Chapter 2
Introduction and Review of literature

INTRODUCTION AND REVIEW OF LITERATURE

2.1. Intrinsically Disordered Proteins:

The majority of proteins contain highly packed hydrophobic cores with a minimum folding of the native structure, as the residues are highly conserved and freely interact with the surrounding environment. Conversely, some proteins lack a conserved hydrophobic core and undergo structural folding, leading to the loss of their dynamic ensemble of conformations, known as Intrinsically Disordered Proteins (IDP). IDP are unstructured proteins characterized by their inability to fold into a stable, well-defined three-dimensional (3-D) structure. They are entirely disordered and fluctuate rapidly through a range of conformations. However, several proteins exist with both ordered and disordered conformers. In the late 1990s, the incidence of IDP emerged, establishing the link between IDP and neurodegenerative disorders, which was the highlight of the scientific community's decision to place IDP as an interest in protein [33–36]. The unique characteristic of IDP is that it exhibits functionality without any secondary structures [37]. In the equilibrium state, IDP forms a dynamic ensemble of conformers that rapidly interconvert [38, 39]. Proteins with Intrinsically Disordered Regions (IDR) play crucial roles in protein interaction networks; transcription factors notably serve as hub proteins in the nucleus and facilitate molecular communication through protein-protein interactions (PPI) [40-42]. Due to their tremendous flexibility and structural plasticity, IDR sample a set of diverse conformational states that may contain different amounts of structure and disorder. IDP have significantly higher polar and charged residues in their native structure, and this coupled with their inadequate hydrophobicity which contributes to their inherent disorder. Since most IDP are prone to aggregation, and their aggregated states resemble those of misfolded globular protein molecules, the level of IDP inside the cell is also highly controlled at the transcriptional and translational levels to prevent certain pathological conditions like cancer and neurodegenerative diseases. Considering all of these factors, it is essential to understand the conformational dynamics and aggregation mechanisms of IDP. IDP or IDR have been reported to be abundant in uncharged and polar amino acids, lack large hydrophobic residues, and exist as dynamic heterogeneous ensembles of folded or extended structures [43–46]. Despite the dynamic nature of IDP structures, the best way to describe their structure is as a series of low-energy conformations [47, 48]. To attain the most stable shape possible under present physiological conditions and in its native state, the polypeptide chain folds itself in a certain manner as shown in **Figure 2.1** [49]. IDP or IDR exhibit the properties of "protein clouds" because they lack a precise equilibrium value for the atomic coordinates and backbone

Ramachandran angles over time [50]. An IDP or IDR changes from a freely bound state with a large amount of conformational freedom to a folded state with a reduced amount of conformational freedom [51]. Thermodynamics plays a crucial role in achieving a high level of complementarity in this interaction. The non-covalent contacts that arise within the IDP or IDR and between the IDP or IDR and partner must contribute enthalpically to the interaction to make up for the loss in entropy [52].

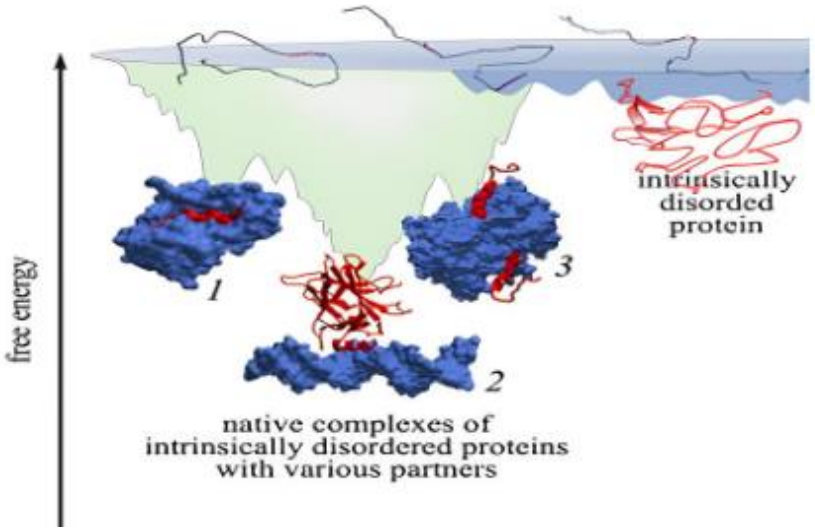


Figure 2.1. *Schematic representation of IDP. '1' indicates native structure, '2' showed intermediate structure and '3' indicated ordered aggregates (Taken from [49])*

2.2. Folding and Misfolding of Proteins:

Protein aggregation is closely linked to diverse protein folding processes, molecular chaperones, and stability. Despite its importance, protein aggregation mechanisms are often disregarded in experiments focusing on protein folding. When proteins misfold, they tend to aggregate, leading to harmful consequences for individuals with conditions like prion diseases, amyloidosis, and other disorders characterized by protein deposition [53]. The process by which a protein attains its native 3-D structure entails the formation of semi-transient intermediates through diverse, dynamic, and heterogeneous processes that are still not well understood at the molecular level. The stability of proteins in their physiological settings [52, 54] influences their folding process within cells, which is governed by both their structure and the surrounding molecular environment [54]. For folding patterns of proteins by ATP-dependent molecular chaperones, cells provide complex assistance [55]. Protein folding is often effective and results in the generation of fully functioning native protein structures [56, 57]. Misfolded proteins are the outcome of ineffective protein folding; nevertheless, folding

errors in proteins cause many illnesses. Misfolding predominantly contributes to the non-functional state of a protein [58]. Polypeptides that diverge from the proper protein folding pathway cause misfolding [59], which can happen in intracellular or extracellular states. The inclusion of LB is the main aggregated form of misfolded protein, which is also one of the main concerns with commercial protein production systems. In human physiology, the presence of aggregated proteins is associated with the pathophysiology of various diseases. These aggregates form when misfolded polypeptides bind together non-specifically [60]. Although reports [61] indicate certain interactions that have a role in protein aggregation, it is unclear if aggregate formation is aided by protein assembly, but some protein motifs are more likely to do so. Molecular chaperones typically identify, refold, or organize incorrectly folded proteins, making them promising contenders for controlling the formation of age-related protein deposits [62]. Nevertheless, protein assembly is vital to cells and serves significant purposes, but limited information is known about the LB formation. The regulation of LB formation and dissolution are influenced by changes in the chemical environment, including pH fluctuations, which can affect the formation of these structures [62]. Chemical modifications, such as phosphorylation states, can also affect the assembly of protein bodies. Furthermore, aggregates and other enzymes facilitate the formation of LB. Uncertainty surrounds the method of incorporation into body development, aided by aggregate. To identify substrates and understand how aggregates form single deposits, further investigation is required [62]. Similar in the manner in which they develop, LB disintegrate either through the action of enzymes or occasionally on their own. These studies have demonstrated that phase alterations in physiological conditions affect the assembly of proteins. Consequently, disruptions in properly functioning protein bodies may occur, potentially leading to pathological protein aggregation. Such protein aggregation can interfere with various biological processes. Misfolding may arise from spontaneous errors or intentional engineering, resulting in aberrant protein aggregation. The accuracy of protein folding is maintained through the degradation of misfolded proteins, which is essential for normal cellular function [63]. Molecules that target various factors that promote protein misfolding and aggregation are among the therapeutic approaches to address the protein folding issue [64]. By strengthening the stability of proteins's natural shapes, therapeutic compounds can increase the thermodynamic barrier against protein misfolding and misassembly [65]. An alternate approach could focus on eliminating previously formed protein aggregates that are improperly assembled and misfolded. It is thermodynamically advantageous for a protein to transition into an aggregated state that is more stable than the original state under specific circumstances, such as denaturation conditions. Other factors that affect protein folding include

post-translational modifications, protein environment changes, increased degradation, and build up of degradation products, and oxidative stress. Proteins that are prone to aggregation tend to aggregate more at high protein concentrations [66]. As shown in **Figure 2.2**, partially folded (or misfolded) intermediates have patches of hydrophobicity on the surface that allow them to assemble and make them more efficient in terms of energy [67]. As a result, these partially folded intermediates assemble into protein aggregates (oligomers), protofibrils, and fibrils [68].

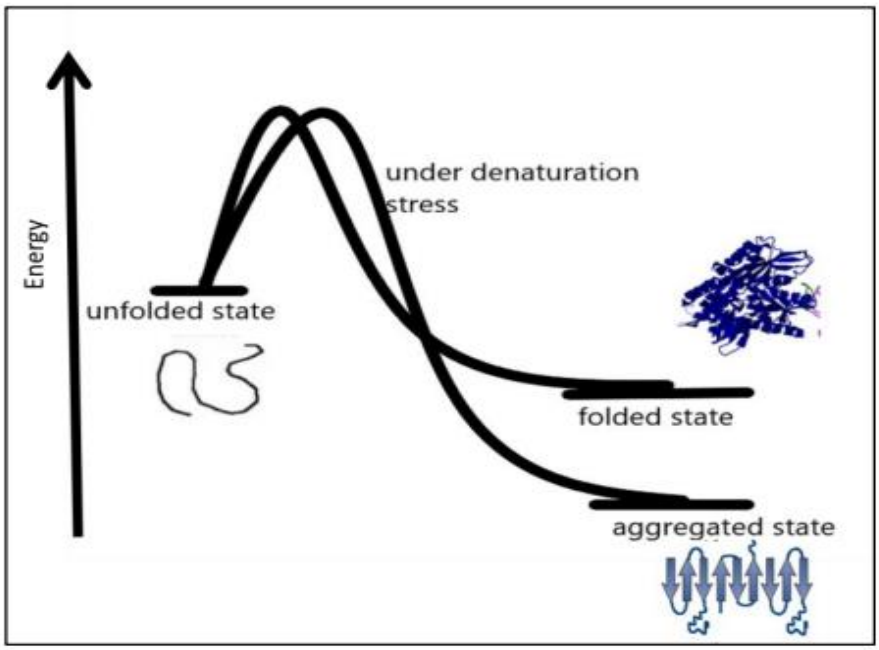


Figure 2.2. *Schematic representation of different states of folding, unfolding, and aggregation of protein to attain a stable state (Taken from [67])*

2.3. Protein aggregation mechanisms:

Neurodegenerative diseases are a type of human illness within the broader category of protein conformational diseases. It results from the proteins failing to take on their normal shape and function properly, leading to aggregation, loss of function, and harmful effects. Certain proteins are more prone to the misfolding, especially with age or in high concentrations [69, 70]. Proteins are prone to unfolding or misfolding when changes occur in the non-covalent interactions among their amino acid residues. These alterations can arise from mutations or post-translational modifications (PTM) that disrupt the protein's core structure. Particularly, IDP [71] are highly prone to misfolding and aggregation because they are primarily unfolded and lack strong, long-lasting intramolecular interactions. Additionally, the native states of IDP encompass a wide range of conformers, some of which may be aggregation-prone by nature

(for example, because of more exposed hydrophobic patches). In these conditions, the misfolded protein may self-associate to form insoluble aggregates if the cell does not help the misfolded protein return to its original functional form or break it down. Presently there is no effective pharmacological therapy for neurodegenerative diseases since it is very challenging and complex to identify and apply substances that can target the multistep protein aggregation process. Drugs that can disrupt the process of protein aggregation are highly desirable [72]. Researchers must pay attention to the pathological protein aggregation of peptides and proteins because it might result in molecular and cellular pathogenicity. The field of protein aggregation and inclusion body formation has witnessed numerous conceptual turns. Natural and functional amyloid have emerged as separate domains of the protein aggregation phenomenon. There is an immense possibility of exploiting amyloids for beneficial purposes, such as natural biomaterial generation materials for various possible applications in industry and medicine. The large-scale production and formulation of therapeutic proteins and peptides face challenges due to protein aggregation. This unintentional formation of aggregates can lead to potentially harmful immune reactions [73, 74], decrease drug effectiveness [75], or increase production expenses. Given the prevalence of protein aggregates, there is a strong need for efficient methods to prevent, detect, and eliminate them. These methods are highly desirable for both the treatment of amyloid-related diseases and the enhancement of the biological properties of biopharmaceuticals. Protein aggregation events have the potential to disrupt cellular functions and accelerate aging. Quality control measures are utilized to identify and remove proteins that are beyond repair. Protein diseases can result from the aggregation of proteins caused by mutations and unfavourable physiological circumstances. Misfolding can occur when a protein finds it difficult to return to its native form. Promising prospects for the development of therapies are the small compounds that bind selectively to the folded state of a protein and stabilize its structure. These small molecules only attach to proteins or their intermediate folding stages because protein aggregation stops protein molecules from folding correctly. They aid in stabilizing the structure and promise to be therapeutic agents for proteins that are prone to spatial misfolding and are associated with a range of human illnesses.

The emergence of partially folded (fragmented or elongated) states and the onset of protein aggregation, predominantly occurring in two forms - amyloidogenic and amorphous - can be induced by intrinsic or extrinsic factors or interacting agents. The process of denaturation in proteins may be essential for triggering the hydrophobic interaction that leads to protein assembly. Various spectroscopic techniques, microscopy, and the binding affinities of dyes

specific to amyloid can all be employed to monitor fibril formation [76, 77]. Across a broad spectrum of proteins, the relative rates of aggregation correlate with physical attributes of the molecules, including charge, secondary structure alignment, and degree of hydrophobicity [78, 79]. The initial phase of amyloid formation involves the generation of small, soluble oligomers through nonspecific interactions. These early prefibrillar stages are often termed "protofilaments" or "protofibrils". It can be challenging to structurally characterize these species using ensemble biophysical approaches since the conversion processes might happen rapidly and may be transitory [80]. Since these early-stage conformations play a critical role in the aggregation landscape, a detailed and comprehensive investigation of their characteristics in the assembly reaction is required **(Figure 2.3)** [81].

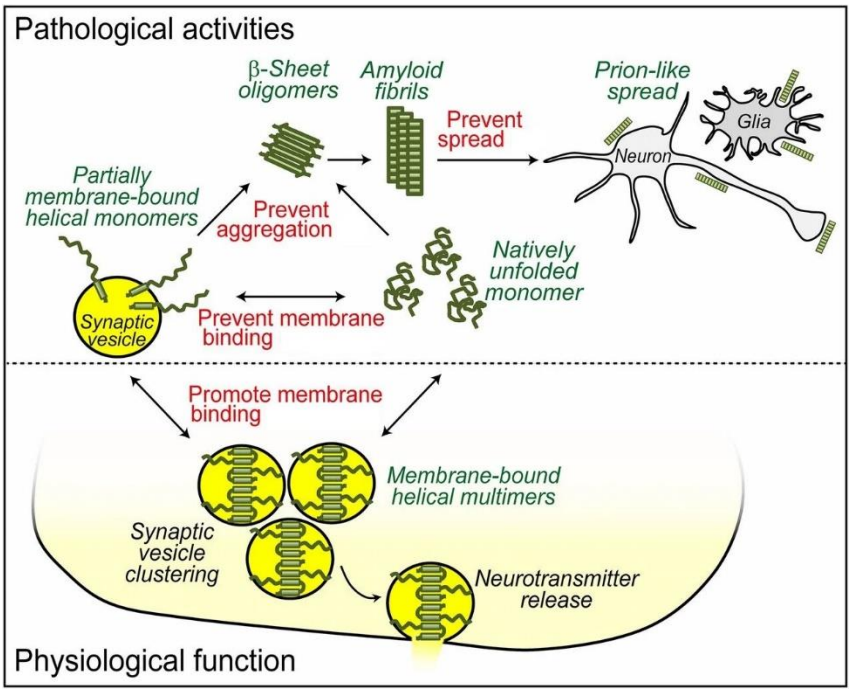


Figure 2.3. *Schematic representation of physiological and pathological conformations of α-Syn (Taken from [81])*

2.4. Parkinson's Disease- Neurodegenerative disorder:

The current statistics shows PD has affected more than 10 million individuals worldwide [82]. Physical symptoms of Parkinsonism include bradykinesia (hindered movement), tremor, rigidity, and instability in posture. Parkinsonism is a clinical syndrome including PD dementia (PDD) and dementia with LB (DLB) are both classified as PD. Nonetheless, PD is the primary condition within Parkinsonism which includes neuronal inclusions made of protein aggregates, such as tubulin and microtubule-associated protein-2 [83], lipids, neurofilaments [84], and

cytochrome c [85], are the primary cause of the distinctive formation of LB. The primary component of LB consists of α-Syn aggregates rich in beta-sheet secondary structure formation.

2.4.1. Epidemiology of Parkinson's Disease:

Estimates of PD incidence based on healthcare organisations vary from 5/100,000 to over 35/100,000 new cases annually [86]. The incidence rises five to ten times between the sixth and ninth decades of life. As people age, the prevalence of PD is also accelerating. The prevalence have arises from less than 1% of men and women in the 45–54 age range to 2% of men and 4% of women in the 85+ age range in a meta-analysis involving North American populations [87]. When PD is identified, mortality does not rise in the first ten years as compared to non-affected persons, but it does show symptoms in the later following years [88]. The prevalence of PD is predicted to rise sharply with the ageing of the world population, doubling over the next 20 years [89]. The social and financial costs of PD will rise in tandem with this rise.

2.4.2. Diagnosis:

PD is more common in adults over the age of 60, and it affects around 1% of those in this age range [90-93]. PD is a degenerative condition that can affect people for longer than 20 years. However, the illness does not begin to manifest symptoms until 10 to 15 years later. Undoubtedly, PD complications can result in a variety of fatalities. Among these are difficulties with swallowing, which can lead to food being aspirated into the lungs and developing pneumonia or other pulmonary problems. There are no definite measures for the diagnosis of PD, but a few tests are available that help in determining the rapid and progressive forms of PD. The following tests may help in the diagnosis: dopamine transporter scan. This scan is not an indicator, but it may help in supporting the medical diagnosis. Also, other scans, such as MRI or CT scans, can show the morphology of the brain and indicate any abnormality in the brain. Another diagnostic aspect that was developed in 2016 is the United Parkinson's Rating Scale. This criteria involved the probability rate of the patient having a PD diagnosis, such as the age factor; determining various other variables, including environmental risks, genetic factors, early diagnostic signs, and the sex factor; and finally, calculating the risk factors to compare with a threshold measure.

Different stages of the development of PD include:

Stage 1: There will be mild changes to a person's walking ability, posture, and face. The majority of the daily tasks will be possible for them to perform.

Stage 2: Tremors will be apparent and affect both sides of the body. Daily activities might get more difficult.

Stage 3: Balancing and coordination issues might be present. More accidents, such as stumbling, falling might occur and dressing assistance could be required.

Stage 4: Full-time care may be required. They could find it challenging to walk on their own.

Stage 5: The person's range of motion will be drastically limited. As an adverse effect of some PD drugs, they may also experience disorientation and hallucinations.

2.4.3. Pathology:

PD is increasingly understood to be a complicated neurodegenerative condition with a distinct course. It appears to impact the olfactory bulbs and nucleus as well as the dorsal motor nucleus of the vagus nerve first, followed by the locus coeruleus and finally the substantia nigra. This is supported by solid data. Later stages of PD affect cortical regions of the brain. Complex pathophysiological changes in multiple neural systems lead to impairments in the motor, cognitive, and neuropsychological domains [93]. Dopamine is released into the synapse from membrane storage vesicles in the presynaptic membrane. Upon crossing the synapse, dopamine attaches to the postsynaptic membrane, activating dopamine receptors. The remaining dopamine in the synapse is taken up by the presynaptic cell, repackaged into storage vesicles, and released back into the synapse. Dopamine degradation occurs as it traverses the synapse between cells, primarily through the action of two enzymes: catechol-O-methyltransferase (COMT) and monoamine oxidase (MAO), rendering it inactive. To prevent dopamine breakdown, a specific treatment involves injecting an MAO inhibitor into the synapse, inhibiting the function of the MAO enzyme. This enhances the quantity of dopamine that can attach to the postsynaptic membrane by allowing more of it to remain in the synapse. The transmission of chemical synapses in the presynaptic cell **(Figure 2.4)** [94].

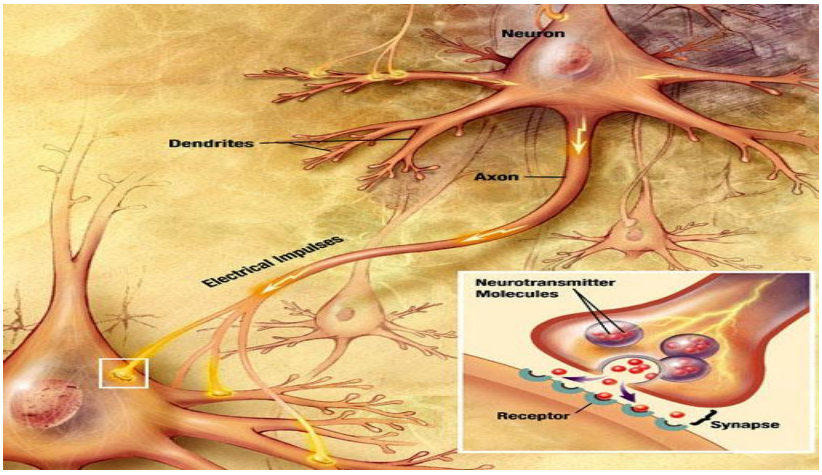


Figure 2.4. *Schematic representation of transmission of chemical synaptic in the presynaptic cell (Taken from [94])*

In the pars compacta, a region of the substantia nigra, dopamine neuron degeneration is particularly noticeable. An important finding is that dopaminergic depletion in the pars compacta increases basal ganglia sensory drive, which impairs voluntary motor control and results in PD symptoms. Starting with levodopa medication, the motor function returns to normal [95]. In PD, there is an abnormal loss of dopamine [95]. The reduction of dopamine exacerbates the severity of the condition and disrupts the function of glutamate, gamma-aminobutyric acid (GABA), serotonin, and other neurotransmitters in the basal ganglia pathways [96]. While certain dopamine cells are affected by PD, some regions of the brain exhibit relatively preserved dopamine-producing neurons, despite the vulnerability of dopamine-producing neurons in the substantia nigra [97] as shown in **Figure 2.5.** [98].

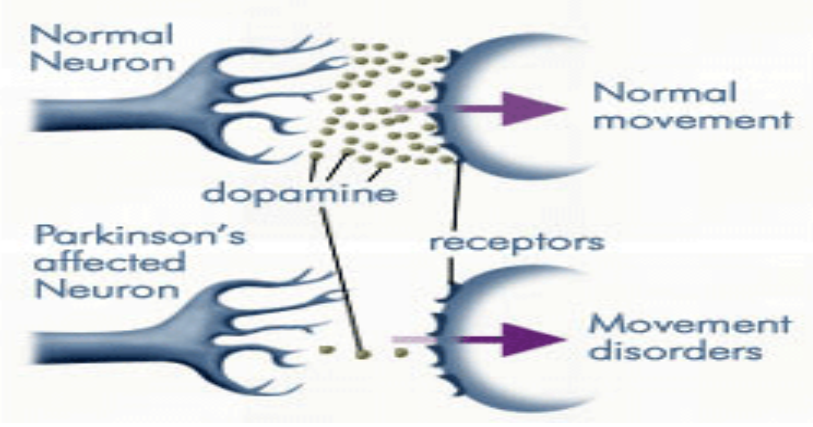


Figure 2.5. *Diagrammatic representation of dopamine levels in a normal and PD affected neurons (Taken from [99])*

2.4.4. Symptoms of Parkinson's disease:

The clinical stage of the disease in the majority of individuals affected by PD exhibits symptoms such as stiffness, postural instability, resting tremor, hypokinesia, and bradykinesia. In the affected areas of the brain, these symptoms cause 80% of the neurons to die. However, some individuals start experiencing symptoms approximately 15 years after the illness first manifests [5, 99]. Since the onset of motor symptoms is caused by a deficiency in dopaminergic neurons, the effect can be restored by modifying dopamine replacement therapy, such as L-3,4-dihydroxyphenylalanine (L-DOPA), as well as by a variety of neurosurgical techniques, like Deep Brain Stimulation (DBS), which can send electrical inputs that would not otherwise be present [97, 100-102]. Even though medical science has made great strides, there is still no cure for PD. Both movement and non-motor symptoms are present in PD [103], as shown in **Figure 2.6.**

Motor symptoms include trembling or shaking at rest, initially often unilateral muscular stiffness or rigidity, which can be painful and affect posture, as well as slowed movement, making tasks like cooking and dressing challenging. Additional symptoms may include alterations in posture, balance difficulties, slower speech, small and difficult-to-read handwriting, swallowing changes, and low blood pressure, especially upon standing up from a seated or lying position. Non-specific early symptoms that are difficult to diagnose include exhaustion, mild depression, agitation, and localized discomfort in the muscles. However, non-motor symptoms of PD include sleep issues (daydreaming and sleep talking), constipation, and cognitive impairment. Although increasing salivation during sleep may alleviate symptoms of depression and anxiety, it does not alleviate fatigue resulting from a diminished sense of smell.

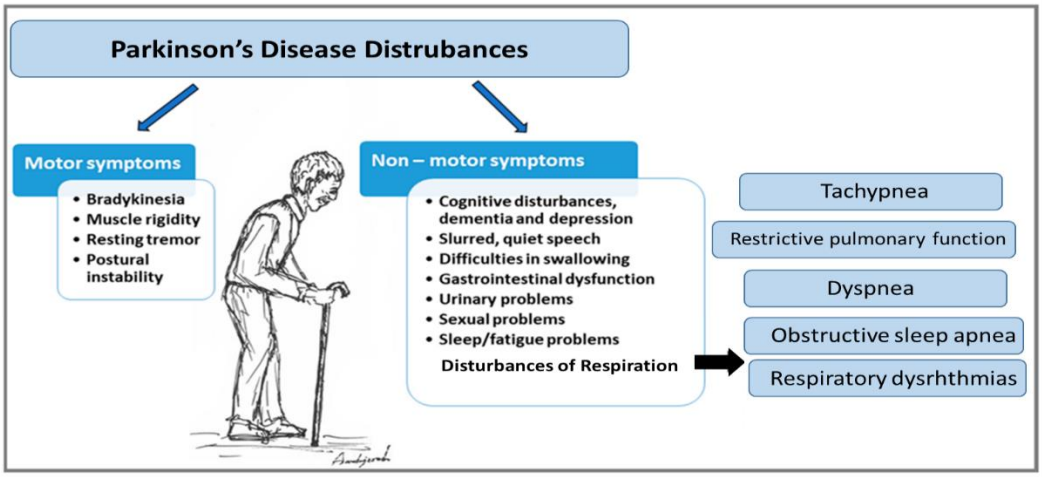


Figure 2.6. *Representation of motor and non-motor symptoms of PD (Taken from [103])*

2.4.5. Causes of Parkinson's disease:

PD affects certain brain neurons, causing progressive degeneration or death of those neurons. The underlying cause of many of the disorders is the loss of brain neurons that produce the chemical messenger dopamine. Decreased mobility and other PD symptoms are caused by aberrant brain activity, which is brought on by low dopamine levels [104].

Although the cause of PD is unknown, several variables seem to be involved such as:

a) **Genes**: Certain genetic variations have been linked to PD by researchers. However, these occurrences are uncommon and only occur in unusual situations where a large population has been affected by PD. Despite this, certain gene mutations seem to raise the chance of PD, but at a very low risk for each of these genetic markers.

b) **Environmental triggers**: If a person is exposed to specific chemicals or environmental factors, their chance of developing PD in the future may be somewhat raised. Also, researchers have observed notable alterations in the brains of PD sufferers, although it's unknown why [103]. Among these changes are:

i) **The existence of LB**: An accumulation of certain chemicals within brain cells is one of the microscopic indicators of PD. These are known as LB, and scientists believe they provide an important clue to the etiology of PD.

ii) **Presence of α-Syn in LB**: Although other compounds are present, it is believed that α-Syn, a widely distributed and naturally occurring protein, is the primary constituent of LB. All LB include it in clumped form, which cells are unable to break down. This is now a topic of interest for PD researchers.

Risk factors of PD:

Genetic counselling may be helpful in assisting a young person with PD in making decisions regarding family planning. For an elderly person with PD, work, social situations, and drug side effects are particularly significant and require special attention.

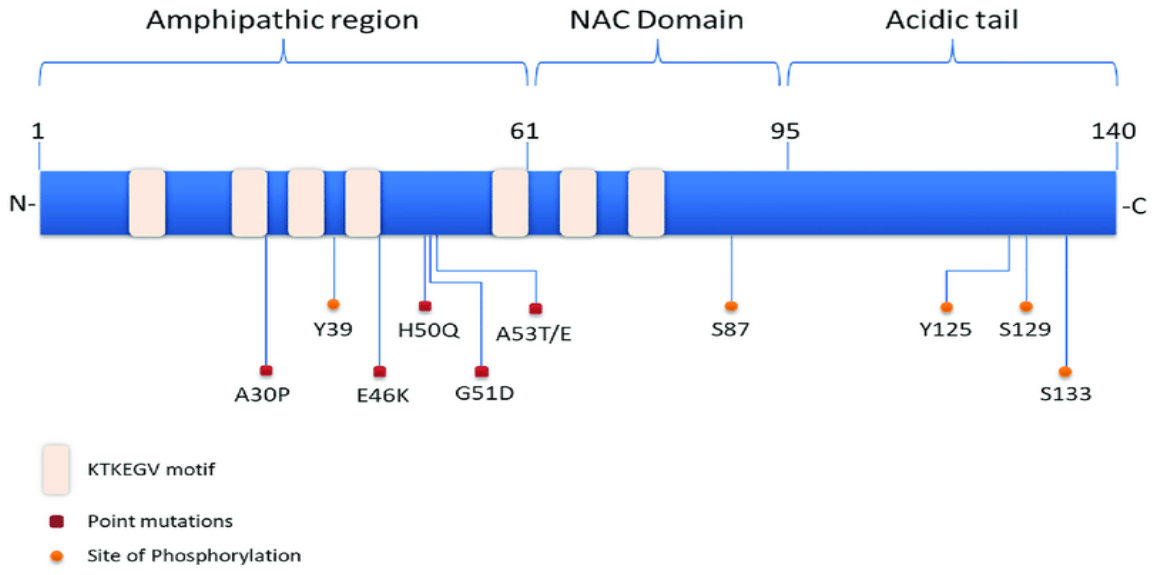
Age factor: Young adults rarely experience PD. The likelihood that it may appear increases with age, usually in middle or later life. People typically develop the illness at 60 years of age or later. The prevalence of PD is higher in males than females.

Genetics: Having a close family member with PD raises your chance of developing the illness. However, the risk is still low unless several family members have PD.

Toxin exposure: Extended use of pesticides and herbicides may raise the chance of PD.

2.5. α-Synuclein- a natively unfolded protein:

The precursor protein responsible for PD development is the α-Syn protein encoded by SNCA gene [1, 9, 105, 106]. α-Syn protein was discovered in the year 1990, but the protein was not named at that time. α-Syn protein was first reported in the electric ray Torpedo and operates as a homolog in the brainstem of rats. Since it was found in synaptic vesicles and close to the nucleus, it was given the name α-Syn [36, 107-116]. The 140 amino acid α-Syn protein (14 kDa), is inherently disordered and highly charged. The SNCA gene encoding α-Syn protein is expressed in the central nervous system [117]. The presynaptic terminals are the primary location of α-Syn, where it binds to synaptic vesicles. The synuclein family, which also contains beta and gamma synucleins (β-Syn and γ-Syn), is comprised of α-Syn. α-Syn makes up about 1% of the total proteins in the cytoplasm of neurons. The amphipathic N-terminal of α-Syn is made up of seven faulty sequence repetitions of eleven residues, and it may have an alpha helix shape that aids in aggregation and lipid binding. Moreover, the C-terminal non-amyloid component (NAC) promotes calcium binding and prevents protein aggregation [118-120] as shown in **Figure 2.7**.



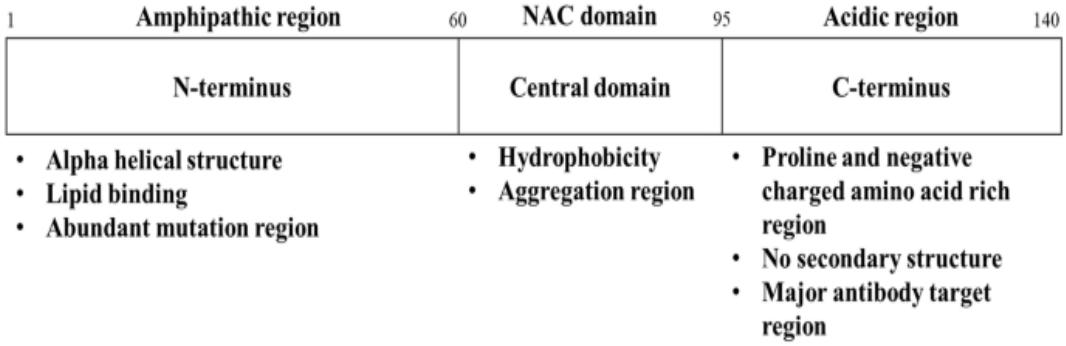


Figure 2.7. *Structure of α-Syn protein showing different domains, mutational sites and phosphorylation sites linked to familial PD (Taken from [119, 120])*

About 140 amino acid residues were found to make up the non-amyloid-beta component of the AD patient's brain plaques. It was thought that they were more comparable to torpedo Synuclein. Furthermore, two homologs comprising around 134 amino acid residues were recognized as β-Syn [121] within the human proteome. Apart from these two proteins, a different kind of Syn protein with around 127 amino acid residues, known as γ-Syn [122, 123], was also found in humans. Furthermore, it was found a homolog of α-Syn found in zebra finches was linked to brain plasticity in later years [124]. As a result, it was proposed that when α-Syn interacts with lipids, its native folded shape changes to a helical secondary structure [125, 126] **(Figure 2.8)**.

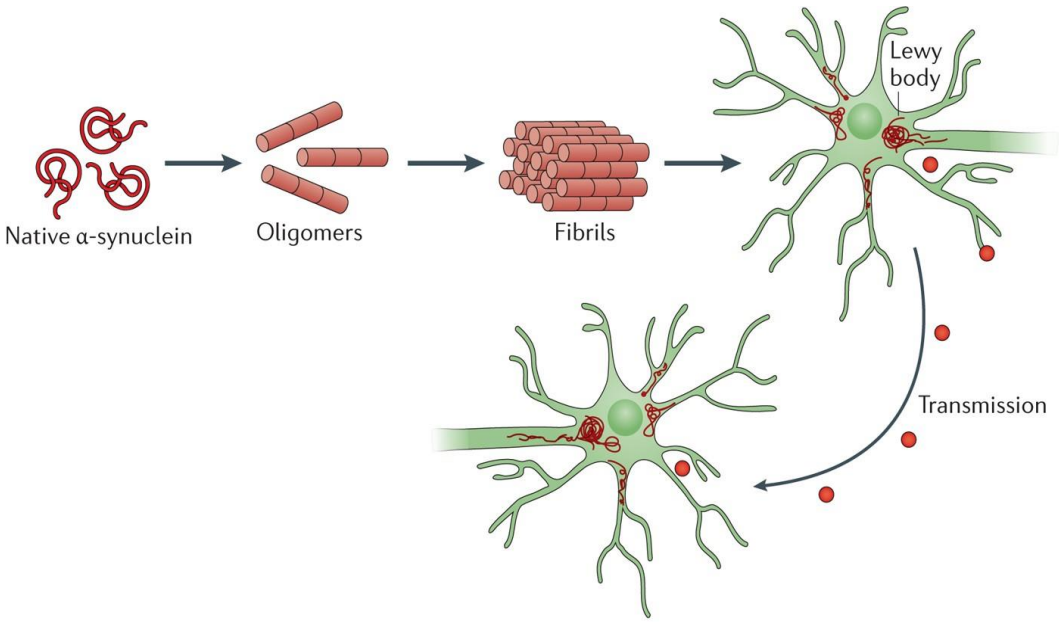


Figure 2.8. *Events of α-Syn monomers followed by pathogenicity of PD (Taken from [126])*

Numerous studies have attempted to investigate the structure under various buffer conditions, such as changes in pH, salt, and lipid content [127]. Others have examined other protein composition alterations (such as phosphorylation, glycation, glycosylation, and acetylation) and their potential impacts on protein structure and function in more recent times [128-130]. Several researchers have examined protein expression and aggregation in mammalian model systems relevant to PD disease to determine and comprehend potential functions of PTM and the surrounding environment on pathology. Though there has been increasing evidence to support the idea that α-Syn is involved in neurotransmitter release and synaptic plasticity, the precise role of α-Syn is still unclear [131, 132]. Similarly, current research indicates that the dynamic regulation of neuronal/synaptic activity governs the physiological release of endogenous α-Syn. Hence, an increase in neuronal activity results in the increasing release of α-Syn protein [133].

2.5.1. Structural details of α-Synuclein:

LB is considered to be the most pathogenic feature of PD. Intraneuronal inclusions known as LB are composed of immunoreactive aggregates of α-Syn, which may also include other neurofilament proteins and proteolysis-related proteins, including ubiquitin. α-Syn aggregation promoting nuclear components, such as histones, are released when nuclear membrane integrity is disrupted, which is the primary cause of cell death. According to Lashuel et al. [133], natural α-Syn normally resides in a dynamic equilibrium between unfolded monomers and α-helically folded tetramers with a low susceptibility to aggregation. The tetramer and monomer ratio are decreasing, leading to an increase in the amount of unfolded monomers of α-Syn, which promotes aggregation [134, 135]. α-Syn assembles into oligomers, protofibrils, and insoluble fibrils, which ultimately aggregate into LB, through a conformational shift that results in the adoption of a β-sheet-rich structure. As a result, α-Syn fibrils seem to be the most effective species at proliferating, aiding in the transmission and advancement of the disease [136, 137]. The majority of research that validates the harmful consequences of distinct α-Syn assemblies has employed *in vitro* generated species; however, it is uncertain to what degree these oligomers replicate the composition and characteristics of those discovered in brain tissue from PD patients [138]. After aggregation begins, α-Syn may diffuse to neighbouring cells directly or indirectly. Around 50–70% of neurons are destroyed in the neighbouring cells in PD patients at the time of death, compared to unaffected normal persons [139-141]. Research [142] has shown that LB play a harmful function in PD, whereas other research [143] implies that LB represent protective strategies for the neuronal cells against the buildup of intracellular

protein aggregates. As a result, there is debate in PD regarding LB. Neuronal dysfunction and cell death pathways may be activated by LB formation [142, 143]. Other important molecular processes in PD include endoplasmic reticulum stress, alteration of dopaminergic neuron signaling pathways, and dysregulation of kinase signaling [144]. Recent advances in the understanding of PD pathology linked to α-Syn are tabulated in **Table 2.1** [145].The 3-D structure consist of three regions- N-terminal , NAC region and C-terminal can be as shown in **Figure 2.9**.

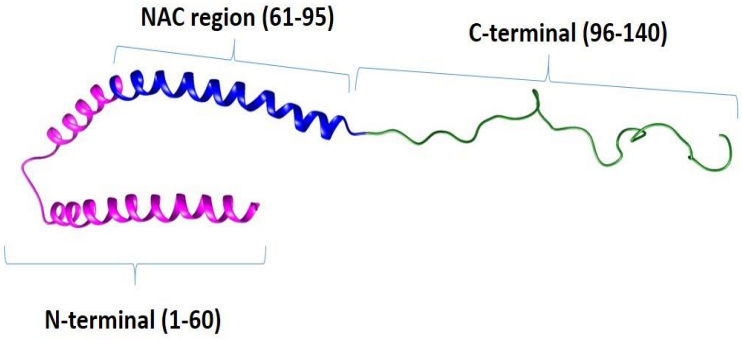


Figure 2.9. *3-D Structure of α-Syn (1XQ8 PDB ID)*

Table 2.1. *Table showing recent research advancements in α-Syn associated PD pathology (Taken from [145])*

S.no	Year of publishing	Author(s)	Title	Key research findings
1	2020	Mellier et al.	The process of Lewy body formation, rather than simply α-synuclein fibrillization, is one of the major drivers of neurodegeneration	• Proposed seeding based model for AS fibrillation. • Elucidated the interaction between AS aggregates and cellular organelles like mitochondria ER etc.
2	2020	Ray et al.	α-Synuclein aggregation nucleates through liquid-liquid phase separation	Provides detailed characterization of AS's phase-separation behavior and its pathogenic transformation into aggregates.
3	2020	Perren et al.	The structural differences between patient-derived α-synuclein strains dictate characteristics of Parkinson's disease, multiple system atrophy, and dementia with Lewy bodies	Characterizes structural variations of pathogenic AD in various synucleinopathies.
4	2020	Stephens et al.	Extent of N-terminus exposure of monomeric alpha-synuclein determines its aggregation propensity	Identifies structural orientations and environmental conditions that furthers monomeric AS's aggregation.
5	2020	Zhao et al.	Parkinson's disease-related phosphorylation at Tyr39 rearranges α-synuclein amyloid fibril structure revealed by cryo-EM	Identifies PD's pathological outcome that reorients AS's 3D structure.
6	2020	Arlehamn et al.	α-Synuclein-specific T cell reactivity is associated with preclinical and early Parkinson's disease	Examines the association between α-syn-specific T cell responses and PD.
7	2020	Niu et al.	A longitudinal study on α-synuclein in plasma neuronal exosomes as a biomarker for Parkinson's disease development and progression	Findings indicate that AS found in plasma neuronal exosomes could server as biomarker in early PD detection and also as prognostic marker for the PD's progression.
8	2019	Bhattacharjee et al.	Mass Spectrometric Analysis of Lewy Body-Enriched α-Synuclein in Parkinson's Disease	Identified 20 AS variant species in PD patients.
9	2019	Levine et al.	α-Synuclein O-GlcNAcylation alters aggregation and toxicity, revealing certain residues as potential inhibitors of Parkinson's disease	Advocates O-GlcNAcylation as a potential therapeutic tool for regulating PD.
10	2019	Tian et al.	Erythrocytic α-Synuclein as a potential biomarker for Parkinson's disease	Finding indicate that AS levels are altered in PD patients' erythrocytesand peripheral erythrocytic could be a potential biomarker.
11	2019	Elfarrash et al.	Organotypic slice culture model demonstrates inter-neuronal spreading of alpha-synuclein aggregates	Characterizes how AS aggregates are formed and spreads across neurons.
12	2019	Landeck et al.	Toxic effects of human and rodent variants of alpha-synuclein *in vivo*	Quantifies the neurodegenerative effects of rodent and human AS variants.
13	2019	Brys et al.	Randomized phase I clinical trial of anti-α-synuclein antibody BIIB054	Delineates the phase I clinical trial outcomes of BIIB054 antibody against AS, which indicates that BIIB054 pose tolerability, safety, and pharmacokinetic propensities in PD patients.
14	2019	Kang et al.	Comparative study of cerebrospinal fluid α-synuclein seeding aggregation assays for diagnosis of Parkinson's disease	Formulates high accuracy AS seeding aggregation assay that can be used for PD diagnosis.
15	2018	Pihlstrom et al.	A comprehensive analysis of SNCA-related genetic risk in sporadic Parkinson's disease	Delineates AS encoding SCNA gene polymorphisms that are associated with sporadic PD.
16	2018	Longhena et al.	Synapsin III is a key component of α-synuclein fibrils in Lewy bodies of PD brains	Identifies Synapsin III as an integral part of AS fibrils in PD patients' brain.
17	2018	Faustini et al.	Synapsin III deficiency hampers α-synuclein aggregation, striatal synaptic damage, and nigral cell loss in an AAV-based mouse model of Parkinson's disease	Constitutes SynapsinIII as a crucial factor for AS aggregation and could be a potential target in mitigating PD.
18	2018	Papagiannakis et al.	Alpha-synuclein dimerization in erythrocytes of patients with genetic and non-genetic forms of Parkinson's Disease	Increased AS dimers were observed in PD patients, which could provide insights into PD prognosis and can be used as biomarker.
19	2018	Jankovic et al.	Safety and Tolerability of Multiple Ascending Doses of PRX002/RG7935, an Anti-α-Synuclein Monoclonal Antibody, in Patients With Parkinson's Disease: A Randomized Clinical Trial	Multiple doses of PRX002 antibodies was found to be safe and tolerable even resulted in robust interaction with peripheral AS.
20	2018	Zhang et al.	A Comprehensive Analysis of the Association Between SNCA Polymorphisms and the Risk of Parkinson's Disease	Delineates SNPs in SCNA gene that pose risk of developing PD rs11931074 and rs2736990 increased the risk in East Asian group rs181489, rs356219, rs356165, rs2737029, and rs11931074 increased the risk for European group.

2.5.2. Structure of membrane-bound α-Synuclein:

According to the present paradigm, the α-Syn protein most likely exists *in vivo* as an equilibrium combination of a statistically disfavoured helical oligomer and unstructured monomer, and may be partly folded at membranes through phospholipid interactions. Stabilizing helical α-Syn is a unique technique for treating PD, as it may be necessary for an undiscovered natural function and is not expected to be harmful. Though small compounds were used to target the protein, Kelly and colleagues' method [146,147] of stabilizing the native fold can be comparable to the proteins in the presence of small compounds. Furthermore, it has been proposed that α-Syn protein has a strong affinity for phospholipid membranes, particularly the protein found on synaptic vesicles, where it prefers membranes with certain membrane microdomains and a high curvature [148]. It has been discovered that α-Syn controls presynaptic terminal size and the dispersion of synaptic vesicles. The α-Syn protein's capacity to bind lipids is specifically aided by the N-terminal alpha helix [149]. Elevation of α-Syn levels has been proposed to reduce glutamine and dopamine neurotransmission by regulating endoplasmic reticulum-golgi vesicular trafficking, lowering synaptic contact, suppressing vesicular priming, and interacting with the SNARE protein complex [150]. It has been demonstrated that overexpression of monomeric wild-type (WT) α-Syn inhibits vesicle endocytosis and degrades neurotransmission. It has been proposed that α-Syn causes PD by interfering with dopamine production, storage, recycling, reuptake, and efflux [137]. It was shown that *in vitro*, α-Syn exhibited significant binding with the lipid membranes of large unilamellar vesicles that had anionic or zwitterion headgroups. This binding is thought to mitigate the α-Syn protein-protein interactions that result in fibrillation. When α-Syn is attached to a membrane, it is more likely to aggregate than when it is in the cytoplasm. Structural alterations may also arise from alterations in the lipid composition or chemical modification of membrane lipids. Oxidative modification is one of these structural alterations that happen to phospholipids and is essential to their aggregation in the membrane. Membrane lipid oxidation can promote the aggregation of α-Syn by generating an environment that is conducive to self-assembly or by oxidatively changing α-Syn, which modifies the protein structure and heightens its vulnerability to aggregation.

2.5.3. Influence of Mutants on Membrane Interaction:

Most studies confirming the detrimental effects of different α-Syn assemblies have primarily utilized *in vitro* produced species, raising uncertainty regarding the extent to which these oligomers mirror the structure and attributes of brain tissue from individuals with PD [2].

Environmental variables, imbalanced production and degradation of α-Syn, post-translational changes, and mutations all affect α-Syn protein's tendency to aggregate. The A53T mutation is linked to an early-onset PD and was the first to be described [151]. There is a high probability of widespread LB and a predisposition to develop severe Parkinsonism with dementia associated with the E46K mutation. Because these mutations change the structure of the α-Syn protein, it can aggregate more easily [151]. Point mutations in the α-Syn gene have been identified as causing autosomal-dominant early-onset PD, while gene overexpression has been associated with late-onset or sporadic PD as shown in **Figure 2.10** [152]. Studies have shown that the mutant α-Syn protein (A30P and A53T disease mutations) associated with familial PD exhibits structural flaws affecting its ability to bind to membranes and altering its binding properties [153]. It has been noted that WT α-Syn forms two distinct dimers, and single point mutations (A30P, E46K, and A53T) may facilitate α-Syn dimerization. Moreover, it has been suggested that the uniform structure of these dimers leads to distinct pathways of aggregation [154]. Recently, the discovery of a novel heterozygous A30G mutation in the SNCA gene associated with familial PD was reported [155], detailing clinical characteristics of affected individuals, genetic findings, and functional implications. Similarly, the G51D alterations on the adjacent residue may hinder fibril formation by diminishing hydrophobicity and flexibility [156]. Additionally, potential charge repulsion with E57, along with the introduction of extra steric bulk at the dimeric fibril interface where the G51 sidechain interposes between V55 and E57 on the opposing chain, further contributes to this effect. A stabilising salt bridge may have formed in most of the structures due to the near closeness of the E46 and K80 side chains [157]. As a consequence of the disruption of this salt bridge in the E46K α-Syn mutant, electrostatic repulsion occurs, leading to destabilization of the Greek key conformation and likely resulting in an increased concentration of smaller oligomers. Also, the A53T and A53E [151] mutant side chains are greater in size and more hydrophilic, they may also prevent the development of hydrophobic zippers between adjacent protofibrils by preventing close contact (A53 inserts between A53 and V55 side chains on the opposite chain). As a result, these modifications probably make the hydrophobic packing inside the steric zipper weaker. Therefore, considering the quickly changing field and the recently discovered structural details of the pathogenic and natural versions of the α-Syn protein. **Table 2.2** shows the effects of various early-onset mutations in the SNCA gene with regard to the WT protein, such as age of onset, lipid binding, and rates of fibril development [158].

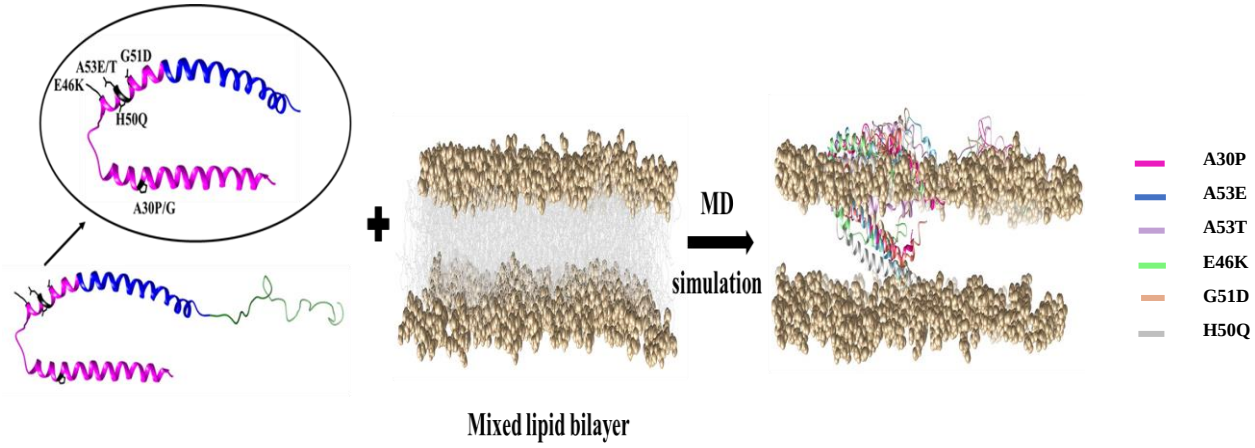


Figure 2.10. *Point missense mutations of α-Syn protein in membrane bound state*

Table 2.2. *Comparison of the consequences of several early onset mutations in the SNCA gene with respect to the WT protein, including age of onset, lipid binding, and fibril development rates (Taken from [158])*

Mutation	Age of onset	Lipid binding	Rate of fibril growth
A30P	54-76	↓	↓
E46K	50-69	↑	↑
H50Q	60-71	≡	↑
G51D	19-61	↓	↓
A53E	36	↓	↓
A53T	20-85	↑	↑

2.5.4. α-Synuclein induced membrane perturbation:

In vivo, α-Syn exists in a partially membrane-bound, soluble, cytosolic, and unstructured state. Notably, it has been suggested that the equilibrium between ordered and disordered conformations of α-Syn at membrane interfaces, or between structured and unstructured states, influences the protein's biological functions and triggers aggregation upon detachment from membranes [159]. The amphipathic N-terminal promotes a conformational shift into a highly helical state, which is necessary for α-Syn to bind to membranes. Moreover, findings revealed that in the amyloid state of α-Syn, the NAC domain exhibited a highly organized beta-sheet conformation, while the C-terminal region of α-Syn was found to be extensively disordered [160]. There is currently agreement that α-Syn facilitates membrane curvature, which aids in synaptic trafficking and vesicle budding. Given the association of α-Syn with presynaptic terminal SNARE complexes [161], this observation holds significance and suggests that α-Syn might play a role in regulating dopamine release. This, in turn, has prompted several investigations into the transmission of protein across synaptic terminals. Additional evidence supports a "prion-like" hypothesis, suggesting that oligomeric α-Syn could propagate across

neurons, leading to the development of LB in both the substantia nigra and other brain regions. Specifically, studies conducted by Bartels et al. [162] and Wang et al. [163] have demonstrated that α-Syn can adopt a stable helical conformation through interactions, forming homotetrameric structures. Subsequent research has shown that this structure can be reproduced by incorporating lipids, resulting in helical multimers and providing further evidence of α-Syn's intrinsic involvement in membrane interactions, particularly in vesicle budding.

2.5.5. Membrane binding induced broken and extended helix:

Significantly, α-Syn interacts with synaptic vesicles anchored to the plasma membrane due to its distinctive helical structure, characterized by two antiparallel membrane-bound helices connected by a non-helical linker [147]. Furthermore, in a study [159] it was reported the analysis of the NMR structure of human α-Syn reveals that amino acids ranging from 3–37 to 45–97 form curved alpha-helices, which are connected by extended linkers and arranged in an unusual anti-parallel fashion. Following this, a noticeably long movable tail ranging from 98 to 140 amino acids is present. The structured alignment of the helical connector implies a specific interaction with lipid surfaces, indicating its potential role as a mediator between the mentioned structure and the previously suggested unbroken helical model, especially when associated with synaptic vesicles of larger diameter [160].

2.6. Post translational modifications of Membrane bound α-Synuclein:

Numerous post-translational modifications (PTM) involving α-Syn have been observed *in vivo,* indicating their significance in the pathophysiology of PD. These modifications primarily encompass acetylation, phosphorylation, and nitration, all of which have been observed to influence the generation of diverse oligomers and the pace of fibril formation. In mammalian cells, N-terminal acetylation is a common post-translational alteration of α-Syn [163]. Studies [164, 165] have shown that it induces a two-fold increase in affinity for lipid vesicles, alongside enhancing helicity in the protein's N-terminal region and reducing aggregation rates. The protein used by Li et al. [166] to determine the cryo-electron microscopy (cryoEM) structure of mature fibrils included this PTM.

2.6.1. Phosphorylation of α-Synuclein:

In recent studies [82, 167] residues pY39, pS87, and pS129 have all been shown to be phosphorylated, however, residue pS129 appears to be the most notable and pathologically significant phosphorylation site. Using cryo-EM [168], researchers elucidated the structure of

the pY39 α-Syn fibril. In this variant, α-Syn folds within the core of the fibril, establishing an electrostatic interaction network with eight charged residues located in the N-terminal region of the protein [168]. Enhanced phosphorylation of pS129 was found to exacerbate toxicity in α-Syn-overexpressing SH-SY5Y cells [169], whereas reduced phosphorylation of pS129 was demonstrated to mitigate neuronal loss in Drosophila melanogaster [170]. In contrast, it has been demonstrated in rat and yeast investigations [171] that knockouts that block pS129 phosphorylation promote beta-sheet rich aggregation formation and α-Syn toxicity. Phosphorylation of the pS87 residue has demonstrated potent aggregation inhibitory effects by enhancing α-Syn's conformational flexibility and lowering its affinity for lipid membranes and vesicles [172].

2.6.2. C-terminal truncation of α-Synuclein:

Truncation of the C-terminal domain (CTD) of α-Syn is found in the brains of PD patients, reducing cell viability and tending to form fibrils. Nevertheless, little is known about the mechanisms underlying the role of C-terminal truncation in the cytotoxicity and aggregation of α-Syn. Using NMR, the truncation alters α-Syn conformation, resulting in an attractive interaction of the N-terminal with membranes and the molecular chaperone, protein disulfide isomerase (PDI). The truncated protein is more toxic to mitochondria than the full-length protein and diminishes the effect of PDI on α-Syn fibrillation [173]. Due to the extraordinary capacity to aggregate and change into pathogenic fibrils, many variants of CTD-truncated α-Syn have been discovered in both normal and PD brains and are the subject of extensive investigation [174, 175]. *In vitro,* CT-α-Syn forms oligomers and fibrils more quickly than FL-α-Syn, the full-length protein. When FL-α-Syn and CT-α-Syn are co-expressed, FL-α-Syn accumulates pathologically [139]. The aggregates generated from α-Syn (1-108) did not show a significant polymorphism in comparison to full-length α-Syn aggregates. The effects of the C-terminus on aggregation point to a specialized function for amino acids 109–140 in the control of aggregation, most likely involving the development of intramolecular interactions, rather than being explained only by a contribution to the protein net charge [176]. The CTD (residues 96–140) is a region rich in proline and very acidic, lacking any discernible structural inclination. To prevent rapid α-Syn fibrillation, the negative charge within the acidic portion of the CTD provides chaperone-like properties and can decelerate the oligomerization process [177].

2.7. Therapeutic Strategies targeting α-Synuclein-lipid membrane Interactions:

As of now, there are no disease-modifying treatments available for PD; nevertheless, palliative care focused on symptom management, such as medication or surgery, may significantly alleviate motor deficits. Therefore, efforts aimed at stabilizing the physiological conformation of α-Syn, reducing its expression, preventing its aggregation, and enhancing intracellular clearance are distinguished from transmission focused strategies. These strategies also involve preventing uptake by neighbouring cells and enhancing extracellular clearance mechanisms in targeting α-Syn [178]. The most promising medication to treat PD motor symptoms is levodopa. Efforts have been undertaken to replace the depleted dopamine at the neurotransmitter level. Carbidopa and levodopa are combined to hinder its peripheral metabolism, thus enabling enhanced penetration of levodopa through the blood-brain barrier (BBB). At least 50% of people who use levodopa experience a variety of involuntary problems with prolonged exposure to the drug, such as five to 10 years [179-181].

However, recent studies [182,183] have shown that peptidomimetic inhibitors like NPT100-18A interact with the CTD of α-Syn, displacing it from the membrane. This action reduces α-Syn oligomer formation, accumulation of α-Syn in neurons, and indicators of cell toxicity. Treatment with NPT100-18A in WT α-Syn transgenic mice improved motor impairments in a dose-dependent manner [182]. Similarly, NPT200-11, akin to NPT100-18A, also inhibits α-Syn aggregation by stabilizing α-Syn conformers and preventing pathological protein misfolding. In another recent study by Perni et al. [183], an aminosterol derivative, Squalamine and its derivative Trodusquemine, antibacterial compounds derived from dogfish sharks, inhibits α-Syn aggregation *in vitro* and toxicity *in vivo* by displacing it from phospholipid membranes. There was a correlation between the removal of α-Syn from liposomes and a reduction in α-Syn aggregation. Also, Squalamine showed a significant degree of binding to α-Syn fibrils, which might account for at least some of the Squalamine-induced suppression of α-Syn aggregation observed [182].

2.8. Scope of thesis:

Numerous strategies were designed to eliminate α-Syn from the membrane, quicken its breakdown and clearance, prevent aggregation, or lessen its synthesis might be useful therapeutic modalities. From the above research findings, it will be helpful for us to refer to a few gaps in the study, such as:

(a) The magnitude of α-Syn aggregation propensity in association with the lipid membrane still needs to be studied in detail.

(b) Even though the interaction between the α-Syn diseased variants and lipid membrane has been studied extensively in vivo and in vitro techniques, the conformational dynamic changes at the atomistic level need to be explored in detail.

(c) Even though the interaction between the α-Syn diseased variants and lipid membrane has been extensively studied in vivo and in vitro, the conformational dynamic changes at the atomistic level need to be explored in detail.

Thus, the conformational stability of membrane-bound α-Syn was studied to understand the α-Syn-membrane interactions that may be useful for developing a new therapeutic approach for treating PD and other neurodegenerative disorders, as discussed in Chapter 4 of my thesis. The amyloidogenic NAC region of α-Syn was observed to expose itself from the lipid bilayer surface, which plays a role in the interconversion between the "extended" and "broken-helix" states and consequently leads to the formation of conformational intermediates that are prone to aggregation.

The co-solute properties of a crowded intracellular environment, along with the excluded volume effect, were characterized to understand the α-Syn dynamics in cells. We have also demonstrated and elaborately discussed in Chapter 5 of my thesis the isolation of the most probable conformer of α-Syn from structural MD analysis based on some critical aspects that emphasize its nature as a potential drug target.

The structure of A30G α-Syn in solution as a free monomer was noticed to be mostly unfolded, but it did show a preference for helical conformation, which may be important in the aggregation of α-Syn into fibrils. Hence, Chapter 6 of my thesis summarizes the effects of the A30G mutation in α-Syn on its association with the lipid membrane and in free solution.

A comparative study of the conformational dynamics of α-Syn mutants (A30P, A53E, A53T, E46K, G51D, and H50Q) and their subsequent aggregation propensity in their membrane-bound state was carried out. Therefore, in Chapter 7 of my thesis, based on the MD trajectory analysis, it was concluded that the membrane-bound H50Q α-Syn showed the highest flexible region in the NAC region, which infers a diverse effect on aggregation propensity.

The potential binding position of the two drugs (NPT100-18A and NPT200-11) on α-Syn and the impact of these two drugs on the α-Syn and lipid membrane interactions at an atomistic

level have been focused on therapeutic strategies against these neurodegenerative disorders in Chapter 8 of my thesis. It was found that in the presence of peptidomimetic and aminosterol inhibitors (Squalamine and Trodusquemine), α-Syn protein adopts well-defined α-helical structures during its interaction with lipid membranes, which inhibits the transition of the α-helical secondary structure into a coiled structure.

The CTD of α-Syn contains the overall negative charge of the amino acid residues (96–140) that remain disordered and flexible even when it is in a membrane-bound state. In Chapter 9 of my thesis, the conformational characteristics of the truncated CTD α-Syn (1-99 and 1-108) and its interaction with the membrane are studied. From our findings, the truncated CTD can be suggested to modulate α-Syn aggregation by interfering with the binding of the α-Syn protein to the membrane and providing support for the pathogenic function of CTD truncation in PD development.

Post-translational modification such as pS129 is capable of stabilizing the propensity of the protein to adopt an α-helical-rich conformation due to a decrease in the hydrophobicity of binding sites. The conformational snapshots of pY39 α-Syn obtained showed a high degree of fluctuations in the N-terminal region that disrupts the helix-2 binding region. In the case of pS87 α-Syn, due to phosphorylation, the C-terminal is flanked out of the membrane, which aids in decreased binding affinity towards the membrane bilayer. In Chapter 10 of my thesis, the MD analysis was performed to give insight into the role of phosphorylation at Tyrosine 39 (pY39), Serine 87 (pS87), and Serine129 (pS129).

2.9. Main objectives of thesis:

- **Objective 1:** To study the structural and dynamic insights of membrane bound α-Synuclein at atomistic level resolution
- **Objective 2:** To study the effects of mutant (A30P, A53E, A53T, E46K, G51D, H50Q and A30G) α-Synuclein on its interaction with membrane and its subsequent aggregation
- **Objective 3:** To design small molecule inhibitors based on the dynamics of membrane bound α-Synuclein protein