid fibrils that are insoluble and can further aggregate into amyloid plaques, while monomeric and oligomeric forms of Aβ are soluble. These different states of Aβ coexist in the AD brain making it difficult to dissect the most relevant and toxic forms concerning pathogenesis. Albeit *in vivo* studies demonstrated that Aβ plaques lead to neuronal loss, neuronal dystrophy and alters their normal neuritic functionality (Meyer-Luehmann et al., 2008, 2009; Shah et al., 2010), different studies have identified soluble oligomeric Aβ species as the toxic drivers responsible for synaptic dysfunction, in particular in the early stage of the disease (Lambert et al., 1998; Shankar et al., 2008; Koffie et al., 2009; Forloni et al., 2016). The fact that the incidence of senile plaques increases with age even in healthy subjects and that the number of plaques often does not correlate with neuronal loss and cognitive decline (Katzman, 1988; Villemagne and Rowe, 2011) nourishes the hypothesis that compact plaques may sequester toxic Aβ oligomers until they reach a saturation point (Esparza et al., 2013; Selkoe and Hardy,

2016). Although the ultimate proof for a causal relationship between fibrillar aggregates and neurodegenerative diseases has not been delivered yet, the "amyloid cascade hypothesis" is still the most prevalent theory (Hardy and Higgins, 1992; Hardy and Selkoe, 2002) with the constrain that Aβ alone is most likely not able to cause the entire damage found in AD patient brains (Ricciarelli and Fedele, 2017). In addition to their different aggregation state, Aβ was detected in the brain of AD patients in distinct misfolded strains with specific propagation properties (Qiang et al., 2017; Condello and Stöehr, 2018), suggesting that also these structural variations may modulate the disease phenotype.

Unlike tau that spreads in a highly predictive pattern as anticipated by computational systems (Fornari et al., 2019), Aβ deposition does not always follow a stereotypic spatio-temporal pattern of progression. Nevertheless, amyloid plaques in general first appear in the neocortex from where they spread into the allocortex and the subcortical regions (Thal et al., 2002; Serrano-Pozo et al., 2011; Grothe et al., 2017). As has been described above for tau, several studies on intracerebral injections of Aβ-rich brain extracts either from AD mice or patients propose that Aβ aggregation can be initiated by prion-like seeding (Kane et al., 2000; Meyer-Luehmann et al., 2006; Eisele et al., 2009; Jucker and Walker, 2013; Ziegler-Waldkirch et al., 2018; Friesen and Meyer-Luehmann, 2019; Katzmarski et al., 2020). These misfolded protein assemblies act as seeds of aggregation to accelerate the polymerization processes of normal proteins (Harper and Lansbury, 1997) that can expand from the injection site to distant regions as well as the contro-lateral side of the brain, thus suggesting a possible spread of seeded pathology *via* neuronal transport along axonally interconnected brain regions (Walker et al., 2002; Rönnbäck et al., 2012; Domert et al., 2014; Ye et al., 2015).

Aβ peptides are collected in intraluminal vesicles within MVBs and, upon fusion with the PM, the intraluminal vesicles are released into the extracellular space as exosomes (Rajendran et al., 2006; Sharples et al., 2008; Hu et al., 2009; **Figure 1**). Furthermore, a recent study reported that Aβ-rich exosomes isolated from AD patients can act as vehicles for cell-to-cell transfer of such toxic species in recipient cultured neurons (Sinha et al., 2018). However, even though the cell-to-cell passage of Aβ represents a plausible hypothesis, substantiated by the fact that the protein is found inside neurons (Wertkin et al., 1993; Turner et al., 1996; Gouras et al., 2000; LaFerla et al., 2007), there is no conclusive evidence for active transport of Aβ along neurons. Transplantations of WT neurons into brains of pre-depositing AD mice revealed that Aβ from the transgenic host tissue can enter and deposit within WT grafts (Meyer-Luehmann et al., 2003; Bachhuber et al., 2015; Espuny-Camacho et al., 2017), thus suggesting a possible passive extracellular diffusion of Aβ from the outside to the inside of the grafts. Although over the last few years many different groups have described glial cells as an alternative source of Aβ (Siman et al., 1989; Joshi et al., 2014) or their involvement in the formation of amyloid plaque deposition (Wisniewski et al., 1990; Venegas et al., 2017; Spangenberg et al., 2019), there are to date no studies that implicate a direct involvement of glial cells in Aβ trafficking across different areas of the brain. Since Aβ40 and Aβ42 circulate in body fluids such as plasma and CSF (Mehta et al., 2000), another potential route of Aβ diffusion from the periphery to the brain may be constituted by the vascular system. Indeed, intraperitoneal or intravenous administration of Aβ-rich extracts in pre-depositing APP23 mice promoted cerebral amyloid angiopathy (CAA; Eisele et al., 2010; Burwinkel et al., 2018) pointing again to a vascular component of circulating immune cell involvement in the spread of Aβ seeds (Cintron et al., 2015).

PROTEIN CROSS-SEEDING

It is well known that different neuropathological lesions such as Aβ, NFTs, or Lewy bodies can co-exist in the brains of AD patients (Braak and Braak, 1997; Hamilton, 2000), predicting cross protein interactions. Indeed, several studies have shown that the interaction between Aβ and tau can exaggerate AD pathology (Ribé et al., 2005; Bennett et al., 2017; He et al., 2018; Vergara et al., 2019) and that amyloid deposition, preceding the NFT formation, can actively influence tau spreading to neocortical regions (Braak and Braak, 1997; Hardy and Selkoe, 2002; Jacobs et al., 2018; Vogel et al., 2020). Furthermore, oligomeric forms of Aβ were found to be abundant in synapses of AD patients early in the disease before the appearance of phospo-tau at later stages, suggesting that soluble Aβ oligomers in synaptic terminals are associated with dementia onset and may initiate a cascade that drives phosphorylated tau accumulation and its synaptic spread (Bilousova et al., 2016).

Nevertheless, the finding that Aβ and tau deposition starts in different brain areas and follows distinct temporal sequences, argues against the idea that tau pathology may be driven exclusively by the presence of amyloid and rather speaks for an Aβ independent pathway (Raj et al., 2015; Jack et al., 2019; van der Kant et al., 2020). Tau aggregation assays with tau isolated from patients containing both lesions showed an enhanced ability to induce tau aggregates when compared to tau isolated from human cases without plaques (Bennett et al., 2017). Similar results have been obtained in double-transgenic mice overexpressing both, mutated APP and tau (Lewis et al., 2001; Hurtado et al., 2010). Furthermore concurrent cortical amyloid deposition in double transgenic mice strongly accelerated interneuronal transfer of tau and boosted its spreading to distal brain regions with an increase in neuronal loss (Hurtado et al., 2010; Pooler et al., 2015).

Intracerebral infusion of Aβ-rich extracts into tau-transgenic mice resulted in significantly more NFT formation compared to tau-rich or WT extracts (Götz et al., 2001; Bolmont et al., 2007; Vasconcelos et al., 2016) indicating that the presence of Aβ triggers the formation of tau pathology and proposing synergistic toxicity on the neuronal network. Inoculation of human AD-tau extracts into the brains of APP transgenic mice that normally do not form NFTs resulted in rapid fibrillization of endogenous tau (Bennett et al., 2017; He et al., 2018). Moreover, ipsi- and contralateral tau propagation was enhanced in tau-injected 5xFAD mice compared to tau-injected WT mice (Vergara et al., 2019).

Transgenic APPPS1+Tau mice, that express WT human tau within a mouse tau-deficient background showed that Aβ and tau can synergistically cooperate to cause a hyperactivity behavioral phenotype and resulted in a downregulation of genes involved in synaptic function (Pickett et al., 2019). Treatment strategies that aim at reducing tau levels in mice that co-express Aβ and human tau prevented neuronal loss (DeVos et al., 2018) as well as excitotoxin-induced neuronal dysfunctions (Roberson et al., 2007) and ameliorated the behavioral and gene expression phenotypes (Pickett et al., 2019). Together, these results demonstrate the therapeutic benefit of tau reduction with a positive impact on the Aβ-induced cytotoxic effects.

Despite all these observational evidence, the mechanism of Aβ and tau interplay remains largely unknown. Further studies are necessary to unravel whether it is a direct interaction between the two pathogenic proteins or instead mediated by other factors.

CONCLUSIONS AND FUTURE PROSPECTIVE

Two of the most remarkable features of AD are: (i) the stereotypic pattern of Aβ and tangle spreading through interconnected areas of the brain (Braak and Braak, 1991) that is closely related to cognitive decline years before the onset of clinical symptoms; and (ii) the ability of pathogenic misfolded Aβ and tau to serve as templates to convert their innocuous counterparts into toxic forms in a prion-like manner (Clavaguera et al., 2009; Jucker and Walker, 2011, 2013). These two aspects, together with the fact that the two hallmarks often coexist in the brain of AD patients and amplify each other's toxic effects downstream (Ittner and Götz, 2011), make the development of an effective therapy challenging. Currently, clinical trials targeting Aβ have reported limited success, implying the notion that the timing of intervention is too late and that directing to only one target might be not sufficient to halt the spreading of both pathogenic proteins and to avoid their synergistic impact on neuronal networks. Furthermore, abundant pieces of evidence have recently highlighted the role that different strains of tau and Aβ may play in modulating the clinical picture of the disease, turning away the possibility to develop therapies against different AD subtypes. For this purpose, a better understanding of the conformational heterogeneity of tau and Aβ is necessary to better design the best intervention methods.

Although in the last years many molecular and cellular mechanisms involved in the formation, aggregation, deposition, and propagation of Aβ and tau were uncovered, many questions remain still open. Which mechanisms guide Aβ diffusion? What is the exact sequence of events leading to clinical symptoms? Which role might other cellular systems or molecular pathways play in promoting the pathological bond of Aβ and tau? These are only some of the questions that need to be clarified to design the best strategies to finally arrest disease progression.

AUTHOR CONTRIBUTIONS

Pd'E and MM-L wrote the manuscript. Pd'E and MM-L read and approved the final manuscript.

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frontiers in Aging Neuroscience

REVIEW
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On the Right Track to Treat Movement Disorders: Promising Therapeutic Approaches for Parkinson's and Huntington's Disease

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Movement disorders are neurological conditions in which patients manifest a diverse range of movement impairments. Distinct structures within the basal ganglia of the brain, an area involved in movement regulation, are differentially affected for every disease. Among the most studied movement disorder conditions are Parkinson's (PD) and Huntington's disease (HD), in which the deregulation of the movement circuitry due to the loss of specific neuronal populations in basal ganglia is the underlying cause of motor symptoms. These symptoms are due to the loss principally of dopaminergic neurons of the substantia nigra (SN) par compacta and the GABAergic neurons of the striatum in PD and HD, respectively. Although these diseases were described in the 19th century, no effective treatment can slow down, reverse, or stop disease progression. Available pharmacological therapies have been focused on preventing or alleviating motor symptoms to improve the quality of life of patients, but these drugs are not able to mitigate the progressive neurodegeneration. Currently, considerable therapeutic advances have been achieved seeking a more efficacious and durable therapeutic effect. Here, we will focus on the new advances of several therapeutic approaches for PD and HD, starting with the available pharmacological treatments to alleviate the motor symptoms in both diseases. Then, we describe therapeutic strategies that aim to restore specific neuronal populations or their activity. Among the discussed strategies, the use of Neurotrophic factors (NTFs) and genetic approaches to prevent the neuronal loss in these diseases will be described. We will highlight strategies that have been evaluated in both Parkinson's and Huntington's patients, and also the ones with strong preclinical evidence. These current therapeutic techniques represent the most promising tools for the safe treatment of both diseases, specifically those aimed to avoid neuronal loss during disease progression.

Keywords: Parkinson's disease, Huntington's disease, neurotrophic factors, pharmacological therapy, gene modifiers, cellular replacement

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INTRODUCTION

Movement disorders are characterized by disabilities in speed, fluency, quality, and ease of motor execution, impairments that could be due to an excess or lack of voluntary movements (Shipton, 2012). Movement is produced by the coordinated action of several cortical and subcortical brain structures such as the spinal cord, brainstem, cerebral cortex, cerebellum, and basal ganglia, which collectively fine-tune voluntary and involuntary movements (Ferreira-Pinto et al., 2018). Particularly, the basal ganglia structure comprises a group of subcortical nuclei including the striatum (subdivided in most mammals in caudate and putamen), internal globus pallidus (GPi) and external globus pallidus (GPe), substantia nigra (SN) pars reticulata (SNpr) and compacta (SNpc), and subthalamic nucleus (STN), that together with the primary motor cortex and the thalamus, comprise the motor circuit involved in the control of voluntary movement (Albin et al., 1989; Obeso et al., 2008; Calabresi et al., 2014). The striatum is composed of medium spiny neurons (MSNs), a type of GABAergic neurons representing 90–95% of striatal neurons (Dubé et al., 1988), cholinergic and GABAergic interneurons (Lapper and Bolam, 1992). MSNs are innervated by glutamatergic (excitatory) inputs from the cortex and thalamus, together with dopaminergic inputs from the SN (Pickel et al., 1992). In turn, MSNs differentially express dopamine (DA) D1 or D2 receptors, which determine their participation in two motor circuits: the direct or indirect pathways. The direct pathway is composed of the striatum, GPi, SNpr, thalamus, and motor cortex, promoting the activation of movement through the inhibition of the GPi, in addition to the consequent disinhibition of the thalamus. The indirect pathway, formed by the striatum, GPe, STN, SNpr, thalamus, and motor cortex favors the inhibition of movement by activating the STN with the consequent inhibition of the thalamus (**Figure 1A**; Calabresi et al., 2014). Alterations in these brain regions are associated with a spectrum of abnormal movement disorders. Among the most studied movement disorders are Parkinson's (PD) and Huntington's disease (HD; **Figures 1B,C**), which are the main focus of this review.

PD is the second most common chronic and progressive neurological disorder with no effective cure (Emamzadeh and Surguchov, 2018) PD incidence is currently around 200 in 100,000 persons (Ball et al., 2019) and the number of PD patients is expected to reach 10 million by 2030 (Dorsey et al., 2007; Ball et al., 2019). PD is mainly an idiopathic disorder with multifactorial etiology, with aging being one of its main risk factors (Hou et al., 2019). Nevertheless, genetic factors have been associated with 5–10% of PD cases (Kim and Alcalay, 2017). Clinical features of PD are a combination of motor symptoms including muscle rigidity, gait difficulty, postural instability, bradykinesia, and tremor at rest (Rees et al., 2018). Also, non-motor symptoms include sensory and sleep alterations, constipation, cognitive impairment, dementia, anxiety, depression, and mood disorders at an early stage of the disease (Munhoz et al., 2015).

One of the two neuropathological criteria required for the diagnosis of PD is the progressive loss of dopaminergic neurons within the SNpc of the basal ganglia (**Figure 1B**; Agid, 1991; Hirsch et al., 1999). However, not all dopaminergic neurons are equally vulnerable: those that project their axons to the putamen are more vulnerable than those that project their axons to cognitive areas. The second pathological criteria is the presence of α-synuclein (α-syn)-positive inclusions accumulated in Lewy bodies in neurons (Double, 2012). Although the main hallmarks of PD are well described, diagnosis before classic clinical features occur is not currently achievable. Motor symptoms are observed when 70% of the striatal dopaminergic neurons terminals are lost and half of the dopaminergic neurons in the whole brain have degenerated (Double, 2012; Surmeier et al., 2017; Fu et al., 2018).

The current PD treatment is a pharmacological therapy that increases DA levels to provide symptomatic relief. Unfortunately, this therapeutic strategy has limited efficacy. However, new therapeutic alternatives under development attempt to modify the pathology of PD by increasing DA production or improving neuronal health. These approaches efficiently delay neurodegeneration as well as PD symptoms in preclinical models and patients. Currently, finding new treatments that modify pathological PD progression, prevent the dopaminergic neuronal loss, counteract aberrant neuronal activity, and delay the appearance of motor symptoms is the principal goal of many investigations.

HD is the world's most common monogenic neurological disorder (Huntington's Disease Collaborative Research Group, 1993), characterized by its autosomal dominant inheritance, midlife onset and progressive course with a combination of motor, cognitive and behavioral features. HD is caused by a mutation in the gene that encodes for the protein huntingtin (HTT), which leads to an expanded CAG trinucleotide, causing an abnormally long polyglutamine (polyQ) tract in HTT. This repetition ranges between 6–35 glutamine units in the normal population. When this tract is $\geq$40 glutamines long, the mutation is highly penetrant, triggering a disease process that leads to the onset of motor symptoms. Mutant HTT (mHTT) exhibits gain-of-toxic properties, causing dysfunction and death of GABAergic MSNs of the striatum, which is particularly vulnerable to mHTT toxicity (**Figure 1C**). Once signs and symptoms begin, they progress inexorably throughout the illness, which is inevitably fatal, with a median survival from motor onset of 18 years (Ross et al., 2014).

We describe current pharmacological therapies for both diseases, cellular replacement strategies to restore lost neuronal populations, administration of Neurotrophic factors (NTFs) to increase neuronal viability and health, electrical neuromodulation to restore movement circuitry lost, and genetic approaches to decrease mHTT and α-syn levels. We focused on studies that have reached clinical testing, highlighting preclinical evidence that supports those clinical trials. The clinical trials mentioned throughout this review are summarized in **Tables 1**, **2**. For those strategies in which clinical trials have not been performed, we present the current state of investigations and remark the important drawbacks that must be solved before these therapeutic approaches jump into clinical trials.

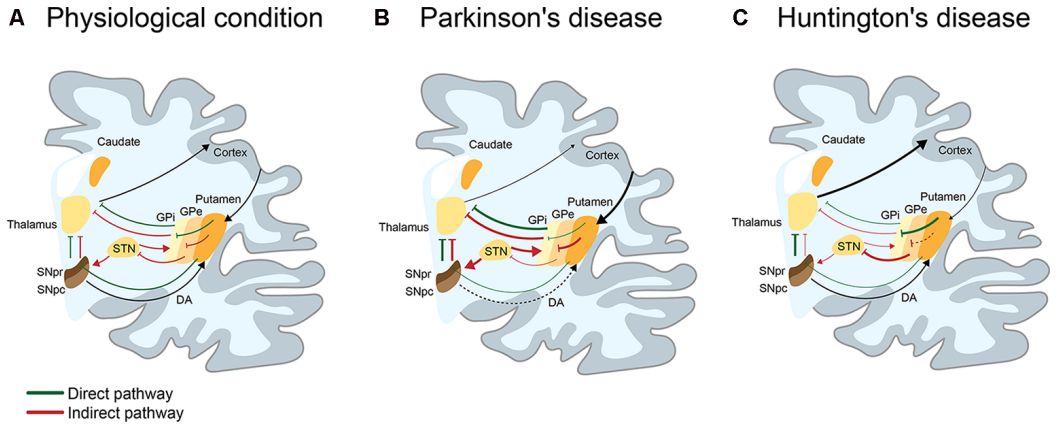

FIGURE 1 | I Schematic representation of the direct and indirect pathways in basal ganglia in physiological, Parkinson's (PD), and Huntington's disease (HD) conditions. **(A)** Physiologically, the direct (green line) pathway participates in the activation of movement. This pathway is engaged when the activation of the cortex produces a release of glutamate into the striatum, activating GABAergic medium spiny neurons (MSNs) of the direct pathway. By releasing GABA to the substantia nigra pars reticulata (SNpr) and the internal globus pallidus (GPi), MSNs inhibit neurons of the SNpr/GPi that are also GABAergic. This causes activation of the glutamatergic neurons present in the thalamus, which projects to the cortex, resulting in the activation of movements. On the contrary, the indirect pathway (red line) participates in the inhibition of movement. When GABAergic MSNs that indirectly project to the SNpr through the external globus pallidus (GPe) and the subthalamic nucleus (STN), release GABA into the GPe, inhibits GABAergic neurons present in the GPe. This leads to the disinhibition of the glutamatergic neurons of the STN, which activates GABAergic neurons of the SNpr/GPi. These neurons inhibit neurons present in the thalamus, resulting in a reduction of movement. The selection and execution of movement reflect a dynamic balance between both pathways. **(B)** In PD, the loss of dopaminergic neurons of the SNpc, induces an overactivation of the indirect pathway and decrease of movement. Consequently, there is an increase of GABAergic activity of the GPi/SNpr over thalamic neurons that project to the cortex, leading to loss of movement (hypokinetic disorder). **(C)** In HD (early stage), MSNs of the indirect pathway appear to be affected before the MSNs of the direct pathway. This induces an increase of GABAergic (or inhibitor) activity of the GPe over the STN, which causes the loss of inhibitory activity of the GPi/SNpr over thalamic neurons that project to cortex, leading to the appearance of choreic movements (hyperkinetic disorder).

CURRENT PHARMACOLOGICAL TREATMENTS TO ALLEVIATE MOTOR SYMPTOMS IN PD AND HD

Currently, no approved drugs can modify the progression of either PD or HD. Available pharmacological therapies only aim to treat motor symptoms to improve the quality of life of patients but, ultimately, these drugs do not mitigate the progressive neurodegeneration.

For PD, pharmacological approaches are designed to reestablish DA levels through: (i) increasing DA availability using DA precursors like levodopa (L-dopa) or dopaminergic agonists like pramipexole, and (ii) inhibiting DA degradation by monoamine oxidase B inhibitors [MAO-BI, like selegiline (Eldepryl®) and rasagiline (Azilect®)] or catechol-O-methyl transferase inhibitors [COMTI, like entacapone (Comtan®)] and tolcapone (Tasmar®; Van de Schyf, 2015; Teijido and Cacabelos, 2018; Carrera and Cacabelos, 2019). A caveat in the chronic administration of anti-parkinsonian drugs is the *"wearing-off"* phenomenon, which produces additional psychomotor and autonomic complications, like levodopa-induced dyskinesia (LID; Fahn et al., 2004; Stacy, 2009; Ammal Kaidery et al., 2013; Cacabelos, 2017). Moreover, L-dopa pharmacokinetics is unpredictable and commonly leads to administration increase, complex regimens, and poor patient compliance. Nevertheless, L-dopa remains as the gold standard pharmacological intervention for motor symptoms in PD patients (Oertel and Schulz, 2016; Fox et al., 2018). Although the pathophysiology of *wearing-off* and dyskinesia is complex and not completely understood, it appears to be linked to the short plasma half-life of L-dopa, as short-acting dopaminergic drugs can induce alterations in brain DA concentrations which lead to motor dysfunction (Olanow et al., 2006; Rajan et al., 2017). To assess this problem, strategies that prolong L-dopa plasma half-life have been developed, including the administration of COMTI. A significant quantity of orally administered L-dopa is metabolized to 3-O-methyldopa (a useless metabolite) by COMT in the gastrointestinal tract. By inhibiting COMT, more L-dopa will be absorbed, increasing its bioavailability and extending its half-life (Oertel and Schulz, 2016). Some of these drugs are tolcapone, entacapone, and opicapone (Parkinson Study Group, 1997; Rinne et al., 1998; Poewe et al., 2002; Cacabelos, 2017). However, the FDA has restricted the use of tolcapone due to hepatic necrosis leading to death (Haasio et al., 2001). Common combinations include L-dopa/carbidopa (an L-dopa decarboxylation inhibitor; Sinemet®) and L-dopa/benserazide (Madopar®), looking to prevent systemic adverse effects related to the peripheral metabolism of L-dopa to DA, including nausea, dyskinesia, motor fluctuations, hypotension, psychiatric symptoms (especially hallucinations) and diaphoresis. Duodopa, an intestinal gel form of L-dopa/carbidopa, provides a more stable response to L-dopa. Stalevo® is a triple-drug, containing carbidopa, L-dopa, and entacapone (Rezak, 2007). Another strategy for prolonging L-dopa half-life is the inhibition of MAO-B, the breakdown-enzyme of DA in the brain, increasing

TABLE 1 | Parkinson's disease (PD) therapies under clinical trials.

Sponsor	CT Identifier	Stage	Administration	Target	Description	Start–completion date
Cellular replacement						
University of Cambridge	NCT01898390	Phase1. Active, not recruiting	Transplant	Striatum	TRANSEURO Open Label Transplant Study in Parkinson's disease (TRANSEURO)	May 2012–March 2021
Chinese Academy of Sciences	NCT03119636	Phase 1–2. Recruiting	Transplant (stereotaxis)	Striatum	Safety and Efficacy Study of Human ESC-derived Neural Precursor Cells in the Treatment of Parkinson's disease	May 2017–December 2021
Hebei Newtherapy Bio-Pharma technology Company Limited	NCT03550183	Phase 1. Recruiting	Intravenous infusion	Not specified	Umbilical Cord Derived Mesenchymal Stem Cells Therapy in Parkinson's disease	January 2018–December 2022
Bundang CHA Hospital	NCT01860794	Phase 1–2. Recruiting	Transplant	Striatum	Evaluation of Safety and Tolerability of Fetal Mesencephalic Dopamine Neuronal Precursor Cells for Parkinson's disease	May 2012–April 2022
Growth factors administration						
North Bristol NHS Trust	NCT03652363	Phase 2. Completed	Bilateral Intraputamenal Infusions of GDNF Administered *via* Convection enhanced delivery	Striatum	GDNF in ideopathic Parkinson's disease	October 2012–April 2016
Herantis Pharma Plc.	NCT03295786	Phase 1–2. Complete	Intraputamenal DDS	Striatum	CDNF brain infusion in Parkinson's disease patients	September 2017–January 2020
Herantis Pharma Plc.	NCT03775538	Phase 1–2. Active, not recruiting	Intraputamenal DDS	Striatum	Safety study of CDNF brain infusion in Parkinson's disease patients	December 2018–January 2020
Newron Sweden AB	NCT02408562	Phase 1–2. Complete	Intracerebroventricular	Not specified	Safety and tolerability study of rhPDGF-BB in Parkinson's disease patients	April 2015–January 2016
Newron Sweden AB	NCT01807338	Phase 1–2. Complete	Intracerebroventricular	Not specified	PDGF-BB in Parkinson's disease patients	March 2013–October 2014
Electrical stimulation						
Northwell Health	NCT04184791	Phase No aplica. Recruiting	DBS	STN	Study the effect of protocol a 60 HZ of STN-DBS in Parkinson's patients with gait disorder	January 2020–December 2021
University of Miami	NCT02022735	Phase No aplica. Active, recruiting yet	DBS	Not specified	Evaluation of different parameters of stimulation for the treatment of gait disorder in Parkinson's patient	December 2013–December 2025
Boston Scientific Corporation	NCT01221948	Phase 2. Completed.	DBS	STN	Evaluation of effectiviness and safe of Boston scientific implantable DBS Vercise system for treatment of moderate to severe idiopathic Parkinson's disease	October 2010–June 2018

(Continued)

TABLE 1 | Continued

Sponsor	CT Identifier	Stage	Administration	Target	Description	Start–completion date
University of Minnesota	NCT00053625	Phase 3. Completed	DBS	STN & GPi	Evaluation of STN-DBS and GPi-DBS in neuropsychological and psychiatric function	February 2003–April 2015
University of Minnesota	NCT02709148	Phase 1. Active, not recruiting	DBS	STN & GPi	Study of relation between brain activity in Parkinson's disease patients and DBS, by recording of LFP in cortex	July 2017–July 2020
Western University, Canada	NCT03079310	Phase Not applicable. Recruiting	SCS	Spinal cord	Thoracic Dorsal Spinal Cord Stimulation for the Treatment of Gait and Balance Impairments in Parkinson's disease	February 2016–April 2020
Gene therapy approaches						
Oxford BioMedica	NCT00627588	Phase 1–2. Complete	Stereotactic injection of ProSavin	Striatum	Safety and efficacy of ProSavin for treatment of idopathic Parkinson's disease patients	March 2008–May 2013
Axovant Sciences Limited	NCT01856439	Phase 1–2. Active, not recruiting	Stereotactic injection of ProSavin	Striatum	Long Term study of ProSavin in Parkinson's disease	May 2013–July 2019

DA levels at synapses. Selegiline, through its metabolite desmethylselegiline, has also shown anti-apoptotic effects (Tatton et al., 1996; Parkinson Study Group, 2005; Cacabelos, 2017). Rasagiline is a significantly more potent MAO-BI that reduces the motor symptoms in PD patients and has disease-modifying potentials (Parkinson Study Group, 2005; Rascol et al., 2005; Olanow et al., 2009). IPX066 (Rytary) is an improved oral formulation of L-dopa approved in 2015 by the FDA containing both an immediate-release and a sustained-release L-dopa. Published phase II and III studies show that IPX066 improved Unified Parkinson's Disease Rating Scale (UPDRS) motor scores compared to placebo in early PD patients and reduced *wearing-off* in advanced PD patients (Kestenbaum and Fahn, 2015). Before L-dopa, anticholinergics were the treatment of choice for PD. However, MAO-BI and modern DA agonists have largely replaced these drugs. Trihexyphenidyl (Artane®), benztropine (Cogentin®), and procyclidine (Kemadrin®) is usually reserved for tremor resistant to dopaminergic agents. The adverse effects of anticholinergics include blurred vision, dry mucus membranes, urinary retention, and cognitive changes (Rezak, 2007).

Dopamine receptor (DR) agonists mimic DA actions in the brain by directly stimulating DRs. Two common DR agonists are pramipexole (Mirapex®) and ropinirole (Requip®). These directly stimulate post-synaptic D2 and D3 receptors in the striatum and are prescribed as monotherapy or combined with L-dopa. Potential side effects of pramipexole and ropinirole include hypotension, sleep, cognitive/psychiatric alterations, dyskinesias, and compulsive behavior. The latter is believed to be a result of DA dysregulation in the limbic and frontal circuits that are connected to the basal ganglia (Evans and Lees, 2004). Polyoxazoline (POZ) polymer conjugation for continuous dopaminergic drug delivery may improve motor symptoms while avoiding side effects. The *in vitro* and *in vivo* pharmacokinetics of POZ-conjugated rotigotine (DA agonist) was characterized, demonstrating that the sustained dopaminergic stimulation profile achieved by POZ-conjugated rotigotine formulations, could represent a significant advance in the treatment of PD. POZ polymer administration can improve motor symptoms in a rat model of PD (Eskow Jaunarajs et al., 2013; Fox et al., 2018). For HD, the only approved pharmacological therapy for the treatment of motor symptoms is tetrabenazine, a vesicular monoamine transporter 2 (VMAT-2) inhibitor that reduces DA neurotransmission *via* its depletion from presynaptic vesicles, resulting in a reduction of chorea manifested by HD patients (Wyant et al., 2017). However, its side effects, which include sedation, anxiety, depression, and suicidality have limited its use (Wyant et al., 2017; Dean and Sung, 2018). In 2017, the FDA approved a deuterated derivative of tetrabenazine, deutetrabenazine, with improved pharmacokinetic profile, allowing a less frequent daily dosage with comparable systemic exposure of the drug, resulting in less adverse events (Dean and Sung, 2018). No other small molecules are currently used for HD treatment, but several therapeutic approaches are in clinical trials, which will be discussed later.

TABLE 2 | Huntington's disease therapies under clinical trials.

Sponsor	CT Identifier	Stage	Administration	Target	Description	Start–completion date
Electrical stimulation						
Heinrich-Heine University, Duesseldorf	NCT02535884	Phase Not Applicable. Recruiting	DBS	GP	Study of efficacy and safety of pallidal DBS in HD patients to improve motor function. Device: ACTIVA® PC neurostimulator (Model 37601)	July 2014–October 2020
DNA targeting approaches						
Ionis Pharmaceuticals, Inc.	NCT02519036	Phase 1–2. Complete	Intrathecal	CNS	Safety, Tolerability, Pharmacokinetics, and Pharmacodynamics of ISIS 443139 in Participants With Early Manifest Huntington's Disease	August 2015–May 2019
Hoffmann-La Roche	NCT03342053	Phase 2. Complete	Intrathecal	CNS	This study will test the safety, tolerability, pharmacokinetics and pharmacodynamics of RO7234292 administered intrathecally to adult patients with Huntington's Disease	November 2017–June 2020
Hoffmann-La Roche	NCT03761849	Phase 3. Recruiting	Intrathecal	CNS	A Study to Evaluate the Efficacy and Safety of Intrathecally Administered RO7234292 (RG6042) in Patients With Manifest Huntington's Disease	January 2019–September 2022
Wave Life Sciences Limited	NCT03225833	Phase 1b–2a. Recruiting	Intrathecal	CNS	Safety and Tolerability of WVE-120101 in Patients With Huntington's Disease (PRECISION-HD1)	July 2017–December 2020
Wave Life Sciences Limited	NCT03225846	Phase 1b–2a. Recruiting	Intrathecal	CNS	Safety and Tolerability of WVE-120102 in Patients With Huntington's Disease (PRECISION-HD2)	July 2017–December 2020
RNA targeting approaches						
UniQure Biopharma B.V.	NCT04120493	Phase 1–2. Recruiting	Stereotaxic	Striatum	Safety and Proof-of-Concept (POC) Study With AMT-130 in Adults With Early Manifest Huntington Disease	September 2019–May 2026

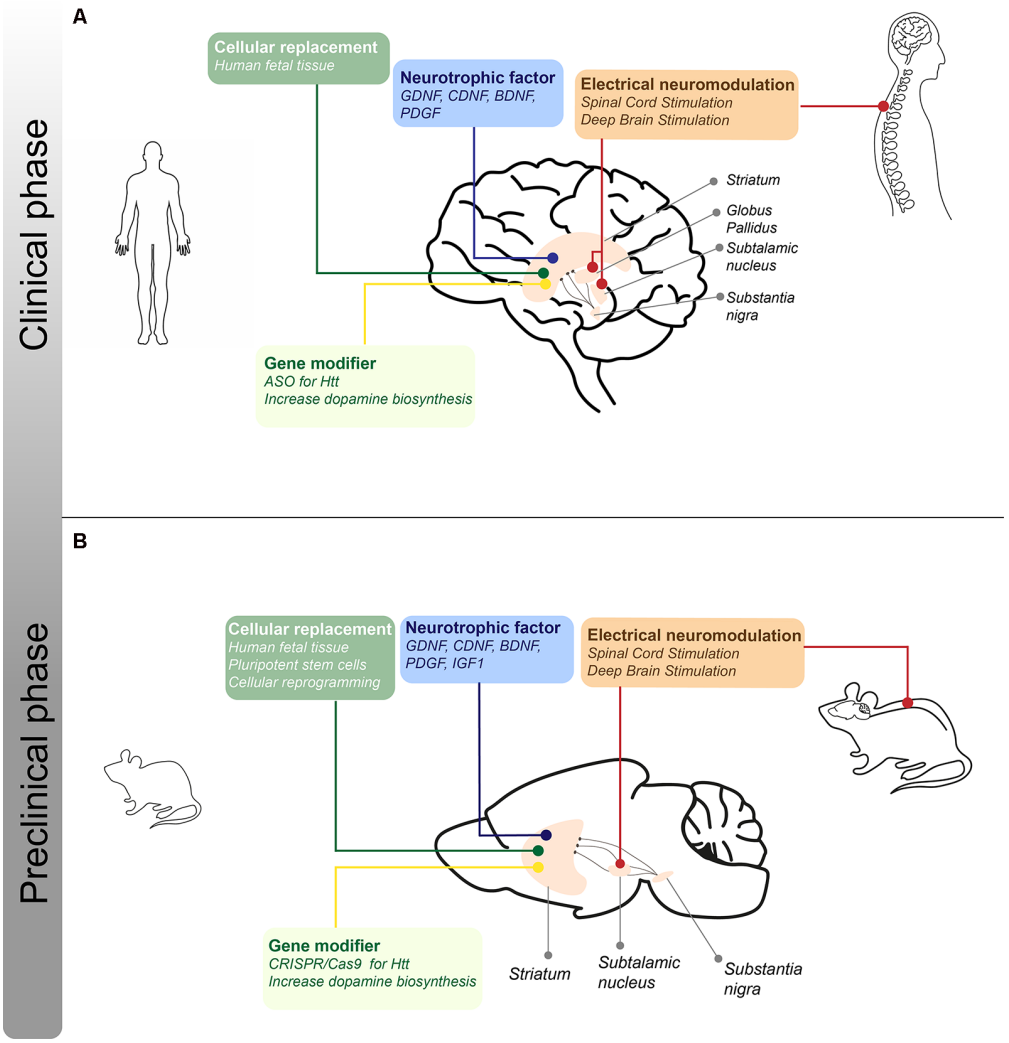

FIGURE 2 | I Schematic representation of the different treatments for motor symptoms for PD and HD under clinical and preclinical phases. **(A)** Approaches regarding cellular, neurotrophic, electrical, and gene-modifying therapies that are in clinical trials or stage of optimization for PD and HD patients. The lines indicate the different brain structures that are the target of the therapeutic strategies mentioned. **(B)** Approaches regarding cellular, neurotrophic, electrical, and disease-modifying therapies in the preclinical phase using rodent models of PD and/or HD. The lines indicate the different brain structures that are the target of the therapeutic strategies mentioned.

CELLULAR REPLACEMENT THERAPIES FOR PD AND HD

In 1967, in an important breakthrough, Cotzias et al. (1967) demonstrated that the administration of a precursor of DA, L-dopa, improved motor function in PD patients, leading to the thought that the cure for PD was discovered. Also in the 1960s, tetrabenazine was introduced as an antipsychotic but also showed beneficial effects for the treatment of hyperkinetic motor symptoms, like chorea in HD patients (Dalby, 1969; Huntington Study Group, 2006). To date, it is known that these drugs do not reverse disease progression and in many cases do not have the desired effects. This has brought the idea that local production of DA and GABA, and therefore the replacement of the neurons that produce it, would be the ideal treatment for these diseases.

The fact that the major symptoms present in PD and HD patients are due to the loss of dopaminergic and GABAergic neurons in specific brain regions, respectively, means that replacing these specific cell types could help relieve some of the symptoms present in patients. This has given rise to different branches of investigations seeking cellular replacement-based therapies, which have shown promising results in animal models for these diseases as well as in affected patients (**Figure 2**).

Human Fetal Tissue as a Source of Progenitor Cells

The first study demonstrating that dopaminergic neurons could be replaced using fetal tissue was performed using 6-hydroxydopamine (6-OHDA)-lesioned rats that were implanted with DA-rich ventral mesencephalic tissue from

rat fetuses (Björklund and Stenevi, 1979; Perlow et al., 1979). These studies were followed by the generation of the first non-human primates PD model: monkeys lesioned with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP; Burns et al., 1983). This model manifested several of the patient's symptoms, and transplanting primate fetal mesencephalic tissue into their striatum showed to alleviate these symptoms (Bakay et al., 1985; Sladek et al., 1987; Taylor et al., 1991). These studies set foot for the first PD cell replacement therapy in humans. These clinical trials were performed using dopaminergic neuron precursors from human fetal tissue, which were transplanted into the striatum of PD patients (Lindvall et al., 1989, 1992; Freed et al., 1990, 1992). Transplanted tissue presented no negative effects at the transplantation site, was functional and survived in the transplanted brain region, but clinical benefits were variable (Freed et al., 1992; Kordower et al., 1998; Hagell et al., 1999; Li et al., 2016).

It is important to understand that dopaminergic neurons engrafted in the striatum are deprived of their SNpc afferents. Instead, when dopaminergic neurons are transplanted in the striatum, they may form connections with cortical, intrastriatal, and thalamic neurons, which are not normally connected with nigral dopaminergic neurons. Moreover, considering the nature of fetal tissue, possibly other types of neurons and glial cells differentiate in the grafted brain area. Additionally, only a portion of the grafted fetal tissue corresponds to cells that needed restoration in the specific brain region. Another important concern is the presence of α-syn aggregates and Lewy body inclusions in the grafted cells in PD patients (Kordower et al., 2008; Li et al., 2008). This highlights the necessity of using a complementary strategy that allows donor cells to be resistant to the spreading of α-syn.

On the other hand, by the mid-1980s, the first studies using fetal tissue were performed in rat models of HD (**Table 4**). These studies demonstrated that the grafted tissue survived, was functional and recovered some of the behavioral alterations present in HD rats (Deckel et al., 1983; Isacson et al., 1984, 1985; Sanberg et al., 1986). The *in vivo* functionality of the grafted striatal fetal tissue was also assessed, showing GABA release upon dopaminergic and glutamatergic inputs (Campbell et al., 1993). Diverse studies further demonstrated the effect of striatal fetal tissue transplantation in diverse pharmacological models of HD (Hantraye et al., 1992; Nakao et al., 1999) and by late 1990s, the first clinical trials in HD patients were performed. HD patients were injected unilaterally or bilaterally with fetal tissue, which was originated from various donated embryos. Given the amount of tissue needed and their origin from different embryos, this strategy causes immune rejection by the patient's immune system. Therefore, complementary immunosuppression therapy is needed, which has shown no adverse effects on patients (Freeman et al., 2000). After fetal cell transplantation, patients presented improved cognitive function and stabilization of motor functions, which worsen a few years after the surgery (Kopyov et al., 1998; Gallina et al., 2008; also see **Table 1**).

Although several clinical trials have been performed using fetal tissue transplants to treat PD and HD patients, this technique has a few but important limitations. First, is important to consider that when using human fetal tissue, tumors can develop, which could be explained by the presence of actively dividing immature neuroepithelial cells (Keene et al., 2009). Second, not only neuronal loss must be corrected, but also the loss of glial cells. Astrocytes are the most abundant cell type found in the brain (Miller, 2018) and play important roles in maintaining brain homeostasis, supporting a neuronal activity, and metabolism. In PD and HD, there is a deregulation of astrocyte activity, including electrophysiological changes, calcium homeostasis, glutamate reuptake, and metabolism, among others (Booth et al., 2017; Garcia et al., 2019; Gray, 2019). Therefore, the replacement of the lost neurons should be accompanied by a replacement or modification of the glial cells that support them.

Pluripotent Stem Cells as a Source of Differentiated Cell

Pluripotent stem cells (PSCs) are an unlimited source of cells with the potential to give rise to any type of cell of the body. Cells differentiated from embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs; Takahashi and Yamanaka, 2006) are widely used as *in vitro* models for many diseases, including neurodegenerative diseases, and also as a source of cell-replacement therapies. Initial studies demonstrated that, when midbrain-derived dopaminergic neurons where grafted in the striatum of rodent models of PD (**Table 3**), long-term survival of these cells was observed, which were tyrosine hydroxylase (TH)-positive neurons, completely reversed amphetamine-induced rotational behavior and lacked neuronal overgrowth (Kriks et al., 2011). Importantly, midbrain human dopaminergic neurons grafted in MPTP-lesioned non-human primates survived in the grafted area, expressed TH, extended fibers to the surrounding striatum, and did not present neuronal overgrowth (Kriks et al., 2011).

However, one limitation regarding the use of PSCs-derived neurons is the greater immune reaction observed with allogeneic grafts compared with isogenic cells (Duan et al., 1995; Morizane et al., 2013). Using both approaches, Hallett et al. (2015) demonstrated that in a non-human primate PD model autologous iPSCs-derived midbrain-like dopaminergic neurons could successfully engraft and survive for as long as 2 years. This led to improving motor function and complete re-innervation in the striatum with extensive axonal outgrowth, and no graft overgrowth, tumor formation or inflammation was observed (Hallett et al., 2015). Also, as clinical trials are underway, establishing the optimal and safest protocol for dopaminergic neurons differentiation is necessary to obtain the adequate number of neurons that permit improvements in patient's motor symptoms. Also, the characterization of the graft is relevant, as the differentiation protocol could give rise to other cell types that may alter the physiological conditions in the grafted site. One important concern about the use of autologous transplantation using iPSC-derived dopaminergic neurons of PD patients is that these cells will carry any intracellular dysfunction related to disease pathogenesis. It is important to remember that dopaminergic neuron replacement is primarily

TABLE 3 | Common animal models of Parkinson's Disease.

Model	Characteristics	Reference
1. Mouse models		
Pharmacological models		
6-OHDA	Stereotaxic injection in medial forebrain bundle	Zigmond and Stricker (1984)
MPTP	IP injection induce loss of dopaminergic neurons of nigrostriatal pathway	Sonsalla et al. (1987) and Sonsalla and Heikkila (1988)
Rotenone	Stereotaxic injection in parenchyma caused damage in dopaminergic nigrostriatal pathway	Heikkila et al. (1985)
Reserpine	Impairment in monoamines storage in intracellular vesicles disrupting motor activity	Spina and Cohen (1989) and Cooper et al. (2004)
Genetically modified models		
A53T	Mutation leads formation of neuronal inclusions leading neurodegeneration	Giasson et al. (2002)
PINK1 transgenic mice (knockout)	Display impaired dopamine release, but not dopaminergic neurons degeneration	Kitada et al. (2007)
Parkin transgenic mice (knockout)	Display abnormalities in dopamine transmission, but not dopaminergic neuron degeneration	Perez et al. (2005)
DJ-1 transgenic mice (knockout)	Display abnormalities in dopamine transmission, but not dopaminergic neuron degeneration	Goldberg et al. (2005)
LRKK2 transgenic mice (overexpression)	Display dopaminergic dysfunction and some behavioral deficits, but not dopaminergic neurons degeneration	Lin et al. (2009)
Recombinant adeno-associated viral vector (AVV) models		
Human WT-α-synuclein	Direct injection in SN induce progressive loss of dopaminergic neuron	St. Martin et al. (2007)
Human-A53T-α-synuclein	Direct injection in SN induce progressive loss of dopaminergic neuron	Oliveras-Salvá et al. (2013)
2. Rat models		
Pharmacological models		
6-OHDA	Direct administration in the brain (striatum, subtantia nigra or median forebrain bundle) cause the loss of dopaminergic neurons	Ungerstedt (1968)
Haloperidol	IP injection block striatal dopamine transmission	Sanberg (1980)
Rotenone	IV or IP administration cause nigrostriatal dopaminergic degeneration	Betarbet et al. (2000)
Recombinant adeno-associated viral vector (AVV) models		
Human WT-α-synuclein Human-A53T-α-synuclein	Direct injection in SN induce progressive loss of dopaminergic neurons and motor impairment	Kirik et al. (2002a,b)
Human A30P-α-synuclein	Direct injection in SN induce progressive loss of dopaminergic neurons and motor impairment	Klein et al. (2002)
3. Large models		
Pharmacological models		
MPTP rhesus monkey	IP injection induce loss of dopaminergic neurons of nigrostriatal pathway	Burns et al. (1983)
MPTP squirrel monkey	IP injection induce loss of dopaminergic neurons of nigrostriatal pathway	Langston et al. (1984)
MPTP marmoset monkey	IP injection induce loss of dopaminergic neurons of nigrostriatal pathway	Jenner et al. (1984)

TABLE 4 | Common animal models of Huntington's disease.

Model	Characteristics	Reference
1. Mouse models		
Genetically modified models		
R6/2	Expresses human exon 1 of HTT with ∼150 glutamine repeats	Mangiarini et al. (1996)
R6/1	Expresses human exon 1 of HTT with ∼115 glutamine repeats	Mangiarini et al. (1996) and Naver et al. (2003)
N171-82Q	Expresses a 171 amino acid mutant HTT fragment with 82 glutamine repeats	Schilling et al. (1999)
YAC128	Expresses full length human HTT with 128 glutamine repeats	Slow et al. (2003) and Van Raamsdonk et al. (2005)
BACHD	Expresess full lenght human HTT with 97 glutamine repeats	Gray et al. (2008)
HdhQ111	knock-in mouse having human *HTT* exon 1 sequence with 111 glutamine repeats	Wheeler et al. (2000)
HdhQ140	knock-in mouse having human *HTT* exon 1 sequence with 140 glutamine repeats	Menalled et al. (2003)
HdhQ150	knock-in mouse having mouse *HTT* exon 1 sequence with 150 glutamine repeats	Lin et al. (2001)
Pharmacological models		
Quinolonic acid (QA)	QA directly administered to the striatum induces striatal neurodegeneration	McLin et al. (2006)
3-Nitropropionic acid (3-NP)	Repeated injections of 3-NP produce excitotoxic-like lesions of the striatum	Gould et al. (1985)
2. Rat models		
Pharmacological models		
Quinolonic acid (QA)	QA directly administered to the striatum induces striatal neurodegeneration	Bordelon et al. (1997)
3-Nitropropionic acid (3-NP)	Repeated injections of 3-NP produce excitotoxic-like lesions of the striatum	Gould et al. (1985)
Kainic acid	Intrastriatal injection produces selective degeneration of neurons	Coyle et al. (1978)
Ibotenic acid (IBO)	Central microinjections induce lesions in the striatum	Smith et al. (1987)
3. Larger animal models		
Pharmacological models		
HD rhesus monkey	Expresses mutant exon 1 HTT with 84 glutamine repeats	Yang et al. (2008)
HD pigs (N208-105Q)	Expresses 208 N-terminal aminoacids of mutant HTT with 105 glutamine repeats	Yang et al. (2010)

focused on the treatment of motor symptoms. Patients in which symptoms like dementia or other cognitive impairment are also present may not be benefited completely with this type of therapy.

One key issue for cellular transplantation into human brains is the necessity of an important amount of cells. Fetal tissue cells not only provide a limited number of cells but also come with ethical and religious concerns. Therefore, the use of iPSCs obtained from somatic cells of patients is excellent for personalized cell-based therapy and to model HD *in vitro*. The first research group to obtain striatal neurons from HD iPSCs-derived neuronal stem cells (NSCs) was Zhang et al. (2010), who used iPSCs derived from an HD patient (Park et al., 2008). These cells not only express MSNs markers but also could be used as an excellent model for drug screening in HD research (Zhang et al., 2010). Using the same HD iPSCs, Zhang et al. (2010) demonstrated that these cells can be corrected for the CAG mutation by replacing the expanded 72 CAG repeat with a normal 20–21 CAG repeat (An et al., 2012). Finally, the corrected iPSCs-derived NSCs could be successfully differentiated into MSNs *in vitro*, and when transplanted into the striatum of R6/2 mice (**Table 4**) they differentiated into MSNs neurons (An et al., 2012). The genetic modification of human iPSCs not only brings us closer to the proper modeling of diseases but also provides a potential therapy. It has been demonstrated that iPSCs-derived neuronal cells from an HD preclinical model develop cellular features of HD cells, which could be rescued by genetic suppression of HTT and pharmacological treatment (Carter et al., 2014). Using human ESCs (hESCs) or human iPSCs (hiPSCs) differentiated into MSNs progenitors, it has been demonstrated that the transplantation of these cells into the striatum of rodent HD models can form functional connections with other cells, and project their axons to other structures involved in the movement circuitry, like the SN (Faedo et al., 2017; Adil et al., 2018).

As highlighted previously, since HD is caused by a genetic mutation, and differentiated MSNs progenitors come from HD patients, it is imperative to correct the mutation present in these cells, along with the replacement of the target neurons and other cell types, like interneurons and glial cells, as they may provide a healthy and functional environment for the new neurons to integrate to the local circuitry and survive. Currently, no clinical trials are assessing the use of PSCs in HD patients.

Cellular Reprogramming for PD and HD

In the adult brain, NSCs are present in the subventricular zone of the lateral ventricle and the subgranular zone of the dentate gyrus. These NSCs are capable of generating neuroblasts, which differentiate into mature neurons (Zhao et al., 2008; Ma et al., 2009). Despite the presence of a niche for the generation of new neurons, these cells have limited migration to remote regions, like the SN and striatum. Hence, the idea to generate new local neurons from preexisting

cells has been studied for the last 10 years. Initial studies have demonstrated that fibroblasts can be reprogrammed to dopaminergic neurons through the ectopic expression of transcription factors (Caiazzo et al., 2011; Kim et al., 2011; Pfisterer et al., 2011). Considering the reprogramming of cells for the treatment of neurodegenerative diseases, astrocytes were initially considered as an attractive alternative and their reprogramming to neurons forming functional synapses was demonstrated (Berninger et al., 2007; Heinrich et al., 2010).

The first studies in which dopaminergic neurons were generated by reprogramming human and mouse fibroblasts using lineage-specific factors for the conversion to dopaminergic neurons (Caiazzo et al., 2011; Kim et al., 2011). The converted neurons were positive for several dopaminergic markers and expressed genes related to the dopaminergic lineage rather than with the fibroblast of origin (Caiazzo et al., 2011; Kim et al., 2011). Converted dopaminergic neurons formed synapses, had synaptic activity in culture, and showed electrophysiological properties similar to dopaminergic neurons (Caiazzo et al., 2011). The transplantation of these cells into the brain of wild type (Caiazzo et al., 2011) and PD mice (Kim et al., 2011) showed that converted neurons integrated with the host tissue expressed dopaminergic markers and had electrophysiological responses, which led to an improvement in mice behavior (Kim et al., 2011).

Also, Addis et al. (2011) were the first to demonstrate that astrocytes could be reprogrammed into dopaminergic neurons *in vitro*. The obtained neurons displayed an up-regulation of genes expressed by dopaminergic neurons, along with electrophysiological properties, including the spontaneous firing of action potentials observed in dopaminergic neurons (Addis et al., 2011). A few years later, the first human astrocytes were directly converted into functionally competent dopaminergic neurons *in vitro* (Rivetti di Val Cervo et al., 2017) and it was described for the first time the *in vivo* conversion of astrocytes into dopaminergic neurons in the 6-OHDA mouse model of PD (Rivetti di Val Cervo et al., 2017). These converted dopaminergic neurons were excitable and expressed dopaminergic neuron markers, which helped relieve the cycling behavior observed in these animals. These and other reports have shown that the cellular reprogramming relies on the expression of lineage-specific transcription factors. However, it has been shown that downregulation of PTB in mouse and human fibroblasts, an RNA-binding protein negatively controlling neuronal induction and maturation, induces the conversion of these cells into functional neurons (Xue et al., 2013, 2016). Considering these finding and that downregulation of PTB occurs during neurogenesis (Hu et al., 2018), Qian et al. (2020) recently demonstrated using the 6-OHDA mouse model of PD that adeno-associated virus (AAV)-mediated downregulation of PTB using an shRNA convert nigral astrocytes into functional dopaminergic neurons. These converted neurons integrate into the nigrostriatal pathway, extending their axons into the striatum and other brain regions. These neurons were electrophysiologically functional and restored the striatal dopamine lost due to the 6-OHDA treatment, leading to a reversal of the motor deficits observed in these mice (Qian et al., 2020). Importantly, these results were also observed using an antisense oligonucleotide against PTB, giving these findings a potentially clinical approach for the treatment of PD in patients (Qian et al., 2020).

Not only glial cells can originate dopaminergic neurons. In an elegant work performed by Niu et al. (2018), authors demonstrated that striatal neurons could be reprogramed to dopaminergic-like neurons in the adult mouse striatum. These neurons, although expressing both dopaminergic and GABAergic markers, have electrophysiological properties like endogenous dopaminergic but no MSNs neurons. These dopaminergic-like neurons were also functionally connected with surrounding neurons, confirmed by the presence of spontaneous postsynaptic currents (Niu et al., 2018). Hence, these results seem to be promising for converting MSNs into dopaminergic neurons under pathological conditions.

Initial studies have shown that striatal astrocytes can be reprogramed into proliferative neuroblasts in young, adult and aged mice brains (Niu et al., 2013), which are interesting especially for HD, a disease of adult-onset. Furthermore, when these neuroblasts were treated with NTFs or histone deacetylase inhibitor, they differentiated into mature neurons with electrophysiological properties (Niu et al., 2013). Using (AAV)-based conversion, striatal GABAergic, and glutamatergic neurons could be originated after reprogramming NG2 glial cells (Torper et al., 2015). Newly generated neurons presented electrophysiological properties of functional neurons, remained stable for a long period, and even integrated into local neuronal circuitry (Torper et al., 2015). Consequently, the use of endogenous glial cells for the regeneration of neuronal population lost under neurodegenerative conditions has emerged as an interesting source, avoiding the use of differentiated external cells and therefore minimizing the possible immunorejection of foreign cells (Li and Chen, 2016; Srivastava and DeWitt, 2016; Barker et al., 2018).

Recently, using AAV-based reprogramming of striatal astrocytes, Wu et al. (2020) demonstrated that astrocytes could be converted to MSNs in the striatum of R6/2 and YAC128 mice. Converted neurons expressed specific MSNs markers, showed electrophysiological properties, and projected their axonal terminals to the GP and SNpr. All these findings were accompanied by a reduction in striatal atrophy, attenuation of the phenotypic deficit, and an extended life span of R6/2 mice with converted MSNs (Wu et al., 2020).

Nevertheless, it is important to complement these reprogram therapies with a therapy that targets the mutation in the HTT gene. Converted neurons will sooner or later express and accumulate mHTT, which will eventually lead to neurodegeneration. Therefore, *in vivo* reprogramming of glial cells into healthy MSNs has an important clinical potential, which must also be combined with gene therapy strategies to reduce or ablate mHTT expression in these new neurons. Also, the application of this approach in the clinic is challenging by the lack of standardized protocols for cellular reprogramming, as well as the efficiency of converted cells. This depends on the donor cell, the type of cell that is needed, the characteristics of patients that must be considered, i.e., age, the severity of the disease, and treatment with other drugs, among others. Although

challenging, *in vivo* cell reprogramming appears as the most promising therapy candidate for cellular replacement for PD and HD patients.

NEUROTROPHIC FACTORS-BASED THERAPIES FOR PD AND HD

NTFs are molecules that promote the differentiation, myelination, and survival of neurons, which are also involved in the neuroinflammatory response (Fernandez and Torres-Aleman, 2012; Labandeira-Garcia et al., 2017). A reduction in the bioavailability of NTFs in the peripheral and central system during aging suggests a role of these factors during neurodegenerative disorders such as PD and HD (Zuccato and Cattaneo, 2007; Gasperi and Castellano, 2010; Procaccini et al., 2016; Salem et al., 2016). NTFs, like Glial cell-line Derived Neurotrophic Factor (GDNF), Brain-Derived Neurotrophic Factor (BDNF), Cerebral Dopamine Neurotrophic Factor (CDNF), Mesencephalic astrocyte-derived Neurotrophic Factor (MANF), Platelet-Derived Growth Factor (PDGF), Insulin-like Growth Factors (IGFs), and others have been through preclinical and clinical trials for PD and HD (**Figure 2**). Here, we describe the recently therapeutic approaches based on the restoration of NTFs levels in the brain to prevent and/or stop the neurodegenerative process describe in PD and HD.

Glial-Derived Neurotrophic Factor (GDNF)

GDNF is considered as a neuro-restorative therapeutic protein that induces the regeneration of dopaminergic neurons given that enhances dopaminergic cell survival and differentiation *in vitro* (Lin et al., 1993; Zurn et al., 2001). Besides, GDNF has shown a protective effect on the survival of noradrenergic neurons in the locus coeruleus (Arenas et al., 1995), an affected region in neurodegenerative diseases such as PD and HD (Zweig et al., 1992; Oertel and Schulz, 2016). The neuroprotective effects of GDNF have prompted preclinical and clinical studies. Chronic infusion of GDNF into the lateral ventricle or the striatum promoted the restoration of the nigrostriatal dopaminergic system and significantly improved motor functions in a rhesus monkey PD model (Grondin et al., 2002). Moreover, GDNF protects nigral dopaminergic neurons from degeneration and improves motor behavior in 6-OHDA rat models of PD (Tereshchenko et al., 2014). However, GDNF has limited use due to its inability to cross the blood-brain barrier (BBB), therefore new administration methods have been explored, including: (1) the delivery of GDNF in biodegradable microspheres (Garbayo et al., 2009); (2) gene therapy using DNA nanoparticle (DNP) technology for the expression of human GDNF (hGDNF) in the striatum (Fletcher et al., 2011); (3) the use of intra-cerebroventricular (ICV) catheters implanted into the basal ganglia (Gill et al., 2003; Nutt et al., 2003); (4) the use of viral vectors (Kordower et al., 2000); and (5) GDNF-producing fibroblasts (Grandoso et al., 2007). ICV administration of recombinant hGDNF to non-human primates showed to significantly improve locomotor activity after 4 months of treatment (Zhang et al., 1997). Also, intraputamenal (Ipu) delivery of GDNF in MPTP-lesioned non-human primates significantly increased DA release (Grondin et al., 2003). Despite the positive results in the survival of dopaminergic neurons and improvements in motor behavior (Gill et al., 2003; Patel et al., 2005; Lang et al., 2006), the invasiveness of the delivery of GDNF to the brain represents a limitation for its use.

The first attempt to probe the benefits of GDNF in PD patients consisted of the ICV administration through catheter implantation in 50 PD patients for 8 months. Patients presented side effects after drug administration, mainly weight loss, nausea, and vomiting. At the end of treatment, patients did not present improvements in the UPDRS motor scores (Nutt et al., 2003). Similarly, delivery through Ipu infusion of recombinant hGDNF in 34 PD patients did not observe significant improvements in UPDRS motor scores (Lang et al., 2006). Furthermore, in a completed clinical study with 42 PD patients, bilateral Ipu GDNF infusions every 4 weeks for 9 months showed that ^{18}F-DOPA analyzed through PET scan imaging had a significant increase in the putamen, but no significant changes in UPDRS scores were registered. However, the extended treatment for 18 months showed significant improvements in the UPDRS motor scores (Whone A. et al., 2019; Whone A. L. et al., 2019). Additionally, a phase I study showed an important improvement in UPDRS motor scores after 1 year of GDNF Ipu therapy (Slevin et al., 2005, 2007). Completed clinical studies have demonstrated the safety and potential efficacy of Ipu GDNF infusion, with no evidence of GDNF-induced toxicity (Slevin et al., 2005). However, antibodies were detected in some patients and device-related problems were reported (Lang et al., 2006; Slevin et al., 2007). It has been shown that the effect of GDNF *in vitro* and *in vivo* requires TGF-β (Peterziel et al., 2002). The combined effect of GDNF-TGFβ showed a strong neuroprotective effect in rodent PD models (Peterziel et al., 2002) and future therapies may include the simultaneous use of both molecules. Finding a non-invasive and safe way to deliver GDNF is key to evaluate this NTF as an effective treatment for PD.

Preclinical studies in rat models of HD have demonstrated the benefits of ICV injection of GDNF in restoring the excitotoxic-induced damage in the striatum, amelioration of amphetamine-induced rotational behavior (Araujo and Hilt, 1997) and locomotor activity improvement (Araujo and Hilt, 1998). However, no registered clinical trials are testing the efficacy of GDNF in HD patients.

Cerebral Dopamine Neurotrophic Factor (CDNF) and Mesencephalic Astrocyte-Derived Neurotrophic Factor (MANF)

In 2003, a protein called mesencephalic astrocyte-derived neurotrophic factor (MANF) was characterized and demonstrated to promote survival of embryonic dopaminergic neurons *in vitro* (Petrova et al., 2003). Then, a homologous protein called CDNF was discovered with a protective role for dopaminergic neurons. Several studies evidence the protective role of CDNF and MANF in dopaminergic neurons against the injury caused by α-syn oligomers (Latge et al., 2015). The intrastriatal injection of CDNF prevents the loss of TH-positive

neurons in a 6-OHDA-lesioned rat model of PD (Lindholm et al., 2007), and protected dopaminergic neurons in 6-OHDA and MPTP mouse models of PD (Lindholm et al., 2007; Voutilainen et al., 2009). MANF has been tested in the 6-OHDA-lesioned rat model showing beneficial effects (Voutilainen et al., 2009). CDNF and MANF diffuse to the brain significantly better than GDNF, and CDNF was more efficient in reducing amphetamine-induced ipsilateral rotations in the 6-OHDA rat PD model in comparison with GDNF treatment (Voutilainen et al., 2011). In 6-OHDA-lesioned monkeys, PET imaging showed a significant increase of DA transporter (DAT) ligand-binding activity in lesioned animals treated with CDNF (Garea-Rodríguez et al., 2016).

The first phase I–II clinical trial using CDNF in PD patients is being conducted since 2017. In this study, an implanted drug delivery system (DDS) for Ipu of recombinant human CDNF is used in patients with idiopathic mild-advanced PD (**Table 1**, NCT03295786). Additionally, another phase I–II clinical trial to evaluate the beneficial effects of CDNF in PD patients is still on course (**Table 1**, NCT03775538). Currently, the delivery of CDNF for HD treatment has not been described.

Brain-Derived Neurotrophic Factor (BDNF)

BDNF is the most abundant NTFs in the brain (Barde et al., 1982), mostly involved in physiological processes including morphological and functional synaptic plasticity, long-term potentiation, learning and memory (Bramham and Messaoudi, 2005; Lu et al., 2014). In the CNS, BDNF binds specifically to tropomyosin-related kinase receptors B (TrkB) receptors and its signaling cascade is involved in neuronal survival (Kaplan and Miller, 2000). Interestingly, MAOI (i.e., rasagiline and selegiline) prevent dopaminergic neuron loss by increasing BDNF expression and other NTFs (Weinreb et al., 2007; Maruyama and Naoi, 2013).

Studies have demonstrated that a decrease of BDNF is implicated in neurological disorders (Siegel and Chauhan, 2000; Takahashi et al., 2012; Lu et al., 2013). Thus, strategies for developing quantification and modulation of BDNF levels represent a viable approach for biomarker and treatment development, respectively, being useful for a variety of neurodegenerative diseases (Lu et al., 2013; Song et al., 2015). Post-mortem studies reveal that BDNF is significantly reduced in nigrostriatal dopaminergic neurons from PD patients (Mogi et al., 1999; Parain et al., 1999; Howells et al., 2000). A decrease of BDNF in serum from PD patients has also been observed (Wang et al., 2016). Also, BDNF offers neuroprotection of striatal neurons, and supporting studies have shown that BDNF levels are decreased in the brains of HD rodent models (Conforti et al., 2008) and patients (Ferrer et al., 2000; Zuccato and Cattaneo, 2007).

A study with 42 HD patients revealed that BDNF serum concentrations were significantly lower in patients compared to healthy controls (Ciammola et al., 2007). However, a later study analyzed 398 blood samples, indicating that mRNA and protein levels of BDNF between HD and healthy controls were not significantly different, questioning its potential as a biomarker for early diagnosis of HD (Zuccato et al., 2011).

Although the contribution of BDNF on PD and HD pathology is robust, no clinical trials are currently testing its safety and efficacy for the treatment of these diseases.

Platelet-Derived Growth Factor (PDGF)

Classic studies indicate that PDGF has diverse functions in organs, including the stimulation of cell proliferation (Heldin and Westermark, 1999). Different isoforms of PDGF can be found in tissues, in which the PDGF-BB isoform has shown a protective effect in cultured dopaminergic neurons (Pietz et al., 1996). After the treatment of rats with 6-OHDA, PDGF-BB was increased, suggesting a compensatory response (Funa et al., 1996) and PDGF-BB injections induced functional recovery and provided neuroprotection of the nigrostriatal system in a PD mouse model (Zachrisson et al., 2011). PDGF-BB might be acting on neural progenitors and stem cells in the subventricular zone, promoting neurogenesis (Zachrisson et al., 2011). A study with 12 PD patients demonstrated that the administration of PDGF-BB into the brain ventricles for 2 weeks was well tolerated with no evident or aggressive side effects, and an increase in DAT binding was noted in the putamen of PDGF-BB-treated patients (Paul et al., 2015). Considering that PDGF-BB can stimulate neurogenesis, it may be possible to evaluate the co-treatment with PDGF-BB and other NTFs or drugs to restore the nigrostriatal pathway and promote neuroprotection in PD.

Both *in* vitro (Nakao et al., 1994) and *in vivo* reports (Sjöborg et al., 1998) have related the effect of PDGF in HD. PDGF-BB exerts trophic effects in developing rat DARPP32-positive striatal neurons in culture, suggesting the possibility that PDGF-BB might participate in the development and maintenance of striatal neurons *in vivo*, and could be used to modulate the neurodegeneration in HD models (Nakao et al., 1994). Then, the same group published the expression profile of PDGF in a rat model of HD, generated by unilateral intrastriatal ibotenic acid injections (**Table 4**). The evidence showed the accumulation of PDGF in astrocytes, suggesting a role of PDGF in a repair process in neurodegeneration (Sjöborg et al., 1998). Currently, no PDGF-based clinical trials are in course for HD treatment.

Insulin-Like Growth Factor Family (IGFs)

The IGF system is composed of insulin, IGF1, and IGF2, and its receptors: IR, IGF1R, and IGF2R, respectively (Cohen et al., 1991). The use of IGFs as therapy might represent a novel tool for the treatment of neurodegenerative disorders (Ebert et al., 2008). Studies *in vitro* and in preclinical models have demonstrated the neuroprotective effects of IGFs (Jarvis et al., 2007). Administration of IGF1 after injury reduced neuronal loss against several stressors such as oxidative stress, excitotoxicity, hypoxia, hypoglycemia, among others (Suh et al., 2013). Several studies using *in vivo* models of PD demonstrated beneficial effects of IGF1 treatment by preventing dopaminergic neuronal loss in the SN (Ebert et al., 2008), improving motor performance in a rat model of PD (Guan et al., 2000; Krishnamurthi et al., 2004).

IGFs have received interest due to its role in preventing and rescuing striatal neuronal damage which is observed in HD (Lewitt and Boyd, 2019). Increased IGF1 plasma levels were

observed in the YAC128 mouse model of HD (Pouladi et al., 2010). Similarly, high IGF1 plasma levels were observed in HD patients and this was associated with cognitive impairment characteristic in this disorder (Saleh et al., 2010), however, the correlation between elevated IGF1 plasma levels and the motor and cognitive impairment in HD remains to be elucidated. Despite the high peripheral levels of IGF1, only small amounts of IGF1 cross the BBB into the brain. In this context, Lopes et al. (2014) showed that intranasal administration of IGF1 significantly improve motor function and restores metabolic changes in YAC128 mice model of HD, demonstrating that intranasal administration allows for the direct delivery of IGF1 into the CNS through the olfactory pathway.

In a recently published study, it has been demonstrated that the AAV administration of IGF2 into the striatum of YAC128 and R6/2 mice decreased the levels of mHTT and increased the levels of DARPP-32, a marker used to assess striatal neurons survival (García-Huerta et al., 2020). Interestingly, neuroprotective effects of IGF2 treatment have been described in preclinical models of others neurodegenerative diseases such as amyotrophic lateral sclerosis (Allodi et al., 2016), spinal muscular atrophy (Brown et al., 2009) and Alzheimer's disease (Pascual-Lucas et al., 2014). However, today no clinical trials are studying the safety and efficacy of IGF2 as a possible treatment for PD or HD.

ELECTRICAL NEUROMODULATION THERAPIES FOR PD AND HD

The cardinal motor symptoms of PD and chorea in HD are caused by the progressive degeneration of dopaminergic neurons in the SNpc (**Figure 1B**) and the loss of MSNs in the striatum (**Figure 1C**), respectively. In both cases, the motor impairment is attributed to alteration of functional connectivity of the striatum, a principal input of the basal ganglia (Albin et al., 1989).

Hyperkinetic movement disorders are characterized by uncontrollable and excessive motor activity, as chorea in HD. Reports published between 1987 and 1989 showed that blocking the activity of the STN produces hyperkinetic motor symptoms. Similar results are observed when the GABAergic inputs from the striatum are blocked, favoring the inhibitory (GABAergic) modulation of the GPe over the STN (Crossman, 1987; Crossman et al., 1988; Robertson et al., 1989). On the other hand, hypokinetic disorders like akinesia and bradykinesia have been described in PD. In this case, the decrease in striatal DA levels, as a result of the decrease in its synthesis, release, and reuptake (Lundblad et al., 2012) alter the corticostriatal balance causing an increase in the activity of the indirect pathway and reducing the activity of the direct pathway, that leads to a breakdown of the internal balance of the basal ganglia, and consequently the loss of movement control (Obeso et al., 2008; Galvan et al., 2015). These symptoms, unlike hyperkinetic movements, are treated with DA agonists as L-dopa (Cotzias et al., 1967, 1969). However, as stated in previous sections, the chronic use of this pharmacological therapy has a limited effect, which in the case of PD can induce a motor complication known as LID and on-off phenomenon (Fahn et al., 2004; Stacy, 2009; Ammal Kaidery et al., 2013; Cacabelos, 2017).

Among the therapeutic alternatives proposed in recent decades, electrical neuromodulation therapies, i.e., Deep Brain Stimulation (DBS) and Spinal Cord Stimulation (SCS) have emerged as interesting options for treatment of neurodegenerative pathologies associated with movement disorders such as PD and HD (**Figure 2**). The effect of stimulation on different basal ganglia nuclei in patients is described below.

Deep Brain Stimulation (DBS)

DBS is a neurosurgical strategy based on the implantation of electrodes on subcortical nuclei that, through electrical signals, can modulate the neuronal activity of different regions of the brain. The use of DBS for the treatment of motor disorders was first proposed in 1971 by Natalia Bekhtereva (Bekhtereva et al., 1972; Hariz et al., 2010). But it was until 1996 when the FDA approved the stimulation of the ventral intermediate nucleus (VIM) of the thalamus for the treatment of essential tremor and severe tremor in PD. Later in 2002, the stimulation of the STN and GPi for the treatment of bradykinesia and rigidity in advanced cases of PD was included (Strotzer et al., 2019). It has been suggested that the stimulation of the STN induce the suppression of aberrant oscillatory synchronization at low frequency (13–35 Hz, beta band) and this contributes to motor amelioration, mainly bradykinesia and rigidity. However, more studies are necessary to understand the mechanisms involved. Currently, a group at the University of Minnesota is performing a clinical trial to investigate how the brain activity in PD patients is related to DBS and pharmacological management, by the chronic recording of local field potential (LFP) in the cortex (**Table 1**, NCT02709148).

The underlying molecular and cellular mechanisms of DBS are still not sufficiently identified and different assumptions about functional principles have been proposed (Jakobs et al., 2019). Studies in neurotoxin pre-clinical models of PD (**Table 3**) have suggested that STN-DBS can induce neuroprotective effects by reducing the loss of dopaminergic neurons (Maesawa et al., 2004; Temel et al., 2006; Wallace et al., 2007; Harnack et al., 2008; Spieles-Engemann et al., 2010; Wu et al., 2012). Additionally, it has been described that STN-DBS induces an increase in the striatal expression of BDNF (Spieles-Engemann et al., 2011), which is described as a powerful anti-apoptotic factor that favors the maintenance and survival of the nigral dopaminergic population (Zhao et al., 2017). As stated before, BDNF would exert its effect by signaling downstream of the TrkB receptor (Fischer et al., 2017). Nevertheless, reports in a genetic model of PD have revealed contradictory results. Musacchio et al. (2017) have reported that DBS reduces the loss of dopaminergic neurons, but it did not rescue the DA deficit. On the other side, Fischer et al. (2017) revealed that STN-DBS cannot counteract the axonopathy and dopaminergic loss progression induced by α-syn overexpression, but the differences in the starting time of application protocol must be considered. Moreover, the application of STN-DBS in pre-clinical models of PD has shown both metabolic and physiological effects on the nigrostriatal

DA system, inducing an increase in the levels of striatal DA and its metabolites (Bruet et al., 2001; Meissner et al., 2003; He et al., 2014). Genetic screening has revealed that high frequency-DBS can induce a differential expression of genes involved in apoptosis, growth, and neuroplasticity (Lortet et al., 2013). Studies also revealed that DBS increased the activity of the enzyme TH (Meissner et al., 2003) and the expression of the D1 receptor (Carcenac et al., 2015).

For this reason, different ongoing clinical trials are assessing the long-term neurological, neurophysiological, and neuropsychological effects of DBS treatment (**Table 1**, NCT00053625, NCT03021031), as well as improving the administration of the treatment (**Table 1**, NCT03021031). The technical difficulty of STN-DBS in PD patients is that a case-by-case parameter adjustment is necessary. Therefore, different studies are looking for electrophysiology biomarkers that can predict and improve the effectiveness of DBS therapy, as well as new stimulation methodologies (directional DBS system) by invasive and non-invasive recording (**Table 1**, NCT03353688). Another limitation of DBS is the incapacity to alleviate the freezing of gait (FoG), a symptom observed in more than half of PD patients. Using electroencephalography (EEG) it is crucial to determine electrophysiological biomarkers present in FoG episodes that can be modulated by GPi-DBS or pedunculopontine nucleus stimulation (PPN-DBS, **Table 1**, NCT02548897). Furthermore, studies are evaluating different parameters of STN-DBS protocols to improve gait disorders (**Table 1**, NCT04184791, NCT02022735).

DBS of the GP is a promising alternative target for the treatment of chorea, motor symptoms classic in the early stages of HD (Albin et al., 1990). DBS has been considered an alternative to pallidotomy, a neurosurgical strategy for the treatment of choreic movement published in 1952, showing good effects in four HD patients (di Cianni et al., 1992). In 2004, Moro and colleagues (Moro et al., 2004) reported the first case of a 43-year-old patient with an 8-year history of HD submitted to bilateral GP stimulation. They found that stimulation at 40 Hz and 130 Hz improved motor symptoms, reducing chorea and dystonia, although the high frequency worsened bradykinesia. The improvement in the patient's performance of a motor task was associated with an increase of the activation of the sensorimotor cortex, premotor cortex, supplementary motor area, and anterior cingulate cortex, although high frequency did not modulate the activity in premotor cortex (Moro et al., 2004). Moreover, the simultaneous stimulation of the STN and GPi has shown beneficial effects (Gruber et al., 2014), however, if the simultaneous stimulation has an additive effect is not clear. To date, two prospective, randomized, and double blind studies have been posted. One published in 2015 (Wojtecki et al., 2015), and others still on course (**Table 2**, NCT02535884). In the first study, six patients, four with chorea-dominant and two with Westphal-variant (rigid-hypokinetic syndrome, often associated with juvenile-onset of HD), underwent DBS treatment for 6 months and showed a reduction of chorea (Wojtecki et al., 2015). Nevertheless, the Westphal-variant patients did not show any improvements, suggesting that GP-DBS cannot ameliorate bradykinesia.

There is no doubt that optimizing DBS systems is a great challenge. Furthermore, understanding the correlation between abnormal brain activity and motor symptoms is necessary to obtain major beneficial results. For a more detailed review of this topic, we suggest some reviews published elsewhere (Anderson and Lenz, 2006; Miocinovic et al., 2013; Fasano and Lozano, 2015).

Spinal Cord Stimulation (SCS)

Generally used for the treatment of chronic pain since 1967 (Shealy et al., 1967; Dones and Levi, 2018), SCS consists of the application of electrical pulses in the dorsal columns of the spinal cord (directly in the epidural space). It has been suggested that the mechanism of action is based on the antidromic activation of the dorsal column fibers, which activate the inhibitory interneurons within the dorsal horn (Yampolsky et al., 2012). However, the exact mechanisms are not fully elucidated.

Currently, the use of this therapeutic strategy has gone beyond nociceptive control. In 2009, Fuentes and colleagues (Fuentes et al., 2009) proposed the use of SCS for the treatment of motor symptoms in PD. Like this report, in the last decade several studies have evaluated SCS effects in advanced cases of PD, showing interesting effects in axial symptoms (gait and postural dysfunction) both in preclinical (Santana et al., 2014) and clinical studies. These benefits are not observed with other treatments like DBS or L-dopa. The mechanism involved in the effects of SCS in motor symptoms of PD is not fully understood. It has been proposed that SCS, by releasing biphasic electrical pulses of high frequencies (300 Hz), would increase locomotor activity mainly through modulation of activity in the cortex and basal ganglia (Fuentes et al., 2009; Santana et al., 2014). This modulation would occur by activating the path of the dorsal columns, which in turn would modulate the activity of the thalamus, and from there to the cortex and striatum, causing the breakdown of aberrant low-frequency oscillations [beta (10–30 Hz)] observed in preclinical models (Fuentes et al., 2009; Santana et al., 2014) and PD patients (Kuhn et al., 2006), similarly as described for DBS treatment. Nevertheless, this does not explain the effect of SCS on gait and postural dysfunction. The participation of the brainstem, specifically the pedunculopontine nucleus (PPN), has been suggested, given its connection with different structures involved in the motor system, summarized by Chambers et al. (2019).

SCS treatment application methodologies differ among reports. The position of the electrodes varies between patients depending on the study. Electrodes can be placed at the cervical or thoracic level (Cai et al., 2020), showing that in general, both strategies ameliorate motor impairments. The first studies assessing SCS as a treatment for motor symptoms involved PD patients with chronic pain as a primary indication. In most cases, SCS reduced the pain and improved motor function, which could reflect a synergistic effect (Thevathasan et al., 2010; Fénelon et al., 2012; Hassan et al., 2013; Nishioka and Nakajima, 2015; Kobayashi et al., 2018; Mazzone et al., 2019). Recently, Samotus et al. (2018) showed the effectiveness of SCS (300–400 μs/30–130 Hz) in the treatment of FoG and gait in five patients without pain for 6 months. This evidence is

encouraging for the search of treatments for symptoms that, to date, have not been satisfactorily controlled. This is the case for gait dysfunctions that affect nearly 40–60% of PD patients and are not improved by dopaminergic therapy (Giladi et al., 2001). Additionally, Pinto de Souza et al. (2017) reported the use and effectiveness of SCS as a complementary strategy to rescue the loss of efficacy of DBS and DA medication. But other studies are currently in a course to evaluate the efficacy of SCS in gait disorders (**Table 1**, NCT03079310).

To date, three studies in preclinical models have suggested that SCS could counteract the progression of PD through a neuroprotective effect. Shinko et al. (2014) showed in rats that SCS applied regularly for 2 weeks (one session of 1 h daily) starting 2 days before 6-OHDA injection, decreases dopaminergic neuronal loss by 20–25%, and increase the expression of VEGF, which could favor neuroprotection given its ability to reduce dopaminergic neuronal death by suppressing apoptosis (Yasuhara et al., 2004). Similarly, Yadav et al. (2014) demonstrated that the application of an SCS protocol for 6 weeks (two sessions of 30 min per week) that started a week post-6-OHDA injection reduced the loss of dopaminergic neurons by 10%. Furthermore, both studies showed a positive effect in preserving the dopaminergic innervation of the striatum, reducing its loss by 30–35%. Recently, an investigation showed that continuous application of SCS (24 h ON SCS), starting immediately after injection of 6-OHDA, reduced the loss of dopaminergic neurons and their striatal projections by ∼35% and ∼32% respectively, while a regular SCS protocol (8 h SCS ON/16 h SVS OFF) counteracted the degeneration of the nigrostriatal dopaminergic pathway only by ∼23% (Kuwahara et al., 2020). Thus, the periodicity of the treatment application reflects on different degrees of neuroprotection (Kuwahara et al., 2020). These data suggest that SCS could have a neuroprotective effect that might contribute to the relief of the observed motor symptoms in PD, given the relevance of dopaminergic projections in modulating the functioning of the circuit of the nuclei of the base through the regulation of cortical and subcortical neuronal activity during movement (Gerfen and Surmeier, 2011; Canessa et al., 2016). Nevertheless, additional studies are necessary to understand the mechanism involved in the beneficial effects associated with SCS in the long-term. More detailed reviews on this topic can be found elsewhere (de Andrade et al., 2016; Cai et al., 2020).

For HD, SCS treatment has not been suggested, probably because SCS has better effects in alleviating gait and posture problems, while DBS is still the best option for the treatment of involuntary movements, such as chorea.

GENE THERAPIES FOR PD AND HD

For the development of new therapies for PD and HD, it is important to include, especially for HD and genetic forms of PD, genetic correction/editing of the mutated gene(s). Nowadays, there are several gene silencing/editing technologies, including RNA interference (RNAi), antisense oligonucleotides (ASO), and clustered interspaced short palindromic repeats (CRISPR/cas9), which can be used as therapies for the treatment of PD and HD. For a more in-depth knowledge of gene therapy delivery systems and other cellular targets, reviews are published elsewhere (Sudhakar and Richardson, 2019; Chen et al., 2020).

As previously stated, PD is characterized by the selective degeneration of dopaminergic neurons in the SN, thus approaches aiming to revert this loss based on the delivery of genes encoding for enzymes required for DA synthesis could be useful. The first enzyme for DA synthesis is TH, which requires the enzyme GTP-cyclohydrolase-1 (GCH-1) to synthesize a cofactor for DA biosynthesis (Daubner et al., 2011). TH converts tyrosine into L-dopa, which finally is converted into DA by the aromatic L-amino acid decarboxylase (AADC; Hadjiconstantinou and Neff, 2008). Therapies to deliver enzymes involved in DA synthesis have been proved in preclinical and clinical studies showing its benefits.

Initially, gene therapy was based on the delivery of separate AAV vectors to transfer two or three enzymes critical for DA biosynthesis. These strategies showed behavioral benefits in rat and non-human primate PD models (Kirik et al., 2002a; Muramatsu et al., 2002). Furthermore, a clinical study in patients with moderate to advanced PD demonstrated the safety and tolerability of a 6 months treatment with a bilateral Ipu of AAV vector encoding for the human AADC gene (AAV-hAADC; Eberling et al., 2008). Importantly, PET scans using an AADC tracer demonstrated an increase in gene expression throughout the study. Similarly, administrating the AAV-hAADC vector in the putamen of PD patients showed to be safe and tolerable (Muramatsu et al., 2010). This study demonstrated the efficacies of AAV vectors for gene delivery, which persisted up to 2 years with a 46% improvement of the UPDRS motor scores (Muramatsu et al., 2010). Another completed study showed that bilateral Ipu infusion of the AAV-hAADC vector improves UPDRS mean scores by 30% in PD patients after 6 months (**Table 1**, NCT00229736). However, this study reported 3 cases in which surgical procedures caused intracranial hemorrhage, showing an important limitation of the surgical procedure, but not necessarily of the therapeutic strategy of gene delivery (Christine et al., 2009). Consequently, a long-term evaluation study of AAV-hAADC demonstrated stable transgene expression over 4 years after vector delivery in PD patients (Mittermeyer et al., 2012).

To potentiate the effects and benefits of the enzyme therapy for DA synthesis, researchers have developed a strategy using a simple vector, which carries the genes that encode for the three key enzymes for DA biosynthesis. One of these strategies uses a lentiviral-based vector derived from the equine infectious anemia virus (EIAV; Azzouz et al., 2002). This tricistronic lentivirus vector encodes TH, AADC and CH1 (Lenti-TH-AADC-CH1) in a single vector (ProSavin). Delivery of Lenti-TH-AADC-CH1 vector into the striatum of a non-human primate PD model restored extracellular concentrations of DA and improved motor function for up to 12 months (Jarraya et al., 2009). Furthermore, ProSavin was administered into the striatum of PD patients, demonstrating a significant improvement in mean UPDRS motor scores at 6 months post-treatment, with no adverse effects detected (**Table 1**, NCT00627588). Additionally, a long-term study of ProSavin showed that the treatment was

well-tolerated and safe, but its clinical benefits are still under observation after 4 years (Palfi et al., 2014).

Other alternatives using gene delivery for PD treatment are focused on lowering α-syn levels in dopaminergic neurons. One study used a ribozyme combined with an AAV vector (rAAV-SynRz). Nigrostriatal injection of rAAV-SynRz in MPP+-treated adult rats, which has increased expression of α-syn (Kühn et al., 2003), resulted in down-regulation of α-syn, preventing its accumulation in the SN, and significantly protected TH-positive neurons (Hayashita-Kinoh et al., 2006). Despite these positive results, no clinical trials using gene therapy for silencing α-syn to treat PD are being conducted.

One important branch of investigation regarding HD therapies is the reduction of HTT DNA, RNA, and/or protein levels. Using a conditional transgenic mouse model of HD, it has been demonstrated that the reduction of mHTT ameliorates motor and psychiatric-like deficits, forebrain weight loss, cortical and striatal volume decrease, presynaptic and postsynaptic markers changes, and electrophysiological changes in these mice (Wang et al., 2014). One possible alternative to reduce mHTT levels is the use of DNA-targeting strategies (Ambrose et al., 1994). Thus, permanent and selective deletion of mHTT gene could be an interesting therapeutic approach for HD with no negative effect on patient health. The CRISPR/Cas9 system has been investigated for its utility for HD therapy but is still in the preclinical stage. Using fibroblasts (Shin et al., 2016; Monteys et al., 2017; Dabrowska et al., 2018) and iPSCs-derived NPCs (Shin et al., 2016) from an HD patient, it has been demonstrated that the selective deletion of a fragment of the mHTT gene using the CRISPR/Cas9 strategy is possible. This approach led to a near-complete reduction of mHTT protein and left intact the wild type HTT gene (Shin et al., 2016). Furthermore, using CRISPR/Cas9 and a transposon-based approach, the precise correction of the CAG expansion in HD iPSCs has been achieved (Xu et al., 2017). These cells retain pluripotency, have a normal karyotype, and could be differentiated into NPCs and MSNs-like neurons, which presented some electrical properties of neurons. Interestingly, the corrected cells were deprived of some phenotypic abnormalities observed in the HD iPSC-derived neurons, including increased susceptibility to growth factor withdrawal, impaired neural rosette formation, and mitochondrial dysfunction (Xu et al., 2017).

Using BACHD mice (**Table 4**) it has been shown that mHTT expression is reduced when Cas9 and a single guide RNA (sgRNA) are delivered using AAVs in the striatum, causing the deletion of a fragment around exon 1 (Monteys et al., 2017). Likewise, AAV-mediated injection of Cas9 and 2 sgRNA into the striatum of HD140Q-KI mice (**Table 4**) achieved a significant reduction in mHTT levels, accompanied by improved motor performance, decrease in reactive astrocytes and attenuation of bodyweight reduction (Yang et al., 2017). Similar results have been found using the R6/2 mice model, in which AAV-mediated delivery of Cas9 and sgRNA into the striatum caused a near 40% reduction in mHTT inclusions and around a 50% decrease in mHTT protein levels (Ekman et al., 2019). These mice presented increased mean survival, better motor performance, and decreased hindlimb clasping, an established indicator of dystonia (Ekman et al., 2019).

Despite the promising results, there is still a need for validation of this approach for human research. Given that many studies use CRISPR/Cas9 technology based in the recognition of short repeat sequences in the DNA, intensive studies must be performed to find these short sequences in the mHTT gene that is not present in another codifying gene sequence, so off-target genomic removal could be avoided. Moreover, single-nucleotide changes can occur in the mHTT gene in every patient affected, therefore sequencing of the mutant gene would be necessary for each patient. Another important issue of using CRISPR/Cas9 technology in humans is that the deletion of the mutated gene is permanent and irreversible. Also, the transduction of Cas9, a protein of bacterial origin, could activate the patients' immune system and edited cells would be effectively eliminated (Wignakumar and Fairchild, 2019). Despite this, CRISPR/Cas9-mediated deletion of the mHTT gene could be perfectly coupled with cell replacement strategies, with a correction of the mutation.

A second strategy for lowering mHTT levels is based on RNA-targeting strategies. One of the most studied methods to achieve this is through RNAi. These include the use of microRNAs (miRNAs) and short hairpin RNAs (shRNAs), all using the Dicer-RISC machinery for the targeted degradation of mRNAs (Ghosh and Tabrizi, 2017). Initially, using shRNAs and HD-N171-82Q HD mice (**Table 4**), it was shown a partial reduction of mHTT mRNA by 50% and decreased mHTT protein accumulation in the striatum, accompanied by a significant improvement in motor performance (Harper et al., 2005). Similarly, using R6/1 HD mice (**Table 4**), it has been demonstrated that AAV-mediated delivery of shRNAs against mHTT significantly reduces mHTT mRNA and protein levels (Rodriguez-Lebron et al., 2005). shRNA-injected striatum had smaller and less intense intranuclear mHTT inclusions, which was accompanied by increased MSNs mRNA markers. However, this group showed just a mild effect on the clasping phenotype observed in these mice (Rodriguez-Lebron et al., 2005).

These and other studies have demonstrated that the reduction of mHTT using RNAi in the brain of HD mice decreases both mHTT mRNA and protein levels, which is accompanied by improved motor behavior. However, in these studies the RNAi was designed to target human mHTT, leaving endogenous mouse HTT unaltered. This represents a problem when we think about patients' treatment, given that both alleles differ in the expanded polyQ in the mutated gene and different single nucleotide polymorphisms (SNPs) present. Even though several SNPs have been identified to be differentially present in the mutant and wild type allele, this accounts just for 80% of the population with HD, leaving an important number of patients without a therapeutic alternative (Pfister et al., 2009). Therefore, RNAi has been tested for the reduction of both mHTT and wild type HTT mRNA. Using an HD mouse model that expresses both mouse wild type and human mutant HTT, non-allele specific miRNA-mediated knockdown of both mRNAs in the striatum can significantly improve motor coordination

(Boudreau et al., 2009). Notably, these mice survived and had no phenotypic changes after a 75% reduction of wild type HTT for 4 months (Boudreau et al., 2009). Using AAV-mediated miRNA injection into the putamen of rhesus monkeys to target both HTT and mHTT mRNAs, it has been demonstrated that a 45% reduction of HTT mRNA did not induce motor deficits in these animals, nor neurodegeneration, astrogliosis, microglial activation or neuroinflammation (McBride et al., 2011). These phenotypes were also maintained in rhesus monkeys treated with AAV-shRNA for HTT mRNA for 6 months (Grondin et al., 2012).

One important limitation of double-stranded RNAs (shRNAs and miRNAs) is that they are not capable of crossing the BBB and do not easily cross the plasma membrane of cells. Given that they demonstrated positive effects when targeted to the brain, methods to deliver these molecules are under study (Chernikov et al., 2019). Despite this limitation, in 2019 the first clinical trial using AAV5-mediated delivery of a miRNA against HTT was initiated (**Table 2**, NCT04120493). Early manifest HD patients will be assessed for motor and cognitive functions, with miRNA and exploratory biomarkers measured in the CSF. The same AAV5-miRNA was proven to lower mHTT mRNA specifically in rat and minipig HD model. Suppression of mHTT mRNA levels was associated with lower mHTT protein levels, inclusion formation, and neuronal dysfunction (Miniarikova et al., 2017; Evers et al., 2018).

Another strategy used for RNA targeting is through ASOs. These are short, synthetic single-stranded DNA molecules that are complementary to a pre-mRNA target sequence in the nucleus, and have been widely studied for the treatment of neurological disorders (Rinaldi and Wood, 2018). Using IONIS-HTTRx against HTT mRNA, Tabrizi et al. (2019) reported the first results obtained from a phase I–II multicenter clinical trial in which this ASO was intrathecally administered in early-manifest HD patients. IONIS-HTTRx demonstrated to be safe and well-tolerated, and importantly, mHTT levels in the cerebrospinal fluid (CSF) of patients treated with IONIS-HTTRx decreased dose-dependently. In this trial, no changes in motor and cognitive functions were observed between the placebo and the treated group, which can be explained by the slow progression of the disease and the narrow window of time in which these changes were measured (Tabrizi et al., 2019). Some of these patients participated in a 15-month extension study, which ended by October 2019 (**Table 2**, NCT03342053). The original clinical trial is now under phase III, with more than 900 patients in 101 locations around the world, which is expected to end by September 2022 (**Table 2**, NCT03761849).

A second and third phase Ib/IIa clinical trials are underway to evaluate single and multiple doses of two ASOs against two specific SNPs found in the mHTT gene. PRECISION-HD1 clinical trial will evaluate the ASO WVE-120101 against the SNP rs362307 (**Table 2**, NCT03225833) and PRECISION-HD2 clinical trial will use the ASO WVE-120102 against the SNP rs362331 (**Table 2**, NCT03225846). These multicenter clinical trials, in which 60 patients with early-manifest HD are participating, started in 2017 and are expected to finish by the end of this year.

The results of the IONIS-HTTRx clinical trial are encouraging and bring us closer to therapy for HD. Given that ASOs bind directly to the DNA sequence, it is less likely that off-target suppression occurs. Moreover, as in the PRECISION clinical trials, unique SNPs found specifically in the mHTT DNA sequence allows for the design of specific ASOs, avoiding off-target suppression. However, the use of ASOs has disadvantages. In the PRECISION clinical trials, the ASOs evaluated target specific SNPs found in the mHTT gene, but they are not present in all HD patients. On the other hand, the IONIS-HTTRx clinical trial targets both wild type and mHTT genes, so it can be used in all HD patients. However, to date, there is no data available on the possible effects of wild type HTT suppression in patients. As mentioned before, non-human primates with sustained suppression of wild type HTT did not show adverse effects in motor and cognitive skills (McBride et al., 2011; Grondin et al., 2012), results that could be escalated to humans. Nevertheless, evaluation of long-term suppression of wild type HTT is key in HD patients. Also, repeated administration of ASOs could be necessary to maintain therapeutic benefits.

DISCUSSION

PD and HD are movement disorders characterized by the presence of aberrant and unwanted involuntary movements. The main cause for the variety of motor symptoms observed in patients is the selective neuronal loss in brain areas implicated in movement fine-tuning. Despite this knowledge, current treatments are not able to stop, reverse, or slow down PD and HD. Current treatments are directed to keep to line the motor symptoms, but with limited efficacy. Thus, continuous strong efforts are being made for the development of new therapeutic strategies for both diseases. Moreover, the development of small molecules for the treatment of these neurodegenerative diseases has a particularly high failure rate. Thus, strong efforts are being made for the development of new therapeutic strategies for both diseases.

Currently, no approved drugs can modify the progression of either PD or HD. Nevertheless, pharmacology provided therapies to reestablish DA levels in PD patients and to reduce DA neurotransmission in HD patients. Unfortunately, different and aggressive side effects appear during these treatments. New formulations have been developed to control the additional psychomotor and autonomic complications produced by some anti-parkinsonians drugs, however, it is important to reduce all the events that can affect or deteriorate the quality life of patients, and maybe the answers are out of the classical pharmacology approach.

Among the first strategies used for the treatment of PD and HD were cellular replacement therapies, in which fetal tissue was transplanted into the patient's brain. Even though some functional recovery was observed, this strategy had several limitations. The number of fetuses used generates important religious and ethical concerns. Besides, in some cases, tumor formation was observed in the site of transplantation, and most of the studies required immunosuppressive therapy. Although new strategies have been developed for obtaining dopaminergic and

GABAergic neurons from ESCs and iPSCs, these have not yet escalated into clinical trials.

Deregulation of NTFs' levels has been reported in PD and HD patients, and the administration of NTFs has shown beneficial effects in patients with these diseases. The reported neuroprotective effect can be explained by the prevention of neurodegeneration, restoring, and delaying the occurrence of motor symptoms. Moreover, therapy with NTFs has proven to be effective not only in preclinical stages but has also shown a positive effect in clinical trials. However, one main limitation of NTFs therapy is its poor brain availability after oral administration, which can only be solved by direct administration to the brain. Clinical studies directly administrating NTFs to the brain have reported side effects such as nausea, vomiting, headache, as well as infection when catheter implantation is used. Despite side effects, these studies have observed promising effects with this therapy, however, improvement and development of new delivery methods are key for successful NTF-base treatments.

Without a doubt, the development of electrical neuromodulatory therapies to treat motor symptoms in PD and HD has brought encouraging news for patients, doctors, and their families. Clinical reports carried out in recent decades have validated the use of DBS or SCS as a complement to classical pharmacological therapies that, due to their chronic use, decrease their effectiveness or induce more complex motor symptoms. Additionally, studies in PD patients have reported that the simultaneous use of DBS and SCS would bring important benefits, since DBS is effective in relieving motor symptoms such as tremor, bradykinesia, and stiffness, while SCS has to relieve symptoms associated with walking and posture. Although this has not been validated in the clinic, it opens the discussion about the possibility of using both strategies for the synergistic treatment of cardinal and axial symptoms of PD. When it comes to HD treatment, DBS has emerged as a promising alternative after showing positive effects similar to pallidotomy, reducing choreic movements and dystonia, but with the advantage of being a flexible option that adjusts to the patient's requirements. Since the mechanisms by which both strategies exert their effect have not been fully elucidated, their use requires personalized patient-to-patient care throughout the treatment, complicating its application in countries with weak health systems. Moreover, given their surgical complexity and high monetary cost, these therapies continue to be considered as a last option, even when reports in pre-clinical models, particularly in the case of PD, have reported encouraging results regarding their effect in counteracting neurodegeneration processes.

Worldwide excitement among HD patients and their families arose after the report of a promising potential therapy (IONIS-$HTTR_x$) that was able to decrease mHTT levels in the CSF of patients. Now, this treatment is under phase III clinical trial, in which patients of more than 100 centers around the world have been recruited. This is by far the closest that we have got to a definite treatment for HD. Nevertheless, many questions need to be answered during this clinical trial to ensure safety for patients. Given that IONIS-$HTTR_x$ targets both HTT and mHTT, one important question is whether long-term suppression of HTT is deleterious for neurons and if patients present improvements in motor and cognitive function.

Along with the development of new therapeutic approaches, like the ones described in this review, comes the important need to assess therapy response and disease progression. A key line of investigation is focused on finding new and reliable biomarkers that allow assessing changes in the disease course, helping in the evaluation of therapeutic response. Several biomarkers have been described for both PD and HD patients, but global multicenter studies are necessary to validate those biomarkers worldwide. Another important aspect to be considered in the development of new therapies is the heterogeneity of symptoms observed in both PD and HD patients, which suggest that not all treatments are the best option for all cases, as well as not all treatments are the best options for the same symptoms, suggesting that future clinical approaches will personalize the treatment or combine the strategies presented in this article to increase their efficacy and/or reduce its adverse effects.

Altogether, the presented data highlights the idea that we are on the right track for the development of new therapeutic strategies for PD and HD, but there is still a long road ahead to find a definitive treatment that may stop the progression or even cure the disease.

AUTHORS CONTRIBUTIONS

PT-E, DS, RP-A, AP, JA, and RV wrote and edited the manuscript. RV and FG critically analyzed the data. PT-E, AP, JA, and RV prepared the figures and tables. RV planned the manuscript. All authors contributed to the article and approved the submitted version.

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The Mitochondrial Unfolded Protein Response: A Hinge Between Healthy and Pathological Aging

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Aging is the time-dependent functional decline that increases the vulnerability to different forms of stress, constituting the major risk factor for the development of neurodegenerative diseases. Dysfunctional mitochondria significantly contribute to aging phenotypes, accumulating particularly in post-mitotic cells, including neurons. To cope with deleterious effects, mitochondria feature different mechanisms for quality control. One such mechanism is the mitochondrial unfolded protein response (UPR^{MT}), which corresponds to the transcriptional activation of mitochondrial chaperones, proteases, and antioxidant enzymes to repair defective mitochondria. Transcription of target UPR^{MT} genes is epigenetically regulated by Histone 3-specific methylation. Age-dependency of this regulation could explain a differential UPR^{MT} activity in early developmental stages or aged organisms. At the same time, precise tuning of mitochondrial stress responses is crucial for maintaining neuronal homeostasis. However, compared to other mitochondrial and stress response programs, the role of UPR^{MT} in neurodegenerative disease is barely understood and studies in this topic are just emerging. In this review, we document the reported evidence characterizing the evolutionarily conserved regulation of the UPR^{MT} and summarize the recent advances in understanding the role of the pathway in neurodegenerative diseases and aging.

Keywords: mitochondrial unfolded protein response, neurodegenerative diseases, aging, mitochondria, epigenetic regulation, stress response

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UPR^{MT} MACHINERY AND MITOCHONDRIAL HOMEOSTASIS REGULATION

Mitochondria are the main energy producers within the cell and the coordinators of several pathways that control essential metabolites, which include not only ATP and NAD^+, but also acetyl-CoA and S-adenosyl methionine for protein acetylation and methylation, respectively (Teperino et al., 2010; Menzies et al., 2016). Mitochondria are unique in that they have an independent genome (mtDNA), which encodes 2 rRNAs, 22 tRNAs, and 13 proteins that constitute the OXPHOS complexes (Wallace and Chalkia, 2013). Remaining mitochondrial proteins are encoded in the nucleus, so the function of the organelle heavily depends on the coordinated regulation of nuclear and mitochondrial genomes (Couvillion et al., 2016). Imbalances in protein expression in any of these two sources activate

an anterograde regulation of mitochondrial function (from the nucleus towards mitochondria) that adjusts its activity to match cellular needs (Cui et al., 2006; Kaarniranta et al., 2018). Mitochondria can also control the expression of nuclear genes through a retrograde regulatory mechanism (Lin and Haynes, 2016). This bidirectional communication between mitochondria and the nucleus forms a molecular network that maintains cellular homeostasis. Part of the network that synchronizes the cellular adaptation to a variety of stressors is termed the mitochondrial unfolded protein response (UPR^{MT}). Thus, UPR^{MT} is the transcriptional program that stabilizes mitochondrial homeostasis and reduces misfolded protein amount in the organelle, increasing the mitochondrial response capability to stress stimuli (reviewed in Jensen and Jasper, 2014; Shpilka and Haynes, 2018; Gomez and Germain, 2019; Tran and Van Aken, 2020). Known activators of UPR^{MT} include the impairment of the Electron Transport Chain (ETC), alteration of mitochondrial dynamics, accumulation of unfolded proteins, deletion of mitochondrial DNA (mtDNA), inhibition of mitochondrial chaperones or proteases, and the increase of reactive oxygen species (ROS) levels (Nargund et al., 2012; Pimenta de Castro et al., 2012; Runkel et al., 2013; Qureshi et al., 2017). Despite the mechanisms underlying the UPR^{MT} are less understood than endoplasmic reticulum UPR (Hetz et al., 2020), this mitochondrial stress pathway is emerging as an important response that guarantees the organelle function.

UPR^{MT} was originally observed in mammalian cells, where mitochondrial stress was induced by mtDNA deletions (Martinus et al., 1996) and by aggregation of mutant ornithine transcarboxylase (ΔOTC) (Zhao et al., 2002). Both stress stimuli upregulated the expression of mitochondrial chaperones Hsp60, Hsp10 under the control of the transcription factor CHOP (Zhao et al., 2002; Horibe and Hoogenraad, 2007). Three nuclear components were then identified in *C. elegans* as UPR^{MT} regulators: ATFS-1, DVE-1, and UBL-5. These proteins are part of the UPR^{MT}-ATF5 axis, an ATFS-1/ATF5 dependent response that is the most characterized UPR^{MT} pathway (**Table 1**, Kenny and Germain, 2017; Ji et al., 2020). ATFS-1, a leucine zipper protein, carries a nuclear localization sequence and a mitochondrial targeting sequence. Under mitochondrial stress, ATFS-1 normal transport towards mitochondria is blocked and translocates instead to the nucleus where it interacts with DVE-1 and UBL-5 (**Figure 1**; Nargund et al., 2012, 2015). In mammals, the CHOP target ATF5 was identified as the functional ortholog for ATFS-1, which also contains targeting sequences for mitochondria and nucleus and upregulates UPR^{MT} genes (Teske et al., 2013; Fiorese et al., 2016). On the other hand, DVE-1 is a DNA binding protein that together with its coregulator UBL-5, interacts with chromatin regions to maintain an ATFS-1-dependent active transcription of UPR^{MT}-related genes (Benedetti et al., 2006; Haynes et al., 2007; Tian et al., 2016). The coordinated action of these three proteins upregulates the expression of mitochondrial chaperones hsp-60, hsp-6, and protease clpp-1 (**Table 1**, Haynes and Ron, 2010).

Two other pathways have been associated with this stress response (**Figure 1**). The UPR^{MT}-ERα axis, a pathway dependent on the activation of the estrogen receptor α (ERα), was described as associated with the accumulation of proteins in the mitochondrial intermembrane space (Papa and Germain, 2011). Mitochondrial stress and ROS production trigger the phosphorylation of the protein kinase AKT and consequently, the activation of ERα. This cascade increases the transcription of protease HTRA2 and the mitochondrial biogenesis regulator NFR1, which translates in an increased proteasome activity independent of activation of the UPR^{MT}-ATF5 axis (**Table 1**, Papa and Germain, 2011). Finally, the UPR^{MT}-SIRT3 axis is based on the activation of Sirtuin 3 that modulates the expression of SOD1, SOD2, and catalase, through activation of FOXO (Papa and Germain, 2014; Kenny et al., 2017). The UPR^{MT}-SIRT3 axis has been validated also in worms and mammalian cells, supporting the high evolutionary conservation of the pathway (Mouchiroud et al., 2013). Importantly, both ERα- and SIRT3-UPR^{MT} axes work independently of CHOP (Papa and Germain, 2014), upholding the idea of three parallel paths coordinating the same stress response (**Figure 1**).

Chromatin remodeling has been shown to play a central role in UPR^{MT} regulation. Histone 3 is a target for methylation catalyzed specifically by methyltransferase MET-2 in *C. elegans* (ortholog of human SETDB1). Activation of UPR^{MT} requires the dimethylation of lysine 3 of histone 3 (H3K9), which translates into a compacted and overall silenced chromatin state. At the same time although, other chromatin portions remain loose, favoring the binding of UPR^{MT} regulators such as DVE-1 (Tian et al., 2016). Also required for UPR^{MT} activation are the conserved demethylases JMJD-3.1 and JMJD-1.2, which reduce the chromatin compaction by removing methylation from H3K9 and H3K27 (**Figure 1**; Merkwirth et al., 2016; Sobue et al., 2017). Interestingly, chromatin remodeling acts independently of ATFS-1, as its downregulation does not affect the nuclear localization of DVE-1 (Tian et al., 2016). It is known that besides genes encoding chaperones and proteases, UPR^{MT} activation increases the expression of glycolysis and amino acid catabolism genes, and represses TCA-cycle and OXPHOS encoding genes (Nargund et al., 2015; Gitschlag et al., 2016; Lin and Haynes, 2016). To date, it is not clear whether UPR^{MT} can activate any other quality control mechanism such as mitochondrial fission, fusion, and mitophagy. It has been reported, however, that the same mitochondrial stressors can activate mitophagy and UPR^{MT} (Nargund et al., 2012; Pimenta de Castro et al., 2012; Jin and Youle, 2013; Runkel et al., 2013; Lin et al., 2016). Organisms that have adapted after constant exposure to low doses of these stressors (misregulation of ETC components and low doses of the UPR^{MT} activator paraquat) exhibit a hormetic phenotype as they show increased longevity despite their mild mitochondrial dysfunction (Yoneda et al., 2004; Owusu-Ansah et al., 2013). This homeostatic regulation is particularly important in post-mitotic cells such as neurons.

THE ROLE AND REGULATION OF UPR^{MT} IN AGING

Aging is defined as the time-dependent functional decline that increases vulnerability to different forms of stress, ultimately

TABLE 1 | Mitochondrial UPR regulators and their function in conserved species.

Name	CE	DM	MM	HS	Function	References
UPR^{MT}-ATF5 axis						
Activating Transcription Factor 5	Atfs-1	crc	Atf5	ATF5	Transcription factor with basic leucine zipper domain. Carries an MTS in the N-term and an NLS in the C-term.	Yoneda et al. (2004), Nargund et al. (2012), Fiorese et al. (2016) and Wu et al. (2018)
Special AT-Rich Sequence-Binding Protein 2	dve	DVE	Satb2	SATB2	DNA binding protein. Stabilizes open chromatin for UPR^{MT}-associated transcription.	Haynes et al. (2007) and Tian et al. (2016)
Ubiquitin Like 5	UBL-5	ubl	Ubl5	UBL5	Protein binding. Binds DVE to activate transcription of Hsp60	Benedetti et al. (2006)
ATP Binding Cassette Subfamily B Member 10	haf-1	CG3156	ABCB10	ABCB10	Mitochondrial inner membrane transporter. Exports peptides from the matrix.	Haynes and Ron (2010) and Yano (2017)
Caseinolytic Mitochondrial Matrix Peptidase Proteolytic	clpp-1	ClpP	ClpP	CLPP	Mitochondrial ATP-dependent protease. Its attenuation reduces the UPR^{MT} activation and the formation of the UBL/DVE complex.	Haynes et al. (2007) and Al-Furoukh et al. (2015)
Translocase of Inner Mitochondrial Membrane 23	timm-23	Tim23	Timm23	TIMM23	Protein transmembrane transporter. Required for full induction of UPR^{MT} mediated by ATFS-1.	Rainbolt et al. (2013)
Lon Peptidase 1	, lonp-1	Lon	Lonp1	LONP1	Mitochondrial protease. Degrades ATFS-1 when imported to mitochondria under stress conditions.	Nargund et al. (2012)
Heat Shock Protein Family D (Hsp60) Member 1	hsp-60	Hsp60A Hsp60B Hsp60C	Hspd1	HSPD1	Mitochondrial heat-shock protease. Upregulated upon mitochondrial stress.	Zhao et al. (2002), Yoneda et al. (2004), Haynes et al. (2007) and Owusu-Ansah et al. (2013)
Heat Shock Protein Family A (Hsp70) Member 9	hsp-6	Hsc70–5	Hspa9	HSPA9	Mitochondrial heat-shock protease. More sensitive to oxidative stress than unfolded protein stress.	Yoneda et al. (2004), Benedetti et al. (2006) and Merkwirth et al. (2016)
UPR^{MT}- ERα axis						
Estrogen Receptor 1	nhr-107	ERR	Esr1	ESR1	Ligand-activated transcription factor. Regulates the expression of Htra2 and NRF1 after Akt phosphorylation.	Papa and Germain (2011) and Riar et al. (2017)
HtrA Serine Peptidase 2	psmd-9	HtrA2	HtrA2	HTRA2	Serine protease. Protein import checkpoint in IMS. Increased expression upon stress.	Papa and Germain (2011)
Nuclear respiratory factor 1	-	-	Nrf1	NRF1	Transcription factor.	Papa and Germain (2011)
UPR^{MT}- SIRT3 axis						
Sirtuin 3	Sir-2.1	Sirt2	Sirt3	SIRT3	NAD$^+$ dependent deacetylase. Regulates the activity of FOXO3 upon oxidative stress in the mitochondria.	Mouchiroud et al. (2013); Papa and Germain (2014); Gariani et al. (2016) and Kenny et al. (2017)
Forkhead box	daf-16	foxo	Foxo3	FOXO3	Transcription factor. Translocate to the nucleus to activate transcription of SOD1, SOD2, and Catalase.	Mouchiroud et al. (2013); Gariani et al. (2016) and Kenny et al. (2017)
Superoxide dismutase 1	sod-1	Sod	Sod1	SOD1	Superoxide dismutase. Soluble cytoplasmic isoenzyme.	Mouchiroud et al. (2013); Gariani et al. (2016) and Kenny et al. (2017)
Superoxide dismutase 2	sod-2	Sod2	Sod2	SOD2	Superoxide dismutase. Mitochondrial isoenzyme.	Mouchiroud et al. (2013); Gariani et al. (2016) and Kenny et al. (2017)

(Continued)

TABLE 1 | Continued

Name	CE	DM	MM	HS	Function	References
UPR^{MT} epigenetic regulators						
SET Domain Bifurcated Histone Lysine Methyltransferase 1	met-2	eggless	Setdb1	SETDB1	Histone methyltransferase. Loci protected by H3K9 contains genes upregulated upon mitochondrial stress.	Tian et al. (2016)
Abnormal cell lineage.65	lin-65	-	-	-	Nuclear co-factor. Highly unstructured protein involved in chromatin remodeling. Necessary for the incorporation of MET-2 to the nucleus.	Tian et al. (2016)
Euchromatic Histone Lysine Methyltransferase 1	SET-6	CG4565	Ehmt1	EHMT1	Histone methyltransferase. Upregulated during aging to inhibit UPR^{MT} activation.	Yuan et al. (2020)
Lysine Demethylase 6A	JMJD-3.1	Utx	Kdm6A	KDM6A	Histone demethylase. Positive regulator of lifespan upon mitochondrial stress. Needed to activate the UPR^{MT}	Merkwirth et al. (2016)
Bromodomain Adjacent to Zinc Finger Domain 2B	Baz-2	tou	Baz2b	BAZ2B	Transcription factor. Neuronal Epigenetic Reader upregulated during aging acting with SAT-6 to inhibit the UPR^{MT} during aging by regulating methylation of H3K9.	Yuan et al. (2020)

CE, Caenorhabditis elegans; DM, Drosophila melanogaster; MM, Mus musculus; HS, Homo sapiens; MTS, mitochondrial targeting sequencing; NLS, nuclear localization signal; IMS, mitochondrial intermembrane space.

leading to death (Kennedy et al., 2014). Aging has particularly severe consequences for organs composed mostly by post-mitotic cells, such as the heart and brain (Kowald and Kirkwood, 2000; Terman et al., 2010). For instance, aging is the major risk factor for the onset of chronic, brain-related, and neurodegenerative diseases (ND). Current studies in the field introduced critical questions aiming to understand the physiological sources of time-dependent damage, the compensatory cellular responses that reestablish homeostasis, and their interconnection to find potential targets to intervene and delay aging. Seven cellular pillars of aging have been described, including among others, alterations to proteostasis, epigenetics, metabolism, and adaptation to stress (Kennedy et al., 2014). Mitochondrial dysfunction is a common factor for these events, suggesting a role of mitochondrial reparative machinery in aging progression. Furthermore, it is accepted that aging in model organisms is functionally associated with mitochondrial decline, contributing to the time-dependent tissue malfunction (Chistiakov et al., 2014; Kim et al., 2018). Therefore, activation of UPR^{MT}, as one of the mitochondrial mechanisms against different forms of aging-causing damage, could be in part bridging the adaptation to stress and other pillars of aging as proteostasis and epigenetics.

Current evidence highlights an age-dependent effect of UPR^{MT} on lifespan. For instance, activation of UPR^{MT} triggered by downregulation of ETC complexes I and IV promotes longevity (Dillin et al., 2002; Durieux et al., 2011; Mouchiroud et al., 2013). Histone demethylases JMJD-1.2 and JMJD-3.1 mediate in part that extension, as their overexpression is sufficient to extend the lifespan of worms (Merkwirth et al., 2016). On the other hand, reducing the expression of nuclear effectors ATFS-1, UBL-5 and DVE-1, or demethylases JMJD-1.2 and JMJD-3.1, suppresses the lifespan extension (**Table 1**, Durieux et al., 2011; Houtkooper et al., 2013; Merkwirth et al., 2016; Lan et al., 2019). It is interesting that UPR^{MT} activation after exposure to mitochondrial stress is strongly responsive only during development and not in later stages of the lifespan (Dillin et al., 2002; Copeland et al., 2009; Durieux et al., 2011; Houtkooper et al., 2013). UPR^{MT} appears less active in adult organisms, so there is no increased lifespan as a response to mitochondrial stressors, as observed in developmental stages in worms and flies (Dillin et al., 2002; Owusu-Ansah et al., 2013; Jensen et al., 2017).

Decreased chromatin accessibility of target UPR^{MT} genes in aged organisms is a potential explanation for the differential UPR^{MT} activation. This was recently confirmed in a study where the methyltransferase SET-6 and the neuronal epigenetic reader BAZ-2, mediated specifically an age-dependent regulation of UPR^{MT}. Both proteins when overexpressed in aged worms increased the levels of H3K9Me3, the triple methylated state of the protein, thus inhibiting UPR^{MT} activation in the H3K9-protected loci (**Figure 1**). Loss of function of SET-6 or BAZ-2 increased healthspan but not longevity, a phenotype that was inhibited downregulating UBL-5 or ATFS-1 (Yuan et al., 2020). Histone 3 methylation appears then as a key epigenetic mediator for UPR^{MT} throughout the lifespan (Merkwirth et al., 2016; Tian et al., 2016; Ono et al., 2017). Longitudinal studies have proved that H3K9Me3 increases during aging in mice

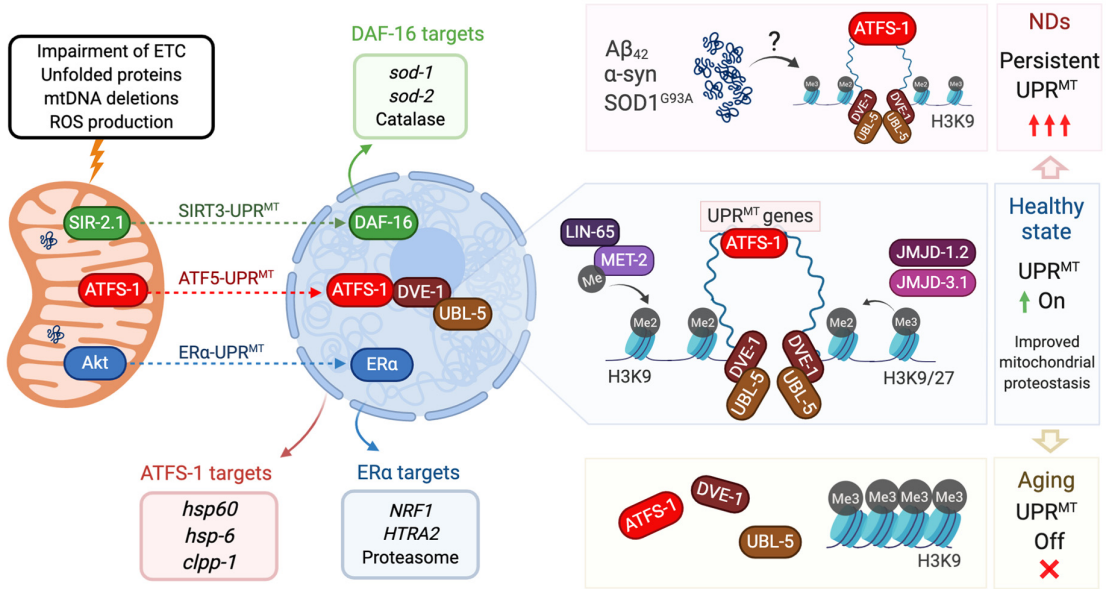

FIGURE 1 | Mitochondrial unfolded protein response (UPR) and its regulation in aging and neurodegeneration. Insults to mitochondria (top left) activate three different axes of the UPR^{MT} program. The SIRT3-UPR^{MT} axis (green arrow) increases the transcription of superoxide dismutases and catalase, after the activation of DAF-16/FOXO3 by the deacetylase SIR-2.1/SIRT3. In the ATF5-UPR^{MT} axis (red arrow), the transcription factor ATFS-1/ATF5 relocalizes from mitochondria to nucleus to upregulate mitochondrial proteostasis related genes (red box) after the interaction with the chromatin stabilizers DVE-1 and UBL-5. In the ERα-UPR^{MT} axis (blue arrow) the estrogen receptor is activated by the kinase Akt, to increase the expression of the protease HTRA2 and mitochondrial biogenesis regulator NRF1. On a healthy state (center right), ATF5-UPR^{MT} activation requires chromatin reorganization. Dimethylation of Histone 3 by MET-2, and presence of demethylases JMJD-1.2 and JMJD-3.1, allow the binding of DVE to facilitate the ATFS-1-dependent expression of UPR^{MT} genes and improve mitochondrial proteostasis. In neurodegenerative states triggered by accumulation of Aβ$_{42}$ (AD), α-syn (PD) or SOD1^{G93A} (ALS, top right), UPR^{MT} could be persistently activated, affecting mitochondria proteostasis and neuron viability. In aging cells (bottom right), Histone 3 is preferentially trimethylated, which blocks DVE and ATFS-1 biding to compacted DNA. Lack of expression of UPR^{MT}-related genes decreases mitochondrial response to aging-causing damage. All protein names are taken from *C. elegans*, except the ones from the ERα axis, which has only been described in mammalian cells.

hippocampus, and inhibition of this methylation state was sufficient to block aging-associated cognitive decline in mice (Snigdha et al., 2016). Advanced knowledge of the loci carrying UPR^{MT} genes on them, will contribute to further understand the lack of UPR^{MT} activation during aging.

UPR^{MT} IN AGING NEURONS AND NEURODEGENERATIVE DISEASES

Several factors influence mitochondrial homeostasis in neurons during aging, such as oxidative damage, neuronal localization, and quality control mechanisms. Compared to mitotic cells, neurons are more sensitive to the accumulation of oxidative damage and defective mitochondria (Kowald and Kirkwood, 2000; Terman et al., 2010). Neuronal unique shape, on the other hand, generates a differential mitochondrial distribution required to provide energy at specific compartments (Obashi and Okabe, 2013). Indeed, evidence shows that at nerve terminals, mitochondria are more prone to age-related dysfunction and oxidative damage compared to non-synaptic mitochondria (Lores-Arnaiz et al., 2016; Olesen et al., 2020). Importantly, aging aggravates the difference between these two populations of mitochondria (Borrás et al., 2010; Lores-Arnaiz et al., 2016). The decreased ability of neurons to renew their pool of healthy mitochondria and the lower activity of quality control mechanisms, act synergistically to trigger deleterious consequences in neurons not only in aging but also at earlier stages. In the etiology of the most prevalent ND, shared critical mitochondrial stressors include misfolded and aggregated proteins, impaired mitophagy, and oxidative stress (Niedzielska et al., 2016; Bakula and Scheibye-Knudsen, 2020; Weidling and Swerdlow, 2020). Considering also the number of ND-causative genes associated with mitochondrial dysfunction (Masters et al., 2015; Hardiman et al., 2017; Poewe et al., 2017), quality control mechanisms such as UPR^{MT} emerge as key intervention targets for age-related diseases. However, compared to other mitochondrial response programs (Pellegrino and Haynes, 2015; Pernas and Scorrano, 2016; Misgeld and Schwarz, 2017) or even UPR^{ER} (Hetz et al., 2020), the studies linking UPR^{MT} and NDs are just emerging.

Parkinson's disease (PD) is caused by decreased dopamine secretion from damaged dopaminergic neurons (reviewed in Poewe et al., 2017). PD pathomechanism is strongly connected to mitochondrial dysfunction and only recently to UPR^{MT} (Franco-Iborra et al., 2018; Chen et al., 2019). Two proteins encoded by PD-causative genes, serine-threonine kinase PINK1 and E3 ubiquitin ligase Parkin, work together to unclutter dysfunctional mitochondria through mitophagy. PINK1 or

Parkin downregulation induces decreased mitochondrial respiration and ATP synthesis, degeneration of dopaminergic neurons, and reduced lifespan (Zhu et al., 2013; Moisoi et al., 2014; Tufi et al., 2014; Choi et al., 2015). In *C. elegans*, the downregulation of their orthologs (*pink-1* and *pdr-1*) activates UPR^{MT} as a mitigation mechanism. Without atfs-1 dependent UPR^{MT} activation, lifespan decreases, and dopamine neurons degenerate (Cooper et al., 2017).

PINK1 also interacts with the ERα target HTRA2, mediating its phosphorylation and activation (Plun-Favreau et al., 2007). Interestingly, mutant alleles of HTRA2 were found in PD patients (Strauss et al., 2005; Unal Gulsuner et al., 2014).

PD pathogenesis is strongly connected to the accumulation of α-synuclein (Poewe et al., 2017). αSyn^{A53T} preferentially accumulates in the mitochondria and interacts with the UPR^{MT}-regulator ClpP, suppressing its peptidase activity. Overexpression of the protease is sufficient to decrease αSyn^{A53T}-associated pathology in mice (Hu et al., 2019). Despite the previous evidence, reports are suggesting a toxic role of UPR^{MT} over-activation. Expression in dopaminergic neurons of an active form of ATFS-1 lacking the mitochondrial target sequence mimics stress conditions with a constant nuclear expression of UPR^{MT} targets. Over-activation of UPR^{MT} shortens lifespan and promotes faulty mitochondria accumulation, a phenotype synergistically increased overexpressing mutant αSyn^{A53T} (Martinez et al., 2017). From the epigenetic point of view, α-synuclein expression in *Drosophila* led to an upregulation of the methyltransferase EHMT2, with an overall H3K9 dimethylation effect (Sugeno et al., 2016). It would be interesting to study whether chromatin remodeling linked to the H3K9Me2 epigenetic mark in this PD model modifies UPR^{MT} activation as previously reported (Merkwirth et al., 2016; Tian et al., 2016).

Amyotrophic lateral sclerosis (ALS) is the most common motor neuron disease and its complex etiology is explained by the almost 30 causative genes that have been linked to familial cases (reviewed in Hardiman et al., 2017). Among these genes, mutations in the superoxide dismutase SOD1 initially unveiled a link between ALS and mitochondrial dysfunction (Rosen et al., 1993). Post-mortem samples of ALS patients show the altered activity of ETC complexes (Bowling et al., 1993), while SOD1 overexpression in transgenic mice causes dysregulated ETC activity, increased ROS production, and diminished mitochondrial Ca^{2+}-buffering (Mattiazzi et al., 2002; Brookes et al., 2004). Mutant SOD1^{G93A} localizes in the mitochondrial intermembrane space, which is sufficient to activate two axes of UPR^{MT} *in vivo* (Gomez and Germain, 2019). CHOP is transiently activated in mice's spinal cord, followed by Akt-dependent phosphorylation of ERα that upregulates NRF1 and proteasome activity (Riar et al., 2017; Gomez and Germain, 2019). This is consistent with recent reports showing that UPR^{MT} activation precedes the onset of ALS and its activity increases throughout disease progression (Pharaoh et al., 2019). Dysregulation of TDP-43, another ALS causative gene, impairs mitochondria in ALS patients, suppresses ETC complex I and activates UPR^{MT} in cellular and animal models. Downregulation of the UPR^{MT} protease LonP1 increased TDP-43 levels, mitochondrial damage and neurodegeneration (Wang et al., 2019). A third ALS-linked mitochondrial protein is CHCHD10, which has an unknown function but its mutant aggregates in mitochondria causing proteotoxic stress, mitochondrial dysfunction and upregulation of the UPR^{MT} regulators CHOP and ATF5 (Anderson et al., 2019). These reports suggest that the accumulation of ALS-associated mutant proteins in mitochondria persistently over activates UPR^{MT}, which could be triggering detrimental effects on already stressed neurons (**Figure 1**).

Alzheimer's disease (AD) is characterized by key neuropathological hallmarks such as the abnormal accumulation of the amyloid-β (Aβ) peptide (reviewed in Masters et al., 2015). Evidence indicates that oxidative damage and mitochondrial dysfunction have a key role in AD pathogenesis (Butterfield and Halliwell, 2019; Weidling and Swerdlow, 2020), but the relationship between UPR^{MT} and AD has only been recently explored. Aβ accumulation activates UPR^{MT} in human cells and mice (Shen et al., 2020). In *C. elegans*, the sirtuin-activator resveratrol reduced the Aβ-induced toxicity on a Ubl-5 dependent manner, decreasing the amount of Aβ aggregates (Regitz et al., 2016). Further characterization of this finding could provide clues of a potential connection between the two UPR^{MT} axes, and their association to AD. On the other hand, deficiency of the mitochondrial protease PITRM1 induces UPR^{MT}, increased Aβ accumulation, and triggered AD-like phenotypes. These were exacerbated by pharmacological inhibition of UPR^{MT} suggesting a protective role of the pathway on Aβ-associated toxicity (Pérez et al., 2020). The expression of UPR^{MT}-related genes appear highly increased in post mortem samples of the prefrontal cortex of AD patients (Beck et al., 2016). It would be noteworthy to determine the temporality of this increased expression to understand whether it is an early program persistently activated throughout the disease progression, or a late response triggered by an overall mitochondrial dysfunction. This is especially relevant considering that the expression of the epigenetic regulators of UPR^{MT} EHMT1 and BAZ2B, and therefore inhibition of UPR^{MT}, correlates positively with the progression of AD (Zhang et al., 2013; Yuan et al., 2020). Therefore, future studies should try to clarify whether both inhibition and persistent activation of UPR^{MT} contribute to ND pathomechanisms.

FUTURE PERSPECTIVES

Mitochondrial dysfunction is a hallmark of aging and age-related neurodegenerative diseases (Kennedy et al., 2014). As UPR^{MT} activation extends mitochondrial function, further characterization of the pathway will provide stronger hints to understand neuronal homeostasis and healthspan extension. So far, it seems that UPR^{MT} activation is partially modulated by the age-dependent methylation levels of Histone 3. As H3K9 is differentially methylated in specific brain regions (Snigdha et al., 2016), regulation of the UPR^{MT} could differ in distinct neuronal types. This fact raises concerns when thinking about therapeutic approaches since systemic inhibition of UPR^{MT}

could be beneficial for cell types with a dysregulated activation of UPR^{MT}, but detrimental for another that requires its activation. Therefore, the fine-tuning of UPR^{MT} in different pathogenic contexts will be a crucial consideration for future studies. In the case of PD, AD and ALS, incipient evidence has emerged in the last years highlighting also an over-activation of UPR^{MT} as contributors of the ND pathomechanisms. Future studies on this topic should focus on determining whether known ND causative genes are associated to UPR^{MT} components on an early neurodegenerative stage, or whether UPR^{MT} is only activated on a late, non-reversible stage as a consequence of an overall neuronal decay. Precise pharmacological modulation of the mitochondrial stress response could bring new alternatives to restore compromised neuronal functions, with a prospective increase in the life quality of ND patients and the elderly population.

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Molecular Chaperones: A Double-Edged Sword in Neurodegenerative Diseases

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Aberrant accumulation of misfolded proteins into amyloid deposits is a hallmark in many age-related neurodegenerative diseases, including Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), and amyotrophic lateral sclerosis (ALS). Pathological inclusions and the associated toxicity appear to spread through the nervous system in a characteristic pattern during the disease. This has been attributed to a prion-like behavior of amyloid-type aggregates, which involves self-replication of the pathological conformation, intercellular transfer, and the subsequent seeding of native forms of the same protein in the neighboring cell. Molecular chaperones play a major role in maintaining cellular proteostasis by assisting the (re)-folding of cellular proteins to ensure their function or by promoting the degradation of terminally misfolded proteins to prevent damage. With increasing age, however, the capacity of this proteostasis network tends to decrease, which enables the manifestation of neurodegenerative diseases. Recently, there has been a plethora of studies investigating how and when chaperones interact with disease-related proteins, which have advanced our understanding of the role of chaperones in protein misfolding diseases. This review article focuses on the steps of prion-like propagation from initial misfolding and self-templated replication to intercellular spreading and discusses the influence that chaperones have on these various steps, highlighting both the positive and adverse consequences chaperone action can have. Understanding how chaperones alleviate and aggravate disease progression is vital for the development of therapeutic strategies to combat these debilitating diseases.

Keywords: neurodegenarative diseases, prion-like spreading, proteostasis, molecular chaperones and Hsps, disaggregation

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INTRODUCTION

A common feature in many neurodegenerative diseases, including Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), amyotrophic lateral sclerosis (ALS), and prion diseases is the age-related formation of amyloid deposits (Chiti and Dobson, 2017). Each disorder is characterized by the misfolding of one or more specific proteins: amyloid-β (Aβ) and Tau (MAPT) in AD, α-synuclein (α-syn/SNCA) in PD, Huntingtin (HTT) in HD, superoxide

dismutase 1 (SOD1), TAR DNA binding protein 43 (TDP-43/TARDBP), FUS RNA-binding protein (FUS) and dipeptide repeat proteins (DPRs) translated from C9orf72-SMCR8 complex subunit (C9orf72) in ALS, and the prion protein (PrP/PRNP) in prion diseases (Dobson, 2017; Eisenberg and Sawaya, 2017). Despite having different structures and functions under physiological conditions, under disease conditions, these proteins adopt a β-sheet-rich conformation with a strong tendency to form highly ordered amyloid fibrils. These fibrils can act as pernicious templates for the native monomeric form of the respective protein to misfold into the amyloid conformation and incorporate into the growing fibrils, which eventually accumulate into large intra- and/or extracellular deposits characteristic for the respective neurodegenerative diseases (Jucker and Walker, 2013).

Protein aggregates usually arise from the failure of the protein quality control (PQC) machinery that maintains cellular protein homeostasis (proteostasis). Molecular chaperones are key components of the PQC network and support cellular proteostasis by regulating the folding of nascent polypeptides, the re-folding of aberrant proteins, or their removal by degradation *via* the ubiquitin-proteasome system (UPS) or autophagy (Bukau et al., 2006; Kampinga and Craig, 2010). When a protein escapes these (re)-folding or clearance mechanisms, misfolded forms accumulate and eventually aggregate (Hartl et al., 2011). An age-related decline in the capacity of the PQC machinery appears to result in a proteostasis collapse (Ben-Zvi et al., 2009), which in turn allows the manifestation of diseases associated with protein misfolding, such as the diseases mentioned above. On a positive note, the age-dependent accumulation of amyloid deposits in neurodegenerative diseases suggests that in younger individuals there are PQC pathways active that can prevent aggregation. Chaperones are key regulators of amyloid formation since they monitor and prevent the misfolding and aggregation of proteins (Kampinga and Bergink, 2016; Wentink et al., 2019). Here, we will highlight the complex ways in which chaperones influence the different stages of prion-like propagation of proteins associated with the most prevalent neurodegenerative diseases. This will contribute to a better understanding, not only of which chaperones could be selected for drug development, but also of when to target these chaperones.

PRION-LIKE PROPAGATION OF PROTEIN MISFOLDING IN NEURODEGENERATIVE DISEASES

The propensity of a protein aggregate to act as a template or "nucleus" or "seed" to promote the aggregation of its native form is central to the prion hypothesis (Prusiner et al., 1998). Prions (proteinaceous infectious particles) are the causative agent in prion diseases including bovine spongiform encephalopathy (BSE), chronic wasting disease (CWD), and scrapie (Prusiner et al., 1998). In prion diseases, disease-associated PrP^{Sc} can propagate itself by templating the conversion of the endogenous PrP^{C} from its normal helical into a β-sheet-rich amyloid conformation (Prusiner, 1998; Glynn et al., 2020). These diseases are truly infectious as they can spread within and between species. While misfolded proteins associated with other neurodegenerative diseases do not seem to be naturally transmitted between individuals, they share many properties of prions, such as the ability to self-propagate, spread from cell to cell, and subsequently induce aggregation of the same protein in neighboring cells (Walker and Jucker, 2015). They are often referred to as "prion-like" to differentiate them from truly infectious prions, but to emphasize the strong similarities concerning the propagation process (Jaunmuktane and Brandner, 2019).

The formation and propagation of amyloids involve several critical steps. The initial conformational rearrangement to an abnormal β-sheet-rich fold favors the assembly of individual proteins into an oligomer (**Figure 1**). The generation of a propagon, a unit with a seeding-competent conformation and size that can self-replicate, is considered the rate-limiting event in amyloid formation (Cox et al., 2003; Iljina et al., 2016; Meisl et al., 2017). Elongation of protofibrillar species, or the templated addition of misfolded proteins, proceeds relatively fast. Although an amyloid fibril is energetically very stable, there is still an equilibrium between different oligomeric, protofibrillar, and fibrillar protein species (Carulla et al., 2005; Baldwin et al., 2011). Fibril growth is further accelerated by secondary nucleation events along the fibril surface and by fragmentation (Törnquist et al., 2018). The latter event promotes amyloid growth by producing more fibril ends to which monomers can be added (Knowles et al., 2009). Also, propagons are further able to spread *via* multiple routes outlined in **Figure 2** and template the aggregation of like proteins in neighboring cells.

This intra- and intercellular propagation of aggregated material seems to underlie the characteristic progressive spreading of pathology in prion and prion-like diseases (Jucker and Walker, 2013). Since molecular chaperones can protect cells from harmful protein aggregates, at least at a young age, they are gaining increasing attention in current research to develop intervention strategies.

THE ROLE OF CHAPERONES IN PRION-LIKE PROPAGATION

Molecular chaperones, first identified as heat shock proteins (Hsps), help fold newly synthesized proteins, inhibit and reverse the misfolding and aggregation, and assist in the degradation of terminally misfolded proteins, thereby maintaining cellular proteostasis under physiological and stress conditions (Klaips et al., 2018). Chaperones recognize hydrophobic motifs in misfolded proteins that are usually hidden in their native folded state. A mere binding activity is considered a holdase function that does not require ATP. However the folding and refolding of proteins often rely on an ATP-dependent cycle that allows the repeated binding and release of chaperones, thereby facilitating (re)-folding processes (Mayer and Bukau, 2005; Liberek et al., 2008). The latter activity is performed by ATP-dependent chaperones, which often require specific co-chaperones that are responsible for regulating the ATP cycle (binding, hydrolysis,

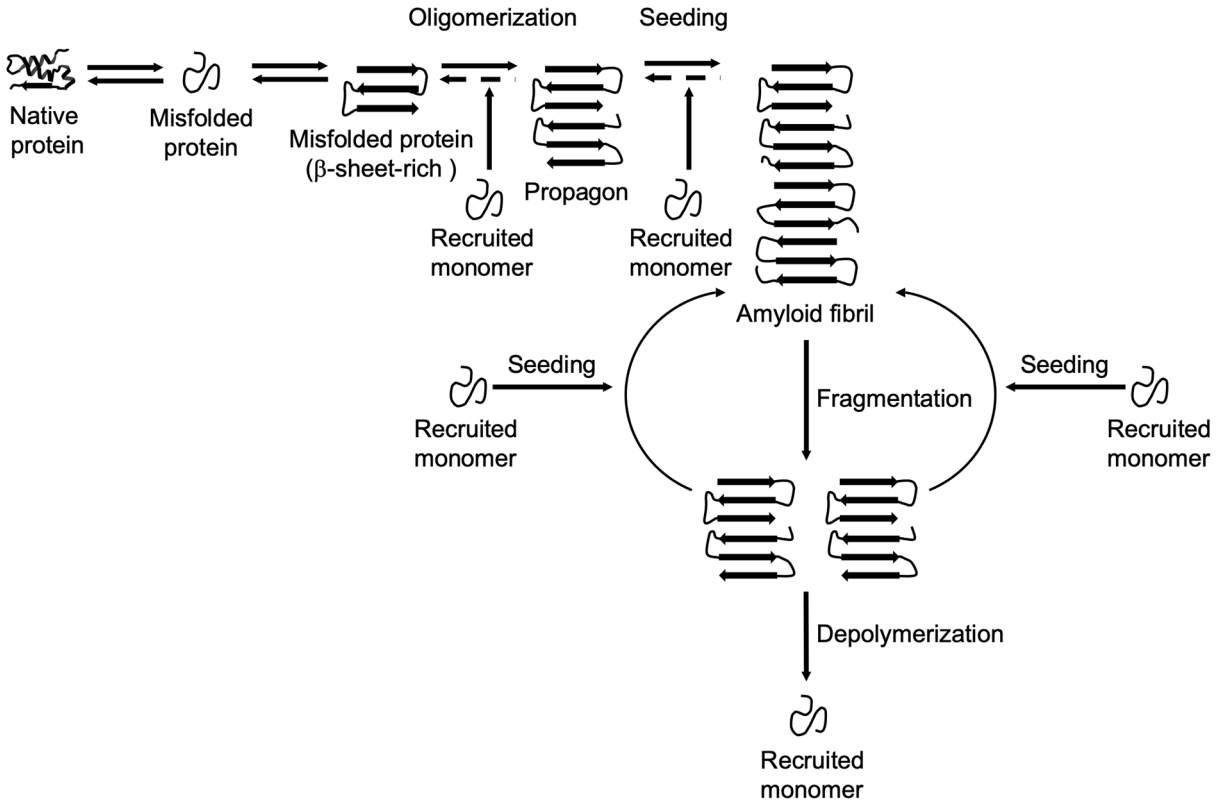

FIGURE 1 | Amyloid formation and propagation at the molecular level. Native proteins oligomerize after adopting an abnormal β-sheet-rich fold, eventually forming a propagon. Propagons are specific units that can recruit and incorporate native monomers, which allows them to grow into amyloid fibrils. Fragmentation events can lead to complete depolymerization into monomers or to the formation of new propagons that in turn provide more ends for recruiting monomers.

and release) and in turn influence substrate specificity and fate (Kampinga and Craig, 2010). Molecular chaperones are classified into different families that were originally named for the molecular weight of the founding member. The four main chaperone families in metazoans are Hsp60s, Hsp70s, Hsp90s, and small Hsps (sHsp).

sHsps lack an ATPase domain and therefore generally act as classical holdases (Webster et al., 2019). They can be found in inactive oligomeric complexes that keep them poised to combat an early misfolding event (Santhanagopalan et al., 2018). Stress conditions can activate sHsps to sequester misfolding proteins and protect the substrates from further aggregation and facilitate their re-folding (Biswas and Das, 2004), often in concert with other chaperones, such as Hsp70s (Mogk et al., 2019).

The Hsp70 family consists of heat shock-inducible (e.g., HSP70-1/HSPA1A) and constitutively expressed (e.g., HSC70/HSPA8) members and has highly assorted functions, including the folding of newly synthesized proteins, refolding of misfolded proteins, disaggregation, membrane translocation, endocytosis, and degradation of terminally misfolded proteins. This functional diversity is provided by a myriad of co-chaperones. The Hsp70 core chaperone typically cooperates with a member of the J-domain protein (DNAJ) family and a nucleotide exchange factor (NEF) that regulate the Hsp70 ATPase cycle (Mayer and Bukau, 2005). The DNAJ family expanded from six DNAJs found in *E. coli* to 49 in *Homo sapiens* (Finka and Goloubinoff, 2013; Bar-Lavan et al., 2016). This increase in complexity may reflect the evolutionary selection pressure for greater versatility of Hsp70 machines. After being processed by Hsp70s and their co-chaperones, clients can be subsequently handed over to chaperonins and Hsp90 family members.

The ATP-dependent Hsp60 family, also commonly referred to as the chaperonins, is divided into two groups: Group I is generally found in eubacteria, but also in evolutionarily derived mitochondria, and Group II is found in archaea and in the eukaryotic cytosol (Hartl and Hayer-Hartl, 2002). The eukaryotic chaperonin, also known as t-complex 1 (TCP1), or chaperonin containing TCP1 (CCT), is a multiprotein complex composed of two rings with eight different but similar subunits. Driven by ATP-binding and hydrolysis, the subunits open and close a central folding chamber that encapsulates substrate proteins. It is essential as it supports the folding of ∼10% of all newly synthesized proteins, in particular actin and tubulin (Yam et al., 2008).

The members of the Hsp90 family are highly conserved and exist in all kingdoms of life except archaea. Similar to the Hsp70 family, there are inducible (e.g., HSP90AA1)

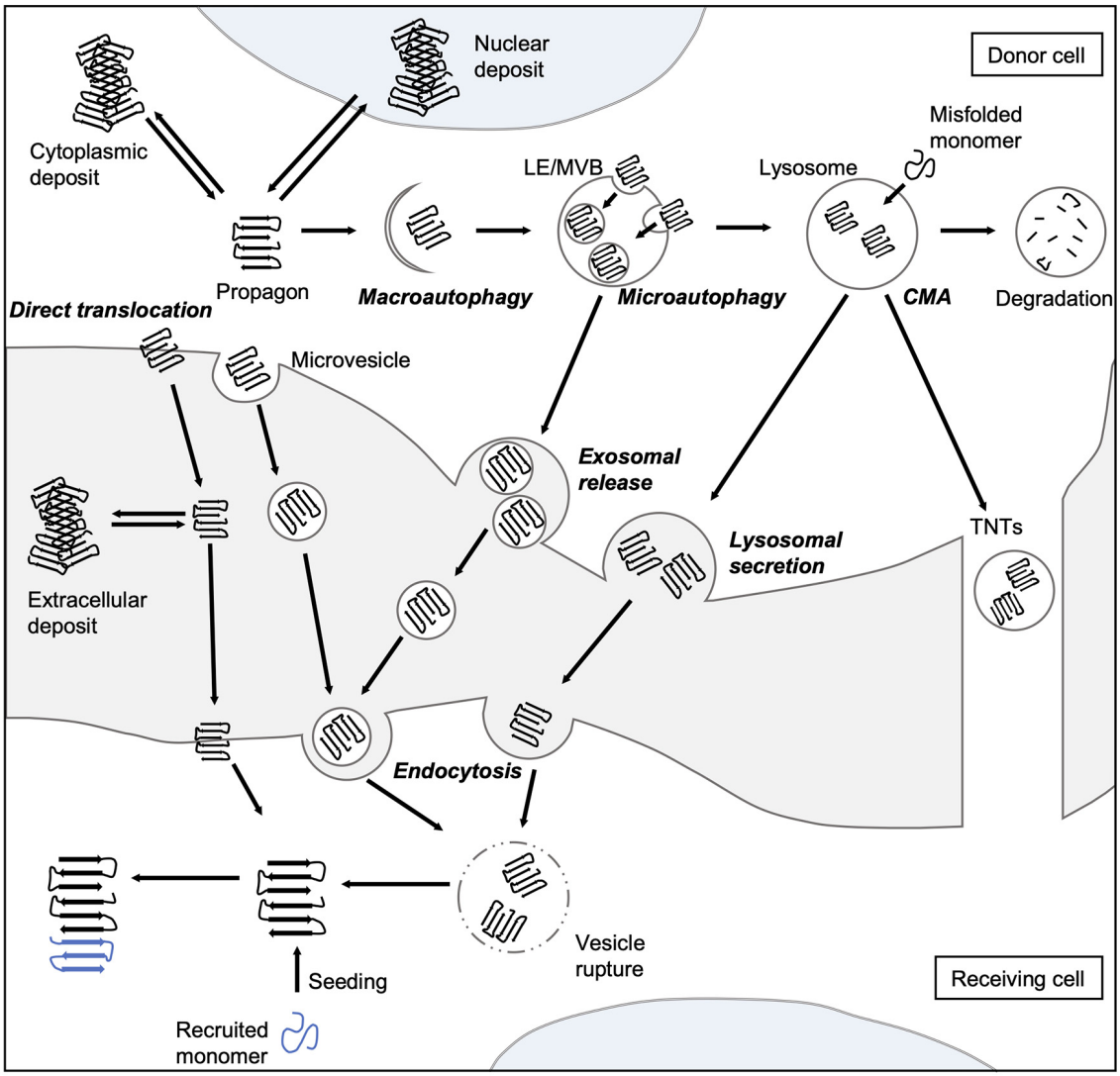

FIGURE 2 | Spreading routes of amyloidogenic proteins. The intercellular transmission of disease proteins can occur *via* several pathways. Some substrates can be translocated directly across the plasma membrane or shed *via* microvesicles. Misfolded proteins can be also targeted by all branches of autophagy, and thereby enter the endo-lysosomal system. Aggregates can either be engulfed *via* bulk or selective (involving adaptor proteins, such as sequestosome 1 (SQSTM1/p62) macroautophagy or taken up into LEs and MVBs through microautophagy. Abnormal proteins may also be directly ingested by lysosomes as a result of CMA. Degradation-resistant aggregates may be released into the extracellular space by lysosomal fusion with the plasma membrane or transported into neighboring cells *via* TNTs. Endocytosis mediates the uptake of either free protein or extracellular vesicles containing the disease-associated protein. To be released into the cytosol of the receiving cell, misfolded proteins can induce endocytic vesicle rupture. Released proteins can then recruit monomers and catalyze their incorporation, which eventually leads to the formation of amyloid aggregates in the receiving cell. CMA, chaperone-mediated autophagy; LE, late endosome; MVB, multivesicular body; TNT, tunneling nanotube.

and constitutively expressed variants (e.g., HSP90B1) that interact with more than 20 co-chaperones and adaptors thereby regulating a multitude of cellular processes (Taipale et al., 2010; Biebl and Buchner, 2019). Since kinases and steroid hormone receptors are major clients of Hsp90s, they are key regulators of many signaling pathways.

In addition to these main chaperone classes, there are several other types of metazoan chaperones for which a relationship with a particular prion-like protein has been established. Details about these chaperones are given in the respective individual sections.

At first, the role of chaperones in prion-like propagation of misfolded proteins might seem obvious, as the main task of chaperones is to support the correct folding of proteins and protect them from misfolding and aggregation. While this is often the case, there are nonetheless also conflicting results where chaperones have been shown to aggravate protein misfolding or toxicity. Although chaperones may interact with the native state

of prion-like proteins also under physiological conditions, we will focus here on the interaction with pathological forms, discussing both the beneficial and detrimental impact chaperones may have on the progression of protein misfolding diseases.

Prion Diseases

Prion diseases or transmissible spongiform encephalopathies (TSEs) are fatal neurodegenerative diseases that affect humans and animals, including BSE (also known as mad cow disease) in cattle, CWD in deer and elk, scrapie in sheep and goats, and Creutzfeldt-Jakob disease (CJD) in humans (Imran and Mahmood, 2011; Collinge, 2016; Scheckel and Aguzzi, 2018). All prion diseases are characterized by the accumulation of PrP^{Sc} in the central nervous system. Cellular PrP^{C} is a glycosylphosphatidylinositol (GPI)-anchored membrane protein and has the highest expression in neurons of the brain and the spinal cord (Stahl et al., 1987; Harris et al., 1993; Tichopad et al., 2003). Proposed functions of PrP^{C} include the maintenance of synapses and neuroprotective signaling (Westergard et al., 2007).

While the exact function of PrP^{C} remains unclear, it is, however, crucial for the propagation of PrP^{Sc}, as mice lacking the *PRNP* gene are resistant to prion infection (Büeler et al., 1993; Sailer et al., 1994; Brandner et al., 1996). Since PrP^{C} is localized on the cell surface, the first interaction and conversion into pathological PrP^{Sc} likely occur at the plasma membrane (Goold et al., 2011). Also, PrP^{C} is endocytosed in a clathrin-dependent manner and delivered from early endosomes and late endosomal multivesicular bodies (MVBs) to lysosomes for degradation. Blocking PrP^{C} endocytosis inhibits the formation of PrP^{Sc}, suggesting that conversion also occurs along the endocytic pathway (Borchelt et al., 1992). PrP^{Sc} is rapidly truncated into a C-terminal PrP27–30 protease-resistant core, which is very stable and accumulates in MVBs and lysosomes. Intercellular transmission proceeds *via* exosomes that are derived from intraluminal vesicles (ILVs) of MVBs and are released into the extracellular space through their fusion with the plasma membrane (Fevrier et al., 2004). In tissue culture cells, PrP^{Sc} was also shown to be able to spread within endocytic vesicles through tunneling nanotubes (TNTs), long membranous tubules that connect the cytosol of two cells (Gousset et al., 2009; Zhu et al., 2015).

Hsp70 family genes are upregulated in CJD patients and prion-infected mice (Kenward et al., 1994; Kovács et al., 2001). Furthermore, several models have shown that manipulation of chaperone levels can influence disease progression, which underlines the relevance of chaperones for prion diseases. When mice that lack heat shock factor 1 (HSF1), the primary transcription factor for the expression of numerous chaperones, are exposed to prions, they succumb to the disease about 20% faster than wildtype animals (Steele et al., 2008). Prion disease progression was also accelerated if cytosolic or endoplasmic reticulum (ER) Hsp70s levels were reduced (Park et al., 2017; Mays et al., 2019). In the opposite direction, the data are less clear. While the induction of HSP70 expression slowed the progression of prion phenotypes in *Drosophila* (Zhang et al., 2014), overexpression of HSP70 had no impact on survival times of prion-infected mice (Tamgüney et al., 2008). Thus, further research is necessary to gain insights into the exact mechanisms by which chaperones influence the course of prion diseases to identify effective therapeutic approaches.

Parkinson's Disease and Other Synucleinopathies

Accumulation of aggregated α-syn/SNCA is a hallmark of PD and other synucleinopathies (Uversky, 2003, 2011). In PD, Lewy bodies containing aggregated α-syn, occur in a predictable manner, which is classified into six distinct stages based on the location of α-syn inclusions seen in postmortem brains (Braak et al., 2003). These observations have led to the hypothesis, that pathological α-syn may propagate like prions. This idea has gained momentum through observations in PD patients who underwent embryonic neuronal cell transplantation. When examined several years later, these patients showed signs of disease development in the grafted tissue, indicating that pathological α-syn had spread from diseased to healthy tissue (Kordower et al., 2008; Li et al., 2008). Various animal and cell culture models have confirmed the existence of such intercellular dissemination of α-syn (Jucker and Walker, 2013; Vasili et al., 2019).

The presence of numerous different chaperones in Lewy bodies suggests a central role of these proteins in α-syn pathology (McLean et al., 2002). Moreover, the importance of chaperones and in particular J-domain proteins in the disease is also evident through the discovery of respective mutations in genome-wide association studies. For instance, DNAJC6 mutations have been linked to juvenile parkinsonism (Lin and Farrer, 2014), while DNAJC13/RME8 mutations have been associated with familial forms of PD (Vilariño-Güell et al., 2014).

Prevention of α-syn aggregation has been shown with multiple sHsps (HSPB1, HSPB2, HSPB3, HSPB5, HSPB6, and HSPB8), the J-domain proteins DNAJB6 and DNAJB8, and with Hsp70s (Rekas et al., 2004; Outeiro et al., 2006; Bruinsma et al., 2011; Cox et al., 2016; Aprile et al., 2017; Bendifallah et al., 2020; Vicente Miranda et al., 2020). Hsp90s also prevent α-syn aggregation but by specifically interacting with oligomeric species rather than monomers or fibrils (Falsone et al., 2009; Daturpalli et al., 2013). The constitutively expressed HSC70, along with HSPB1 and HSPB5, can also bind α-syn fibrils, and coating of the fibrillar surface reduced toxicity (Waudby et al., 2010; Pemberton et al., 2011; Redeker et al., 2012; Cox et al., 2018).

Chaperones not only interfere with early nucleation and fibril elongation events but are also able to depolymerize mature α-syn fibrils (Duennwald et al., 2012; Gao et al., 2015). This disaggregation function is dependent on the specific cooperation of the core HSC70 with a class B J-domain protein, DNAJB1, and an Hsp110-type NEF, HSPA4/APG-2 (Gao et al., 2015). HSPB5/αB-crystallin can assist in the depolymerization of α-syn fibrils by stimulating the Hsp70 disaggregase (Duennwald et al., 2012). These *in vitro* observations indicated that α-syn disaggregation might be beneficial and cytoprotective since fibrillar α-syn was eventually dissolved. However, a recent study

reported the opposite effect *in vivo*. Diminishing disaggregation activity by knocking down the only cytosolic Hsp110-type NEF, HSP-110 significantly reduced the accumulation of toxic α-syn species in *C. elegans* (Tittelmeier et al., 2020). Moreover, α-syn particles generated by the Hsp70 disaggregase were preferred substrates for intercellular transfer. Hence, the Hsp70 disaggregation machinery seems to be involved in the prion-like propagation of α-syn by generating seeding-competent α-syn species, and blocking this activity is beneficial with regard to amyloidogenic substrates (Tittelmeier et al., 2020). While this result seems counterintuitive at first, it is reminiscent of the crucial role of Hsp104 in the propagation of yeast prions, where depletion of Hsp104 leads to a rapid loss of yeast prions (Chernoff et al., 1995). Hsp104 cooperates with the Hsp70 chaperone system and promotes prion replication by extracting monomers from prion fibrils, which leads to their fragmentation and the increased generation of propagons (Jones and Tuite, 2005; Tessarz et al., 2008; Tipton et al., 2008). Hexameric AAA+ Hsp100-type disaggregates such as Hsp104 are absent in metazoans, but the Hsp70 system has evolved to provide this function (Shorter, 2011; Rampelt et al., 2012). While chaperone-mediated disaggregation seems to significantly contribute to the toxicity associated with pathological α-syn, it is essential for the maintenance of cellular proteostasis, as reducing HSP-110 levels compromised the overall cellular protein folding environment (Tittelmeier et al., 2020). For this reason, complete inhibition of this activity is not a suitable intervention strategy. Rather, the modulation of individual isoforms or more specific components may be a promising therapeutic approach. The observed adverse side effects could be minimized, e.g., by the only temporary intake of drugs that inhibit the machinery. Also, the human chaperome is more redundant (there are e.g., three Hsp110-type NEFs compared to only one in *C. elegans*, Brehme et al., 2014) and the reduction of a single-player would probably reduce rather than eliminate disaggregation activity and result in fewer side effects. Nevertheless, more studies are needed to explore the usefulness of this approach.

Another way in which chaperones can help prevent the spreading of α-syn is by facilitating the elimination of aberrant species. Turnover of α-syn can be mediated by the UPS, with the HSC70 co-chaperone carboxyl terminus of Hsp70-interacting protein (CHIP) governing this degradation pathway (Shin et al., 2005). Another process, described as chaperone-mediated autophagy (CMA), also involves HSC70, which targets misfolded α-syn and translocates it into lysosomes for degradation (Cuervo et al., 2004). However, CMA and lysosomal degradation are often impaired in PD (Pan et al., 2008; Chu et al., 2009).

The exact mechanisms of intercellular transfer of α-syn are not yet fully understood, but current research suggests several parallel transmission routes (Brundin and Melki, 2017; Vasili et al., 2019). First, α-syn must exit the donor cell. As a cytosolic protein, α-syn is not released by the conventional secretory pathway *via* the ER and Golgi apparatus. Instead, there is growing evidence that the endo-lysosomal system is involved in α-syn spreading in addition to its role in the degradation of the protein. Endosomal α-syn can be either directly transported to neighboring cells *via* TNTs (Abounit et al., 2016; Rostami et al., 2017; Victoria and Zurzolo, 2017), or it can eventually be released *via* unconventional secretion pathways involving secretory lysosomes or exosomes (Emmanouilidou et al., 2010; Danzer et al., 2012; Ngolab et al., 2017; Tsunemi et al., 2019). Another recently described mechanism for the release of aberrant protein species is the misfolding-associated protein secretion (MAPS) pathway (Lee et al., 2016). Here the ER-associated deubiquitinase USP19 recruits misfolded cytosolic proteins, such as α-syn, to the ER surface and then transfers them to DNAJC5 and HSC70 localized at late endosomes (LEs), which finally fuse with the plasma membrane and release their content to the extracellular space (Fontaine et al., 2016; Xu et al., 2018). Spreading also relies on the uptake of extracellular α-syn into the recipient cell. To this end, clathrin-mediated endocytosis is involved in the uptake of free or exosomal α-syn (Oh et al., 2016; Ngolab et al., 2017). As part of this process, HSC70 cooperates with DNAJC6/Auxillin and an Hsp110-type NEF to disassemble clathrin coats (Sousa and Lafer, 2015). After uptake, misfolded α-syn species must enter the cytosol to be able to template the aggregation of native α-syn in the recipient cell. Indeed, α-syn has been shown to escape from endocytic vesicles by rupturing the endosomal membrane (Flavin et al., 2017).

Alzheimer's Disease and Other Tauopathies

Tau

More than 20 different neurodegenerative diseases are associated with the progressive accumulation of Tau inclusions in different brain areas and cell types which are collectively referred to as tauopathies, including AD and frontotemporal dementia (FTD; Goedert et al., 2017a). The sequential appearance of Tau aggregates in the brain during disease progression follows a stereotypic distribution pattern, categorized into six "Braak stages" according to the prevalence of Tau pathology in different brain regions (Braak and Braak, 1991; Jucker and Walker, 2013). Intriguingly, the extent of Tau deposition in the different brain regions is a good correlate for the disease stage (Jucker and Walker, 2013). Tau's capacity to propagate in the brain is further supported by extensive research in mouse models. Injection of recombinant or patient-derived Tau aggregates into mouse brains causes the formation of Tau inclusions both at the injection site and in distant interconnected brain areas (Narasimhan and Lee, 2017). Therefore, it is assumed that seeding-competent Tau material is transported to other parts of the brain in a connectivity-dependent manner where it induces the aggregate formation of native Tau (Goedert et al., 2017a).

Although we are mainly focusing here on the effect of chaperone action on pathological Tau species, it is worth mentioning that under healthy conditions, various chaperones control the homeostasis of native Tau, such as its loading onto microtubules or degradation *via* the proteasome and autophagy pathways (Miyata et al., 2011; Young et al., 2018).

NMR studies with monomeric Tau have identified binding sites for various chaperones that are either close to or within

the repeat domains that contribute to the β-sheet structures in amyloid Tau fibrils (Jinwal et al., 2013; Mok et al., 2018). By interacting with this region chaperones can stabilize soluble Tau and thereby prevent its assembly into amyloid fibrils. Several Hsp70 family members, their co-chaperones DNAJA1 and DNAJA2, chaperonin, various sHsps, as well as the extracellular chaperone clusterin (CLU) delay the aggregation of wildtype and aggregation-prone Tau mutants *in vitro* (Patterson et al., 2011; Mok et al., 2018). Additionally, it has been shown that Hsp70s suppress Tau aggregation by stabilizing Tau oligomers to inhibit further seeding (Kundel et al., 2018) and by preventing fibril elongation into larger assemblies (Patterson et al., 2011; Baughman et al., 2018). This prevention of Tau aggregation and fibril growth observed *in vitro* presumably delays disease onset and progression, as supported by studies in *in vivo* models, in which the absence of particular chaperones led to accelerated Tau aggregation and toxicity (Eroglu et al., 2010). In line with this, HSPB1 overexpression decreased Tau levels and rescued the Tau mediated damage in a mouse model (Abisambra et al., 2010). Interestingly, HSP90 stabilizes aggregation-prone conformations of Tau and promotes oligomer formation *in vitro* (Weickert et al., 2020). However, the fate of Tau is highly dependent on the specific HSP90 co-chaperone (Shelton et al., 2017). For instance, overexpression of the co-chaperone FKBP prolyl isomerase 5 (FKBP51) in a mouse model increases Tau oligomers at the cost of fibril formation and at the same time enhances neurotoxicity (Blair et al., 2013).

Chaperones do not only suppress or delay the initial aggregation of monomeric and oligomeric Tau species but are also capable of dissolving Tau fibrils. The aforementioned trimeric human Hsp70 disaggregation machinery (HSC70, DNAJB1, HSPA4) readily disassembles a variety of amyloid Tau aggregates ranging from *in vitro* assembled fibrils to aggregates extracted from a cell culture model to brain material of AD patients (Nachman et al., 2020). Thus, the Hsp70 disaggregation machinery is capable of disaggregating pathologically relevant forms of Tau. Although mainly monomers were released the liberated Tau species were nevertheless seeding-competent and induced self-propagating Tau aggregates in a cell culture model (Nachman et al., 2020). In subsequent research, it needs to be determined whether disaggregation of amyloid Tau fibrils may exacerbate neurotoxicity *in vivo*. However, it is tempting to speculate that chaperone-mediated disaggregation might promote the prion-like propagation of amyloid Tau aggregates and eventually increase the overall amyloid burden *in vivo*, especially considering its effect on α-syn aggregation and toxicity in *C. elegans* discussed above (Tittelmeier et al., 2020). Interestingly, the co-chaperone DNAJB4 can substitute for DNAJB1 in the Hsp70 disaggregase, while class A J-domain proteins are unable to support disaggregation of Tau, indicating specificity, but also a certain redundancy in the recognition of amyloid Tau (Nachman et al., 2020). Interfering with the specific interaction between these class B J-domain proteins and amyloid fibrils could be an effective treatment strategy to reduce unfavorable amyloid disaggregation without affecting the processing of other substrates.

As Tau is a cytoplasmatic protein that deposits intracellularly, the spreading of Tau requires release and uptake of seeding-competent Tau material from the cytosol of donor and receiving cells. Similar to α-syn, Tau is also a substrate of the MAPS pathway (Fontaine et al., 2016; Lee et al., 2016; Xu et al., 2018), where HSC70 together with its co-chaperone DNAJC5 promotes the release of Tau into the extracellular space both in cell culture and in a mouse model. However, it remains unknown which Tau species get released *via* this pathway and whether this material can then seed the aggregation of native Tau molecules in recipient cells. Following its release, extracellular Tau can then be taken up by neighboring cells by similar routes described for α-syn (Goedert et al., 2017b). However, it is not yet clear to what extent clathrin-mediated endocytosis and the chaperones involved could contribute to Tau propagation (Yoshiyama et al., 2007; Holmes et al., 2013; Calafate et al., 2016).

Aβ

AD is characterized by the deposition of both intracellular Tau aggregates and extracellular senile plaques consisting of the Aβ peptide in the brain (Goedert and Spillantini, 2006). The Aβ peptide is generated by endoproteolytic cleavages within the transmembrane protein amyloid-β precursor protein APP (De Strooper and Annaert, 2010). Similar to the stereotypical deposition of Tau and α-syn aggregates, the appearance of Aβ plaques follows a predictable pattern that sequentially affects certain areas of the brain during disease progression (Jucker and Walker, 2013). The prion-like behavior of Aβ has been confirmed in numerous rodent models (Meyer-Luehmann et al., 2006; Eisele et al., 2010). Moreover, it has been shown that cadaveric pituitary-derived human growth hormone, which was contaminated with Aβ seeds, caused a plaque-like pathology in treated patients, suggesting an iatrogenic transmission of Aβ pathology (Jaunmuktane et al., 2015; Purro et al., 2018).

In vitro studies have identified several cytosolic chaperones, such as the sHsps HSPB1, HSPB5, HSPB6, and HSPB8, the J-domain protein DNAJB6, as well as chaperonin that suppress Aβ aggregation, either by inhibiting initial aggregation or recruiting oligomeric species into larger structures (Lee et al., 2006; Wilhelmus et al., 2006; Shammas et al., 2011; Månsson et al., 2014a; Mangione et al., 2016; Vilasi et al., 2019). As the sequestration of oligomers reduces the number of particles that can act as seeds this mechanism could help to limit the incorporation of monomers by templated misfolding. Almost all amino acids in the Aβ peptide are incorporated into its amyloid fold (Kollmer et al., 2019). In contrast, larger proteins, such as α-syn and Tau have N- and C-terminal extensions forming a fuzzy coat around their amyloid cores (Scheres et al., 2020; Schweighauser et al., 2020). The lack of available binding sites flanking the amyloid core is probably the reason why the canonical Hsp70 chaperone machinery does not interact with Aβ assemblies (Kakkar et al., 2014; Wentink et al., 2019). In contrast, the mitochondrial chaperonin HSPD1 can bind to Aβ oligomers, which reduces Aβ-mediated neurotoxicity by preventing Aβ oligomers from interacting with membranes (Marino et al., 2019; Vilasi et al., 2019). It is tempting to speculate that this function might be conserved by the

homologous cytosolic chaperonin CCT, which could help to reduce Aβ toxicity by preventing disruption of intracellular membranes (Julien et al., 2018). Yet, the physiological relevance of these findings regarding cytosolic chaperones remains to be investigated. Aβ aggregates form in the endosomal-lysosomal pathway and plaques deposit in the extracellular space (Peric and Annaert, 2015). However, a contribution of cytoplasmic Aβ oligomers to amyloid toxicity and transmission has been demonstrated in cell culture models (Nath et al., 2012). It will be interesting to test whether cytosolic chaperones directly interact with these cytoplasmic Aβ species and modulate their properties *in vivo*.

The amyloid formation can also be accelerated by secondary nucleation events on amyloid surfaces as their interaction with monomers catalyzes the formation of new seeds (Törnquist et al., 2018). By shielding such surfaces, the BRICHOS domain inhibits Aβ aggregation *in vitro* by interfering with oligomerization and secondary nucleation (Willander et al., 2012). The BRICHOS domain is found in the proteins Bri2 and Bri3 that co-localize with extracellular Aβ plaques in the early stages of disease (Del Campo and Teunissen, 2014; Dolfe et al., 2018), which hints to a potential role of these proteins in containing the spreading of pathology by shielding the plaques. The extracellular chaperone CLU is a well-established genetic risk factor for AD (Foster et al., 2019). CLU prevents Aβ aggregation *in vitro* by sequestering and stabilizing oligomeric species (Narayan et al., 2012; Beeg et al., 2016). While several studies have found CLU reduces the uptake of Aβ oligomers and fibrils into neurons and microglia in cell culture models (Nielsen et al., 2010; Mulder et al., 2014), others have observed enhanced Aβ uptake in the presence of CLU (Yeh et al., 2016). A contribution of CLU to cellular uptake of Aβ would directly impact Aβ transmission. Thus, to be able to exploit CLU as a potential therapeutic target, it is essential to further explore its role in the intercellular dissemination of Aβ.

Polyglutamine Diseases

There are nine different adult-onset autosomal dominantly inherited diseases that are caused by an expansion of a trinucleotide (CAG) repeat encoding a polyglutamine (polyQ) tract, including HD and six forms of spinocerebellar ataxia (Lieberman et al., 2019). The disease-associated proteins are not related to each other, but they all contain a polyQ tract with a length of 10–35 glutamine repeats in healthy individuals. A stretch of 40 or more glutamines will eventually cause disease, with a longer expansion correlating with an earlier age of onset (Scherzinger et al., 1999). PolyQ expansion is associated with the formation of amyloid aggregates, which can be localized in the nucleus or in the cytoplasm (Scherzinger et al., 1997; Reddy et al., 1999). HD is the most prevalent of these diseases, where polyQ expansion occurs in HTT.

Genetic screens for modifiers of polyQ aggregation have identified several chaperones (Krobitsch and Lindquist, 2000; Nollen et al., 2004; Silva et al., 2011). Furthermore, activation of HSF1 ameliorates the toxicity of polyQ *in vivo* (Fujikake et al., 2008; Kumsta and Hansen, 2017). Many sHsps, including HSPB1, HSPB4, HSPB6, HSPB7, HSPB8, and J-domain proteins, including DNAJB1, DNAJB2, DNAJB6, and DNAJB8, were shown to prevent the initial aggregation of polyQ (Kazemi-Esfarjani and Benzer, 2000; Willingham et al., 2003; Carra et al., 2005; Hageman et al., 2010; Vos et al., 2010; Labbadia et al., 2012; Månsson et al., 2014b), while the chaperonin CTT prevented polyQ aggregation by capturing smaller oligomeric species (Tam et al., 2009; Shahmoradian et al., 2013). DNAJB6 emerges as a key protective co-chaperone for polyQ containing sequences and has been shown to very efficiently inhibit the primary nucleation step in polyQ amyloid formation by directly binding to the polyQ tract (Gillis et al., 2013; Kakkar et al., 2016). Moreover, recent research revealed that during differentiation of pluripotent stem cell lines from HD patients into neurons, there is a loss of expression in DNAJB6, which leads to aggregation of polyQ (Thiruvalluvan et al., 2020). This could explain why pathological aggregates are predominantly present in neurons and why stem cells are protected.

The Hsp70 system plays a multifaceted role in polyQ aggregation and toxicity. HSP70 is involved in the prevention of polyQ aggregation alone (Muchowski et al., 2000; Monsellier et al., 2015), as well as in collaboration with the J-domain protein DNAJB1 (Wacker et al., 2004). HSP70 is also capable of sequestering smaller polyQ species into larger non-toxic fibrillar structures thereby preventing their toxic interaction with other cellular components (Behrends et al., 2006). This function is mediated by HSP70 with the help of CCT and a J-domain protein (Behrends et al., 2006). Short Q-rich peptides can also shield the interactive surfaces of polyQ proteins, altering the interaction of other prion-like proteins, directing them into nontoxic aggregates (Ripaud et al., 2014).

The HSC70-DNAJB1-HSPA4 disaggregation machinery is not only able to disintegrate α-syn and Tau fibrils as described above (Gao et al., 2015; Nachman et al., 2020), but also disentangle polyQ fibrils (Duennwald et al., 2012; Scior et al., 2018), which demonstrates the high versatility of this chaperone machinery. Similar as in the case of α-syn, compromising this disaggregase leads to fewer aggregates and rescues the toxicity of polyQ in *C. elegans* (Tittelmeier et al., 2020). This is in agreement with observations in yeast, where deletion of the yeast disaggregase Hsp104 also leads to a decrease in polyQ aggregates (Krobitsch and Lindquist, 2000). These data imply that chaperone-mediated disaggregation can handle many different types of amyloid aggregates and as a result, it could play a central role in the prion-like propagation of aggregates in many diseases, rendering it a highly attractive therapeutic target.

While polyQ aggregates can replicate by inducing the aggregation of native proteins through a templated seeding mechanism, like the other prion-like proteins, the relevance of intercellular spreading of protein aggregates in disease pathogenesis is unclear, especially because of the strong genetic component of these diseases. There is currently also no evidence for the involvement of chaperones in the intercellular spreading of polyQ proteins besides a potential indirect effect on vesicle trafficking.

However, multiple studies are implying non-cell-autonomous effects in these diseases, such as excitotoxicity, where neurons

die as a result of disturbances in the surrounding supporting cells. Models, where polyQ is expressed exclusively in the most vulnerable neurons, fail to elicit many disease symptoms, which are seen when polyQ is expressed not only in neurons but also in glial cells (Gu et al., 2005; Sambataro and Pennuto, 2012). This suggests that some aspects of disease pathology are due to non-cell-autonomous toxicity. For example, the selective vulnerability of neurons has been linked to aberrant activation of glutamate. Normally, this is regulated by the uptake of glutamate into glial cells, however, this process is altered in glial cells expressing disease-associated polyQ proteins (Liévens et al., 2001). Targeting this interconnection seen between neurons and glial cells may be a practical goal in the treatment of these diseases. Recently, this idea was explored using a *Drosophila* model with polyQ expressed in neurons and DNAJB6 expressed in glial cells. The exclusive expression of DNAJB6 in glial cells results in the non-cell-autonomous protection against neurodegeneration and prolongs lifespan (Bason et al., 2019). A deeper understanding of how chaperones could alleviate the non-cell-autonomous effects of prion-like proteins could reveal an exciting new therapeutic approach.

Amyotrophic Lateral Sclerosis

ALS is a fatal, rapidly progressing disease characterized by the degeneration of upper and lower motor neurons. Typically, motor symptoms manifest at mid-adulthood and begin in a restricted part of the body, which varies from patient to patient and then spreads to neighboring areas. This implies a prion-like pathomechanism based on neuronal connectivity (Ravits and La Spada, 2009; Sibilla and Bertolotti, 2017). The speed at which symptoms spread from one area to another correlates with disease duration (Ravits, 2014).

In motor neurons of patients with both familial and sporadic forms of the disease, protein inclusions have been found postmortem that usually contains either SOD1 or ubiquitinated TDP-43 (Kwong et al., 2007). Moreover, various missense mutations have been identified in SOD1, TDP-43, and FUS, as well as a hexanucleotide repeat expansion in C9orf72, which increase the aggregation propensity of these proteins and are associated with familial ALS (fALS) accounting for 10% of all ALS cases (Sibilla and Bertolotti, 2017). Also, exome sequencing of a large ALS patient cohort identified several mutations in the J-domain protein DNAJC7 that led to reduced protein levels in patient-derived fibroblasts (Farhan et al., 2019). This finding directly links chaperone activity to ALS etiology. Although further work is required to elucidate how the loss of DNAJC7 function causes ALS, this underlines the importance of the PQC system for these protein misfolding diseases.

SOD1

The prion-like behavior of SOD1 has been established over the last decade employing *in vitro* and cell culture systems as well as mouse models. Recombinant SOD1 aggregates act as seeds accelerating the aggregation of natively folded SOD1 *in vitro* (Chattopadhyay et al., 2008). In cell culture, SOD1 aggregates can spread intercellularly within exosomes or *via* direct release into the extracellular space (Münch et al., 2011; Grad et al., 2014; Silverman et al., 2016). The released SOD1 species are taken up from the medium by the receiving cells *via* micropinocytosis (Münch et al., 2011). Subsequently, the SOD1 aggregates escape into the cytoplasm where they seed aggregation of the native SOD1 molecules forming self-propagating foci (Münch et al., 2011). Also, SOD1 aggregate pathology can be transmitted between mice through the injection of brain homogenate (Ayers et al., 2014).

Members of the Hsp70 family and their J-domain partner protein and sHsp chaperones have been found to colocalize with SOD1 inclusions in patient tissues and rodent ALS models and to interact with mutant SOD1 in cell culture models, indicating that aggregated SOD1 is recognized as a substrate by the PQC system (Shinder et al., 2001; Watanabe et al., 2001; Howland et al., 2002; Liu et al., 2005; Matsumoto et al., 2005). However, even though the sHsps HSPB1 and HSPB5 reduce SOD1 aggregation *in vitro* (Yerbury et al., 2013), overexpression of HSPB1 in a $SOD1^{G93A}$ mouse model only delayed the onset of motor symptoms and did not affect the overall survival of these mice (Sharp et al., 2008), suggesting that increasing the levels of individual chaperones does not always lead to a beneficial outcome. Accordingly, while increased levels of Hsp70 family members reduced SOD1 aggregation and toxicity in cultured mouse primary motor neurons expressing $SOD1^{G93A}$ (Bruening et al., 1999), this had no impact on either survival or onset of motor symptoms in several SOD1 mutant mouse models, including $SOD1^{G93A}$ (Liu et al., 2005). Interestingly, Serlidaki et al. (2020) have recently demonstrated in cell culture that the effect of Hsp70s on the aggregation of the $SOD1^{A4V}$ mutant depends on the particular Hsp70 variant, which is overexpressed. While HSPA1A suppresses SOD1 aggregation, its close homolog HSPA1L enhances aggregate formation. The differences in client fate seem to result from the fact that Hsp110-type co-chaperones prefer to interact with HSPA1A rather than HSPA1L (Serlidaki et al., 2020). More specifically, HSPA1A requires the NEF HSPA4 (APG-2) to inhibit SOD1 aggregation, while the aggregation promoting activity of HSPA1L does not depend on this NEF. However, it remains unknown which specific molecular mechanisms determine the affinity of Hsp110 family members for different Hsp70s in the cellular context.

It is therefore not surprising that NEF overexpression has also varying consequences. Overexpression of BAG1 in $SOD1^{G93A}$ transgenic mice did not improve their survival or the onset of ALS-like phenotypes (Rohde et al., 2008). In contrast, overexpression of the Hsp110-type NEF HSPA4L (APG-1) in $SOD1^{G85R}$ transgenic mice extended the lifespan of these animals (Nagy et al., 2016). However, the molecular mechanism underlying the beneficial effect of HSPA4L overexpression remains unclear. While the authors speculate that elevated levels of HSPA4L might lead to an increase in Hsp70 disaggregation capacity, the opposite is also conceivable. It has been shown that excessive concentrations of NEFs can poison the Hsp70 system (Nollen et al., 2000; Kampinga and Craig, 2010; Gao et al., 2015). Usually, NEFs function in sub-stoichiometric amounts relative to Hsp70, and increasing their concentrations beyond this optimal

ratio will overstimulate the ATPase cycle and consequently lead to too rapid dissociation of the substrate. As a result, the client protein would be released prematurely. Consequently, NEF overexpression would disrupt rather than promote the Hsp70 function. Further research is, therefore, necessary to shed light on this important aspect.

Currently, there is no evidence that chaperones are directly involved in the intercellular transmission of SOD1. However, chaperones are capable of modulating SOD1 aggregation and toxicity, which could at least indirectly affect the spreading. Moreover, since it has been shown that the sHsp HSPB8 together with HSC70, BAG3, and CHIP, mediates the autophagic degradation of misfolded SOD1 and thus directs it into the endo-lysosomal pathway (Crippa et al., 2010), this could promote the dissemination of SOD1 assuming that it follows similar routes as α-syn and Tau (Uemura et al., 2020).

Future research is needed to gain a more comprehensive picture of which sets of chaperones and co-chaperones act together to suppress or enhance SOD1 aggregation. Furthermore, it is necessary to evaluate the effect of modifying individual chaperone levels in *in vivo* models to predict the overall effect on SOD1 pathology.

TDP-43

TDP-43 is the main component of the characteristic protein inclusions in the central nervous system (CNS) of patients suffering from sporadic ALS (sALS) (Neumann et al., 2006). TDP43 pathology can be observed in about 95% of all sALS cases but is also frequently found in other neurodegenerative diseases, such as frontotemporal lobar degeneration (FTLD), in which ubiquitin-positive Tau-negative TDP-43 inclusions occur. These diseases are collectively referred to as TDP-43 proteinopathies (Arai et al., 2006).

Although the disease-associated aggregation and spreading of TDP-43 have not yet been studied in as much detail as other prion-like proteins, such as Tau or α-syn, there is nevertheless strong indication for a prion-like pathomechanism of TDP-43. The progressive spreading of TDP-43 pathology between interconnected brain areas upon injection of patient-derived pathological TDP-43 was demonstrated in a mouse model (Porta et al., 2018). In cell culture models, both recombinant TDP-43 aggregates and patient-derived detergent-insoluble TPD-43 are taken up from the medium and seed the aggregation of endogenous TDP-43 in the cytoplasm (Furukawa et al., 2011; Nonaka et al., 2013). The intercellular transmission of seeding-competent TDP-43 species in these model systems occurs at least in part *via* exosomes (Nonaka et al., 2013; Iguchi et al., 2016). Intriguingly, exosomes containing seeding-competent TDP-43 are also present in the cerebrospinal fluid (CSF) of ALS patients, which could contribute to the spreading of pathology during disease progression (Iguchi et al., 2016).

TDP-43 is a client of several chaperone families. Consequently, enhanced chaperone expression due to HSF1 activation reduces TDP-43 aggregation and restores TDP-43 solubility (Chen et al., 2016; Wang et al., 2017). While Chen et al. (2016) attributed this observation to the induction of Hsp70s and the co-chaperone DNAJB2a, a similar approach by Wang et al. (2017) identified DNAJB1 and HSPB1 as the major downstream factors of HSF1. *In vitro* assays will be required to identify the specific molecular mechanisms by which each of these chaperones interact with TDP-43 and at what stage during TDP-43 aggregation they act.

Also, TDP-43 is degraded by chaperone-assisted selective autophagy (CASA), where the sHsp HSPB8 works together with HSC70, BAG3, and CHIP to deliver substrates to autophagy. Inducing HSPB8 in a cell culture model increases TDP-43 turnover and overexpression of the *Drosophila* HSPB8 ortholog suppresses TDP-43-mediated neurotoxicity (Crippa et al., 2010, 2016; Gregory et al., 2012). The presence of TDP-43 in the lysosomal fraction isolated from the rodent brain also indicates autophagy-mediated turnover (Ormeño et al., 2020). Moreover, both recombinant wildtype TDP-43 and an aggregation-prone mutant are degraded by isolated lysosomes *in vitro* and TDP-43 is a substrate of CMA interacting with the major CMA components in cell culture (Huang et al., 2014; Ormeño et al., 2020). Although TDP-43 aggregation upregulates CMA, it simultaneously disturbs the membrane integrity of LAMP2A-positive lysosomes compromising the autophagolysosomal pathway (Ormeño et al., 2020). It is therefore highly likely that the disruption of lysosomal membranes leads to the release of seeding-competent TDP-43 species and thus contributes to TDP-43 spreading similarly as previously shown for α-syn and Tau (Flavin et al., 2017). In addition to α-syn and Tau, TDP-43 is also a client of the MAPS pathway (Fontaine et al., 2016), but whether this HSC70/DNAJC5-dependent release of TDP-43 contributes to the spreading of TDP-43 pathology in ALS and FTD patients remains to be shown.

Taken together, TDP-43 shares significant characteristics of a prion-like protein, and chaperones are involved at several key steps of TDP-43 turnover, which could be critical for the propagation of TDP43 pathology. However, to obtain a comprehensive picture of the specific mechanisms by which individual chaperones or combinations of chaperones influence TDP-43 aggregation, *in vitro* studies investigating the direct effect of chaperones on TDP-43 aggregation kinetics using recombinant proteins are required. This would be of great value for the discovery of potential drug targets.

FUS and C9orf72

While for the other ALS-related proteins such as SOD1 and TDP-43 a prion-like behavior is well established, data indicating an intercellular spreading mechanism for FUS and C9orf72-derived DPRs is only recently emerging (Nomura et al., 2014; Feuillette et al., 2017; Zhou et al., 2017; Morón-Oset et al., 2019). However, since chaperones are known to be able to modulate the toxicity of FUS and C9orf72 (Deng et al., 2015; Cristofani et al., 2018), it is highly likely that they would also affect their transmission in one way or the other.

The Role of Chaperones in the Homeostasis of Membraneless Compartments

Over the last decade, it has become increasingly clear that there is a relationship between neurodegenerative diseases, in particular ALS and FTD, and abnormal formation of

membraneless cellular compartments (Alberti and Dormann, 2019). Prion-like proteins, including TDP-43 and FUS, contain intrinsically disordered domains that can undergo liquid-liquid phase separation (LLPS), which is crucial for the formation of membraneless cellular compartments, such as nucleoli and stress granules (SGs; Alberti and Dormann, 2019). During phase separation a homogenous solution of macromolecules partitions into two distinct phases; specific macromolecules accumulate to form a denser phase, while the remaining phase is depleted of these components (Hyman et al., 2014). This dense phase is not solid but behaves liquid-like, i.e., it can undergo droplet fusion and is characterized by high internal dynamics as the enriched components rapidly move in and out of this phase (Alberti et al., 2019).

Under healthy conditions, TDP-43 and FUS co-localize with RNA-containing SGs, which are normally formed transiently upon cellular stress and rapidly dissolve as the stress subsides (Aulas and Velde, 2015). However, disease-associated mutations in these proteins alter their phase separation characteristics towards more solid states, which has been shown to impede SG dissociation and promote the maturation from liquid droplets into immobile aggregates both *in vitro* and in cell culture models (Patel et al., 2015). Moreover, non-RNA-binding proteins implicated in other proteinopathies, such as Tau (Wegmann et al., 2018) and α-syn (Ray et al., 2020) may also undergo LLPS, suggesting that this could represent a pathway for the formation of amyloid aggregates in general.

Since reduced dynamics and a more rigid consistency within phase-separated compartments ultimately promote amyloid fibril assembly, their formation and disintegration need to be tightly controlled by the cellular PQC machinery (Alberti and Dormann, 2019). Several *in vitro* and cell culture studies have shown that molecular chaperones are recruited into SGs and regulate SG formation and stability (**Figure 3**).

Under normal conditions, DNAJB6 and HSPA1A bind to TDP-43 and control its accumulation into SGs (Udan-Johns et al., 2014). However, upon heat shock, the availability of these chaperones becomes limited, which favors the formation of insoluble TDP-43 aggregates. DNAJB6 also helps to resolve nuclear TDP-43 SGs which form under healthy conditions in response to a heat shock (Stein et al., 2014). In this study, a DNAJB6 harboring a mutation in the G/F domain is unable to promote SG dissolution, leading to the persistence of TDP-43 aggregates. The fact that a mutation in DNAJB6 is associated with limb-girdle muscular dystrophy which in turn is accompanied by pathological TDP-43 aggregates, indicates a protective function of DNAJB6 against dysregulated TDP-43 SG formatio (Harms et al., 2012).

In addition to TDP-43, aggregation-prone SOD1 variants can also cause SGs to transition to a more solid state (Mateju et al., 2017). In this study, Hsp70 family members and the sHsp HSPB1 were enriched in SOD1 containing SGs. Likewise, HSPB1 regulates the LLPS behavior of FUS depending on the cellular stress state, thus preventing aberrant FUS amyloid

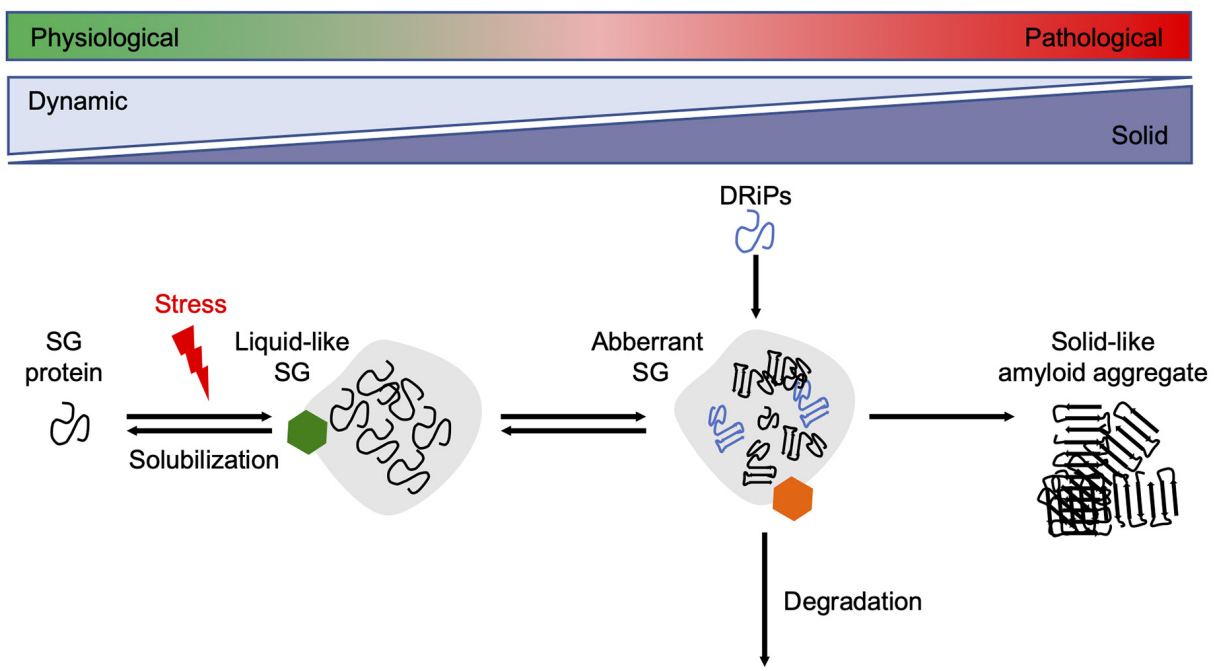

FIGURE 3 | Chaperones maintain SG dynamics. Proteins containing intrinsically disordered domains can undergo phase separation upon cellular stress, such as heat stress, and form liquid-like SGs. Under healthy conditions, the SGs are dissolved when the stress subsides. Chaperones (green hexamer) such as Hsp70s, sHsps, and DNAJB6 are involved in the resolubilization process and thus regulate SG homeostasis. Disease-associated mutations in SG associated proteins, the accumulation of DRiPs, or prolonged exposure to stress conditions reduce the fluidity of SGs, leading to a more solid-like structure that promotes amyloid formation over time. The HSPA1A-BAG3-HSPB8 chaperone network (orange hexamer) targets aberrant SGs for degradation. SG, stress granule.

formation within SGs (Liu et al., 2020). Moreover, defective ribosomal products (DRiPs), i.e., prematurely terminated nascent polypeptides, accumulate in cytosolic SGs leading to a reduction in SG fluidity (Seguin et al., 2014; Ganassi et al., 2016). A chaperone complex consisting of HSPA1A, BAG3, and HSPB8 monitors SG composition and mediates the degradation of DRiPs to restore SG dynamics (Ganassi et al., 2016).

The involvement of chaperones in regulating the dynamics of membraneless compartments is further supported by their function in the nucleolus. The phase-separated nucleolus serves as a PQC compartment which sequesters misfolded protein species (Mediani et al., 2019). Prolonged exposure to stress or a failure to dissolve the liquid-like phase causes proteins in the nucleolus to transition into an amyloid state (Azkanaz et al., 2019; Frottin et al., 2019; Mediani et al., 2019). Their resolubilization during the recovery period depends on the refolding activity of Hsp70 family members that re-localize to the nucleolus (Audas et al., 2016; Azkanaz et al., 2019; Frottin et al., 2019; Mediani et al., 2019).

In addition to conventional chaperones, several other proteins exhibit chaperone-like activity as they suppress abnormal phase separation of certain RNA-binding proteins. For example, the nuclear import receptor transportin-1 (TNPO1) shifts disease-associated FUS variants to a more dispersed state thus preventing their assembly into granules that inhibit local mRNA translation within the axonal compartment (Qamar et al., 2018).

Taken together, chaperones control properties of phase-separated compartments by modulating their internal dynamics during physiological and stress conditions. They are vital to maintaining their liquid-like state by mediating their disassembly or autophagic degradation (Alberti et al., 2017). This surveillance mechanism prevents abnormal aggregation and amyloid formation within membraneless organelles and thus serves as a cellular defense strategy in protein folding diseases to delay disease onset.

CONCLUSIONS AND FUTURE PERSPECTIVES

The main task of molecular chaperones is to maintain cellular proteostasis. Interactions of chaperones with abnormal protein species are therefore aimed to remove them. In principle, the following basic mechanisms of action of chaperones during prion-like propagation of disease proteins can be distinguished (**Figure 4**). On the one hand, chaperones can stabilize misfolded protein species, thus preventing their further accumulation (Hartl et al., 2011). Also, they can dissociate protein aggregates by extracting individual monomers (Mogk et al., 2018). Both these processes can eventually help the substrates to regain their native folding state. If this does not succeed, chaperones can control the sequestration of misfolded protein species in a way that prevents harmful interactions with the rest of the proteome (Miller et al., 2015). Finally, they can also mediate their degradation by the UPS (Kästle and Grune, 2012) or autophagy (Menzies et al., 2017). Besides their role in eliminating abnormal protein species, chaperones are also involved in multiple cellular processes, such as endocytosis (Sousa and Lafer, 2015).

Although all chaperone activities are generally "well-intentioned" and many of them have purely positive effects, some also have disadvantages. For instance, the action of the Hsp70 disaggregation machinery can have two outcomes, one beneficial and one harmful. This chaperone system is supposed to dissolve protein aggregates and in the case of amorphous aggregates that are not seeding-competent, it usually accomplishes their dissociation (Rampelt et al., 2012; Tittelmeier et al., 2020). In the case of amyloid substrates, however, this activity seems to liberate more toxic and seeding- and spreading-competent species (Nachman et al., 2020; Tittelmeier et al., 2020), which accelerates prion-like propagation. Also, delivery to the UPS or autophagy is intended to degrade misfolded proteins, but if this fails, these pathways could also generate seeding- and spreading-competent fragments of disease proteins or, in the case of autophagy, facilitate their delivery to neighboring cells. The respective outcome might depend on the state of the cellular proteostasis network. While in young individuals the proteostasis capacity is high and e.g., the products of the disaggregation reaction can either be refolded or degraded, in older individuals the proteostasis capacity is increasingly impaired and the disaggregated material can no longer be efficiently removed. Moreover, chaperones are also involved in trafficking pathways, which are linked to the intercellular spreading of prion-like proteins. Thus, chaperones can also contribute indirectly to the dissemination of propagons by sustaining the cellular pathways required for cell-to-cell spreading, which are hijacked by disease proteins.

A cautionary note is advised when interpreting results from investigating the impact of chaperones. Due to the interconnectivity of the proteostasis network, manipulating expression levels of one chaperone can have unforeseen effects. On the one hand, inhibition of a central chaperone can lead to compensatory upregulation of other chaperones within the network (Sannino et al., 2018). On the other hand, high concentrations of certain chaperones can also have an inhibitory effect. For example, both *in vitro* and *in vivo* work suggest that not only low levels of the HSP110 NEF, but also too high levels can poison the HSP70 disaggregation activity (Nollen et al., 2000; Kampinga and Craig, 2010; Rampelt et al., 2012). Therefore, it would be very beneficial for future studies to characterize not only the expression level of the protein of interest but also the fluctuations of the entire chaperone network; this would allow a more differentiated interpretation of the results.

Taken together, the studies discussed here show that chaperones play an ambivalent role in neurodegenerative diseases. When considering chaperone therapy, it is therefore important to bear in mind that chaperone action is not *per se* beneficial in the context of proteinopathies. Nevertheless, the recent literature established a strong relationship between molecular chaperones and the propagation and spreading of prion-like proteins, suggesting that chaperones are a promising therapeutic target to interfere with the progression of neurodegenerative diseases. While boosting chaperone activity to prevent the initial aggregate formation in early phases of the disease would be the most effective strategy, this might not be feasible as these initial aggregation events

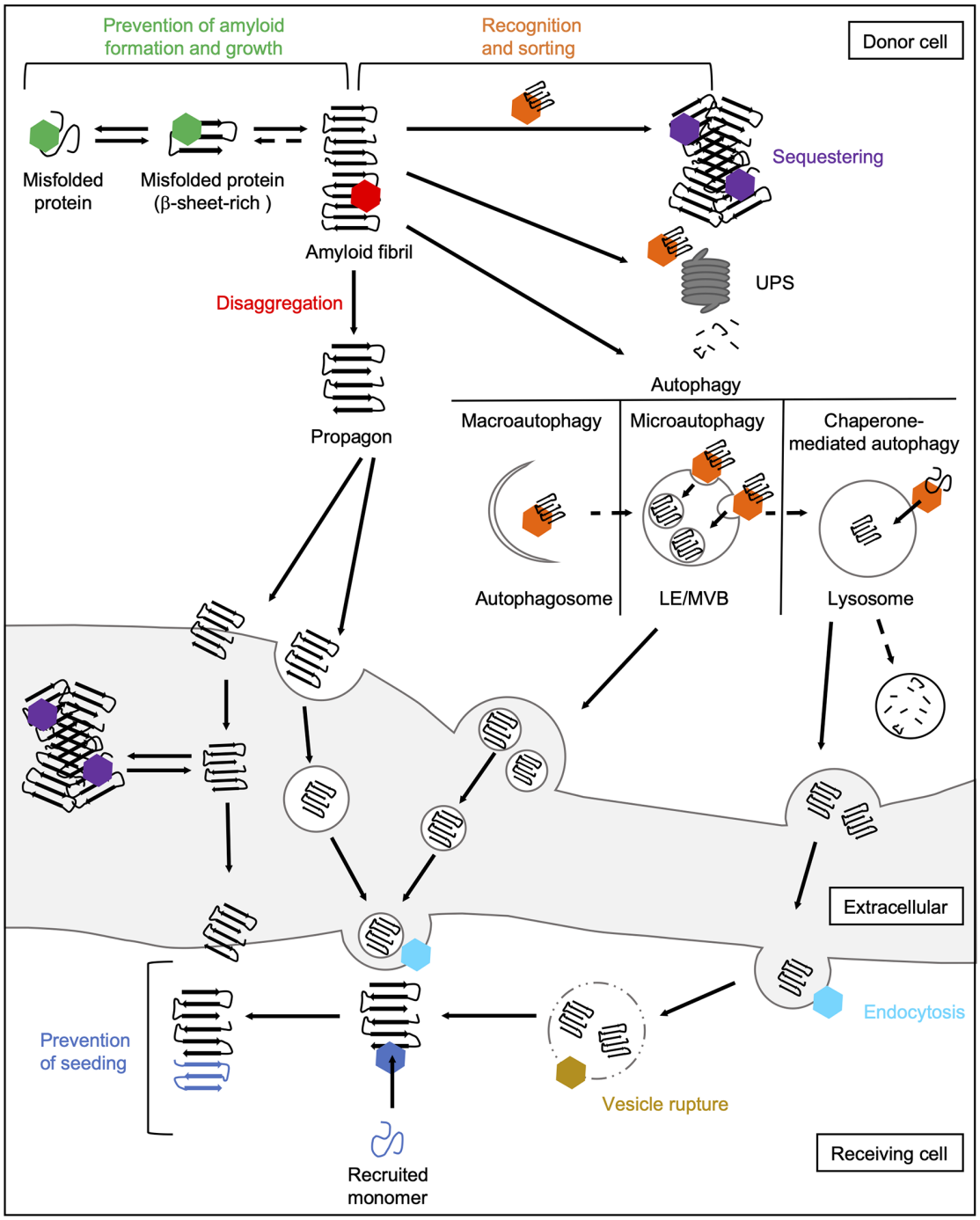

FIGURE 4 | Chaperone interactions during prion-like propagation of disease proteins. The folding and refolding activity of chaperones helps destabilized or misfolded protein species to resume their native state. These transient interactions with misfolded proteins or small oligomers prevent the formation of a seeding-competent propagon (green hexamer). In contrast, the Hsp70 disaggregation machinery can fragment large fibrils leading to the formation of smaller seeding- and spreading-competent species (red hexamer). Chaperones also recognize and sort terminally misfolded forms (orange hexamer) and either mediate their sequestration into an inert deposit (purple hexamer) or deliver them to degradation pathways. Sequestration may reduce the accessibility of fibril ends and thus prevent the further incorporation of native proteins into the amyloid fibril. Extracellular chaperones can also sequester amyloidogenic proteins into large deposits making uptake into receiving cells more difficult. The delivery of amyloidogenic proteins to macroautophagic isolation membranes for their selective clearance is mediated by HSC70 and an sHsp, HSPB8, together with the NEF BAG3 (Gamerdinger et al., 2009). In microautophagy, constitutively expressed HSC70 targets substrates to LEs/MVBs (Sahu et al., 2011). In CMA, HSC70 translocates clients directly across the lysosomal membrane (Tekirdag and Maria Cuervo, 2018). Lysosomes and MVBs can fuse with the plasma membrane releasing either free proteins or exosomes to the extracellular space. In the receiving cell, HSC70 and DNAJC6 are involved in the internalization of misfolded proteins *via* clathrin-mediated endocytosis by uncoating clathrin-coated vesicles (light blue hexamer;

(*Continued*)

FIGURE 4 | Continued
Sousa and Lafer, 2015). By rupturing the endosomal membrane, disease-associated proteins are released from the endocytic vesicle into the cytosol (Flavin et al., 2017), which might be prevented by lysosomal or cytosolic Hsps (yellow hexamer). In the cytosol of the receiving cell, chaperones can finally interfere with the seeding of naïve species by the released propagon (dark blue hexamer). CMA, chaperone-mediated autophagy; LE, late endosome; MVB, multivesicular body; NEF, nucleotide exchange factor; UPS, ubiquitin-proteasome system.

usually remain undetected for a long period. During more advanced disease stages, when the proteostasis capacity is already significantly impaired, it might therefore be more effective to interfere with specific chaperone activities to prevent the dissemination or generation of propagons. Since it is not yet fully understood which aggregate species (oligomers, prefibrillar assemblies, or amyloid fibrils) mediate neurotoxicity and which specific variants spread from cell to cell during disease progression (and whether these are the same or distinct species; Ries and Nussbaum-Krammer, 2016), more research is needed to determine which specific chaperone actions are overall beneficial or detrimental *in vivo* as this will determine therapeutic strategies.

Another important aspect to consider in chaperone therapy is that the fate of a certain amyloidogenic protein species depends not only on a single chaperone but also on its interactions with various co-chaperones. Furthermore, it is also determined by the cellular environment. These findings emphasize the complexity within the chaperone network *in vivo*, which cannot be inferred easily from *in vitro* data. More research using *in vivo* models is therefore required to fully understand how chaperone cooperation ultimately determines the fate of certain aggregate species.

Finally, proteinopathies are characterized by disease-specific patterns of neurodegeneration, which mainly affect certain cell types and brain regions during the disease, while others are spared (Jackson, 2014; Fu et al., 2018). There is growing evidence that not only prions but also other prion-like proteins can form fibrils with different conformations, so-called strains, which can affect different brain regions to different degrees (Jackson, 2014; Melki, 2015). These observations raise the question of whether differences in the proteostasis capacity of these cells could be the reason for this selective susceptibility (Jackson, 2014; Labbadia and Morimoto, 2015). In yeast, chaperone activity helps to maintain the conformational diversity of prion strains (Stein and True, 2014; Killian et al., 2019). Thus, it is tempting to speculate that mammalian chaperones could as well promote the structural identity of amyloid conformers and contribute to the amplification of disease-specific strains by generating distinct seeding-competent propagons. Whether and which role differences in the chaperone repertoire play in this process is still completely unclear and an exciting question for the future.

AUTHOR CONTRIBUTIONS

JT, EN, and CN-K: conceptualization, investigation (literature review), writing—original draft preparation, and writing—review and editing. JT: visualization. CN-K: supervision, project administration, and funding acquisition. All authors contributed to the article and approved the submitted version.

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frontiers in Aging Neuroscience

REVIEW
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Axonal Degeneration in AD: The Contribution of Aβ and Tau

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Alzheimer's disease (AD) represents the most common age-related neurodegenerative disorder, affecting around 35 million people worldwide. Despite enormous efforts dedicated to AD research over decades, there is still no cure for the disease. Misfolding and accumulation of Aβ and tau proteins in the brain constitute a defining signature of AD neuropathology, and mounting evidence has documented a link between aggregation of these proteins and neuronal dysfunction. In this context, progressive axonal degeneration has been associated with early stages of AD and linked to Aβ and tau accumulation. As the axonal degeneration mechanism has been starting to be unveiled, it constitutes a promising target for neuroprotection in AD. A comprehensive understanding of the mechanism of axonal destruction in neurodegenerative conditions is therefore critical for the development of new therapies aimed to prevent axonal loss before irreversible neuronal death occurs in AD. Here, we review current evidence of the involvement of Aβ and tau pathologies in the activation of signaling cascades that can promote axonal demise.

Keywords: Alzheheimer's disease, protein misfolding, axonal degeneration, amyloid β, tau, necroaxoptosis

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INTRODUCTION

Alzheimer's disease (AD) is an adult-onset neurodegenerative disorder and the leading cause of dementia in aged people. While most AD cases are sporadic, less than 5% of the cases are caused by mutations in the amyloid precursor protein (APP) gene or presenilin 1 or 2 genes, leading to excessive production and accumulation of amyloid-β peptide (Aβ; Citron et al., 1992; Hendriks et al., 1992; Mullan et al., 1992; Suzuki et al., 1994; Harvey et al., 2003; Goate, 2006). Many risk factors have been associated with the development of sporadic AD, including the apolipoprotein E ε4 allele (Agosta et al., 2009), female gender (Koran et al., 2017), cardiovascular disease risk factors (Samieri et al., 2018), traumatic brain injury (TBI; LoBue et al., 2017) and aging, which is the most important one (Oh et al., 2014). Currently, there is no cure for AD and available treatments can only modestly and briefly alleviate symptoms (Alzheimer's Association, 2020). The progression of AD, from alterations that include only changes in biomarkers but without the involvement of cognitive decline, to changes that indeed translate into cognitive impairment, follows a continuum that comprises three phases: preclinical AD, mild cognitive impairment (MCI), and dementia due to AD. Clinical symptoms of the disease include deficits in short term memory and language difficulties, as well as behavioral symptoms such as personality changes and depression. Progressive cognitive decline characterized by severe memory loss occurs as the disease progresses. Basic vital functions -such as swallowing- are altered at later stages of the disease, leading to death (Lopez and Dekosky, 2008; Alzheimer's Association, 2020).

Protein misfolding and accumulation are prominent hallmarks of the disease, with Aβ plaques and tau tangles being the neuropathological signature of AD brains (Wang et al., 2016). Numerous studies have demonstrated that these toxic structures do not act independently and that rather, the neurodegenerative process in AD depends on the interaction between both Aβ and tau (Götz et al., 2001; Lewis et al., 2001; Rapoport et al., 2002; King et al., 2006; Roberson et al., 2007; Hurtado et al., 2010; Ittner et al., 2010; Vossel et al., 2010; Nussbaum et al., 2012; Zempel et al., 2013; Wang et al., 2016). Progressive build-up of these abnormal aggregates is associated with synaptic disruption (Shankar et al., 2008; Koffie et al., 2009; Moreno et al., 2011; Zempel et al., 2013; Rajmohan and Reddy, 2017; Pickett et al., 2019) and neuronal loss (Kadowaki et al., 2005; Jawhar et al., 2012; DeVos et al., 2017; Fu et al., 2017), leading to atrophy of specific brain regions (Spires-Jones and Hyman, 2014; Ferreira et al., 2017; Ten Kate et al., 2018).

Remarkably, the evidence indicates that accumulation of Aβ and tau is slow and that initiates more than two decades before clinical symptoms appear (Jack et al., 2009; Braak et al., 2011; Bateman et al., 2012; Villemagne et al., 2013), which has important diagnostic and therapeutic implications. In this context of early pathological changes during AD, axonal degeneration constitutes a common, initial event in several neurodegenerative conditions (Salvadores et al., 2017). Supporting evidence comes from imaging analyses of individuals with MCI -which are subjects at risk of developing AD- showing a significant decrease in white matter volume. Importantly, these results suggest that atrophy due to disruption of white matter fibers might contribute to memory decline (Kalus et al., 2006; Stoub et al., 2006; Rogalski et al., 2009; Ihara et al., 2010; Bozzali et al., 2011). Additionally, the use of diffusion tensor imaging to examine the microstructural integrity of white matter has revealed a pattern of alterations characteristically observed in axon-related pathologies. These changes correlate with cognitive impairment and are consistent with loss of brain connectivity (Huang and Auchus, 2007; Power et al., 2019).

Acute axonal degeneration, as the result of a traumatic lesion in the central nervous system, is a rapid process that takes hours to days depending on the organism and degenerative stimuli (Court and Coleman, 2012). However, in the case of neurodegenerative diseases, the degeneration of axons might take much longer periods (Lingor et al., 2012). Indeed, histopathological analyses using a mouse model of AD showed that, despite extreme dystrophy, axons maintain continuity throughout the disease course for several months (Adalbert et al., 2009). Moreover, computer modeling using data on neuron loss and neurofibrillary tangle (NFT) formation on AD brains, revealed that NFT bearing neurons can survive for up to 20 years (Morsch et al., 1999). This evidence suggests that neurodegeneration is a slow process in AD, thus providing the opportunity to target degenerating axons as an early therapeutic intervention. Compelling experimental and pathological studies have demonstrated that neurons in AD follow a dying-back pattern of neurodegeneration, where axonal terminals and then axons progressively degenerate toward the neuronal cell body (Bell and Claudio Cuello, 2006; Kalus et al., 2006; Stoub et al., 2006; Huang and Auchus, 2007; Adalbert et al., 2009; Rogalski et al., 2009; Gilley et al., 2011; Nishioka et al., 2019). Despite the difference in timing, axonal degeneration in the context of both acute lesions and neurodegenerative diseases, share morphologic features that include axonal swelling, microtubule disruption, and fragmentation of neuronal processes (Wang et al., 2012), suggesting that they correspond to similar processes. Recent studies have shed light into the mechanisms that govern acute axonal degeneration, revealing the dependence of NAD^+ in this process, as well as the involvement of mitochondrial dysfunction and necroptosis activation (Barrientos et al., 2011; Osterloh et al., 2012; Neukomm et al., 2017; Hernández et al., 2018; Arrázola et al., 2019; Ko et al., 2020; Loreto et al., 2020; Oñate et al., 2020). Notably, all these pathways are associated to the hallmarks of aging (Kennedy et al., 2014; Sun et al., 2016; Deepa et al., 2018; Haas, 2019; Lautrup et al., 2019; Royce et al., 2019; McReynolds et al., 2020). In the context of AD, different pathogenic pathways have been shown to contribute to axon demise, including calcium signaling imbalance, mitochondrial dysfunction, alterations in axonal transport, and increased oxidative stress (Bamburg and Bloom, 2009; Yu et al., 2009; Ye et al., 2012; Cioffi et al., 2019; Guo et al., 2020). As stated above, the accumulation of misfolded proteins constitutes a salient feature of AD neuropathology and a large body of evidence linking Aβ and tau pathologies with the disruption of axons has been published. In this review, we will present the pathways that contribute to the mechanism of acute axonal degeneration. Then, we will critically evaluate the evidence associating Aβ and tau pathologies with the disruption of axons in AD.

CELLULAR MECHANISMS ASSOCIATED WITH AXONAL DEGENERATION

Wallerian Degeneration

The degeneration of axons corresponds to a process activated in response to several stimuli including chemotherapy drugs, infection, inflammation, toxins, and mechanical injury, among others. Recent advances in the study of the molecular mechanisms that govern axon demise have contributed to uncovering essential components of the axon degeneration program. A schematic representation of the steps associated with axonal degeneration is presented in **Figure 1**.

Initial studies performed by August Waller to study axonal degeneration following nerve transection (Waller, 1850) led to the discovery of an ordered process in which three distinctive phases are typically observed. Initially, a latent period of about 36 h occurs, where the distal injured nerve fiber remains intact. A rapid phase then takes place, where the cytoskeleton is disrupted, and axons undergo fragmentation, which *in vivo* is associated with glial activation (Catenaccio et al., 2017). Finally, axonal disintegration and myelin degradation occurs, followed by macrophage infiltration and clearance of cell debris. This process is known as Wallerian degeneration (Coleman, 2005; Court and Coleman, 2012).

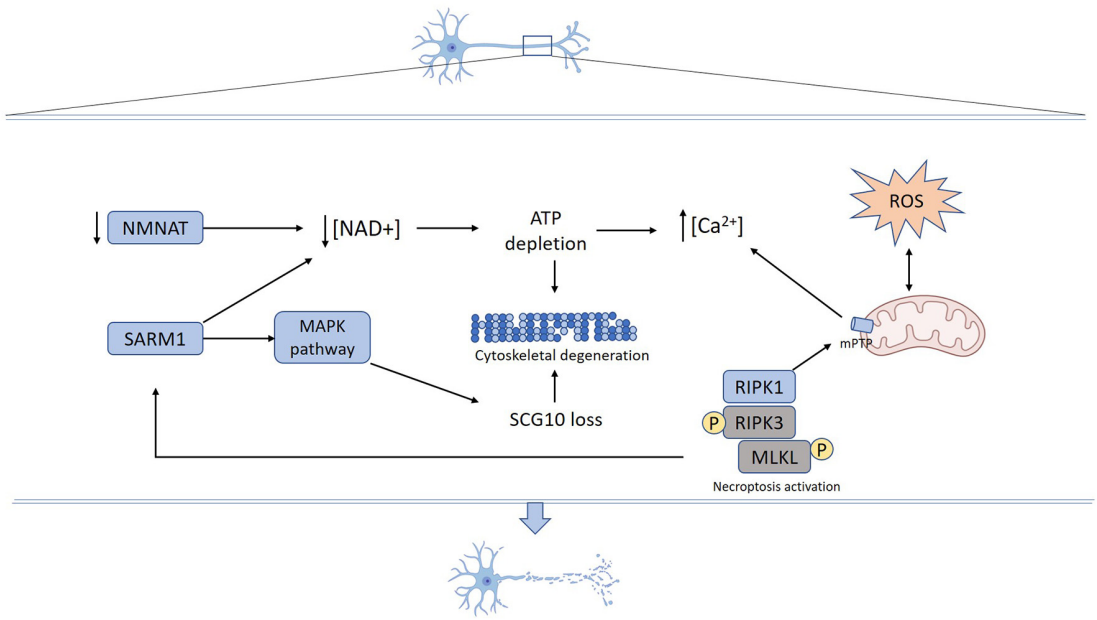

FIGURE 1 | Mechanisms underlying axon degeneration. Mechanical or toxic injury lead to nicotinamide mononucleotide adenylyltransferase (NMNAT) axonal loss and SARM1 activation resulting in NAD+ depletion. Activated SARM1 promotes NAD+ destruction and NMAT loss decreases NAD+ synthesis. Reduced axonal NAD+ levels lead to energy failure and ATP depletion. SARM1 also activates MAPK signaling pathways, which promotes SCG10 proteolysis. Increased reactive oxygen species (ROS) production promotes mPTP formation that also can be triggered by necroptosis activation. Energy failure derived from both NAD+ depletion and mitochondrial damage contributes to calcium unbalance, ROS production, and mPTP formation. Cumulative activation of mechanisms and structural damage ultimately result in cytoskeleton fragmentation and axon degeneration.

NAD$^+$ Metabolism in Axonal Degeneration

More than a 100 years later, studies performed on the Wallerian degeneration slow (*Wld^s*) mutant mice uncovered that upon axotomy, denucleated axons actively execute their own destruction, which is mediated by an evolutionarily conserved signaling pathway (Lunn et al., 1989; Perry et al., 1990, 1991; Lyon et al., 1993). Moreover, the discovery of the *Wld^s* strain supported the idea that axonal degeneration and cell body death are two events regulated by different molecular mechanisms (Lunn et al., 1989; Deckwerth and Johnson, 1994). In *Wld^s* mice, the distal denucleated axon remains functional for 3–4 weeks after nerve injury, suggesting that the *Wld^s* gene confers a protective effect intrinsic to the axon. *Wld^s* phenotype is caused by the overexpression of a chimeric *Wld^s* gene, encoding the full-length nicotinamide mononucleotide adenylyltransferase (NMNAT1) and a short region of a ubiquitin assembly protein (UFD2; Conforti et al., 2000). The two components of the *Wld^s* gene suggest the involvement of the ubiquitin-proteasome system (UPS) and NAD$^+$ metabolism in the process of axon degeneration. Although genetic and pharmacological inhibition of UPS activity delays axon degeneration, overexpression of NMNAT1 alone can prevent axonal degeneration (Zhai et al., 2003; Araki et al., 2004). In injured axons, NAD$^+$ levels decrease, and preventing this axonal NAD$^+$ decline by exogenous application of NAD+ protects axons from degeneration (Wang et al., 2005). Since NAD$^+$ is essential for glucose-dependent ATP production, reduced levels of NAD$^+$ impair axonal energy production that contributes to axon degeneration (Gerdts et al., 2016). These data suggest that NAD$^+$ metabolism plays a crucial role in axon degeneration.

SARM1 Mediates Axonal Loss

Mammals harbor three different isoforms of NMNAT proteins (NMNAT 1–3) that differ in subcellular localization and kinetic activity. NMNAT2 is the most labile isoform and it is constantly replenished in axons through fast axonal transport. Upon axotomy, NMNAT2 fails to be transported toward the axons and its levels rapidly drop in axons before Wallerian degeneration occurs (Gilley and Coleman, 2010; Gerdts et al., 2016). Thus, specific depletion of NMNAT2 is sufficient to induce Wallerian-like degeneration of uninjured axons. Moreover, the overexpression of NMNAT3, which is predominantly located in mitochondria, confers axonal protection after injury (Sasaki et al., 2006). The loss of NAD$^+$ is suppressed in sterile alpha and TIR motif-containing 1 (SARM1) knockout (ko) axons both *in vitro* and *in vivo*, suggesting that SARM1 is a key mediator of axon destruction (Gerdts et al., 2013, 2015; Gilley et al., 2015). SARM1 contains a C-terminal Toll-interleukin receptor domain, which dimerizes and mediates the rapid breakdown of NAD$^+$ (Gerdts et al., 2016). Axonal defects and embryonic lethality observed in *Nmnat2* ko mice are suppressed by *Sarm1* ablation, as *Nmnat2/Sarm1* double-ko mice are healthy. This indicates a relationship between NMNAT2 and SARM1 in the control of NAD$^+$ metabolism and axon degeneration. It has been hypothesized that loss of NMNAT2 induces a decline in NAD$^+$ levels, which in turn might lead to SARM1-dependent NAD$^+$

destruction, and consequently to a disastrous loss of NAD^+ in the axon (Gerdts et al., 2016).

Although current data indicates that NAD^+ depletion occurs downstream SARM1 activation after injury, additional evidence suggests the participation of other signaling pathways in axonal degeneration. Forced dimerization of the SARM1 TIR domain which results in NAD^+ depletion and axon degeneration, also triggers MAPK signaling activation. Moreover, in damaged axons, SARM1 is required for activation of MAPK and this signaling disrupts axonal energy homeostasis leading to ATP depletion (Yang et al., 2015). Thus, the deletion of mitogen-activated protein kinase kinase 12 (MAP3K12), also known as DLK, significantly delays the degeneration of distal axons (Miller et al., 2009). DLK signals through the downstream target of MAPK JNK and pharmacological inhibition of JNK leads to axon protection similar to *Dlk* ablation. Although *Dlk* ablation confers axon protection, this protective effect is weaker than the one resulting from overexpression of *Nmnat* and *Wlds* genes. Also, after injury activation of two additional members of the MAPK cascade has been identified, MEKK4 (MAP3k4) and MLK2 (MAP3K10), which promote axon degeneration. Genetic ablation of all three MAP3Ks leads to robust axonal protection (Yang et al., 2015). All these MAP3Ks converge in the activation of the JNK pathway, which induces UPS-dependent degradation of SCG10 (stathmin 2) after axon injury (Shin et al., 2012).

The Role of Mitochondria in Axonal Degeneration

As aforementioned, a crucial role of NMNAT in the axonal degeneration cascade has been proven. Supporting studies demonstrated that NMNAT3 overexpression prevents axonal loss mediated by oxidative damage induced by reactive oxygen species (ROS) exposure (Press and Milbrandt, 2008). Additional evidence supported this data and suggested that mitochondrial localization of NMNAT activity has a key role in NMNAT-mediated axonal protection (Yahata et al., 2009). Prompted by this evidence and considering the involvement of mitochondrial permeability transition (mPT) on neurodegenerative conditions (Forte et al., 2007; Du et al., 2008; Martin et al., 2009), Barrientos et al. (2011) sought to determine the role of the mPT pore (mPTP) on the mechanism of axonal degeneration. The researchers showed that degeneration of axons induced by vincristine or nerve transection—in *ex vivo* and *in vitro* models—was associated with activation of the mPTP and targeting the mPTP component Cyclophilin D (CypD), either by pharmacological or genetic means, significantly delayed axonal disintegration (Barrientos et al., 2011). These results identify the mPTP as a key effector of axonal degeneration, as well as a potential target to prevent axonal loss triggered by both mechanical and toxic stimuli. Further studies revealed that upon axonal injury, mPTP formation is mediated by calcium release from the axonal endoplasmic reticulum, constituting an early step in the mechanism of axonal degeneration (Villegas et al., 2014). Moreover, many studies have demonstrated that following axotomy, an increase in intra-axonal Ca^{2+} occurs, constituting a common step to activate the axonal degeneration cascade (George et al., 1995; Adalbert et al., 2012; Avery et al., 2012; Mishra et al., 2013; Vargas et al., 2015). Besides mitochondrial calcium overload, mPTP opening can also be triggered by oxidative stress (Brockemeier et al., 1992). In accordance, *in vivo* work performed in both *C. elegans* and mice, recognized ROS as key intermediates in the mechanism of axonal degeneration, as increasing the anti-oxidative capacity of the neuron efficiently prevented axon demise and functional loss triggered by the hyperactivated degenerin channel MEC-4d (Calixto et al., 2012). Moreover, *in vivo* studies carried out in a mouse model of Charcot-Marie-Tooth 2A disease demonstrated an uncoupling between ATP and ROS production in axonal mitochondria. Similarly, *in vivo*-induced demyelination triggered reduced levels of ATP, along with increased ROS production in axonal mitochondria. These data suggest that mitochondrial ATP and ROS imbalances may contribute to axonal degeneration (van Hameren et al., 2019).

Necroptosis Involvement in the Mechanism of Axonal Demise

Mitochondrial dysfunction, ROS production, and intracellular calcium increase, which as mentioned above are key events that mediate axonal degeneration, have also been associated with activation of the necroptosis signaling pathway (Vandenabeele et al., 2010). To test the involvement of necroptosis in the mechanism of axonal degeneration, Hernández et al. (2018) used an *in vitro* model of glutamate-induced excitotoxicity in hippocampal neurons seeded in microfluidic devices. The authors showed that axonal degeneration proceeds by necroptosis, which involved the activation of the mPTP as well as calcium dyshomeostasis in axons. Pharmacological inhibition of the necroptotic kinase RIPK1 using Nec-1s, or genetic downregulation of *Ripk3* or *Mlkl*, significantly prevented axonal degeneration and neuronal death (Hernández et al., 2018). In the same line, it was also demonstrated that axonal demise induced by mechanical and toxic stimuli *in vitro*—by axotomy or vincristine, respectively—is dependent on RIPK1, as Nec-1s prevented the degeneration of axons under those conditions. This protective effect was also observed by genetic inhibition of *Ripk*3 or *Mlkl*. Notably, the loss of the electrophysiological nerve function was also prevented by blocking the necroptotic machinery (Arrázola et al., 2019). Furthermore, investigating the mechanisms of axonal degeneration in *in vitro* and *in vivo* models of Parkinson's disease, we also uncover the role of the necroptosis signaling pathway in this process. Neurons treated with 6-OHDA exhibited significant axonal degeneration, as well as elevated expression levels of necroptotic markers, which was prevented by Nec-1s or the pMLKL inhibitor GW80. Similarly, intracerebral injection of 6-OHDA in mice triggered axonal degeneration, which was accompanied by RIPK3 and pMLKL upregulation. Nec-1s administration decreased axonal degeneration and improved motor performance of 6-OHDA injected mice, and similar results were obtained by *Ripk3* or *Mlkl* ko (Oñate et al., 2020). Together, these studies confirm that necroptosis activation takes place to mediate the degeneration of axons under different pro-degenerative stimuli, a process we have named necroaxoptosis (Oñate et al., 2020). Recently, a link between necroptosis and SARM1 was discovered using

a neuroinflammatory model of glaucoma. TNF-α was injected into the vitreous cavity of wild type mice, triggering axonal and cell body loss, which was prevented in the SARM1 ko mice. Increased levels of necroptosis markers in optic nerves of both wild type and SARM1 ko mice suggested that SARM1 functions downstream of necroptosis mediate axonal degeneration. Additional experiments where necroptosis was induced by direct MLKL dimerization, showed that necroptosis-mediated calcium influx, loss of mitochondrial potential, and axon degeneration were blocked in SARM1 ko axons, integrating previous steps of the axonal degenerative pathway (Press and Milbrandt, 2008; Barrientos et al., 2011; Villegas et al., 2014; Arrázola et al., 2019; Ko et al., 2020). Therefore, necroaxoptosis seems to be a common mechanism for axonal degeneration after a variety of insults.

THE ROLE OF PROTEIN MISFOLDING IN AXONAL DEGENERATION IN AD

Axonal degeneration and dysfunction are prominent features of AD brains. White matter alterations revealed by *in vivo* imaging can be observed at the early stages of the disease, even in MCI patients. Importantly, these changes correlate with clinical measures of cognitive disability (Kalus et al., 2006; Stoub et al., 2006; Rogalski et al., 2009; Ihara et al., 2010; Bozzali et al., 2011). Accumulation of misfolded proteins is also an early pathological signature during AD, and cumulative evidence documenting a link between these two neuropathological events has been published, which is the focus of this section. A schematic representation of the signaling cascades triggered by Aβ and tau that contribute to axonal degeneration is presented in **Figure 2**.

Axonal Disruption Associated With Tau Pathology

The first report describing tau pathology showed that neurofibrillary changes in the form of NFT and neuropil threads (NT) exhibit a characteristic distribution pattern affecting vulnerable brain regions such as the cerebral cortex and hippocampus (Braak and Braak, 1997). These lesions begin with misfolded phospho-tau in the proximal axon and then spreads into the somatodendritic compartment (Braak and Del Tredici, 2011). Deterioration of the cytoskeleton in individual neurons reveals a sequence of changes occurring in neuronal processes suggesting that the disruption of microtubules containing tau may cause the degeneration of axons (Kowall and Kosik, 1987; Braak et al., 1994; Braak and Del Tredici, 2011). Moreover, analysis of hippocampal regions showed that tau inclusions within dystrophic neurites correlate with several measures of the mini-mental state examination, suggesting that these pathological lesions contribute to cognitive dysfunction (Ghoshal et al., 2002).

The Role of Tau-Mediated Axonal Transport Disruption in Axonal Degeneration

Due to their polarized nature, neurons rely on an efficient axonal transport system for delivering proteins, lipids, and organelles from the cell body to the axon and synapses. The proper function of axonal transport depends on the correct assembly and functioning of all components including microtubules and motor proteins. Therefore, alterations in axonal transport render neurons vulnerable to the loss of synapses and can trigger axonal degeneration (Mandelkow et al., 2003). Indeed, chemical interventions that directly or indirectly affect axonal transport result in a dying-back form of axonal degeneration (Fukuda et al., 2017). Disruption of axonal transport as well as morphological alterations of the axons occur early in the course of AD and can be detected even a year before other neuropathological abnormalities develop, including amyloid deposition (Stokin, 2005). Defects in axonal transport have been extensively studied in the context of AD and it has been suggested to have a causative role in the disease (Muresan and Muresan, 2009; Vicario-Orri et al., 2014).

In AD, pathological changes associated with tau begin as the granular accumulation of phosphorylated tau in the cytoplasm, axon, and dendrites. Then, tau gradually aggregates to form NT and NFT, which affect the neuronal process that eventually undergoes degeneration (Braak et al., 1994; Braak and Del Tredici, 2011). Tau is a microtubule-associated protein involved in microtubules dynamics, which function as a track for axonal transport (Gao et al., 2018). It has been hypothesized that an imbalance in intracellular signaling causes excessive tau phosphorylation and its subsequent detachment from microtubules. This, in turn, promotes microtubule destabilization and impairment of axonal transport. Several studies have shown that tau overexpression in neurons increases tau phosphorylation and inhibits axonal transport (Stamer et al., 2002; Mandelkow et al., 2003; Chee et al., 2005; Thies and Mandelkow, 2007). As a result of tau-dependent axonal transport inhibition, organelles and vesicles are reduced in cell processes, which lead to energy depletion, decreased oxidative defense, and dying-back of neurites (Stamer et al., 2002; Mandelkow et al., 2003). The NAD^+ biosynthetic enzyme NMNAT2, a well-known pro-survival factor that inhibits axonal degeneration after injury, depends on constant replenishment by anterograde axonal transport (Gilley and Coleman, 2010). Interestingly, in human AD brains as well as in rTg4510 mice, NMNAT2 expression is reduced, suggesting a possible mechanism by which tau pathology can contribute to axonal degeneration (Ljungberg et al., 2011; Ali et al., 2016). Thus, disruption of axonal transport-associated to abnormal tau phosphorylation might lead to decreased axonal NMNAT2 levels. The unbalance of the NAD^+/NMN index due to loss of NMNAT2 could lead to SARM1-dependent NAD^+ destruction in the axon and ultimately axon degeneration. However, this remains to be established.

Also, to regulate microtubule dynamics, tau modulates the function of the motor proteins dynein and kinesin, modifying the axonal transport of proteins and organelles. Enhanced tau phosphorylation also causes synaptic dysfunction as a consequence of energy depletion, associated with a reduced number of mitochondria in the pre-synapsis (Chee et al., 2005). This reduced density of mitochondria in axons and presynaptic terminals directly affects the energy status and calcium buffering. Additionally, it has been shown that tau may directly affect the endoplasmic reticulum-mitochondria interactions and therefore

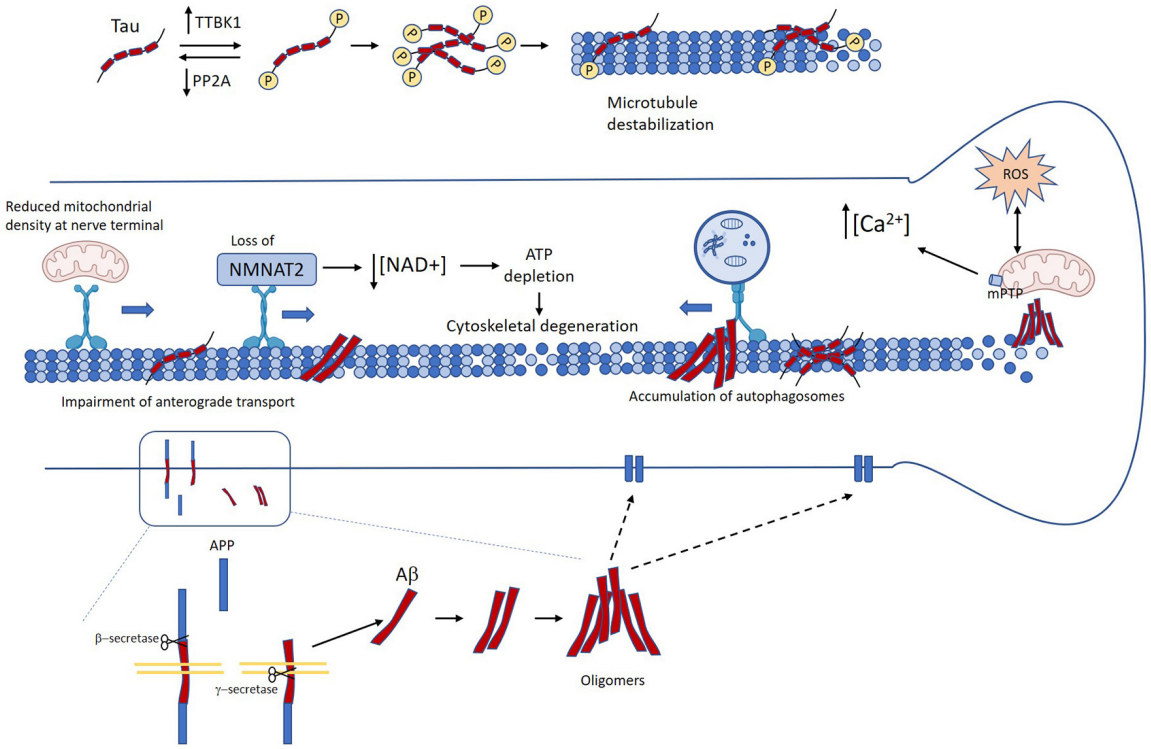

FIGURE 2 | Mechanisms linking Tau and amyloid-β (Aβ) in Alzheimer's disease (AD) to axon degeneration. Unbalance between kinases and phosphatases leads to the accumulation of abnormally phosphorylated tau, which in turn induces detachment form microtubules and microtubule destabilization (Köpke et al., 1993). Several age-related factors contribute to the accumulation of Aβ oligomers. Accumulation of pathological tau and Aβ promote axonal transport impairment (Calkins and Reddy, 2011; Tang et al., 2012; Wang et al., 2015; Sadleir et al., 2016; Zhang et al., 2018). This in turn causes loss of axonal NMAT2, decreased mitochondrial density at the nerve terminal, and autophagy flux impairment (Gilley and Coleman, 2010; Ljungberg et al., 2011; Ali et al., 2016). Neuron loss and neurofibrillary tangle (NFT) and Aβ oligomers also promote mitochondrial dysfunction, oxidative stress, and calcium dyshomeostasis (Stamer et al., 2002; Chee et al., 2005; Cieri et al., 2018; Mata, 2018; Albensi, 2019). All these events drive the failure of critical mechanisms for axonal functioning and maintenance that lead to loss of axonal homeostasis, and ultimately axon degeneration.

calcium handling (Cieri et al., 2018). Calcium unbalance may lead to activation of calcium-dependent proteases, calpains, which are implicated in the granular disintegration of the axonal cytoskeleton, a hallmark of Wallerian degeneration. Indeed, calpains are abnormally activated in AD brains, and they have been implicated in the development of tau pathology (Mahaman et al., 2019).

Phosphorylation plays a critical role in the regulation of tau functions, including the regulation of microtubule stabilization and assembly. Phosphorylation of tau is increased in AD brains, suggesting an unbalance in tau-associated kinases and phosphatases (Köpke et al., 1993). The expression of tau-tubulin kinase1 (TTBK1), a brain-specific tau kinase, is significantly up-regulated in the frontal neocortical region of the AD brain and colocalizes with NFT-positive neurons (Sato et al., 2006, 2008). Additionally, transgenic mice expressing human TTBK1 show increased tau phosphorylation and significant axonal degeneration in the entorhinal cortex (Sato et al., 2008; Ikezu et al., 2020). On the other hand, the activity of PP2A, the main tau phosphatase, is reduced in AD brains, and the inhibition of PP2A activity in mice models of AD result in tau pathology and cognitive impairment (Wang et al., 2010; Louis et al., 2011; Braithwaite et al., 2012). Strikingly, PP2A activity can be regulated by NMNAT2, hence reduced NMNAT2 expression observed in AD might down-regulate PP2A activity resulting in tau hyperphosphorylation (Cheng et al., 2013). Thus, in AD pathogenesis the unbalance between kinases and phosphatases may lead to abnormally hyperphosphorylated tau, which disrupts axonal transport, triggering axonal degeneration.

Axons depend on the constant remodeling of damaged proteins and organelles to maintain their correct functioning and connectivity (Maday and Holzbaur, 2016). This remodeling depends on homeostatic/degradation systems such as the UPS and autophagy (Korhonen and Lindholm, 2004; Kulkarni and Maday, 2018). Acetylation of tau inhibits the degradation of phosphorylated tau by the UPS, and the accumulation of acetylated tau has been identified in AD brains (Min et al., 2010, 2015). Moreover, the loss of Sirtuin-1, which deacetylate tau, is closely associated with the accumulation of tau in the cerebellar cortex (Julien et al., 2009). Although the UPS is responsible for the degradation of up to 80–90% of proteins including tau, misfolded proteins and aggregates are too large to be processed through the proteasome barrel and impede UPS function by physical occlusion (David et al.,

2002; Chung et al., 2019). However, *in vitro* studies have demonstrated that tau aggregates can be degraded by autophagy. Ultrastructural analysis of post-mortem brain samples from AD patients showed the accumulation of autophagic vesicles within swollen and dystrophic neurites. Interestingly, autophagic vesicles were found more frequently in neurons bearing NFT (Nixon et al., 2005). Inducing autophagy by pharmacological interventions in different models of AD results in lower tau accumulation and better cognitive performance (Li et al., 2017). These observations suggest that autophagy impairment is associated with the deposition of pathological tau and contributes to neuronal demise in AD. Transport of autophagic vesicles loaded with unfolded proteins and organelles from distal axonal domains relies on a well-functioning retrograde transport to reach the soma, where autophagy degradation occurs (Maday and Holzbaur, 2014; Kulkarni and Maday, 2018). Dysregulations of tau observed in AD can impair dynein-retrograde axonal transport (Wang et al., 2015), which may lead to autophagy disruption and accumulation of autophagic vesicles within axons. Ultimately all these events might cause the loss of axonal homeostasis that gradually dye back to the soma.

Oxidative stress is another event associated with axonal degeneration in AD at the early stages of the pathologic process (Alavi Naini and Soussi-Yanicostas, 2015). Increased susceptibility to oxidative stress is linked to tau hyperphosphorylation, which leads to peroxisome depletion in neurites due to the inhibition of microtubule transport (Stamer et al., 2002). On the other hand, oxidative stress leads to increased tau phosphorylation in neuronal cultures and animal models of AD (Melov et al., 2007; Su et al., 2010). Indeed, antioxidant treatment reduces oxidative stress, tau pathology, and improves cognitive performance in 3xTg-AD mice (Clausen et al., 2012). An imbalance between pro-oxidants and antioxidants at the early stages of AD leads to increased oxidative stress, which may promote dysregulation of tau phosphorylation. This in turn might promote disruption of axonal transport that exacerbates oxidative stress and tau phosphorylation. Thus, tau phosphorylation and oxidative stress interplay is a key component of a vicious circle that plays a crucial role in the pathological process of AD. Then, ROS production might trigger mitochondrial dysfunction and necroptosis activation, two well-established mechanisms of axonal degeneration.

Analysis of postmortem brain samples suggests that tau pathology begins before the formation of NFT, as the accumulation of hyperphosphorylated tau is observed in young AD patients (Braak et al., 2011). Several lines of evidence suggest that abnormal phosphorylation of soluble tau causes synapse loss, impaired synaptic function, disrupted axonal transport, and cognitive deficits (DeKosky and Scheff, 1990; Callahan and Coleman, 1995; Mandelkow et al., 2003; Thies and Mandelkow, 2007; Hoover et al., 2010). Therefore, it has been hypothesized that NFT occurs as a protective cellular response, where NFT might scavenge the toxic monomeric or oligomeric tau. However, these tau aggregates might sequester other cell components or even cause an axonal clogging that ultimately leads to axonal degeneration (Mandelkow et al., 2003). Thus, tau pathology in AD likely initiates with a signaling dysregulation leading to hyperphosphorylation of tau, which causes microtubule destabilization and tau mislocalization. These events may drive the failure of critical mechanisms for axonal functioning and maintenance that lead to loss of axonal homeostasis. Finally, phospho-tau assembles into NFT, and the aggregation of these tau oligomers eventually contributes to axonal degeneration.

Evidence Linking Aβ to Axonal Disruption

Aβ-Related Axonal Dystrophy

In the first report describing the neuropathology of the postmortem brain of an AD patient, the appearance of altered neuronal processes (referred to as dystrophic neurites) was described (Alzheimer, 1907; article translated in Stelzmann et al., 1995). Subsequently, numerous studies examining AD brain tissue, as well as transgenic animal models of the disease, have revealed an intimate association between dystrophic neurites (i.e., axons and dendrites) and Aβ deposition (reviewed in Spires and Hyman, 2004; Woodhouse et al., 2005; Bell and Claudio Cuello, 2006; Mokhtar et al., 2013). Dystrophic neurites are defined as abnormal neuronal processes, characterized by the presence of thick, tortuous, as well as swollen segments (Su et al., 1993). The morphology of these irregular neuronal processes, which correspond to regions with cytoskeletal alteration and organelle accumulation, may change as the disease progresses (Vickers et al., 1996; Su et al., 1998; Woodhouse et al., 2009; Sanchez-Varo et al., 2012).

Although most research in AD has focused on the study of the loss of dendrites and synapses associated with Aβ pathology, as there is compelling evidence linking these alterations with cognitive decline (Davies et al., 1987; DeKosky and Scheff, 1990; Terry et al., 1991; Scheff et al., 2006; Bastrikova et al., 2008; Jackson et al., 2019), Aβ-related dystrophic axons are widespread in diseased brains and several studies suggest that they contribute to synaptic damage (Adalbert et al., 2009; Sanchez-Varo et al., 2012).

In vivo evidence of the temporal course of Aβ-related axonal pathology underwent by different AD mouse models—using two-photon imaging—has revealed that the environment around plaques is not uniform and that rather, it promotes a continuous remodeling, suggesting that axonal dystrophies associated with Aβ plaques are highly plastic structures, with a morphology that varies over time (Tsai et al., 2004; Blazquez-Llorca et al., 2017). Notably, although a re-growth phenomenon has been observed, were newly formed axonal segments were visualized in dystrophic axons, the elimination rates of neurites near amyloid aggregates are significantly higher than the formation rates, thereby leading to a progressive net loss over time (Tsai et al., 2004). As the formation of dystrophic axons seems to be a very dynamic process, with alterations appearing and disappearing within short periods, it has been suggested that early treatment to prevent amyloid accumulation may induce the recovery of the neuronal networks (Blazquez-Llorca et al., 2017). In agreement, different research groups have demonstrated that Aβ clearance by immunotherapy can attenuate Aβ-related axonal

degeneration in AD transgenic models (Lombardo et al., 2003; Brendza et al., 2005; Liu et al., 2011).

Axonal Transport Disruption Associated With Aβ

Although there is a general notion that the development of dystrophic axons occurs as a consequence of nearby amyloid deposition, a growing body of evidence supports the converse view of an axonal origin of Aβ substrates for extracellular plaque formation, at sites were dystrophies are formed. Thus, the cause and effect relationship between amyloid plaques and axon damage remains a matter of debate.

Altered axonal transport constitutes a typical feature seen in the brain of TBI patients (who are at high risk of developing AD), as well as in TBI animal models (Choe, 2016). Immunohistochemical assessment of TBI brain samples shows increased levels of intra-axonal APP at swelling sites, and this has been attributed to the neuroprotective functions of this protein, as well as the products of its proteolytic processing (Plummer et al., 2016). However, there is well-documented evidence showing that widespread diffuse Aβ plaque deposition occurs in TBI survivors, and this has also been demonstrated in animal models of TBI (Johnson et al., 2012; Shishido et al., 2016; Edwards et al., 2017; Abu Hamdeh et al., 2018). Importantly, longitudinal studies performed to these individuals have revealed that NFT and amyloid plaque pathologies persist, and are associated with cognitive impairment (Johnson et al., 2012). Moreover, compelling studies have demonstrated that alterations in APP transport can promote the local release of Aβ, which can further impair axonal transport (Rodrigues et al., 2012; Mórotz et al., 2019). Thus, just as alterations in axonal transport can lead to amyloid pathology, there is also extensive evidence that Aβ itself can cause microtubule-based transport defects (Pigino et al., 2009; Zhao et al., 2010; Calkins and Reddy, 2011; Tang et al., 2012; Cruz et al., 2018; Zhang et al., 2018). In this line, and considering the role of microtubules in axonal transport, several studies have focused on the effects of Aβ on the integrity of the microtubule network (Fifre et al., 2006; Gevorkian et al., 2008; Silva et al., 2011; Mota et al., 2012; Pianu et al., 2014; Wang et al., 2018; Gao et al., 2019). For instance, Sadleir et al. (2016) hypothesized that presynaptic dystrophies were triggered by Aβ-mediated microtubule disruption. Using live-cell imaging of primary neurons, the authors observed that exposure to Aβ oligomers causes microtubule depolymerization, neuritic beading, and altered axonal trafficking. These data were validated in brain tissue from an AD mouse model as well as in human AD samples, where dystrophic axons and terminals in the proximity to Aβ deposits, displayed aberrant localization of tubulin, as well as evidence of decreased lysosomal function and autophagic vesicles accumulation, suggesting alterations in the microtubule-based transport. Notably, elevated β-secretase-1 and APP levels were also observed in peri-plaque dystrophies, which caused local Aβ generation that may further exacerbate extracellular amyloid pathology (Sadleir et al., 2016). Together, this evidence points towards a mechanism in which Aβ-mediated axonal defects, and the contribution of axonal dystrophies to Aβ plaque formation, are not isolated processes. Rather, a vicious circle seems to occur, where axons in the vicinity of amyloid deposits undergo alterations that further enhance Aβ accumulation.

As discussed above, defects in axonal transport occur early in the onset of AD. Considering the architecture of axons, transport along this structure is critical to preserve its integrity, as a failure of intra-axonal trafficking can lead to deprivation of cargoes that are essential for axon survival and can also impair the communication with the cell body or other cells (Coleman, 2011; Kanaan et al., 2013). Accordingly, disruptions on this vital cellular process can eventually lead to axonal degeneration (Ferri et al., 2003; Coleman, 2005, 2011; Pigino et al., 2009; Kanaan et al., 2013). In this regard, Aβ-mediated disturbances in microtubule-based cellular transport block the trafficking of vital cargoes to synapses. For example, many works reporting impairment of mitochondrial transport induced by Aβ have been published (Rui et al., 2006; Calkins and Reddy, 2011; Kim et al., 2012; Umeda et al., 2015; Zhang et al., 2018). Mitochondria are required at pre- and postsynaptic terminals for the correct function of neurotransmission, as it has essential roles in ATP production and buffering synaptic calcium (Li et al., 2004; Sheng and Cai, 2012). Thereby, the abnormal function of axonal transport induced by Aβ can block mitochondria trafficking, triggering synaptic alterations, which can initiate retrograde degeneration of axons. However, the exact mechanism that mediates this process remains unclear. As previously mentioned, alterations in NMNAT2 are associated with AD (Ali et al., 2016). Although a direct link between Aβ and NMNAT2 has not been proven, it is likely that, in the course of events leading to the activation of the axon death cascade, tau -which indeed has been related to NMNAT2 downregulation (Ljungberg et al., 2011)- acts downstream Aβ.

Aβ and the Mechanism of Axonal Degeneration

As previously stated, white matter alterations, reflecting axonal degeneration and dysfunction, are present not only in AD brains but also in MCI patients. Growing data from *in vivo* imaging studies have documented a relationship between such white matter abnormalities and Aβ deposition (Collins-Praino et al., 2014; Hoy et al., 2017; Schilling et al., 2018; Vipin et al., 2019; Weaver et al., 2019; Caballero et al., 2020). Importantly, recent longitudinal studies on the association between white matter integrity and amyloid pathology in cognitively normal individuals, revealed that Aβ aggregates build-up in white matter fibers known to be affected in AD, in an age-dependent manner (Vipin et al., 2019; Caballero et al., 2020). These studies underscore the impact of Aβ load on early white matter alterations in normal aged subjects at risk of AD. For this reason, a thorough understanding of the signaling that mediates axonal degeneration is crucial, as this would allow the identification of potential therapeutic targets.

Several lines of investigation have demonstrated that rather than senile plaques, Aβ oligomers are the most cytotoxic form of the aggregates and that plaques might serve as reservoirs from which oligomers can diffuse, reaching cellular targets in their vicinity. Aβ oligomers can exert toxicity through several different pathways, and many of them are involved in

the process of axonal degeneration, including mitochondrial dysfunction (Swerdlow, 2018; Albensi, 2019), oxidative stress (Butterfield and Boyd-Kimball, 2018; Cheignon et al., 2018) and calcium dyshomeostasis (Mata, 2018; Popugaeva et al., 2018; Wang and Zheng, 2019). As discussed, previous work by our group demonstrated that axonal degeneration triggered by distinct insults is dependent on mitochondrial dysfunction and activation of the mPTP (Barrientos et al., 2011). In this line, Aβ oligomers were shown to target the mPTP regulator cyclophilin D (CypD) in a transgenic mouse model of AD as well as in AD brain samples. In this animal model, CypD deficiency prevented Aβ-induced mitochondrial swelling and permeability transition, improved calcium buffering capacity, and decreased mitochondrial ROS (Du et al., 2008, 2011). *in vitro*, the absence of CypD protected neurons from Aβ- and oxidative stress-mediated cell death. Remarkably, inhibiting the mPTP by genetic ablation of CypD improved cognitive and synaptic function in the transgenic AD model (Du et al., 2008, 2011). Furthermore, Aβ-induced impairment of axonal mitochondrial trafficking depends on CypD-mediated mPTP activation. Genetic deletion of CypD suppressed Aβ-mediated activation of the p38/MAPK signaling pathway, reversed axonal mitochondrial alterations, improved synaptic function, and prevented synapse loss (Guo et al., 2013). These studies shed light on the mechanism by which Aβ triggers mitochondrial dysfunction, which may promote axonal degeneration in AD.

As mentioned, both oxidative stress and calcium dyshomeostasis have shown to mediate axonal degeneration, and these two events have been largely studied in the context of Aβ-mediated neurodegeneration. However, most of the works have focused on the neuron as a whole, instead of studying the subcellular mechanisms that are activated in response to Aβ. Thus, to comprehend the exact pathways that trigger the loss of axonal structures, the use of compartmentalized microfluidic devices, which allow the isolation of axons from cell bodies and dendrites, represent a useful tool. In this regard, Song et al. (2006) used a compartmented chamber to study the differential effects of Aβ in somas and axons of sympathetic neurons. The researchers demonstrated that exposure of axons to Aβ triggered a caspase-independent mechanism of degeneration, which lead to nuclear apoptosis, as caspase inhibitors prevented apoptosis but did not protect neurons from axonal degeneration. Interestingly, the treatment of axons with calpastatin to inhibit calpains, which have been shown to mediate axonal cytoskeleton disintegration during Wallerian degeneration, not only protected axons from degeneration but also prevented nuclear apoptosis (Song et al., 2006). Using the same approach, another research group found that exposure of axons from hippocampal neurons to Aβ oligomers activated intra-axonal translation and induced local ATF4 synthesis. Retrograde transport of the protein to the soma induced ATF4-dependent gene expression and cell death. Similarly, intracerebral injection of Aβ in mice led to ATF4 translation in cholinergic axons, which was required for the retrograde transmission of Aβ-induced neurodegeneration (Baleriola et al., 2014; Walker et al., 2018).

As described here, recent studies have unveiled that axonal degeneration depends on necroaxoptosis activation (Hernández et al., 2018; Arrázola et al., 2019; Ko et al., 2020; Oñate et al., 2020). Although necroaxoptosis has not been demonstrated to occur in the context of AD, the evidence of necroptosis activation in AD brains (Caccamo et al., 2017) suggests that—similar to the findings in PD (Oñate et al., 2020)—this process may indeed contribute to axonal demise in AD.

Synergistic Contribution of Aβ and Tau to Axonal Degeneration

Mounting data has proven not only a synergistic but also a dependent neurotoxic effect of Aβ and tau pathologies (Bloom, 2014). To *in vivo* assess the pathological mechanism of Aβ and tau interaction on axonal degeneration, Nishioka et al. (2019) used a transgenic tau model and injected Aβ aggregates into the axonal terminals of retinal ganglion cells. Diffusion tensor imaging revealed a progressive white matter loss that correlated with the histopathological observation of retrograde axonal degeneration. Moreover, Aβ exposure triggered tau phosphorylation that preceded axon loss, and treatment with the microtubule-stabilizing compound Epothilone D inhibited tau phosphorylation and prevented axonal degeneration (Nishioka et al., 2019). This work emphasizes the cross-talk between Aβ and tau proteins that trigger the degeneration of axons and represents the first study to *in vivo* demonstrate the influence of this interaction in the mechanism of axonal degeneration. However, the exact signaling pathway activated downstream Aβ and tau interaction, and responsible for the disintegration of the axonal cytoskeleton, has not yet been explored.

Previous works have shown that reductions in tau can prevent Aβ-induced neurodegeneration and cognitive alterations in AD mouse models (Roberson et al., 2007; Ittner et al., 2010). Considering both the evidence of Aβ-mediated axonal transport deficits, as well as the interaction between tau and Aβ to induce neuronal alterations, Vossel et al. (2010) studied the effects of Aβ on axonal transport of mitochondria and TrkA, in hippocampal neurons from tau deficient and wild type mice. The researchers found that Aβ-dependent inhibition of axonal transport of mitochondria and TrkA was prevented in $tau^{-/-}$ neurons, stressing that a dependent degenerative effect between both proteins might take place in AD (Vossel et al., 2010). In line with this, i*n vitro* and *in vivo* experiments showed that Aβ trimers derived from postmortem human AD brains, induced conformational changes in tau that led to reduced expression of the kinesin-1 light chain and increased intra-axonal APP, suggesting disruption of axonal transport (Sherman et al., 2016).

Although axonal transport deficits associated with misfolded amyloid and tau have been largely studied in AD brains and models, the signaling cascades acting downstream these alterations that might activate axonal degeneration pathways are not clearly defined. Thus, the evidence demonstrates that axonal degeneration is an early event in AD, where Aβ and tau pathologies interplay contributes to the degenerative process. Nevertheless, besides the reduced NMNAT2 levels found in AD brains, there is no evidence of the involvement of key molecules that govern Wallerian degeneration—such as necroptosis-associated proteins or SARM1—in the context of AD. Moreover, whereas Wallerian degeneration proceeds rapidly, the evidence

indicates that axonal degeneration in AD rather constitutes an extended process that may take months or even years. Hence, it is possible that, although some common players are indeed involved, a mechanism different from the one activated during Wallerian degeneration mediates axonal degeneration in AD.

CONCLUDING REMARKS

Aging is considered the main risk factor contributing to the development of neurodegenerative diseases, including AD. The age-dependent decrease of homeostatic systems such as chaperones, degradation systems, and antioxidant defense considerably contributes to the accumulation of misfolded proteins in the brain. Although deposition of Aβ and tau has been the center of AD drug development research, many clinical trials on Aβ and tau clearing have been conducted, with disappointing outcomes (Congdon and Sigurdsson, 2018; Huang et al., 2020). As discussed in this review, a large body of evidence has uncovered a link between the development of Aβ and tau pathologies and the process of axonal degeneration. Nonetheless, a clear mechanism that integrates the known signaling cascades that activate axonal death, with the pathways that have been shown to mediate axonal degeneration in the context of AD, remains elusive. Many reports have documented that significant decreases in NAD^+ occur during aging (Lautrup et al., 2019; McReynolds et al., 2020) and supplementation of this metabolite has been suggested as a potential treatment for age-related diseases including AD (Braidy et al., 2018; Hou et al., 2018). Despite the importance of NAD^+ in the mechanism of axonal degeneration, its role in axon demise in AD is undefined. Furthermore, whether necroaxoptosis is activated in AD, is still unknown. Axonal loss in AD appears to be a slow process that initiates early during disease progression, thus providing a window for therapeutic intervention. An in-depth understanding of the mechanism that governs axonal degeneration is critical for the development of new therapies that allow to halt axonal loss and therefore prevent cell death and cognitive decline in AD.

AUTHOR CONTRIBUTIONS

NS planned and structured the manuscript, researched, and wrote the review. CG-O researched and wrote the review. FC revised the manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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Amyotrophic Lateral Sclerosis: Proteins, Proteostasis, Prions, and Promises

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Amyotrophic lateral sclerosis (ALS) is characterized by the progressive degeneration of the motor neurons that innervate muscle, resulting in gradual paralysis and culminating in the inability to breathe or swallow. This neuronal degeneration occurs in a spatiotemporal manner from a point of onset in the central nervous system (CNS), suggesting that there is a molecule that spreads from cell-to-cell. There is strong evidence that the onset and progression of ALS pathology is a consequence of protein misfolding and aggregation. In line with this, a hallmark pathology of ALS is protein deposition and inclusion formation within motor neurons and surrounding glia of the proteins TAR DNA-binding protein 43, superoxide dismutase-1, or fused in sarcoma. Collectively, the observed protein aggregation, in conjunction with the spatiotemporal spread of symptoms, strongly suggests a prion-like propagation of protein aggregation occurs in ALS. In this review, we discuss the role of protein aggregation in ALS concerning protein homeostasis (proteostasis) mechanisms and prion-like propagation. Furthermore, we examine the experimental models used to investigate these processes, including *in vitro* assays, cultured cells, invertebrate models, and murine models. Finally, we evaluate the therapeutics that may best prevent the onset or spread of pathology in ALS and discuss what lies on the horizon for treating this currently incurable disease.

Keywords: amyotrophic lateral scelerosis, proteostasis, protein aggregation, prion-like, *in vitro* models, invertebrate models, mouse models, therapeutics

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INTRODUCTION

Proteostasis and Prion-Like Propagation

A major pathological component of neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, frontotemporal lobar degeneration (FTLD), and amyotrophic lateral sclerosis (ALS) is the prion-like propagation of misfolded and aggregated proteins in the central nervous system (CNS; for recent reviews in each case see—Hock and Polymenidou, 2016; Watts and Prusiner, 2018; McAlary et al., 2019b; Vargas et al., 2019). The idea of prion-like propagation

of protein misfolding and aggregation in these diseases offers a plausible explanation for their idiopathic nature, progressive anatomical spread, the selective vulnerability of specific CNS regions, characteristic pathologies, and age-associated onset (Prusiner, 2001). In each disease, particular proteins are thought to misfold into a conformation that is capable of propagating throughout the CNS like the infectious prion protein (PrP; Vaquer-Alicea and Diamond, 2019).

PrP is typically found in a conformation composed primarily of alpha-helices (PrP^{C}); however, it can be converted into a β-sheet-rich pathogenic conformation (PrP^{Sc}) that can recruit and convert normal PrP to become pathogenic PrP^{Sc} through a template-directed manner (Prusiner, 1982; Pan et al., 1993). This conversion may be the result of aberrant biosynthesis and processing in cells (reviewed in Chakrabarti et al., 2009). There is no defined structure for PrP^{Sc} as it exists in a continuum from monomers to large amyloid-like polymeric assemblies (McKinley et al., 1991; Ceroni et al., 1996). Similar to PrP^{Sc}, proteins implicated in the etiology of many neurodegenerative diseases are known to form amyloid *in vitro* and deposit into intracellular inclusions in an amyloid conformation (Chiti and Dobson, 2017). The amyloid state is defined by its cross-β fiber architecture (Eisenberg and Jucker, 2012; Eisenberg and Sawaya, 2017), and is of critical importance to the pathogenic mechanisms of prions and prion-like proteins.

In vitro, the formation of amyloid is thought to proceed from the initial misfolding and aggregation of protein monomers into an ensemble of soluble non-native oligomeric states, with some oligomers forming proto-fibrils capable of elongating through template-directed monomer addition (Arosio et al., 2015). Amyloid fibrils are typically highly thermodynamically stable but vary in their mechanical stability (Yoon et al., 2013). Amyloid fibrils with low mechanical stability are more likely to fragment, leading to the exposure of more ends from which they can elongate in a repetitive cycle of fragmentation and growth (Knowles and Buehler, 2011; **Figures 1A,B**). Indeed, fragmentation is a highly important characteristic of the infectivity and replication propensity of prions and prion-like proteins (Marchante et al., 2017). Furthermore, microstructural heterogeneity in the initial population of misfolded protein and oligomers is thought to give rise to a range of conformationally distinct amyloid fibrils with different physical properties, often termed polymorphs (Petkova, 2005; Kodali et al., 2010; Safar et al., 2015). Over time, the fibril polymorphs with properties that permit efficient self-replication are selected for, giving rise to a dominant "strain" of amyloid fibril (**Figure 1A**). The notion of different strains of amyloid and prions provides a highly plausible explanation for the pathologic and clinical heterogeneity observed in neurodegeneration, as different strains can spread and recruit benign substrates at different rates (Telling et al., 1996; Safar et al., 1998; Morales, 2017). Supporting this, cryo-electron microscopy of amyloid isolated from human brain tissue has shown structural diversity in fibrils isolated from a single person (Kollmer et al., 2019) and in different neurodegenerative prion-like diseases (Fitzpatrick et al., 2017; Strohäker et al., 2019).

Although amyloid formation is often simplified as a relatively rapid kinetic reaction involving only the constituent amyloidogenic protein (Arosio et al., 2015; **Figure 1B**), the *in vivo* formation of these assemblies is notably slower and more complex (Owen et al., 2019). This additional complexity is primarily due to the existence of cellular mechanisms to maintain protein homeostasis (proteostasis; Yerbury et al., 2016; **Figure 1A**). Proteostasis is the concept of the maintenance of the proteome in the correct concentration (balance of protein synthesis and degradation), in the correct conformation (chaperones), and at the right location at the right time (trafficking). The importance of proteostasis in neurodegenerative disease is highlighted by the fact that disturbed proteostasis mechanisms are strongly associated with aging and neurodegeneration (Cheng et al., 2018; Kurtishi et al., 2019; Lehtonen et al., 2019; Yerbury et al., 2020). Mutations can predispose a protein to become more aggregation prone, and mutations in proteostasis components can impair proteostasis, where both are capable of leading to an earlier onset of neurodegenerative disease (**Figure 1C**). It is suggested that proteostasis capacity declines with age, with this being due to a combination of genetic and environmental factors (Hipp et al., 2019), making efforts into how determining proteostasis is maintained an important part of neurodegenerative disease research.

The key parts of the proteostasis network that are related to protein quality control are molecular chaperones and protein degradation. Molecular chaperone proteins are found throughout cell compartments, with the endoplasmic reticulum (ER) containing many chaperones due to the role it plays in protein synthesis. Protein disulfide isomerases (PDI) are a family of molecular chaperone proteins, localized primarily to the ER, that have oxidoreductase activity, which allows them to reduce and oxidize disulfide bonds in the proteins they are chaperoning (Hatahet and Ruddock, 2009). The important role of PDI's in chaperoning newly synthesized proteins is signified by their growing involvement in several neurodegenerative diseases, including ALS (Perri et al., 2016). Another class of molecular chaperones are heat shock proteins, which act to prevent protein misfolding and aggregation by binding unstable proteins to either aid in their refolding or delivering them to cellular protein degradation machinery (Goldberg, 2003; Hartl et al., 2011; Balchin et al., 2016). Some molecular chaperone proteins are also suggested to aid in the mechanical stabilization of amyloid fibrils by binding along their solvent-exposed surfaces (Shammas et al., 2011; Binger et al., 2013; Cox et al., 2018). Molecular chaperones can even break apart amyloid assemblies (Baughman et al., 2018; Scior et al., 2018), making them easier to degrade or potentially promoting their propagation (Jones and Tuite, 2005). If a protein is terminally misfolded or aggregated, it is delivered to protein degradation machinery, such as the ubiquitin-proteasome system and autophagy-lysosomal system, for proteolytic degradation (Goldberg, 2003; Yerbury et al., 2016). The ubiquitin-proteasome degrades misfolded proteins (Kleiger and Mayor, 2014), but is not capable of dealing with larger aggregates, which are typically degraded, along with damaged cellular components, through autophagy (Dikic and Elazar, 2018).

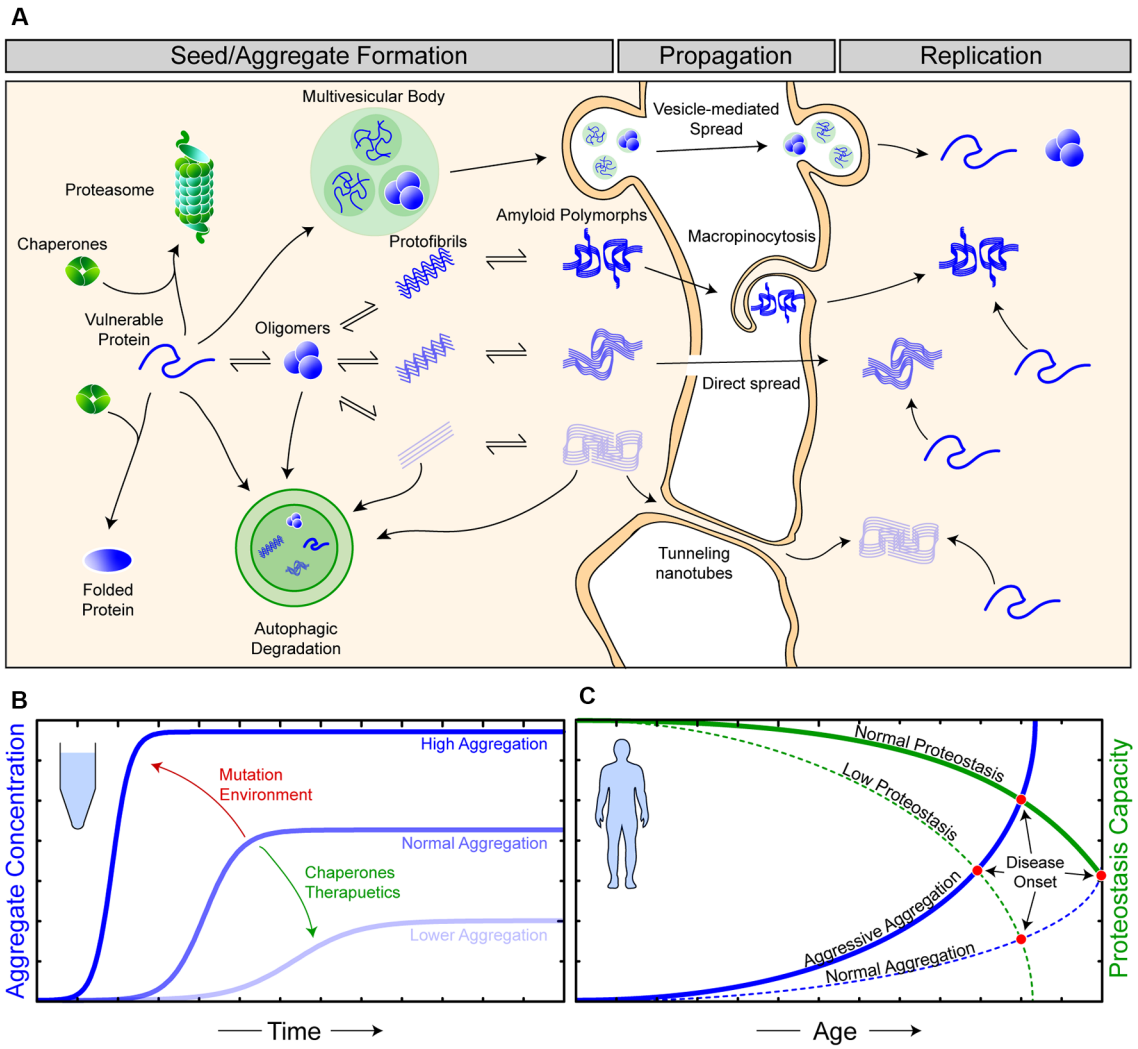

FIGURE 1 | The relationship between proteostasis and prion-like protein propagation. **(A)** The proteostasis network (green objects) is composed of molecular chaperone proteins, degradation pathways (proteasomal and autophagic), and the trafficking of proteins. Chaperones act to protect vulnerable proteins from becoming misfolded and aggregating, potentially through the amyloid pathway (blue). During seed/aggregate formation, proteins vulnerable to amyloid aggregation can form polymorphic assemblies through template-directed growth, eventually, elicit different biological and pathological effects dependent on the polymorphic assembly (strain). Amyloid assemblies are thought to propagate from cell-to-cell through exocytosis in vesicles and exosomes, through membrane breakages, macropinocytosis, and tunneling nanotubes. Once an amyloid assembly has been transferred to a naive cell, replication continues as the amyloid assemblies can now recruit vulnerable protein within this cell. **(B)** *In vitro* experiments have shown that amyloid formation can be augmented *via* changes in environmental conditions or mutations (red) in substrate proteins to become more aggressive. Likewise, the addition of molecular chaperones and/or therapeutics (green), such as small molecules or antibodies, can suppress amyloid aggregation. **(C)** Amyloid aggregation in humans is a stochastic process, occurring over long time scales and, in simplistic terms, is an interplay of proteostasis capacity (green) and protein aggregation propensity (blue). Mutations and/or environmental features can result in both aggressive aggregation and/or a lower proteostasis capacity, ultimately resulting in earlier disease onset in affected individuals.

The relationship between prion-like propagation and the proteostasis network is still being elucidated. Although the proteostasis network acts to suppress protein misfolding and aggregation, some prion-like particles eventually evade these defensive systems. Indeed, some prion-like strains may be resistant to neutralization by proteostasis mechanisms. Over time, these resistant strains could outcompete other strains, leading to the formation of the pathology observed post-mortem. It may well be that the polymorphic amyloid assemblies currently being identified *ex vivo* (Fitzpatrick et al., 2017; Kollmer et al., 2019) represent those that are most capable of inducing disease and, therefore, may present as viable therapeutic targets in the face of the extreme patient heterogeneity observed in neurodegenerative diseases. It is also possible that dynamic prion-like propagation of misfolded proteins may place further stress on the proteostatic machinery, resulting in additional errors or even collapse (Yerbury et al., 2016). A combined understanding of proteostasis and the prion-like mechanisms

involved in some conditions could potentially shed further light on pathogenesis. Therefore, this review focuses on the examination of both proteostasis and prion-like propagation in amyotrophic lateral sclerosis, a disease in which there is strong evidence to suggest a combination of prion-like propagation and dysfunctional proteostasis is occurring simultaneously.

Amyotrophic Lateral Sclerosis—Pathology and Genetics Related to Prion-Like Propagation and Proteostasis

Amyotrophic lateral sclerosis (ALS) is characterized by the progressive degeneration of motor neurons in both the motor cortex, brain stem, and spinal cord (Van Es et al., 2017). Motor symptoms have been observed to occur from a focal point of onset from which proximal motor neurons become affected in an orderly manner (Ravits et al., 2007; Ravits and La Spada, 2009). The site of onset can be highly heterogeneous, resulting in varied symptom presentation, which ultimately leads to difficulty in making a correct diagnosis (Chiò et al., 2009a). Pathology is also thought to begin at a focal point within the CNS and, like other neurodegenerative diseases, is associated with the deposition of proteins into insoluble inclusions. ALS-associated inclusions are found primarily within motor neurons, but can also form in the surrounding oligodendrocytes and astrocytes (Mori et al., 2008; Zhang et al., 2008; Philips et al., 2013; Brettschneider et al., 2014; Fatima et al., 2015) as well as cell populations outside the pyramidal motor system (Geser et al., 2008; Ince et al., 2011; Braak et al., 2013; Brettschneider et al., 2013). Histopathological studies have suggested that the spread of pathology from a focal point occurs through connected cells in stages (Mori et al., 2008; Brettschneider et al., 2013), similar to other neurodegenerative diseases and prion diseases.

The key proteins associated with pathological inclusion formation are transactive response DNA-binding protein 43 (TDP-43; Arai et al., 2006; Neumann et al., 2006), superoxide dismutase-1 (SOD1; Shibata et al., 1993), and fused in sarcoma (FUS; Kwiatkowski et al., 2009; Vance et al., 2009). A significant majority of ALS cases (∼97%) are associated with TDP-43 positive inclusions, where the remainder are associated with either SOD1 (∼2%) or FUS (∼1%) inclusions (Ling et al., 2013). TDP-43 is a DNA/RNA-binding protein that has many functions in RNA metabolism (Buratti et al., 2001, 2005; Hefferon et al., 2004; Mercado et al., 2005). FUS is also a DNA/RNA-binding protein that has functions related to both RNA metabolism (Belly et al., 2005; Fujii and Takumi, 2005; Andersson et al., 2008) and the DNA-damage response (Wang W.-Y. et al., 2013). SOD1 is the primary antioxidant enzyme of the cell and functions to convert superoxide anion to less harmful molecular oxygen or hydrogen peroxide (Mccord and Fridovich, 1969). Importantly, TDP-43, SOD1, and FUS are capable of forming amyloid fibrils *in vitro* (Chattopadhyay et al., 2008; Chen et al., 2010; Nomura et al., 2014), and there is also strong evidence to suggest that, for at least SOD1 and TDP-43, they adopt an amyloid formation *in vivo* (Kato et al., 2000; Bigio et al., 2013; Robinson et al., 2013), although there is not yet a consensus as classical amyloid stains are often negative in ALS cases (Neumann et al., 2006; Kerman et al., 2010).

The most common identified cause of fALS cases is hexanucleotide (G_4C_2) repeat expansions in the *C9ORF72* gene, which result in the transcription of large (tens to thousands of repeats) hexanucleotide repeat RNA molecules and subsequent repeat-associated non-AUG (RAN) translation into dipeptide repeat (DPR) proteins (Freibaum and Taylor, 2017). The DPRs translated from the G_4C_2 repeat RNA include Gly-Arg (GA), Pro-Gly (PG), Pro-Arg (PR), Gly-Ala (GA), and Pro-Ala (PA) polypeptides. These DPRs are thought to elicit different levels of toxicity and react differently within the cell based on their physicochemical properties (Freibaum and Taylor, 2017). For example, PR and GR repeats are suggested to aberrantly interact with RNA and nucleolar proteins, resulting in disruption to ribosomal biogenesis (White et al., 2019). On the other hand, GA repeats have been suggested to bind and trap multiple proteins responsible for the trafficking of biomolecules between the nucleus and cytoplasm (Zhang et al., 2016). Regardless of the effects of the DPRs, the major pathological hallmark of C9ORF72-associated ALS remains TDP-43 mislocalization and aggregation (Cook et al., 2020).

Although TDP-43 mislocalization, phosphorylation, and aggregation in motor neurons is the major pathological hallmark of ALS, few patients carry TDP-43 mutations. Similar to most other neurodegenerative diseases and prion diseases, the majority of ALS cases are sporadic (sALS), accounting for roughly 90% of cases. The remaining cases are familial (fALS) and, although some of the underlying genetic causes remain unidentified, are typically associated with a family history of the disease (Taylor et al., 2016). Only ∼5% of patients with fALS carry TDP-43 mutations, SOD1 accounts for ∼20% of fALS cases, and FUS accounts for ∼5% (Laferriere and Polymenidou, 2015). The identification of hexanucleotide repeats in *C9ORF72* as being ALS-causative was a significant discovery to the field as these mutations account for approximately 40% of fALS cases (Dejesus-Hernandez et al., 2011; Renton et al., 2011). Importantly, over 20 fALS-associated genes have been identified, where these genes are broadly associated with proteostasis mechanisms including protein degradation, protein production (RNA metabolism), and protein trafficking (Taylor et al., 2016; Yerbury et al., 2020). Comprehensive reviews of the genetics and resulting pathomechanisms of ALS have been reviewed elsewhere (Matus et al., 2013; Renton et al., 2014; Carrì et al., 2015; Medinas et al., 2017b; Nguyen et al., 2018; Burk and Pasterkamp, 2019; Mejzini et al., 2019; Vicencio et al., 2020).

A brief example of the complexity of ALS genetics and mechanisms can be found with ubiquilin-2 (UBQLN2). UBQLN2 is an fALS-associated protein responsible for many interactions that facilitate protein degradation *via* the proteasomal and autophagic pathways (Kleijnen et al., 2000; Kim et al., 2008; N'Diaye et al., 2009). As such, pathogenic mutations in ubiquilin-2 can result in dysfunctional protein degradation (Deng et al., 2011; Chang and Monteiro, 2015; Osaka et al., 2016; Renaud et al., 2019). However, UBQLN2 may also play a role in the dissolution of the cytoplasmic ribonucleoprotein complexes known as stress granules (Dao et al., 2018), implicating

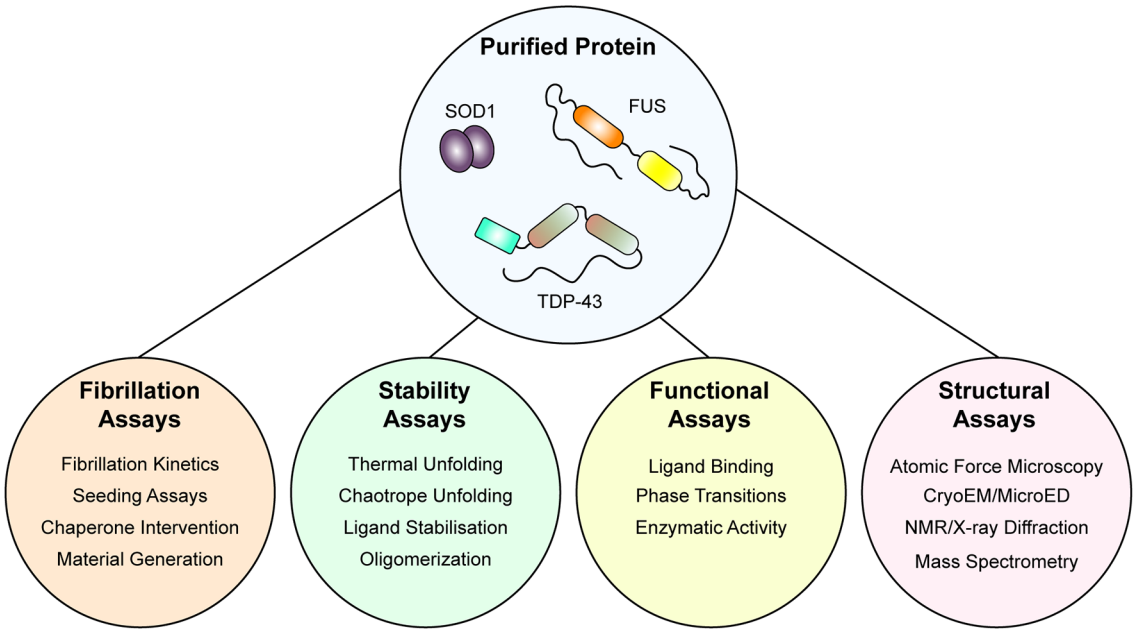

FIGURE 2 | An integrative approach using purified protein to assaying the mechanisms of protein unfolding, aggregation, and prion-like behavior. Once purified, both wild-type and mutant protein is amenable to a suite of assays that can report on folding stability, function, fibrillation, and structure. Application of such techniques has allowed for the determination of the effect of the mutation on protein stability and fibrillation, the role of specific domains in fibrillation, and even the structure of fibrils themselves.

dysfunctional RNA metabolism for UBQLN2 mutants. Furthermore, mutations in genes such as *UBQLN2* may exacerbate the already metastable supersaturated states of TDP-43, SOD1, and FUS (Ciryam et al., 2017), hence, leading to the downstream pathological aggregation of these proteins in particular.

Collectively, the pathological aggregation and prion-like spread of specific proteins as a response to numerous potential mutations in the proteostasis network highlights the critical relationship between proteostasis and prion-like propagation in ALS. As such, researchers have exploited various experimental models to investigate these mechanisms in disease, including the *in vitro* study of purified proteins and cultured cells, as well as *in vivo* in invertebrate models (*Caenorhabditis elegans, Drosophila melanogaster*) and mouse models. For reviews that incorporate zebrafish (*Danio rerio*) models, see Babin et al. (2014), Van Damme et al. (2017), and Morrice et al. (2018). Here, we focus specifically on models that have used either SOD1, TDP-43, or FUS.

IN VITRO MODELS TO EXAMINE PROTEOSTASIS AND PRION-LIKE FEATURES OF AMYOTROPHIC LATERAL SCLEROSIS

Purified Protein Systems

Much of the knowledge that forms the foundation of our understanding of proteostasis collapse, and how this may result in the exponential replication of a prion-like particle in disease, has been supported and initiated by studies of purified protein. The simplistic and reductionist nature of experiments using purified protein affords significant advantages in the specific elucidation of protein structure and function. Through precise control and modulation of the environment (temperature, pH, oxidative/reducing, osmolyte concentration, denaturants) and the proteins (post-translational modification, mutation, binding partners, chaperones) in an assay, a more fundamental understanding of the processes underlying protein misfolding and prion-like propagation can be achieved.

Superoxide Dismutase-1

As a result of being the first protein found to harbor ALS-associated mutations (Rosen et al., 1993), SOD1, in its purified form, has been extensively studied from the perspectives of protein folding, aggregation, and prion-like propagation (McAlary et al., 2019b; Wright et al., 2019; Trist et al., 2020). SOD1 protein is most often expressed and purified using either bacteria (*E. coli*) or yeast (*S. cerevisiae*, Hallewell et al., 1985, 1987). Considering the extensive post-translational modification (PTM) that SOD1 must undergo before reaching its native conformation, which includes metal-binding, disulfide formation, and N-terminal acetylation, yeast are the expression vector of choice as this system is capable of imparting all of these PTMs (Hallewell et al., 1987). A bacterial expression strategy to facilitate correct metal input and disulfide formation is to co-express SOD1 with its co-chaperone the copper chaperone

for SOD1 (CCS) in the presence of excess copper and zinc (Lindberg et al., 2002).

A SOD1 monomer is an 8-stranded β-barrel with two major loops, the metal-binding loop (loop IV) and electrostatic loop (loop VII), being responsible for the coordination of metals or guidance of superoxide substrate to the enzymatic site, respectively. Furthermore, a conserved intramolecular disulfide, that stabilizes the tertiary structure and promotes dimerization, is formed between residues Cys57 and Cys146 (Wright et al., 2019). The native conformation of SOD1 is an impressively stable homodimer, evidenced by resistance to thermal denaturation and proteolytic digestion (Senoo et al., 1988; Rodriguez et al., 2002). For this reason, SOD1 homodimers are sometimes used as a non-aggregating control protein in some assays (Gregory et al., 2017). Although the native conformation of SOD1 is highly stable, the maturation (sequential acquisition of post-translational modifications) folding landscape of SOD1 is highly susceptible to destabilization and off-folding pathways, especially when mutated (Lindberg et al., 2002; Rodriguez et al., 2002; Luchinat et al., 2014, 2017; Sekhar et al., 2015, 2016; McAlary et al., 2016; Culik et al., 2018). Given that ALS-associated mutations in SOD1 were found to elicit a gain-of-toxic function effect (Bruijn et al., 1998), and that protein misfolding and aggregation is a key hallmark of SOD1-associated ALS (Durham et al., 1997; Shinder et al., 2001), much work has focused on the effect that ALS-associated mutations had on SOD1 folding stability (Wright et al., 2019). In particular, studies have focused on dissecting the differences between mutant and wild-type SOD1 across its maturation landscape, with a focus on monomer stability (Lindberg et al., 2005; McAlary et al., 2016) and dimer stability (Lindberg et al., 2005; Redler et al., 2011; McAlary et al., 2013; Capper et al., 2018; Chantadul et al., 2020). Using both wild-type and variants of SOD1 with its free cysteines replaced (Lepock et al., 1990), SOD1 folding and misfolding has been rigorously probed *via* circular dichroism (Lindberg et al., 2002, 2005; Byström et al., 2010; Wright et al., 2013), nuclear magnetic resonance (Arnesano et al., 2004; Sekhar et al., 2015, 2016; Culik et al., 2018), and calorimetry (Rodriguez et al., 2002; Broom et al., 2015, 2016; Anzai et al., 2017).

Some evidence, however, shows that certain ALS-associated mutations, such as D101N, do not significantly alter the folding stability of the SOD1 maturation states that are thought to be likely disease precursors (Byström et al., 2010; Vassall et al., 2011). Indeed, some mutations confound attempts to directly relate folding stability to the variable patient survival observed for patients with different SOD1 mutations (Byström et al., 2010; Vassall et al., 2011; McAlary et al., 2016). More recently, the role of macromolecular crowding in tuning protein folding stability has become clearer. Macromolecular crowding refers to a high concentration of macromolecules (proteins, nucleic acids, lipids, carbohydrates) in solution. Contrary to previous ideas, macromolecular crowding can destabilize proteins that are typically considered as stable when assessed at dilute concentrations (Miklos et al., 2011; Sarkar et al., 2012). SOD1 is no exception to this as evidence is emerging that even wild-type SOD1, in its metal-free and/or reduced form, is destabilized at a physiological temperature under crowded conditions (Bille et al., 2019; Takahashi et al., 2020). Indeed, in-cell nuclear magnetic resonance experiments of SOD1 have revealed that destabilization is common to both wild-type and ALS-associated mutants (Luchinat et al., 2014; Danielsson et al., 2015). In regards to both proteostasis and prion-like propagation, the consensus of this work suggests that ALS-associated mutations can have specific destabilizing effects on SOD1 structure, resulting in a greater proportion of the protein populating an ensemble of non-native states and, therefore, is susceptible to aggregation and prion-like conversion (McAlary et al., 2019b; Wright et al., 2019). Furthermore, it would be interesting to revisit the biophysical studies of the effects of ALS-associated mutations on SOD1 folding stability with our greater understanding of the effect of macromolecular protein crowders. By better understanding the effects of protein crowding on SOD1 stability, we may better understand how specific mutations promote cytotoxic aggregation and the relationship between SOD1 variant stability and patient phenotype.

Similar to the above-mentioned studies of SOD1 folding stability, the examination of the fibrillation of SOD1 has seen extensive use of purified protein. Due to the high stability of SOD1, some assays have induced fibrillation of purified protein using harsh thermal or chemical conditions (DiDonato et al., 2003; Stathopulos et al., 2003); however, the most physiologically relevant assays have simply utilized chelating and reducing conditions to promote SOD1 fibrillation (Chattopadhyay et al., 2008). Typical SOD1 aggregation assays induce the fibrillation of the protein under shaking conditions and measure an increase in β-sheet content using Thioflavin-T (Naiki et al., 1989). Shaking is considered to increase the rate at which fibrils fragment to create new ends from which to polymerize. Although the fibrillation of SOD1 has been described as being mostly fragmentation-assisted *in vitro* and *in vivo* (Lang et al., 2015), a recent study reported the formation of SOD1 fibrils under quiescent conditions (Khan et al., 2017), suggesting alternative pathways by which SOD1 can fibrillate and that certain strains may be preferentially selected depending on the method used. Using the Thioflavin-T assay, it has been shown that even wild-type SOD1, in its demetallated and reduced form, is capable of forming amyloid fibrils (Chattopadhyay et al., 2008; Furukawa et al., 2008; Lang et al., 2012; Chan et al., 2013; Ivanova et al., 2014; Abdolvahabi et al., 2016; McAlary et al., 2016). Furthermore, ALS-associated mutations augment the fibrillation of SOD1 to different degrees (Furukawa et al., 2008, 2010; Lang et al., 2012; Chan et al., 2013; Abdolvahabi et al., 2016, 2017; McAlary et al., 2016), whereas some *de novo* mutations and PTMs have been shown to prevent (Abdolvahabi et al., 2015; Rasouli et al., 2017; Pokrishevsky et al., 2018) or enhance fibrillation (Shi et al., 2013). The Thioflavin-T assay has also been used to examine if SOD1 fibrillation can be lowered using chaperone proteins (Yerbury et al., 2013) or small molecules (Bhatia et al., 2015, 2020: Malik et al., 2019).

N-terminal acetylation of α-synuclein is reported to significantly alter the secondary structure of the protein (Trexler and Rhoades, 2012) and therefore, its aggregation kinetics and structure (Guerrero-Ferreira et al., 2019; Watson

and Lee, 2019). Importantly, many of the studies examining SOD1 fibrillation have utilized bacterially expressed protein, which is not N-terminally acetylated like it is typically found in eukaryotic systems. Although no direct comparisons have been performed between N-terminally acetylated SOD1 and non-acetylated SOD1, there are some notable differences in the identified cores of the amyloid fibril structures produced by the two forms (Furukawa et al., 2010; Chan et al., 2013). There is currently no high-resolution structure of SOD1 fibrils composed of the full-length protein, unlike tau (Fitzpatrick et al., 2017), α-synuclein (Li B. et al., 2018), and amyloid-β (Schmidt et al., 2015). Current high-resolution structures of SOD1-related fibrils are restricted to synthetically produced peptides of small amyloidogenic regions of the protein (Ivanova et al., 2014; Sangwan et al., 2017). The amyloidogenic regions of SOD1 are $_{14}$VQGIINFE$_{21}$, $_{30}$KVWGSIKGL$_{38}$, $_{101}$DSVISLS$_{107}$, and $_{147}$GVIGIAQ$_{153}$ (Ivanova et al., 2014). However, limited proteolysis of fibrils produced from recombinant SOD1 has identified the N-terminus of the protein, comprising residues 1–63, as the potential amyloidogenic core (Furukawa et al., 2010; Chan et al., 2013). Other regions of the SOD1 molecule have also been detected, suggesting polymorphism in the fibril structures generated. It would be valuable to determine the structure of SOD1 fibril polymorphs to understand better the relationship between SOD1 aggregation and the prion-like spread observed in patients.

Protein fibrillation assays have been a key tool in understanding the underlying prion-like propagation of SOD1 aggregation. The presence of seed in fibrillation assays essentially bypasses the need for the nucleation of a fibrillation-competent protein assembly in the assay, thus shifting the kinetics almost entirely to fibril elongation (Cohen et al., 2011). Through the addition of preformed protein aggregates (from recombinant sources, cells, or whole organisms) to fibrillation assays, it can be elucidated whether or not the seed is present within a sample (Schmitz et al., 2016), the susceptibility of protein variants to seeded fibrillation (Pokrishevsky et al., 2018), or the ability of different treatments to prevent seeded fibrillation (Sievers et al., 2011). Indeed, the first evidence that SOD1 may have prion-like properties first came from fibrillation assays of recombinant purified SOD1 seeded with spinal cord extracts from mice overexpressing human SOD1-G93A (Chia et al., 2010). From seeded fibrillation assays, it has been determined that loss of the intramolecular disulfide is a significant contributor to increasing the susceptibility of SOD1 to undergo seeded fibrillation (Chattopadhyay et al., 2015) and that this is enhanced by mutation. Other studies examining the seeded fibrillation of SOD1 have shown that some *de novo* mutants of SOD1 are resistant to seed-induced fibrillation (Pokrishevsky et al., 2018), and that specific amyloidogenic segments of SOD1 contribute more significantly to seeded aggregation (Ivanova et al., 2014).

Overall, it is clear that ALS-associated mutations in SOD1 lower the folding stability of the protein and permit the sampling of more aggregation-prone conformations. These destabilized conformations are capable of both nucleating the formation of amyloid fibrils and acting as a substrate for the further polymerization of existing fibrils. Future inquiries in this area focusing on the elucidation of the structure and polymorphism of SOD1 fibrils would be useful to not only understand the fibrillation of SOD1 but also to design therapies that may prevent it. Besides, advances in the sensitivity of detecting fibrillar species with prion-like properties from clinical sources using fibrillation assays (also called the real-time quaking-induced conversion assay; Schmitz et al., 2016) is currently unexplored for SOD1-associated ALS and ALS in general. Application of the sensitivity of the Thioflavin-T-based fibrillation assays to ALS patient tissue would be invaluable in elucidating the relationship between patient prognosis and the prion-like nature of the disorder.

Transactive Response DNA-Binding Protein 43

There has been less research performed utilizing recombinant purified TDP-43, which is primarily due to the difficulty in purifying soluble TDP-43, and the difficulty handling the purified product. Some advances have recently been made in this space with the usage of solubility tags, replacement of tryptophan residues with alanine, and exhaustive processing of recombinant protein extracts (Li et al., 2017; Vivoli Vega et al., 2019; Wright et al., 2020). Owing to the difficulty associated with purification of the full-length protein, the biophysical understanding of TDP-43 has been obtained predominantly by analyses of purified single domains of the protein, including the N-terminal domain (NTD; Kuo et al., 2009; Mompeán et al., 2016), RNA-recognition motifs (RRM1 and RRM2; Kuo et al., 2009; Lukavsky et al., 2013), and the C-terminal low-complexity domain (LCD; Conicella et al., 2016). The intrinsically high aggregation propensity of the LCD is likely why the examination of full-length TDP-43 has been difficult, as other domains are reported to be soluble when expressed in heterologous systems (Kuo et al., 2009; Lukavsky et al., 2013; Mompeán et al., 2016). As well as affording TDP-43 a high propensity to aggregate, the LCD is also the domain that contains the majority of ALS/FTLD-associated mutations (Abel et al., 2013) and forms the major component by mass of the ALS/FTLD-associated fragments of TDP-43 (Neumann et al., 2006). As such, the LCD has been a primary focus of TDP-43-focused ALS/FTLD research.

The TDP-43 LCD is defined as a prion-like domain due to its enrichment in asparagine, glutamine, glycine, and tyrosine residues, making it similar in sequence composition and physicochemical properties to the yeast prion protein Sup35 (King et al., 2012). Recent studies have highlighted the capability of low-complexity domains in DNA/RNA-binding proteins (not all of them prion-like) to facilitate the formation of physiologically relevant biomolecular condensates through the process of liquid-liquid phase separation (LLPS) *in vitro* (Banani et al., 2017). Evidence for *in vivo* formation and dysfunction of biomolecular condensates is starting to emerge (Zhang et al., 2020). The TDP-43 LCD has been shown to undergo LLPS, with this being primarily mediated by a transiently-populated α-helix (residues 321–330; Conicella et al., 2016) as well as aromatic residues spread throughout the domain (Li H.-R. et al., 2018). The majority of ALS/FTLD-associated

mutations in TDP-43 are localized to the LCD. Considering this, a recent effort has been applied to examining the role of biomolecular condensates, such as stress granules, as a potential site in which the initial aggregation of TDP-43 (and other RNA-binding proteins) may occur (reviewed in Nedelsky and Taylor, 2019). *In vitro* evidence suggests that LLPS of the TDP-43 LCD promotes amyloid fibril formation, likely at the condensate-solution interface (Babinchak et al., 2019) however, whether these fibrils are formed within condensates remains unclear. It is thought that disease-associated mutations may exacerbate the gradual loss of internal molecular rearrangement within condensates and promote a liquid-to-solid transition of the protein components, although this hypothesis is currently not fully tested, especially in regards to amyloid formation, and requires further investigation in both protein only assays and living cells.

Although the relationship between TDP-43 LCD LLPS and aggregation is not well understood, the capacity of the TDP-43 LCD, and smaller segments of it, to form amyloid fibrils *in vitro* is well-established (Chen et al., 2010; Guo et al., 2011; Jiang et al., 2013, 2016; Mompeán et al., 2014; Sun C.-S. et al., 2014; Zhu et al., 2014; Babinchak et al., 2019; Cao et al., 2019). Cross-β fibers are formed by several small segments of the TDP-43 LCD (Guenther et al., 2018a) which may comprise the cores of amyloid polymorphs formed by larger segments and the entire LCD itself (Chen et al., 2010; Guo et al., 2011; Jiang et al., 2013, 2016; Mompeán et al., 2014; Babinchak et al., 2019; Cao et al., 2019; Shenoy et al., 2020). Cryo-electron microscopy has been utilized to examine the polymorphic nature of synthetic TDP-43 LCD segment assemblies composed of amino acid residues 311–360 or 286–331 (Cao et al., 2019). Although these structures are the result of *in vitro* assays of LCD segments, they provide a starting point by which to understand the amyloid state of TDP-43 and how it might confer different pathogenic severities in patients. Indeed, it is clear that TDP-43 can adopt an amyloid-like state in relevant tissues from ALS and FTLD patient samples as shown by immunoelectron microscopy (Lin and Dickson, 2008; Thorpe et al., 2008) and Thioflavin-S staining of carefully processed samples (Bigio et al., 2013; Robinson et al., 2013). It is important to note that confirmation of amyloid in tissue samples has classically relied upon Thioflavin-S and Congo-red staining, however, evidence has emerged that in some cases protein fibrils may not effectively bind these dyes (Wang Y.-T. et al., 2013; Rasouli et al., 2017), making electron microscopy the tool of choice to definitively assess aggregate morphology (amorphous or fibrillar).

Unlike the LCD, the TDP-43 NTD is a well-structured and stable domain (Qin et al., 2014; Mompeán et al., 2016) that is capable of undergoing extensive multimerization (Chang et al., 2012) and polymerization (Afroz et al., 2017; Mompeán et al., 2017; Tsoi et al., 2017; Wang et al., 2018) *in vitro*. These biophysical studies, using measures of stability, protein-protein interactions, and structural techniques, have suggested several roles for the NTD in both TDP-43 function and pathology. Namely, the NTD promotes the formation of TDP-43 dimers and multimers, likely through head-to-tail interactions (Afroz et al., 2017; Wang et al., 2018; Wright et al., 2020; although there is a dispute about the contact interfaces). The NTD-mediated interaction of separate TDP-43 molecules may prevent the interaction of the aggregation-prone LCDs between interacting TDP-43 molecules (Afroz et al., 2017), suggesting a protective role for the NTD in ALS/FTLD. This notion of NTD-derived protection is supported by the cleavage of the NTD to give rise to C-terminal pathological fragments of TDP-43 in ALS/FTLD (reviewed in Berning and Walker, 2019). In contrast, others have suggested that NTD-mediated interactions promote the association and subsequent aggregation of the TDP-43 LCD (Tsoi et al., 2017).

Similar to the NTD and LCD, the biophysical study of the TDP-43 RRMs has resulted in furthering the understanding of how TDP-43 may gain pathogenic properties through misfolding. A key finding from biophysical analyses of purified RRM domains or full-length TDP-43 is that DNA/RNA-binding to the RRMs increases their stability and decreases the propensity of TDP-43 to aggregate (Huang et al., 2013; Sun Y. et al., 2014; Zacco et al., 2018, 2019). This information highlights the importance of the RRMs in understanding TDP-43 proteinopathy and also provides a means for which potential therapies may be explored by using DNA/RNA oligos to stabilize cytoplasmic TDP-43, a strategy that is currently being pursued (Mann et al., 2019). However, some care must be taken, as the disease-associated D169G mutation that occurs within RRM1 of TDP-43 has been shown to increase the stability of the domain but make TDP-43 more susceptible to caspase-mediated cleavage (Chiang et al., 2016). It is currently unknown what effect RNA/DNA-binding has on the proteolytic cleavage of TDP-43. RRM2 of TDP-43 has also been examined concerning its potential amyloidogenic properties, with data suggesting that it may play an active role in TDP-43 aggregation and amyloid formation. Most studies of the amyloidogenic properties of TDP-43 RRM2 have used small synthetic peptides of the domain (Saini and Chauhan, 2011, 2014; Wang Y.-T. et al., 2013; Shimonaka et al., 2016; Guenther et al., 2018b), but some work has investigated the ability of the entire RRM2 domain to form amyloid (Shimonaka et al., 2016; Garnier et al., 2017).

Collectively, this evidence supports a role for RRM2 in the fibrillation of TDP-43, however, there are still unanswered questions. The use of small peptides, whilst informative, is not suitable for the examination of the heterotypic interactions that typically underlie the formation of diverse fibril polymorphs (Fitzpatrick et al., 2017; Li B. et al., 2018; Guerrero-Ferreira et al., 2019; Kollmer et al., 2019). Considering the number of potential amyloidogenic regions proposed in the TDP-43 LCD (Guenther et al., 2018a), and potential amyloid properties of RRM2, it may well be that the number of possible fibril polymorphs formed by heterotypic interactions within TDP-43 is substantial. Furthermore, the disease-associated truncation of TDP-43 into C-terminal fragments may further diversify the amyloid structures that it can form. This being said, small peptides do give the advantage of allowing for the examination of the seeding properties of highly structurally pure amyloid samples. This has been demonstrated by using fibrils formed from small segments of the TDP-43 LCD to

seed TDP-43 aggregation in cultured overexpressing TDP-43 transgenes (Shimonaka et al., 2016).

Fused in Sarcoma

Like TDP-43, purified FUS protein has not been as well explored compared to studies of purified SOD1. The N-terminus of FUS is a functional prion-like domain, followed by several RGG rich regions encompassing a single RRM, a zinc finger domain, and finally a nuclear localization signal at the far C-terminus (Iko et al., 2004). Most purification strategies involve the expression of FUS in bacteria as a fusion to tags (sometimes fluorescent and/or solubilizing) on either the N- or C-terminus (Han et al., 2012; Kato et al., 2012; Kwon et al., 2013; Nomura et al., 2014; Patel et al., 2015; Monahan et al., 2017; Alberti et al., 2018; Hofweber et al., 2018; Maharana et al., 2018; Qamar et al., 2018; Murthy et al., 2019).

Also similar to TDP-43, a focus has been placed on the ability of FUS to undergo LLPS to form biomolecular condensates or hydrogels (Han et al., 2012; Kato et al., 2012; Kwon et al., 2013; Patel et al., 2015; Monahan et al., 2017; Alberti et al., 2018; Hofweber et al., 2018; Maharana et al., 2018; Qamar et al., 2018; Murthy et al., 2019). The ability of FUS to undergo LLPS *in vitro* can be enhanced by the ALS-associated G156E mutation (Patel et al., 2015), or it can be suppressed by chaperones and/or post-translational modifications (Monahan et al., 2017; Hofweber et al., 2018; Qamar et al., 2018). Concerning proteostasis, these findings suggest that the aberrant condensation of FUS, or simply condensation of FUS disease mutants, may result in the formation of FUS aggregates in disease. Indeed, FUS condensates are suggested to solidify (mature) over time and also perhaps form larger fibrous structures (Patel et al., 2015), although some work has suggested that careful control of *in vitro* conditions (removal of bubbles, usage of unscratched surfaces) prevents excessive FUS fibril formation in condensation experiments *in vitro* (Alberti et al., 2018).

Indeed, current understanding of ALS as a prion-like disorder is supported strongly by experimentation of both SOD1 and TDP-43, however, experimentation with FUS is in early stages (McAlary et al., 2019b). Experiments examining only smaller regions of FUS, including the RGG-rich regions and the N-terminal prion-like domain, have established the ability of FUS to form labile amyloid-like fibers, distinguished from typical amyloid by their lower thermal stability (Kato et al., 2012; Murray et al., 2017; Hughes et al., 2018). Also, initial experiments examining the amyloidogenicity of purified FUS found that GST-tagged full-length FUS protein showed very rapid increases in solution turbidity, suggesting that LLPS was potentially occurring here rather than aggregation (Nomura et al., 2014). Nomura et al. (2014) also show GST-tagged FUS-G156E samples were Thioflavin-T positive and that fibrils were present by electron microscopy, but it is unclear if these were labile amyloid-like structures or *bona fide* highly stable amyloid. In the future, experiments designed to examine if a population of the amyloid assemblies formed by purified FUS (whether through LLPS or simple agitation assays) is resistant to detergent denaturation and proteolytic degradation would be useful to determine if purified FUS can form pathogenic amyloids. For a visual summary of the assays that can be performed with purified protein, see **Figure 2**.

Cultured Cells

Although studies utilizing purified protein have provided substantial insight into the misfolding, chaperoning, and amyloid aggregation of ALS-associated proteins, there is a significant lack of biological complexity in these systems. Cultured cells, in the form of immortalized cell lines, have formed a foundation for understanding proteostasis and prion-like propagation in ALS/FTLD. The types of systems used are diverse in their design and application, so we have elicited to focus primarily on those systems that have best helped understand protein homeostasis and prion-like propagation within the cellular environment (reviews on iPSC culture modeling of ALS see Guo et al., 2017; Van Damme et al., 2017).

Standard cell lines in any biological experimentation involving ALS/FTLD often include human embryonic kidney cells (HEKs—often various clones), HeLa cells, SH-SY5Y cells, Neuro-2a, and NSC-34, however, other cell lines are used too. NSC-34 cells are a hybrid cell line generated from a fusion of murine neuroblastoma cells and murine embryonic spinal cord enriched in motor neurons (Cashman et al., 1992), making them a particularly useful immortalized model for studying ALS/FTLD.

Chosen cell lines are often genetically manipulated to express mutant forms of ALS/FTLD-associated proteins *via* transient transfection methods. This has several advantages over examining endogenous proteins as it allows for the delivery of mutant genes, fusion constructs, and overexpression. A common strategy involves the use of fluorescently tagged SOD1 mutants (Turner et al., 2005; Prudencio et al., 2009; Stevens et al., 2010; Münch et al., 2011; Polling et al., 2014; Farrawell et al., 2015, 2019; McAlary et al., 2016; Ayers et al., 2017; Pokrishevsky et al., 2017, 2018; Zhong et al., 2017; Crosby et al., 2018), TDP-43 mutants and fragments (Zhang et al., 2009; Yang et al., 2010; Walker et al., 2013; Farrawell et al., 2015; Zeineddine et al., 2017; Chen and Cohen, 2019; Chen et al., 2019; Sackmann et al., 2020), and FUS (Mastrocola et al., 2013; Britton et al., 2014; Yang et al., 2014; Farrawell et al., 2015; Patel et al., 2015; Bogaert et al., 2018; Maharana et al., 2018). Genetically encoded fluorophore tags, such as green fluorescent protein (GFP), are a highly useful method to specifically interrogate a protein of interest in cells (Day and Davidson, 2009), and have formed an important part of understanding the dynamics of ALS/FTLD mutant proteins in the physiologic environment.

At least for SOD1, there is good evidence to suggest that the fusion of GFP to it's C-terminus does not significantly augment its ability to fold or dimerize (Stevens et al., 2010). Folding or dimerization of tagged TDP-43 or FUS has not been assessed; however, fluorescent protein-tagged versions of either of these proteins appear to localize correctly in cells, suggesting that tagging is not overly detrimental. Some researchers use smaller non-fluorescent affinity tags to overcome non-physiological interactions induced by fluorescent protein

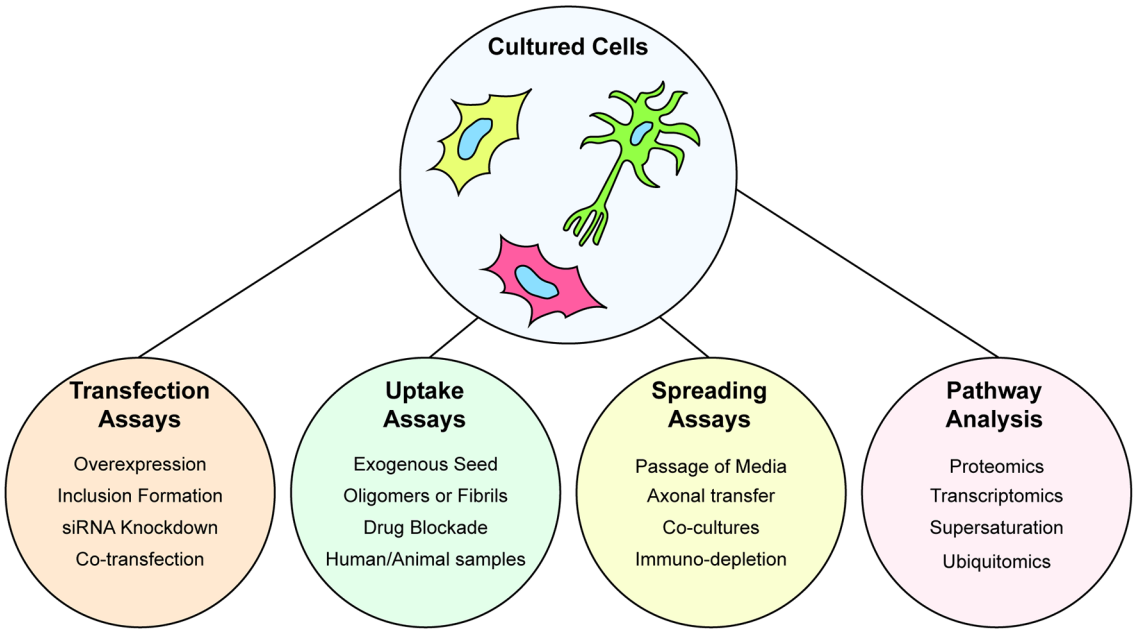

FIGURE 3 | Understanding protein aggregation and prion-like spread using cultured cells. Cultured cells are amenable to genetic manipulation *via* transient or stable transfection, or gene editing to express fluorescently tagged wild-type or mutant forms of proteins. Cultured cells are used to study the ability of cells to uptake preformed protein aggregates in the form of purified aggregates, aggregates from other cultures, or extracts from organisms. Spreading of prion-like particles can also be assayed by serial passage of conditioned media, axonal spread through the use of microfluidic co-cultures. Furthermore, cultured cells are amenable to multiple types of omics that can provide information on wide-scale alterations to the proteome or transcriptome.

tags. Another advantage associated with fluorescent protein tagging of aggregation-prone proteins is that the localization of proteins into inclusions can sometimes mask epitopes detected by commercial antibodies (Prudencio and Borchelt, 2011). Since the fluorescent protein itself is incorporated into protein inclusions when tagged to aggregation-prone proteins, it is much easier to ascertain the presence of inclusions in cell populations using this system. One can then effectively utilize fluorescence microscopy or flow cytometry to detect the presence of fluorescent protein inclusions within cells (Ramdzan et al., 2012), or from cell extracts (Whiten et al., 2016; Zeineddine et al., 2017).

An advantage of fluorescent tagging of aggregation-prone proteins in cells is the capability to perform biophysical analyses by exploiting the photophysical properties of the fluorophore itself, such as fluorescence recovery after photobleaching (FRAP; Axelrod et al., 1976) or fluorescence loss in photobleaching (Lippincott-Schwartz et al., 2003). A major disadvantage of immunodetection using either affinity epitopes or endogenous epitopes is that this requires the fixation of cells, making it challenging or impossible to analyze dynamic events. Combined usage of both fluorescent tagging and FRAP has revealed that specific aggregation-prone proteins can localize to distinct inclusion types (Kaganovich et al., 2008) and that the mobility of SOD1, TDP-43, and FUS in and outside inclusions and other compartments can be examined using photobleaching (Matsumoto et al., 2005, 2006; Weisberg et al., 2012; Farrawell et al., 2015, 2018; Park et al., 2017).

Key findings from the usage of fluorescently tagged SOD1 in immortalized cell lines related to protein homeostasis include that: (1) disease-associated mutant forms of SOD1 are significantly more aggregation-prone than wild-type SOD1 (Prudencio et al., 2009; Prudencio and Borchelt, 2011; McAlary et al., 2016; Ayers et al., 2017; Crosby et al., 2018; Pokrishevsky et al., 2018; Farrawell et al., 2019; Crown et al., 2020); (2) SOD1 mutants are a target of ubiquitin proteasome degradation and partition exclusively to the juxtanuclear quality control compartment that triages proteins for proteasomal degradation (Matsumoto et al., 2005, 2006; Weisberg et al., 2012; Farrawell et al., 2015; Ayers et al., 2017; Park et al., 2017); and (3) the aggregation and associated toxicity of SOD1 mutants can be alleviated *via* overexpression of components of the cellular quality control machinery or small molecules (Walker et al., 2010; Xia et al., 2011; Park et al., 2017; Pokrishevsky et al., 2017, 2018; Farrawell et al., 2019). For TDP-43 and FUS, fluorescent tagging has been similarly successful, especially when examining the dynamics of FUS and TDP-43 biomolecular condensates (Patel et al., 2015; Maharana et al., 2018; Qamar et al., 2018; Gasset-Rosa et al., 2019; Mann et al., 2019). Considering the growing understanding of the number of diverse and transient structures formed in cells (Nedelsky and Taylor, 2019), experiments investigating protein aggregation in cells must be carefully interpreted when examining fixed samples. What is thought to be an aggregate may simply be a transient structure in some cases.

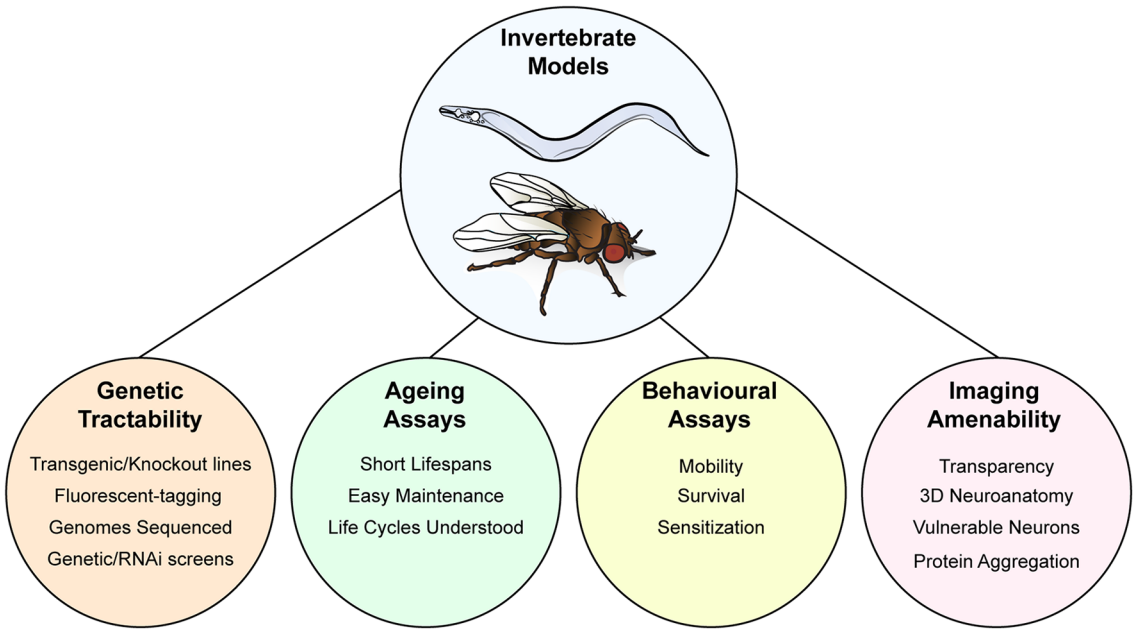

FIGURE 4 | Invertebrate models of protein aggregation disorders. Invertebrate models are cheap and efficient systems that are highly amenable to genetic manipulation through the delivery of transgenes in the forms of human genes or fluorescent proteins, as well as knockout of endogenous genes. The short lifespan of these models makes them a powerful tool for assaying development and aging. Behavioral analysis can be performed to determine the effect of protein aggregation in these models, linking observed pathology to clinical phenotypes. Last, these models are highly accessible to imaging and provide access to well-understood neuroanatomy to assay prion-like spread.

Immortalized cell lines are a highly useful model to examine the prion-like propagation of protein misfolding and aggregation in cells, particularly when paired with purified protein samples. Indeed, a combination of transiently expressed GFP-tagged SOD1 variants, and *in vitro* aggregated purified SOD1 protein was utilized to determine the prion-like properties of SOD1 aggregates (Münch et al., 2011). The ability to use exogenous aggregates that can be pure amyloidogenic peptides from a protein, or have the amyloidogenic region(s) deleted, is a powerful assay for understanding the structure-function relationship of prion-like proteins. As such, this system has been used to examine the seeding of GFP-tagged TDP-43 by various exogenously added TDP-43 peptide-derived amyloid fibrils (Shimonaka et al., 2016) or fibrils composed of full-length TDP-43 (Furukawa et al., 2011). The power of assays that utilize transgene expression of proteins in cultured cells is exemplified by the ability to seed aggregation of the reporter proteins *via* the addition of samples from human or animal *in vivo* sources (Nonaka et al., 2013; Pokrishevsky et al., 2017; Porta et al., 2018; Laferrière et al., 2019). Furthermore, the capability of prion-like proteins to replicate and infect can be easily examined in cells by the simple passage of the conditioned media to new naive cultures as has been shown for prion-like SOD1 conformations (Grad et al., 2014). This spread of protein aggregates between cultured cells can be examined by using dual fluorescent reporter, or split reporter, strategies. In these assays, two separate populations of cells are respectively transfected with prion-like proteins with different color fluorescent tags or split fluorescent/luminescence tags. Mixing of the separate cell populations and subsequent co-culturing allows for protein transfer between the populations which can be detected using fluorescence microscopy, flow cytometry, or a plate reader (Feiler et al., 2015; Zeineddine et al., 2017; Sackmann et al., 2020). For a visual summary of the assays that can be performed with cultured cells, see **Figure 3**.

To complement tractable and scalable cell-based experiments, animal experiments are required to examine the effect of prion-like protein aggregation on physiology and disease pathogenesis.

INVERTEBRATE MODELS OF ALS

The genetic power and experimental tractability of small animal models (worm, fly, and fish) provide unique opportunities to investigate the pathological role of ALS-associated protein aggregates. Here we will discuss models for neurodegeneration using *Caenorhabditis elegans* and *Drosophila melanogaster*, focusing on insights gained on pathological mechanisms of SOD1, TDP-43, and FUS concerning proteostasis mechanisms and prion-like propagation.

Caenorhabditis elegans

The nematode (roundworm) *C. elegans* has been used for decades in the study of neuronal development (Sulston et al., 1983), anatomy (White et al., 1986), and cellular/molecular function (Hobert, 2013; Walker et al., 2019). This extensive study has

led to *C. elegans* becoming one of the most well-characterized experimental nervous systems, being the only adult animal for which the synaptic connectivity of all its ∼300 neurons is known (Cook et al., 2019). More recently, the worm model has become increasingly popular for studies of neurodegeneration and pathology, in part due to its short lifespan (2–3 weeks), genetic tractability, transparent nature, amenability to high-throughput genetic or pharmacological screens (Kaletta and Hengartner, 2006), and the fact that >80% of the *C. elegans* proteome has human homologs (Lai et al., 2000). In particular, the genetic accessibility of *C. elegans* permits the rapid generation of transgenic lines (within 1–2 weeks) with expression restricted to specific cells or tissues, including the remarkable ability to express transgenes in individual neurons. Also, the transparent nature of *C. elegans* enables imaging of fluorescence-tagged proteins in specific tissues/cells within a whole organism (Heppert et al., 2016), which has been invaluable in making mechanistic links between protein aggregation and behavioral deficits. Moreover, the short lifespan of *C. elegans* facilitates aging experiments to be performed rapidly and cost-effectively.

In this section, we will focus on studies that use *C. elegans* transgenic lines to investigate the mechanisms through which ALS-associated mutations in SOD1, TDP-43, and FUS contribute to protein aggregation, neurodegeneration, and behavioral defects concerning proteostasis mechanisms and prion-like propagation. Other aspects of ALS (and other neurodegenerative disease) pathology are covered in detail in reviews elsewhere (Dimitriadi and Hart, 2010; Li and Le, 2013; Therrien and Parker, 2014).

Superoxide Dismutase-1

Most studies of SOD1 using *C. elegans* involve the transgenic expression of human wild-type (WT) or mutant SOD1 (hSOD1) tagged with fluorescent proteins to facilitate visualization of protein localization (i.e., whether diffuse in the cell or aggregated). These studies have shown that expression of disease-associated mutant forms of SOD1 (usually G85R, G93A) is associated with increased aggregation and behavioral deficits. The formation of aggregates during aging is correlated with defects in the proteostasis network (Hipp et al., 2019). For example, expression of hSOD1-G85R in all neurons resulted in locomotor deficits and inclusion-like structures of hSOD1-G85R-YFP (yellow fluorescent protein) in the cell bodies of motor neurons along the ventral nerve cord, observed *via* fluorescence microscopy and electron microscopy (Wang et al., 2009a). These observations correlated with reduced numbers, fluorescence intensity, and motility of pre-synaptic markers such as GFP-tagged synaptobrevin. Interestingly, despite hSOD1-YFP fluorescence being observed in most neurons, some individual neurons did not display aggregates at the fourth larval stage (the last larval stage before the adult moult; Wang et al., 2009a), although later time points were not analyzed in this study. This indicates that there are neuron-subtype-specific differences in how misfolding-prone SOD1 is sequestered in individual cells. Interestingly, expression of human SOD1 (hSOD1) mutants G85R, G93A, and G127X in *C. elegans* muscle showed limited toxic effects (after assaying motility, myofilament organization, muscle development, and egg-laying) despite all hSOD1(mutant) lines showing clear aggregate formation (Gidalevitz et al., 2009).

Wang et al. (2009a) used their hSOD1 *C. elegans* model to perform a genome-wide RNAi screen for genes that when knocked down led to an increase in fluorescence aggregates; these genes were therefore postulated to normally suppress or delay aggregation formation. Eighty-one hits were identified, of which 27.2% were factors involved in proteostasis (protein chaperones, misfolding, and turnover) including heat shock factor 1 (HSF1), Hsp110, a DnaJ family member A2 (DNJ-19), an Hsp70 family member (STC-1), E3 ubiquitin ligase components SEL-10 and RBX-1, and proteins involved in SUMOylation [small ubiquitin-like modifiers (SUMOs)] UBC-9 and GEI-17. Several of these hits were validated through genetic crosses (see Wang et al., 2009a for details).

Later studies examining the effects of transgenic hSOD1-G93A expression in GABAergic motor neurons of the ventral nerve cord (using the *unc-25* promoter) confirmed earlier findings, showing that expression of GFP-tagged hSOD1-G93A is correlated with reduced locomotion and the formation of large aggregates (Li et al., 2013, 2014). Interestingly, critical defects were observed to a similar extent in hSOD1-WT and hSOD1-G93A transgenic lines, such as age-dependent locomotor dysfunction and motor neuron degeneration; hSOD1-WT also formed aggregates in motor neurons—albeit to a lesser extent than hSOD1-G93A (Li et al., 2014). As toxicity associated with $hSOD1^{WT}$ expression was not observed in the hSOD1 pan-neuronal lines (Wang et al., 2009a), these authors suggested that motor neurons may be more susceptible to hSOD1-linked toxicity, or that their transgene expression levels were simply higher compared with pan-neuronal lines. In both $hSOD1^{WT}$ and hSOD1-G93A lines, cell-autonomous over-expression of key autophagy initiator UNC-51 partially alleviated axon guidance defects observed with hSOD1 expression (Li et al., 2014). UNC-51 is required for proper localization of the Netrin receptor UNC-5, a critical player in axon guidance (Ogura and Goshima, 2006), which may explain its involvement in this phenotype—although a role specifically in autophagy has not yet been explored.

Conventional microinjection protocols for generating transgenic *C. elegans* result in the formation of multi-copy transgene arrays (Mello et al., 1991), which express at high levels. Over-expression of hSOD1 has been linked to deleterious effects even for wild-type hSOD1 in *C. elegans* (Li et al., 2014). This makes it challenging to differentiate the effects of protein overexpression from that of ALS mutations, or to identify interventions that address one or the other. To overcome this issue, Baskoylu et al. (2018) generated single-copy insertions of *C. elegans* wild-type SOD-1 and A4V, H71Y, and G85R mutants (by comparison of *C. elegans* and human SOD1 sequences) *via* Mos1-mediated single copy insertion (MosSCI). Toxic effects were more subtle in MosSCI transgenic lines compared with conventional multi-copy arrays expressing hSOD1 (Baskoylu et al., 2018). One of the most pronounced effects was the formation of $hSOD1^{WT}$-YFP inclusions when this transgene was co-expressed in the

same neurons expressing single-copy insertions of mutant *C. elegans* SOD-1 (ceSOD1): single-copy insertions of mutant ceSOD1 showed significantly higher hSOD1^{WT}-YFP inclusions compared with ceSOD1^{WT} or the empty vector control. The authors theorize that the hSOD1^{WT}-YFP inclusions were possibly seeded by misfolded mutant *C. elegans* SOD1 protein in MosSCI models, suggesting a prion-like propagation effect (Baskoylu et al., 2018).

Mislocalization of SOD1 to the nucleus is linked to toxicity (Gertz et al., 2012). Mutations introduced in SOD1 to disrupt the consensus Nuclear Export Signal (NES) showed nuclear localization of GFP/YFP-tagged SOD1 in cultured cells and *C. elegans* neurons (Gertz et al., 2012). Although the NES-disrupting mutation L42Q by itself did not cause severe lifespan defects, in combination with the G85R mutation, expression of hSOD1$^{L42Q/G85R}$ in *C. elegans* neurons led to significantly reduced lifespan compared with hSOD1^{WT} controls (Zhong et al., 2017).

Transactive Response DNA-Binding Protein 43

C. elegans is an excellent model system to assess prion-like spreading due to its well-defined, stereotyped anatomy and transparent nature, enabling the visualization of fluorescently tagged proteins across multiple tissues in a living organism (Nussbaum-Krammer and Morimoto, 2014). Like for SOD1, numerous studies have used overexpression of wild-type or ALS-linked variants of human TDP-43 (hTDP-43) to model aspects of pathology in *C. elegans*. Pan-neuronal expression of YFP-tagged hTDP-43(WT), ALS-associated mutant forms (Q331K and M337V), or the C-terminal 25 kDa fragment (hTDP-C25, residues 219–414) led to severe locomotor defects (Zhang et al., 2011). Analogous to human pathological data (Arai et al., 2006; Neumann et al., 2006), hTDP-C25 was found predominantly in the insoluble fraction, suggesting a high propensity for aggregation. Since an elevated temperature was linked to increased accumulation of hTDP-C25-YFP in both soluble and insoluble fractions, Zhang et al., 2011 used RNAi to knockdown components of the protein quality control network, finding that HSF1 was a major protective factor in toxicity in hTDP-43 protein aggregation. *HSF-1* partial loss-of-function mutations enhanced the locomotor defects observed in hTDP-C25-YFP transgenic lines. Besides, the insulin-like/IGF1 signaling (IIS) receptor DAF-2, which requires HSF1 for pro-longevity effects (Morley and Morimoto, 2004), also modulates the toxicity of hTDP43. *daf-2* mutants live twice as long as WT animals (Kenyon et al., 1993), and genetic crosses between *daf-2(-)* and hTDP-43 transgenic lines improved locomotion and reduced hTDP-43-YFP aggregation (Zhang et al., 2011). These data suggest that molecular chaperones and protein quality control play an important role in attenuating hTDP43 aggregation and related behavioral deficits.

Neuronal expression of untagged wild-type and mutant (G290A, A315T, and M337V) hTDP-43 also showed similar locomotion defects, with some evidence of aggregate formation in the nucleus of *C. elegans* neurons (assayed *via* immunostaining) in all transgenic lines (Liachko et al., 2010). Although both WT and mutant hTDP-43 lines showed reduced locomotion on day one of the assays, this was more severe in ALS-linked mutant lines and deteriorated more rapidly in hTDP-43(mutant) lines with increasing age. hTDP-43(mutant) lines also showed degeneration of GABAergic motor neurons, whereas hTDP-43^{WT} animals did not show significant differences compared to non-transgenic controls (Liachko et al., 2010). The authors also show that phosphorylation at S409/410 is important for toxicity in *C. elegans* models, as mutating the S409/410 sites to alanine in hTDP-43(mutant) lines was able to partially rescue the locomotion defects observed in G290A and M337V transgenic animals (Liachko et al., 2010). As TDP-43 phosphorylated at S409/410 is linked to pathological inclusions in both sporadic and fALS, as well as FTLD (Inukai et al., 2008; Neumann et al., 2009), this demonstrates that the *C. elegans* models generated in these studies recapitulate aspects of TDP-43 pathology observed in humans.

Restricting expression of wild-type and mutant (A315T) hTDP-43 to GABAergic motor neurons of *C. elegans* led to adult-onset, age-dependent paralysis and progressive motor neuron degeneration in hTDP-43(mutant) lines (Vaccaro et al., 2012b). This is distinct from the models described earlier, which already showed significant locomotor defects on the first day of adulthood (Liachko et al., 2010; Zhang et al., 2011), suggesting that motor neuron transgenic lines are not affected developmentally nor show general deficiencies. Nonetheless, motor neuron-restricted transgenic lines showed hTDP-43^{A315T} aggregate formation in the cytoplasm and nucleus of neurons in day 1 adults. The authors suggest that their model, with its strong age-dependent phenotypes, can be used for medium-throughput screens for genetic modifiers or small molecule inhibitors of hTDP-43 toxicity (Vaccaro et al., 2012b). Indeed, the compound TRVA242 was found to potently rescue locomotor defects of this *C. elegans* model in a screen of 3,765 novel small molecule derivatives of pimozide (Bose et al., 2019), a neuroleptic proposed to be used as a therapeutic for ALS (Patten et al., 2017). In addition to Methylene Blue, a compound that enhances oxidative stress responses (Stack et al., 2014), compounds known to act specifically on endoplasmic reticulum (ER) stress [such as salubrinal (Boyce et al., 2005) and guanabenz (Tsaytler et al., 2011)] also protected against hTDP-43^{A315T}-mediated locomotor deficits in the *C. elegans* model (Vaccaro et al., 2013). This result adds to growing evidence that ER stress may be an attractive therapeutic target for ALS (Medinas et al., 2017a).

In a study focusing on the *C. elegans* ortholog of TDP-43 (TDP-1, referred to here as ceTDP-43), the authors showed that ceTDP-43 knockout was linked to sensitivity to oxidative and osmotic stress, as well as increased lifespan at moderate (20°C) temperatures. Unlike effects on hTDP-43 transgenic animals, the effects of the IIS pathway on ceTDP-1-mediated stress responses and lifespan are less clear—two distinct DAF-2 insulin receptor mutants have opposing effects, making it more difficult to derive a conclusive mechanistic link. Nonetheless, ceTDP-43 does appear to be required for the stress resistance in DAF-2 mutants as well as age-dependent proteotoxicity (Vaccaro et al., 2012c).

Fused in Sarcoma

Pan-neuronal expression of wild-type and mutant human FUS (hFUS) in *C. elegans* leads to cytoplasmic mislocalization, aggregation, and locomotor defects (Murakami et al., 2012). Murakami et al. (2012) generated a series of GFP-tagged hFUS transgenic lines, including WT FUS, clinical mutations (R514G, R521G, R522G, R524S, and P525L), and C-terminal truncations (FUS513 and FUS501). The C-terminal truncations aim to resemble human C-terminal splicing/frame-shifting truncation mutations associated with severe ALS phenotypes (Dejesus-Hernandez et al., 2010; Belzil et al., 2011). GFP-tagged hFUS R522G, R524S, P525L, FUS513, and FUS501 showed clear mislocalization of hFUS in the cytoplasm of neurons in the head and body of *C. elegans*, whereas WT hFUS and R514G or R521G mutations showed primarily nuclear localization. Cytoplasmic mislocalization of FUS is correlated with behavioral deficits, with only the strains showing cytoplasmic FUS also displaying age-dependent locomotor defects. These strains also showed a reduced lifespan compared with controls, as well as colocalization of FUS-GFP variants with cytoplasmic stress granule markers following acute heat stress (Murakami et al., 2012). To test whether mutant FUS can, under stress conditions, trigger the mislocalization of FUS^{WT} from the nucleus to the cytoplasm, the authors co-expressed TagRFP-$hFUS^{WT}$ together with GFP-FUS^{P525L}. After heat shock, TagRFP-WT-hFUS remained in the nucleus whereas GFP-FUS^{P525L} could be observed in both the nucleus and cytoplasm (in cytoplasmic structures resembling stress granules). The authors conclude that mutated FUS is likely to be pathogenic through a gain-of-function effect, rather than through titrating WT-FUS from the nucleus. In future work, it would be interesting to assess whether the colocalization of FUS with stress granules can be initiated by stressors other than acute heat stress (Huelgas-Morales et al., 2016), such as oxidative stress (Vance et al., 2013) or in the context of aging (Cao et al., 2020). *C elegans* is an ideal model system to explore the biology of stress signaling during aging due to its short lifespan and ease of growth/maintenance (Olsen, 2006).

In a *C. elegans* model expressing wild type and S57Δ mutant hFUS in GABAergic motor neurons, hFUS transgenic lines showed adult-onset, age-dependent locomotion deficits (Vaccaro et al., 2012b). These age-dependent behavioral effects correlate with motor neuron degeneration, which is more severe in $hFUS^{S57\Delta}$ transgenic animals compared with $hFUS^{WT}$ (Vaccaro et al., 2012b). Using the same model, the neuroprotective agent Methylene Blue was able to alleviate behavioral defects in $hFUS^{S57\Delta}$ animals in a dose-dependent manner, potentially by protecting against oxidative stress (Vaccaro et al., 2012a). Given the link between oxidative stress, aging, and proteostasis, further investigation into how oxidative stress responses modulate FUS pathology will be of significant interest.

Drosophila melanogaster

Research using the fly *Drosophila melanogaster* has made significant contributions to our understanding of nervous system development, function, and disease (Bellen et al., 2010; Owald et al., 2015). *Drosophila* has been used as a model organism for over 100 years: its genetic power combined with a relatively small brain (∼250,000 neurons in adult) and complex array of behaviors that can be rapidly assessed experimentally, provides a powerful model animal to pursue investigations into the mechanisms of neuronal dysfunction (Casci and Pandey, 2015). Like *C. elegans*, *Drosophila* is amenable to genetic manipulation *via* RNAi (Dietzl et al., 2007) as well as tissue-specific transgene expression *via* the commonly-used Gal4/UAS (Upstream Activation Sequence) system (Brand and Perrimon, 1993). *Drosophila* is also cheap to grow and maintain, has a short generation time (∼10 days), and is a more practical and ethical system than mammalian models to perform large-scale genetic or pharmacological screens (St Johnston, 2002; Hales et al., 2015). Another key advantage of *Drosophila* models is the high degree of genetic conservation with the human genome (∼60%; Dietzl et al., 2007). Last, sophisticated imaging approaches have been developed for systematic investigation of *Drosophila* neurons, both in terms of neuroanatomy (Jenett et al., 2012) as well as "functional imaging" of neuronal activity, i.e., calcium/voltage sensors and optogenetics (Simpson and Looger, 2018). Indeed, current advances in mapping the synaptic connectivity of the *Drosophila* nervous system are well underway, and early data releases are showing exciting promise for neurobiology research in this model animal (Zheng et al., 2018).

Superoxide Dismutase 1

Early fly models, like those in *C. elegans*, were based on the expression of human SOD1 in *Drosophila* neurons. Flies expressing wild-type hSOD1 as well as A4V and G85R mutant forms in motor neurons showed no gross developmental or lifespan defects but showed age-dependent progressive motor defects in all lines when compared with a control line expressing *Drosophila* SOD1 (dSOD1). Interestingly, although the hSOD1-G85R line showed the most significant locomotor defect, this did not correlate with a gross reduction in the number of motor neurons in the thoracic ganglia or with the formation of insoluble species in these neurons (Watson et al., 2008). Also, expression of mutant or wild-type hSOD1 led to upregulation of Hsp70 expression in surrounding glial cells—whether this glial response is protective to motor neurons is not known (Watson et al., 2008). Indeed the role of the heat shock response in glial cells in ALS pathogenesis is not yet clear and is an area of active study (Robinson et al., 2005; Liddelow et al., 2017; San Gil et al., 2017).

Ubiquitous expression of zinc-deficient hSOD1-D83S in *Drosophila* resulted in age-dependent locomotor defects but no substantial loss of neurons or reduction in lifespan compared with non-transgenic or $hSOD1^{WT}$ transgenic flies (Bahadorani et al., 2013). Tissue-specific expression of hSOD1-D83S in all neurons or in glia had the most potent effect on locomotion, with motor neuron-restricted expression having a moderate impact. However, no defects were observed upon expression in muscle, and no reduction in lifespan was seen compared to $hSOD1^{WT}$ transgenic animals. These defects correlated with increased sensitivity to the oxidative stressor paraquat and dysfunction of the mitochondrial respiratory chain (Bahadorani et al., 2013). These results agree with the previously mentioned

purified protein and cell culture data, suggesting that structural destabilization of SOD1 is a crucial factor in the formation of toxic species and aggregates in cells.

dSOD1 is highly similar to hSOD1, being identical at 104/153 residues. Şahin et al. (2017) engineered the ALS-associated mutations G37R, H48R, H71Y, and G85R into the endogenous locus of dSOD1 to generate mutant "knock-in" lines. These lines showed varying effects: compared with dSOD1(WTLoxP) controls, homozygous dSOD1 H71Y showed reduced lifespan, reduced fertility, and increased sensitivity to oxidative stress. dSOD1-G85R and dSOD1 H48R lines were developmentally lethal as homozygotes, but dSOD1-G85R heterozygotes showed normal lifespan and oxidative stress responses. dSOD1-G37R animals were phenotypically normal compared to controls. In larvae, dSOD1-G85R and dSOD1-H71Y homozygotes showed severe climbing defects that were suppressed in heterozygotes of each transgene. These were not correlated with a gross reduction in motor neuron number. However, dSOD1-G85R and dSOD1-H71Y animals showed reduced dSOD1 protein levels and enzymatic activity (Şahin et al., 2017). These models, which express ALS-linked mutant SOD1 from the endogenous dSOD1 locus, will be useful tools for future analysis or high-throughput genetic/pharmacological screens.

Transactive Response DNA-Binding Protein 43

Expression of a repeated form of amino acids 342–366 (12xQ/N) of TDP-43 in the *Drosophila* eye leads to the formation of insoluble aggregates that were by themselves not strongly neurotoxic (Cragnaz et al., 2014). Over-expression of the *Drosophila* ortholog of TDP-43 (TBPH, referred to here as dTDP-43) in the eyes leads to significant levels of tissue degeneration. This toxicity was completely reverted by the co-expression of EGFP-tagged 12xQ/N. The authors proposed that dTDP-43 may be sequestered in aggregates formed by EGFP-12xQ/N, thereby titrating excess dTDP-43 (Cragnaz et al., 2014). In this case, the formation of aggregates is protective. When EGFP-12xQ/N is expressed in fly neurons, it results in an age-associated locomotor defect and a shortened lifespan (Cragnaz et al., 2015). In the neuronal model, the authors suggest that the locomotor defect is correlated with reduced levels of endogenous dTDP-43 (protein and mRNA levels), due to dTDP-43 being sequestered in EGFP-12xQ/N aggregates (Cragnaz et al., 2015). This can be compensated in younger animals by increased protein production, but in older animals, the lower capacity for protein synthesis (Liang et al., 2014; Anisimova et al., 2018) may lead to a loss-of-function effect due to reduced dTDP-43 levels.

Over-expression models where wild-type or human hTDP-43 is expressed in fly neurons have also provided interesting insights into TDP-43-linked ALS/FTLD pathology. Miguel et al. (2011) generated *Drosophila* models expressing FLAG-tagged hTDP-43^{WT}, hTDP-43 with a defective NLS (hTDP-$43^{\Delta NLS}$—predominantly cytoplasmic localization) or hTDP-43 with a defective NES (hTDP-$43^{\Delta NES}$—restricted to nuclear localization). Neuron-specific expression of these transgenes was larval lethal, so an inducible system was used to bypass developmental effects (Miguel et al., 2011). Compared with controls, all inducible transgenic flies showed a reduced lifespan. Biochemical analysis (immunoblot) demonstrated that hTDP-43, hTDP-$43^{\Delta NES}$, and hTDP-$43^{\Delta NLS}$ proteins could be detected in monomeric form or as high molecular weight (HMW, potentially aggregated) species, although hTDP-$43^{\Delta NES}$ and hTDP-$43^{\Delta NLS}$ expression showed more HMW species compared with hTDP-43^{WT}. When probed with an antibody against phospho-TDP-43(S409/410), hTDP-$43^{\Delta NES}$ HMW species, but not those of hTDP-43^{WT} or hTDP-$43^{\Delta NLS}$, were strongly labeled by the pTDP-43 antibody. Interestingly, immunostaining did not reveal the presence of inclusions formed by the expression of any of these transgenes, suggesting that toxicity (reduced survival) does not require the formation of inclusions. This study also demonstrated cell-type-specific toxicity resulting from the expression of hTDP-43 transgenes: in adult neurons, both nuclear and cytoplasmic accumulations of TDP-43 result in toxicity, whereas in muscle and glial cells, only the accumulation of cytoplasmic species of TDP-43 was toxic (Miguel et al., 2011). This could be due to differences in proteostasis mechanisms in different tissues, such as the capacity for protein production, differences in post-translational modification, or sequestration of aggregated/oligomeric forms (Vilchez et al., 2014).

TDP-43 autoregulates its own levels through binding to a TDP-43 binding region (TDPBR) in its 3′ UTR (Ayala et al., 2011). Expression of hTDP-43 containing the TDPBR in *Drosophila* retinal cells led to significantly reduced protein and mRNA levels for *hTDP-43* (Pons et al., 2017), similar to observations in cultured cells and mammalian models (Ayala et al., 2011; Polymenidou et al., 2011). This *hTDP43_TDPBR* transgenic line can then be used to perform genetic crosses with *Drosophila* mutant strains to identify other factors that modulate *hTDP-43* levels through the TDPBR. Pons et al. (2017) used this method to identify *Drosophila* splicing factors that modulated *hTDP-43* levels, and in a follow-up study, identified the protein CG42724 [and its human orthologue Transcription elongation regulator 1 (TCERG1)] as a modulator of *hTDP-43* protein levels (Pons et al., 2018).

TDP-43 has been shown to associate with human Hsc70 in cultured mammalian cells (Freibaum et al., 2010). In *Drosophila*, one of the Hsc70 proteins (Hsc70–4) is highly expressed in neurons and functions in synaptic vesicle exo- and endocytosis (Bronk et al., 2001; Chang et al., 2002). Coyne et al. (2017) showed that *Drosophila* Hsc70–4 associates with transgenic hTDP-43 in fly motor neurons. As a result, over-expression of hTDP-43 in motor neurons sequesters Hsc70–4, leading to reduced synaptic expression. This ultimately inhibits synaptic vesicle endocytosis and results in defects in synaptic transmission (Coyne et al., 2017). Interestingly, expression of ALS-linked mutant forms of hTDP-43 leads to locomotor defects that can be rescued by over-expression of wild-type Hsc70–4, but not mutant forms that are defective in chaperone (Hsc70–4^{D10N}) or microautophagy functions (Hsc70–4^{3KA}). This suggests that hTDP-43 compromises both chaperone and microautophagy activities of Hsc70–4 (Coyne et al., 2017).

Fused in Sarcoma

Drosophila models have also been useful in investigating the pathological roles of ALS-associated protein FUS. Transgenic *Drosophila* lines were generated expressing $hFUS^{WT}$ and two disease-related variants $hFUS^{P525L}$ and $hFUS^{R495X}$ (Jäckel et al., 2015). Expression in motor neurons resulted in viability defects and locomotor defects in $hFUS^{WT}$ and $hFUS^{P525L}$, but not in $hFUS^{R495X}$-expressing flies. Immunostaining showed that $hFUS^{WT}$ localized to the nucleus, whereas $hFUS^{P525L}$ showed an additional cytoplasmic staining, and $hFUS^{R495X}$ was observed at relatively equal levels in the nucleus and cytoplasm. The lack of phenotype observed in $hFUS^{R495X}$ flies is surprising because the R495X mutation is linked to a highly aggressive form of ALS (Chiò et al., 2009b). The pathological role of $hFUS^{R495X}$ in this *Drosophila* model could not be associated with its partial cytoplasmic mislocalization, which is thought to be a critical disease mechanism (Kim and Taylor, 2017), suggesting that disease-modifying factors are missing in this model.

FUS expression can autoregulate its levels in cultured cells (Zhou et al., 2013; Dini Modigliani et al., 2014). Similarly, in *Drosophila*, over-expression of hFUS in fly motor neurons leads to decreased levels of the endogenous Caz protein (the *Drosophila* ortholog of FUS—dFUS; Machamer et al., 2014; Jäckel et al., 2015). These changes are correlated with alterations in neuromuscular junction structure and defects in synaptic transmission. In contrast, Caz/dFUS null alleles show enhanced synaptic transmission as demonstrated by increased excitatory junction potential (EJP) amplitude (Machamer et al., 2014). Therefore, although hFUS over-expression (both WT and mutant) leads to lower levels of Caz/dFUS, this is likely not the mechanism by which hFUS triggers synaptic dysfunction.

Both TDP-43 and FUS are components of the biomolecular condensate compartment known as stress granules through interactions in their respective LCDs (Harrison and Shorter, 2017). A *Drosophila* study performed a systematic domain deletion screen of FUS, to identify toxicity-mediating domains (Bogaert et al., 2018). Deletion of the N-terminal QGSY prion-like LCD partially rescued toxicity. The C-terminal RGG-2 region was also essential for toxicity, and the two domains appear to act synergistically. Interestingly, the prion-like QGSY domain is not found in Caz/dFUS, which may differentiate between toxicity caused by human and fly orthologs (Bogaert et al., 2018). The nuclear-import receptor Kap-β2 (Karyopherin-β2) inhibits phase-transition and fibrillization of FUS *in vitro*. Also, transgenic expression of Kap-β2 and could rescue the shortened lifespan of *Drosophila* expressing $hFUS^{R521H}$, thereby mitigating hFUS induced motor-neuron toxicity *in vivo* (Guo et al., 2018).

For a visual summary of the advantages of using invertebrate models, see **Figure 4**.

Mouse Models of ALS

Mouse models of ALS have been a key system by which our understanding of the underlying molecular pathology of ALS develops. In this section, we will describe the current mouse models used to examine both protein homeostasis and prion-like propagation in ALS.

Superoxide Dismutase-1

Since the seminal discovery identifying the association of SOD1 mutations with fALS (Rosen et al., 1993), several SOD1 transgenic mouse models have been generated. Despite the discovery of several other genes and a relatively small percentage of patients exhibiting SOD1 mutations, SOD1 mouse models are still the most extensively used model to investigate the pathogenesis and treatment of ALS. The first transgenic SOD1 mouse model expressed hSOD1 with the G93A mutation (denoted SOD1-G93A; Gurney et al., 1994). Transgenic SOD1-G93A mice exhibit phenotypic symptoms at 3–4 months of age and subsequently develop ubiquitinated SOD1 deposits, similar to those found in fALS post-mortem tissue (Bruijn et al., 1997). Years after its initial generation, this model provided the first *in vivo* clues indicating that SOD1 acts in a prion-like fashion. Deng et al. (2006) observed that crossing human wild-type SOD1 mice with SOD1-G93A transgenic mice accelerated ALS-like phenotypes. Furthermore, soluble human wild-type SOD1 was converted to an aggregated and detergent-insoluble conformation. Also, spinal cord homogenates from SOD1-G93A mice were used to seed recombinant purified SOD1 to form amyloid fibrils (Chia et al., 2010).

The studies described above hinted at the ability of SOD1 to propagate in a prion-like manner but lacked animal-to-animal transmission. Utilizing experimental paradigms from the prion field, Ayers et al. (2014) were the first to provide *bona fide in vivo* evidence of SOD1 prion-like propagation. Heterozygous mice expressing SOD1-G85R mutant C-terminally tagged with a yellow fluorescent protein (SOD1-G85R:YFP) do not develop an ALS phenotype (Wang et al., 2009b). However, when spinal cord homogenates from paralyzed SOD1-G93A mice were injected into the spinal cord of SOD1-G85R:YFP mice at PN0 (postnatal day 0), some (∼50%) of these mice subsequently developed ALS-like phenotypes, including paralysis as early as 6 months of age, as well as reduced life-span (Ayers et al., 2014). However, when spinal cord homogenates from SOD1-G93A inoculated SOD1-G85R:YFP mice were subsequently re-passaged into newborn SOD1-G85R:YFP mice, all of these mice developed ALS symptoms, and at an earlier time-point. Furthermore, SOD1-G93A recipient mice exhibited SOD1-G85R:YFP protein aggregates in the spinal cord as well as in the brain stem, suggesting prion-like spread distal to the site of injection. Also, second passage inoculation of naïve adult SOD1-G85R:YFP mice resulted in paralysis of the ipsilateral hind-limb of the injection site within 1 month, followed by contralateral leg paralysis after 2 months and reaching end-stage paralysis within 3 months post-injection. Furthermore, post-mortem analysis of mice at several time points post-injection showed inclusion pathology in both neurons and astrocytes in the dorsal root ganglion, cervical spinal cord, and brain stem (Ayers et al., 2016), suggesting focal spreading throughout the CNS, a classic hallmark of pathological prion propagation. It is important to note that YFP-tagged SOD1 is not required as a template for seeding, as other work has shown untagged mutant SOD1 expressed in mice can be efficiently seeded by injection of preformed SOD1 aggregates

(Bergh et al., 2015; Lang et al., 2015; Bidhendi et al., 2016; Bidhendi et al., 2018).

Whilst it is unclear how SOD1 spreads throughout the CNS, *in vitro* studies highlight the important role of exosomes in the extracellular transmission, yet *in vivo* experiments supporting such transmissibility have yet to be explored or reported. These studies may be of use to aid in the design of anti-propagation therapeutic avenues. In line with this, *in vitro* work has shown small molecules that interact with or near the Trp32 residue of SOD1 can reduce its aggregation propensity. Furthermore, Trp to Ser substitutions at the 32 position residue can suppress intra- and intercellular propagation (Grad et al., 2011, 2014), suggesting its involvement in SOD1 prion-like propagation. However, some research showed that recombinant SOD1 with a Trp32Ser (W32S) substitution produced a similar disease frequency and earlier disease onset compared to WT SOD1 when seeded in G85R-SOD1:YFP mice (Crown et al., 2020), suggesting that fibrillation through other amyloidogenic regions within SOD1 may also confer disease. The Trp32 residue is conserved to human SOD1 in beta-sheet 3 and not present within mice. To date, there are no reports of a transgenic SOD1 mouse model expressing modifications to the Trp32 residue, however, such a model may lead to further understanding its role in SOD1 aggregation and prion-like propagation.

Various prion isolates can develop a heterogeneous pathology giving rise to prion strains that are determined by variability within their tertiary conformation. Similarly, fALS SOD1 mutations display a heterogeneous disease pathology, which has been proposed in part due to their tertiary structure and propensity to aggregate (Prudencio et al., 2009). Using binary epitope mapping with several anti-peptide antibodies covering the entire SOD1 protein, Bergh et al. (2015) used insoluble material obtained from wild-type and mutant (G85R, D90A, and G93A) SOD1 mice to show evidence of two distinct strains (referred to as A and B by the authors). Strain A aggregates were observed in all SOD1 mutant mice investigated, while strain B was only observed in D90A mice. *In vitro*, biochemical/physical examination of strain A and B showed variation in their aggregation structure, kinetics, and fragility, with strain B showing increased aggregation kinetics and fragility (Lang et al., 2015). Furthermore, injection of strain A and B in the lumbar spinal cord of adult $SOD1^{G85R}$ mice showed both strains were capable of seeding and transmissibility, with the rostral spreading of aggregates, subsequently causing end-stage death ∼100 days following injection (Bidhendi et al., 2016). Later, injection of spinal cord homogenate from a patient carrying the SOD1-G127X mutation into transgenic mice carrying the same mutation showed propagation of strain A into the mice, suggesting that this aggregate conformer may exist in humans (Bidhendi et al., 2018).

Whilst the evidence and modeling are still in the early days, these data collectively support the ability of SOD1 to undergo prion-like propagation. Future experiments using SOD1 mice with various amyloidogenic regions within SOD1 deleted and/or the use of synthetic peptide samples composed of a single amyloidogenic segment of SOD1 would be useful to reveal the mechanistic details of this pathology.

Transactive Response DNA-Binding Protein 43

TDP-43 has been extensively implicated in ALS, in addition to FTLD, due to the high majority of both fALS and sALS patients exhibiting insoluble and ubiquitinated TDP-43 cytoplasmic inclusions within motor neurons and glia (Neumann et al., 2006). Several TDP-43 transgenic mouse models have been generated which show motor deficits, neuronal loss, and mislocalized TDP-43 inclusions (Lee et al., 2012). However, overexpression of WT TDP-43 can cause similar pathology, making it difficult to distinguish the pathological effects of various mutations without adequate control over transgene expression. To overcome these issues, several groups have produced sophisticated knock-in models to control for transgene overexpression. These models include introducing Hemi- and homologous TDP-43 transgene mutations at G298S, Q331, and M337V (Fratta et al., 2018; White et al., 2018; Ebstein et al., 2019; Gordon et al., 2019). Interestingly, these mice homologous for these transgenes show motor deficits and in some cases, motor neuron loss in the absence of mislocalized TDP-43, suggesting TDP-43 mislocalization may not be the cause of motor neuron toxicity in ALS (Fratta et al., 2018; White et al., 2018; Ebstein et al., 2019; Gordon et al., 2019).

Observations from ALS and FTLD post-mortem tissue suggests that TDP-43 also exhibits prion-like propagation and spreading throughout neuroanatomical tracts. Until recently there has been a lack of *in vivo* investigation into the prion-like propagation of TDP-43 using animal models. Employing a cell-based screening system, Porta et al. (2018) were able to apply sarkosyl-insoluble frontal cortex homogenates from FTLD-TDP subjects to identify those with seeding capacity. Subsequently, samples that showed *in vitro* seeding capacity were injected into the brains (neocortex, hippocampus, and thalamus) of doxycycline-regulatable transgenic mice expressing a human $TDP\text{-}43^{\Delta NLS}$ in forebrain neurons (under a CAMKIIa promoter). Recipient mice displayed pTDP-43 inclusions within the ipsilateral injection site within 1-month post-injection, with relatively few inclusions observed on the contralateral side. Furthermore, spatiotemporal immunohistochemical analysis of mice injected with TDP-43 seeding material showed a transneuronal-like spreading pattern of TDP-43 pathology in the 1–9 months following injection. Unsurprisingly, a large majority of TDP-43 aggregates were observed within forebrain neuronal cells where the CAMKIIa promoter controls the expression of $TDP\text{-}43^{\Delta NLS}$. However, TDP-43 aggregates were also found within the subcortical regions including the nucleus accumbens and caudate-putamen, suggesting regional spreading.

Furthermore, similar to ALS/FTLD post-mortem samples, TDP-43 aggregates were also sparsely observed in oligodendrocytes and astrocytes, suggesting local cell-to-cell transmission that is not dependent on the subcellular mislocalization of TDP-43. Further supporting this, seeding was also observed in wild-type mice injected with FTLD-derived material, however, the capacity was less than NLS-mice. Furthermore, injection of FTLD material in mice overexpressing cytoplasmic TDP-43 C-terminal fragments did not show TDP-43 pathology, indicating a role for the N-terminal sequence in TDP-43 in prion-like seeding. While this study provides

initial *in vivo* evidence for the prion-like characteristics of TDP-43, further research is required to identify the specific regions of TDP-43 that are important for seeding. As mentioned previously, full-length TDP-43 and its CTFs have multiple amyloidogenic regions (Guenther et al., 2018a,b). Assessing the contribution of each of these segments would be essential to determine the sequence region by which TDP-43 aggregates propagate. This information would allow for the design of therapies that may block templating or cell-to-cell transmission of TDP-43 prion-like particles.

Fused in Sarcoma

FUS knockout mouse models do not show evidence of motor impairments or ALS associated histopathology, suggesting that a FUS loss-of-function mechanism is not responsible for ALS pathology. However, several groups have developed human wild-type FUS (hFUS) overexpression mouse models under various promoters. Mitchell et al. (2013) were the first to produce a FUS overexpression model, whereby hFUS levels were ∼1.9 times higher than wild-type mice. These mice develop paralysis at 10–12 weeks of age (Mitchell et al., 2013). Furthermore, overexpression of hFUS resulted in the cytoplasmic mislocalization of the ectopic protein in the brain and spinal cord. However, cell loss was only observed in the spinal cord, indicating that lower motor neurons may be prone to FUS overexpression.

FUS mutations are inherited in an autosomal dominant fashion, with FUS-R521C mutations commonly observed in FUS-associated fALS patients. Qiu et al. (2014) developed a transgenic FUS-R521C mouse model that exhibited aggressive motor function deficits and reduced life span. These transgenic mice showed alterations in the transcription and splicing of the growth factor BDNF, which is regulated by FUS. Spinal cord homogenates from FUS-R521C mice showed a high propensity to form complexes with FUS-WT, consistent with experiments in HEK293T cells co-expressing mutant- and FUS-WT. Together with the autosomal dominant inheritance of FUS mutations, these data support a gain of function property of mutant FUS, which may sequester FUS-WT from carrying out its physiological role.

Recently, Zhang et al. (2020) employed two-photon imaging in combination with a knock-in mouse model of FUS-R521C. They showed that mutant FUS was incorporated and potentiated stress granule formation following arsenite challenge. Expression of mutant FUS also caused the cytoplasmic mislocalization of mutant FUS and upregulation of ubiquitin, which appeared to be also co-localized. Also, neurons that showed an inability to clear or disassemble stress granules died within days following arsenite challenge, highlighting the role of stress granule formation in the pathogenesis of ALS.

To distinguish the pathological role of FUS aggregates without the confounding effects on RNA metabolism, Shelkovnikova et al., developed a transgenic mouse expressing a truncated human FUS protein (residues 1–359), which lacked the RNA recognition motif and NLS but still retained an N-terminal QGSY prion-like domain (Shelkovnikova et al., 2013). These mice developed paralysis, exhibited motor neuron loss, and reduced life-span. Furthermore, the truncated FUS protein formed aggregates, and subsequent FUS inclusions containing endogenous murine FUS were observed in the motor cortex and lower motor neurons. Collectively this study suggests that mutant FUS may exhibit a seeding capacity, which is capable of causing motor neuron degeneration in the absence of its role in RNA processing. For a visual summary of the key features of murine models, see **Figure 5**.

THERAPEUTIC STRATEGIES TO TARGET PROTEOSTATIC IMPAIRMENT AND PRION-LIKE PROPAGATION IN ALS

Currently, only two therapies exist for the treatment of ALS in humans. The first, riluzole, interrupts glutaminergic signaling (Bryson et al., 1996) potentially through preventing glutamate release (Kretschmer et al., 1998). Riluzole treatment led to increased motor function and survival in an initial clinical trial (Bensimon et al., 1994; Lacomblez et al., 1996); however, improvements in survival were not observed in a follow-up clinical study. Also, riluzole only extends lifespan by 3–6 months, and only in patients with moderate impairment (Bensimon et al., 1994; Lacomblez et al., 1996). The second compound, edaravone, is a free-radical scavenger (Yoshino and Kimura, 2006), which is primarily sold to promote recovery from stroke in Japan due to its ability to prevent edema, neuronal damage, and neurological defects in rats (Edaravone Acute Infarction Study Group, 2003). Edaravone is offered as a therapeutic to treat ALS in Japan, South Korea, the US, and Canada, but is not being used elsewhere (Yoshino, 2019). Clinical trials using edaravone did not demonstrate a benefit for the majority of patients, with a follow-up clinical study suggesting that edaravone only benefits patients with mild impairment [Yoshino and Kimura, 2006; Abe et al., 2014; Writing Group; Edaravone (MCI-186) ALS 19 Study Group, 2017]. The above highlights how the only two drugs currently offered to treat ALS symptoms are limited in scope, and there is a dire need for new therapies that potentially target one or more ALS-associated aggregating proteins.

In silico and *In vitro* High-Throughput Identification of Potential Therapeutics

With improved computing power and rapidly-evolving methods to better examine protein structure, computational modeling is being utilized as a means for drug discovery. Due to the lack of need for biological material, and bioinformatic nature, computational screening of drugs can be carried out rapidly and efficiently. Additionally, high-throughput screens using *in vitro* models that recapitulate some aspects of ALS pathology can be utilized to rapidly screen potential drugs to determine their biological activity and therapeutic potential. A recent review of high-throughput screens investigating drugs that alter ALS pathology was published by McGown and Stopford (2018). This review describes studies that investigated reducing SOD1 transcription, protein aggregation, and oxidative stress as a therapeutic strategy, as well as the potential of anti-glutamatergic and cytoprotective drugs for the treatment of ALS. Studies focusing solely on drug candidates that modify

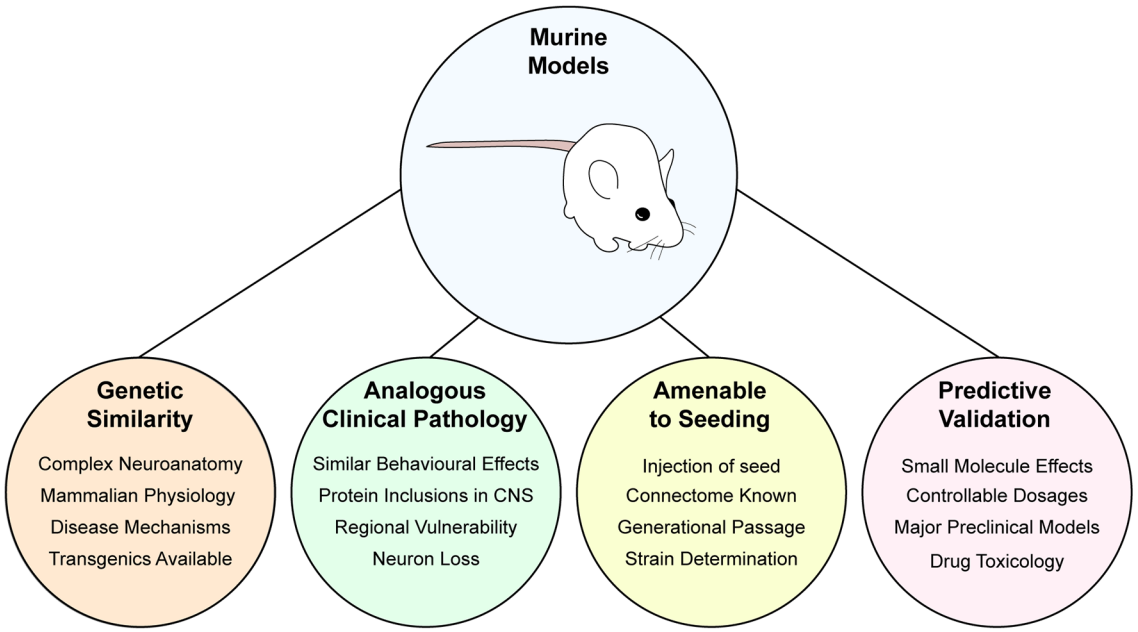

FIGURE 5 | Murine models of protein aggregation and prion-like propagation. Murine models form the crux of our pathobiological understanding of the effects of protein aggregation and prion-like propagation in mammals. This is due to their genetic similarity with humans, which translates into similar physiology, similar disease susceptibility, and similar neuroanatomy. The input of disease-associated genes into mice often results in similar clinicopathological features including behavioral deficits, protein aggregation, and loss of neurons in specific tissues. Prion-like propagation can be assayed through the injection of seed protein aggregates into specific locations, and the passage of extracted prion-like seeds can be used to define strains. Finally, these models are the gateway to the clinic and are mostly used for the testing of drugs and therapies that are aimed at alleviating human disease.

protein aggregation that was described briefly in the review, and studies published since are discussed below.

Superoxide Dismutase-1

A computational screen found 16 novel compounds that bound mutant SOD1-A4V and prevented its aggregation *in vitro* (Ray et al., 2005), however, these compounds did not exert this effect in the presence of blood plasma (Nowak et al., 2010). Subsequent computational work identified 6 different novel compounds that bound SOD1 in the presence of plasma (Nowak et al., 2010), but these are yet to be tested *in vitro* or *in vivo*. Using *in vitro* aggregation assays of SOD1-G37R protein, Anzai et al. (2016) tested 640 FDA-approved drugs and identified seven hit compounds, with six able to completely suppress SOD1 aggregation (measured by turbidity). Three of these were statins (simvastatin, lovastatin, and mevastatin), another three were vitamin D derivatives (alfacalcidol, calcidiol, and calcitriol), and the final drug was miltefosine, an antimicrobial. A computational screen of ∼4,400 compounds revealed that quercitrin, quercetin-3-β-D-glucoside, and epigallocatechin gallate (EGCG) bind directly to SOD1-A4V and inhibit ROS-induced SOD1 aggregation (Ip et al., 2017), but only the latter has been tested *in vitro* and *in vivo*. EGCG administered in SOD1-G93A mice improved disease compared to controls, delaying means symptom onset (128 days vs. 115 days), locomotor deficits measured by rotarod test (140 days vs. 120 days), and mortality (mean survival time 143 days vs. 123 days), however the mechanism was not described (Koh et al., 2006), and this drug has not progressed to clinical trials.

Using high-throughput *in vitro* screening using an inducible SOD1-G37R PC12 cell line, Broom et al. (2006) screened 116,680 compounds from various libraries and found 67 hit compounds. However, most compounds exhibited cytotoxicity or failed to have their effect replicated, and only 2 hits (no description available) are being investigated further. Rather than using SOD1 aggregation as a readout, Wright et al. (2012) instead investigated SOD1 transcription using PC12 cells transfected with SOD1-GFP to screen 30,000 compounds, with the most potent inhibitor of SOD1 transcription being *N*-[4-[4-(4-methyl benzoyl)-1-piperazinyl]phenyl]-2-thiophenecarboxamide. *In vivo* experiments using a SOD1-G93A mouse model showed that the drug was well tolerated, with toxicity estimated to be 75 mg/kg. These mice also showed a significant decrease in SOD1 protein levels, but aggregation was not described (Wright et al., 2012).

Finally, using iPSC-derived motor neurons from fALS patients, Imamura et al. (2017) screened 1,416 compounds and determined the Src/c-Abl tyrosine kinase inhibitor bosutinib improved iPSC-derived motor neuron survival, potentially through clearance of SOD1 aggregates by up-regulation of autophagy pathways. Intraperitoneal injection of bosutinib (5 mg/kg) into transgenic SOD1-G93A mice daily starting at

8 weeks of age for 5 weeks, reduced misfolded SOD1 and motor neuron degeneration in spinal cords, as well as delaying disease onset by 10.8 days and extending survival by 7.8 days (Imamura et al., 2017).

Transactive Response DNA-Binding Protein 43

Boyd et al. (2014) generated a doxycycline-inducible TDP-43-GFP expressing PC-12 cell line to screen ∼75,000 small molecules to identify those that reduce sodium arsenite-induced TDP-43 aggregates as measured by microscopy. One compound, termed LDN-0130436, reduced TDP-43 aggregation in a concentration-dependent manner and was confirmed by immunoblotting. LDN-0130436 restored behavioral defects and protected against TDP-43-induced cell loss in *C. elegans* expressing mutant TDP-43^{A315T}, however, effects on protein aggregation in this model were not reported (Boyd et al., 2014).

Using a luciferase-based fluorescence assay to measure TDP-43 inclusions in the murine Neuro-2a neuroblastoma cell line, Oberstadt et al. (2018) screened the LOPAC1280 library (Sigma–Aldrich) and found 16 active compounds that reduced TDP-43 dimerization by 30–82%. In an initial screen, riluzole, one of the two therapies offered to ALS patients, reduced N-terminal TDP-43 interactions by 82%, whilst chelerythrine and auranofin reduced interactions by 64% and 63%, respectively. During validation, 25 μM auranofin significantly reduced 98% of TDP-43 interactions, whilst 25 μM chelerythrine reduced 90% of TDP-43 interactions but exhibited a toxic effect at higher concentrations (Oberstadt et al., 2018). Fang et al. (2019) screened 3,350 compounds in HEK293 cells transfected with TDP-43 and 5,910 compounds in neural progenitor cells to identify drugs that affect stress granule formation. In this study, Anisomycin, mitoxantrone, and GSK2656157 significantly reduced stress granule formation, and interestingly mitoxantrone inhibited TDP-43$^{\Delta NLS}$ colocalization with stress granules (Fang et al., 2019).

Using patient-derived iPSCs differentiated into spinal motor neurons, Egawa et al. (2012) investigated the impact of four compounds on arsenite-induced TDP-43 inclusion formation and cell death. Only anacardic acid reduced TDP-43 mRNA expression, inclusion formation, and prevented cell death. Additionally, bosutinib (mentioned above for SOD1) demonstrated a similar reduction in TDP-43 inclusions in iPSC-derived motor neurons. Another study utilized a high-throughput image analysis method in iPSC-derived motor neurons from sALS patients to screen 1,757 bioactive compounds for the reduction of TDP-43 aggregation and expression levels (Burkhardt et al., 2013). This screen identified 38 hits that reduced TDP-43 aggregation, but not TDP-43 expression, and subsequent validation characterized four classes of compounds: cyclin-dependent, and c-Jun N-terminal kinase inhibitors, triptolide, and cardiac glycosides. Another study using iPSC-derived motor neurons from fALS patients expressing TDP-43 mutations screened 1,232 drugs using a high-throughput imaging system and identified the dopamine D2 receptor antagonist ropinirole as a potential therapeutic (Fujimori et al., 2018). Ropinirole prevented neurite regression, stress granule formation, TDP-43 aggregation, and prevented cell death of fALS-derived iPSCs.

Patten et al. (2017) screened 3,850 compounds in a transgenic *C. elegans* model expressing mutant TDP-43^{A315T} and identified several neuroleptics, which rescued paralysis. Further screening in a transgenic zebrafish model expressing mutant TDP-43^{G348C}, led to the identification of pimozide as a potential therapeutic, which rescued paralysis, as well as motor neuron axon branching and neuro-muscular junction structures. The authors also describe that pimozide is already FDA-approved and the use of pimozide (1 mg daily) in a randomized double-blind clinical trial in Poland, led to a reduction in ALS severity (Szczudlik et al., 1998), but this is yet to be investigated elsewhere.

In a transgenic *Drosophila* model of ALS, in which motor neurons express mutant TDP-43^{D169G}, TDP-43^{G298S}, TDP-43^{A315T}, or TDP-43^{N345K}, resulting in complete mortality, Joardar et al. (2015) screened 1,200 FDA-approved drugs to determine if any improve survival. This screen identified several antidiabetic drugs such as thiazolidinediones, sulfonylureas, and biguanides, with the most potent effect exhibited by the thiazolidinedione pioglitazone. Pioglitazone prevented locomotor deficits and glial death, but could not prevent the death of motor neurons, and was ineffective in preventing disease pathology in a transgenic SOD1-G85R model. This finding contradicts two studies which have shown the benefit of pioglitazone to rescue locomotor deficits, prevent motor neuron death, and improve survival in transgenic SOD1-G93A mice, however, there was no reduction in SOD1 expression, and SOD1 aggregation was not characterized (Kiaei et al., 2005; Schütz et al., 2005).

Using *C. elegans* expressing mutant TDP-43-A315T, Bose et al. (2019) screened a library containing 3,765 novel molecules and identified 27 hit compounds. Validation in a toxic gain-of-function TDP-43-G348C zebrafish model identified four of these hits which improved locomotion. One compound, TRVA242 was identified as the most effective compound and rescued locomotion, improved motor neuron survival, and rescued neuromuscular junction deficits in the aforementioned *C. elegans* and zebrafish model, as well as in SOD1 and C9ORF72 zebrafish models of ALS, and in SOD1-G37R mice.

Fused in Sarcoma

High-throughput screening to investigate therapies targeting FUS in ALS using iPSC-derived motor neurons have been performed. A GFP-tagged FUS^{P525L} expressing iPSC cell line treated with sodium arsenite was used to screen ∼1,000 small molecules to investigate stress granule formation *via* imaging (Marrone et al., 2018). This screen identified 69 compounds, and further assessment validated 13 molecules targeting the PI3K/AKT/mTOR pathway, with the mTOR inhibitor rapamycin reducing stress granule formation and promoting survival of motor neurons generated from the iPSCs. Whilst the effect of rapamycin on protein aggregation was not characterized, rapamycin did upregulate autophagy, a mechanism important in the clearance of protein aggregates (Ramesh and Pandey, 2017). As mentioned above, the dopamine receptor antagonist ropinirole prevented ALS pathology in

iPSC-derived motor neurons from fALS patients with TDP-43 (M337V or Q343R) mutations, and similarly, ropinirole prevented neurite regression, stress granule formation, FUS aggregation, and prevented cell death in iPSC-derived motor neurons from fALS patients with FUS (H517D) mutations (Fujimori et al., 2018).

Currently, there are no high-throughput studies *in vivo* focusing solely on FUS. However, as mentioned above the *C. elegans*, *D. melanogaster* and zebrafish models of FUS represent ideal models to investigate potential drugs, with Vaccaro et al. (2012a) demonstrating methylene blue prevented oxidative stress and improved locomotor defects in both a *C. elegans* and zebrafish model. Additionally, Joardar et al. (2015) did demonstrate that pioglitazone rescues locomotor function in transgenic FUS^{P525L} *Drosophila*, but does not impact survival.

Diacetylbis(*N*(4)-Methylthiosemicarbazonato)-CuII (CuATSM)

Superoxide Dismutase-1

Numerous *in vitro* models exist to explore the role of SOD1 aggregation in ALS pathogenesis. In NSC-34 cells, CuATSM decreased aggregation and increased survival in cells transiently transfected with GFP-tagged mutant A4V, C6G, E100G, G37R, G93A, and V148G SOD1, but not those transfected with H46R, G85R, or G127X (Farrawell et al., 2019). Importantly, the mutants that were not responsive to CuATSM treatment reduce or ablate the ability of SOD1 to bind copper (Carrì et al., 1994). This data suggests that the activity of CuATSM in this model is to facilitate the delivery of copper to SOD1 and thereby limit the pool of immature copper-free SOD1 that is more prone to aggregate (Tokuda et al., 2018).

Dermal application of CuATSM improved survival in a SOD1-G93A mouse model, with CuATSM-treated mice living a median 155 days, compared with vehicle-treated controls with a median lifespan of 133 days (Williams et al., 2016). Oral administration of CuATSM delayed neurological damage and improved latency to fall using a rotarod test, with CuATSM mice displaying these symptoms at 113 days compared with 100 days in vehicle-treated mice. CuATSM treated mice also demonstrated an improved mean survival time of 143 days compared with 129 days in vehicle-treated mice (Hilton et al., 2017). Conversely, CuATSM-treated mice demonstrated significantly increased relative abundance of mutant SOD1-G93A, SOD1 activity, and copper in spinal cords compared with vehicle-treated mice. This increase can be attributed to CuATSM facilitating increased copper delivery to SOD1 *via* CCS, which can improve copper-dependent SOD1 activity, an effect that protects motor neurons and improves disease outcomes (Soon et al., 2011; Roberts et al., 2014; Hilton et al., 2017). This is supported by evidence that ligand-free ATSM does not exert protective effects compared to CuATSM (Vieira et al., 2017).

Similar to the SOD1-G93A mouse model, CuATSM also improves locomotor function (rotarod test) and median survival time (212–248 days depending on dose) in SOD1-G37R mice compared to vehicle control (median survival time 196 days). Administration of riluzole in conjunction with CuATSM did not exhibit any additive effect (McAllum et al., 2013). In a more severe SOD1-G93A mouse model, where mice co-express human CCS, SOD1-G93AxhCCS mice exhibit a mean survival time of 36 days, compared to 226 days in SOD1-G93A mice (Son et al., 2007). Administration of CuATSM in SOD1-G93AxhCCS mice daily from day 5 increased lifespan to >1 year, whereas suspending treatment after 21 days of age until disease onset (80 days) generally led to reduced symptom severity, although some mice developed symptoms and survived for 302–377 days of age (Williams et al., 2016). Complete withdrawal of treatment (after 21 days of age) had a smaller effect on lifespan, with these mice surviving for 122–132 days of age (Williams et al., 2016).

Transactive Response DNA-Binding Protein 43

Similar to SOD1, CuATSM has also been explored as a therapy for preventing TDP-43-associated toxicity. In SH-SY5Y neuroblastoma cells, CuATSM protected against paraquat-induced cell death, inhibited aggregation of TDP-43 CTFs, and prevented stress granule formation (Parker et al., 2012). Oral administration of CuATSM also reduced both cytoplasmic accumulation of full-length and C-terminal fragments of TDP-43 in SOD1-G93A mice (Soon et al., 2011) suggesting this therapy may be effective in sporadic ALS patients demonstrating SOD1 and/or TDP-43 pathology.

Clinical Trials

CuATSM is currently under investigation in clinical trials, with one phase I study complete (NCT02870634), a continuation of the study ongoing (NCT04313166), and a placebo-controlled study currently recruiting (NCT04082832) at the time of publication.

Anti-sense Oligonucleotides

Anti-sense oligonucleotides (ASO) can bind to mRNA and reduce protein expression, representing a potential therapeutic strategy (Bennett and Swayze, 2010), and using ASOs targeted at proteins such as SOD1 and TDP-43 can prevent protein aggregation and ALS.

Superoxide Dismutase-1

Smith (2006) designed ISIS 333611, an ASO which exhibits a high binding affinity for SOD1. Intraperitoneal injection of ISIS 333611 reduced both mRNA and protein levels of SOD1 in transgenic rats expressing human SOD1-G93A. Also, injection of ISIS 333611 extended the survival of transgenic SOD1-G93A rats to 132 days, compared with rats injected with sham controls, with a mean survival of 122 days. Finally, the transfection of fibroblasts sourced from a human SOD1-A4V ALS patient with ISIS 333611 demonstrated degradation of SOD1 protein (Smith, 2006).

The ASO Tofersen, which targets and degrades SOD1 mRNA, prevented neuron loss in SOD1-G93A mice extending survival by 40 days, and more than 50 days in SOD1-G93A rats (McCampbell et al., 2018). Tofersen was recently used in a Phase-I/II clinical trial, which reduced SOD1 mRNA concentrations at the highest Tofersen concentration administered (100 mg), but

was not sufficiently powered to report on motor improvements or survival (Miller et al., 2020), and requires further investigation.

Transactive Response DNA-Binding Protein 43

TDP-43 is an essential protein for cellular function, therefore, TDP-43 expression cannot be completely prevented or cell death will occur (Sephton et al., 2012). However, some therapeutic strategies have overcome this by targeting important interactors of TDP-43, such as ataxin-2. Crossing an ataxin-2 deficient mouse strain with a TDP-43 transgenic line led to reduced TDP-43 inclusion formation, reduced stress granule formation, and prolonged survival (Becker et al., 2017). Furthermore, a single intracerebroventricular injection of an ASO that targets ataxin-2 (3 μl of a 15 μg/μl) resulted in reduced ataxin-2 mRNA levels with no effect on TDP-43 mRNA expression. However, there were reduced numbers of TDP-43 inclusions in spinal cords, as well as improved locomotor function and significantly increased mean survival time (>300 days) compared with vehicle-treated mice (29 days).

Clinical Trials

Intrathecal injection of ISIS 333611 (doses of 0.15, 0.5, 1.5, and 3 mg) over 11.5 h in phase I, randomized trial of SOD1 fALS was demonstrated to be well tolerated, however, the therapeutic benefits were not measured (Miller et al., 2013). With regards to ASO targeting ataxin-2, due to the injection route chosen and the timing of injection in mice (neonatal), the feasibility of this ASO needs to be carefully considered.

CLR01

Protein folding in cells is dysregulated in ALS, resulting in protein aggregation. The design of "molecular tweezers" that interact with assembling proteins and acts as a pharmacological chaperone represents a strategy to stabilize this process and prevent aggregation (Klärner and Kahlert, 2003). The molecular tweezer CLR01 inhibits amyloid-protein self-assembly (Fokkens et al., 2005), is well-tolerated in mice (Attar et al., 2014), and represents a potential therapy for ALS.

Superoxide Dismutase-1

Malik et al. (2019) demonstrated that CLR01 binds to SOD1 and, using *in vitro* fibrillation assays, showed that CLR01 treatment reduced aggregation of wild-type SOD1 as well as mutant SOD1-E21K, SOD1-H46R, SOD1-D76Y, and SOD1-G93A. Immunohistochemical analysis demonstrated that CLR01 reduced SOD1 aggregates in microglia, but failed to improve locomotor function or survival in SOD1-G93A mice irrespective of the dose used (0.5 or 5 mg/kg—daily subcutaneous injection; Malik et al., 2019).

Transactive Response DNA-Binding Protein 43

In a recent study, Monaco et al. (2020) demonstrated that TDP-43 is present in amyloid aggregates in a murine model of the lysosomal storage disease mucopolysaccharidosis (MPS) type IIIA, and aggregates could be reduced by CLR01; however, there was no evidence of CLR01 directly interacting with TDP-43.

4,5-BIS[(*N*-CARBOXY METHYL IMIDAZOLIUM)METHYLACRIDINE] DIBROMIDE (AIM4)

Transactive Response DNA-Binding Protein 43

Prasad et al. (2016) screened a range of acridine derivatives to determine the effect on TDP-43 aggregation. They found that the acridine derivative, [4,5-bis[(N-carboxy methyl imidazolium)methylacridine] dibromide (AIM4) reduced aggregation of C-terminal TDP-43 in yeast. A recent follow-up study found that AIM4 could prevent the fibrillation of purified TDP-43$^{(194–414)}$ (Girdhar et al., 2020). However, further validation of the therapeutic potential and assessment of the toxicity of this drug *in vivo* has not been conducted at this time.

THERAPIES TARGETING PROTEOSTATIC IMPAIRMENT

Arimoclomol

Superoxide Dismutase-1

Hydroxylamine derivatives are a family of compounds that up-regulate the expression of heat shock proteins (HSPs; Vígh et al., 1997). The hydroxylamine derivative arimoclomol differs from other derivatives in that it up-regulates HSP expression only in cells under stress (Hargitai et al., 2003), and works by activating heat shock factor 1 and promoting up-regulation of HSP70 and HSP90 (Kalmar et al., 2014). The up-regulation of these HSPs by arimoclomol reduces SOD1 aggregation. Intraperitoneal injection of arimoclomol into SOD1-G93A mice improved motor neuron survival and increased mean survival to 153 days, compared with untreated SOD1-G93A mice (with a mean survival of 125 days; Kieran et al., 2004; Kalmar et al., 2008). Importantly, this effect was observed if treatment was administered early or at disease onset, and the effect was observed regardless of sex (Kieran et al., 2004; Kalmar et al., 2008).

Transactive Response DNA-Binding Protein 43

Activation of HIF-1 is important in maintaining the solubility of TDP-43 and preventing aggregation, as well as maintaining cell survival in SH-SY5Y neuroblastoma cells (Chen et al., 2016; Lin et al., 2016). In a murine model of TDP-43-acetylation, which causes TDP-43 to localize in the cytosol and form inclusions, activation of HIF-1 reduced TDP-43 inclusion formation. The authors suggest this could be achieved with the use of arimoclomol, however, these experiments are yet to be performed (Wang et al., 2017).

Fused in Sarcoma

In iPSC-derived spinal motor neurons, arimoclomol was able to retain FUS in the nucleus, and restore the DNA-damage response in cells expressing ALS-mutant FUS^{R521H} (Kuta et al., 2020).

Clinical Trials

Arimoclomol has been used in clinical trials for ALS and is well-tolerated in doses up to 200 mg thrice daily, however, the evidence of a benefit in patients remains inconclusive (Benatar et al., 2018). For a recent review of proteostasis-targeting therapies in clinical trials see McAlary et al. (2019a).

Pridopidine

The Sigma-1 receptor (S1R) is an important chaperone that prevents protein aggregation (Hong et al., 2017), suppresses ROS production (Meunier and Hayashi, 2010), and maintains cell survival (Hayashi and Su, 2007). S1Rs are reduced in spinal cords of ALS patients (Prause et al., 2013), and S1R KO mice exhibit locomotor deficiencies and MN loss (Bernard-Marissal et al., 2015). Pridopidine is a Sigma-1 receptor agonist (Sahlholm et al., 2015), and represents a potential therapeutic for ALS.

Superoxide Dismutase-1

Ionescu et al. (2019) demonstrated that incubation of primary SOD1-G93A motor neuron cultures with pridopidine reduces MN death and restores axonal transport and MN junction activity. Furthermore, subcutaneous administration of pridopidine (daily, 3 or 30 mg/kg) in SOD1-G93A mice reduced SOD1 aggregation in spinal cords, prevented muscle fiber atrophy, and preserved NM junctions, but pridopidine treatment alone did not lead to improved survival of SOD1-G93A mice (Ionescu et al., 2019).

Clinical Trials

Pridopidine is well tolerated (45, 67.5, 90, and 112.5 mg taken orally twice daily) in Huntington's disease patients (Reilmann et al., 2019). A clinical trial of pridopidine in ALS is set to commence at Massachusetts General Hospital.

CONCLUSION

Years of intensive study into the disease mechanisms of ALS strongly implicates a pathological role for both declining proteostasis and prion-like propagation. There are intimate links between both of these concepts that explain much of the pathological manifestation of ALS. For example, the observation of proteinaceous inclusions in affected tissues, the apparent spread of pathology through neuroanatomically connected tracts, and the preferential degeneration of motor neurons over other cell types. SOD1 and TDP-43 can misfold and aggregate into conformations that can propagate within and between cells in a prion-like manner, as evidenced by the studies mentioned above ranging from biophysical assays of protein stability to whole-organism studies of aggregate seeding. Indeed, this knowledge of proteostasis dysfunction and protein aggregation has led to the development and examination of multiple therapeutics for ALS. The questions that remain, however, are what the conformation of these spreading strains are, the precise mechanism by which they contribute to pathology, and if targeted therapies can be designed to prevent their formation or spread. Also, a question of significant importance to the field is whether FUS can spread pathology from cell-to-cell in a pathological prion-like manner. This knowledge would unify our understanding of the underlying pathomechanisms in ALS.

AUTHOR CONTRIBUTIONS

LM directed the manuscript. All authors wrote and edited the manuscript. NRC and JJY conceptualized the manuscript. All authors contributed to the article and approved the submitted version.

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Cytoplasmic Expression of the ALS/FTD-Related Protein TDP-43 Decreases Global Translation Both *in vitro* and *in vivo*

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TDP-43 is a major component of cytoplasmic inclusions observed in neurodegenerative diseases like frontotemporal dementia (FTD) and amyotrophic lateral sclerosis (ALS). To further understand the role of TDP-43 in mRNA/protein metabolism and proteostasis, we used a combined approach with cellular and animal models overexpressing a cytoplasmic form of human TDP-43 (TDP-43-ΔNLS), recapitulating ALS/FTD features. We applied in HEK293 cells a method for labeling *de novo* translation, surface sensing of translation (SUnSET), based on puromycin (PURO) incorporation. While control cells displayed robust puromycilation, TDP-43-ΔNLS transfected cells exhibited reduced ongoing protein synthesis. Next, by using a transgenic mouse overexpressing cytoplasmic TDP-43 in the forebrain (TDP-43-ΔNLS mice) we assessed whether cytoplasmic TDP-43 regulates global translation *in vivo*. Polysome profiling of brain cortices from transgenic mice showed a shift toward non-polysomal fractions as compared to wild-type littermates, indicating a decrease in global translation. Lastly, cellular level translational assessment by SUNSET was performed in TDP-43-ΔNLS mice brain slices. Control mice slices incubated with PURO exhibited robust cytoplasmic PURO signal in layer 5 neurons from motor cortex, and normal nuclear TDP-43 staining. Neurons in TDP-43-ΔNLS mice slices incubated with PURO exhibited high cytoplasmic expression of TDP-43 and reduced puromycilation respect to control mice. These *in vitro* and *in vivo* results indicate that cytoplasmic TDP-43 decreases global translation and potentially cause functional/cytotoxic effects as observed in ALS/FTD. Our study provide *in vivo* evidence (by two independent and complementary methods) for a role of mislocalized TDP-43 in the regulation of global mRNA translation, with implications for TDP-43 proteinopathies.

Keywords: TDP-43, frontotemporal dementia (FTD), amyotrophic lateral sclerosis (ALS), transgenic mice, protein synthesis, animal model, proteinopathy

INTRODUCTION

TAR DNA-binding protein 43 (TDP-43) is a ubiquitously expressed, predominantly nuclear protein with multiple roles in RNA processing (Buratti et al., 2001; Gao et al., 2018; Nussbacher et al., 2019). In neurodegenerative diseases such as amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD), a key finding is that pathological TDP-43 accumulates in the cytoplasm, forming aggregates (Arai et al., 2006; Neumann et al., 2006; Ito et al., 2017). Nevertheless, how this mislocalization contributes to pathogenesis is still poorly understood.

Although the most extensively described function of TDP-43 has been its role in mRNA splicing, there has been reports consistent with a role for this protein in translational regulation. A proteomic study in HEK293 cells showed that TDP-43 interact extensively with proteins of the translational machinery (Freibaum et al., 2010) and *in vitro* downregulation of TDP-43 alters global translational yield through a SKAR-dependent mechanism (Fiesel et al., 2012). Moreover, the ribosomal protein RACK1 seems to be a key mediator of the interaction of TDP-43 with the translational machinery (Russo et al., 2017).

Using inducible transgenic mice, we demonstrated that expression of a cytoplasmic form of TDP-43 (termed TDP-43-ΔNLS) recapitulates multiple features of TDP-43 proteinopathies of the ALS/FTD spectrum, including progressive neurodegeneration, gliosis, global gene expression changes and behavioral abnormalities encompassing the motor, cognitive and social domains (Igaz et al., 2011; Alfieri et al., 2014). Other animal models with cytoplasmic TDP-43 expression, covering from *Drosophila* to mammalian systems, also support the idea that this mislocalization event might be pathogenically relevant (Miguel et al., 2011; Walker et al., 2015; Wang et al., 2015; Yin et al., 2019).

While most studies have focused on the regulation of global or specific translation using cell lines or primary neurons in culture, the consequences of *in vivo* TDP-43 manipulation (specifically, of its cytoplasmic form) are virtually unknown. In this work, we investigated both *in vitro* and *in vivo* if cytoplasmic expression of TDP-43 modifies global protein synthesis, using two different approaches, polysome profiling and surface sensing of translation (SUnSET) technique.

MATERIALS AND METHODS

Surface Sensing of Translation (SUnSET) in Cultured Cells

Cell Culture and Transfection

Myc epitope-tagged hTDP-43-ΔNLS construct was generated in pcDNA 5/To plasmid (Invitrogen) using restriction sites BamHI and XbaI, as previously described (Winton et al., 2008). Human embryonic kidney HEK293T cells were grown in high glucose (4.5 g/L glucose) Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% fetal bovine serum, penicillin/streptomycin (100 units/ml and 100 μg/ml respectively, Thermo Fisher Scientific), and 110 mg/L of sodium pyruvate (Thermo Fisher Scientific) in a 37°C humidified incubator containing 5% CO_2. Cells (5×10^5 per well) were plated on poly-D-lysine-coated 12 mm glass coverslips in a 6-well plate, and grown for 24 h before transfection. Lipofectamine 2000 reagent (Invitrogen) was used for transfection of constructs (empty vector as control or hTDP-43-ΔNLS), according to the manufacturer's instructions. After 48 h, medium was replaced with fresh medium with or without puromycin (10 μg/ml, P7255, Sigma) for 20 min and washed with PBS.

Immunocytochemistry

Cells on coverslips were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 10 min, permeabilized with 0.2% Triton X-100 (Sigma) in PBS for 10 min, blocked with 5% powdered milk in PBS for 2 h, and incubated overnight with primary antibody at 4°C. Primary antibodies used in this study (diluted in 5% powdered milk in PBS) were as follows: anti-total-TDP-43 [1:5000, rabbit polyclonal anti-TDP-43 1038C (Igaz et al., 2008)] and anti-puromycin (1:1000 dilution, MABE343, clone 12D10, Millipore). Primary antibodies were visualized with secondary antibodies conjugated with Alexa Fluor 488 and Alexa Fluor 594 (1:500, donkey anti-mouse and donkey anti-rabbit IgG, respectively, Jackson Immunoresearch), and nuclei were detected using Hoescht 33258 (Sigma). Coverslips were mounted in gelatin-coated slides using Fluoro-gel (Thermo Fisher Scientific) and stored at 4°C. Images were obtained with a Zeiss AxioImager 2 microscope equipped with APOTOME 0.2 structured illumination, using a Hamamatsu Orca Flash 4.0 camera. Four areas per image were analyzed using ImageJ software (version 1.52a). Mean puromycin and TDP-43 signals were calculated and normalized to empty vector (+PURO) group signal.

Animals

hTDP-43-ΔNLS line was generated as described in Igaz et al. (2011). Monogenic tetO-TDP-ΔNLS mice were bred to Camk2a-tTA mice (Jackson Laboratory) generating non-transgenic, tTA monogenic, single tetO-TDP-43 transgenic mice, and bigenic mice expressing hTDP-43-ΔNLS. Breeding mice and pups were treated with 0.2 mg/ml doxycycline hyclate (DOX; sc-204734A; Santa Cruz Biotechnology) in drinking water, to avoid pre-natal and post-natal developmental effects. Transgene expression was activated at weaning (post-natal day 28) by removing DOX from water and mice were analyzed after ∼1 month (at post-natal day 60). Tail DNA was used for genotyping, employing primers described in Igaz et al. (2011). The experimental protocol for this study was approved by the National Animal Care and Use Committee of the University of Buenos Aires (CICUAL). All animal procedures were performed according the NIH Guide for the Care and Use of Experimental Animals.

Abbreviations: ACSF, artificial cerebrospinal fluid; ALS, amyotrophic lateral sclerosis; Ctrol, control; DOX, doxycycline; FTD, frontotemporal dementia; IF, immunofluorescence; mRNA, messenger ribonucleic acid; NP, non-polysomal fraction; P, polysomal fraction; PBS, phosphate buffered saline; PURO, puromycin; RBP, RNA binding protein; RNA, ribonucleic acid; RNP, ribonucleoprotein; SUnSET, surface sensing of translation; TDP-43, TAR DNA-binding protein 43.

Polysome Profiling

Sucrose Gradient Preparation

Cortices of control (non-transgenic) or hTDP-43-ΔNLS animals were homogenized in cold lysis buffer [15 mM Tris-HCl, pH 8.0, 300 mM NaCl, 15 mM $MgCl_2$, 1% Triton X-100, 1 mg/ml heparin, protease inhibitors (Roche) and 0.1 mg/ml cycloheximide (Sigma-Aldrich Inc.)] at a ratio of 500 µl buffer /10 mg tissue. Homogenates were then placed on ice for 10 min and centrifuged at 5,000 rpm for 10 min at 4°C. Supernatant, consisting in cytosolic extract containing polysomes, was collected and protein concentration was determined by bicinchoninic acid (BCA) method (Pierce). Sucrose gradient, ranging from 15 to 50%, were prepared in gradient buffer (15 mM Tris-HCl, pH 8.0, 300 mM NaCl, 5 mM $MgCl_2$, 1 mg/ml heparin and 0.1 mg/ml cycloheximide). Briefly, 2.4 ml 50% sucrose solution was added to a tube and chilled for 30 min at −80°C. The procedure was repeated with layers of sucrose solution at lower concentration (41.25, 32.5, 23.75, and 15%) and stored at 4°C if used the next day. 0.5 to 1 mg of cytosolic protein extract were added to each gradient, and centrifuged in a SW40 rotor at 35,000 rpm for 2–4 h, at 4°C. Five hundred microliters aliquots were separated and absorbance at 260 nm was measured (Eppendorf Biophotometer Plus, Germany). A peak area ratio between polysomal (P) and non-polysomal (NP, comprising RNPs and monosomes) fractions was determined for each group.

Western Blot

Five hundred microliters of sample from each fraction was subjected to methanol/chloroform protein extraction method and resuspended in sample buffer. Equal amounts of samples were resolved on 10% sodium dodecyl Sulfate polyacrilamyde gel electrophoresis (SDS-PAGE) and transferred onto PVDF membranes (Immobilon-P; Millipore). After transfer, membranes were stained with Ponceau S for total protein load assessment, then blocked in 5% powdered milk in 0.1% Tween-TBS, and incubated in anti-total TDP-43 primary antibody overnight (1038C, 1:20000). Detection of primary antibody was performed with horseradish peroxidase-conjugated secondary antibody (Jackson Immunoresearch), and ECL was used for blot development. Digital images were acquired using a G-BOX Imager (Syngene). Ponceau S staining was used as a control for equal protein loading in our immunoblot analysis of polysomal fractions, since typical housekeeping genes are barely detectable in polysome fractions (Reschke et al., 2013).

Surface Sensing of Translation (SUnSET) in Brain Slices

Tissue Processing

SUnSET labeling in brain slices was adapted from Cook et al. (2014), with some modifications. Brain coronal slices were prepared from 8-week old (1 month off-DOX) hTDP-43-ΔNLS or control mice littermates. Slices were cohort and age matched. Mice were anesthetized with isoflurane and decapitated; brain was removed and chilled ice-cold in 95 O_2-5% CO_2-saturated, sucrose-based artificial cerebrospinal fluid (ACSF, 220 mM sucrose, 2.5 mM KCl, 1 mM $CaCl_2$, 6 mM $MgCl_2$, 1.25 mM NaH_2PO_4, 26 mM $NaHCO_3$, and 10 mM dextrose). The brain was next glued onto a vibratome stage and acute sagittal slices (400 µm) were cut. Then, the slices were recovered at room temperature for an hour in ACSF (125 mM NaCl, 2.5 mM KCl, 2 mM $CaCl_2$, 1.1 mM $MgCl_2$, 1.25 mM NaH_2PO_4, 26 mM $NaHCO_3$, and 10 mM glucose, pH 7.4). After recovery, slices were placed on a fine grid mesh in a petri dish and incubated in ACSF (C- group) or ACSF supplemented with 5 µg/µl puromycin (P7255, Sigma-Aldrich Inc.; C+ or ΔNLS+ groups) at a final volume of 30 ml. Incubation time was 45 min under continuous aeration with carbogen (95% O_2/ 5% CO_2). Slices were then rinsed in ACSF and fixated in 4% paraformaldehyde (Sigma-Aldrich Inc.) in 0.1 M PBS for 24 h at 4°C. Next day, slices were washed three times in 0.01 M PBS for 10 min each under continuous agitation in an orbital shaker, incubated with 10% sucrose in 0.01 M PBS for 2 h at 4°C and stored in 30% sucrose-0.01 M PBS at −20°C until use.

Immunofluorescence

Brain slices containing the motor cortex [approximately bregma 1.18 mm, (Paxinos and Franklin, 2008)] were selected and washed three times in 0.01 M PBS for 10 min. Permeabilization step was performed with 0.01 M PBS-1% Triton X-100 for 1 h at room temperature. Next, slices were incubated with blocking solution containing 5% normal goat serum in 0.01 M PBS-0.3% Triton X-100 for 1 h at room temperature. Sections were immunolabeled with rabbit polyclonal anti-TDP-43 (1038C, 1:15000) and mouse monoclonal anti-puromycin (MABE343 clone 12D10, Millipore, 1:4000) as primary antibodies; incubation was performed first at room temperature for 3 h followed by 48 h at 4°C. Staining against the neuronal marker NeuN (mouse monoclonal MAB377, Millipore, 1:1000) or human TDP-43 protein (mouse monoclonal 60019-2, Proteintech, 1:10000) was performed in 50 µm slices as described in Alfieri et al. (2014). Then, slices were washed three times for 10 min each with 0.01 M PBS-0.04% Triton X-100 and incubated with Alexa Fluor 594 goat anti-rabbit IgG and Alexa Fluor 488 goat anti-mouse IgG (each at 1:500 dilution, Jackson laboratories) for 4 h at room temperature in darkness. Slices were washed three times for 10 min with 0.01 M PBS-0.04% Triton X-100 and subjected to Hoescht staining (1:2000 dilution in 0.01M PBS) for 30 min at room temperature in darkness. Finally, sections were washed in 0.01 M PBS two times (10 min each) and mounted in gelatin-coated glass slides with 30% glycerol in 0.01 M PBS, covered with glass coverslips, sealed and stored in darkness at 4°C until use. All incubation and wash steps were performed in a 12-well plate under continuous agitation in an orbital shaker.

Image Detection and Analysis

Images were obtained with a Zeiss AxioImager 2 microscope equipped with APOTOME.2 structured illumination, using a Hamamatsu Orca Flash 4.0 camera. Two sections of layer 5 cells of motor cortex were analyzed using ImageJ software (version 1.52a). In non-transgenic (control, C) mice incubated with puromycin (C+), each cellular area was defined in the green channel corresponding to puromycin signal, as well

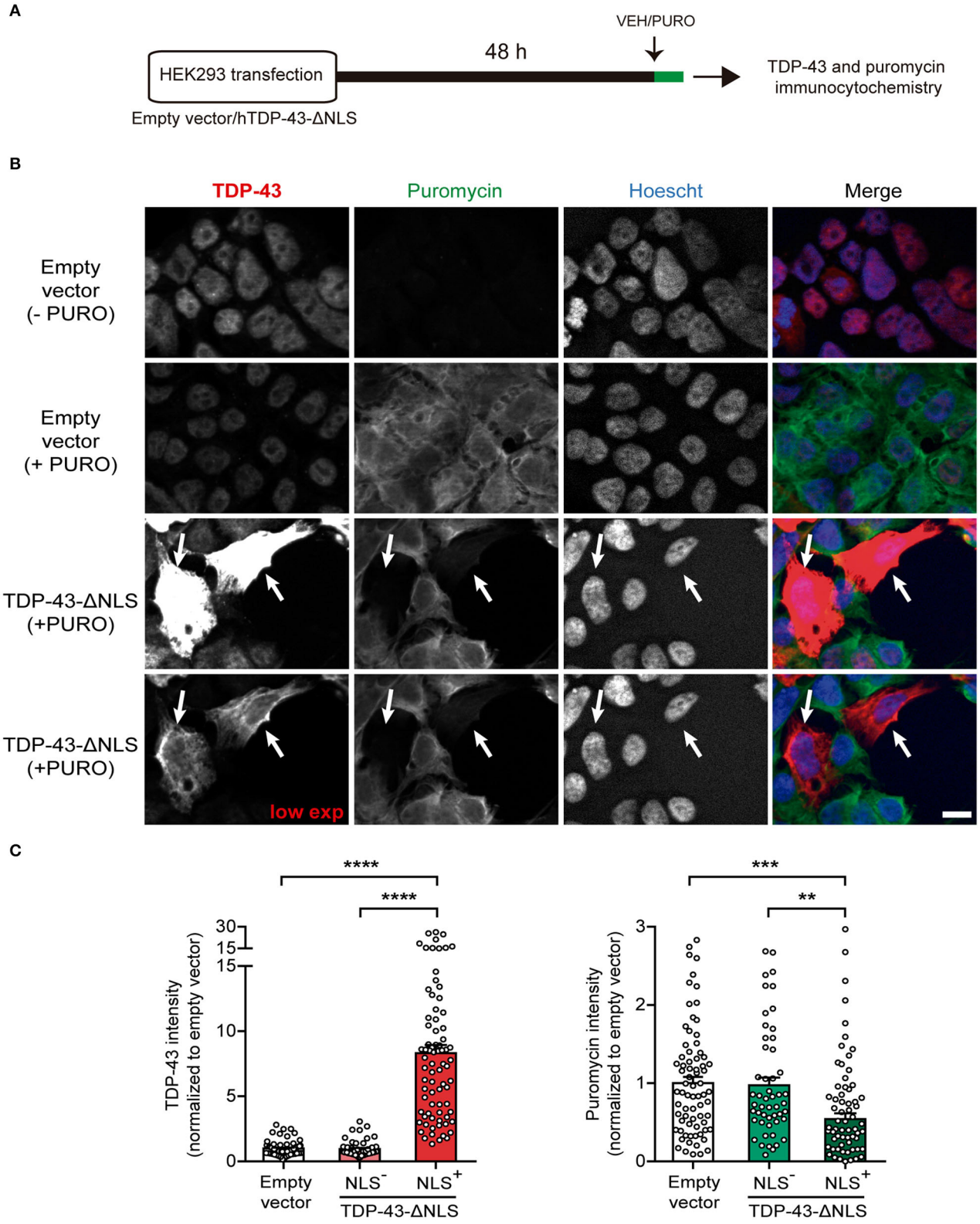

FIGURE 1 | Overexpression of a cytoplasmic form of TDP-43 affects global protein synthesis in HEK293 cells. **(A)** Workflow diagram of single cell puromycilation measurement by SUnSET. HEK293 cells were transfected with empty vector or TDP-43-ΔNLS, and 72 h later, a single 20 min puromycin (+ PURO) or Vehicle (VEH, -PURO) pulse was applied to the cultures. Then, cells were subjected to total TDP-43 and puromycin IF, and Hoescht 33258 was used as a nuclear marker.

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FIGURE 1 | (B) Representative micrographs of total TDP-43 (red) and puromycin (green) IF staining. Control (empty vector) cells treated with vehicle (- PURO) served to assess non-specific staining and no puromycin signal was detected, while control (+ PURO) cells exhibited robust puromycilation. In both control groups, TDP-43 labeling was nuclear (as expected for endogenous expression), while TDP-43-ΔNLS transfected cells showed intense, cytoplasmatic expression. A lower exposure for the TDP-43 channel is also shown. Puromycin staining is visibly decreased in TDP-43-ΔNLS transfected cells (arrows). Scale bar: 10 μm. **(C)** Quantitative analysis of puromycilation signal revealed that cells with cytoplasmic TDP-43 (NLS+) exhibit a decreased ongoing translation. Staining intensity of TDP-43-ΔNLS immunopositive cells show significant differences when compared to puromycin-labeled control cells and non-transfected (NLS-) cells. TDP-43 levels were significantly higher in NLS+ than in the control or non-transfected groups (as expected for transgene overexpression). Data represent mean ± SEM; $n = 75$ cells per condition (from three replicates). One-way ANOVA/Tukey's multiple comparison test: $^{****}P < 0.0001$, $^{***}P < 0.001$, $^{**}P < 0.01$.

as background areas between cells; the same approach was employed in hTDP-43-ΔNLS animals incubated with puromycin (ΔNLS+) but in the red channel (total TDP-43 signal). For non-transgenic mice incubated in ACSF (C-), a circular area centered in each red nucleus and background areas between cells were quantified. Background signal was normalized to individual cell areas, and a ratio between raw cell intensity signal and normalized background signal was calculated. The signal from individual cell populations and averaged signal per animal were compared between experimental groups.

Statistical Analysis

Data are expressed as means ± SEM. Statistical analysis was performed using a standard unpaired two-tailed student's *t*-test when comparing only two groups on one measure. A one-way ANOVA followed by Tukey's multiple-comparison *post hoc* test was used when comparing three groups. Prism Version 8 (GraphPad Software) was used for statistical analysis and differences were considered to be statistically significant when the probability value was < 0.05.

RESULTS

As a first approach to study at the cellular level the effect of cytoplasmic TDP-43 expression on protein synthesis, we adapted the SUnSET methodology (Schmidt et al., 2009) to measure single-cell puromycilation by immunocytochemistry (**Figure 1**). Puromycin incorporation into nascent polypeptides enables direct visualization of actively translating ribosomes. We transfected either empty vector or TDP-43-ΔNLS in HEK293 cells and, during the last 20 min of the experiment, applied puromycin (+ PURO) or vehicle (-PURO) to the cultures (**Figure 1A**). Representative micrographs show immunofluorescence staining using antibodies against total TDP-43 or puromycin (**Figure 1B**). Control (empty vector) cells treated with vehicle (-PURO) were used to assess non-specific staining and showed virtually no puromycin signal, while control (+PURO) cells displayed robust puromycilation. As expected, TDP-43 staining showed nuclear, endogenous TDP-43 for both control groups, while TDP-43-ΔNLS transfected cells demonstrate abundant, cytoplasmic expression in a proportion of HEK293 cells. Remarkably, analysis of puromycilation signal in TDP-43-ΔNLS transfected cells demonstrates that cells with cytoplasmic TDP-43 (NLS^+) show a decrease in ongoing protein synthesis (arrows, **Figure 1B**). Quantification of puromycin staining intensity in TDP-43-ΔNLS positive cells show significant differences against both the puromycin-labeled control cells and TDP-43-ΔNLS negative (non-transfected) cells (**Figure 1C**). As expected, TDP-43 levels are significantly higher in TDP-43-ΔNLS cells than in the control or non-transfected groups (**Figure 1C**). Overall, these results demonstrate at cellular resolution that cytoplasmic TDP-43 decreases ongoing protein synthesis in cultured cells.

Next, we wanted to extrapolate these results *in vivo*, taking advantage of our inducible human TDP-43-ΔNLS mouse model with CamKIIα-driven transgene expression enriched in forebrain neurons (Igaz et al., 2011). We first aimed to study global protein synthesis by polysome profiling (**Figure 2**), a method that allows for translational state assessment by differential separation of mRNA-protein complexes in a sucrose gradient (Chasse et al., 2017). TDP-43-ΔNLS or control mice were generated (**Figure 2A**) and transgene induction was started at P28 to avoid developmental deficits. Animals were sacrificed at P60 (after 1 month of transgene expression), a time point when these mice already develop ALS/FTD-like phenotypes (Igaz et al., 2011; Alfieri et al., 2014; Walker et al., 2015) (**Figure 2B**). Immunofluorescence analysis of TDP-43-ΔNLS mice demonstrate robust cytoplasmic transgene expression in cortical neurons, as revealed by an antibody that specifically stains human TDP-43 (i.e., the transgene product) but not endogenous mouse TDP-43 (**Figure 2C**). Polysome profiles from control (non-transgenic) or TDP-43-ΔNLS mice cortical homogenates show an increase in the free ribonucleoprotein (RNP) fraction (low density) and a decrease in translationally active fractions of high density (polysomes) in transgenic mice (**Figure 2D**). Interestingly, immunoblots using total TDP-43 antibody show that overexpression of TDP-43-ΔNLS leads to an increased presence of TDP-43 in most gradient fractions, but especially in those with higher density. Quantification of the ratio between areas under the curve for polysomal and non-polysomal (i.e., non-translationally active) fractions demonstrate a significant reduction in transgenic mice (**Figure 2E**), indicating that cortical global protein synthesis is reduced *in vivo* due to cytoplasmic TDP-43 expression.

Our polysome profiling data strongly suggest that global translation was decreased in the brains of TDP-43 mice, but this technique does not allow for examination of cell-specific populations. To elucidate if TDP-43-ΔNLS mice display altered ongoing protein synthesis *in vivo* at the neuronal level, we applied the SUnSET method in slices from control or transgenic mice and studied puromycilation levels in neurons from layer 5 of the motor cortex (**Figure 3**). We evaluated protein synthesis after 1 month of TDP-43-ΔNLS expression, using acute thick slices (400 μm) from coronal vibratome

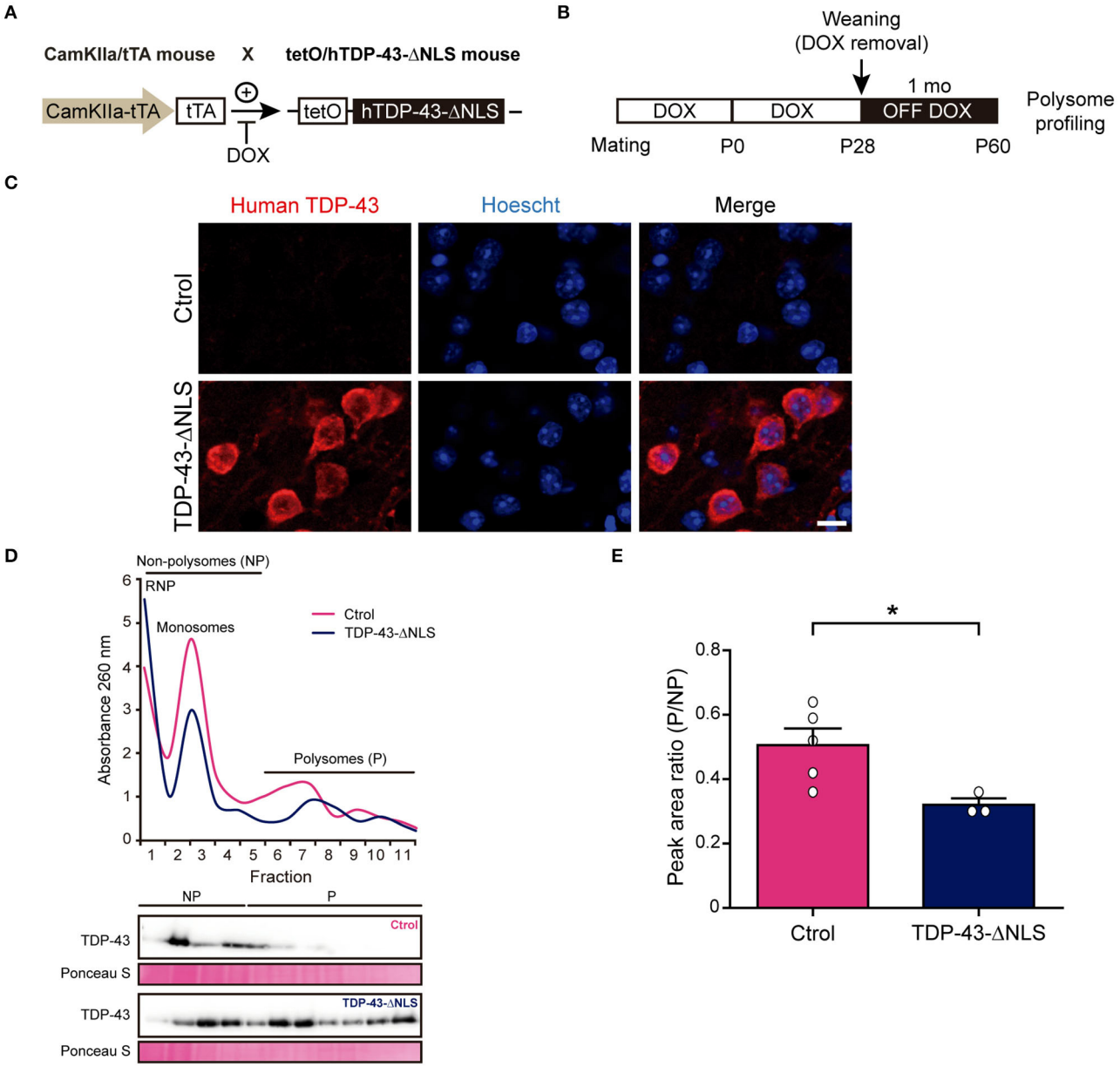

FIGURE 2 | Transgenic TDP-43-ΔNLS mice have an altered polysome profile in brain cortex. **(A)** Generation of hTDP-43-expressing mice. CamKIIa/tTA mice are crossed with tetO/hTDP-43-ΔNLS mice. In bigenic mice, doxicycline (DOX) inhibits tTA binding to the tetracycline–responsive element (tetO), thus repressing hTDP-43 expression. Non-transgenic littermates were used as controls. **(B)** Timeline and experimental workflow. Mating and breeding of animals was carried in the presence of DOX to avoid developmental effects. Transgene expression was induced at weaning (P28) by removing DOX from water (OFF DOX), and mice were analyzed at ∼1 month post-induction (P60). Then, brain cortices were subjected to polysome profiling. **(C)** Representative micrographs of human-specific TDP-43 antibody IF in control (Ctrol) and TDP-43-ΔNLS mice at 1 month off DOX. Transgenic animals show robust cytoplasmic expression of the transgene in cortical neurons. Hoescht 33258 was used as a nuclear marker. Scale bar: 10 μm. **(D)** Representative cortical homogenate polysome profiles in non-transgenic (Ctrol) and transgenic (TDP-43-ΔNLS) mice. In TDP-43-ΔNLS animals, the free ribonucleoprotein (RNP) fraction is increased while the translationally active fractions (polysomes, P) are decreased. Immunoblots against total TDP-43 using protein isolated from the individual fractions revealed that overexpression of TDP-43-ΔNLS increases the presence of total TDP-43, especially in the polysomal fractions. Ponceau S was used as a loading control. **(E)** Quantification of the ratio between the areas under the curve for polysomal (P) and non-polysomal (NP) fractions. Transgenic mice display a significant reduction, indicating that global translation in brain cortex is decreased due to cytoplasmic TDP-43 expression. Data represent mean ± SEM; $n = 5$ for Ctrol, $n = 3$ for TDP-43-ΔNLS. $^*P < 0.05$, significantly different from Ctrl mice (Student's *t*-test).

sections. After a short incubation with puromycin, nascent peptides were labeled and detected at the cellular level with anti-PURO immunofluorescence (**Figure 3A**). Additionally, we used an anti-TDP-43 antibody to identify and evaluate both cells expressing endogenous TDP-43 protein (nuclear), and neurons expressing transgenic TDP-43-ΔNLS (cytoplasmic) for further

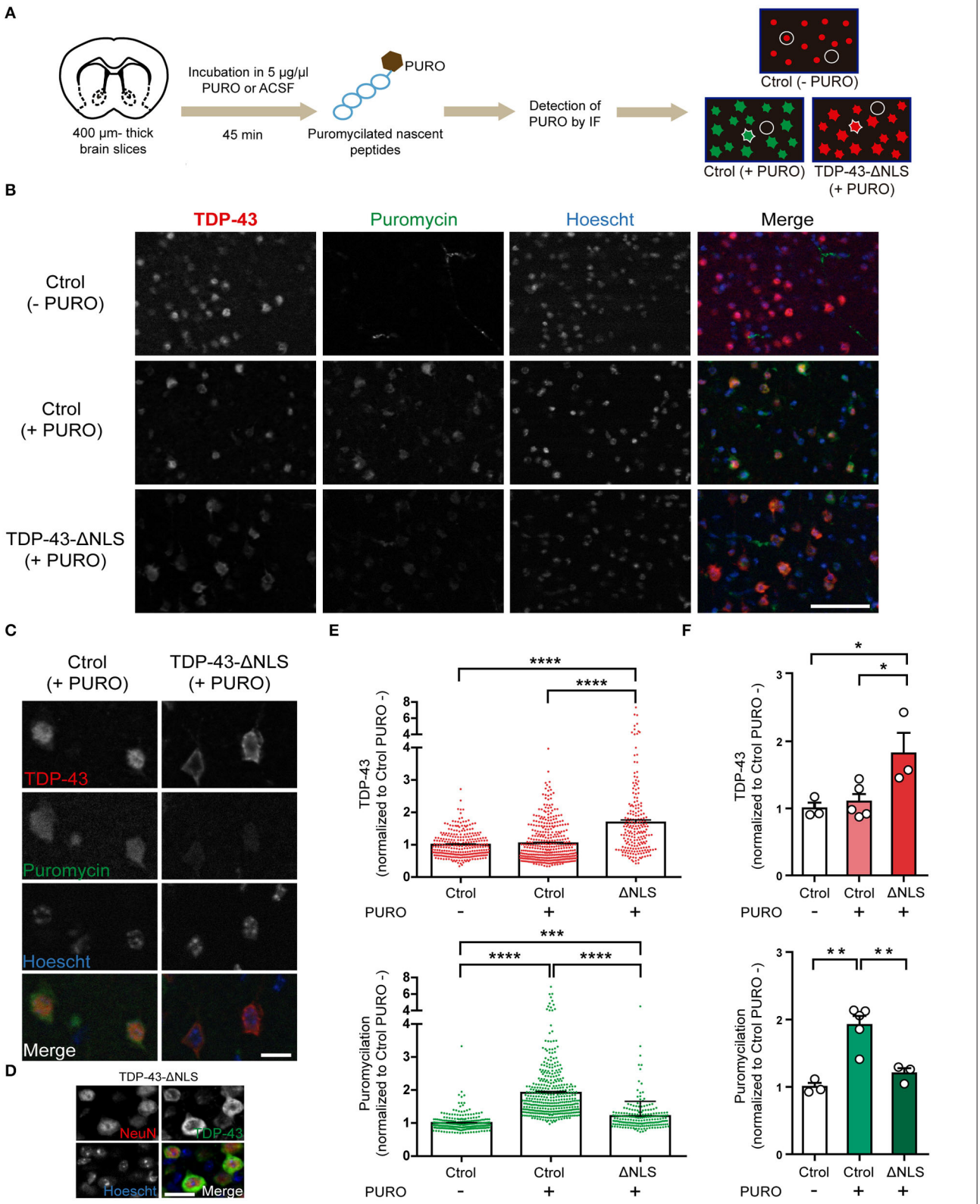

FIGURE 3 | Cortical motor neurons from TDP-43-ΔNLS mice show decreased ongoing protein synthesis *in vivo*. **(A)** Diagram of the puromycilation experiment (SUnSET technique). Coronal brain slices from non-transgenic (Ctrol) and transgenic (TDP-43-ΔNLS) mice were incubated in 5 µg/µl puromycin (+ PURO) or ACSF (- PURO) for 45 min. Detection of nascent puromycilated peptides and total TDP-43 was done by immunofluorescence (IF). Microscopy images of motor cortex

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FIGURE 3 | (layer 5) were analyzed. In Ctrol (+PURO) mice, cellular areas were defined in the green channel (puromycin signal, cell contour), as well as background areas between cells (circle); for TDP-43-ΔNLS (+PURO) animals the same approach was employed but using the red channel (total TDP-43 signal). For Ctrol (-PURO) mice, an area centered in the red nucleus (circle around the red dot) and background areas (circle) were registered. **(B)** Puromycin (green) and TDP-43 (red) IF in acute cortical slices from Ctrol (-PURO and +PURO) and ΔNLS (+PURO) mice. Hoescht 33258 was used as a nuclear marker. Scale bar: 50 μm. **(C)** Higher magnification images of representative cells as described in **(B)**. Scale bar: 10 μm. **(D)** Representative micrographs of double IF using the neuronal marker NeuN (red) and TDP-43 (green) in cortical sections from TDP-43-ΔNLS mice at 1 month off DOX. NeuN staining show that cells with cytoplasmic TDP-43 staining are neurons. Hoescht 33258 was used as a nuclear marker. Scale bar: 10 μm. **(E)** Fold change (respect to Ctrol -PURO) analysis of TDP-43 (top) and puromycin (bottom) cellular labeling. $n = 278$ for Ctrol (-PURO), $n = 396$ for Ctrol (+PURO), $n = 203$ for ΔNLS (+PURO). **(F)** Average TDP-43 (top) and puromycin (bottom) cell signal for each experimental mice. $n = 3$ for Ctrol (-PURO), $n = 5$ for Ctrol (+PURO), $n = 3$ for ΔNLS (+PURO). One-way ANOVA /Tukey's multiple comparison test; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$. Data represent mean ± SEM.

quantification. Low magnification immunofluorescence images form Layer 5 of the motor cortex show that non-transgenic, control mice (Ctrol) incubated with vehicle (ACSF) instead of puromycin (-PURO) showed normal nuclear staining of endogenous TDP-43 (**Figure 3B**). As expected, anti-PURO signal was negligible, although some non-specific staining of blood vessels can be observed (this is likely due to remaining IgG in the slices reacting with the anti-PURO Ab raised in mice). Slices from control mice incubated with puromycin display robust cytoplasmic signal in most cells, indicating proper labeling of ongoing protein synthesis. As expected, TDP-43 endogenous levels are also nuclear and with intensity comparable to control (-PURO) group (**Figure 3B**). TDP-43-ΔNLS mice slices incubated with PURO show the expected pattern of increased cytoplasmic expression of TDP-43, while these cells display a reduction in puromycilation (**Figure 3B**). A high-magnification view of cortical motor neurons from layer 5 clearly show that cells overexpressing cytoplasmic TDP-43 display reduced levels of anti-PURO signal (**Figure 3C**). Neuronal identity of cells with cytoplasmic TDP-43 was corroborated by NeuN and TDP-43 double immunofluorescence on thin cortical slices from TDP-43-ΔNLS mice (**Figure 3D**). Quantification at the cellular level demonstrates that TDP-43-ΔNLS mice display a significant increase in TDP-43 signal intensity compared to both control groups, and that PURO treatment does not affect TDP-43 levels (**Figure 3E**). Cellular analysis of puromycilation in those same neurons exhibits the expected significant increase in control groups treated with (+ PURO) vs. without (-PURO) puromycin. Importantly, neurons in TDP-43-ΔNLS mice display a drastic reduction in protein synthesis as revealed by anti-PURO signal (**Figure 3E**). These results are also evident when we pool data from each individual animal, with a significant reduction of puromycin signal in neurons from transgenic mice (**Figure 3F**).

DISCUSSION

Protein synthesis is an essential process for all cellular types, but neurons are particularly sensitive to proper control of this metabolic process due to their high polarization and the demands of plasticity mechanisms (Kapur et al., 2017). Several studies have suggested a role for translational dysfunction in a wide range of neurodegenerative diseases, including Alzheimer's, tauopathies, Huntington's, Prion disease, and the ALS/FTD spectrum (Ding et al., 2005; Halliday et al., 2015; Sun et al., 2015; Meier et al., 2016; Russo et al., 2017; Kamelgarn et al., 2018; Zhang et al., 2018; Joag et al., 2019; Koren et al., 2019). In this report, we show both in cultured cells and, more importantly, in cortical neurons *in vivo* that cytoplasmic TDP-43 leads to altered protein synthesis, expanding our understanding of the possible role of this RNA-binding protein (RBP) in ALS/FTD pathogenesis.

There are other examples of RBPs involved in neurological disease (Thelen and Kye, 2019), including FMRP (related to Fragile X syndrome) and FUS (associated with ALS/FTD) (Donlin-Asp et al., 2017; Zhou et al., 2018). FMRP regulates activity-dependent translation of its mRNA cargoes (Davis and Broadie, 2017), and some of its functions are performed in cooperation with other RBPs, including TDP-43 and Staufen 1 (Chu et al., 2019). FUS is similar in several aspects to TDP-43, and recent reports demonstrated that disease-related mutations in FUS can lead to suppression of global (Kamelgarn et al., 2018) or axonal translation (Lopez-Erauskin et al., 2018). However, most of these studies exploring the relationship between neurodegenerative disease-related proteins and translation were performed either in cultured cell lines or in primary cultures derived from different transgenic mouse models. A remarkable *in vivo* study recently showed that TDP-43 and DISC1 co-aggregate in frontotemporal lobar degeneration human samples and animal models, leading to disruption of dendritic local translation and aberrant behavior (Endo et al., 2018).

We show here that TDP-43-ΔNLS expression reduces the amount of puromycin incorporated into translating peptides, and found that although TDP-43-ΔNLS is abundantly localized to polysome fractions, there are fewer polysome-associated mRNAs. This raises the possibility that mislocalized TDP-43 may disrupt mRNA recruitment to polysomes. In addition, the direction of change in translation opposes that seen with TDP-43 knockdown (Fiesel et al., 2012), suggesting that rather than loss of function, there is a gain of toxic function that might be mediated by TDP-43 abnormal expression/localization. Further studies investigating these possibilities are warranted.

What could be the potential players involved in this translational effect mediated by TDP-43-ΔNLS? A report using SH-SY5Y cells has shown that cytoplasmic TDP-43 interacts with both RACK1 and ribosomal protein S6 (rpS6) as demonstrated by immunoprecipitation, and that TDP-43-ΔNLS co-localizes in the cytoplasm with RACK1 and rpS6, suggesting that TDP-43

associates to translational machinery (Russo et al., 2017). RACK1 is an associated ribosomal protein and functions as a docking site for several proteins on the translational machinery; in addition, it has been shown to accumulate in TDP-43 positive inclusions in motor neurons of ALS spinal cord cases (Russo et al., 2017). Other potential candidates are those identified in a proteomic study using HEK293T cells in which a main cluster of TDP-43-interacting proteins include a cytoplasmic "translational" cluster, which contains multiple translation initiation and elongation factors, and ribosomal subunits (Freibaum et al., 2010). Although it is a predominantly nuclear protein, it has been previously shown that TDP-43 shuttles between the nucleus and cytoplasm (Winton et al., 2008), and alteration of cytoplasmic TDP-43 levels might lead to abnormal formation of complexes involved in translation, due to an altered stoichiometric ratio between TDP-43 and its protein and RNA partners. Lastly, TDP-43 has been shown to regulate the alternative splicing of SKAR (Fiesel et al., 2012), a protein that positively affects cap-dependent protein translation (Ma et al., 2008). TDP-43 downregulation leads *in vitro* to a shift in SKAR α/β isoform ratio, which in turn increases global translation (Fiesel et al., 2012). Interestingly, Yang et al. (2014) used a mouse model to show that partial TDP-43 knock-down increased SKAR isoforms with exon 3 excluded, leading to a lower α/β isoform ratio in brain and spinal cord due to altered alternative splicing.

Different mechanisms induced by ALS/FTD-related proteins may lead to translational repression. Pathological forms of these proteins can trigger stress granule formation and/or can activate ER stress-induced pathways (i.e., PERK) leading to translational modulation events such as eIF2α phosphorylation (Cestra et al., 2017). High levels of misfolded proteins stimulate the Unfolded Protein Response (UPR), which tend to stabilize proteostasis and may lead to protein synthesis inhibition. Chronic, sustained activation of these pathways can lead to cell death (Li et al., 2006; Sano and Reed, 2013). ER stress markers have been detected in samples from ALS patients and animal models (Matus et al., 2013); remarkably, ER-stress induced HIPK2 activation and neurodegeneration has been described in TDP-43-ΔNLS mice (Lee et al., 2016). Alleviation of disease-related phenotypes can be achieved in neurodegenerative disease models by pharmacological and genetic interventions which lead to protein translation restoration (Moreno et al., 2013; Halliday et al., 2017). In this line, targeting UPR signaling is an attractive goal, given the variety of proof-of-concept drugs that act at different steps, such as PERK activation (i.e., GSK2606414) or eIF2α signaling (ISRIB), potentially enabling translational recovery (Costa-Mattioli et al., 2007; Kim et al., 2014; Halliday et al., 2015; Radford et al., 2015).

Given that our TDP-43-ΔNLS mouse model recapitulates several FTD/ALS-related behavioral phenotypes (Alfieri et al., 2014), our present results showing global translational inhibition suggest that this alteration can be part of a pathogenic mechanism affecting not only neuronal survival but also multiple behavioral domains. An important step in future studies will be to apply strategies that tend to restore protein synthesis levels and evaluate if the neurodegenerative and behavioral phenotypes of TDP-43 animal models can be ameliorated.

In conclusion, we have shown here by two different approaches that expression of cytoplasmic TDP-43 leads to a decrease in global translation. Although the role of TDP-43 in protein synthesis regulation has been discussed and explored recently, our *in vivo* results underscore its importance as a mechanistic link between the possible pathogenic molecular changes and the clinical and pathological manifestations of TDP-43 proteinopathies.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by National Animal Care and Use Committee of the University of Buenos Aires (CICUAL).

AUTHOR CONTRIBUTIONS

SC, LL, and LI designed the study. SC and AV performed the cell culture experiments. LL performed the polysome profiling and immunoblot experiments. SC performed *in vitro* and *in vivo* SUnSET studies, image processing, and data analysis. MB provided key reagents, funding, and helped design and analyze the *in vitro* experiments. SC and LI wrote the manuscript. LI conceived and supervised all aspects of the project and obtained study funding. All authors read and approved the final manuscript.

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frontiers in Aging Neuroscience

REVIEW
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Impact of Chaperone-Mediated Autophagy in Brain Aging: Neurodegenerative Diseases and Glioblastoma

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Brain aging is characterized by a time-dependent decline of tissue integrity and function, and it is a major risk for neurodegenerative diseases and brain cancer. Chaperone-mediated autophagy (CMA) is a selective form of autophagy specialized in protein degradation, which is based on the individual translocation of a cargo protein through the lysosomal membrane. Regulation of processes such as proteostasis, cellular energetics, or immune system activity has been associated with CMA, indicating its pivotal role in tissue homeostasis. Since first studies associating Parkinson's disease (PD) to CMA dysfunction, increasing evidence points out that CMA is altered in both physiological and pathological brain aging. In this review article, we summarize the current knowledge regarding the impact of CMA during aging in brain physiopathology, highlighting the role of CMA in neurodegenerative diseases and glioblastoma, the most common and aggressive brain tumor in adults.

Keywords: CMA, LAMP2, physiological aging, neurodegenerative diseases, glioblastoma

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INTRODUCTION TO AUTOPHAGY

The term "autophagy" comes from the Ancient Greek meaning "self-eating," which refers to the catabolic processes that use the cell to recycle its own constituents within the lysosome (Yang and Klionsky, 2010; Boya et al., 2013). The first discoveries were based on the bulk trapping of the cargo in double-membrane autophagosomes that fuse with lysosomes, which afterward was named "macroautophagy" (De Duve, 1963). Further studies demonstrated also the existence of another type of autophagy named "microautophagy," which involves the uptake of soluble or membrane-bound material directly into the lysosome by invagination (De Duve and Wattiaux, 1966; Marzella et al., 1981). However, it was not until Dice et al. (1986) described a protein-selective form of autophagy, identifying a pentapeptide region on ribonuclease A required for its enhanced degradation during serum deprivation. This form of autophagy, nowadays known as "chaperone-mediated autophagy" (CMA), was at that time the unique selective form of autophagy. Since then, selective forms of macroautophagy and microautophagy have been described (Klionsky et al., 2016), with a special interest in "chaperone-assisted selective autophagy" (CASA), which participates in the selective trapping of ubiquitin-positive protein aggregates *via* autophagosomes (Arndt et al., 2010) and "endosomal-microautophagy," in which cytosolic proteins enter endosomal compartments inside vesicles generated at the surface of the late endosomes (Sahu et al., 2011).

However, among all forms of autophagy, CMA is still the only process that enables the direct translocation of an individual cargo protein across the lysosomal membrane for its degradation (Kaushik and Cuervo, 2018). Thus, herein we will review the principal components and mechanisms involved in CMA and highlight its impact on physiological aging of the brain, with special emphasis on neurodegenerative disorders and glioblastoma.

CMA PROCESS

Mechanism of Action

Biochemical and genetic approaches have characterized CMA as a rigorous process where the cargo protein must overcome several stages to reach proper lysosomal degradation (**Figure 1**).

Cargo Protein Selection

For a specific protein to be degraded *via* CMA, it first has to bind to the heat shock cognate 71 kDa protein (HSC70), a chaperone that is going to target it to the lysosomal membrane (Dice, 1990). For the recognition of this cargo protein, a "KFERQ" motif is needed (Kaushik and Cuervo, 2018), which comprises a sequence of amino acids with specific charge and hydrophobicity. Overall, it is known that approximately 40% of the mammalian proteome could contain this canonical motif (Kaushik and Cuervo, 2018). However, post-translational modifications, such as phosphorylation, acetylation, or even ubiquitination, within incomplete motifs can confer specific properties, recreating a complete KFERQ-like motif (Kaushik and Cuervo, 2018). Not only inside the motif, but post-translational modifications outside the motif can even expose or mask an existing one, by conformational changes of the protein (Ferreira et al., 2015; Kaushik and Cuervo, 2018). Thus, CMA substrates can vary substantially. In this line, a free web-based resource called "KFERQ finder" has been developed, which directly identifies KFERQ-like motifs in any protein sequence (Kirchner et al., 2019).

HSC70 Binding and Targeting to Lysosomal Membrane

Hidden KFERQ motifs become accessible to HSC70 after: (I) protein conformational changes; (II) multiprotein complex disassembly; or (III) releasing from the subcellular membranes (Cuervo and Wong, 2014). Once bound to the chaperone, the substrate is delivered to the surface of the lysosome where it interacts with the cytosolic tail of the lysosome-associated membrane protein type 2A (LAMP2A; Cuervo and Dice, 1996). It is important to highlight that HSC70's role in autophagy has been assigned not only to CMA but also to CASA (Arndt et al., 2010) and endosomal-microautophagy (Sahu et al., 2011).

LAMP2A Targeting and Substrate Translocation

LAMP2A is one of the three splicing variants of the *LAMP2* gene, which contains a cytosolic tail that differs from the other isoforms and is crucial for CMA to occur (Cuervo and Dice, 2000b). Substrates can bind to LAMP2A in a folded state, but they must be unfolded to be translocated to the lumen of the lysosome (Salvador et al., 2000). In this aspect, HSC70 and other co-chaperones located in the membrane of the lysosome are thought to be mediating this process (Cuervo and Wong, 2014; Rout et al., 2014). During this step, LAMP2A monomers are multimerized, while the heat shock cognate 90 kDa (HSC90) chaperone stabilizes the complex from the luminal part, hiding the protease-sensitive regions of LAMP2A (Bandyopadhyay et al., 2008). When the translocation complex, which comprises 700 kDa, is formed and the cargo protein is unfolded, the substrates enter the lumen of the lysosome. Moreover, there is a form of HSC70 located within the lysosome (lys-HSC70), which reinforces the translocation of the substrate pulling it and preventing its return into the cytosol (Agarraberes and Dice, 2001). A recently published bioinformatic study demonstrated that several domains of the *LAMP2* gene present positive selective pressure in placental mammals, giving new insights regarding the molecular evolution of the key protein of CMA (Jalali and Parvaz, 2020). Future work should define the functional impact of those selective substitutions in CMA and autophagy, particularly in the brain. Nevertheless, dependence on LAMP2A is the best criteria to determine whether a specific protein is degraded by CMA (Kaushik and Cuervo, 2018).

Regulation of CMA Function

The CMA process is tightly regulated and this facilitates the maintenance of cell and organism homeostasis. As LAMP2A is the main effector of CMA, its expression, trafficking, and stabilization are key aspects for CMA to occur. Next, we summarize the regulators of these processes and we provide recent data regarding epigenetic regulation of chaperones and CMA (**Figure 2**).

De novo LAMP2A Synthesis

The first transcription factor identified in the regulation of CMA was the Nuclear Factor of Activated T Cells 2 (NFATC2), also known as NFAT1, which, after activation by calcium-calcineurin, is recruited to the *Lamp2* promoter to induce its expression, mediating the response to reactive oxygen species (ROS) in activated murine T cells (Valdor et al., 2014). Together with NFAT1, nuclear factor erythroid 2-related factor 2 (NRF2) transcription factor has been also described as a modulator of *LAMP2A* expression and consequently, CMA activity in both human and mouse cell types, as the *LAMP2* gene presents binding sequences for NRF2 (Pajares et al., 2018). Interestingly, the study of *de novo LAMP2A* expression modulation has been extended from specific transcription factors to other signaling pathways. Among them, the PI3K/AKT/mTOR signaling pathway should be pointed out, since it has been described as a negative regulator of *LAMP2A* and *HSC70* expression (Li et al., 2017b), but also macroautophagy activity (Heras-Sandoval et al., 2014). However, the detailed mechanism of how this pathway modulates *LAMP2A* expression remains to be elucidated.

LAMP2A Trafficking to the Lysosome

Different subcellular distributions of LAMP2 splicing variants have been assigned largely by the COOH-terminal amino acid residue (Gough and Fambrough, 1997). Besides this regulatory level, other key effectors in LAMP2A trafficking have been

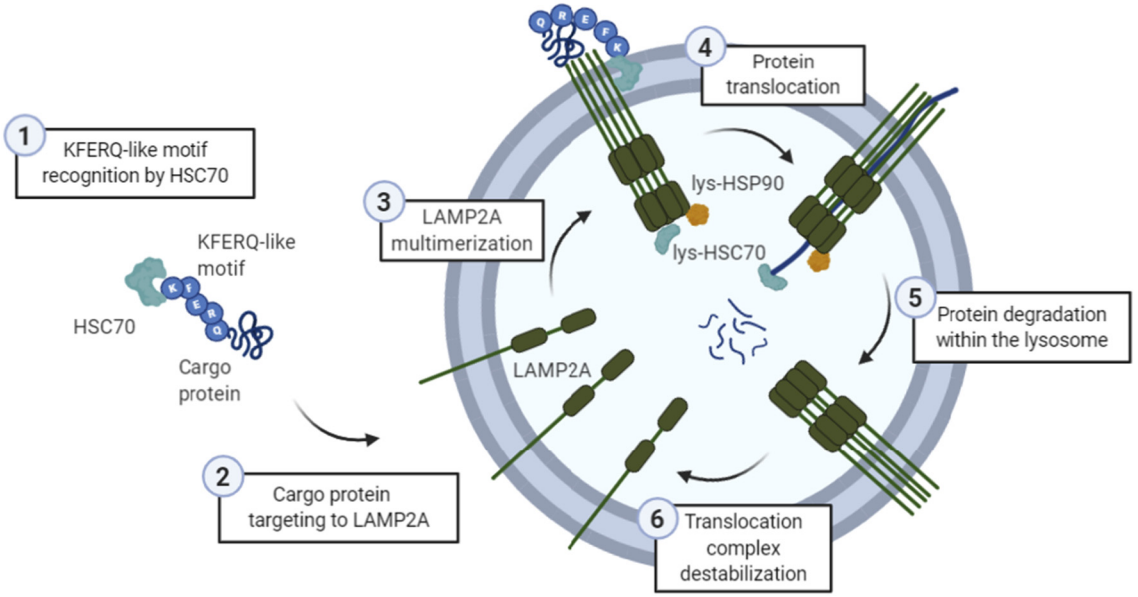

FIGURE 1 | General scheme of the chaperone-mediated autophagy (CMA) process. (1) The KFERQ-like motif of a cargo protein is detected by HSC70, (2) which is the one targeting it into the lysosomal membrane protein lysosome-associated membrane protein type 2A (LAMP2A). Then, (3) LAMP2A monomers are assembled into multimeric structures, forming the translocation complex. Once this structure is correctly formed, (4) protein is translocated through the lysosomal membrane, while lysosomal HSC70 (lys-HSC70) pulls it into the lumen and avoid the return out of the membrane. Lysosomal HSP90 (lys-HSP90) is important in LAMP2A stabilization since it masks lysosomal protease binding sequences, and thus, prevents its degradation during this transition. Finally, (5) protein is degraded inside the lysosome, and (6) translocation complex is disassembled.

discovered in a cystinosis model. Herein, cystinosin (CTNS) has been associated with the regulation of LAMP2A trafficking, as Ctns−/− cellular model presents reduced co-localization of lysosomal membrane protein LAMP1 and LAMP2A, and a significantly increased number of LAMP2A-positive vesicles with motility restriction (Zhang et al., 2017). Indeed, up-regulation of Rab11 or Rab7/RILP leads to the correction of the LAMP2A mislocalization in Ctns−/− cells, which shows their regulatory role in LAMP2A trafficking, at least, in this model (Zhang et al., 2017). Future studies should evaluate the role of CTNS, Rab11, and RILP in LAMP2A trafficking under physiological conditions.

LAMP2A Stability in the Lysosomal Membrane

Levels of LAMP2A at the lysosomal membrane are mainly modulated by changes in its distribution and half-life. A percentage of LAMP2A is located in lipid-enriched membrane microdomains during resting conditions, and its organization into multimeric complexes for substrate translocation only occurs outside these regions (Kaushik et al., 2006). Loading of lysosomes with cholesterol significantly reduces CMA, mainly due to the increased susceptibility of LAMP2A to be cleaved by proteases such as cathepsin A and posterior degradation in the lysosomal lumen (Cuervo et al., 2003). In this line, sorting nexin (SNX)-10 deficiency upregulates LAMP2A and so, CMA activation, probably due to a dysregulation of cathepsin A trafficking into lysosomes and thus, protecting LAMP2A from its degradation (You et al., 2018).

Translocation Complex Stability

GTP presents an inhibitory effect on CMA by altering the stability of the translocation complex, mediated by glial fibrillary acidic protein (GFAP) and elongation factor 1α (EF1α), in mouse and rat models (Bandyopadhyay et al., 2010). In the absence of GTP stimulus, GFAP stabilizes the multimeric translocation complex, whereas GTP-mediated release of EF1α from the membrane of the lysosome promotes self-association of GFAP, reducing the stabilization of the complex and consequently diminishing CMA activity (Bandyopadhyay et al., 2010). An additional study shows that the interaction between Akt and mTORC2/PHLPP1 also controls the dynamics of the assembly and disassembly of the translocation complex (Arias et al., 2015). In particular, when associated with the lysosomal membrane, the phosphatase PHLPP1 and the kinase mTORC2 regulate the phosphorylation state of Akt, modulating thus the stability of the translocation complex (Arias et al., 2015). Moreover, Humanin, a mitochondria-associated peptide, interacts with HSP90, and stabilizes the binding of this chaperone to CMA substrates (Gong et al., 2018).

Post-translational Regulation of HSC Chaperones

Several studies have recently shown new functions of deacetylase and methyltransferase enzymes on the activity of chaperones within CMA. An initial study revealed that histone deacetylase 6 (HDAC6) regulates HSP90 acetylation, thus controlling its interaction with LAMP2A and HSC70, under the hypoxia-ischemia-stress induced by acute spinal cord injury

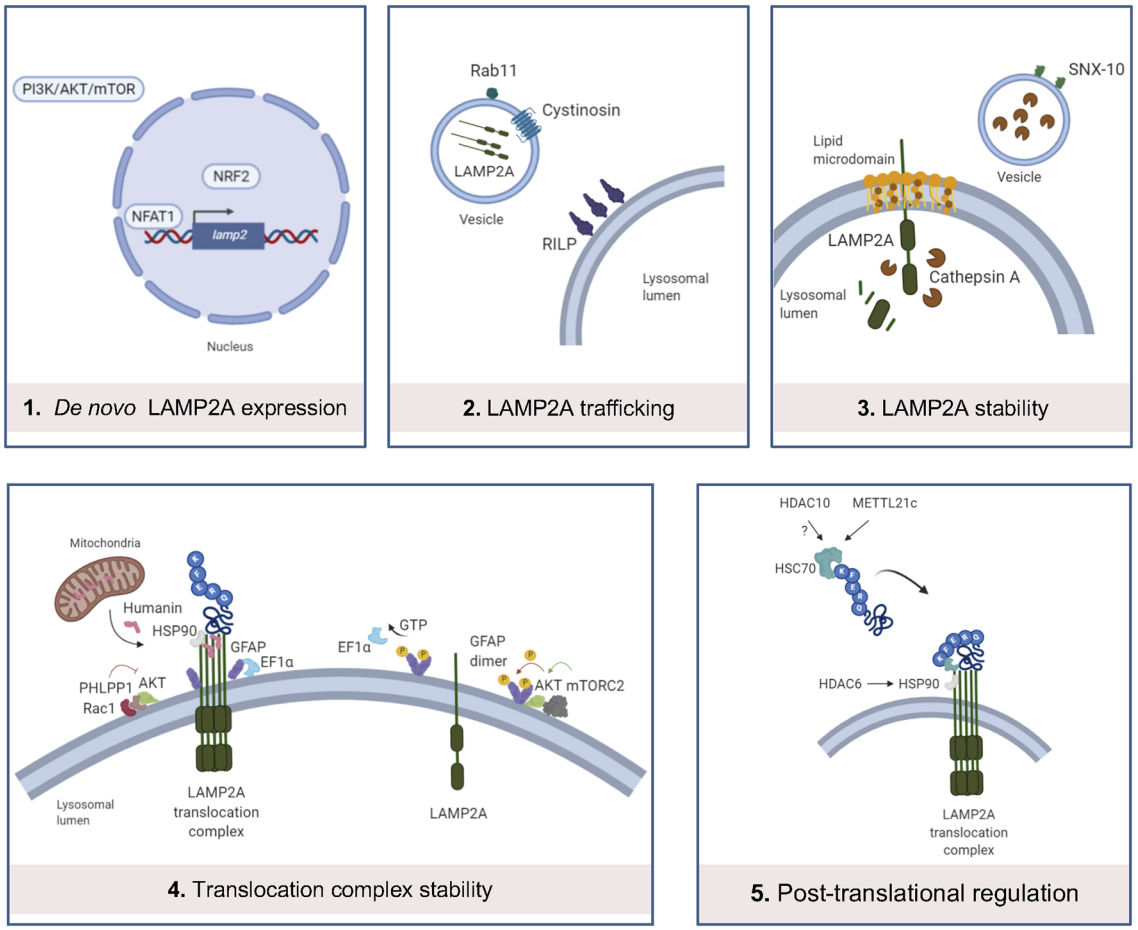

FIGURE 2 | Main levels of regulation of CMA. (1) *LAMP2A de novo* expression can be regulated by the binding of NFAT1 to the promoter of *LAMP2* gene, binding of NRF2 and PI3K/AKT/mTOR pathway; (2) LAMP2A trafficking into the lysosomal membrane is altered by cystinosin and Rab11 and RILP protein dysfunctions; (3) in resting conditions, LAMP2A monomers are located in lipid microdomains, where LAMP2A is more sensitive to cathepsin A-mediated degradation. SNX-10 deficiency upregulates LAMP2A expression, probably by dysregulation of cathepsin A trafficking into the lysosomes; (4) Rac1/PHLPP1 complex inhibits Akt, enabling glial fibrillary acidic protein (GFAP)-mediated translocation complex stabilization. On the contrary, this complex is destabilized by GTP-mediated release of Elongation factor 1α (EF1α) from the lysosomal membrane and mTORC2/Akt phosphorylation of GFAP, promoting GFAP self-association. Humanin, a mitochondria-associated peptide, interacts with HSP90 and stabilizes the binding of this chaperone to CMA substrates; (5) recent studies demonstrate that METT21c and potentially HDAC10 regulate HSC70 action by post-transcriptional regulation. HDAC6 regulates HSP90 acetylation and activity.

(Su et al., 2016). Other studies showed that histone deacetylase 10 (HDAC10) deacetylates HSC70 (Oehme et al., 2013) and that HDAC10 knock-out accumulates LAMP2A positive lysosomes around the nucleus, activating CMA (Obayashi et al., 2020). In the same line, an additional study described that the newly classified methyltransferase-like 21c (METTL21c) trimethylates HSP70 at Lys-561 to enhance its stability, inducing its function in CMA in mice (Wang et al., 2019a). All these recent data exhibit the potential that epigenetic enzymes may have in CMA regulation.

Crosstalk With Other Types of Protein Degradation Systems

Although three isoforms of the *LAMP2* gene present a common luminal domain, their different cytosolic and transmembrane domains (Cuervo and Dice, 2000b) give them unique properties that hamper compensation of all functions; while LAMP2A is the only isoform that can work in CMA, LAMP2B is associated to macroautophagy (Nishino et al., 2000; Chi et al., 2019) and LAMP2C to a selective type of autophagy called "RNautophagy" (Fujiwara et al., 2013).

Compensatory mechanisms between different proteolytic systems within the cell have been widely described for the correct proteostasis of the tissue (Park and Cuervo, 2013; Wu et al., 2015; Dong and Cui, 2018). Indeed, a recent proteome-wide study identified a globally harmonized rhythm for basal macroautophagy, CMA, and proteasomal activity for proteolysis during the daytime (Ryzhikov et al., 2019).

Regarding macroautophagy, it can be upregulated in CMA-defective conditions to maintain normal rates of long-lived protein degradation, although is unable to compensate for all CMA features (Massey et al., 2006). Supporting this original

evidence, a study showed that the upregulation of CMA mediated by the stress caused by hepatitis C, caused selective degradation of beclin1, thus impairing autophagosome-endosome fusion, a key step in the macroautophagic process (Aydin et al., 2018). In the same way, the blockage of macroautophagy leads to upregulation of CMA (Kaushik et al., 2008), which has been associated with resistance to death induced by oxidative stress from menadione or UV light (Singh and Czaja, 2008). Another evidence that supports this mutual regulation of macroautophagy and CMA is that protein kinase Cα (PKCα) mediated phosphorylation of ULK1, on the one hand, prevents autolysosome formation, but on the other hand, enhances its interaction with HSC70 and increases its degradation through CMA (Wang et al., 2018a). However, macroautophagy and CMA can also work together in a coordinated way, as it is demonstrated in a study where, to occur hydrolysis of lipid droplets (LD) during starvation, CMA degradation of PLIN2 and PLIN3 was enhanced, concurrent with elevated levels of macroautophagy proteins on LDs (Kaushik and Cuervo, 2015). The ubiquitin-proteasome system (UPS) is the main non-lysosomal degradative pathway for ubiquitinated proteins (Pandey et al., 2007). Notably, it has been described as the upregulation of CMA (Koga et al., 2011b) and an accumulation of proteasomal subunits in lysosomes in response to inhibition of the proteasome (Cuervo et al., 1995; Goebel et al., 2020). On the contrary, in the first stages of CMA blockage, the activity of both macroautophagy and UPS has been identified to be impaired, precipitating the intracellular accumulation of altered components and making the cell unable to eliminate them even after the subsequent restoration of normal UPS and activation of macroautophagy (Massey et al., 2008).

Interestingly, some of these mechanisms have been described in the brain. Firstly, ubiquilins, highly conserved ubiquitin-binding proteins, have been recently described as integrators of UPS and lysosomal degradation for neuronal maintenance, *via* mTOR signaling (Šentürk et al., 2019). Indeed, a previous study demonstrated that ubiquilin is present in autophagosomes from brain tissue of mice and that it is degraded during both macroautophagy and CMA (Rothenberg et al., 2010). Not only ubiquilins but also HDAC6, which interacts with polyubiquitinated proteins, has been identified as an essential mechanistic link between UPS and autophagy in a neurodegenerative model (Pandey et al., 2007).

CMA DURING PHYSIOLOGICAL AGING

The important regulatory role of CMA highlights its relevance in organ functionality and tissue maintenance. Indeed, a functional decline of CMA during physiological aging has been found in the last decades (Cuervo and Dice, 2000a; Zhou et al., 2017). Interestingly, it has been described that not its transcriptional rate, but the stability of LAMP2A within the lysosomal membrane is altered with age (Kiffin et al., 2007), which indeed can be further degenerated after chronic exposure to a high-fat diet (Rodriguez-Navarro et al., 2012). On the contrary, mice with an inducible exogenous extra copy of *LAMP2A* in the liver displayed reduced damaged proteins and improved organ function (Zhang and Cuervo, 2008), reinforcing its action on physiological aging. In line with this, resveratrol, an agent with anti-aging activity, upregulates *LAMP2* in hepatocytes (Lee et al., 2019). Liver lysosomes from long-lived both Pou1f1/Pit1 mutant (Snell) mice and growth hormone receptor (ghr) knockout mice also present increased CMA substrate uptake activity (Endicott et al., 2020), further associating the beneficial impact of maintaining CMA with healthspan and longevity.

CMA DURING PHYSIOLOGICAL AGING IN THE BRAIN

The impact of autophagy and specifically CMA, in physiological brain aging remains largely unknown, although some evidence is supporting the downregulation of relevant proteins involved in it (**Table 1**, **Figure 3**). Almost all of the studies published so far remain correlative, suggesting an impaired regulation of *de novo* LAMP2A synthesis. Among them, a study showed a significant decline of HSC70 (Loeffler et al., 2016) and little changes in LAMP2 (Loeffler et al., 2018) concentration in cerebrospinal fluid (CSF). Interestingly, neuronal nitric oxide synthase (nNOS) has been associated with neuronal aging in rodents, causing LAMP2A reduction and consequent accumulation of CMA substrates (Valek et al., 2019). In the same way, CMA impairment is detrimental for neuronal viability *in vivo* (Xilouri et al., 2016) and identified PARK7/DJ-1 and DPYSL2/CRMP-2 as the most significantly altered proteins upon LAMP2A downregulation in primary rat cortical neurons (Brekk et al., 2019). Interestingly, PARK7/DJ-1 contains overlapping canonical and acetylation-generated KFERQ-like

TABLE 1 | Main studies revealing chaperone-mediated autophagy (CMA) downregulation in brain homeostasis and aging.

Molecule	Role in CMA	Finding	Model of study	Reference
LAMP2A	Key effector	Its reduction induces progressive loss of nigral dopaminergic neurons, increased astro-microgliosis and motor deficits.	Rat substrantia nigra *in vivo*	Xilouri et al. (2016)
		Alters the expression of PARK7/DJ-1 and DPYSL2/CRMP-2 proteins, with measurable effects in neuronal homeostasis and phenotype.	Primary rat cortical neurons	Brekk et al. (2019)
LAMP2	Gene coding *LAMP2A*	Downregulation in the expression in aged patients.	Human CSF	Loeffler et al. (2018)
HSPA8	Effector	Downregulation in the expression in aged patients.	Human CSF	Loeffler et al. (2016)
nNOS	Regulator	Associated to neuronal aging, causes an accumulation of CMA substrates and loss of LAMP2A.	Human neuroblastoma cell line	Valek et al. (2019)

CFS, cerebrospinal fluid.

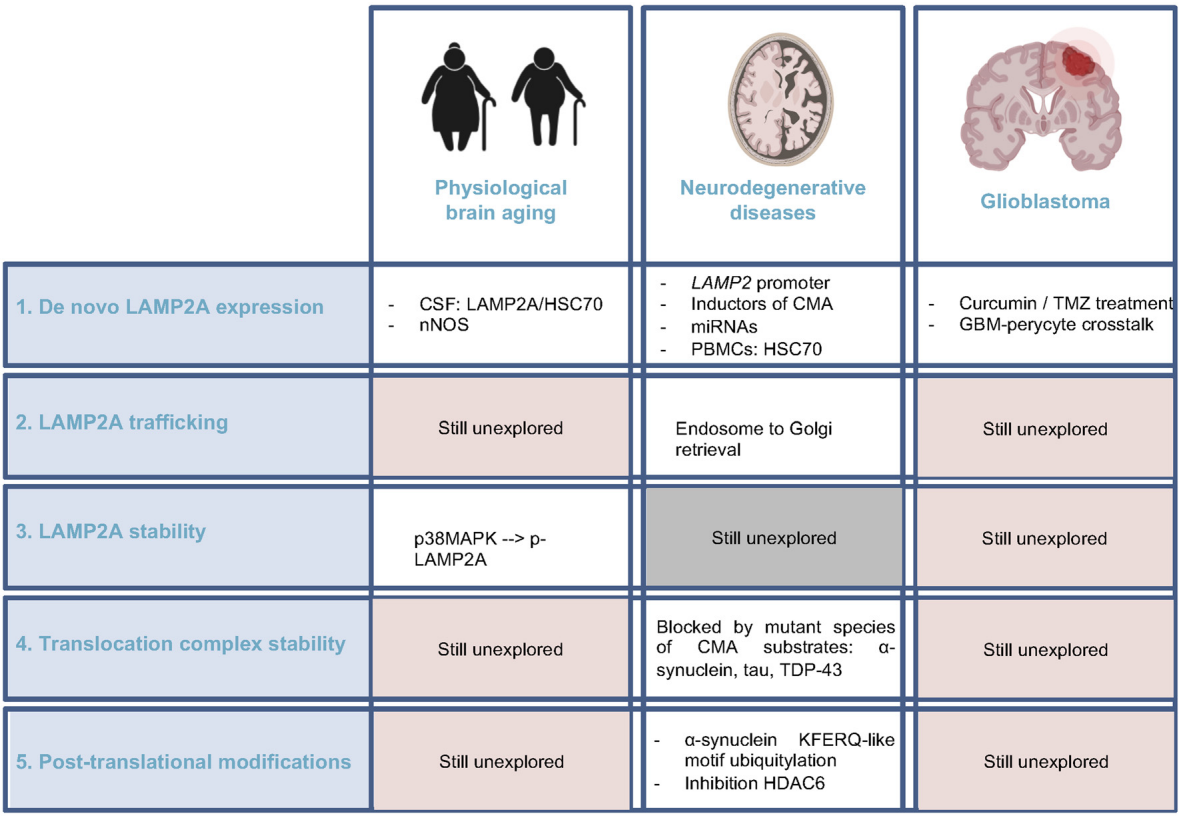

FIGURE 3 | Current knowledge regarding CMA regulators alteration in brain aging, neurodegenerative diseases, and glioblastoma. Comparison of the pieces of evidence among these three physiopathological conditions of the brain, highlighting the unexplored fields. CSF, cerebrospinal fluid; nNOS, neuronal nitric oxide synthase; PBMC, peripheral blood mononuclear cell.

motifs 10 residues upstream of ^{106}C shown to be key for the activation of this protein by oxidative stress. It has been postulated that the oxidative status of ^{106}C may change the exposure of the motif to HSC70 binding and subsequent CMA-mediated degradation, or prevent it by ubiquitylation (Kirchner et al., 2019). As oxidative stress has been associated with aging, this evidence makes it attractive to propose that post-translational modifications in KFERQ-like motifs of CMA substrates could have an impact on the aging phenotype.

Studies regarding altered regulation of LAMP2A stability in lysosomal membrane could also be associated with brain aging. In this line, p38MAPK, which phosphorylates LAMP2A causing its lysosomal membrane accumulation and active conformational change (Li et al., 2017a), has been recently identified as a regulator of neuronal and neural stem cell (NSC) activity during brain aging (Moreno-Cugnon et al., 2019, 2020), which support the idea that alterations in CMA regulation may be associated to brain aging.

Besides, a decline in the integrity of the blood-brain barrier (BBB) is a major feature of brain aging, and it has been speculated that downregulation of endothelial annexin A1, a CMA regulator (Liu et al., 2018), maybe partially responsible for the aged BBB phenotype (McArthur et al., 2016).

Considering all these ideas, it is reasonable to suggest that CMA activity could decrease in brain physiological aging, although still very preliminary (**Figure 3**). Thus, future studies should be directed to the elucidation of this hypothesis, focusing on different cell types among the brain and the crosstalk with UPS or macroautophagy, with a special interest in *in vivo* models.

PHYSIOLOGICAL ROLES OF CMA IN THE BRAIN

Protein degradation by CMA regulates diverse cellular processes so that its balanced activity is crucial, especially in the brain. Several studies have linked the onset of aging and age-associated neurodegenerative diseases to the downregulation of CMA (Cuervo and Dice, 2000a; Alfaro et al., 2018; Loeffler, 2019). Regarding malignant transformation, it has been described that CMA presents anti-oncogenic effects in untransformed healthy cells (Arias and Cuervo, 2020) suggesting that brain aging-associated CMA decline could be a risk factor for brain tumorigenesis. Once the damage is accumulated, tumor cells activate CMA constitutively, promoting brain cancer progression (Valdor et al., 2019).

It is widely accepted that both aging and cancer are two manifestations of the accumulation of cellular and DNA damage

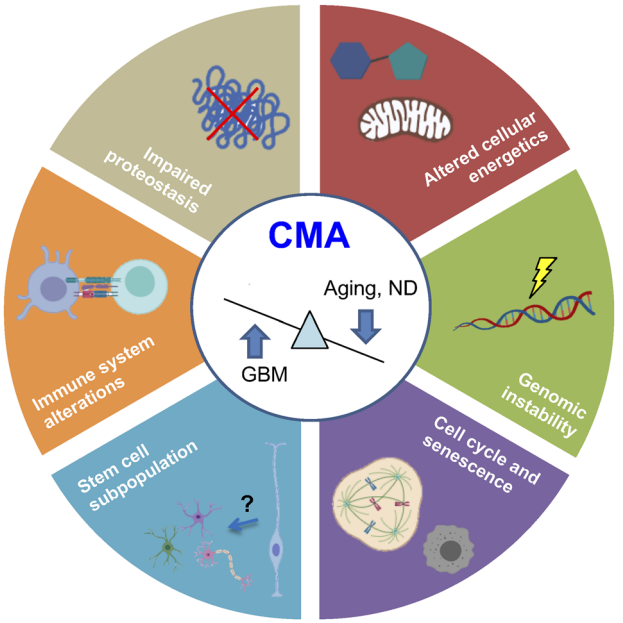

FIGURE 4 | Common hallmarks of aging and cancer regulated by CMA. Recent evidence demonstrates that physiological aging and ND are associated with CMA downregulation, whereas glioblastoma (GBM) presents an upregulated activity. Processes regulated by CMA are represented in the figure. ND, neurodegenerative diseases.

(López-Otín et al., 2013) so that CMA acts as a cell survival mechanism. In particular, CMA participates in the regulation of various common hallmarks of aging (López-Otín et al., 2013) and cancer (Hanahan and Weinberg, 2011), where, the existing data could suggest that, while brain cancer cells use CMA for survival, age-dependent CMA decline prevents recovery and restoration of damaged tissues (**Figure 4**).

Altered Proteostasis

The most characterized role of CMA is the maintenance of a correct proteostasis, which has been widely described to be impaired in aging and neurodegenerative diseases (López-Otín et al., 2013) and must be maintained in cancer cells to survive (Yang et al., 2019). In this sense, oxidative stress (Law et al., 2016; Issa et al., 2018) and hypoxic stress (Dohi et al., 2012) have been described to activate CMA to reduce protein damage in the brain.

Impaired Cellular Energetics and Metabolism

Besides quality control, there is currently evidence that supports that protein damage is not a requirement for CMA targeting (Kaushik and Cuervo, 2018). Cellular energetics, another common hallmark of aging and cancer, has been linked to CMA since its discovery when it was described that starvation-induced activation (Dice et al., 1986). This evidence was associated for a long time with a mechanism in which a pool of free amino acids was needed. However, recent studies show that the role of enzymes degraded by CMA in metabolism could be responsible for cellular energetic changes (Tasset and Cuervo, 2016). Interestingly, lipid and carbohydrate metabolic alterations caused by altered CMA have been described to be associated with physiological aging of the brain (Hallett et al., 2018) and neurodegenerative diseases (Alfaro et al., 2018). Not only that, but macrophage-specific LAMP2A deficient mice also exhibited significant intracellular lipid accumulation (Qiao et al., 2020), extending the impact of CMA to immune cells, which has been linked to brain homeostasis and disease (Li and Barres, 2018; Dulken et al., 2019). Regarding amino acid metabolism, the accumulation of cystine within the lysosome occurred in cystinosis, a disorder that affects also the brain (Jonas et al., 1987), which is associated with CMA activity (Zhang et al., 2017).

Augmented Genomic Instability

Genotoxic insults together with oxidative stress and starvation are the main stimulus which activates CMA (Park et al., 2015). In this regard, CHK1 participates in genome quality control, and it has been associated with physiological neuronal differentiation (Oshikawa et al., 2017) and resistance of glioblastoma cells to chemo/radiotherapy (Patties et al., 2019). Interestingly, CHK1 has been described as a CMA substrate, as it presents various KFERQ-like motifs in different domains (Kirchner et al., 2019). Indeed, phosphorylation of ^{345}S, separated only by four amino acids from the canonical KFERQ-motif, produces conformational changes, altering HSC70 recognition, and subsequent degradation *via* CMA (Kirchner et al., 2019). The abnormal nuclear accumulation of phosphorylated CHK1 after a genotoxic insult in CMA-deficient condition alters proteins involved in cell cycle regulation and DNA repair, inducing levels of DNA damage marker p-γH2AX (Park et al., 2015). Indeed, ubiquitylation events in the putative motif of the catalytic region may also modulate the degradation of still inactive CHK1 (Kirchner et al., 2019), suggesting that many post-translational modifications within KFERQ-like motifs may modulate CHK1 degradation *via* CMA and consequently, regulate DNA repair response.

Cell Cycle Dysregulation and Senescence

Some studies describe the impact of CMA on cell cycle and senescence. On the one hand, tumor suppressor proteins such as p73 (Nguyen et al., 2020) or mutant p53 (Vakifahmetoglu-Norberg et al., 2013), and transcription factors MEF2A (Zhang et al., 2014) and MEF2D (Yang et al., 2009), previously associated to embryonic and adult neurogenesis processes, have been denominated as CMA substrates. Besides, it has been proposed that CMA dysfunction during aging promotes cellular senescence (Moreno-Blas et al., 2018). In contrast, CMA also participates in ferroptosis (Wu et al., 2019b), a specific necrotic cell death that has been linked to diverse age-associated brain diseases (Weiland et al., 2019).

Changes in Stem Cell Subpopulation

Alterations in stem cell subpopulations have been widely described in brain pathologies, with an age-dependent physiological decline of the NSC pool (Schultz and Sinclair,

2016) and the presence of malignant cancer stem cells in glioblastoma (Garros-Regulez et al., 2016; Arrizabalaga et al., 2017). Interestingly, old quiescent NSCs display defects in their lysosomal activity, with an increased accumulation of protein aggregates and reduced ability to be activated (Leeman et al., 2018). Many studies have focused on macroautophagy (Revuelta and Matheu, 2017; Sotthibundhu et al., 2018; Ryskalin et al., 2019), but the impact of CMA in NSC maintenance remains mostly elusive. To date, some studies could give insights about the possible link between stem cell regulation and the CMA process. On the one hand, retinoic acid has been associated with neural and astroglial differentiation during development (Hádinger et al., 2009; Tan et al., 2015), adulthood (Jacobs et al., 2006; Mishra et al., 2018), and glioblastoma pathogenesis (Ying et al., 2011) and this signaling pathway inhibits CMA (Anguiano et al., 2013). In the same way, hypoxia promotes NSC proliferation *via* Hypoxia-inducible factor 1-α (HIF1α; Hubbi and Semenza, 2015), which is targeted for lysosomal degradation by CMA (Ferreira et al., 2013; Hubbi et al., 2013). Moreover, mice with an extra copy of Arf/p53 display delayed exhaustion of NSCs and enhanced cognitive activity (Carrasco-Garcia et al., 2015, 2017). However, further research would be needed to test these associations and get direct evidence regarding CMA impact, if any, in stem cell maintenance.

Immune System Alterations

The first evidence that linked CMA to the immune system was that LAMP2A participates in the display of cytoplasmic epitopes *via* class II molecules in antigen-presenting cells (Zhou et al., 2005). Further research has shown a positive association between CMA and activation of T cells (Valdor et al., 2014) and tumor-associated macrophages in hepatocellular carcinoma (Guo et al., 2017) and breast cancer (Wang et al., 2019b). However, very little is known regarding the physiological role of CMA in this organ, although several studies have described the role of other types of autophagy in the brain's immune system (Plaza-Zabala et al., 2017; Molina et al., 2019). Thus, CMA degrades IKKβ and thereby reduces TNFα expression in microglia (Liu et al., 2018) and CMA inhibition contributes to mesenchymal stem cell immunosuppressive function (Zhang et al., 2020). Further studies are needed to elucidate the role of CMA in the brain's immune system and its crosstalk with other cell types.

CMA IN NEURODEGENERATIVE DISEASES

It was neurodegenerative Parkinson's disease (PD) the first disorder associated with CMA (Cuervo et al., 2004). Since then, research has been extended to other prevalent neurodegenerative disorders, demonstrating a reduction in CMA activity in most of them (**Table 2**, **Figure 3**).

Parkinson's Disease (PD)

PD is the second most common neurodegenerative disorder after Alzheimer's disease. It is characterized by an extensive and progressive loss of dopaminergic neurons within the substantia nigra and aggregation of the protein α-synuclein in Lewy bodies. PD is mostly sporadic, except in a 5–10% familial, and mainly related to α-synuclein and leucine-rich repeat kinase 2 (LRRK2) mutations (Campbell et al., 2018; Tolosa et al., 2020). It has been shown that α-synuclein contains a KFERQ-like motif (Cuervo et al., 2004), whereas LRRK2 contains a total of 8 putative motifs (Orenstein et al., 2013). These pieces of evidence are accompanied by both *in vitro* and *in vivo* studies demonstrating that they can be degraded by CMA (Cuervo et al., 2004; Mak et al., 2010; Orenstein et al., 2013). Interestingly, this motif is masked in the structure of α-synuclein fibers, supporting the evidence that, once in an oligomeric state, the protein is no longer available for CMA degradation (Kirchner et al., 2019). Indeed, ubiquitylation in ^{96}K and protein partners binding KFERQ-like motif may prevent degradation by CMA of free soluble α-synuclein (Kirchner et al., 2019). In the same line, pathogenic mutant or even dopamine-modified species of α-synuclein and LRRK2 are not able to be degraded by CMA but present a significantly higher affinity to bind to LAMP2A protein than other CMA substrates, blocking the degradation of the later ones (Cuervo et al., 2004; Martinez-Vicente et al., 2008; Orenstein et al., 2013). These data could suggest that changes in the KFERQ-like motif sequence or even any post-translational modification modifying it may be responsible for this augmented affinity of pathogenic forms to reach the lysosomal membrane. Currently, there is not any evidence regarding this idea, so future studies should be intended to determine its impact on CMA disruption in PD.

A familial PD-associated mutation in ubiquitin C-terminal hydrolase L1 (UCH-L1) has been also described as an inhibitor of CMA activity, by promoting an induced aberrant interaction with LAMP2A, HSC70, and HSP90 (Kabuta et al., 2008). Conversely, studies regarding alterations in *de novo* LAMP2A expression regulation have been also performed. In this line, mutations in *LAMP2* gene promoter itself have been also associated with sporadic PD patients (Pang et al., 2012), and there is a decrease in LAMP2A and HSC70 expression in the substantia nigra and amygdala of PD brains compared to control samples (Alvarez-Erviti et al., 2010). Moreover, inductors of PD animal models such as 6-hydroxydopamine (6-OHDA) and rotenone, or even methamphetamine exposure, alter the expression of both Lamp2a and Hsc70, leading to neurotoxicity caused by α-synuclein accumulation (Gao et al., 2014; Sala et al., 2016; Wang et al., 2018b; Sun et al., 2019). Interestingly, several studies have also identified a set of miRNAs that regulate negatively LAMP2A and HSC70 in PD models (Alvarez-Erviti et al., 2013; Li et al., 2014).

In addition to expression alterations, impairment on LAMP2A trafficking has been also found in PD. In particular, it has been suggested that the impaired endosome-to-Golgi retrieval of Lamp2a in VPS35-deficient dopaminergic neurons may contribute to the accumulation of α-synuclein (Tang et al., 2015).

On the contrary, re-activation of CMA by upregulating *lamp2a* in neurons ameliorate α-synuclein-induced dopaminergic neurodegeneration in mice (Xilouri et al., 2013). Similarly, overexpression of CMA regulators such as *nrf2* (Gan et al., 2012) or hDJ-1 in astrocytes (De Miranda et al., 2018)

TABLE 2 | Main studies revealing CMA downregulation in neurodegenerative diseases.

Disease type	Role in CMA	Molecule	Finding	Model of study	Reference
PD	Gene coding *LAMP2A*	*LAMP2*	Study of *LAMP2* promoter variants and its association with sporadic PD	Human peripheral leukocytes genomic DNA	Pang et al. (2012)
			Expression diminishment in female LRRK2 PD	Human CFS	Klaver et al. (2018)
	Effector	LAMP2A	Reduction in the expression of LAMP2A and Hsc70 in PD patients	Substantia nigra pars compacta and amygdala of PD and control brains	Alvarez-Erviti et al. (2010)
			Several miRNA regulate negatively LAMP2A in PD	Neuroblastoma cell line and human PD brains	Alvarez-Erviti et al. (2013)
			Impaired endosome-to-golgi retrieval of LAMP2A accumulate α-syn	VPS35 mutant mice	Tang et al. (2015)
			Overexpression of LAMP2A causes decreased α-syninduced dopaminergic neurodegeneration	Human neuroblastoma cells, rat primary cortical neurons *in vitro*, and nigral dopaminergic neurons *in vivo*	Xilouri et al. (2013)
		Hsc70	Reduction of the expresion in sporadic PD	Human PBMCs	Sala et al. (2014)
	CMA substrate	α-syn	First evidence of α-syn degradation by CMA. Effect of mutant forms in CMA perforrmance	Rat ventral midbrain neuronal cultures	Cuervo et al. (2004)
			in vivo degradation of α-syn under normal and enhanced protein burden conditions	C57BL/6 and transgenic mice overexpressing wild-type mouse α-syn	Mak et al. (2010)
			Dopamine-modified α-syn effect in CMA	Mouse ventral medial neuron culture, neuroblastoma cell line	Martinez-Vicente et al. (2008)
		LRRK2	First evidence of LRRK2 degradation by CMA. Effect of G2019S mutant form in CMA perforrmance	BAC transgenic for WT and mutant LRRK2, patient-derived iPSCs, human brain tissue (DMV)	Orenstein et al. (2013)
	Regulator	UCH-L1	UCH-L1 interaction with LAMP2A, Hsc70 and Hsp90. Effect of I93M UCH-L1 mutation	Human and murine fibroblasts	Kabuta et al. (2008)
		Nrf2	Nrf2 in astrocytes delays CMA dysfunction observed in the hSYN(A53T) mice	GFAP-Nrf2/hSYN(A53T) mice strain	Gan et al. (2012)
		hDJ-1	Human DJ-1 in astrocytes protects from rotenone-induced neurodegeneration	hDJ-1 vector delivery to the substantia nigra and striatum of adult Lewis rats	De Miranda et al. (2018)
		HDAC6	Tubastatin A reduces astrocyte reactivity and neurodegeneration	Wilstar rat α-syn model	Francelle et al. (2020)
AD	CMA substrate	Tau	Tau degradation by CMA. Effect of mutant forms in CMA performance	Murine neuroblastoma cell line	Wang et al. (2009)
		β-amyloid	Oligomers tagged with KFERQ motifs enter in the lysosomes	iPSC cortical neurons derived from AD patient fibroblasts	Dou et al. (2020)
			Amyloid precursor protein (APP) contains KFERQ motif associated to its processing	Neuroblastoma cell line	Park et al. (2016)
HD	CMA inductor?	HTT	Mutant HTT induces the expression of *LAMP2A* and *HSC70*	18QHtt and 111QHtt knock-in mice. Striatum neuronal cultures from HD94 Human lymphoblast from HD patients	Koga et al. (2011a,b)
			Attachment of KFERQ motifs to HTT induces it degradation by CMA	Murine neuroblastoma cell line, R6/2 HT mice	Bauer et al. (2010)
SCA7	CMA substrate?	Ataxin-7	May be degraded by CMA and poliQ expanded ataxin-7 blocks degradation of other CMA substrates	Mouse Purkinje cells	Duncan et al. (2010)

(Continued)

TABLE 2 | Continued

Disease type	Role in CMA	Molecule	Finding	Model of study	Reference
ALS/FTLD	CMA substrate	TDP-43	TDP-43 degradation by CMA. Effect of mutant forms in CMA performance	Liver and brain of Sprague–Dawley rats	Ormeño et al. (2020)
Prion disease	CMA substrate	PrP	Downregulation of LAMP2A and HSC70. PLK3 induces degradation of PrP by CMA	263K-infected hamster and SMB-S15 cell line	Wang et al. (2017)

PD, Parkinson's disease; AD, Alzheimer's disease; HD, Huntington's disease; SCA7, spinocerebellar ataxia 7; ALS, Amyotrophic lateral sclerosis; FTLD, Frontotemporal lobal degerenation; α-syn, α-synuclein; CFS, cerebrospinal fluid; DMV, Dorsal motor nucleus of the vagus nerve.

presented also a decreased α-synuclein accumulation. These studies reinforce the role of CMA in PD and highlight the benefits of CMA restoration.

The idea of using CMA effectors as biomarkers for the diagnosis of PD has been also investigated. Indeed, it has been shown that LAMP2 concentrations are decreased in female LRRK2 PD patients compared to healthy individuals (Klaver et al., 2018) in CSF samples. Besides, a significant reduction of HSC70 has been identified in peripheral blood mononuclear cells (PBMCs) of patients of sporadic PD, but no differences in LAMP2A were detected (Sala et al., 2014). Further research should be needed to explore in more detail the potential role of CMA as a PD trait biomarker.

Pharmacological activation of CMA has been postulated as a therapeutic strategy (Campbell et al., 2018). In support of this, co-culture of human pluripotent stem cell (iPSC)-derived astrocytes and neurons from control and PD patients, showed that chemical enhancement of CMA protected both from degeneration (di Domenico et al., 2019). In line with this, the treatment with the selective inhibitor of HDAC6 Tubastatin A reduces both astrocyte reactivity and neuron degeneration induced by α-synuclein in a rat PD model, increasing Lamp2a and Hsc70 expression and partially inducing α-synuclein acetylation (Francelle et al., 2020). Crosstalk between different protein degradation systems is also noteworthy in PD pathogenesis. It has been described that not only CMA but also UPS can participate in the degradation of soluble α-synuclein (Webb et al., 2003). As protein aggregation occurs, macroautophagy is activated as a secondary mechanism to compensate for CMA/UPS blockage and alleviate these conditions (Webb et al., 2003). Interestingly, the connection between autophagy and UPS is not limited to the removal of cytosolic proteins but also involves the removal of organelles. In this line, it has been described that ubiquitination of constituent proteins in the membrane of peroxisomes mediates their macroautophagy, whereas the familial form of PD-associated ubiquitin ligase parkin ubiquitinates mitochondrial proteins, inducing mitophagy (Wong and Cuervo, 2010).

Alzheimer's Disease

Alzheimer's disease (AD) is the most common form of dementia in elderly people, characterized classically by the presence of β-amyloid plaques and neurofibrillary tangles of hyperphosphorylated and cleaved forms of the microtubule-associated protein tau (Weller and Budson, 2018). In AD, the translocated mutant tau products at the lysosomal membrane hamper the binding and translocation of other CMA substrates, decreasing their activity (Wang et al., 2009). Not only this, but reduced targeting to the lysosomes has been also observed in a mutant form of tau (Caballero et al., 2018), corroborating a general impaired CMA activity in AD. Interestingly, a regulator of calcineurin 1 (RCAN1) is elevated in AD and contains KFERQ motifs that habilitate it to be degraded by CMA (Liu et al., 2009). As RCAN1 is an inhibitor of calcineurin-dependent dephosphorylation of tau protein, it would be worthy to test the hypothesis that increased degradation of RCAN1 by activation of CMA could be mediating, at least in part, the reduction of the formation of tau aggregates. Together with this, AD-associated ubiquilin is degraded by CMA (Rothenberg et al., 2010), reinforcing the impact of this type of autophagy in AD pathogenesis. Interestingly, a recent study showed that tagging β-amyloid oligomers with multiple KFERQ motifs promoted their entering in endosomes and lysosomes, protecting human primary cultured cortical neurons from neurotoxicity (Dou et al., 2020). Indeed, amyloid precursor protein (APP) by itself, where β-amyloid is derived from, contains a KFERQ motif at its C-terminus which, intriguingly, has been associated with the correct APP processing, not to CMA-dependent degradation (Park et al., 2016). However, deletion of this motif increases the amount of APP cleaved products, suggesting that other KFERQ motif-dependent protein degradation system may be responsible for this effect.

Huntington's Disease

Huntington's disease (HD) is a dominantly inherited pathology caused by the accumulation of mutant huntingtin protein (HTT) that contains an expanded N-terminal polyglutamine (polyQ) tract (Bauer et al., 2010). HD is the only neurodegenerative disease with elevated LAMP2A and HSC70 expression (Koga et al., 2011a), as a result of both UPS and macroautophagy failure. The use of an adaptor that contains two copies of polyQ binding sequences and two different KFERQ motifs, directs specifically mutant HTT to the CMA machinery for its degradation, ameliorating symptoms, and extending the life span of an HD mouse model (Bauer et al., 2010). However, the efficiency of this compensatory mechanism may not be sufficient to control the evolution of the disease (Kon et al., 2011), due to the age-dependent CMA activity decline. Thus, the effect of high levels of CMA regulators and whether pharmacological activation of CMA

could be beneficial in the evolution of the disease should be investigated.

Other Neurodegenerative Disorders

Associations between CMA alterations and other neurodegenerative disorders have been also described in the last few years. On the one hand, some studies showed that both wild-type and pathological forms of TAR DNA binding protein 43 kDa (TDP-43), whose accumulation in neurons is associated with amyotrophic lateral sclerosis and frontotemporal lobar degeneration, are substrates of CMA and can affect this degradative system (Ormeño et al., 2020). Interestingly, macroautophagy and endocytosis connection is also important in models of frontotemporal dementia. Impaired formation of multivesicular bodies due to ESCRT-III dysfunction in the membrane of the late endosomes reduces autophagy activity and induces autophagosome accumulation (Wong and Cuervo, 2010). This idea demonstrates the crosstalk between multiple types of lysosomal processes in this neurodegenerative disorder.

On the other hand, animal models for mutant prion protein (PrP) disease present downregulation of LAMP2A and HSC70 proteins, and that the overexpression of polo-like kinase 3 (PLK3) induces PrP degradation by CMA (Wang et al., 2017).

Overall, either by the negative effect of the mutant proteins or by the reduction on the expression of the machinery itself, a downregulation of CMA has been described in most neurodegenerative diseases. Further research directed to the study of the other regulatory levels of CMA discussed in previous sections in this work would contribute to the comprehensive view of the altered physiological mechanisms leading to CMA downregulation in ND (**Figure 3**). Together with this, the potential of CMA as a diagnosis/prognosis biomarker and its use as a therapeutic target remains to be elucidated.

CMA IN GLIOBLASTOMA

Glioblastoma (GBM) represents the most common and aggressive primary brain tumor and it is classified as grade IV glioma, by the World Health Organization (WHO; Louis et al., 2016). Recent pieces of evidence support that CMA is hyper-activated in GBM (**Table 3**). Moreover, surgical samples from patients treated with temozolomide (TMZ) chemotherapy present induced expression of LC3B, LAMP1, and LAMP2A compared to the initial sample (Natsumeda et al., 2011). Curcumin treatment, which has shown anti-tumor effects in GBM, downregulates the expression of LAMP2A in GBM cell lines (Maiti et al., 2019) suggesting a cell-autonomous effect of CMA in GBM. In this direction, GBM-associated proteins such as TP53, EGFR, or HIF1α have been identified as CMA substrates (Vakifahmetoglu-Norberg et al., 2013). However, the implication of their altered CMA-mediated degradation in GBM remains to be elucidated. A quiescent subset of endogenous glioma cells named glioma stem cells (GSC) is responsible for sustaining long-term tumor growth and therapy resistance (Chen et al., 2012). The impact of macroautophagy in GSCs has been widely described (Nazio et al., 2019), but the role of CMA fully remains to be investigated. The fact that CMA substrates such as EGFR or HIF1α have been associated with GSCs population (Emlet et al., 2014), and that curcumin treatment potentially may target GSC subpopulation (Fong et al., 2010), indirectly links CMA to GSC activity. However, other studies claim that blocking CMA-mediated HIF1α degradation induces resistance to TMZ (Lo Dico et al., 2018) and that TMZ resistant cells are unable to increase cytoplasmic ROS levels and activate CMA, preventing thus GBM cell toxicity (Lo Dico et al., 2019). In this sense, it has been described that the inhibition of macroautophagy increases the susceptibility of GSCs to TMZ by augmenting ferroptosis, a CMA-associated necrotic cell death mechanism (Buccarelli et al., 2018). Thus, further studies are needed to elucidate the role of CMA in GSC subpopulation and therapy resistance.

Interestingly, a GBM mouse model grafted *in vivo* with CMA-defective pericytes shows reduced GB proliferation and effective immune response compared to the ones grafted with control pericytes (Valdor et al., 2019). This fact, together with other studies associating CMA to tumor-associated macrophages in other types of cancer (Guo et al., 2017; Wang et al., 2019b), highlights the potential role of CMA in the crosstalk between the tumor cells and the microenvironment.

Overall, studies regarding CMA in GBM have focused on the impact of the modulation of LAMP2A expression by itself (**Figure 3**). Nevertheless, the status of LAMP2A trafficking or stability in the lysosomal membrane or even post-translational

TABLE 3 | Main studies revealing CMA upregulation in glioblastoma (GBM).

Target	Role in CMA	Finding	Model of study	Reference
LAMP2A	Key effector	CMA defective pericytes reduce proliferation of GB.	Murine primary pericytes and U373/U87 GB cell lines.	Valdor et al. (2019)
		Curcumin reduces LAMP2A expression.	U-87MG, GL261, F98, C6-glioma, and N2a cells.	Maiti et al. (2019)
		TMZ treatment induce expression of LAMP2A	pre-/post-TMZ treatment human tumors.	Natsumeda et al. (2011)
		Block of CMA-mediated HIF1α degradation induces resistance to TMZ.	U251, U87, T98, U138 GB cell lines.	Lo Dico et al. (2018)
		TMZ resistant cells are unable to increase ROS levels and activate CMA, preventing GB cell toxicity	U251 and T98 cell lines.	Lo Dico et al. (2019)
Ferroptosis	Process in which CMA participate.	Macroautophagy inhibition increases susceptibility of GSCs to TMZ by inducing ferroptosis	Patient-derived GSCs and deparaffinized human tumor sections.	Buccarelli et al. (2018)

modifications of LAMP2A, HSC70, or even KFERQ-like motifs of CMA substrates has not been explored.

Of note, it is known that GBM cells need to maintain balanced proteostasis for their survival and progression, by complex crosstalk between protein synthesis, unfolded protein response, stress response pathways, autophagy, and UPS (Yang et al., 2019). However, future studies will be necessary to assess the possible association of CMA with other protein degradation systems in GBM, as it is still a young field and no evidence has been published so far.

THERAPEUTIC TARGETING OF CMA

The impact of autophagy as a therapeutic target in human brain pathologies has been studied mainly in terms of macroautophagy, where many preclinical studies in PD and GBM have been developed (Towers and Thorburn, 2016; Moors et al., 2017; Taylor et al., 2018). Indeed, few initial clinical trials in GBM with the lysosomal inhibitor chloroquine have been also performed, but with no significant overall survival improvement due to dose-limiting toxicity (Sotelo et al., 2006; Rosenfeld et al., 2014). Importantly, macroautophagy modulators such as 3-methyladenine, LY294002, vinblastine, or rapamycin do not alter CMA activity (Finn et al., 2005). Although to date, there are not specific modulators of CMA, several compounds have demonstrated to alter key effectors of this process. On the one hand, compounds such as geldanamycin (Pedrozo et al., 2013), 6-aminonicotinamide (Finn et al., 2005), glucose-6-phosphate dehydrogenase inhibitor (Finn et al., 2005), silymarin (Tripathi et al., 2020), chronic caffeine (Luan et al., 2018), manganese (Yan et al., 2019), trehalose (Rusmini et al., 2019), β-Asarone (Huang et al., 2016) and other compounds extracted from natural medicinal plants (Wu et al., 2019a), or even combination treatments with bortezomib and suberoylanilide hydroxamic acid (SAHA; Watanabe et al., 2010) increase LAMP2A levels and thus, CMA activity. This therapeutic strategy could be beneficial for neurodegenerative diseases, where, as mentioned before, CMA activity is downregulated.

On the other hand, treatment with compounds such as P140 (Macri et al., 2015), vitamin E (Cao et al., 2009), protein synthesis inhibitors anisomycin and cycloheximide (Finn et al., 2005) or p38MAPK inhibitor (Finn et al., 2005) downregulate CMA, a strategy that may be beneficial for cancer context. Overall, as mentioned before, the modulatory effects of all these compounds are not specific for CMA machinery, they have many other targets. Thus, novel strategies must be developed to get new insights regarding CMA-specific and thus, safer therapeutic approaches. For this, many advances have been done regarding both *in vitro* and *in vivo* models for the study of CMA (Juste and Cuervo, 2019). Among them, *in vitro* methods based on the use of a photoconvertible fluorescent reporter (Koga et al., 2011b) or a "GAPDH-HaloTag" fusion protein (Seki et al., 2012; Sato et al., 2016), and a recently described transgenic reporter mouse that allows dynamic measurement of CMA activity *in vivo* (Dong et al., 2020) will facilitate further advances in the field.

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

CMA is a protein selective type of autophagy with increasing evidence revealing a role in homeostasis and pathology of a wide variety of organs, with special emphasis on aging and cancer. Regarding the brain, and in line with the protective role of CMA against protein damage and formation of toxic aggregates, much of the evidence gathered so far is focused on neurodegenerative diseases. Alterations in the expression of CMA regulators and the blocking effect of mutant CMA substrates show the reduced activity of this process in disorders such as PD or AD. The study of CMA in physiological brain aging is still a relatively young field, but recent pieces of evidence support the hypothesis that CMA could be involved in the decline in brain homeostasis and regeneration capacity. To consolidate this idea, future studies should be directed to understanding the impact of CMA in different cell types, with special interest on the neurogenic niches, whose impairment has been linked to age-associated cognitive and functional decline. Intriguingly, some evidence demonstrates that glioblastoma presents an upregulated activity of CMA, not only affecting tumor cells but also their crosstalk with pericytes. Similarly, recent studies in PD also show a CMA-mediated modulation of the crosstalk between astrocytes and neurons, highlighting its promising role in intercellular communication. Thus, further efforts should be done to elucidate the impact of CMA in the brain microenvironment during physiological and pathological aging.

Overall, CMA alterations in brain aging, neurodegenerative diseases, or glioblastoma could be interpreted in two directions. On the one hand, it could be postulated that CMA-mediated degradation of key disease-relevant proteins can be modulated, so that while aged cells are not able to degrade mutant species of physiological CMA substrates, cancer cells use CMA to eliminate proteins such as tumor suppressor. On the contrary, it may be suggested that it is the entire CMA process that is altered, being downregulated in aging and upregulated in glioblastoma progression. These ideas encourage further research to understand the specific mechanisms altering CMA activity in brain aging and glioblastoma, as well as to develop selective modulators of CMA as new strategies against age-related pathologies.

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JA-I wrote the draft and AM reviewed it and supervised the work.

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REVIEW
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Emerging Evidence Highlighting the Importance of Redox Dysregulation in the Pathogenesis of Amyotrophic Lateral Sclerosis (ALS)

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The cellular redox state, or balance between cellular oxidation and reduction reactions, serves as a vital antioxidant defence system that is linked to all important cellular activities. Redox regulation is therefore a fundamental cellular process for aerobic organisms. Whilst oxidative stress is well described in neurodegenerative disorders including amyotrophic lateral sclerosis (ALS), other aspects of redox dysfunction and their contributions to pathophysiology are only just emerging. ALS is a fatal neurodegenerative disease affecting motor neurons, with few useful treatments. Hence there is an urgent need to develop more effective therapeutics in the future. Here, we discuss the increasing evidence for redox dysregulation as an important and primary contributor to ALS pathogenesis, which is associated with multiple disease mechanisms. Understanding the connection between redox homeostasis, proteins that mediate redox regulation, and disease pathophysiology in ALS, may facilitate a better understanding of disease mechanisms, and lead to the design of better therapeutic strategies.

Keywords: redox dysregulation, ALS pathogenesis, oxidative stress, PDI—protein disulfide isomerase, SOD1, ROS—reactive oxygen species

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INTRODUCTION

Amyotrophic lateral sclerosis (ALS), or motor neuron disease (MND), is a fatal neurodegenerative disorder associated with aging, with an average survival time of 2–5 years following diagnosis. ALS is characterized by the degeneration of both upper motor neurons, which project from the cortex to the brainstem and the spinal cord, and lower motor neurons, which project from the brainstem or spinal cord to muscle. ALS commonly begins in late adulthood and many patients present with spinal onset ALS, characterized by muscle weakness in the limbs. However, in others, ALS begins in the bulbar muscles, which is characterized by difficulties in speech and swallowing. Patients develop progressive paralysis and the disease advances rapidly until at end-stage, only support and palliation are available. Death usually occurs from respiratory failure. ALS also shares neuropathological similarities with frontotemporal dementia (FTD). In fact, ALS and FTD represent two opposite ends of the same disease spectrum, with overlapping clinical symptoms and genetics (Shahheydari et al., 2017).

The cellular redox state, or balance between cellular oxidation/reduction reactions, serves as a vital antioxidant defence system that is linked to all important cellular activities (Calabrese et al., 2010). Redox regulation is, therefore, a fundamental cellular process for aerobic organisms. The imbalance between the production and accumulation of reactive species in cells leads to oxidative and nitrosative stress, producing reactive oxygen species (ROS) and reactive nitrogen species (RNS). Dysregulation of redox conditions alters the cellular redox state, modifies redox-sensitive proteins, and disrupts redox-regulated mechanisms.

Redox homeostasis controls multiple cellular signaling pathways including proper functioning of the mitochondria and endoplasmic reticulum (ER) compartments, calcium regulation, axonal transport, autophagy, protein folding, and proteostasis (Görlach et al., 2006; Cao and Kaufman, 2014; Li P. et al., 2016; Yoboue et al., 2018; Guerrero-Gómez et al., 2019). Not surprisingly, dysregulation in the cellular redox state is implicated in many diseases, including ALS (Parakh et al., 2013; Mcbean et al., 2015; Pinho et al., 2020). Furthermore, during the normal aging processes, the ability of cells to maintain their normal redox state diminishes (Go and Jones, 2017; Castelli et al., 2019). Whilst this is true of all cell types, neurons are particularly susceptible to redox dysregulation due to their large size and high consumption of oxygen. Furthermore, they produce significant quantities of ROS and RNS (Kato et al., 2005). Moreover, disruption to redox dysregulation is increasingly implicated as an important driver of neurodegeneration in the pathogenesis of many age-related neurodegenerative disorders, including ALS. Also, several pathogenic mechanisms linked to ALS involve redox-sensitive proteins, including protein disulfide isomerase (PDI), thioredoxin, and glutathione (GSH), and recent evidence highlights their importance in neurodegeneration. However, whilst redox dysfunction has been associated with ALS for some time (Harraz et al., 2008; Cohen et al., 2012; Conrad et al., 2013; Parakh et al., 2020), a precise understanding of how cellular redox conditions are dysregulated has been lacking. Nevertheless, recent evidence implies that redox homeostasis is a central and primary mechanism in ALS and it may have greater importance than previously recognized (Sbodio et al., 2019; Parakh et al., 2020). Here, we provide a comprehensive review of the evidence linking redox dysfunction to ALS. We discuss the cellular redox system, the major pathological proteins and pathways associated with ALS, and their relationship to redox homeostasis. We also describe how redox modifications are associated with ALS-like phenotypes.

AMYOTROPHIC LATERAL SCLEROSIS (ALS)

ALS is a multifactorial neurodegenerative disease (Taylor et al., 2016). Most cases (∼90%) have no previous family history and hence are termed "sporadic ALS (sALS)." Specific environmental factors may increase the risk of developing ALS, including smoking, air pollution, agricultural and industrial pollutants, β-N-methyl amino-L-alanine (BMAA) toxicity, and physical activity. However, previous studies have yielded conflicting results, so the role of environmental factors involved in triggering ALS remains unclear (Bozzoni et al., 2016; Wood, 2016; Yu and Pamphlett, 2017). Genetic mutations account for the remaining approximately 10% of cases, termed "familial" ALS (fALS; Taylor et al., 2016). ALS shares clinical and pathological attributes with FTD, which is characterized by deterioration in behavior, personality, and/or language. In FTD, neurons in the frontal and temporal lobes of the brain primarily degenerate and die (Neumann et al., 2006; Burrell et al., 2016). ALS and FTD are closely related genetically and pathologically, and these two conditions are now considered to be at opposite ends of the same disease continuum (Shahheydari et al., 2017). Like other neurodegenerative diseases, ALS is a protein misfolding disorder and abnormal misfolded protein inclusions are present in motor neurons and glia.

Most familial cases of both ALS (∼40%) and FTD (25%) are associated with hexanucleotide (G4C2) expansions in the *Chromosome 9 open reading frame 72 (C9orf72)* gene (Dejesus-Hernandez et al., 2011; Renton et al., 2011; Bigio, 2012; Lee Y.-B. et al., 2013; Ling et al., 2013; Balendra and Isaacs, 2018), followed by mutations in *superoxide dismutase 1* (*SOD1*; 20%), *TARDBP* (4–5%), encoding TAR DNA-Binding Protein-43 (TDP-43), and *Fused in Sarcoma* (*FUS*; 5%). Interestingly, genetic mutations in sALS patients have also been described, in *C9orf72* (7% of cases), *TARDBP, SOD1, FUS, VCP, p62, PFN-1*, MATR3, *OPTN, UBQLN2*, *CHCHD10, TBK1*, *TUBA4A, NEK1, C21orf2*, and *CCNF*, together accounting for 15% of sporadic ALS cases (Taylor et al., 2016; Chia et al., 2018). **Table 1** summarizes the genetic mutations identified in both familial and sporadic ALS patients.

Mutations in SOD1, TDP-43, C9orf72, and FUS therefore together account for a large proportion of fALS cases and have been extensively studied *in vitro* (Taylor et al., 2016). Furthermore, disease models based on the transgenic expression of ALS-associated mutations *in vivo* have been used extensively to investigate ALS pathogenesis and motor neuron degeneration (Taylor et al., 2016). From these studies, many pathogenic mechanisms have been described, including dysfunction to redox homeostasis (Taylor et al., 2016; Hardiman et al., 2017; Mejzini et al., 2019). Transgenic mice expressing human SOD1 mutants, particularly $SOD1^{G93A}$, are the most extensively studied animal used for ALS.

Most of the ALS mutations are point mutations or truncations. However, C9orf72 contains a hexanucleotide repeat expansion, hence it is distinct from the other ALS/FTD mutations. Both losses of normal cellular function and gain of aberrant toxic functions (or both together) are implicated as pathogenic mechanisms in ALS, depending on the protein involved. Both loss and gain of functions have been proposed for mutant TDP-43 and mutant FUS, whereas gain of a toxic function is the preferred mechanism associated with mutant SOD1. Interestingly, two possible gain of functions mechanisms are thought to be induced by the C9orf72 repeat expansion; toxicity from the long repeat RNA or the production of dipeptide repeat proteins (DPR). These DPRs are generated by repeat-associated non-ATG translation (RAN translation), and five distinct

TABLE 1 | Genetic mutations identified in familial and sporadic forms of amyotrophic lateral sclerosis (ALS).

Gene	Locus	Protein encoded	sALS (%) Frequency	fALS (%) Frequency	Cellular function	References
SOD1	21q22.1	Superoxide dismutase 1	1–2	12–23.5	Redox regulation and antioxidant activity	Rosen et al. (1993) and Andersen et al. (2003)
DAO	12q22-23	D-amino acid oxidase	NA	Unknown	Enzyme that catalyzes the oxidative deamination of D-amino acids	Mitchell et al. (2010)
C9orf72	9p21.2	Chromosome 9 open reading frame72	7	30–50	Rab mediated cellular trafficking, autophagy, autoimmunity, indirect oxidative stress, and vesicular trafficking	Dejesus-Hernandez et al. (2011) and Renton et al. (2011)
TARDBP	1p36.22	TAR DNA-binding protein-43	1	2–5	RNA metabolism, DNA repair	Rutherford et al. (2008), Sreedharan et al. (2008), and Kirby et al. (2010)
FUS	16p11.2	Fused in Sarcoma	1	5	RNA metabolism, DNA repair	Kwiatkowski et al. (2009) and Vance et al. (2009)
VCP	9p13.3	Valosin-containing protein	1	<1	Protein degradation, intracellular membrane fusion and protein quality control	Shaw (2010)
TBK1	12q14.2	Tank-binding kinase1	<1	<1–5	Kinase involved in autophagy, inflammation	Cirulli et al. (2015), Freischmidt et al. (2015) and Gijselinck et al. (2015)
CHCHD10	22q11.23	Coiled-coil-helix-coiled-coil-helix domain containing 10	<1	3.6	Mitochondrial regulation	Bannwarth et al. (2014) and Zhang et al. (2015)
SQSTM1/p62	5q35	Sequestosome 1/p62	<1	1.8	Autophagy receptor involved in selective autophagy, UPS	Fecto et al. (2011) and Le Ber et al. (2013)
CCNF	16p13.3	Cyclin F	2	0.6–3.3	Regulator of cell cyclin transitions, a component of the E3 Ubiquitin protein ligase linked to protein homeostasis, UPS	Williams et al. (2016)
PFN1	17p13	Profilin-1	<1	<1	Actin binding protein involved in regulating actin dynamics	Wu et al. (2012)
ALS2	2q33	Alsin	NA	Unknown	Cellular trafficking, neuroinflammation, redox regulation	Yang et al. (2001)
KIF5A	12q13.3	Kinesin family of proteins	NA	<1	Molecular motor protein involved in axonal transport	Brenner et al. (2018) and Nicolas et al. (2018)
OPTN	10p13	Optineurin	<1	2.6	Maintenance of Golgi complex, membrane trafficking, and autophagy receptor	Maruyama et al. (2010) and Pottier et al. (2015)
UBQLN2	Xp11.21	Ubiquilin 2	<1	0.5–2	Macroautophagy and the UPS	Deng et al. (2011), Synofzik et al. (2012), Williams et al. (2012), and Gellera et al. (2013)
NEFH	22q12	Neurofilament heavy chain		Rare	Component of the cytoskeleton that provides structural support and facilitates axonal transport	Figlewicz et al. (1994)
TUBA4A	2q36.1	Tubulin α-4A chain	<1	<1	Microtubule subunit involved in intracellular transport and DNA segregation	Smith et al. (2014)

peptides are produced (GA, GP, GR, PR, and PA) resulting from translation of both sense and antisense strands (Dejesus-Hernandez et al., 2011; Renton et al., 2011). Haploinsufficiency, due to reduced expression of C9orf72 in ALS patients, is also implicated in pathogenesis (Sellier et al., 2016).

Interestingly, the proteins encoded by these genes display diverse functions. The normal cellular function of C9orf72 is related to vesicular trafficking and autophagy (Farg et al., 2014) whereas SOD1 is an antioxidant enzyme that mediates detoxification of the superoxide anion radical (O_2^-;

Mccord and Fridovich, 1969). In contrast, both TDP-43 and FUS are RNA and DNA binding proteins found predominantly in the nucleus, where they regulate transcription, the DNA response, mRNA splicing, RNA stability, micro-RNA biogenesis, translation and transport, and stress granule formation (Cohen et al., 2011; Loughlin and Wilce, 2019; Birsa et al., 2020). TDP-43 is an important pathological protein because the abnormal accumulation of misfolded, wildtype (WT) cytoplasmic TDP-43 into inclusions is the characteristic pathological hallmark of ∼97% of ALS patients (sALS and fALS; Neumann et al., 2006). Similarly, mislocalization of WT, misfolded FUS into the cytoplasm was recently implicated as a key pathological hallmark of ALS (Neumann et al., 2006; Tyzack et al., 2019). TDP-43 and FUS, therefore, share strong structural, functional, and pathological similarities, distinct from C9orf72 and SOD1. However, as well as TDP-43 and FUS, WT SOD1 has also been described in the inclusions in sporadic ALS motor neurons (Tokuda et al., 2019).

Disease Mechanisms in ALS

Whilst the proteins associated genetically or pathologically with ALS are diverse in function and structure, similar disease mechanisms are implicated. Moreover, there are a plethora of different processes known to be dysregulated in ALS. However, ultimately it is imperative to elucidate the primary, upstream mechanisms responsible for neurodegeneration in ALS so that effective therapeutics can be designed. These mechanisms include impaired axonal transport (Collard et al., 1995; Williamson and Cleveland, 1999), neurofilament aggregation (Al-Chalabi et al., 1995; Xiao et al., 2006; Xu Z. et al., 2016), protein misfolding (Kopito, 2000; Basso et al., 2006), abnormal RNA processing (Chen et al., 2010; Dejesus-Hernandez et al., 2011; Parisi et al., 2013; Droppelmann et al., 2014), lipid peroxidation (Shibata et al., 2001) and cholesterol esterification (Cutler et al., 2002; Chaves-Filho et al., 2019), defects in nucleocytoplasmic transport (Boeynaems et al., 2016), induction of DNA damage (Konopka and Atkin, 2018; Naumann et al., 2018; Konopka et al., 2020), cytoplasmic mislocalization of nuclear proteins (Neumann et al., 2006), mitochondrial dysfunction (Albers and Beal, 2000), glutamate excitotoxicity (Shaw and Ince, 1997), proteasomal and autophagic dysfunction (Chen et al., 2012), ER stress (Nagata et al., 2007; Walker et al., 2010), mitochondrial associated membrane (MAM) dysfunction (Watanabe et al., 2016), ER-Golgi transport defects (Atkin et al., 2014; Soo et al., 2015), autophagy dysregulation and apoptosis (Ravits et al., 2013; Robberecht and Philips, 2013; Gao et al., 2017; Mandrioli et al., 2020). For a detailed discussion of these mechanisms, please see several excellent reviews (Zarei et al., 2015; Taylor et al., 2016; Weishaupt et al., 2016; Mejzini et al., 2019). Here we will focus only on those mechanisms linked to cellular redox processes.

Mechanisms of redox regulation are a vital antioxidant defense system that underpins many important cellular activities. Not surprisingly, redox dysfunction is implicated as an important pathogenic mechanism in ALS. Mutations in many ALS-associated genes are associated with cellular redox dysregulation, particularly *SOD1*, *C9orf72, FUS*, *TDP-43*, *CHCHD10*, and *ALS2*. Furthermore, mutations in other ALS genes disrupt the cellular redox balance and induce oxidative stress (Carter et al., 2009). Also, mutations in genes encoding proteins that are involved in maintaining redox homeostasis are present in ALS patients, such as SOD1 and D-amino acid oxidase (DAO; Mitchell et al., 2010; Kondori et al., 2018). Moreover, dysregulation of redox homeostasis is also present in sALS patient tissues (Kato et al., 2005), thus placing redox dysregulation onto the pathophysiology of the most common forms of ALS. The fundamental processes underlying control of the cellular redox environment will now be discussed.

THE CELLULAR REDOX SYSTEM

The cellular redox state refers to the balance between oxidation and reduction reactions (Calabrese et al., 2010; Sies et al., 2017; Sies and Jones, 2020). Redox regulation involves proteins and cofactors that maintain the appropriate redox environment for proper functioning of the cell (Ray et al., 2012), ensuring that there is a balance between the production of ROS, RNS, and their consequent elimination by antioxidant enzymes and smaller molecules. Cells have developed a sophisticated antioxidant system to protect against these oxidative insults, consisting of enzymes that either convert superoxide radicals into hydrogen peroxide (H_2O_2, SOD1, and catalases) or H_2O_2 into water and oxygen [peroxiredoxin (Prx) and glutathione peroxidase (GPx); Fridovich, 1986; Espinosa-Diez et al., 2015]. Prx and GPx require secondary enzymes and cofactors to function efficiently and regulate the cytoplasmic redox system (Chae et al., 1994).

The overall cellular redox state is determined by two cellular disulfide reductase systems (Das and White, 2002; Ren et al., 2017). First, the thioredoxin system comprises thioredoxin (Trx), thioredoxin reductase (TrxR), thioredoxin peroxidase (TrxP), and nicotinamide adenine dinucleotide phosphate (NADPH; Lee S. et al., 2013; Lu and Holmgren, 2013). Trxs are ubiquitous antioxidant enzymes containing thiol groups (-SH) that are present in cysteine residues (Collet and Messens, 2010). Thiol groups are important components of redox-mediated processes due to their unique chemistry, involving nucleophilicity, metal binding, and the ability to form protein disulfide bonds (Ulrich and Jakob, 2019). Hence, whilst cysteine is one of the less common amino acids, it is often highly conserved within protein functional groups (Marino and Gladyshev, 2010). Second, the glutathione system comprises NADPH, glutathione reductase (GR), glutathione peroxidase (GPx), and glutathione (GSH; Ursini et al., 2016; Ren et al., 2017). GSH is a tripeptide consisting of cysteine, glutamic acid, and glycine, and it is an important cellular antioxidant. In fact, it is the most abundant low molecular weight thiol-containing compound produced in cells. NADPH is the principal reductant used to maintain the redox states of both the Trx and GSH systems. Trx and GSH control redox signaling and regulate cellular H_2O_2 concentrations through Prxs and GPxs. Trx and GSH are found in several different subcellular compartments, including the nucleus, cytoplasm and mitochondria, in both neuronal and glial cells (Ren et al., 2017).

The reduced form of glutathione (reduced GSH) is the biologically active species that regulates the cellular antioxidant defense system. GSH also maintains the intracellular redox milieu to preserve the thiol-disulfide redox states of proteins (Morgan et al., 2013). Interestingly, the proper folding of proteins and the formation of protein disulfide bonds both depend on the redox status within the ER. Importantly, the lumen of the ER represents a more oxidizing environment than the cytoplasm, with a higher ratio of oxidized to reduced glutathione (GSSG/GSH; Van Der Vlies et al., 2003). In the ER, the oxidoreductase enzymes endoplasmic oxidoreductin 1 (Ero1) and PDI facilitate disulfide bond formation in substrate proteins. Redox dysfunction can affect their activity, and in particular reduced GSH or protein thiols react with and generate ROS, inducing ER stress (Hatahet and Ruddock, 2009; Ramming and Appenzeller-Herzog, 2012), which dysregulates cellular redox homeostasis (Espinosa-Diez et al., 2015). **Figure 1** summarizes the major cellular redox systems.

REDOX DYSREGULATION

Oxidative stress results from elevated intracellular levels of ROS, and nitrosative stress results from increased levels of RNS, and both events can significantly damage cells. Even slight modulations in the cellular redox state can produce neurotoxic species. ROS includes free radicals (superoxide (O_2^-) and hydroxyl radicals ($\cdot OH$), whereas RNS includes nitric oxide (NO) and nitrogen dioxide (NO_2; Valko et al., 2007). Redox homeostasis ensures that cells respond to these redox stressors efficiently. However, when it is disturbed, neurodegeneration can result (Parakh et al., 2013; Mcbean et al., 2015). The primary sources of ROS production in the central nervous system (CNS) are mitochondrial proteins, NADPH oxidase, Rac1, and SOD1 (Nayernia et al., 2014; Di Meo et al., 2016). The generation of ROS can be activated by endogenous factors, such as the mitochondrial electron transport chain (ETC), NADPH oxidases (NOX), lipoxygenases (LOX), cytochrome P450, and xanthine oxidase (XO), or by exogenous causes such as pollutants, chemicals/drugs, radiation and heavy metals (Moussa et al., 2019). Oxidation of GSH to GSSG results in dysregulation of the intracellular redox imbalance (decreased GSH:GSSG ratio), which is associated with oxidative stress and DNA damage in fibroblasts (Asensi et al., 1999).

Modification of cysteine thiols by ROS and RNS has emerged as an important mechanism of altering protein

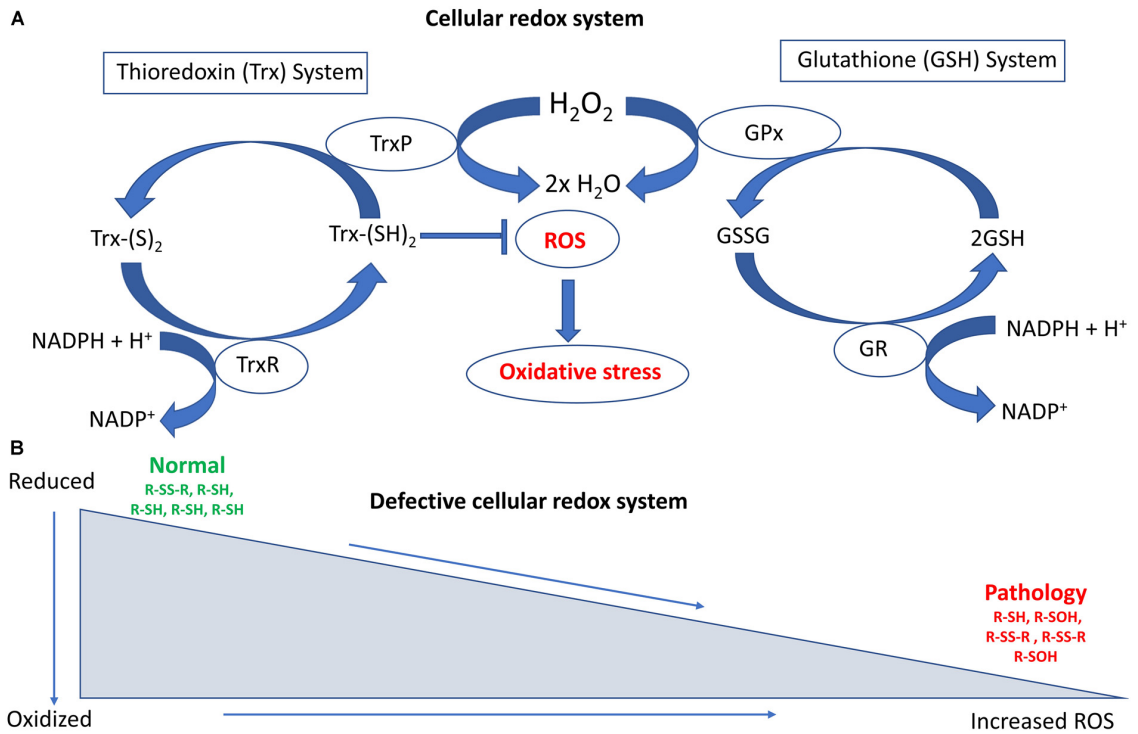

FIGURE 1 | Schematic diagram illustrating the major cellular redox systems. **(A)** Cellular redox processes include the thioredoxin (Trx) and glutathione (GSH) systems that reversibly regulate thiol modifications. The overall redox state of the cell is determined by these two cellular disulfide reductase systems. The thioredoxin system comprises thioredoxin reductase (TrxR), nicotinamide adenine dinucleotide phosphate (NAPH), thioredoxin peroxidase (TrxP), and Trx. TrxR is involved in the conversion of Trx-$(S)_2$ (oxidized form of Trx) into Trx-$(SH)_2$ (reduced form of Trx), whereas TrxP is involved in the conversion of Trx $(SH)_2$ into Trx-$(S)_2$. The glutathione system comprises GR (Glutathione reductase), NADPH, GSH, and GPx (Glutathione peroxidase). GR is involved in converting GSSG (Glutathione disulfide) to GSH whereas GPx is involved in converting GSH to GSSH. Overloading of the Trx system can increase reactive oxygen species (ROS) accumulation and oxidative stress. **(B)** Defective cellular redox conditions can produce increased levels of ROS. This can lead to irreversible thiol modifications such as sulfinic or sulfonic acid ($-SO_3H$) formation, as well as induce protein degradation and associated pathological events in amyotrophic lateral sclerosis (ALS).

structure and function (Cai and Yan, 2013). Many proteins undergo reversible thiol modifications during physiological redox signaling processes as part of cellular defense mechanisms against oxidative and nitrosative damage (Brandes et al., 2009). There is a broad range of possible alterations to these thiols, including S-nitrosylation (-SNO), S-sulfenylation (sulfenic acid, -SOH), S-glutathionylation (-SSG), disulfide formation (-S-S-), S-sulfhydration (−S-SH), and S-sulfinylation (−SO_2H; Hawkins et al., 2009; Mieyal and Chock, 2012; Finelli, 2020). However, in addition to these reversible modifications, several cysteine adducts can form irreversibly due to prolonged exposure of cysteine residues to ROS and RNS, such as sulfinic or sulfonic acids (Chouchani et al., 2011; Paulsen and Carroll, 2013). These irreversible thiol modifications can lead to protein degradation and loss of function and are present in neurodegenerative diseases (Ren et al., 2017).

DIRECT EVIDENCE FOR A ROLE OF REDOX DYSREGULATION IN ALS

In this section, we discuss evidence demonstrating that the central cellular redox regulatory mechanisms and associated proteins are perturbed in ALS (**Table 2**). This includes NOX, apurinic/apyrimidinic endonuclease 1 (APE1), Prx, thioredoxin (TRX)-related transmembrane-2 (TMX2), activator protein 1 (AP-1), PDI, and SOD1. Later we discuss more indirect evidence for dysregulation to redox homeostasis in ALS.

Induction of Oxidative Stress

It is well established that oxidative and nitrosative stress markers are upregulated in ALS patients and disease models (Yang et al., 2001; Cereda et al., 2006; Babu et al., 2008; Lee et al., 2009; Cozzolino et al., 2012; D'Amico et al., 2013). Increased ROS, RNS, and products of oxidation, have been observed both in post-mortem human samples and in $SOD1^{G93A}$ mice (Carrí et al., 2003). Oxidative stress has also been linked to the abnormal accumulation of misfolded SOD1 in ALS patients, and in transgenic *C. elegans* expressing mutants $SOD1^{A4V}$, $SOD1^{G37R}$, or $SOD1^{G93A}$ (Oeda et al., 2001). Furthermore, oxidative stress has been extensively studied in cells expressing mutant SOD1 as well as in transgenic $SOD1^{G93A}$ mice, revealing that various SOD1 mutants increase oxidative stress and dysregulate redox homeostasis (Ferri et al., 2006; Marden et al., 2007; Fukai and Ushio-Fukai, 2011).

An ALS clinical trial administering 600 mg of GSH reported a slightly decreased rate of disease progression (Chili et al., 1998). A significantly lower level of GSH was detected in serum of human sALS patients compared to controls (Ehrhart et al., 2015). Furthermore, reduction in the levels of intracellular GSH increases oxidative stress, mitochondrial dysfunction, and apoptosis in $SOD1^{G93A}$ mice models (Chi et al., 2007). Decreased levels of GSH in WT SOD1 expressing mice were associated with fewer motor neurons and a shorter average lifespan (Killoy et al., 2018). Oxidative stress induced by GSH depletion also reproduces pathological features of TDP-43 in neuronal cells; phosphorylation, cytoplasmic re-distribution, and aggregation (Iguchi et al., 2012). The addition of GSH or expression of Grxs 1 and 2 significantly improves mutant SOD1 solubility in cell culture, whereas reducing the levels of intracellular GSH decreases SOD1 solubility, suggesting that GSH reduction promotes the aggregation of mutant SOD1 (Guareschi et al., 2012).

Treating cells with L-buthionine sulfoximine (BSO), an inhibitor that blocks the synthesis of GSH, renders neuronal SH-SY5Y cells expressing mutant valosin-containing protein (VCP) R487H more susceptible to oxidative stress (Hirano et al., 2015) and increases mutant SOD1 toxicity (Alvarez-Zaldiernas et al., 2016; Bakavayev et al., 2019). Furthermore, BSO induces the misfolding of mutant TDP-43 and mutant SOD1 (see "Protein Folding" section). Similarly, expression of mutant TDP-43 in cellular, yeast, and *Drosophila* models increases markers of oxidative stress, including protein carbonylation and glutathione S transferase D1 (Duan et al., 2010; Braun et al., 2011; Zhan et al., 2015). Furthermore, several micro-RNAs known to regulate the expression of genes involved in counteracting ROS/RNS are differentially regulated in ALS patients. Specific micro-RNAs were upregulated; miR-27a, miR-338-3p, miR-155, whereas other micro-RNAs were downregulated in ALS patients; miR-142-5p, and miR-34a (Koval et al., 2013; Waller et al., 2017; Ricci et al., 2018; Li C. et al., 2019). A meta-data analysis of oxidative stress biomarkers from 41 studies involving a total of 4,588 ALS patients and 6,344 control subjects, revealed a significant increase in malondialdehyde, 8-hydroxyguanosine and advanced oxidation protein products in ALS patients compared to controls (Wang Z. et al., 2019). However, the levels of other oxidative stress markers, uric acid, and GSH were significantly reduced in ALS patients (Wang Z. et al., 2019). Furthermore, no significant changes in the levels of other markers were observed; blood Cu, SOD1, glutathione peroxidase, ceruloplasmin, triglycerides, total cholesterol, low-density lipoprotein, high-density lipoprotein, coenzyme-Q10, and transferrin l; (Wang Z. et al., 2019).

There is also evidence that FUS is involved in the cellular response to oxidative DNA damage, and it is well established that FUS has significant functions in DNA repair (Wang et al., 2013; Naumann et al., 2018). Immunoprecipitation studies revealed an increased association of FUS with XRCC1, LigIII, and PARP-1, but not with other base excision repair (BER) proteins in ALS patient-derived iPSC lines carrying FUS mutations (R521H and P525L). This implies the presence of defects in DNA nick ligation and oxidative damage, and DNA repair mechanisms in FUS-associated ALS (Wang H. et al., 2018). Recently, TDP-43 was also shown to have a role in DNA repair (Mitra et al., 2019; Konopka et al., 2020), which is linked to oxidative stress (Guerrero et al., 2019). Collectively, these studies suggest that defects in DNA damage are a component of dysregulated redox homeostasis in ALS.

C9orf72 is a key regulator of lipid metabolism under conditions of cellular stress (Liu Y. et al., 2018). Loss of C9orf72 during starvation leads to dysregulated autophagy and increased *de novo* fatty acid synthesis (Liu Y. et al., 2018). Increased levels of free fatty acids and liquid droplets were

TABLE 2 | Redox related proteins associated dysregulation in ALS.

Protein Name	Function	Abnormalities in ALS	References
SOD1	This cytosolic enzyme facilitates the conversion of the superoxide anion to hydrogen peroxide and oxygen.	Mutant SOD1 disrupts redox homeostasis in ALS by abnormal production of ROS and RNS, and by the formation of misfolded protein aggregates.	Mccord and Fridovich (1969), Bruijn et al. (2004), and Wu et al. (2006)
NOX	NOX produces the substrate superoxide anion for the reaction catalyzed by SOD1 and controls the production of pro-inflammatory cytokines.	Elevated levels of NOX2, O_2^- and inactivation of NOX1 were observed in $SOD1^{G93A}$ mice. Deletion of NOX improves the survival of $SOD1^{G93A}$ mice. Mutant SOD1 alters NOX-dependent redox stress.	Mccord and Fridovich (1969) and Dunckley et al. (2007)
APE1	Regulates multiple transcription factors; NF-κB, STAT3, AP-1, HIF-1, and p53. APE1 interacts with proteins involved in both DNA repair and redox regulation.	APE1 is upregulated in motor neurons, astrocytes, and spinal cords of ALS patients. Mutant $SOD1^{G93A}$ restricts the localization of APE1 and inhibits redox homeostasis.	Shaikh and Martin (2002) and Kim et al. (2020)
Peroxiredoxins	Group of antioxidant enzymes that regulate peroxide and peroxynitrite.	Prx 3 is downregulated in $SOD1^{G93A}$ mice, SOD1, and SALS patients. Prx1, 2, and 6 were upregulated in $SOD1^{G93A}$ mice. Prx3 and Prx5 were found in mutant SOD1 aggregates.	Kato et al. (2005), Wood-Allum et al. (2006), and Knoops et al. (2016)
TMX2	Important sensor required for the maintenance of cellular redox homeostasis.	TMX2 is a protective modifier against C9orf72 DPR toxicity. Depletion of TMX2 suppresses PR_{20} induced cellular toxicity.	Kramer et al. (2018)
AP-1	A redox-sensitive transcription factor that regulates gene expression.	Mutant SOD1 upregulates AP-1 and AP-1 complex proteins like JUN and FOSL1.	Gomez Del Arco et al. (1997), Bhinge et al. (2017), and Kale et al. (2018)
PDI	A chaperone involved in protein folding. It also regulates the cellular redox state and signaling.	PDI levels are upregulated in transgenic mouse models of ALS and ALS patients. Mutations in PDI are also associated with the risk of developing ALS. The redox activity of PDI is protective in cellular models and zebrafish.	Walker et al. (2010), Woehlbier et al. (2016), and Parakh et al. (2020)

detected in iPSC-derived motor neurons from C9orf72 ALS/FTD patients, suggesting the presence of dysregulated lipid metabolism by free fatty acid synthesis (Liu Y. et al., 2018). Similarly, increased levels of Lysosomal-associated membrane protein 1 (LAMP1) and NOX2 were detected in C9orf72 ALS/FTD patient iPSC-derived motor neurons, and in spinal cords of C9orf72 ALS patients (Liu Y. et al., 2018). Also, NOX2 was upregulated in embryonic fibroblasts obtained from C9orf72 knockout mice, suggesting that cellular redox conditions are dysregulated by depletion of C9orf72 (Liu Y. et al., 2018). From these studies, it is therefore tempting to speculate that C9orf72 is involved in the regulation of redox homeostasis through NOX2.

Proteomic analysis of the frontal cortex (area 8) in C9orf72 FTD patients revealed abnormal expression of proteins linked to the synthesis of ROS, suggesting the presence of redox dysregulation in these patients (Andrés-Benito et al., 2019). Also, modifiers of poly GR_{100} toxicity identified from an unbiased genome-wide nonessential yeast gene knockout study revealed dysregulation of mitochondrial and NADPH related metabolic pathways (Chai and Gitler, 2018). Consistent with these findings, another study demonstrated that increased oxygen and ATP consumption increased ROS, and induced mitochondria hyperpolarization in C9orf72 ALS patient-derived fibroblasts (Onesto et al., 2016). Overall, whilst these findings imply that increased production of ROS is associated with C9orf72-ALS, it is unclear whether loss of C9orf72 or expression of hexanucleotide repeat expansions disturbs redox homeostasis.

SOD1

SOD1 is an important 32 kDa cytosolic antioxidant enzyme that facilitates the conversion of a superoxide anion radical (O_2^-) to H_2O_2 and oxygen (O_2);

$$2O_2^- + 2H+ \rightarrow H_2O_2 + O_2$$

(Mccord and Fridovich, 1969). Furthermore, SOD1 undergoes the cyclic reduction and oxidation of copper ions (Mccord and Fridovich, 1969), and it also inhibits oxidative inactivation of nitric oxide to prevent peroxynitrite formation (Harraz et al., 2008). More than 180 ALS-associated mutations in *SOD1* have been identified, and almost all these mutations are autosomal dominant. SOD1 was the first gene linked to ALS (Rosen et al., 1993), thus there has been intensive research into pathogenic mechanisms associated with mutant SOD1 (Rosen et al., 1993).

SOD1 normally regulates the NADPH oxidase-dependent production of O_2^- by inhibiting the Rac1 signaling pathway. Rac1 is a member of the Rac family of guanosine triphosphate (GTP) phosphohydrolases (GTPases), which bind both guanosine diphosphate (GDP) and GTP, leading to its inactivation or activation, respectively. Rac1 normally cycles between the GTP and GDP bound states, depending on the redox state of Rac1. The interaction between SOD1 and Rac1 serves as a redox sensor for the regulation of NADPH oxidase (Harraz et al., 2008), and itself is redox-sensitive. Under oxidizing conditions, the SOD1-Rac1 GTP interaction is inhibited. However, during reducing conditions, SOD1 efficiently binds to Rac1 and activates the NOX2 signaling pathway (Harraz et al., 2008). However, the normal uncoupling of SOD1 from Rac1 is defective in $SOD1^{G93A}$

mice, leading to Rac1 activation and hence inactivation of NADPH oxidase (Harraz et al., 2008).

The antioxidant properties of SOD1 and its relationship to neurodegeneration have been extensively studied in ALS (Proescher et al., 2008; Karch et al., 2009). Whilst loss of the normal SOD1 antioxidant enzymatic activity was initially proposed as a cause of neurodegeneration, further research implicated gain of a toxic function instead (Hu et al., 2003). Several studies have shown that misfolded, mutant SOD1 disrupts redox homeostasis in ALS by the abnormal production of ROS and RNS, inducing oxidative stress (Poon et al., 2005; Harraz et al., 2008). This is implicated in perturbing many cellular processes in both motor neurons and non-neuronal cells, including neuroinflammation (Bruijn et al., 2004; Wu et al., 2006; Marden et al., 2007). Also, both mutant and WT SOD1 produce cytotoxic levels of H_2O_2 *via* a cysteine redox regulation system (Bakavayev et al., 2019). Also, $SOD1^{G93A}$ mice show significantly increased protein carbonyl levels in the spinal cord, including elevated carbonylation of mutant SOD1, which has been linked to motor neuron degeneration (Poon et al., 2005).

The gain-of-function of mutant SOD1 toxicity has been also related to its ability to generate oxidants, as well as to a higher sensitivity of the enzyme to oxidants. Oxidation of the solvent-exposed W32 residue in SOD1 in particular has been associated with aggregation of mutant SOD1 *in vitro* and *in vivo* (Taylor et al., 2007; Coelho et al., 2014; Duval et al., 2019). Furthermore, W32 can be oxidized by the carbonate radical produced by SOD1 bicarbonate-dependent peroxidase activity, leading to the formation of a SOD1 covalent dimer cross-linked by a di-tryptophan bond. The di-tryptophan cross-link may weaken the non-covalent bonds between the SOD1 monomers, triggering enzyme unfolding, oligomerization, and aggregation (Coelho et al., 2014). Finally, recent studies have suggested that the oxidation of lipids (such as polyunsaturated fatty acids and cholesterol) generates electrophilic compounds that modify Lys residues in SOD1, inducing its aggregation (Dantas et al., 2020).

ALS-associated SOD1 mutants produce free radicals ($ONOO^-$ or OH^-) and some mutants lose its catalytic activity, which in turn produces highly unstable intermediate products and tyrosine (Abe et al., 1997; Raoul et al., 2006). Nitro-tyrosine and nitrated proteins have also been detected in the CSF of both sALS and fALS patients, indicating the presence of redox imbalance in these tissues. Reversible phosphorylation of the SOD1 residue Ser39 in yeast and Thr40 in human cell lines (HEK293, Hep3B, A549, and MCF7 cell) by mTOR signaling moderates ROS levels, prevents oxidative damage, and regulates redox-dependent growth and survival (Tsang and Zheng, 2018; Tsang et al., 2018). Furthermore, a recent study comparing global to muscle-specific knockout of SOD1 in mice demonstrated differentially altered neuromuscular integrity and dysregulated redox pathways in both the nerve and muscle of these animals (Sakellariou et al., 2018). This study supports the notion of impaired redox signaling, rather than oxidative damage, in peripheral nerves playing a key role in muscle loss and muscle sarcopenia during aging (Sakellariou et al., 2018).

NADPH Oxidase

Dysregulation of multiple transmembrane NOX proteins are implicated in ALS (Bedard and Krause, 2007; Marrali et al., 2014). Seven members of the NOX family of enzymes are known to exist in humans; NOX1, NOX2/gp91phox, NOX3, NOX4, NOX5, Duox1, and Duox2 (Bedard and Krause, 2007), and each has a specific tissue distribution and mechanism of activation (Leto et al., 2009). Importantly, activation of NOX generates the substrate O_2^- for the reaction catalyzed by SOD1 (Mccord and Fridovich, 1969). NOX also controls the production of pro-inflammatory cytokines interleukin-1-β (IL-1β), and tumor necrosis factor-α (TNFα), which are elevated in the plasma and CSF of ALS patients (Poloni et al., 2000; Dengler et al., 2005) and in spinal motor neurons of $SOD1^{G93A}$ (Hensley et al., 2003) and $SOD1^{G37R}$ (Nguyen et al., 2001) mice. This correlates with enhanced activation of nuclear factor κ-light chain enhancer of activated B cells (NFκB; Nguyen et al., 2001), suggesting that NOX regulates neuroinflammation in ALS. NOX1 and NOX2 were also linked to dysregulation of redox homeostasis in $SOD1^{G93A}$ mice (Marden et al., 2007). Similarly, NOX2 was also upregulated in $SOD1^{G93A}$ mice and sALS patients (Kato et al., 2005). Importantly, deletion of *NOX2* and *NOX1* significantly delays disease progression and prolongs survival in $SOD1^{G93A}$ mice (Bruijn et al., 2004; Poon et al., 2005; Marden et al., 2007). Furthermore, NOX2 activity was downregulated in peripheral neutrophils of ALS patients, which also correlated with improved survival and disease outcomes (Marrali et al., 2014). Also, elevated levels of NOX2, O_2^- and inactivation of NOX1 in $SOD1^{G93A}$ mice, induced by neuroinflammation, prolonged survival, and led to reduced ROS levels and oxidative stress in spinal cords of these animals (Wu et al., 2006). However, contradictory findings were obtained in another recent study, where genetic deletion of *NOX1* or *NOX2* did not improve survival in $SOD1^{G93A}$ mice (Seredenina et al., 2016). Hence, whether deletion of NOX improves survival of $SOD1^{G93A}$ mice remains unclear.

Whole-genome analysis of sALS patients identified *NOX4* as a potential genetic risk factor in sALS (Dunckley et al., 2007). This finding is intriguing because NOX4 also regulates ROS production, and lowering the levels of NOX4 decreases ROS production (Hordijk, 2006; Bedard and Krause, 2007). NOX4 is highly expressed in neurons but it is activated by different mechanisms compared to NOX1 and NOX2 (Hordijk, 2006). However, similar to NOX1 and NOX2, NOX4 can also be regulated by Rac1 and Akt, and protein kinase B signaling pathways (Gorin et al., 2003). Altered NOX-dependent redox stress induced by mutant SOD1 is also thought to be a secondary event associated with neuroinflammation and microgliosis (Boillée et al., 2006). The interplay between NOX, mutant SOD1, and microglia in the production of superoxide is therefore potentially important, and more studies in this area are warranted (Valdmanis et al., 2008; Zhou et al., 2020). However, conflicting evidence exists regarding the use of NOX as a therapeutic target in ALS (Marrali et al., 2014; Seredenina et al., 2016). More studies using specific NOX inhibitors or small molecules are therefore required to examine this possibility in more detail.

Apurinic/Apyrimidinic Endonuclease

Apurinic/apyrimidinic endonuclease (APE1) is also known as redox effector factor 1 (RF1), human AP endonuclease 1 (HAP1), or apurinic/apyrimidinic endodeoxyribonuclease 1 (APEX1; Liu et al., 2005). APE1 is a 37 kDa ubiquitous multifunctional protein involved in the regulation of multiple transcription factors, including NF-κB, STAT3, AP-1, hypoxia-inducible factor-1 (HIF-1), and tumor protein 53 (p53; Chiueh, 2010). It has two major functions: DNA repair and redox regulation (Angkeow et al., 2002), and it is particularly important in neurons because they are highly susceptible to oxidative DNA damage (Coppedè, 2011). APE1 is particularly important in BER, a specific mechanism of DNA repair that eliminates damaged bases (oxidized or alkylated) generated by ROS (Hayward et al., 1999). APE1 is therefore considered to be neuroprotective because of its dual role in oxidative DNA damage and redox regulation (Coppedè, 2011).

APE1 is upregulated in motor neurons of ALS patients compared to age-matched controls (Shaikh and Martin, 2002). In addition, APE1 was also upregulated in astrocytes and spinal cord white matter in ALS patients (Shaikh and Martin, 2002). Furthermore, activity of the BER pathway was significantly increased in sALS patients (Kisby et al., 1997; Coppedè, 2011). In contrast, however, another study concluded that the activity of APE1 was reduced in sALS patients (Kisby et al., 1997). Furthermore, loss of immunoreactivity to APE1 was observed in spinal cords of pre-symptomatic $SOD1^{G93A}$ mice, suggesting that reduced levels of APE1 in motor neurons precede neurodegeneration (Manabe et al., 2001; Nagano et al., 2002). The evidence for APE1 in ALS is therefore somewhat conflicting. Genomic DNA analysis identified several variants of APE1 (L104R, E126D, R237A, D283A, D148E, G306A, and G241R) that were over-represented in ALS patients compared to controls. However, these variants did not affect the DNA repair activity of APE1, and they do not contribute to the risk of developing sALS (Hayward et al., 1999; Hadi et al., 2000; Tomkins et al., 2000; Coppedè et al., 2010). APE1 also negatively regulates nuclear factor erythroid-related factor 2 (NRF2), a prominent antioxidant that is protective against oxidative damage triggered by injury and inflammation (Fishel et al., 2015).

APE1 is also associated with DNA damage induced by C9orf72 mutations. APE1 co-precipitates more with nucleophosmin (NPM1) in C9orf72 patient tissue lysates compared to controls (Farg et al., 2017), and overexpression of NPM1 inhibits apoptosis in neuronal cells expressing poly GR_{100} and poly PR_{100} (Farg et al., 2017). Also, genetic modifiers of poly GR toxicity in *Drosophila* identified APE1 and other DNA repair proteins (Ku80 and ERCC1) as suppressors of GR toxicity (Lopez-Gonzalez et al., 2019). A recent study also demonstrated mislocalization of APE1 from the nucleus to the cytoplasm as a possible trigger of oxidative DNA damage in spinal motor neurons expressing mutant $SOD1^{G93A}$, despite upregulation of multiple DNA repair enzymes, suggesting that restricted localization of APE1 could inhibit redox homeostasis (Li J. et al., 2019).

APE1 interacts with several proteins involved in both DNA repair and redox regulation. Furthermore, the motor cortex of ALS patients contains epigenetic hypomethylation of the APE1 promoter (Kim et al., 2020). Apurinic/Apyrimidinic (AP) sites in the brain, spinal cord, and brainstem of ALS patients are vulnerable to DNA lesions induced by free radicals and intermediates (Kim et al., 2020). ROS can produce 50,000–200,000 AP sites in the genome of a mammalian cell every day, and it is estimated to produce significantly more AP sites in the brain (Atamna et al., 2000). Therefore, small molecules that mimic APE1 or elevate APE1 expression may be protective against DNA damage and dysregulated redox homeostasis in ALS. However, whilst this possibility has not been examined in detail, overexpression of human APE1 in brain and spinal cord motor neurons was protective against apoptosis and axotomy, in two independent mouse models of injury-induced neurodegeneration (Martin and Wong, 2017). The repair function of APE1 is protective by switching on antioxidant and cell survival mechanisms after oxidative DNA damage to neurons (Jiang et al., 2009).

Peroxiredoxins

Prxs are a ubiquitous family of antioxidant enzymes that regulate peroxide and peroxynitrite levels in mammalian cells (Sanchez-Font et al., 2003). They are arguably the most important family of enzymes involved in peroxide metabolism because they are capable of reducing the levels of cellular H_2O_2 by 90% (Rhee, 2016). The family members in humans are classified, based on sequence homology and structural data, into six subfamilies, namely, Prx1, Prx2, Prx3, Prx4, Prx5, and Prx6.

There are now several lines of evidence that Prxs are dysregulated in ALS. Prx3, which is found in mitochondria, is downregulated in cells expressing $SOD1^{G37R}$ and $SOD1^{G93A}$ mutants, and in $SOD1^{G93A}$ transgenic mice (Wood-Allum et al., 2006). Quantitative real-time PCR (Q-PCR) analyses also revealed downregulation of Prx3 in spinal cords of sALS and mutant SOD1 ALS patients, suggesting loss of redox regulation and these antioxidant defense mechanisms in ALS (Wood-Allum et al., 2006). However, Prx6 was upregulated in spinal motor neurons of $SOD1^{G93A}$ mice (Strey et al., 2004) and Prx 1 was upregulated in NSC-34 cells expressing $SOD1^{G93A}$ or $SOD1^{G37R}$ mutants (Allen et al., 2003). Similarly, Prx2, Prx3, catalase, and Prx6 were upregulated in $SOD1^{G93A}$ mice compared to WT controls (Pharaoh et al., 2019). In contrast, Prx3 and Prx4 were downregulated in the presence of mutant $SOD1^{G93A}$ in NSC-34 cells (Kirby et al., 2005), and immunohistochemical analysis of motor neurons from sALS and fALS patients revealed negative immunoreactivity for Prx2 and glutathione peroxidase-l (Kato et al., 2005). These findings together imply that upregulation of Prxs renders motor neurons less susceptible to neurodegeneration, whereas breakdown of this redox system at late disease stages induces neuronal degeneration and accelerates disease progression (Kato et al., 2005). Prx3 and Prx5 were also present in $hSOD1^{G93A}$ aggregates in cells, suggesting that Prxs may also be involved in protein folding (Wood-Allum et al., 2006; Knoops et al., 2016). Further evidence for this notion comes from observations that Prx5 downregulation correlates with downregulation of molecular chaperones, and upregulation of proteins associated

with neuroinflammation (Knoops et al., 2016). Therefore, these studies reveal that several peroxiredoxins are dysregulated in ALS models, but how this contributes to pathology is unknown.

TMX2

TMX2, which is localized in the MAM compartment, acts as an important sensor of redox conditions and is crucial for the maintenance of cellular redox homeostasis (Vandervore et al., 2019). TMX2 functions in neuronal differentiation, dendritic, and axonal growth, and its dysregulation is linked to severe developmental abnormalities in the brain (Vandervore et al., 2019). CRISPR-Cas9 whole genome-wide gene-knockout screens for suppressors and enhancers of C9orf72 DPRs in human cells revealed TMX2 as an important protective modifier against toxicity (Kramer et al., 2018). This study suggested that depletion of TMX2 using single guide (sg) RNA in K562 cells and mouse primary cortical neurons was sufficient to suppress PR_{20} induced cellular toxicity (Kramer et al., 2018). Furthermore, transcriptomics performed from TMX2 deleted PR_{20} and GR_{20} expressing neurons revealed upregulation of pro-survival unfolded protein response (UPR) pathway genes and downregulation of calcium-binding and apoptotic genes. These findings, therefore, suggest that loss of TMX2 is protective against DPR toxicity by modulating ER stress (Kramer et al., 2018).

Activator Protein 1

AP-1 is a redox-sensitive transcription factor that regulates gene expression in response to various stimuli, including cytokines, growth factors, and cellular stress. It is controlled by the MAP kinase cascade (Gomez Del Arco et al., 1997) and is therefore involved in a range of cellular processes, including cell differentiation, growth, and proliferation. Activation of AP-1 by mutant SOD1 in NSC-34 cells mediates upregulation of Bcl2-A1, which regulates apoptosis (Kale et al., 2018). This finding, therefore, suggests that AP-1 drives the regulation of apoptosis in motor neurons (Iaccarino et al., 2011). AP1 is a complex of several proteins including JUN, which is upregulated in motor neurons derived from SOD1 patients compared to other neurons, providing potential mechanistic insights into the selective degeneration of motor neurons in ALS (Bhinge et al., 2017). Furthermore, another component of the API complex, FOSL1, is highly expressed in IPSC-derived motor neurons from SOD1 patients carrying the E100G mutation (Bhinge et al., 2017) compared to isogenic controls, suggesting that the AP1 complex is a driver for neurodegeneration. Also, the AP1 complex FBJ osteosarcoma oncogene (c-FOS) was upregulated in neuronal cells expressing SOD1 mutations, suggesting dysregulation of these antioxidant response proteins (Kirby et al., 2005). However, a direct relation between AP-1 and redox regulation in ALS has not been defined.

PDI Family of Proteins

The ER is a redox-regulated organelle that maintains redox homeostasis and facilitates protein folding (Sevier and Kaiser, 2008). However, protein misfolding within the ER triggers ER stress, which induces the UPR, a distinct signaling pathway that aims to relieve this stress (Matus et al., 2013; Hetz and Saxena, 2017). While initially protective, prolonged UPR induces apoptosis. PDIA1 (also known as PDI) is the prototype of the PDI family of ER chaperones which are induced during the UPR. The redox regulation of PDI is a crucial component of the maintenance of a balanced redox environment, and inhibition of its enzymatic activity will lead to important consequences for the cell. PDI has now been implicated in several neurodegenerative disorders, including ALS (Perri et al., 2016). Recent studies showing the protective effect of the redox activity of PDI in cellular and zebrafish models of ALS have placed redox dysregulation centrally in ALS, implying that it has a much broader role than previously realized.

As well as general chaperone activity, PDI family members possess oxidoreductase activity, which mediates the formation of protein disulfide bonds by cysteine residues located within its active site. Hence PDI proteins play a critical role in regulating the intracellular redox state, redox signaling, and in preventing protein misfolding and/or aggregation (Wang et al., 2015). The cysteine residues of PDI family members such as PDI, ERp57, and ERp72 contain redox-sensitive side chains and may become oxidized during redox dysregulation (Valle and Carrì, 2017). These residues are also actively modified by post-translational regulation, including disulfide formation and S-nitrosylation (Paulsen and Carroll, 2013; Fra et al., 2017). Both the chaperone activity and the overall conformation of human PDI are redox-regulated. Conformational changes in PDI alter its compact conformation and expose the normally shielded hydrophobic regions, which regulates its chaperone activity (Wang et al., 2012, 2015). GSH also regulates PDI functions and facilitates the redox activity of PDI during protein folding (Chakravarthi et al., 2006).

There is now growing evidence for a role of PDI in ALS (Parakh and Atkin, 2015; Parakh et al., 2020). PDI levels are upregulated in transgenic models of ALS and spinal cord tissues of ALS patients (Walker et al., 2010; Honjo et al., 2011; Jeon et al., 2014). Novel roles for PDI proteins were also recently identified in neurons, in mediating motor function and neuronal connectivity (Castillo et al., 2015; Woehlbier et al., 2016). Mutations in PDI and ERp57 were also described in ALS patients, but are thought to be more of a risk factor than directly causative of neurodegeneration (Woehlbier et al., 2016). Recently, the redox function of PDI, in contrast to its chaperone function, was shown to be protective against multiple cellular processes that dysfunction in ALS; protein misfolding, mislocalization of TDP-43 to the cytoplasm, ER stress, inhibition of ER-Golgi transport, and apoptosis, in neuronal cells expressing pathological forms of TDP-43 or SOD1 (Parakh et al., 2020). Furthermore, the redox activity of PDI, but not its chaperone function, rescued motor dysfunction and axonopathy in zebrafish models of ALS expressing mutant SOD1, together implying that PDI has an important role both *in vitro* and *in vivo* (Parakh et al., 2020). In contrast, the PDI ALS-mutants (D292N and R300H) lack this redox activity and were not protective against ALS phenotypes, further confirming the importance of the redox activity of PDI in ALS (Parakh et al., 2020). These findings, therefore, implicate redox homeostasis as a central and dominant feature of ALS and suggest that regulation

of the neuronal redox environment has a much broader link to neurodegeneration than previously recognized.

The upregulation of PDI in ALS suggests that a cellular defensive mechanism is triggered against redox dysfunction. However, there is evidence that the normal protective function of PDI is inhibited in neurodegeneration (Walker et al., 2010). Modifications of active site thiol groups by two redox-dependent aberrant post-translational modifications, S-glutathionylation and S-nitrosylation, lead to inactivation of the normal enzymatic activity of PDI (Walker et al., 2010). Both of these direct oxidation processes affect crucial active site cysteine residues and result in a loss of enzymatic activity (Halloran et al., 2013). S-nitrosylation involves the transfer of NO to one or more cysteine thiol groups and it occurs when there is an increased production of RNS during oxidative stress (Conway and Harris, 2015). This process represents a prominent redox reaction mediating NO signaling under both physiological and pathophysiological conditions (Halloran et al., 2013). PDI is S-nitrosylated in lumbar spinal cords of sporadic ALS patients (Walker et al., 2010), which abrogates PDI-mediated attenuation of neuronal cell death triggered by ER stress or misfolded proteins (Uehara et al., 2006). Furthermore, in the presence of S-nitrosylated PDI, the formation of mutant SOD1 aggregates increases *in vitro* (Jeon et al., 2014). These findings suggest that loss of PDI functional activity can directly lead to apoptosis, or indirectly, to a range of cellular abnormalities, such as oxidative stress, protein misfolding, as well as cell death (Jeon et al., 2014). Hence, these data imply that loss of PDI function contributes to pathophysiology in ALS and that PDI controls the cellular redox environment in the development of neurodegeneration.

INDIRECT EVIDENCE FOR A ROLE OF REDOX DYSREGULATION IN ALS

As well as the studies described above, there is also evidence that cellular processes associated with redox homeostasis are dysregulated in ALS (**Figure 2**). These more indirect mechanisms associated with redox perturbations in ALS are described in detail below.

CELLULAR PROCESSES LINKED TO REDOX IMBALANCE THAT ARE DYSREGULATED IN ALS

Protein Misfolding

The presence of misfolded proteins is known to induce oxidative stress. However, there is also evidence that oxidative stress induces protein misfolding. Mutant SOD1 forms inclusions in the presence of oxidative stress and WT SOD1 misfolds when the redox environment is dysregulated (Oeda et al., 2001). BSO inhibits glutathione synthesis (Hamilos and Wedner, 1985; Spitz et al., 1995) and treatment of neuronal cells expressing mutant TDP-43 with BSO leads to increased inclusion formation (Parakh et al., 2020). Importantly, WT forms of both SOD1 and TDP-43 form inclusions following BSO treatment. Similarly, SOD WT misfolds and develops a similar conformation to mutant SOD1, leading to aggregation and the gain of toxic functions *in vitro* (Bosco et al., 2010; Guareschi et al., 2012; Parakh et al., 2020). These studies, therefore, highlight redox dysregulation as an important trigger for protein misfolding, which is central to neurodegeneration in ALS.

Redox dysregulation is also linked to protein misfolding by the production of aberrant, non-native disulfide bonds in both mutant SOD1 and TDP-43, which leads to the formation of inclusions and induces toxicity. In mutant SOD1, these aberrant disulfide bonds involve cysteine residues Cys 6 and Cys111, and in both mutant and WT TDP-43, cysteines Cys173, 175, 198, and 244 in the RNA-recognition motif (RRM) are involved (Cohen et al., 2012; Shodai et al., 2013). Compared with WT SOD1, disease-linked mutant SOD1 proteins readily form monomers by reduction of the disulfide bond between Cys-57 and Cys-146 or by demetallation at the dimer interface (Tiwari and Hayward, 2003; Rakhit et al., 2004). Due to this monomerization, mutant SOD1 has an increased propensity to misfold (Rakhit et al., 2004; Kerman et al., 2010). Several previous studies have concluded that aberrant, non-native disulfide bonds involving Cys-6 and Cys-111 result in inclusion formation and disulfide reduction (Deng et al., 2006; Furukawa et al., 2006; Wang et al., 2006; Niwa et al., 2007) and the induction of both ER stress and toxicity (Alvarez-Zaldiernas et al., 2016; Xu G. et al., 2016; Perri et al., 2020). Moreover, these aberrant disulfide bonds have also been identified *in vivo* (Karch et al., 2009; Medinas et al., 2018). Similarly, aberrant disulfide cross-linking leads to misfolding and subcellular mislocalization of TDP-43 (Barmada et al., 2010; Cohen et al., 2012), which is induced by dysregulation of redox conditions. Hence, oxidative stress promotes the formation of these non-native disulfide bonds which leads to aggregation of both mutant SOD1 and TDP-43 (Cohen et al., 2012; Fukai and Ushio-Fukai, 2011).

Another link between protein misfolding and redox mechanisms is illustrated by the presence of important redox proteins in the misfolded protein inclusions in ALS. PDI associates with misfolded protein inclusions in patients with ALS (Honjo et al., 2011; Parakh et al., 2018a) cellular models (Farg et al., 2012; Jeon et al., 2014), and canine degenerative myelopathy (DM; Chang et al., 2019), and both PDI and ERp57 inhibit the formation of mutant SOD1 inclusions in neuronal cells (Walker et al., 2010; Parakh et al., 2018a). Furthermore, Keap1, a cysteine rich protein which binds to NRF2 and regulates oxidative and electrophilic stress, was co-localized with intracellular misfolded protein inclusions in motor neurons in the spinal cord of ALS patients. Moreover, in the motor cortex of ALS patients, the levels of NRF2 mRNA and protein were reduced, whereas Keap1 mRNA expression was increased compared to control patients (Sarlette et al., 2008; Tanji et al., 2013), suggesting that the NRF2-EpRE pathway is dysfunctional in ALS.

Mitochondrial Damage

Mitochondria are the major cellular site of ROS production and damage to mitochondrial structure or function increases oxidative stress (Albers and Beal, 2000; Carrí et al., 2003).

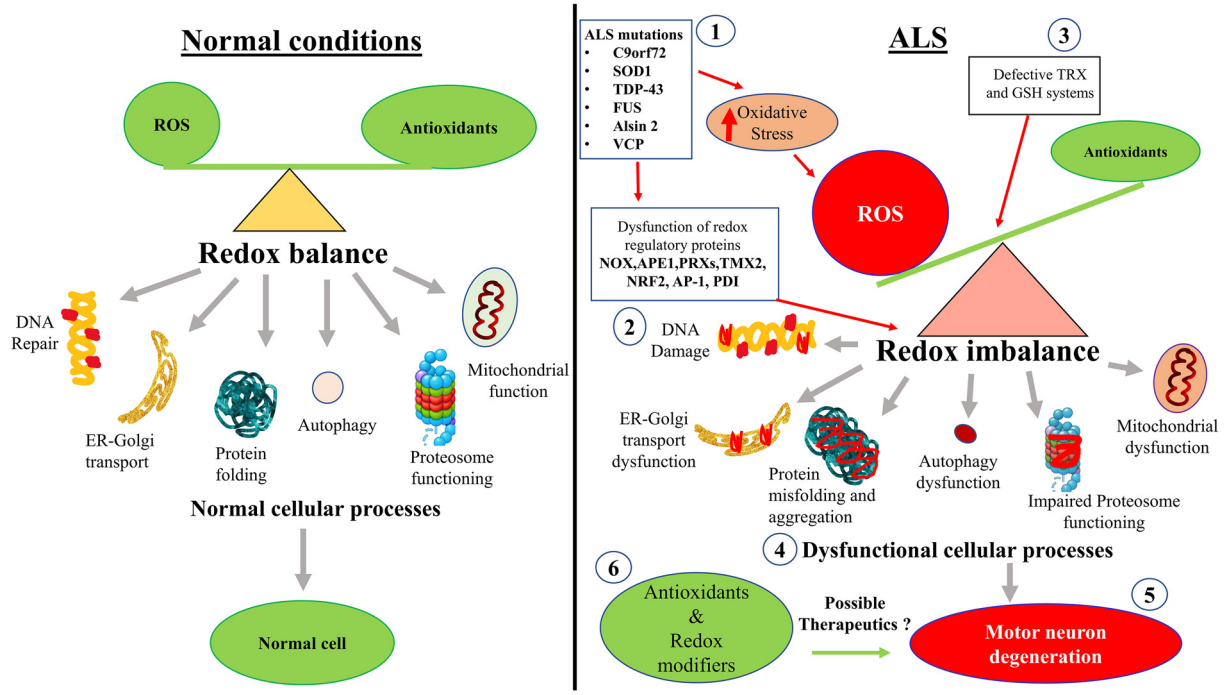

FIGURE 2 | Hypothetical schematic diagram illustrating how redox imbalance may induce neurodegeneration in ALS. (1) ALS mutant proteins induce oxidative stress and damage, which (2) dysregulates redox regulatory proteins and (3) impairs the TRX and GSH systems. This leads to increased ROS production and redox imbalance in neurons. (4) Redox imbalance subsequently induces dysfunction to mitochondria, the proteasome and autophagy, cellular trafficking, protein misfolding and aggregation, and inhibits DNA repair. (5) Dysregulation of these cellular processes induces motor neuron degeneration in ALS. (6) Redox modifiers and antioxidants have been trialled clinically. However, interventions that control redox regulatory processes may be more beneficial than administering the antioxidants themselves. Diagram not to scale.

Mitochondrial impairment and dysregulation of mitochondrial proteins are present in postmortem brain and spinal cord tissues of ALS patients and SOD1^{G93A} mice (Carrí et al., 2003; Ferri et al., 2006; Tan et al., 2014). Similarly, WT TDP-43 interacts with several mitochondrial proteins that are crucial for mitophagy and mitochondrial dynamics (Davis et al., 2018). Expression of ALS-associated mutant TDP-43^{A315T} also leads to mitochondrial abnormalities in cell culture (Gao et al., 2019; Wang P. et al., 2019). Furthermore, mitochondrial damage is present early in disease course in TDP-43^{A315T} mice and increases motor neuron vulnerability (Gautam et al., 2019), consistent with dysregulation of the redox system early in neurodegeneration. ALS-associated mutant TDP-43 is known to be aberrantly localized in mitochondria, but suppressing its mitochondrial localization protects against neurotoxicity in TDP-43^{A315T} mice (Wang et al., 2016). Similarly, motor and cognitive functions in TDP-43^{A315T} mice are improved by inhibiting the mitochondrial localization of TDP-43 (Wang et al., 2017). However, contradictory findings were obtained in a recent study which concluded that mutant TDP-43^{A315T} does not impair mitochondrial bioenergetics *in vitro* and *in vivo* (Kawamata et al., 2017).

The C9orf72 DPRs also interact with mitochondrial proteins, resulting in mitochondrial dysfunction, inflammation, and neurotoxicity (Gendron and Petrucelli, 2018). Elevated production of ROS in C9orf72-associated ALS is also associated with abnormalities in mitochondrial function and neuroinflammation (Briehl et al., 2014; Alvarez-Zaldiernas et al., 2016). C9orf72 ALS/FTD-associated poly $(GR)_{80}$ DPRs interact with Atp5a1, which compromises mitochondrial functions in mice due to increased ROS production (Choi et al., 2019). Also, poly $(GR)_{80}$ interacts with mitochondrial ribosomal proteins, inducing mitochondrial dysfunction, in motor neurons differentiated from C9orf72 patient iPSCs, compared to controls (Lopez-Gonzalez et al., 2016). Induction of oxidative stress also induced more DNA damage in iPSC-derived C9orf72 motor neurons than controls, in an age-dependent manner (Lopez-Gonzalez et al., 2016). Furthermore, in the same study, expression of poly $(GR)_{80}$ in neurons increased ROS levels, inducing oxidative stress and dysregulating redox conditions in C9orf72 iPSCs (Lopez-Gonzalez et al., 2016). Pharmacological reduction of oxidative stress by administering Trolox, a water-soluble antioxidant and vitamin E analog, also partially rescued DNA damage and cellular toxicity in *Drosophila* expressing C9orf72 DPRs (Lopez-Gonzalez et al., 2016). Furthermore, myogenic progenitors derived from C9orf72 ALS patients displayed increased susceptibility to oxidative stress and dysregulation of mitochondrial genes, and these events were associated with mitochondrial abnormalities and toxicity (Lynch et al., 2019).

Mitochondria connect to the ER at multiple contact sites to form the MAMs. Mutations in *C9orf72, TDP-43, VAPB, VCP*, and *FUS* are known to disturb these ER-mitochondria associations and signaling (Stoica et al., 2014; Zhang et al., 2017; Lau et al., 2018). FUS is known to interact with HSP60, which is associated with mitochondrial abnormalities, and these defects have been identified in FUS-FTLD patients (Deng et al., 2015). Mutant FUS^{R495X} dysregulates the expression of genes associated with oxidative mitochondrial metabolism in neurons, and significantly reduces the size of mitochondria, which induces neurotoxicity (Nakaya and Maragkakis, 2018). Similarly, ER-mitochondria associations and VAPB-PTPIP51 interactions are disrupted by ALS-associated mutant FUS (Stoica et al., 2016), which results in increased production of ROS. The ATP synthase beta subunit, a mitochondrial enzyme involved in redox regulation, associates with FUS, inducing the mitochondrial UPR in cellular and transgenic *Drosophila* models of ALS (Deng et al., 2018). Similarly, abnormal accumulation and aggregation of mitochondria in the inter-myofibrillar space was detected in iPSCs derived from an ALS patient bearing a VCP mutation (Bartolome et al., 2013; Hall et al., 2017). Furthermore, heterozygous knock-in of mutant VCP^{R155H} in mice leads to alterations in mitochondrial respiratory complex activity (Yin et al., 2012). Fibroblasts obtained from ALS patients carrying the CHCHD10 mutation S59L display respiratory chain deficiency, ultrastructural alterations, and fragmentation of the mitochondrial network (Bannwarth et al., 2014; Mccann et al., 2020). Therefore, together these studies imply that mutant proteins linked to ALS induce damage to mitochondrial structure, impair its function, dysregulate energy metabolism, and disturb redox homeostasis.

Neuroinflammation

Although motor neurons specifically degenerate in ALS, increasing evidence implies that non-neuronal cells, such as astrocytes (Lee et al., 2016), microglia (Henkel et al., 2009), and oligodendrocytes (Li J. et al., 2016), directly contribute to neurodegeneration by a non-cell-autonomous mechanism (Radford et al., 2015). This results in the appearance of reactive microglia and astroglia which is referred to as neuroinflammation (Radford et al., 2015). Mutant ALS proteins trigger microglial activation, increase the levels of ROS, and induce neurotoxicity (Henkel et al., 2009). Microglia exist in two states, resting and activated, and their activation represents a continuum between the two classical phenotypes; neuroprotective M2 vs. neurotoxic M1 (Liao et al., 2012; Chiu et al., 2013). Two different microglial phenotypes have also been described in $SOD1^{G93A}$ transgenic mice. Mutant $SOD1^{G93A}$ and $SOD1^{G85R}$ activate microglia and increase the levels of ROS and other pro-inflammatory cytokines (Zhao et al., 2010). Transcriptome analysis of microglia isolated from $SOD1^{G93A}$ mice revealed increases in activated pro-inflammatory *Igf1*, Progranulin, *Trem2*, cytokines, and neurotoxic factor MMP-12 (Chiu et al., 2013). Also, mutant $SOD1^{G93A}$ and $SOD1^{L8Q}$ stimulate Rac1-GTP activation of NOX and the production of ROS, unlike WT SOD1 (Boillée et al., 2006; Beers et al., 2011). Members of the NOX enzyme family catalyze the formation of ROS and are implicated as mediators of neurodegeneration induced by neuroinflammation in ALS (Calvo et al., 2014). Therefore, together these findings suggest the interplay between NOX, ROS, the redox state of Rac1 and SOD1 contributes to neuroinflammation and associated toxicity in ALS.

M1 microglial cells are also activated by inflammatory stimuli. Mutant TDP-43 activates M1 microglia and upregulates pro-inflammatory mediators NOX2, TNF-α, and IL-1β (Beers et al., 2011; Liao et al., 2012; Chiu et al., 2013). Consistent with these findings, glial cells express more endogenous TDP-43 after treatment with lipopolysaccharide (LPS) or ROS, and produce more pro-inflammatory cytokines and neurotoxic mediators (Swarup et al., 2011). Furthermore, expression of WT, ALS-associated mutant, or truncated forms of TDP-43, promote CD14-mediated activation of microglia through NF-κB signaling and NLRP3 inflammasomes (Zhao et al., 2015). WT FUS also activates NF-κB, the master regulator of inflammation, and induces expression of both pro-inflammatory markers and redox signaling proteins in microglia (Frakes et al., 2014; Geloso et al., 2017; Ajmone-Cat et al., 2019).

C9orf72 repeat expansions activate microglia and astrocytes, as well as initiate the formation of innate immune inflammasome complexes and activation of intracellular receptors responsible for inducing inflammation. Astrocytes derived from C9orf72 and sporadic ALS patients were found to be toxic *in vitro* and *in vivo* to motor neurons, by a non-cell-autonomous mechanism (Di Giorgio et al., 2007; Yamanaka et al., 2008; Haidet-Phillips et al., 2011). This toxicity involved either secretion of neurotoxic factors or loss of astrocytic support functions (Meyer et al., 2014; Madill et al., 2017). Post-mortem analyses of spinal cord tissue sections from sALS patients revealed increases in markers of lipid peroxidation and protein glycoxidation, resulting in oxidative damage in both neurons and non-neuronal cells (Shibata et al., 2001). However, a recent study revealed that iPSC-derived astrocytes obtained from C9orf72 ALS patients display increased oxidative stress and senescence (Birger et al., 2019). Furthermore, motor neurons cultured using conditioned media from iPSC-derived C9orf72 astrocytes exhibit increased oxidative stress (Birger et al., 2019). Also, mutant C9orf72-derived astrocytes downregulate secretion of several antioxidants and induce cellular senescence (Birger et al., 2019). Despite these findings, however, it remains unclear whether neurotoxicity is induced by astrocyte disturbances to redox homeostasis in C9orf72 ALS. Furthermore, whilst loss of C9orf72 in mice models disturbs microglial function, resulting in age-related neuroinflammation, this was not sufficient to cause neurodegeneration in C9orf72 knockout mice (Lall and Baloh, 2017). Collectively, these studies imply mutant ALS proteins produce inflammatory cytokines, disturb redox homeostasis, and increase neuroinflammation and toxicity. However, it remains unclear whether activation of inflammatory cytokines in C9orf72 ALS models is directly linked to redox regulation.

Cellular Trafficking Defects

Haploinsufficiency is increasingly implicated as a disease mechanism in ALS, which is characterized by reduced expression

of C9orf72 in ALS patients. C9orf72 is a member of the DENN domain family of proteins (Zhang et al., 2012) and it was initially found to interact with Rabs 1, 5, 7 and 11, in the regulation of endocytosis and autophagy in neuronal cells (Farg et al., 2014). However, several later studies have now shown that C9orf72 also interacts with many other Rabs; Rab 1a, 1b, Rab3 (a, b, c, d), Rab5a, Rab7a Rab7L1, 8a, 8b, 10, 13, 15, 18, 19, 27a, 38, 40a and 42 (Gitler and Tsuiji, 2016; Tang, 2016; Webster et al., 2016; Aoki et al., 2017; Gao et al., 2017). C9orf72 was also shown to be a Rab guanine exchange factor (GEF) involved in the modulation of Rab activity (Iyer et al., 2018), which regulates NOX2 recruitment to phagosomes in dendritic cells (Jancic et al., 2007). C9orf72 also forms a complex with SMCR8 and WDR41 in the regulation of autophagy, and it is also implicated in autoimmunity, immune dysregulation, endocytosis, and lysosome homeostasis (Atanasio et al., 2016; O'Rourke et al., 2016; Sullivan et al., 2016; Webster et al., 2016; Yang et al., 2016; Corrionero and Horvitz, 2018; Zhang et al., 2018). Lysosomal accumulation was observed in SMCR8 deficient macrophages from Smcr8$^{-/-}$mice, possibly because of increased ROS (Mcalpine et al., 2018). Rab-mediated cellular trafficking defects are also known to be induced by mutant forms of SOD1, TDP-43, and FUS (Soo et al., 2015; Parakh et al., 2018b).

The *ALS2* gene is mutated in autosomal recessive forms of juvenile-onset ALS (Yang et al., 2001). *ALS2* encodes alsin, which functions as a guanine nucleotide exchange factor (GEF) for the small GTPase Rab5. There are two isoforms, long and short. The long isoform consists of three independent GEF-like domains (RCC1 domain, PH domain, and VPS9 domain) whereas the short contains the RCC1 domain (Yang et al., 2001). The longer isoform alone is known to act as a Rab GEF or Rab activating protein for Rab5 and Rac1GTPases, and it functions in endocytic mechanisms, endosomal dynamics, and micropinocytosis (Topp et al., 2004; Otomo et al., 2008). An important aspect of redox signaling is the localization of redox-active processes within distinct microenvironments of the cell, and redox-active endosomes (redoxosomes) are one key example of this. Redoxosomes contain redox proteins that are involved in transmitting ROS signals from interior to outer membranes, and they regulate ROS as a secondary messenger (Oakley et al., 2009).

Interestingly, alsin and SOD1 are both effectors of Rac1GTPases (Kanekura et al., 2004). The interplay between SOD1, alsin, and Rac1 in endosome formation and trafficking is particularly intriguing in the context of NOX dependent production of IL-1β and TNFα. Hence this is also relevant to SOD1-mediated redoxosomal signaling defects in ALS (Kanekura et al., 2005; Otomo et al., 2008; Li et al., 2011). Furthermore, alsin also interacts with mutant SOD1 and Rac1 to inhibit hyperactivation of NOX and reduce ROS production (Jacquier et al., 2006; Li et al., 2011). It is therefore tempting to speculate that defects in the interaction between SOD1, Rac1, and alsin 2 influence redox signaling at the endosomal level.

There is also evidence that alsin is protective against oxidative stress and may be involved in redox regulation. First, overexpression of the alsin long isoform protects against motor neuron toxicity induced by expression of A4T, or mutant SOD1^{G85R} or SOD1^{G93A} (Kanekura et al., 2004). Second, knockout of alsin in mice is not sufficient to trigger motor neuron degeneration, but neurons cultured from these animals are more susceptible to oxidative stress (Cai et al., 2005). Furthermore, these mice exhibit defects in endosomal trafficking (Devon et al., 2006; Hadano et al., 2010), indicating that alsin could be a component of the redox-sensing mechanisms that inhibit NOX signaling. Together these findings, therefore, suggest that alsin protects cells from oxidative stress and could be a redox sensing protein, similar to Rab5, Rac1, and SOD1.

MODIFIERS OF REDOX REGULATION AS A THERAPEUTIC TARGET FOR ALS

Only two drugs are currently approved by the USA Food and Drug Administration (US FDA-FDA) for ALS treatment. Given the extensive evidence linking redox dysfunction to ALS, it is not surprising that several redox-active molecules have been trialed as potential therapeutic agents. However, whilst multiple compounds targeting redox regulation have been reported to slow disease progression in SOD1^{G93A} mice (Dash et al., 2018), they have subsequently failed to enhance survival or improve motor function in ALS patients in clinical trials. Efforts to modulate GSH directly have failed, due to limits of solubility, absorption, stability, and the short half-life of GSH. Moreover, direct administration of cysteine to increase GSH is not a viable option, because of its poor absorption and toxicity at high doses (Johnson et al., 2012). Nevertheless, the second FDA-approved drug for ALS, edaravone, is a strong antioxidant that inhibits oxidative stress and is a potent scavenger of free radicals, highlighting the importance of redox regulation in disease. The ALS Functional Rating Scale-Revised (ALSFRS-R) is a scale that determines the progression and severity of ALS patients and is widely used in clinical trials (Rooney et al., 2017). Unfortunately, edaravone can only be used in a small subset of early-stage ALS patients (grade 1 or 2 in the Japan ALS Severity Classification, scoring at least 2 points on all 12 items of ALSFRS-R; Dash et al., 2018). Furthermore, it is not yet available orally. Riluzole was the first FDA-approved compound for ALS (in 1995), which inhibits glutamatergic neurotransmission and thus inhibits excitotoxicity (Dharmadasa and Kiernan, 2018; Dash et al., 2018). However, administration of riluzole statistically only improves survival in ALS patients up to 60 days. Hence both compounds are not particularly effective (Dharmadasa and Kiernan, 2018; Dash et al., 2018). There is therefore a current need to develop much more successful therapeutics. The section below will discuss therapeutics strategies that have targeted modifiers of redox regulation in pre-clinical models of ALS and/or clinical trials. **Table 3** summarizes the studies discussed below.

CLINICAL TRIALS

Vitamin E is known to regulate redox balance, and a randomized placebo-controlled clinical trial examined the effect

TABLE 3 | List of potent redox modifiers trialled in ALS.

Drug	Properties	Mechanism of action	Trial/Study	Results	References
(A) CLINICAL TRIALS					
Alpha-tocopherol (Vitamin E) 500 mg	Antioxidant	Reduces oxidative stress	Randomized placebo-controlled clinical trial (RCT)	No significant improvement in disease progression or survival	Desnuelle et al. (2001) and Graf et al. (2005)
Higher doses of Vitamin E (5,000 mg)	Antioxidant	Reduces oxidative stress	Phase III RCT	No significant improvement in disease progression or survival	Graf et al. (2005)
Nanocurcumin or Vitamin E as add-on therapy with Riluzole	Anticancer agent and antioxidant	Vitamin E reduces oxidative stress and Riluzole increases glutamate uptake	Pilot RCT	No significant improvement in motor function of ALS patients	Ahmadi et al. (2018)
Vitamin C and Carotenoids Supplementation	Antioxidant	Reduces oxidative stress	Pooled results from 5 different cohort studies	Does not reduce the risk of developing ALS	Fitzgerald et al. (2013)
Coenzyme Q10 (CoQ10)	Antioxidant and co-factor in the ETC	Reduces oxidative stress and mitochondrial impairment	Open-label dose-escalation trial	No significant improvement in ALSFRS-R score	Ferrante et al. (2005)
Dexpramipexole	Antioxidant and apoptosis inhibitor	Reduces oxidative stress	Phase III RCT	No significant improvement in motor function of ALS patients	Cudkowicz et al. (2013)
Combination of Vitamin C, E, selegiline, selenium, and L-methionine	Combination of antioxidants	Reduces oxidative stress	All randomized or quasi-randomized controlled trials	No significant improvement in disease progression or survival in ALS patients	Orrell et al. (2008)
Edaravone	Strong antioxidant	Reduces oxidative stress	Phase 1, II, III RCTs	Improves motor function by 33% compared to control patients. Significant improvement in survival in a subset of ALS patients	Writing Group on Behalf of the Edaravone (MCI-186) ALS 19 Study Group (2017a), Writing Group; Edaravone (MCI-186) ALS 19 Study Group (2017b), Abe et al. (2014), and Takei et al. (2017a)
Curcumin (600 mg/day, Brainoil)	Antioxidant, anti-inflammatory, anti-cancer agent	Reduces oxidative stress and neuroinflammation	Double-blind controlled trial	Reduces oxidative stress and improves aerobic metabolism	Chico et al. (2018)
EH301, a combination of PT and NR	Combination of antioxidant and anti-aging agent	Improves mitochondrial oxidative metabolism	Pilot RCT	Slows disease progression in a small number of ALS patients	De La Rubia et al. (2019)
(B) PRE-CLINICAL MODELS					
H_2S	Antioxidant	Increases Ca^{2+} levels, improves mitochondrial functions, and inhibits SOD1 aggregation	$SOD1^{G93A}$ mice	Effective against mitochondrial dysfunction	Pratt et al. (2012) and Paul and Snyder (2018)
Fisetin and 7,8-Dihydroxyflavone	Antioxidant	Reduces ROS production and activates the ERK signaling pathway	$SOD1^{G93A}$ mice	Reduces ROS production and neurodegeneration	Korkmaz et al. (2014)
Resveratrol	Antioxidant, Anti-aging agent	Inhibits oxidative stress	$SOD1^{G93A}$ mice	Significant improvement in motor function and survival	Mancuso et al. (2014) and Song et al. (2014)
Epigallocatechin	Antioxidant	Inhibits oxidative stress and ROS production	$SOD1^{G93A}$ mice	Significant improvement in motor function and survival	Koh et al. (2004, 2006) and Xu et al. (2006)
CPN-9	NRF2 activator	Inhibits ROS production	$SOD1^{H46R}$ mice	Improves motor function and delays disease progression	Kanno et al. (2012)
NR, a form of Vitamin B_3	Antioxidant	Inhibits oxidative stress, activates mitochondrial UPR and SIRT6 expression, reduces neuroinflammation	$SOD1^{G93A}$ mice	Delays motor neuron degeneration, reduces neuroinflammation in the spinal cord, and slightly prolongs survival	Zhou et al. (2020)

(Continued)

TABLE 3 | Continued

Drug	Properties	Mechanism of action	Trial/Study	Results	References
Arimoclomol	Inducer of heat shock proteins, indirect antioxidant	Reduces mutant SOD1 aggregation and enhances protein folding	$SOD1^{G93A}$ mice	Protective against toxic mutant SOD1 aggregates, delays disease progression, and improves motor functions	Kieran et al. (2004), Kalmar et al. (2008), and Lanka et al. (2009)
(C) CELL CULTURE AND DROSOPHILA MODELS OF ALS					
γ-Oryzanol	Antioxidant, Anti-inflammatory	Reduces oxidative stress and neuroinflammation	$SOD1^{G85R}$ *Drosophila* and cell culture models	Reduces oxidative stress ad neurotoxicity	Zhang et al. (2019a)
Urate	Antioxidant	Reduces astrocyte induced toxicity and oxidative stress	Mutant $SOD1^{G93A}$ cell culture and Drosophila model of ALS	Neuroprotection against motor neuron toxicity induced by mutant $SOD1^{G93A}$	Bakshi et al. (2018) and Zhang et al. (2019b)
Lipoic acid	Antioxidant, Anti-inflammatory	Inhibit oxidative stress and reduces neuroinflammation associated toxicity	$SOD1^{G93A}$ and $SOD1^{G85R}$ *Drosophila* and cell culture models	Attenuates oxidative stress and protects against neurotoxicity	Moura et al. (2015), Saleh et al. (2017), and Wang T. et al. (2018)
Diallyl trisulfide	Antioxidant	Increases the expression of HO-1 and NQO1 to prevent oxidative stress	TDP-43 ^{Q331K} and TDP-43 ^{M337V} cell culture models of ALS	Protects from motor neuron toxicity	Liu C. et al. (2018)

of administering 500 mg alpha-tocopherol (the primary form of vitamin E used by humans) to ALS patients, along with riluzole. However, no beneficial effects on disease progression or survival were observed in these patients, despite changes in biochemical markers of oxidative stress (Desnuelle et al., 2001; Galbussera et al., 2006). Similarly, another phase III clinical trial using higher doses of vitamin E (5,000 mg) did not improve the quality of life or disease progression in ALS patients compared to controls (Graf et al., 2005). Similarly, the use of the antioxidant nanocurcumin (or vitamin E) as an add-on therapy to riluzole in ALS patients did not improve motor function (Graf et al., 2005; Ahmadi et al., 2018). Increased intake of dietary vitamin C and carotenoids, known to be powerful antioxidants, also did not reduce the risk of developing ALS (Fitzgerald et al., 2013). EH301, a combination of two antioxidants; pterostilbene (PT), an analog of resveratrol (found in red wine and berries), and nicotinamide riboside (NR; a form of vitamin B3 promoted as an anti-aging supplement), slows disease progression and improved primary outcomes [ALSFRS-R and forced vital capacity (FVC)] in ALS patients. However, this phase III trial was criticized for its small sample size, short duration, and high rate of patient dropout (De La Rubia et al., 2019). A double-blinded clinical trial, in which oral curcumin (600 mg/day, Brainoil) was administered to ALS patients reduced oxidative stress, and improved aerobic metabolism. These promising results warrant future studies examining the effect of these compounds on motor dysfunction and other primary outcomes in ALS patients (Chico et al., 2018).

An open-label dose-escalation trial found administering another antioxidant and cofactor in the ETC, coenzyme Q10 (CoQ10; Ferrante et al., 2005), resulted in no significant improvement in ALSFRS-R score (Levy et al., 2006). Also, a phase III randomized placebo-controlled trial demonstrated that another compound protective against oxidative stress, dexpramipexole, did not improve motor function in ALS patients (Cudkowicz et al., 2013). Similarly, trials in which a combination of several antioxidants was administered (vitamins C, E, selegiline, selenium, and L-methionine) also failed to show any improvement in ALS patients (Orrell et al., 2008). Therefore, collectively, these studies imply that these antioxidant strategies are not effective in ALS.

Despite the negative outcomes of these studies, edaravone (Radicava), was recently approved by the USFDA (Takei et al., 2017b; Dash et al., 2018). Edaravone slows down the loss of motor function in ALS patients by 33% compared to controls [Writing Group on Behalf of the Edaravone (MCI-186) ALS 19 Study Group, 2017a; Writing Group; Edaravone (MCI-186) ALS 19 Study Group, 2017b; Takei et al., 2017a,b; Bhandari et al., 2018], and inhibits disease progression during the early stages [Writing Group on Behalf of the Edaravone (MCI-186) ALS 19 Study Group, 2017a; Takei et al., 2017b]. A phase II open-label clinical trial demonstrated that edaravone significantly reduced nitrated tyrosine levels in the CSF of ALS patients (Sawada, 2017). Whilst one phase III trial did not reveal a significant difference in ALSFRS-R score when additional patient inclusion criteria were applied [Oakes et al., 2017; Edaravone (MCI-186) ALS 16 Study Group, 2017; Sawada, 2017], another confirmatory phase III placebo-controlled trial showed that edaravone treatment resulted in a statistically significant change in ALS FRS-R primary endpoint over 24 weeks in a subset of ALS patients (Writing Group; Edaravone (MCI-186) ALS 19 Study Group, 2017b; Sawada, 2017; Takei et al., 2017a). However, edaravone causes hypersensitivity and allergic reactions in some patients and may be effective in less than 5% of the ALS population [Writing Group; Edaravone (MCI-186) ALS 19 Study Group, 2017b]. Despite these concerns, edaravone is effective in a subset of ALS patients. Hence, this raises the possibility that other antioxidants and potent modifiers of redox regulation, which have shown promise in pre-clinical models of ALS, should be re-examined in clinical trials (**Table 3**).

PRE-CLINICAL STUDIES

Most previous preclinical studies have used transgenic mice overexpressing human mutant SOD1. However, these mice do not display the TDP-43 pathology that is present in almost all cases of ALS, and therefore they have been increasingly criticized as a disease model. Urate, also known as uric acid, is a major endogenous antioxidant that is neuroprotective against both astrocyte-induced toxicity in $SOD1^{G93A}$ mice and H_2O_2 induced oxidative stress in cells expressing mutant $SOD1^{G93A}$ (Bakshi et al., 2018). Similarly, urate is also neuroprotective by enhancing GSH expression through activation of the Akt/GSK3β/NRF2/GCLC pathway in mutant $SOD1^{G85R}$ cellular and *Drosophila* models (Zhang et al., 2019b). Also, another oxidant, hydrogen sulfide (H_2S), was effective against mitochondrial dysfunction in $SOD1^{G93A}$ mice by increasing the levels of Ca^{2+} and inhibiting SOD1 aggregation (Pratt et al., 2012; Paul and Snyder, 2018). Therapeutics based on mitochondrial dysfunction and oxidative stress, such as mitochondria-targeted antioxidant [10-(4,5-dimethoxy-2-methyl-3,6-dioxo-1,4-cyclohexadien-1-yl)decyl]triphenylphosphonium methanesulfonate (MitoQ), modify disease progression by slowing the decline of mitochondrial function and disease progression in the $SOD1^{G93A}$ mouse model of ALS (Miquel et al., 2014).

Recent studies have shown that treatment of $SOD1^{G93A}$ mice with naturally occurring flavonoids fisetin and 7,8-dihydroxyflavone, inhibits ROS production and neurodegeneration by activating the ERK signaling pathway (Korkmaz et al., 2014; Wang T. H. et al., 2018). Also, resveratrol improved motor function and survival in $SOD1^{G93A}$ mice through activation of Sirtuin1 (SIRT1) and suppression of oxidative stress (Mancuso et al., 2014; Song et al., 2014). Similarly, epigallocatechin, a naturally occurring antioxidant present in fruits, nuts, and tea, inhibited oxidative stress, induced motor neuron degeneration, and prolonged survival of $SOD1^{G93A}$ mice (Koh et al., 2004, 2006; Xu et al., 2006). Furthermore, lipoic acid, an antioxidant with anti-inflammatory properties, attenuated oxidative stress and neurotoxicity in $SOD1^{G93A}$ and $SOD1^{G85R}$ *Drosophila* and cell culture models (Moura et al., 2015; Wang T. et al., 2018).

NAD^+, the co-enzyme of reduced NADPH, plays a critical role in redox reactions, energy metabolism, mitochondrial function, calcium homeostasis, DNA repair, and *SIRT1* gene expression (Yaku et al., 2018; Yoshino et al., 2018). However, precursors of NAD+; nicotinamide mononucleotide (NMN), nicotinamide (NAM), nicotinic acid (NA), and NR; are more stable and more easily able to penetrate neurons compared to NADPH, and are protective in $SOD1^{G93A}$ mice and axotomy mice models (Sasaki et al., 2006; Harlan et al., 2016). Enhancing NAD^+ levels in astrocytes expressing mutant $SOD1^{G93A}$ attenuates toxicity (Harlan et al., 2016). Supplementation of NR activates *SIRT6* expression and delays motor neuron degeneration, reduces neuroinflammation in the spinal cord, and slightly prolongs survival of $SOD1^{G93A}$ mice (Harlan et al., 2016). NR also activates the mitochondrial UPR and prolongs the survival of $SOD1^{G93A}$ mice (Zhou et al., 2020). Furthermore, low doses of the compound diallyl trisulfide, which increases expression of the antioxidant enzymes heme oxygenase1 (HO-1) and NAD(P)H quinone dehydrogenase (NQO1), protects motor neurons from toxicity induced by mutant $TDP\text{-}43^{Q331K}$ and $TDP\text{-}43^{M337V}$ (Liu C. et al., 2018). Therefore, collectively, these findings suggest that administering NAD^+ precursors prevents oxidative stress and mitochondrial dysfunction, and hence may be a useful therapeutic target for ALS.

Deletion of NOX by genetic approaches or using NOX inhibitors, is protective against neurotoxicity in ALS disease models (Sorce et al., 2017; Barua et al., 2019). Inactivation of NOX decreases ROS production and prolongs survival in $SOD1^{G93A}$ mice (Seredenina et al., 2016), and may modify survival in ALS patients (Marrali et al., 2014). Furthermore, CPN-9, a novel NRF2 activator, is neuroprotective against mutant $SOD1^{H46R}$ motor deficits and disease progression in mice (Kanno et al., 2012). Also, γ-oryzanol, a mixture of lipids derived from rice, inhibits oxidative stress and neurotoxicity in $SOD1^{G85R}$ *Drosophila* and cell culture models (Zhang et al., 2019a). Several independent studies have also shown that treatment with arimoclomol, a co-inducer of heat shock proteins, is protective against toxic mutant SOD1 aggregates, delays disease progression and improves motor function in $SOD1^{G93A}$ mice (Kieran et al., 2004; Kalmar et al., 2008; Lanka et al., 2009).

CuII [atsm; diacetylbis(4-methylthiosemicarbazonato) copper] is a redox-active molecule involved in redox cycling between oxidized CuII and reduced CuI, that is showing promise as a potential therapeutic in ALS (Hilton et al., 2020). In $SOD1^{G93A}$ mice, CuII (atsm) improves survival by 26% (Hilton et al., 2017) and was therefore more effective than riluzole (3% survival improvement) in this model (Mcallum et al., 2013; Roberts et al., 2014). Moreover, it is the only candidate drug for ALS to be independently validated by the ALS Therapy Development Institute (Soon et al., 2011). A recent Phase I clinical trial found administering CuII (atsm) in ALS patients was safe and well-tolerated (Lincoln, 2018). Phase II trials evaluating the efficacy of CuII (atsm), involving 80 ALS participants, are ongoing.

In summary, at first glance, these studies suggest antioxidant strategies are not effective in ALS, but this may reflect the specific unfavorable pharmacological properties of the antioxidants trialled so far. Harnessing mechanisms that regulate the cellular redox state, rather than the redox molecules themselves, might be more effective in the future. Furthermore, most of the potential redox modifiers described above were only efficacious in $SOD1^{G93A}$ mice, and these mice do not develop the TDP-43 pathology present in almost all ALS cases. Therefore, it will be worthwhile to assess the therapeutic potential of antioxidants and modifiers of redox regulation in TDP-43, C9ORF72, and FUS models of ALS in the future. However, edaravone (Radicava) is one of only two USA-FDA approved treatments currently available for ALS, highlighting the potential importance of restoring redox homeostasis in ALS.

DISCUSSION/CONCLUSION

Although links between redox dysregulation and ALS are becoming well documented in the literature, the directionality of these links and their underlying cause is still unclear. However, recent evidence suggests that redox dysregulation may be an important or even primary trigger of a cascade of events leading to neurodegeneration. Notably, TDP-43 pathology is present in almost all ALS cases and specific features can be induced by redox dysregulation; inclusion formation, subcellular localization, and insolubility (Cohen et al., 2012). Reduction of GSH, a vital redox modifier, affects multiple ALS associated proteins. GSH reduction is associated with TDP-43 inclusion formation in sALS patients (Valle and Carrì, 2017) and WT forms of TDP-43 and SOD1 can be induced to misfold and aggregate in neuronal cells (Iguchi et al., 2012; Parakh et al., 2020), thus linking redox dysregulation to protein misfolding and sporadic ALS. Moreover, reduction of GSH also inhibits the protective activity of PDI, which is increasingly implicated in ALS (Parakh et al., 2020).

The clinical manifestations of ALS usually appear between 50–60 years of age, suggesting that neurons die through cumulative damage to normal cellular processes. Given that oxidative stress is associated with aging, prolonged redox dysregulation may therefore induce neurodegeneration, either directly or indirectly (Parakh et al., 2013; Mcbean et al., 2015). Furthermore, redox dysregulation may act as a double-edged sword, inducing a cascade of cellular events that lead to neurodegeneration and simultaneously inactivating protective thiols such as PDI by post-translational modifications. It is therefore imperative to find therapeutics that balance the cellular redox state and regulate redox-regulated proteins associated with key cellular functions.

Redox homeostasis underlies all important cellular activities and ALS is a systemic disease that affects multiple cellular processes. Several studies collectively support the hypothesis that redox dysregulation is central to ALS pathogenesis, particularly in genetically predisposed individuals. Modifiers of redox regulation may therefore be a potential therapeutic target for ALS. Many studies have shown potentially useful effects of antioxidants and modifiers of redox regulation in TDP-43, C9orf72, SOD1, and FUS mice models of ALS. However, previous studies have failed to demonstrate a convincing beneficial effect for redox-active compounds in clinical trials. Therefore, both pre-clinical and clinical studies need to be carefully designed to consider the sample size, primary endpoint, duration of the trial, and animal model used or ALS patient population. It is also possible that the redox modulators previously examined are not the most effective, and additional studies trialling other components of redox regulation may yield more promising findings in the future.

AUTHOR CONTRIBUTIONS

CJ wrote the section on direct and indirect evidence for the role of redox dysregulation in ALS, modifiers of redox regulation as a therapeutic target for ALS, contributed to all the figures and tables, and edited the manuscript throughout. SP wrote the introduction, section on cellular redox system, PDI family of proteins, parts of the Discussion, and edited the manuscript throughout. JA conceived the article, contributed additional text, and edited the manuscript throughout for content and style consistency. All authors contributed to the article and approved the submitted version.

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frontiers in Aging Neuroscience

REVIEW
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The Potential Role of Protein Kinase R as a Regulator of Age-Related Neurodegeneration

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There is a growing evidence describing a decline in adaptive homeostasis in aging-related diseases affecting the central nervous system (CNS), many of which are characterized by the appearance of non-native protein aggregates. One signaling pathway that allows cell adaptation is the integrated stress response (ISR), which senses stress stimuli through four kinases. ISR activation promotes translational arrest through the phosphorylation of the eukaryotic translation initiation factor 2 alpha (eIF2α) and the induction of a gene expression program to restore cellular homeostasis. However, depending on the stimulus, ISR can also induce cell death. One of the ISR sensors is the double-stranded RNA-dependent protein kinase [protein kinase R (PKR)], initially described as a viral infection sensor, and now a growing evidence supports a role for PKR on CNS physiology. PKR has been largely involved in the Alzheimer's disease (AD) pathological process. Here, we reviewed the antecedents supporting the role of PKR on the efficiency of synaptic transmission and cognition. Then, we review PKR's contribution to AD and discuss the possible participation of PKR as a player in the neurodegenerative process involved in aging-related pathologies affecting the CNS.

Keywords: double-stranded RNA-dependent protein kinase, integrated stress response, neurocognitive functions, Alzheimer's disease, Parkinson's disease, Huntington's disease, aging

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INTRODUCTION

Neurocognitive functions rely on neuronal cell adaptation mechanisms during the nervous system lifespan. Neurons respond continuously to changing cellular contexts to achieve these adaptive dynamics. An increasing evidence has shown a reduced capacity in adaptive homeostasis mechanisms in aging and aging-related neurodegenerative diseases. One of the signaling pathways that allow cell adaptation is the integrated stress response (ISR). The ISR comprises four kinases/sensors: double-stranded RNA-dependent protein kinase [protein kinase R (PKR)], heme-regulated inhibitor (HRI), the general control non-derepressible-2 (GCN2), and the PKR-like endoplasmic reticulum (ER) resident protein kinase (PERK). Depending on the stimulus, one of the ISR kinases becomes active and phosphorylates the eukaryotic translation initiation factor 2 (eIF2α) on Ser51 (Harding et al., 2003; Pakos-Zebrucka et al., 2016). This phosphorylation event not only leads to a decrease in global protein synthesis but also induces the expression of selected genes, including the one coding for the activating transcription factor 4 (ATF4) (Harding et al., 2003; Pakos-Zebrucka et al., 2016). Once translated, ATF4 translocates to the nucleus

and promotes genetic programs involved in essential cellular processes/functions, including autophagy, redox homeostasis, and amino acid metabolism. However, under chronic overactivation, ISR can also induce apoptosis (Pakos-Zebrucka et al., 2016). Although ISR signaling events have been described in dividing cells, the consequences of ISR activation, specifically in neurons, remain poorly explored in physiological conditions. However, there are strong pieces of evidence that suggest a role for ISR in central nervous system (CNS) pathophysiology. For instance, there is significant information about the role of PERK in neurodegeneration. PERK is a sensor that can be activated directly by mis- or unfolded proteins at the ER (Wang et al., 2018). Several neurodegenerative diseases are characterized by the presence of aggregated unfolded or misfolded proteins, including Huntington's disease (HD), Parkinson's disease (PD), and AD, and the contribution of PERK has been largely discussed in other articles (Bell et al., 2016; Hughes and Mallucci, 2019). This review is focused on the kinase PKR and the consequences of its activation in the CNS. PKR is a stress sensor first identified as a kinase responding against viral infection (Meurs et al., 1990). Today, PKR is considered a significant regulator of central cellular processes, including mRNA translation, transcriptional control, apoptosis regulation, growth regulation, and cell proliferation (He et al., 2013; Zhou et al., 2014; Shinohara et al., 2015; Gal-Ben-Ari et al., 2018). In the CNS, PKR controls protein synthesis and regulates synaptic and cognitive function. PKR activation has also been involved in neurological conditions, including AD (Peel and Bredesen, 2003; Couturier et al., 2010; Dumurgier et al., 2013; Hugon et al., 2017) (reviewed in Hugon et al., 2017), PD (Bando et al., 2005), and HD (Peel et al., 2001; Bando et al., 2005), suggesting a role of PKR in pathologies associated to aging affecting the CNS.

Here, we reviewed the antecedents supporting PKR and the consequences of its activation as a key player in physiological conditions and its contribution in the neurodegenerative process involved in aging-related pathologies affecting the CNS.

PKR-eIF2α ROLE ON CNS PHYSIOLOGY

Protein kinase R is conserved in vertebrates, and it has not been found in plants, fungi, protists, and invertebrates (Taniuchi et al., 2016). PKR was initially described to be activated by viral double-stranded RNAs (dsRNA) (Hovanessian, 2007). Once the stimulus is detected, the activation of PKR is mediated by homodimerization and trans-autophosphorylation at threonine 446 and threonine 451 (Romano et al., 1998). Activated PKR induces the phosphorylation of eIF2α (p-eIF2α), leading to general protein synthesis inhibition (Romano et al., 1998). In parallel, PKR activation can also promote p-eIF2α-dependent translation of messenger RNAs (mRNAs) containing specific 5′ untranslated region (UTR) regulatory regions (Chesnokova et al., 2017). One of the proteins synthesized after ISR activation is ATF4, which leads to apoptosis in several cell types (Wek et al., 2006; Hussain and Ramaiah, 2007; Lee et al., 2007). It has been recently reported that PKR activation is negatively regulated by its interaction with sphingosine kinase 1 (SPHK1), which reduces apoptosis execution on cell lines (Qiao et al., 2021). It has been reported that PKR can also be activated in the absence of virus (dsRNA) by endogenous cellular stresses such as oxidative stress, intracellular calcium increase, or ER stress (Romano et al., 1998; Zhu et al., 2011; He et al., 2013). Additionally, interferon-gamma (IFNγ), tumor necrosis factor α (TNFα), heparin, platelet-derived growth factor, and inosine–uracil mismatches have also been described as PKR activators (Deb et al., 2001; Page et al., 2006; Anderson et al., 2011; McAllister et al., 2012; Husain et al., 2015; Reimer et al., 2018). Thus, the PKR-eIF2α branch may potentially be activated in response to a diversity of inductors depending on a particular cellular context. Moreover, a specific PKR activation mechanism has been described, in the absence of dsRNA, through the PKR associated protein activator (PACT) (in humans) or its murine homolog RAX. Thus, PACT/RAX has been proposed as physiological activators of PKR (Patel and Sen, 1998; Ito et al., 1999). A recent detailed study of PKR-activating dsRNAs found that most of the PKR-interacting RNA repertoires are mitochondrial RNAs (mtRNAs) that can form intermolecular dsRNAs (Youssef et al., 2015; Kim et al., 2018). mtRNAs interaction with PKR was found to induce this kinase activation, eIF2α phosphorylation, and subsequent control cell translation (Youssef et al., 2015; Kim et al., 2018). This promiscuity of PKR as a sensor of endogenous and exogenous stimuli suggests that PKR could be a negative regulator of protein synthesis in several cellular physiological and pathological contexts (see **Figure 1**).

As mentioned earlier, ISR activation can lead to cell adaptation or apoptosis, which has been extensively reviewed elsewhere (Pakos-Zebrucka et al., 2016). This paradoxical role of the ISR is supported by signaling assays on proliferating cells with spatially homogeneous dynamics. An ISR modulating stimulus is applied to a whole cell, and survival readouts are studied on these assays. In neuronal models, it has been established that the activation of the PKR eIF2 a branch also mediates apoptosis (Vaughn et al., 2014). However, it is worth considering that neuronal cells show a significantly higher degree of complexity to support their local cytoarchitecture. This particularity of neurons diversifies the potential roles of homeostasis regulation on neuronal functions. For example, it has been established that local protein synthesis, a process directly regulated by PKR, is necessary not only to maintain local axonal phenotype (merotrophism) and axonal integrity but also to execute axonal degeneration in an apoptotic independent manner (Alvarez et al., 2000; Whitmore et al., 2003; Hillefors et al., 2007; Pazyra-Murphy et al., 2009). Thus, local protein synthesis regulates neuronal integrity. Consequently, homeostasis regulation mechanisms may be essential not only for neuronal survival but also for local integrity maintenance in neurons. However, the participation of PKR in local integrity maintenance in neurons remains unknown.

The role of PKR-eIF2α in the CNS has been studied using genetic strategies. The mouse in which part of the PKR coding gene has been deleted (PKR-KO) or the knock-in mouse bearing an eIF2α allele with the S51A mutation (eIF2αS/A) does not show qualitative changes on CNS overall morphology and histological patterns of axonal or synaptic markers (Costa-Mattioli et al., 2007; Zhu et al., 2011) compared

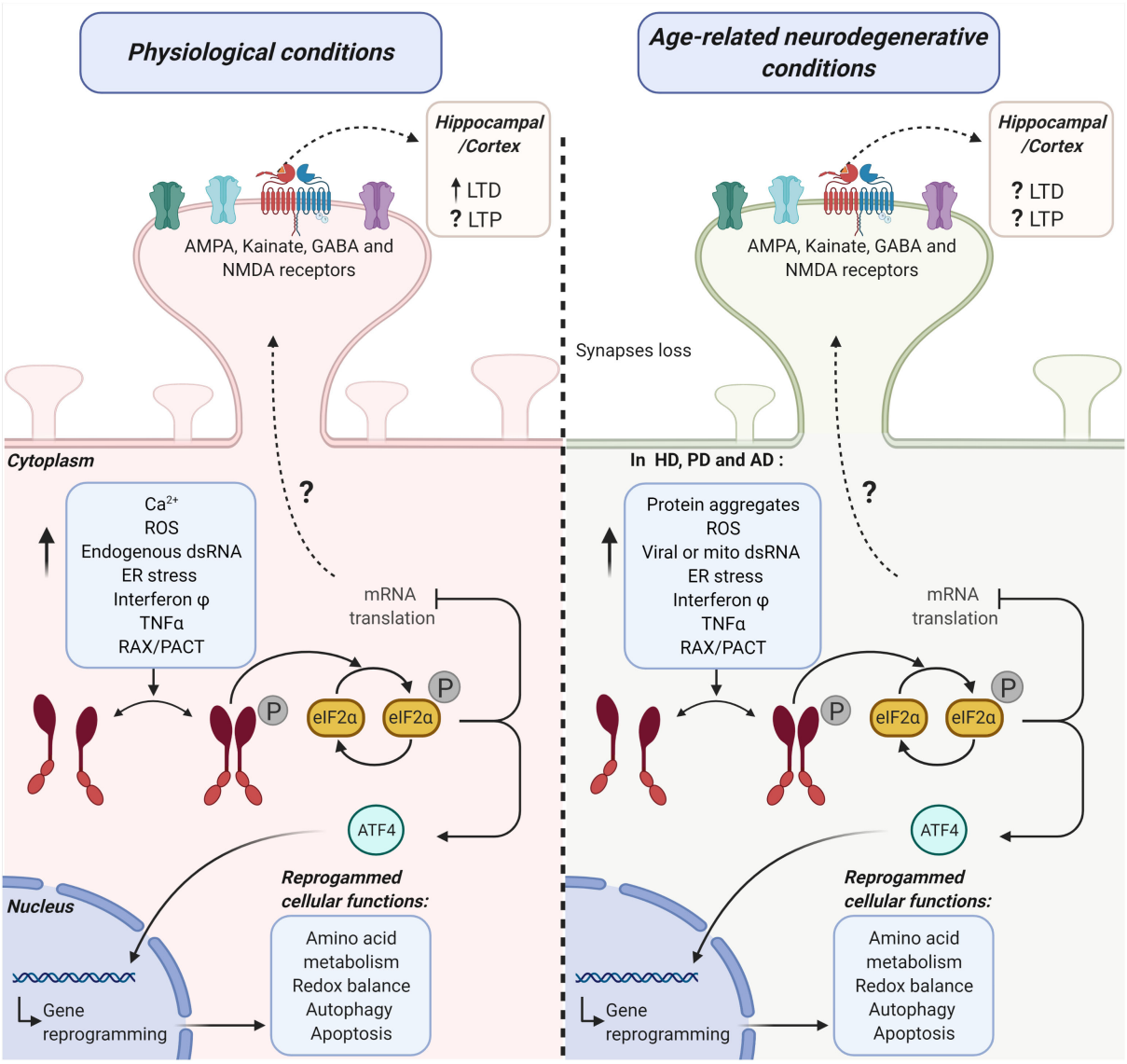

FIGURE 1 | Possible mechanistic links between the activation of the PKR-eIF2α branch of the ISR and hippocampal/cortical plasticity under physiological and age-related neurodegenerative conditions. The diagram represents the potential role of the double-stranded RNA protein kinase [protein kinase R (PKR)] in synaptic transmission efficiency under physiological conditions (left side) and age-related neurodegenerative conditions (right side). PKR-activating stimuli induce a significant increase in the phosphorylation (P) of the alpha subunit eukaryotic initiation factor 2 (eIF2α) in a dsRNA-dependent manner or via its endogenous activator RAX/PACT. This pathway activation promotes the inhibition of mRNA translation and ATF4 dependent gene reprogramming of several cellular functions. Altogether, PKR-eIF2α ISR branch activation may lead to cell adaptation or cell death. PKR-eIF2α ISR branch loss of function (LOF) under physiological conditions (left side) significantly modifies cortical and hippocampal receptor-mediated synaptic transmission. Consequently, long-term potentiation (LTP) and long-term depression (LTD) are also affected. Under age-related neurodegenerative conditions (right side), common stimuli could participate in the activation of PKR-eIF2α ISR branch activation in Huntington's diseases (HD), Parkinson's disease (PD), and Alzheimer's disease (AD). Under this activation, ATF4 translocates to the nucleus and mediates degenerative reprogrammation. On the other hand, the eIF2α phosphorylation decreases mRNA translation. This decrease in translation induces modifications in the hippocampal and cortex LTP and LTD through an unknown mechanism.

to wild-type (WT) mice. Specifically, PKR-KO mouse brain shows no gross abnormalities on Nissl staining and presynaptic or postsynaptic structures, visualized by synaptic markers such as the vesicular glutamate transporter 1 (VGLUT1), the postsynaptic density protein 95 (PSD95), and the glutamic acid decarboxylase 67 (GAD67), a GABAergic terminal marker (Zhu et al., 2011). Additionally, eIF2αS/A knock-in mouse has no detectable differences in the hippocampus overall morphology, based on axonal or presynaptic marker stains (Small et al., 2000; Costa-Mattioli et al., 2007). In spite of this general preservation of CNS tissue under PKR-eIF2α ISR branch loss of function (LOF), a growing body of evidence shows that PKR participates in neuronal essential functions, survival, and integrity.

PKR in Neuronal Function

A major functional expression of synaptic plasticity is the efficiency of synaptic transmission. In turn, an efficient synaptic transmission supports neurocognitive functions. The relationship between synaptic plasticity and neurocognitive functions has been extensively reviewed before (Martinez et al., 2018). Importantly, synapses loss and dendritic atrophy are observed in the aging human brain and correlate with neurocognitive dysfunctions progression (Uylings, 2000). Consequently, altered synaptic transmission is also a common characteristic of age-related neurodegenerative diseases (Kumar and Foster, 2007). The widest used model for modifiability of synaptic transmission is the hippocampal field activity measurement, based on the synaptic plasticity role on cognitive memory mechanisms. There, brief high-frequency neuronal firings induce an increase in the efficacy of neuronal synapses that sustains for hours, known as long-term potentiation (LTP) (Connor and Wang, 2016; Peters et al., 2018). This physiological adaptive mechanism requires postsynaptic Ca^{2+} significant entry, activation of metabotropic glutamate receptors, and the generation of diffusible intercellular messengers (Landfield et al., 1986; Barnes et al., 1992; Rosenzweig et al., 1997; Connor and Wang, 2016; Peters et al., 2018). On the other hand, repeated low-frequency firing, which results in a modest rise in Ca^{2+}, induces long-term depression (LTD), provoking a significant, sustained decrease in the efficacy of neuronal synapses. Healthy aging and age-related neurodegeneration are associated with a decrease in synaptic plasticity, leading to impaired synaptic transmission (Rosenzweig et al., 1997). Consequently, reduced ability to enhance synaptic transmission through LTP or an LTD imbalance over LTP would trigger a decrease in synaptic transmission in aged animals (Landfield et al., 1986; Barnes et al., 1992; Rosenzweig et al., 1997; Temido-Ferreira et al., 2019). LTP and LTD are the major mechanistic insights that have been proposed to correlate with performance on mouse models of cognitive memory, which has been already reviewed (Landfield et al., 1986; Barnes et al., 1992; Rosenzweig et al., 1997; Connor and Wang, 2016; Figlioli et al., 2019).

An accumulating evidence suggests that the PKR-eIF2α signaling pathway negatively regulates hippocampal synaptic transmission efficiency in non-pathological conditions. Earlier studies on hippocampal slices showed that a significant decrease in eIF2α phosphorylation levels correlates with a lower threshold for long-lasting LTP (LTP that requires protein synthesis) (Takei et al., 2001; Costa-Mattioli et al., 2005). This result suggests that the eIF2α pool phosphorylation state modulates the establishment of a sustained increase in the efficacy of neuronal synapses. Furthermore, the eIF2αS/A mouse, which shows reduced eIF2α phosphorylation levels (by ∼50%) relative to WT mice, does not display any difference in basal transmission compared to WT mice (Costa-Mattioli et al., 2007). Under these significantly reduced eIF2α phosphorylation levels, Schaffer collateral/commissural fibers' stimulation with a short-lasting inducing LTP protocol (which does not require protein synthesis) (Kandel, 2001; Kelleher et al., 2004) elicits the expected result on WT hippocampal slices. Notably, the same protocol elicits a long-lasting LTP on hippocampal slices from eIF2αS/A mice littermates, which is sensitive to protein synthesis inhibitors (Costa-Mattioli et al., 2005, 2007). These results strongly suggest that eIF2α phosphorylation negatively regulates the transition from short- to long-lasting LTP at the hippocampus. Notably, previous reports have shown that eIF2α phosphorylation is necessary for LTD promotion at hippocampal slices (Di Prisco et al., 2014). Taken together, these results suggest that eIF2α phosphorylation could simultaneously modulate both LTP and LTD at the hippocampus. Consequently, ISR sensor kinases may potentially play a role in transmission efficiency by modulating the eIF2α phosphorylation state.

Acute pharmacological inhibition of PKR induces aberrant activity on the neocortex of free-moving adult mice, studied by electroencephalography (EEG), without changes in ongoing behavior (Zhu et al., 2011). These results suggest that eIF2α-mediated regulation of synaptic efficiency could be downstream of PKR signaling. Coincidently, authors have determined that PKR genetic deficiency in mice leads to aberrant hyperactivity of neuronal networks by reducing GABA-mediated inhibitory synaptic transmission (Zhu et al., 2011). In this model, PKR LOF led to a significantly higher number of spikes reaching a higher excitatory postsynaptic potential (ceiling), presumably because PKR-mediated inhibition was impaired (Zhu et al., 2011). Thus, the authors have proposed that the physiological role of PKR over transmission efficiency is to maintain a relatively low level of excitability by enhancing GABAergic synaptic transmission with a lack of change in postsynaptic receptor-related mechanisms.

As mentioned earlier, eIF2α phosphorylation is necessary to induce LTD (Di Prisco et al., 2014). Notably, PKR activation is sufficient to induce sustained LTD mediated by the phosphorylation of eIF2α (Di Prisco et al., 2014). LTD induction by PKR activation has been studied in a transgenic mouse in which a drug can activate PKR in certain neurons in the CNS. Specifically, the mouse expresses a transgene encoding a drug-dependent conditional PKR that dimerizes and activates at hippocampal CA1 neurons. The pharmacological induction of PKR activation led to eIF2α phosphorylation and sustained LTD selectively on neurons expressing the transgene (Di Prisco et al., 2014). Together, these data support the idea that PKR and eIF2α are major regulators of transmission efficiency. Thus, the regulation mediated by PKR-eIF2α also participates in CNS physiology at a functional level, beyond neuronal survival and integrity. Concomitantly, loss of function of eIF2α and PKR also induces significant changes over neurocognitive functions.

Since the modulation of eIF2α phosphorylation impacts transmission efficiency, it is plausible to expect that it also affects the performance of mice when paradigms evaluating cognitive memory are applied. In fact, it has been shown that pharmacological inhibition of eIF2α dephosphorylation mediates memory consolidation of drug-paired stimuli (Huang et al., 2016). In a model in which basolateral amygdala (BLA)-dependent cocaine addiction is established, rats are exposed to freely choose between saline paired side or cocaine paired side of a chamber. The difference in the time spent on the cocaine-paired side versus the saline-paired side is calculated as the cocaine place preference. Authors have found that eIF2α

phosphorylation and ATF4 levels diminish in the BLA after a re-exposure to a previously cocaine-paired context (Huang et al., 2016). Notably, local injection in the BLA of a selective inhibitor of eIF2α dephosphorylation after this memory retrieval protocol disrupts drug-paired stimulus-induced craving (Huang et al., 2016). Moreover, eIF2α also plays a role in spatial learning and spatial memory when studied by the Morris water maze paradigm (Costa-Mattioli et al., 2007). In this test, mice are trained during consecutive days to swim, find a platform, and memorize its location in a pool. Then, the time required for the mice to find the hidden platform ("escape latencies") and the time spent on the platform's quadrant is measured (Morris et al., 1982). Using this setup, authors have found that eIF2αS/A knock-in mice reached the platform significantly faster than their WT littermates (Costa-Mattioli et al., 2007). In addition, eIF2αS/A mice had a significantly greater preference for the platform quadrant (Costa-Mattioli et al., 2007). These results strongly suggest that eIF2α partial LOF enhances hippocampal-dependent spatial learning and spatial memory. Furthermore, the eIF2α's role over memory function has also been explored in several experimental paradigms of protein synthesis-dependent long-lasting memory such as auditory and contextual fear conditioning, conditioned taste aversion (CTA), and latent inhibition (LI) of CTA (Rosenblum et al., 1993; Bourtchouladze et al., 1998; Schafe et al., 1998) in eIF2αS/A mice. Remarkably, partial loss of eIF2α function (eIF2αS/A) induces a significant improvement on evocative parameters at all those above experimental long-lasting memory paradigms (Costa-Mattioli et al., 2007). Thus, eIF2α phosphorylation state modulation regulates long-term memory function beyond its effect on the transmission efficiency discussed before.

Similarly, acute pharmacological targeting of PKR using the inhibitor C16 has been evaluated in a taste learning paradigm. Local stereotaxic injection into the insular cortex or intraperitoneal (i.p.) injection of C16 before applying an aversive taste stimulus results in enhanced cortical-dependent novel taste learning and CTA in rats (Ariffin et al., 2010; Stern et al., 2013). This effect of acute inhibition has been proposed as indicative of PKR involvement in cognitive processing. To evaluate this, authors had used acute pharmacological inhibition of PKR before contextual and auditory fear conditioning and studied the incorporation of neurons to functional circuits induced by learning through the expression of a specific marker gene, immediate-early gene (Egr-1) (Hall et al., 2000; Frankland et al., 2004; Zhu et al., 2011). On this experimental setup, both contextual and auditory long-term fear memories were enhanced under PKR pharmacological inhibition in mice (Zhu et al., 2011). Concomitantly, in the same group of experiments, long-lasting memory in PKR-KO mice correlated with Egr-1 levels at hippocampal CA1 neurons. Authors have proposed that PKR LOF not only improves long-lasting memory but also participates in the recruitment of CA1 neurons into the cognitive encoding process (Zhu et al., 2011). Furthermore, the PKR-KO mouse also shows significantly improved hippocampus-dependent spatial memory assayed by the Morris water maze test. Even more, the PKR-KO mouse also shows improved auditory and contextual long-term fear memories compared to WT littermates when tested through Pavlovian fear conditioning (Zhu et al., 2011). Thus, genetic deletion of PKR strengthens long-term spatial memory.

PKR on Glial Cells

Several authors have reported the presence of the components of the PKR-eIF2α branch on another type of cells present in the CNS, the glial cells (Auch et al., 2004; Ong et al., 2005; Alirezaei et al., 2007; Vantelon et al., 2007; Flores-Mendez et al., 2013). However, the role of the PKR-eIF2α branch on glial functions under physiological conditions remains mostly unexplored. In this context, emerging reports suggest that eIF2α activation controls protein a synthesis rates on glial cells. Specifically, protein synthesis rates increase in astrocytes correlate with Eif2 a phosphorylation, in response to lactic acid (Vantelon et al., 2007). It has also been reported that eIF2α activation mediates protein synthesis rate changes in response to glutamate neurotransmitter on Bergmann glial cells (Flores-Mendez et al., 2013). In turn, PKR mediates an increase in nitric oxide (NO) production on *in vitro* human astrocytes in response to dsRNA by inducing inducible nitric oxide synthase (iNOS) expression (Auch et al., 2004). Altogether, these reports suggest that the PKR-eIF2α branch may have a relevant role in CNS functions regulated by glial cells. Despite this, the antecedents available describing the role of the components of the PKR-eIF2α branch on glial biology have been performed in the context of pathological inflammation, which we will review in the next section.

Altogether, an accumulated evidence suggests that the PKR-eIF2α signaling pathway participates in CNS morpho-functionality *per se* under physiological conditions: at the neuronal cell level through survival and integrity, through glial regulation, at the transmission efficiency of circuits, grounded on synaptic plasticity, and finally, at the neurocognitive level by regulating long-term memory (LTM). This role of PKR and eIF2α at several organizational and functional levels under physiological conditions points to a potentially relevant role in the early stages of age-related neurodegenerative diseases (**Figure 2**).

PKR ROLE IN AGE-RELATED NEURODEGENERATIVE DISEASES

Based on the extended role described for PKR and eIF2α on CNS morpho-functional levels that we reviewed in the previous section, we identified the potential for this pathway to play a role as a significant regulator of age-related neurodegenerative diseases. We reviewed the possible role of PKR-eIF2α pathway in neurodegenerative diseases in which pathological hallmarks, including progressive neuronal cell death, transmission efficiency defects, and neurocognitive functions decline, can be detected.

Mechanistic Link Between AD and PKR

The PKR and eIF2α roles over age-related neurodegenerative diseases have been mostly studied for AD in the context of the amyloid cascade hypothesis experimental models. We reviewed

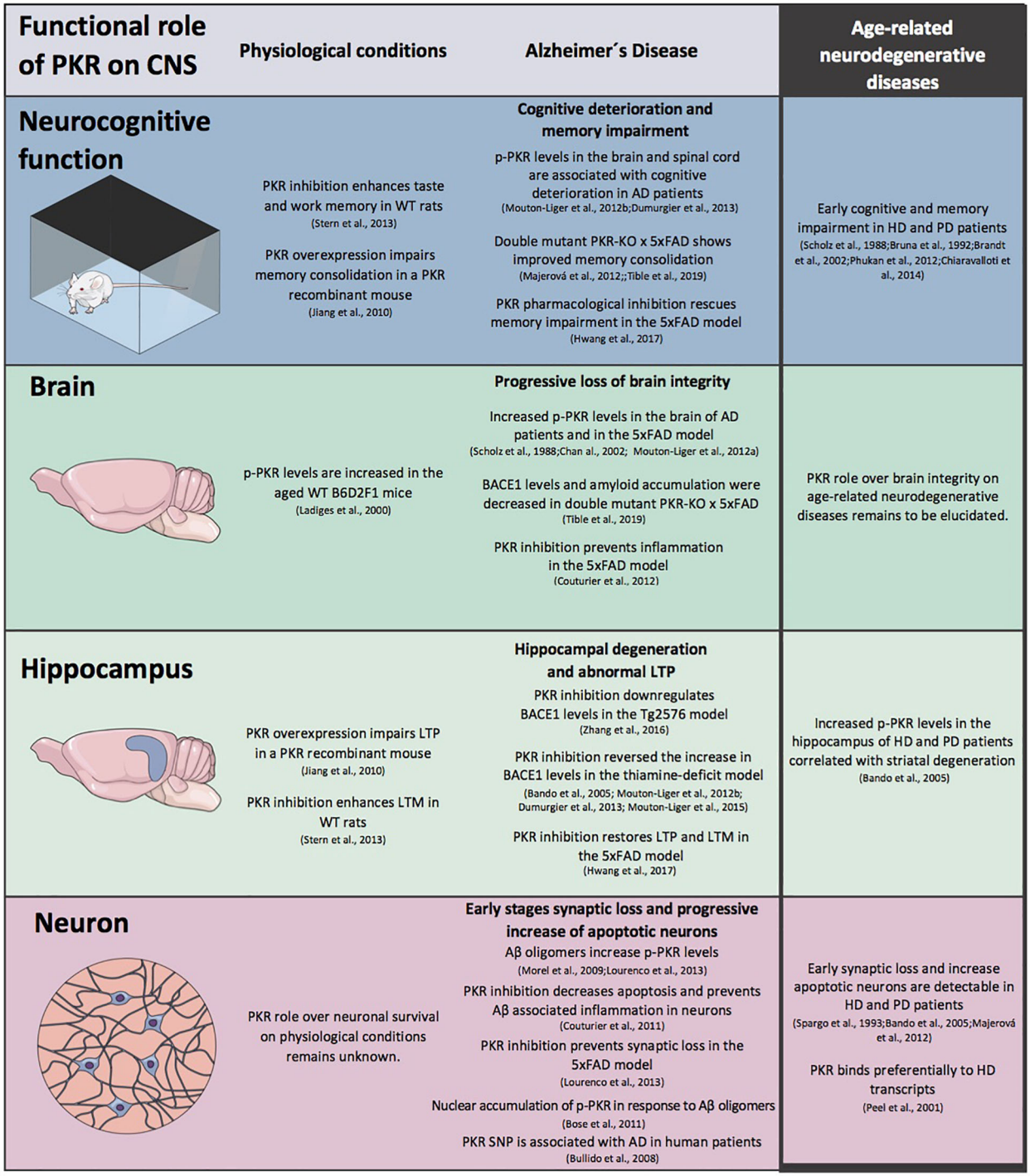

FIGURE 2 | The role of protein kinase R (PKR) in CNS physiology and pathophysiology. A schematic view of the modulation exerts by PKR in CNS morpho-functional integrity at different levels under physiological conditions and in Alzheimer's (AD) disease. The information available for age-related neurodegenerative diseases is included.

the evidence on the role of PKR-eIF2α over AD pathogenic hallmarks. We detailed the studies related to the role of PKR on AD pathogeny in **Table 1**. A detailed description of the AD models, characteristics, and progression has been extensively detailed elsewhere (De-Paula et al., 2012; Cheng et al., 2018; Bouteiller et al., 2019; Castellani et al., 2019).

Alzheimer's disease is characterized by the presence of extracellular senile plaques of the amyloid-beta (Aβ) aggregated

TABLE 1 | Studies involving protein kinase R (PKR) in Alzheimer's disease.

AD model (*in vitro*/*in vivo*)	PKR active/LOF/GOF	Variable relationship	Neurodegeneration readouts/outcome	References
AD patients	Active	Correlative	PKR accumulation at nuclei in *postmortem* AD brain tissue	Onuki et al. (2004)
5xFAD transgenic mouse	Active	Correlative	p-PKR and p-eIF2α levels are increased in cortical tissue, by immunoblotting	Mouton-Liger et al. (2012a)
Aβ neurotoxicity over primary neuronal culture	PKRi	Functional	PKR pharmacological LOF prevented $A\beta_{1-42}$-induced activation of inflammatory pathways, release of TNFα and interleukin (IL)-1β, and inhibited apoptosis	Couturier et al. (2011)
5xFAD transgenic mouse and $A\beta_{1-42}$-injected mouse	PKRi	Functional	PKR pharmacological LOF restores deficits in LTM and LTP in both mouse AD models	Hwang et al. (2017)
Aβ toxicity over SH-SY5Y cells and AD patients	PKRi	Functional and correlative	PKR colocalizes with neuronal GSK-3β and tau in AD brains. PKR modulates Aβ induced GSK-3β activation, tau phosphorylation, and apoptosis in neuroblastoma cells	Bose et al. (2011)
AD patients	Active	Correlative	p-PKR in aged brains histology negatively correlates with cognitive scores	Taga et al. (2017)
AD patients	Active	Correlative	A SNP (rs2254958) on the PKR coding gene correlates with AD progression	Bullido et al. (2008)
Thiamine-deficient diet *ad libitum*	PKR-KO mouse or PKRi	Functional	PKR LOF (genetical and pharmacological) reverses Aβ oligomers levels increase in thalamus nuclei, motor deficits, and neurodegeneration induced by thiamine	Mouton-Liger et al. (2015)
$A\beta_{25-35}$ neurotoxicity over primary neuronal culture	Active	Correlative	Purified $A\beta_{25-35}$ induces PKR phosphorylation	Yu et al. (2007)
$A\beta_{25-35}$ neurotoxicity over primary culture of cortical neurons	PKR siRNA	Functional	Purified $A\beta_{25-35}$ induces PKR phosphorylation	Lai et al. (2006)
FAD-mutant hAPP mouse and AD patients	Active	Correlative	p-PKR associates with plaques in the FAD-mutant hAPP mouse brain. p-PKR in the hippocampus and the neocortex of AD patients associates with amyloid plaques	Peel and Bredesen (2003)
APPSwe/PS1DE9 mouse and monkeys (*Macaca fascicularis)* exposed to Aβ oligomers	PKRi	Functional	Amyloid-β induces PKR and eIF2α phosphorylation in the brain of mouse and monkeys. Activated PKR correlates with synapse loss and memory impairment	Couturier et al. (2012)
AD patients	Active	Correlative	p-PKR levels at CSF strongly correlates with the severity of cognitive impairment	Dumurgier et al. (2013)
5xFAD mouse	PKR-KO mouse	Functional	PKR LOF (genetic) in the 5xFAD mouse shows reduced BACE1 and Aβ levels, synaptic alterations, neurodegeneration, and neuroinflammation and improves memory defects	Tible et al. (2019)
APPSL/PS1 KI mouse	Active	Correlative	p-PKR and p-eIF2α levels are increased in the cortex of APP_{SL}/PS1 KI mouse	Couturier et al. (2010)
Mouse overexpressing the Swedish mutation of 101 amyloid precursor protein (Tg2576)	PKRi	Functional	p-PKR and p-eIF2α levels are increased in the brain of Tg2576 mouse. PKR LOF (pharmacological) alleviates memory deficits in the Tg2576 mouse	Zhang et al. (2016)
Four-month-old ApoE3 and ApoE4 mice	PKRi	Functional	Pharmacological PKR LOF (locally injected) rescues memory impairment and attenuates ATF4 mRNA increased translation in the ApoE4 mouse	Segev et al. (2015)
$A\beta_{1-42}$ peptide neurotoxicity over primary neuronal cultures and SH-SY5Y cells	PKR siRNA	Functional	PKR LOF (siRNA) inhibits $A\beta_{1-42}$ induced pro-neurodegenerative signaling in nuclei	Morel et al. (2009)
AD patients	Active	Correlative	PKR and eIF2α levels in lymphocytes of AD patients correlates with cognitive and memory test scores	Paccalin et al. (2006)

AD, Alzheimer's disease; Aß, amyloid-beta protein; LOF, loss of function; LTM, long-term memory; LTD, long-term depression; TNFα, tumor necrosis factor alpha; IL, interleukin; SNP, single-nucleotide polymorphism; APP, amyloid precursor protein; FAD, familial Alzheimer's disease; p-PKR, phosphorylated PKR; eIF2α, eukaryotic initiation factor alpha 2; PKRi, PKR pharmacological inhibitor (C16); SH-SY5Y, thrice-cloned subline of bone marrow biopsy-derived line; GSk-3b, glycogen synthase kinase 3 beta; CSF, cerebrospinal fluid; ATF4, activating transcription factor 4; ApoE4, apolipoprotein E4.

protein, intracellular neurofibrillary tangles (NFTs) composed of hyperphosphorylated tau protein, and neuroinflammation (De-Paula et al., 2012; Cheng et al., 2018; Bouteiller et al., 2019; Castellani et al., 2019; Diaz-Zuniga et al., 2020). Senile plaques are composed of Aβ peptides generated after amyloid precursor protein (APP) proteolysis through the amyloidogenic pathway (Chow et al., 2010). Shortly, β-site APP cleaving enzyme 1 (BACE1) cleaves APP, shedding its ectodomain and leaving in the membrane a fragment of 99 amino acids (C99) (Vassar et al., 1999; Bennett et al., 2000). Then, a γ-secretase complex cleaves C99, generating variants of Aβ peptides, being the peptide of 42 residues ($A\beta_{1-42}$), the major component of the amyloid plaques and the one with toxic properties (Barrow and Zagorski, 1991; Miller et al., 1993; Klein et al., 1999; Vassar et al., 1999; Bennett et al., 2000; Cai et al., 2001; Luo et al., 2001). Several studies have shown that BACE1 levels and its proteolytic activity are increased in postmortem AD brain samples (Fukumoto et al., 2002; Holsinger et al., 2002; Zhao et al., 2007). According to the amyloid hypothesis (Hardy and Selkoe, 2002), the accumulation and aggregation of Aβ is the triggering event leading to neurodegeneration in AD. These antecedents suggest that elevated BACE1 levels could participate in AD onset or progression. Altogether, these pathological characteristics participate in the neurotoxic mechanism that ultimately leads to a progressive decline of memory function and other cognitive skills (De-Paula et al., 2012; Bouteiller et al., 2019; Castellani et al., 2019).

Protein kinase R can control the levels of BACE1 protein in human neuroblastoma cells exposed to oxidative stress (Mouton-Liger et al., 2012a,b; Taga et al., 2017). The same group has reported that phosphorylated (activated) PKR (p-PKR), p-eIF2α, and BACE1 levels are increased in the AD brain. Moreover, a significant correlation between BACE1 with phosphorylated eIF2α was found (Mouton-Liger et al., 2012b; Taga et al., 2017). These antecedents suggest that PKR-eIF2α could modulate Aβ production. However, little is known about the role of PKR over the specific mechanisms associated with the amyloid hypothesis of AD.

Protein kinase R has also been involved in the mechanism of tau protein phosphorylation. An analysis performed on AD brains and transgenic mouse models found that the distribution of p-PKR matched the distribution of abnormally phosphorylated tau in adjacent sections (Peel and Bredesen, 2003). It has been established that cell lines with reduced PKR expression through RNA interference (RNAi) strategies significantly reduce tau phosphorylation at the 12E8 epitope (serine 262/serine 356), a disease-related phosphorylation site (Azorsa et al., 2010). Notably, a recent work has reported that PKR overexpression and knockdown increase and decrease tau protein and mRNA levels in cell lines, respectively (Reimer et al., 2021). Moreover, the same study showed that PKR directly phosphorylates multiple abnormal and disease-related residues within tau protein (Reimer et al., 2021). Furthermore, this PKR-mediated phosphorylation induces tau displacement from microtubules, promoting a pathological role for tau. Based on this, several authors have proposed that PKR activation links Aβ and tau mechanisms of neurodegeneration (Bose et al., 2011; Amin et al., 2015; Reimer et al., 2021).

Protein kinase R is overexpressed in the brain of patients with AD (Chang et al., 2002; Peel and Bredesen, 2003; Onuki et al., 2004). Activated PKR has been found in neuron cytoplasm, in granule-vacuolar degeneration sites, neuronal nuclei, and around senile plaques by immunohistological analysis of brains derived from AD mouse models and human AD patients postmortem biopsies (Peel et al., 2001; Chang et al., 2002; Peel and Bredesen, 2003; Hugon et al., 2017). In these studies, AD cases showed prominent granular p-PKR immunoreactivity in association with neuritic plaques and pyramidal neurons in the hippocampus and neocortex compared to samples from subjects without dementia (Peel et al., 2001). Interestingly, p-PKR immunoreactivity has also been found distributed within and around the periphery of senile plaques of the Aβ-aggregated protein (Peel et al., 2001; Chang et al., 2002; Peel and Bredesen, 2003; Hugon et al., 2017). Whether this PKR activation patterns and effect over Aβ production are an early event in the disease process or a late consequence of neurodegeneration has not been established. On the other hand, authors have suggested that mutations in the PKR gene are related to an early onset of AD in human patients (Bullido et al., 2008). A 5′ UTR SNP (rs2254958) of the EIF2AK2 (PKR coding gene) has been associated with susceptibility for developing AD at an early age (Bullido et al., 2008). More specifically, this polymorphism is commonly found in AD patients. Compared to other genotypes, the homozygotes of rs2254958 showed earlier (around 3.3 years) onset of AD (Bullido et al., 2008). Consequently, PKR aberrant expression may predispose to AD progression (Peel and Bredesen, 2003). Interestingly, reports from *in vitro* models of AD suggest that PKR is in turn activated by the Aβ peptide (Peel et al., 2001; Hugon et al., 2017).

An interesting report showed that p-PKR is significantly increased in cerebrospinal fluid (CSF) of AD patients when compared with sex-paired and age-matched patients without dementia (Dumurgier et al., 2013). Moreover, when p-PKR was cross-sectionally associated with a standardized cognitive test, the Mini-Mental State Exam (MMSE), it was found that higher levels of p-PKR over the follow-up were correlated with cognitive deterioration (Dumurgier et al., 2013). Interestingly, while CSF $A\beta_{1-42}$ levels and p-Tau 181/Tau ratio were also cross-sectionally associated with the MMSE score at the diagnosis, only p-PKR was determined as a biomarker of cognitive decline during the progression of AD. Based on this, authors have proposed that a higher level of CSF p-PKR can predict a faster rate of cognitive decline at the time of AD diagnosis (Dumurgier et al., 2013). This suggests that CNS cellular components actively extrude activated PKR to the extracellular milieu in the context of a progressive worsening of AD.

The role of endogenous activators of PKR in the context of AD has been poorly explored. However, Paquet et al. (2012) reported that PACT and p-PKR have significantly higher colocalization on AD patients' brains postmortem in comparison to age-matched controls. Furthermore, levels of activated PKR (normalized p-PKR levels) strongly correlate with PACT protein levels on the same samples (Paquet et al., 2012). Interestingly, $A\beta_{1-42}$ peptides

induce a timely coordinated significant increase in both PKR activation levels and PACT protein levels *in vitro* on the SH-SY5Y human neuroblastoma cell line (Paquet et al., 2012). These data suggest that PACT and PKR could participate in a common cellular response to AD-related neurotoxicity. However, further research is needed to characterize a specific mechanism.

On the other hand, authors have proposed a potential role for dsRNAs, well-described PKR activators, in the context of AD progression. In brief, transcripts with repetitive elements can unspecifically form dsRNA on the cytoplasm (Scheckel et al., 2016). Interestingly, changes in chromatin and epigenetic modifications associated with age-related neurodegenerative diseases promote the derepression of repetitive element transcription due to changes in heterochromatin (Scheckel et al., 2016). Based on this, it has been suggested that this derepression may lead to an increased accumulation of intracellular dsRNA. Notably, RNA-seq-based analysis of global transcriptomes from AD patients versus age-matched controls shows increased levels of transcripts from multiple classes of repetitive elements (Saldi et al., 2019). Furthermore, adenosine-to-inosine RNA editing, a posttranscriptional marker of dsRNA, is also comparatively increased on the same AD patients' global transcriptomes (Saldi et al., 2019). Thus, a potential role for dsRNAs on the onset or progression of AD has been proposed. However, cellular mechanistic links between dsRNA, PKR, and AD pathogenic markers are currently unknown. In **Figure 1**, possible stimuli participating in PKR activation are shown on AD context and other age-related neurodegenerative conditions.

As mentioned, it has been widely reported that the activation of the PKR-eIF2α branch of the ISR leads to apoptosis on several cell types, including neurons (Lee and Esteban, 1994; Der et al., 1997; Gil and Esteban, 2000; Scheuner et al., 2006). This proapoptotic role of PKR-eIF2α has been reported to be executed mostly through the canonical apoptotic pathway (Lee and Esteban, 1994; Der et al., 1997; Gil and Esteban, 2000; Scheuner et al., 2006). It has also been shown that PKR inhibition suppresses apoptosis execution in neural cells. Specifically, overexpression of a negative dominant form of PKR (K296R) on neuroblastoma cells inhibits pharmacological ER stress (induced with tunicamycin) and the induction of the apoptotic markers caspase-3 and C/EBP homologous protein (CHOP, also known as GADD153). This apoptosis inhibition correlates with a delay in eIF2α phosphorylation and ATF4 expression (Vaughn et al., 2014). In addition, the role of PKR activation in neuronal survival has been explored on the paradigm of ethanol-induced apoptosis (Qi et al., 2014; Li et al., 2015). This model has shown that ethanol exposure causes neuronal apoptosis in mice's developing cerebellum (Olney et al., 2002; Qi et al., 2014). There, PKR pharmacological inhibition preserves cell survival under ethanol toxicity in cultured cerebellar granule neurons (Ke et al., 2009). Furthermore, when PKR activity dependence on its endogenous activator RAX is absent through a genetical deletion of the domain of interaction between endogenous activator RAX and PKR (the deficient RAX-binding domain in PKR mouse), Purkinje and granule neurons densities are significantly preserved in response to ethanol when compared to WT mice (Li et al., 2015). Altogether, these results suggest that PKR dynamics regulate neuronal survival in response to neurotoxicity by modulating apoptotic cell death.

To our interest, several reports suggest that the PKR-eIF2α signaling pathway also modulates neuronal apoptosis on AD models. For instance, it has been found that pharmacological or genetic inhibition of PKR significantly reduces Aβ-induced apoptosis (Page et al., 2006). Initially, it was reported that in an AD *in vitro* model based on Aβ deposition, increased tau phosphorylation and neuronal death induced by Okadaic Acid correlates with PKR and eIF2α activation (Kim et al., 2010). Other authors have also reported that cortical neurons from PKR-KO mice exhibit significantly lower apoptotic cell death levels in response to Aβ (Gourmaud et al., 2016). This protective effect of PKR genetic LOF was correlated with significantly lower levels of apoptosis executors, including cleaved poly ADP-ribose polymerase (PARP) and cleaved caspase 3. Levels of Fas-associated protein with death domain (FADD), an adaptor that bridges death receptor signaling to the caspase cascade indispensable for the induction of extrinsic apoptotic cell death, are significantly increased on the cortex of a mouse model of Alzheimer's disease (5xFAD) at a presymptomatic stage when compared to WT littermates *in vivo* (Couturier et al., 2010). Furthermore, co-immunoprecipitation assays showed that PKR and FADD physically interact in cortex extracts derived from 5xFAD mice, and no interaction is detectable at WT littermates (Couturier et al., 2010). Notably, the authors have determined that $A\beta_{1-42}$ induces p-PKR phosphorylation, increases FADD levels, and promotes physical interaction between PKR and FADD in the nucleus of neuroblastoma cells. Even more, PKR gene silencing (RNAi) or treatment with the specific PKR inhibitor, C16, significantly inhibits PKR activation in neuroblastoma cells and inhibits downstream activities of caspase-3 and caspase-8. Taken together, these antecedents suggest that PKR activation promotes neuronal apoptotic cell death in the context of Aβ neurotoxicity in models of Alzheimer's disease.

Another major characteristic of AD pathogenic hallmark is the glia-mediated neuroinflammation. Briefly, microglial cells execute the innate immunity in the CNS and participate in regulating synaptic plasticity and neuronal circuits activity (Hasan and Singh, 2019; Ikegami et al., 2019; Konishi et al., 2019). Notably, microglia and astrocytes react to pathological stressors by producing and releasing inflammatory mediators that aim to resolve the pathological state. Age-related neurodegenerative diseases operate as chronic pathological stressors over glial cells, which promotes a phenotypical change (glial activation) characterized by a significant increase in the release of inflammatory mediators from glia (Pawate et al., 2004; Lassmann, 2020). The role of glial cells has been largely described for neurodegenerative diseases, including HD (Hsiao and Chern, 2010) and PD (Przedborski and Goldman, 2004). However, a direct contribution of PKR in glial cells has not been explored in HD or PD.

Histological studies of brains from AD patients and AD animal models show a strong colocalization of reactive glial cells with senile plaques and neurofibrillary tangles (Parachikova et al., 2007; Hickman et al., 2008; Lopez-Gonzalez et al., 2015). The

inflammatory cascade mechanism during AD associated with Aβ toxicity has been largely reviewed before (Bruni et al., 2020; Kim et al., 2020; Merlo et al., 2020; Webers et al., 2020). In brief, microglia and astrocyte activation participates in Aβ clearance on AD progression's earlier steps (Ries and Sastre, 2016). However, further AD progression is characterized by an increase in microglial activation. This increase in inflammatory signaling in the latter stages of AD correlates with a significant decrease in Aβ clearance by microglia (Lee and Landreth, 2010; Solito and Sastre, 2012). In turn, Aβ peptide triggers microglial cell activation and induces the release of proinflammatory cytokines (Khandelwal et al., 2011). Specifically, microglia overexpress proinflammatory cytokines such as interleukin (IL)-1β, IL-6, and TNFα, and this promotes neurodegeneration in the later stages of AD (Yamamoto et al., 2007; Di Bona et al., 2008; Forlenza et al., 2009; Wang et al., 2015). Brain inflammation has been reported on several histological analysis of postmortem AD samples (McGeer and McGeer, 2010; Zotova et al., 2010). Astrocytic and microglial cell reactions are often detected surrounding senile plaques. Based on these observations, it has been suggested that glial inflammation may increase synaptic integrity loss and neuronal degeneration during AD.

Notably, the increase in glial proinflammatory cytokines has also been correlated with a significant decrease in hippocampal LTP on several models of AD (Hickman et al., 2008; Ojala et al., 2008; Chakrabarty et al., 2010; Park and Bowers, 2010; Kitazawa et al., 2011; Spooren et al., 2011; Zhao et al., 2011; Sutinen et al., 2012). Thus, inflammatory signaling may be directly related to neurocognitive dysfunction that characterizes AD. Interestingly, proinflammatory cytokines have also been involved in the improvement of neurocognitive functions. Specifically, it has been reported that lipopolysaccharides (LPS) infusion into the insular rat cortex enhances associative taste learning through the increase in glutamatergic AMPA receptors expression and trafficking at synapses (Delpech et al., 2015). Altogether, the relationship between inflammation, transmission efficiency, and neurocognitive functions is highly complex and beyond this review's scope.

Local and systemic administration of a PKR activator, the dsRNA analogous polyinosinic:polycytidylic acid (poly:IC), has been extensively used as a neuroinflammation model (White et al., 2016). Under this treatment, poly:IC activates inflammatory antiviral responses on neurons and glial cells via Toll-like receptors signaling (Carpentier et al., 2008; Botos et al., 2009; Trudler et al., 2010; Chen et al., 2019). Importantly, it has been found that repeated consecutive peripheral poly:IC injections during 7 days induce a sustained significant increase in hippocampal Aβ levels on mice up to 21 days after last administration (White et al., 2016). Even more, the Aβ increase induced by poly:IC injections strongly correlates with significantly lower performance on the contextual memory test (freezing test) (White et al., 2016). However, a functional relationship between neurotoxicity of Aβ oligomers induced by poly:IC and PKR-eIF2α ISR branch remains unexplored.

Interestingly, authors have proposed that the components of PKR-eIF2α branch participate in CNS response to Aβ-oligomers associated neuroinflammation. Specifically, blocking the TNFα function through a TNFα neutralizing monoclonal antibody (Infliximab) significantly inhibits PKR activation (normalized p-PKR) and eIF2α phosphorylation triggered by Aβ oligomers in neuronal cultures (Lourenco et al., 2013). Notably, TNFα receptor 1 (TNFR1) genetic LOF significantly inhibits hippocampal phosphorylation of PKR and eIF2α in response to local intracerebroventricular (i.c.v.) injection of $A\beta_{1-42}$ oligomers in comparison to WT mice (Lourenco et al., 2013). Concomitantly, TNFR1 genetic LOF completely prevents Aβ-oligomer-induced synapse degeneration on hippocampal neurons *in vitro*. These results suggest that Aβ-dependent activation of TNFα receptors lies upstream of PKR and p-eIF2α *in vivo*. Based on this, authors have theorized that TNFα receptors and the activation of PKR-eIF2α induced by TNFα signaling could participate in memory impairment in response to Aβ oligomers (Lourenco et al., 2013). However, further research is needed to establish a functional link and determine whether this is a neuronal-specific or neuro-glial mechanism. On the other hand, PKR downregulation prevents hippocampal LPS-induced microglial activation and cytokines production (Khandelwal et al., 2011). Specifically, PKR genetic LOF significantly inhibits microglial activation, detected by levels of ionized calcium-binding adaptor molecule 1 (Iba1) and astrocytosis, detected by glial fibrillary acidic protein (GFAP) on the hippocampus of LPS-injected mice in comparison to WT mice (Khandelwal et al., 2011). Concomitantly, PKR genetic LOF also significantly inhibits the increase in brain TNFα and IL-6 induced by LPS (Khandelwal et al., 2011). Thus, PKR participates in a positive feedback between inflammatory signaling related to pathology on the brain. It has been previously reported that LPS i.p. injections promote a significant increase in Aβ peptide on mice brain (Lee et al., 2008). Notably, PKR genetic LOF significantly inhibits LPS-induced Aβ, and BACE1 hippocampal increases protein levels compared to WT mice under the same treatment (Khandelwal et al., 2011). Altogether, these results show that the PKR-eIF2α branch participates in AD-related neuroinflammation as a mediator of Aβ neurotoxicity, where PKR activation is promoted by inflammatory signaling and also promotes an increase in cytokines. Thus, these results suggest that the PKR-eIF2α branch could be a core regulator axis of inflammatory signaling on the memory-related regions of the brain.

Synapse degeneration is another relevant step in AD's onset and progression (Briggs et al., 2017). Interestingly, synaptic dysfunction occurs first, at presymptomatic stages of AD (Briggs et al., 2017), presumably because of the presence of soluble oligomeric assemblies of Aβ protein (Clare et al., 2010; Robinson et al., 2014; Forner et al., 2017). Furthermore, cognitive dysfunction during AD is strongly correlated with synaptic loss (Forner et al., 2017). Specifically, the morphometric assessment of synapses number in AD demonstrates that synapse loss is the major indicator that correlates with cognitive impairment in patients (Robinson et al., 2014). It has been possible to detect early deficits in synaptic function and plasticity in mouse models of Alzheimer's disease (Wishart et al., 2006; Hanus and Schuman, 2013). This early synaptic function alteration suggests that structural and functional modifications at synapses may be responsible for the early cognitive decline observed

in human patients (Wishart et al., 2006; Hanus and Schuman, 2013). Synapse degeneration and dysfunction are also key pathological events in other dementias and may contribute to the cognitive decline observed during aging and aging-related neuropathologies (Cohen et al., 2013; Namjoshi and Raab-Graham, 2017). However, the role of the PKR-eIF2α branch over synaptic integrity during AD progression remains unknown. A growing body of evidence has proposed that PKR activity inhibition significantly reduces transmission efficiency defects and neurocognitive dysfunction in the context of murine models of AD *in vivo*. For example, pharmacological inhibition of PKR by i.p. injection of C16 in the context of AD model ApoE4 mouse significantly improves the long-term contextual memory compared with ApoE4 vehicle-treated mice (Segev et al., 2015). It is worth mentioning that this pharmacological inhibition of PKR was induced as a pretreatment before memory consolidation training (Segev et al., 2015). In addition, the role of PKR genetic LOF has been functionally assayed over Aβ-oligomer-induced cognitive dysfunction. Specifically, authors have found that i.c.v. injection of Aβ oligomers induces a significant decrease in freezing events on the contextual fear conditioning test in WT mouse (Lourenco et al., 2013). Notably, PKR genetic LOF completely inhibits the decrease in freezing events induced by local Aβ neurotoxicity (Lourenco et al., 2013). Altogether, these results suggest that PKR activation participates in long-term contextual memory impairment induced by the AD pathogenic hallmark mediators.

The role of PKR and eIF2α in transmission efficiency and neurocognitive functions in age-related neurodegenerative diseases has been mostly explored in the 5xFAD AD mouse model. In brief, these 5XFAD mice co-overexpress human APP and presenilin 1 (PS1) carrying five familiar Alzheimer's disease-related mutations (FAD mutations) in APP and PS1 transgenes (the Swedish mutation: K670N, M671L; the Florida mutation: I716V; the London mutation: V717I) and PS1 (M146L; L286V) driven by the Thy-1 promoter (Oakley et al., 2006). There, it has been described that the Swedish mutation increases Aβ production, and the other mutations contribute to increasing the production of $A\beta_{1-42}$. Consequently, in this mouse, five FAD mutations act together to additively increase levels of cerebral $A\beta_{1-42}$ neurotoxic peptides (Oakley et al., 2006; Ohno et al., 2006, 2007). Thus, the 5XFAD mouse model develops amyloid deposits (senile plaques) around 2 months of age, consistent with their accelerated $A\beta_{1-42}$ production compared to other AD transgenic mouse models (Eriksen and Janus, 2007). At 2 months of age, amyloid deposition begins and accumulates in the hippocampus' subiculum and specific cortex layers in this mouse model. Then, a sustained increase in $A\beta_{1-42}$ deposits fills the hippocampus and cortex of the brain of the 5XFAD mouse up to 6 months of age (Oakley et al., 2006). Concomitantly, 5xFAD mice develop comparatively earlier onset and more aggressive symptoms within amyloid mouse models (Oakley et al., 2006; Ohno et al., 2006, 2007). Related to the participation of PKR in this mouse model, the authors have assayed the effect of pharmacological inhibition of PKR or genetic deletion of PKR over hippocampal LTP and LTD (Lourenco et al., 2013; Zhang et al., 2016; Hwang et al., 2017; Tible et al., 2019). In addition, LTM has been assayed by several authors in this model under the same conditions. It is worth mentioning that authors have described a lack of NFTs in 5xFAD histological samples (Sasaguri et al., 2017). Then, conclusions about the role of PKR and eIF2α obtained on this model under pharmacological or genetic modulations are giving clues mainly about Aβ neurotoxicity as AD pathogenic hallmark. Notably, it has been established that PKR pharmacological and genetic LOF significantly modulates transmission defects and neurocognitive dysfunction during the pathological progression on 5xFAD mouse (Hwang et al., 2017; Tible et al., 2019). There, authors have recently found that genetic PKR LOF in the context of 5xFAD background achieved by double mutation significantly improves LTP on hippocampal slices compared to 5xFAD mouse (Tible et al., 2019). Milder PKR LOF by pharmacological inhibition also significantly improves transmission efficiency on the 5xFAD mice (Hwang et al., 2017). Interestingly, PKR genetic LOFs' protective effect over synaptic transmission efficiency strongly correlates with memory function maintenance during presymptomatic and symptomatic AD stages in the 5xFAD mouse (Hwang et al., 2017; Tible et al., 2019).

Altogether, the evidence mentioned above reveals an extended role of PKR in AD pathology, where this kinase participates in the demise of neuronal integrity and dysfunctional synaptic transmission efficiency that leads to neurocognitive impairment. This extended role points to interesting pathogenic mechanisms that could be modulated beyond the context of AD.

The Possible Role of PKR in Huntington's Disease

Huntington's disease is a progressive, debilitating, and fatal neurological disorder. Its main symptoms include involuntary or uncontrollable dance-like movements (chorea movements), cognitive and memory impairment, and other psychiatric changes (Ha and Fung, 2012). HD is inherited in an autosomal dominant manner (Cepeda et al., 2007). The mutated gene contains an expansion in the number of CAG repeats in the huntingtin gene (*HTT*) on chromosome 4 (Walker, 2007). HD is typically a late-onset disease, although juvenile variants occur (Cepeda et al., 2007).

Neuropathological changes in HD are characterized by a prominent loss and atrophy of medium spiny projection neurons (MSNs) in the striatum (caudate and putamen) (Vonsattel and DiFiglia, 1998; Gutekunst et al., 2002; Rubinsztein, 2003). Besides, abnormalities in neurons in the cerebral cortex (Vonsattel et al., 1985), substantia nigra, and thalamus, which input to striatal projection neurons (SPN), have been described (Reiner and Deng, 2018). The output tracts to the globus pallidus and substantia nigra from SPN also show abnormalities (Reiner and Deng, 2018). Neuronal cell loss is also evident in other brain regions, including the hippocampus (Vonsattel et al., 1985; Spargo et al., 1993; Utal et al., 1998). The striatal neuron loss is non-prominent in premanifest HD (Vonsattel et al., 1985; Albin et al., 1991; Vonsattel and DiFiglia, 1998), and the early symptoms seem to be driven by striatal neuron circuit connectivity loss and dysfunction.

The striatum, which is highly affected in the disease, is the basal ganglia's main input nucleus and transforms thalamic and cortical inputs into two output streams, called the direct and the indirect pathways. Although these two pathways' actions are complex, the basal ganglia circuit's simplified model proposes that the direct pathway facilitates directed movements, and the indirect pathway terminates or suppresses movements (Smith et al., 1998). Imbalances in the two pathways' activity are hypothesized to underlie numerous movement disorders, including the ones observed in HD (Galvan and Wichmann, 2007). Medium spiny projection neurons (MSNs) of the striatum, which are GABAergic inhibitory neurons (Lighthall and Kitai, 1983), determine the direct and indirect pathways' activation.

Another pathological landmark of HD is the presence of aggregated forms of mutant huntingtin protein (mHtt) in neurons. These aggregates include intranuclear and cytoplasmic inclusions, as well as microaggregates. The contribution of these inclusions in neuronal loss observed in HD has not been completely elucidated, but essential processes affected in the disease have been identified (Walker, 2007). The expansion CAG repeats can be bidirectionally transcribed (Cho et al., 2005; Moseley et al., 2006), and several of these mutated genes have been shown to produce aberrant proteins, which are synthesized from multiple reading frames in the absence of AUG in a process called repeat-associated non-AUG (RAN) translation (Zu et al., 2011). One of the disease processes is related to the chronic production of misfolded mHtt, which overwhelms the proteostatic machinery (chaperons, proteasome, and autophagy), leading to a global collapse of the proteostasis network (Soares et al., 2019). Alterations in cell–cell interactions have also been described, including axonal transport and delivery of trophic factors (Soares et al., 2019).

The discovery of the HD gene in 1993 led to the development of genetic models of the disease, which provided material for studies in the earliest stages of disease pathogenesis and mechanistic studies (Cepeda et al., 2010). We address recent findings concerning PKR involvement in HD and its contribution to synaptic transmission efficiency and synaptic integrity. The activation of PKR has been found in tissues derived from HD patients' postmortem samples. Increased p-PKR levels were detected in the hippocampal tissue of patients with HD, suggesting an association of PKR activation with extrastriatal degeneration (Bando et al., 2005). This result is particularly interesting because the hippocampus's role in HD pathology in the last years has gained importance (Harris et al., 2019). HD patients showed significant deficits in hippocampal-dependent spatial cognition. Moreover, a correlation was found between the CAG repetitions and the severity of the symptoms, suggesting that deficits relate to HD's disease process.

Huntington's disease mouse models have shown alterations in striatal and cortical synaptic transmission. Specifically, the R6/2 mouse model, which carries a fragment of the HD gene (exon 1) and contains 150 CAG repeats (Mangiarini et al., 1996), exhibits a consistent decrease in the frequency of spontaneous excitatory postsynaptic currents (EPSCs) (Cepeda et al., 2003) in striatal MSNs and an increase in spontaneous inhibitory postsynaptic currents (IPSCs) in MSNs (Cepeda et al., 2004, 2010). The increased inhibition of GABAergic neurons would reduce striatal output along the indirect pathway. This may lead to disinhibition of the external globus pallidus and could explain some HD symptoms. Another study performed an electrophysiological analysis of striatal interneurons in the heterozygous Q175 mouse model of HD that contains human *HTT* allele with the expanded CAG repeat (∼179 repeats) (Holley et al., 2019). They found increased excitability of the fast-spiking interneurons (FSIs) and low-threshold spiking (LTS) (Holley et al., 2019). The increase in excitability of FSIs lead to increase in IPSC of MSNs (Koos and Tepper, 1999); interestingly, FSIs are GABAergic cells that provide inhibitory inputs to spiny neuron and degenerate in HD (Gittis et al., 2010). In contrast, LTS neurons, associated with a modulatory role on excitatory synaptic input, are spared from degeneration (Ferrante et al., 1985). This evidence suggests that the increase in the inhibitory activity of MSNs is a common symptom of HD pathology, and a possible role of PKR in mediating the inhibitory activity of GABAergic inhibition on the cortex and striatal MSNs can be considered.

Presymptomatic Huntington's disease patients often exhibit cognitive deficits before the onset of typical symptoms (Lawrence et al., 1998; Brandt et al., 2002). Learning and memory are believed to depend on changes in synaptic efficacy in certain key brain regions, including the hippocampus and other regions. LTP and LTD, the most studied form of synaptic plasticity, have been largely described to be altered on several HD models (Lione et al., 1999; Lynch et al., 2007; Simmons et al., 2009; Brooks et al., 2012). Indeed, the R6/2 mice displayed age-related alterations in synaptic plasticity at CA1 and dentate granule cell synapses and impaired spatial cognitive performance in the Morris water maze (Murphy et al., 2000). Supporting the premature occurrence of cognitive impairment in HD, LTP has been shown to be reduced in hippocampal slices from presymptomatic Hdh(Q92) and Hdh(Q111) knock-in mice (Lynch et al., 2007). Indeed, the LTP impairment in an early-onset HD mouse model was related to the reduced ability of excitatory synapses in cortical areas to fully respond under low stimulus rates (Usdin et al., 1999). Mouse HD models expressing full-length human mHTT (YAC46 and YAC72) (Hodgson et al., 1999) showed early electrophysiological abnormalities and LTP impairment before any noticeable behavioral abnormalities and any evidence of neurodegeneration or aggregate formation (Hodgson et al., 1999). Moreover, R6/1 HD mice, carrying 115 CAG repeats (Mangiarini et al., 1996), showed reduced hippocampal LTP (Milnerwood et al., 2006). Defects in memory consolidation and cognitive behavior have also been demonstrated in a transgenic HD monkey, including progressive impairment in motor functions and cognitive decline, recognition memory, and spatial memory (Chan et al., 2014). As mentioned, hippocampus-associated behavioral task is impaired in humans affected by the disease (Harris et al., 2019). Hippocampal cell loss, synaptic plasticity abnormalities, and memory impairment are initial events on HD's pathology. PKR and eIF2α phosphorylation participates in memory consolidation in a bidirectional manner on CA1 hippocampal slices of eIF2α knock-in mice and recombinant PKR-expressing mice (Costa-Mattioli et al., 2007; Jiang et al., 2010). This information strongly suggests that increased PKR levels in HD brains can

be partially involved in the impaired LTP and aberrant synaptic plasticity on the hippocampus through eIF2α phosphorylation. An unsolved question in the role of PKR in physiological or pathological conditions is the stimulus that is activating the kinase. In this context, the binding and activation of PKR to RNAs containing another triplet repeat, the CUG sequence, which is the genetic basis of myotonic dystrophy, have been described (Tian et al., 2000).

Protein kinase R also binds preferentially mutant *huntingtin* RNA transcripts containing CAG repeats (Peel et al., 2001), raising the possibility that, in the HD pathological context, PKR activation described in HD mouse models and postmortem samples could be mediated by the binding of expansion of trinucleotide repeat regions. The RAN translation, a pathological phenomenon described in HD, has recently been described as a process regulated by PKR and its phosphorylation (Zu et al., 2020). Genetic deletion of PKR or the expression of a dominant-negative form of PKR inhibits RAN translation. Moreover, metformin, a drug widely used for treating type 2 diabetes and recently tested in neurodegenerative disorders (Gantois et al., 2019; Martinez et al., 2020), showed an inhibiting effect on RAN translation and PKR activation (Zu et al., 2020). It remains to be elucidated how metformin inhibits PKR and its possible effects in neurodegenerative diseases. We detail studies on the role of PKR on HD pathogeny in **Table 2**.

Overall, the evidence shows a role of PKR in HD, either as a synaptic transmission modulator or controlling translation, which reinforces the potential therapeutic role of this kinase in HD.

A Link Between PD and PKR

Parkinson's disease is one of the most common age-related brain disorders. PD is defined primarily as a movement disorder, with the typical symptoms being resting tremor, rigidity, bradykinesia, and postural instability. PD is pathologically characterized by degeneration of nigrostriatal dopaminergic neurons and abnormal aggregates of α-synuclein protein, called Lewy bodies, in the surviving neurons (Kalia and Lang, 2015). The presence of these abnormal aggregates of α-synuclein protein is called Lewy pathology. PD patients also display non-motor symptoms, such as cognitive impairment (Muslimovic et al., 2005), recognition memory deficits (Aarsland et al., 2017), and impaired learning (Foltynie et al., 2004; Muslimovic et al., 2005; Aarsland et al., 2009; Elgh et al., 2009). Remarkably, PD and HD patients showed similarities in long-term memory impairment (Scholz et al., 1988); this indicates that similar mechanisms modulate the memory deficits in both neurodegenerative diseases.

In addition to the classic nigrostriatal α-synuclein misfolding and dopaminergic neuronal loss, several mechanisms contribute to the brain changes described in PD, including synaptic dysfunction and loss, mitochondrial dysfunction, retrograde signaling impairment, and altered neurotransmitter activity, among others (Aarsland et al., 2017). Compared with the motor symptoms, little is known about the mechanisms underlying cognitive decline in PD, and several key questions remain unresolved. The evidence from postmortem studies indicates that Lewy body pathology in limbic and cortical areas is the main pathological hallmark of PD's cognitive impairment. The model proposed is that α-synuclein pathology spreads from areas in the lower brainstem or olfactory bulb (or extra-CNS territories like the gut or other areas innervated by the vagus nucleus) to the midbrain, forebrain, and limbic structures, and, finally, neocortical regions (Braak et al., 2003; Sauerbier et al., 2016).

The contribution of PKR in PD has been poorly explored. However, an interesting set of evidence suggests that PKR may play a role in PD pathogenesis. Specifically, postmortem biopsies from PD patients show a significant increase in activated PKR in hippocampal neurons compared to age-matched controls. Furthermore, activated PKR was also significantly increased on nuclei from hippocampal lysates from the same patients (Bando et al., 2005). Interestingly, murine models of PD reproduce this significant increase in activated PKR at the hippocampus (Deguil et al., 2010). Specifically, parkinsonism in mice induced by intraperitoneal injection of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) correlates with a significant increase in activated PKR at the hippocampus (Deguil et al., 2010). Interestingly, a preclinical treatment against PD significantly reduces activated PKR at the hippocampus and improves spatial memory on the Morris water maze paradigm (Deguil et al., 2010). Furthermore, additional strong evidence can be considered to involve PKR as kinase participating in PD. It has been described that the extent of amyloid

TABLE 2 | Reports of protein kinase R (PKR) and age-related neurodegenerative diseases.

AD model (*in vitro*/*in vivo*)	Tissue	Finding	References
HD patients	Brain tissue from HD patients and htt YAC mice	PKR preferentially binds to mutant huntingtin RNA transcripts. p-PKR immunolocalizes with degenerated areas in HD model	Peel et al. (2001)
HD patients	Hippocampal tissue from HD patients	p-PKR is significantly higher and forming aggregates in the nuclei of the CA1, CA2, and CA3 hippocampal regions	Bando et al. (2005)
PD patients	Hippocampal tissue from PD patients	p-PKR is significantly higher CA2 and CA3 hippocampal regions	Bando et al. (2005)
C57Bl/6 mouse treated with MPTP (parkinsonism)	Striatum, midbrain containing the substantia nigra, hippocampus, frontal cortex samples	PKR levels are increased in the striatum and hippocampal tissue and eIF2α phosphorylation is increase in the striatum in response to MPTP	Deguil et al. (2010)

YAC, yeast artificial chromosome; p-PKR, phosphorylated PKR; CA, hippocampal cornu ammonis; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; Ser, serine; OLN, oligodendroglial permanent cell line; MSA, multiple system atrophy; SH-SY5Y ASYN, alpha-synuclein overexpressing SH-SY5Y neurons; OLN-AS7, oligodendroglial cells AS7.

plaque pathology observed in PD is a significant contributor to the cognitive decline observed in the disease (Compta et al., 2011; Aarsland et al., 2017), suggesting a possible common mechanism driving cognitive impairment observed in AD and PD. Another common aspect can be found between AD and PD. Synaptic dysfunction followed by synaptic loss is likely to be the early and key events in AD (Terry et al., 1991). Emerging evidence has shown synaptic alterations in PD patients (Matuskey et al., 2020). Using *in vivo* high-resolution positron emission tomographic imaging and postmortem autoradiography derived from patients' samples, the authors showed decreased levels of synaptic vesicle glycoprotein 2A (SV2A) in PD patients (Matuskey et al., 2020). Notably, most of the genes implicated in PD (e.g., *SNCA*, *LRRK2*, *DJ-1*, *PINK1*, and *PRKN*) have a critical role in synaptic function, and knockout mouse for each of the genes has demonstrated disruption of synaptic plasticity and neurotransmitter function (Plowey and Chu, 2011; Belluzzi et al., 2012; Abeliovich and Gitler, 2016).

Cognitive impairment is also detected in mouse models of the disease, in which partial lesions of dopaminergic and noradrenergic inputs to the striatum and hippocampus are induced with 6-hydroxydopamine. This mouse displayed reduced long-term novel object recognition and decreased LTP, predominantly in the dentate gyrus (Bonito-Oliva et al., 2014). Concomitantly, the application of extracellular α-syn oligomers in rat hippocampal brain slices impairs LTP (Martin et al., 2012). On the other hand, overexpression of α-syn induced impairment in short-term memory and spatial learning in rats principally due to α-syn accumulation primarily in the CA2 region. Thus, processes controlled by PKR are also altered in PD mouse models. We detail studies on the role of PKR on PD pathogeny in **Table 2**.

The phosphorylation of eIF2α in the substantia nigra has been described in the postmortem tissue from PD cases, and in the same study, the activation (phosphorylation) of another ISR kinase, the ER stress sensor PERK (Hoozemans et al., 2007), was also detected. On the other hand, another study showed strong induction of phosphorylated PKR in hippocampal neurons (Bando et al., 2005), but the phosphorylation of eIF2α was not analyzed. It is possible to consider that PKR could be mediating the phosphorylation of eIF2α in other regions, i.e., substantia nigra, and driving cell death events observed in the disease.

The stimulus activating PKR in PD is still unknown. In this context, an association between viral infections mediated by herpes simplex virus (HSV) and influenza virus A and increased PD incidence has been found (Olsen et al., 2018). It has been proposed that HSV influenza virus A infections may lead to PD pathology. Viral transcripts, possibly detected by PKR, could be part of the mechanism underlying the association proposed.

CONCLUDING REMARKS

The role of PKR seems to be relevant in physiological and pathological conditions but with completely different consequences. PKR could be considered as a regulator of synaptic efficiency transmission and, consequently, a neurocognitive regulator. The identity of the stimulus that activates the kinase in normal conditions remains to be determined. In pathological conditions, PKR seems to be a cell death regulator, resulting in an interesting candidate for therapeutics strategies. The abnormalities observed in aged-related neurodegenerative diseases could have a common regulator, PKR, mediating apoptotic signals, synaptic transmission deficiencies, and neurocognitive dysfunction.

AUTHOR CONTRIBUTIONS

NM, FG, and SM contributed equally to the final manuscript. All authors contributed to the article and approved the submitted version.

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