PROTEIN MISFOLDING AND SPREADING OF PATHOLOGY IN NEURODEGENERATIVE DISEASES

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PROTEIN MISFOLDING AND SPREADING OF PATHOLOGY IN NEURODEGENERATIVE DISEASES

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EDITORIAL
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Editorial: Protein Misfolding and Spreading Pathology in Neurodegenerative Diseases

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Editorial on the Research Topic

Protein Misfolding and Spreading Pathology in Neurodegenerative Diseases

A common pathological hallmark among different neurodegenerative diseases is the accumulation of aggregated proteins that might cause cellular dysfunction and, eventually, lead to cell death. Amyloid-beta, Tau, alpha-synuclein, TDP-43, or the prion protein, are just a few examples of proteins that can aggregate and contribute to the pathogenesis of neurodegenerative diseases with diverse clinical manifestations (Alzheimer's disease, frontotemporal lobar degeneration, Pick's disease, Parkinson's disease, Lewy body dementia, multiple system atrophy, amyotrophic lateral sclerosis among the most common). Emerging evidence suggests that the progression of symptoms in patients affected by such disorders correlates with the spreading of pathology through the brain, but the molecular mechanisms underlying aggregation and propagation of protein aggregates are still obscure. This Research Topic focuses on the structural and molecular characteristics of aggregation-prone proteins and resumes new aspects of pathology spreading. A series of 10 articles provides an exciting up-to-date overview of core biological features of prion and prion-like neurodegenerative disorders.

Protein aggregation is a common feature among numerous neurodegenerative diseases, and is thought to culminate with detrimental effects for the cells where the proteins accumulate. Despite the commonalities of protein aggregation and cell dysfunction, the pathobiological bases of these diseases may differ. For example, the two characteristic protein deposits in Alzheimer's disease (AD) are the extracellular senile plaques, whose main constituent are amyloid-β (Aβ) fibrils, and intraneuronal neurofibrillary tangles (NFT), composed of hyperphosphorylated Tau protein (Brion et al., 1985, 1986; Kirschner et al., 1986; Sisodia et al., 1990). Similarly, fibrillary alpha-synuclein (aSyn) is the main protein component of Lewy bodies (LB) which are the key neuropathological hallmarks of Parkinson's disease (PD) and Lewy body dementia (DLB) (Spillantini et al., 1997), and can be found within glial cytoplasmic inclusions in the brain of patients affected by multiple system atrophy (MSA) (Wakabayashi et al., 1998). Finally, TAR-DNA binding protein 43 (TDP-43)- or tau-positive inclusions can be found in the brain or spinal cord in, e.g., frontotemporal lobar degeneration (FTLD) or amyotrophic lateral sclerosis (ALS) (Arai et al., 2006; Hasegawa et al., 2008; Kabashi et al., 2008; Sreedharan et al., 2008; Da Cruz and Cleveland, 2011).

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Recently, Aβ, Tau, aSyn, and TDP-43 were proposed to display several properties similar to those exhibited by the prion protein (PrP). In particular, their physiological conformation can shift to a self-replicating pathological strain which can spread from a neuron to another across different brain regions.

Intriguingly, there seems to be a cross-talk between PD and AD, as aSyn pathology is frequent in AD brains, and Tau accumulation is common in PD with dementia (Lippa et al., 1998; Hamilton, 2000; Irwin et al., 2013). In a similar vein, Aβ plaques are a common feature of DLB, where aSyn aggregates are the predominant feature (McCann et al., 2014).

Remarkably, the prion-like properties of Aβ and aSyn demonstrated in experimental models, have been corroborated by studies that have suggested that human transmission of cerebral amyloid, and Aβ pathology contaminated material, occurs upon certain clinical procedures (Purro et al., 2018; Banerjee et al., 2019; Spinazzi et al., 2019). Furthermore, host to graft propagation of LB pathology has been suggested to have occurred in patients who received fetal neuron transplants over one decade prior to death and neuropathological examination (Kordower et al., 2008a,b; Chu and Kordower, 2010; Li et al., 2010; Kurowska et al., 2011). Whether these clinical observations with Aβ and aSyn pathology represent authentic prion spreading of pathology remains controversial, and this issue calls for caution due to the tremendous implications it might have.

Therefore, using prion-like terminology for all neurodegenerative diseases requires a deeper understanding of the molecular mechanisms involved. There are currently no disease-modifying treatments available for these diseases, and therefore it is desirable to design strategies that could directly target the aggregation of these proteins or modulate their ability to propagate from one brain region to another. Ongoing clinical trials with immunotherapy are focused on clearing the aggregates when they are in the extracellular space, and can be viewed as a strategy to limit prion-like spreading of the pathology (Gallardo and Holtzman, 2017; Sigurdsson, 2018; Panza et al., 2019).

In this Research Topic of Frontiers in Molecular Neuroscience, Terry and Wadsworth review the importance of prion structure for pathogenicity. They highlight and discuss new findings differentiating the architecture of authentic infectious lethal prions from that of PrP amyloidosis as the pillar for a critical re-evaluation of the structure of other prion-like proteins associated with other neurodegenerative diseases (Terry and Wadsworth). In the second paper, Lim reviews the analogy between prion protein and other aggregation-prone proteins, such as aSyn, Aβ, and Tau, focusing on the likelihood of these proteins to cross-seed and to adopt different conformations, and on the importance of understanding the molecular basis that drive the different conformations. Next, Vasili et al. discuss overlapping aspects between PD and AD, and elaborate on the mechanisms involved in the transfer/spreading of aSyn and Tau. They provide a thorough overview of the current cell and animal models to assess the mechanisms of spreading of pathology (Vasili et al.). On the same line, Friesen and Meyer-Luehmann summarize the literature on Aβ seeding in mouse models of AD, and their application for the study of cerebral amyloidosis and associated pathologies.

Prasad et al. present a comprehensive review about the role of TDP-43 in ALS. They discuss the imbalances on TDP-43 homeostasis, implicated in miss-regulation of RNA and cytotoxicity mechanisms. McAlary et al. provide a detailed review of the current evidence on idiopathic ALS as a prion-like disorder. They focus on key proteins and genes involved in the disease (TDP-43, SOD1, FUS, and C9orf72), and discuss the current evidence from biophysical to *in vivo* studies (McAlary et al.).

Chen et al. review the consequences of gut inflammation on PD, highlighting how it may initiate and promote enteric aSyn pathology deposition and spreading into the brain.

The putative contribution of calcium channels to PD etiopathogenesis and progression, putting the accent on how they might contribute to aSyn aggregation and secretion in synucleinopathies, is addressed and discussed in the article by Leandrou et al..

New translational insights on neurodegenerative disorders characterized by prion-like protein spreading are provided by Singh et al., which describe that increased levels of SIRT2 correlate with circulating aSyn in early PD. They propose SIRT2 as potential biomarker for early detection of the disease (Singh et al.). Finally, Nam et al. discuss the efficacy of ALWPs, a combination of oriental herbal medicines with proven efficacy in diabetes mellitus, immune modulation, and owning both neurotrophic and anti-inflammatory action in decreasing Aβ plaque load, as well as Tau hyperphosphorylation in the cortex and hippocampus of the 5XFAD mouse model of AD.

In short, the articles in this Research Topic provide new up-to-date insights into our understanding of protein aggregation and spreading of pathology in prion and prion-like neurodegenerative disorders. Addressing a wide variety of topics, they introduce thought-provoking concepts and clues about the relevance of laboratory findings to the clinical arena. Hopefully, a greater understanding of the prion-like propagation of protein aggregate pathology will lead to novel therapeutic strategies that slow disease progression.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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REVIEW
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Molecular Mechanisms of TDP-43 Misfolding and Pathology in Amyotrophic Lateral Sclerosis

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TAR DNA binding protein 43 (TDP-43) is a versatile RNA/DNA binding protein involved in RNA-related metabolism. Hyper-phosphorylated and ubiquitinated TDP-43 deposits act as inclusion bodies in the brain and spinal cord of patients with the motor neuron diseases: amyotrophic lateral sclerosis (ALS) and frontotemporal lobar degeneration (FTLD). While the majority of ALS cases (90–95%) are sporadic (sALS), among familial ALS cases 5–10% involve the inheritance of mutations in the *TARDBP* gene and the remaining (90–95%) are due to mutations in other genes such as: *C9ORF72, SOD1, FUS,* and *NEK1* etc. Strikingly however, the majority of sporadic ALS patients (up to 97%) also contain the TDP-43 protein deposited in the neuronal inclusions, which suggests of its pivotal role in the ALS pathology. Thus, unraveling the molecular mechanisms of the TDP-43 pathology seems central to the ALS therapeutics, hence, we comprehensively review the current understanding of the TDP-43's pathology in ALS. We discuss the roles of TDP-43's mutations, its cytoplasmic mis-localization and aberrant post-translational modifications in ALS. Also, we evaluate TDP-43's amyloid-like *in vitro* aggregation, its physiological vs. pathological oligomerization *in vivo*, liquid-liquid phase separation (LLPS), and potential prion-like propagation propensity of the TDP-43 inclusions. Finally, we describe the various evolving TDP-43-induced toxicity mechanisms, such as the impairment of endocytosis and mitotoxicity etc. and also discuss the emerging strategies toward TDP-43 disaggregation and ALS therapeutics.

Keywords: amyotrophic lateral sclerosis (ALS), TDP-43, mitotoxicity, liquid-liquid phase separation (LLPS), endocytosis, frontotemporal lobar degeneration (FTLD), prion, ALS therapeutics

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INTRODUCTION

TAR DNA binding protein-43 (TDP-43) was identified in 1995 as a repressor protein associated with HIV-1 transcription, which binds to the trans-active response element DNA sequence of the viral genome and is critical for the regulation of the viral gene expression (Ou et al., 1995). In 2001, TDP-43 was also reported to be involved in RNA splicing of cystic fibrosis transmembrane conductance regulator (CFTR) exons (Buratti and Baralle, 2001). It is a highly conserved and ubiquitously expressed RNA/DNA-binding protein which belongs to the large heterogeneous nuclear ribonucleoprotein (hnRNP) family, where the members of the family show ability to bind to RNA with considerable sequence-specificity achieved through the presence of one or more, highly conserved, RNA recognition motifs (RRMs) (Sephton et al., 2010, 2012; Geuens et al., 2016). TDP-43 has since then been also shown to regulate mRNAs involved in the development of neurons and embryos (Polymenidou et al., 2011; Sephton et al., 2011; Tollervey et al., 2011).

In 2006, TDP-43 was identified as a key component of the insoluble and ubiquitinated inclusions in the brains of patients suffering from amyotrophic lateral sclerosis (ALS) and frontotemporal lobar degeneration (FTLD or FTLD-TDP) diseases (Arai et al., 2006; Neumann et al., 2006). Other diseases involving TDP-43 pathological developments are primary lateral sclerosis and progressive muscular atrophy, and together these four diseases are known as TDP-proteinopathies (**Figure 1**) (Dugger and Dickson, 2017). Both ALS and FTLD-TDP are late-onset neurodegenerative disorders with several common clinical, neuropathological and genetic features, however, they affect distinct regions of the nervous system (Neumann et al., 2006; Spires-Jones et al., 2017; Tan et al., 2017). Strikingly, ∼97% of the ALS cases and ∼45% of all FTLD cases (called: FTLD-TDP) involve TDP-43's aggregation (Ling et al., 2013; Tan et al., 2017).

ALS is a fatal neurodegenerative disease characterized by progressive degeneration of both the upper and lower motor neurons, which display cytoplasmic inclusions (Rowland and Shneider, 2001; Kiernan et al., 2011). The degradation of the upper motor neurons leads to spasticity and hyper-excitability, while the death of the lower motor neurons causes weakness, fasciculations and eventually muscular atrophy followed by progressive paralysis. The earliest symptoms include cramping and stiffness of muscles leading to muscle weakness affecting the arms and legs. The patients display slurred speech and difficulty in chewing or swallowing (Mitchell and Borasio, 2007; Rothstein, 2009). Finally, death of the patient occurs due to complications involving respiratory failure and pneumonia within about 3–5 years after the onset of disease symptoms. The average age of onset of the disease is ∼50 years (Logroscino et al., 2007; Chio et al., 2009). The disease has a prevalence of ∼5 individuals out of 100,000 each year worldwide. While the majority of the ALS cases (∼90–95%) are considered as sporadic (sALS) with unknown cause, ∼5–10% cases involve Mendelian pattern of inheritance of familial gene mutations and are known as familial ALS (fALS) (Renton et al., 2014; Taylor et al., 2016).

In addition to the TDP-43 encoding *TARDBP* gene, mutations in several other genes have also been linked with ALS such as: *SOD1* (Superoxide dismutase 1) (Rosen, 1993; Kunst et al., 1997), *FUS* (Fused in sarcoma) (Kwiatkowski et al., 2009; Vance et al., 2009), *C9ORF72* (Hexanucleotide repeat expansion in C9ORF72) (Dejesus-Hernandez et al., 2011; Renton et al., 2011), *ATXN2* (Ataxin-2) (Elden et al., 2010; Ross et al., 2011), *OPTN* (Optineurin) (Maruyama et al., 2010), *VCP* (Valosin-containing protein) (Johnson et al., 2010; Koppers et al., 2012), *PFN1* (Profilin 1) (Wu et al., 2012; Tanaka et al., 2016), *UBQLN2* and *UBQLN4* (Ubiquilin 2 and Ubiquilin 4) (Deng et al., 2011; Edens et al., 2017), *NEK1* (NIMA-like kinase 1) (Brenner et al., 2016), *MATR3* (Matrin 3) (Johnson et al., 2014b), *CHCHD10* (Coiled-coil-helix-coiled-coil-helix domain containing 10) (Woo et al., 2017), *SETX* (Senataxin) (Hirano et al., 2011), *TBK1* (TANK-binding kinase 1) (Oakes et al., 2017), and *KIF5A* (Kinesin heavy chain isoform 5A) (Nicolas et al., 2018) etc. The corresponding proteins with mutations in these genes are involved in the pathogenesis of ALS by various mechanisms.

FTLD is a progressive neuronal disease associated with the degeneration of the frontal and temporal lobes with neuronal intranuclear and cytoplasmic inclusions (Mackenzie et al., 2007; Dugger and Dickson, 2017). Unlike ALS, which rarely involves dementia, FTLD is the second most prevalent cause of dementia after the Alzheimer's disease, in individuals <65 years of age, with an estimated prevalence of ∼15–22 per 100,000 (Van Langenhove et al., 2012; Onyike and Diehl-Schmid, 2013). It is characterized by significant personality and behavioral changes, as well as gradual impairment of the language skills. Strikingly, TDP-43 inclusions in FTLD-TDP are also hyper-phosphorylated, ubiquitinated and N-terminally truncated as observed in ALS (Neumann et al., 2007a; Hasegawa et al., 2008; Igaz et al., 2008). Also, mutations in the *TARDBP* gene can lead to ALS as well as the FTLD-TDP disease.

STRUCTURE OF TDP-43

The TDP-43 protein contains 414 amino acids and the encoding gene *TARDBP* is located on the chromosome number 1. It comprises of an N-terminal region (aa 1–102) with a nuclear localization signal (NLS, aa 82–98), two RNA recognition motifs: RRM1 (aa 104–176) and RRM2 (aa 192–262), a nuclear export signal (NES, aa 239–250), a C-terminal region (aa 274–414) which encompasses a prion-like glutamine/asparagine-rich (Q/N) domain (aa 345–366) and a glycine-rich region (aa 366–414) (**Figure 2**) (Cohen et al., 2011; Lukavsky et al., 2013; Kuo et al., 2014; Qin et al., 2014; Jiang et al., 2016; Mompeán et al., 2016b). TDP-43 is predominantly localized in the nucleus but also shuttles to the cytoplasm for some of its functions (Ayala et al., 2008). In ALS, there is an increase in the cytoplasmic TDP-43 concentration leading to cytoplasmic inclusion formation (Neumann et al., 2006; Winton et al., 2008a). Mitochondrial localization of TDP-43 depends on internal motifs M1 (aa 35–41), M3 (aa 146–150), and M5 (aa 294–300), which consists of continuous stretch of hydrophobic amino acids (Wang et al., 2016). Owing to its poor *in vitro* solubility and high aggregation propensity, the complete structure of TDP-43 has remained elusive thus far. Several groups, however, have determined high resolution structures of some of its domains (**Figure 2**) and its holistic structure is now evolving.

N-Terminal Domain (NTD)

Accumulating evidence suggests that TDP-43 is natively dimeric or at least exists in a monomer-dimer equilibrium under normal physiological conditions (Shiina et al., 2010; Zhang Y. J. et al., 2013). TDP-43's dimerization apparently occurs through interactions of the N-terminal residues and while several reports suggest that TDP-43 N-terminal domain's (NTD) dimerization is necessary for its physiological functions like RNA splicing. Others have also argued that the NTD's dimerization may, in fact, be involved in its aggregation (Shiina et al., 2010; Zhang Y. J. et al., 2013; Afroz et al., 2017). Notably, TDP-43's N-terminal region exhibits an ubiquitin-like fold, which consists of one α-helix and six β-strands in the β1-β2-α1-β3-β4-β5-β6 arrangement (**Figure 2**) (Qin et al., 2014; Mompeán et al., 2016b). The homodimerization of TDP-43 molecules occurs by head-to-head interaction of the two NTDs while the RRM2 domains are extended outwards (Wang Y. T. et al., 2013). In

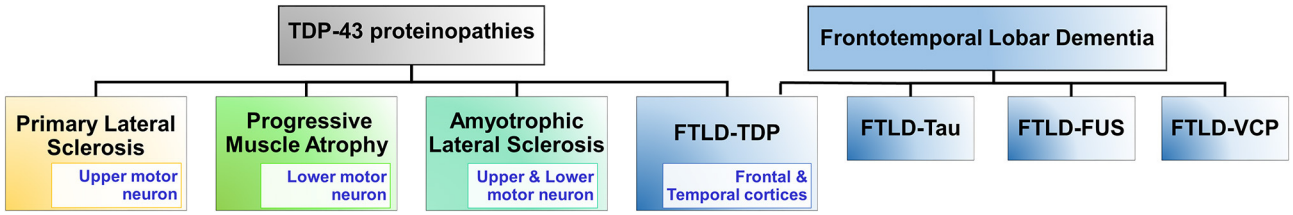

FIGURE 1 | TDP-43 proteinopathies. TDP-43 proteinopathies refer to the diseases where TDP-43 is implicated and it includes: amyotrophic lateral sclerosis (ALS), frontotemporal lobar degeneration (FTLD-TDP), primary lateral sclerosis, and progressive muscular atrophy. FTLD is a group of disorders principally of the frontal temporal lobes of the brain causing dementia. Other forms of FTLD disorders are FTLD-Tau, FTLD-FUS, and FTLD-VCP. FTLD-Tau is associated with mutations in the *MAPT* gene which encodes microtubule associated protein, Tau. Tau's misfolding and aggregation lead to loss of microtubule-binding function and formation of neuronal and glial inclusions (Irwin et al., 2015). FTLD-FUS is associated with mutations in the RNA-binding protein FUS, which results in disruption of its nuclear localization and leads to its accumulation into inclusion bodies (Mackenzie et al., 2011). FTLD-VCP is associated with mutations in the valosin-containing protein (VCP). FTLD-VCP manifests ubiquitin and TDP-43-positive neuronal intranuclear and cytoplasmic inclusions. FUS, fused in sarcoma; TDP-43, TAR DNA binding protein 43; VCP, valosin containing protein.

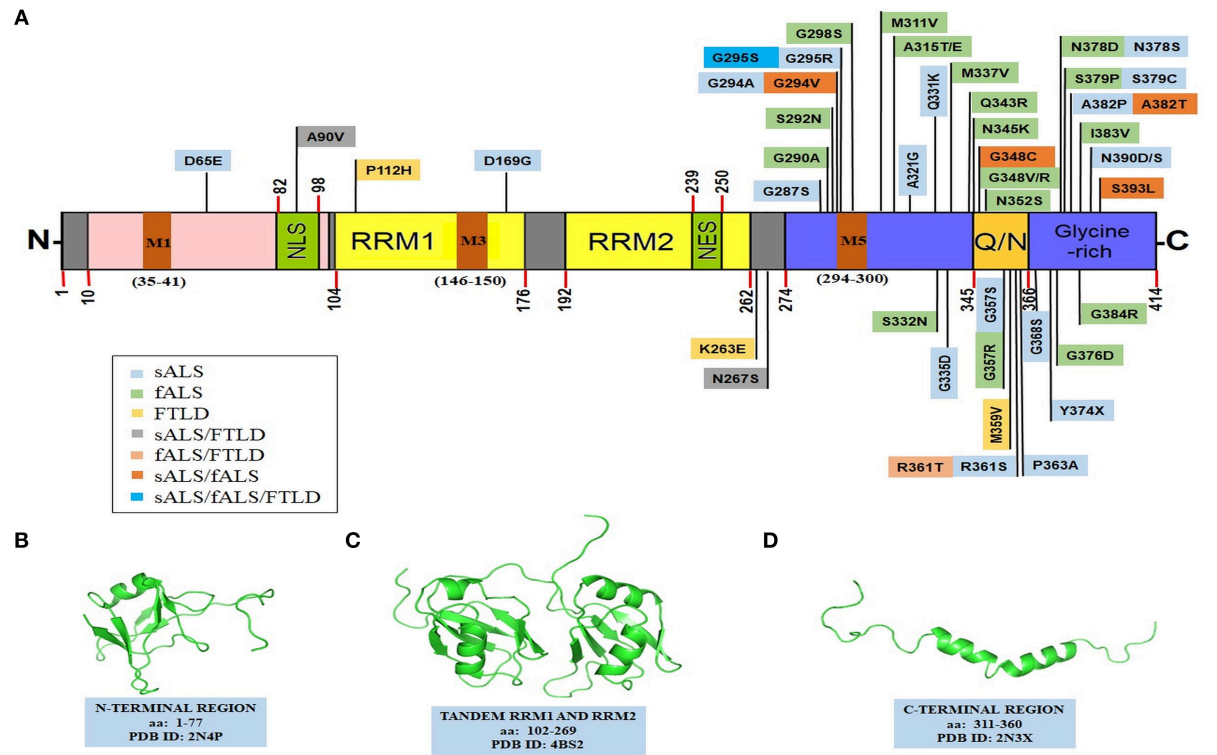

FIGURE 2 | Structural features of TDP-43. **(A)** TDP-43's domain organization depicting ALS and FTLD-linked mutations. TDP-43 comprises of an NTD domain, two RRM domains, a nuclear export signal (NES), a nuclear localization signal (NLS), a prion-like disordered C-terminal domain (with glutamine/asparagine-rich (Q/N) and Glycine-rich regions) and mitochondrial localization motifs (M1−35–41; M3−146–150; M5−294–300). Sporadic mutations and familial gene mutations generating amino acid substitutions in TDP-43 are classified. Several TDP-43 mutations overlap between ALS and FTLD as well as between sALS and fALS (Baumer et al., 2009; Xiong et al., 2010; Fujita et al., 2011; Janssens et al., 2011; Budini et al., 2012; Chiang et al., 2012; Cruts et al., 2012; Lattante et al., 2013; Moreno et al., 2015). PDB structures of: **(B)** N-terminal region (PDB id-2N4P); **(C)** a tandem RRM1 and RRM2 segment (PDB id-4BS2); **(D)** a C-terminal region (aa: 311–360) (PDB id-2N3X). Structures in the **(B–D)**, have been adapted with permissions respectively from: John Wiley and Sons (Mompeán et al., 2016b); Springer Nature (Lukavsky et al., 2013); and Springer Nature (Jiang et al., 2016, creative commons attribution 4.0 license). fALS, familial amyotrophic lateral sclerosis; NES, nuclear export signal; NLS, nuclear localization signal; NTD, N-terminal domain; Q/N, glutamine/asparagine; RRM, RNA recognition motif; sALS, sporadic amyotrophic lateral sclerosis; TDP-43, TAR DNA binding protein 43.

fact, Zhang et al. have reported that the first ten residues of the NTD are crucial for the formation of the functional homodimers and are also involved in the aggregation of the full-length TDP-43 (Zhang Y. J. et al., 2013). Of note, the N-terminal region can promote self-oligomerization in a concentration-dependent manner, which modulates its nucleic acid binding

properties (Chang et al., 2012). Recently, using single-molecule fluorescence technique, evidence has also been provided that the NTD undergoes reversible oligomerization, which enhances the propensity of the intrinsically disordered C-terminal region to aggregate (Tsoi et al., 2017).

In contrast, it has also been argued that the TDP-43's dimerization *via* NTD allows for the interactions with the partner proteins and the target RNAs, thereby possibly preventing its aggregation. Indeed, the dimeric TDP-43 NTD has been shown to enhance its pre-mRNA splicing activity, improve solubility and protect against the formation of the cytoplasmic TDP-43 inclusions (Jiang et al., 2017). Recently, a 2.1 Å resolution structure of the 1–80 residues of the TDP-43 NTD has revealed the presence of dynamic solenoid-like structure which spatially separates the aggregation-prone C-terminal region and probably reduces the pathological aggregation (Afroz et al., 2017). Deletion or mutation in the nuclear localization signal (NLS) sequence in the NTD induces cytoplasmic relocalization and aggregation of TDP-43 (Winton et al., 2008a; Barmada et al., 2010). In fact, the ALS-associated A90V mutation present in the nuclear localization signal (NLS) can sequester the endogenous TDP-43 into insoluble cytoplasmic aggregates (Winton et al., 2008b).

RNA Recognition Motifs (RRMs)

RNA binding proteins (RBPs) contain highly conserved RNA recognition motifs (RRMs), which are among the most abundant protein domains in the eukaryotes (Romano and Buratti, 2013; Gerstberger et al., 2014; Marchese et al., 2016; Conlon and Manley, 2017). These proteins are involved in several RNA metabolic processes like mRNA processing, RNA export and RNA stability. Some RBPs, such as TDP-43, are also implicated in neurodegenerative diseases which therefore hints of disturbances in the RNA metabolism as a causative factor (Maris et al., 2005; Lunde et al., 2007; Clery et al., 2008). TDP-43 contains two RRM domains (RRM1 and RRM2) that are separated by 15 amino acids (Kuo et al., 2009, 2014; Lukavsky et al., 2013). These RRM domains comprise of five β-strands and two α-helices arranged in the β1-α1-β2-β3-α2-β4-β5 pattern (Lukavsky et al., 2013; Sun and Chakrabartty, 2017). Both of the TDP-43 RRM domains are involved in binding with cognate RNA/DNA molecules with higher specificity toward short UG/TG-rich sequences of the RNA/DNA molecules (Lukavsky et al., 2013; Kuo et al., 2014). Several mutations in the RRMs are shown to disrupt the RNA binding capability while not significantly interfering with the RNA recognition (Lukavsky et al., 2013). Notably, two ALS-linked missense mutations have also been identified in this region: the P112H and the caspase cleavage susceptible, D169G (Buratti, 2015; Moreno et al., 2015; Chiang et al., 2016). Proposedly, the RRM2 domain may also contribute to the dimerization of the TDP-43 protein (Kuo et al., 2009). Binding to single-stranded DNA (ssDNA) or single-stranded RNA (ssRNA), and not to double-stranded DNA (dsDNA), has been shown to enhance the TDP-43's solubility and expectedly also prevent its aggregation (Huang et al., 2013; Sun and Chakrabartty, 2017). Importantly, TDP-43 actively binds to the 3′ untranslated regions (UTRs) of several thousand mRNA transcripts, and even to its own mRNA as an autoregulation mechanism to control its own cellular concentration and possibly also its solubility (Ayala et al., 2011).

C-Terminal Domain (CTD)

The C-terminal region of TDP-43 (aa 277–414) is highly disordered and comprises of a glycine-rich region and also a segment enriched in uncharged polar amino acids, glutamine and asparagine (Q/N) (**Figure 2**). This unusual composition resembles the prion-like domains of several yeast proteins, such as Sup35, Rnq1, and Cyc8 etc. (Patel et al., 2009; King et al., 2012; Liebman and Chernoff, 2012). The prionogenic domain-containing yeast proteins can switch from a disordered conformation to a self-templating, cross-β sheet-rich amyloid-like conformation, sometimes as an adaptive physiological response (Liebman and Chernoff, 2012). Strikingly, out of nearly 240 human proteins that harbor a potential prion-like domain, about 70 of them are RNA/DNA-binding proteins containing an RRM motif, several of which, including TDP-43, FUS, hnRNPs, TATA-box binding protein associated factor 15 (TAF15), and EWS RNA binding protein 1 (EWRS1) etc., are being implicated in the pathogenesis of various neurodegenerative diseases (March et al., 2016; Harrison and Shorter, 2017). The C-terminal region of TDP-43 seems of special relevance to the pathological behavior of TDP-43. Firstly, alike prion-like domains, it is intrinsically disordered and aggregation-prone (Santamaria et al., 2017). Secondly, it harbors most of the ALS-associated *TARDBP* mutations and phosphorylation sites. Thirdly, certain C-terminal fragments of sizes ∼25–35 kDa produced from TDP-43 through aberrant activity of caspases, are highly cytotoxic and are the prominent species found in the inclusion bodies identified from the ALS-affected brains (Zhang et al., 2007, 2009). The C-terminal region of TDP-43 also contains a short, highly dynamic and unstable helix-turn-helix region (aa 311–360) (Jiang et al., 2013, 2016). Peptides from this region can efficiently form amyloid-like fibrils *in vitro*, which can exhibit prion-like infectious seeding ability to cells expressing the soluble TDP-43 (Chen et al., 2010; Guo et al., 2011; Jiang et al., 2013). Interestingly, TDP-43 C-terminal region can also undergo liquid-liquid phase separation (LLPS) to form dynamic protein droplets. Within these droplets, the C-terminal residues show mild transient interactions, that appear crucial for stress granule formation (Conicella et al., 2016). Mutations, persistent stress conditions, or aging, are proposed to cause these droplets to undergo a liquid-to-solid phase separation (LSPS), thereby forming irreversible pathological aggregates (Patel et al., 2015).

PHYSIOLOGICAL FUNCTIONS OF TDP-43

TDP-43-RNA Interactions

TDP-43 has versatile functions and it is involved in several steps of RNA metabolism such as: transcription, translation, mRNA transport, mRNA stabilization, microRNA (miRNA) and long non-coding RNA (lncRNA) processing etc. (Ling et al., 2013; Coyne et al., 2017) (**Figure 3**). Using genome-wide RNA immunoprecipitation techniques (CLIP-seq), more than 6,000 mRNA targets were identified to associate with TDP-43, which

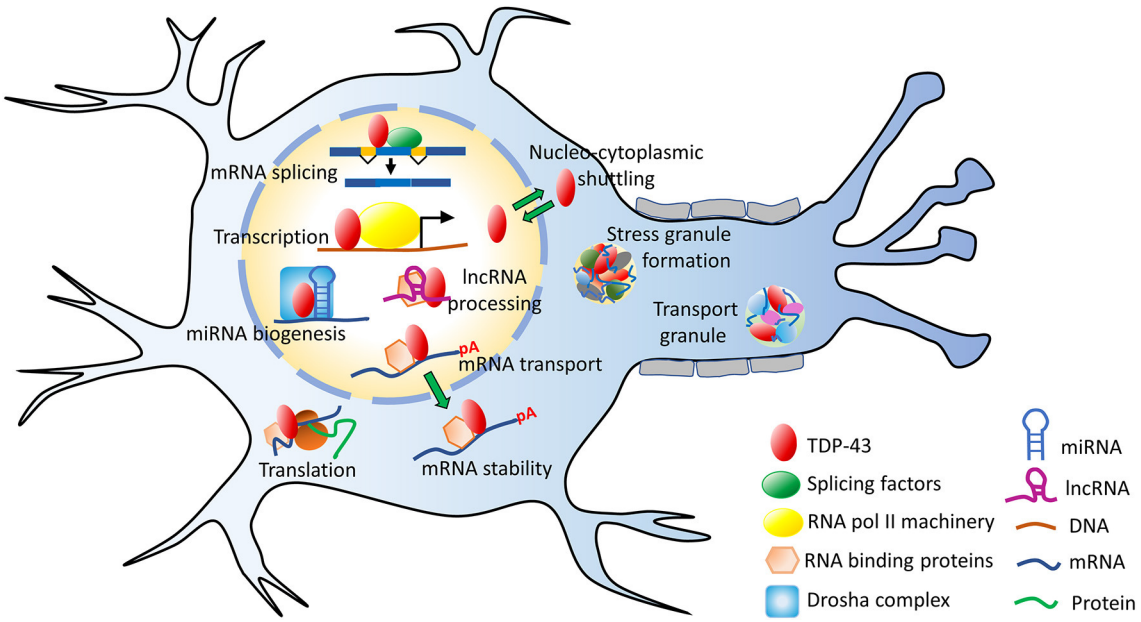

FIGURE 3 | Functions of TDP-43. TDP-43 performs several mRNA-related processes in the nucleus, such as transcription, splicing, maintaining RNA stability as well as miRNA and lncRNA processing. It is predominantly a nuclear protein but also shuttles between the nucleus and the cytoplasm. In the cytoplasm, TDP-43 participates in the stress granule formation, ribonucleoprotein (RNP) transport granule formation, translation and other processes. lncRNA, long non-coding RNA; miRNA, microRNA; mRNA, messenger RNA; pA, poly-A mRNA tail; TDP-43, TAR DNA binding protein 43.

would be nearly 30% of the entire transcriptome (Polymenidou et al., 2011; Tollervey et al., 2011; Xiao et al., 2011). Earlier conventional RNA immunoprecipitation methods, have also revealed specific RNA targets (Buratti and Baralle, 2001; Sephton et al., 2011). While TDP-43 binds with high specificity to the UG-rich sequences of RNAs, it mostly binds to the 3′ UTRs of mRNAs/pre-mRNAs when localized to the cytoplasm (Colombrita et al., 2012). This suggests a broad role of TDP-43 in maintaining mRNA stability, maturation and transport (Tollervey et al., 2011; Colombrita et al., 2012).

mRNA Transcription and Splicing

TDP-43 is absent from the areas of silent heterochromatin but localizes to the sites of transcription and splicing (Casafont et al., 2009). It regulates the splicing patterns of transcripts of several important genes, such as Cystic fibrosis transmembrane conductance regulator (*CFTR*), *TARDBP*, *FUS*, *SNCA* (α-synuclein), *HTT* (Huntingtin), and *APP* (Amyloid precursor protein) etc. (Buratti and Baralle, 2001; Polymenidou et al., 2011, 2012). In fact, nuclear depletion of TDP-43 results in mRNA splicing aberrations (Arnold et al., 2013; Highley et al., 2014; Yang et al., 2014). Likewise, over-abundance of TDP-43 could form dysfunctional complexes, due to limited supply of the binding partner proteins. Indeed, imbalances caused by the overexpression of TDP-43 are detrimental to the neuronal cells (Cannon et al., 2012; Heyburn and Moussa, 2016; Lu et al., 2016). The nuclear depletion of TDP-43 was also found to trigger widespread dysregulation of the splicing events in the motor neurons (Highley et al., 2014). Two ALS-associated mutations in TDP-43, Q331K, and M337V, have also been shown to alter mRNA splicing processes in a transgenic mice model (Polymenidou et al., 2011, 2012; Lagier-Tourenne et al., 2012; Arnold et al., 2013).

mRNA Maturation and Stability

By binding with mRNA transcripts, TDP-43 regulates stabilities of several mRNAs, including that of its own mRNA (Strong et al., 2007; Volkening et al., 2009; Ayala et al., 2011; Colombrita et al., 2012; Costessi et al., 2014). TDP-43 interacts with regulatory 3′ UTR sequences of these mRNAs and affects their half-life, either positively, as observed for the human low molecular weight neurofilament mRNA, or negatively, as documented for the vascular endothelial growth factor and progranulin mRNA transcripts (Strong et al., 2007; Volkening et al., 2009; Ayala et al., 2011; Colombrita et al., 2012; Costessi et al., 2014).

mRNA Transport

TDP-43 associates with the RNA molecules to produce ribonucleoprotein (RNP) granules which transport mRNA to distant locations. In the axonal cells, RNP granules are trafficked with assistance from microtubules (Alami et al., 2014). In fact, ALS-associated TDP-43 mutants were found to impair the transportation of the RNP granules (Wang et al., 2008; Alami et al., 2014).

mRNA Translation

Proteomics has revealed the TDP-43's global protein interaction profile which has also identified several partner proteins involved in the RNA metabolism, such as splicing and translation.

Several of these interactions were unperturbed by the ALS-linked mutations, A315T and M337V (Freibaum et al., 2010; Kim et al., 2010). Recent studies in *Drosophila*, have reported that TDP-43 regulates localization and translation of the *Futsch* (ortholog of *Map1b*) mRNA at the neuromuscular junctions (Coyne et al., 2014). TDP-43 can also form complexes with other proteins involved in the translation machinery, for example: the ribosomal protein, receptor for activated C kinase 1 (RACK1) (Russo et al., 2017). In one study, an increase in cytoplasmic TDP-43 caused repression of the global protein synthesis in the neuroblastoma cells, which could be rescued by the over-expression of RACK1 (Russo et al., 2017). TDP-43 can also alter the translation of several mRNAs *via* sequestration of the translation factors into stress granules (Aulas and Vande Velde, 2015).

Stress Granule Formation

Eukaryotic cells have developed several mechanisms that protect cells against diverse cellular insults. The formation of stress granules (SG), the membrane-less cytoplasmic foci of sizes $\leq 5\,\mu m$, ensues quickly upon exposure to stresses like: oxidative stress, heat shock, viral infection, and chemical exposure etc. (Anderson and Kedersha, 2009; Aulas and Vande Velde, 2015). SGs are usually safe "storage and sorting stations" for RNA binding proteins, translationally stalled mRNAs and arrested pre-initiation complexes. The formation of SG is a reversible process and SGs dissolve after the stress is over (Anderson and Kedersha, 2008). Neuronal cells are quite vulnerable to stress, and a defective stress response may facilitate the conversion of SGs into pathological inclusion bodies as seen in the ALS and FTLD-affected brains (Wojcik et al., 2006; Van Damme et al., 2008; Colombrita et al., 2009; Dormann and Haass, 2011). TDP-43 is capable of assembling into stress granules, indicating its protective role against cellular insults (Colombrita et al., 2009; Aulas and Vande Velde, 2015). In fact, TDP-43 is involved in both assembly and maintenance of SGs, and it also regulates the expression of key SG nucleating proteins, rasGAP SH3 domain binding protein 1 (G3BP) and T cell-restricted intracellular antigen-1 (TIA-1) (McDonald et al., 2011). ALS-linked mutations can influence stress granule dynamics. Under sorbitol-induced osmotic stress, the G348C mutant TDP-43 was found to be localized into progressively larger stress granules (Dewey et al., 2011). On the contrary, the R361S mutant of TDP-43 was shown to disrupt the stress granule assembly (McDonald et al., 2011). The abnormal effects of several other ALS-associated mutations on stress granule dynamics is discussed furthermore in the "role of TDP-43 mutations" section of this review.

miRNA and lncRNAs Processing

TDP-43 also promotes biogenesis and processing of the non-coding RNAs, such as microRNA (miRNA) (Kawahara and Mieda-Sato, 2012). Recent studies have confirmed of the interactions of TDP-43 with the Drosha and Dicer complexes (Ling et al., 2010; Kawahara and Mieda-Sato, 2012). TDP-43 associates with the nuclear Drosha complex and binds directly to the primary miRNAs to facilitate the production of a subset of precursor miRNAs (pre-miRNAs) (Kawahara and Mieda-Sato, 2012). In human embryonic kidney 293 cells (HEK293), it is found that the cytoplasmic TDP-43 interacts with the Dicer complex and promotes pre-miRNA processing (Kawahara and Mieda-Sato, 2012). In fact, TDP-43's down-regulation leads to altered expression of several miRNAs in the cultured HeLa cells, rodent neurons and induced pluripotent stem cells (iPSC)-derived human neurons (Buratti et al., 2010; Zhang Z. et al., 2013). In genome-wide studies, several long non-coding RNAs (lncRNAs), which are transcripts of >200 nucleotides, that do not encode for proteins but regulate gene expression through various mechanisms, such as nuclear enriched abundant transcript 1 (NEAT1) and metastasis associated in lung adenocarcinoma transcript 1 (MALAT1), were found to bind with TDP-43. Interestingly, NEAT1 and MALAT1 are also found at elevated levels in FTLD-TDP (Tollervey et al., 2011).

TDP-43 Protein-Protein Interactions

A global interactome study has revealed that TDP-43 interacts with proteins involved in diverse physiological functions (Freibaum et al., 2010). In a recent study, Blokhuis et al. have performed an interactome analysis to identify binding partners of ALS-associated proteins in neuronal cells using immunoprecipitation, pull down assays and mass spectrometry. Many DNA- and RNA-binding proteins were detected in the interactome of TDP-43 which are involved in RNA processing, gene expression, RNA splicing, post-transcriptional regulation of gene expression and translation (Blokhuis et al., 2016). TDP-43 has either direct physical interactions, or RNA-dependent interactions, with several proteins and some of the key interactions have been outlined in the **Table 1**.

TDP-43 PATHOLOGY IN ALS

The pathological hallmarks of TDP-43 proteinopathies include nucleus to cytoplasmic mislocalization, deposition of ubiquitinated and hyper-phosphorylated TDP-43 into inclusion bodies, protein truncation leading to formation of toxic C-terminal TDP-43 fragments, and protein aggregation. Sporadic or familial mutations can aggravate these detrimental effects and cause early disease-onset. In this section, we review these disease mechanisms in detail.

Role of TDP-43 Mutations

Numerous mutations in the *TARDBP* gene have been identified to be associated with ALS and FTLD (Sreedharan et al., 2008; Buratti, 2015) (**Figure 2**). The effects of these mutations on the TDP-43 protein include: increased propensity to aggregate, enhanced cytoplasmic mislocalization, altered protein stability, resistance to proteases or modified binding interactions with other proteins etc. The role of TDP-43 mutations have also been comprehensively reviewed earlier elsewhere (Pesiridis et al., 2009; Lattante et al., 2013; Buratti, 2015). Dedicated online databases are also available that provide detailed information about geographical prevalence of these mutations (Pinto et al., 2011; Cruts et al., 2012; Abel et al., 2013). Most of the ALS-associated mutations appear in the exon 6 of the *TARDBP*

TABLE 1 | Key interactions of TDP-43 protein with other proteins.

Protein	Remarks	Reference(s)
RNA-BINDING PROTEINS		
FUS	TDP-43 interacts with a small fraction of FUS. ALS mutations in TDP-43 enhance interaction with FUS. Perturbation of this interaction was observed to reduce the expression of histone deacetylase 6 (HDAC6) mRNA.	Kim et al., 2010; Ling et al., 2010; Kabashi et al., 2011
hnRNPA1 and hnRNPA2/B1	hnRNPs interact with TDP-43 C-terminal region and regulate mRNA splicing and TDP-43's feedback auto-regulation.	Buratti et al., 2005; D'ambrogio et al., 2009; Romano et al., 2014; Blokhuis et al., 2016
TIA1	TIA1 is involved in stress granule (SG) formation and participates in direct physical or RNA-dependent association with TDP-43 in SGs. TIA1 mutations identified in ALS increase its phase separation propensity, disrupt the normal disassembly of SGs and promote the accumulation of non-dynamic SGs containing the TDP-43 protein.	Liu-Yesucevitz et al., 2010; McDonald et al., 2011; Mackenzie et al., 2017
RBM45	RBM45 accumulates in inclusion bodies in ALS and FTLD patients. RBM45 co-localizes with TDP-43's cytoplasmic aggregates. No RBM45 mutations in ALS have been reported yet. Mutations in RBM45 show propensity to form cytoplasmic aggregates which recruit TDP-43, and impair mitochondrial functions.	Collins et al., 2012; Li Y. et al., 2015; Mashiko et al., 2016
Ataxin-2	Poly-glutamine expansion in Ataxin-2 are genetic risk factor for ALS. Ataxin-2 with 22 glutamines is normal, while 27–33Qs impart ALS risk and if present with >34Qs, it is involved in spinocerebellar ataxia type 2 (SCA2). Ataxin-2 and TDP-43 physically interact in an RNA-dependent manner. Poly-glutamine expansions in ataxin-2 that have been identified in ALS enhance its stability and increase the TDP-43's cleavage and phosphorylation.	Elden et al., 2010; Ross et al., 2011; Hart and Gitler, 2012; Nihei et al., 2012; Kim et al., 2014
Matrin3	Co-immunoprecipitation experiments have revealed that Matrin3 and TDP-43 interact in an RNA-dependent manner. Matrin3's S85C mutation enhances its interaction with TDP-43.	Johnson et al., 2014b; Gallego-Iradi et al., 2015; Boehringer et al., 2017
IMMUNE RESPONSE		
p62 and p65 (NFκB)	TDP-43 interacts with NFκB and acts as a co-activator of NFκB in ALS patient's glial and neuronal cells inducing the production of pro-inflammatory cytokines and neurotoxic mediators.	Swarup et al., 2011
HEAT SHOCK RESPONSE AND PROTEOSTASIS		
Hsp40 and Hsp70	Hsp40/Hsp70 co-chaperone/chaperone system interact with TDP-43's C-terminal region and suppress heat-shock-induced TDP-43 aggregation. Heat-shock protein DNAJB2 associates with Hsp70 and regulates TDP-43's clearance by maintaining it in soluble state. Overexpression of the yeast Hsp40 homolog Sis1 reduces TDP-43 toxicity in the yeast model.	Udan-Johns et al., 2014; Chen H. J. et al., 2016; Park et al., 2017
DNAJB1 and DNAJB6	Overexpression of DNAJB1 (Hsp40 protein, mammalian Sis1 homolog) was found to reduce TDP-43-mediated toxicity in primary cortical neurons of rodent. Overexpression of DNAJB6 suppresses the formation of heat-shock-induced TDP-43 nuclear aggregates. DNAJB6 interacts with the disordered C-terminal domain of TDP-43 and modulates TDP-43 aggregation and also influences its interaction with other RNA binding partners.	Udan-Johns et al., 2014; Park et al., 2017
PDI	The chaperone PDI interacts with mutant TDP-43 and co-localizes in the spinal cord neuronal cells. PDI might also be involved in preventing the abnormal cysteine cross-linking of TDP-43.	Walker et al., 2013
Parkin	The E3-ubiquitin ligase Parkin ubiquitinates TDP-43 and forms a multi-protein complex with HDAC6 and induces sequestration of TDP-43 into cytosolic inclusions.	Hebron et al., 2013; Wenqiang et al., 2014
Ubiquilin1 and Ubiquilin2	Mutations in ubiquilin proteins are involved in aberrations in the proteasomal and autophagy pathways. Ubiquilin2 binds with high affinity to TDP-43 and induces accumulation of poly-ubiquitinated inclusions in the neuronal cells.	Kim et al., 2009; Hanson et al., 2010; Cassel and Reitz, 2013; Osaka et al., 2016
Optineurin	Optineurin mutations cause blindness and glaucoma. Recently, optineurin was found to associate with TDP-43 in ALS and sporadic inclusion body myositis.	Yamashita et al., 2013; Li C. et al., 2015
OXIDATIVE STRESS RESPONSE		
SOD1	ALS-linked SOD1 mutants interact with TDP-43 into detergent-insoluble fractions. Mutant SOD1 and TDP-43 co-operatively modulate the neurofilament mRNA stability.	Volkening et al., 2009; Higashi et al., 2010
CHCHD10	CHCHD10 is a mitochondrial protein found at the cristae junction in the intermembrane spaces that regulates mitochondrial structure and oxidative phosphorylation. TDP-43 interacts with CHCHD10 and induces its nuclear localization while the CHCHD10 dysfunction promotes the TDP-43's cytoplasmic accumulation.	Johnson et al., 2014a; Woo et al., 2017

CHCHD10, coiled coil helix coiled coil helix domain containing protein 10; DNAJB1, DnaJ homolog subfamily B member 1; DNAJB6, DnaJ homolog subfamily B member 6; FUS, fused in sarcoma; HDAC6, histone deacetylase 6; hnRNP A1 and A2/B, heterogeneous nuclear ribonucleoprotein A1 and A2/B; Hsp, heat shock protein; NFκB, nuclear factor kappa-light-chain-enhancer of activated B cells; PDI, protein disulfide isomerase; RBM45, RNA-binding motif protein 45; SCA2, spinocerebellar ataxia type 2; SOD1, superoxide dismutase 1; TIA1, T cell-restricted intracellular antigen-1.

gene which encodes for the C-terminal glycine-rich region of TDP-43. The most commonly occurring missense mutations are A382T and M337V and some of the most well-studied mutations are A315T, Q331K, M337V, D169G, G294A/V, and Q343R etc., for which several ALS-disease models have also been established (Buratti, 2015). TDP-43 mutations including A90V and N267S are observed in both cases of sporadic ALS as well as FTLD whereas R361T was reported in a

patient case of fALS and FTLD. Mutations, such as G294V, G348C, A328T, and S393L are found in both the sporadic as well as familial cases of ALS. Interestingly, TDP-43 mutation G295S encompasses various pathological conditions including sALS, fALS, and FTLD (Baumer et al., 2009; Xiong et al., 2010; Fujita et al., 2011; Janssens et al., 2011; Budini et al., 2012; Chiang et al., 2012; Cruts et al., 2012; Lattante et al., 2013; Moreno et al., 2015). Of interest, a fALS associated phosphorylation-prone TDP-43 mutant, which contains G298S mutation in the mitochondrial localizing internal motif M5, was found to have increased import into the mitochondria (Wang et al., 2016).

Mutations in the TDP-43's C-terminal region enhance its intrinsic aggregation propensity (Johnson et al., 2009). Recombinantly expressed TDP-43 protein harboring the ALS-linked mutations, such as Q331K, M337V, Q343R, N345K, R361S, and N390D, were found to have increased aggregation *in vitro* and also promoted cytotoxicity in the yeast cells (Johnson et al., 2009). Peptides from the TDP-43's putative amyloidogenic core region (aa 286–366) containing the ALS-associated mutations were also found to efficiently form amyloid-like fibrils (Chen et al., 2010; Guo et al., 2011; Sun et al., 2011; Zhu et al., 2014) (**Table 2**). Interestingly, Zhu et al. have reported that an aggregated TDP-43 peptide with the A315E mutation is capable even of cross-seeding the aggregation of the amyloid-β 1–40 peptide (Zhu et al., 2014). Also, Guo et al. have shown that TDP-43 A315T forms amyloid fibrils *in vitro* and causes neuronal death when added to the cultured neuronal cells (Guo et al., 2011). Certain mutations in TDP-43 like G294V, A315T, M337V, A382T, and G376D, are also found to enhance the cytoplasmic mislocalization of TDP-43 (Barmada et al., 2010; Mutihac et al., 2015; Mitsuzawa et al., 2018).

TDP-43 protein is intricately associated with stress granule dynamics (Liu-Yesucevitz et al., 2010; Walker et al., 2013). Quantification of the TDP-43 levels accumulated in the stress granules, has revealed that the ALS-linked D169G and R361S mutants accumulate in larger quantities than the wild-type TDP-43 (McDonald et al., 2011). Additionally, TDP-43 with the G348C mutation forms significantly larger stress granules, and is incorporated into the stress granules earlier than the wild-type TDP-43, although eventually, the wild-type TDP-43 expressing cells form more number of stress granules per cell, albeit, the granule sizes remain unchanged (Dewey et al., 2011). Additionally, the aggregation-enhancing A315T and Q343R mutations have been shown to increase TDP-43-containing RNA granule's average sizes, decrease their distribution density and also hamper their mobility in the neuronal cells (Liu-Yesucevitz et al., 2014). The mutations, D169G, G294A, Q343R, N390D, Q331K, and M337V, were found to enhance the formation of TDP-43-positive inclusion bodies in the neuronal cell line, SH-SY5Y (Nonaka et al., 2009a).

A plausible pathological mechanism is the alteration of the TDP-43 protein's stability by the mutations. In one study, the ALS-linked TDP-43 with the mutations G298S, Q331K, and M337V, showed longer half-life and higher stability than the wild-type TDP-43 (half-life: ∼24–48 h vs. 12 h for the wild-type TDP-43) in an isogenic cell line (Ling et al., 2010). Further evidence from the works of Watanabe et al. (2013) and Austin et al. (2014), has shown that the accelerated disease onset in the familial ALS patients is related to the TDP-43 mutations (such as: A315T, Q343R, N352S, M337V, G298S, G348C, A382T, D169G, and K263E) possibly *via* increase in the protein half-lives and the aggregation propensities, which may further influence their own mRNA's processing and cause misregulation of the TDP-43's translation (Watanabe et al., 2013; Austin et al., 2014).

Certain mutations also confer increased susceptibility of TDP-43 to protease-mediated degradation (Nonaka et al., 2009b). Calpain-I could fragment the recombinant TDP-43 A315T and M337V mutant proteins more rapidly than the wild-type TDP-43, whereas the D169G mutant TDP-43 was more efficiently cleaved by caspase-3 *in vitro* (Yamashita et al., 2012; Chiang et al., 2016). Interestingly, another mutation A90V in TDP-43 imparts partial resistance to the digestion by caspase-3 (Wobst et al., 2017).

Nuclear Depletion and Cytoplasmic Accumulation of TDP-43

One of the prominent features of ALS and FTLD-TDP, is the loss of functional TDP-43 in the nucleus and its increased deposition into cytoplasmic inclusion bodies in the brain and spinal cord neurons (Arai et al., 2006; Neumann et al., 2006). While TDP-43 is predominantly nuclear, it also shuttles between the nucleus and the cytoplasm thereby engaging in diverse functions (Ayala et al., 2008). In fact, TDP-43 interacts with several proteins involved in the mRNA splicing and other RNA metabolisms in the nucleus, and also interacts with several cytoplasmic proteins, such as those involved in the mRNA translation (Freibaum et al., 2010; Ling et al., 2013). TDP-43's cellular concentration is therefore tightly auto-regulated to maintain its steady levels *via* a negative-feedback mechanism (Ayala et al., 2011). The precise sequence of events abetting the pathological TDP-43 mislocalization is debated, however, nuclear TDP-43 depletion appears to precede the inclusion body formation (Lee et al., 2011; Xu, 2012). Notably however, it is argued that the TDP-43-associated disturbances in the mRNA metabolism may be more central, as compared to the cytoplasmic accumulation and aggregation of TDP-43, toward the pathogenesis of ALS and FTLD-TDP. It is accepted that the cytoplasmic accumulation and the aggregation of TDP-43 into inclusion bodies confer both a loss-of-function as well as a gain-of-toxic-function (Vanden Broeck et al., 2015; Ederle and Dormann, 2017). Numerous studies have supported the detrimental effects of the TDP-43's cytoplasmic aggregation in the neuronal cells (Igaz et al., 2009; Pesiridis et al., 2011; Yang et al., 2011; Wang Y. T. et al., 2013). The cytoplasmic accumulation into inclusion bodies reduces the amount of TDP-43 necessary for mRNA transport. Recently, TDP-43 has also been found to function as a translational repressor by interacting with the ribosomal protein, receptor of activated protein C kinase 1 (RACK1), thereby resulting in the global protein synthesis inhibition (Russo et al., 2017). Interestingly,

TABLE 2 | Observations on amyloid-like aggregation and oligomerization of TDP-43 and its peptides.

TDP-43 protein or its peptides	Tools used	Observation(s)	Reference(s)
AMYLOID-LIKE AGGREGATION			
FL TDP-43 (1–414)	ThT, CR, TEM	Amyloid-like fibrils.	Johnson et al., 2009
FL TDP-43, FL TDP-43 M337V, FL TDP-43 A382T, 193–414, 193–414 M337V, 193–414 A382T	ThT, TEM	Amyloid-like fibrils. Formation of thin fibrils that stack together to form thick bundles. Moderately bind to the ThT dye.	Furukawa et al., 2011
287–322, 287–322 A315T, 287–322 G294A, 287–322 G294V, 287–322 G294P, 287–322 G295S, 292–322, 297–322, 302–322, 307–322	ThT, TEM, CD, FTIR	Amyloid-like fibrils. Peptides 287–322 (wt and A315T) form β-sheet-rich ThT-negative fibrils. Mutants G294A, G294V, and G295S form ThT-staining fibrils. Further delineation of the amyloidogenic 287–322 fragment, shows amyloidogenic propensity of smaller peptides. Peptide 307–322 exerts disruption of membrane integrity. All peptides impart neurotoxicity.	Chen et al., 2010; Liu et al., 2013; Sun et al., 2014
286–331, 286–331 A315T	ThT, TEM, AFM	Amyloid-like fibrils. Both these 46 amino acid peptides form ThT-positive amyloid fibrils which are toxic to the neuronal cells. The A315T mutation enhances fibril formation and toxicity.	Guo et al., 2011
103–183, 103–183 C173S, 103–183 C175S	ThT, AFM	Amyloid-like fibrils. The RRM1 domain forms large globular particles and fibrillar aggregates. Cysteine to serine substitution enhances amyloidogenicity.	Shodai et al., 2013
208–265	ThT, TEM, CD, SAXS	Truncated RRM2 domain forms ThT-negative fibrillar aggregates.	Wang Y. T. et al., 2013
307–319, 307–319 A315T, 307–319 A315E	ThT, AFM, CD, FTIR	Amyloid-like fibrils. Mutant TDP-43 peptides are capable of cross-seeding the aggregation of the Alzheimer's amyloid-β peptide.	Zhu et al., 2014
246–258, 311–323, and smaller peptides from these regions.	ThT, TEM, SLS	Amyloid-like fibrils. ThT-positive amyloid aggregates. Regions, 246–255 (EDLIIKGISV) and 311–320 (MNFGAFSINP) are important sequence determinants of the TDP-43 C-terminal aggregation. DLII and NFGAF are the shortest amyloidogenic sequences.	Saini and Chauhan, 2011, 2014
318–343, 311–360, 311–360 Q331K, 311–360 G335D, 311–360 M337V	ThT, CD, AFM	Amyloid-like fibrils. Amyloidogenic core region 311–360 forms ThT-positive amyloid fibrils. G335D mutation enhances aggregation and inclusion body formation. G335D forms a loop linker between the two α-helices in this region and promotes α-to-β transition.	Jiang et al., 2013, 2016
341–357	ThT, CR, TEM, CD, XRD	Amyloid-like fibrils. Putative amyloidogenic core 341–357 peptide forms amyloid fibrils ∼ 15–25 nm wide and several hundred nanometers long with marked tendency to associate laterally.	Mompean et al., 2015
193–414	ThT, AFM, CD	Amyloid-like fibrils. ThT-positive fibrillar aggregates having varying lengths (∼1 to 2 μm) with an average height of ∼20–25 nm. β-sheet content is found to be ∼ 45%.	Furukawa et al., 2011; Prasad et al., 2016, 2018
234–273, 274–313, 314–353	ThS, TEM	Amyloid-like fibrils. 234–273 peptide formed short fibrillar aggregates. The peptides 274–313 and 314–353 formed longer, straight, or twisted fibrils (10–15 nm diameter) and displayed prion-like behavior.	Shimonaka et al., 2016
102–269	ThT, TEM, DLS	Amyloid-like fibrils. Zinc binds to RRM 1 and 2 regions and results in aggregation into rope-like aggregates.	Garnier et al., 2017
TDP-43 RRM2 region: 247–252, 247–255, 247–256, 247–257, 248–253, 248–256, 248–257, 250–259, 252–257, 252–259, 253–259	XRD, MicroED, Cryo-EM	The RRM peptide 247-DLIIKGISVHI-257 forms an array of amyloid polymorphs, which fit into different classes of steric zippers and adopt different backbone conformations.	Guenther et al., 2018b

(Continued)

TABLE 2 | Continued

TDP-43 protein or its peptides	Tools used	Observation(s)	Reference(s)
TDP-43 LCD region: 300–306, 321–326, 328–333, 333–343, 370–375, 396–402	XRD, MicroED, TEM	These segments form amyloid steric zipper structures.	Guenther et al., 2018a
TDP-43 LCD region: 312–317, 312–317 A315E, 312–317 A315T	XRD, MicroED, TEM	These segments form kinked beta-sheet structure and are involved in hydrogels and protein droplet formations alike to as observed in the membrane less organelles.	Guenther et al., 2018a
OLIGOMERIZATION			
FL TDP-43	TEM, AFM, DLS, Immuno-labeling	Oligomers. Spherical oligomers with particle sizes of ∼40–60 nm. Oligomers are conformationally and functionally distinct from the native TDP-43 and are neurotoxic.	Fang et al., 2014
FL TDP-43 (Tandem dimer) (Amino acid residues 1–414 x2)	Immuno-blotting	Dimers. Dimeric TDP-43 induces accumulation of high molecular weight TDP-43 aggregates.	Shiina et al., 2010
FL TDP-43, TDP-43 NTD	Chemical cross-linking, TEM, NMR spectroscopy	Oligomers. N-terminal domain (NTD)-mediated TDP-43's oligomerization into solenoid-like structures. Resistant to cellular stress (cysteine-independent).	Afroz et al., 2017
NLS-TDP-25, TDP-25	FRET, FRAP, FCS, Super-resolution Fluorescence Microscopy	Oligomers. NLS-TDP-25 oligomers are ordered and non-toxic whereas TDP-25 oligomers are disordered and toxic. NLS-TDP-25 does not incorporate into cytoplasmic inclusion bodies.	Kitamura et al., 2017
FL TDP-43	Immuno-labeling, TEM	Oligomers. TDP-43 forms neurotoxic spherical oligomers in the human brain. TDP-43 oligomers are abundant in the FTLD-TDP type C brain.	Kao et al., 2015

AFM, atomic force microscopy; CD, circular dichroism; CR, Congo red; Cryo-EM, cryo-electron microscopy; FCS, fluorescence correlation spectroscopy; FL, full-length; FRAP, fluorescence recovery after photobleaching; FRET, fluorescence resonance energy transfer; FTIR, fourier-transform infrared spectroscopy; LCD, low complexity domain; MicroED, micro-electron diffraction; NLS, nuclear localization signal; NMR, nuclear magnetic resonance spectroscopy; NTD, N-terminal domain; SAXS, small-angle X-ray scattering; SLS, static light scattering; TEM, transmission electron microscopy; ThS, thioflavin-S; ThT, thioflavin-T; XRD, X-ray diffraction. The tools used for the analyses of the amyloid-like aggregates/aggregation have been briefly described in ***Box 1****.*

RACK1 was also found to be sequestered into the TDP-43 inclusions in the motor neurons of the ALS patients (Russo et al., 2017). Cytoplasmic TDP-43 is also proposedly involved in the mitochondrial impairment, which is further discussed here in a later section (Wang et al., 2016).

The TDP-43's signal sequences, nuclear localization signal (NLS) and nuclear export signal (NES), regulate the nucleocytoplasmic shuttle of TDP-43 (Winton et al., 2008a). Deletion of the nuclear localization signal (NLS) [or nuclear export signal (NES)] sequence impairs TDP-43's functions. Expectedly, TDP-43 with the nuclear localization signal (NLS) deletion accumulates as cytoplasmic aggregates and can even sequester the native TDP-43 thereby further depleting the TDP-43 pool, which consequently alters the transcripts that regulate chromatin assembly and histone processing (Winton et al., 2008a; Amlie-Wolf et al., 2015). Likewise, a TDP-43 mutant with nuclear export signal (NES) deletion manifested a propensity to form nuclear aggregates (Winton et al., 2008a). Only one familial ALS-linked mutation in the nuclear localization signal (NLS) has been identified till date i.e., A90V, although several C-terminal mutations can also drive increased cytoplasmic localization, however, how this ensues remains to be fully elucidated (Barmada et al., 2010; Mutihac et al., 2015). Thus, factors influencing nucleocytoplasmic transport, such as the role of nuclear importins, transport-partners and effects of mutations on the TDP-43 conformation during the transit, need further investigation (Archbold et al., 2018).

C-Terminal Fragmentation of TDP-43

The generation of C-terminal fragments of TDP-43 *via* proteolytic cleavages by the caspase and calpain proteases seems to be one of the prominent toxicity generating mechanisms, as we have discussed, previously in the "C-terminal domain" section (Zhang et al., 2007, 2009; Dormann et al., 2009; Igaz et al., 2009; Johnson et al., 2009; Yang et al., 2011; Xu, 2012; Buratti, 2015).

Post-translational Modifications

The two most pathologically significant common post-translational modifications (PTMs) in TDP-43 are phosphorylation and ubiquitination (Arai et al., 2006; Neumann et al., 2006, 2009; Hasegawa et al., 2008; Inukai et al., 2008). While the TDP-43's phosphorylation from TDP-43-positive inclusions in brain samples has been well-characterized owing to the availability of highly specific antibodies to detect the TDP-43's

BOX 1 | Tools for the analysis of amyloid-like aggregates.

AFM (Atomic Force Microscopy): AFM provides the surface contour by scanning using a molecular size cantilever and it provides surface topology of amyloid aggregates or fibers. AFM images can provide height features of amyloid fibrils/aggregates.

CD (Circular Dichroism Spectroscopy): By measuring the differential absorbance of circularly polarized light, CD is widely used to characterize the protein's secondary structural elements. Amyloid-like aggregates tend to show higher β-sheet structure in comparison with the soluble, monomeric protein molecules and exhibits a negative peak around 215 nm.

CR (Congo Red Birefringence): Upon binding with amyloid aggregates, absorbance maximum of CR shifts from 490 to 540 nm. Macroscopic amyloid aggregates bound to CR display apple-green birefringence when observed under cross-polarized light.

Cryo-EM (Cryo-Electron Microscopy): An electron microscopic technique used for imaging frozen-hydrated specimens at cryogenic temperatures, where the specimens remain in their native state without the need for dyes or fixatives, allowing structure determination at high resolution. Cryo-EM generated micrographs have been used to distinguish various structural classes of amyloids.

DLS (Dynamic Light Scattering): Fluctuation of intensity of scattered light with time due to Brownian motion of particles in solution are analyzed to detect diffusion of the molecules. DLS provides hydrodynamic radii of particles and can be used to evaluate the presence of amyloid aggregates and estimate their sizes.

FCS (Fluorescence Correlation Spectroscopy): FCS records fluctuations in fluorescence intensity, providing information, such as diffusion coefficient and hydrodynamic radius which are used as a measure of size and concentration of monomers and aggregates in a solution.

FRAP (Fluorescence Recovery After Photobleaching): A spectroscopic technique which is used to measure the diffusion of a population of fluorescently labeled molecules after photobleaching. It gives valuable insights into the mobility of intracellular aggregated species.

FRET (Fluorescence Resonance Energy Transfer): FRET measures energy transfer from a donor fluorophore to acceptor fluorophore and can be used to detect the presence of small sub-population of oligomeric assemblies of misfolded proteins.

FTIR (Fourier Transform Infrared Spectroscopy): Composition of secondary structural elements are determined by FTIR by measuring molecular bond vibrational frequencies. FTIR spectra can provide structural features of protein misfolding intermediates where the larger and rigid amyloids absorb near 1,620 cm^{-1} whereas the small and disordered fibers absorb at ∼1,635 cm^{-1}.

MicroED (Micro- Electron Diffraction): A new method of cryo-EM where diffraction patterns are collected from submicron-thick 3D crystals using a focused low-dose electron beam under cryogenic temperatures and are deployed to visualize amyloid crystals with dimensions of few hundred nanometers.

NMR spectroscopy (Nuclear Magnetic Resonance Spectroscopy): NMR is a spectroscopic technique to determine the molecular structure, dynamics and chemical environment of molecules by measuring magnetic fields of certain atomic nuclei. Since amyloids exhibit favorable nuclear spin relaxation, NMR is used in characterization of the overall symmetry of cross–β structures.

SAXS (Small-Angle X-ray Scattering): SAXS is used to determine the average particle size, shape, distribution, and surface-to-volume ratio by analyzing the elastic scattering of X-rays at small angles when passed through a specimen. This technique is widely used to characterize structural variations in amyloid fibrils.

SLS (Static Light Scattering): SLS uses time-averaged intensity of scattered light to estimate molecular weight of particles in a solution and thereby helps in identifying the presence of higher molecular weight amyloid-like aggregates.

Super-resolution Fluorescence Microscopy: In super-resolution microscopy, temporal or spatial modulation of the excitation or activation light helps to overcome the resolution limit to extract higher resolution information of the samples and provides detailed information on species morphology of oligomeric and fibrillary structures.

TEM (Transmission Electron Microscopy): TEM provides morphological visualization of amyloid aggregates or fibers. First, the amyloid samples are negatively stained using metal compounds, such as uranyl acetate, before imaging.

ThS (Thioflavin-S Fluorescence): Binding of ThS with amyloid aggregates displays a sharp fluorescence emission peak at ∼520 nm when excited at 440 nm. It is also used to stain amyloid aggregates present in tissue sections and cell culture.

ThT (Thioflavin-T Fluorescence): Binding of the planar dye Thioflavin-T to amyloid-like aggregates increases its fluorescence emission intensity at ∼485 nm when excited at 445 nm.

XRD (X-Ray Diffraction): Subjecting amyloid fibers to X-ray results in the display of a specific diffraction pattern known as the cross-β pattern in which β-strands run perpendicular to the fiber axis and β-sheets extend parallel to the fiber axis.

phosphorylation at different sites, the TDP-43's ubiquitination is now being investigated profusely. Recently, other PTMs like acetylation, poly ADP-ribosylation and cysteine oxidation, have also been identified from the ALS patients. Detailed characterization of the PTMs, has the potential of unearthing novel TDP-43 toxicity mechanisms in ALS (Kametani et al., 2016) (**Figure 4**).

Phosphorylation

TDP-43 has 41 serine, 15 threonine and 8 tyrosine residues, which may act as potential phosphorylation sites. The casein kinases, CK1 and CK2, have been shown to mediate phosphorylations at Ser-379, Ser-403, Ser-404, and especially Ser-409/Ser-410, which are now considered a signature of ALS pathology (Neumann et al., 2006, 2009). Another kinase, glycogen synthase kinase (GSK3) is also found to be involved in the TDP-43's phosphorylation (Sreedharan et al., 2015). The TDP-43's phosphorylation is associated with its increased cytoplasmic mislocalization and aggregation in the neuronal cells (Nonaka et al., 2009a, 2016; Barmada et al., 2010; Liachko et al., 2010; Choksi et al., 2014). Notably, distinctly phosphorylated TDP-43 inclusions have been reported in the brain cortex vs. the spinal cord cells of the ALS and FTLD patients. While affected brain cortex shows accumulation of phosphorylated C-terminal fragments, the spinal-cord cells show a predominant deposition of the phosphorylated full-length TDP-43 (Neumann et al., 2009). Antibodies developed against the phosphorylated TDP-43 have shown potential as tools for rapid detection of the TDP-43 inclusions.

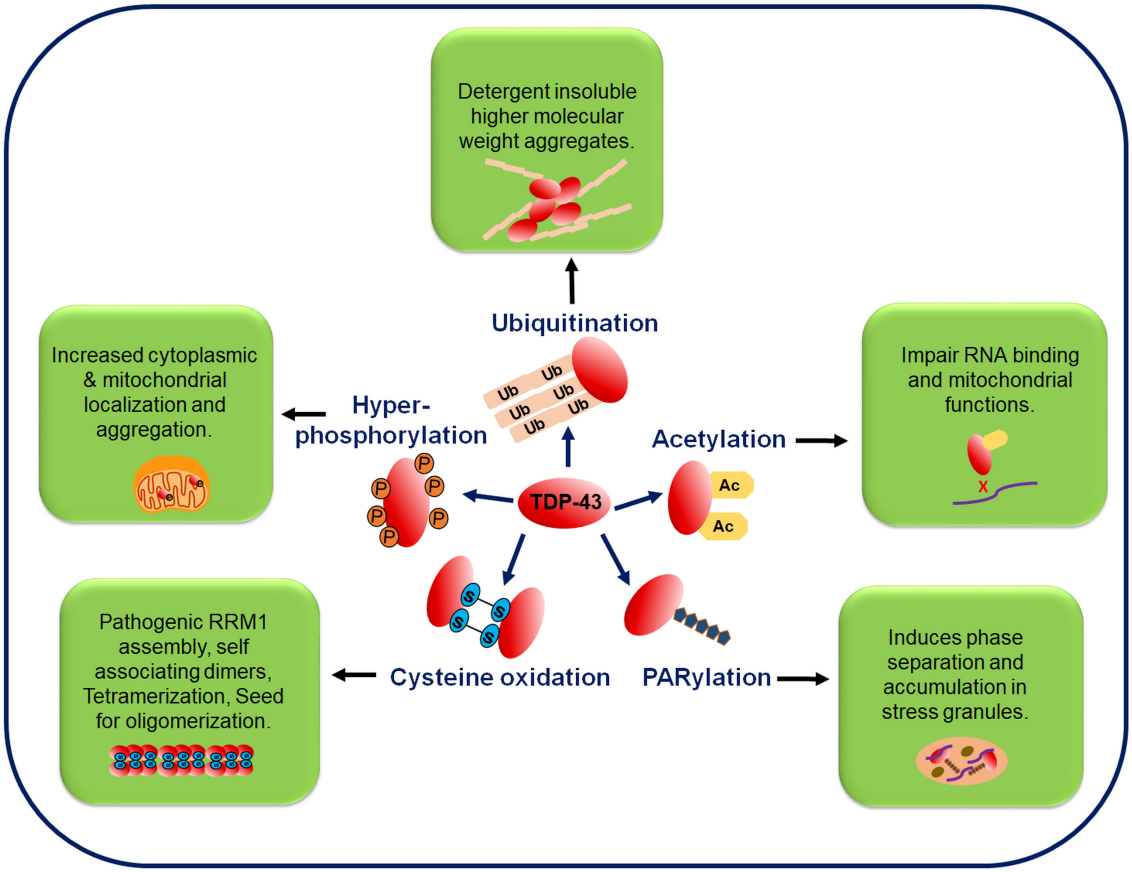

FIGURE 4 | Post-translational modifications in the TDP-43 protein. TDP-43 undergoes several post-translational modifications, such as phosphorylation, ubiquitination, acetylation, PARylation, and cysteine oxidation. Phosphorylation of the full-length and C-terminal fragments of TDP-43 is a pathological hallmark of ALS and is associated with its increased cytoplasmic mislocalization. In FTLD and ALS brain inclusions, pathological TDP-43 is found in the ubiquitinated state and mutations at the ubiquitination sites decrease the TDP-43 aggregation. Acetylation promotes accumulation of the insoluble and hyper-phosphorylated TDP-43 aggregates. PARylation promotes the phase separation of TDP-43 into stress granules. Oxidative stress mediated cysteine oxidation promotes the oligomerization and aggregation. Ac, acetylation; P, phosphorylation; PARylation, poly ADP ribosylation; Ub, ubiquitination.

Ubiquitination

TDP-43 has also been found in ubiquitinated state in the ALS and FTLD brain inclusions (Neumann et al., 2006, 2007b). The E3 ubiquitin ligase (Parkin) is shown to ubiquitinate TDP-43 *via* the ubiquitin lysines, K-48, and K-63. This facilitates the TDP-43's cytoplasmic accumulation into inclusions without any detectable evidence of its protein degradation (Seyfried et al., 2010; Hebron et al., 2013). The ubiquitin-conjugating enzyme UBE2E3 and ubiquitin-isopeptidase Y (UBPY) were identified, in a yeast two-hybrid screen, to interact with TDP-43 and this interaction is proposed to enhance the ubiquitination and accumulation of its insoluble high molecular weight aggregates (Hans et al., 2014). Notably, an FTLD-associated TDP-43 with K263E mutation was observed to be excessively ubiquitinated, possibly as a consequence of its misfolding due to the substitution of the positively charged lysine residue with a negatively charged aspartate residue in the RRM2 domain (Hans et al., 2014). Strikingly, Scotter et al. have demonstrated that the full-length TDP-43 aggregates are labeled by both K-48- and K-63-linked polyubiquitin chains and subsequently directed toward different fates: ubiquitin proteasomal-mediated degradation of TDP-43 for the K-48-linked polyubiquitin chains, and autophagic removal of the TDP-43 with K-63-linked polyubiquitin chains (Scotter et al., 2014). Additionally, using proteomics, several ubiquitination sites have also been identified near the TDP-43's RRM1 domain and about 35 proteins, including the RNA binding proteins rasGAP SH3 domain binding protein 1 (G3BP), poly(A)-binding protein cytoplasmic 1(PABPC1), and eukaryotic initiation factor 4A1 (eIF4A1), were found in the detergent-insoluble fractions containing the ubiquitinated TDP-43 (Dammer et al., 2012). Moreover, mutations at these ubiquitination sites were also found to decrease the TDP-43's accumulation thereby implicating the ubiquitination in modulating the TDP-43 aggregation (Dammer et al., 2012).

Acetylation

There are 20 lysine residues in TDP-43, some of which are prone to acetylation, such as the K-145 and K-192 (Cohen et al.,

2015; Wang P. et al., 2017). Using an acetylation mimic, where lysine was mutated to glutamine residue, the TDP-43 acetylation was shown to impair RNA binding, disturb mitochondrial functions, and promote accumulation of the insoluble and hyper-phosphorylated TDP-43 aggregates in the neuronal cell cultures (Cohen et al., 2015). In another study, arsenite-induced oxidative stress could trigger the TDP-43's acetylation and formation of aggregates of ∼75–250 kDa (Cohen et al., 2015; Wang P. et al., 2017). Additionally, an antibody Ac-K145 raised against the acetylation at the lysine 145 could, in fact, identify the lesions positive for acetylated TDP-43 in the ALS patient's spinal cord (Cohen et al., 2015; Wang P. et al., 2017). It remains to be examined whether any other lysines are prone to acetylation *in vivo* and if so, what are their effects on the TDP-43's aggregation. Understandably, even non-specific multi-site *in vivo,* or *in vitro* acetylation mediated through acetylating agents like aspirin, would dramatically alter the TDP-43's net charge, which can affect its aggregation propensity through electrostatic repulsions (Abdolvahabi et al., 2015; Ayyadevara et al., 2017; Prasad et al., 2018).

Poly ADP-Ribosylation

Poly ADP-ribosylation (or PARylation) is a post-translational modification that appears rapidly at the DNA damage sites, and has implications in cancer, cell cycle regulation, DNA repair pathways, and chromatin reorganization, etc. (Bai, 2015). Poly (ADP-ribose) polymerase (PARP) enzymes attach the ADP-ribose unit *via* an ester bond to the carboxyl group of the acidic residues, such as glutamate and aspartate on the target proteins. Polymeric PAR chains are formed when subunits are linked to one another *via* ribose-ribose bonds (Leung, 2014). The negative charge on PAR can alter the structure of the target proteins and modify the protein-DNA/RNA and the protein-protein interactions. In fact, PARylation has been found to induce phase separation of the intrinsically disordered proteins involved in ALS (Altmeyer et al., 2015). Preliminary data (Duan et al., 2018) suggests that hnRNPA1 and TDP-43 can both be PARylated and bind to PARylated proteins. The PARP enzyme, tankyrase, was shown to reduce the TDP-43's aggregation by non-covalently attaching PAR *via* PAR-binding motif present in the TDP-43 nuclear localization signal (NLS) sequence. PAR binding was found to promote the TDP-43's phase separation *in vitro* and was also shown to be essential for the TDP-43's accumulation in the stress granules in the mammalian cells and neurons (Mcgurk et al., 2018).

Cysteine Oxidation

In addition to the disulfide bridging for proper folding of proteins, cysteine residues also play an essential role in the maintenance of the cellular redox state. Altered cellular redox balance and oxidative stress have been proposed as contributory factors to the ALS pathology. Thus, cysteine oxidation may represent a crucial pathological pathway in ALS (Valle and Carri, 2017; Buratti, 2018). Using the *in vitro* and cell-based studies, Cohen et al. have reported that oxidative stress promotes the TDP-43's cross-linking *via* cysteine oxidation into disulfide bond formation. Among the six cysteine residues (C39, C50, C173, C175, C198, and C244) present in the TDP-43 protein, four cysteine residues at the positions 173, 175, 198, and 244, are highly conserved (in human, mouse, *Drosophila* and zebrafish) and can undergo oxidation and disulfide bond formation (Cohen et al., 2012). Importantly, the cysteine-generating ALS-linked missense mutations (G358C, S379C, and G295C) introduce additional cysteines which can potentially enhance the abnormal TDP-43 disulfide cross-linking. Notably, the inter- and intra-molecular cross-links can also result in alterations in the TDP-43's subcellular localization and solubility (Cohen et al., 2012). Structure-function analysis of the RRM1 domain has suggested that the cysteines (C-173 the C-175) in this domain, are crucial for the TDP-43's conformation and these are also involved in the pathogenic RRM1 self-assembly (Shodai et al., 2013). In another study, cysteines in the RRM2 domain (C-198 and C-244) could form self-aggregating disulfide-linked dimers upon oxidation and assembled into aggregated species (Rabdano et al., 2017). Notably, oxidation of the two N-terminal cysteines (C39 and C50) can contribute to oligomerization possibly by priming the process (seeding). Significant reduction in the oligomer formation, was observed when mutations were introduced at these positions (Bozzo et al., 2017). Another study has found that the intermolecular N-terminal cysteine disulfides result in the tetramerization of TDP-43 by formation of NTD homodimers first, and both the dimers and the tetramers inhibit the TDP-43 aggregation (Jiang et al., 2017). Cysteine residues are present in the NTD, RRM1, and RRM2 domains, can all be oxidized and result in the loss-of-function and aggregation of TDP-43 under both the *in vitro* and *in vivo* conditions. Proposedly, the oxidation-induced conformational change of RRM1 seems more crucial for the TDP-43's aggregation and the ALS pathology than the cysteine oxidation of the NTD and RRM2 domains (Chang et al., 2013). Recently, we have shown that a recombinantly purified TDP-43 C-terminal fragment, which encompasses the RRM2 domain, can spontaneously form cysteine-linked homodimers and can convert into amyloid-like aggregated species (Prasad et al., 2018).

AGGREGATION OF TDP-43

Amyloid-Like Aggregation of TDP-43

Whether TDP-43 deposited in the neuronal cells has amyloid-like aggregate features, is still debated. Early reports had suggested that the filament-like structure of TDP-43 found in the ALS-affected brains do not stain with the amyloid-specific dyes, thioflavin-T (ThT) and Congo red (Neumann et al., 2006; Johnson et al., 2009). From some ALS cases, thioflavin-S (ThS)/ThT-staining amyloid aggregates have now been reported (Bigio et al., 2013; Robinson et al., 2013). Considerable interest, therefore, exists in deciphering any potentially amyloidogenic behavior of TDP-43 both *in vivo* and *in vitro*.

Recombinantly expressed full-length TDP-43 has been shown to form smooth granulo-filamentous, ThT-negative aggregates *in vitro*, similar to those found in the degenerating neurons of the ALS and FTLD patients (Johnson et al., 2009; Furukawa et al., 2011). TEM has revealed a stacking of thin fibers into thicker bundles, which also exhibit sarkosyl insolubility (Furukawa et al.,

2011). Protease treatment of these full-length TDP-43 fibrillar aggregates, followed by mass spectrometry showed that the fibril core structure comprises of different C-terminal fragments spanning from the RRM1 to the C-terminal end (Furukawa et al., 2011). In yet another study, following the overexpression of TDP-43 in the bacterial cells, the TDP-43 inclusion bodies formed, were found also to be ThT-negative (Capitini et al., 2014).

However, in certain other studies, both wild-type and ALS-associated mutant TDP-43's peptides have been shown to efficiently form β-sheet-rich, ThT-positive fibrillar aggregates suggestive of their amyloid-like nature (Chen et al., 2010; Guo et al., 2011; Sun et al., 2011; Zhu et al., 2014) (**Table 2**). Different amyloidogenic cores for the TDP-43's aggregation have been defined from its C-terminal region, including the sequences: 286–331, 311–360, and 342–366 (Chen et al., 2010; Guo et al., 2011; Saini and Chauhan, 2011; Mompean et al., 2015; Jiang et al., 2016). The shortest peptides from TDP-43 that are shown to form amyloid-like aggregates are DLII (247–250) and NFGAF (312–316), which bear resemblance to the amyloidogenic core sequence of the human islet amyloid polypeptide (IAPP) (Furukawa et al., 2011; Saini and Chauhan, 2011, 2014; Prasad et al., 2016). Notably, TDP-43 peptides containing the ALS-linked mutations like A315T and G335D have been found to enhance amyloid-like aggregation with self-seeding and cross-seeding abilities (Guo et al., 2011; Jiang et al., 2016). It has been argued that the familial mutations in the C-terminal region increase the propensity of the short α-helices toward β-sheet structural transition (Sun and Chakrabartty, 2017).

High resolution structures have been obtained of the amyloidogenic peptides from the RRM2 domain and the low complexity domain (LCD) of TDP-43, which could adopt the characteristic amyloid steric zipper structures (Guenther et al., 2018a,b). An RRM2 peptide, aa 247–257, was shown to form distinct types of amyloid aggregates that fit into different classes of steric zipper structures. This polymorphic ability was attributed to its ability to adopt different backbone conformations (Guenther et al., 2018b). Furthermore, a peptide from the LCD region, aa 312–317, and its ALS-linked mutant variants, A315E and A315T, were also shown to form kinked β-sheet structures which promote the formation of phase separated droplets and hydrogels, unlike several other peptides of this LCD region (Guenther et al., 2018a).

Alike to as previously reported for the Amyloid β (Aβ)-42 peptide's amyloid aggregation, a low net charge on the TDP-43 protein decreases its solubility and improves its aggregation, whereas, with high net charge the electrostatic repulsions dominate, which can impede the aggregation of TDP-43 (Mompeán et al., 2016a). We have, in fact, recently explored the *in vitro* amyloidogenic aggregation of a C-terminal fragment (aa 193–414) of TDP-43 in the presence of different Hofmeister series anions. We found that kosmotropic anions greatly accelerate whereas the chaotropic anions impede its amyloid-like aggregation rates (Prasad et al., 2018). Amyloid fibril morphological features also varied in the presence of the kosmotropic vs. the chaotropic anions. Furthermore, *in vitro* aspirin-mediated non-specific lysine acetylations, which would mask the lysine's charges, significantly reduced the TDP-43's C-terminal fragment's amyloid-like aggregation (Prasad et al., 2018).

Physiological vs. Pathological Oligomerization of TDP-43

For several neurodegenerative diseases like the Alzheimer's, Parkinson's and prion diseases, the neuronal cytotoxicity is proposedly exerted through oligomeric forms of the aggregating proteins/peptides (Kayed et al., 2003; Haass and Selkoe, 2007). Recently, several studies have also examined TDP-43's oligomerization and its potential neurotoxic properties (**Table 2**). Evidence suggests that in the normal brain, TDP-43 exists in dimeric form predominantly in the neuronal cell nucleus (Kuo et al., 2009; Shiina et al., 2010; Afroz et al., 2017). The NTD region, especially its first 10 amino acids, appear to be indispensable for the dimerization (Chang et al., 2012; Zhang Y. J. et al., 2013; Mompean et al., 2017). Recently, cross-linking experiments have revealed that in the normal human brain, TDP-43 can exist not only as dimers, but rather in a spectrum of oligomeric species viz. dimers, trimers, tetramers and multimers (Afroz et al., 2017). This oligomerization is proposed to be important for the TDP-43's functional roles in the RNA binding, probably by its increased affinity and specificity for its RNA targets, and/or *via* optimal recruitment of the other RNA splicing factors.

In contrast, pathological forms of TDP-43 oligomers have also been reported (**Table 2**), which may be structurally distinct from the nuclear TDP-43 oligomers. Shiina et al. have reported that the N-terminal region (aa 3–183) acts as an intermolecular interacting domain in an 86 kDa dimeric form of TDP-43 overexpressed in the cells. Thus, they have proposed that the dimeric TDP-43 may seed the formation of the pathological higher molecular weight TDP-43 aggregates (Shiina et al., 2010). Indeed, expression of a tandem TDP-43 construct expressing TDP-43 repeat as an 86 kDa protein in the HEK293 cells, induced the accumulation of TDP-43 aggregates. Furthermore, an 86 kDa species was also observed in an immunoblot of extracts from the deceased ALS brains (Shiina et al., 2010).

Fang et al. have reported that the full-length TDP-43 forms spheroidal and ring-like oligomeric structures with cytotoxicity to the neuronal cells (Fang et al., 2014). Following purification of recombinantly expressed full-length TDP-43 by size exclusion chromatography, DLS and TEM analyses have shown that the fractions containing oligomeric TDP-43 have a size distribution of 40–400 nm. The TDP-43 oligomers also manifest a propensity to cross-seed Aβ-42 peptide thereby demonstrating a structural inter-convertibility among the common amyloid oligomeric structures (Kayed et al., 2003; Fang et al., 2014). TEM analysis of gold immunolabelled FTLD-TDP brain fractions has revealed TDP-43 oligomers with a diameter of $\sim$ 50 nm (Fang et al., 2014; Kao et al., 2015). Furthermore, polyclonal antibodies raised against the TDP-43 oligomers (TDP-O) could not only detect the oligomeric aggregates obtained *in vitro*, but more importantly also the oligomers from the brain sections of the TDP-43 mice

model and also those from the FTLD-TDP affected patients. This is a step forward toward the development of TDP-43 oligomer detection as a biomarker for ALS.

In a recent study, beneficial forms of TDP-43 oligomers have been identified in the skeletal muscles (Vogler et al., 2018). These SDS-resistant oligomers were found to be distinct from those observed in stress granules, and were termed as myo-granules. Furthermore, the myo-granules exhibited amyloid-like characteristics. X-ray diffraction of the lyophilized myo-granules showed a diffraction pattern with a 4.8 Å reflection indicating a β-sheet-rich structure, however they lacked a 10 Å reflection which suggests that these myo-granules lack the typical cross β-sheet arrangement. The TDP-43 myo-granules seem functionally significant as they contain the mRNAs that encode for proteins involved in the formation of sarcomeres (Becker and Gitler, 2018; Vogler et al., 2018).

Prion-Like Behavior of TDP-43 Aggregates

The fatal human neurodegenerative diseases Creutzfeldt-Jakob Disease (CJD) and Kuru involve deposition of the infectious prion protein PrP in aggregated amyloid-like conformation in the affected brains (Aguzzi et al., 2008; Aguzzi and Calella, 2009). Prions were first proposed by Stanley Prusiner to be novel "protein-only" infectious agents (Prusiner, 1982). Fungi, such as yeast and *Podospora* have also been found to harbor prion-like elements (Wickner, 1994; Derkatch et al., 2001; Maddelein et al., 2002; Patel et al., 2009; Liebman and Chernoff, 2012). Several of the fungal prions have been vividly shown to infect in a "protein only" fashion (King and Diaz-Avalos, 2004; Tanaka et al., 2004; Patel and Liebman, 2007). The transmissibility of the infectious prion aggregates is attributed to their exceptional protease and detergent resistance and to their ability to propagate from cell-to-cell and organism-to-organism by "seeding" to induce more pathological aggregates (Caughey et al., 2009; Cobb and Surewicz, 2009). In fact, several yeast prions can also influence the aggregation and/or toxicity of certain human amyloidogenic proteins, such as poly-glutamine, transthyretin and TDP-43 etc., proposedly *via* heterologous cross-seeding or by influencing the chaperone availability (Derkatch et al., 2001; Meriin et al., 2002; Park et al., 2017; Verma et al., 2018).

Accumulating evidence suggests that several other proteins previously considered as non-prion proteins, including Aβ-42, α-synuclein, and TDP-43 etc., can exhibit prion-like behavior both *in vitro* and in the disease models (Brundin et al., 2010; Hock and Polymenidou, 2016). In the case of TDP-43, Furukawa et al. have reported that the transduction of pre-formed, sarkosyl-insoluble, fibrillar aggregates of the recombinantly expressed full-length TDP-43 into the HEK293T cells expressing TDP-43, induces the aggregation of the endogenous TDP-43 into detergent-insoluble and ubiquitinated inclusions, similar to those observed in the ALS patients (Furukawa et al., 2011). In another pivotal study, Nonaka et al. have identified different strains of the TDP-43 aggregates from the ALS/FTLD diseased brains (Nonaka et al., 2013). When these TDP-43 aggregates were introduced into SH-SY5Y human neuroblastoma cells expressing TDP-43, seed-dependent formation of the insoluble TDP-43 inclusions was observed, resembling the pathological profiles of the parent TDP-43 seeds used. Also, the TDP-43 aggregates could be propagated between the cells over serial passages thereby further supporting their prion-like behavior. Additionally, the seeding ability of the insoluble TDP-43 was unaffected by heat or proteinase treatment, but was abrogated by formic acid, indicating that the β-sheet structure of these aggregates is important for the seeding capability (Nonaka et al., 2013).

In another study, oligomeric TDP-43 obtained from sorbitol-induced osmotic stress in the HEK293 cells, as well as oligomers derived from the ALS brain lysates, were found to show seeding ability and intercellular transmission *via* microvesicles/exosomes (Feiler et al., 2015). Additionally, Smethurst et al. have identified a diverse range of TDP-43 inclusion bodies, such as skeins, dot-like, and granular etc., upon seeding with the pathological TDP-43 aggregates from different ALS patient's brains into the cells expressing full-length TDP-43, possibly indicating strain-like propagation characteristic of prions. In fact, repeated inoculation with the TDP-43-containing insoluble fractions showed a consistent increase in the accumulated TDP-43 thereby supporting prion-like seeded aggregation and a capability of intercellular transmission (Smethurst et al., 2016).

In a recent study, Ishii et al. have demonstrated a time-course microscopy of formation of phosphorylated and ubiquitinated TDP-43 aggregates in electron-dense granules and intercellular spreading of these TDP-43-positive granules into the cells subjected to MG-132-induced stress and expressing wild-type and C-terminal region of TDP-43 (Ishii et al., 2017). In a separate study, 40 amino acid long peptides (spanning aa 274–313 and 314–353) could form amyloid-like fibrils *in vitro* and transduction of these amyloid fibrils induced inclusion body formations that contained phosphorylated C-terminal TDP-43 fragments in the SH-SY5Y cells expressing the wild-type TDP-43 (Shimonaka et al., 2016). Furthermore, prion strain-like behavior was also observed when trypsin digestion of the TDP-43-positive, sarkosyl-insoluble fractions, was found to show different TDP-43 band patterns from the 274–313 and 314–353 fibril-treated cells, indicating template-dependent aggregation (Shimonaka et al., 2016). The TDP-43 oligomers involved in the seeded aggregation and prion-like transmission can be therapeutic targets hence how they propagate need detailed elucidation.

Phase Separation of TDP-43

An increasingly recognized process being implicated in several neurodegenerative diseases is the formation of membraneless liquid droplet-like organelles by the proteins containing prion-like domains through a process called liquid-liquid phase separation (LLPS) (**Figure 5**) (Shin and Brangwynne, 2017). Several RNA binding proteins like TDP-43, FUS, hnRNPA1 and hnRNPA2/B1 etc., contain intrinsically disordered regions and can undergo phase separation through transient intermolecular interactions (Burke et al., 2015; Lin et al., 2015; Molliex et al., 2015; Patel et al., 2015; Conicella et al., 2016; Batlle et al., 2017; Gopal et al., 2017; Li et al., 2017; Sun and Chakrabartty, 2017; Uversky, 2017). Proteins with a prion-like low complexity

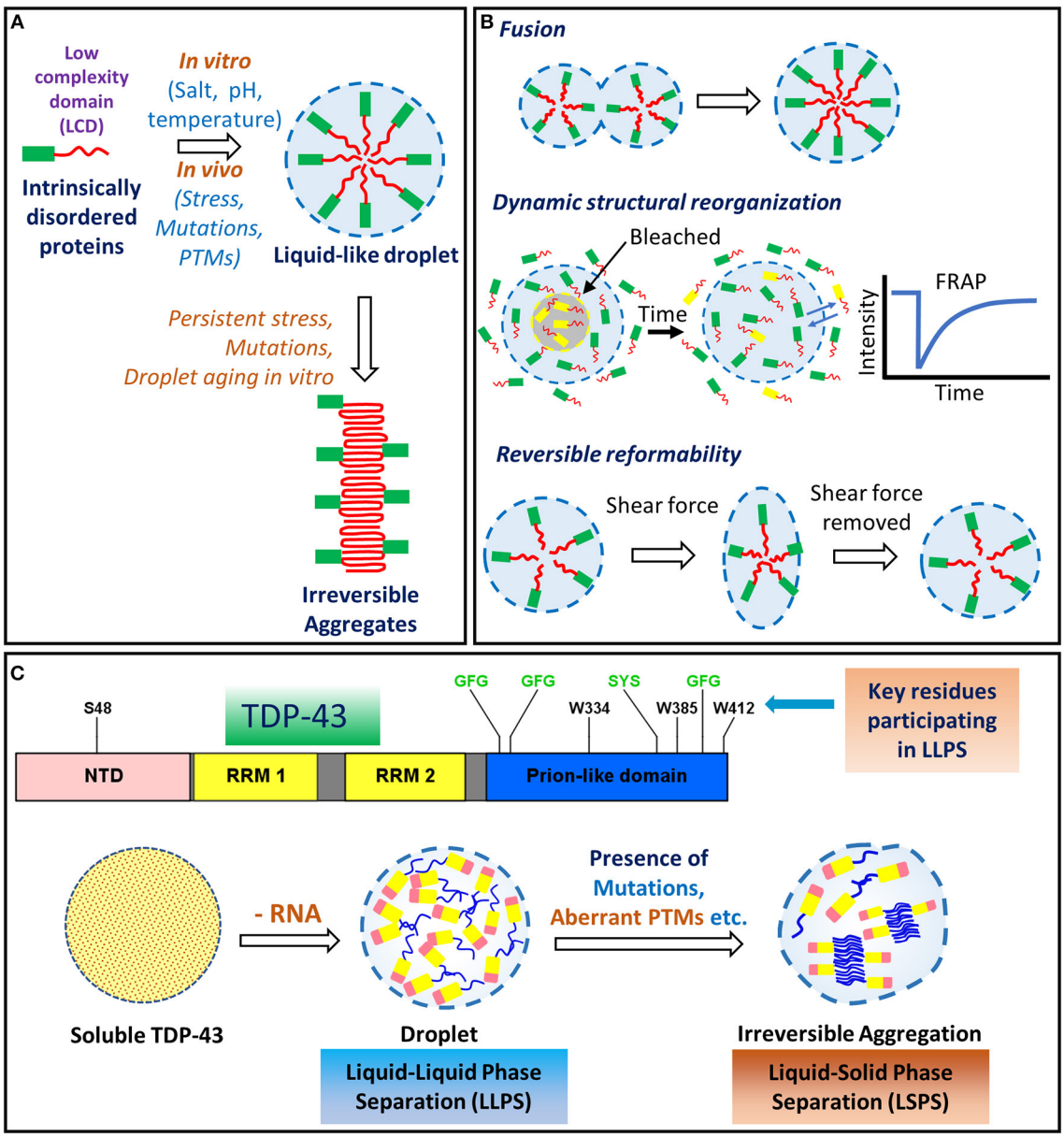

FIGURE 5 | Liquid-liquid phase separation (LLPS) and liquid-solid phase separation (LSPS) of TDP-43. **(A)** Proteins containing low complexity/prion-like domains undergo phase-separation into membrane-less, spherical compartments, often aided by the presence of salt, pH changes or temperature changes. Persistent stress, mutations and droplet-aging, might induce irreversible aggregation into pathological structures, such as the amyloid-like aggregates. **(B)** Liquid droplet-like properties are manifested by the intrinsically disordered proteins, such as: the ability of the smaller droplets to freely fuse into a larger droplet; transient intermolecular interactions allowing the dynamic rearrangement of the internal structural components; and reversible reformability upon removal of the external shear forces. **(C)** Liquid-liquid phase separation (LLPS) of TDP-43 is influenced by both hydrophilic and hydrophobic residues. The (G/S)-(F/Y)-(G/S) motifs (highlighted in green) promote the phase separation through transient interactions in several intrinsically disordered proteins (Li et al., 2018). The tryptophan residues promote LLPS by hydrophobic interactions (Li et al., 2018). Depletion of the TDP-43's interactions with RNA molecules, upon high protein: RNA ratio, can lead to irreversible aggregation *via* Liquid-solid phase separation (LSPS) (Maharana et al., 2018). ALS-linked mutations are also proposed to lead to the formation of the irreversible aggregates. FRAP, fluorescence recovery after photobleaching; LCD, Low complexity domain; LLPS, liquid-liquid phase separation; LSPS, liquid-solid phase separation; NTD, N-terminal domain; PTM, post-translational modification; RRM, RNA recognition motif.

domain (LCD), exhibit in this region, an over-representation of polar and charged amino acids including arginine, lysine, glutamine, serine, glutamic acid and occasionally glycine, alanine and proline with interspersed aromatic residues, particularly tyrosine and phenylalanine (Shin and Brangwynne, 2017). LLPS behavior appears to be driven by transient intermolecular interactions, such as the hydrophobic, cation-pi and pi-pi interactions, as well as the charge patterning of the polar and

charged amino acids in the prion-like LCD domains (Shin and Brangwynne, 2017; Simon et al., 2017).

Phase-separated droplets of the ALS-linked FUS mutants were found to display a propensity to mature into amyloid-like fibrillar aggregates (Patel et al., 2015). Hence, LLPS appears to be an immense risk factor as the transient localization of the intrinsically disordered proteins into the droplets under stress conditions, possess the peril of their conformational transitions within the liquid compartments into pathological irreversible aggregates. The phase separation behavior of the RNA binding proteins, seems closely associated with their propensity to form stress granules (Molliex et al., 2015; Protter and Parker, 2016; Riback et al., 2017).

In one study, although mutant TDP-43 droplets did show irregular morphologies, the ThT staining was not indicative of amyloid-like features (Conicella et al., 2016). Conicella et al. have reported that prion-like TDP-43's C-terminal region (aa 276–414) undergoes phase separation *in vitro* in the presence of salt and RNA. Interestingly, certain ALS-associated TDP-43 mutations, such as A321G, Q331K, and M337V, have been found to decrease the phase separation ability and increase the propensity to aggregate with irregular morphology (Conicella et al., 2016). Structural analysis has shown that a tryptophan residue, W334, in the α-helical segment (aa: 320–340) is crucial for the TDP-43's prion-like domain's phase separation (Li et al., 2017, 2018). Wang et al. suggest that a phosphomimetic substitution at S48 in the NTD disrupts the TDP-43's LLPS and decreases the NTD's polymerization, and thus, it is a conserved phosphorylation site found to be phosphorylated at low levels in the ALS *in vivo* models (Wang et al., 2018). Strikingly, the poly(ADP-ribose) polymerase, tankyrase, was found to modify TDP-43 by adding negatively charged poly(ADP-ribose) polymer to its nuclear localization signal sequence, which promoted LLPS and facilitated the TDP-43's accumulation into stress granules in the neuronal cells (Mcgurk et al., 2018).

Recently, Gopal et al. have shown that TDP-43 containing RNP transport granule, in the axonal cells, display droplet-like properties, such as spherical shape, fusion, deformability upon shear force, rapid internal TDP-43 redistribution and sensitivity to disruption of the weak hydrophobic interactions by 1,6-hexanediol treatment. Also, ALS-linked TDP-43 mutations like M337V and G298S were found to display increased granule viscosity and disrupted axonal transport functions (Gopal et al., 2017). Strikingly, depletion of the TDP-43's interaction with the RNA molecules in cells, upon high protein:RNA ratio, was recently found to cause TDP-43's irreversible aggregation *via* liquid-solid phase separation (LSPS) (Maharana et al., 2018). Thus, finding modulators of the phase separation may have tremendous therapeutic potential.

EMERGING MECHANISMS OF TDP-43-INDUCED CYTOTOXICITY

Dysregulation of TDP-43 Protein Turnover

Protein homeostasis in a cell is maintained *via* ubiquitin-proteasome system (UPS), autophagy and ER stress-activated unfolded protein response (UPR). Abnormal turnover of TDP-43 caused by mislocalization and aggregation appears as a key event for ALS and aberrations in the neuronal proteostasis have been identified in ALS (Braun, 2015; Budini et al., 2017; Ramesh and Pandey, 2017) (**Figure 6**).

TDP-43 has been found to be involved in the regulation of autophagy by associating with the mRNA of a key autophagy-associated protein ATG7 (autophagy related 7), however, some of the ALS-linked TDP-43 mutations can abolish its ATG7 mRNA binding ability (Bose et al., 2011). TDP-43 can also affect the localization of the transcription factor TFEB (transcription factor EB) which regulates the expression of several autophagy lysosomal pathway proteins in the neuronal cells (Xia et al., 2016). Inclusion bodies positive for autophagy markers like LC3 and p62/SQSTM1, have been identified in the ALS and FTLD patients' spinal cords suggesting the involvement of autophagy in the ALS disease progression (King et al., 2010a; Budini et al., 2017). The ALS-associated mutations in *UBQLN2* cause impaired autophagy and induce increased overall TDP-43 levels and promote the TDP-43 aggregation in the neuronal cells (Osaka et al., 2016). Araki et al. have found that the disease-associated TDP-43 mutants like G298S and A382T, are more rapidly turned over than the wild-type protein, through the ubiquitin-proteasome system, thus highlighting the pathological relevance of the TDP-43 proteolysis and clearance (Araki et al., 2014).

The role of autophagy in rescuing TDP-43-associated toxicity might be a complex process as suggested by conflicting data showing that autophagy can either accelerate or slow down disease progression (Barmada et al., 2014). In a systematic genetic screen in the yeast cells expressing TDP-43, it was found that the vacuolar fusion machinery and the endo-lysosomal pathways are critical for the TDP-43 clearance and for maintaining the cell survival. Strikingly, the autophagy pathway that contributed to the TDP-43 clearance was also found to increase cytotoxicity (Leibiger et al., 2018). Filimonenko et al. have reported that TDP-43 accumulation increases in the cells with defective autophagy processes. The endosomal sorting complexes required for transport (ESCRT) are important proteins involved in the autophagy pathway. Depletion of ESCRT subunits results in the formation of multivesicular bodies (MVBs) with abnormal morphology. In ESCRT-depleted cells, TDP-43 was found to accumulate in the ubiquitin-positive inclusions (Filimonenko et al., 2007).

The full-length TDP-43 and its fragments, are also known ubiquitin substrates that are directed for degradation either through the ubiquitin-proteasome system (UPS) or autophagy. Early studies suggested that the soluble as well as the aggregated TDP-43 are cleared by both the ubiquitin-proteasome system (UPS) and autophagy (Urushitani et al., 2009; Wang et al., 2010; Zhang et al., 2010). Recently, Scotter et al. have shown that the soluble TDP-43 is mainly degraded by the ubiquitin-proteasome system (UPS), whereas the cytotoxic aggregated forms of TDP-43, are preferentially removed through autophagy (Scotter et al., 2014). Barmada et al. have identified potent compounds from a pharmacophore library that can significantly stimulate neuronal autophagy and enhance TDP-43 turnover, thereby improving the

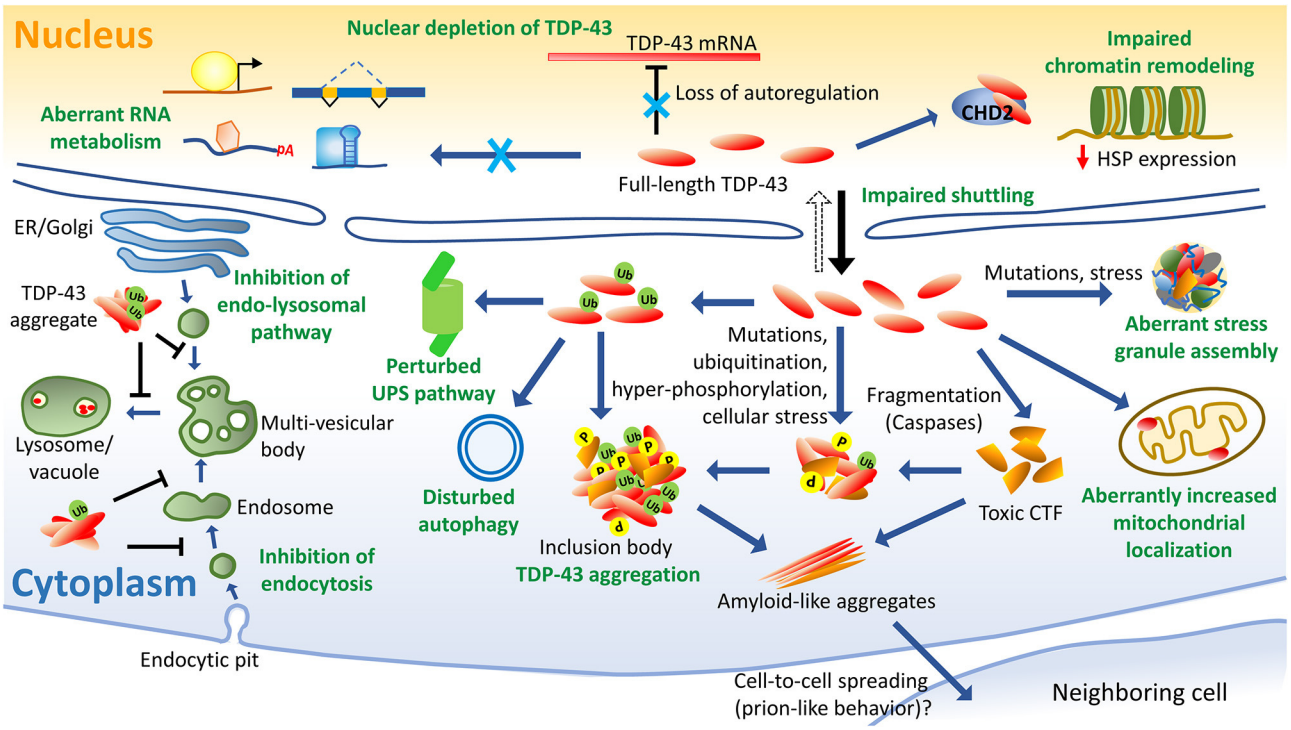

FIGURE 6 | Schematics of TDP-43-induced pathology. Several aspects of TDP-43-linked cellular dysfunctions have been identified in ALS, such as nuclear depletion which leads to aberrant RNA metabolism and a loss of autoregulation of TDP-43 levels. Cytoplasmic accumulation of the hyper-phosphorylated and ubiquitinated TDP-43 are ALS disease hallmarks. Fragmentation of TDP-43 leads to the formation of toxic and aggregation-prone C-terminal fragments (CTFs). TDP-43 mutations can lead to abnormal stress granule assembly and release. Aberrantly increased mitochondrial localization of TDP-43 impairs its function. TDP-43 is also associated with the misregulated autophagy and proteosomal processes. TDP-43 expression perturbs the endocytosis process possibly by altering the expression of key endocytic components. Also, the TDP-43 aggregates have been identified as an inhibitor of the endolysosomal pathway. TDP-43 interacts with chromatin remodeling protein CHD2 and perturbs the chromatin dynamics which prevents the expressions of heat shock proteins. Prion-like inter-cellular propagation of detergent-resistant, β-sheet-rich aggregates of TDP-43, has also been demonstrated in the neuronal cell models. CHD2, chromodomain helicase DNA binding protein 2; CTF, C-terminal fragments; ER, endoplasmic reticulum; HSP, heat shock protein; P, phosphorylation; Ub, ubiquitination; UPS, ubiquitin-proteasome system.

growths of the primary neurons, human iPSC-derived neurons and astrocytes (Barmada et al., 2014).

Impairment of Endocytosis

Defective endocytosis might be a contributory factor for the TDP-43's toxicity in ALS. Abnormal levels of TDP-43 inhibit endocytosis by co-localizing with the endocytosis-associated proteins in the yeast cells and cellular models, and such co-localization was also observed in an ALS patient's frontal cortex tissue (Liu et al., 2017). Impaired endocytosis has been linked with increased TDP-43 aggregation, while enhancing endocytosis was found to reverse the TDP-43 toxicity and the motor neuron dysfunction (Liu et al., 2017). In another study, TDP-43 knockdown was found to specifically reduce the number and motilities of recycling endosomes in the human iPSC-derived neurons, whereas TDP-43 overexpression caused the opposite effect (Schwenk et al., 2016). Furthermore, TDP-43 knockdown was also seen to affect the dendrite growth by decreasing the expression of the cell surface receptors crucial for neuronal growth and survival (Schwenk et al., 2016). Leibiger et al. have also shown that the endocytosis and the endo-lysosomal pathway are markedly disturbed by TDP-43 expression (**Figure 6**). The endo-lysosomal pathway could also contribute to TDP-43 clearance independent of autophagy. The importance of the endo-lysosomal pathway in TDP-43 pathology is highlighted by the observation that certain ALS-associated genes encode for components of this pathway, e.g., charged multivesicular body protein 2B (CHMP2B) (Leibiger et al., 2018).

Aberrantly Increased Localization of TDP-43 to Mitochondria and Associated Toxicity

Neuronal vulnerability to the mis-localized and/or aggregated proteins, has often been found to be mediated *via* dysfunctional mitochondria (Saxena and Caroni, 2011). Post-mitotic neurons have high demands for ATP for the maintenance of the ionic gradients across cell membranes and for the intracellular communication (Kann and Kovacs, 2007; Verkhratsky et al., 2014). Hence, defects in the mitochondrial transport, mitochondrial length, intracellular Ca^{2+} levels, mitochondrial respiration and ATP production, can severely impede the proper functioning of neurons and can accelerate neurodegeneration [reviewed in (Lin and Beal, 2006; Reddy, 2009; Johri and

Beal, 2012; Lezi and Swerdlow, 2012; Smith et al., 2017)]. Mitochondrial dysfunction has been recorded in both *in vivo* and *in vitro* models expressing the wild-type TDP-43 or its mutants, thus implicating the mitochondria as a route for mediating the TDP-43 toxicity (Braun et al., 2011; Wang W. et al., 2013; Stribl et al., 2014) (**Figure 7**).

Over-expression of TDP-43, or its mutants, in the primary motor neurons has been found to result in reduction of the mitochondrial length and impaired mitochondrial movement which could be reversed upon the co-expression of the mitochondrial fusion protein, mitofusin-2 (Mfn2) (Wang W. et al., 2013). Also, in the mouse model, expression of the mutant TDP-43 resulted in abnormal mitochondrial transport and distribution (Magrane et al., 2014). Furthermore, expression of TDP-43 in the fly model was documented to enhance mitochondrial fission and fragmentation thereby suggesting an impairment of the mitochondrial dynamics (Altanbyek et al., 2016). In the yeast model, TDP-43 expression was found to cause increased oxidative stress and formation of peri-mitochondrial TDP-43 aggregates. Importantly, presence of functional mitochondria was observed to exacerbate the deleterious effects of TDP-43 hence implicating mitochondria as a target for mediating the TDP-43 toxicity (Braun et al., 2011; Braun, 2012). Recently, TDP-43 and its disease-associated mutants have been found to significantly enhance the mitochondrial abnormalities across various models thereby reflecting the mitochondrial dysfunction observed in the ALS patients (Wang W. et al., 2013; Wang et al., 2016).

Mitochondria are the known primary sites for the production of reactive oxygen species (ROS) and also as the major target of the ROS-induced damage. Protein oxidation/carbonylation, lipid peroxidation, depletion of anti-oxidants like glutathione, increase in the intracellular free iron content and damage to DNA, are widely used markers of oxidative stress (Farrugia and Balzan, 2012) (**Figure 7**). Strikingly, mutant TDP-43 was found to induce oxidative damage and cause increased accumulation of the anti-oxidant response modulator, Nrf2, in the nucleus (Duan et al., 2010). Subsequently, it was also found that, although TDP-43 increased the localization of Nrf2 to the nucleus, the total expression of Nrf2 was, in fact, markedly decreased and the Nrf2/ARE pathway was impaired in the NSC-34 cell lines resulting in reduced neurites and increased lipid peroxidation products (Tian et al., 2017). Also, expression of TDP-43 in *Drosophila*, was recorded to increase the levels of protein carbonylation and glutathione S-transferase D1 (Carri et al., 2015; Zhan et al., 2015). Recently, by developing an easy red/white color assay, we have confirmed that the TDP-43 aggregation also induces oxidative stress in the yeast TDP-43 aggregation model (Bharathi et al., 2016, 2017).

TDP-43 aggregation and oxidative stress seem to mutually abet each other. Depletion of glutathione using ethacrynic acid increases the insolubility of TDP-43 and also promotes the fragmentation of TDP-43 in the primary cortical neurons (Iguchi et al., 2012) (**Figure 7**). Consistent with this, modification of TDP-43 with the product of lipid peroxidation, 4-hydroxynonenal, has been observed to result in a considerable increase in the insolubility and cytosolic localization of TDP-43 in COS-7 cells (Kabuta et al., 2015). Recently, increasing intracellular reduced glutathione (GSH) by treating the TDP-43 mutant expressing cells with GSH monoethyl ether, has been shown to reduce the aggregate formation, ROS generation and cell death (Chen et al., 2018). Furthermore, on subjecting the TDP-43 expressing cells to various oxidants, it was found that the cysteine oxidation and disulfide bond formation promoted the aggregation of TDP-43 (Cohen et al., 2012). In agreement, oxidation of cysteine residues in the RRM1 domain enhanced protein aggregation and inhibited the nucleic-acid binding ability of TDP-43 (Chang et al., 2013). In summary, the interplay of TDP-43 aggregation and oxidative stress instigate the toxicity of TDP-43 as well as its deleterious effects on the mitochondria.

Interestingly, superoxide dismutase 1 (SOD1), which is also implicated in ALS pathology, is transported to the mitochondria *via* translocase of the outer membrane (TOM) complex, even though SOD1 lacks a mitochondrial localization signal. Mutant SOD1 accumulates in the intermembrane space (IMS) and matrix of mitochondria and elicits toxicity (Zeineddine et al., 2017). Misfolded SOD1 also aggregates on the outer mitochondrial membrane (OMM) and is involved in mitochondria dependent apoptosis. Of note, the addition of exogenous mutant SOD1 aggregates has been reported to cause cytoplasmic mislocalization of TDP-43 and enhance its aggregation (Zeineddine et al., 2017) (**Figure 7**). Also mutant SOD1 expression has been found to increase the C-terminal fragmentation and phosphorylation of TDP-43 and the interaction of the mutant SOD1 with TDP-43 fragments has been speculated to mediate toxicity *via* apoptosis (Jeon et al., 2018).

The mechanistic details of how TDP-43 damages the function of mitochondria are now being uncovered. Expression of mutant TDP-43 disrupts the ER-mitochondrial connection by disturbing the interaction of the ER protein Vesicle associated membrane protein (VAPB) and the mitochondrial protein tyrosine phosphatase interacting protein (PTPIP51) and it also reduces the uptake of calcium by mitochondria, which has detrimental effects on the Ca^{2+}-dependent ATP synthesis pathway and the transportation of mitochondria in the neuron (Stoica et al., 2014). Notably, the loss of mitochondria-ER contact *via* the loss of VAPB-PTPIP51 contact, stimulates autophagy (Gomez-Suaga et al., 2017). It is known that reduced fusion and simultaneously increased mitochondrial fission can have damaging effects on the post-mitotic neurons. Of note, the overexpression of TDP-43 also promotes mitochondrial fragmentation with a concurrent increase in the levels of mitochondrial fission factors, dynamin related protein 1 (Drp1) and fission 1 (Fis1) (Xu et al., 2010). ALS patient-derived fibroblast cells carrying TDP-43 mutations have been reported to exhibit significantly increased Drp1 recruitment to the mitochondria and enhanced mitochondrial fragmentation. In fact, a selective peptide inhibitor of Fis1/Drp1 called P110 was found to greatly reduce this mitochondrial dysfunction thereby directly implicating the high levels of Drp1 in mitochondrial toxicity (Joshi et al., 2018) (**Figure 7**).

Cytoplasmic accumulation of TDP-43, which is a pathological feature of ALS, results in unsolicited interaction with various cellular organelles, primarily the mitochondria (Scotter et al.,

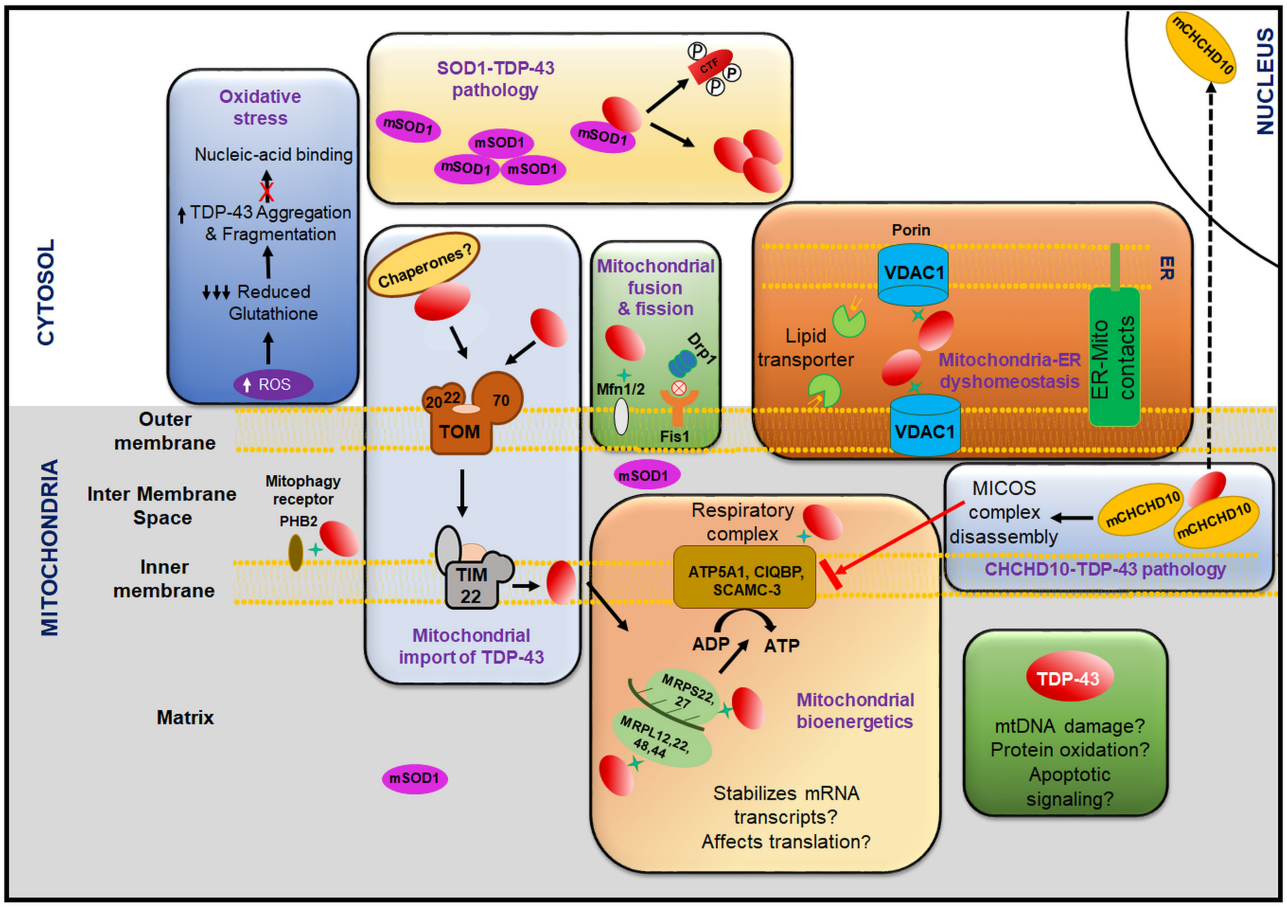

FIGURE 7 | Role of mitochondria in the TDP-43 pathology. TDP-43 mediated dysfunction of the mitochondria leads to increase in the production of ROS that causes decline in the reduced glutathione levels which in turn can increase the aggregation of TDP-43 and also inhibit TDP-43 from binding to the nucleic acid. Mutant SOD1 can cause cytoplasmic mislocalization, fragmentation, phosphorylation and aggregation of TDP-43. Inhibition of the interaction of the mitochondrial fission proteins Drp1 and Fis1 greatly reduces the mitochondrial dysfunction caused by the TDP-43 overexpression/aggregation. TDP-43 also disrupts the ER-mitochondrial contacts which can have potential implications to the calcium signaling, ATP production and lipid transport. TDP-43 is imported into mitochondria *via* the outer membrane complex (TOM70) and across the inner membrane *via* TIM22. Several factors like chaperones and mitochondrial membrane potential might play a role in the TDP-43 import. After internalization, TDP-43 is found to interact with several proteins involved in the mitochondrial translation machinery (MRPS22, 27 and MRPL12, 22, 48, 44) and the mitochondrial respiratory complex (ATPA, CIQBP, SCAMC-3). TDP-43 also perturbs the translation of ND3/6 of the respiratory complex I and thus severely impairs the mitochondrial bioenergetics and reduces the ATP production. TDP-43 overexpression alters the CHCHD10 localization from the mitochondria to the nucleus and the loss-of-function mutations in *CHCHD10* are associated with MICOS complex disassembly and may negatively regulate the assembly of the respiratory complex. TDP-43 also interacts with other crucial mitochondrial proteins including the mitochondrial fusion protein Mfn2 and the mitophagy receptor PHB2. TDP-43 is depicted here by the red oval structure. TDP-43 interaction with the mitochondrial proteins are depicted *via* green star. Inhibition is denoted by red cross mark. ATPA, ATP synthase subunit A; CHCHD10, coiled-coil-helix-coiled-coil-helix domain containing 10; CIQBP, complement component 1Q binding protein; CTF, C-terminal fragments; Drp1, dynamin related protein 1; ER-Mito contacts, endoplasmic reticulum (ER)-mitochondria contacts; Fis1, fission 1 (mitochondrial); Mfn2, mitofusin-2; MICOS, mitochondrial contact site and cristae organizing system; MRPL, mitochondrial ribosomal protein (large subunit); MRPS, mitochondrial ribosomal protein (small subunit); ND, NADH dehydrogenase; P, phosphorylation; PHB2, prohibitin-2; ROS, reactive oxygen species; SCAMC-3, small calcium-binding mitochondrial carrier protein 3; SOD1, superoxide dismutase 1; TIM, translocase of inner membrane; TOM, translocase of outer membrane.

2015). In 2012, full length and truncated TDP-43 proteins expressed in the motor neuron-like NSC-34 cells, were found to localize with mitochondria and cause its dysfunction (Hong et al., 2012) (**Figure 7**). Earlier in 2009, a proteomic study aimed to identify the interacting partners of TDP-43, revealed interactions with several mitochondrial ribosomal proteins (both the small and large subunit proteins) and several mitochondrial respiratory complex proteins including the alpha subunit-1 of mitochondrial F1 complex of ATP synthase (ATP5A1) (Freibaum et al., 2010). Recently, a proteomic screen has also revealed several TDP-43-interacting mitochondrial proteins including: mitochondrial porin, voltage-gated anion channel 1 (VDAC1), a crucial mitophagy receptor, prohibitin 2 (PHB2), mitochondrial fusion protein, mitofusin-2 (MFN2) and the mitochondrial respiratory complex proteins, ubiquinol-cytochrome creductase core protein I and II (UBQCRC1 and UBQCRC2) (Davis et al., 2018).

Inhibition of the TDP-43′s mitochondrial localization has been shown to rescue the TDP-43-mediated toxicity (Wang et al., 2016). Unlike the antioxidant enzyme SOD1-mediated

ALS pathology, which is mostly attributed to the mitochondrial dysfunction, TDP-43 is believed to cause toxicity also by its RNA/DNA-binding regions (Bozzo et al., 2017). However, the observed presence of TDP-43 in the inner mitochondrial membrane fraction, and its preferential binding to the mitochondrial ND3 and ND6 mRNAs that encodes for the respiratory complex I subunits, have brought the focus back on the role of mitochondrial pathways in the TDP-43 toxicity (Wang et al., 2016). In fact, in a transgenic mouse model expressing the TDP-43 M337V mutant, inhibition of the mitochondrial localization could relieve the cognitive dysfunction and restore the mitochondrial function (Wang W. et al., 2017). This consolidates the interaction of TDP-43 with mitochondria as one of the crucial mechanisms in eliciting toxicity.

Mutations in the coiled helix domain containing 10 (CHCHD10) protein are linked to ALS, and the mutant CHCHD10 protein molecules are localized to the intermembrane space of mitochondria and are also found to interact with TDP-43 (Lehmer et al., 2018). CHCHD10 protein is involved in organizing of the cristae morphology and thereby playing a vital role in the mitochondrial integrity (Woo et al., 2017). Loss of function mutations in CHCHD10 are associated with the disassembly of mitochondrial contact site and cristae organizing system (MICOS) which has negative impact on the assembly of respiratory chain complex (Genin et al., 2016) (**Figure 7**). TDP-43 overexpression alters the CHCHD10 localization from the mitochondria to the nucleus and loss-of-function mutations in CHCHD10 induces cytoplasmic accumulation of TDP-43 (Woo et al., 2017). Interestingly, loss of mitochondrial integrity caused by mutations in CHCHD10 has been shown to be independent of the mitochondrial localization of TDP-43 (Genin et al., 2018).

Recently, when the A315T TDP-43 mutant was expressed in the mice model, though mitochondrial localization was detected there was no significant alteration in the mitochondrial bioenergetics, especially the oxidative phosphorylation (Kawamata et al., 2017). On the contrary, increased mitochondrial calcium uptake was observed, the potential implications of which need further investigation. As TDP-43 has been shown to bind to and stabilize the intermediates of the mitochondrial transcripts, including the electron transport chain transcripts, and as a considerable amount of TDP-43 is transported into the mitochondria even under normal conditions, furthermore studies are required to unearth the details of the molecular mechanisms of TDP-43 function and toxicity in relation to mitochondria (Izumikawa et al., 2017).

Dysregulation of Metal Ion Homeostasis

The dysregulation of metal ion homeostasis has been implicated in a number of neurodegenerative diseases (Gaeta and Hider, 2005; Lovejoy and Guillemin, 2014; Chen P. et al., 2016). Increased metal ion levels can impart physiological insults like oxidative stress, mitochondrial dysfunction, protein misfolding, DNA damage, and ER stress etc. (Roos et al., 2006; Wright and Baccarelli, 2007; Dang et al., 2014). Strikingly, increased iron and iron-associated protein levels have been found in the ALS patients' brain cortex and blood sera (Veyrat-Durebex et al., 2014; Yu et al., 2014). Recently, in a TDP-43 mice model expressing the TDP-43 A315T mutant, a significant increase in the levels of zinc, manganese and copper ions were observed as compared to the control mice expressing the wild-type TDP-43 (Dang et al., 2014). The mechanism of the metal dysregulation caused by this mutant variant and the reason for the involvement of the spinal cord cells are unclear, however, the increment in the levels of these metal ions could be attributed to the oxidative stress and mitochondrial dysfunction, as observed by the elevated amounts of oxidized proteins in the spinal cord (Dang et al., 2014). In another study, zinc ions have also been shown to increase the TDP-43 aggregation propensity *in vitro* (Caragounis et al., 2010). On the contrary, certain copper-based complexes, such as Cu^{II}(atsm) and Cu^{II}(gtsm), have shown potential to significantly improve the phenotypes of the TDP-43- and SOD1-associated toxicity in the transgenic mice and the neuronal cell models (Parker et al., 2012; Roberts et al., 2014; Williams et al., 2016). Notably, the zinc ions could induce inclusion bodies formation and aggregation in the neuronal cell cultures, and this effect was not observed with copper or iron, indicating zinc-specific effects (Caragounis et al., 2010). In another study, a TDP-43 fragment with the RRM 1–2 domain *via* its histidine, cysteine, and glutamate residues that usually show affinity for zinc ions, was shown to aggregate in the presence of the zinc ions into ThT-staining rope-like aggregates (with hydrodynamic diameters: 300–1,000 nm) and also into small oligomeric structures (20–30 nm) (Garnier et al., 2017). Recently, Ash et al. demonstrated that heavy metals, such as lead, mercury and tin, can trigger aggregation and formation of nuclear inclusions of TDP-43 in the PC12 cell lines (Ash et al., 2018). The exposure to lead and methyl mercury was found to disrupt the TDP-43's homeostasis in the neuronal cells and dysregulate its splicing activity. Also, lead could decrease the TDP-43 solubility and promote the phase separation of TDP-43 *in vitro* in a dose-dependent manner (Ash et al., 2018). Thus, the relationship between metal ion content and the TDP-43 functions and aggregation need thorough investigation.

Interference With Chromatin Remodeling

Notably, epigenetic processes, such as chromatin remodeling, histone modifications, and DNA methylation etc., are involved in several aspects of the neuronal function and development (Bastle and Maze, 2019). In fact, altered chromatin regulation might also be involved in the pathology of neurodegenerative diseases including the Alzheimer's, Huntington's and ALS diseases (Berson et al., 2018; Bastle and Maze, 2019). In an important study, TDP-43 was found to impair nucleosomal dynamics (Berson et al., 2017). Here, knockdown of the chromodomain helicase DNA binding protein 1 (CHD1), which is a nucleosome remodeling factor, in *Drosophila*, was shown to be associated with an increase in the number and size of stress granules, and also the percentage of cells exhibiting visible stress granules. TDP-43 was linked with impaired expression of heat shock response proteins, thereby decreasing survival, whereas the upregulation of CHD1 could restore their survival. Also, alteration of chromatin dynamics by TDP-43 due to abnormal histone clearance could be relieved upon CHD1 overexpression. In fact, co-immunoprecipitation showed that TDP-43 physically

interacts with CHD1, and limits its recruitment to the chromatin machinery, possibly due to its aggregation-prone nature. Strikingly, the cytoplasmic accumulation of TDP-43 in ALS and FTLD brain cortex, was also found to be associated with decreased levels of its human ortholog CHD2, thereby indicating that aberrant chromatin remodeling might be involved in ALS (Berson et al., 2017) (**Figure 6**). Additionally, levels of the functional subunits of neuronal Brg1-associated factor (nBAF), which is another chromatin remodeling protein critical for the neuronal differentiation, dendritic extension and synaptic function, was reduced by mutant TDP-43 in the cultured motor neurons and in the ALS spinal motor neurons (Tibshirani et al., 2017). Also, the nuclear loss of TDP-43, or FUS, has been linked with concomitant nuclear retention of the mRNAs encoding the proteins involved in the formation of the nBAF complex. Furthermore, an ALS-linked TDP-43 M337V mutant, was shown to decrease the global expression levels of a key epigenetic marker, H3S10Ph-K14Ac in neuronal cells, whereas, the overexpression of wild-type TDP-43 caused an increase in the histone H3K9Me3 levels (Masala et al., 2018). In summary, TDP-43's role in epigenetics may have importance to the pathogenesis of ALS.

TDP-43-POSITIVE INCLUSIONS IN OTHER NEURODEGENERATIVE DISEASES

Although, pathologically ubiquitinated and phosphorylated TDP-43 inclusions are commonly linked to neurodegeneration in ALS and FTLD-TDP patients (Arai et al., 2006; Neumann et al., 2006; Kwong et al., 2008), TDP-43-positive inclusion cross-immunoreactive pathology, has also been observed in the patients of several other neurological diseases such as: dementia with Lewy bodies (Higashi et al., 2007; Nakashima-Yasuda et al., 2007; Lin and Dickson, 2008), corticobasal degeneration (Uryu et al., 2008), progressive supranuclear palsy (PSP) (Yokota et al., 2010; Koga et al., 2017), dementia Parkinsonism ALS complex of guam (G-PDC) (Hasegawa et al., 2007; Geser et al., 2008), Pick's disease (Freeman et al., 2008; Lin and Dickson, 2008; Dardis et al., 2016), hippocampal sclerosis (Amador-Ortiz et al., 2007; Yokota et al., 2010), Alzheimer's disease, Huntington's disease and Parkinson's disease (Nakashima-Yasuda et al., 2007; Schwab et al., 2008; King et al., 2010b). Notably however, the localization of the TDP-43 aggregates in the brain varies among these different pathological conditions. While in the ALS, FTLD and G-PDC patients the TDP-43 inclusions are widespread in the brain, those in the Alzheimer's disease, Parkinson's disease and PSP patients are more prominent in the limbic region (Baloh, 2011).

Immunohistochemistry has revealed the presence of TDP-43 in the majority of the Huntington's disease patient cases, where it was found to be co-localized with the huntingtin protein in the dystrophic neurites and the intracellular inclusions but was absent from the intra-nuclear regions (Schwab et al., 2008). Schwab et al., using immuno-staining with phosphorylation-specific TDP-43 antibodies, have shown the presence of pathological and phosphorylated TDP-43 in the Huntington's disease samples (Schwab et al., 2008). In another study, TDP-43 was found to co-localize with the poly-glutamine aggregates in the cell culture models where a glutamine/asparagine-rich (Q/N) region from the C-terminal region of TDP-43, present between amino acids 320 and 367, was found to interact with the poly-glutamine aggregates (Fuentealba et al., 2010). Further studies from the nematode and cell culture models, have found that the poly-glutamine's toxicity can, in fact, be reduced by the suppression of the TDP-43 expression, proposedly due to a downstream effector protein, progranulin (PGRN) (Tauffenberger et al., 2013). The TDP-43 and PGRN-mediated effects on the huntingtin toxicity need further investigation using mammalian models and Huntington's disease patients (Tauffenberger et al., 2013).

Notably, immunoreactivity of TDP-43 has also been detected in the Alzheimer's disease brain, in about 75% of the patients (Amador-Ortiz et al., 2007; Higashi et al., 2007; Uryu et al., 2008; King et al., 2010b; Josephs et al., 2014). Immuno-histochemical analysis has found the presence of TDP-43 inclusions co-existing with the tau-positive neuro fibrillar tangles (NFTs) which suggests of its Aβ-42 independent role in the Alzheimer's disease cases (Higashi et al., 2007). However, *in vitro* studies have found that pre-formed TDP-43 aggregates, in fact, can prevent the maturation of the aggregating Aβ-42 into fibrils and rather arrest it into spherical oligomeric species (Fang et al., 2014). Notably, oligomeric Aβ-42 has already been implicated to be of high relevance to the neuro-toxicity in the Alzheimer's disease patients (Selkoe and Hardy, 2016). In another study, Herman et al., using mice Alzheimer's disease models, have found that the Aβ-42 amyloid can trigger the TDP-43 pathology, thus, the TDP-43 and Aβ-42 oligomers/aggregates appear to be capable of cross-seeding each other into toxic species (Herman et al., 2011; Fang et al., 2014; Chang et al., 2016).

Furthermore, TDP-43 proteinopathy has also been detected in the Parkinson's disease patients and also in the transgenic mice Parkinson's disease models, and the toxicity of α-synuclein to the dopaminergic neurons was found to be instigated by the concomitant over-expression of TDP-43 (Arai et al., 2009; Tian et al., 2011). Strikingly, TDP-43 has also been found to form cytoplasmic and sarcoplasmic inclusions in several other diseases such as: the Inclusion body myopathy with early-onset Paget disease and frontotemporal dementia (IBMPFD), sporadic IBM, myofibrillar myopathies, oculopharyngeal muscular dystrophy (OPMD) and distal myopathies with rimmed vacuoles (DMRV) (Weihl et al., 2008; Kusters et al., 2009; Olive et al., 2009; Salajegheh et al., 2009). In addition, TDP-43-positive inclusions have also been described in the skeletal muscles of the patients with sporadic inclusion body myositis (sIBM) and in IBM with mutations in the valosin-containing protein (VCP) (Weihl et al., 2008; Baloh, 2011). Considering the spectrum of diseases with TDP-43-positive inclusions, further investigation is required to illuminate whether the TDP-43 inclusions are indeed disease triggering, or rather merely an induced by-product effected by the other implicated primary aggregating proteins, such as Aβ-42 or α-synuclein etc.

THERAPEUTIC STRATEGIES FOR ALS

Targeting ALS-Related General Toxicity Mechanisms

Therapeutics of ALS is highly challenging as it is a complex disorder which involves numerous mechanisms linked with progressive motor neuron degeneration including the glutamate-mediated excitotoxicity, protein aggregation, increased oxidative stress, endoplasmic reticulum stress, mitochondrial dysfunction, neuro-inflammation and gene dysregulation etc. (Dugger and Dickson, 2017; Tan et al., 2017). In spite of the decades of extensive research, the sequence of events involved in the neuronal dysfunction in ALS remains largely unclear. Therapeutic options for ALS are very limited and thus far, no effective cure or diagnostic biomarkers have been developed for ALS (Mitsumoto et al., 2014; Petrov et al., 2017). Several efforts toward the development of therapeutics for ALS are in progress. These involve the identification of small molecules targeting the specific mechanisms causing the cellular dysfunction, some of which are discussed below.

Glutamate-Mediated Excitotoxicity

Glutamate is an important neurotransmitter in the mammalian nervous system. Excessive stimulation of glutamate receptors results in increased influx of Na^{+} and Ca^{2+} ions, which causes excitotoxicity, leading to neuronal injury or neuronal death (Heath and Shaw, 2002). A small molecule, riluzole, can inhibit glutamate excitotoxicity by regulating the release of glutamate, suppressing post-synaptic receptor activation and blocking voltage-sensitive sodium channels. In 1995, riluzole became the first FDA-approved drug for the treatment of ALS. However, it is only modestly effective in slowing the ALS disease progression, showing no effects on the disease symptoms and only improving the lifespan of ALS patients by 2–3 months (Bensimon et al., 1994; Miller et al., 2012). Also, a sodium channel blocker, mexiletine, which reduces the neuronal hyperexcitability and another glutamate antagonist, memantine, are presently under clinical trials for ALS treatment (De Carvalho et al., 2010; Weiss et al., 2016).

Oxidative Stress

Oxidative stress contributes to the motor neuron degeneration in ALS, and also affects the other cellular pathological mechanisms, such as the mitochondrial dysfunction and protein aggregation etc. (Barber et al., 2006). In 2017, a new anti-oxidant drug, edaravone (also called: radicava), became the first new FDA-approved drug for the treatment of ALS, in over two decades since riluzole. It is a free-radical scavenger and a potent anti-oxidant that alleviates the oxidative stress on the nerves and the vascular endothelial cells (Yoshino and Kimura, 2006; Takei et al., 2017).

Neuro-Inflammation

Evidence indicates that neuroinflammatory responses contribute to the progressive degeneration of neuronal cells in the ALS patients. An increase in the number of mast cells is associated with denervation of the neuromuscular junctions caused by degranulation of the mast cells, which release a mixture of serine proteases, histamine, and serotonin, etc. Masitinib is a selective tyrosine kinase inhibitor that mainly targets type III growth factor receptors like c-Kit, Lyn, and Fyn kinases, and is particularly effective in controlling the survival, differentiation, and degranulation of mast cells. Masitinib has been found to prevent the CNS neuro-inflammation by targeting the degranulation of the mast cells accumulating around the degenerating neuronal axons and by decreasing the release of inflammatory cytokines (Trias et al., 2016; Hammam et al., 2017). It can even target the microglia cells which are the resident macrophages of the brain. Masitinib has entered phase III trials for ALS therapeutics in 2017, as an add-on to riluzole (Mora and Hermine, 2017). Another small molecule immunomodulator, NP001, which reverses the pro-inflammatory response of the activated macrophages by producing intra-cellular chloramines, has also entered phase II trials for ALS treatment (Miller et al., 2014, 2015).

Muscle Troponin Activation

Muscular atrophy and decline of muscle strength, especially respiratory muscles, are among the key insults for ALS pathology. The functioning of muscle sarcomere depends upon the binding of myosin to actin which is regulated by the actin-associated proteins, tropomyosin, and troponin. The release of calcium ions from the sarcoplasmic reticulum and its binding to the troponin complex plays an important role in the fast skeletal muscle contraction. A small molecule, tirasemtiv can selectively sensitize the fast skeletal muscle sarcomere troponin to calcium ions, and slow down their release from the regulatory troponin complex, thereby amplifying the response of the muscles to neuromuscular inputs which improves the muscle function and muscle strength (Russell et al., 2012; Hansen et al., 2014; Hwee et al., 2014). Tirasemtiv had reached phase III trials for the ALS treatment however, the results were disappointing as the ALS patients reported poor tolerance. Recently, yet another next generation fast skeletal muscle activator, CK-2127107, has entered phase III trials and may potentially address the limitations of tirasemtiv (Andrews et al., 2017; Nace, 2017).

Heat-Shock Response Activation

Heat shock proteins, or chaperones, promote cell survival by refolding the misfolded proteins into their native functional conformations. The heat shock transcription factor 1 (HSF1) is a master regulator of the expression of several heat-shock proteins during stress conditions (Neef et al., 2011). A small molecule, arimoclomol, is a potent activator of HSF1 which also amplifies Hsp70 and Hsp90 expressions. In a recent study, arimoclomol showed HSF1-mediated reduction in the TDP-43 aggregate levels (Kieran et al., 2004; Kalmar et al., 2014; Wang P. et al., 2017). Arimoclomol has also shown promising results in the phase II trials for ALS.

Autophagy Induction

The cellular protein degradation machinery and autophagy pathways play a crucial role in clearing misfolded and aggregated proteins. The mammalian target of rapamycin (mTOR) kinase is

an important protein involved in the regulation of cell signaling, protein synthesis, and autophagy pathway. Several small molecules, like trehalose and rapamycin, can induce protective autophagy and improve the neuronal health. Rapamycin, a small molecule inhibitor of mTOR, stimulates autophagy through the formation of autophagosomes from the phagophore and enhances protein degradation (Ravikumar et al., 2004; Bachar-Wikstrom et al., 2013). Rapamycin was shown to induce autophagy, improve memory and rescue motor dysfunction in a TDP-43 mouse model which manifested a decrease in the caspase-3 levels and the amount of cytoplasmic TDP-43 inclusions (Wang et al., 2012). Efficacy of rapamycin for the ALS treatment is being monitored in phase II clinical trials (Mandrioli et al., 2018).

Targeting TDP-43's Aggregation and Clearance

Small Molecule Inhibitors of TDP-43 Aggregation

Small molecule interventions of the TDP-43 associated pathology need to aim at its aggregation behavior, stress granule dynamics, nucleo-cytoplasmic shuttling and caspase-resistance etc. Small molecule inhibitors of the amyloid-like aggregation, as well as stabilizers of the non-pathogenic native monomers or oligomers, may alleviate the neuronal toxicity. Tafamidis is the only, so far, FDA approved anti-amyloidogenic drug which is used for the treatment of transthyretin amyloidosis and it acts by arresting transthyretin into homo-tetrameric species (Bulawa et al., 2012). We have recently identified a TDP-43 aggregation inhibitor molecule which is an acridine-imidazole derivative (AIM4), using *in vitro* and yeast model (Prasad et al., 2016; Raju et al., 2016). In another study, using high-throughput screening, several compounds were identified that decrease the aggregation of TDP-43 into inclusion bodies and rescue the toxicity in the rat PC12 cells (Boyd et al., 2014). Furthermore, 4-aminoquinoline derivatives have been shown to alter the TDP-43's nucleic acid binding properties and enhance its caspase-mediated cleavage (Cassel et al., 2012). Also, inhibition of the TDP-43's accumulation into stress granules and inhibition of the C-terminal fragment aggregation, were reported in the ALS models treated with copper complexes Cu^{II}(btsc) and Cu^{II}(atsm), which proposedly act by slowly releasing the Cu^{II}-ions within certain sub-cellular compartments like the stress granules (Donnelly et al., 2008; Crouch et al., 2009; Parker et al., 2012).

Heat Shock Proteins

In yeast, the over-expression of the chaperone Hsp104 has been vividly shown to efficiently dissolve certain yeast prion aggregates (Chernoff et al., 1995; Shorter and Lindquist, 2005; Liebman and Chernoff, 2012). Hsp104 has recently been used in the yeast models of several neurodegenerative disorders as a potential disaggregase (Jackrel and Shorter, 2014; Jackrel et al., 2014; Sweeny et al., 2015; Sweeny and Shorter, 2016; Torrente et al., 2016). Using random mutagenesis, engineered Hsp104 variants were obtained which showed capability of dissolving the aggregates of TDP-43, FUS, and α-synuclein (Jackrel et al., 2014). In fact, the mutants of Hsp104, A503V/S/C and V426L, could reduce the toxicity, suppress the aggregation and promote the nuclear localization of wild-type TDP-43 and an ALS-linked TDP-43 M337V mutant. Also, Hsp104 A503V and A503S variants, but not the wild-type Hsp104, displayed a propensity to dissolve the short TDP-43 filaments and amorphous structures *in vitro*, and similar observations were also documented for the FUS and α-synuclein fibrillar aggregates (Jackrel and Shorter, 2014; Jackrel et al., 2014). The cryo-EM structure of the hexameric Hsp104 is now available, which has revealed the mechanistic aspects of the substrate binding and disaggregation, and this may help in further optimization of its disaggregase activity (Gates et al., 2017). Following overexpression of Sis1, a yeast Hsp40 chaperone, reduction in the deleterious effects of the TDP-43 aggregation, was observed (Park et al., 2017). In fact, Sis1 could rescue the yeast cells from the TDP-43-associated toxicity, improve growth and survival, as well as protect from abnormal cellular morphologies, although there was no evidence for a direct physical association between Sis1 and TDP-43. Furthermore, overexpression of the mammalian Sis1 homolog, DNAJB1, in the primary rodent neurons could also relieve the TDP-43-mediated toxicity, suggesting that Sis1 and its related homolog might have neuroprotective effects for ALS (Park et al., 2017). Previously, heat shock has been shown to induce the reversible nuclear aggregation of TDP-43 (Udan-Johns et al., 2014). Interestingly, overexpression of DNAJB6, another Hsp40 protein, was found to suppress the formation of heat shock-induced TDP-43 nuclear aggregates. DNAJB6 was shown to be associated with the disordered C-terminal prion domain of TDP-43 and could possibly regulate not only its aggregation behavior but also its interaction with the other RNA binding partners (Udan-Johns et al., 2014).

Nuclear Import Receptors

Nuclear import receptors (NIRs), which are part of the nuclear pore machinery, have been shown to act as chaperones and disaggregases (Chook and Suel, 2011). In fact, karyopherin-β1 has shown an ability to decrease and reverse TDP-43 fibrillization possibly by associating with its classic nuclear localization signal (cNLS) sequence. Similarly, karyopherin-β2 could also decrease the fibrillization of some other RNA binding proteins—FUS, TATA-box binding protein associated factor 15 (TAF15), EWS RNA binding protein 1 (EWSR1), hnRNPA1, and hnRNPA2, by interacting with their proline-tyrosine nuclear localization signal (PY-NLS) sequences (Guo et al., 2018). Karyopherin-β2 was also found to manifest a propensity to dissolve the phase-separated droplets and aberrant fibril-containing hydrogels formed by FUS and hnRNPA1. Also, karyopherin-β2 could prevent the accumulation of these RNA-binding proteins into stress granules and restore their nuclear localization and cellular functions. Thus, condiserable interest exists in the nuclear importins as promising therapeutic targets (Hofweber et al., 2018; Yoshizawa et al., 2018).

Heat Shock Factors

Heat shock transcription factors have a significant role in maintaining the cellular proteostasis (Neef et al., 2011; San Gil et al., 2017). In an elegant study, heat shock factor1 (HSF1)

was shown to reduce the levels of insoluble TDP-43 in the cell culture and mice ALS models (Chen H. J. et al., 2016). HSF1-mediated TDP-43 clearance was found to be closely associated with the chaperone Hsp70 and its co-chaperone DNAJB2a. Chen et al. suggest that DNAJB2a recognizes the insoluble TDP-43 aggregates and directs them toward Hsp70 for refolding and solubilization (Chen H. J. et al., 2016). Strikingly, activating HSF1 showed high transcriptional induction of Hsp40 and Hsp70 chaperones which significantly suppressed the TDP-43 aggregation into the inclusions bodies. Thus, harnessing the disaggregation potential of the HSPs and HSFs, as well as small molecule activators of HSF1 seem to be exciting prospects for TDP-43's anti-aggregation and therapeutics (Wang P. et al., 2017).

CONCLUSIONS

The TDP-43 protein, by virtue of its versatile functions in RNA metabolism and homeostasis, has emerged as a vital protein for cellular health. Supporting its importance, aberrations in the TDP-43 homeostasis due to imbalance in its nucleocytoplasmic distribution, genetic mutations, aberrant post-translational modifications or aggregation, is increasingly being accepted as a causative of mis-regulation of RNA homeostasis and cytotoxicity. A few facets of the TDP-43-induced toxicity, such as mitotoxicity and proteosomal overload etc. have been unearthed, but how and in which sequence the toxicity mechanisms ensue leading to the neurodegeneration, remain poorly understood. The presence of TDP-43-positive inclusions in several other neurodegenerative diseases, in addition to ALS and FTLD, suggest of a more widespread and vital role of TDP-43 in the general process of neurodegeneration. Thus, targeting the TDP-43 dyshomeostasis, may hold the key to finding common therapeutics, applicable to a multitude of neurodegenerative diseases.

AUTHOR CONTRIBUTIONS

BP directed the manuscript preparation. AP, VB, VS, AG, and BP wrote the manuscript.

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REVIEW
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Spreading of α-Synuclein and Tau: A Systematic Comparison of the Mechanisms Involved

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Alzheimer's disease (AD) and Parkinson's disease (PD) are age-associated neurodegenerative disorders characterized by the misfolding and aggregation of alpha-synuclein (aSyn) and tau, respectively. The coexistence of aSyn and tau aggregates suggests a strong overlap between tauopathies and synucleinopathies. Interestingly, misfolded forms of aSyn and tau can propagate from cell to cell, and throughout the brain, thereby templating the misfolding of native forms of the proteins. The exact mechanisms involved in the propagation of the two proteins show similarities, and are reminiscent of the spreading characteristic of prion diseases. Recently, several models were developed to study the spreading of aSyn and tau. Here, we discuss the mechanisms involved, the similarities and differences between the spreading of the two proteins and that of the prion protein, and the different cell and animal models used for studying these processes. Ultimately, a deeper understanding of the molecular mechanisms involved may lead to the identification of novel targets for therapeutic intervention in a variety of devastating neurodegenerative diseases.

Keywords: alpha-synuclein, Parkinson's disease, tau, Alzheimer's disease, spreading

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INTRODUCTION

Alzheimer's disease (AD) and Parkinson's disease (PD) are progressive, age-associated neurodegenerative disorders. Recent epidemiological studies revealed that around 50 million people worldwide are living with AD, and more than 10 million people above 60 years old with PD, respectively (Karlawish et al., 2017; Tysnes and Storstein, 2017). The prevalence of both diseases is increased in the highest age groups and the number will escalate rapidly the coming years (Karlawish et al., 2017; Tysnes and Storstein, 2017). While the clinical features are quite distinct between the two diseases, at the molecular level they are characterized by the misfolding, aggregation, and deposition of proteins in characteristic types of inclusions (Brion et al., 1985; Kosik et al., 1986; Iwai et al., 1995; Spillantini et al., 1997). Accumulation of aggregated tau is a hallmark of AD and related tauopathies and the accumulation of alpha-synuclein (aSyn) aggregates is the hallmark of PD and related synucleinopathies (Brion et al., 1985, 1986; Kosik et al., 1986; Wood et al., 1986; Wischik et al., 1988; Spillantini et al., 1997; Baba et al., 1998; Bayer et al., 1999). aSyn and tau are abundant brain proteins, both known as intrinsically disordered proteins (IDPs) with prion-like properties, as they can misfold, seed, and spread the misfolded conformation to normal monomeric forms of each protein (Uversky and Fink, 2004; Eliezer, 2009; Bartels et al., 2010; Wu and Baum, 2010; Coelho-Cerqueira et al., 2013).

Different strains of aSyn and tau display different cell binding and penetration properties, resulting in transmission of pathology between cells (Clavaguera et al., 2009; Guo and Lee, 2011; Hansen et al., 2011; Angot et al., 2012; Kfoury et al., 2012; Masuda-Suzukake et al., 2013; Wu et al., 2013; Recasens and Dehay, 2014; Sanders et al., 2014; Grozdanov and Danzer, 2018). It is currently thought that distinct protein conformations account for differences in seeding potency. Interestingly, several studies revealed the accumulation of abnormal tau aggregates in numerous cases of aSyn deposition, and vice versa (Brion et al., 1985; Kosik et al., 1986). The coexistence of aSyn and tau aggregates suggests a strong cross-talk between tauopathies and synucleinopathies, and raises the hypothesis that cross-seeding might take place, thereby contributing to disease progression (Kosik et al., 1986; Spillantini et al., 1997; Cabrales Fontela et al., 2017). Furthermore, the interaction between aSyn and tau appear to promote the oligomerization and solubility of each other *in vitro* and *in vivo*, thereby disrupting cytoskeletal organization, impairing axonal transport, and compromising synaptic organization (Masliah et al., 2001; Giasson et al., 2003; Kotzbauer et al., 2004; Bellani et al., 2010; Cabrales Fontela et al., 2017; Sotiropoulos et al., 2017; Biswas and Kalil, 2018; Ordonez et al., 2018; Prots et al., 2018; Tuerde et al., 2018; Yuan et al., 2018). However, the exact molecular mechanisms involved in the cross-talk between the two proteins, and in the propagation of pathology, are still obscure. Here, we discuss the current knowledge about the mechanisms involved in transmission of both proteins, focusing on similarities and differences between the different spreading mechanisms.

aSyn STRUCTURE AND FUNCTION

Alpha-synuclein (aSyn) is a 14.5 kDa acidic protein of 140 amino acid residues, encoded by the *SNCA* gene (Chen et al., 1995), and is strongly implicated in PD. aSyn belongs to the synuclein family, together with beta- and gamma-synuclein.

aSyn was first isolated from the synaptic vesicles and nuclei of the electric organ of *Torpedo californica* (Maroteaux et al., 1988). In 1997, aSyn was identified as the major protein component of Lewy bodies (LBs) and Lewy neurites (LNs), the pathognomonic deposits in PD (Spillantini et al., 1997). In the same year, the first point mutation in the *SNCA* gene was associated with autosomal-dominant forms of PD, demonstrating the role of genetics in the disease (Polymeropoulos et al., 1997). Furthermore, the identification of families with duplications and triplications of the *SNCA* locus confirmed that increased levels of aSyn can cause disease (Singleton et al., 2003). These findings, along with a plethora of *in vitro* and *in vivo* studies, suggest that aSyn is a central player in a group of neurodegenerative disorders known as synucleinopathies.

aSyn is classified as an intrinsically disordered protein (IDP) as it lacks defined secondary structure (Uversky, 2003, 2011a,b; Bernado et al., 2005; Breydo et al., 2012). Although the precise physiological function of aSyn is still unclear, several studies suggest that aSyn is involved in the regulation of synaptic membrane processes and in neurotransmitter release through interactions with members of the SNARE family (Tsigelny et al., 2012; Bellucci et al., 2016). Surprisingly, studies in aSyn knockout mice revealed that aSyn is not essential for synapse formation and cell survival (Bisaglia et al., 2009).

The primary sequence of aSyn can be divided in three distinct domains: the amino-terminal domain (N-terminal, residues 1–60), the central domain (residues 61–95) and the carboxy-terminal domain (C-terminal domain, residues 96–140). The N-terminal domain includes four repeats of the 11 amino acid alpha-helical lipid-binding motif (KTKEGV) (**Figure 1Ai**, R1–4), enabling the formation of amphipathic α-helical structures upon interaction with lipid membranes (Jao et al., 2004, 2008; Georgieva et al., 2008). The lipid composition of membranes is critical for aSyn binding. aSyn specifically prefers the binding in membranes characterized by high concentrations in cholesterol and sphingolipids, known also as lipid rafts. It seems that lipid rafts serve as a platform, which promotes aSyn binding and oligomerization (Davidson et al., 1998; Jo et al., 2000; Fortin et al., 2004; Zabrocki et al., 2008; Middleton and Rhoades, 2010; Fabelo et al., 2011; Hellstrand et al., 2013).

Importantly, all the known mutations associated with familial forms of PD are clustered within the N-terminal region of aSyn (Polymeropoulos et al., 1997; Kruger et al., 1998; Zarranz et al., 2004; Appel-Cresswell et al., 2013; Lesage et al., 2013; Proukakis et al., 2013; Pasanen et al., 2014), reinforcing the hypothesis that changes in the lipid binding domain may be linked to aSyn pathology. Interestingly, aSyn was reported to be acetylated at the N-terminus in cells, an essential modification that protects its native conformation against pathological aggregation (Iyer et al., 2016; Bu et al., 2017). The central domain, also known as NAC domain (non-amyloid-β component) (**Figure 1Ai**), is enriched in hydrophobic residues and is involved in the pathologic aggregation of the protein due to conformational changes (El-Agnaf et al., 1998; Giasson et al., 2001; Bellucci et al., 2012). Interestingly, one phosphorylation site is present in the NAC domain—the S87 residue. S87 phosphorylation is increased in synucleinopathies, leading to inhibition of aSyn oligomerization which influences synuclein-membrane interactions (Paleologou et al., 2010). The carboxy-terminal domain (C-terminal domain) is characterized by a non-defined structure (Bisaglia et al., 2009) and incorporates most of the posttranslational modification sites (PTMs), including the most common phosphorylation at S129 (Fujiwara et al., 2002; Oueslati, 2016). The importance of phosphorylation is emphasized by a study showing that in DLB brains, approximately 90% of insoluble aSyn is phosphorylated at S129, compared with only 4% in soluble cytosolic aSyn (Anderson et al., 2006). This suggests the implication of phosphorylation in the aggregation propensity. These PTMs may act by modulating the structure, the physiological functions and the toxicity of aSyn. Furthermore, they can modulate protein-protein interactions, interaction with metal ions (Paik et al., 1999; Brown, 2007; Bisaglia et al., 2009), including Ca^{2+} binding (Nielsen et al., 2001), polyamine complexes binding, modulation of phospholipid-binding (Paleologou et al., 2008; Visanji et al., 2011), affecting the aggregation propensity of the protein. Moreover, the region plays a protective role against aggregation, due to the presence of all the five proline (Pro)

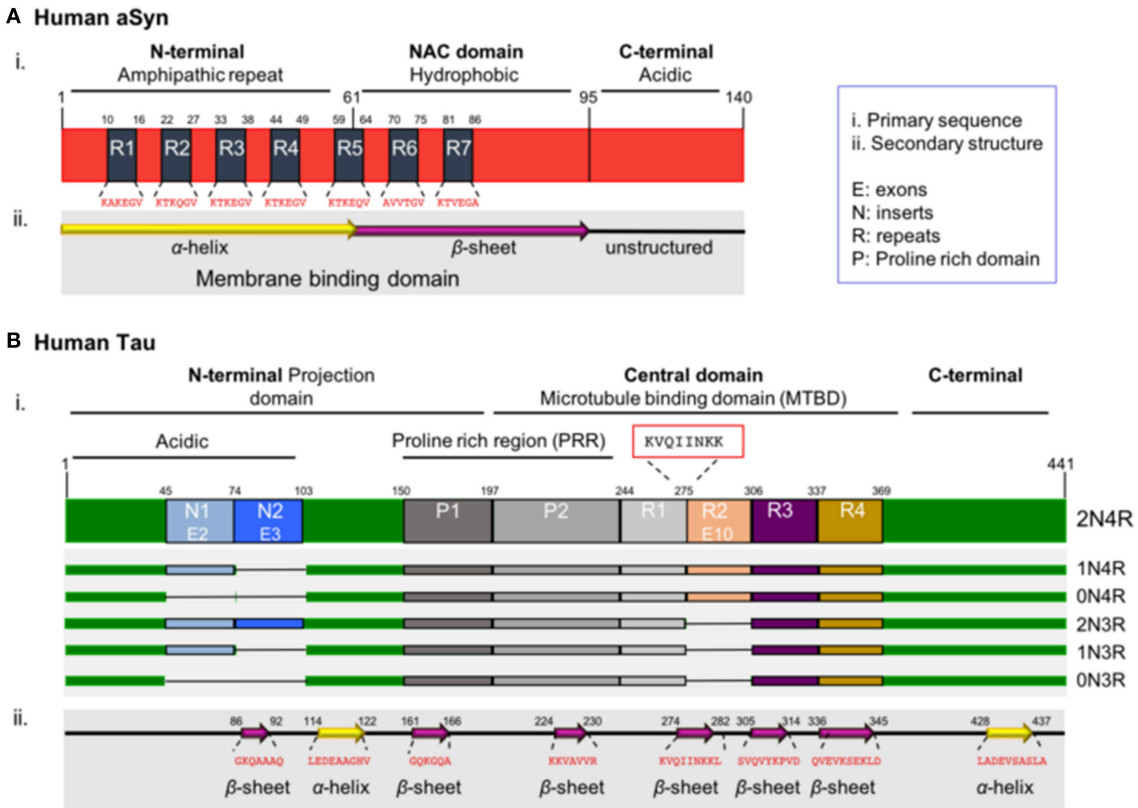

FIGURE 1 | Schematic illustration of aSyn and tau proteins. **(A)** aSyn is encoded by the *SNCA* gene. The primary sequence of aSyn can be divided in three distinct domains: the amino-terminal domain (N-terminal, residues 1–60), the central domain also known as NAC domain (residues 61–95), and the carboxy-terminal domain (C-terminal domain, residues 96–140). The N-terminal domain includes four repeats (R1–R4) of the 11 amino acid alpha-helical lipid-binding motif (KTKEGV). This region has propensity to form amphipathic α-helical structures upon interacting with lipid membranes. The NAC domain (non-amyloid-β component), contains three additional repeats (R5–R7) of the lipid-binding motif, is enriched in hydrophobic residues, leading to the formation of cylindrical β-sheets and amyloid-β fibrils. Both the N-terminal and NAC domain are characterized part of the membrane binding domain. The C-terminal domain is rich in acidic residues (15 acidic amino acids: 10 Glu and 5 Asp residues) and lacks defined secondary structure. **(B)** Tau is encoded by the *MAPT* gene. Alternative splicing of the *MAPT* gene results in six isoforms known as 2N/4R, 1N/4R, 0N/4R, 2N/3R, 1N/3R, and 0N/3R, depending on the presence or absence of exon 10 (4R or 3R) and on the numbers of amino-terminal inserts (0N, 1N, and 2N) encoded by exons 2 and 3. The primary sequence of the full-length human tau isoform can be divided in the N-terminal domain also known as projection domain, the central domain which is the microtubule binding domain (MTBD) and the C-terminal tail. The N-terminal consists of the acidic part encoded by exons 2 and 3 (E2-3) called inserts 1 and 2 (N1-2), followed by the proline-rich region (PRR). The MTBD in the longest isoform contains four repeats (R1-4). The region with the strongest propensity for microtubule polymerization is the oligopeptide "KVQIINKK" (residues 274–281), located in the sub-region between the R1–R2 repeats (red box). The C-terminal tail is enriched in positively charged residues. Notably, the region with the strongest propensity for microtubule binding is located in the sub-region between the R1–R2 repeats and, more specifically, is the oligopeptide "KVQIINKK" (residues 274–281), which is included only in the 4R tau isoform, providing a stronger binding affinity when compared to the 3R isoforms (Brandt and Lee, 1993; Goode and Feinstein, 1994; Sergeant et al., 2005).

residues of the protein (Meuvis et al., 2010). Changes in the charge or the hydrophobicity by residue substitution as well as deletion of the C-terminal lead to accelerated aggregation of aSyn *in vitro* (Hoyer et al., 2004). As mentioned above, aSyn has the ability to bind to acidic membranes. This binding is mediated by the amphipathic α-helix in the N-terminal domain. Under physiological conditions aSyn exists in a dynamic equilibrium between the unfolded cytosolic and the membrane–bound state (Burre et al., 2014). In contrast, under pathological conditions, aSyn adopts a β-sheet–rich amyloid conformation, which leads to the fibril formation and subsequently aSyn deposition into LBs (Pineda and Burre, 2017). Importantly, the α-helical part is responsible for the formation of the different types of oligomers, the species currently considered to be most toxic. However, the exact nature of those toxic species remains unknown, and is still unclear whether aggregation initiates from its lipid-bound part or from the unstructured cytosolic protein (Trexler and Rhoades, 2012; Chen et al., 2015; Ghosh et al., 2015; Gallea et al., 2018).

TAU STRUCTURE AND FUNCTION

Tau was first discovered associated with microtubules, together with other microtubule-associated proteins (Weingarten et al., 1975; Kolarova et al., 2012). For this reason, it was included in the family of microtubule-associated proteins (MAPs). There are six different isoforms of tau in the central nervous system, generated from the *MAPT* gene, as a result of alternative splicing. These isoforms range from 352 to 441 amino acids (Neve et al.,

1986). Each of the six tau isoforms differs in their primary structure due to the content of three (3R) or four repeats (4R) of the microtubule binding domains in the C-terminal region, in combination with the presence or absence of one (N1) or two (N2) amino acid inserts in the N-terminal part of the protein. The six isoforms are known as 0N/3R (352 residues, 60 kDa), 1N/3R (381 residues, 64 kDa), 2N/3R (410 residues, 69 kDa), 0N/4R (383 residues, 64 kDa), 1N/4R (412 residues, 69 kDa), and 2N/4R (441 residues, 74 kDa) (**Figure 1 Bi**), all showing higher apparent molecular masses than the predicted ones. Since the isoforms are differentially expressed in the brain during development, and stimulate microtubule assembly with different efficiencies, possibly possess particular physiological roles and implicated at different biological activities (Utton et al., 2001; Stanford et al., 2003). The shortest isoform is also known as "fetal tau isoform" because it is expressed also in the fetal brain, while all of them are detected in the human adult brain (Kosik et al., 1989; Stanford et al., 2003).

Importantly, in neurodegenerative diseases such as AD and PD, modified proportions of the different tau isoforms have been observed (Bre and Karsenti, 1990; Avila et al., 2004). Tau stabilizes the polymerization of microtubules through the three or four MTBR repeats in case of the longest isoform (Drubin and Kirschner, 1986; Maccioni et al., 1989). Under physiological conditions, in mature neurons, all tau protein is likely to be microtubule bound (Ackmann et al., 2000), and it is considered a dipole protein since the two ends of the protein have opposite charges (Sergeant et al., 2008).

The primary sequence of tau consists of the N-terminal domain, half of which is enriched in acidic residues, followed by a proline-rich region and the positively charged C-terminal tail.

Tau, like aSyn, is an intrinsically disordered protein, since it contains regions without defined secondary structure that are inserted between very short β-sheets and α-helices (**Figure 1Bii**). The protein can also undergo different types of PTMs like phosphorylation, ubiquitination, acetylation, glycation, methylation, truncation of the N- or C-terminal regions or nitration (Avila et al., 2004; Garcia-Sierra et al., 2008; Avila, 2009; Morris et al., 2015; Huang et al., 2016; Iqbal et al., 2016), that likely modulate its normal function and lead to pathological features. Notably, tau contains a high number of potential phosphorylation sites (80 serines/threonines and 5 tyrosines) (Grundke-Iqbal et al., 1986b; Bancher et al., 1989; Wang and Mandelkow, 2016). Most of these sites are located within the proline-rich region in close proximity to the MTBR domains and in the C-terminal tail (**Figure 1Bi**) (Buee et al., 2000; Sergeant et al., 2008). The phosphorylation state of the protein affects the secondary structure and, subsequently, regulates all the normal and abnormal functions like development, interaction with different protein partners such as microtubules, localization, aggregation, and spreading (Camero et al., 2014; Multhaup et al., 2015; Wang and Mandelkow, 2016). In principle, a normal and strictly controlled level of phosphorylation is required for the appropriate function of the protein, while the pathological state is characterized by hyperphosphorylation that leads the tau to lose its biological activity (Kopke et al., 1993).

The deposition of hyperphosphorylated tau in insoluble filaments in the brain is a pathological hallmark not only of AD but also of related neurodegenerative diseases, known as tauopathies, including frontotemporal dementias (FTD) like Pick's Disease and argyrophilic grain disease, progressive supranuclear palsy, and corticobasal degeneration (Grundke-Iqbal et al., 1986b). Major differences between tauopathies are the deposition of different isoforms of tau (Rademakers et al., 2004) and the occurrence of different structures of tau aggregates (Gerson et al., 2014; Dujardin et al., 2018). In AD, all the six-tau isoforms are hyperphosphorylated and aggregated into paired helical filaments (PHF) (Grundke-Iqbal et al., 1986a,b). In Pick's disease 3R isoforms are predominant, and the arrangement of tau is different than that in inclusions found in AD (Falcon et al., 2018). On the other hand, in the other tauopathies only the 4R isoform is present in the filaments (Goedert, 2015). In AD brains, the abnormally hyperphosphorylated tau is presented in the cytosol inhibiting the assembly of tubulin and disrupting microtubules. Furthermore, as a result of self-assembly is accumulated into neurofibrillary deposits in neurons and glial cells (Iqbal et al., 2010). In sporadic and familial FTD, several mutations have been identified in the tau gene. Some of these mutations are thought to disrupt the normal binding of tau to tubulin resulting in pathological deposits of hyperphosphorylated tau (Rademakers et al., 2004), or makes tau more vulnerable to self-aggregation (S422E, ΔK280, R5L, P301L, and R406W) (Haase et al., 2004; van Swieten et al., 2007; Mutreja et al., 2019). As with aSyn, it is believed that in a variety of tauopathies, the most toxic species are oligomeric, but the controversy is still not fully resolved. These oligomeric species are non-fibrillar, multimeric, soluble forms of the protein (Haase et al., 2004; Ghag et al., 2018). Examples of tauopathies where oligomers have been proposed as the toxic species include AD, corticobasal degeneration, Pick's disease, and progressive supranuclear palsy (Maeda et al., 2006; Patterson et al., 2011; Gerson et al., 2014).

PRIONS AND PRION-LIKE SPREADING OF PATHOLOGY

Prion diseases are infectious diseases that can be transmitted horizontally between individuals of the same or even different species (Costanzo and Zurzolo, 2013; Kizhakke et al., 2017). In these diseases, PrP^{C} misfolds and converts into the pathogenic form PrP^{Sc}. PrP^{Sc} then acts as a template, converting endogenous PrP^{C} into additional PrP^{Sc}, thereby spreading pathology in the brain (Brandner et al., 1996). Other proteins may manifest prion-like behavior. The prion-like behavior of amyloid-β has been broadly studied (Walker et al., 2016; Ruiz-Riquelme et al., 2018; Sarnataro, 2018). Alterations in amyloid-β conformation lead to aggregation and the formation of plaques, and it has been reported that amyloid-β can reach the brain form outside the CNS (Eisele et al., 2014).

The stable propagation of different misfolded protein conformations was established as a defining feature of the prion paradigm and of the prion-like spreading of pathology

(Jucker and Walker, 2013). Importantly, both aSyn and tau appear to spread in a prion-like manner (Holmes et al., 2013). However, different structural features may affect the way they propagate. Each protein has a characteristic core that undergoes conformational changes and may lead to aggregation. In particular, these are the NAC region in aSyn and the MTBR (together with the final part of the poliproline region) domain in tau (El-Agnaf et al., 1998; Ackmann et al., 2000; Giasson et al., 2001). Differences in these regions may lead to differences in the aggregated species formed. Whether aSyn and tau spreading have an infectious nature like that of the prion protein remains unclear. In general, protein infectivity depends on several factors, such as irreversibility of misfolded protein assemblies, the efficiency by which precursor polypeptides are recruited into aggregates, the clearance of the aggregates, and the efficiency of the spreading of misfolded protein proteins (Brundin et al., 2010).

Spreading of aSyn Pathology

aSyn neuropathology typically progresses in a predictable manner throughout the brain. Post mortem analysis of human brains revealed progression of neuropathology in a series of stages (Braak et al., 2003a). Initially, the lesions start in the olfactory bulb, anterior olfactory nucleus, and dorsal motor nucleus of the vagus (Ordonez et al., 2018) in what is considered the first stage. During the second stage, pathology spreads to the lower raphe nuclei, the magnocellular portions of the reticular formation and the locus coeruleus (Prots et al., 2018). In the third stage, the pathology reaches the midbrain, affecting fundamentally the substantia nigra pars compacta (Braak et al., 2003a). Pathology spreads then to the cortex during the fourth stage. In this stage the mesocortex is affected whilst the neocortex is unaffected (Braak et al., 2003a,b). In the last two stages, pathology reaches the neocortex. Initially affecting the prefrontal neocortex and then moving to the premotor areas, the primary sensory areas and the primary motor field (Braak et al., 2003a).

aSyn can cross the blood-brain barrier (Peelaerts et al., 2015) and was shown to reach the central nervous system (CNS) after gastrointestinal administration (Holmqvist et al., 2014). It has been found in the choroid plexus, where it may be produced by the choroid cells that participate in its transport between the blood and cerebrospinal fluid (Bellani et al., 2010).

Additional studies showed the spreading of aSyn from diseased to healthy tissue. Several PD patients underwent embryonic neuronal cell transplantation developed the disease years after the surgery. The postmortem analysis of the tissue showed signs of PD, including the presence of LB and LN, in the grafted tissue. Interestingly, in these studies, the presence of cytosolic aSyn phosphorylated at S129 was also shown (Kordower et al., 2008; Li et al., 2008).

These studies, together with the Braak staging hypothesis, were considered strong evidence in favor of the prion-like spreading of aSyn pathology in the brain (Olanow and Prusiner, 2009).

Following these findings, several new studies revealed that aSyn can propagate from host to grafted tissue (Desplats et al., 2009; Angot et al., 2012; Reyes et al., 2014). A different set of studies showed how the administration of brain lysates from multiple system atrophy (MSA) patients into TgM83 mice brain leads to transmission in a way that is reminiscent of the transmission of the prion protein in chimpanzee brains in a model of Kuru (Gajdusek et al., 1966; Watts et al., 2013). This process was proposed to happen through cell-to-cell transmission following not only cell connectivity, and may reach parts of the CNS away from the injection site (Luk et al., 2012). Furthermore, 9 months after the inoculation of pathological aSyn from sarkosyl-insoluble fractions from cortical brain tissue from MSA patients, aSyn aggregates were found in the side contralateral to the administration (Bernis et al., 2015). Interestingly, in all these experiments human material leads to disease in different species. The transmission among different species is one of the main characteristics of prion proteins. Another argument in favor of the prion-like behavior of aSyn is that, under certain conditions, aSyn can assemble aberrantly forming prion strains (Guo et al., 2013; Peelaerts et al., 2015). When aSyn fibrils are inoculated in Wistar rats they act as seeds imprinting their intrinsic structures, turning monomeric aSyn into fibrils. When aSyn ribbons were injected, endogenous aSyn in Wistar rats acquired this specific conformation (Breydo et al., 2012). Furthermore, when two different strains of aSyn pre formed fibrils were inoculated in mice, endogenous aSyn acquired the structure of the strain inoculated (Peelaerts et al., 2015). Also, assemblies such as fibrils and ribbons can cross the blood-brain barrier and reach the CNS after intravenous injection (Uversky, 2011b).

Several mechanisms have been put forward to explain the spreading of aSyn between cells. These include membrane pores (Stockl et al., 2013), passive diffusion (Ahn et al., 2006; Grozdanov and Danzer, 2018), receptor mediated endocytosis (Mao et al., 2016), through exo- and endocytosis (Lee et al., 2005), exosomal transport (Emmanouilidou et al., 2010), tunneling nanotubes (Abounit et al., 2016a,b; Dieriks et al., 2017), and the possibility of transport through carrier proteins (Sung et al., 2001; Yang et al., 2017) (**Figure 2**).

aSyn is present in extracellular fluids, as it has been found in human plasma and cerebrospinal fluid and in medium from cultured human M17 neuroblastoma cell line (El-Agnaf et al., 2003). The release of aSyn has also been shown in other cell lines such as SH-SY5Y cells, H4, MES cells, and in primary neurons (Lee et al., 2005; Danzer et al., 2011; Yamada and Iwatsubo, 2018). In these cells, aSyn is released through a calcium-dependent non-conventional pathway. Treatment with compounds that interfere with the normal function of endosomes result to significant changes in the extracellular levels of aSyn. Thus, the release pathway of aSyn is dependent on the integrity of the endosomal compartment (Emmanouilidou et al., 2010; Alvarez-Erviti et al., 2011; Emmanouilidou and Vekrellis, 2016). Release can also happen through exosomes. Exosomes are vesicles of $<$100 nm of diameter that facilitate intercellular communication transporting proteins or RNA (Jansen et al., 2017; Mutreja and Gamblin, 2017). They can be released through budding forming a small vesicle or formed inside the multivesicular body, which fuses then with the cell membrane releasing its vesicles into the extracellular space (Beaudoin and Grondin, 1991; Denzer et al., 2000; Rustom

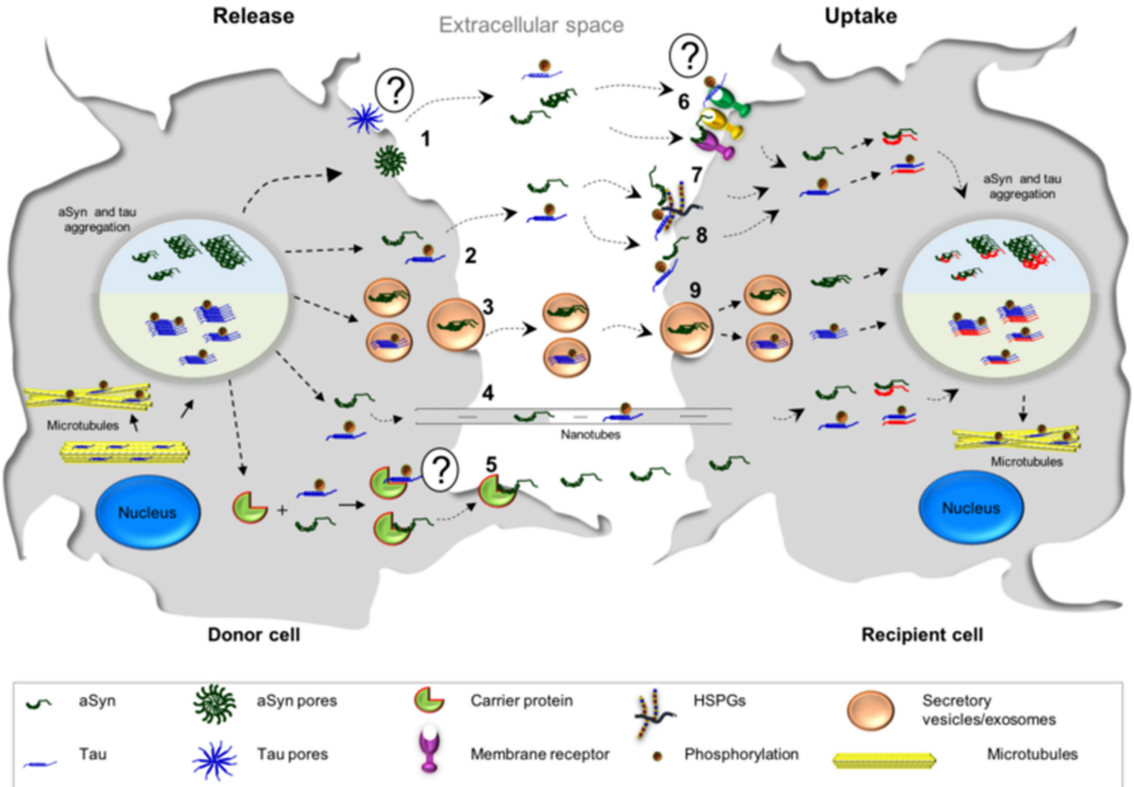

FIGURE 2 | Possible mechanisms associated with the cell-to-cell transmission of aSyn and tau. The release of tau and aSyn is thought to take place via different mechanisms: (1) aSyn oligomers may form pore-like structures that penetrate the plasma membrane. These structures may act as non-selective channels, leading to the release of aSyn. At the present moment, there is less evidence in support of such mechanism for tau; (2, 8) Direct penetration of the plasma membrane may lead to protein release through passive diffusion and, consequently, passive uptake from the extracellular space, a common mechanism for both proteins; (3) aSyn and tau monomers and oligomers may be released in exosomes/secretory vesicles; (4) transmission of aSyn and tau may also occur via tunneling nanotubes which are membrane bridges between the cells composed by F-actin; (5) another possibility is that aSyn interacts with a possible carrier protein, which mediates the transfer in the plasma membrane and subsequently the release—it is still not known if tau could be released in this manner; (6) the release of both proteins may also take place from dying cells and the uptake of aSyn and tau could be mediated by cell surface receptors; (7) Heparan sulfate proteoglycans (HSPGs) may facilitate the internalization of aSyn and tau. Both proteins (monomers or fibrils) bind HSPGs at the cell surface, and then get internalized; (9) both proteins may be taken up by endocytosis.

et al., 2004). Membrane carrier proteins like the secretory carrier membrane protein 5 can also participate in the release of aSyn through exosomes (Yang et al., 2017). These release processes can happen from either the cell soma or the synaptic button, as aSyn is also transmitted trans-synaptically (Danzer et al., 2011; Freundt et al., 2012; Yamada and Iwatsubo, 2018), in processes that may or may not require axon-dendrite contacts (Freundt et al., 2012). Interestingly, mutant forms of aSyn such as the H50Q and the G51D are more prone to be released via exosomes and other types of extracellular vesicles than the wild type protein (Falcon et al., 2018). In addition, aSyn can also reach the extracellular space, not only by active mechanisms, but also passively by leakage through damaged cell membranes or by cell impairments. This process can be exacerbated by aSyn itself, as its interactions and fibrillization may disrupt cell membrane integrity (Volles and Lansbury, 2002; Chaudhary et al., 2014).

Internalization can then happen through the aforementioned mechanisms. During pinocytosis, aSyn is internalized in a dynamin-dependent process which seems to be more relevant for monomeric than for aggregated aSyn (Hansen et al., 2011). Furthermore, the endocytic process of aSyn internalization is a dynamin-dependent process, but not clathrin-dependent (Uversky, 2011a).

Tunneling nanotubes are F-actin containing membranous bridges that connect the cytoplasm of remote cells, first described in PC12 cells (Abounit and Zurzolo, 2012). Fibrillar aSyn can spread from cell to cell in a prion-like way through tunneling nanotubes by mechanisms such as intercellular trafficking of lysosomes (Abounit et al., 2016a; Dieriks et al., 2017). This happens not only among neuronal cells, but also in pericytes and astrocytes (Dieriks et al., 2017; Rostami et al., 2017), and from one type of cell to another (Sun et al., 2012).

Changes in aggregation propensity like the ones mentioned above lead to changes in the internalization and clearance of aSyn (Lee et al., 2008).

It has been proposed that aSyn can cross the cell membrane through pore-like structures such as the β-barrel voltage-dependent anion channel (Hoogerheide et al., 2017).

These pores can be formed by aSyn itself in its oligomeric form, as it crosses the cell membrane leading to the formation of octameric ring structures, being the mutant A53T more prone to do it (Ma et al., 2017). The formation of these pores by aSyn is related with its binding properties to membranes and lipid layers. The association with these membranes leads to changes in membrane conductance which result to changes in its pore activity formation (Tosatto et al., 2012). The association with membranes is dependent on the presence of the KTKEGV repeat motif (Jao et al., 2004). In fact, binding of aSyn to the cell membrane causes permeabilization of the cell membrane by decreasing the lipid order (Stockl et al., 2013). This process also facilitates passive diffusion of the protein. Passive diffusion is a mechanism that allows exclusively the internalization of monomers (Lee et al., 2008). It is noteworthy that changes in the amphipatic N-terminus lead to an altered binding of aSyn to membranes. This results in abnormal vesicle interactions and changes the conformation of the vesicles, affecting aSyn spreading (Taneva et al., 2012; Dettmer et al., 2017). Out of the three known membrane proteins described to interact with aSyn, only the LAG3 and PrP^C mediate in its internalization through endocytosis (Chen et al., 2015; Mao et al., 2016; De Cecco and Legname, 2018). Activation of N-Methyl-D-Aspartate receptor (NMDAR) leads to the clathrin-mediated internalization of the receptor (Chen et al., 2017). Interestingly, activation of PrPc receptor by extracellular aSyn oligomers also leads to the phosphorylation of Fyn kinase activating the NMDAR receptor in a process that is independent of pore formation and impairs hippocampal long term potentiation leading to cognitive impairment (Diogenes et al., 2012; Chen et al., 2015).

Spreading of Tau Pathology

Tau also progresses in a predictable manner throughout the CNS. In AD, tau starts its propagation in the transentorhinal region and progresses through the hippocampus, cortex and the superior temporal gyrus, finally reaching the neocortex (Uversky, 2003; Braak et al., 2006). Nonetheless, particularly in FTD cases with Pick's disease type of tau pathology, atrophy progression starts in the frontal lobe and the hippocampus, then the temporal lobe and the insula, and finally pathology reaches areas of the parietal lobe (Broe et al., 2003; Gallea et al., 2018).

In mice, tau isoforms 2N4R, 2N3R, and 0N4R were found to readily and bidirectionally cross the blood-brain barrier (Ghosh et al., 2015).

Tau propagates trans-synaptically, mostly based on connectivity and not on proximity (Ahmed et al., 2014). This propagation happens mostly through afferent connections (Iba et al., 2015) and is mediated by trans-synaptic mechanisms (Dujardin et al., 2014), where transmission was proposed to take place through exosomes (Wang et al., 2017). Although propagation happens mainly through afferent connections, small tau species can be transported anterogradely and retrogradely in neurons (Wu et al., 2013). In P301S transgenic mice, tau pre-formed fibrils lead to templated misfolding of tau in a prion-like manner along neuronal connections (Stancu et al., 2015). When injected into the brain, aggregated P301S tau present in brain homogenates from P301S mice induces the spreading of filamentous tau pathology in ALZ17 mice (Clavaguera et al., 2009). Interestingly, the P301S mutant spreads at least five times faster than the wild type (WT) tau (Kundel et al., 2018).

In normal conditions, purified human tau does not form protein assemblies (Crowther et al., 1994). Polyanionic substances, especially glycosaminoglycans such as heparin or heparan sulfate proteoglicans (HSPGs), promote tau aggregation, thereby accelerating the formation of amyloid tau fibrils (Montejo de Garcini et al., 1986; Friedhoff et al., 1998). All six tau isoforms are able to aggregate even though, *in vitro,* the 4R isoforms are more prone to aggregation than the 3R isoforms (Zhong et al., 2012). Once the protein forms assemblies, these can act as seeds for the generation of new assemblies, even when they are applied extracellularly, spreading subsequently to other cells (Goedert and Spillantini, 2017).

Tau spreads from cell to cell through several different putative mechanisms, similar to those proposed for aSyn (Guo and Lee, 2011; Tardivel et al., 2016; Katsinelos et al., 2018; Polanco et al., 2018) (**Figure 2**). A mechanism that was proposed to be more frequent for tau than for aSyn, is the internalization of the protein via macropinocytosis (Lee et al., 2008; Holmes et al., 2013) (**Figure 2**). Macropinocytosis is an endocytic process driven by actin and involves the formation of the macropinosome in response to the direct actions of cargo/receptor molecules that coordinate the activity and recruitment of specific effector molecules, and subsequently fuse with degradative compartments of the cell (Kirkham and Parton, 2005; Kerr and Teasdale, 2009). Different types of HSPGs have been described to facilitate cellular internalization of aSyn and tau *in vitro* and *in vivo* and blocking their expression diminishes the internalization of tau and aSyn monomer and aggregates (Holmes et al., 2013; Gerson et al., 2014; Dujardin et al., 2018). Oligomers and short fibrils which bind to membranes can be internalized through receptor-independent mechanisms. On the other hand, monomers, long fibrils, or long filaments, are more dependent on receptor-mediated mechanisms (Wu et al., 2013). Extracellular tau can also affect the accumulation of endogenous tau in a way that is dependent on the tau isoform found in the extracellular space. In addition, it has been shown that oligomeric 0N4R tau induces the accumulation of endogenous tau to a small extent, while oligomeric 0N3R, 1N3R, and 1N4R tau do not stimulate accumulation of intracellular tau (Swanson et al., 2017).

Detachment of tau from microtubules, e.g., due to hyperphosphorylation, increases the levels of free intracellular protein, which can then cross the membrane through translocation mechanisms (Katsinelos et al., 2018). Certain extracellular forms of tau, especially soluble forms composed mostly of monomers and small oligomers, appear to be cytotoxic through muscarinic receptor activation, involving the tissue non-specific alkaline phosphatase (Sebastian-Serrano et al., 2018). Extracellular tau, mainly truncated forms, can also contribute to synaptic dysfunction (Brandt et al., 1995; Sebastian-Serrano et al., 2018). In fact, at least 75% of tau in the synapse of AD patients is C-terminally truncated, and can be released from cortical synapses and affect its physiological role (Sokolow et al.,

2015). Interestingly, these truncated forms have been reported to undergo truncation from the C-terminus to inner regions and have been linked to the pathogenesis of AD (Basurto-Islas et al., 2008; Garcia-Sierra et al., 2008). So far, two major sites of truncation have been studied, especially truncation at residues E391 and D421. Interestingly, the truncation at D421 is not only found in AD brains, but also in Pick's disease brains, where C-terminal truncated tau has been proposed to be the main isoform found in exosomes (Mena et al., 1995; Gamblin et al., 2003; Basurto-Islas et al., 2008; Mondragon-Rodriguez et al., 2008; Kanmert et al., 2015). Furthermore, overexpression of the projection domain of tau suppresses neuronal growth factor-induced neurite formation, contributing to synaptic dysfunction (Brandt et al., 1995).

CELL MODELS FOR STUDYING THE SPREADING OF aSyn AND TAU

The mechanisms by which aSyn and tau spread through the central nervous system are of utmost importance to understand the progression of PD and AD, respectively. To study these mechanisms, several cell models of aSyn and tau spreading have been used in the last years.

Different cell lines have been used to study specific mechanisms underlying the spreading of aSyn and tau pathology. For instance, the mouse neuroblastoma N2a cell line has been extensively used to study cell-to-cell spreading through exosomes (Wang et al., 2017). Other cell lines, such as the HEK293, SH-SY5Y, and B103, or primary neuronal cultures, were also used

TABLE 1 | Cell models used to study aSyn spreading.

Model	Mutation	Result	References
SH-SY5Y	None	aSyn can be transferred from one cell to another via exosomes	Alvarez-Erviti et al., 2011
N2a; primary hippocampal neuron; N2aprnp$^{-/-}$	None	Prp facilitates the accumulation and spreading of aSyn aggregates in N2a cells and primary hippocampal neurons	Aulic et al., 2017
HEK293T	None	Used to titer the viral vectors	Azeredo da Silveira et al., 2009
Primary cortical neurons; primary astrocytes; human Lewy body incubation	None	Quantification of aSyn uptake in neurons and astrocytes	Cavaliere et al., 2017
SH-SY5Y; primary cortical neurons; mouse cortical NSCs; aSyn expression using recombinant AdV	None	aSyn uptaken by cells; observed cell-to-cell propagation	Desplats et al., 2009
SH-SY5Y	None	Cell to cell transfer in a 3D human neuron-like cell model	Domert et al., 2016
SH-SY5Y; Tet-off system	None	aSyn is secreted through an endosomal pathway	Emmanouilidou et al., 2010
HEK293; SH-SY5Y; SKMe15: N2A/SKMe15 coculture; CM with aSyn oligos	None	aSyn can transfer between cells and seed the assembly of soluble aSyn	Hansen et al., 2011
Primary oligodendrocites; MN9D; Oli-neu; OLN93	A53T	Oligodendrocytes, Oli-neu, and OLN-93 uptake aSyn. Uptake is inhibited when clathrin heavy chain is silenced	Kisos et al., 2012
Primary cortical neurons; SH-SY5Y; KG1C; PC12	A30P; A53T	Incorporated aSyn oligomers form cytoplasmic inclusions	Konno et al., 2012
SH-SY5Y	None	aSyn is secreted through exocytosis	Lee et al., 2005
SH-SY5Y	A30P; A53T	High level of expression	Lo Bianco et al., 2002
Primary neurons; primary astrocytes; fibril administration	None	Transfer from neurons to astrocytes	Loria et al., 2017
Primary neuron, microglial and astrocyte culture; COS-7; HeLa; HEK293FT; SH-SY5Y; PFF administration; PFF with biotin; aSyn-biotin PFF administration	LAG3$^{-/-}$	Pathologic aSyn transmission and toxicity is initiated by binding to LAG3 and that neuron-to-neuron transmission of pathological aSyn involves the endocytosis of exogenous ASyn PFF by the engagement of LAG3 on neurons	Mao et al., 2016
B103; human exosome incubation	None	Exosomes internalized by endocytosis	Ngolab et al., 2017
HEK293; infected with MSA and PD samples	A53T	Samples from MSA patients could be transmitted to cultured HEK cells but not samples from PD	Prusiner et al., 2015
OLN-93	None	Capture of monomers, oligomers and fibrils	Reyes et al., 2014
N2a; neuronal iPS; CM	None	Transfer from neuron to neuron	Reyes et al., 2015
HEK293; PC12	None	High level of expression	Yamada et al., 2004

CM, conditioned media; MSA, Multiple System Atrophy; PFF, Pre-formed fibril.

to study exosomal-mediated release, but less frequently (Alvarez-Erviti et al., 2011; Polanco et al., 2016; Ngolab et al., 2017). The cell-to-cell transmission through tunneling nanotubes has been studied in the cathecolaminergic CAD cell line (Abounit et al., 2016a). The C17.2 neural precursor cell line was used to study macropinocytosis (Holmes et al., 2013) (**Tables 1**, **2**).

TABLE 2 | Cell models used to study tau spreading.

Model	Mutation	Result	References
CAD; HeLa; h tau fibrils administration	None	Tau fibrils are internalized and appear inside tunneling nanotubes	Abounit et al., 2016b
Rat primary neuronal cultures; V5-Tau-LV or eGFP-LVs treatment	P301L	Cell-to-cell transfer of WT V5-Tau protein via axonal transport from the primary neurons; transferred species mainly in a dephosphorylated state	Dujardin et al., 2014
HEK293T; HEK-TREx-293; h tau transfection; sarkosyl insoluble tau and total brain lysate from TgP301S mice administration	P301S	Native Tau aggregates enter Cells through the same mechanism as recombinant Tau aggregates, consistent with macropinocytosis	Falcon et al., 2015
QBI-293; human WT, P301L, T43, t40/deltaK280,T40/P301L, and T40/R406W transfection; myc-tau and K18 fibril administration	P301L; deltaK280; R406W	Spontaneous fibril uptake is mediated by endocytosis	Guo and Lee, 2011
C17.2; HEK293; tau RD-CFP, tau RD-YFP, and FRET biosensor for tau; aSyn-488, Htt exon 1 (Q50) administration	P301L; V337M	Tau fibrils enter cells via macropinocytosis; HSPGs are receptors for cell uptake of tau and α-synuclein	Holmes et al., 2013
Mice primary neuronal cultures; HEK293T; liposome transduction of tau seeds; treatment with tau, synuclein and Htt(Q50) seeds; tau RD-CFP, tau RD-YFP, and FRET biosensor tau cell line	P301S	Seeding with tau seeds; interaction with synuclein	Holmes et al., 2014
HEK293; tau RD-CFP, tau RD-YFP, and FRET biosensor for tau	deltaK280; P301L; V337M; I227P; I308P	Repeat domain aggregates transfer between cells, induce aggregation and propagate misfolding between cells; transfer within cell medium	Kfoury et al., 2012
M1C; NB2a/d1; pRcCMV and pcDNA/V5-DEST transfection	None	Tau exon 2 insert inhibits tau secretion	Kim et al., 2010
Primary neuronal cultures; primary microglial cultures; primary astrocyte cultures; COS-7; HeLa; HEK293FT; SH-SY5Y; PFF administration; PFF with biotin; aSyn-biotin PFF administration	LAG3 -/-	Tau PFF do not bind LAG3	Mao et al., 2016
HEK293; HEK293T; tau RD-CFP, tau RD-YFP, and FRET biosensor tau cell line	P301L	Transfer of seeds through exosomes; P301L tau-containing exosome-like EVs carry tau seeds that are capable of inducing aggregation of endogenous tau after being taken up by recipient tau biosensor cells	Polanco et al., 2016
Primary neuronal cultures; mCherry-CD9-10 and Dendra2-CD9-10 transfection; exosome administration	P301L	Exchange of exosomes by interconnected neurons	Polanco et al., 2018
Primary neuronal cultures	None	Stimulation in release by neuronal activity; probably through a pre-synaptic mechanism rather than by extrusion of exosomes	Pooler et al., 2013
HEK293; tau RD-YFP, aSyn-YFP, htt exon 1 (Q25)-YFP	P301S	Seeding dependent on beta-sheet structure; tau propagates different strains; propagates to naïve cells after lysate transduction	Sanders et al., 2014
SH-SY5Y; h 3R1N, 4R1N, and HA-4R1N tau transfection; h APP-695 wild-type (WT), F690P, KM670/671NL, V717F, V717G, and APP-C99 transfection; h tau fibrils administration	F690P (for APP)	Overexpressed APP on the cell surface associates with tau fibrils and accelerates intracellular tau aggregation; transient expression of APP may increase the activity of cellular endocytosis and metabolism of APP	Takahashi et al., 2015
CAD; V5-hTau1N4R, mCherry-tubulin and GFP-actin LV infection	None	Extracellular tau species activate the formation of TNTs; tau is transported through TNTs via actin	Tardivel et al., 2016
N2A; GFP-Tau, RFP-Tau, GFP-flotillin	deltaK280	Release of seeding prone tau through exosomes; mutants for FTDP more prone; synaptic contacts are required for exosome-mediated transmission of tau	Wang et al., 2017
Mice primary neuronal cultures; HeLa; low MW aggregates administration	$MAPT^{-/-}$	Tau aggregates are taken up in tau KO mice neurons; small tau aggregates internalized and anterogradely transported in neurons and non-neuronal cells	Wu et al., 2013
Mice primary neuronal cultures; RD-P301S YFP inoculation	P301L; $MAPT^{-/-}$:GFP	Transfer through cell medium	Wu et al., 2016

h, human; KO, Knockout; APP, Amyloid precursor protein; FTDP, Frontotemporal Dementia; TNT, Tunneling nanotube; WT, wild type; MW, molecular weight.

TABLE 3 | Animal models used to study aSyn spreading.

Model	Site of injection	Material injected	Mutation	Result	References
Sprague-Dawley rats	Substantia nigra	AAV2/6-haSyn, transplanted rat VM DAnergic neurons	None	Propagation from host tissue to transplanted dopaminergic neurons	Angot et al., 2012
Mice: $Prnp^{-/-}$; $prnp^{+/+}$	Substantia nigra	Recombinant aSyn fibrils	None	Spreading of aSyn may be facilitated by PrP	Aulic et al., 2017
Wistar rats	Substantia nigra	AAV2/6-CMV:-aSyn, -S129A-aSyn, -S129D-aSyn, -A30P-aSyn, -A30P-S129A-aSyn, -A30P-S129A-aSyn	S129A; S129D; A30P	S129A leads to the formation of smaller aggregates, S129D to larger	Azeredo da Silveira et al., 2009
Tg(SNCA)1Nbm/J	Left striatum	MSA and ILBD brain lysates inoculation Insoluble fraction of human cerebral cortices	$aSyn^{-/-}$	Transmission of aggregates to the contralateral side 9 months after inoculation	Bernis et al., 2015
Rats: Sprague-Dawley, Wistar, Lewis; mice: C57BL/6J, SAMP8, SAMR1 *Callithrix jacchus*	Substantia nigra (rodents)	AAV2/9-p.A53T-haSyn pAAV2-CMVie/hSyn-synA53T-WPRE-pA	A53T	Spreading to striatum and throughout the whole mesencephalon in rats 16 weeks after surgery. Absence of spreading for phosphor-aSyn in rats and marmosets	Bourdenx et al., 2015
Sprague-Dawley rats	Substantia nigra	rAAV2/1-aSyn	G2019S (LRRK2); h aSyn OX	Neurodegeneration in the contralateral side	Daher et al., 2015
Sprague-Dawley rats	Substantia nigra	rAAV6-aSyn-WPRE, rAAV6-aSyn+WRPE, or rAAV-CBA-mutant aSyn	h aSyn OX	Retrograde progression of neurodegeneration	Decressac et al., 2012
(Thy1)-hαSYN transgenic mice	Hippocampus	Lentiviral GFP, stem cells, MCNSC cells	h aSyn	Transmission of aSyn from host to grafted NSCs; Inclusion body formation via cell-to-cell transmission of aSyn	Desplats et al., 2009
Callithrix jacchus	Substantia nigra	rAAV2/5-CBA-aSyn	A53T	Presence of aggregates in the CP 1 year after transduction in the SN	Eslamboli et al., 2007
(Thy1)-h[A30P]aSyn transgenic mice	None	None	A30P	Widespread presence of aSyn aggregates after 12 months	Freichel et al., 2007
Sprague-Dawley rats; WT C57BL/6J mice	Right cortex	Recombinant aSyn monomers, oligomers and fibrils	h aSyn	aSyn can transfer between cells and seed the assembly of soluble aSyn	Hansen et al., 2011
C57BL/6J mice; C57BL/6JOlaHsd mice	Left vagus	AAV2/6-aSyn, AAV2/6-GFP	KO of SNCA gene; h aSyn OX	Spreading from medulla oblongata to rostral brain regions	Helwig et al., 2016
Sprague-Dawley rats	Intestine wall of the stomach and duodenum	PD brain lysate, recombinant aSyn monomers, oligomers and fibrils	None	Spreading from stomach and duodenum to CNS	Holmqvist et al., 2014
BDF1 mice	Striatum	DLB brain lysate sarkosyl soluble/ insoluble fractions	h aSyn OX	Inoculation fractions of human LBD in mice leads to CNS pathology	Jones et al., 2015
Sprague-Dawley rats	Right substantia nigra	rAAV-CBA-GFP, rAAV-CBA-aSyn, rAAV-CBA-μaSyn	A53T	Overexpression of wt or mutant aSyn induce a progressive neurodegenerative pathology in the nigrostriatal DA neurons	Kirik et al., 2002
Callithrix jacchus	Substantia nigra	rAAV-CBA-aSyn, rAAV-CBA-μaSyn, rAAV-CBA-GFP	h aSyn OX	Spreading from medulla oblongata to rostral brain regions. Release do not enhance interneuronal hα-syn propagation	Kirik et al., 2003
A53T aSyn tg mice; Clathrin silencing	None	None	A53T	Oligodendrocytes, Oli-neu and OLN-93 uptake aSyn. Uptake is inhibited by silencing the expression of clathrin heavy chain	Kisos et al., 2012

(Continued)

TABLE 3 | Continued

Model	Site of injection	Material injected	Mutation	Result	References
Sprague-Dawley rats	Substantia nigra	AAV-pTR-UF12, AAV-GFP, and AAV-pSyn30	A30P	Accumulation of aSyn in SN after 12 months; presence of Lewy-like neurites in the SN and the striatum	Klein et al., 2002
Sprague-Dawley rats	Substantia nigra	AAV1/2-A53T, AAV1/2-GFP, AAV1/2-EV	A53T	Transmission to the striatum from the SN	Koprich et al., 2010
Cynomolgus macaque	Substantia nigra	AAV1/2-A53T, AAV1/2-aSyn, AAV1/2-GFP, AAV1/2-WPRE-bGH-polyA	A53T; scrambled A53T	Age-dependent increase in the accumulation of A53T; higher levels and more widespread degeneration with A53T	Koprich et al., 2016
F344 rats	Striatum and ventral mesencephalon	AAV6-aSyn, AAV6-GFP	None	Fetal DA neurons grafted into the striatum of 6-OHDA lesioned rats can retrogradely transfer h aSyn from the host into the graft	Kordower et al., 2011
Rats	Right substantia nigra	VSV-G-A30P, VSV-G-A53T, VSV-G-HWT	A30P; A53T; wt	Overexpression of wt or mutated h aSyn leads to dopamine neuronal cell death in rodents not only in the site of injection	Lo Bianco et al., 2002
TgM83 transgenic mice	Somatosensory cortex and dorsal neostriatum	M83 mice brain lysate and recombinant aSyn fibrils	A53T; aSyn$^{-/-}$	Pathological aSyn propagated along major CNS pathways to regions far beyond injection sites	Luk et al., 2012
Sprague-Dawley rats	Above the right substantia nigra	rAAV6-aSyn, rAAV6-GFP	None	Development of degenerative changes in the nigrostriatal axons and terminals and DA release impairments	Lundblad et al., 2012
C57BL/6 mice; CD1 mice	Striatum	Recombinant aSyn fibrils	A53T; LAG3 -/-	Pathologic aSyn transmission and toxicity initiated by binding to LAG3, endocytosis of exogenous aSyn PFFs by the engagement of LAG3 on neurons	Mao et al., 2016
C57BL/6J mice	Substanstia nigra	Recombinant aSyn monomers and fibrils	None	aSyn is deposited in neurons through a prion-like mechanism or by seed-dependent aggregation by crossing the species barrier	Masuda-Suzukake et al., 2013
TgM83 transgenic mice	Intracerebral inoculation	M83 mice brain lysate	A53T; aSyn$^{-/-}$	Data consistent with prion-like propagation of aSyn	Mougenot et al., 2012
WT C57BL/6J × DBA/2F1 mice	Hippocampus	DLB exosomes	None	Exosomes may play a role in aSyn pathogenesis, possibly through the seeding of toxic forms of aSyn	Ngolab et al., 2017
C57BL/6 mice	Right substantia nigra	rAAV2/7-haSyn	A53T	Progressive nigral dopaminergic neuron loss, presence of aSyn-rich inclusions in the surviving cell bodies	Oliveras-Salva et al., 2013
Wistar rats	Right substantia nigra and striatum	A53T α-SYN rAAV2/7, recombinant aSyn monomers, oligomers and fibrils	h aSyn OX	aSyn crosses the blood brain barrier; propagation in a stain-dependent manner	Peelaerts et al., 2015
TgM83 transgenic mice	Right parietal lobe	PD and MSA brain lysate, M83 mice brain lysate	A53T	Transmission of MSA prions requires Tg A53T aSyn mice. Unsuccessful transmit PD to TgM83+/– mice	Prusiner et al., 2015
Mice: WT C57BL/6J; C57Bl6Sv129; *Macaca fascicularis*	Above the substantia nigra (mice) and motor striatum (monkeys)	PD brain lysate	aSyn$^{-/-}$	Whether injected into the striatum or the SNpc, LB-induced degeneration was detected earlier and more extensively at the level of striatal dopaminergic axon terminals rather than SNpc cell bodies.	Recasens and Dehay, 2014

(Continued)

TABLE 3 | Continued

Model	Site of injection	Material injected	Mutation	Result	References
C57BL/6J mice; Sprague-Dawley rats	Cortex	Recombinant aSyn monomers, oligomers and fibrils	h aSyn OX	Transfer from host brain to grafted oligodendrocytes; oligodendrocytes take up recombinant aSyn monomers and oligomers	Reyes et al., 2014
(Thy1)-h[A30P]aSyn transgenic mice	None	None	A30P	Nutritional factors can have a significant impact on α-synucleinopathy	Rotermund et al., 2014
Sprague-Dawley rats	Intravagal	AAV2/6-aSyn	h aSyn OX	Transfer to medulla oblongata from higher brain regions	Rusconi et al., 2018
Mice: $M20^{+/+}$; BL6C3HF1	Lateral to the lateral ventricles	Recombinant Δ71-82 and full lenght aSyn fibrils	h aSyn OX	Non-amyloidogenic Δ71-82 aSyn induce pathology. Amyloidogenic h aSyn shows limited induction of neuronal aSyn inclusions	Sacino et al., 2013
Mice: $M83^{+/+}$; $M83^{+/--}$; $M20^{+/+}$; C3H/C57BL6 n	Biceps femoris	Recombinant Δ71-82 and full lenght aSyn fibrils	A53T; h aSyn OX	Hindlimb intramuscular injection of fibrillar aSyn lead to CNS inclusion pathology	Sacino et al., 2014b
Thy-aSyn mice	Oral administration	PD gut microbiota	aSyn OX	Microbiota are required for the motor and GI dysfunction, postnatal gut-brain signaling by microbial molecules impact neuroinflammation and αSyn aggregation	Sampson et al., 2016
(Thy1)-h[A30P]aSyn transgenic mice	None	None	A30P	Pathological aSyn species can impair synaptic plasticity	Schell et al., 2012
Callithrix jacchus	Caudate nucleus and putamen	Recombinant human and mouse aSyn monomers and fibrils	None	Retrograde progression of neurodegeneration	Shimozawa et al., 2017
C57BL/6	Substantia nigra	rAAV GFP and rAAV aSym	h aSyn OX	Slow disease progression that mimics human disease and allows for earlier points of characterization and/or intervention	St Martin et al., 2007
Wistar rats	Left substantia nigra	AVV2: -aSyn, A56P-aSyn, -A30P, -A30P/A56P/A76P, -EGFP	A30P; A56P; A30P/A56P/A76P	Fibrillar and prefibrillar aSyn variants secreted from rat primary cortical neurons	Taschenberger et al., 2012
C57BL/6J × CBA/ca hybrid mice	None	None	Truncated 120 aa	h aSyn120 under the control of the TH promoter led to the formation of pathological inclusions in SN and OB	Tofaris et al., 2006
Rats; mice	Substantia nigra	AAV: -aSyn, -EGFP	Truncated 120 aa	Development of inclusions in axons	Tozzi et al., 2016
Sprague-Dawley rats	Right substantia nigra	rAAV5-αsynΔ110+rAAV5-GFP, rAAV5-αsynFL+rAAV5-GFP, or rAAV5-αsynFL+rAAV5-αsynΔ110	Truncated 110 aa	Mixture of truncated and full length, lead to increased accumulation and pathological aSyn in the striatum	Ulusoy et al., 2010
Sprague-Dawley rats	Left vagus	rAAV6: -aSyn, -GFP	h aSyn OX	Spreading from medulla oblongata to rostral brain regions	Ulusoy et al., 2013
Sprague-Dawley rats	Left vagus	rAAV6: -aSyn, -GFP	h aSyn OX	Spreading from medulla oblongata to rostral brain regions	Ulusoy et al., 2015
Sprague-Dawley rats	Substantia nigra	AAV: -aSyn, -EGFP	None	DMV nerve has a role in the transmission of aSyn from the brain to peripheral tissues	Ulusoy et al., 2017
Wistar rats	Substantia nigra	rAAV: 2/1, 2/2, 2/5, 2/6.2, 2/7, 2/8, and 2/9 eGFP	None	High widespread transgene expression	Van der Perren et al., 2011

(Continued)

TABLE 3 | Continued

Model	Site of injection	Material injected	Mutation	Result	References
TgM83 transgenic mice	Right parietal lobe	MSA brain lysate, M83 mice brain lysates	A53T; GFAP-Luc	Lethality upon transmission to animals and similar transmission to that of kuru, CJD, and related diseases	Watts et al., 2013
Sprague-Dawley rats	Left substantia nigra	rAAV-aSyn, rAAV-EGFP	None	Progressive dopaminergic cell loss and aggregation	Yamada et al., 2004
Mice; *Macaca fascicularis*	Substantia nigra (rodents and monkeys)	Lentiviral A53T and GFP	A53T	Age-dependent increase in the accumulation of A53T; higher levels of degeneration in monkeys than in mice	Yang et al., 2015
1K and 3K mice	None	None	E35K, 346K, 361K	3K mutation causes shift from cytosolic to membrane associated aSyn	Nuber et al., 2018
M83, M47, and haSyn mice	Hippocampus and cortex	Recombinant human, mouse A53T and E36K aSyn fibrils	A53T, E46K	Spreading of inclusion pathology only in M83 mice after 4 months	Sacino et al., 2014a

OX, overexpressing; CP, Cerebral Palsy.

ANIMAL MODELS FOR STUDYING THE SPREADING OF aSyn AND TAU

In recent years, animal models based in the administration of different forms of aSyn and tau in specific brain and peripheral areas have been used (**Table 3**). The use of animal models enables us to address specific aspects of the spreading of aSyn or tau pathology, in particular those related to neuronal connectivity, or to the spreading between different organs (Braak and Braak, 1991; Delacourte et al., 1999; Zaccai et al., 2008). For aSyn, both mice and rats have been extensively used (Hansen et al., 2011; Masuda-Suzukake et al., 2013; Holmqvist et al., 2014; Recasens and Dehay, 2014), while in the case of tau most studies employed mice (Dujardin et al., 2014).

Non-human primates have already been used to study the spreading of aSyn pathology (Kirik et al., 2003; Eslamboli et al., 2007; Shimozawa et al., 2017), but have not been reported thus far for the study of tau spreading. Interestingly, a lamprey model has been used to study the effects of several tau mutations in the progression of neurodegeneration (Lee et al., 2009).

Transgenic mice expressing mutant forms of aSyn, such as A53T and A30P, have also been used, and are proving useful for studying other synucleinopathies including MSA (Giasson et al., 2002; Prusiner et al., 2015). For tau, the rTg4510 mouse line, harboring the P301L mutation, has been widely used (Barghorn et al., 2000).

Additionally, injections of viral vectors have been also extensively used to induce aSyn and tau overexpression. In the case of aSyn, several studies reported the mimicking of relevant PD features, the progressive nature, and the spreading of pathology, in mice, rats, and non-human primates (Kirik et al., 2002, 2003; St Martin et al., 2007; Low and Aebischer, 2012). In the case of tau there are also models based on the use of viral vectors (Klein et al., 2008), including models for the study of rapid tau propagation (Asai et al., 2015) (**Tables 3**, **4**).

CONCLUSIONS

Tau and aSyn pathologies spread in a manner that is reminiscent of the process of prion spreading in prion diseases, whereby misfolded forms of the proteins can act as templates and induce the misfolding of normally structured proteins. The altered proteins can spread from cell to cell throughout the CNS, and possibly also between different organs through neuronal connections.

In contrast to prion diseases, in AD and PD there is still no definitive evidence for horizontal transmission of tau or aSyn pathologies, as these proteins have not been shown to be infectious. Additionally, the spreading of pathology in synucleinopathies and tauopathies seems to be slower than that observed in prion diseases, but the reason for this is still unknown (Yekhlef et al., 2003; Peden et al., 2004; Schofield et al., 2005; Desplats et al., 2009; Angot et al., 2012; Reyes et al., 2014; Mabbott, 2017).

The putative mechanisms involved in the spreading of both tau and aSyn are thought to be similar. One difference is that tau appears to have greater propensity to be internalized through macropinocytosis. Another difference is that, thus far, no putative direct receptors have been involved in mediating tau internalization, whereas for aSyn several putative "receptors" have been reported.

From an anatomical point of view, aSyn and tau appear to spread from and to different locations, in patterns that are relatively predictable (McCann et al., 2016; Hoenig et al., 2018; Schwarz et al., 2018).

In conclusion, through the use of different model systems, it is becoming evident that the spreading of aSyn and tau pathologies share similar mechanisms, and there is hope that

TABLE 4 | Animal models used to study tau spreading.

Animal model	Site of injection	Material injected	Mutation	Result	References
C57BL/6J mice	Left hippocampus	Mice brain extract	P301S	Spreading through connectivity	Ahmed et al., 2014
PS19 tau mice; microglial depletion	Medial entorhinal cortex, dentate gyrus	AAV2/6-SYN1, AAV2/6-SYN1-GFP, AAV2/6-GFP. Exosomes from microglial culture, exosomes from mouse brain lysates	P301L	Participation of microglia in the spreading of tau; mediation of exosomes	Asai et al., 2015
Wistar rats	Striatum	lentiviral hTau46WT, hTauP301L and GFP	P301L	AT8 immunoreactivity extends from the CA1 (Cornus Ammonis area 1) to the cortex in rats injected with LV-hTau46WT, whereas it is restricted to the hippocampal formation in rats injected with LV-hTau46P301L, even 8 months p.i	Caillierez et al., 2013
C57BL/6J mice; ALZ17 mice	Hippocampus and overlying cerebral cortex	Mice brain homogenate	P301S	Transmission of hyperphosphorylated tau in mice transgenic for human wt tau	Clavaguera et al., 2009
ALZ17 mice; APP23 mice	Hippocampus and overlying cerebral cortex	AD, tangle only dementia, Pick's Disease, argyrophilic grain disease, progressive supranuclear palsy, and corticobasal degeneration human brain extracts	P301S; h APP	Self-propagation of tau inclusions independently of other pathological mechanisms	Clavaguera et al., 2013
C57BL/6J mice	Intraperitoneal	P301S and WT mice brainstem homogenate	P301S	Tauopathies can be seeded in the brain by tau aggregates delivered peripherally	Clavaguera et al., 2014
rTG tau EC mice	None	None	P301L	Spreading of the pathology to downstream connected neurons	de Calignon et al., 2012
Wistar rats	Striatum	lentiviral V5-hTau46WT, hTau46WT, hTauP301L, and eGFP	P301L	Spreading of Tau throughout the brain 8 months after post-injection; transmission through connected areas; trans-synaptic transmission	Dujardin et al., 2014
C57BL/6J mice	Cortex	FL-Tau-488 fibrils	None	Tau fibrils enter cells via macropinocytosis; HSPGs are receptors for cell uptake of tau and aSyn	Holmes et al., 2013
B6C3 mice; C57BL/6J mice	None	None	P301S	Tau seeding activity detected at 1.5 months, before any changes in histopathology; hyperphosphorylated tau accumulation	Holmes et al., 2014
PS19 tau mice	Locus coeruleus	Recombinant tau fibrils	P301S	The pattern of spreading did not match neurofibrillary tangles staging in h AD brains, developed tau pathology more rapidly after tau PFF injections into the LC.	Iba et al., 2015
Lamprey	Hindbrain	WT htau23 and htau24 plasmids	P301L; G272V; V337M; R406W	All mutations accelerate progression	Lee et al., 2009
Neuropsin-tTA-Tau	None	None	P301L	Spreading from the entorhinal cortex	Liu et al., 2012
Tg tau P301L mice	Hippocampus	Recombinant tau fibrils	P301L	Single injection of tau PFFs in the hippocampus or frontal cortex of young tauP301L mice acts as a seed to induce spreading of tau pathology throughout the mouse brain; neuron loss in the hippocampus	Peeraer et al., 2015
rTg4510 mice	None	None	P301L	Transfer of seeds through exosomes; P301L tau-containing exosome-like EVs carry tau seeds that are capable of inducing aggregation of endogenous tau after being taken up by recipient tau biosensor cells	Polanco et al., 2016
B6C3 mice	Hippocampus	Mice brain homogenate	P301S; h 4R1N	Spreading from the left hippocampus after 5 weeks to the entorhinal cortex, retrospenial cortex and contralateral hippocampus	Sanders et al., 2014

(Continued)

TABLE 4 | Continued

Animal model	Site of injection	Material injected	Mutation	Result	References
rTg4510 mice	Cerebral cortex	Recombinant tau short filaments	P301L	Tau aggregates taken up in tau KO mice neurons; tau aggregates internalized and anterogradely transported between brain cells	Wu et al., 2013
rTg4510 mice; neuropsin-tTA-Tau	Medial entorhinal cortex and hippocampus	AAV5 CamKII.hM3Dq-mCherry, AAV9/CamKIIa.hChR2-mCherry, AAV9/CamKIIa.hChR2-mCherry	P301L; h tau; KO	Stimulation of release and spreading by increased neuronal activity	Wu et al., 2016
JNPL3; Tg4510 mice	Intraperitoneal	Pan-tau Abs DA9, and DA31, PHF-tau CP13, and RZ3 antibodies; and 1 conformation-specific antibody MC1	P301L	Tau detected in serum	d'Abramo et al., 2016
mice JNPL3	Hippocampus	AAV5-scFv-MC1, AAV5-eGFP	P301L	Diffusion to distant areas	Vitale et al., 2018
P19 mice	Right lateral ventricle	Human antisense oligonucleotides	P301S	Decrease in human tau mRNA reverses tau seeding	DeVos et al., 2017
SHR24, SHR72 rats	Motor cortex	Sarkosyl insoluble SHR72 and SHR24 brain homogenate	Truncated aa 151-391 3R and 4R tau	Spreading only of the SHR72 4R tau variant	Levarska et al., 2013
SHR72 rats	Hippocampus	AD brain insoluble fraction, human brain extract	Truncated aa 151-391 4R tau	Exogenous human AD tau was able to spread from the area of injection and induce tau pathology	Smolek et al., 2019

a deeper understanding of those mechanisms may lead to the identification of novel targets for therapeutic intervention in various neurodegenerative diseases.

AUTHOR CONTRIBUTIONS

EV and AD-M reviewed the literature and contributed equally to the design and writing of the manuscript. TO reviewed the final manuscript.

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Elevated Serum SIRT 2 May Differentiate Parkinson's Disease From Atypical Parkinsonian Syndromes

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Atypical Parkinson syndromes (APSs) often have symptoms that overlap with those of Parkinson's disease (PD), especially early in the disease, making these disorders difficult to diagnose. Previous studies have demonstrated an association of oligomeric α-synuclein (α-Syn), a key element in the pathogenesis of PD, with Sirtuin (SIRT)2 proteins for modulating PD. We aimed to evaluate SIRT protein expression in serum of PD patients and compare it with APSs and normal elderly control (GC) and to correlate this with α-Syn. SIRT protein expression was evaluated in sera of 68 PD; 34 APS and 68 GC without any neuro-psychiatric illness as controls by surface plasmon resonance (SPR). SIRT2 expression was correlated with α-Syn in PD and GC. Significant ($p < 0.0001$) differences were observed between serum SIRT2 concentration in PD and APS and GC as well as between APS and GC. Receiver operating characteristic (ROC) analysis revealed the strong cut-off value to differentiate PD from APS and GC and also APS from GC. Significant correlation was observed among SIRT2 levels in early PD patients with Unified Parkinson's Disease Rating Scale (UPDRS), Hoehn & Yahr (H & Y) and increased duration of disease. In addition, a strong positive correlation of SIRT2 with α-Syn ($p < 0.0001$) was observed. However, no such difference was detected for serum SIRT1 in cases of PD and APS or for GC. The present study is the first to report elevated serum SIRT2 in PD. The study also provided a simple test to distinguish PD from APS and may have translational utility for diagnosis.

Keywords: Parkinson's disease, atypical parkinsonian syndromes, protein marker, α-synuclein, sirtuin, SPR

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INTRODUCTION

Parkinson's disease (PD) is a common movement disorder of the aging population. It is the second most common neurodegenerative disorder after Alzheimer's disease. It affects 1–2 per 1,000 of the population and its prevalence increases with age to affect 1% of the population above 60 years of age (Tysnes and Storstein, 2017). The pathological changes in PD are well established as being the loss of dopaminergic neurons in the substantia nigra and the presence of cytoplasmic inclusions of Lewy bodies (LBs). LBs primarily consist of α-synuclein protein (α-Syn). α-Syn is primarily expressed by neurons and constitutively released into the extracellular space (Stefanis, 2012).

The N-terminal acetylation of α-Syn significantly reduces oligomerization of the protein and thus disrupts intermolecular H-bonds, thereby slowing the initiation of the process (Bu et al., 2017). Recently, it was found that Sirtuin-2 (SIRT2) deacetylates α-Syn (de Oliveira et al., 2017). Sirtuins are NAD^+-dependent protein deacetylases that mediate calorie restriction and promote longevity. The overexpression of SIRT1 reduces the aggregation of α-Syn in SHSY-5Y cells (Singh et al., 2017). SIRT2 deacetylates α-tubulin, an important pathway involved in the neurodegeneration of PD, promoting the buildup of its toxic complex with α-Syn oligomers (Kazantsev and Kolchinsky, 2008). Several reports have supported this observation by the inhibition of SIRT2 and reduction in the formation of the toxic complex and neurotoxicity (Outeiro et al., 2007; Chen et al., 2015). The inactivation of SIRT2 results in a rescue effect similar to that observed in the activation of SIRT1. Hence, it would be useful to examine the expression of SIRT1 and SIRT2 in PD in view of their opposite roles in the same cycle of α-Syn.

The main symptoms of PD are tremor, gait disturbance and movement. The diagnosis of PD is often difficult, especially at early stage due to overlapping symptoms. Phenotypically similar Atypical Parkinsonian Syndromes (APSs)—namely multiple system atrophy (MSA) and progressive supranuclear palsy (PSP)—can pose diagnostic challenges, even though the pathogenesis and treatment response are dissimilar. No gold standard diagnostic tests for PD are related to the underlying biological process, which necessitates research on biomarker development.

In view of the need for diagnostic tests with biological relevance in pathogenesis that can be performed easily, blood-based biomarkers have caught the imagination of researchers (Baird et al., 2015).

This study quantified SIRT protein levels using the real time label-free surface plasmon resonance (SPR) technique (Kim et al., 2006), which is well established for biomarker discovery in the picomolar range. This study evaluates the level of SIRT1 and SIRT2 protein expression in the serum of PD patients and compares it with APSs and normal elderly controls (GC) in addition to showing the correlation with serum α-Syn levels.

MATERIALS AND METHODS

Study Participants

One-hundred and two patients with PD and similar movement disorders and various stages of disease progression were recruited from the Department of Neurology and Geriatric Medicine of the All India Institute of Medical Sciences, New Delhi, India. Diagnosis of PD, PSP and MSA was carried out as per established clinical criteria by way of the UK Parkinson's Disease Society Brain Bank Criteria for PD (Hughes et al., 1992), NINDS criteria for PSP (Litvan et al., 1996) and consensus criterion for MSA (Osaki et al., 2009). There were 68 cases of PD and 34 cases of APS (20 MSA and 12 PSP) in the sample. Among these PD patients, 22 with PD whose disease duration was $\leq$3 years were considered as the early stage cohort as was reported earlier by Hansson et al. (2017). Sixty-eight individuals over 60 years of age with good health (no chronic disease like hypertension, diabetes and no history of any disorder of the central nervous system) were invited for participation in the study through advertisements or general announcements at the hospital.

Demographic details, duration of symptoms up to recruitment to the study, age at onset of illness, family history and co-morbidities were recorded. Detailed clinical assessment for Parkinson's Disease using the Unified PD Rating Scale (UPDRS, part III motor score) and Hoehn & Yahr staging systems was carried out. Venous blood (2 mL) was collected from the participants in yellow vacutainer (Vacutainer® SST™ Tubes) and was allowed to settle for 1 h at room temperature. The buffy coat was removed from the blood and centrifuged at 3,000 rpm for 20 min. Serum was collected and stored at 70°C in multiple aliquots.

Ethical Statement: The study was approved by the Ethics Committee, All India Institute of Medical Sciences, New Delhi, India (IECNP-221/05.06.2015, RP-01/2015) and written informed consent was obtained from each participant.

Estimation of Serum Sirtuins and α-Syn

All SPR measurements were performed at 25°C using the BIAcore-3000 (Wipro GE Healthcare, Upasala, Sweden), which is a biosensor-based system for real-time label-free specific interaction analysis. SPR is one of the most powerful technologies to determine specificity, affinity and kinetic parameters during the binding of macromolecules, including protein-protein, protein-DNA, enzyme-substrate or inhibitor, receptor-drug, lipid membrane-protein and protein-polysaccharide interactions. This technique measures the refractive index changes in the vicinity of thin metal layers in response to bimolecular interactions and the real-time response of the experiment is usually presented in the form of a sensorgram.

Mouse anti-SIRT1 IgG (Santa Cruz Bio-technology, Santa Cruz, CA, USA), goat anti-SIRT2 IgG (Santa Cruz Bio-technology, Santa Cruz, CA, USA) and mouse anti-α-syn IgG (Santa Cruz Bio-technology, CA, USA) were used for immobilization on different flow cells of a CM5 sensor chip using the amine coupling kit (Wipro GE Healthcare, Upasala, Sweden). The system was equilibrated with running buffer, i.e., HBS-EP buffer (Wipro GE Healthcare, Upasala, Sweden), and maintained at a flow rate of 5 μL/min. The experimental flow cell dextran matrix was activated using a 1:1 volume mixture of N-ethyl-N′-3-diethylaminopropylcarbodiimide (EDC; 75 μg/μl) and N-hydroxysuccinimide (NHS; 11.5 μg/μl). All three antibodies (100 μg/mL) were diluted with 10 mM sodium acetate (pH 5) and injected over the activated chip surface on three different flow cells, respectively. Unreacted groups were blocked by ethanolamine (pH 8.5).

Standard graphs were prepared by passing different known concentrations of pure recombinant SIRT1 proteins (0.7, 3.5, 7, 10.5, 14, 17.5, 21, 28 ng/μL), SIRT2 proteins (1.7, 8.5, 17, 25.5, 34, 42.5, 51, 68 ng/μL) and α-syn proteins (5, 10, 20, 40, 80, 160, 320 ng/μL) over corresponding antibody on the respective flow cells of sensor chips, and

response unit (RU) values were recorded. Recombinant SIRT1, SIRT2 and α-Syn proteins were cloned, expressed and purified in bacterial systems. Serum samples were diluted (1:70) with HBS-EP buffer, passed over immobilized antibodies and RU was obtained for each sample. Each sample was analyzed in triplicate. The concentrations of SIRT1, SIRT2 and the α-Syn of study groups were determined from respective standard curves by extra polating different RU values obtained for each sample.

By Western Blot

Serum samples collected from the PD, APS and GC groups were subjected to removal of major interfering proteins using an Albumin OUT kit according to the manufacturer's protocol (G-Biosciences, St. Louis, MO, USA). Total protein concentration was determined using a bicinchoninic acid assay (BCA) with bovine serum albumin as the standard. Total protein (40 μg) was separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). After electrophoresis, proteins were transferred to polyvinylidenedifluoride (PVDF) membranes (MDI Membrane Technologies, India). The membranes were then blocked over BSA 5% w/v with 0.05% Tween-20 prepared in TBS (10 mm Tris pH 7.5, 150 mm NaCl) for 2 h and subsequently incubated with primary antibodies diluted with TBS at 4°C overnight. The following antibodies and titers were used: mouse anti-human SIRT1 IgG (1:300) and goat anti-human SIRT2 IgG (1:300). After washing with TBS-T (20 mm Tris pH 7.5, 500 mm NaCl, 0.1% Tween-20), membranes were incubated with HRP-conjugated secondary antibodies—goat anti-mouse IgG (1:4,000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and Donkey Anti-Goat IgG (1:1,000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA)—at room temperature for 1 h. After washing with TBS-T, bands were visualized using SuperSignal West Pico Chemiluminescent substrate (Pierce Biotechnology; Thermo Scientific, Rockford, IL, USA). This experiment was also performed with recombinant α-syn protein to check the specificity of sirtuin (anti-SIRT1 and anti-SIRT2) antibody. Quantification of band intensity was performed using myImageAnalysis software (Thermo Scientific, Rockford, IL, USA).

Statistical Analysis

Statistical analysis was done using STATA version 14.2 (Stata. Corp. LP, College Station, TX, USA) and GraphPad Prism 5 (La Jolla, CA, USA). All the experiments were done in triplicate and mean ± SD was determined by GraphPad Prism 5. Apart from descriptive statistics, to find the association between qualitative variables, chi square test or Fisher exact test was used. To measure the correlation, Pearson correlation or Spearman rank correlation was used as required. Baseline comparison of quantitative measures between two groups was made using independent *t*-test or the Wilcoxon rank sum test and for more than two groups one-way ANOVA with Bonferroni correction was used. Receiver operating characteristic (ROC) curves were constructed to determine best cut-off, and area under the curve (AUC) was used to compare predictive ability of proteins. The best cut-off point was the point where the sum of specificity and sensitivity were maximized, when equal weight was given to both. A p-value less than 0.05 was considered as statistically significant.

RESULTS

Demographic Data of the Study Groups

Descriptive statistics of cases and controls are presented in **Table 1**. Mean age and body mass index (BMI) were similar among cases and controls. Eleven (16.17%) PD cases had positive family history and the rest were sporadic PD. In PD, the mean Hoehn & Yahr stage was 2.15 ± 0.67 with a UPDRS part III motor score of 23.29 ± 10.79.

Quantitative Analysis of Serum Sirtuins and α-Syn

Estimation by SPR

The SPR signals (RU value) for immobilization of human SIRT1, SIRT2 and α-syn antibodies over sensor chip CM5 were 6,164.6, 6,075.8 and 6,046.4, respectively **(Supplementary Figures S1A–C)**. The binding pattern of the ligands, i.e., pure recombinant SIRT1, SIRT2 and α-Syn proteins, plotted in

TABLE 1 | Descriptive statistics of the study population.

Variables (N)	GC (68)	PD (68)	APS (34)	*p*-value
Male (%)	51.47	86.76	61.76	0.000[a]
Family History (%)	Nil	16.17	Nil	
Age (years), Mean ± SD	64.76 ± 5.99	65.51 ± 4.71	67.18 ± 6.15	0.2159[b]
Disease duration (years) Mean ± SD	Nil	6.11 ± 4.66	3.92 ± 4.13	0.0058[c]
BMI kg/m^2, Mean ± SD	23.14 ± 4.30	23.67 ± 3.18	24.69 ± 3.17	0.0919[b]
SIRT1 Mean ± SD (95% CI) ng/μL	6.16 ± 1.13 (5.90–6.44)	5.89 ± 0.70 (5.73–6.07)	5.84 ± 0.90 (5.30–6.40)	OA = 0.3319[b] GC*PD = 0.099 GC*APS = 0.239 PD*APS = 0.961
SIRT2 Mean ± SD (95% CI) ng/μL	11.18 ± 2.35 (10.62–11.76)	16.19 ± 2.78 (15.52–6.86)	13.86 ± 2.56 (12.97–14.76)	OA = <0.0001[b] GC*PD = $p < 0.0001$ GC*APS = $p < 0.0001$ PD*APS = $p = 0.0001$

*BMI, body mass index; OA, Overall p value, GC*PD; GC vs. PD, GC*APS; GC vs. APS, PD*APS; PD vs. APS. a, chi square test; b, one-way ANOVA with Bonferroni correction; c, Wilcoxon rank sum test.*

a standard curve were in the linear range **(Supplementary Figures S1D–F)**.

The concentration of the sirtuins in serum samples were evaluated from the standard curve and observed mean concentration of serum SIRT2 was significantly ($p < 0.0001$) higher in case of PD compared to APS and GC (**Table 1**, **Figure 1A**). However, no significant difference existed in the concentration of serum SIRT1 between cases of PD and APS and GC (**Figure 1B**). Further, no significant ($p = 0.2389$) difference was observed in SIRT1 level between APS and GC (**Table 1**).

Based on the SPR data, the area under ROC was computed to measure the utility of both SIRT1 and SIRT2 as potential markers for PD between two diagnostic groups to evaluate how well a parameter can distinguish between them. In this study, higher levels were associated with disease condition; hence, the ROC curves were constructed to detect PD. ROC analysis showed that SIRT2 could differentiate PD from GC (AUC = 0.929; 95% CI: 0.89–0.97; cutoff: 13.50 ng/μL) with high sensitivity and specificity (**Figure 1C**). SIRT2 could differentiate PD from APS with good sensitivity and specificity (AUC = 0.713; 95% CI: 0.61–0.81; cut-off: 15.35 ng/μL; **Figure 1D**) as well as APS from GC (AUC = 0.787; 95% CI: 0.68–0.89; cut-off: 13.07 ng/μL; **Figure 1E**). However, SIRT1 did not have the strength to discriminate the disease from GC (AUC = 0.413; 95% CI: 0.31–0.51) and APS (AUC = 0.490; 95% CI: 0.36–0.62). No difference was noted in relation to family history of disease ($p = 0.4609$).

In PD patients with early-stage disease duration (≤3 years), a correlation of serum SIRT2 concentration was noted with UPDRS part III motor score ($r = 0.605$, $p = 0.0028$), duration ($r = 0.54$, $p = 0.0101$) and H&Y stage ($r = 0.41$, $p = 0.05$; **Figures 2A–C**). However, no significant correlation of SIRT2 with UPDRS or H&Y was observed with total PD in the study cohort.

α-Syn serum concentrations showed the same pattern as SIRT2 and were significantly ($p < 0.0001$) elevated in PD (38.34 ± 6.32 ng/μL, 95% CI: 36.81–39.87) as compared to GC (32.92 ± 4.81 ng/μL, 95% CI: 31.75–34.08; **Supplementary Figure S2A**). AUC of α-Syn from ROC curve was found to be 0.755 (95% CI: 0.67–0.84, cut-off: 35.05 ng/μL; **Supplementary Figure S2B**). Correlation coefficients indicated a highly positive correlation between α-Syn levels and serum SIRT2 ($r = 0.723$, $p < 0.0001$) within PD patients (**Figure 2D**). The comparative ROC of SIRT2 and α-Syn is illustrated in **Figure 2E**, which shows that predictive performance of SIRT2 was significantly better ($p < 0.0001$). Further, we did not observe significant correlation of α-Syn levels with age ($r = 0.083$, $p = 0.5028$), H&Y stage ($r = 0.109$, $p = 0.376$), UPDRSIII score ($r = 0.2$, $p = 0.101$) or

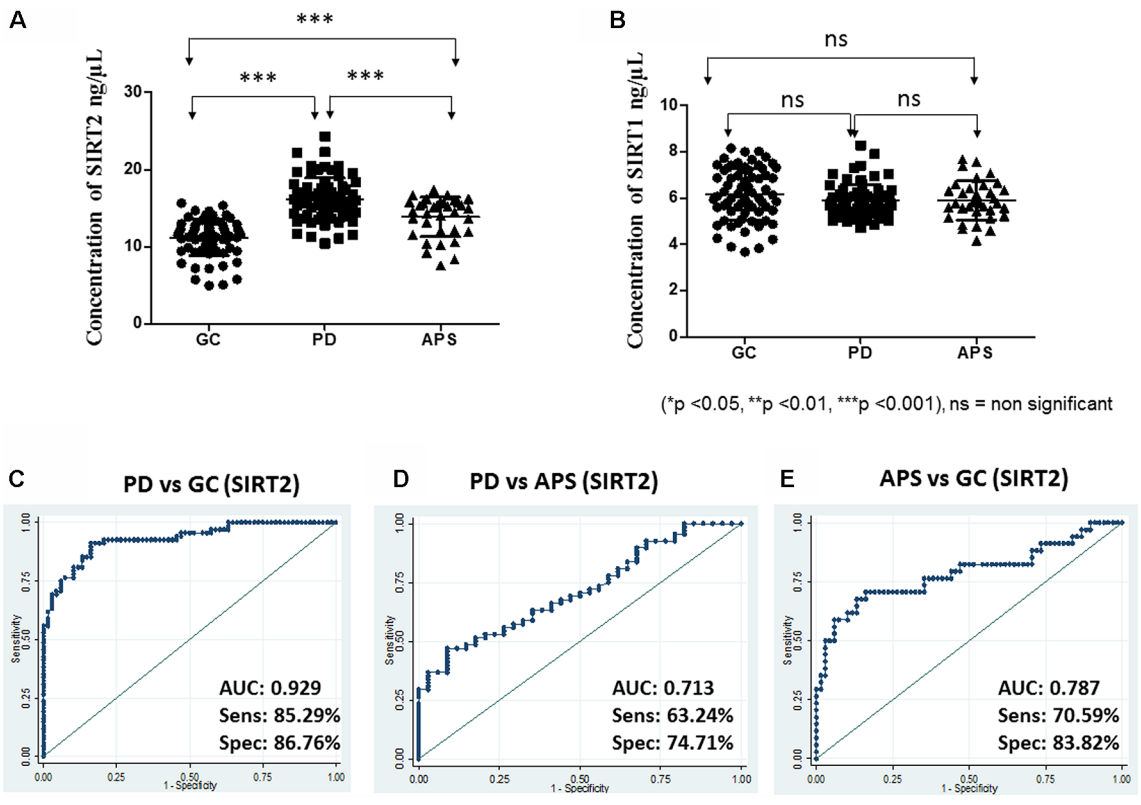

FIGURE 1 | Scatter plot of serum **(A)** Sirtuin 2 (SIRT2) and **(B)** SIRT1 concentration in elderly control (GC), Parkinson's disease (PD) and atypical parkinson syndrome (APS) by one-way ANOVA with Bonferroni correction. Receiver operating characteristic (ROC) curves of serum SIRT2 between different groups: **(C)** PD vs. GC, **(D)** PD vs. APS and **(E)** APS vs. GC. ***$p < 0.001$; ns, non-significant.

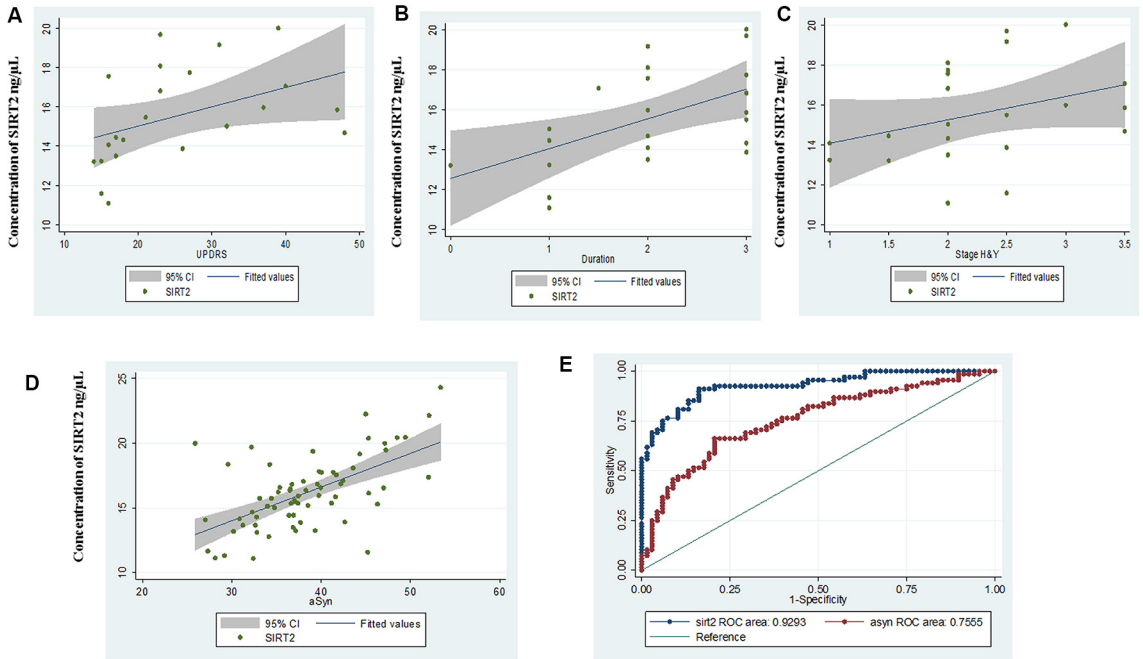

FIGURE 2 | Scatter plot for correlation of serum SIRT2 with **(A)** Unified Parkinson's Disease Rating Scale III (UPDRS III), **(B)** duration, **(C)** Hoehn & Yahr (H&Y) in early disease group, **(D)** α-synuclein (α-syn) and **(E)** comparative ROC of SIRT2 and α-syn.

disease duration (r = 0.1, p = 0.405). Also, the levels of serum SIRT1 did not correlate with serum α-Syn levels (r = 0.142, p = 0.2459) or SIRT2 (r = 0.110, p = 0.3718).

Western Blot

Western blot analysis of the serum samples was used to validate the differential expression of SIRT1 and SIRT2 in PD, APS and GC. Results were consistent with the band density of SIRT1 and SIRT2 in PD when compared to APS and GC (**Figures 3A,B**) as demonstrated by SPR data.

DISCUSSION

In this study, SIRT1 and SIRT2 were estimated for the first time in the serum of PD patients and compared with APSs and GCs. Sporadic PD cases are 85%–90% natural and the rest are familial in origin. In the present study only 16% cases were of familial origin, in line with previous reports (Singleton et al., 2013). Although the present study is not an epidemiological study, the lower number of APSs does reflect their relative rarity compared with PD, as reported earlier (Osaki et al., 2011).

SIRT2 concentration was high in cases of PD and differentiated from APSs and GCs. The level of SIRT2 in PD patients was found to be increased irrespective of the other covariates. This study found a significant correlation of SIRT2 levels in early PD patients (disease duration ≤3 years) with different parameters, such as UPDRS, H & Y and an increased disease duration. Serum SIRT2 level can also differentiate PD from APS patients according to an ROC analysis with efficient cut-off values. However, no significant difference in SIRT1 was observed among the study groups.

Knowledge about the role of sirtuins in complicated biological systems that influence many regulator molecules and pathways in health and disease is still incomplete. Few studies of SIRT proteins in humans are available for neurodegeneration such as PD. In our earlier study with sirtuin in Alzheimer's disease, which is another of the most common neurodegenerative diseases, we found the downregulation of SIRT1 in serum compared with mild cognitive impairment as well as the control group (Kumar et al., 2013). The present study with PD surprisingly showed no effect on serum SIRT1 and overexpressed SIRT2 compared with the control. Hence, two isoforms of the same protein family are altered in a different manner in two of the most prominent neurodegenerative diseases with very high sensitivity and specific statistical parameters.

The periodic updating of diagnostic criteria reflects the continuity of efforts to understand the disease. It also increases knowledge on the critical pathophysiology of PD to establish a gold standard for diagnosis criteria based on the pathology of the disease state. Ideally, the body fluid closest to the organ involved provides the best indication of the pathological process. However, it may not always be easy to access such body fluid repeatedly, namely CSF, without the risk of harm, especially in older subjects. Thus, the analysis of other body fluid proteomes also appears to be a promising approach to identify the protein biomarkers of diseases. The human protein circulates in the serum due to secretion and leakage from a variety of tissues (Taylor, 1969). It was reported earlier that these circulated proteins reflect human physiological or pathological conditions

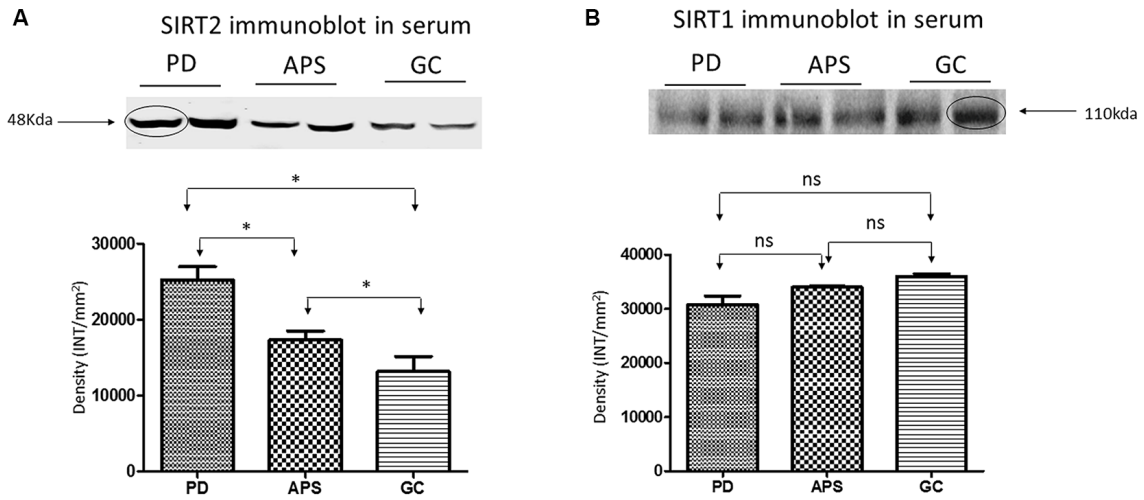

FIGURE 3 | Western blot and density analysis by one-way ANOVA with Bonferroni correction of **(A)** SIRT2 and **(B)** SIRT1 in the serum of PD, APS and GC. Lane 1–2 (PD), Lane 3–4 (APS), Lane 5–6 (GC). Histograms representing normalized mean integrated density ± standard deviation values. *$p < 0.05$; ns, non-significant.

(Anderson and Anderson, 2002). Blood-based markers offer many advantages due to their easy accessibility compared with CSF. In this study, SPR technology is used for the assay. SPR is an optical phenomenon that is sensitive to changes in the optical parameter of the medium close to a metal surface *via* a quantum mechanical aspect to determine specificity, affinity and kinetic parameters during the study of biomolecular interactions. SPR has proven to be useful in the discovery of biomarkers due to being label-free, its real-time nature, low amount requirement, reproducibility, robustness and high sensitivity. It has been successfully applied in detection and validation of biomarkers for numerous diseases (Cooper, 2003; Homola, 2003; Nguyen et al., 2015). The SPR technique offers several advantages over other immunological experiments such as ELISA for the reusability of the antibody over a large number of samples.

In the present study, circulating α-Syn was found to be overexpressed in PD compared with controls, in line with earlier reports. In recent years, substantial progress has been made in identifying the events, pathways and proteins involved in the pathogenesis of PD. The current research interest focuses on α-Syn, which has been implicated in the development of familial as well as sporadic forms of PD. Both structurally normal as well as abnormal α-Syn have been considered to be involved in the genesis of PD (Stefanis, 2012). The abnormal α-Syn increased the toxicity of brain cells, which can trigger neurodegeneration in critical areas of the brain, leading to the development of the disease, suggesting the possibility of it being a prion protein and PD a prion disease. The variable a-Syn was reported in CSF and blood levels of PD patients by different groups in the literature. Supporting this hypothesis, increased (Aasly et al., 2014) and decreased (Mollenhauer et al., 2011) α-Syn concentrations in CSF have been observed in PD, while normal (Gupta et al., 2015) and upregulated (Duran et al., 2010) α-Syn concentrations were reported in plasma of PD patients.

The linkage between α-Syn and sirtuins in PD is interesting. SIRT1 localized mainly in the nucleus (Tanno et al., 2007) and SIRT2 is localized in the cytoplasm (North et al., 2003). SIRT2 deacetylates α-tubulin, which stabilizes microtubules, and microtubules are found throughout the cytoplasm. The possible hypothesis involved in the pathophysiology of PD may be either the interaction of the deacetylated α-tubulin oligomer by SIRT2 with an α-Syn oligomer or the promoting initiation of α-Syn oligomerization by deacetylating the N-terminal of α-Syn by SIRT2. Hence, SIRT2 might be associated with early events of pathogenesis. SIRT2 is indirectly associated with the cellular processes involved in the pathophysiology of neurodegenerative disorders such as autophagy, oxidative stress and inflammation by deacetylating FOXO1 to mediate autophagy in the context of neurodegeneration (Salih and Brunet, 2008). Previous studies have shown the rescue of neuronal death by the inhibition of SIRT2 in PD models. This may be due to the formation of a larger LB-like inclusion by reducing the deacetylation of α-tubulin (Outeiro et al., 2007). The exact role of SIRT1 and SIRT2 in PD is not yet clear. This result also correlates with the overexpressed α-Syn protein in the serum of PD patients. The elevated level of α-Syn in PD supports previous findings reported in the literature.

PD remains a clinical diagnosis even 200 years after its first description, as no gold standard biomarker has evolved for pre-mortem diagnosis. Thus, with the best of clinical abilities and use of guidelines, deficiencies in diagnosis remain (Rizzo et al., 2016). Being a chronic disease of subtle symptoms and signs, diagnosis is often delayed, and the course of the disease is prolonged with the progression of disease pathology. A small subset of patients with movement disorder disease has extensive involvement of the brain with atypical manifestations and poorer response to treatment. Most PD patients are diagnosed at a late stage when vulnerable dopaminergic neurons in the substantia nigra have already been lost, and it is nearly impossible to detect PD by any screening test before the appearance of motor

symptoms. To date, lower uric acid, lower plasma ApoA1 and lower EGF levels have been identified as blood-based biomarkers in independent cohorts of PD patients (Chahine et al., 2014). However, none of these is a potential marker for the early detection of the disease. The proposed protein marker (SIRT2) can be the starting point of establishing diagnostic tests for PD, alone or in combination with other biomarkers.

The strength of the study is the label-free highly sensitive SPR technique used for the quantification of sirtuin proteins. This can detect the binding target molecule in the picomolar range. The technique is very sensitive as the minimum detection limit is 1 pg/mL. This study for the first time reported the serum sirtuin level in PD, APS and GC, which can be used in a panel of biomarkers for the early detection of diseases as well as to differentiate PD from APSs.

The novelty of the study is that for the first time, an increase in circulating SIRT2 is reported in PD and correlated with circulating α-Syn. The cut-off value, sensitivity and specificity of increased circulating SIRT2 obtained from the ROC analysis allowed us to distinguish PD from GC as well as APS. Its correlation with α-Syn suggests its association with early events in the pathogenesis of PD. It can be concluded from this study that SIRT2 may be a diagnostic marker for PD. A larger sample of PD as well as APSs, the use of CSF and follow-up assessment could provide further information on SIRT2 as a biomarker of PD.

LIMITATION

The limitation of the present study is its cross-sectional nature and small APS sample size.

DATA AVAILABILITY

No datasets were generated or analyzed for this study.

ETHICS STATEMENT

The study was approved by the Ethics Committee, All India Institute of Medical Sciences, New Delhi, India (IECNP-221/05.06.2015, RP-01/2015) and written consent was obtained from each participant.

AUTHOR CONTRIBUTIONS

AS did major part of the experiment. GR, AD and MB provided all blood samples after diagnosis of the disease. VS and SDw performed the statistical analysis. TB was involved in α-synuclein purification. SDe conceptualized the research and wrote the manuscript. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnmol.2019.00129/full#supplementary-material

FIGURE S1 | Immobilization profile of **(A)** SIRT1, **(B)** SIRT2 and **(C)** α-syn antibody on CM5 sensor chip. Standard curve plotted between Resonance unit (RU) and known concentrations of purified recombinant **(D)** SIRT1 **(E)**, SIRT2 and **(F)** α-Syn.

FIGURE S2 | (A) Scatter plot by independent *t*-test for serum α-Syn between PD vs. GC and **(B)** ROC curves of serum α-Syn PD vs. GC.

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Diverse Misfolded Conformational Strains and Cross-seeding of Misfolded Proteins Implicated in Neurodegenerative Diseases

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Numerous neurodegenerative diseases including prion, Alzheimer's and Parkinson's diseases are characterized by accumulation of protein aggregates in brain. Prion disease is unique in that the natively folded prion protein forms diverse misfolded aggregates with distinct molecular conformations (strains), which underlie different disease phenotypes. In addition, the conformational strains are able to self-propagate their unique conformations by recruiting normal protein monomers and converting their conformations to misfolded conformers. There is an increasing body of evidence that suggests other aggregation-prone proteins including tau and α-synuclein associated with Alzheimer's and Parkinson's diseases, respectively, also behave like a prion that has conformational strains with self-propagation (seeding) property. Moreover, misfolded protein aggregates can promote misfolding and aggregation of different proteins through cross-seeding, which might be associated with co-occurrence of multiple neurodegenerative diseases in the same patient. Elucidation of diverse conformational strains with self-propagation capability and of molecular basis for the cross-talk between misfolded proteins is essential to the development of effective therapeutic intervention.

Keywords: misfolding, prion, cross-seeding, α-synuclein, tau, conformational strain

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INTRODUCTION

The hallmark of numerous neurodegenerative diseases is extra- or intra-cellular deposits of misfolded protein aggregates in the central nervous systems (CNS; Sacchettini and Kelly, 2002; Jahn and Radford, 2008; Eisenberg and Jucker, 2012; Knowles et al., 2014; Chiti and Dobson, 2017). Protein misfolding and aggregation involves conformational changes from native polypeptides to aggregation-prone misfolded conformers, which self-assemble into fibrillar aggregates. The fibrillar protein assemblies adopt cross-β structures in which β-strands are arranged in an orientation perpendicular to the aggregation axis (Tycko, 2006; Riek and Eisenberg, 2016). More than 20 polypeptides have been identified to undergo misfolding transition associated with diverse human disorders including Alzheimer's and Parkinson's diseases, amyloidosis, type 2 diabetes, and prion diseases (Campioni et al., 2010; Eisenberg and Jucker, 2012; Chiti and Dobson, 2017).

The unique characteristics of prion protein is the ability to form diverse molecular conformations (strains) associated with different disease phenotypes (Collinge, 2010; Frost and Diamond, 2010; Westermark and Westermark, 2010; Jucker and Walker, 2013).

Misfolded prion aggregates are also capable of propagating their unique conformations and spreading pathological conditions between cells and tissues. Growing evidence suggests that other aggregation-prone proteins have prion-like properties of conformational strains associated with distinct disease phenotypes and self-propagation of the conformations through seeding (Frost and Diamond, 2010; Kim and Holtzman, 2010; Lee et al., 2010; Westermark and Westermark, 2010; Gerson et al., 2016; Goedert et al., 2017). Recent high-resolution structural studies using solid-state NMR and cryo-EM has begun to reveal near-atomic resolution structures of several filamentous aggregates (Colvin et al., 2016; Tuttle et al., 2016; Fitzpatrick et al., 2017; Falcon et al., 2018; Guerrero-Ferreira et al., 2018; Li B. et al., 2018). The high-resolution structures showed that a single polypeptide can adopt distinct filamentous aggregates (strains) with different molecular structures. In addition, misfolded strains with different molecular structures induce distinct disease phenotypes in animal models (Peelaerts et al., 2015), supporting the prion hypothesis for the neurodegenerative diseases.

Traditionally, misfolding and aggregation of a single protein was believed to be involved in each pathological process. For example, intracellular deposition of misfolded α-synuclein is linked to Parkinson's disease (PD), while intraneuronal accumulation of filamentous tau is associated with Alzheimer's diseases (AD). However, there is mounting evidence that suggests AD and PD pathologies are significantly overlapped presumably due to synergistic interactions between tau and α-synuclein, highlighting the complexity of Alzheimer's diseases and related dementia (ADRD) pathogenesis (Clinton et al., 2010; Moussaud et al., 2014; Castillo-Carranza et al., 2018). Understanding molecular basis for the misfolded conformational strains with self-propagation properties and cross-seeding between the misfolded proteins would, therefore, be crucial to the development of therapeutic interventions for the debilitating human disorders.

MISFOLDED CONFORMATIONAL STRAINS

Main structural feature of misfolded protein aggregates is the cross-β fold in which β-strands are stacked in an orientation perpendicular to the aggregation axis (bottom in **Figure 1A**). Major driving force for the formation of cross-β structure is the hydrogen bonding interactions between the main chain carbonyl oxygen and amide hydrogen (Sawaya et al., 2007; Lu et al., 2013; Riek and Eisenberg, 2016). Misfolded filamentous aggregates share the common structural motif irrespective of amino acid sequence. However, recent high-resolution structural studies revealed that misfolded filamentous aggregates can adopt diverse molecular structures within the common cross-β fold (Eichner and Radford, 2011; Tycko, 2015; Annamalai et al., 2016; Iadanza et al., 2018).

A majority of aggregation-prone proteins are intrinsically disordered under the physiological condition. These proteins include β-amyloid (Aβ) peptides, α-synuclein, and tau associated with various age-related neurodegenerative diseases. Intrinsically disordered proteins adopt a heterogeneous ensemble of conformations. The diverse conformers in the conformational ensemble might be induced to form distinct misfolded aggregates (strains) with different molecular conformations depending on experimental conditions (**Figure 1A**). Indeed various pathological proteins including Aβ peptides, α-synuclein and tau were shown to be able to adopt distinct strains with different molecular structures, which might be linked to phenotype diversities of the neurodegenerative diseases (Guo et al., 2013; Riek and Eisenberg, 2016; Goedert et al., 2017; Falcon et al., 2018; Peng et al., 2018a,b).

It is, therefore, of critical importance to characterize various strains with diverse molecular conformations to understand the molecular basis for the prion-like propagation and spreading of pathological protein aggregates. In this review article, diverse high-resolution structures of filamentous aggregates formed by three aggregation-prone proteins (Aβ peptides, α-synuclein, and tau) will be discussed.

Aβ Peptides

Aβ peptides are 36–43 residue peptides derived by the cleavage of amyloid precursor protein. Major components of the amyloid plaques observed in Alzheimer's patients are Aβ(1–40) and Aβ(1–42) peptides. Over the last two decades, structural studies have been predominantly focused on misfolded aggregates of the two Aβ peptides. The first structural model for Aβ(1–40) fibrillar aggregates was reported on the basis of solid-state NMR constraints (Petkova et al., 2002; Tycko, 2011; Lu et al., 2013). Later, higher resolution structures were determined by solid-state NMR and cryo-EM for the Aβ(1–42) fibrillar aggregates (Lu et al., 2013; Colvin et al., 2016; Wälti et al., 2016; Gremer et al., 2017).

The atomic resolution structure of Aβ(1–42) fibrillar aggregates revealed that the cross-β fold is stabilized by various interactions (**Figure 1B**). First, the β-strands stacked along the aggregation axis are held together by extensive hydrogen bonding interactions between amide hydrogens and carbonyl carbons in the peptide backbones (**Figure 1C**, left). Second, hydrophobic interactions between bulky hydrophobic sidechains play an important role in stabilizing the cross-β fold (**Figure 1B**, right). A variety of hydrophobic interactions through sidechain interdigitations (steric zipper) have been observed in the fibrillar aggregates. Third, salt bridges between acidic and basic side chains help maintain fibrillar structures (**Figure 1C**, right).

Polymorphic nature of fibrillar aggregates has been also observed for Aβ peptides incubated under different experimental conditions (Tycko, 2015). Two distinct Aβ(1–40) fibrillar aggregates were formed when the samples were incubated with gentle circular agitation and quiescent (unstirred) conditions (Petkova et al., 2005). The agitated fibrils exhibited straight ribbon-type morphologies where protofilaments are laterally associated. On the contrary, quiescent fibrils adopt twisted morphologies in which protofilaments are twisted around each other. Intriguingly, Aβ aggregates seeded by brain extracts from different AD patients were also shown to exhibit distinct molecular structures (Tycko, 2015; Qiang et al., 2017). These experimental results suggest that distinct misfolded strains

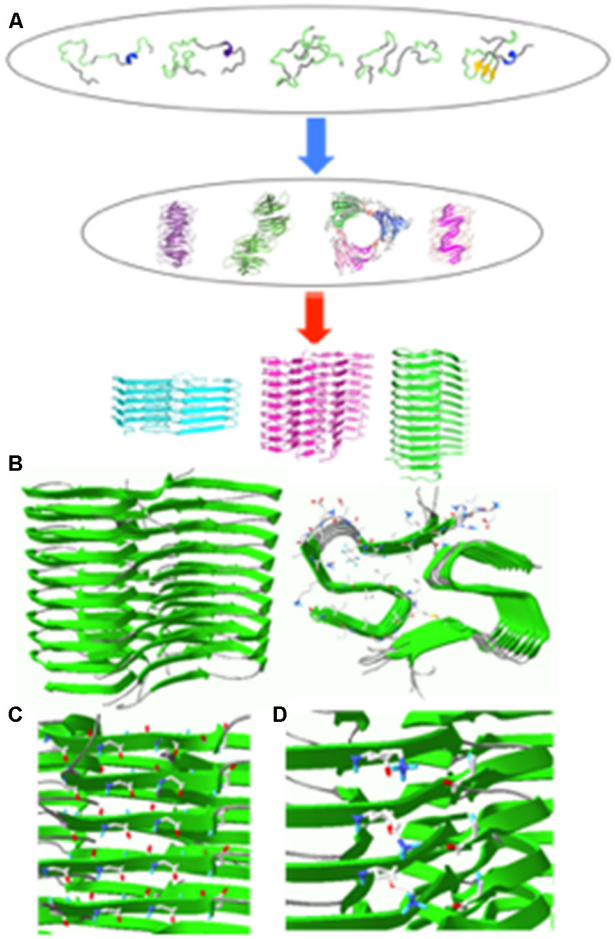

FIGURE 1 | (A) Schematic diagram of aggregation process for an intrinsically disordered protein. Some of the aggregation-prone conformers in the conformational ensemble may form various oligomers that can self-assemble into fibrillar aggregates with different molecular conformations depending on different environments (Jahn and Radford, 2005). **(B)** Solid-state NMR structure of Aβ(1–42) filaments (sideview, left; top view, right). **(C)** Hydrogen bonding interactions between carbonyl carbon (red) and amide hydrogen (blue). **(D)** Salt bridges between NH3+ of K28 (blue) and COO^- of A42 (red) in Aβ(1–42) filaments. The filament structures were drawn with PDB accession number (5KK3).

might be associated with different disease phenotypes. Further investigation of molecular structures of diverse misfolded Aβ aggregates and their biological activities will help understand structure-function relationship of misfolded Aβ aggregates.

α-Synuclein

α-synuclein is a 140-residue intrinsically disordered protein that is primarily localized at presynaptic terminals of the CNS. Intracellular inclusions containing misfolded α-synuclein aggregates are a hallmark of several neurodegenerative diseases such as PD and dementia with Lewy bodies, collectively termed synucleinopathies (Goedert, 2001; Lashuel et al., 2013).

The presynaptic protein consists of three main regions: the amphipathic N-terminal (1–64), hydrophobic non-Aβ-component (NAC; 65–95), and acidic C-terminal region (96–140). The hydrophobic NAC region that plays a central role in α-synuclein aggregation was shown to be protected by long-range interactions between the N- and C-terminal regions in the natively disordered state of α-synuclein (Bertoncini et al., 2005b; Dedmon et al., 2005). Thus, perturbations of the long-range interactions by single-point mutations and interactions with small molecules led to the formation of filamentous α-synuclein aggregates (Bertoncini et al., 2005a).

Recent structural studies of misfolded α-synuclein aggregates revealed that filamentous α-synuclein aggregates also have different molecular structures depending on the experimental conditions such as pH and salt concentration. Under low salt conditions, α-synuclein filaments have ribbon-type morphology, while twisted morphology was observed at high salt conditions (Bousset et al., 2013). The two distinct filaments were also shown to exhibit distinct disease phenotypes in mice models (Peelaerts et al., 2015).

Very recently, high-resolution structures were determined by solid-state NMR and cryo-EM (**Figures 2A–E**; Tuttle et al., 2016; Guerrero-Ferreira et al., 2018; Li B. et al., 2018; Li Y. et al., 2018). Solid-state NMR structure of α-synuclein filaments revealed that the filament core consisting of residues 38–97 adopts a Greek-key topology (**Figure 2A**; Tuttle et al., 2016). Near-atomic high-resolution structures were also solved by cryo-EM at resolution of 3–4 Å (**Figures 2B–E**). Two cryo-EM structures exhibit a Greek-key type fold similar to that in solid-state NMR structure (**Figures 2B,C**; Guerrero-Ferreira et al., 2018; Li B. et al., 2018). However, detailed molecular structures such as the location of the turn and sidechain orientations differ in the structures. For example, residues 51–67 are disordered in the solid-state NMR structure, while those regions are well defined in the cryo-EM structure. The sidechain of residue A53 points into the opposite directions. In addition, distinct cross-β folds were observed in α-synuclein filaments formed in the presence of tetrabutylphosphonium bromide (15 mM; **Figures 2D,F**; Li B. et al., 2018). In particular, the bent b-arch fold in **Figure 2E** has distinct inter facial regions (residues 46–56), compared to that (residues 68–78) in **Figure 2D**. The different molecular structures also led to the formation of distinct morphologies, rod and twisted polymorphsin **Figures 2D,E**, respectively (Li Y. et al., 2018). Filamentous aggregates with different molecular structures and morphologies (surface structure) may interact with the monomers in a different way, leading to differential (cross-) seeding activities. Additional structural studies of the filamentous aggregates and elucidation of their (cross-) seeding activities are, therefore, required to better understand the differential functional properties of the diverse α-synuclein filaments.

Tau

Tau is an intrinsically disordered 352–441 residue microtubule-binding protein that is abundantly present in the CNS. An alternative splicing of the tau gene leads to six isoforms of the tau protein. The six isoforms consist of the N-terminal region with 0, 1, 2 inserts (0N, 1N, and 2N), prolin-rich domain (PRD), microtubule binding domain with three or four repeats

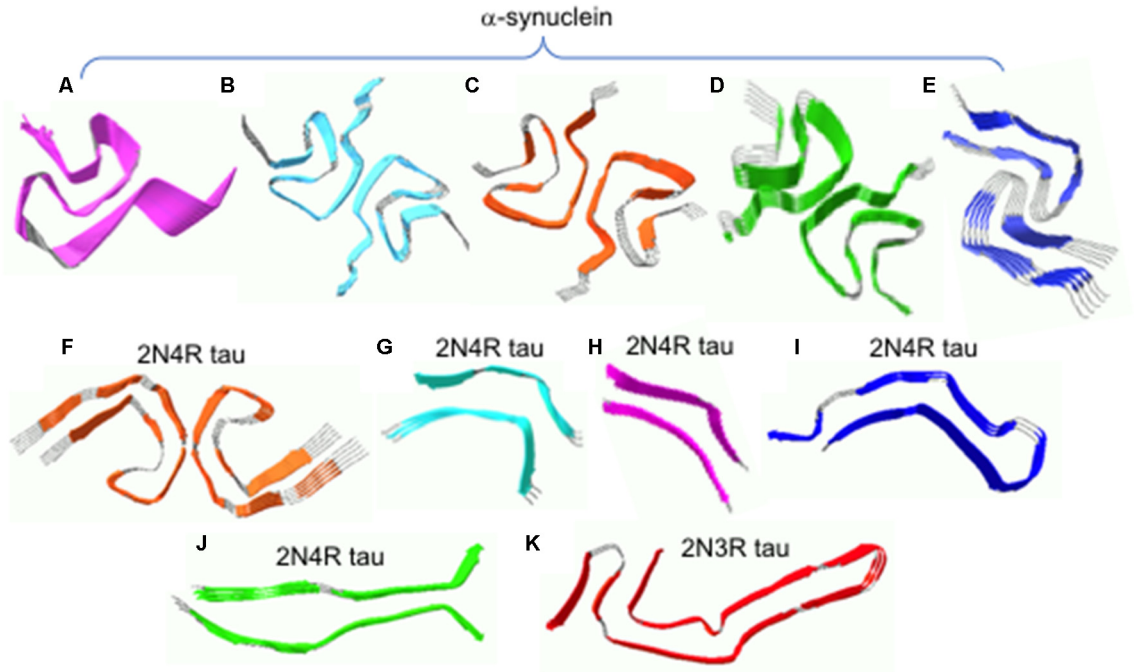

FIGURE 2 | (A) Solid-state NMR structure of α-synuclein filaments. **(B–E)** Cryo-EM structures of α-synuclein filaments. **(F)** Cryo-EM structures of tau filaments extracted from an AD patient. **(G–J)** Cryo-EM structures of tau filaments induced by heparin. **(K)** Cryo-EM structures of tau filaments extracted from a PiD patient. The filament structures were drawn with PDB accession numbers, 2N0A **(A)**, 6A6B **(B)**, 6FLT **(C)**, 6CU7 **(D)**, 6CU8 **(E)**, 5O3L **(F)**, 3QJM **(G)**, 3QJP **(H)**, 3QJH **(I)**, 3QJQ **(J)**, and 6GX5 **(K)**.

(3R or 4R), and C-terminal region. Intraneuronal accumulation of filamentous tau protein is a hallmark of a wide range of neurodegenerative diseases such as AD and frontotemporal dementia, collectively termed tauopathy (Ballatore et al., 2007; Goedert et al., 2017). The filamentous tau aggregates in different tauopathy patients were shown to have different composition. For example, all six isoforms were observed in AD patients, while only 4R-or 3R-isoform of tau was detected exclusively in progressive supranuclear palsy (PSP) and Pick's disease (PiD), respectively (Goedert et al., 2017).

Recently, near-atomic resolution structures of tau filaments were determined by cryo-EM (Fitzpatrick et al., 2017; Falcon et al., 2018; Zhang et al., 2019). **Figure 2F** shows high-resolution structure of 2N4R tau filaments derived from an AD brain tissue (Fitzpatrick et al., 2017). Residues 306–378 form a core of the filament which adopts the cross-β fold with β-helix structures, while the rest of the residues are disordered and form the fuzzy coat. The two protofilaments form twisted filaments with interfacial region of residues 332–336. Tau filaments (2N4R) induced by the addition of heparin were shown to have at least four distinct molecular structures (**Figures 2G–J**), which are quite different from that in AD (**Figure 2F**; Zhang et al., 2019). In addition, tau filaments (2N3R) from patients with PiD have a distinct cross-β fold consisting of residues 254–378 (**Figure 2K**; Falcon et al., 2018), highlighting structural diversities of tau filaments. The distinct molecular conformations of tau filaments may have different biological activities associated with different disease phenotypes. More detailed investigation of biological activities including toxicity and self-propagation properties of diverse tau filaments will lead to a deeper understanding of the phenotype diversities of tauopathies. Finally, it is worth emphasizing that the heparin-induced *in vitro* tau filaments (**Figures 2G-J**) adopt completely different structures from those of *in vivo* tau filaments (**Figures 2F,K**), highlighting the importance of using physiological conditions for the biophysical studies of misfolded protein aggregates. This also raises an important question whether recombinant aggregation-proneproteins including tau and α-synuclein can form disease-associated filamentous aggregates observed *in vivo*, and thus special care must be taken when developing therapeutic intervention on the basis of *in vitro* filament structures.

SELF-PROPAGATION OF MISFOLDED STRAINS IN A PRION-LIKE MANNER

Misfolded protein aggregates are initially found in a distinct brain region depending on the type of diseases. At a later stage, the protein aggregates gradually spread in a predictable manner (Frost and Diamond, 2010; Kim and Holtzman, 2010; Lee et al., 2010; Westermark and Westermark, 2010; Goedert et al., 2017). For example, tau aggregates are initially found in the locus coeruleus, entorhinal cortex and hippocampal formation, which are associated with the memory, in Alzheimer's patients. Over time, the misfolded tau aggregates begin to appear in

the neighboring regions of the temporal and frontal lobes, which affect cognitive functions such as learning and speech. In addition, the intracerebral injection of brain extracts containing misfolded protein aggregates into transgenic mice induced the formation of misfolded tau aggregates at near injection site (Clavaguera et al., 2009, 2013; Iba et al., 2013). The induced aggregates propagate systematically from the injection site and spread to distant regions, promoting disease phenotypes similar to those of the corresponding human diseases (Meyer-Luehmann et al., 2006). It was also demonstrated that misfolded tau aggregates can be taken by neurons in cell culture and the tau aggregates were able to stimulate tau aggregation inside the neurons. In addition, recent studies revealed that tau aggregates propagate from neuron to neuron, suggesting that the misfolded aggregates spread through neuronal networks (Gibbons et al., 2019).

The prion-like behavior was also observed in other misfolded proteins such as Aβ and α-synuclein. Intracellular injection of brain extracts containing misfolded α-synuclein aggregates into transgenic mice induced motor dysfunction similar to those of PD (Sacino et al., 2014). In addition, misfolded aggregates derived by purified recombinant α-synuclein were shown to be able to induce α-synuclein aggregation *in vivo* (Peng et al., 2018). Injection of the brain extracts including Aβ plaques as well as synthetic Aβ aggregates seeded Aβ deposition in mice (Meyer-Luehmann et al., 2006). These experimental evidences clearly suggest that the aggregation-prone pathological proteins have the ability of propagating their pathological conformations like a prion. Elucidation of the molecular mechanism of the self-propagation is critical to developing therapeutic agents that can inhibit the propagation process.

During the self-propagation process, the misfolded protein aggregates recruit soluble monomeric proteins and induce conformational changes to misfolded conformers. The end of the filamentous aggregates may interact with the soluble proteins or surface of the aggregates may also recruit the monomeric proteins, promoting intermolecular associations of the monomeric proteins. Distinct strains with different molecular structure and morphologies may interact with monomeric proteins in different ways to induce distinct conformational changes to propagate their unique molecular conformations. Elucidation of the molecular basis of the interactions between the misfolded strains and monomeric proteins would be of great importance in developing therapeutic strategies to block the self-propagation process. Recently solved high-resolution structures of the misfolded filamentous aggregates will be of great use in investigating interactions between the filaments and monomeric proteins.

CROSS-TALK BETWEEN MISFOLDED PROTEINS

Traditionally, misfolding and aggregation of a single protein is thought to be implicated in each neurodegenerative disorder with an independent pathological process. There is, however, an increasing body of evidence that suggests multiple neurodegenerative diseases are significantly overlapped. First, co-deposition of α-synuclein and tau was observed in ADRD (Clinton et al., 2010; Irwin et al., 2013; Moussaud et al., 2014; Nonaka et al., 2018). For example, misfolded α-synuclein aggregates were found in more than 50% of AD, Down's syndrome, and familial AD cases (Lippa et al., 1998, 1999; Hamilton, 2000). Tau aggregates were also detected in patients with Parkinson's disease dementia and the amount of tau aggregates is correlated well with cognitive decline (Forman et al., 2002; Irwin et al., 2013). Second, it was previously shown that αsynuclein and tau can synergistically promote their mutual aggregation, suggesting that synergistic interactions between the two pathological proteins might exacerbate ADRD pathology (Giasson et al., 2003; Oikawa et al., 2016). In particular, misfolded α-synuclein aggregates with distinct molecular conformations (strains) were shown to differentially induce the formation of distinct tau strains *in vivo* (cross-seeding; Guo et al., 2013). These results indicate that AD and PD pathologies are significantly overlapped presumably due to synergistic interactions between tau and α-synuclein, highlighting the complexity of ADRD pathogenesis.

Recent studies have also shown that patients with type 2 diabetes have a higher risk for AD. In addition, about 80% of Alzheimer's patients develop type 2 diabetes, suggesting that AD might be linked to type-2 diabetes (Jucker and Walker, 2011). Misfolding and aggregation of a small 37-residue peptide (islet amyloid polypeptide, IAPP) is associated with type-2 diabetes. Misfolded IAPP aggregates were also shown to be able to seed Aβ aggregation *in vitro* as well as *in vivo* (Hu et al., 2015; Moreno-Gonzalez et al., 2017). Intracellular injection of pancreas IAPP aggregates into transgenic mice induced higher extracellular Aβ deposition in brain, resulting in more severe learning and memory deficits (Moreno-Gonzalez et al., 2017). These experimental results indicate the presence of synergistic interactions between the two aggregation-prone proteins, aggravating the diseases.

Despite the growing evidence of the cross-talk among protein misfolding disorders, molecular mechanism of the synergistic interactions remains to be determined. Aggregation-prone proteins may interact directly with each other to promote mutual aggregations, as was demonstrated *in vitro*. It is, however, unclear how the filamentous aggregates mutually promote aggregation of the other aggregation-prone proteins. Even though the misfolded filamentous aggregates of the three proteins are stabilized by similar interactions such as hydrogen bonding, salt-bridge, and hydrophobic interactions, they adopt completely different cross-b folds as shown in **Figures 1B, 2**. These different structures suggest that disordered regions rather than the amyloid core may interact with the monomers of different aggregation-prone proteins, accelerating aggregation. For example, negatively charged C-terminal region (101–140; pI, 3.4) that is not involved in α-synuclein filament core may disrupt long-range interactions present in positively charged tau (pI, 11.4; Mukrasch et al., 2009), destabilizing tau monomers and subsequently promoting aggregation, and vice-versa. Determination of high-resolution structures of filamentous aggregates cross-seeded by other misfolded aggregates will be required to better understand the

cross-seeding activities. Misfolded protein aggregates may also indirectly induce aggregation of the other pathological proteins by interfering protein quality control systems, increasing cellular vulnerability to misfolding and aggregation.

CONCLUDING REMARKS

It is increasingly evident that aggregation-prone proteins behave like a prion that adopts diverse molecular conformations with self-propagation properties and cross-seeding activities. Recent advances in cryo-EM and solid-state NMR techniques allowed high-resolution structures of misfolded filamentous aggregates to be determined at near-atomic resolutions, which revealed diverse molecular conformations. Additional structural studies of filamentous aggregates cross-seeded by another misfolded proteins and *in vivo* filaments extracted from patient's brains will greatly enhance our understanding of molecular basis for the diverse molecular conformations. Investigation of their biological activities such as toxicity and cross-seeding ability will also provide more detailed insights into structure-function relationship of the conformationally diverse misfolded strains. Finally, it is important to note that the high-resolution structures described in this review were determined only for highly ordered filamentous protein aggregates. Oligomeric intermediate states that are believed to be real cytotoxic species may have different molecular structures. Structural studies of the oligomers using cryo-EM and solid-state NMR are, however, of great challenge due to their heterogeneous, transient nature. A recent study showed that oligomeric species dissociated from filamentous aggregates exhibited cytotoxic activities (Ghag et al., 2018), and thus high-resolution structures of the amyloid filaments described above may help investigate structural features of the cytotoxic oligomers.

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frontiers in Molecular Neuroscience

MINI REVIEW
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Recent Advances in Understanding Mammalian Prion Structure: A Mini Review

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Prions are lethal pathogens, which cause fatal neurodegenerative diseases in mammals. They are unique infectious agents and are composed of self-propagating multi-chain assemblies of misfolded host-encoded prion protein (PrP). Understanding prion structure is fundamental to understanding prion disease pathogenesis however to date, the high-resolution structure of authentic *ex vivo* infectious prions remains unknown. Advances in determining prion structure have been severely impeded by the difficulty in recovering relatively homogeneous prion particles from infected brain and definitively associating infectivity with the PrP assembly state. Recently, however, images of highly infectious *ex vivo* PrP rods that produce prion-strain specific disease phenotypes in mice have been obtained using cryo-electron microscopy and atomic force microscopy. These images have provided the most detailed description of *ex vivo* mammalian prions reported to date and have established that prions isolated from multiple strains have a common hierarchical structure. Misfolded PrP is assembled into 20 nm wide rods containing two fibers, each with double helical repeating substructure, separated by a characteristic central gap 8–10 nm in width. Irregularly structured material with adhesive properties distinct to that of the fibers is present within the central gap of the rod. Prions are clearly distinguishable from non-infectious recombinant PrP fibrils generated *in vitro* and from all other propagating protein structures so far described in other neurodegenerative diseases. The basic architecture of mammalian prions appears to be exceptional and fundamental to their lethal pathogenicity.

Keywords: Alzheimer's disease, prion disease, prion, prion-like, prion structure, amyloid beta, α-synuclein, tau

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INTRODUCTION

Prion diseases are a closely related group of neurodegenerative conditions which affect both humans and animals. They include bovine spongiform encephalopathy (BSE) in cattle, scrapie in sheep and goats, chronic wasting disease (CWD) in deer and elk, and the human prion diseases, kuru, Creutzfeldt-Jakob disease (CJD), variant CJD (vCJD), fatal familial insomnia (FFI) and Gerstmann–Sträussler–Scheinker disease (GSS; Prusiner, 1998; Collinge, 2001; Wadsworth and Collinge, 2011; Haïk and Brandel, 2014; Greenlee and Greenlee, 2015; Benestad and Telling, 2018; Rossi et al., 2019). They are exceptional pathogens (devoid of

significant coding nucleic acid) and comprise infectious polymeric assemblies of misfolded host-encoded prion protein (PrP; Prusiner, 1998; Collinge and Clarke, 2007; Collinge, 2016). Prions propagate by means of seeded protein polymerization, which involves recruitment of PrP monomers to fibrillar assemblies followed by fragmentation of these structures to generate more "seeds." Different prion strains produce different disease phenotypes in the same inbred host and appear to be encoded by distinct misfolded PrP conformations and quaternary assembly states (Prusiner, 1998; Collinge and Clarke, 2007; Collinge, 2016).

While lacking the overt infectivity of prions, many other proteins are also capable of seeded protein misfolding and the generation of self-propagating polymeric or amyloid protein assemblies now appears to be widely involved in the pathogenesis of many other human diseases. Consequently "prion-like" mechanisms and the prion strain phenomena have become a major research focus in other neurodegenerative conditions, in particular, in Alzheimer's disease (AD) and Parkinson's disease where propagating assemblies of amyloid-β, tau and α-synuclein are being studied (Prusiner, 2013; Goedert, 2015; Collinge, 2016; Walker, 2016; Qiang et al., 2017; Condello et al., 2018; Peng et al., 2018; Vaquer-Alicea and Diamond, 2019). Notably, in the case of tau, structurally distinct fibrillar assemblies from brain have recently been characterized in AD, Pick's disease and chronic traumatic encephalopathy (CTE) strongly suggesting that distinct strains of propagating tau assemblies are contributing to different disease phenotypes in humans (Fitzpatrick et al., 2017; Falcon et al., 2018; Falcon et al., 2019).

Significantly, while iatrogenic transmission of neurodegenerative diseases was thought to be restricted to prions, there is now considerable evidence for human transmission of cerebral amyloid angiopathy and amyloid-β protein pathology resulting from discontinued medical practices involving treatment with human cadaveric pituitary-derived growth hormone or cadaveric dura mater grafting (Jaunmuktane et al., 2015; Frontzek et al., 2016; Ritchie et al., 2017; Cali et al., 2018; Purro et al., 2018; Banerjee et al., 2019). These findings now underscore the importance of fully understanding the prion-like properties of proteopathic seeds generated in other neurodegenerative diseases and systematically establishing their potential risks for iatrogenic transmission.

At present there is considerable debate regarding the nomenclature that should be used in describing the propagation of non-PrP protein assemblies (to distinguish them from lethal PrP prions) with terms such as propagons and prionoids being proposed (Collinge, 2016; Kara et al., 2018; Scheckel and Aguzzi, 2018; Duyckaerts et al., 2019; Eraña, 2019). Biological criteria that a propagating protein assembly must fulfill in order to be regarded as truly "prion-like" have yet to be defined (Kara et al., 2018; Scheckel and Aguzzi, 2018; Duyckaerts et al., 2019; Eraña, 2019) and structural classification of propagating protein assemblies remains a key goal. Indeed, in this context, it should be noted that propagation and spread of assemblies of amyloid-β, tau and α-synuclein in animal models of other neurodegenerative diseases rarely result in lethal neurodegeneration, suggesting that the basic architecture of mammalian prions may be unique and central to their lethality (Collinge, 2016; Terry et al., 2019).

Here, we now highlight recent advances in prion isolation and structural characterization that have provided the first meaningful opportunity to compare the basic architecture of authentic infectious mammalian prions with the structures of protein assemblies from other neurodegenerative diseases. Current available data indicate that mammalian prions have unique structural features that readily distinguish them from propagating assemblies of amyloid-β, tau and α-synuclein that have been described in other neurodegenerative diseases.

KEY MOLECULAR FEATURES OF PRION DISEASES

The central feature of prion diseases is the aberrant misfolding of PrP which can adopt distinct conformations and assembly states (Prusiner, 1998; Collinge and Clarke, 2007; Rodriguez et al., 2017). The normal form of the protein, referred to as PrP^{C} (the cellular isoform) is a highly conserved cell surface glycosylphosphatidylinositol (GPI)-anchored sialoglycoprotein with an ordered C-terminal domain containing three α-helices, a short anti-parallel β-sheet and a flexible disordered N-terminal domain (Wüthrich and Riek, 2001; Rodriguez et al., 2017) and is soluble in detergents and sensitive to digestion with proteases. In contrast, disease-associated isoforms of PrP that comprise infectious prion assemblies are found only in prion-infected tissue and are composed of detergent-insoluble polymeric PrP structures some of which become protease-resistant and are classically termed PrP^{Sc} (the scrapie isoform; Meyer et al., 1986; Prusiner, 1987, 1991, 1998). PrP^{Sc} is derived from PrP^{C} by conformational rearrangement and neither systematic study of known covalent post-translational modifications nor amino acid sequencing have shown any consistent variations between PrP^{Sc} and PrP^{C} (Prusiner, 1991, 1998; Riesner, 2003). The structural transition of PrP^{C} to fibrillar assemblies of PrP^{Sc} involves acquisition of a beta sheet rich configuration (Pan et al., 1993; Caughey et al., 1998; Prusiner, 1998; Riesner, 2003; Rodriguez et al., 2017) likely to be similar to amyloid where beta sheets stack perpendicular to the fiber axis forming a cross-beta structure (Eisenberg and Sawaya, 2017; Iadanza et al., 2018).

To date, the critical molecular events during infection that generate prototypical PrP^{Sc} and how this causes neurodegeneration remains poorly defined. Notably, in many prion strain/host combinations the majority of disease-related PrP and prion titre is destroyed by protease-treatments that are typically used to detect prototypical PrP^{Sc} (Safar et al., 1998, 2005; Cronier et al., 2008; D'Castro et al., 2010; Tixador et al., 2010; Sandberg et al., 2014). These findings indicate that the term PrP^{Sc}, often used interchangeably with prion infectivity, should be restricted to material as classically biochemically defined (infectious detergent-insoluble, protease-resistant PrP assemblies). Such prototypical PrP^{Sc} comprises a small proportion of total disease-related PrP isoforms and while it is clearly an infectious structure its specific contribution to other aspects of disease pathogenesis remains unclear (Sandberg et al., 2014). Notably in this context, it is now thought

that a distinct oligomeric or monomeric PrP isoform designated PrP^{L} (for lethal) may comprise the neurotoxic species, and that prototypical PrP^{Sc} and indeed prions *per se* may not themselves be highly neurotoxic (Collinge and Clarke, 2007; Sandberg et al., 2011, 2014; Collinge, 2016). Determining the structural relationship between infectious and neurotoxic PrP species and whether protease-sensitive and protease-resistant infectious PrP assemblies are simply different-sized particles of essentially the same PrP structure has yet to be resolved. Consequently, it is now clear that a complete understanding of prion disease pathogenesis will require knowledge not only of infectious PrP structures but also the role of other PrP assemblies that may be variably generated during prion disease pathogenesis (Collinge and Clarke, 2007; Collinge, 2016).

BRIEF OVERVIEW OF HISTORICAL STUDIES ON PRION STRUCTURE

High resolution structural analysis of infectious mammalian prions has been obstructed by two central problems. First, the difficulty in recovering relatively homogeneous particles from affected tissue whose composition and PrP assembly state can be directly correlated with infectivity, and second, the failure to reproducibly generate high-titre synthetic prions from fully defined constituents. Although the formation of prions *in vitro* from recombinant PrP or isolated PrP^{C} preparations (either alone or in combination with non-protein cofactors) has been reported, specific-infectivities are generally too low for meaningful structural analysis (Collinge and Clarke, 2007; Diaz-Espinoza and Soto, 2012; Schmidt et al., 2015; Collinge, 2016) and preparations with high prion titre (for example Moudjou et al., 2016) have not yet been structurally characterized. Consequently, the goal of solving infectious prion structure continues to rely upon the isolation of high-titre *ex vivo* prions in a form suitable for detailed structural study.

Scrapie associated fibrils (SAFs; Merz et al., 1981) and prion rods (Prusiner et al., 1983) were first described in prion-enriched isolates from infected brain tissue more than 35 years ago. While contemporary comparison of SAFs and prion rods now suggest they are synonymous, at the time of their discovery (before the PrP gene was identified) they were interpreted very differently. While Merz et al. (1984) proposed that SAFs may represent a new class of filamentous animal virus, Prusiner et al. (1983) proposed that prion rods were infectious protein assemblies (prions) composed of a protein designated PrP 27–30 (subsequently established to be proteolytically truncated PrP^{Sc}) and that the morphology of the prion rods was incompatible with a uniform virus structure (DeArmond et al., 1985); in particular, that the length of the prion rods was not essential for preservation of prion infectivity (Barry et al., 1985; Prusiner, 1987). Subsequently, Prusiner (1991, 1998) proposed that the prion rods were an artifact of purification and suggested that protease-truncation of PrP^{Sc} to PrP 27–30 in the presence of detergent facilitated the assembly of prion rods from smaller infectious oligomers of PrP^{Sc} (McKinley et al., 1991). While this proposal at the time clearly excluded a viral etiology for prion diseases, this situation also left the field having to contend with the idea that large fibrillar PrP assemblies associated with prion infectivity might not actually represent authentic biologically relevant structures. Consequently, many researchers chose not to pursue structural characterization of the prion rods and instead focused on either trying to isolate smaller infectious oligomers of PrP^{Sc} from infected brain or generating synthetic prions from bacterially expressed recombinant PrP. While numerous studies have now proposed various possible PrP structures as the authentic infectious prion assembly state (Silveira et al., 2005; Sim and Caughey, 2009; Wille et al., 2009; Requena and Wille, 2014; Vázquez-Fernández et al., 2016, 2017) none of these have been convincingly correlated with high specific prion infectivity and no international consensus has been reached on their *in vivo* relevance (Baskakov et al., 2019).

RECENT PROGRESS IN PURIFYING AND CHARACTERIZING MAMMALIAN PRIONS

The availability of cell-based prion bioassays (Klöhn et al., 2003; Mahal et al., 2007; Schmidt et al., 2015) has recently enabled the development of novel procedures for isolating extremely pure, intact high-titre infectious prions from mammalian brain (Wenborn et al., 2015). Misfolded PrP in these preparations is highly protease-resistant and is assembled into rod-like structures, PrP rods (akin to prion rods identified by Prusiner and colleagues), which faithfully transmit prion strain-specific phenotypes when inoculated into mice (Wenborn et al., 2015). PrP rods are intrinsically infectious in cell culture infectivity assays and form aggregates whose size and number appear to determine the number of infectious units available to cells at inoculation (Terry et al., 2016). Filtration of the rod preparations showed no evidence for the presence of small infectious oligomers of PrP^{Sc} and thermal and chemical inactivation profiles of prion infectivity indicated the destruction of the same infectious structures in isolated PrP rod preparations or starting brain homogenate (Terry et al., 2016). Importantly, exploration of the origin of the PrP rods showed no evidence for their artifactual generation during purification as they could be isolated from brain without using detergent and the dimensions and morphology of the rods from crude brain homogenate were not noticeably influenced by variable exposure to protease and detergent (Terry et al., 2016). Differences in the length of propagating infectious PrP rods in brain (in which the ends of the rod may comprise the infectious surface) can readily explain variance in specific prion infectivity with respect to PrP monomers and also account for the widespread distribution of infectious prion particles that is seen when prion-infected brain homogenate is fractionated by sedimentation velocity ultracentrifugation (Prusiner et al., 1987).

INFECTIOUS *EX VIVO* PrP RODS HAVE UNIQUE STRUCTURAL FEATURES

Examination of infectious PrP rods isolated from multiple prion strains by negative-stain electron microscopy (EM), negative-stain electron tomography, cryo-EM and atomic force microscopy (AFM) have recently revealed a common

three-dimensional architecture (Terry et al., 2016, 2019). Infectious PrP rods are ∼20 nm wide and are composed of two fibers (each with a double helical substructure) separated by a distinct gap of 8–10 nm in width. AFM showed that the central gap contains irregularly-structured material that appears to be compositionally distinct from the surface of the individual fibers. This finding is consistent with the idea that PrP N-linked glycans in the gap may be contributing to the overall stability of the rod and as a consequence its infectivity (Terry et al., 2016, 2019). The overall architecture of the infectious PrP rods is very distinct to the structure of non-infectious PrP fibrils generated *in vitro* from recombinant PrP which comprise long, single fibers (10 nm wide) formed by a double helical arrangement of two protofilaments (Tattum et al., 2006; Terry et al., 2016, 2019; **Figure 1**).

Notably, because of their helical twist, PrP rods when imaged on a surface alternate between narrower, edge-on and wider, face-on views of the structure. Consequently, the overall architecture of this twisted assembly is often hard to distinguish in EM images, which are two-dimensional density projections. However, the twist of the paired fibers in the rod, as well as a twisted two stranded structure within each fiber, becomes apparent when the three dimensional structure of the assembly is resolved by tomography (Terry et al., 2016). Significantly, visualization of the PrP rods by tomography and the ensuing recognition of their basic architecture (Terry et al., 2016, 2019) now enables the structural features of the PrP rods to be readily seen in most published EM images of SAFs and prion rods from earlier studies, including those from CJD brain (Merz et al., 1984). Collectively, these new data overturn previous dogma and firmly establish *ex vivo* PrP rods as the authentic infectious prion assembly state that should now be targeted in future high resolution imaging studies.

At present without a high-resolution three-dimensional structure of infectious PrP rods, the detailed arrangement of secondary structure components of PrP within the fibers of the rod remains unknown. Knowledge of the structures of alternative single fiber PrP amyloid fibrils (either generated *in vitro* or isolated from mice expressing mutant PrP) cannot be applied with any certainty to the PrP rods (Terry et al., 2016, 2019). From the various PrP fibrillar structures that have been characterized to date, two major structural models for prions have been proposed; the parallel in-register intermolecular β-sheet (PIRIBS) architectures and the 4-rung beta solenoid model (Baskakov et al., 2019). Determining whether either of these models applies to infectious PrP rods is now dependent on obtaining their high resolution structure.

While strain-specific structural differences in infectious PrP rods may become apparent with future application of higher resolution imaging methods (such as cryo-tomography and subtomogram averaging) their basic architecture can now be compared with fibrillar assemblies of other proteins that propagate in other neurodegenerative diseases (**Table 1**). Based upon the available data the structure of infectious PrP rods can be

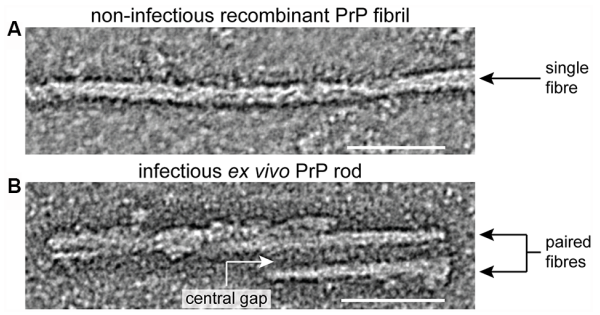

FIGURE 1 | Structural differences between infectious *ex vivo* PrP rods and non-infectious recombinant prion protein (PrP) fibrils generated *in vitro*. Panels **(A,B)** show sections from negative stain electron tomography reconstructions that were originally published in (Terry et al., 2016), scale bars, 50 nm. Non-infectious recombinant PrP fibrils **(A)** appear as single fibers ∼10 nm wide comprised of two closely intertwined protofilaments (Tattum et al., 2006; Terry et al., 2016). In contrast infectious *ex vivo* PrP rods **(B)** are ∼20 nm wide and are composed of two fibers (each with a double helical substructure) separated by a central gap of 8–10 nm in width which is filled with irregularly structured material (Terry et al., 2016, 2019).

TABLE 1 | Studies reporting the structures of fibrillar protein assemblies from patients with various neurodegenerative diseases other than prion diseases.

Neurodegenerative disease (assembled protein)	Structural method	Tissue source	Reference
Alzheimer's disease (AD; Amyloid-β)	Negative stain EM	Brain from patients with AD	Paravastu et al. (2009)
Alzheimer's disease (Amyloid-β)	Negative stain EM	Brain from patients with AD	Lu et al. (2013)
Alzheimer's disease (Tau)	Cryo-EM	Brain from patients with AD	Fitzpatrick et al. (2017)
Pick's disease (Tau)	Cryo-EM	Brain from patients with Pick's disease	Falcon et al. (2018)
Chronic traumatic encephalopathy (CTE; Tau)	Cryo-EM	Brain from patients with CTE	Falcon et al. (2019)
Parkinson's disease/dementia with Lewy bodies (α-synuclein)	Negative stain EM	Brain from patients with dementia with Lewy bodies	Spillantini et al. (1998)
Amyotrophic lateral sclerosis (ALS; SOD1)	Negative stain EM	Spinal cord from patients with familial ALS	Kato et al. (1997, 2000)
Frontotemporal lobar degeneration (FTLD-U) and amyotrophic lateral sclerosis (TDP-43)	Negative stain EM	Brain from patients with FTLD-U and ALS	Lin and Dickson (2008)
Frontotemporal lobar degeneration (TDP-43)	Negative stain EM	Brain from patients with FTLD with TDP-43 proteinopathy	Thorpe et al. (2008)
Amyotrophic lateral sclerosis and frontotemporal lobar degeneration (TDP-43)	Negative stain EM	Brain from patients with ALS and FTLD-with TDP-43 proteinopathy	Nonaka et al. (2013)
Frontotemporal lobar degeneration (TDP-43)	Negative stain EM	Brain from patients with FTLD with TDP-43 proteinopathy	Laferrière et al. (2019)

readily distinguished from fibrillar structures of tau, amyloid-β, α-synuclein, TDP-43 and SOD1 because none of these possess a prominent central gap region that resembles the PrP rods.

CO-PROPAGATION OF INFECTIOUS PrP RODS AND TRANSMISSIBLE PrP AMYLOID

Prion-infected transgenic mice expressing mutant GPI-anchorless PrP replicate authentic prions (that are transmissible to wild-type mice) but they also develop intense PrP amyloid plaques in their brain which are not seen in the brain of prion-infected wild-type mice (Chesebro et al., 2005, 2010). Based upon the currently available evidence (summarized in Terry et al., 2019) it appears that prion infection in these mice leads to the propagation of infectious PrP rods which account for the transmissible prion infectivity and structurally distinct single PrP fibers (10 nm wide; Vázquez-Fernández et al., 2016) which account for the striking PrP amyloid plaque deposits that distinguish these mice.

Importantly, such co-propagation of infectious paired-fiber PrP rods and distinct single-fiber amyloid PrP assemblies may also be occurring in some inherited prion diseases in particular in patients with GSS disease phenotypes in which amyloid plaques are a prominent neuropathological feature (see Terry et al., 2019). This could readily explain why biochemically-distinct PrP assemblies from GSS patients with the P102L PrP mutation can transmit different phenotypes to experimental mice resulting in either a clinically silent PrP amyloidosis or a lethal spongiform encephalopathy (Piccardo et al., 2007; Barron et al., 2016; Barron, 2017). Variation in the substructure of infectious PrP rods or distinct amyloid PrP fibrils (governed by the specific PrP missense mutation) would be expected to dictate highly specific strain transmission properties *via* conformational selection (Collinge, 1999, 2016; Collinge and Clarke, 2007; Wadsworth et al., 2010) as has recently been demonstrated for *PRNP* P102L and A117V mutations (Asante et al., 2009, 2013, 2015). Notably, while patients with different *PRNP* missense mutations that produce full-length mutant PrP (for example P102L) would be expected to be capable of co-propagating both authentic prions (infectious PrP rods) and alternative transmissible PrP amyloid assemblies, patients with different *PRNP* stop mutations which produce C-terminally truncated PrP devoid of N-linked glycans (for example Y163X; Mead et al., 2013) may only be capable of propagating transmissible PrP amyloids giving rise to distinct disease phenotypes (Mead and Reilly, 2015).

CONCLUDING REMARKS

Prion-infected brain contains multiple disease-related PrP assemblies. Infectious PrP rods comprise authentic prions that generate a lethal transmissible spongiform encephalopathy when inoculated into a susceptible host. Neurotoxicity accompanying the propagation of authentic prions is thought to involve the generation of a distinct toxic PrP species whose steady-state level may determine the rate of neurodegeneration. In some inherited prion diseases transmissible PrP amyloids only may be generated while in others transmissible PrP amyloids may variably co-propagate with authentic prions and act as a major modifier of clinicopathological phenotype. Studying the transmission properties of synthetic prion preparations is complex and experiments should be carefully designed and interpreted in order to differentiate between the generation of authentic lethal prions and transmissible PrP amyloids. At present, propagating fibrillar assemblies of proteins in other neurodegenerative diseases appear to have biological and structural properties that are more closely aligned with transmissible PrP amyloids rather than authentic lethal prions.

AUTHOR CONTRIBUTIONS

CT and JW wrote the article.

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The remaining author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Molecular Neuroscience

ORIGINAL RESEARCH
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ALWPs Improve Cognitive Function and Regulate Aβ Plaque and Tau Hyperphosphorylation in a Mouse Model of Alzheimer's Disease

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Recently, we reported that ALWPs, which we developed by combining Liuwei Dihuang pills (LWPs) with antler, regulate the LPS-induced neuroinflammatory response and rescue LPS-induced short- and long-term memory impairment in wild-type (WT) mice. In the present study, we examined the effects of ALWPs on Alzheimer's disease (AD) pathology and cognitive function in WT mice as well as 5x FAD mice (a mouse model of AD). We found that administration of ALWPs significantly reduced amyloid plaque levels in 5x FAD mice and significantly decreased amyloid β (Aβ) levels in amyloid precursor protein (APP)-overexpressing H4 cells. In addition, ALWPs administration significantly suppressed tau hyperphosphorylation in 5x FAD mice. Oral administration of ALWPs significantly improved long-term memory in scopolamine (SCO)-injected WT mice and 5x FAD mice by altering dendritic spine density. Importantly, ALWPs promoted spinogenesis in primary hippocampal neurons and WT mice and modulated the dendritic spine number in an extracellular signal-regulated kinase (ERK)-dependent manner. Taken together, our results suggest that ALWPs are a candidate therapeutic drug for AD that can modulate amyloid plaque load, tau phosphorylation, and synaptic/cognitive function.

Keywords: Aβ, tau, Alzheimer's disease, amyloid plaque, long-term memory, dendritic spines

INTRODUCTION

Alzheimer's disease (AD) is an age-related neurodegenerative disease (Deary and Whalley, 1988; Hardy and Selkoe, 2002) with several major pathological hallmarks reflecting the disease mechanism. Recent clinical evidence strongly supports the involvement of amyloid β (Aβ) and tau in AD (Deary and Whalley, 1988; Lee et al., 2010). Specifically, abnormal hyperphosphorylation of tau and accumulation of Aβ plaques can lead to dendritic spine loss,

synaptic dysfunction, neuronal cell death, and memory impairment (Hardy and Selkoe, 2002). For instance, several studies have demonstrated that soluble Aβ isolated from AD brains or Aβ oligomer impairs long-term potentiation (LTP) and increases long-term depression (LTD) through an α-amino-3-hydroxy-5-methyl-4-isoxazolepropionics acid (AMPA) receptor-dependent signaling pathway (Li et al., 2013; Reinders et al., 2016). In addition, aggregation of tau causes dendritic spine loss and impairment of synaptic function by inducing the disassembly of microtubules (Thies and Mandelkow, 2007; Nisbet et al., 2015). Thus, both Aβ and tau appear to be involved in dendritic spine loss and synaptic dysfunction, which lead to cognitive decline in AD, and drugs that can reduce both Aβ plaques and tau phosphorylation are therefore therapeutic candidates for AD.

Liuwei Dihuang pills (LWPs) are a traditional oriental herbal medicine for the treatment of diabetes mellitus and malfunctions of the immune system (Park et al., 2005; Lee et al., 2012). LWPs contain six different herbal components: steamed *Rehmanniae radix*, *Discoreae radix*, *Corni fructus*, *Hoelen*, *Moutan cortex radices*, and *Alismatis radix* (Sangha et al., 2012). Several studies have demonstrated that LWPs affect learning and memory in D-galactose-induced aging and ibotenic acid-induced amnesia rodent models (Kang et al., 2006; Zhang et al., 2011). However, whether LWPs can modulate AD pathology and synaptic function has not been examined in detail.

Antler is widely used as a traditional oriental medicine and affects several biological functions. For instance, molecules secreted from antlers can facilitate neurite outgrowth and axonal growth (Gray et al., 1992; Li et al., 2007; Pita-Thomas et al., 2010). In addition, antler can regulate neuroinflammatory responses and cognitive performance (Lee et al., 2010; Dong et al., 2018). Based on the literature on LWPs and antler, we hypothesized that ALWPs containing antler and LWPs might have synergistic effects on LPS-induced neuroinflammation and LPS-induced memory impairment. Indeed, we recently demonstrated that ALWPs have additive effects on LPS-mediated neuroinflammatory responses compared with the individual components of ALWPs (Lee et al., 2018). In addition, we found that oral administration of ALWPs improved short-term and long-term memory in LPS-injected wild-type (WT) mice (Lee et al., 2018).

In the present study, we further examined whether ALWPs can affect AD pathology (including Aβ plaque and tau hyperphosphorylation) and found that oral administration of ALWPs significantly decreased amyloid plaque number as well as tau hyperphosphorylation in the cortex and hippocampus of 5x FAD mice, a model of AD. In addition, oral administration of ALWPs to scopolamine (SCO)-injected WT mice and 5x FAD mice rescued deficits in long-term memory and promoted dendritic spine number. ALWPs treatment promoted dendritic spine formation in both primary hippocampal neurons and WT mice. Importantly, ALWPs increased dendritic spine number in an extracellular signal-regulated kinase (ERK)-dependent manner in primary hippocampal neurons. Taken together, these data suggest that ALWPs may be a useful potential drug for preventing and/or treating AD.

MATERIALS AND METHODS

Ethics Statement

All experiments were approved by the institutional biosafety committee (IBC) of the Korea Brain Research Institute (KBRI, approval no. 2014-479).

Cell Lines and Culture Conditions

COS7 (monkey kidney) cells were maintained in DMEM-high glucose (HyClone, USA) supplemented with 10% fetal bovine serum (FBS, HyClone, USA) in a 5% CO_2 incubator. Amyloid precursor protein (APP)-H4 cells (H4 cells overexpressing human APP and producing high levels of Aβ were maintained in DMEM-high glucose supplemented with 10% FBS and gentamycin in a 5% CO_2 incubator.

Wild-Type Mice

All procedures were approved by the Institutional Animal Care and Use Committee (IACUC-2016-0013) of KBRI. Adult WT C57BL6/J male mice (8 weeks old, 25–30 g; Orient-Bio Company, Gyeonggi-do, South Korea) were used in the experiments. The animals were housed under a 12-h light/dark cycle with food and water *ad libitum* in a pathogen-free facility. For all experiments, mice were randomly assigned to the control (PBS) or treatment (ALWPs) group. We used 40 or 37 mice for each of the Y-maze and novel object recognition (NOR) tests. After adaptation for 1 week, the mice were randomly divided into three groups: Y-maze: (1) control (PBS, $n = 13$); (2) scopolamine (SCO, 1 mg/kg; $n = 13$); and (3) ALWPs (200 mg/kg) + SCO (1 mg/kg; $n = 14$); NOR: (1) control (PBS, $n = 12$); (2) SCO (1 mg/kg; $n = 12$); and (3) ALWPs (200 mg/kg) + SCO (1 mg/kg; $n = 13$). The doses of ALWPs were determined based on our previous report (Lee et al., 2018). The mice were orally administered PBS or ALWPs (200 mg/kg) daily for 11 days (days 1–11). Beginning on day 3, the mice were injected with SCO (1 mg/kg) daily for 9 days (days 3–11), and the Y-maze test was conducted on day 10. On day 11, training sessions for NOR were conducted. After NOR training, SCO (1 mg/kg) was injected, and the NOR test was performed on the following day. Mice that showed a low interaction time (<7 s in NOR training were regarded as insufficiently trained and eliminated from further testing. The *in vivo* experimental design is summarized in **Figure 1**.

5x FAD Mice

All animal experiments were performed in accordance with approved animal protocols and guidelines established by KBRI (IACUC-2016-0013). 5x FAD transgenic (Tg) mice (stock number 008730, B6SJL-Tg APPSwFlLon, PSEN1*M146L*L286V6799Vas/Mmjax) were purchased from Jackson Laboratory, and only male mice were used for this study. For the behavior experiments, 5x FAD mice were orally administered PBS or ALWPs (200 mg/kg) daily for 14 days, and Y maze and NOR tests were conducted. The mice were randomly divided into two groups: Y-maze: (1) control (PBS, $n = 8$); and (2) ALWPs (200 mg/kg; $n = 8$); NOR: (1) control (PBS, $n = 8$); and (2) ALWPs (200 mg/kg; $n = 8$). After the behavior experiments, we conducted Golgi staining to measure

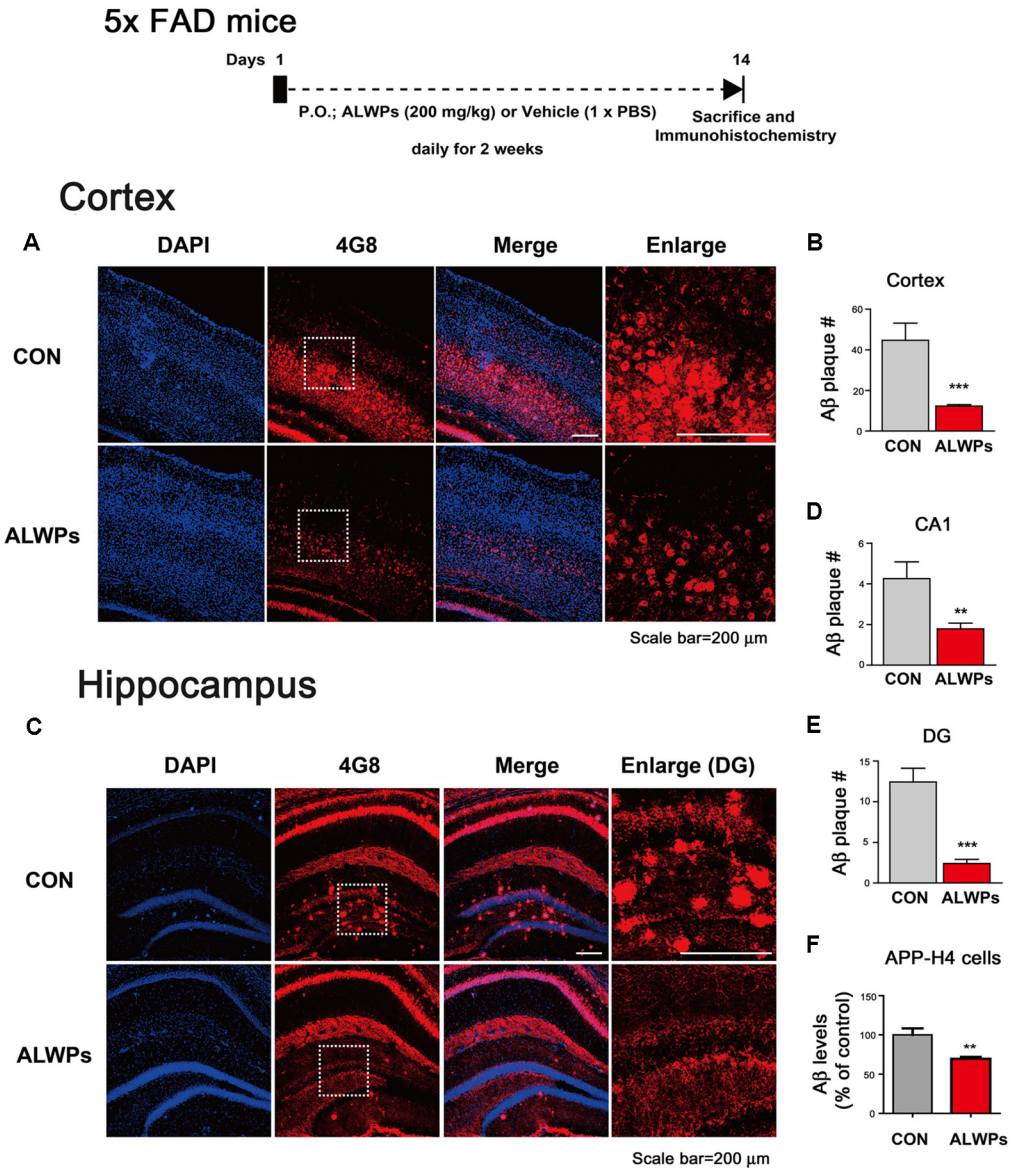

FIGURE 1 | ALWPs significantly decrease amyloid plaque levels in 5x FAD mice. **(A)** 5x FAD mice were orally administered ALWPs (200 mg/kg, p.o.) or PBS daily for 2 weeks, followed by immunostaining of brain slices with an anti-4G8 antibody. Representative images of the cortex are shown. **(B)** Quantification of data from (**A**; con, $n = 6$ mice; ALWPs, $n = 6$ mice). **(C)** Representative images of the hippocampus of 5x FAD mice administered ALWPs (200 mg/kg, p. o.) or PBS daily for 2 weeks. **(D,E)** Quantification of data from (**C**; CA1 and DG; con, $n = 6$ mice; ALWPs, $n = 6$ mice). **(F)** Amyloid precursor protein (APP)-H4 cells (H4 cells overexpressing human APP and producing high levels of Aβ were treated with ALWPs (500 μg/ml) or PBS for 24 h, and human Aβ ELISA was performed (con, $n = 22$; ALWPs, $n = 22$). $^{**}p < 0.01$, $^{***}p < 0.0001$.

dendritic spine density ($n = 8$ mice/group). To examine the effects of ALWPs on Aβ plaque levels, 5x FAD mice were orally administered PBS or ALWPs (200 mg/kg, p.o.) daily for 2 weeks, and immunohistochemistry was conducted with an anti-4G8 antibody ($n = 6$ mice/group). In addition, 5x FAD mice were orally administered PBS or ALWPs (200 mg/kg, p.o.) daily for 2 weeks, and immunohistochemistry was conducted with various anti-tau antibodies to determine the effects of ALWPs on tau phosphorylation ($n = 3$ mice/group). Data were analyzed in a semi-automated manner using ImageJ software, and the results were confirmed by an independent researcher who did not participate in the current experiments.

Y-Maze and NOR Tests

Y-maze and NOR tests were performed to assess cognitive function as described previously (Lee et al., 2018). All experiments were performed under low red light (4–5 lux). For the Y-maze test, mice were placed in a Y-shaped maze with three arms at 120° angles (36.5 cm × 7 cm × 15.5 cm). The mice were allowed to explore for 3 min, and the number of arms visited

and their sequences were recorded using SMART 3.0 software (Panlab Harvard Apparatus). The percentage of alternation (%) was calculated using the following formula: number of alternation triplets/(total number of arm entries − 2) × 100. For the NOR test, mice were placed in an experimental apparatus (30 cm × 30 cm × 25 cm) containing two identical objects for training. They were allowed to freely move to explore the objects for 3 min. On the following day, the mice were placed in the same apparatus with two objects for 5 min. One was a familiar object, which was the same object experienced previously, and the other was a novel object, which was a new item that the mice had never experienced before. All trials were video-recorded, and the interaction preference (%) was calculated using the following formula: interaction time with the novel object/(total interaction time with two objects) × 100.

Antibodies

We used the following primary antibodies for this study: mouse anti-4G8 (1:1,000, BioLegend, San Diego, CA, USA), mouse anti-6E10 (1:1,000, BioLegend, San Diego, CA, USA), rabbit anti-APP N-terminus (1:1,000, Sigma, Ronkonkoma, NY, USA), rabbit anti-ADAM8 (1:200, LSBio, Seattle, WA, USA), rabbit anti-ADAM9 (1:200, Abcam, UK), rabbit anti-ADAM10 (1:200, Abcam, UK), rabbit anti-ADAM12 (1:200, Abcam, UK), rabbit anti-ADAM17 (1:200, Abcam, UK), mouse anti-BACE1 (1:200, Abcam, UK), rabbit anti-neprilysin (1:200, Millipore, Burlington, MA, USA), mouse anti-AT100 (1:500, Invitrogen, Carlsbad, CA, USA), mouse anti-AT180 (1:500, Invitrogen, Carlsbad, CA, USA), and mouse anti-Tau5 (1:500, Invitrogen, Carlsbad, CA, USA).

Preparation of ALWPs

ALWPs included LWPs (Yukmijuhwang-tang: *Rehmannia glutinosa*, *Cornus officinalis*, *Discoreae rhizoma, Paeonia suffruticosa*, *Poria cocos*, and *Alisma orientale*), *Lycium chinense*, *Polygala tenuifolia*, *Acorus gramineus*, and *Antler*. ALWPs were prepared through a three-step extraction process. First, *Lycium chinense* (1,000 g), *Rehmannia glutinosa* (1,200 g), *Dioscorea rhizoma* (500 g), *Cornus officinalis* (500 g), *Paeonia suffruticosa* (360 g), *Alisma orientale* (360 g), *Poria cocos* (360 g), and *Antler* (240 g) were boiled for 12 h at 120°C. Next, the heated mixture was sifted through a filter to remove debris. *Polygala tenuifolia* (180 g), *Acorus gramineus* (180 g), and *Poria cocos* (90 g) were added to the reaction mixture from the first step, followed by boiling for 1 h to prepare a cohesive agent. Finally, the cohesive agent was air-dried for 72 h and molded into pills. The pills were stored at 4°C, and the extract was dissolved in 1× PBS before conducting experiments. Ultra-high-performance liquid chromatography (UHPLC) was previously performed to determine each component, the content of principal markers, and the concentration of each component in the ALWPs formula, and the results of the quantitative analysis of four markers were presented in a previous study (Lee et al., 2018).

Immunocytochemistry

Primary hippocampal neurons from E18 Sprague–Dawley rats were cultured as described previously (Brewer et al., 1993). To examine the effects of ALWPs on ADAMs, BACE1, or NEP levels, primary hippocampal neurons were transfected with GFP plasmid DNA for 24 h, followed by treatment with ALWPs (500 μg/ml) or PBS for 24 h. The primary hippocampal neurons were then fixed in ice-cold methanol for 8 min, washed with 1× PBS three times, and incubated with primary antibodies (α-ADAM 8, α-ADAM 9, α-ADAM 10, α-ADAM 12, α-ADAM 17, α-BACE1, or α-NEP) in GDB buffer (0.1% gelatin, 0.3% Triton X-100, 16 mM sodium phosphate pH 7.4, and 450 mM NaCl). The next day, the cells were washed three times with 1× PBS and incubated with secondary antibodies (Alexa Fluor 555 or Alexa Fluor 488) for 1 h at room temperature. Images were acquired using a confocal microscope (63×, Nikon, Japan), and ADAMs or BACE1 intensity was analyzed using ImageJ software. To measure the levels of ADAM17 and BACE1 in primary hippocampal neurons after ALWPs treatment, we used 18–19 neurons in four sister cultures per group to measure levels of ADAM17 (con, n = 67 dendrites; ALWPs, n = 71 dendrites) or BACE1 (con, n = 97 dendrites; ALWPs, n = 94 dendrites). To determine the effects of ALWPs on NEP levels in primary hippocampal neurons, we used 30 neurons in six sister cultures per group (con, n = 90 dendrites; ALWPs, n = 90 dendrites). To quantify the expression of ADAM17, BACE1, or NEP, the area of secondary dendritic segments was measured by drawing a region of interest (ROI) in a GFP fluorescence image using ImageJ software. Selected ROIs were overlaid on matching ADAM17, BACE1, or NEP images, and ADAM17, BACE1, or NEP fluorescence intensity inside the overlaid ROI was measured. ADAM17, BACE1, or NEP levels are presented as the measurement of the fluorescence intensity divided by the selected ROI area (Nwabuisi-Heath et al., 2012). To quantify the levels of ADAM8, ADAM9, ADAM10, or ADAM12 in primary hippocampal neurons after ALWPs treatment, we used 20 neurons in four sister cultures per group (ADAM8, AMAM9, ADAM10, ADAM12; con, n = 60 dendrites; ALWPs, n = 60 dendrites). Area of secondary dendritic segments were measured by drawing a ROI in red fluorescence image using ImageJ software. Fluorescence intensity inside the drawn ROI were measured, and ADAM8, ADAM9, ADAM10, or ADAM12 levels are presented as the measurement of the fluorescence intensity divided by the measured ROI area.

Immunohistochemistry

ALWPs- or PBS-treated animals were perfused and fixed with 4% paraformaldehyde (PFA) solution, and brain tissues were flash-frozen and dissected using a cryostat (35 μm thickness). Sections were permeabilized for 1 h at room temperature in PBS with 0.2% Triton X-100 and 0.5% BSA, followed by incubation with primary antibodies (i.e., 4G8, AT100, AT180, Tau5) at 4 °C overnight. The sections were then washed three times with 0.5% BSA and incubated with secondary antibodies (Alexa Fluor 555 or Alexa Fluor 488, 1:500, Molecular Probes, Eugene, OR, USA) for 1 h at room temperature. The brain sections were mounted on gelatin-coated cover glass and covered with DAPI-containing mounting solution (Vector Laboratories, Burlingame, CA, USA). Images of the stained tissue sections were captured using a confocal microscope (TI-RCP, Nikon, Japan), and immunoreactivity was analyzed using ImageJ software. To

measure the effects of ALWPs on cerebral amyloid plaque levels, we used six male 5x FAD mice per group, 12 in total. Three to four slices of each brain from −1.70 mm to −2.30 mm relative to the Bregma in stereotaxic coordinates were used to quantify amyloid plaques in the cortex and hippocampus. In addition, data were analyzed in a semi-automated manner using ImageJ software, and the results were confirmed by an independent researcher who did not participate in the current experiments. To determine whether ALWPs can modulate tau phosphorylation, 5x FAD mice (3 months old) were orally administered ALWPs (200 mg/kg, p.o.) or PBS daily for 2 weeks. After 2 weeks, we performed immunohistochemistry with anti-AT100, anti-AT180 or anti-Tau-5 antibodies. Images were acquired by a Nikon TI-RCP confocal laser microscope (10×). For this study, we used three male 5x FAD mice per group (six total), and three slices of each brain from −1.70 mm to −2.30 mm relative to the Bregma were used to quantify tau phosphorylation in the cortex and hippocampus. To quantify the levels of AT100, AT180, and Tau-5 in the cortex and hippocampus, the area of each region was measured by drawing a ROI in a DAPI fluorescence image using ImageJ software (n = 3 mice/group). The selected ROIs were overlaid on matching red fluorescence images, and the fluorescence intensity inside the overlaid ROIs was measured. The levels of AT100, AT180, and Tau-5 are presented as the measurement of the fluorescence intensity divided by the selected ROI area (Jensen, 2013).

Cell-Surface Biotinylation and Live Cell-Surface Staining

APP-H4 cells (H4 cells overexpressing human APP and producing high levels of Aβ) were treated with ALWPs (500 μg/ml) or PBS for 24 h. Surface proteins were then labeled with sulfo-NHS-SS-biotin under gentle agitation at 4 °C for 30 min. Next, quenching solution (50 μl) was added, and the surface-labeled cells were lysed, disrupted by sonication, incubated for 30 min on ice, and clarified by centrifugation (10,000× g, 10 min). The supernatant was added to immobilized NeutrAvidin^{TM} gel and incubated for 1 h at room temperature. The samples were washed with wash buffer (50 Mm Tris-HCl (pH 7.4), 0.15 M NaCl, 1% Nonidet P-40, and protease inhibitors) three times and incubated for 1 h in SDS-PAGE sample buffer including DTT at room temperature. Then, western blotting was performed with an antibody recognizing the N-terminus of APP. The effect of ALWPs on cell-surface levels of APP in primary hippocampal neurons was measured by live cell-surface staining as described previously (Hoe et al., 2006).

APP Processing

APP-H4 cells (H4 cells overexpressing human APP and producing high levels of Aβ were treated with ALWPs (500 μg/ml) or PBS for 24 h, and conditioned medium and cell lysates were harvested. Secreted APP and APP C-terminal fragments (CTFs) were measured by western blotting with anti-sAPP alpha and anti-c1.6.1 antibodies (to detect full-length APP and APP CTF).

Human Aβ ELISA

To examine the effects of ALWPs on human Aβ levels, we detected soluble human Aβ in conditioned medium with human Aβ 40 ELISA kits (KHB3481, Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

Golgi Staining and Dendritic Spine Analysis

To analyze dendritic spine density in the brain, mice were orally administered PBS or ALWPs (200 mg/kg, p.o.) daily for 2 weeks. After 2 weeks, Golgi staining was performed using the FD Rapid GolgiStain Kit (FD NeuroTechnologies, Columbia, MD, USA) according to the protocol provided by the manufacturer. Briefly, PBS- or ALWPs-injected animals were submerged in solutions A and B for 2 weeks in the dark. After 2 weeks, brains were transferred to solution C in the dark for 24 h. Solution C was replaced after the first 24 h, and after an additional 24 h, individual mouse brains were sliced into coronal sections with a thickness of 150 μm using a VT1000S vibratome (Leica, USA). To quantify the dendritic spine density and morphologies in the cortical layer V and hippocampus CA1 regions, we used 8 slices of each mouse brain from −1.70 mm to −2.30 mm relative to the Bregma. Dendritic spines with a length of 0.4–3.8 μm and spine heads with a width of 0.3–2.9 μm were analyzed in this study.

Statistical Analysis

All data were analyzed using either unpaired two-tailed t-tests with Welch's correction for comparisons between two groups or one-way analysis of variance (ANOVA) for multiple comparisons with Prism 6 (GraphPad Software, San Diego, CA, USA). *Post hoc* analyses were completed with Tukey's multiple comparison test, with significance set at $p < 0.05$. Data are presented as the mean ± standard error of the mean (SEM; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$).

RESULTS

ALWPs Significantly Decrease Amyloid Plaque Levels in 5x FAD Mice

Several studies have shown that microglia are a possible cause of AD and other neurological disorders (Wee Yong, 2010; Hirsch et al., 2012; Asai et al., 2015; Heneka et al., 2015). In addition, we recently reported that ALWPs regulate LPS-induced neuroinflammation as well as LPS-induced cognitive function impairment (Lee et al., 2018). Based on the literature and our findings, we hypothesized that ALWPs may regulate AD pathology by modulating neuroinflammatory responses.

To test our hypothesis, we initially examined whether ALWPs can alter Aβ plaque levels. For this experiment, 5x FAD mice (3 months old) were orally administered ALWPs (200 mg/kg, p.o.) or PBS daily for 2 weeks. After 14 days, we performed immunohistochemistry with an anti-4G8 antibody (to detect amyloid plaques). Interestingly, we observed that oral administration of ALWPs in 5x FAD mice significantly reduced the amyloid plaque number in the cortex (**Figures 1A,B**) and hippocampus CA1 and DG (**Figures 1C–E**). These data suggest that ALWPs can modulate amyloid plaque loads in the cortex and hippocampus of 5x FAD mice.

Next, we tested whether ALWPs can alter Aβ levels. We transiently transfected COS7 cells (a monkey kidney fibroblast cell line) with a human APP expression construct for 24 h, followed by treatment with ALWPs (500 μg/ml) or PBS for 24 h, and Aβ ELISA was conducted. Surprisingly, ALWPs did not alter Aβ levels in COS7 cells compared to the control treatment (**Supplementary Figures S1A,B**). We then examined whether ALWPs can regulate Aβ levels in APP-H4 cells (H4 cells overexpressing human APP and producing high levels of Aβ APP-H4 cells were treated with ALWPs (500 μg/ml) or PBS for 24 h, and human Aβ ELISA was performed. We found that ALWPs significantly reduced human Aβ levels in APP-H4 cells compared to the control treatment (**Figure 1F**). These data suggest that ALWPs may differentially regulate human Aβ levels depending on the cell type or APP expression level (transiently overexpressed APP vs. stably overexpressed APP).

In addition, we examined whether ALWPs can alter APP processing to alter human Aβ levels. For this experiment, APP-H4 cells were treated with ALWPs (500 μg/ml) or PBS for 24 h, conditioned medium and cell lysates were collected, and western blotting was performed with anti-secreted APP alpha (sAPPα) and anti-c1.6.1 (to detect full-length APP and APP-CTF) antibodies. Interestingly, we observed that ALWPs-treated APP-H4 cells significantly increased sAPPα but did not alter the full-length APP and APP CTFs (**Supplementary Figures S1C–F**). These data suggest that ALWPs modulate α -secretase enzyme levels and lead to decreased Aβ levels/Aβ plaque load in APP-H4 cells.

ALWPs Significantly Increase Cell-Surface Levels of APP

To determine the molecular mechanisms by which ALWPs regulate Aβ plaque load and Aβ levels, we first investigated the effects of ALWPs on cell-surface levels of APP, as several studies have shown that the α -secretases responsible for APP cleavage (i.e., ADAM17 and ADAM10) are mostly expressed on the cell surface (Cui et al., 2015). Thus, we hypothesized that ALWPs may affect cell-surface levels of APP, thereby promoting cleavage of APP by α -secretase and reducing Aβ levels. To test this hypothesis, APP-H4 cells were treated with ALWPs (500 μg/ml) or PBS for 24 h, and cell-surface levels of APP were measured using a cell-surface biotinylation assay. We found that ALWPs dramatically increased cell-surface levels of APP in APP-H4 cells (**Figures 2A,B**), but the total levels of APP were unchanged (**Figures 2A,C**).

To further confirm our findings, primary hippocampal neurons were transfected with GFP plasmid DNA (to visualize dendritic spines) for 24 h and treated with ALWPs (500 μg/ml) or PBS for 24 h. Then, live cell-surface immunostaining was conducted with an antibody targeting the N-terminus of APP. Again, ALWPs significantly increased cell-surface levels of APP in primary hippocampal neurons compared with the control treatment (**Figures 2D,E**). These data suggest that ALWPs may affect APP trafficking, resulting in decreased Aβ levels.

Since ALWPs decrease Aβ levels and increase cell-surface levels of APP, we further examined whether ALWPs alter levels of critical enzymes for APP processing. For these experiments,

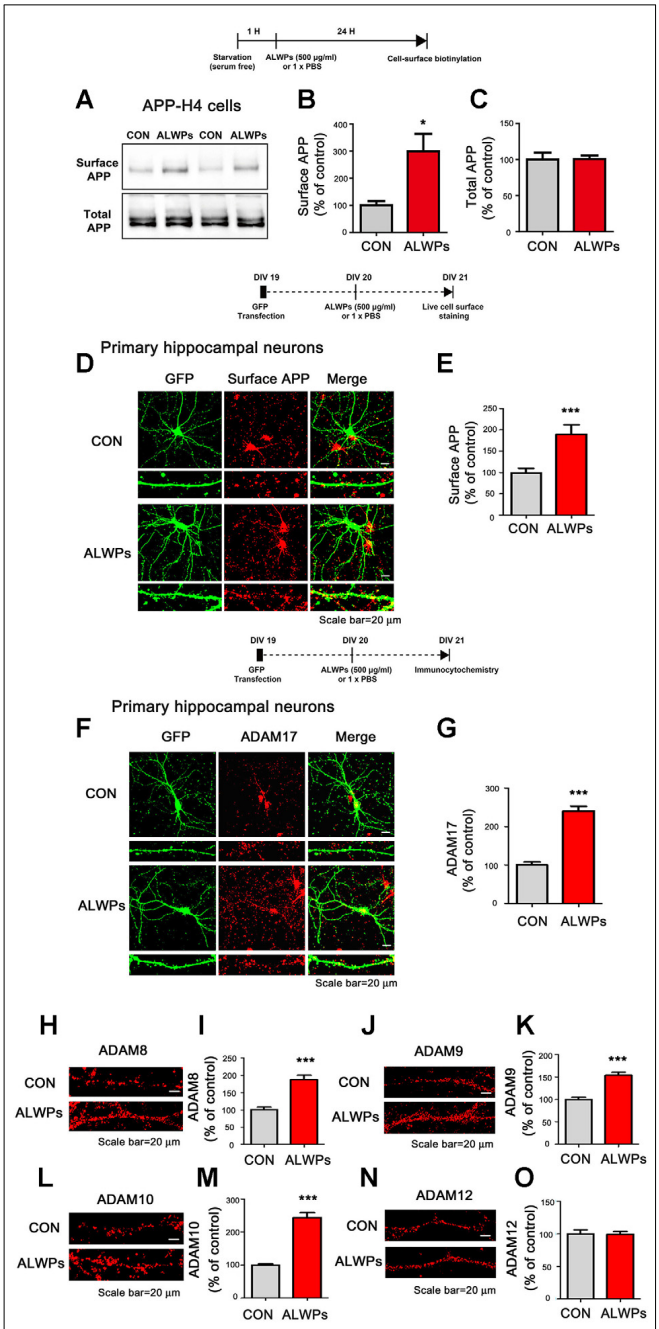

FIGURE 2 | ALWPs significantly increase cell-surface levels of APP. **(A)** APP-H4 cells were treated with ALWPs (500 μg/ml) or PBS for 24 h, and cell-surface biotinylation assays were conducted with an anti-6E10 antibody. **(B,C)** Quantification of data from (**A**; surface APP and total APP; con, $n = 8$; ALWPs, $n = 8$). **(D,E)** Primary hippocampal neurons were transfected with GFP plasmid DNA for 24 h, followed by treatment with ALWPs (500 μg/ml) or PBS for 24 h and live cell-surface staining (con, $n = 134$ dendrites; ALWPs, $n = 120$ dendrites). **(F)** Primary hippocampal neurons were transfected with GFP plasmid DNA for 24 h, treated with ALWPs (500 μg/ml) or PBS for 24 h, and immunostained with an anti-ADAM17 antibody. **(G)** Quantification of data from (**F**; con, $n = 67$ dendrites; ALWPs, $n = 71$ dendrites). **(H,J,L,N)** Primary hippocampal neurons were treated with ALWPs (500 μg/ml) or PBS for 24 h and immunostained with anti-ADAM8, ADAM9, ADAM10, or ADAM12 antibodies. **(I,K,M,O)** Quantification of data from (**H,J,L,N**; con, $n = 60$ dendrites; ALWPs, $n = 60$ dendrites). $^{*}p < 0.05$, $^{***}p < 0.0001$.

primary hippocampal neurons were transfected with GFP plasmid DNA for 24 h, treated with ALWPs (500 μg/ml) or PBS for 24 h, and immunostained with anti-ADAM17 (α-secretase) or anti-BACE1 (β-secretase) antibodies. We found that ALWPs significantly increased ADAM17 levels (**Figures 2E,F**) but not BACE1 levels (**Supplementary Figures S2A,B**).

We then examined whether ALWPs can alter the levels of other ADAMs with APP α-secretase cleavage activity (i.e., ADAM8, ADAM9, and ADAM10) as well as ADAM12, which is known to mediate Aβ neurotoxicity (Asai et al., 2003; Malinin et al., 2005; Vingtdeux and Marambaud, 2012). For these experiments, primary hippocampal neurons were treated with ALWPs (500 μg/ml) or PBS for 24 h and immunostained with anti-ADAM8, anti-ADMA9, anti-ADAM10, or anti-ADAM12 antibodies. We found that ALWPs significantly increased ADAM8, ADAM9, and ADAM10 levels compared to the control treatment (**Figures 2H–M**), but not ADAM12 levels (**Figures 2N,O**). These results indicate that ALWPs may modulate Aβ plaques by up-regulating α-secretase enzyme levels.

As another mechanism of action for decreasing Aβ plaques, we examined whether ALWPs can alter the level of the Aβ degradation enzyme neprilysin (NEP). For these experiments, primary hippocampal neurons were transfected with GFP plasmid DNA for 24 h, treated with ALWPs (500 μg/ml) or PBS for 24 h, and immunostained with an anti-NEP antibody. Interestingly, we observed that treatment with ALWPs increased NEP levels in primary hippocampal neurons (**Supplementary Figures S2C,D**).

Next, we tested whether ALWPs regulate the levels of Aβ degradation enzymes to alter Aβ plaque load and Aβ levels *in vivo*. 5x FAD mice (3 months old) were orally administered ALWPs (200 mg/kg, p.o.) or PBS daily for 2 weeks. After 14 days, we performed immunohistochemistry with an antibody against NEP. Interestingly, ALWPs increased NEP levels in the cortex (**Supplementary Figures S2E,F**) but not the hippocampus (**Supplementary Figures S2G–I**) compared with PBS-treated 5x FAD mice. These data suggest that ALWPs may modulate α -secretase enzyme levels and/or Aβ degradation enzyme levels in specific brain regions to alter Aβ pathology.

Oral Administration of ALWPs Decreases Tau Phosphorylation in 5x FAD Mice

As shown in **Figures 1**, **2**, ALWPs reduce Aβ plaque load and Aβ levels by increasing cell-surface levels of APP. We, therefore, examined whether ALWPs can modulate tau phosphorylation, another hallmark of AD. 5x FAD mice (3 months old) were orally administered ALWPs (200 mg/kg, p.o.) or PBS daily for 2 weeks. After 14 days, immunohistochemistry was performed with anti-AT100, anti-AT180 or anti-Tau5 antibodies. Interestingly, we found that ALWPs significantly reduced tau phosphorylation at Thr212/Ser214 (AT100) in the cortex (**Figures 3A,B**) and hippocampus (**Figures 3C–E**). In addition, ALWPs significantly decreased immunoreactivity associated with tau phosphorylation at Thr231 (AT180) in the cortex (**Figures 3F,G**) and hippocampus (**Figures 3H–J**). However, ALWPs had no effect on total tau levels (anti-Tau5) compared to the control treatment (**Figures 3K–M**) in 5x FAD mice. These data suggest that ALWPs affect tau phosphorylation in a mouse model of AD.

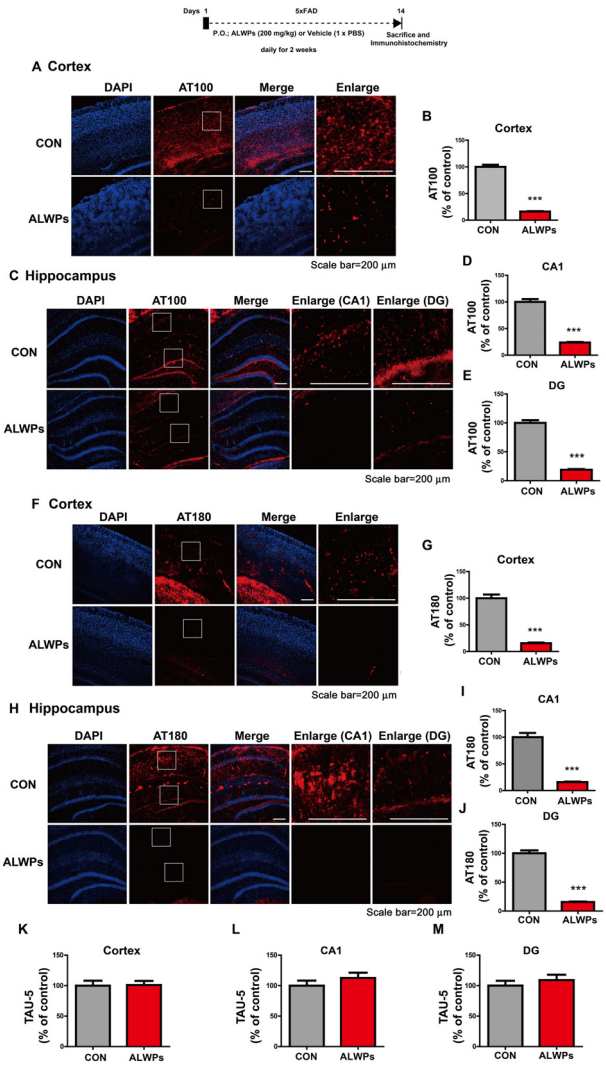

FIGURE 3 | ALWPs significantly decrease tau phosphorylation in 5x FAD mice. **(A,C)** Representative images of the cortex **(A)** and hippocampus **(C)** of 5x FAD mice administered ALWPs (200 mg/kg, p.o.) or PBS immunohistochemically stained with an anti-AT100 antibody. **(B,D,E)** Quantification of data from (**A**; cortex; con, $n = 3$ mice; ALWPs, $n = 3$ mice) and (**C**; CA1 and DG; con, $n = 3$ mice; ALWPs, $n = 3$ mice). **(F–J)** Representative images of the cortex **(F)** and hippocampus **(H)** of 5x FAD mice immunohistochemically stained with an anti-AT180 antibody. **(G,I,J)** Quantification of data from (**F**; cortex; con, $n = 3$ mice; ALWPs, $n = 3$ mice) and (**H**; CA1 and DG; con, $n = 3$ mice; ALWPs, $n = 3$ mice). **(K–M)** Representative images of the cortex (**K**, con, $n = 3$ mice; ALWPs, $n = 3$ mice) and hippocampus CA1 (**L**, con, $n = 3$ mice; ALWPs, $n = 3$ mice) and DG (**M**, con, $n = 3$ mice; ALWPs, $n = 3$ mice) of 5x FAD mice immunohistochemically stained with an anti-Tau5 antibody. $^{***}p < 0.0001$.

ALWPs Block the Impairment of Long-Term Memory by SCO in WT Mice

Several studies have demonstrated that enhanced pro-inflammatory cytokine levels and neuroinflammation

can lead to synaptic dysfunction and memory impairment, which eventually result in neurodegenerative diseases (Fang et al., 2010; Tweedie et al., 2012). We recently reported that compared to the individual components, ALWPs have additive effects on LPS-induced neuroinflammation and LPS-mediated cognitive function impairment (Lee et al., 2018). Here, we further examined whether ALWPs can affect short-term and long-term memory in a SCO-induced amnesia animal model. SCO is a nonselective muscarinic receptor antagonist that induces learning and memory deficits similar to the aging-related and dementia-related symptoms of cognitive impairment (Lee et al., 2010).

To investigate whether ALWPs can attenuate the learning and memory impairment induced by SCO, WT mice were orally administered a control (PBS) or ALWPs (200 mg/kg, p.o.) daily for 11 days. Beginning on day 3, SCO [1 mg/kg, intraperitoneal injection (i.p.)] was injected daily for 9 days (days 3–11), and Y-maze and NOR tests were conducted on days 10 and 12, respectively. In the Y maze, spontaneous "alternation triplet" behavior, defined as successive visits of the three arms in order (e.g., A-B-C or B-A-C or C-A-B), was quantified to examine short-term memory. There was no difference in alternation behavior in the Y-maze between the groups (**Figure 4A**). For the NOR test, the "interaction preference" for the novel object, which is the relative interaction time with the novel object compared to the total interaction time with both the novel and familiar objects, was determined 24 h after the NOR training session to examine the level of long-term object recognition memory. We found that WT mice injected with SCO exhibited significantly decreased long-term memory compared with control WT mice (**Figure 4B**). Interestingly, treatment of WT mice with ALWPs prior to SCO injection blocked the impairment of long-term memory compared with SCO-injected WT mice (**Figure 4B**).

We then tested whether ALWPs can alter dendritic spine formation, which is associated with learning and memory. WT mice were orally administered a control (PBS) or ALWPs (200 mg/kg, p.o.) daily for 11 days. Beginning on day 3, SCO (1 mg/kg, i.p.) was injected daily for 9 days (days 3–11), and Golgi staining was performed. Interestingly, we observed that the dendritic spine density was significantly reduced in SCO-injected WT mice compared to control WT mice in hippocampus CA1 (**Figures 4C–G**). However, pretreatment of WT mice with ALWPs before SCO treatment significantly rescued the dendritic spine number in hippocampus CA1 compared to SCO-injected WT mice (**Figures 4C–G**). In addition, pretreatment with ALWPs also significantly recovered the dendritic spine number in the cortical layer V region compared to SCO-injected WT mice (**Figures 4H–J**). These results indicate that ALWPs can regulate cognitive performance by altering dendritic spine density in SCO-injected WT mice.

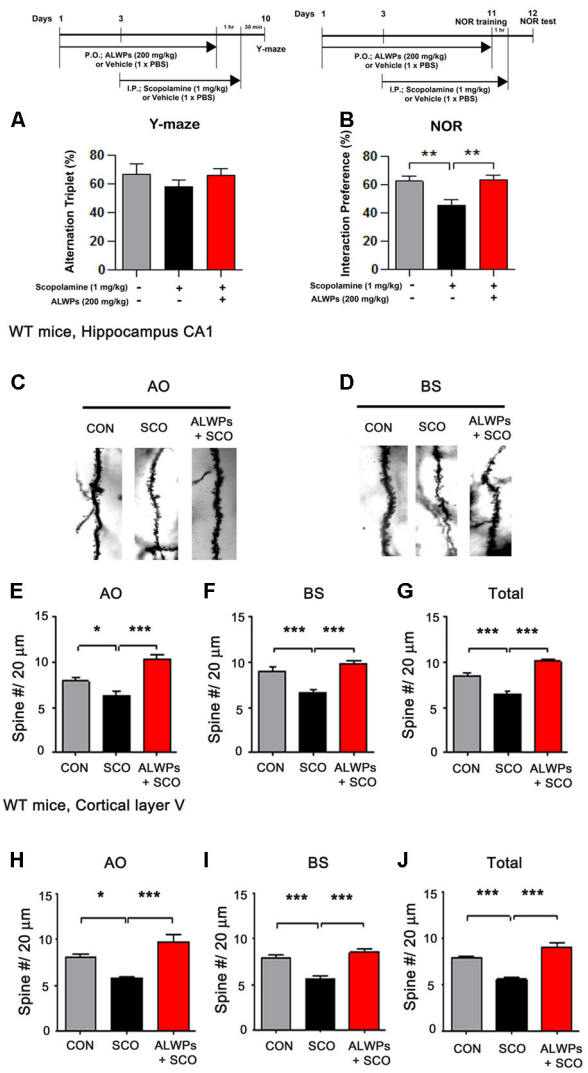

FIGURE 4 | ALWPs reverse scopolamine (SCO)-induced long-term memory impairment in wild-type (WT) mice. **(A,B)** WT mice were orally administered ALWPs (200 mg/kg, p.o.) or PBS daily for 11 days. Beginning on day 3, the mice were injected with a control (PBS) or SCO (1 mg/kg) daily for 9 days, and Y-maze and novel object recognition (NOR) tests were performed on days 10 and 12, respectively (Y-maze: CON, n = 13; SCO, n = 13; SCO+ALWPs, n = 14; NOR: CON, n = 12; SCO, n = 12; SCO+ALWPs, n = 13). **(C,D)** Representative AO and BS dendrites from the hippocampal CA1 regions of mice treated with PBS (control) or ALWPs as indicated. **(E)** Dendritic spine density in hippocampal AO dendrites. **(F)** Dendritic spine density in hippocampal BS dendrites. **(G)** Total average dendritic spine density in hippocampal dendrites (n = 4 mice/group, con, n = 27–28/neurons ; SCO, n = 27–30/neurons ; ALWPs + SCO, n = 27–28/neurons). **(H)** Dendritic spine density in cortical layer V AO dendrites. **(I)** Dendritic spine density in cortical layer V BS dendrites. **(J)** Total average dendritic spine density in cortical layer V dendrites (n = 4 mice/group, con, n = 27–29/neurons ; SCO, n = 32–33/neurons ; ALWPs + SCO, n = 29–30/neurons). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.0001$.

ALWPs Promote Long-Term Memory and Dendritic Spine Density in 5x FAD Mice

As shown in **Figure 4**, ALWPs can regulate cognitive function and dendritic spine formation. We next examined whether ALWPs can affect cognitive performance in a mouse model of AD. For this experiment, 5x FAD mice (3 months old) were administered vehicle (PBS) or ALWPs (200 mg/kg, p.o.) orally daily for 2 weeks, and Y-maze and NOR

tests were conducted. Interestingly, 5x FAD mice treated with ALWPs exhibited increased short-term memory and significantly improved long-term memory compared with PBS-administered 5x FAD mice (**Figures 5A,B**). These data suggest that ALWPs can also modulate cognitive function in a mouse model of AD.

Next, we investigated whether ALWPs can regulate dendritic spine number in a mouse model of AD. For this experiment, 3-month-old 5x FAD mice were administered ALWPs (200 mg/kg, p.o.) or PBS orally daily for 2 weeks. After 14 days, we conducted Golgi staining and measured dendritic spine number and spine morphology. We found that ALWPs significantly increased dendritic spine density in the hippocampus (**Figures 5C–G**) and in cortex layer V (**Figures 5H–L**). We then examined whether dendritic spine morphology was altered in ALWPs-treated 5x FAD mice and found shorter and narrow spines in apical oblique (AO) dendrites of hippocampus CA1 compared to the control group (**Supplementary Figure S3**). However, treatment with ALWPs did not alter dendritic spine morphology in basal (BS) dendrites of hippocampus CA1 or cortical layer V (**Supplementary Figure S3**), suggesting that ALWPs selectively affect dendritic spine morphology and density in specific brain regions in a mouse model of AD.

ALWPs Significantly Increase Dendritic Spine Number in Primary Hippocampal Neurons and Wild-Type Mice

As ALWPs affect cognitive function as well as dendritic spine formation in SCO-injected WT mice and 5x FAD mice, we next examined whether ALWPs can alter dendritic spine formation under normal conditions. For this experiment, primary hippocampal neurons (DIV19) were transfected with GFP plasmid DNA (to visualize dendrite segments and spines) and treated with ALWPs (500 μg/ml) or control (PBS) for 24 h. Dendritic spine density was significantly increased in 500 μg/ml ALWPs-treated primary hippocampal neurons (**Figures 6A,B**). However, ALWPs (500 μg/ml) did not alter spine morphology (i.e., dendritic spine head width and spine length) in primary hippocampal cultures (**Figures 6C,D**).

We then tested whether a higher concentration of ALWPs can alter dendritic spine number. Primary hippocampal neurons (DIV19) were transfected with GFP plasmid DNA and treated with ALWPs (750 μg/ml) or control (PBS) for 24 h. After 24 h, dendritic spine density was measured. Dendritic spine density was significantly increased in primary hippocampal neurons treated with 750 μg/ml ALWPs (**Figures 6E,F**), but spine head width and spine length were not altered (**Figures 6G,H**). These data suggest that ALWPs can increase the number of dendritic spines in primary hippocampal neurons in mature stages.

To further examine whether ALWPs can alter dendritic spine density in WT mice, WT mice (3 months old) were orally administered ALWPs (200 mg/kg, p.o.) or PBS daily for 2 weeks. After 14 days, we conducted Golgi staining and measured dendritic spine number and spine morphology. Treatment with ALWPs significantly increased the dendritic spine number in AO and BS dendrites in the hippocampus (**Figures 7A–E**). In

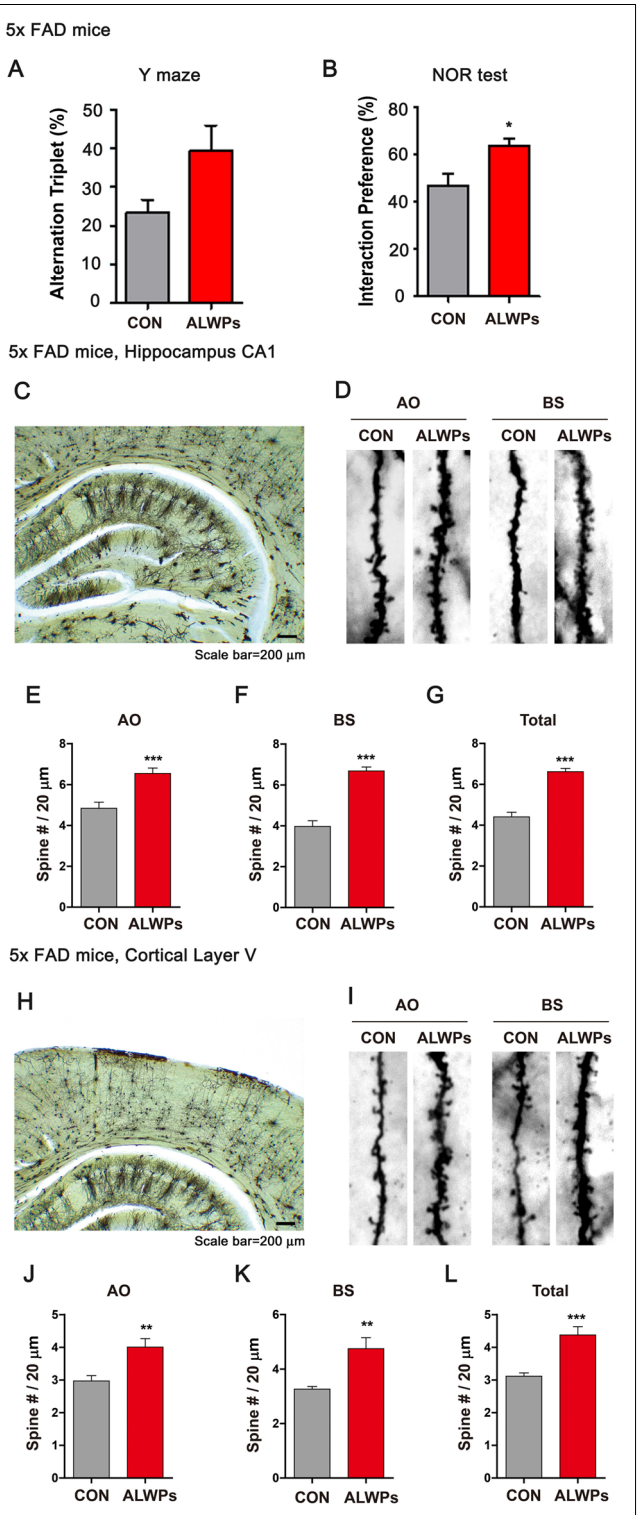

FIGURE 5 | Oral administration of ALWPs to 5x FAD mice significantly enhances long-term memory and dendritic spine number. **(A,B)** 5x FAD mice were orally administered ALWPs (200 mg/kg, p.o.) or PBS (control) daily for 2 weeks. After 12 days, Y-maze (5x FAD CON, n = 8 mice; 5x FAD ALWPs, n = 8 mice) and NOR (5x FAD CON, n = 8 mice; 5x FAD ALWPs, n = 8 mice) tests were performed. **(C,D)** Representative AO and BS dendrites

(*Continued*)

FIGURE 5 | Continued
from hippocampal CA1 neurons of 5x FAD mice treated with PBS or ALWPs (200 mg/kg, p.o.) as indicated. **(E–G)** Dendritic spine density in hippocampal AO dendrites **(E)** and BS dendrites **(F)** and total average spine density **(G)** in hippocampal dendrites of 5x FAD mice (n = 8 mice/group, CA1: con, n = 58–72/neurons; ALWPs, n = 68–83/neurons). **(H,I)** Representative AO and BS dendrites from cortical layer V neurons of 5x FAD mice treated with PBS or ALWPs (200 mg/kg, p.o.) as indicated. **(J–L)** Dendritic spine density in cortical layer V AO dendrites **(J)** and BS dendrites **(K)** and total average spine density **(L)** in cortical dendrites of 5x FAD mice (n = 8 mice/group, con, n = 36–42/neurons ; ALWPs, n = 36–37/neurons). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.0001$.

addition, ALWPs-treated WT mice had longer and wider spines in hippocampal AO dendrites but not hippocampal BS dendrites compared with control WT mice (**Figures 7F–I**).

We then tested whether ALWPs can affect dendritic spine density and morphology in cortex layer V. The dendritic spine density in cortical layer V was significantly increased in ALWPs-treated WT mice (**Figures 7J–N**). Moreover, the spines in cortical layer V AO dendrites were longer and wider in ALWPs-treated WT mice compared with control WT mice (**Figures 7O,P**). However, neither the length nor width of dendritic spines changed in cortical layer V BS dendrites (**Figures 7Q,R**). Taken together, these data suggest that ALWPs can also affect dendritic spine number and spine morphology in WT mice.

ALWPs Promote Dendritic Spine Density in an ERK-Dependent Manner

Ras/ERK signaling is involved in dendritic spine formation (Thomas and Huganir, 2004; Tang and Yasuda, 2017). Therefore, we tested whether ALWPs affect ERK signaling. To address this question, we transfected primary hippocampal neurons with GFP plasmid DNA, treated the neurons with ALWPs (500 μg/ml) or control (PBS) for 24 h, and immunostained the neurons with an anti-p-ERK antibody. p-ERK levels were significantly increased in ALWPs-treated primary hippocampal neurons compared to the control treatment (**Figures 8A,B**). As a complementary study, primary cortical neurons were treated with ALWPs (500 μg/ml) or control (PBS) for 24 h, and western blotting was performed with anti-p-ERK and anti-ERK antibodies. Again, we observed that treatment with ALWPs significantly increased p-ERK levels in primary cortical neurons (**Figures 8C,D**), whereas the total levels of ERK were unchanged (**Figures 8C,E**).

Next, we examined whether ALWPs require ERK signaling to alter dendritic spine density. For this experiment, primary hippocampal neurons were transfected with GFP plasmid DNA for 24 h, pretreated with an ERK inhibitor (PD98059, 10 μM) for 1 h, and treated with ALWPs (500 μg/ml) or control (PBS) for 24 h. After 24 h, the dendritic spine number was measured. Treatment with ALWPs significantly increased the dendritic spine number, consistent with our previous findings (**Figures 8F,G**). By contrast, ALWPs did not increase the dendritic spine number in neurons pretreated with the ERK inhibitor (**Figures 8F,G**). These data suggest that ERK signaling is necessary for ALWPs to regulate dendritic spine formation.

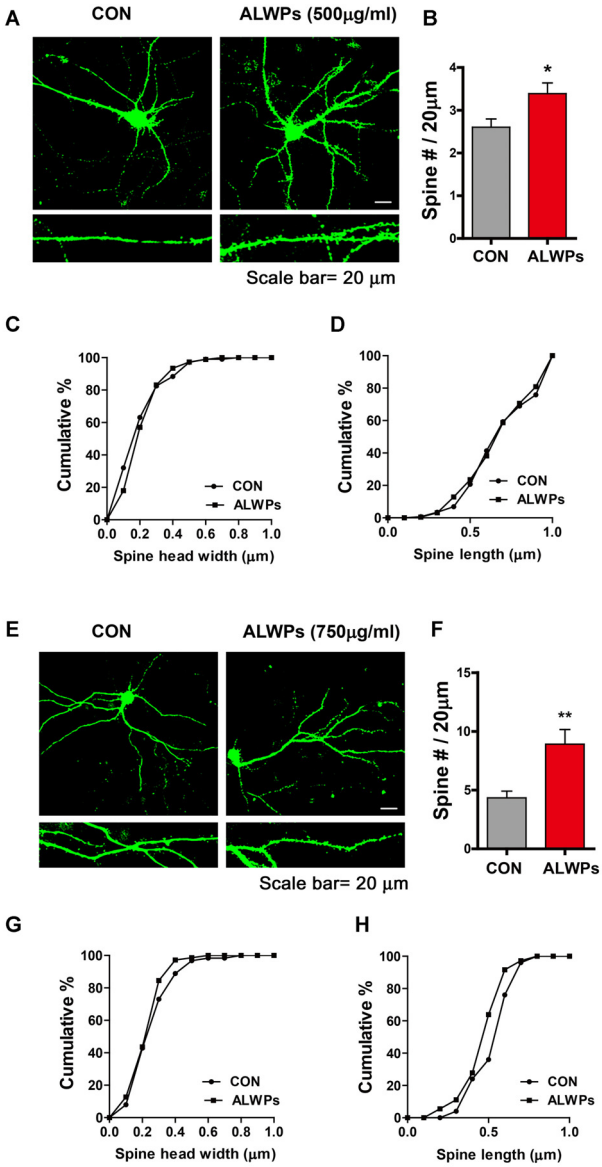

FIGURE 6 | ALWPs significantly promote spinogenesis in primary hippocampal neurons. **(A)** Primary hippocampal neurons were transfected with GFP plasmid DNA, followed by treatment with ALWPs (500 μg/ml) or PBS for 24 h and measurement of dendritic spine density. **(B)** Quantification of data from (**A**; CON, n = 29; ALWPS, n = 27). **(C,D)** The cumulative distribution percentage of spine head width and spine length in primary hippocampal neurons treated with ALWPs (500 μg/ml) or PBS for 24 h (Kolmogorov–Smirnov test). **(E)** Primary hippocampal neurons were transfected with GFP plasmid DNA, followed by treatment with ALWPs (750 μg/ml) or PBS for 24 h and measurement of dendritic spine density. **(F)** Quantification of data from (**F**; CON, n = 34; ALWPS, n = 26). **(G,H)** The cumulative distribution percentage of spine head width and spine length in primary hippocampal neurons treated with ALWPs (750 μg/ml) or PBS for 24 h (Kolmogorov–Smirnov test). $^{*}p < 0.05$, $^{**}p < 0.01$.

We then investigated whether ALWPs can affect Rap signaling, which is involved in dendritic spine retardation, to modulate dendritic spine formation (Lee K. J. et al., 2011; Morel et al., 2018). Primary cortical neurons were treated with ALWPs

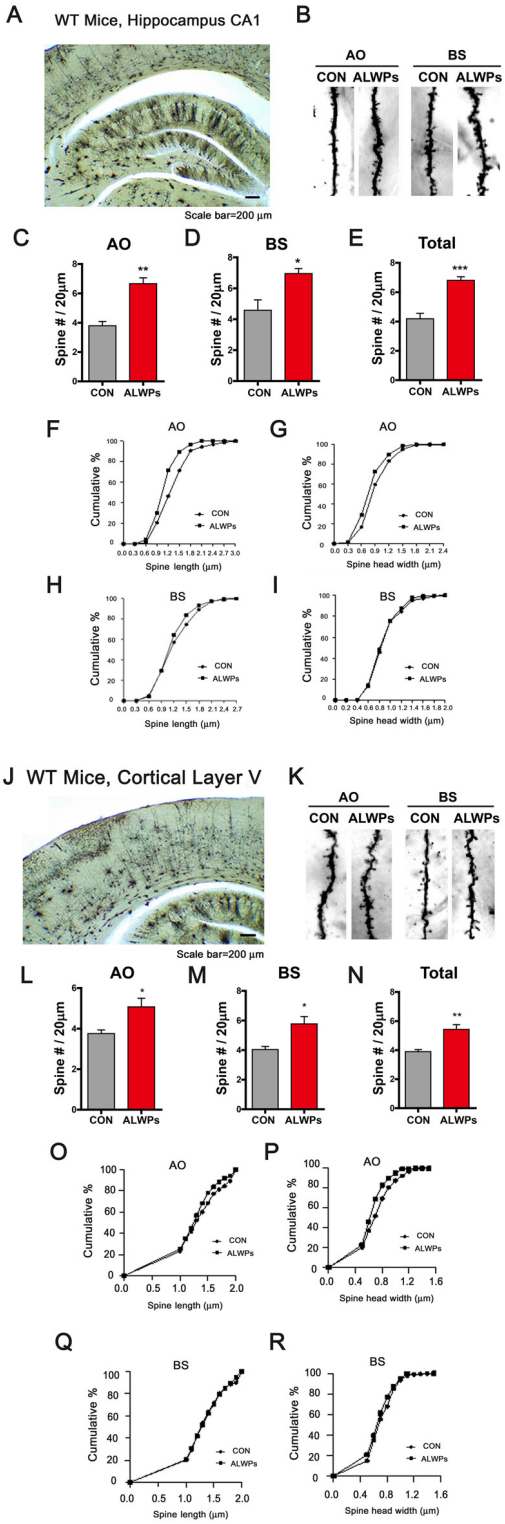

FIGURE 7 | Oral administration of ALWPs to WT mice significantly promotes dendritic spine formation. **(A,B)** Representative AO and BS dendrites from hippocampal CA1 neurons of WT mice treated with the control or ALWPs (200 mg/kg, p.o.) as indicated. **(C–E)** Dendritic spine density in hippocampal AO dendrites **(C)** and BS dendrites **(D)** and total average spine density (*Continued*)

FIGURE 7 | Continued
(E) in hippocampal dendrites of WT mice (n = 4 mice/group, con, n = 20–37/neurons ; ALWPs, n = 27–36/neurons). **(F,G)** The cumulative distribution percentage of spine length **(F)** and spine head width **(G)** in hippocampal CA1 AO dendrites of WT mice (Kolmogorov–Smirnov test). **(H,I)** The cumulative distribution percentage of spine length **(H)** and spine head width **(I)** in hippocampal CA1 BS dendrites of WT mice (Kolmogorov–Smirnov test). **(J–K)** Representative AO and BS dendrites from cortical layer V neurons of WT mice treated with the control or ALWPs (200 mg/kg, p.o.), as indicated. **(L–N)** Dendritic spine density in cortical layer V AO dendrites **(L)** and BS dendrites **(M)** and total average spine density **(N)** in cortical dendrites of WT mice (n = 4 mice/group, con, n = 42–44/neurons ; ALWPs, n = 42–43/neurons). **(O,P)** The cumulative distribution percentage of spine length **(O)** and spine head width **(P)** in cortical layer V AO dendrites of WT mice (Kolmogorov–Smirnov test). **(Q–R)** The cumulative distribution percentage of spine length **(Q)** and spine head width **(R)** in cortical layer V BS dendrites of WT mice (Kolmogorov–Smirnov test). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.0001$.

(500 μg/ml) or control (PBS) for 24 h, and western blotting was performed with anti-PLK2, anti-RapGEF, and anti-p-JNK/JNK antibodies. We found that ALWPs did not alter any Rap signaling pathway in primary cortical neurons (**Supplementary Figure S4**).

DISCUSSION

In traditional oriental herbal medicine, LWPs have been used for more than a 1,000 years to improve or restore declining cognitive function related to the aging process and geriatric diseases (Wee Yong, 2010). LWPs have been shown to modulate the neuronal and synaptic function and to enhance cognitive function by improving functional neurotransmission between neurons (Dong et al., 2018; Lee et al., 2018). Another traditional herbal medicine, antler, is one of the most effective drugs in ameliorating the effects of aging and fatigue and is believed to have many health benefits (Wu et al., 2013). Based on the literature, we recently developed ALWPs by combining LWPs with antler and reported that ALWPs can regulate the LPS-induced neuroinflammatory response and rescue LPS-induced short-and long-term memory impairment (Lee et al., 2018). In the present study, we further examined whether ALWPs can affect AD pathology and cognitive function under normal and pathological conditions. Interestingly, we observed that ALWPs altered both Aβ plaque load and tau phosphorylation in a mouse model of AD. In addition, ALWPs regulated cognitive function by modulating dendritic spine number in SCO-injected WT mice and in 5x FAD mice. Moreover, ALWPs affected dendritic spine formation through ERK signaling in primary hippocampal neurons. Taken together, our results suggest that ALWPs may hold therapeutic drug potential for AD by modulating AD pathology and cognitive function impairment.

Several studies have reported that the individual components of LWPs have effects on Aβ pathology (Sangha et al., 2012). For example, *Corni fructus* has inhibitory activity against BACE1 (β-secretase) and decreases Aβ levels (Youn and Jun, 2012). In another study, Yangxue Qingnao (YXQN), which shares some of the components of ALWPs, significantly decreased cerebral Aβ

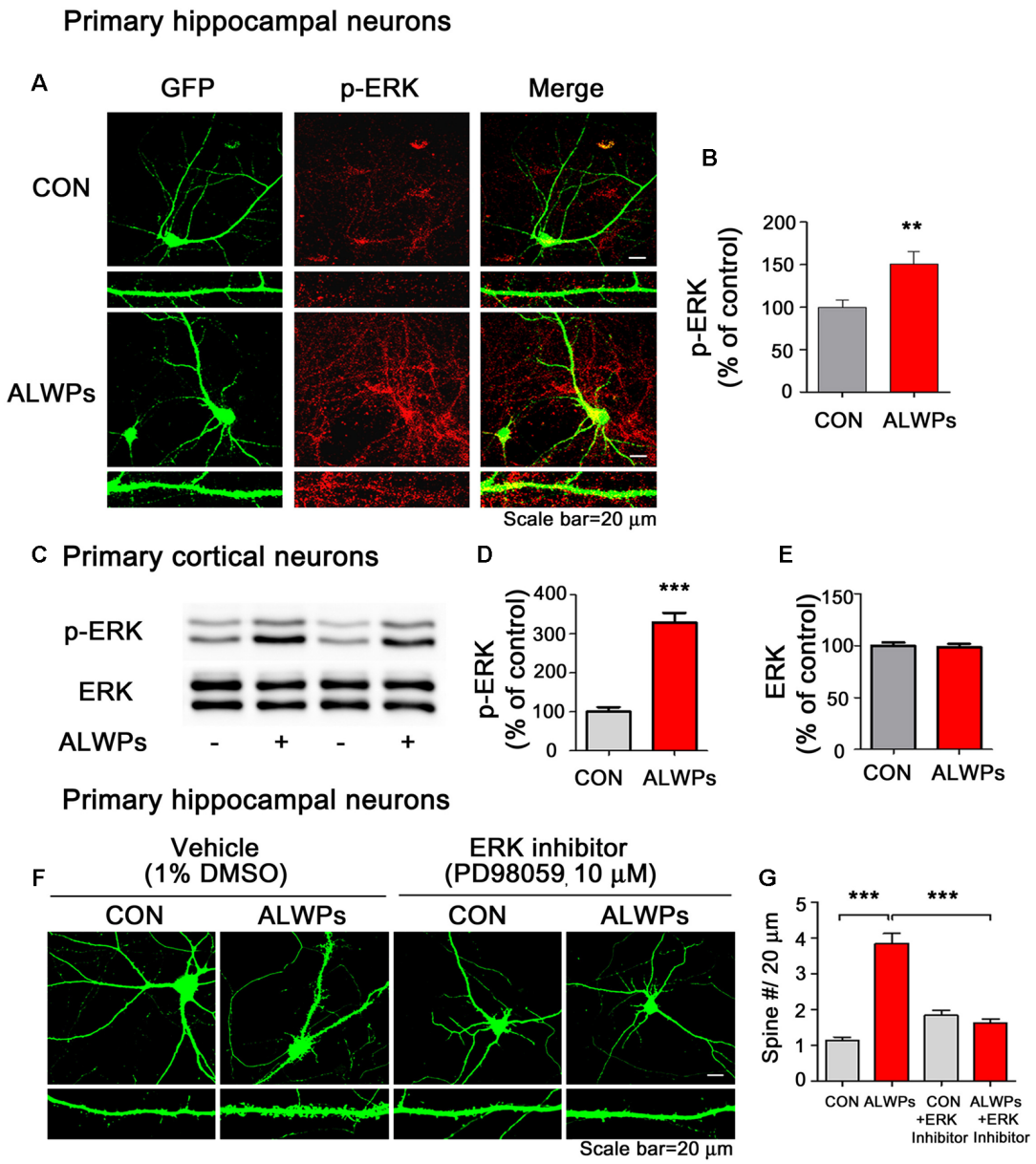

FIGURE 8 | ALWPs require extracellular signal-regulated kinase (ERK) signaling to modulate dendritic spine formation. **(A)** Primary hippocampal neurons were transfected with GFP plasmid DNA for 24 h, treated with ALWPs (500 μg/ml) or PBS for 24 h, and immunostained with an anti-p-ERK antibody. **(B)** Quantification of data from (**A**; con, $n = 88$ dendrites; ALWPs, $n = 100$ dendrites). **(C)** Primary cortical neurons were treated with ALWPs (500 μg/ml) or PBS for 24 h and immunoblotted with anti-p-ERK or anti-ERK antibodies. **(D,E)** Quantification of data from (**C**; p-ERK, $n = 8$; ERK, $n = 4$). **(F)** Primary hippocampal neurons were transfected with GFP plasmid DNA for 24 h, pretreated with an ERK inhibitor (PD98059, 10 μM) or vehicle (1% DMSO) for 1 h, and treated with ALWPs (500 μg/ml) or PBS for 24 h, followed by measurement of dendritic spine density. **(G)** Quantification of data from (**F**; con, $n = 86$; ALWPs, $n = 71$; PD98059, $n = 84$; ALWPS+PD98059, $n = 91$). $^{**}p < 0.01$, $^{***}p < 0.0001$.

plaque loads by attenuating the activity of BACE1 (Wang et al., 2017). However, whether the individual components of LWPs or ALWPs are able to affect AD pathology and the associated molecular mechanisms have not been explored in detail. Here, we demonstrated that ALWPs treatment significantly reduced amyloid plaque loads in 5x FAD mice (**Figure 1**) and Aβ levels in APP-H4 cells (**Figure 1**).

How do ALWPs regulate Aβ pathology? Based on our findings and the literature, one possible mechanism is that ALWPs increase cell-surface levels of APP, leading to increased cleavage of α-secretase on the cell surface and thereby regulating Aβ levels. Indeed, we found that ALWPs significantly enhanced cell-surface levels of APP in APP-H4 cells and primary hippocampal neurons (**Figure 2**). In addition, ALWPs increased

ADAM8, ADAM9, ADAM10, and ADAM17 (α -secretase) but not BACE1 (β-secretase) and ADAM12 levels in primary hippocampal neurons (**Figure 2**, **Supplementary Figure S2**), suggesting that ALWPs regulate Aβ pathology by modulating APP trafficking and/or Aβ pathology-related enzymes/proteins. Another possible mechanism by which ALWPs regulate Aβ pathology is by regulating the levels/activity of Aβ degradation enzymes, specifically NEP, to reduce Aβ pathology. Several studies have shown that NEP, which is also known as neutral endopeptidase, play important roles in AD pathogenesis (Iwata et al., 2001; Turner et al., 2001). For instance, NEP expression levels decline with aging, leading to decreased Aβ clearance (Grimm et al., 2013), and NEP mRNA and protein levels are significantly lower in AD brains than in age-matched normal control brains (Wang et al., 2010). This decline in NEP levels is considered an important factor in the progression of AD. In the present study, we observed that ALWPs treatment increased NEP levels in primary hippocampal neurons. In addition, we found that oral administration of ALWPs to 5x FAD mice significantly increased NEP levels in the cortex but not the hippocampus (**Supplementary Figure S2**). The duration of treatment of 5x FAD mice with ALWPs may not have been sufficient to greatly affect NEP levels in the brain; thus, future studies will use longer treatment times to examine the effects of ALWPs on NEP levels in the brain. Overall, our data suggest that ALWPs alter Aβ pathology by regulating APP trafficking and/or APP processing enzyme levels/activity.

Since, we observed that ALWPs can regulate Aβ pathology, we examined the effects of ALWPs on tau phosphorylation. Tau has many sites of phosphorylation with distinct functions. For example, tau phosphorylation at Thr212, Thr231, and Ser262 produces a toxic tau molecule, induces caspase activation, and leads to neurodegeneration (Alonso et al., 2010). Tau phosphorylation at Thr212/Ser214 (AT100) may facilitate microtubule-based trafficking of organelles by causing detachment of tau from microtubules (Stamer et al., 2002; Ksiezak-Reding et al., 2003). In addition, neurofibrillary tangle (NFT) formation is tightly correlated with the phosphorylation of tau at Thr212/Ser214 (AT100) and Thr 231 (AT180) and can induce many neurodegenerative diseases (Augustinack et al., 2002; Congdon and Sigurdsson, 2018). Although the precise pathophysiology is not fully known, evidence indicates that tau phosphorylation at Thr212/Ser214 and Thr231 may be an important therapeutic target for AD. Interestingly, we found that ALWPs significantly decreased tau phosphorylation at Thr212/Ser214 (AT100) and Thr231 (AT180) in 5x FAD mice (**Figure 3**).

How do ALWPs regulate both Aβ pathology and tau phosphorylation? Several recent studies suggest that Aβ and tau can act synergistically in AD. For instance, Aβ promotes tau phosphorylation at specific tau epitopes as well as neurodegeneration (Jin et al., 2011). Consistent with these findings, another recent study demonstrated that Aβ upregulates tau hyperphosphorylation as well as tau kinases, including GSK-3β, cyclin-dependent kinase 5 (CDK-5), proline-directed kinase (PDK), and casein kinase II, *in vitro* and in a model of AD (Takashima et al., 1993; Vintém et al., 2009; Oliveira et al., 2015; Wu et al., 2018). Conversely, other studies have shown that tau can affect Aβ pathology. For example, Leroy et al. (2012) showed that APP/PS1 mice in which the tau protein was deleted exhibited a significantly reduced Aβ plaque number compared with APP/PS1/Tau transgenic mice. Supporting these findings, Rapoport et al. (2002) found that Aβ did not induce neurite degeneration in primary neurons lacking tau, whereas treatment with human tau protein rescued the Aβ sensitivity of primary neurons lacking tau. However, the mechanisms by which Aβ and/or tau modulates tau phosphorylation and/or Aβ pathology have not been comprehensively studied. In the present study, we observed that ALWPs decreased both Aβ plaque burden and tau phosphorylation in 5x FAD mice. Based on the literature and our findings, there are several possible routes by which ALWPs might regulate both Aβ and tau phosphorylation. One possibility is that ALWPs first affect Aβ pathology, which in turn leads to regulation of tau kinase activity (i.e., CDK5, GSK-3B) and modulation of tau phosphorylation. A second possibility is that ALWPs may alter tau kinase activity as well as tau phosphorylation, with subsequent modulating effects on Aβ pathology. It is also possible that ALWPs regulate Aβ or tau pathology *via* independent mechanisms by modulating tau- or Aβ-related downstream targets. Thus, future studies will explore how ALWPs influence the relationship between Aβ and tau pathology as well as the molecular mechanisms by which ALWPs regulate Aβ and tau pathology.

The processes of learning and memory formation depend on synaptic plasticity, which appears to mediate a behavioral change caused by an experience and the process for acquiring memory. Dendritic spines in the hippocampal and cortical regions are considered the major factor in synaptic plasticity for memory formation (Leuner et al., 2003; Restivo et al., 2009). The relationship between dendritic spine density and memory behaviors has been demonstrated using various disease models and different behavioral assessments (Chen et al., 2010; Jiang et al., 2015; Roy et al., 2016, 2017). The major symptoms observed in aging and AD patients include memory decline or synapse and dendritic spine loss, possibly due to progressive impairments of synaptic circuits or plasticity (Knobloch and Mansuy, 2008; Dorostkar et al., 2015). The potential use of traditional herbal materials to improve memory function related to aging and neurodegenerative diseases has received extensive attention (Shih et al., 2000; Li et al., 2016; Kuboyama et al., 2017). For instance, LWPs and their analogs (i.e., LDW and LDW-AFC) modulate the synaptic function and ameliorate impaired learning and memory performance in animal models of cognitive deficit such as AD. Other studies have demonstrated that LWPs facilitate the induction of LTP in SAMP8 hippocampal slices by inhibiting voltage-dependent calcium channels (VDCCs; Huang et al., 2012). In addition, *Rehmanniae glutinosa*, a component of LWPs, attenuates SCO-induced dementia and accompanying learning and memory impairment *via* regulation of cholinergic systems in rat (Lee B. et al., 2011). *Rehmanniae radix* (Qifu-yin), a component of LWPs, has also been shown to affect cognitive function in mice (Wang et al., 2014). Another traditional herbal medicine, PLAG (a component of antler),

ameliorates SCO-induced memory impairment in mice *via* acetylcholinesterase inhibition and LTP activation (Jeon et al., 2017). However, whether LWPs or other traditional herbal medicines can regulate dendritic spine formation, which is associated with learning and memory, has not been well-studied. In the current study, we examined whether ALWPs can affect learning and memory as well as dendritic spine formation. We observed that ALWPs attenuated the impairment of long-term memory in SCO-injected WT mice (**Figure 4**) and in 5x FAD mice (**Figure 5**). In addition, ALWPs rescued dendritic spine density in SCO-injected WT mice (**Figure 4**) and in 5x FAD mice (**Figure 5**), promoted dendritic spine formation in cultured hippocampal neurons (**Figure 6**), and increased the dendritic spine number in WT mice (**Figure 7**). Interestingly, we observed altered dendritic spine morphology in hippocampal AO dendrites but not hippocampal BS dendrites in ALWPs-treated 5x FAD or WT mice (**Supplementary Figure S3**, **Figure 6**). These phenotypes suggest that several pathways may be regulated by ALWPs in hippocampal AO dendrites. Further study of the differences in dendritic spine morphology between hippocampus CA1 AO and BS dendrites in mice orally administered ALWPs is needed.

To elucidate the molecular mechanism by which ALWPs regulate the formation of dendritic spines to affect learning and memory processes, we first tested whether ALWPs can modulate the Ras and Rap signaling pathways. Ras and Rap signaling are closely related to dendritic spine formation/retardation (Lee K. J. et al., 2011; Tang and Yasuda, 2017), and several studies have shown that Ras activity is involved in Aβ pathology as well as synaptic function (Passafaro et al., 2003; Qin et al., 2005; Gu and Stornetta, 2007; Szatmari et al., 2013). Therefore, it is important to develop therapeutic agents that improve learning and memory by modulating both the Ras and Rap signaling pathways. Here, we found that ALWPs significantly upregulated levels of ERK, an important molecule in the Ras signaling pathway (**Figure 8**). In addition, treatment with an ERK inhibitor blocked the effects of ALWPs on dendritic spine formation, suggesting that ALWPs may regulate dendritic spine formation *via* ERK signaling. We also tested whether ALWPs alter the Rap signaling pathway to regulate dendritic spine formation as another possible mechanism. We found that ALWPs did not alter any Rap signaling molecule, including Plk2, RapGEF, and JNK (**Supplementary Figure S4**). Overall, our data suggest that ALWPs regulate dendritic spine formation through the ERK signaling pathway rather than the Rap signaling pathway.

CONCLUSION

In summary, our findings showed that ALWPs regulated Aβ plaque load and tau phosphorylation in a mouse model of AD. In addition, ALWPs rescued the impairment of long-term memory in SCO-injected WT mice and 5x FAD mice. Moreover, ALWPs promoted dendritic spine formation in cultured hippocampal neurons and significantly increased dendritic spine density in WT mice, SCO-injected mice, and 5x FAD mice. Finally, ALWPs altered dendritic spine formation through an ERK-dependent mechanism. We, therefore, advocate ALWPs as a useful therapeutic agent for the treatment of AD.

ETHICS STATEMENT

All animal experiments were performed in accordance with approved animal protocols and guidelines established by KBRI (IACUC-2016-0013).

AUTHOR CONTRIBUTIONS

YK, JWK, Y-MW, and H-SH: study conception and design. YN, BJ, YK, and JK: acquisition of data. YN, BJ, J-YL, K-MH, and K-YR: preparation of figures. YN, BJ, YK, JK, JWK, Y-MW, and H-SH: writing of manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnmol.2019.00192/full#supplementary-material

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REVIEW
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Gut Inflammation in Association With Pathogenesis of Parkinson's Disease

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Parkinson's disease (PD) is a neurodegenerative disease that is generally thought to be caused by multiple factors, including environmental and genetic factors. Emerging evidence suggests that intestinal disturbances, such as constipation, are common non-motor symptoms of PD. Gut inflammation may be closely associated with pathogenesis in PD. This review aims to discuss the cross-talk between gut inflammation and PD pathology initiation and progression. Firstly, we will highlight the studies demonstrating how gut inflammation is related to PD. Secondly, we will analyze how gut inflammation spreads from the gastro-intestine to the brain. Here, we will mainly discuss the neural pathway of pathologic α-syn and the systemic inflammatory routes. Thereafter, we will address how alterations in the brain subsequently lead to dopaminergic neuron degeneration, in which oxidative stress, glutamate excitotoxicity, T cell driven inflammation and cyclooxygenase-2 (COX-2) are involved. We conclude a model of PD triggered by gut inflammation, which provides a new angle to understand the mechanisms of the disease.

Keywords: gut inflammation, Parkinson's disease, α-synuclein, oxidative stress, cyclooxygenase-2, glutamate excitotoxicity, T-cell

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INTRODUCTION

Parkinson's disease (PD) is a neurodegenerative disease that is characterized by the degeneration of dopaminergic neurons in the substantia nigra pars compacta (SNpc) (Hostiuc et al., 2016) and the presence of Lewy bodies (LBs) (Engelhardt, 2017), in which the primary protein component is misfolded and aggregated α-synuclein (α-syn) (Spillantini et al., 1998). PD patients typically exhibit motor symptoms, such as tremor, stiffness, unstable posture, and slowness of movement (Jankovic, 2008), which are accompanied, and often preceded by a series of non-motor symptoms, such as intestinal dysfunction (Mukherjee et al., 2016), sleep disorders (Partinen, 1997; Ciric et al., 2018), depression (Taylor et al., 1986) and cognitive impairment (Starkstein et al., 1989). Among these non-motor symptoms, intestinal dysfunction has been paid special attention to, not only because it often appears prior to the motor symptoms (Suzuki et al., 2019), but also because α-syn aggregates have been detected in the gastrointestinal tract years before the motor-symptom onset (Cersosimo, 2015; Stokholm et al., 2016; Kim et al., 2017; Lu et al., 2017). LBs present in the gastrointestinal tract were first described in 1986 by Qualman et al. (1984) in which LBs were detected in the esophagus and colon in 2 PD patients with dysphagia. Since then, more reports reinforced the discoveries of pathological α-syn in the ENS (Wakabayashi et al., 1988, 1990; Braak et al., 2006). Braak et al. (2003) postulated that LBs were first localized in the dorsal motor nucleus of the vagus (DMNV) and then spread to the upper brain regions, which inspired more researches on the time course of the presence of LBs in the gut and brain. Stokholm et al. (2016) found that phosphorylated α-syn

positive profiles were seen in 22 of 39 (56%) prodromal PD subjects and 30 of 67 (45%) prodromal tissue blocks, which were significantly higher compared to control subjects. Rota et al. (2019) found that the gastrointestinal α-syn pathology precedes in the CNS at least by 6 month in human-A53T α-syn transgenic mice. These evidences consolidate the view that PD patients and animal models of PD are characterized by enteric α-syn pathology at the early stages of the disease. Therefore, it has been proposed that PD may be initiated from the gut (Braak et al., 2004).

Crohn's disease (CD) and ulcerative colitis (UC) are the two main inflammatory bowel diseases (IBD) with UC mainly affecting the colon and rectum, and CD impacting the small and large intestine (Baumgart and Carding, 2007; Baumgart and Sandborn, 2007). Coincidentally, IBD shares some features with PD, including a negative correlation with smoking (Hernán et al., 2001, 2002; James, 2003; Breckenridge et al., 2016; Kridin et al., 2018; Salih et al., 2018; Wang et al., 2018) and several shared risk genes, such as LRRK2 (Miyake et al., 2010; Hui et al., 2018) and CARD15 (Bialecka et al., 2007). Interestingly, Devos et al. (2013) reported the inflammatory responses in the gut since the early stages of PD, by analyzing the ascending colonic biopsies of PD patients, they found that the pro-inflammatory cytokines (TNF-α, IF-γ, IL-6, and IL-1β) and glia markers GFAP and Sox-10 were significantly elevated, and some of them (IL-6, IL-1β, and Sox-10) were negatively correlated with disease duration. Cote et al. (2011) treated MYD 88 knockout mice with MPTP intraperitoneal administration, they found that MYD 88 knockout mice protected against MPTP induced TH-immunoreactive neuron degeneration in the myenteric plexus of distal ileum, although they detected no macrophage density changes compared with MPTP treated WT mice, the MYD 88 knockout mice exhibited a predominant pro-repair phenotype. Moreover, Cote et al. (2015) found clear presence of M1 monocytes and increased IL-1βand IL-6 in the gut, while in the partial depletion of M1 monocytes the mice protected against MPTP induced TH expression in the gut but not in the striatum, in the meanwhile the microglia activation showed no difference in microglia activation in the brain. In addition, several studies have reported the causal relationship between IBD and PD in recent years (Lin et al., 2016; Fujioka et al., 2017; Wan et al., 2018; Zhu et al., 2019). Here, we analyzed the recent studies on the relationship between PD and intestinal disorders, and highlighted the potential underlying mechanisms of gut inflammation triggering PD.

RELATIONSHIP BETWEEN GUT INFLAMMATION AND PD

In the earlier years, most of the findings were supportive of a relationship between PD and IBD. Lin et al. (2016) demonstrated that IBD was associated with an increased incidence of PD, especially in CD, in a retrospective clinical cohort from 2000 to 2011. In a Danish nationwide cohort study (1977–2014), Villumsen et al. (2019) also found that patients with IBD had a 22% increased risk of developing PD, compared to non-IBD individuals. However, the increased risk of PD was significantly higher in the patients with UC, but not significantly different among patients with CD. These findings were questioned by Weimers et al. (2018), however, after a thorough re-examination, the same conclusions were drawn (Villumsen et al., 2018). In contrast to these favorable reports, some studies have challenged the view on the association between PD and IBD. Fujioka et al. (2017) identified only 2 patients with CD among 876 PD patients, which was comparable to the incidence in the general population. Moreover, PD was even inversely associated with either CD or UC, in some of the newly diagnosed PD cases (Camacho-Soto et al., 2018). Although Weimers et al. (2019) found that IBD was associated with a higher risk of PD, the correlation appeared to be caused by a surveillance bias (Weimers et al., 2018).

In a systematic review and meta-analysis, Wan et al. (2018) found that IBD patients did not show increased risk of PD, however, a subgroup analysis showed a significant difference in the more aged patients (>60 years old). Zhu et al. (2019) suggested that both CD and UC patients have an increased risk of PD compared to the control subjects. To date, although no consensus has been reached, among all of the researches, the most recent data implied that IBD exacerbates PD (Peter et al., 2018). However, the underlying mechanisms on how IBD could trigger PD pathogenesis are still unclear.

EVIDENCE OF GUT INFLAMMATION SPREADING TO THE BRAIN

As we hypothesized, gut inflammation was able to trigger PD symptoms, the three key factors, (1) the initiator from gut inflammation, (2) the pathways, and (3) the subsequent effects of gut inflammation in the brain must be analyzed step by step. In the gut the inflammatory response induces the disrupted intestinal mucosal barrier, resulting in the exposure to microbiota. Thus, the enteric nervous system, immune system, and microbiota interplayed, which is considered to be a mutually integrated interaction network (Yoo and Mazmanian, 2017). Then, the products or stimulation of these comprehensive interactions can spread to the brain, which may summarize as the microbiota-immune-neuro gut-brain axis. Considering that pathologic α-syn was reported as triggers of PD, we analyzed the inflammatory response and microbiota induced α-syn pathology in the gut. In addition, the inflammatory response itself was also estimated as the pathological process. Therefore, for the pathways, we will mainly introduce (1) the systemic inflammatory routes that spread proinflammatory factors and (2) the pathologic α-syn propagation pathway.

The Crosstalk Among Microbiota, Inflammatory Response and Pathological α-Syn in the Enteric Microenvironment

Although researches have demonstrated that anti-inflammatory treatment is effective to ease PD symptoms (Gagne and Power, 2010; Thome et al., 2015, 2016), there is a lack of evidence of gut inflammation induced pathologic α-syn directly. However,

increased inflammation in the gut, can increase gut permeability, thereby leaking intruders from the gut lumen, such as microbiota and their metabolites, may trigger aggregation of α-syn (Chorell et al., 2015; Bhattacharyya et al., 2019). Interestingly, the dysbiosis in PD is widely reported (Scheperjans et al., 2015; Cassani et al., 2015) and the fecal microbiota transplant is proven to ease symptoms of PD (Ananthaswamy, 2011), suggesting an important role of microbiota in the initiation of PD. A growing body of evidence highlight the potential role of microbiota inducing pathologic α-syn (Fitzgerald et al., 2019). The first proposed mechanism is conceived from the gut lumen harbors *Escherichia coli* which can produce Curli, an extracellular bacterial amyloid protein. In a study conducted by Chen et al. (2016) rats exposed to curli-producing bacteria displayed increased neuronal α-syn deposition in both gut and brain compared to rats exposed to either mutant bacteria unable to synthesize curli, or to vehicle alone. They also found that α-syn-expressing *Caenorhabditis elegans* fed on curli-producing bacteria expressed enhanced α-syn aggregation (Chen et al., 2016). The key element of Curli, CsgA, contains amyloidogenic peptide repeat motifs shared by human and yeast prions (Cherny et al., 2005; Evans et al., 2015), indicating that the α-syn deposition induced by curli may result from the cross-seeding effects between with α-syn and CsgA in the gut (Chen et al., 2016). Besides, the chaperon-like proteins CsgC and CsgE encoded by the Curli operon are also found to modulate α-syn amyloid formation (Chorell et al., 2015). Another mechanism is that α-syn aggregation could be induced by lipid structure in lipopolysaccharide (LPS) (Bussell and Eliezer, 2003), one of the triggers of PD (Qin et al., 2007), which is mainly in the outer membrane of gram-negative bacteria. By semi-quantitative analysis using nuclear magnetic resonance (NMR), Bussell and Eliezer (2003) proved that unbroken helical α-syn structure adopted an unusual, slightly unwound, α11/3 helix conformation to bind the lipid surface, indicating that α-syn can bind to the lipid structure of LPS. More substantial evidence supports this theory. Kim et al. (2016) found that α-syn monomers after being incubated with LPS, showed a strong thioflavin T fluorescence. When these α-syn fibrils were injected into the striatum of mice (C57BL/6J), phosphorylated-α-syn pathology was found throughout the brains, including the striatum, SNpc, amygdala, and auditory cortex (Kim et al., 2016), demonstrating that LPS induced α-syn fibrils are toxic. Besides, in the study of Bhattacharyya et al. (2019) LPS was found to modulate the overall aggregation kinetics of α-syn in a concentration-dependent manner.

The microbiota is also involved in the inflammatory responses (Hooper et al., 2012). It was reported that *Bacteroides fragilis*, can induce colitis through the activation of STAT3 and Th17 response (Wu et al., 2004) via the NF-κB pathway, which leads to an increase of IL-8 production by intestinal epithelial cells (Wu et al., 2009). Prindiville et al. (2000) described that 19.3% of IBD patients with active disease have enterotoxigenic *B. fragilis* in their stool specimen, while control subjects did not show this subset of bacteria. However, the changes of *Bacteroides* in PD is still of controversy: Hasegawa et al. (2015) reported a decrease in PD patients, while Keshavarzian et al. (2015) reported an increase. The inconsistent reports may result from the different stages of the patients. In a 2-year follow-up study, Minato et al. (2017) reported that the deteriorated group (worsening of UPDRS I scores) had lower counts of Bifidobacterium, *B. fragilis*, than the stable group at year 0 but not at year 2. Besides, the intestinal inflammation was reported to promote the overgrowth of Enterobacteriaceae (Lupp et al., 2007; Zeng et al., 2016), of which the *E. coli* strains is able to induce IL-1β through NLR family pyrin domain containing 3 (NLRP3) -dependent mechanism in PD patients (De la Fuente et al., 2014). Moreover, the Prevotellaceae that reported to be decreased in both intestinal inflammation and PD, is also involved in the inflammation. Prevotellaceae is one of main source of short chain fatty acids (SCFAs), which is reported to provide primary energy for intestinal epithelial cells to maintain the stability of the intestinal barrier, contribute to the development of colonic regulatory T (Treg) cells to limit local inflammation and engage the G protein-coupled receptor GPR43 on neutrophils to diminish their infiltration into tissues (Maslowski et al., 2009; Smith et al., 2013; Morrison and Preston, 2016).

Neural Pathway of Pathologic α-Syn Spreading

After intensive examination of tissues from the peripheral nervous system and the brain, Braak and his colleagues proposed that the α-syn pathologies may be initiated from the olfactory system and low brain stem, which is connected to the peripheral tissues, such as the gut, via the vagal nerve. Pathological studies with postmortem tissues from the brain and the peripheral tissues suggest that pathological α-syn nucleation and aggregation may occur in the enteric neurons of the gastrointestinal tract and can propagate from the gut to the brain (Braak et al., 2003). According to this hypothesis, LBs may first be initiated in the gut (Braak et al., 2006) and then spread via the vagal nerves to the DMNV, locus coeruleus (LC), substantia nigra (SN) and cortex in sequence (Braak et al., 2003). We and others have provided direct evidence of this route (Holmqvist et al., 2014; Uemura et al., 2018). We injected a human PD brain lysate containing different forms of α-syn or different aggregated forms of recombinant α-syn into the intestinal wall of Sprague Dawley rats and found that the exogenously delivered human α-syn could be rapidly transported via the vagal nerve and reach the DMNV in the brainstem in a time-dependent manner (Holmqvist et al., 2014). Uemura et al. (2018) found phosphorylated α-syn-positive LB-like aggregates in the DMNV 45 days after α-syn preformed fibrils (PFFs) were injected into the mouse gastric wall. Although these studies did not examine whether the pathological a-syn in the DMNV were of exogenous or endogenous origin, they could support a prion-like spreading of a-syn whereby α-syn replicates through a mechanism of self-propagating conformation and assembles into filaments, which can act as a seed to recruit the soluble form of the protein and enhance filament load through the seed extension (Goedert et al., 2010; Hansen and Li, 2012). Very recently, Kim et al. (2019) further reinforced the evidence for gut-to-the-brain α-syn pathology spread (Kim et al., 2019). After injecting α-syn PFFs into the muscular layer of the mouse

duodenum and pylorus, the authors detected phosphorylated α-syn in DMNV, LC, SNpc and up to upper brain regions (Kim et al., 2019). More importantly they observed dopaminergic degeneration and motor and non-motor behavioral deficits in response to α-syn PFF injections in the gut (Kim et al., 2019). Vagotomy, which interrupts the spreading of α-syn PFFs from the gut the brain, alleviated the severity of morphological and behavioral alterations (Kim et al., 2019). Interestingly, no pathology was observed when the recipient mice are in α-syn knock-out background (Kim et al., 2019). This study provides strong evidence that α-syn pathology can be spread from the gut to the brain via the route of the vagal nerve, can induce neuronal degeneration, and can cause respective neuronal dysfunction and behavioral defects.

SYSTEMIC INFLAMMATORY ROUTES

Not only can pathological α-syn induced by intestinal inflammation be transmitted to the brain, but also the inflammatory response itself in the gut can influence the brain. The inflammatory response in the gut may affect the brain through two routes, the neuroimmune pathway and the humoral pathway.

Neuroimmune Pathway

The neuroimmune pathway of gut inflammation transmitting to brain is mainly conducted by the vagal nerve. It was reported that vagotomized mice and rats presented attenuated social exploration and depression in social investigation induced by intraperitoneal injection of recombinant rat IL-1β (Bluthe et al., 1996a,b). Besides, vagal nerve stimulation could decrease the inflammatory response and improve survival in experimental sepsis, hemorrhagic shock, ischemia reperfusion injury, and other conditions of cytokine excess (Borovikova et al., 2000; Johnston and Webster, 2009; Huffman et al., 2019).

Up to now, the specific mechanisms of the neuroimmune pathway are not clear, but some studies have suggested that the vagal nerve plays a dual role in inflammatory regulation both through its afferent and the efferent fibers (Bonaz et al., 2017). The vagal afferents target the hypothalamic-pituitary-adrenal (HPA) axis. Vagal afferents activate neurons from the A2 noradrenergic group in the nucleus tractus solitarius (NTS). These neurons project to the parvo-cellular paraventricular nucleus of the hypothalamus (PVH), where the corticotrophin-releasing factor (CRF) neurons are stimulated to release CRF, which in turn induces the pituitary to release adrenocorticotropic hormone (Bonaz et al., 2016). Adrenocorticotropic hormone then stimulates the adrenal glands to release glucocorticoid, which plays a role in the inhibition of peripheral inflammation (Bonaz et al., 2017). In addition, Lubbers et al. (2010) showed that activating cholecystokinin-1 receptors on vagal afferents can also regulate inflammation. The vagal efferents are involved in the cholinergic anti-inflammatory pathway. This pathway regulates systemic inflammation through the release of acetylcholine (Ach) by the vagal nerve (Zhai et al., 2017). The details of this pathway remain obscure, but studies have showed that a subunit of the α7 nicotinic Ach receptor (α7nAchR), one of the Ach receptors, is expressed on macrophages, and the α7nAchR-agonist, GTS, is able to restrain systemic inflammation (Wang et al., 2003; Cai et al., 2009).

Humoral Pathway

Humoral pathways of gut inflammation spreading to brain are mainly involved in the leakage of the Blood–Brain Barrier (BBB), which can be divided into disruptive and non-disruptive approaches, respectively, reflecting the physical conditions of the BBB (Varatharaj and Galea, 2017).

Disruptive BBB change is usually evident in the structural alterations, and can be detected using inserted tracers (Varatharaj and Galea, 2017). A considerable number of reports have shown that the BBB is damaged in PD patients (Pisani et al., 2012; Gray and Woulfe, 2015). In addition, 60% of LPS induced PD models exhibited disrupted BBB (Varatharaj and Galea, 2017). In the event that the blood-brain barrier is damaged, proinflammatory cytokines and immune cells such as T cells (Engelhardt and Ransohoff, 2012) and mast cells (Jones et al., 2019) from peripheral inflammation are able to enter the brain.

Non-disruptive BBB changes usually occur at a molecular level, and are not visible in histological architecture (Varatharaj and Galea, 2017). The changes can be mediated by special transporters (Xaio et al., 2001; Osburg et al., 2002; Hartz et al., 2006; Pan et al., 2008; Jaeger et al., 2009; Wittmann et al., 2015), cytokines (Herkenham et al., 1998; Skelly et al., 2013), prostaglandins (PGs) (Vasilache et al., 2015) and cellular transmigration (Bohatschek et al., 2001; Wang et al., 2008; Banks et al., 2012). In addition, substances can also enter the brain through the areas that lack the BBB, such as the circumventricular organs (Ferrari and Tarelli, 2011). Therefore, proinflammatory cytokines such as IL-1α, IL-1β, IL-6, TNF-α, etc., can enter the brain and activate microglia or astrocytes to induce inflammatory response (Vallieres and Rivest, 1997; Osburg et al., 2002; Sato et al., 2012; Neniskyte et al., 2014).

SUBSEQUENT EFFECTS OF GUT INFLAMMATION IN THE BRAIN

Pathological α-syn, which could be triggered by intestinal bacterial components, is the main component of LB, whose neurotoxicity is related to its structure and post-translational modification, such as phosphorylation at Ser129 (Arawaka et al., 2017) and nitration (He et al., 2019). These pathological proteins, on the one hand, recruit normal α-syn and disrupt its physiological functions; on the other hand, their toxicity can act as environmental stress to increase inflammation, oxidative stress, and interfere with other physiological effects (Xu et al., 2013; Zhang et al., 2017). Another subsequent effect is that the immune cells and proinflammatory cytokines in the brain can cause additional release of inflammatory and neurotoxic molecules, contributing to chronic neuroinflammation and neuronal death (Ferrari and Tarelli, 2011; Tufekci et al., 2012). The underlying mechanisms of these subsequent effects inducing neurodegeneration are unclear, however, they may be involved in a series of molecular mechanisms.

It should be emphasized that the pro-inflammation and pathological α-syn propagation may not work alone, they are interrelated both in the brain and the gut. For example, marmosets with colitis show significantly increased phosphorylated α-syn in the colonic myenteric ganglia (Resnikoff et al., 2019). In the brain, fibrillar α-syn is found to produce pro-inflammatory mediators, such as IL-1β. IL-1β via the activation of the microglial NLRP3 inflammasome (Codolo et al., 2013; Gordon et al., 2018). α-Syn is also found to activate pro-inflammatory TLR4 pathways in astrocytes (Rannikko et al., 2015) and disrupt the anti-inflammation of Dopamine D2 receptor (Du et al., 2018). In contrast, in a study conducted by Horvath et al. (2018) pro-inflammatory factor S100A9 is also reported to induce α-syn aggregation.

Oxidative Stress

Oxidative stress occurs when excessive oxygen free radicals are produced within cells. When the concentration of these active substances is not controlled by internal defense mechanisms, such as antioxidants or oxygen free radical removal enzymes, protein lipids, and DNA are oxidized causing damage (Gagné, 2014). Studies have shown that the production of high levels of reactive oxygen species (ROS) and reactive nitrogen species (RNS) and the reduction of antioxidant substances lead to neuronal cell death in neurodegenerative diseases (Farooqui and Farooqui, 2009; Melo et al., 2011).

Oxidative stress in the brain is usually found along with inflammatory responses such as activated immune cells, cytokines and other inflammatory mediators (Leszek et al., 2016). These inflammatory responses activate microglia, then microglial activation with gliosis results in an oxidative burst, which releases ROS, including superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), the highly reactive hydroxyl radical ($HO^{\cdot}$) and RNS such as nitric oxide (NO) into the environment (Tufekci et al., 2012). In addition, NO can react with O_2^-, producing peroxynitrite ($ONOO^-$), which is a powerful oxidant and may decompose to form $HO^{\cdot}$ (Melo et al., 2011).

Pathological α-syn is also reported to be involved in causing oxidative stress in a large number of reports (Esteves et al., 2009; Renella et al., 2014; Deas et al., 2016; Perfeito et al., 2017; Russo et al., 2019). Oxidative stress-induced toxicity depends on the structures of α-syn. Deas et al. (2016) found that although both oligomeric and fibrillar α-syn can induce free radicals, only oligomeric forms of a-syn cause neurotoxicity and endogenous glutathione reduction. They also found that oligomer-induced ROS depends on the presence of metal ions, because the addition of metal chelators can block oligomer-induced ROS and reduce neuronal death (Deas et al., 2016). Interestingly, α-syn also mediates oxidative stress caused by metal ions: down-regulation of the α-syn protein significantly increases cell viability and reduced oxidative stress in maltose-aluminated cells (Saberzadeh et al., 2016). Therefore, the oxidative stress induced by metal ions or α-syn, is probably the result of the two co-factors.

Glutamate Excitotoxicity

Glutamate is the main cognitive neurotransmitter in the brain, inducing an excitatory response when binding to its receptors (Dong et al., 2009). In order to maintain a high signal-to-noise ratio outside the synapse, the extracellular concentration of glutamate is required to be very low. The overspill of glutamate and excessive activation of glutamate receptors can lead to neuronal dysfunction and cell death, known as excitatory toxicity (Dong et al., 2009).

Pathological α-syn has been shown to affect all of the glutamate receptors: N-methyl-D-aspartic acid (NMDA), α-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA) and kainic acid (KA) receptors (Dong et al., 2009). Firstly, prolonged exposure to α-syn oligomers are reported to increase basal synaptic transmission through NMDA receptor activation, triggering enhanced contribution of calcium-permeable AMPA receptors (Diogenes et al., 2012). Secondly, nanomolar concentrations of large α-syn oligomers, formed by incubating α-syn with organic solvent and Fe (3+) ions, altered both pre- and post-synaptic mechanisms of AMPA-receptor-mediated synaptic transmission (Huls et al., 2011). Furthermore, Chang et al. (2012) found that melatonin attenuates KA-induced neurotoxicity through the reduction of KA-increased α-syn aggregation, which indicates that KA-induced neurotoxicity may be mediated by α-syn aggregation. Thus, pathological α-syn may lead to the activation of ionotropic receptors, contributing to glutamate excitotoxicity.

It has also been widely demonstrated that inflammation can induce glutamate excitatory toxicity. Firstly, monocyte-derived macrophages and activated microglia are able to induce glutamate excitotoxicity, which may result from their function of extruding glutamate into the extra synaptic space in exchange for cystine via the cystine/glutamate exchanger (Xc) – transporter (Kigerl et al., 2012). Secondly, the astrocyte function of clearing, buffering and containing glutamate abilities can be decreased by inflammatory factors, such as TNF-α, IL-1β, and IF-γ (Haroon and Miller, 2017; Haroon et al., 2018). In addition, immune activation can increase glutamate-like molecular, quinolinic acid, which (1) over excites the NMDA receptor, (2) inhibits glutamine synthetase, a critical enzyme in the glutamate-glutamine cycle in astrocytes, keeping the stable glutamate level and (3) promotes glutamate release (Guillemin, 2012).

T-Cell Driven Inflammation

The central nervous system was thought to be isolated from the adaptive immune system for a long period of time (Carson et al., 2006). However, Louveau et al. (2015) discovered the lymphatic system in the brain of mice, which were verified in monkeys and humans thereafter (Absinta et al., 2017). In addition, inflammatory factors from peripheral inflammation, such as IL-1β, TNF-α can also act on the blood–brain barrier to allow peripheral lymphocytes to enter the brain.

It is well established that, T cells, especially the CD4+ T-cells, are involved in PD. Brochard et al. (2009) found a significant increase of T-cells but not B-cells in post-mortem brains of PD patients. Later, they identified that lacking CD4+ cells, but not CD8+ cells, led to an attenuated response to MPTP-induced dopaminergic cell death in mice (Brochard et al., 2009). Additionally, Reynolds et al. (2010) identified that the subsets of CD4+ cells, T-helper-1 (Th1) and T-helper-17 (Th17), are the

main causers of MPTP-induced cell death. The mechanism by which gut inflammation causes PD through CD4+ T-cells can be seen. First of all, peptides derived from two regions of α-syn (the Tyr 39 and phosphorylated Ser129 region) can act as antigenic epitopes (Sulzer et al., 2017). Therefore, pathological α-syn can be captured at the lymph node, and be presented to CD4+ T-cells by antigen presenting cells (APCs) (Campos-Acuña et al., 2019). If naïve CD4+ cells are activated, they transform into their subtypes: Th1 and Th17. When the BBB is damaged, Th1 and Th17 infiltrate the brain, where the microglia act as local APCs, presenting α-syn antigen via MHC II, polarizing CD4+ T-cells to the Th1 and Th17 subtypes (Campos-Acuña et al., 2019). The Th1 and Th17 cells produce a large number of inflammatory factors, such as, TNF, IFN-γ, IL-1, IL-2, and IL-21 (Kaiko et al., 2008), which in turn re-stimulate glial cells (M1 microglia) to produce large amounts of glutamate, inflammatory factors, ROS and RNS (Gonzalez et al., 2015), thereafter recruiting leukocytes from the blood and exaggerating more inflammatory reactions (Mosley et al., 2012).

COX-2

Cyclooxygenase-2 (COX-2), also known as prostaglandin-endoperoxide synthase 2, is one of the three cyclooxygenases (COX-1, COX-2, and COX-3) (Teismann, 2012) that primarily induces the synthesis of prostaglandins from arachidonic acid (Kirkby et al., 2016). COX-2 is mainly detected in distal dendrites and dendritic spines, especially in excitatory neurons (Kaufmann et al., 1997). In general, COX-2 is not detected in dopaminergic structures such as the SN and striatum (Teismann et al., 2003). However, in PD patients and MPTP-induced mouse models, the immunopositive reaction of COX-2 in dopaminergic neurons is intense (Teismann et al., 2003). Therefore, there exists a high possibility that COX-2 may be related with dopaminergic neuron death.

The mechanisms through which COX-2 damages neurons may be through two mechanisms. (1) The first one is via oxidative stress. Arachidonic acid can be converted to PGH_2 in two steps. Firstly, arachidonic acid reacts with $2O_2$ to form the prostaglandin G_2 (PGG_2). Secondly, PGG_2 is converted by the cyclooxygenases to form prostaglandin H_2 (PGH_2) (Smith et al., 2000). The second step of COX-2 induction reacts far more rapidly than COX-1, however, the COX-1 reaction involves the reduction of two electrons of superoxide substrate, while about 40% of the COX-2 conversion just reduces one electron of superoxide substrate (Teismann, 2012). In this situation, the leaked electrons can react with oxygen to produce reactive oxygen species (Teismann, 2012). (2) Another possible cause of COX-2 induced neuron death is the crosstalk between cytokines and PGs, one of COX-2's metabolized products (Yao and Narumiya, 2019). Traditionally, PGs are mostly reported to have an inhibitory effect on acute inflammation (Narumiya and Furuyashiki, 2011). However, the expression of COX-2 are widely found in chronic inflammation, such as IBD (Wallace, 2001), rheumatoid arthritis (Mikos, 1976; Jasani and Bach, 1979; Svendsen et al., 1985; Fattahi and Mirshafiey, 2012; Kirkby et al., 2016), and multiple sclerosis (Mirshafiey and Jadidi-Niaragh, 2010). Based on this, studies found that PGs can crosstalk with cytokines and amplify the cytokines' effects. On the one hand, PGs induce expression of relevant cytokine receptors, which is typically observed in Th1 cell differentiation and Th17 cell expansion (Yao et al., 2009); on the other hand, PGs and cytokines synergistically activate NF-κB to induce expression of inflammation-related genes, including chemokines and COX-2 itself (Yao and Narumiya, 2019). These signals amplify chronic immune inflammation and exacerbate

TABLE 1 | Summary of intestinal function and inflammatory alterations in Parkinson's disease (PD) animal models of intranigral injection of 6-OHDA or LPS.

Model (species)	Functional evidence	Inflammatory evidence	References
Bilateral intranigral injection of 6-OHDA (rat)	Delayed gastric empty; Impaired gastric motility;		Zheng et al., 2011
	Delayed gastric empty; Impaired gastric motility;		Zheng et al., 2014
	Delayed gastric empty and intestinal transport; decreased fecal pellets and content		Feng et al., 2019
Unilateral intranigral injection of 6-ohda (rat)	Delayed gastric empty;		Toti and Travagli, 2014
	Delayed gastric empty; Constipation		Vegezzi et al., 2014
	Decreased weigh and water Content of fecal matter;		Zhu et al., 2012
	Impaired colonic transit;		Fornai et al., 2016
	Impaired colonic transit;	GFAP↑(colon); TNF-α↑(colon); IL-1β↑(colon); Eosinophils↑ (colon); Mast cells↑ (colon)	Pellegrini et al., 2017
	Colonic relaxation defect; Decreased intraluminal pressure; Decreased frequency of peristalsis		Colucci et al., 2012
		TNF↑ (colon); IL-1β↑ (colon); Eosinophils↑ (colon); Mast cells↑ (colon)	Pellegrini et al., 2016
Bilateral intranigral injection of LPS (rat)	Impaired gastric motility		Zheng et al., 2013

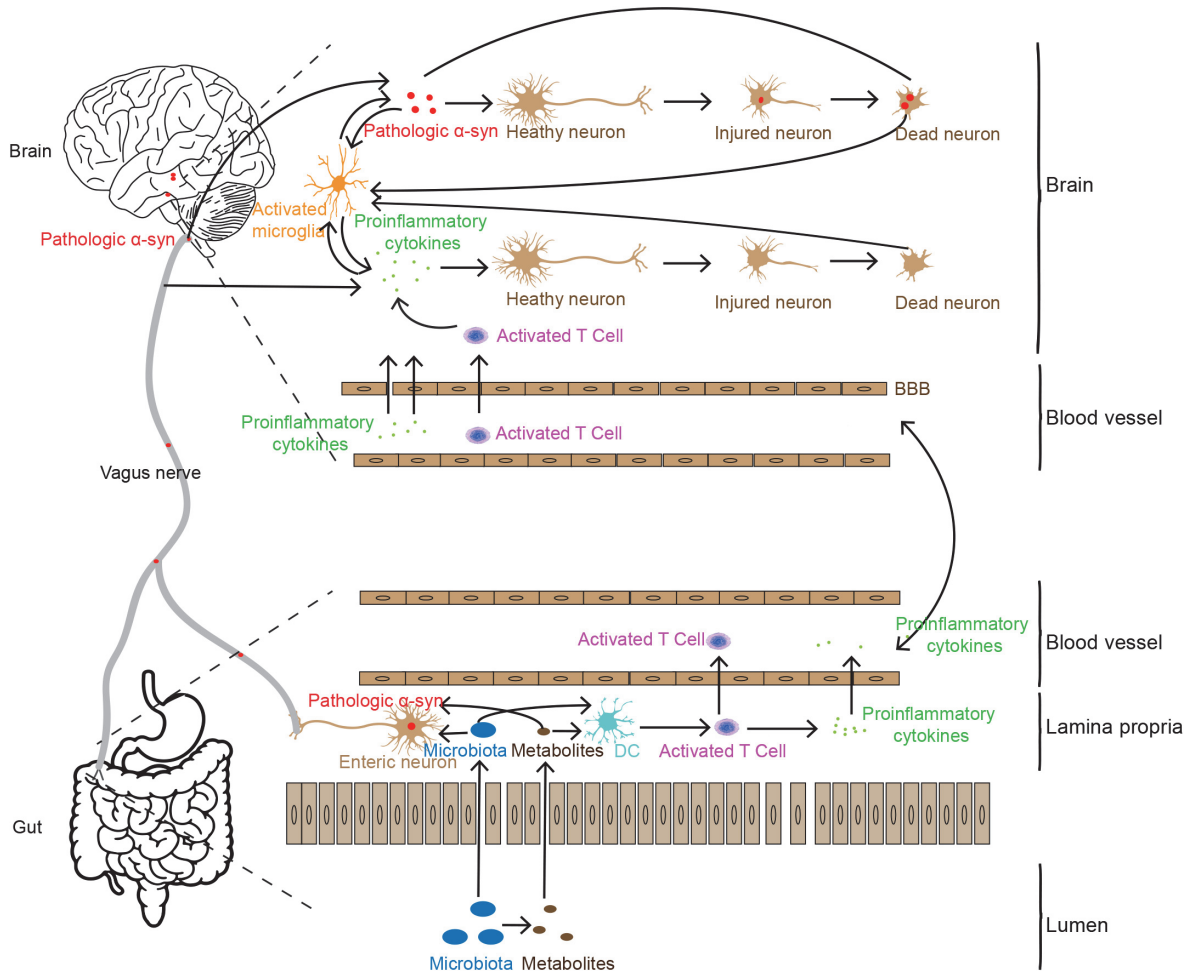

FIGURE 1 | Possible pathways involved in gut inflammation induced neuron death in the brain. Gut inflammation may increase intestinal permeability, allowing the leakage of bacteria and their metabolites which may trigger pathologic α-syn aggregation and pro-inflammatory cytokine production and release in the ENS. Pathologic α-syn is propagated to the brain via the vagal nerve, and inflammatory cytokines are transported to the brain through the humoral pathway or stimulate the vagal nerve to produce pro-inflammatory factors in the brain. Pro-inflammatory cytokines and synucleinopathies in the brain may induce neuronal injury and death, which in turn enhance more severe inflammatory responses.

neuronal death. In addition, the overexpression of COX-2 in dopaminergic neurons also plays a role in α-syn accumulation (Bartels and Leenders, 2010).

THE REACTION OF CENTRAL NEUROINFLAMMATION IN GUT

Among the various pre-clinic PD animal models, the intranigral injections of 6-OHDA or LPS directly act on the nigra-striatal system, thus, they could be used to elucidate the effect of nigral-striatal degeneration on the gut, comparing to the peripheral administration which may affect the gut first or at the same time. In the 6-OHDA induced PD model, gastrointestinal dysfunctions have widely been reported (see **Table 1**), such as delayed gastric empty (Zheng et al., 2011, 2014; Toti and Travagli, 2014; Vegezzi et al., 2014; Feng et al., 2019), impaired gastric motility (Zheng et al., 2011, 2014; Vegezzi et al., 2014; Feng et al., 2019), impaired colonic transit (Fornai et al., 2016; Pellegrini et al., 2017), decreased weight and water content of the feces (Zhu et al., 2012), colonic relaxation defect (Colucci et al., 2012), decreased intraluminal pressure (Colucci et al., 2012), decreased frequency of peristalsis (Colucci et al., 2012). In addition, in the studies of Pellegrini et al., inflammatory evidence is also presented in the model of intranigral injection of 6-OHDA, such as the increased GFAP, TNFα, IL-1β, eosinophils and mast cell in colon. In addition, in the study of intranigral injection of LPS, the impaired gastric motility is also observed. Further, in a study conducted by Ulusoy et al. (2017) the pathological α-syn was transported from the brain to the stomach. Therefore, the central neuroinflammation and nigrostriatal degeneration could, in turn, spread to the ENS and contribute to exacerbated intestinal inflammatory responses and gastrointestinal dysfunction via brain-to-the-gut descending pathways, thus generating a positive loop that

could drive the chronicization of the ongoing central and peripheral neuroinflammatory and neurodegenerative processes and contribute to gut motor dysfunctions.

CONCLUSION

Based on the analyses above, we summarize the potential association of intestinal inflammation in PD pathogenesis, as shown in **Figure 1**. Increased intestinal permeability caused by gut inflammation induces the leakage of flora and its metabolites into the body (Berg, 1995; Camilleri et al., 2012; Mu et al., 2017), stimulating the production of pathologic α-syn or pro-inflammatory cytokines (Chen et al., 2016; Kim et al., 2016; Fukui, 2017). Pathologic α-syn can be spread to the brain via the vagal nerve and pro-inflammatory cytokines and immune cells transmit to the brain through the humoral system. In addition, the leakage of flora and its metabolites from gut lumen can also activate immune cells, such as T cells. These immune cells can infiltrate into the brain via the disrupted BBB caused by the pro-inflammatory cytokines. In the brain, the two factors, pathologic α-syn and pro-inflammatory cytokines and immune cells enhance the dysfunction and degeneration of dopaminergic neurons. They, together with tissue debris or diseased proteins released from lysed cells, trigger the cascade and feedback loop of inflammation, including microglial activation, and neuronal dysfunction and cell death.

Of note, although a large body of evidence has implied the relationship between PD and gut inflammation, details on how the process takes place are still largely unknown. Further investigations are required to clarify the mechanisms of the mutual transformation between intestinal inflammation, microbiota and pathological α-syn or other PD-related pathogens.

Overall, we have briefly addressed the mechanisms on how gut inflammation is transmitted to the brain and how it causes damage in the brain. We hope that this will provide some clues for further studies in this interesting field of research.

AUTHOR'S NOTE

This material has not been published in whole or in part elsewhere. The manuscript is not currently being considered for publication in another journal. All authors have been personally and actively involved in substantive work leading to the manuscript, and will hold themselves jointly and individually responsible for its content.

AUTHOR CONTRIBUTIONS

Q-QC and J-YL conceived of the review and drafted the manuscript. CH and WL edited the manuscript. All authors approved the manuscript.

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Aβ Seeding as a Tool to Study Cerebral Amyloidosis and Associated Pathology

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Misfolded proteins can form aggregates and induce a self-perpetuating process leading to the amplification and spreading of pathological protein assemblies. These misfolded protein assemblies act as seeds of aggregation. In an *in vivo* exogenous seeding model, both the features of seeds and the position at which seeding originates are precisely defined. Ample evidence from studies on intracerebal injection of amyloid-beta (Aβ)-rich brain extracts suggests that Aβ aggregation can be initiated by prion-like seeding. In this mini-review article, we will summarize the past and current literature on Aβ seeding in mouse models of AD and discuss its implementation as a tool to study cerebral amyloidosis and associated pathology.

Keywords: Alzheimer's disease, cerebral amyloidosis, amyloid plaques, Aβ seeding, cross-seeding

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HISTORY OF SEEDING: FROM *IN VITRO* TO *IN VIVO*

Protein aggregation is a common feature of many neurodegenerative diseases that is assumed to play a central role in the pathogenesis. The aggregation of Aβ has been described as a nucleation-dependent polymerization process, including an initial slow nucleation phase, also called lag-phase, followed by a rapid growth phase (Jarrett and Lansbury, 1993; Jarrett et al., 1993; Harper and Lansbury, 1997; Walsh et al., 1997). The nucleus or seed formation in the nucleation phase is the rate-limiting step and thermodynamically unfavorable (Jarrett and Lansbury, 1993). The addition of stable seeds, e.g., aggregates generated by fragmentation of fibrils (Jarrett and Lansbury, 1993; Falsig et al., 2008; Knowles et al., 2009; Xue et al., 2009), accelerate the polymerization process and shorten significantly the lag-phase in a process termed "seeding" (Jarrett and Lansbury, 1992, 1993; Harper and Lansbury, 1997). These preformed seeds serve as a template for polymerization of respective aggregates (Jarrett and Lansbury, 1992) and can either have the same nature as the nuclei leading to a homologous seeding or be made from a different protein inducing heterologous seeding (Morales et al., 2009, 2013). Several *in vitro* and *in vivo* aggregation studies have provided the essential proof for a seeded-nucleation model of Aβ (Jarrett et al., 1993; Lomakin et al., 1996, 1997; Harper and Lansbury, 1997; Kane et al., 2000; Walker et al., 2002; Petkova et al., 2005; Meyer-Luehmann et al., 2006; Knowles et al., 2009; Paravastu et al., 2009; Eisele et al., 2010; Cohen et al., 2013).

The nucleation-dependent polymerization process gives rise to several Aβ assemblies such as oligomers, protofibrils and fibrils (Glenner and Wong, 1984; Harper et al., 1997; Lambert et al., 1998). Soluble Aβ oligomers were proposed to be the most toxic species of Aβ (Walsh et al., 2002; Wang et al., 2002; Cleary et al., 2005; Lesné et al., 2006; Townsend et al., 2006; Shankar et al., 2008), as it was already demonstrated for small assemblies of the prion protein (PrP; Silveira et al., 2005). Intracerebroventricular injections of media containing naturally secreted Aβ oligomers into rats led to inhibition of hippocampal long-term potentiation (LTP) and disrupted cognitive function (Walsh et al., 2002; Cleary et al., 2005). Moreover, soluble Aβ oligomers such as dimers isolated from human AD brains and Aβ*56 isolated from APP-transgenic (APP-tg) mouse brain were shown to impair synaptic plasticity and memory when administered to rodents and rat hippocampal slices (Lesné et al., 2006; Shankar et al., 2008). Hyperaggregation of soluble Aβ into higher aggregates was shown to be protective against Aβ mediated toxicity (Cohen et al., 2009). Despite the fact that soluble oligomers represent the most toxic species of Aβ, the presence of insoluble Aβ amyloid plaques leads to disruption of neocortical synaptic transmission, neuronal deformation and neuronal dysfunction (Stern et al., 2004; Tsai et al., 2004; Meyer-Luehmann et al., 2008, 2009).

The intracerebral injections of PrP-containing human brain homogenates into animals have led to the discovery of the transmissible prion disease approach (Gajdusek et al., 1966; Gibbs et al., 1968; Gajdusek, 1977; Hadlow et al., 1980; Prusiner, 1982). Since PrP and the Aβ peptide share similar biological and molecular features regarding the pathogenic self-assembly, misfolding and spreading within the brain, the question whether this concept could also be applied to other neurodegenerative diseases such as Alzheimer's disease with Aβ peptide as potential trigger of the disease has been under discussion for quite some time (Prusiner, 1984; Rasmussen et al., 2017a).

Initial *in vivo* Aβ seeding experiments were performed by intracerebral inoculation of brain extract from AD patients in non-human primates that yielded inconsistent results (Goudsmit et al., 1980; Manuelidis and Manuelidis, 1991). Later attempts to seed Aβ pathology were successful in wild-type marmosets (Baker et al., 1993, 1994; Ridley et al., 2006), a New World monkey that express human-type sequence of Aβ (Heuer et al., 2012). Intracerebral infusion of brain tissue material from an AD patient resulted in Aβ deposits that could not be observed in control animals (Baker et al., 1993, 1994; Ridley et al., 2006). Interestingly, the distribution pattern of exogenously induced plaques was similar to those of elderly uninjected controls that developed cerebral amyloidosis (Maclean et al., 2000). However, the use of marmosets as a model for AD was considered impractical (Baker et al., 1993, 1994; Maclean et al., 2000; Ridley et al., 2006).

It is worth highlighting that compared to other mouse models for α-synuclein and tau, it is not possible to induce cerebral amyloid deposition in non-transgenic mice within its normal life span (Meyer-Luehmann et al., 2006; Luk et al., 2012; Guo et al., 2016), due to three amino acid difference between the mouse- and human-derived Aβ sequence (Otvos et al., 1993).

The strongest piece of evidence for "prion-like" seeding of misfolded Aβ aggregates *in vivo* was documented in experiments carrying out the inoculation of diluted brain extracts derived from confirmed AD patients into young, pre-depositing APP-tg mice (Kane et al., 2000; Walker et al., 2002; Meyer-Luehmann et al., 2006). APP-tg mice inoculated with AD brain extracts displayed remarkable Aβ deposition as well as mice injected with brain homogenate from control patients due to similar Aβ production levels (Kane et al., 2000; Walker et al., 2002; Meyer-Luehmann et al., 2006; Szaruga et al., 2015), while attempts to seed Aβ pathology *in vivo* using cerebrospinal fluid (CSF) from AD patients failed, although Aβ concentrations were significantly higher than the ones present typically in brain homogenates (Fritschi et al., 2014b). Whether some cofactors or a specific conformation of Aβ is missing in CSF compared to brain homogenates is currently not clear (Fritschi et al., 2014b). Further studies confirmed that APP-tg mouse brain extracts were equally efficient to seed Aβ aggregation in APP-tg mice as the ones prepared from human AD brains (Meyer-Luehmann et al., 2006; Eisele et al., 2009; Watts et al., 2011; Morales et al., 2012; Rosen et al., 2012). Most of those Aβ seeding studies are carried out in tg mice overexpressing mutant human APP, although Aβ deposits can also be induced *de novo* in rodents after comparable longer incubation periods that would never exhibit Aβ plaque pathology spontaneously within their normal lifespan (Morales et al., 2012; Rosen et al., 2012). This is a strong indication that exogenously applied seeds act as a template for misfolding of endogenous Aβ and that seeding is not solely promoting the premature deposition of amyloid. Importantly, the overexpression of APP seems not essential for the prion-like propagation of seeded Aβ aggregates within the brain (Ruiz-Riquelme et al., 2018).

NATURE OF Aβ SEEDS AND SEED-INDUCED Aβ DEPOSITS

The injection of autopsy-derived, brain extracts from young control individuals into APP-tg mice with no seed-induced Aβ deposits suggested that the presence of Aβ in the brain extract is crucial for *in vivo* seeding (Kane et al., 2000; Walker et al., 2002). To substantiate this hypothesis, depletion of Aβ and Aβ aggregates from the brain extracts prevented induction of Aβ deposits in host mice as well as formic acid treatment of the inoculum (Meyer-Luehmann et al., 2006; Duran-Aniotz et al., 2014). On the other hand, synthetic, multimeric Aβ fibrils have also been used to determine the essential seeding factor. Original experiments using synthetic Aβ in different composition displayed only poor seeding capacity (Meyer-Luehmann et al., 2006), while higher amounts of synthetic Aβ fibrils were indeed seed-competent but not as efficient as Aβ-containing human or mouse brain homogenates (Stöhr et al., 2012, 2014). Using hippocampal slice culture model, Novotny et al. revealed that synthetic Aβ can be converted into seeds able to induce β-amyloidosis *in vivo* (Novotny et al., 2016). Whether a particular conformation of the Aβ or additional factors is needed to induce seeding is not yet clear. To assess the stability of Aβ seeds *in vivo*, APP-null mice have been

used as recipient mice that fail to induce Aβ pathology because of absent Aβ production. Six-months post-incubation, brain homogenates of these mice successfully induced Aβ deposition in APP-tg hosts, suggesting that Aβ seeds are highly robust and are able to retain their seeding activity for months (Ye et al., 2015a). Furthermore, Aβ-rich extracts prepared from human AD and APP-tg mice brains after formaldehyde treatment for 1–2 years were still able to seed Aβ deposits in APP-tg mice (Fritschi et al., 2014a). Nonetheless, the question still remains which Aβ species in brain homogenates is essential for the seeding activity in APP-tg mice and what cofactors are required. Indeed, another study revealed that Aβ seeds do not consist of only one type of Aβ aggregate, but are rather a mixture of small soluble or insoluble (in 100,000× *g* ultracentrifuged supernatant or pellet fraction) and proteinase-K (PK)-sensitive or PK-resistant Aβ species (Langer et al., 2011). Interestingly, sonication and thus fragmentation of the insoluble fraction into smaller and more soluble Aβ seeds, enhanced the seeding activity of the inoculum, consistent with results from fragmentation studies (Jarrett and Lansbury, 1993; Falsig et al., 2008; Knowles et al., 2009; Xue et al., 2009). In line with the previous mentioned studies, Aβ oligomers seem to be important for the initiation of Aβ aggregation especially for the early phase of the seeding process (Katzmarski et al., 2019). According to another study, the Aβ seeding potency of the brain extracts is highest at the very early stage of cerebral amyloidosis (Ye et al., 2017).

As demonstrated before, Aβ can aggregate into polymorphic shapes (Fändrich et al., 2009; Levine and Walker, 2010; Eisenberg and Jucker, 2012). Aβ isolated from AD patients were shown to trigger synthetic Aβ to adopt corresponding structural "strains" (Lu et al., 2013; Qiang et al., 2017). Also molecular differences in Aβ between non-demented and AD cases were pointed out (Piccini et al., 2005; Portelius et al., 2015), supporting the hypothesis of defined Aβ "strains" that probably correspond to the development of AD pathology. "Strain-like" variations of Aβ were observed in *in vitro* (Petkova et al., 2005; Nilsson et al., 2007; Paravastu et al., 2008; Meinhardt et al., 2009; Spirig et al., 2014) as well as *in vivo* studies using AD mouse models (Meyer-Luehmann et al., 2006; Heilbronner et al., 2013; Stöhr et al., 2014; Watts et al., 2014; Condello et al., 2018). Moreover, these "strain-like" variations in the molecular structure of Aβ aggregates were shown to be transmissible between APP-tg donor and recipient (Meyer-Luehmann et al., 2006; Heilbronner et al., 2013). Interestingly, the morphology and the biochemical composition of the seed-induced Aβ deposits represented histopathological features of the plaques present in donor and host mice. The respective brain region and thus the local environment seem to play a crucial role with regard to the morphology of the exogenously induced Aβ plaques (Eisele et al., 2009; Ye et al., 2015b). While infusion of brain homogenate into the hippocampus yielded compact and diffuse plaques, injections into striatum led to the formation of merely diffuse plaques (Eisele et al., 2009). Finally, "strain-like" features of Aβ aggregates present in different human familial AD cases could be partially recapitulated in mouse models by exogenous seeding (Watts et al., 2014; Rasmussen et al., 2017b; Condello et al., 2018) that were even maintained after multiple passages (Watts et al., 2014).

SPREADING

The induction of Aβ plaque pathology as consequence of exogenous seeding appears initially in the proximity to the injection site, where the highest concentration of seeds due to administration has been assumed (Kane et al., 2000; Walker et al., 2002; Meyer-Luehmann et al., 2006; Eisele et al., 2009; Hamaguchi et al., 2012; Ye et al., 2015b). Within the first day, injected material is still measurable before the Aβ signal usually becomes immunohistochemically undetectable for several months (Ye et al., 2015b). Importantly, seed-induced Aβ deposits present after several months of incubation were evidently not the injectate itself (Kane et al., 2000; Meyer-Luehmann et al., 2006; Eisele et al., 2009). Moreover, the diffusion of the injected Aβ material away from the injection site within a 7 day time-frame revealed that the initial Aβ distribution pattern in the affected regions resembled the pattern of seeded aggregation appearing 5 months after injection (Walker et al., 2002). Although seed-induced Aβ deposition close to the injection site has been shown to increase over time, it expands also to more distant and axonally interconnected brain regions (Kane et al., 2000; Walker et al., 2002; Meyer-Luehmann et al., 2006; Hamaguchi et al., 2012; Rönnbäck et al., 2012; Ye et al., 2015b), suggesting that neuronal pathways are essential for the trafficking of Aβ seeds through the brain. It was further postulated that Aβ was traveling non-randomly and depositing mostly along structures corresponding to the limbic connectome (Ye et al., 2015b). So far, there is no evidence for active transport of Aβ along neurons *in vivo*, even though it is thought to be a plausible mechanism for the spreading of Aβ pathology based on results from *in vitro* studies (Nath et al., 2012; Domert et al., 2014; Song et al., 2014; Brahic et al., 2016). Furthermore, the occurrence of intraneuronal Aβ in mice, as well as human brains, supports the hypothesis of neuronal involvement in Aβ pathology dissemination (Gouras et al., 2000; LaFerla et al., 2007). Most likely the mechanism responsible for the distribution of seeded Aβ deposits is a combination of both, passive diffusion and active transport mechanisms (Eisele and Duyckaerts, 2016). The exact cellular mechanisms involved in spreading of Aβ have not yet been elucidated. Recent studies suggested the involvement of endosomes/lysosomes or intracellular assemblies of Aβ to be crucial factors (Hu et al., 2009; Marzesco et al., 2016).

Since the transmission of Aβ seeds resembles a prion-like mechanism, the systemic routes relevant for transmission of prion diseases from periphery to CNS were also taken into account (Blättler et al., 1997; Mabbott and MacPherson, 2006; Aguzzi et al., 2008). Induction of Aβ pathology by means of oral, intraocular or intranasal routes was excluded due to absent Aβ seeding in inoculated APP-tg mice (Eisele et al., 2009). However, intraperitoneal (Eisele et al., 2010, 2014) or intravenous (Burwinkel et al., 2018) administration of Aβ-rich brain extracts induced intracerebral β-amyloidosis especially in

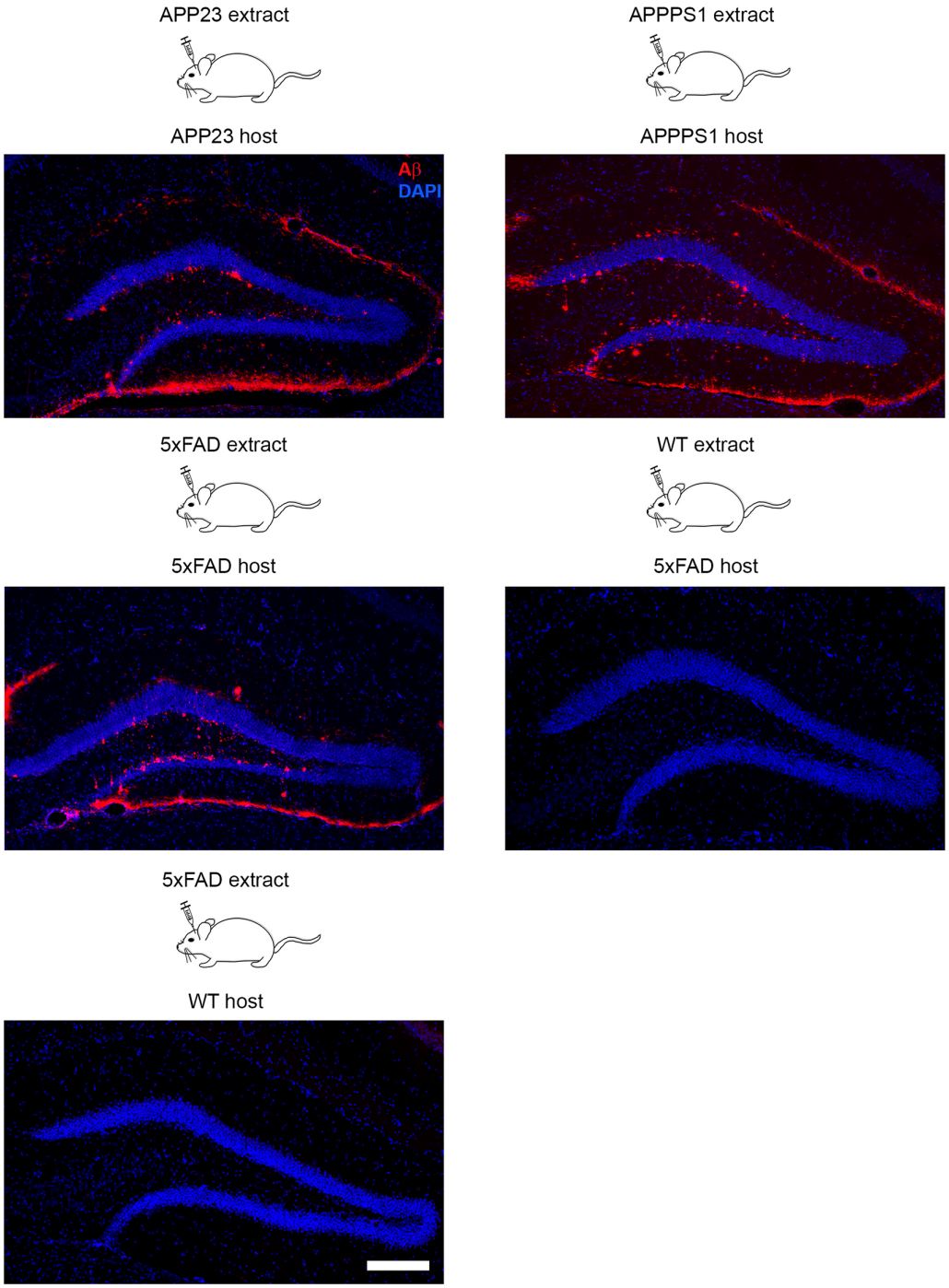

FIGURE 1 | Intracerebral inoculation of brain extracts from APP23, APPPS1 and 5xFAD mice into the hippocampus of young APP-transgenic (APP-tg) host mice with genetically identical background induced seeding of Aβ pathology. In contrast, infusion of brain homogenate from a wild-type mouse into a 5xFAD tg host and vice versa failed to induce cerebral β-amyloidosis due to the lack of Aβ either in the brain extract or host animal. Fluorescence microscopy of Aβ (6E10, red) and cells (DAPI, blue). Scale bar represents 100 μm.

the walls of the blood vessels in form of cerebral amyloid angiopathy (CAA). In contrast to CAA, Aβ aggregates become manifest in amyloid plaques in the brain parenchyma (Thal et al., 2015). CAA is an age-related vessel disorder that can occur in the brain of AD patients as well as non-demented elderly people and is associated with vascular dementia (Thal et al., 2012, 2015). The occurrence of CAA after administration of Aβ-rich extracts in spatially different brain regions indicates as

well a role for the vascular system (Meyer-Luehmann et al., 2006; Eisele et al., 2009) or perivascular drainage channels (Weller et al., 1998; Thal et al., 2007) as possible propagation route for Aβ seeds. Whether parenchymal and vascular seed-induced Aβ deposits are generated by the same or *via* different pathways is still unknown. Nevertheless, these results provided sufficient evidence for the spread of Aβ seeds possibly *via* vascular routes. Transport of Aβ from the periphery to the brain by immune cells such as macrophages was also proposed (Eisele et al., 2014; Cintron et al., 2015), but the precise mechanisms remained unclear.

CROSS-SEEDING

Several studies have proven the overlap of different neuropathological lesions such as neurofibrillary tangles (NFTs; tau), Lewy bodies (α-synuclein) or prions with Aβ pathology in brains of patients with neurodegenerative diseases like AD, PD, Dementia with Lewy Bodies (DLB) and Creutzfeldt-Jakob disease (CJD) (McKeith et al., 1996; Braak and Braak, 1997; Hamilton, 2000; McKeith, 2000, 2006; Ferrer et al., 2001; Tsuchiya et al., 2004; Debatin et al., 2008; Hyman et al., 2012; Jaunmuktane et al., 2015). *In vitro* and *in vivo* studies have demonstrated that tau, α-synuclein and prion proteins can interact with Aβ and thus influence the onset and course of the respective disease (Hamilton, 2000; McKeith, 2000; Götz et al., 2001; Lewis et al., 2001; Masliah et al., 2001; Tsigelny et al., 2008; Lasagna-Reeves et al., 2010; Morales et al., 2010).

Since several years, there has been a debate on the interaction between Aβ peptide and tau protein and its influence on the pathogenesis of AD. Both pathologies, amyloid plaques and NFTs containing hyperphosphorylated tau, are necessary for the accurate diagnosis of AD (Hyman et al., 2012). Tau pathology was also shown to be directly inducible *in vivo* in wild-type and tau tg mice after injections of tau-containing brain extracts or synthetic tau fibrils (Clavaguera et al., 2009, 2013; Lasagna-Reeves et al., 2012; Iba et al., 2013; Guo et al., 2016). According to the amyloid cascade hypothesis, amyloid deposition precedes NFT formation and posits that changes in amyloid-β lead to widespread tau pathology (Hardy and Selkoe, 2002). Early results from *in vitro* studies gave already a hint on the potency of Aβ to induce phosphorylation and aggregation of tau (Busciglio et al., 1995; Ferrari et al., 2003; Pennanen and Götz, 2005; Lasagna-Reeves et al., 2010). Infusion of synthetic Aβ fibrils, pre-aggregated Aβ or Aβ-rich extracts into the hippocampus of tau-transgenic (tau-tg) mice indeed resulted in enhanced NFT formation (Götz et al., 2001; Bolmont et al., 2007; Vasconcelos et al., 2016), similar to the effects seen in double-transgenic mice exhibiting both Aβ and tau pathology (Lewis et al., 2001; Pooler et al., 2015). Injections of human derived-tau in mice with a high Aβ plaque load developed enhanced induced tau pathology, suggesting that Aβ plaques might trigger the propagation of tau (He et al., 2018).

Furthermore, in about 50% of AD patients the presence of α-synuclein aggregates has been verified (Hamilton, 2000; Uchikado et al., 2006). Previous studies have implicated a direct interaction of Aβ and α-synuclein (Tsigelny et al., 2008). It has also been demonstrated that Aβ can trigger α-synuclein polymerization *in vitro* and overexpression of human APP/Aβ fostered the accumulation of α-synuclein and associated disease phenotype *in vivo* (Paik et al., 1998; Masliah et al., 2001). Besides, α-synuclein has been identified as a major protein accumulating in neurites in APP-tg mice, implying that Aβ might be causal for this aggregation (Yang et al., 2000). Surprisingly, APP-tg mice intracerebrally injected with α-synuclein-derived extract were devoid of seeded Aβ deposition, suggesting that α-synuclein is not able to cross-seed Aβ plaques *in vivo* (Bachhuber et al., 2015). Instead, the presence of α-synuclein even hampered amyloid plaque formation in APP-tg mice (Bachhuber et al., 2015). Moreover, brains of APP-tg mice lacking α-synuclein exhibited a significant increase in Aβ plaque load, suggesting as well a suppressive role of α-synuclein on the progression of Aβ plaque pathology (Kallhoff et al., 2007).

Finally, a role for prion protein to interact with Aβ aggregates has also been proposed. Intraperitoneal injection of prion proteins into APP-tg mice seemed to promote mature Aβ plaque formation and prion pathology was enhanced in presence of Aβ (Schwarze-Eicker et al., 2005; Morales et al., 2010). However, no cross-seeding was observed after intracerebral inoculation of infectious prions into the hippocampus of APP-tg mice (Rasmussen et al., 2018).

CONCLUSION

The seeding model of AD pathology in mice is a widely used tool to study plaque formation *in vivo* at its very early stage and within a defined time period (Kane et al., 2000; Meyer-Luehmann et al., 2006). In general it is a very robust model that was applied in many different APP-tg mouse models (Tg2576, APP/PS1, APP23, 5xFAD) with only slight variation with regard to the onset of seeding (first sign of seed-induced Aβ deposits after intracerebral inoculations) and affected brain areas (Kane et al., 2000; Meyer-Luehmann et al., 2006; Duran-Aniotz et al., 2014; Ziegler-Waldkirch et al., 2018) (**Figure 1**). Although seeding experiments were performed mainly in the hippocampus, the induction of Aβ deposits was additionally demonstrated in several different brain areas such as parietal cortex, striatum or olfactory bulb (Eisele et al., 2009). The greatest advantage of this model is that the accelerated Aβ plaque formation reduces the incubation time (Meyer-Luehmann et al., 2006). Furthermore, the age of the newborn plaques is easily determinable due to the predictability of the model. Characterization of seed-induced Aβ deposition and its effect on the environment at the injection side as well as areas affected by spreading of Aβ seeds can be studied as well. Recently, the consequence of Aβ seeding on adult neurogenesis and cell death was demonstrated (Ziegler-Waldkirch et al., 2018), supporting the notion that seed-induced Aβ deposits may also be a source of toxicity. Future experiments will need to test the consequences of Aβ seeding on other cell types and unravel in detail the origin of Aβ-mediated toxicity. Finally, *in vivo* seeding as a model can assist to identify factors impacting or accelerating AD progression such as microglia-derived ASC specks (Venegas et al., 2017) or the Trem2 receptor

on microglia (Parhizkar et al., 2019) that might in turn contribute to the development of appropriate AD treatment.

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frontiers in Molecular Neuroscience

MINI REVIEW
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Voltage-Gated Calcium Channels and α-Synuclein: Implications in Parkinson's Disease

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Alpha-synuclein (α-syn) is biochemically and genetically linked to Parkinson's disease (PD) and other synucleinopathies. It is now widely accepted that α-syn can be released in the extracellular space, even though the mechanism of its release is still unclear. In addition, pathology-related aggregated species of α-syn have been shown to propagate between neurons in synaptically connected areas of the brain thereby assisting the spreading of pathology in healthy neighboring neuronal cells. In neurons, calcium channels are key signaling elements that modulate the release of bioactive molecules (hormones, proteins, and neurotransmitters) through calcium sensing. Such calcium sensing activity is determined by the distinct biophysical and pharmacological properties and the ability of calcium channels to interact with other modulatory proteins. Although the function of extracellular α-syn is currently unknown, previous work suggested the presence of a calcium-dependent mechanism for α-syn secretion both *in vitro*, in neuronal cells in culture, and also *in vivo*, in the context of a *trans*-neuronal network in brain. Mechanisms regulating extracellular α-syn levels may be of particular importance as they could represent novel therapeutic targets. We discuss here how calcium channel activity may contribute to α-syn aggregation and secretion as a pathway to disease progression in synucleinopathies.

Keywords: alpha-synuclein, Parkinson's and related diseases, protein aggregation, secretion, calcium, voltage gated Ca^{2+} channel, neurodegeneration

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INTRODUCTION

The affected neurons in Parkinson's disease (PD) brains contain dense filamentous inclusions called Lewy bodies that primarily consist of the presynaptic protein α-synuclein (α-syn), a small neuronal protein that is abundant throughout the central nervous system under normal conditions (Goedert et al., 2017). The causal role of α-syn, a protein physiologically present in

Abbreviations: α-syn, alpha-synuclein; 6-OHDA, 6-hydroxydopamine; CaM, calmodulin; DLB, dementia with Lewy bodies; DMV, dorsal motor nucleus of the vagus; ER, endoplasmic reticulum; HVA, high-voltage activated; iPSC, induced pluripotent stem cell; ISF, interstitial fluid; LVA, low-voltage activated; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; NAC domain, non-amyloid component domain; PD, Parkinson's disease; SERCA, sarco/endoplasmic reticulum Ca^{2+}-ATPase; SNpc, substantia nigra pars compacta; STN, subthalamic nuclei; VGCCs, voltage-gated calcium channels; VTA, ventral tegmental area.

all neurons, in the pathogenesis of PD is further highlighted by human genetic studies that have linked multiplications and coding-region point mutations of the *SNCA* gene encoding for α-syn with familial PD (Houlden and Singleton, 2012). Increased levels of aggregated α-syn species have been strongly correlated with neuronal cell death partly due to a potent impairment of the degradation capacity of cellular proteolytic systems. Apart from acting in the cytoplasm, α-syn is normally secreted in the interstitial fluid (ISF) of the brain and can be taken up by neuronal cells (reviewed in Stefanis et al., 2019). High burden of extracellular α-syn has been shown to promote the production and transfer of pathology-linked, possibly hyper-aggregated, α-syn material. Under pathological conditions, such as PD, these pathologically relevant species of α-syn have the ability to propagate along interconnected neuronal networks leading to a progressive degeneration of neuronal function and ultimately cell death (Bieri et al., 2018).

It is now established that α-syn binds membranes with high affinity and this binding determines its functions especially at the synaptic sites. In particular, α-syn has been implicated in the process of neurotransmitter release where a number of potential roles of the protein in the maturation of synaptic vesicles, vesicle docking, priming and fusion have been proposed. The suggested mechanisms involve in part a direct or indirect interaction with the SNARE complex, but whether α-syn promotes or disrupts the formation of SNARE complex is still under debate (reviewed in Huang et al., 2019). How aggregated α-syn assemblies interfere with neurotransmitter release thereby affecting the functionality of neuronal synapses also remains elusive. In PD brains, deposits of small oligomers/aggregates of α-syn can be detected in the presynaptic sites prior to the formation of Lewy bodies and synaptic deficits seem to precede cell death in the course of the disease (Calo et al., 2016). The presence of abnormal α-syn assemblies extracellularly could modify neurotransmission and drastically affect the network dynamics of interconnected microcircuits and enhance the progression of disease pathology by facilitating the cell-to-cell transfer of pathological synuclein species.

Aggregation in the extracellular space may be favored by high local accumulation of α-syn. As such, the regulation of extracellular α-syn levels could be used therapeutically providing we understand the mechanism(s) of α-syn secretion and how this is triggered. Previous work indicated the presence of a Ca^{2+}-dependent mechanism for α-syn secretion both *in vitro*, in neuronal cells in culture, and also *in vivo*, in the context of a *trans*-neuronal network in mouse brain, where α-syn release is mediated by presynaptic Ca^{2+} channels (Emmanouilidou et al., 2010, 2016). It is possible that abnormal function of the specific Ca^{2+} channel(s) regulating α-syn secretion could alter the levels of α-syn released to the extracellular space in a manner that favors the local aggregation of the protein at least in certain extra-synaptic sites. The aggregated assemblies may then be internalized by neighboring neurons acting as seeds to recruit the intracellular α-syn and facilitate the templating and subsequent release of pathological α-syn species by a cell-to-cell propagation mechanism.

The main presynaptic Ca^{2+} channels are the voltage-gated calcium channels (VGCCs) and the ligand-gated ion channels. The VGCCs consist of ten calcium channel isoforms, nine of which are widely expressed in the nervous system. VGCCs are responsible for calcium influx thereby controlling neuronal calcium homeostasis. As summarized in **Table 1**, dysregulation of VGCCs expression or activity has been linked with many neurological disorders such as Alzheimer's disease, Parkinson's disease, Multiple Sclerosis, iron, and zing neurotoxicity (Cataldi, 2013). In this review we focus on the role of VGCCs in PD and especially in neuronal degeneration that is apparent in PD. Up to date, there is no direct evidence that presynaptic calcium channels regulate α-syn propagation. In the paragraphs that follow, we try to analyze the role of Ca^{2+} signaling in α-syn aggregation and secretion, as they depict the major precursor mechanisms for α-syn propagation.

CALCIUM AND α-SYNUCLEIN

Calcium homeostasis is important for the maintenance of neuronal integrity, since it is involved in synaptic transmission, neuronal plasticity, and cell survival. Activation of calcium signaling cascades is a result of intracellular calcium elevation, either via calcium influx or via calcium release from intracellular stores, such as the endoplasmic reticulum (ER) (reviewed in Yang et al., 2019). Among the different events that trigger α-syn pathology, the disruption of calcium homeostasis stands out as a possible mediator of aggregation and abnormal secretion of α-syn.

α-syn aggregation can be enhanced by alterations in intracellular calcium concentration in a direct or indirect manner. It has been shown that a transient increase of intracellular calcium leads to an increase in α-syn aggregates in human cell lines expressing α-syn (Nath et al., 2011; Follett et al., 2013). The mechanism through which calcium promotes α-syn aggregation is not clear. It has been shown that subsequent to calcium binding, the calcium-binding protein, calmodulin (CaM), changes its conformation and binds to α-syn inducing α-syn fibrillization (Lee et al., 2002; Martinez et al., 2003). CaM along with calbindin are calcium-binding proteins that are used by the cell as buffering proteins to maintain intracellular calcium homeostasis. The interplay between the presence of calbindin and the progression of PD was initially proposed after the discovery of a subgroup of calbindin-positive dopaminergic neurons that exhibited less pathologic features compared to calbindin-negative dopaminergic neurons in rat substantia nigra pars compacta (SNpc) and postmortem human brain material from PD patients (Gerfen et al., 1985; Yamada et al., 1990). Furthermore, a study in human brain tissues from dementia with Lewy bodies (DLB) patients showed that the Lewy bodies were exclusively formed in calbindin-negative neurons, a result that was confirmed using the mouse rotenone model, where α-syn aggregation was evident primarily in calbindin-negative neurons (Rcom-H'cheo-Gauthier et al., 2016). In the same line of evidence, striatal administration of a calbindin-expressing adenoviral vector in macaque

TABLE 1 | Voltage-gated calcium channel blockers and their effect on PD pathophysiology.

VGCCs	Blocker	Experimental model	Effect	References
N-type P/Q-type	ω-conotoxin ω-agatoxin	Sprague–Dawley rats	Decreased the dopamine release from striatal terminals	Bergquist et al., 1998
R-type	SNX-482	Sprague–Dawley rats	Decreased the somatodendritic DA release in SN	Bergquist and Nissbrandt, 2003
T-type	Ni^{2+} mifebradil	STN slices + Wistar rats	Reduced the burst activity in STN neurons and improved the locomotor deficits in 6-OHDA lesioned rats	Tai et al., 2011
	ML218	iPSCs derived from dopaminergic neurons of PARK-2 patients	Ameliorated the effect of rotenone treatment by rescuing the neuronal apoptotic phenotype	Tabata et al., 2018
N-type	ω-conotoxin	Rat primary cortical neurons	Diminished the elevation of intracellular calcium and dopamine release that was triggered after extracellular α-synuclein application	Ronzitti et al., 2014
L-type N-type	Nifedipine ω-conotoxin	SH-SY5Y cells	Diminished the elevation of intracellular calcium that was observed when extracellular α-synuclein was applied to the cells	Melachroinou et al., 2013
	Isradipine	Adult brain slices + C57BL/6 mice	Reversed the rotenone and MPTP-induced TH-loss and motor deficits	Chan et al., 2007
	Nimodipine	Primary dopaminergic neurons	Reduced the increased levels of cytosolic dopamine that are observed after L-DOPA administration	Mosharov et al., 2009
	Isradipine	C57B1/6 mito-roGFP transgenic mice	Reduced the mitochondrial oxidant stress in SNc dopaminergic neurons	Guzman et al., 2010
	Isradipine	C57BL/6 mice	Increased the survival of SNc dopaminergic cells after 6-OHDA-induced degeneration	Ilijic et al., 2011
L-type	Isradipine Nimodipine	Primary dopaminergic neurons	Prevented the MPP^{+}-induced intracellular calcium elevation in SN but not in VTA.	Lieberman et al., 2017
	Isradipine	6-OHDA-treated mice	Failed to achieve neuroprotection of SNc neurons, due to low selectivity for Cav1.3 VGCCs	Ortner et al., 2017
	Isradipine	TH-mito-roGFP transgenic mice	Decreased mitochondrial oxidant stress was achieved by reducing Ca^{2+} oscillations in SNc	Guzman et al., 2018
	Isradipine	Ventral mesencephalic primary neurons	Reversed the clustering of a-synuclein positive vesicles and α-synuclein aggregation that is observed after dopamine administration	Lautenschläger et al., 2018

monkeys protected the nigrostriatal dopamine system from 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced degeneration (Inoue et al., 2019). These data suggest that α-syn aggregates are more prone to be formed in neurons with decreased calcium buffering capacity possibly due to irregular intracellular calcium levels.

Apart from calcium binding proteins, calcium-dependent proteases, such as calpain, seem to play a role in α-syn aggregation. In particular, increase in intracellular calcium concentrations lead to pathologically increased calpain activity. Mice deficient for calpastatin, a calpain-specific inhibitor, that were crossed with A30P α-syn expressing mice, exhibited higher calpain activity and increased α-syn aggregated species (Diepenbroek et al., 2014).

Finally, it has been proposed that calcium binds directly to the C-terminal of α-syn (Nielsen et al., 2001). Using time-resolved circular dichroism spectroscopy and infrared spectroscopy, it has been shown that calcium binding leads to exposure of the non-amyloid component (NAC) domain of the monomeric protein promoting the formation of β-sheet structures and thus accelerating the formation of α-syn aggregates (Han et al., 2018). In a different point of view, application of recombinant α-syn monomers or oligomers to primary neuronal cultures induced an increase in cytosolic transient $[Ca^{2+}]$ linking α-syn-induced neurotoxicity with increased intracellular calcium signaling (Angelova et al., 2016). By using the planar lipid bilayer approach, the authors concluded that the interaction of α-syn with the plasma membrane could facilitate calcium influx via affecting membrane permeability (Angelova et al., 2016). Alternatively, it has been proposed that α-syn aggregates can bind to the Ca^{2+} pump sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) on the endoplasmic reticulum modulating the intracellular calcium concentration. In this study, blockage of SERCA ameliorated the α-syn aggregated-induced cell death in neuronal cells in culture (Betzer et al., 2018).

Alpha-synuclein is considered to be secreted from neuronal cells though a stimulus-dependent mechanism that is regulated by the levels of intracellular Ca^{2+}. It has been shown that an increase in intracellular calcium stimulate the secretion of α-syn in α-syn expressing SH-SY5Y cells (Emmanouilidou et al., 2010). Importantly, the secretion of α-syn in mouse striatum is thought to be regulated by the operation of presynaptic calcium channels as has been shown using a reverse microdialysis approach (Emmanouilidou et al., 2016). This is in accordance with a recent study that proposes, using a similar experimental setting, that the release of α-syn is dependent on neuronal activity *in vivo* (Yamada and Iwatsubo, 2018). The above studies support the idea that α-syn can be found in extracellular milieu not only as a monomer but also as an oligomeric conformer that may be internalized by neuronal cells.

The work described so far supports a multifactorial relationship of α-syn with calcium signaling where elevations in intracellular Ca^{2+} can result in the aggregation and release of α-syn or vice versa (**Figure 1**).

CHANNELOPATHIES AND NEURODEGENERATION IN PD

In search of the mechanisms of disease progression in PD, previous work has focused on the connection between α-syn secretion, neurodegeneration, and alterations in calcium influx, mainly via the VGCCs. In neurons, VGCCs are key signaling elements that modulate the release of bioactive molecules (hormones, proteins, and neurotransmitters) through Ca^{2+} sensing. Following neuronal spiking and K^{+} channels opening, VGCCs modulate Ca^{2+} influx during repolarization back to the resting membrane potential. In most cases, VGCCs open at high voltages and close slowly during repolarization, leaving a time window where calcium influx occurs. The amount of calcium ions that enter the cell depend on the duration of the spike. Neurons regulate the amount of calcium influx by reducing the duration of the spikes (less than 1 ms) and by expressing calcium binding proteins (reviewed in James Surmeier et al., 2012). The VGCCs are divided to high-voltage activated (HVA) channels, which are activated at high membrane depolarization, or low-voltage activated (LVA) channels, which open at voltages near the resting membrane potential (Armstrong and Matteson, 1985; Bean, 1985). The HVA channels include L-type(Cav1 family), P-, Q-, and R-type (Cav2 family) VGCCs, whereas LVA channels include only T-type channels (Cav3 family) (reviewed in Zamponi, 2016).

It is noteworthy that in PD degeneration occurs preferentially in SNc dopaminergic neurons, while ventral tegmental area (VTA)-residing dopaminergic neurons remain mostly unaffected. It is proposed that differences in the regulation of calcium balance in these two neuronal populations could contribute to the onset and progression of neurodegeneration in PD. In particular, the adult SNc neurons mostly rely on L-type VGCCs for their basal activity and, more specifically, on the Cav1.3 subtype of L-type channels that open at relatively negative

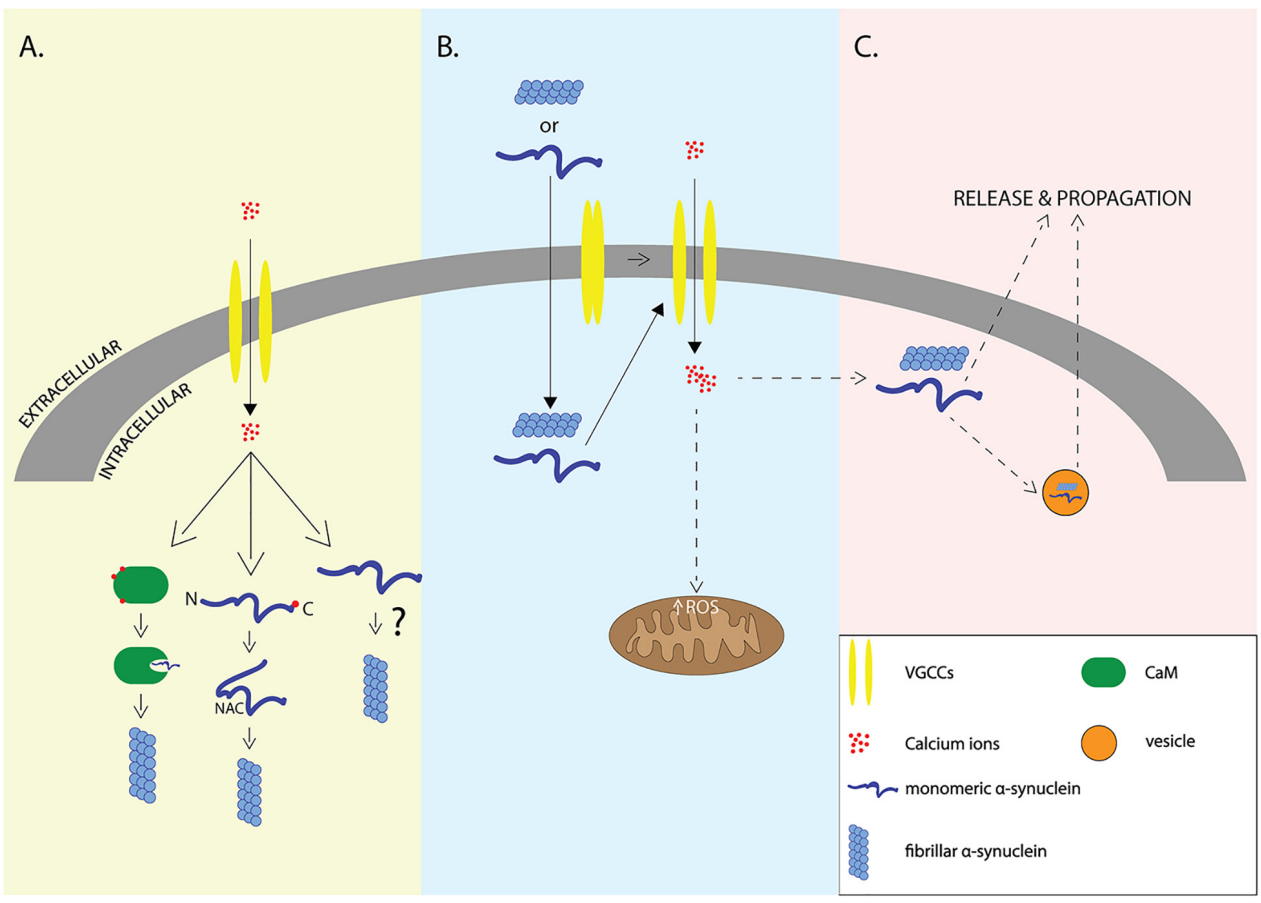

FIGURE 1 | Interplay of α-syn and Ca^{2+}. Elevation of intracellular Ca^{2+} levels through voltage-gated calcium channels (VGCCs) may lead to aggregation of α-syn via indirect interactions with calcium binding proteins such as Calmodulin, or via direct binding of Ca^{2+} to α-syn that leads to exposure of NAC domain **(A)**, elevation of intracellular amounts of α-syn may lead to VGCCs opening and calcium influx that results in increased formation of reactive oxygen species (ROS) and neurodegeneration **(B)**. It still remains unclear whether elevation of intracellular Ca^{2+} is the cause or the result of α-syn aggregation which promotes the release and propagation of α-syn -the release is accomplished either through exosomes or through other proposed secretory mechanisms **(C)**.

membrane potential (Mercuri et al., 1994). SNc dopaminergic neurons also express calcium binding proteins at lower level compared to VTA dopaminergic neurons (Foehring et al., 2009). These differences seem to be developmentally coordinated; it has been shown that during the embryonic stage and until postnatal week 3, the SNc dopaminergic neurons rely mostly on voltage-dependent Na^+ channels, until a developmental switch renders Cav1.3-VGCCs the primary voltage-gated channels responsible for their pacemaking activities (Chan et al., 2007). In support for a critical role of VGCCs in the susceptibility of dopaminergic neurons, application of nimodipine, a Cav1.2/Cav1.3 blocker, in cultured L-DOPA-treated midbrain neurons decreased the levels of cytosolic dopamine suggesting a role of L-type channels in dopamine metabolism and neuronal survival (Mosharov et al., 2009). In addition, the L-type VGCCs were shown to be responsible for the elevation of intracellular calcium in primary dopaminergic neurons exposed to MPP^+. Such calcium elevation was not observed in α-syn knock-out cultures after MPP^+ exposure, suggesting that α-syn is implicated in intracellular calcium changes under stress conditions (Lieberman et al., 2017). Interestingly, increased intracellular calcium in SNc neurons overexpressing α-syn led to increased mitochondrial oxidation and neurotoxicity in these neurons, but not VTA neurons, probably due to the fact that VTA neurons do not depend on Cav1.3 VGCCs for neuronal firing (Lieberman et al., 2017). In support to these observations, a recent study depicted the importance of a fine balance between intracellular α-syn and intracellular calcium. In mesencephalic neurons, calcium binding in α-syn mediates the localization of the protein in synaptic vesicles and, under conditions of increased calcium or α-syn, this localization promotes synaptic vesicle clustering and α-syn aggregation. In this system, isradipine treatment reversed α-syn aggregation and improved neuronal survival (Lautenschläger et al., 2018). Several other studies using pharmacological inhibition emphasized on the importance of L-type channels in degeneration. Blockage of L-type calcium channels with the L-type inhibitor isradipine significantly reduced mitochondrial oxidation, indicating that calcium influx via the L-type VGCCs during pacemaking plays an important role in mitochondrial oxidant stress (Guzman et al., 2010). Furthermore, systemic administration of isradipine protected the striatal dopaminergic terminals, as well as the somata of dopaminergic neurons, after intrastriatal injection of 6-hydroxydopamine (6-OHDA) in mice (Ilijic et al., 2011).

Apart from the degeneration of dopaminergic neurons in SNpc of PD patients, the pathology is also evident in nuclei of the brainstem and olfactory bulbs (Jellinger, 2008). Cholinergic neurons in the dorsal motor nucleus of the vagus (DMV) in the caudal medulla have been shown to exhibit early Lewy body formation (Braak and Del Tredici, 2009). Interestingly, these neurons are autonomous slow pacemakers receiving an increased intracellular calcium load via the L-type VGCCs and they express low levels of calcium binding proteins. To induce mitochondrial stress, Goldberg et al. (2012) diminished DJ-1 from cholinergic DMV neurons and showed that pharmacological blockage of L-type VGCCs ameliorates the induced mitochondrial stress.

Further addressing a key role of L-type channels in PD, examination of post mortem material has revealed significant differences in the expression levels of these channels in PD patients compared with healthy subjects suggesting a possible role of the L-type family in the process of neurodegeneration, probably, via increased calcium influx that may lead to excitotoxicity. Immunostaining for Cav1 channels revealed that the expression ratio of Cav1.3/Cav1.2 of Cav1 was increased in early-stage PD brains compared to healthy controls (Hurley et al., 2013). Furthermore, two independent drug epidemiological studies targeting Cav1.3 channels concluded that administration of dihydropyridines lead to a reduced risk of developing PD (Ritz et al., 2010; Pasternak et al., 2012). However, since both Cav1.2 and Cav1.3 channels have very similar structural and pharmacological properties, the selectivity of 1,4-dihydropyridines is very low and the application of these L-type inhibitors could also block Cav1.2 channels (Xu and Lipscombe, 2001). In this context, isradipine-a well-known anti-hypertensive drug- is currently in phase III clinical trials to determine whether it can be effective against the progression of PD (Biglan et al., 2017). Isradipine has previously been used against neurodegeneration, but its selectivity is still under debate (Ortner et al., 2017; Guzman et al., 2018). In general, the variability in the blocking properties of dihydropyridines during different membrane depolarization states of dopaminergic neurons raises concerns about the usage of these drugs for PD treatment, since higher doses of dihydropyridines might not be tolerated during long-term treatment (Ortner et al., 2017).

Specific VGCCs have been implicated in α-syn-induced neurotoxicity. In SH-SY5Y cells, treatment with either nifedipine or ω-conotoxin, specific inhibitors for the L-type and N-type VGCCs, respectively, diminished the intracellular calcium raise induced by extracellular α-syn (Melachroinou et al., 2013). In rat cortical neurons, Ronzitti et al. (2014) showed that the calcium influx following application of extracellular α-syn was abolished by ω-conotoxin, but not by nifedipine or ω-agatoxin (specific inhibitors for L-type and P/Q type, respectively) indicating a possible role for the N-type VGCCs on α-syn-induced calcium increase.

Finally, targeting of T-type VGCCs has recently been considered a neuroprotective strategy for neurodegeneration and, more specifically, PD (Kopecky et al., 2014; Yang et al., 2014). Tabata et al. (2018) highlighted the importance of T-type VGCCs using induced pluripotent stem cell (iPSC) dopamine neurons derived from PARK2 patients by applying pharmacological or genetic silencing of T-type channels. In this study, the specific T-type antagonist ML218 ameliorated the effects of rotenone-induced mitochondrial stress by rescuing the apoptotic phenotype thereby leading to neuroprotection. Similar results were obtained after silencing of T-type VGCCs with specific siRNAs, while overexpression of T-type VGCCs led to the opposite effects. It has also been suggested that T-type VGCCs play a role in locomotor deficits accompanied after 6-OHDA lesion in rats. Specifically, *in vitro* as well as *in vivo* studies on subthalamic nuclei (STN) neurons revealed that blockage of T-type channels by the T-type specific inhibitors, Ni^{2+} and mifebradil, reduced the pathologically increased oscillations of

STN that may be responsible for the tremor and other motor deficits present in PD. The above finding was also confirmed by behavioral experiments, where 6-OHDA lesioned rats showed a significant improvement in open field locomotor test after direct microinjection of either Ni^{2+} of mifebradil in STN (Tai et al., 2011).

CONCLUDING REMARKS

We can conclude that all different types of VGCCs have been implicated in the progressive neurodegeneration present in PD. This is highlighted by a plethora of studies in which specific VGCCs are pharmacologically targeted in dopaminergic neurons to assess their role in preserving normal dopamine release and promoting cell survival under conditions of cellular stress. Several parameters could contribute to the discrepancies observed among the different studies. The different model systems used, ranging from *in vitro* cell models, such as cell lines and primary neurons, to the living rodent brain, could affect the magnitude and interpretation of the effects observed following the pharmacological manipulation of each VGCC. It is also possible that the different α-syn species (monomers, oligomers, and fibrils) have the ability to act through independent molecular mechanisms to trigger alterations in intracellular calcium. Finally, the pharmacological inhibition of certain VGCCs could stimulate compensatory mechanisms in which other calcium channels operate synergistically to regulate calcium levels adding further complexity to the interpretation of the results obtained so far.

Calcium influx can trigger α-syn aggregation thus providing an alternative pathway to PD neurodegeneration. There is also evidence that VGCCs can facilitate α-syn secretion under normal or pathological conditions, even though the mechanism for the stimulation of this process is still elusive. Abnormal function of these specific VGCCs may cause local accumulation of aggregated α-syn material into the extracellular space which could be taken up by recipient neurons thereby promoting the cell-to-cell spreading of disease pathology. As such, VGCCs that regulate α-syn properties could indicate specific molecular pathways to target as alternative therapeutic approaches for PD.

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All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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REVIEW
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Prion-Like Propagation of Protein Misfolding and Aggregation in Amyotrophic Lateral Sclerosis

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The discovery that prion protein can misfold into a pathological conformation that encodes structural information capable of both propagation and inducing severe neuropathology has revolutionized our understanding of neurodegenerative disease. Many neurodegenerative diseases with a protein misfolding component are now classified as "prion-like" owing to the propagation of both symptoms and protein aggregation pathology in affected individuals. The neuromuscular disorder amyotrophic lateral sclerosis (ALS) is characterized by protein inclusions formed by either TAR DNA-binding protein of 43 kDa (TDP-43), Cu/Zn superoxide dismutase (SOD1), or fused in sarcoma (FUS), in both upper and lower motor neurons. Evidence from *in vitro*, cell culture, and *in vivo* studies has provided strong evidence to support the involvement of a prion-like mechanism in ALS. In this article, we review the evidence suggesting that prion-like propagation of protein aggregation is a primary pathomechanism in ALS, focusing on the key proteins and genes involved in disease (TDP-43, SOD1, FUS, and *C9orf72*). In each case, we discuss the evidence ranging from biophysical studies to *in vivo* examinations of prion-like spreading. We suggest that the idiopathic nature of ALS may stem from its prion-like nature and that elucidation of the specific propagating protein assemblies is paramount to developing effective therapies.

Keywords: amyotrophic lateral sclerosis, protein misfolding, protein aggregation, prion, proteostasis

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NEURODEGENERATIVE DISEASE: A COMMON PATHOPHYSIOLOGY

Neurodegenerative diseases are characterized by the selective and progressive loss of neurons, leading to the development of neurological dysfunction which can include memory, behavioral, and movement deficits (Erkkinen et al., 2018). Neurodegenerative diseases include Alzheimer's disease (AD), Parkinson's disease (PD), frontotemporal dementia (FTD), amyotrophic lateral sclerosis (ALS), spinocerebellar ataxia's, Huntington's disease, multiple system atrophy, dementia with Lewy bodies, corticobasal degeneration, chronic traumatic encephalopathy, and prion diseases [e.g., Creutzfeldt-Jakob Disease (CJD), fatal familial insomnia, or kuru]. Collectively, these diseases

exact a significant and growing toll on human well-being both socially and economically (Prince et al., 2013), making the development of effective therapies essential to societal health and well-being.

Although all of the above listed diseases have in common the progressive degeneration of neurons, they typically manifest clinically with different symptoms due to a characteristic set of neuronal subtypes specifically vulnerable in each condition (Erkkinen et al., 2018). For example, a patient suffering from canonical ALS will exhibit preferential degeneration of motor neurons in the spinal cord/brainstem and motor cortex, however, neurons in other central nervous system (CNS) regions will generally remain unaffected (Ravits and La Spada, 2009). Another example is that in PD dopaminergic neurons within the substantia nigra preferentially degenerate (Brichta and Greengard, 2014). Although the vulnerability of certain neurons to undergo death appears to be disease-specific, the key markers for this death are observed across many diseases (Chi et al., 2018). One such key marker is mitochondrial dysfunction, which can be a consequence of the accumulation of misfolded proteins like SOD1 (Magrané et al., 2012). Maintenance of mitochondrial protein homeostasis is especially important for neurons, owing to their large size and high energy requirements (reviewed in Baker et al., 2011).

A unifying feature of all the above mentioned neurodegenerative diseases is that the cell and neuroanatomical region specificity of degeneration is often accompanied by the aberrant misfolding, aggregation, and deposition of specific proteins (Chiti and Dobson, 2017). Protein misfolding is defined as the adoption of a non-native conformation by a protein; Protein aggregation is the aberrant accumulation of a protein into multimeric soluble or insoluble non-native structures; Protein deposition is the formation of large insoluble deposits of proteins (Chiti and Dobson, 2017). Examples of specific disease-associated aggregating proteins include amyloid-β (Glenner and Wong, 1984) and τ-protein (Grundke-Iqbal et al., 1986) in AD, α-synuclein in PD (Spillantini et al., 1997) and multiple system atrophy (Tu et al., 1998; Wakabayashi et al., 1998), poly-Q expansions in huntingtin protein in Huntington's disease (DiFiglia et al., 1997), and either Cu/Zn superoxide dismutase (Rosen et al., 1993), TAR-DNA-binding protein of 43 kDa (TDP-43; Neumann et al., 2006), or fused in sarcoma (FUS; Kwiatkowski et al., 2009; Vance et al., 2009) in ALS.

It was previously thought that the aggregation of specific proteins is exclusively associated with specific disorders, however, there is evidence for some overlap between misfolding and aggregating proteins and various syndromes (Jellinger, 2012). Indeed, aberrant localization and aggregation of TDP-43 are being associated with an increasing number of neurodegenerative diseases (reviewed in Gao et al., 2018), and SOD1 positive inclusions have been reported in PD cases (Trist et al., 2017). Furthermore, the fact that patients suffering from FTD can have protein inclusion pathology that is positive for either TDP-43, τ-protein, or FUS (Ling et al., 2013), and that ∼15% of ALS patients are estimated to develop cognitive deficits meeting FTD criteria (Ringholz et al., 2005), is striking evidence of both pathological and symptomatic overlap. This has led to the suggestion that both ALS and FTD perhaps exist as extreme syndromes of a single disease spectrum (Ling et al., 2013).

AMYOTROPHIC LATERAL SCLEROSIS—CLINICAL AND PATHOLOGICAL MANIFESTATION

ALS is a neuromuscular disorder in which the upper (motor cortex) and lower (spinal) motor neurons, responsible for motor function, progressively degenerate, ultimately resulting in patient mortality *via* asphyxiation or inanition (Hardiman et al., 2017). The disease is considered to comprise two main categories on the basis of familial history of disease or lack thereof. Sporadic ALS (sALS) accounts for approximately 90% of cases (Taylor et al., 2016), and the remaining 10% are classed as familial (fALS). Collectively, ALS is estimated to have an annual incidence rate of 2.4 per 100,000 people in Europe, and is suggested to affect a susceptible population rather than being generally related with aging (Logroscino et al., 2010). Clinical diagnosis of ALS is often difficult due to significant variability in patient presentation and prognosis, as well as lack of a definitive biomarker for disease (Al-Chalabi et al., 2016; Grad et al., 2017). Generally, ALS patients will initially show symptoms of either lower motor neuron degeneration (weakness with muscle wasting, fasciculation, cramps) or upper motor neuron degeneration (weakness and spasticity), however, both regions of the CNS are ultimately affected (Tartaglia et al., 2007; Van den Berg-Vos et al., 2009). A characteristic feature of ALS is that often, regardless of the initial site of onset, symptoms spread to nearby contiguous anatomical regions in the CNS in a spatiotemporal manner (Ravits et al., 2007; Ravits and La Spada, 2009). There are cases in which the spread of symptoms has been reported to be discontiguous, however, they remain a minority (Walhout et al., 2018; Zhenfei et al., 2019). To better understand the relationship between symptoms and pathology, it will be important to examine discontiguous and contiguous spread in relation to the patterns of pathology in patients.

A key pathological hallmark of ALS is the deposition of proteins into ubiquitinated, and sometimes hyperphosphorylated, cytoplasmic inclusions in motor neurons and glia in the spinal cord/brainstem and motor cortex. Other hallmarks include the degeneration of motor neurons in the spinal cord and motor cortex, as well as atrophy of the corresponding denervated skeletal muscles. The pathological protein inclusions observed in patients are immunoreactive for either TDP-43 (Neumann et al., 2006), SOD1 (Rosen et al., 1993), or FUS (Kwiatkowski et al., 2009; Vance et al., 2009), of which the vast majority of cases show TDP-43 (97% of cases), rather than SOD1 (2%) or FUS (1%), pathology (Ling et al., 2013). Notably, aggregates of SOD1 or FUS are associated with mutations in cognate genes, whereas TDP-43 aggregates can be made from wild-type protein in sALS as well as associated with mutations in TDP-43 in fALS. TDP-43 pathology can also be observed as a downstream consequence of other ALS-implicated gene mutations (e.g., C9orf72).

GENETICS OF ALS—INTERPLAY OF RNA METABOLISM, CYTOSKELETAL DYNAMICS, AND PROTEOSTASIS

Both sALS and fALS are clinically indistinguishable from each other (Hardiman et al., 2011), whereas the genetics of ALS are heterogeneous (Taylor et al., 2016), involving numerous genes that control various biochemical processes and cellular pathways (Taylor et al., 2016). As our understanding of the functions of these genes has increased, it has become apparent that they can be broadly classified into three major groups; genes associated with RNA metabolism, genes associated with cytoskeletal dynamics, and genes associated with protein homeostasis (proteostasis; Taylor et al., 2016). There is also a great deal of overlap between these important cellular processes, making it difficult to designate causal pathomechanism(s).

Disturbances in RNA metabolism in ALS have been keenly studied owing to the discovery that TDP-43 and FUS, which are RNA-binding proteins (RBPs), form neuronal inclusions and harbor mutations that are ALS causative (Neumann et al., 2006; Kwiatkowski et al., 2009; Vance et al., 2009). An increasing number of RNA metabolism-related genes have been identified to carry ALS-associated mutations, including heterogeneous nuclear ribonucleoprotein A1 (hnRNPA1) and heterogeneous nuclear ribonucleoprotein A2B1 (hnRNPA2B1; Kim et al., 2013), matrin-3 (Johnson et al., 2014), TIA-1 cytotoxic granule-associated RNA binding protein (Mackenzie et al., 2017), and TATA-box binding protein associated factor 15 (TAF-15; Couthouis et al., 2011; Ticozzi et al., 2011). Furthermore, the most common identified cause for fALS are hexanucleotide (GGGGCC) repeat expansions in the chromosome 9 open reading frame 72 (*C9orf72*) gene (DeJesus-Hernandez et al., 2011; Renton et al., 2011), that potentially lead to toxicity caused by the synthesis of large non-coding RNAs that form sense and anti-sense foci in cells that can sequester RBPs and prevent them from carrying out their biological functions (Donnelly et al., 2013; Gendron et al., 2013; Lagier-Tourenne et al., 2013; Mizielinska et al., 2013; Zu et al., 2013). Many of the above mentioned ALS-associated RBPs take part in the formation of ribonucleoprotein (RNP) granules and biomolecular condensates in cells (Banani et al., 2017), and there is growing evidence that mutations affect the dynamics of formation and dissolution of these structures (Murakami et al., 2015; Patel et al., 2015; Lee et al., 2016; Lin et al., 2016; Martinez et al., 2016; Boeynaems et al., 2017; Mackenzie et al., 2017). It is suggested that this dysregulation of RNP granules can result in significant alterations to alternative splicing, mRNA transcription, and RNA transport, which can result in widespread toxicity in cells (Butti and Patten, 2019). An overlap with proteostasis mechanisms is also possible with ALS-associated mutations in RBPs as they can act to promote a liquid-to-solid transition of biomolecular condensates, leading to the formation of pathological aggregates in cells (Murakami et al., 2015; Patel et al., 2015; Mackenzie et al., 2017; Sun and Chakrabartty, 2017; Gasset-Rosa et al., 2019). Cytoskeletal dynamics can also be affected by ALS-associated RBP mutants, for example, ALS-associated mutations in TDP-43 can alter the transport dynamics and viscosity of transport RNPs (Alami et al., 2014; Gopal et al., 2017), potentially resulting in impaired delivery of mRNA to the axons of motor neurons.

Motor neurons are significantly larger than other cell types, owing to the requirement to extend axons anatomically long distances throughout the motor cortex, spinal cord and periphery. Therefore, disruption of the mechanisms that traffic protein and nucleotides to distal cellular compartments would be expected to impair proper cellular functioning. Indeed, ALS-associated mutations have been identified in cytoskeletal components and regulatory elements, such as heavy neurofilament (Al-Chalabi et al., 1999), peripherin (Gros-Louis et al., 2004), NIMA-related kinase 1 (NEK1; Kenna et al., 2016), profilin-1, and kinesin family member 5A (KIF5A; Brenner et al., 2018), amongst others (Puls et al., 2003; Wu et al., 2012; Smith et al., 2014). The mechanisms by which mutations in cytoskeletal components can cause ALS are not fully understood but are thought to be a combination of loss-of-function and gain-of-toxic-function that can overlap with both RNA metabolism and proteostasis (Hensel and Claus, 2017). In the case of profilin-1, in its normal role it binds to actin and it affects the overall structure of the cytoskeleton. In terms of RNA metabolism, it was found that ALS-associated profilin-1 mutants differentially localized to stress granules and that stress granule dynamics were altered (Figley et al., 2014). In the case of proteostasis, expression of ALS-associated profilin-1 mutants has been shown to lead to the formation and seeding of TDP-43 positive proteinaceous aggregates in cultured cells (Wu et al., 2012; Smith et al., 2015; Tanaka and Hasegawa, 2016).

That proteinaceous aggregates are the primary characteristic marker for ALS suggests that, at the very least, proteostasis is a key mechanism affected in ALS. Indeed, there are several identified ALS-associated mutations in genes associated with autophagy (Maruyama et al., 2010; Fecto et al., 2011), and the ubiquitin proteasome system (UPS; Johnson et al., 2010; Deng et al., 2011; Williams et al., 2016). Furthermore, autophagy and the UPS have been suggested to be altered in sALS on the basis of studies of post-mortem tissue (Sasaki, 2011; Kabashi et al., 2012). Interestingly, TDP-43, SOD1, and FUS, which are the primary components of inclusions in ALS patient tissue (Ling et al., 2013), are shown to be supersaturated (expressed at levels higher than their predicted solubility) in spinal motor neurons (Ciryam et al., 2017). Following this notion, it has been suggested that the average supersaturation of the entire proteome of spinal motor neurons is greater than that of the ALS-resistant ocular motor neurons (Yerbury et al., 2019), providing important physico-chemical insight into the selective vulnerability of spinal motor neurons to proteotoxicity. Again, overlap is observed between proteostasis and both RNA metabolism and cytoskeletal dynamics. For example, the UPS protein ubiquilin-2 has been observed to control the dynamics of both stress granule formation and RNA/FUS complexes (Alexander et al., 2018), and mutant SOD1 can interact with the stress granule forming protein Ras GTPase-activating protein-binding protein 1 (G3BP1) to impair proper stress granule formation (Gal et al., 2016).

Collectively, RNA metabolism, cytoskeletal dynamics, and proteostasis should not be viewed as separate pathologically altered pathways, but as an interconnected network. However, regardless of whether RNA metabolism, cytoskeletal dynamics, or proteostasis are perturbed in ALS, the most prominent pathology is the formation of proteinaceous aggregates in neurons and glial support cells composed of TDP-43, or SOD1, or FUS in a mutually exclusive manner (Farrawell et al., 2015). This is supported by the finding that TDP-43 and FUS, despite both being RNA binding proteins participating in stress granule formation, generally do not appear to co-localize in pathological ALS inclusions (Ling et al., 2013). Furthermore, the neuroanatomical spread of pathology and symptoms indicates that there are propagating agents in ALS (**Table 1**). Owing to the spatiotemporal spread of pathology in ALS, as well as PD and AD, it has been suggest that a key mechanism in neurodegenerative disease pathobiology is the "prion-like" spread of protein misfolding and aggregation, pathways of which would be different for the three major inclusion forming proteins (Farrawell et al., 2015) to account for the exclusivity noted above.

PRION DISEASE AND PRION THEORY

Classical understanding of progressive and infectious diseases mandated that a nucleic acid component be present to facilitate replication of the infectious particles. This idea has since been challenged owing to the discovery that a class of neurological disorders, known as the prion diseases, possess an infectious life cycle and spread in the CNS in the absence of any microbial nucleic acid component. The landmark finding was that the infectious particle responsible for scrapie, a neurodegenerative disease that affects sheep and goats, was proteinaceous in origin, resulting from the misfolding of prion protein (PrP^{C}) into a pathological conformation (PrP^{Sc}; Prusiner, 1982). Furthermore, PrP^{Sc} was found to be able to catalyze the misfolding of PrP^{C}, thus generating more conformational copies of PrP^{Sc} that can then spread throughout cells, tissues, and even between organisms. This notion of the propagation of protein misfolding has been subsequently applied to neurodegenerative diseases, providing a possible explanation for the protein aggregation pathology and progressive spatiotemporal degeneration observed in these diseases.

Prion diseases include CJD, Bovine Spongiform Encephalopathy, Fatal Familial Insomnia, Kuru, and Gerstmann–Sträussler–Scheinker disease, amongst others (Geschwind, 2015). The clinical manifestations and pathological profiles of prion diseases are highly similar to those of other neurodegenerative diseases (Prusiner, 2001). In both cases they are invariably fatal; The clinical symptoms are mainly associated with neurological functioning and can include dementia, ataxia, involuntary movements, weakness, and spasticity, although, specific prion diseases will manifest distinct symptoms from this list (Glatzel et al., 2005). The neuropathological similarities of prion diseases with other neurodegenerative diseases include substantial neuronal loss, accumulation of proteins into aggregates, gliosis, and general cerebral atrophy (Prusiner, 2001).

TABLE 1 | Evidence supporting the prion-like characteristics of amyotrophic lateral sclerosis (ALS)-associated proteins.

Protein/Gene	*in vitro* fibril formation	*in vitro* fibril seeding	Cell seeding with *in vitro* protein	Cell-to-cell propagation	Human to cell propagation	*in vitro* to animal propagation	Animal to animal propagation	Human to animal propagation
SOD1	✓ Chattopadhyay et al. (2008)	✓ Chia et al. (2010)	✓ Münch et al. (2011)	✓ Grad et al. (2014)	✓ Pokrishevsky et al. (2017)	✓ Ayers et al. (2016a)	✓ Ayers et al. (2014)	✓ Ekhtiari Bidhendi et al. (2018)
TDP-43	✓ Johnson et al. (2009)	✓ Furukawa et al. (2011)	✓ Furukawa et al. (2011)	✓ Nonaka et al. (2013)	✓ Nonaka et al. (2013)	–	–	✓ Porta et al. (2018)
FUS	✓ Nomura et al. (2014)	✓ Nomura et al. (2014)	–	–	–	–	–	–
C9orf72 (DPRs)	–	–	–	✓ Westergard et al. (2016)	–	–	–	–

Note: ✓ *= confirmed;* – *= no published evidence.*

Biophysical examination of PrP^{C} has suggested that its aggregation is preceded by a conformational change from a structure composed mostly of α-helical elements to a β-sheet rich conformation, where the β-sheet rich conformation is thought to be the disease-associated PrP^{Sc} form (Pan et al., 1993). The β-sheet rich form is capable of interacting with and converting the normal α-helix rich form to the β-sheet rich form in a template-directed manner. Furthermore, the β-sheet rich form of PrP is capable of polymerizing into amyloid-like fibrils (McKinley et al., 1991). This is a similar kinetic process that occurs to amyloid-β peptides to generate amyloid fibrils (Arosio et al., 2015). The process is understood as an initial monomeric misfolding event, followed by the formation of small disordered oligomers, where oligomers nucleate the formation of the larger ordered amyloid fibrils. Interestingly, *in vitro* studies have shown that many neurodegenerative disease-associated proteins are capable of forming amyloid-like fibrils (Eisenberg and Jucker, 2012; Knowles et al., 2014). In ALS this includes TDP-43 (Chen et al., 2010; Guo et al., 2011; Saini and Chauhan, 2011, 2014; Jiang et al., 2013; Wang et al., 2013; Zhang et al., 2013; Mompeán et al., 2014; Sun et al., 2014; Cao et al., 2019), SOD1 (Chattopadhyay et al., 2008, 2015; Münch and Bertolotti, 2010; Lang et al., 2012; Chan et al., 2013; Ivanova et al., 2014; Abdolvahabi et al., 2016; McAlary et al., 2016), FUS (Kato et al., 2012; Murakami et al., 2015; Patel et al., 2015; Murray et al., 2017; Luo et al., 2018), amongst others (Molliex et al., 2015; Mackenzie et al., 2017).

Within the schema of prion theory, there exists a notion that different structural conformations of the prion agent may confer specific modes of spread, different abilities to recruit normal protein, and different levels and mechanisms of cellular toxicity to recipient species (Morales, 2017). Together, these characteristics are used to define different "strains" of prion. An integral part of the prion strain idea is that the ability of a prion strain to transmit disease is dependent on the conformation of the prion particle, the amino acid sequence of the template-competent substrate, post-translational modification of the substrate, and the cell type (substrate expression level and cellular capacity for degradation of misfolded/aggregated protein; Telling et al., 1996; Safar et al., 1998; Legname et al., 2006). Conformation of the prion assembly is especially important, as it has been established that strains that are more likely to fragment into a greater number of seeds are the most capable of spreading (Tanaka et al., 2006). In relation to ALS, it is interesting to note that the main aggregating proteins (SOD1, TDP-43, FUS) are supersaturated in spinal motor neurons (Ciryam et al., 2017), suggesting that motor neurons may provide an efficient environment for ALS-associated prion-like seeds to form and propagate. Indeed, the combination of the spatiotemporal spread of symptoms in ALS with both the evidence of protein aggregation and ability of the aggregating proteins to form amyloid-like structures has led to the suggestion that a prion-like mechanism is active in ALS (Ayers and Cashman, 2018). Below we review the evidence for prion-like characteristics of SOD1, TDP-43, FUS, and *C9orf72* associated dipeptide repeats, as they relate to ALS.

SUPEROXIDE DISMUTASE-1

SOD1 Structure and Function

Superoxide dismutase-1 (SOD1) was the first gene linked to familial ALS (Rosen et al., 1993), and since this initial discovery, there have been over 160 fALS-associated mutations identified in the *Sod1* gene (Abel et al., 2013). In its natively folded state, SOD1 is a 32 kDa homodimer, where each 153 amino acid subunit coordinates a zinc ion, copper ion, and contains an intramolecular disulfide bond. The maturation pathway (**Figure 1A**) of SOD1, from nascent polypeptide to native dimeric conformation, is complex for such a small protein and has been extensively studied (Banci et al., 2012, 2013; Wright et al., 2016; Luchinat et al., 2017; Sala et al., 2019). Following synthesis, SOD1 assumes a partially folded state with no metal cofactors and reduced cysteines [E, E(SH)]. This nascent state is further stabilized by the binding of a zinc (Zn) ligand to form E, Zn(SH). The copper chaperone for SOD1 (CCS) then associates with the Zn-bound intermediate monomer, forming a heterodimer that facilitates Cu delivery and intramolecular disulfide formation to form a mature SOD1 monomer [Cu, Zn(SS)]. Interestingly, this interaction can be prohibited or stalled by SOD1-fALS mutations (Wright et al., 2016). Disulfide formation *via* CCS can also occur through a Cu-independent pathway (Banci et al., 2013) and can be promoted using small molecules (Capper et al., 2018). Recent work has also suggested that CCS performs a chaperone function to stabilize E, E-$SOD1^{2SH}$ SOD1 and promote Zn-binding mediated by its own SOD-like domain (Luchinat et al., 2017). The final step is dimerization of mature monomers to form the highly stable enzyme. Once natively folded, SOD1 acts to catalyze the dismutation of superoxide anions to hydrogen peroxide and molecular oxygen.

Loss of Function or Gain of Toxic Function?

Owing to the role of SOD1 as an antioxidant enzyme, it was initially thought that the fALS-associated mutations in SOD1 were inducing a loss of enzymatic function, leading to toxicity mediated by increased concentrations of oxygen radicals in cells. However, continued research revealed that this was not the case as overexpression of human mutant SOD1-G93A in a transgenic mouse line showed increased dismutase activity combined with rapid mortality in comparison to wild-type mice (Gurney et al., 1994). Another study knocked out SOD1 from mice, showing slightly increased neuronal cell death following axonal injury, but normal motor neuron development (Reaume et al., 1996), indicating that SOD1 is not essential to cell survival or development, and its antioxidant activity may be compensated for by other proteins (including SOD2 and SOD3). Furthermore, *in vitro* studies of several SOD1-fALS mutants found no correlation between relative enzymatic activity and clinical pathology (Borchelt et al., 1995; Ratovitski et al., 1999), and that many SOD1-fALS mutants possess significant enzymatic activity (Borchelt et al., 1994). Ruling out a loss-of-function mechanism for SOD1-fALS led to research into a gain-of-toxic-function

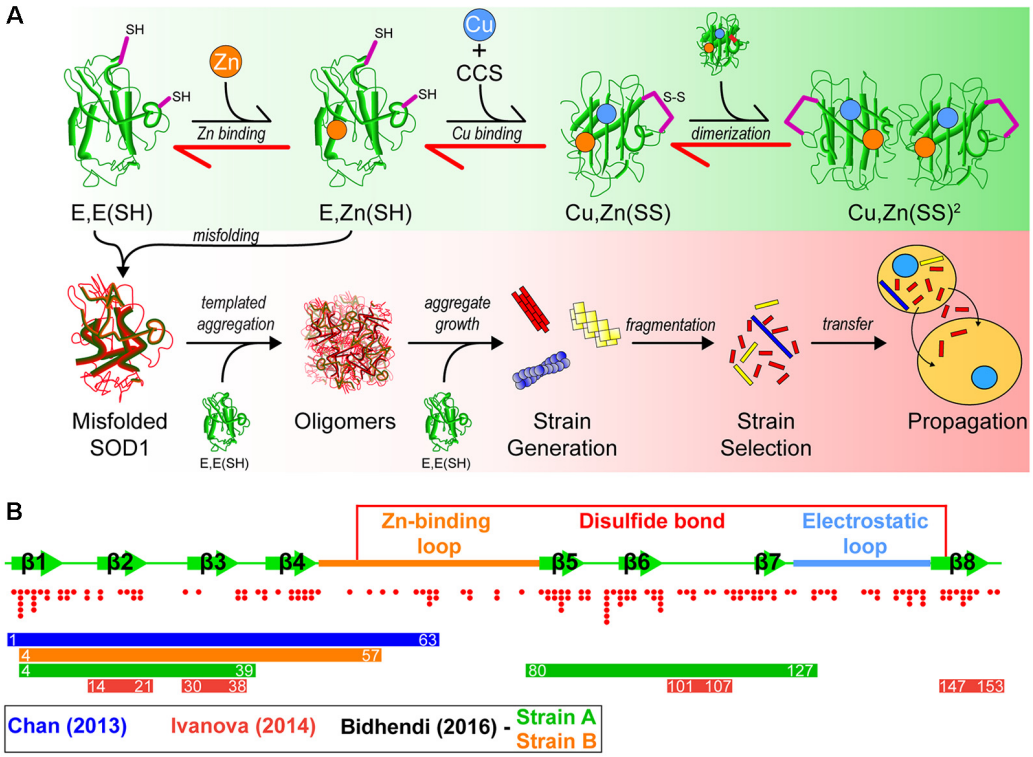

FIGURE 1 | A model of superoxide dismutase-1 (SOD1) aggregation and prion-like propagation. **(A)** The native folding (green background) and off-folding pathways (red background) of SOD1. When undergoing native folding, SOD1 is initially in a metal-free intermediate state [E, E(SH)] which is primed for Zn binding. Following Zn binding, the copper chaperone for SOD1 (CCS) heterodimerizes with a SOD1 monomer to implant Cu and facilitate the oxidation of the disulfide bond between Cys57 and Cys146 to form a mature SOD1 monomer [Cu, Zn(SS)]. Subsequent dimerization of two mature monomers gives the fully folded SOD1 enzyme. In the off-folding pathway (red background) amyotrophic lateral sclerosis (ALS)-associated mutations have the ability to push the folding back towards the nascent intermediates (red arrows), which are more aggregation prone than mature states. Misfolded SOD1 is capable of recruiting nascent SOD1 states to form oligomers. Oligomers can grow in size to form larger aggregates that may have strain-like properties. Strains that are prone to fragment are the most likely to propagate to adjacent cells and seed naïve SOD1. **(B)** Graphic representation of the SOD1 primary structure with important features. SOD1 is composed of 8 beta-strands and contains two major loop regions. The Zn-binding loop is responsible for coordination of Zn and, to a lesser extent, Cu. The electrostatic loop is responsible for guidance of superoxide substrate to the active site of the enzyme. A disulfide bond is formed between Cys57 and Cys146 in the mature protein. Red dots below the graphic represent the location and number of ALS-associated mutations that have been identified to date within SOD1. Coloured bars represent potential cores or contributing regions of the protein to the formation of aggregate strains from corresponding colour coded publications.

mechanism, focused on the misfolding and aggregation of SOD1 (**Figure 1A**).

SOD1 Pathological Features in Patients

Immunohistochemical studies of post-mortem tissue from both fALS and sALS patients with SOD1 misfolding-specific antibodies have revealed a potential role for the misfolding of WT SOD1 in all ALS (Bosco et al., 2010; Forsberg et al., 2010, 2011, 2019; Pokrishevsky et al., 2012; Paré et al., 2018), however, this is a contentious idea (Liu et al., 2009; Kerman et al., 2010; Da Cruz et al., 2017). Regardless, discrete SOD1 inclusions are a key component of fALS with SOD1 mutations. Currently, there are discrepancies as to the exact structural nature of the SOD1 inclusions found in patient tissue. Electron microscopy imaging of protein inclusions harvested from ALS patients suggests that they are distinct from classic amyloid, having an amorphous or granular structure (Kato et al., 2000) that does not stain with Congo red, and stains relatively weakly with thioflavin-S (Kerman et al., 2010). Whether amyloid-like elements exist in inclusions composed of SOD1 in SOD1-fALS patients remains to be fully elucidated, although it is important to note that SOD1 has the ability to form amyloid-like fibrils *in vitro* (Chattopadhyay et al., 2008, 2015; Münch and Bertolotti, 2010; Lang et al., 2012; Chan et al., 2013; Ivanova et al., 2014; Abdolvahabi et al., 2016; McAlary et al., 2016). Furthermore, aggregated SOD1 in human mutant SOD1-expressing transgenic mice is observed to have an ordered microscopic and molecular structure (Ayers et al., 2014, 2016b; Bergh et al., 2015; Lang et al., 2015; Bidhendi et al., 2016, 2018).

Amyloid Aggregation of SOD1

The first evidence that SOD1 may act in a prion-like manner came from protein only *in vitro* assays utilizing recombinant wild-type (WT) SOD1 and fALS-mutants. Initially, SOD1 aggregation assays utilized high temperatures, extreme pH, and organic solvents to induce aggregate assembly

(DiDonato et al., 2003; Stathopulos et al., 2003; Banci et al., 2008), conditions with little relevance to the intracellular environment. Modern recombinant SOD1 *in vitro* aggregation protocols rely upon destabilizing SOD1 *via* reduction of the disulfide and chelation of the metals under physiological salt and pH (Chattopadhyay et al., 2008; Lang et al., 2012; Chan et al., 2013; Ivanova et al., 2014; Abdolvahabi et al., 2016; McAlary et al., 2016), which are events more likely to occur in the reducing and metal-poor intracellular environment. Utilizing denaturing conditions, Chia et al. (2010) demonstrated that preformed recombinant SOD1 fibrils or spinal cord homogenates from multiple SOD1 transgenic mice were capable of seeding the aggregation of recombinant WT or mutant Cu, Zn-SOD1^{SS} *in vitro*. This requirement to destabilize SOD1 state to promote fibril formation and seeding suggests that immature SOD1 may be the major molecular species prone to aggregation and a primary template for SOD1 seeded aggregation. Indeed, careful investigations into the *in vivo* aggregation of SOD1 mutants in transgenic mice have been shown to have a striking similarity to these cell-free recombinant systems, suggesting E, E(SH) SOD1 is the primary aggregation substrate, and that aggregate growth is fragmentation dependent both *in vivo* and *in vitro* (Lang et al., 2015).

Interestingly, SOD1 has multiple sequence segments that have characteristics favorable to form amyloid fibrils (Ivanova et al., 2014). These include amino acid sequence segments $_{14}$VQGIINFE$_{21}$, $_{30}$KVWGSIKGL$_{38}$, $_{101}$DSVISLS$_{107}$, and $_{147}$GVIGIAQ$_{153}$, as determined by using the 3D profile method (see Münch and Bertolotti, 2010). The 3D profile method uses a sliding window of every six amino acid peptides from a queried protein sequence to generate structures of these peptides, where these peptide structures are computationally mapped onto ensembles created from the amyloidogenic NNQQNY peptide sequence from Sup35 yeast prion-protein. Peptides with strong binding energies are considered to be potentially amyloidogenic (Thompson et al., 2006). Assays on these synthesized peptides have shown that all have the ability to form fibrils *in vitro*, and seed full-length SOD1 aggregation (Ivanova et al., 2014). Of particular note is segment $_{30}$KVWGSIKGL$_{38}$ which has been recently shown to be able to form oligomers that are neurotoxic (Sangwan et al., 2017, 2018), and also contains the only tryptophan residue in SOD1 (W32), which is implicated in its aggregation and prion-like propagation (Taylor et al., 2007; Grad et al., 2011, 2014; Pokrishevsky et al., 2018). That SOD1 can form fibrils which may be nucleated by several different regions of the protein or controlled by specific residues, has implications for the existence of SOD1 prion-like strains (**Figure 1B**).

Intra- and Inter-cellular Prion-Like Spread of SOD1 Aggregation

The ability of cellular SOD1 to misfold and/or aggregate in a prion-like manner in living cells was first demonstrated using the addition of recombinant SOD1 aggregates to cultured cells overexpressing ALS mutant SOD1 variants (Münch et al., 2011). It was suggested that the exogenous SOD1 aggregates were entering the cells *via* macropinocytosis and escaping the macropinosomes to seed aggregation of cytosolic SOD1 (Münch et al., 2011), a finding that was substantiated in following publications (Holmes et al., 2013; Grad et al., 2014; Zeineddine et al., 2015). Shortly following the first study reporting intracellular seeding of SOD1, another study demonstrated the ability of endogenous wild-type SOD1 to become misfolded when ALS-associated mutant SOD1 was overexpressed in cultured cells (Grad et al., 2011). It was also determined that the prion-like propagation of SOD1 misfolding required the unique tryptophan at position 32 in both seed and substrate molecules (Grad et al., 2011). However, although a potential mechanism of cellular uptake and seeding was suggested, it was not known if or how aggregated or misfolded SOD1 may exit cells and spread. Grad et al. (2014) demonstrated the intercellular propagation of SOD1 misfolding and aggregation, and established two main mechanisms by which spread was occurring: release of aggregates from dying cells, and/or packaging of misfolded protein seed into or on extracellular vesicles called exosomes. More recently, it has been shown that extracellular vesicles purified from the neural tissues of SOD1-G93A mice carry misfolded SOD1 (Silverman et al., 2019), providing further evidence that this mechanism may play an important role in prion-like propagation of protein aggregation in ALS. Additionally, homogenates generated from spinal cord samples of SOD1-fALS patients were recently found to seed the aggregation of ALS mutant SOD1-GFP protein expressed in human embryonic kidney cells (Ayers et al., 2014; Pokrishevsky et al., 2017).

In vivo Seeding and Spread of SOD1 Aggregation

Evidence showing the prion-like propagation of SOD1 *in vivo* shortly followed the cultured cell experiments, providing information on the existence of different transmissible strains of SOD1. Initially, transgenic mice expressing low levels of the SOD1-G85R ALS mutant C-terminally tagged with yellow fluorescent protein (SOD1-G85R:YFP) were used as a model owing to them not developing SOD1-associated ALS-like pathology or symptoms when the transgene was heterozygous (Wang et al., 2009). Spinal cord homogenates harvested from mice overexpressing SOD1 mutants were injected into the sciatic nerve, cervical spinal cord, or cerebral ventricles of SOD1-G85R:YFP mice in an attempt to induce ALS-like pathology and symptoms (Ayers et al., 2014). Potent seeding was observed following injection into the mice, resulting in the formation of SOD1-G85R:YFP aggregates in neurons in spinal cord and motor cortex. The formation of SOD1-G85R:YFP aggregates was accompanied by the typical ALS phenotypes of motor dysfunction and paralysis. Later, this same model was used to examine the anatomical pathways by which SOD1 aggregates may traverse the CNS, finding that aggregation spread through synaptically connected cells in the spinal cord (Ayers et al., 2016b), providing useful information on possible mechanisms of prion-like transmission in ALS.

Considering that slight differences in the conformation of PrP^{Sc} can result in different pathological activities in prion diseases (Prusiner, 2013), it has been suggested that this could also occur in regards to SOD1 aggregation in fALS. Evidence

for the existence of SOD1 strains *in vivo* was first shown by binary epitope mapping the insoluble material from spinal cords of transgenic mice overexpressing WT, G85R, D90A, or G93A SOD1 variants (Bergh et al., 2015), in which two strains (denoted strain A or strain B) were identified on the basis of differential epitope exposure. Mice expressing WT, G85R, and G93A were found to contain strain A aggregates, whereas D90A expressing mice contained either strain A or B aggregates (Bergh et al., 2015). Additionally, strain B aggregates correlated with a more rapid disease progression in mice and were more structurally unstable than strain A aggregates, suggesting aggregate fragmentation as an important mechanism in prion-like spreading (Bergh et al., 2015; Lang et al., 2015). These strains were found to be transmissible to SOD1-G85R transgenic mice through injection of fractions enriched with insoluble SOD1 from strain A or B into the spinal lumbar spinal cord, resulting in strain propagation as measured by binary epitope mapping and ALS-like symptoms (Bidhendi et al., 2016). Similar results were also obtained using the aforementioned SOD1-G85R:YFP mice, where injections of recombinant WT SOD1 protein resulted in aggregates with a thread-like morphology in recipient mice, in comparison to more amorphous features from injections using homogenates from other SOD1-fALS mice (Ayers et al., 2016a). More recently, transmission of strain A was found to occur when enriched insoluble material from the spinal cord of a SOD1-G127X patient was injected into transgenic mice harboring the same mutation (Ekhtiari Bidhendi et al., 2018). Collectively, the combined cell-free, *in vitro*, and *in vivo* studies strongly suggest that SOD1 acts in a prion-like manner in SOD1-fALS.

TDP-43 PRION EVIDENCE

TDP-43 Structure and Function

As mentioned previously, aggregated cytoplasmic TDP-43 is observed in the majority of ALS cases (Ling et al., 2013). TDP-43 is a DNA/RNA-binding protein that has various roles in RNA metabolism, including trafficking, splicing, and degradation (Buratti et al., 2001, 2005; Hefferon et al., 2004; Mercado et al., 2005). The protein contains both a nuclear export signal (NES) and a nuclear localization signal (NLS), however, it is predominantly localized to the nucleus. Structurally, the protein is composed of 4 domains; an N-terminal domain (NTD) with a ubiquitin-like fold (Mompeán et al., 2016), tandem RNA-recognition motifs (RRM), and a prion-like (on the basis of sequence properties similar to yeast prion proteins) C-terminal low-complexity domain (LCD) rich in Gln/Asn residues (**Figure 2A**). The architecture of TDP-43 plays a significant role in its ability to interact with other TDP-43 molecules and other proteins. For example, the NTD can mediate the formation of physiological TDP-43 multimers (Afroz et al., 2017; Mompeán et al., 2017; Tsoi et al., 2017; Wang et al., 2018), and the LCD mediates TDP-43's ability to form or take part in biomolecular condensates (Conicella et al., 2016; Li et al., 2018), such as stress granules (Bentmann et al., 2012).

TDP-43 Pathological Features in Patients

Pathologically, TDP-43 is observed to be mislocalized from the nucleus to the cytoplasm in degenerating neurons, where it forms large ubiquitinated and hyperphosphorylated cytoplasmic inclusions (Neumann et al., 2006). Although there is some debate, strong evidence indicates that at least some of the TDP-43 inclusions observed in patient tissue contain amyloid structures (Mori et al., 2008; Bigio et al., 2013; Nonaka et al., 2013; Porta et al., 2018; Laferrière et al., 2019). Indeed, immunohistochemical examination of sALS patient tissue suggests that maturation of some TDP-43 inclusions proceeds first through small linear "wisps" in the cytoplasm that then go on to form thicker thread-like aggregates, which then grow to form skein-like inclusions (Mori et al., 2008). Additionally, more exhaustive processing of patient tissue to reduce background fluorescence shows that TDP-43 inclusions in ALS are strongly thioflavin-S fluorescent (Bigio et al., 2013). Furthermore, inclusions in sALS patient tissue that are observed to be non-linear at the micron scale are found to have a linear filamentous structure at the scale of tens of nanometers (Lowe et al., 1988; Lowe, 1994), suggesting potential amyloid-like aggregation is associated with TDP-43-related ALS. Biochemical characterization of the insoluble fractions of ALS patient CNS tissue showed that TDP-43 is aberrantly cleaved into 35 kDa and 25 kDa C-terminal fragments (Neumann et al., 2006) which are aggregation-prone and toxic in cell overexpression models (Igaz et al., 2009; Zhang et al., 2009; Yang et al., 2010; Nonaka et al., 2013). Although the TDP-43 C-terminal fragments are associated with ALS and FTD, what causes their fragmentation and what their pathological role might be is still debated (reviewed in Berning and Walker, 2019). It is interesting to note that genetic screening has identified more than 50 mutations in TDP-43 that are ALS or FTD-associated, with most of these mutations being localized to the LCD (Abel et al., 2013), a domain which comprises the bulk mass of the pathological C-terminal fragments.

Amyloid Aggregation of TDP-43

Regarding the aggregation propensity of TDP-43, the LCD is of particular interest since it is predicted to be a prion-like domain (PrLD) on the basis of similarity to the yeast prion protein Sup35 sequence physicochemical properties, which is enriched for asparagine, glutamine, tyrosine and glycine residues (King et al., 2012). Some PrLDs are capable of forming amyloid owing to their amino acid sequence being enriched for polar and uncharged amino acids such as glycine, glutamine, tyrosine, and asparagine (Alberti et al., 2009; Toombs et al., 2010; King et al., 2012). Indeed, application of the 3D-profile method (Thompson et al., 2006) to the TDP-43 LCD identified several regions that could potentially form amyloid fibrils, with subsequent crystallography experiments showing that 6 of these segments ($_{300}$GNNQGSN$_{306}$, $_{321}$AMMAAA$_{326}$, $_{328}$AALQSS$_{333}$, $_{333}$SWGMMGMLASQ$_{343}$, $_{370}$GNNSYS$_{375}$, and $_{396}$GFNGGFG$_{402}$) could form steric zippers (Guenther et al., 2018a), a structure that is characteristic of the spines of amyloid fibrils (Sawaya et al., 2007; Eisenberg and Jucker, 2012). Other studies, using longer peptide segments, have also identified

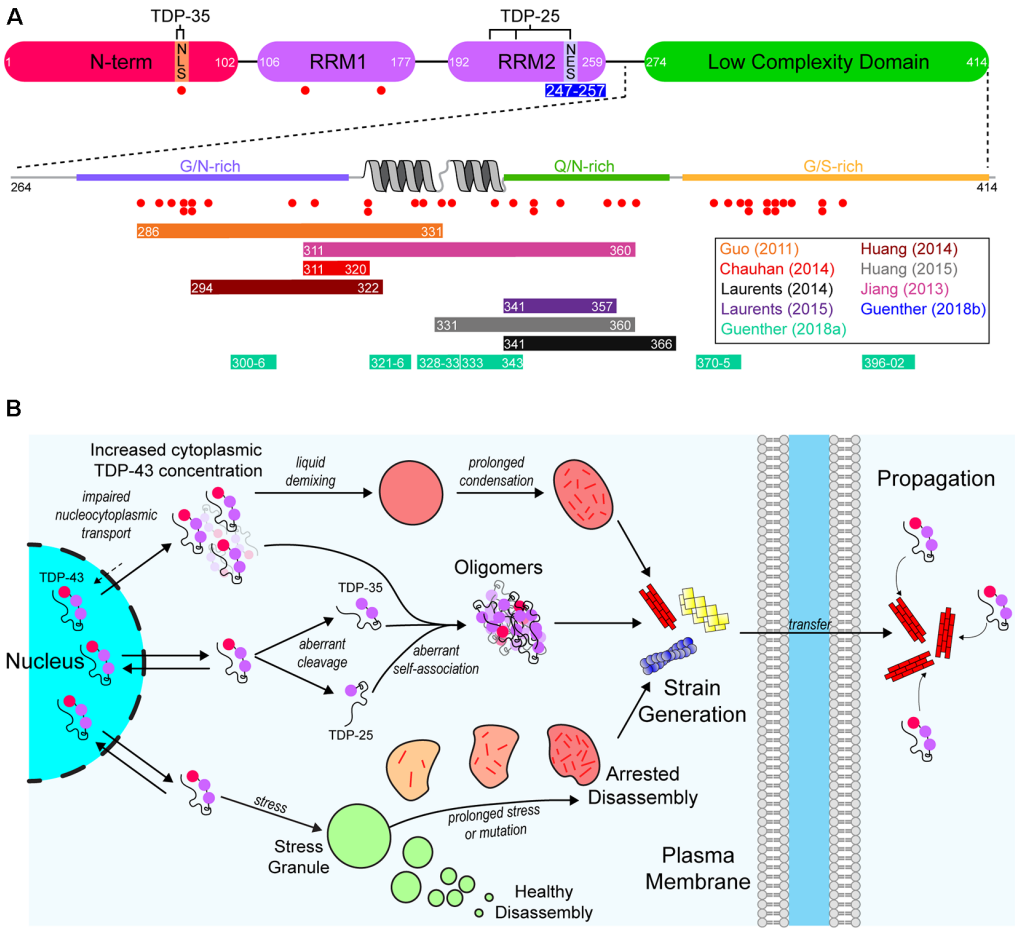

FIGURE 2 | A model of the aggregation and prion-like propagation of TAR DNA-binding protein of 43 kDa (TDP-43) in ALS. **(A)** TDP-43 is composed of four domains which include the ubiquitin-like N-terminal domain (NTD), tandem RNA-recognition motifs (RRM), and a C-terminal low complexity domain (LCD). The NTD contains the nuclear localization signal (NLS) of TDP-43, whereas the nuclear export signal (NES) is in RRM2. The pathological 35 kDa (TDP-35) and 25 kDa (TDP-25) are formed *via* aberrant caspase-mediated cleavage at the NLS and in RRM2 respectively. The blue bar below RRM2 represents an amyloidogenic segment. The LCD is a prion-like domain due to it being enriched for G/N/Q/S residues. It also contains a helix-turn-helix motif which is important for association with other TDP-43 LCDs. Red dots below both the main TDP-43 graphic and the LCD expansion represent ALS-associated mutations. Colored bars represent experimentally identified regions of the protein that are capable of forming amyloid-like structures. Bar colors correspond to the publications listed on the right. **(B)** TDP-43 is a primarily nuclear-localized protein, however, it shuttles between the nucleus and cytoplasm. Impairment of nucleocytoplasmic transport (top) can lead to the cytoplasmic concentration of TDP-43 increasing, leading to liquid demixing to form biomolecular condensates distinct from stress granules. Prolonged residency of TDP-43 in these condensates can lead to the formation of aggregates. Aberrant cleavage (middle) leads to the formation of the highly aggregation-prone TDP-35 and TDP-25 pathological fragments. These fragments, and even full-length TDP-43 can aberrantly self-associate to form oligomers that may grow to become larger aggregates. Also, TDP-43 is known to inhabit stress granules (bottom). Given prolonged stress or genetic mutations, stress granules can persist for longer than necessary and can fail to disassemble, which leads to the formation of aggregates. Each of these possible pathways may also result in strains of TDP-43 aggregates that can propagate from cell-to-cell and seed further aggregation.

amyloid-forming regions within the TDP-43 LCD (Chen et al., 2010; Guo et al., 2011; Jiang et al., 2013, 2016; Mompeán et al., 2014; Cao et al., 2019; **Figure 2A**). A recent study has used cryo-electron microscopy to determine the structure of several TDP-43 LCD polymorphic fibrillar assemblies occurring from two segments of the LCD corresponding to amino acid residues 311–360 and 286–331 (Cao et al., 2019). This has implications for the prion-like nature of TDP-43 as different fibrillar polymorphs may have different prion-like characteristics.

Other than the LCD, a segment of RRM2 ($_{247}$DLIIKGISVHI$_{257}$) has been shown to form several steric zipper polymorphs (Guenther et al., 2018b), suggesting this domain may also contribute to pathological aggregation at least when RRM2 is partially folded (Tavella et al., 2018). Additionally, the size of the TDP-43 fragments (35 kDa or 25 kDa) influences the aggregation and degradation pathways of TDP-43 (Kasu et al., 2018). On the other hand, the majority of evidence for TDP-43 to form amyloid comes from purified protein studies utilizing C-terminal fragments that are either pathological fragments (35 or 25 kDa) or just the LCD (amino acids 274–414), as well as just the RRMs or small segments thereof (Guenther et al., 2018b; Agrawal et al., 2019; Cao et al.,

2019). Less work has been carried out examining the ability of full-length TDP-43 to form amyloid in these assays (Furukawa et al., 2011; Vogler et al., 2018), which may be due to the difficulty in purifying and handling full-length recombinant TDP-43. It appears as though the full-length protein is capable of forming amyloid at low micromolar concentrations (Furukawa et al., 2011), and also capable of being seeded by cell culture-derived TDP-43 positive myo-granules (Vogler et al., 2018). Collectively, the above evidence suggests that, at the least, the pathologic fragments of TDP-43 are capable of forming amyloid and that the LCD makes a significant contribution to the formation of TDP-43 amyloid-like fibrils (**Figure 2A**).

Contribution of Phase Transitions to TDP-43 Amyloid Formation

Considering the importance of the TDP-43 LCD to the formation of both amyloid (Chen et al., 2010; Guo et al., 2011; Jiang et al., 2013, 2016; Mompeán et al., 2014) and biomolecular condensates (Conicella et al., 2016; Li et al., 2018), it is interesting to consider the relationship between these phenomena. Previous studies investigating ALS-associated mutations in both FUS (Murakami et al., 2015; Patel et al., 2015) and hnRNPA1 (Molliex et al., 2015) have highlighted their capacity to mature/age from a liquid-like state to more solid gels composed of fibrillar structures under conditions that promote phase transition. In the case of TDP-43, recent work has suggested that phase transitions of purified TDP-43 LCD promote the formation of amyloid-like fibrils (Babinchak et al., 2019), however, it is important to note that in some cases fibrils generated from the phase transition of PrLDs can be sensitive to sodium-dodecyl-sulfate (SDS; Lin et al., 2015), whereas typical amyloid is resistant to SDS denaturation (Kryndushkin et al., 2003; Halfmann and Lindquist, 2008). Indeed, recent work has highlighted the contribution of small sequence segments containing aromatic residues within PrLDs, called "low-complexity aromatic-rich kinked segments" (LARKS), to reversible phase transition and fiber formation (Hughes et al., 2018). Importantly, LARKS can form fibers that are calculated to have weaker binding energies than the steric zippers that form amyloid spines, evidenced by the LARKS-formed fibrils being susceptible to dissolution by gentle heat (Hughes et al., 2018). It has been suggested that post-translational modification or mutation of the TDP-43 LCD can result in the strengthening of LARKS interactions to become irreversible (Guenther et al., 2018a), although these experiments were performed using small peptide segments of the TDP-43 LCD. It remains to be established if full-length TDP-43 that has undergone maturation in its liquid-liquid phase-separated state forms typical steric zipper amyloid structures, although it is highly likely.

Cell culture studies of the ability of TDP-43 to form biomolecular condensates, and examination of the consequences of condensate formation, have been difficult for several reasons. First, the induction of stress granules requires stressors such as oxidative stress, heat stress, or osmotic stress (Aulas et al., 2017). These stressors not only affect cell health, making the long term monitoring of condensate maturation into aggregates difficult but also induce condensates with different protein constituents dependent on the stressor or cell type used (Aulas et al., 2017). Methods to overcome this have recently been introduced with the usage of light-sensitive proteins to promote protein-protein interactions of proteins capable of undergoing phase transitions (Shin et al., 2017, 2018; Dine et al., 2018; Bracha et al., 2019; Zhang et al., 2019). Replacement of the dimerization domain on G3BP1 with the light-sensitive Cryptochrome-2 (CRY2) protein allowed for the light-inducible generation of biomolecular condensates with similar biochemical and physical properties to stress granules without the addition of stressors (Zhang et al., 2019). The authors found that repetitive or sustained induction of the light-inducible condensates resulted in their evolution to inclusions with the pathological hallmarks of ALS, such as the deposition of SQSTM1 and the hyperphosphorylation of TDP-43 (Zhang et al., 2019). This work suggests that the initial site of TDP-43 aggregate nucleation may be in stress granules or perhaps another biomolecular condensate (**Figure 2B**), although, the authors did not examine if the inclusions formed in this assay were capable of transmitting aggregation pathology to naïve cells. A more recent study has suggested that either addition of preformed TDP-43 amyloid-like fibrils or higher than typical TDP-43 levels in the cytoplasm can provoke the formation of cytoplasmic TDP-43 condensates independent of stress granules (Gasset-Rosa et al., 2019), suggesting that nucleation of TDP-43 aggregates may occur in the absence of typical membraneless-organelles. It remains to be established if the aggregates generated by phase transitions can seed the further aggregation of TDP-43.

Intra- and Inter-cellular Prion-Like Spread of TDP-43 Aggregation

Overexpression of untagged or fluorescently-tagged TDP-43 in cultured mammalian cells can result in its mislocalization and aggregation in the cytoplasm (Johnson et al., 2009; Nonaka et al., 2009; Yamashita et al., 2009; Zhang et al., 2009, 2011; Barmada et al., 2010; Wang et al., 2010, 2013; Xu et al., 2010; Yang et al., 2010; Che et al., 2011, 2015; Furukawa et al., 2011; Gregory et al., 2012; Hong et al., 2012; Chang et al., 2013; Walker et al., 2013; Farrawell et al., 2015; Jiang et al., 2016; Tanaka and Hasegawa, 2016; Afroz et al., 2017; Zeineddine et al., 2017). Furthermore, disruption of the TDP-43 NLS leads to its accumulation and aggregation in the cytoplasm (a mutant denoted as TDP-43$^{\Delta NLS}$; Winton et al., 2008). Indeed, the first evidence for the seeded aggregation of TDP-43 in cells came from experiments utilizing an overexpression of TDP-43 with a C-terminal hemagglutinin (HA) tag (Furukawa et al., 2011). Furukawa et al. (2011) generated recombinant full-length TDP-43 and induced its fibrillar aggregation by vigorous agitation and subsequently introduced these fibrils to cells using lipofection. They found that introduction of TDP-43 aggregates to cells induced the aggregation of the HA-tagged TDP-43 in the cell cytoplasm, and that these cellular aggregates were polyubiquitinated and sarkosyl insoluble (Furukawa et al., 2011). The first evidence of the intercellular transmission of TDP-43 aggregation and relevance to patient pathology came when Nonaka et al. (2013) induced the aggregation of overexpressed HA-tagged TDP-43 in a neuronal cell line through lipofection of the insoluble protein

fraction of human sALS or TDP-43 positive FTD patient brain samples. They observed that addition of either the FTD or sALS samples to cells resulted in the formation of HA-tagged positive TDP-43 positive inclusions. Additionally, they determined that the inclusions formed by the brain fractions in cultured cells could be used to seed further aggregation in cultures of naïve cells (Nonaka et al., 2013), providing substantial evidence supporting a pathological prion-like propagation of TDP-43 in FTD and ALS. Later studies showed similar results for the cell-to-cell spread of aggregation using stable cultures (Porta et al., 2018; Laferrière et al., 2019) overexpression cultures (Smethurst et al., 2016), co-cultures (Zeineddine et al., 2017), and even showed the axonal uptake and transport of TDP-43 seeds (Feiler et al., 2015).

Currently, cell cultured-based experiments provide the most well-established examinations of pathologic TDP-43 prion-like strains. Sequential deletion of amino acid segments, 20 residues in size, from the C-terminal region of TDP-43 highlighted amino acids 274–313 or 314–353 as being important for the cellular aggregation of TDP-43 (Shimonaka et al., 2016), suggesting that separate regions of the protein could be responsible for aggregation. Indeed, injection of amyloid fibrils composed of synthetic peptides composed of residues 274–313 or 314–353 resulted in the template-directed aggregation of wild-type TDP-43 or TDP-43$^{\Delta NLS}$ indicating that more than one region of the C-terminal region of the protein was capable of seeding pathologic aggregation (Shimonaka et al., 2016), however, the authors of this study did not note differences in aggregation characteristics. Although this provides evidence of possible strain-like characteristics of TDP-43, synthetic peptides provide little relevance to the pathological structures that may exist in disease. More recently, strong evidence for the existence of TDP-43 strains in human FTD patients was provided by Laferrière et al. (2019) when they carefully processed human tissue from FTD patients and ALS patients to enrich for pathological TDP-43. Examination of the size of the enriched TDP-43 assemblies from patients using velocity sedimentation showed that disease subtypes had different sized assemblies present, and that material from different disease subtypes as capable of inducing different levels of toxicity in HEK cells stably expressing HA-tagged TDP-43 and primary mouse cortical neurons (Laferrière et al., 2019). Most importantly, the authors showed that the toxicity of the TDP-43 assemblies to cultured cells correlated with the severity of the disease determined in the donor patient. It will be interesting to see if the disease severity will correlate with an *in vivo* model of TDP-43 prion-like propagation in future work.

In vivo Seeding and Spread of TDP-43 Aggregation

Evidence supporting the prion-like propagation of TDP-43 aggregation *in vivo* occurred only recently. Porta et al. (2018) screened the prion-like properties of FTD patient brain extracts using cultured cells. Samples that had seeding activity were directly injected into the brains of mice expressing human TDP-43 with a mutated NLS signal under a doxycycline controllable promoter in their forebrain neurons (Igaz et al., 2011; Alfieri et al., 2014). It was found that injection of FTD patient-derived TDP-43 enriched samples led to the formation of pathologically relevant TDP-43 inclusions in the recipient mice (Porta et al., 2018). Additionally, careful immunohistochemical analysis of the injected mice showed that pathology appeared to spread through connected neuroanatomical tracts in a time-dependent manner, and interestingly, that in some cases endogenous mouse TDP-43 was colocalized with human TDP-43 inclusions. The authors then went on to inject seeding-competent, human-derived FTD material into non-transgenic wild-type mice, finding that the endogenous mouse TDP-43 could be seeded also, but to a lesser extent comparative to the doxycycline-regulated human TDP-43 transgenic mice (Porta et al., 2018). It was also determined that mice expressing a C-terminal fragment under a doxycycline regulatable element had a lower efficiency of seeding after injection with the FTD patient-derived samples, suggesting that the N-terminal regions of TDP-43 play an important role in its self-recognition and prion-like aggregation. Although the study by Porta et al. (2018) provides strong evidence for the prion-like role of TDP-43 in FTD, the experimental transmissibility of ALS patient-derived TDP-43 aggregates remains to be established.

FUSED IN SARCOMA AND C9orf72

Fused in Sarcoma

Other than TDP-43 or SOD1, FUS plays an active role in the etiology of ALS owing to its presence in inclusions in a small percentage of ALS patients (Ling et al., 2013), and it containing several ALS-associated mutations (Kwiatkowski et al., 2009; Vance et al., 2009). Pathologically, FUS mostly forms basophilic inclusion bodies and was first found to play a role in a form of FTD called basophilic inclusion body disease (Munoz et al., 2009). Previous immunohistochemical analysis of basophilic inclusions in aytpical ALS patient tissue identified key constituents of stress granules [poly(A)-binding protein, TIA-1, and ribosomal protein S6] but not markers of other biomolecular condensates such as processing bodies or ribonucleoprotein particles (Fujita et al., 2008). Importantly, this work showed for the first time that stress granules play an important role in ALS pathogenesis.

Similar to TDP-43, FUS is an RBP which plays crucial roles in RNA metabolism (Belly et al., 2005; Fujii and Takumi, 2005; Andersson et al., 2008), but is also important for the DNA-damage repair response (Wang et al., 2008, 2013). Structurally, FUS belongs to the ten-eleven translocation (TET) protein family which are characterized by their N-terminal QSGY-rich domains, conserved RRMs, RGG-rich regions, and a C-terminal zinc finger motif (Morohoshi et al., 1998; Iko et al., 2004). Importantly, the N-terminal QSGY-rich domain (residues 1–165) of FUS is predicted to be a prion-like domain (PrLD; King et al., 2012), suggesting that this domain plays a role in the pathological aggregation of FUS observed in patients. However, the majority of ALS-associated mutations identified in FUS are localized to the N-terminal RGG-rich region (residues 165–276) or a short stretch (residues 495–526) of the C-terminal RGG-rich region (residues 449–526) which contains the FUS NLS (Abel

et al., 2013); only one ALS-associated mutation (G156E) has been identified in the PrLD.

Examination of purified FUS PrLD has shown that it can undergo a phase transition to form hydrogels composed of amyloid-like fibrils (Han et al., 2012; Kato et al., 2012; Kwon et al., 2013). Additionally, EGFP-tagged full-length FUS can form biomolecular condensates in which the G156E mutation can exacerbate a transition from liquid to fibrous solid (Patel et al., 2015). Interestingly, although the ability of purified FUS to form aggregates is established, evidence suggesting that these assemblies can seed the aggregation of soluble FUS protein has only recently been established (Nomura et al., 2014). Nomura et al. (2014) generated purified GST-tagged FUS wild-type and the G156E mutant, finding that the G156E mutant was more prone to form fibrils and that these fibrils were capable of seeding purified wild-type FUS. They then went on to transfect rat-hippocampal primary neurons with both G156E FUS and wild-type FUS constructs, finding that inclusions would only form for the mutant and that these inclusions would recruit the plasmid derived wild-type FUS (Nomura et al., 2014). Other than the work by Nomura et al. (2014), there has been little research carried out on examining the prion-like characteristics of FUS. Future work examining if FUS has bona-fide prion-like characteristics (seeding from exogenous aggregates, cell-to-cell propagation) will be important in determining if a prion-like mechanism is common across the main aggregation-prone proteins in ALS (SOD1, TDP-43, FUS).

C9orf72

Hexanucleotide repeat (GGGGCC) expansions in *C9orf72* were first identified in DeJesus-Hernandez et al. (2011) and Renton et al. (2011) and are now thought to be responsible for approximately 40% of fALS cases (Majounie et al., 2012). Typically, the number of repeats in the healthy population is $\leq$11, whereas the number of repeats in an individual suffering from *C9orf72*-associated fALS can number in the thousands (van Blitterswijk et al., 2013; Suh et al., 2015). Although exact functions for the protein encoded by *C9orf72* is yet to be fully elucidated, there is evidence to suggest that it has a role as a guanine nucleotide exchange factor for small GTPases (Wu et al., 2011; Levine et al., 2013; Iyer et al., 2018). The protein encoded by *C9orf72* is also implicated in other cellular functions such as autophagy (Sellier et al., 2016; Webster et al., 2016) and vesicular transport (Aoki et al., 2017). Although there is little known about the function(s) of the C9orf72 protein, understanding of the mechanisms by which disease arises consequently from repeat expansions has proceeded rapidly.

There are now thought to be several mechanisms by which repeat expansions in *C9orf72* cause toxicity, including a loss of function, transcription into long repetitive RNA that forms foci composed of sense or antisense RNA (Donnelly et al., 2013; Gendron et al., 2013; Lagier-Tourenne et al., 2013; Mizielinska et al., 2013; Zu et al., 2013), and the translation of repeat RNA into five different dipeptide repeat (DPR) proteins (poly-GR, poly-GP, poly-GA, poly-PR, and poly-PA; Mori et al., 2013). Currently, there is little evidence to suggest a prion-like propagation of any of the DPRs generated in C9orf72-associated ALS. One study by Westergard et al. (2016) used a battery of cell culture experiments to examine DPR spread between cells. They determined that all DPRs, with the exception of poly-PR, were capable of transfer between cells through anterograde and retrograde mechanisms, and that this transfer can occur through both exosome-dependent and exosome-independent mechanisms Modes of transfer of prion-like material is summarized in **Figure 3**. Although this work provides strong evidence for the cell-to-cell transfer of certain DPRs, it is still not established if DPRs can assume a conformation with prion-like characteristics or if they can seed further aggregation of other DPRs.

PERSPECTIVE AND CONCLUSION

It is now established that prion-like mechanisms play key roles in the cell-specific nature and cell-to-cell spread of pathology in neurodegenerative disease (Prusiner, 2013). In particular, ALS shows the hallmarks of prion-like propagation owing to the spread of both symptoms and pathology throughout affected tissues (evidence is summarized in **Table 1**). Different types of research including biophysical, biochemical, and biomedical studies have increased our understanding of the contribution of prion-like mechanisms to ALS. Cell-free *in vitro* examination of the aggregation and seeding characteristics of prion-like proteins associate with ALS have provided useful information on the kinetics of aggregation and the biophysical traits of the substrate and seed species. Cell culture experiments have helped establish the cellular mechanisms by which proteinaceous aggregation may occur, and how cells may facilitate the transmission of the prion-like particles. Research using animal models has been effective at establishing the biochemical and phenotypic profiles of some prion-like proteins and strains in ALS and has also helped in determining if material from patients suffering ALS or FTD have prion-like (PrP-like) properties (Ayers et al., 2014, 2016a,b; Bergh et al., 2015; Lang et al., 2015; Bidhendi et al., 2016, 2018; Laferrière et al., 2019).

Although great progress has been made in the understanding of the prion-like nature of ALS, what remains to be elucidated are high-resolution structural models of specific ALS-associated prion strains that are related to disease pathology. While recent work has identified several fibrillar polymorphs of the TDP-43 low complexity domain (Cao et al., 2019), it remains to be established if these conformational assemblies have relevance to pathogenicity *in vivo*, missing a crucial link between conformation of the amyloid-like assemblies and their toxicity. If we are to develop effective therapeutics against such polymorphic protein assemblies, we need to know what to target. Future experiments investigating the roles of prion-like propagation in human disease should combine *in vivo* models of seeding and neuroanatomical spread, cell culture studies of seeding potency of different strains, and the structural resolving power of cryo-electron microscopy for structure determination.

The existence of prion-like conformational strains in ALS provides not only a challenging hurdle to overcome experimentally and therapeutically, but also provides opportunities to develop effective therapies. With access to

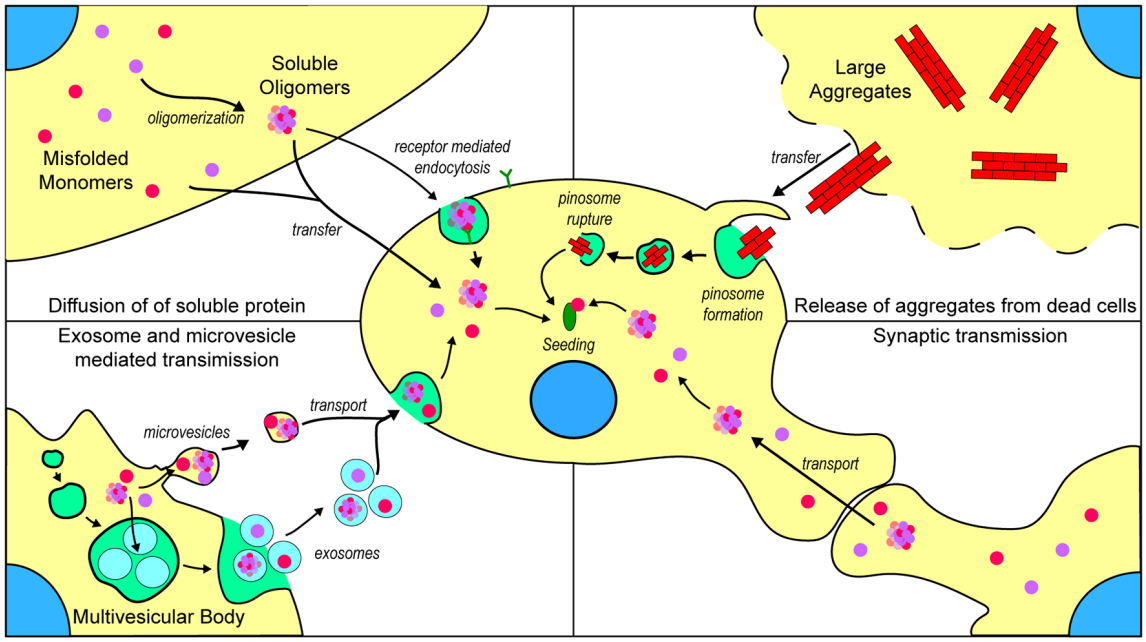

FIGURE 3 | Mechanisms by which prion-like ALS-associated protein aggregates may transfer between cells. (Top left) Once formed, misfolded monomers and soluble oligomers can potentially exit the cell through diffusion and enter nearby cells either through diffusion or receptor-mediated endocytosis. (Top right) Cells that are dead or dying can potentially release larger insoluble aggregates that can be taken up into nearby naïve cells through micropinocytosis. (Bottom left) Misfolded and oligomeric proteins are capable of being loaded into either microvesicles or exosomes for transport to nearby cells. (Bottom right) Misfolded monomers or soluble oligomers are capable of being transported across axon terminals to naïve cells.

disease-specific structural models of prion-like aggregates, researchers have the capability to perform large-scale structure-based small molecule docking screens, which may identify compounds that could alleviate or prevent the occurrence or spread of pathology in prion-like diseases (Jiang et al., 2013). Furthermore, structure-based design of monoclonal antibodies targeted against misfolding specific epitopes is a growing space with great potential. Given a prion strain structure, an antibody could be rationally designed to target a specific conformation of protein assembly to prevent further spread or promote clearance (Peng et al., 2018; Silverman et al., 2018). An advantage of this approach is that the use of monoclonal antibodies would overcome target distraction through their high binding specificity.

Lastly, the role of prion-like propagation of pathologic proteins in ALS is likely tied to the mostly idiopathic nature of the disease. Whilst there are mutations that are ALS-causative, the majority of cases remain sporadic, suggesting that an environmental trigger may exist. From this, a question that must be asked is whether or not all, or a set, of sporadic cases of ALS are the result of a still-unidentified prion-like agent in the environment. Recent research has provided evidence supporting the Braak hypothesis (Braak et al., 2003) that pathologically aggregated α-synuclein can propagate from the gut, through the vagus nerve, to the ventral midbrain (Kim et al., 2019). Furthermore, some patients who received human growth hormone from patients suffering from Creutzfeld-Jakob disease (CJD) developed CJD themselves (Gibbs et al., 1985). Surprisingly, some of the patients who received growth hormone from CJD sufferers were found to have amyloid-β pathology, suggesting that amyloid-β had also propagated in these cases (Jaunmuktane et al., 2015). Considering the above evidence, there is a possibility for similar mechanisms to be occurring in ALS, however, there is currently no strong evidence supporting this hypothesis. Epidemiological studies may provide further insight into links between the populations that develop sporadic ALS beyond just genetic background.

AUTHOR CONTRIBUTIONS

LM wrote the initial manuscript. SP edited and revised the manuscript. JY edited and revised the manuscript. NC conceived, edited and revised the manuscript.

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CORRECTION
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Corrigendum: Prion-Like Propagation of Protein Misfolding and Aggregation in Amyotrophic Lateral Sclerosis

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Prion-Like Propagation of Protein Misfolding and Aggregation in Amyotrophic Lateral Sclerosis

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In the original article, there was a mistake in **Table 1** as published. The incorrect original research articles were referenced for some of the table cells. The corrected **Table 1** appears below.
The authors apologize for this error and state that this does not change the scientific conclusions of the article in any way. The original article has been updated.

TABLE 1 | Evidence supporting the prion-like characteristics of ALS-associated proteins.

Protein/Gene	*in vitro* fibril formation	*in vitro* fibril seeding	Cell seeding with *in vitro* protein	Cell-to-cell propagation	Human to cell propagation	*in vitro* to animal propagation	Animal to animal propagation	Human to animal propagation
SOD1	✓ Chattopadhyay et al. (2008)	✓ Chia et al. (2010)	✓ Munch et al. (2011)	✓ Grad et al. (2014)	✓ Pokrishevsky et al. (2017)	✓ Ayers et al. (2016a)	✓ Ayers et al. (2014)	✓ Ekhtiari Bidhendi et al. (2018)
TDP-43	✓ Johnson et al. (2009)	✓ Furukawa et al. (2011)	✓ Furukawa et al. (2011)	✓ Nonaka et al. (2013)	✓ Nonaka et al. (2013)	–	–	✓ Porta et al. (2018)
FUS	✓ Nomura et al. (2014)	✓ Nomura et al. (2014)	–	–	–	–	–	–
C9orf72 (DPRs)	–	–	–	✓ Westergard et al. (2016)	–	–	–	–

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