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DNA induced folding/fibrillation of alpha-synuclein: new insights in Parkinson's disease

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1. ABSTRACT

Emerging evidences on the nuclear localization of alpha-Synuclein in neurons and a close look in to its primary sequence/structural organization led us to examine its DNA binding ability. Subsequently, we first time demonstrated the interaction of DNA with alpha-Synuclein which was also confirmed by others. We recently showed that double-stranded oligos induce partial folding in alpha-Synuclein and promote its aggregation, where as single-strand circular DNA and supercoiled plasmid DNA induced a helix-rich conformation and protected the protein from fibrillation. In turn, alpha-Synuclein modulates DNA conformation from B- to an altered B-form, which may affect DNA transactions. Interestingly, amyloid-beta peptides and prion proteins implicated in Alzheimer's disease and Prion diseases respectively, were also shown to have DNA binding activity which suggests that DNA binding may be a common property of many amyloidogenic proteins associated with various neurodegenerative disorders. In this review, we debate the biological significance of DNA-alpha-Synuclein interactions; it's beneficial vs. toxic role in relevance to Parkinson's disease.

2. INTRODUCTION

alpha-Synuclein is a highly conserved protein of yet undetermined function, which has been implicated in the pathogenesis of several neurodegenerative diseases, including Parkinson's disease (PD), dementia with Lewy bodies (DLB) and multiple system atrophy (1-7). The protein accumulates in intracellular inclusions and abnormal neurites (Lewy bodies and Lewy neurites) that are characteristic of PD, the second common neurodegenerative disorder (4). However, the role of alpha Synuclein in neuropathology leading to degeneration of neurons is not clearly understood.

Although alpha-Synuclein was originally believed to be a presynaptic protein and its accumulation was predominantly cytosolic, interestingly, several recent studies have shown the presence of the protein in the cell nucleus (8-16). A recent study involving semiquantitative analysis in different subcellular compartments revealed that a significant fraction of alpha-Synuclein is in nucleus of neuronal cells in rat brain (16). In addition to the normal localization in nucleus, the increased permeability of nuclear membrane in the neurons of PD affected brain

regions, and conditions of oxidative stress could result in non-specific translocation of alpha-Synuclein in to nucleus (13). With these evidences for localization in nucleus and the knowledge of primary sequence of alpha-Synuclein, where it has the positively charged amino acids clustered towards N-terminal end, we hypothesized that alpha-Synuclein may be having a DNA binding role in nucleus (17). Subsequently, we have recently provided the first evidence for DNA-binding activity of alpha-Synuclein (17-19). We showed that alpha-Synuclein alters the helicity of supercoiled plasmid DNA *in vitro* (17), and single-strand circular DNA induces an alpha-helical conformation in alpha-Synuclein, while various other linear DNA sequences induce partially folded conformations (19). We also showed that DNA binding significantly modulates fibrillation properties of alpha-Synuclein (19). This was also further shown independently by other groups (20).

In this article, we will extensively review the DNA binding property of alpha-Synuclein and debate its potential significance in the pathophysiology of PD. We will also explore the potential utilization of DNA binding property for protection from alpha-Synuclein aggregation and/or toxicity in relevance to PD and related alpha-Synucleinopathies.

2.1. General characteristics of PD

PD was first formally described in "An Essay on the Shaking Palsy," published in 1817 by a London physician named James Parkinson (21). It is a common progressive neurological disorder that results from degeneration of nerve cells in a region of the brain called '*substantia nigra*' (SN) that controls balance and coordinates muscle movement. This degeneration creates a shortage of dopamine, a neurotransmitter, which causes impaired movement. In the United States alone, about a million people are believed to suffer from PD, and about 50,000 new cases are reported every year (22-24). Because the symptoms typically appear later in life, these figures are expected to grow as the average age of the population increases over the next several decades.

There is no cure for PD to date. Available drugs suppress symptoms early in PD, but progressively fail as more nerve cells die. The emergence of drug-induced dyskinesias and motor fluctuations often limits drug benefits. Developing therapies to prevent PD, to suppress symptoms, to halt disease progression, and to repair damage are all fundamental goals in modern day research, besides early diagnosis of PD. The preclinical diagnosis of PD is critical, so that neuroprotective therapies might be administered during the early stage and efficiently slow down the diaease progression. To achieve therapeutic goals, new and innovative studies are required, from basic research advances to translating the same in to animal testing, and safety studies in human patients.

2.1.1. Pathology: Lewy bodies

Pathologically, PD is characterized by the loss of the pigmented dopaminergic neurons from the substantia nigra pars compacta (SNpc). These nerve cells, for reasons that are not fully understood, are especially vulnerable to damage of various sorts, including drugs, disease, and head trauma. These neurons project to the striatum and their loss leads to alterations in the activity of the neural circuits within the basal ganglia that regulate movement. Disruption of dopamine along the non-striatal pathways likely explains much of the neuropsychiatric pathology associated with PD. Excessive accumulations of iron, which are toxic to nerve cells, are also typically observed in conjunction with the protein inclusions. Other pathological events include the presence of extracellular melanin (a dark pigment), released from degenerating neurons, reactive gliosis (increase in numbers of glial or support cells), and pink-staining cellular inclusions known as *Lewy Bodies* (LBs) in the remaining SNpc neurons (25). The LB, which was first described by Frederick Lewy in 1913, is present in essentially all cases of PD (25). The major protein constituent of LBs is alpha-Synuclein, a natively unfolded protein having high propensity for fibrillation/aggregation. The mechanism by which the brain cells in Parkinson's are lost may consist of an abnormal accumulation of the protein alpha synuclein bound to ubiquitin in the damaged cells.

2.2. Oxidative stress and DNA damage in PD

Substantial evidence implies that redox imbalance or oxidative stress following overproduction of reactive oxygen/nitrogen species overwhelming the protective defense mechanism of cells contributes to the pathogenesis of PD (26-35). Nigral dopaminergic neurons in human brain are particularly exposed to oxidative stress because the metabolism of dopamine gives rise to various molecules that can act as endogenous toxins if not handled properly (36, 37). In PD, nigral cells seem to be further under a heightened state of oxidative stress, as indicated by elevations in by-products of lipid, protein and DNA oxidation, and by compensatory increase in antioxidant systems (38-43). The level of iron, which is significantly higher in the normal SN than in other regions owing to its binding affinity to neuromelanin, was further increased in the SN of PD further contributing to oxidative stress (41, 44-49).

One of the consequences of redox imbalance is apoptosis and/or necrosis which are associated with neurodegeneration in PD (50-56). Studies have also shown that the levels of the nucleoside, 8-hydroxy -2'-deoxyguanosine (8-OHdG), a product of free radical attack on DNA were generally increased and differentially distributed in PD brains with highest levels in caudate, putamen, SN and cerebral cortex (42). Features of apoptosis based on histochemical methods to mark endonuclease-induced DNA fragmentation by *in situ* terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling-TUNEL/ISEL (57) or *in situ* nick translation (58) have been reported in SN in PD (30, 51, 53, 59-63).

We have recently shown increased DNA fragmentation and decreased DNA stability in affected human brain regions of PD (64). Similarly, we also showed an altered DNA conformation in hippocampus of human brains affected with Alzheimer's disease (AD) (65)

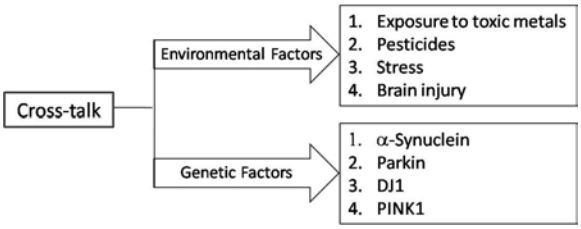

Figure 1. The 'c*ross-Talk*' or interplay of environmental and genetic causative factors for Parkinson's disease.

In addition to DNA damage, extensive RNA damage has been reported in PD brain (66). Nunomora *et al.,* (67) demonstrated using immunoreactivity to 8-hydroxyguanosine in neurons that RNA was a major site of nucleic acid oxidation in DLB. The authors suggested that normal RNA oxidation might represent one of the fundamental abnormalities in age-associated neurodegeneration including PD and AD.

Furthermore, studies suggested that an alteration in the genetic material within mitochondria in PD, including a common 4977-bp deletion in mitochondrial DNA (68, 69). However, Zhang *et al.,* (70) recently demonstrated that this 4977-bp deletion is associated with normal ageing and there is no particular association with neurodegeneration.

2.3. Cause: cross-talk of environment and genome

The precise causes of PD remain undetermined. The causes are likely to include both genetic (Parkin and alpha-Synuclein) and environmental factors (metals, pesticides etc). However, very few cases of PD have pure genetic or environmental etiology; while in vast majority both genetic and environmental factors are involved. Understanding this 'cross-talk' between genetic and environmental factors is important in PD research.

During the past decade, genetic approaches to the study of PD have resulted in major insights. The number of genes implicated in the pathogenesis of PD has been constantly increasing, and includes genes encoding for alpha-Synuclein, Parkin, DJ-1 and PINK1 (71). These genes are thought to be involved in the proteasomal protein degradation pathway, in the cell's response to oxidative stress, and in mitochondrial function, respectively (71). Over the last few years, several genes for rare, monogenically inherited forms of PD have been mapped and/or cloned. In dominant families, mutations have been identified in the gene for alpha-Synuclein. Although most people do not inherit PD, studying the genes responsible for the inherited cases is advancing our understanding of both common and familial PD.

Evidence has accumulated steadily to support the view that PD can originate from long-term, subclinical damage to the nervous system caused by environmental toxins (72-75). In fact, several studies have implicated such environmental factors as pesticides, herbicides, and heavy metals in the PD origin (30, 76-82). Our lab recently showed that trace metal homeostasis is significantly affected in serum samples from PD affected human subjects and there is a direct link between disturbance of trace metal levels in serum and brain (83), suggesting important role played by metals in PD pathology (84-87).

There is interaction between the environment and the genome; in some disorders inheritance establishes susceptibility and environment triggers pathology (88). Hence, the recent trend to study PD is to look at the interplay or *cross-talk* between genetics and environmental triggers (Figure 1). Hence, it is important to understand/explore the complex interactions between genetic predisposition and environmental influences that probably cause most cases of PD.

3. ALPHA-SYNUCLEIN AND PD

The synucleins are a family of proteins whose function in normal cell is not well understood. The first of the synuclein proteins described in 1988 was alpha-Synuclein. The name 'synuclein' was chosen because the protein was found in both synapses and nuclear envelope (8). Later, it was also named the non-amyloid component (NAC) of beta-amyloid plaque precursor protein. The NAC peptide was isolated from amyloid-rich senile plaques of brains of patients with AD. Amyloid plaques are one of the hallmarks of AD. NAC peptide was shown to be identical to a certain part of alpha-Synuclein. The second member of the synuclein family is known as beta-synuclein. Both these proteins are found in the presynaptic terminals of neurons and many researchers believe they may be involved in synaptic function. The third member of synuclein family is gama-synuclein. All synucleins have in common a highly conserved alpha-helical lipid-binding motif with similarity to the class-A2 lipid–binding domains of the exchangeable apolipoproteins (89). Synuclein family members are not found outside vertebrates, although they have some conserved structural similarity with plant 'late-embryo-abundant' proteins. The alpa and beta Synuclein proteins are found primarily in brain tissue. The gama-Synuclein is found primarily in the peripheral nervous system and retina, but its expression in breast tumors is a marker for tumor progression. While alpha-Synuclein has been implicated in neurodegenerative disorders mainly PD, until recently there has been no evidence to suggest a role for the other synucleins in neurodegeneration. alpha-Synuclein forms fibrillar aggregates known as LBs in PD brain, these insoluble protein aggregates are morphologically similar to the amyloid fibrils found in AD neuritic plaques and in protein deposits associated with other amyloidogenic diseases (90, 91).

Three missense mutations in the alpa-Synuclein gene have been reported to be associated with families susceptible to inherited forms of PD (92-94). These mutations cause alterations in the amino acid sequence of alpa Synuclein (at residues Ala30Pro or Ala53Thr or Glu46Lys) in regions predicted to influence the secondary structure of alpha-Synuclein. The substitutions may disrupt the structure of alpa-Synuclein, rendering the protein more prone to self- aggregation (95, 96)

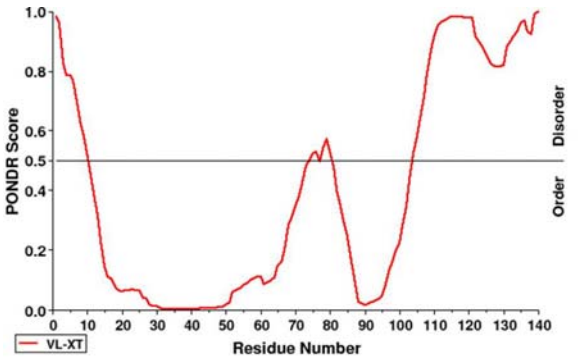

Figure 2. PONDR plot of the predicted secondary structure of alpha-Synuclein.The protein sequence is obtained from NCBI database. PONDR score of 0.5 and higher indicates disordered structures (114). The C-terminal ~40-50 residues in alpha-Synuclein are disordered. The lower panel shows alpha-Synuclein aminoacid sequence, the regions with disoreder propensity are underlined.

Several lines of converging evidence directly implicate alpha-Synuclein in mechanisms underlying the onset/ progression of PD (97). They are: (i) Missense mutations in the alpha-Synuclein gene (A53T, A30P and E46K) cause familial PD in rare kinds (92-94); (ii) Antibodies to alpha-Synuclein specifically detect LBs, (3, 4, 98-104); (iii) LBs purified from PD brains contain abnormally aggregated alpha-Synuclein and insoluble forms of alpha-Synuclein (4, 98). The precise mechanism whereby such aggregates of alpha-Synuclein cause degeneration of dopaminergic neurons is not known. The aggregates may be merely a normal reaction by the cells as part of their effort to correct a different pathological event, as-yet unknown. This issue is dealt with in detail in the latter part of the article.

An important feature of alpha-Synuclein primary structure is six imperfect repeats within the first 95 residues. This brings the similarity of alpha-Synuclein with the amphipathic lipid-binding α-helical domains of apolipoproteins (105, 106), which show variation in hydrophobicity with a strictly conserved periodicity of 11. alpha-Synuclein shares the defining properties of the class A2 lipid-binding helix, distinguished by the clustered basic residues at the polar-apolar interface, positioned $\pm 100^0$ from the center of apolar face; predominance of lysines relative to arginines among these basic residues; and several glutamate residues at the polar surface (107-109). In agreement with the above structural features, alpha-Synuclein binds specifically to synthetic vesicles containing acidic phospholipids (109, 110). Further, this binding was shown to be accompanied by a dramatic increase in alpha-helix content.

Recently, attempts have been made to analyze the structure of alpha-Synuclein using NMR studies (111-113). It was shown that the conformation of alpha-Synuclein consists of two alpha-helical regions that are interrupted by a short break (112). NMR study of free monomeric alpha-Synuclein revealed that the first 100 residues in N-terminus region of free alpha-Synuclein have an overall preference for helical structure and there may be the presence of a transient helical structure from residues 6 to 37. In contrast, the final 40 residues of free alpha-Synuclein exhibited secondary shifts indicative of highly unfolded and extended form (113). We used the predictor of naturally disordered regions (PONDR, Molecular Kinetics, Inc.) (114) software to alpha-Synuclein (Figure 2). This shows that about 40-50 residues in in alpha-Synuclein C-terminus is relatively disordered.

NMR data of alpha-Synuclein in presence of unilamellar vesicles suggested that the N-terminal region is responsible for lipid binding and the boundary for this region occurs between residues 102 and 103. The shifts in C^{alpha} chemical shifts clearly indicated that there is the formation of helical structure upon alpha-Synuclein association with unilamellar vesicles. It was noted that it is only the N-terminal region of the protein containing the amphipathic apolipoprotein helical motifs, which binds and adopts a helical conformation. The C-terminal region remains in the same conformation as in the free alpha-Synuclein and does not bind to the lipid vesicle surface (113).

alpha-Synuclein folding and fibrillation have been found to be promoted on binding to long chain fatty acids (115) and also upon its interaction with lipid droplets (116). It was also shown that membrane interactions induce a large conformational change from random coil to alpha-helix in alpha-Synuclein and these interactions may be physiologically important (117). On the basis of these observations, it has been assumed that alpha-Synuclein may exist in two structurally different isoforms *in vivo*: a helix-rich, membrane-bound form and a disordered, cytosolic form, with the membrane-bound alpha-Synuclein generating nuclei that seed the aggregation of the more abundant cytosolic form (118, 119). The partially folded intermediate of alpha-Synuclein is more prone for aggregation. The aggregation of alpha-Synuclein depends upon extent of folding induced by the membrane interaction but the mechanism is not clear.

It is suggested that the misfolded or partially folded alpha-Synuclein is more cytotoxic than the protein aggregates. The intermediate partially folded or misfolded form may be entropically rich in energy and may bind to other components in the cell and may be a cause for neurodegeneration. Transgenic animal models expressing human alpha-Synuclein had shown neurodegeneration, without fibrillar alpha-Synuclein (120, 121). In that sense, the formation of aggregates could be a protective measure adapted by the cell against the toxicity of this intermediate. However, it is still a matter of debate regarding the toxic form of the protein (monomeric or oligomeric?) in neurodegenerative disorders (122).

3.1. Potential normal functions of alpha-Synuclein

Dispite of strong evidence implicating alpha-Synuclein in the pathogenesis of several meurodegenerative diseases, its physiological function remains poorly understood. The difficulty in determining the functions of alpha-Synuclein is because inactivation of the alpha-Synuclein gene does not lead to a significant neurological phenotype. However, overexpression of alpha-Synuclein in rat substantia nigra was shown to cause loss of dopaminergic neurons, but is limited to the targeted region and does not mimic the broad pathology observed in the disease (123). Furthermore, mouse models based on overexpression of alpha-Synuclein through genetic methods lead to a wide variety of phenotypes accompanied by non-existant, late onset, or non-specific neurodegeneration (124). Understanding the role of alpha-Synuclein in normal cell life might be critical importance since disruption of its normal function might indirectly result in neurodegeneration (125). The association with membrane lipids and its functional homology with 14-3-3 chaperone proteins suggested that alpha Synuclein may play a role in cell signaling pathways (126). It was also suggested that alpha-Synuclein may modulate tau function. alpha-Synuclein was detected in axons and developing pre-synaptic terminals after their formation in rat embryonic hippocampal cells in culture, suggesting a possible role in synaptic development and maintenance. Alpha-Synuclein may contribute to neuronal differentiation as well (127-129). The involvement of alpha-Synuclein in synaptic plasticity and neuronal differentiation may be mediated by the selective inhibition of Phospholipase D2 by alpha-Synuclein (130, 131). When alpha-Synuclein expression was markedly reduced in cultured rat neurons (132) or abolished in alpha-Synuclein knock out mice (133), the number of vesicles in the distal pool of the pre-synaptic terminal is reduced indicating a role for alpha-Synuclein in vesicular dynamics. According to Cole and Murphy (133) alpha-Synuclein's involvement in lipid metabolism cannot be ruled out, given its propensity to bind molecules with high hydrophobic content or exposed hydrophobic domains. Thus persuasive evidence of a role of alpha-Synuclein in any pathway or function requires multiple approaches (133). The structure, expression and functions of alpha-Synuclein have been recently reviewed by Dev *et al.*, (134).

3.2.alpha-synuclein toxicity in diffuse Lewy body disease

Diffuse Lewy Body Disease is the second most common cause of dementia after AD. It is also commonly reffered to as Dementia with LBs (DLB). DLB usually presents with a neurobehavioral syndrome that may include hellucinations, delutions, and psychosis, eventually leading to dementia. DLB overlaps in clinical, pathological, and genetic features with AD and PD. Pathologically DLB demonstrate prominent cortical and subcortical LB formation, which differentiates it from PD (135). In DLB, unlike PD, LBs are distributed widely through out paralimbic and neocortical regions (136). These LBs generally coexist plaques similar to the ones predominant in AD.

Similar to PD, LBs in DLB are rich in alpha-Synuclein protein aggregates. Infact the neuritic alpha-Synuclein accumulation, density of cortical LBs and AD-type pathology (senile plaques and hippocapal neurofibrillary tangles) are more intense in DLB than PD (137). Pathologically, most PD cases have minimal or no senile plaques and neurofibrillary tangles, while they are present in DLB. It is also suggested that alpha-Synuclein and amyloid beta interact in DLB (138).

4. EXPRESSION AND SUBCELLULAR DISTRIBUTION OF ALPHA-SYNUCLEIN

alpha-Synuclein appears to be expressed ubiquitously throughout the brain (139). In the early weeks of development, alpha-Synuclein redistributes from cell bodies to synaptic terminals (127, 128, 140). The transcription of alpha-Synuclein is developmentally regulated. The levels increase during development and are sustained at fairly high levels throughout adulthood (141). Furthermore, various cellular treatments have been shown to affect synuclein levels, including nerve growth factor (129), 1-methyl-4-phenyl-1,2,3,6-tetrahydropiridine (MPTP) (142, 143), certain inflammatory cytokines, cellular stress, and during megakaryocyte differentiation (143). However, a clearer understanding of the transcriptional and translational regulation of synuclein expression is needed before we can understand how any changes in these mechanisms may affect the disease process (133).

Recently Zhang *et al.*, examined the subcellular localization and relative amounts in different subcellular pools in rat brain neurons (16). They showed that alpha-Synuclein was unevenly distributed in axons, presynaptic terminals, cytoplasm and nucleus in neurons. The density was more in presynaptic terminal and nucleus, compared to other subcellular compartments. Interestingly, alpha-Synuclein was also present in mitochondria.

4.1. Nuclear localization of alpha-Synuclein

Several studies have shown the presence of alpha-Synuclein in the neuronal nuclei. However, no definite nuclear functions have been attributed to alpha-Synuclein to date. It is not known if nuclear localization is the common property of alpha-Synuclein or it is cause/consequence of PD pathology. Overexpression of alpha-Synuclein in neuronal cell lines showed diffused nuclear staining (144). The term alpha Synuclein was first coined by Maroteaux based on its localization in the synaptic region and nucleus (8). Mori *et al.*, showed localization in the nucleus of substantia nigra and pontine nucleus neurons of rat brain (145). Further, nuclear localization of alpha Synuclein was shown in cultured primary neurons (146) and cell lines (147). Nuclear inclusions of neurons and oligodendroglia of multiple system atrophy contain the alpha-Synuclein protein (15). Yu *et al.*, showed nuclear localization of alpha-Synuclein in the rat brain by immunoelectron microscopy using colloidal gold probes (147, 148). Mono and oligomeric forms of alpha-Synuclein were observed in the nuclear fractions of human dopaminergic neuroblastoma SH-SY5Y cells (12).

Extensive nuclear localization of alpha-Synuclein indicates that it might play important role in the nucleus (16-19).

Although the above observations do not suggest what the function of alpha-Synuclein in nucleus is, Leng *et al.*, (12) predicted that alpha-Synuclein may play a role in regulating processes in the PI- cycle in the nucleus and phosphatidyl inositol-linked activities may also occur in nucleus.

4.1.1. Nuclear transport of alpha-Synuclein

The mode of appearance of alpha-Synuclein into the neuronal nuclei and functions of alpha-Synuclein in nuclei is still obscure. According to Maroteaux *et al.*, (8) the mode of localization of alpha-Synuclein in the nucleus could involve a lateral diffusion along the endoplasmic reticulum and outer nuclear membrane (149) or more conventional transport through nuclear pores. They proposed that alpha-Synuclein family proteins may be involved in coordinating nuclear and synaptic events. However, under PD conditions, the nuclear localization of alpha-Synuclein could be enhanced due to non-specific transportation through oxidatively damaged nuclear membrane (17). The highly oxidative cytological environment in PD brain, because of increase in paramagnetic ferrous and other free radical generating metals, are known to disrupt the biological membranes leading to translocation of alpha-Synuclein in to the nucleus. Sangchot *et al.*, (13) have provided new evidences for nuclear membrane disruption by lipid peroxidation caused by increase in iron and consequent translocation of alpha-Synuclein aggregates in to perinuclear and endonuclear regions of human dopaminergic neuroblastoma SK-N-SH cell lines. Leng *et al.*, (12) also observed alpha-Synuclein both in monomeric and oligomeric forms in nuclear fractions of human dopaminergic neuroblastoma SH-SYSY cell cultures.

4.2. alpha-Synuclein genotoxicity

With several evidences showing the presence of alpha Synuclein in neuronal nuclei, as discussed above, the question arises about its function/role in the nuclein. Further, it is interesting to discuss whether nuclear alpha-Synuclein is an active or passive response to PD pathology. It was shown that alpha-Synuclein interacts with histones *in vitro* and this interaction if confirmed *in vivo,* might alter the gene transcription (14). To support this, when, transfected to cell lines, alpha-Synuclein changes the expression of many genes (150). alpha-Synuclein alters gene expression changes of stress response genes, transcription regulators, apoptosis inducers, transcription factors, membrane bound proteins and protein involved in the dopamine synthesis. In alpha-Synuclein transfected dopamenergic cell lines, tyrosine hydroxylase was inhibited (147). Furthermore, alpha-Synuclein over expression in PC12 cells showed enhanced proliferation and enrichment of cells with S-phase, again suggesting enhanced gene expression (118).

alpha-Synuclein nuclear targeting accelerated the neurodegenerative process in the dopaminergic neurons of flies (151). Histone deacetylase inhibitors prevented this neurodegenerative process when administrated. This may be explained assuming that alpha-Synuclein induces its toxicity by inhibiting the histone acetylation. alpha-Synuclein was also shown to associate with histone H3 and inhibit its acetylation.

Further, widespread DNA damage is observed in the brain regions affected with synucleinopathies in which neurodegeneration is observed (64, 152). These brain regions have been quite often linked to excess iron accumulation. In presence Fe (II) alpha-Synuclein can generate reactive oxygen species and damage DNA indirectly in the neuronal cells (153, 154). Although, this is an indirect indication, the fact that ncreased DNA damage is observed in cells transfected with wild type alpha-Synuclein and mutants A30P, A53T after treating with Fe (II), strengthens this hypothesis. The above observations suggest that alpha-Synuclein contributes to genotoxicity in various ways.

Our lab reported the ability of alpha-Synuclein to directly bind to DNA molecule that results in altered DNA conformation and damage which will be discussed later in this review.

5. ALPHA-SYNUCLEIN DNA INTERACTIONS: A NEW CONCEPT

We recently made an interesting observation that alpha-Synuclein has DNA binding property which has created a new opportunity in understanding role of alpha-Synuclein in PD pathology (17-19). Previously we also showed for the first time that amyloid beta peptides implicated in AD can also bind to DNA (155, 156). The origin of the above concept and subsequent progress are discussed below.

5.1. Our hypothesis on DNA binding of alpha-Synuclein- genesis of model

As stated earlier, several studies showed that alpha-Synuclein is localized in the chromatin region of nuclei in the brain (8-16). This strongly indicated the association of alpha-Synuclein with chromatin in the nucleus. It was also shown previously that several cationic and anionic ligands interact with alpha-Synuclein such as polyamines and metals (157, 158). Furthermore, a close look into the peculiar primary sequence/structure of alpha-Synuclein shows presence of several positively charged lysine residues at its N-terminus, suggesting a possible DNA binding property. It was recently observed that alpha-Synuclein interacts with histone proteins, a major component of chromatin, which modulates its conformation and aggregation properties (14). We thought it is interesting to investigate the DNA binding property of alpha-Synuclein and study the effect of DNA binding on alpha-Synuclein folding/conformation and aggregation properties. Moreover, we had previously observed DNA binding of amyloid beta peptides, which led us to examine alpha-Synuclein in similar lines (155, 156). We also proposed that understanding the effect of DNA binding on alpha-Synuclein stability, conformation and fibrillation could lead to a better understanding of PD pathogenesis and could also

be exploited for DNA binding based therapeutic interventions.

5.2. New evidence for DNA binding property of alpha-Synuclein

We first time demonstrated that alpha-Synuclein binds to DNA *in vitro*, a new and novel property of alpha-Synuclein (17-19). This was independently confirmed by other groups as well (20). This is the first report on DNA binding property/ability of alpha-Synuclein and presents an interesting curiosity about the implications of this property in PD. The association of DNA with alpha-Synuclein is not limited to wild-type protein. Familial mutants A53T and A30P also showed DNA binding (20). Numerous studies have demonstrated that various intracellular factors affect folding and fibrillation properties of alpha-Synuclein. Histones, one of the important components of chromatin was shown to specifically interact with alpha-Synuclein and significantly stimulate its aggregation (14). DNA being another component of chromatin, its interaction with alpha-Synuclein strongly suggests an important role of alpha-Synuclein in the nucleus. The possible mechanisms and implications of alpha-Synuclein-DNA interactions are discussed below.

5.3. alpha-Synuclein affects DNA conformation

Circular dichroism (CD) spectra of alpha-Synuclein-supercoiled DNA complex demonstrated a strong binding of alpha-Synuclein to supercoiled DNA, causing a conformational change from the B-form of DNA to an altered B-form (17). It was further shown that alpha-Synuclein uncoils supercoiled DNA to open cicular form. Differential sensitivity of synuclein-supercoiled DNA complex to chloroquine induced topoisomers separation compared to DNA alone suggested destabilization of DNA by alpha-Synuclein (17). The modulation of DNA conformation and stability by alpha-Synuclein could be important in PD pathology as it may affect DNA transactions such as replication and transcription and hasten accumulation of DNA damage. However, considering that alpha Synuclein is expressed ubiquitously in the brain, the question arises, if this interaction could eventually take place in any other brain region not affected during PD? Or is there brain region selectivity?. Our recent observations show that the most pathological, misfolded form of alpha Synuclein found in dopaminergic neurons exhibit significantly higher DNA binding and damage activity compared to the native monomeric form found in normal brain which suggests that the DNA interaction of alpha Synuclein might be higher in PD affected brain regions (18, 122); Hegde *et al., unpublished observation*).

A plausible scenario for DNA binding to alpha-Synuclein could be as follows: It appears that initially on mixing with alpha-Synuclein in solution, alpha-Synuclein monomers interact electrostatically with DNA phosphate groups. DNA interacts possibly with the positively charged lysine side chains located predominantly in the N-terminal and partly in the central region of alpha-Synuclein sequence. Because it is highly unlikely to bind to the C-terminal end of alpha-Synuclein which is rich in negatively charged amino acid residues (20). These electrostatic interactions may lead to (i) formation of non-sequence specific complex of alpha-Synuclein with DNA, and (ii) increase in the local concentration of alpha-Synuclein on DNA (20). Once alpha-Synuclein binds to DNA by electrostatic forces, there could be a conformational change in alpha-Synuclein making the protein enzymatically bind to DNA.

5.4. DNA induced folding of alpha-Synuclein

We observed that various DNAs significantly modulate conformation and fibrillation properties of alpha-Synuclein. Single strand circular DNA binding to native, random coiled alpha-Synuclein resulted in about 80% increase in alpha-helix content of the protein (19). Although, double strand circular DNA also bound to alpha-Synuclein, it did not change its conformation, indicating specificity of single strand DNA binding. However, supercoiled plasmid DNA caused a biphasic conformational transition in alpha-Synuclein. On immediate mixing of the DNA and alpha-Synuclein a partial folding was induced in alpha-Synuclein, while alpha-helix conformation was formed on long term incubation (19).

We also provided interesting insight on sequence specific binding affinity of DNA to alpha-Synuclein. Poly d(GC).d(GC) caused a partially folded conformation, where as poly d(AT).d(AT) binding to alpha-Synuclein did not result in any such conformational transition. It was further observed using GC- and AT-specific 8-mer oligonucleotides that only d(GCGCGCGC) induced a partial folding in alpha-Synuclein. Interestingly, d(GCATGCAT) also induced a partial folding in alpha-Synuclein,while, d(ATATATAT) did not. Closer examination of the CD data indicated that the folding induced by d(GCGCGCGC) was more in magnitude compared to d(GCATGCAT).

The effect of binding of large genomic DNA (lamda and Calf-thymus DNA) on alpha-Synuclein conformations showed that both these genomic DNA caused the formation of a partially folded structure in alpha-Synuclein. However, the amount of folding induced by lamda DNA was more when compared to calf-thymus DNA. The GC content of calf-thymus DNA is ~70%, while for lamda DNA it is ~42% which should explain the differential ability of calf-thymus and lamda DNA in inducing conformational transition in alpha-Synuclein.

The above studies from our lab indicated specificity of single stranded DNA and GC sequence in inducing folding in alpha-Synuclein. It appears that the DNA binding to alpha-Synuclein is mediated through electrostatic interaction between negatively charged phosphate groups of DNA and the epsilon amino group of lysine aminoacids in alpha-Synuclein. The DNA molecule is richly negatively charged on its surface as it is laced with phosphate groups, where as alpha-Synuclein has 15 basic lysine residues which are mostly clustered in the N-terminal of its sequence. The neutralization of basic charge on epsilon amino group side chain of lysine residues will reduce the repulsion between the like charges in the N-

terminal end of alpha-Synuclein and this appears to be the driving force in inducing DNA mediated folding in the protein. Studies have shown that the N-terminal half of alpha-Synuclein sequence has a very high propensity to form ordered conformation (112).

5.5. alpha-Synuclein aggregation and DNA binding

Previous studies have shown that the transformation of alpha-Synuclein into a partially folded conformation (induced by pH or temperature or metal ions) is strongly correlated with the enhanced formation of alpha-Synuclein fibrils (157, 159). alpha-Synuclein is a natively unfolded protein with little or no ordered structure under physiological conditions. At neutral pH, it is calculated to have 24 negative charges (15 of which are localized in the last third of the protein sequence), leading to a strong electrostatic repulsion, which hinders the folding of alpha-Synuclein (157). As a consequence of the structural flexibility of alpha-Synuclein, many diverse ligands change its conformation and modulate its aggregation property (20). Generally, transition from random coiled alpha-Synuclein to partially folded conformation accelerates the fibrillation reaction, while stabilizing alpha-Synuclein in to alpha-helix-rich conformation delays fibrillation. Aggregation or self-association is a characteristic property of a partially folded (denatured) proteins and most aggregating protein systems probably involve a transient partially folded intermediate as the key precursor of fibrillation (160, 161). It has also been shown that in some cases the self-association induces additional structure and stability in the partially folded intermediates.

Recently it was shown that double stranded DNA promotes aggregation of alpha-Synuclein (20). They showed that the morphology of the fibrils remains unchanged in the presence of linear double stranded DNA. In this context, we analyzed the aggregation propensity of alpha-Synuclein in the presence of different DNAs which induce partially folded conformation and also alpha-helix. Our studies showed that DNA induced aggregation of alpha-Synuclein correlated with the ability of that DNA to induce partially folded conformation in alpha-Synuclein (19). DNA which induced partial folding in alpha-Synuclein such as GC-rich oligonucleotides resulted in a very substantial acceleration of the kinetics of aggregation indicated by a shorter lag time and a larger rate of fibril formation compared to alpha-Synuclein alone. However, single-strand circular DNA which formed alpha-helix conformation in alpha-Synuclein delayed the aggregation significantly by nearly ~25 hrs. The structure of alpha-Synuclein aggregates/ fibrils were qualitatively similar in the presence or absence of DNA.

Similar observations were made by Uversky *et al*, (162), where they showed that trimethylamine-N-oxide (TMAO) induces a partial folding and acceleration of fibrillization in alpha-Synuclein at low concentrations, where as, at high concentrations causes the formation of alpha-helix conformation and inhibits aggregation to a considerable extent. Our results are in agreement with Uversky *et al.*, (162). Hence, it appears that a partially folded intermediate conformation is a very critical step in alpha-Synuclein aggregation pathway.

The possible mechanisms of double stranded DNA promoting alpha-Synuclein fibrillation has been proposed recently by Cherny *et al.*, (20). The authors observed that neuronal nuclear inclusions potentially account for a significant fraction of the total amount of alpha-Synuclein in a cell. Hence, minute variations in local alpha-Synuclein concentrations or the presence of factors enhancing its fibrillation, *e.g.,* DNA or histones, may stimulate the aggregation of alpha-Synuclein significantly. It was further proposed that effective mechanisms preventing occasional conversion of a soluble alpha-Synuclein into insoluble isoforms must exist in both cytoplasm and nucleus (20). We provided a comprehensive picture of DNA binding effect on alpha-Synuclein fibrillation using different DNAs such as double and single stranded DNA, AT and GC sequence specific DNA of different sizes etc and showed that only those DNA which induce a partial folding in alpha-Synuclein promote its aggregation, while, single strand circular DNA forms alpha-helix conformation in alpha-Synuclein and inhibits aggregation to a considerable extent. Hence, we feel that extrapolation of *in vitro* results on DNA binding property of alpha-Synuclein to *in vivo* system in PD has to be more cautiously done.

We used effect of osmolytes on alpha-Synuclein conformation to understand the mechanism of DNA induced folding/fibrillation of alpha-Synuclein (19). Osmolytes such as TMAO, Betaine, sarcosine converted natively unfolded alpha-Synuclein to partially folded form which accelerated the kinetics of fibrillation. The ability of DNA and osmolytes in inducing conformational transition in alpha-Synuclein, indicates that two factors are critical in modulating alpha-Synuclein folding: (i) Electrostatic interaction as in the case of DNA, and (ii) Hydrophobic interactions as in the case of osmolytes.

6. OUR MODEL ON ALPHA-SYNUCLEIN GENOTOXICITY

We propose alpha-Synuclein-mediated genotoxicity as one of the key underlying mechanisms of disease progression in the PD. Normally alpha-Synuclein is in random coil conformation in aqueous solutions *in vitro.* It is suggested that free alpha-Synuclein in neuronal cytoplasm may also be in random coil conformation. However, in association with membranes alpha-Synuclein is in alpha-helix-rich conformation. alpha-Synuclein is diversly/unevenly distributed in various subcellular mileu such as cytoplasm, presynaptic terminus, nucleus, endoplasmic reticulum and mitochondria (16). The factors that govern alpha-Synuclein distribution in cell are not fully understood. However it is known that oxidative stress and other cytological scenario that exists in PD such as metal toxicity may modulate alpha-Synuclein subcellular translocation significantly, especially the nuclear alpha-Synuclein, because these factors greatly affect the permeability of membranes.

Several studies have reported higher levels of iron (Fe) and other transition metals in PD brain substantia nigra, the main target of PD (41). However, how a specific increase in the total Fe content of SN should occur in PD is not understood (163, 164). It has been argued that the increased Fe levels with the severity of neuropathological changes in PD are presumably due to increased transport through the BBB (165). Furthermore, in PD the increased total Fe level in SN was not associated with a compensatory increase in ferritin; instead the brain ferritin immunoreactivity was decreased (166). Hence the increased Fe load in PD may exceed the storage capacity of available ferritin, leading to excess reactive Fe, driving free radical generation. In the presence of these metals alpha-Synuclein acquires a misfolded or partially folded conformation and promote aggregation *in vitro* (159). We hypothesized that the partially folded or misfolded alpha-Synuclein induced by metals may not bind to vesicle membrane lipids as it does in normal brain. In addition, it was observed that one of the familial mutant alpha-Synucleins, A30P completely abolished membrane-binding property of alpha-Synuclein (105). Hence, the disruption in membrane binding resulting from increase in metals and mutations in familial PD would result in the increase in free alpha-Synuclein (partially folded or unfolded native conformation) levels in cell. This possibly triggers the increase in precursor for alpha-Synuclein aggregation in PD (17). In addition to the accumulating evidence for normal nuclear localization of alpha-Synuclein, the increased oxidative stress and altered permeability of nuclear membrane could ensure significant amount of alpha-Synuclein in the nucleus. In the nucleus it exerts toxic role by altering chromatin organization or by directly binding to DNA or by both. alpha-Synuclein can bind to the histone proteins and affect their normal functioning of maintaining the chromatin integrity. As histones loss their function, chromatin will open up exposing DNA to alpha-Synuclein and other targets. Now the transcription factors or inhibitors can bind to DNA altering the gene expression. alpha-Synuclein can itself bind to DNA and relax the supercoils in the DNA molecule and can induce a conformational change, which may further affect the gene expression profile. The altered gene expressions finally lead to altered neuronal cell metabolism leading to cell death. Besides, DNA induced partial folding in alpha-Synuclein enhances its toxicity to the cell. Several studies have shown that partially folded intermediate form of alpha-Synuclein is more toxic than monomers or aggregates. Partially folded alpha-Synuclein has higher aggregation propensity and in PD the presence of metals and other free radicals can further stimulate the aggregation process. The aggregated protein can disrupt several processes in the nucleus including gene expression and DNA functioning. Our model on alpha-Synuclein Genotoxicity is represented in Figure 3.

7. IS DNA BINDING COMMON PROPERTY OF MANY AMYLOIDOGENIC PROTEINS?

It has been widely reported by us and others, that nucleic acids interact with different amyloid peptides such as beta-amyloid, tau protein, prion peptides and alpha-Synuclein and modulate their folding and aggregation kinetics (19, 155, 156, 167-175). In many cases double stranded DNA accelerated the kinetics of fibrillation. However, an extensive study by us on alpha-Synuclein showed that the effect was dependent on the structure of DNA (19). Nandi group by several well designed studies showed that nucleic acids can induce structural changes to beta-sheet rich conformation in prion peptides by forming stable complexes, which catalyzes/modulates their polymerization (168, 172, 173). Association of Abeta (1-40) and Abeta (25-35) with double stranded DNA was detected (169). Abeta (25-35) was shown to cause formation of open circular DNA from supercoiled DNA in presence of ferrous ions (170). We have recently observed binding of Abeta (1-42) and Abeta (1-16) peptides with supercoiled DNA and their ability to convert supercoiled DNA into open circular form (155). Our lab showed that Abeta (1-42) can directly inflict DNA nicking which could contribute DNA damage associated in AD brain (156, 176). In similar lines latest studies from our lab shows DNA single-strand breaks directly induced by alpha-Synuclein in its partially folded form (19); Hegde *et al.*, unpublished observation).

The above scenario suggests that DNA binding could be a normal property of many amyloid-forming proteins associated with diverse neurodegenerative disorders. However, at this stage it is hard to pin down whether DNA binding contributes to PD pathology as a major causative phenomenon or if it is just a consequence of the disease process where non specific nuclear transportaion of amyloid proteins result in DNA binding. We feel that a parallel approach to DNA binding of these amyloid proteins in several neurodegenerative diseases may yield better results. In addition, the finding of insoluble protein-containing materials in different neuronal and glial cell populations in a broad range of syndromes suggests that many of these disorders have something in common (177). Even though these syndromes express different symptoms and lesions, the mechanisms underlying filament formation may be similar. The assembly of normally soluble protein subunits into insoluble filaments in these diseases does not normally occur in healthy brain. Hence, another way to approach these disorders is to consider the disease state as one of an abnormality in protein metabolism. Future research efforts will pursue molecular analyses of shared protein abnormalities across several disorders. This approach should provide insights into disease mechanisms underlying one or more degenerative disorders characterized by abundant filamentous lesions.

8. BIOLOGICAL SIGNIFICANCE OF DNA BINDING OF ALPHA-SYNUCLEIN

Structurally, purified alpha-Synuclein is a natively unfolded protein (17, 113, 159, 178). This lack of folding has been shown to correlate with the specific combinations of low overall hydrophobicity and large net charge (179-181). *In vitro,* alpha-Synuclein readily assembles in to fibrils, with morphologies and staining characteristics similar to those of fibrils extracted from PD affected brain (90, 91, 119, 159, 182-188).

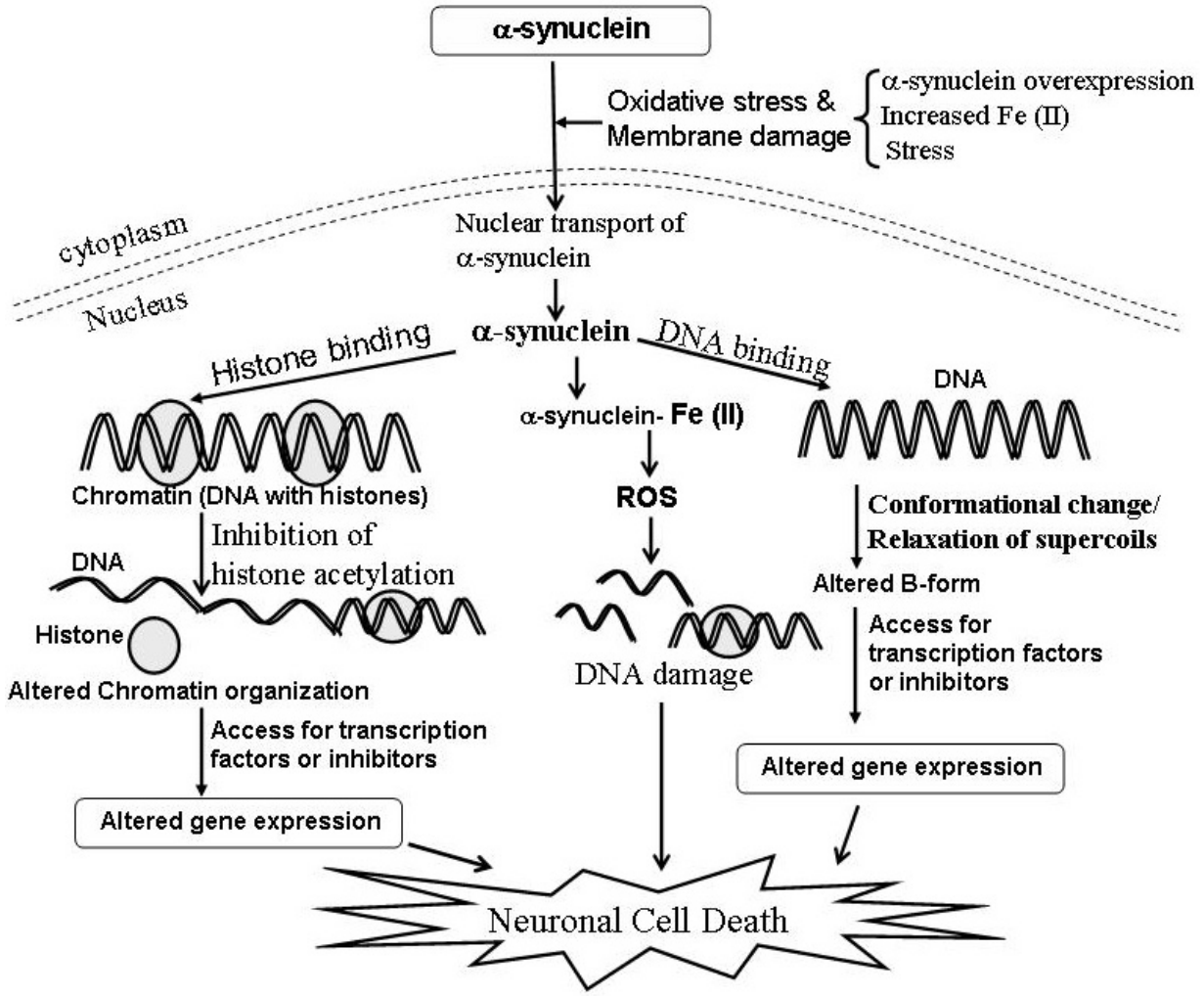

Figure 3. Our model on genotoxicity of alpha-Synuclein: During stress conditions, there is increased transportation of alpha-Synuclein into the nucleus. In the nucleus, alpha-Synuclein can directly interact with histones or inhibits histone acetylation affecting the chromatin organization. alpha-Synuclein can bind to DNA and alter the conformation of DNA, relax supercoiling of DNA. Change in chromatin organization, conformation of DNA and supercoil relaxation may lead to altered gene expression. alpha-Synuclein in the presence of Fe(II) can generate reactive oxygen species and induce DNA damage. Altered gene expression and DNA damage lead to neuronal cell death.

The physiological significance of DNA induced alpha-Synuclein conformation and modulation of its assembly/ fibrillation is unclear at the present time. However, emerging lucid evidences for the presence of alpha-Synuclein in neuronal nuclei indicates that the DNA binding activity of alpha-Synuclein may not be a mere non-specific phenomenon and may have very significant role to play in neuronal cell death in PD through DNA instability. It will be evocative to speculate the potential implications of the *in vitro* findings on DNA binding of amyloid proteins to neurodegenerative changes associated with PD. Goers *et al.*, (14) provided evidence for the co-localization of alpha-Synuclein with histones in the nuclei of nigral neurons from mice exposed to a toxic insult. The authors observed that histones stimulate alpha-Synuclein fibrillation *in vitro* (14). These studies further strongly suggested association of alpha-Synuclein with chromatin.

Cherny *et al.*, proposed that alpha-Synuclein may interact with histone-free, transcriptionally active DNA segments and hence may lead to a decreased transcriptional activity of some genes responding to environmental stimuli (20). It is suggested that the interactions of alpha-Synuclein with DNA and histones may function to regulate gene expressions.

Interestingly, a recent study involving semi-quantitaive analysis of alpha-Synuclein in subcellular pools of rat brain neurons showed that there is a significant fraction of alpha-Synuclein in the nuclear compartment (16). They used immunogold electron microscopic technique with a C-terminal specific antibody. It was shown that alpha-Synuclein-positive gold particles were unevenly distributed in different subcellular compartnets. The density was relatively greater in presynaptic terminals

and nucleus. In this perspective, association of alpha-Synuclein with chromatin attains significance. alpha-Synuclein-induced changes in DNA conformation may affect gene expression pattern in affected neurons. In addition, DNA induced folding and modulation of fibrillation property may have special pathophysiological significance and contribute enormously to the accumulation DNA damage in degenerative neurons and lead to cell death.

9. ALTERNATIVE VIEW: ALPHA-SYNUCLEIN AND NEUROPROTECTION

Several lines of evidences suggest alpha-Synuclein toxicity in PD, however, an alternative debate for the neuroprotective role of alpha-Synuclein in PD is emerging (189, 190). Although, alpha-Synuclein accumulation in the form of aggregates in dopaminergic neurons is a common pathological feature in PD, the precise mechanism of how this aggregation process is triggered? Or how the protein aggregates cause neuronal degeneration is still obscure. Furthermore, some studies have failed to show consistant results for neurotoxicity of alpha-Synuclein (191-194) and few studies also suggested that alpha-Synuclein may play a neuroprotective role (191, 195).

In other words, there is a school of thoughts which argues that alpha-Synuclein has a normal function in normal brain, but in response to environmental or endogenous stimulus it aggregates as a neuroprotective response or as a passive response to pathological events. For instance, oxidative stress caused by the herbicide paraquat results in alpha-Synuclein aggregation in the brains of experimental animals and this increased expression and aggregation of alpha-Synuclein was neuroprotective (195). Studies showed that various neurotoxins including MPTP and rotenone increase alpha-Synuclein expression in brain (196, 197). These observations lead a group of researchers to suggest that the increased alpha-Synuclein expression may represent an adaptive homeostatic regulatory response to toxic stimuli (189). In support of this, overexpression of alpha-Synuclein in transgenic mice does not consistantly result in neuronal damage (192, 193), nor does it mimic MPTP induced neurodegeneration completely (198).

Similarly, other amyloidogenic proteins involved in neurodegenerative pathologies, such as, amyloid beta peptides in AD and prion proteins in prion diseases could have neuroprotective properties. These observations need to be considered when developing therapies to PD and other neurodegenerative diseases. In other words, therapy should be targetted at the cause of the disease rather than the end result (protein aggregates), unless it is conclusively proved that dissolving/eradicating protein aggregation improves the disease symptoms.

As discussed elsewhere in this article, it is not clear whether the recently discovered DNA binding property of alpha-Synuclein contributes to the cause of PD pathology or it is a passive secondary response of neurons affected by PD. These studies have to be addressed as toxic vs. protective responses in PD.

10. PERSPECTIVES AND FUTURE DIRECTIONS

DNA binding effect on alpha-Synuclein fibrillation using different DNAs such as double and single stranded DNA, AT and GC sequence specific DNA, of different sizes, genomic DNA etc, showed that only those DNA which induce a partial folding in alpha-Synuclein (GC* rich DNA) promote its aggregation, while, single-strand circular DNA forms alpha-helix conformation in alpha-Synuclein and also inhibit aggregation to a considerable extent.

We propose two dimensions to the DNA binding property of alpha-Synuclein. Firstly, it could imply an important pathological role for nuclear translocated alpha-Synuclein, irrespective of whether alpha-Synuclein enters nucleus by active process or by non-specific means during PD pathology. Secondly, stabilization of alpha-Synuclein in helix-rich conformation by single-strand circular DNA that delays aggregation kinetics and or reduces the formation of toxic partially folded intermediates may be of significance in engineering DNA-chip based therapeutic approaches to PD and other amyloid disorders.

Future studies should focus on establishing the DNA binding of alpha-Synuclein and other amyloidogenic proteins *in vivo*, in cells and also using animal models. Studies may also be done in post mortem human brain tissue. It is also essential to understand the mechanism of the property more thoroughly using different DNAs, which will help design DNA based or similar DNA mimicking ligands to protect from amyloid toxicity. We also propose that it is important to have a parallel approach to study neurodegenerative disorders as several features from cause to pathology are common to them.

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A**bbreviations**: PD: Parkinson's disease, AD: Alzheimer's disease, LBs: Lewy bodies, SN: substantia nigra, 8-OHdG: 8-hydroxy -2'-deoxyguanosine, NAC: non-amyloid component of beta-amyloid precursor protein, MPTP: 1-methyl-4-phenyl-1,2,3,6-tetrahydropiridine, CD: Circular dichroism

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