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Misfolded Protein Linked Strategies Toward Biomarker Development for Neurodegenerative Diseases

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Abstract

The progressive loss of structure and function of neurons causes various neurodegenerative diseases which need to be examined using measurable indicators, known as biomarkers. Proteins are the building blocks for the cell and are essential as they participate in many processes in the cells. When biologically essential proteins are impaired, it leads to devastating consequences in humans and mammals among which the most prominent is neurodegenerative disease. Proteins conform to three-dimensional structures to enable their functions. Besides, some proteins have the tendency to form self-assembly structures. When these self-assembly proteins assume abnormal conformation, they accumulate and cause pathological conditions. The genetic and molecular origins of protein misfolding in association with their relationship with neurodegeneration and aging are being studied to better understand and develop treatments. Accumulations of these misfolded proteins form aggregates which is considered as the most prominent cause of many neurodegenerative diseases. This article reviews the misfolded proteins in various neurodegenerative diseases and analyzes the diverse aspects of protein misfolding as a potential agent of biomarkers with an approach for finding an inhibitor for misfolding.

Keywords Misfolded protein · Neurodegenerative diseases · Biomarkers · Inhibitors

Background

Advancements in medical science and technology address mostly the diseases; however, there are few diseases in which no dramatic developments have occurred. Understanding the mechanisms and finding a cure for neurodegenerative diseases need to be an area of focus in forthcoming years. Basic research has addressed the root cause for some neurodegenerative diseases (Alzheimer's disease, Parkinson's disease, Huntington's disease, and amyotrophic lateral sclerosis) and extensive research has been carried out worldwide to address these issues. Protein misfolding is generally considered as a prominent cause of neurodegenerative diseases. Figure 1 shows the protein folding inside the cell and differentiates between normal and misfolded protein [1].

The abnormal conformation and folding pathway of proteins, also known as protein misfolding, results into neurodegenerative diseases like Alzheimer's and Parkinson's disease. Protein misfolding in Alzheimer's disease (AD) is generally due to the amyloid precursor protein (APP), apolipoprotein E gene (ApoE), and protein from the ApoE. Overexpression of proteins like presenilin-1 (PS1), presenilin-2 (PS2), and leucine-rich repeat kinase 2 (LRRK2) signifies Parkinson's disease (PD) and the aggregation of the superoxide dismutase 1 (SOD1) is a primary factor in the development of amyotrophic lateral sclerosis (ALS).

Advancements in science and technology in the field of neurodegenerative diseases have occurred over the past 20 years, however is limited in the area of prevention. The biomarkers

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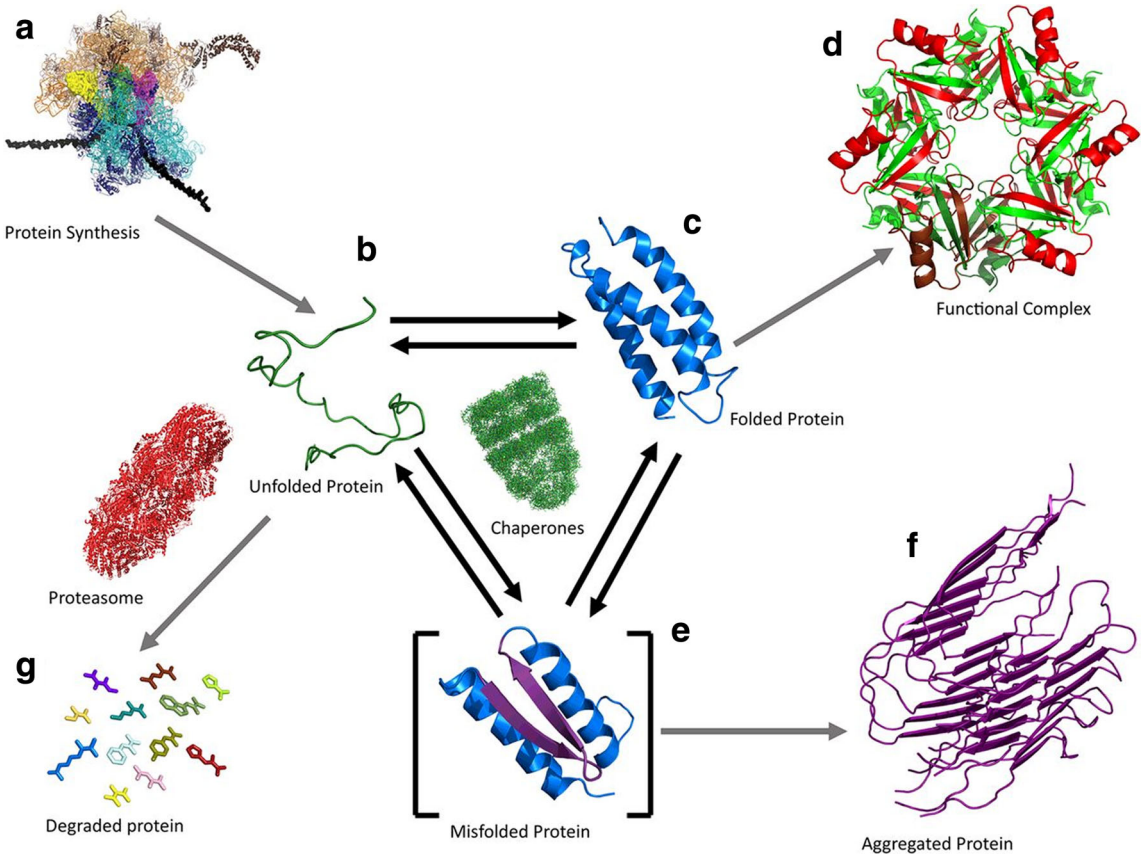

Fig. 1 Protein folding inside the cell: a new protein is synthetized at the ribosome as determined by the activation of gene expression. The nascent chain is typically bound to chaperones that keep it unfolded until the process is complete and the chain is released (**a**). The recently synthesized unfolded protein folds autonomously and reversibly, establishing a dynamic equilibrium between the unfolded ensemble (**b**) and the native state (**c**). This conformational equilibrium determines the protein's ability to perform its function, either directly as a monomer or by further assembling into larger macromolecular complexes (**d**). The half-life of its biological activity is also controlled by the folded–unfolded equilibrium because the proteasome machinery eliminates targeted proteins from their unfolded state (**g**). Likewise, the transient formation of partially folded conformations can lead to misfolding events (**e**) that feed the formation of aberrant and/or toxic protein aggregates (e.g., amyloids) (**f**). *This extract/figure/table was originally published in Ref.* [1]

which have been found in the past two decades can only diagnose disease after onset; early detection is still not possible. Diagnosing neurodegenerative disorders at the nascent stage and preventing their development has not been accomplished. Since disease-causing proteins and interacting proteins are the starting point, it is proposed to perform multiple proteolysis and make a library of short peptides. The methods used are (i) determining the active part of the library peptides, (ii) designing the mimicking peptides/with additional derivatives in the peptides, (iii) synthesizing or cloning the peptides and overexpressing them, (iv) labeling the modified peptides, and (v) using the modified peptides as the marker or inhibitor. In these hits, more scientific evidence and new early biomarkers and early inhibitors to stop the progression of the disease can be explored.

Protein Biomarkers as a Main Target

The proteolytic cleavage of amyloid precursor protein (APP) and its aggregation creates amyloid plaques which are composed primarily of amyloid-beta (Aβ) and considered as a main factor in the development of Alzheimer's disease (AD). Present diagnostic methods can only characterize the disease after its onset; early detection is still not achievable. In life, it is too late to prevent the pathogenesis after the clinical signs begin and diagnosis is made. The presently available treatments only slow progression for a time and cannot cure or reverse the disease. During the onset of AD, some brain function and memory is lost and the treatment only maintains the remaining brain function and memory for a time. Retention of brain function and memory and their sustainability depends on many factors and current medications only work for a few years.

The improper cleavage of Aβ4 by γ-secretase at C-terminus of Aβ sequence resulted two major isoforms of Aβ: Aβ40 (40 residue long) and Aβ42 (42 residue long). The increase in concentration of plasma Aβ40 and Aβ42 and their interplay have been considered to play essential role in AD. Increase ratios of Aβ42/Aβ40 have direct correlation to early onset of AD [2]. When plasma Aβ42 exceeds a limit in AD patients, there is an increased chance of mortality [3]. Contrasting studies show that

the high concentration of Aβ42 in the plasma of normal aging does not cause AD [4–7], but an increased concentration of Aβ42 in cerebrospinal fluid (CSF) is directly correlated to the progression of AD [8]. Earlier studies clearly show that a panel of 18 predictors which belong to cell-signaling proteins lead to a more than 80% probability of developing AD with aging [9]. This study identified AD-specific signature from more than 120 proteins. Computational network analysis using Ingenuity Pathway Analysis, identified two network of proteins, one involving tumor necrosis factor (TNF)-a and monocyte-colony stimulating factor (M-CSF), while the second pathway involving epidermal growth factor (EGF). The noticed imbalance of signaling pathways represented by these 18 predictors possibly predict early onset of AD. The "cellular communicome" consisting of specific signature may exist for other neurodegenerative diseases which potentially create early diagnosis.

Alzheimer's Disease and the Associated Protein

Alzheimer's disease (AD) is a chronic neurodegenerative disease which initially starts with a low impact and progresses over time. Full-blown AD can reflect symptoms like memory loss, disorientation, problems with speech and language, self-mismanagement, behavioral issues, etc. The progression of this disease can vary from person to person with high chances of mortality at the full-blown stage. More than 70% cases of AD are caused by a genetic disorder with several genes involved [10] and the remaining cases are due to other factors.

Based on cognitive and functional impairment, AD is characterized by four primary stages: stage 1: pre-dementia, stage 2: early AD, stage 3: moderate AD, and stage 4: advanced AD. The pre-dementia stage is linked with thoughtfulness, scheduling, suppleness, and abstract judgment/semantic memory [11]. The early stage is marked with difficulties in language, executive functions, and execution of movements [12]. In the moderate stage, the progression of disease is rapid and can be clearly seen with speech difficulties and dependency on others for activities of daily living. The advanced stage is a full-blown disease stage and the patient is totally dependent on others for day to day activities with loss of speech [12]. There are various hypotheses derived for AD like the cholinergic hypothesis, amyloid hypothesis, Tau hypothesis, and others; here, we will discuss two most important ones namely amyloid hypothesis and Tau hypothesis.

Amyloid Hypothesis

Amyloid precursor protein (APP) is an important protein which helps in neuronal growth, its survival and healing in the event of damage [13, 14]. However, the physiological role of APP remains unknown but knockdown of APP using siRNA shows abnormalities in neuronal migration in mouse embryos [15]. Due to proteolytic cleavage of the APP by secretases, protein misfolding occurs and results in amyloid-beta fibrils [16]. The proteolytic processing of APP by secretases releases secretory fragments. The ectodomain released due to the activity of α-secretase cleavage, produces no amyloidogenic formation. On the other hand, cleavage by β-secretase (BACE1) led to improper fragmentation of APP, in which the ectodomain contains additional 16 amino acids at the C-terminus. Interestingly, Nikolaev et al. [17] established that the during growth factor deprivation, BACE1 generated ectodomain interacts with death receptor 6 (DR6) causing neuronal degeneration. Current researches note that extracellular amyloid-beta (Aβ) aggregations are the main cause for the occurrence of AD [18, 19]. This postulate is also supported by the site of the gene on chromosome 21 for the amyloid precursor protein (APP). Apart from this, there is one more isoform of apolipoprotein, the APOE4, which is also considered as a high-risk factor for this disease. Protein misfolding caused by the Aβ protein results in the aggregation of protein and plaque formation. The aggregations are made up of small peptides ranging from 39 to 43 amino acids and are called Aβ which is a portion of the APP. Figure 2 illustrates the cleavage of APP molecule and formation of amyloid-beta plaques.

Tau Hypothesis

Tau is a microtubule-associated protein that stabilizes the assembly of tubulin. It is located in the chromosome 17 and has been reported to exist as six isoforms in human brain. Several laboratories have established the formation of neurofibrillary tangles (NFTs) with tau in AD pathologies [20]. Under tau hypothesis, the principle cause for AD is tau. Accumulation of hyperphosphorylated tau has been reported in the AD brains [21–24]. Interestingly, accumulation of tau in the neuronal cell bodies due to failure of tau protein sorting has been proposed for AD [25] and using genetic models, it was demonstrated that extracellular accumulation of Aβ fibrils is not toxic to nerve cells; further formation of NFTs is also abolished in these model animals. Kim et al. have demonstrated that overexpression of Aβ42 in BRI2-Aβ mice did not induce neuronal loss or impairment of cognitive functions [26, 27]. These results indicate that Aβ42 oligomers and amyloid fibrils are not neurotoxic; further immunotherapies targeting Aβ decreased the Aβ deposition in mice brains but did not alter accumulation of tau [28–31]. Abnormalities in tau have also been reported in several other neurodegenerative disorders such as frontotemporal dementia, Parkinson's disease, Pick's disease, etc. Several mutations in the tau gene results in the accumulation of tau; however, in sporadic cases including AD, the initial cause for the accumulation of wild-type tau is unclear. In total, tau pathology causes extensive damage to the neuronal cell by disrupting cellular machinery such as cytoskeletal, transport, and signaling systems.

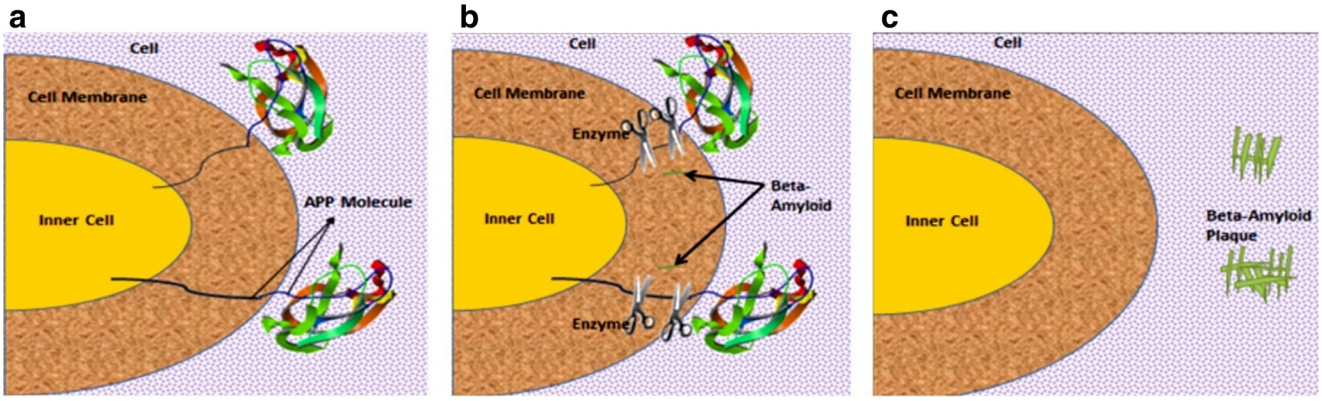

Fig. 2 **a**–**c** Illustration of the cleavage of the APP molecule and formation of amyloid-beta plaques

Parkinson's Disease and the Associated Proteins

Parkinson's disease (PD) is another neurodegenerative disease which causes degeneration of central nervous system. The dopamine-producing cells are located in the substantia nigara in the mid-region of the brain. Due to unknown reasons, the dominie generating cell death occurs and results in Parkinson's disease (PD). This disease initially presents with difficulty in movement like trembling, stiffness, slow movement, and trouble in walking and gait. The disease progresses with time and the patient faces difficulties with thinking, dementia, sleep disorders, and emotional imbalance.

PD—Genetic or Non-genetic?

In general, PD is considered as a non-genetic disease and only a small portion (~15%) of the patients are associated with a genetic disorder. The cause of PD in most patients is idiopathic. Studies have shown that gene mutations in parkin (PRKN), alpha-synuclein (SNCA), ATP13A, PTEN-induced putative kinase-1 (PINK1), DJ-1, and leucine-rich repeat kinase-2 (LRRK2) can cause this disease [32].

Role of Protein in PD

Proteins like alpha-synuclein, PS1, PS2, LRRK2, Htra1, pink1, and DJ1 are involved in Parkinson's disease; alpha-synuclein and DJ1 function show that these two proteins play an active role in PD [33].

Alpha-synuclein protein is one of the most important proteins present in CSF in monomer form with appropriate concentration. This protein is secreted from the cells and functions in neuronal plasticity which codes presynaptic proteins after mutations in α-synuclein [34, 35]. When there is an insufficient concentration of α-synuclein monomer protein, the chances of developing Parkinson's disease is high. After misfolding of the α-synuclein protein, the protein oligomerises and there is an increased concentration of the oligomer in cerebrospinal fluid (CSF) and blood of Parkinson's patients.

Another protein, Parkinson disease protein 7, causes Parkinson's disorder (autosomal recessive, early onset) and is encoded by the PARK7 gene. This protein has interesting functions like (1) working as an up-regulator of androgen receptor dependent transcription, (2) redox-sensitive chaperon, and (3) protecting neurons against oxidative stress and cell death. Due to defects in the PARK7 gene, autosomal recessive onset of PD 7 occurs [36].

Treatment of Parkinson's Disease

The therapies available currently can only delay the onset of symptoms and there is no permanent treatment or cure for this disorder. These therapies are mainly focused on increasing the dopamine level in the brain or inhibiting its breakdown by prolonged dopamine action. Levodopa is an effective medicine which is converted into dopamine in order to delay the onset of PD [37]. Long-term incorporation of levodopa can cause side effects, but the combination of carbidopa and lower amount of levodopa reduces potential side effects [38]. There are other options like surgery for patients who cannot be managed with medication. Surgery includes the implantation of electrodes to activate the area of the brain involved in movement [39]. Another therapy which was recently developed is stem cell therapy which can create a dopamine-producing cell. Research is still ongoing to develop this therapy and provide better management. Two important study that showed intracerebral grafting of fetal mesencephalic dopamine-rich tissue in rats ameliorated signs of PD laid foundation for the cellular therapy for PD [40, 41]. Several studies in human showed initial promise but were discontinued due to lack of efficacy and adverse effects [42–44]. Stem cells (neural stem cells, embryonic stem cells, and induced pluripotent stem cells) were proposed to generate dopaminergic neurons for cell-based therapy for PD. Purified

(cell sorting) dopaminergic neurons could be achieved from the stem cells using established protocols [45–47]. Kriks et al. [48] reported an efficient dopaminergic neuron differentiation methodology and transplantation of these cells resulted in improved condition in rats. Follow-up studies using various cell sources showed progress in the cell therapy area for PD [49–52].

Amyotrophic Lateral Sclerosis—a Mystery?

Amyotrophic lateral sclerosis (ALS) disorder is known to cause neuronal death and is also called Lou Gehrig's or Charcot disorder [53]. Only 5 to 10% of the cases are genetic and mostly 90 to 95% of cases the cause for the disease is unknown; there is no cure for this disorder [54]. In genetic cases, one of two specific genes is responsible. Some 2% of cases reported overall are due to a defect on chromosome 21 that codes superoxide dismutase [55–57]. ALS symptoms include muscle twitching, muscle stiffness, weakness in the limbs, difficulty in chewing and swallowing, and slurred speech.

There is no clear hypothesis or any evidence to date on the cause of ALS. However, there are research reports about gene mutation, which results in production of SOD1 enzyme which is one of the main causes for this disease. However, there is no concrete evidence that the SOD1 gene may cause motor neuron degeneration [58]. It has been postulated that SOD1 mutants, which are made to promote trimerization, cause an increase in cell death and there exists a relationship between the misfolded oligomer's presence and neuron death [59]. There are many conformations that lead mutant SOD1 protein to turn toxic and to date, more than 12 mutations have been identified. Mutation in ALS changes the processing of RNA molecules that can lead to motor neuron degradation. There is research which reveals that the defect in c9 or f72 is not only present in the subset of ALS patients but also in frontotemporal dementia (FTD). Figure 3 shows the comparison between normal and ALS-affected nerve cell and muscle. When ALS affects the motor neurons that provide voluntary movement and muscle power, the muscles of the arms and legs are affected. It does not affect the muscles in heart and digestive system but there is no clear evidence until now for this.

Huntington's Disease

Huntington's disease (HD) is a neurodegenerative disease caused by a genetic disorder which alters muscle coordination and generates a mental decline and behavioral symptoms. The genetic disorder is stated to be the main cause for this disease and it affects involuntary writhing movements called chorea. HD is also called as Huntington's chorea. In either one of the autosomal dominant mutations, Huntington's disease is caused in any one of the individual two copies of a gene known as Huntingtin.

Huntingtin protein function is still unknown but supposed to have a major role in the brain nerve cells. Huntington's disease is caused by an *HTT* mutation which leads to the formation of a CAG trinucleotide repeat. This trinucleotide segment is made up of three DNA building blocks (cytosine, guanine, and adenine) that appear numerous times in a row. In the normal gene, CAG repetition is 10 to 35 times while in a Huntington diseased gene, CAG is repeated 36 to 120 times. The signs and symptoms of HD may develop when repetition is more than 40 times. Since a CAG repeat produces a longer fragment, the elongated fragment is not sustainable and gets cleaved to smaller fragments. The cleavage is accumulated into toxic fragments and overlays onto neurons and thus disrupts the normal function of cells. Due to toxic fragment overgrowth, loss of the neurons in certain regions of the brain takes place and results in Huntington's disease. Huntingtin protein has an important role in growth and its absence is lethal [60]. This protein lacks the sequence homology when compared with other proteins and is expressed highly in humans and rodents neurons and testis [61, 62]. There is a study which shows that Huntingtin positively regulates the expression of BDNF at transcription factor point, but the gene expression mechanism of regulation has not been determined. The association of Huntingtin is found with vesicles and microtubules primarily; it has been studied by electron microscopy, immunohistochemistry, and subcellular fraction studies of the molecule [63, 64].

Protein Misfolding—a Major Cause for Neurodegenerative Disease?

Cellular physiological status of the cell is determined by mainly proteins. The proteome of a cell needs to be balanced between synthesis and degradation. Several biological functions of the protein are determined by the conformation of the protein. For example, ligand binding and release depends on the three-dimensional conformation of the protein molecule. Conformation of the protein molecule is determined by the amino acid sequence and formation of interactions between specific amino acids. Any change in the key amino acid sequence might impact the conformation of the synthesized protein. Since 10,000 to 20, 000 proteins are actively being synthesized in a cell, the process is error prone resulting in misfolded proteins. A molecular mechanism consisting of several chaperones exist in cells to verify the nascent polypeptide during translation by ribosomes. However, parallel mechanisms exist in cooperation with chaperone to clear the misfolded proteins [65]. Protein misfolding leads to pathologies either by loss of function or toxic accumulation. Majority

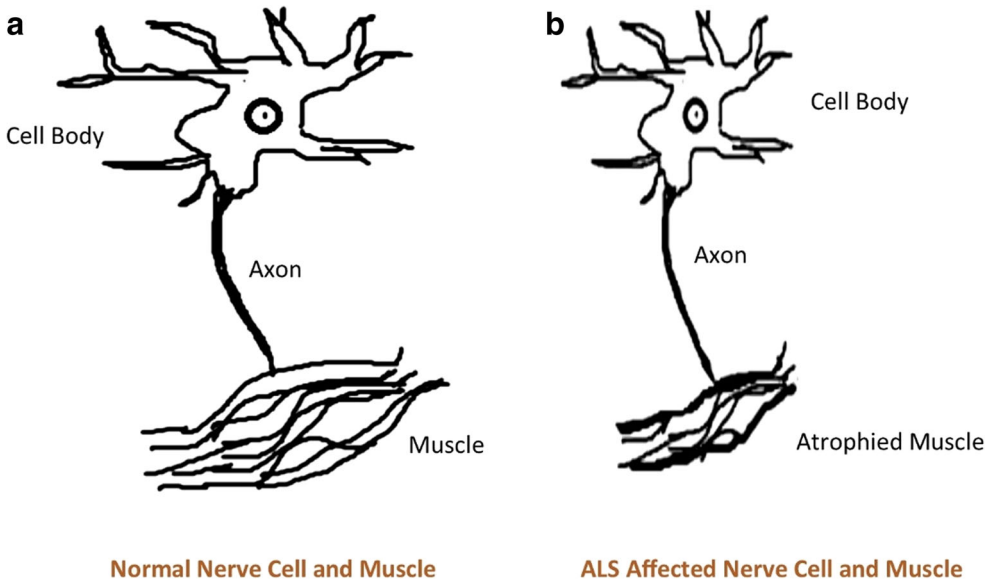

Fig. 3 Comparison of normal (**a**) and ALS-affected (**b**) nerve cell and muscle

of neurodegenerative diseases are caused by the aggregation of misfolded proteins; key examples include AD, PD, and HD. In addition, the aggregation of misfolded proteins causes downregulation of proteolytic pathways [66–71]. Further proteolytic activities are generally lower in aging neurons, which further contributes to the accumulation of misfolded proteins in pathological conditions [67, 68, 71]. Interestingly, small molecules targeting proteolytic pathways were successfully demonstrated to clear aggregated misfolded proteins [72–77]. One study shows that the potential mechanism of cell-to-cell transmission is also considered to be one of the reasons for onset of neurodegenerative disorders [78]. Protein misfolding is caused by various factors like mutations, protein concentration, pH of the protein, and ionic strength. Other factors such as Aβ, metal ions, immune activation, and excitotoxicity resulting into oxidative stress are also causes.

Monomeric or oligomeric proteins cause organelle membrane damage that could also contribute to these diseases. Induction of membrane curvature and membrane damage by α-synuclein, after incubation with artificial phospholipid vesicles of these proteins, extensive tubulation, and vesiculation were observed [79]. The intrinsic mitochondrial apoptotic pathway is also one of the causes for neurodegeneration cell death. Caspase-9 activation controls the discharge of cytochrome-C from intermembrane space (IMS) of mitochondria. Mitochondrial respiratory activity leads to the formation of reactive oxygen species (ROS) as byproducts. Mitochondrial antioxidants such as glutathione peroxidase and manganese superoxide dismutase (SOD2) control ROS concentration in the surrounding environment. When ROS (oxidative stress) is produced in excess, it may also lead to neurodegeneration disorders. Moreover, with the generation of ROS, mitochondria have also been involved in life-sustaining tasks like mitochondrial membrane lipid concentration, programmed cell death (PCD), calcium homeostasis, fission and fusion of mitochondria, and the mitochondrial permeability transition. In addition to neurodegenerative diseases caused by mitochondria, it is likely to involve dysfunction or misbalance of one or more of abovementioned mitochondrial life-sustaining functions [80]. Four neurodegenerative disease pathogenesis, Alzheimer's diseases (AD), Parkinson's disease (PD), Huntington's disease (HD), and amyotrophic lateral sclerosis (ALS), are caused by mitochondrial dysfunction and oxidative stress plays a major role and there is strong evidences to support this statement [81, 82].

Disruption of axonal transport triggers to a degenerative pathway called Wallerian-like degeneration [83]. Swellings of axons and spheroids have been observed in most degenerative disorders which demonstrate that faulty axons are not only present in diseased neurons but also cause certain pathological injury due to their aggregation in organelles. The axonal transport is disrupted by a mechanism like kinesin and cytoplasmic dynein microtubules, cargoes, and mitochondria [84].

Programmed Cell Death

Programmed cell death (PCD) is mediated by an intercellular program that activates the neurodegeneration [85]. It is generally classified into three classes: (1) apoptosis (type I), (2) autophagic (type II), and (3) cytoplasmic (type III). Programmed cell death in a multicellular organism is known as apoptosis. Due to a series of biochemical changes, which leads to a major change in characteristic cell morphology, apoptosis occurs. This cause is among the primary types of apoptosis and the pathways are classified as extrinsic apoptotic pathways and intrinsic apoptotic pathways. Figure 4 illustrates the programmed cell death and the extrinsic and intrinsic apoptotic pathways.

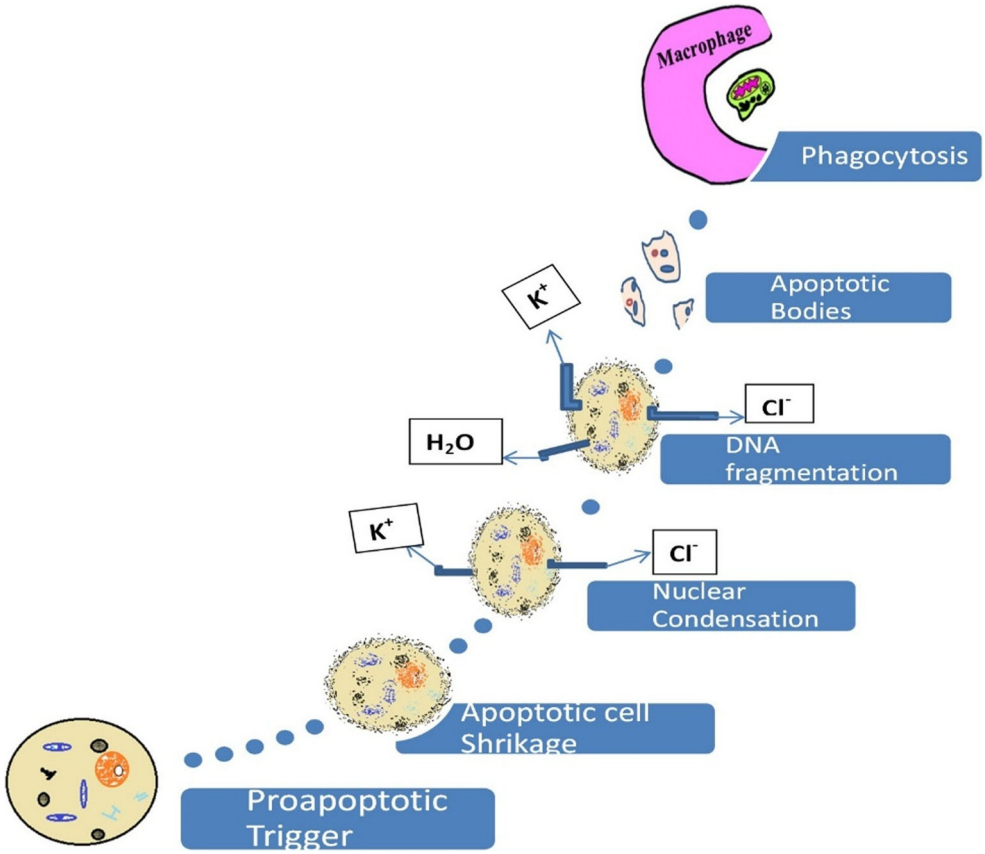

Fig. 4 Programmed cell death and the extrinsic and intrinsic apoptotic pathways

Autophagy is a type of phagocytosis (intracellular) in which autophagosomes are formed when damaged organelles or misfolded proteins are encapsulated after engulfing and fuse with a lysosome to demolish the same. Several neurodegenerative diseases show unusual protein aggregates. Cytoplasmic cell death is a type III PCD and the mechanism is through non-apoptotic processes. There is no clear evidence for this mechanism and possible causes are due to cytotoxins that stimulate PCD to cause necrosis and trophotoxicity.

There are several aspects of neurodegenerative disease, but the present study is primarily focused on protein misfolding as the main cause due to strong scientific theory and supporting evidence.

Proteolysis of the Proteins Involved in the Neurodegenerative Disease

Ubiquitin proteasome pathway (UPP) is derived from the protein catabolism mechanism in the nucleus and cytosol of cells. The process in any pathway should be regulated and the defect occurs when highly regulated UPP affects the cellular process and substrates in the system, thereby leading to the pathogenesis of many human diseases. Figure 5 shows the process of ubiquitination and protein degradation.

UPP is the main control mechanism which regulates almost all cellular process including immune response and inflammation, antigen processing, PCD, cell cycle and division, cell surface receptor modulation, organelle biogenesis, DNA transcription and repair, stress and extracellular modulator response, neural and muscular degeneration, neural network morphogenesis, the secretory and ion channel pathways, viral infection, and ribosome biogenesis. Catalytic 20S core and the 19S regulator (degradation) together form the 26S proteasome. A targeted protein degrades with covalent modification of lysine residue by the proteasome and requires three enzymes: (1) ubiquitin activating enzyme, (2) ubiquitin conjugate enzyme, and (3) ubiquitin ligase. After the protein ubiquitination, it is recognized by the 19S regulatory unit in an ATP-dependent binding step [86]. After entering the inter portion of the 20S unit, the substrate protein comes in contact with the proteolytic active sites.

Substrates should be at least partially unfolded when entering the core because the central channel of the 20S unit is constricted and also gated by the N-terminal tails of α ring subunits. This process is called translocation in which unfolded substrates pass into the center and it happens after deubiquitination.

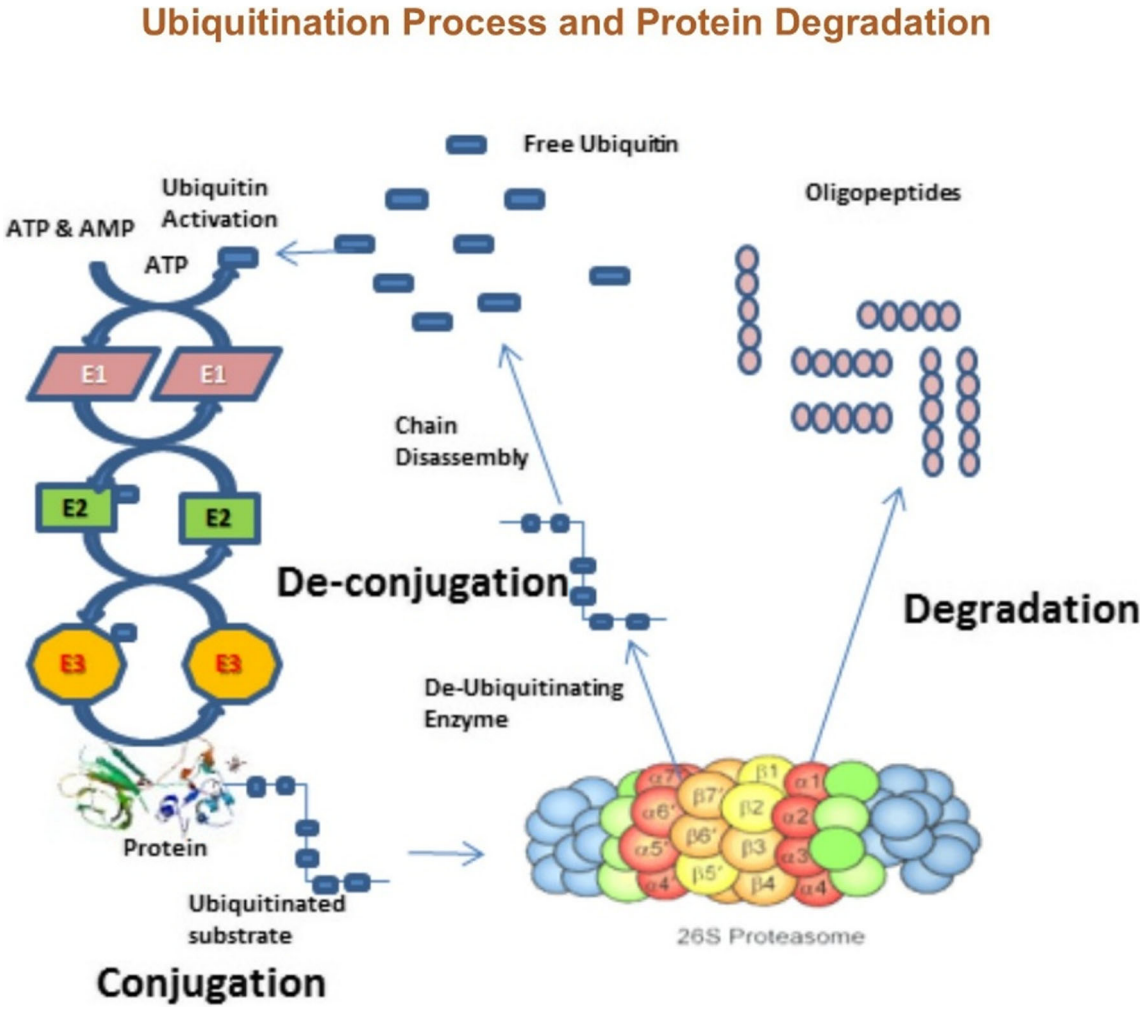

Fig. 5 The process of ubiquitination and protein degradation

Failure of Autophagy in Brain

Autophagy is a lysosome-mediated degradation process that assists in recycling faulty cellular components like the removal of damaged organelles and protein aggregates. Using several mechanisms, endosomes as well as autophagosomes are formed after delivery of substrate containing lysosomes. The autophagic and endocytic pathways digest the constituents completely after their effective interactions. As aging occurs, neurons are susceptible to this interface becoming disordered. The autophagy regulating gene mutations may cause neurodegenerative diseases across a broad age range with inimitable rate of recurrence. Neurodegenerative disease such as AD, ALS, and familial PD originate at various stages of the autophagy pathway and possess a few repercussions for pathogenesis and treatment. The principle of the autophagosomal-lysosomal network may be critical in the progression of aging. Impairment and failure of autophagy clearance is a main cause for the progression of neurodegenerative disease. Figure 6 shows the autophagy impairment and Alzheimer's disease [87]. Table 1 lists significant cases of neurodegenerative diseases with associated protein misfolding.

Biomarker Development

How to Narrow Down the Biomarkers Using Proteolytic Fragment of Targeted Proteins

It is evident that we have only fewer biomarkers that are clinically used to diagnose neurodegenerative diseases. The key issue is the commonality of the disease features. Thus, the current diagnosis and evaluation of the disease entirely depend on clinical/pathological examination. Hence, it is important to use currently available high-throughput screening approaches that can profile the disease at different stage of progression and to find new specific biomarkers for these diseases [109]. One such approach is using various methodologies of proteomics to profile the changes in specific protein(s) of interest. Biochemical biomarkers, which broadly include proteins and metabolites, can be quantitated in tissues or biofluids from patients. Most of the biochemical biomarkers utilize blood or cerebrospinal fluid; however, due to the presence of blood-brain barrier, the true profiling of brain-related neuronal disease are difficult. To overcome this, brain imaging using

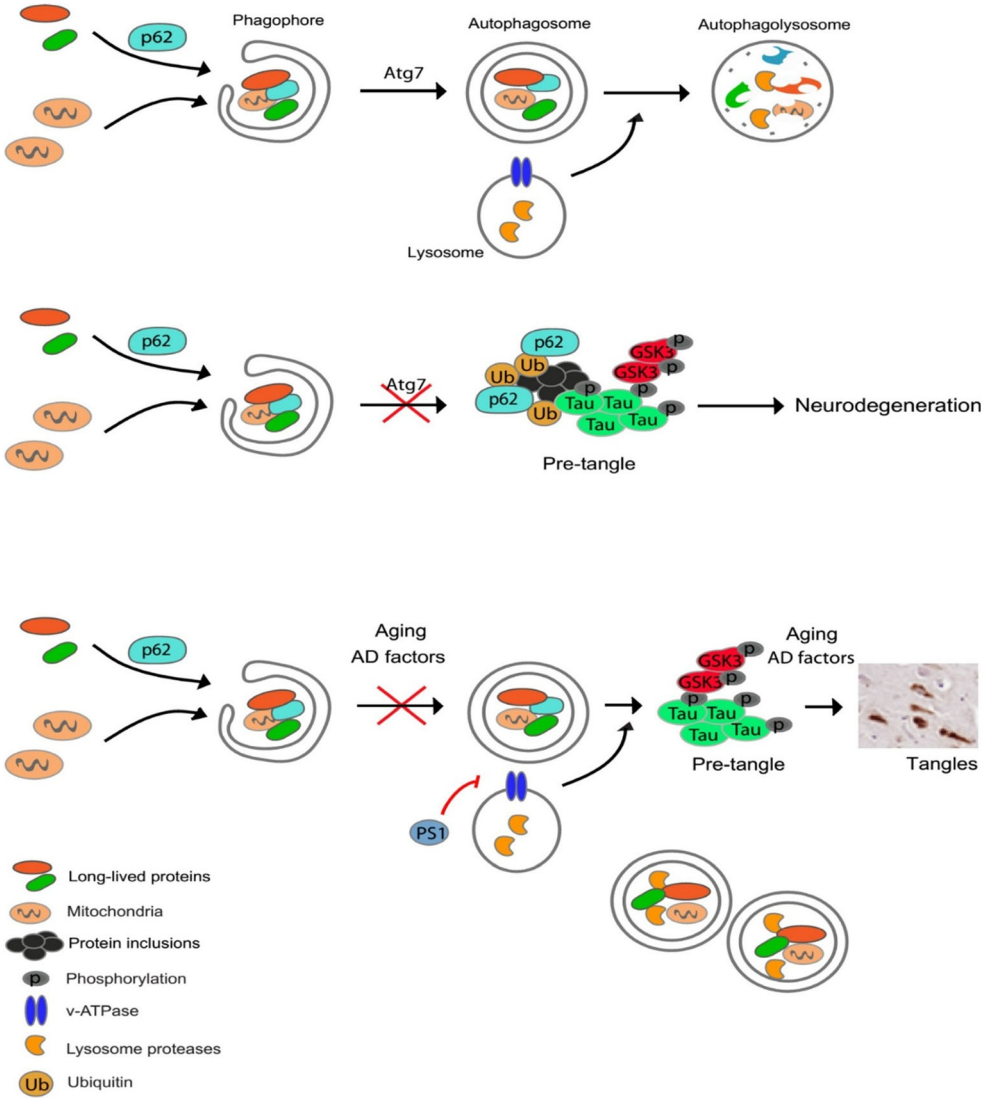

Fig. 6 Autophagy impairment and Alzheimer's disease. **a** Diagrammatic demonstration of the common autophagy pathway steps. **b** Ablation of the essential autophagy regulator Atg7 in neurons triggers spontaneous neurodegeneration, leading to the aggregation of ubiquitin-positive inclusions in a p62-dependent manner. **c** Speculative model: in Alzheimer's disease (AD), disease in the autophagy pathway due to genetic mutations, environmental factors, and/or aging may contribute to the accumulation of abnormal protein aggregates and possible phospho-Tau in a pre-tangle state. *Adapted from Ref.* [87]

various methods has been used as biomarker for several brain diseases. For example, dopamine transporter protein-binding ligands have been used in the diagnosis of PD [110]. It is also important to mention that majority of the biomarker screening in the past uses target-specific candidates, while the importance of an unbiased biomarker screening has been realized recently. In the past, gene expression analysis has been used to study the neurodegenerative diseases. However, in general, the gene expression profile does not always correlate with protein profile indicating that stabilization of mRNAs and post-translational modifications contribute to the disease status [111, 112]. Several methods are available to perform differential proteomic profiling. A simple two-dimensional gel electrophoresis (2-DE) has been routinely used to identify differential "spots." Further, the proteins in the spots are identified through matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDITOF-MS) [113, 114]. For example, using this method, more than 1000 proteins have been identified in AD [115]. Further, with great effort, proteome maps of different regions of human brains have been developed using 2-DE [116–119]. The most recent methods in proteomics include stable isotope labeling with amino acids in cell culture (SILAC) [120] and isobaric tags for relative and absolute quantification (iTRAQ) [121] to identify post-translational modifications and protein-protein interactions. In the future, single-cell differential proteomics methods will be used to identify disease severity and to understand molecular mechanisms in neurodegenerative diseases. Disease progression cannot be realized in the early stages so early biomarkers will be a boon for the patients suffering from neurodegenerative diseases. With the disease-causing proteins and interacting proteins as a starting point, it is prudent to mimic the peptides in proteins and make a library of short peptides. The mimicking protein/peptide can be labeled and used as a potential marker which can be the primary approach. Figure 7 shows the proposed method of analysis, imaging, and the proteomic study of the proteolytic fragments.

Table 1 Significant cases of neurodegenerative diseases with associated protein misfolding

Disease	Defect	Donor cell	Age/sex	Reprogramming	Description	Ref.
Alzheimer's disease	Sporadic	Dermal fibroblasts	N/A	Episomal	Accumulation of Aβ oligomers, endoplasmic reticulum and oxidative stress, drug response compared with familial AD	[88]
		Dermal fibroblasts	83/F and 83/M	Retrovirus; OSKM	Increased Aβ40 secretion, increased Tau and GSK3β activity, presence of large early endosomes	[89]
	APP	Dermal fibroblasts	NA	Episomal; OKSM	Accumulation of Aβ oligomers, oxidative stress, compared with sporadic AD	[88]
		Dermal fibroblasts	51/M and 60/F	Retrovirus; OSKM	Dominant, increased Aβ40 secretion, increased Tau, GSK3β	[89]
	PS1	Dermal fibroblasts	65/F and 64/F	Retrovirus; OSKM	Elevated levels of Aβ42/Aβ40; Efficacy of the non-steroidal anti--inflammatory drugs examined; various small molecules and γ-secretase modulators tested	[90]
	PS2	Coriell Institute	N/A	Retrovirus; OSKM	Increased levels of Aβ42/Aβ40; response to γ-secretase inhibitors	[91]
	Trisomy 21	Dermal fibroblasts	1/M	Lentivrus; OKSMN	Increased secretion of Aβ40, accumulation of amyloid aggregates, MAPT phosphorylation	[92]
Fragile X syndrome	FMR1	Coriell Institute	4/M, 3/M and 26/M	Retrovirus; OSKM	CpG methylation of FMR1 gene, loss of FMRP expression, neuronal differentiation affected	[93]
Huntington's disease	HTT	Coriell Institute	3 to 18 years of both male and female	Lentivirus and episomal; OKSM and OKSMNL	Differences in energy metabolism, cell attachment, decreased survival of neurons	[94]
	HTT	Dermal fibroblast	20/F	Lentivrus; OKSMN	Disease phenotype reversed with gene correction, neurons respond to TGFβ and BDNF	[95]
Amyotrophic lateral sclerosis and frontotemporal dementia	Sporadic	Dermal fibroblast	64/M and 67/M	Lentivirus; OKSM	Sensitivity to stress increased	[96]
	PGRN	Dermal fibroblast	64/M and 67/M	Lentivirus; OKSM	Reduced expression of PGRN, disease phenotype reversed with exogenous PGRN	[96]
	SOD1	Dermal fibroblast	44/F and 60/F	Retrovirus; OKS	Reduced number of neurons, increased apoptosis, mitochondrial defects rescued by gene correction	[97]
	–	Coriell Institute	50/F	Retrovirus; OKSM	Neurofilament aggregation, axonal degeneration	[98]
	C9orf72	Dermal fibroblast	47–51/M	Episomal; OKSML	Altered membrane excitability, antisense oligos reverse disease phenotype	[99]
	TARDBP	Dermal fibroblast	56/M	Retrovirus; OKSM	Accumulation of TDP-43, decreased survival of astrocytes	[100]
Parkinson's disease	Sporadic	Epidermal keratinocytes, dermal fibroblasts	7 patients (age 46–66, M and F)	Retrovirus; OKS	Number of neurites and neurite length reduced, high autophagosomes, normal α-synuclein	[101]
	PARKIN	Dermal fibroblasts	N/A	Lentivirus; OKSM	Increased spontaneous dopamine release, decreased dopamine uptake, mitochondrial dysfunction, α-synuclein accumulation	[102]
	PINK1	Dermal fibroblasts	N/A	Retrovirus; OKSM	Impairment of PARKIN translocation, abnormalities in mtDNA	[103]
	SNCA	Dermal fibroblasts	48/M	Retrovirus; OKSM	Increased α-synuclein expression, aberrant mitochondrial activity	[104]
Rett syndrome	MECP2	Dermal fibroblasts	6/F	Retrovirus; OKSM	Inactive X-chromosome, aberrant neuronal morphology, gene correction reversed disease phenotype	[105, 106]
Schizophernia	Unknown	Dermal fibroblasts	5 patients (22–27 age both M and F)	Lentivirus; OKSML	Decreased neuronal connectivity, decreased neurites and synaptic proteins	[107]
Timothy syndrome	CACNA1C	Dermal fibroblasts	N/A	Retrovirus; OKSM	Abnormal neuronal differentiation, defects in calcium signaling	[108]

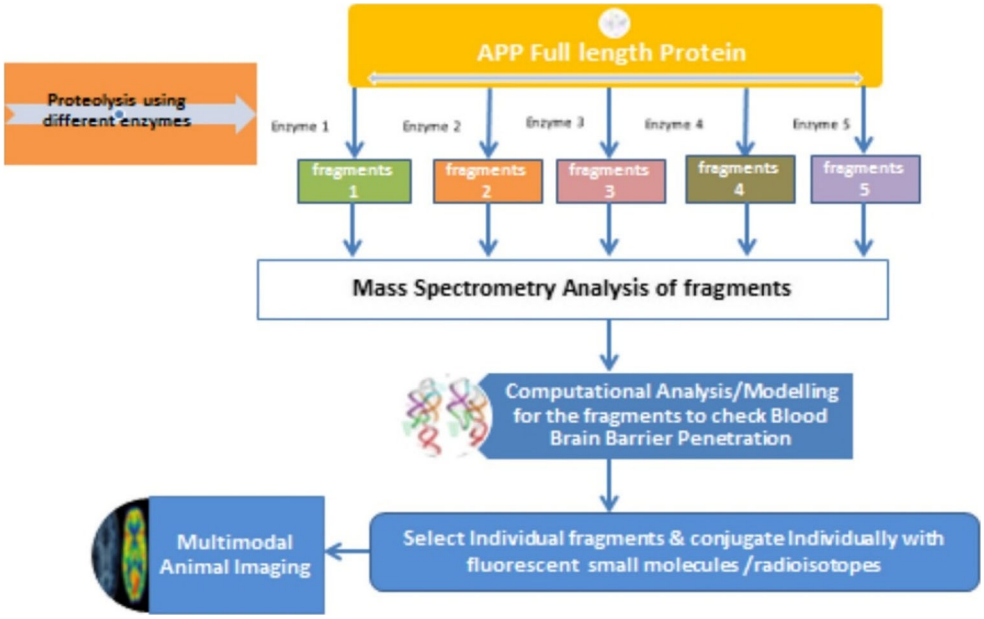

Fig. 7 The proposed method of analysis, imaging, and the proteomic study of the proteolytic fragments

Proteomics approach is used to identify specific peptides from target proteins such as, APP, APoE, PS1, PS2, Tau, and ubiquitin ligases as imaging agents. This approach is used in order to mimic AD pathology for early detection of Alzheimer's and to dope the identified peptide probes with nanoparticles to enhance their permeability across blood-brain barrier (BBB) while not altering the etiology.

Radiolabeling of proteins is an emerging trend in basic and clinical research. To date, radiolabeling of proteins involves chemical derivatives resulting in multiple additions of radionuclides in random sites. This method is designed to specifically localize the radionuclides to the targeted sites.

The fluorescent small molecules are excellent optical probes with high quantum yield, photostability, tunable emission, and broad excitation spectra. Due to its excellent biocompatibility, abundant nature, and potential for large-scale green synthesis, it is attracting more and more attention in the bioimaging community as an alternative to heavy-metal-containing quantum dots [122]. The doped fluorescent small molecules are safer; they are10-nm amorphous nanoparticles.

Will Antibody Development from Proteolytic Cleavage Help in Early Biomarker Development or Act as an Inhibitor?

An antibody (AB), also called an immunoglobulin (Ig), is a comparatively large protein of its class and its shape is similar to the letter Y. Immunoglobulins (Ig) are generated by plasma cells and their function is identification and neutralization of bacterial and viral pathogens.

The antibody identifies an antigen that is a distinct molecule of the toxic agent through its variable region [123, 124]. The tip of Y has the paratope and is specific to a particular epitope on an antigen; these two structures bind each other. The binding mechanism can be used for the antibody to bind with the infected cells while attaching the immune system and can also neutralize the target directly.

When a specific antibody is produced for the plaque forming proteins/peptides, the antigen can readily bind to the specific antibody but the permeability into the blood-brain barrier is unknown. If the permeability of a specific antigen into blood-brain barrier is not an issue, then the specific antibody can be conjugated with fluorescent small molecules or radionuclides and can be used as an early biomarker. In some cases, the antibody-antigen affinities are weak and the possibility of using it as an inhibitor is remote as the interactions may be reversible. Another possibility is that when the specific antibody binds to the pathogen, it has the tendency to agglutinate. The agglutination may induce toxicity to the cells and cause disease progression.

The Inhibitor/Drug Target for the Neurodegenerative Disease

Capsase-6, a member of cysteine protease family, is well known for its role in "Programmed Cell Death". Caspase-6 can act as a contributor to the neurodegenerative pathology associated with Huntington's disease (HD), Alzheimer's disease (AD), aging and stroke. There are no defined mechanisms behind the role of caspase-6-mediated neurodegeneration but evidence suggests activation of caspase-6. This activation results in cleavage of caspase-6 substrates such as Huntingtin, amyloid precursor protein (APP), Tau, CBP/p300, NF-kB, and Caspase-8, and makes inhibition of caspase-6 an appealing therapeutic target for neurodegenerative disease.

Another interesting enzyme is the c-Jun N-terminal kinase (JNK); specifically, JNK3 has been identified as an important enzyme in the pathology of various neurodegenerative disorders. The peptides which mimic JNK3 can be a potential inhibitor for neurodegenerative diseases.

Hypothesis for the Biomarker Development

A body of evidence indicates that neurodegeneration (NDD) occurs in response to excessive accumulation, abnormal aggregation, and deposition of various proteins and peptides, including toxic oligomers, amyloid and tau proteins, and misfolded proteins in the brain. These processes indicate seriously altered metabolic mechanisms, resulting in an increased production (synthesis) and/or decreased elimination (cleavage) of critically important molecules (peptides, proteins), including, among others, APP and Aβ. The above process clearly explains the misfolding of the proteins and how it can be diagnosed early using the combination of misfolding fragments and other agents.

Over the past decade, imaging methods have been used as diagnostics and prognostics for various diseases. Real-time imaging capabilities of cellular function can identify pathological changes at cellular level. Bioimaging has advantage over other biomarker studies in being non-invasive. Several advanced approaches have been adopted to enhance the resolution of the imaging. Magnetic resonance imaging (MRI) can image deep tissues and have advantage of not exposing patients to radiation. Novel nanoparticles have been synthesized to improve tissue contrast and specificity. The physiochemical properties of nanoparticles are considered as highly useful characteristics in developing probes for imaging. One such important characteristics of nanoparticles is the functionalization of nanoparticles with specific targeting ligands to target-specific cell/organ. For example, van Kasteren et al. [125] have established that iron oxide nanoparticles functionalized with glycan could be used to study brain disease. In vivo "molecular bio marking" of these pathological proteins in the brain is possible at this time with molecular imaging methods, such as positron emission tomography using appropriate radio ligands. The challenge with the presently available approaches is that they can demonstrate the presence of the "end products" of the altered process, e.g., the accumulation and deposition of Aβ amyloid. Consequently, the presently available molecular imaging biomarkers are "late biomarkers" and their diagnostic importance is hampered by the fact that the clinical diagnosis is usually already evident before the molecular imaging approaches, such as positron emission tomography (PET) scans, can demonstrate the presence of the pathological proteins in the brain. There is a need to develop "early bio marking approaches," which would indicate sensitively and reliably very early on the alterations in the critical metabolic pathways.

Here, we propose "to challenge the system" by introducing the concept of multifunctional imaging probes which contain (i) a core (or carrier) structure (a nanoparticle), (ii) functional attachments ("mimicking protein," e.g., amyloid Aβ molecule or its fragments) which will enter the relevant metabolic pathway and thereby lead to the accumulation of the imaging probes in cells with critically altered metabolism (or, alternatively, their lack in these structures, as compared to the normal situation, will have clinical significance), and (iii) a "dopant," serving as a "beacon," which will be responsible for the signal emission (let it be an optical, fluorescent, or radioactive signal) (Fig. 8). A critical issue is, of course, that these constructs should be able to pass through the blood-brain barrier and reach the critically altered cells in the brain parenchyma, if we would like to use them as "central" (i.e., CNS) imaging markers. With the help of such constructs, we can challenge early on the critical metabolic pathways, participating in the pathogenesis of NDDs: depending on the functional attachments of the probe, after their administration, they will accumulate in cells due to the increased activity of the synthetic enzymes or due to the decreased activity of enzymes responsible for the cleavage of the pathogenic protein, resulting in increased or decreased signals detectable by the appropriate imaging methods, e.g., optical, fluorescent, or PET).

Disease Modeling Using Induced Pluripotent Stem Cells

Neural disorders are difficult to study mainly because of the non-availability of biopsy samples. Most currently available information on neurodevelopmental and neurodegenerative diseases in humans has been accumulated from samples obtained from deceased patients who are at the end-stage of the disease. Understanding these diseases in a laboratory setup is a challenge; there has been little success in using relevant mouse models [126–129]. Disease modeling with rodents does not always recapitulate the pathological conditions observed in human diseases.

A major breakthrough in stem cell research is discovery of induced pluripotent stem cells (iPSCs) [130]. iPSCs were developed with transfection of uniquely expressed transcription factors identified to be associated with the stemness of embryonic stem cells. Following its success in the mouse, reprogramming was performed using human somatic cells [131]. Since the inception of the iPSC technology, it has received overwhelming interest in the regenerative medicine field. Several methodologies were developed to create safe clinical grade iPSCs (Fig. 9).

With the concept of reprogramming somatic cells obtained from a specific donor, one could have disease-tailored pluripotent stem cells to study the development of diseases in a Petri dish [132]. Human embryonic stem cells (hESCs) have long been used as a tool to study various diseases. Various diseases such as Huntington disease and fragile X-chromosome

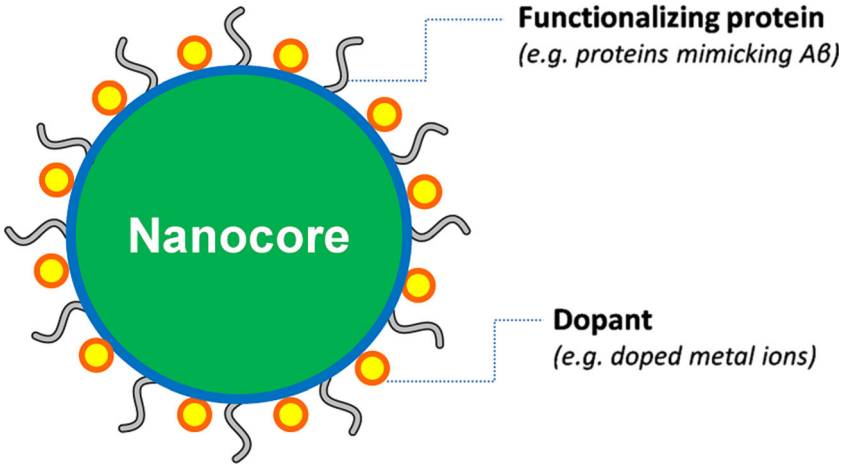

Fig. 8 A schematic structure of the functionalized nanoparticle with its core, the "dopants" (e.g., doped metal ions) and functionalizing proteins (e.g., proteins mimicking Aβ)

diseases were studied with hESCs derived from these disease associated embryos [133, 134]. The difference between the use of hESCs and hiPSCs in disease modeling is overwhelming. The most compelling advantage of iPSCs over hESCs is that the hESC-based disease models need genetic manipulation and also they do not have a correlation between the genotype and phenotype [135].

In neurodegenerative diseases, the functional capabilities of neurons are compromised due to chronic and progressive pathological conditions. The loss of neuronal functions, as in neurodegenerative disorders, has many common features such as mitochondrial dysfunction, degradation pathways of proteins, defects in biochemical transportation, and autophagy [136]. A comparative study between the neural lineage cells differentiated from the patient-derived iPSCs and control iPSCs will shed light on various mechanism of the neural disease progression which will help in the identification of the new biomarkers for these diseases and in drug discovery. Some tools used in comparative studies include high-content imaging, gene expression analysis by microarray, metabolic profiling, micro-RNA profiling, proteomics or phosphoproteomics and epigenetics (Fig. 10). There are several well-established protocols which exist for the differentiation of iPSCs into motor neurons [137], dopaminergic neurons [138], and oligodendrocytes [139].

Mutations in the gamma-secretase coding genes PS1 and PS2 were found to be possible candidates for familial Alzheimer's disease [140–143]. iPSCs were generated from fibroblasts of familial Alzheimer's disease with the PS1 mutation A246E and the PS2 mutation N141I. These cells were successfully differentiated to neurons. The secretion of Aβ42 in the patient-derived neurons was reported to be at increased levels. Moreover, these neurons were used to test several potential compounds that inhibit the secretion of Aβ42 [91]. In the case of sporadic Alzheimer's disease (SAD), the disease has been linked to different genetic risk factors and the environment. There are hundreds of genetic alterations associated with this disease [144]. The biochemical and molecular analysis of SAD comes from post-mortem samples because it is impossible to obtain brain tissue from living patients. Predisposition to SAD involves interactions between patient-specific genomic variants and environmental factors. Researchers have used iPSCs to examine genetic variants that may contribute to SAD via SORL1 gene variants, which creates a SAD risk with reduced response to BDNF treatment. Additionally, knockdown of the SROL1 gene with shRNA also produces a SAD phenotype which could be used in disease modeling [145].

Multiple sclerosis (MS) is a complex inflammatory neural disorder which occurs due to mutations in a wide variety of susceptible genes leading to destruction of the myelin-

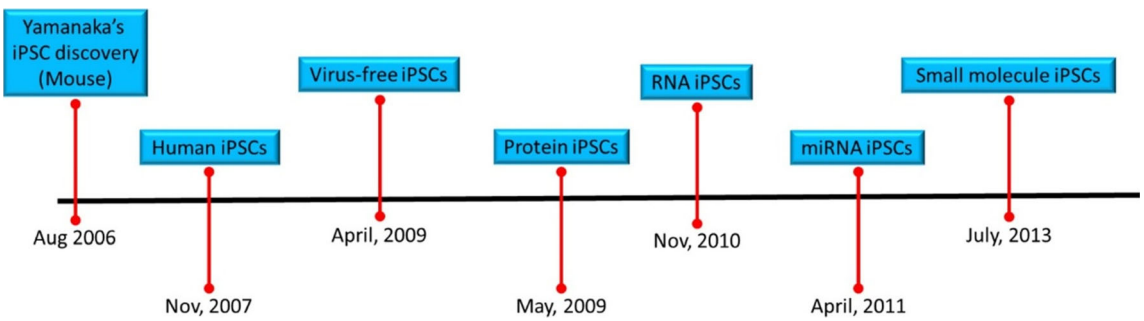

Fig. 9 Methodologies developed over time to create safe clinical grade iPSCs

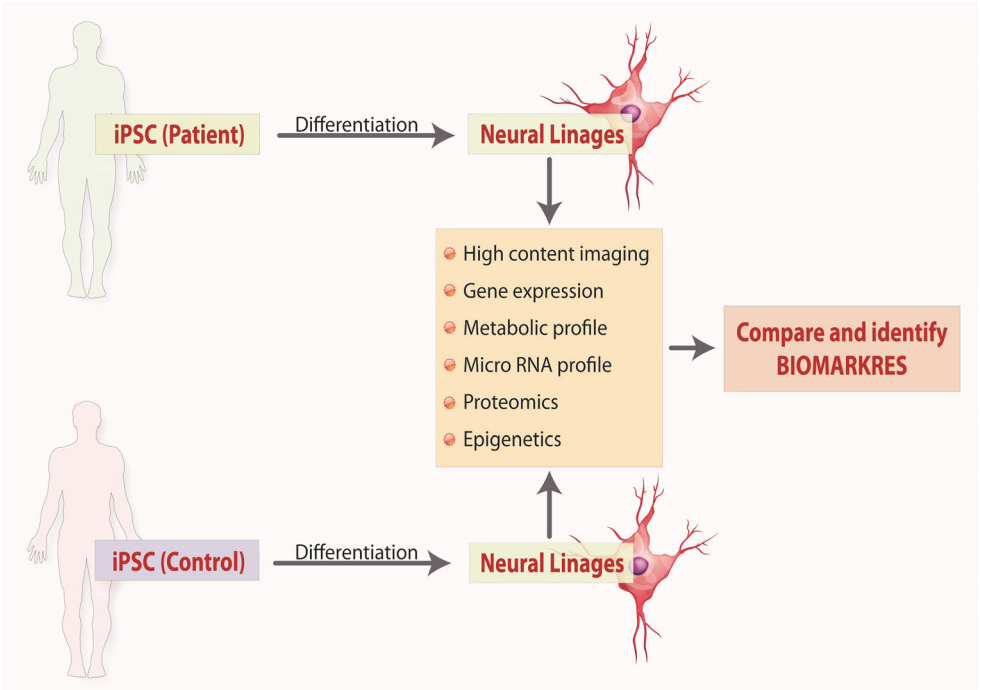

Fig. 10 The approach and tools for comparative study between neural lineage cells differentiated from patient-derived iPSCs and control iPSCs

producing cells by the immune system [146]. There are no specific genetic variants associated with MS disease. The extent to which MS affects other neural cell types was also examined. For example, Baranzini et al. [147] have studied glutamate stimulation in neurons from MS patients. Results of this study suggest that variance in neurotransmitter activity is associated with MS and contributes to the heterogeneity of this disease [107]. It is still unclear whether MS patient-derived iPSCs differentiated to neurons will have any genetic defects. This topic has not yet been addressed; it would be interesting to investigate the functionalities of neurons derived from iPSCs generated from MS patient fibroblasts. It would be interesting to note that in other neurodegenerative diseases such as schizophrenia, neurons differentiated from the patient-derived iPSCs, showed reduced synaptic activity [107].

Similarly, our perception of the molecular mechanism of PD is limited by the scarcity of human dopaminergic neurons. The etiology of PD is not clear. However, one of the severe consequences of the disease is the gradual loss of motor activity, which gives the typical rigidity and tremor [148]. The factors that initiate PD remain unclear. Mutations in GBA, LRRK2, PARK2, PARK7, PINK1, SNCA, or UCHL1 increase the chances of PD, suggesting links between these proteins and the disease [35]. Overexpression studies of these genes in a variety of organisms shed light on the basic understanding of the disease [149–151]. However, these studies did not yield clear mechanisms for the disease. Human iPSCs were used to create disease models of PD; however, most of these models simulated the early onset of the disorders rather than late onset [104, 152–157].

In fact, most of the neurodegenerative disease models developed using iPSCs are early-onset disease models [158–165]. One drawback that continues to confound studies on neurodegenerative diseases is the aging factor. Most neurodegenerative diseases are the outcome of aging, and incorporation of the aging factor into the iPSC-based disease model is challenging at the current stage [166]. Moreover, using patient-derived iPSCs, the biochemical and molecular interactions of these phenotypes with other neural lineages (such as astrocytes) would shed more light on the perception of the disorder and lead to improvement of novel drugs that can target these phenotypes [132]. The iPSC disease model with more complex culture systems such as a co-culture system and three-dimensional (3-D) culture systems will additionally help move forward drug discovery and biomarker identification in these neurodegenerative diseases [167]. 3-D culture with astrocytes and oligodendrocytes provides an exciting platform for biomarker identification and drug discovery [139, 168]. Even though iPSC technology has revolutionized the regenerative medicine field, it still needs to mature to overcome certain drawbacks highlighted above. Recent reports suggest that the OKSM-derived iPSCs may have a slightly lower differentiation potential when compared to hESCs or ESCs derived by somatic cell nuclear transfer (SCNT) [169–175]. Figure 11 lists iPSC-based neurodegenerative disease modeling. Given to the context of the known variability in human genomes, biochemical cues, and physiology, inclusion of proper controls is mandatory in iPSC disease model systems. The more ideal control is the one with a defined genetic background or isogenic cell lines [176].

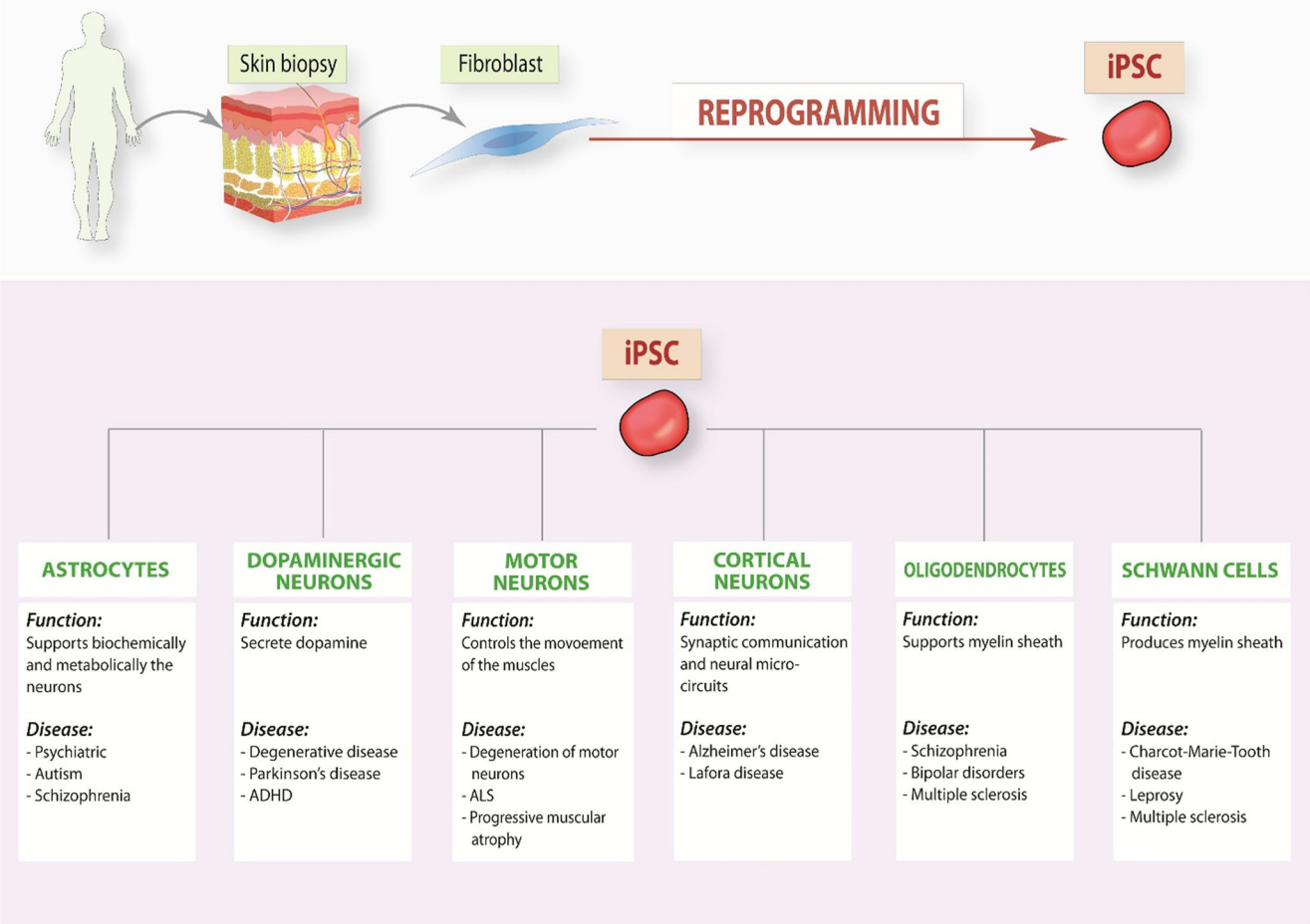

Fig. 11 iPSC-based neurodegenerative disease modeling

Conclusion

The review of pertinent literature presented the role of proteins in various neurodegenerative abnormalities including Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, and Huntington's disease with their associated pathways. Further, the abnormal conformation and misfolding of proteins is explored for neurodegeneration disorders with focus on causes and factors. Also, the approach of early biomarker development by mimicking the peptides in protein and labeling is successfully demonstrated followed by suggestions for recognizing inhibitors of protein misfolding. Also, the concept of disease modeling with iPSCs helped to explore neurodegeneration through various angles. Thus, it can be concluded that the misfolding of proteins can suitably guide the development of biomarkers and help us understand many aspects of various neurodegenerative diseases.

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Author Contributions SK, NK, and SM contributed in preparing layout, writing, and editing the manuscript. PP, GR, and BG contributed in reviewing the content. All authors read and approved the final manuscript.

Compliance with Ethical Standards

Conflict of Interest The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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