

## Mini Review

# Lewy Body in Parkinson's Disease: Causes or Scars of Neurodegeneration?

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## Abstract

Parkinson's disease is an age related neurodegenerative and movement disorder affecting 1-3% of individuals above the age of 60 years. Parkinson's disease is characterized by the degeneration of dopaminergic neurons in the substantia nigra pars compacta and the accumulation of Lewy bodies in the surviving neurons. Lewy bodies are present in the central and sympathetic nervous systems, and it is a type of alpha-synucleinopathy, since alpha-synuclein is the main constituent of Lewy body.

The long disputed role of Lewy bodies in causing neurodegenerative diseases is poorly understood which stands as the main hurdle in developing neuroprotective therapies. In this review, we explore in depth the characteristics of Lewy body with a specific focus on alpha-synuclein, the protein chiefly responsible for the formation of Lewy bodies. We also throw light on the role of Lewy body in therapies for Parkinson's disease and the relationship between dopamine homeostasis and protein misfolding. The discovery of several genes involved in Parkinson's disease has led to the hypothesis that misfolding of proteins and dysfunction of the ubiquitin-proteasome pathway are vital to the neurodegenerative process. Mitochondrial dysfunction and oxidative stress have also been known to have an important role in affecting the dopaminergic neurons and in the accumulation of misfolded proteins.

The body of evidence suggests that intermediate stage oligomers and protofibrils of alpha-synuclein are toxic and are part of the process of Lewy body formation. *In vitro* and *in vivo* drosophila and mouse work have shown that the pathway of protein misfolding and aggregation plays a key role in unraveling how, when and why neuronal apoptosis occurs. The factors influencing metabolism of alpha-synuclein and pathways influencing its aggregation would further enhance our understanding on the physiological role of Lewy body.

## Parkinson's Disease

Parkinson's Disease (PD) is a slowly progressing neurodegenerative disorder affecting about 2% of the population above 65 years of age [1,2]. Pathological hallmarks of PD are the degeneration of nigro-striatal Dopamine neurons (DA) and the presence of  $\alpha$ -synuclein-containing inclusions called Lewy Bodies (LB) in afflicted brain regions [3,4]. The symptoms include rigidity, postural instability, tremor at rest and slowness or absence of voluntary movement, but also neuropsychiatric symptoms [1,2,5]. These symptoms have been long attributed to the degeneration of dopamine neurons in the substantia nigra (SNc), resulting in less dopamine production in the striatum [2,3]. Various hypotheses have been implicated to have a role in the pathogenesis of PD such as oxidative stress, mitochondrial dysfunction, impairment of the ubiquitin proteasome pathway and defective autophagy process [6-8]. However, the exact mechanisms leading to neuronal death are unknown. One main hypothesis is the missense mutations of alpha-synuclein gene ( $\alpha$ -synuclein), which are responsible for autosomal dominant early-onset form of PD [9,10,4]. In addition,  $\alpha$ -synuclein gene duplication and triplication also plays a direct causal role in causing familial PD [11-13],  $\alpha$ -synuclein epitopes are also associated with other diseases such as multiple system atrophy [14].

## Dementia with Lewy Bodies

Dementia with Lewy Bodies (DLB) is also known as Lewy Body Disease, and is also one of the most common causes of dementia in the elderly [15,16]. DLB is characterized by changes in alertness and attention, confusion, loss of memory, inability to move smoothly and rigidity [17,18]. These symptoms are also similar to that of PD, and hence it is sometimes difficult to differentiate the two diseases [18]. DLB is characterized by the presence of LBs and ubiquitin in neurons [19].

L-3,4-dihydroxyphenylalanine (L-DOPA) has been used for parkinsonism in patients with dementia with Lewy bodies [20]. Since the use of antipsychotic agents causes severe neuroleptic sensitivity, use of pharmacological agents is thought to be beneficial [19]. Fujishiro et al. in 2013 reported the improvement of behavioral and psychological symptoms of dementia through L-DOPA treatment patient with DLB [20].

## Lewy bodies

LB's are cytosolic eosinophilic inclusions that displace other cell components, comprising of two types: the classical brain stem LB and cortical LB [21]. LBs tend to accumulate many proteins

such as ubiquitin, neurofilaments, parkin and Ubiquitin C-terminal Hydrolase 1 (UCHL1) but, the main component is  $\alpha$ -synuclein, a fibrillary component, which appears in both brain stem and cortical LBs [22-24]. LBs are thought to be a result of altered protein handling in PD [25,23],  $\alpha$ -synuclein is the most widely studied of all the proteins in LBs because it has been closely linked to dopamine homeostasis [11,12]. This suggests that  $\alpha$ -synuclein plays an important role for *in vivo* pathogenesis of PD.

### $\alpha$ -synuclein

$\alpha$ -synuclein is a 14kD presynaptic protein that is expressed at high levels in the brain and has three components: (1) an amino terminal region (1-60 residues), which include the sites where mutations in PD occur; (2) a central region; (3) a carboxy terminal region [15]. These three regions are thought to have different functions; the amino terminal is responsible for membrane interactions [26]; the carboxyl terminal is responsible for protein-protein interactions, and also required for the chaperone-like activity of  $\alpha$ -synuclein [26].  $\alpha$ -synuclein is encoded by the Parkinson Disease 1 (PARK1) (also known as SNCA) gene [27]. The non-amyloid component of  $\alpha$ -synuclein is known as NAC, which is a fragment of its precursor protein, NACP [27,28]. Full-length  $\alpha$ -synuclein exists in a natively unfolded state [29,30]. Evidence suggests that in its unfolded state, it is prone to aggregation [1,30,31]. Fibrillization is an important notion underlying the toxicity of  $\alpha$ -synuclein [1]. Fibrillar  $\alpha$ -synuclein is protease-resistant, a chief trait that is associated with other A $\beta$  fibrils [32]. The carboxy terminal region has residues that are acidic in nature and proline-rich [33,34].  $\alpha$ -synuclein protofibrils can also form  $\beta$ -sheet rich rings and are able to permeabilize membranes [33,34], suggesting that it is indeed the protofibrils and not monomeric  $\alpha$ -synuclein that is toxic in the pathogenesis of PD. Recent *in vivo* study suggest that  $\alpha$ -synuclein oligomers are toxic [35] and intermediate state oligomers exist in cytosolic inclusions that are in a different conformation to the non-aggregated soluble protein [35].

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### Effects of mutations in leucine-rich repeat kinase 2 (LRRK2)

LRRK2 is the most commonly mutated gene and have been long associated with in inherited and idiopathic PD [2,36]. Proteins that interact with LRRK2 are associated with several pathological pathways that could be targeted in the development of new therapies [5]. Carriers of LRRK2 mutation display a varied neuropathology, including  $\alpha$ -synuclein and tau inclusions, suggesting a vital role for LRRK2 in protein aggregation [2,36]. The accumulation of proteins due to mutations is not a new phenomenon: it was shown recently that over expression of mutant LRRK2 promotes aggregation of  $\alpha$ -synuclein due to an inability to clear proteasomes properly via the Ubiquitin Proteasome Pathway (UPP) [37]. Typically, mutations in the genes for fibrillar proteins are associated with early-onset familial forms of these diseases [9]. This implies that protein unfolding, oligomerization and/or fibril formation could be promoted by these mutations. But it is unclear whether the neurotoxicity lies in the prefibrillar intermediate, fibrillization, or the actual fibril itself.

### Different pathogenic models

Aggregation of misfolded proteins has been suggested to be characteristic feature of the neurodegenerative process. Significant evidence points to the aggregates occurring in the onset and progression of age-related neurodegenerative diseases [32,38]. LBs are thought to be toxic and analogous to the toxicity of amyloid plaques in Alzheimer's disease [22]. The frequency of LBs is proportional to the severity of Dementia with LBs [20], suggesting the neurotoxic nature of these fibrillary components. Environmental toxins and proteasome inhibitors have also been used to induce Parkinson's in animal models, resulting in  $\alpha$ -synuclein aggregation [39]. It has been confirmed that dopaminergic neurons degenerate in  $\alpha$ -synuclein transgenic animals, thus confirming that animal models are feasible to study the formation of LBs in [40]. On the other hand, LBs could simply be epiphenomenon, or scars of neurodegeneration that have been induced by neuronal death [41]. Post-mortem studies have showed that there is a negative correlation between amyloid deposits and the severity of the neurodegenerative disease [42]. It has also been suggested that LBs are part of a physiologic response to sequester/deactivate a neurotoxic species, namely dopamine-quinone [8,43,44]. Mutations in  $\alpha$ -synuclein reduce the number of vesicles available for dopamine storage, which results in an abundance of neurotoxic by-products such as dopamine-quinone, superoxide radicals and hydrogen peroxide, and an increased level of oxidative stress [7,8,44,45]. There have also been debates about whether PD is a prion-like disease, like Creutzfeldt-Jakob, as this could lead to better treatments in PD. This has been noticed because  $\alpha$ -synuclein behaves similarly to the prion precursor and propagates between cells [46]. However, there is no evidence to say that PD is a prion-like disease despite the genetic similarities and gain-of-function mutant protein between the two diseases [6].

The non-amyloid component NAC of  $\alpha$ -synuclein is thought to be the precursor to the non-amyloid precursor NACP in Alzheimer's disease [28]. Two missense mutations identified in the gene encoding for  $\alpha$ -synuclein G209A and G88C are believed to be responsible for the formation of LBs [2,9,47]. Immunochemical studies done on LBs, Pale Bodies (PBs), and Lewy-Related Neuritis (LRN) in PD and in Dementia with LB (DLB) were shown to be reactive for anti-NACP antibodies [16,48], suggesting that these mutations are indeed responsible for the formation of Lewy-related bodies and neuritis. In addition, two mutant forms of  $\alpha$ -synuclein called Ala53Thr and Ala30Pro quickened the fibrillization of LB, suggesting that mutant NACP is also responsible for the formation of  $\alpha$ -synuclein aggregates, and therefore LBs [15,28,48,49].

### Effects of mutations in PARK2

Mutations in Parkin RBR(RING-Between rings-RING),(PARK2) are responsible for the mutation of parkin, which is an E3 ubiquitin ligase and is involved in the degradation of misfolded or damaged proteins by the Ubiquitin-Proteasome Pathway (UPP) [50,51]. These mutations in the Parkin gene, on the other hand, are responsible for causing autosomal recessive PD [2,52,53]. Patients with parkin mutations have either pure nigral degeneration or typical LBs [36,43,45].

### Therapeutic approach for PD and the role of Lewy bodies

Grafting techniques using brain tissues have been in the limelight

recently to treat neurological diseases, especially PD [14]. Kordower et al, in 2008 showed that though the grafted neurons were at an age when PD does not usually develop, Lewy bodies were found in the brain tissue that was grafted to PD patients more than ten years ago [14]. It is speculated that misfolded  $\alpha$ -synuclein transfers from the host neurons to the neurons in the grafted brain tissue and once in the new cells they trigger the formation of Lewy bodies in a prion-like manner [54]. *In vitro* studies have shown that  $\alpha$ -synuclein can spread between cells in an endocytosis-dependant manner and also that it could be transported through axons. This not only indicates that the misfolded  $\alpha$ -synuclein spreads from the host cells to the grafted tissues through axonal transport but also explains the spreading of LBs throughout the brain in patients with PD [55]. Even though it was observed that the functionality of the grafts were not affected by the small proportion of LBs present but the grafts showed decreased levels of Dopamine transporter and tyrosine hydroxylase [47]. The above-mentioned studies validate that the brain tissue grafts are indeed affected by  $\alpha$ -synuclein pathology of PD and arises questions on neural grafting being a potential therapy for PD.

## Conclusion

Small oligomers and protofibrils are responsible for the death of neurons [26]. Formation of  $\alpha$ -synuclein has been discovered as a causal factor in apoptosis, suggesting that  $\alpha$ -synuclein aggregates are toxic at certain stages during LB formation, such as sequestering neurotoxic species like dopamine-quinone. LBs are influenced by various factors, namely  $\alpha$ -synuclein mutations, mitochondrial dysfunction and environmental factors. Further elucidation of their interaction will provide further clues to their pathophysiologic role. Future work should include determining the importance of ubiquitination of  $\alpha$ -synuclein, as this could be an important link to the  $\alpha$ -synuclein aggregation pathway. Also, it must be determined where in the pathway ubiquitination occurs.

While there is no unequivocal answer to the relative cytotoxic or cytoprotective role of LBs, they are definitely the end product of a very long and complex pathway, thereby making them scars but definitive characteristic index markers of PD.

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## References

- Dauer W, Przedborski S. Parkinson's disease: mechanisms and models. *Neuron*. 2003; 39: 889–909.
- Tan, E. K. & Skipper, L. M. Pathogenic mutations in Parkinson disease. *Hum. Mutat.* 2007; 28: 641–653.
- Davie CA. A review of Parkinson's disease. *Br. Med. Bull.* 2008; 86: 109–127.
- Forno LS. Neuropathology of Parkinson's disease. *J Neuropathol Exp Neuro.* 1996; 55: 259–272.
- Chan SL, Angeles DC, Tan EK. Targeting leucine-rich repeat kinase 2 in Parkinson's disease. *Expert Opin. Ther. Targets.* 2013; 17: 1471–1482.
- Dawson TM, Ko HS, Dawson VL. Genetic animal models of Parkinson's disease. *Neuron*. 2010; 66: 646–661.
- Angeles DC, Gan BH, Onstead L, Zhao Y, Lim KL, Dachselt J, et al. Mutations in LRRK2 increase phosphorylation of peroxiredoxin 3 exacerbating oxidative stress-induced neuronal death. *Hum. Mutat.* 2011; 32: 1390–1397.
- Angeles DC, Ho P, Chua LL, Wang C, Yap YW, Ng C, et al. Thiol-peroxidases ameliorate LRRK2 mutant-induced mitochondrial and dopaminergic neuronal degeneration in *Drosophila*. *Hum. Mol. Genet.* 2014; 23: 3157–3165.
- Polymeropoulos MH, Lavedan C, Leroy E, Ide SE, Dehejia A, Dutra A, et al. Mutation in the alpha-synuclein gene identified in families with Parkinson's disease. *Science.* 1997; 276: 2045–2047.
- Chartier-Harlin MC, Kachergus J, Roumier C, Mouroux V, Douay X, Lincoln S, et al. Alpha-synuclein locus duplication as a cause of familial Parkinson's disease. *Lancet.* 2004; 364: 1167–1169.
- Lashuel HA, Overk CR, Oueslati A, Masliah E. The many faces of  $\alpha$ -synuclein: from structure and toxicity to therapeutic target. *Nat. Rev. Neurosci.* 2013; 14: 38–48.
- Kasten M, Klein C. The many faces of alpha-synuclein mutations. *Mov. Disord.* 2013; 28: 697–701.
- Ibáñez P, Bonnet AM, Débarges B, Lohmann E, Tison F, Pollak P, et al. Causal relation between alpha-synuclein gene duplication and familial Parkinson's disease. *Lancet.* 2004; 364: 1169–1171.
- Petit GH, Olsson TT, Brundin P. The future of cell therapies and brain repair: Parkinson's disease leads the way. *Neuropathol Appl Neurobiol.* 2014; 40: 60–70.
- Arima K, Uéda K, Sunohara N, Arakawa K, Hirai S, Nakamura M, et al. NACP/alpha-synuclein immunoreactivity in fibrillary components of neuronal and oligodendroglial cytoplasmic inclusions in the pontine nuclei in multiple system atrophy. *Acta Neuropathol.* 1998; 96: 439–444.
- Morra LF, Donovick PJ. Clinical presentation and differential diagnosis of dementia with Lewy bodies: A review. *Int. J. Geriatr. Psychiatry.* 2013; 6: 569–576.
- Neef D, Walling AD. Dementia with Lewy bodies: an emerging disease. *Am. Fam. Physician.* 2006; 73: 1223–1229.
- Kosaka K, Manabe Y. The first autopsied case of diffuse Lewy body disease (DLBD): Re-examination by recent immunostaining methods: The 50th Anniversary of Japanese Society of Neuropathology. *Neuropathology.* 2010; 30: 458–462.
- McKeith IG. Dementia with Lewy bodies. *Br J Psychiatry.* 2002; 180: 144–147.
- Fujishiro H, Iseki E, Nakamura S, Kasanuki K, Chiba Y, Ota K, et al. Dementia with Lewy bodies: Early diagnostic challenges. *Psychogeriatrics.* 2013; 13: 128–138.
- Singleton AB, Farrer M, Johnson J, Singleton A, Hague S, Kachergus j, et al. alpha-Synuclein locus triplication causes Parkinson's disease. *Science.* 2003; 302: 841.
- Tenreiro S, Eckermann K, Outeiro TF. Protein phosphorylation in neurodegeneration: friend or foe? *Front. Mol. Neurosci.* 2014; 7: 42.
- McNaught KSP, Belzaira R, Isacson O, Jenner P, Olanow CW. Altered proteasomal function in sporadic Parkinson's disease. *Exp. Neurol.* 2003; 179: 38–46.
- Tompkins MM, Basgall EJ, Zamrini E, Hill WD. Apoptotic-like changes in Lewy-body-associated disorders and normal aging in substantia nigral neurons. *Am. J. Pathol.* 1997; 150: 119–131.
- Farrer M, Chan P, Chen R, Tan L, Lincoln S, Hernandez D, et al. Lewy bodies and parkinsonism in families with parkin mutations. *Ann. Neurol.* 2001; 50: 293–300.
- Volles MJ, Lansbury PT. Relationships between the sequence of alpha-synuclein and its membrane affinity, fibrillization propensity, and yeast toxicity. *J Mol Biol.* 2007; 366: 1510–1522.
- Li H, Guo M. Protein degradation in Parkinson disease revisited: it's complex. *J. Clin. Invest.* 2009; 119: 442–445.
- Culvenor JG, McLean CA, Cutt S, Campbell BC, Maher F, Jäkälä P, et al.

- Non-Abeta component of Alzheimer's disease amyloid (NAC) revisited. NAC and alpha-synuclein are not associated with Abeta amyloid. *Am. J. Pathol.* 1999; 155: 1173–1181.
29. Tofaris GK, Razaq A, Ghetti B, Lilley KS, Spillantini MG. Ubiquitination of alpha-synuclein in Lewy bodies is a pathological event not associated with impairment of proteasome function. *J Biol Chem.* 2003; 278: 44405–44411.
30. Dawson TM, Dawson VL. Molecular pathways of neurodegeneration in Parkinson's disease. *Science.* 2003; 302: 819–822.
31. Tofaris GK, Spillantini MG. Physiological and pathological properties of alpha-synuclein. *Cell Mol Life Sci.* 2007; 64: 2194–2201.
32. Soto C. Unfolding the role of protein misfolding in neurodegenerative diseases. *Nat Rev Neurosci.* 2003; 4: 49–60.
33. Uversky VN, Eliezer D. Biophysics of Parkinson's disease: structure and aggregation of alpha-synuclein. *Curr. Protein Pept. Sci.* 2009; 10: 483–499.
34. Crowther RA, Jakes R, Spillantini MG, Goedert M. Synthetic filaments assembled from C-terminally truncated alpha-synuclein. *FEBS Lett.* 1998; 436: 309–312.
35. Winner B, Jappelli R, Maji SK, Desplats PA, Boyer L, Aigner S, et al. In vivo demonstration that alpha-synuclein oligomers are toxic. *Proc Natl Acad Sci U S A.* 2011; 108: 4194–4199.
36. Tan EK, Peng R, Teo YY, Tan LC, Angeles D, Ho P, et al. Multiple LRRK2 variants modulate risk of Parkinson disease: A Chinese multicenter study. *Hum. Mutat.* 2010; 31: 561–568.
37. Lichtenberg M, Mansilla A, Zecchini VR, Fleming A, Rubinsztein DC. The Parkinson's disease protein LRRK2 impairs proteasome substrate clearance without affecting proteasome catalytic activity. *Cell Death Dis.* 2011, 2.
38. Winklhofer KF, Tatzelt J, Haass C. The two faces of protein misfolding: gain- and loss-of-function in neurodegenerative diseases. *EMBO J.* 2008; 27: 336–349.
39. Vernon AC, Natesan S, Modo M, Kapur S. Effect of chronic antipsychotic treatment on brain structure: a serial magnetic resonance imaging study with ex vivo and postmortem confirmation. *Biol Psychiatry.* 2011; 69: 936–944.
40. Feany MB, Bender WW. A Drosophila model of Parkinson's disease. *Nature.* 2000; 404: 394–398.
41. Popescu A, Lippa CF, Lee VM, Trojanowski JQ. Lewy bodies in the amygdala: increase of alpha-synuclein aggregates in neurodegenerative diseases with tau-based inclusions. *Arch Neurol.* 2004; 61: 1915–1919.
42. Gutekunst CA, Li SH, Yi H, Mulroy JS, Kuemmerle S, Jones R, et al. Nuclear and neuropil aggregates in Huntington's disease: relationship to neuropathology. *J Neurosci.* 1999; 19: 2522–2534.
43. Lotharius J, Brundin P. Pathogenesis of Parkinson's disease: dopamine, vesicles and alpha-synuclein. *Nat. Rev. Neurosci.* 2002; 3: 932–942.
44. Zhi DZ, Fathima SR, Shao PX, Shin HN, Christine HSC, Patrick Ghim HH, et al. Mutant PINK1 upregulates tyrosine hydroxylase and dopamine levels, leading to vulnerability of dopaminergic neurons. *Free Radic. Biol. Med.* 2014; 68: 220–233.
45. Chan SL, Chua LL, Angeles DC, Tan EK. MAP1B rescues LRRK2 mutant-mediated cytotoxicity. *Mol. Brain.* 2014; 7: 29.
46. Hilker R, Brotchie JM, Chapman J. Pros and cons of a prion-like pathogenesis in Parkinson's disease. *BMC Neurol.* 2011; 11: 74.
47. Chu Y, Kordower JH. Lewy body pathology in fetal grafts. *Ann N Y Acad Sci.* 2010; 1184: 55–67.
48. Arima K, Ueda K, Sunohara N, Hirai S, Izumiyama Y, Tonozuka-Uehara H, et al. Immunoelectron-microscopic demonstration of NACP/alpha-synuclein-epitopes on the filamentous component of Lewy bodies in Parkinson's disease and in dementia with Lewy bodies. *Brain Res.* 1998; 808: 93–100.
49. Krüger R, Kuhn W, Müller T, Woitalla D, Graeber M, Kösel S, et al. Ala30Pro mutation in the gene encoding alpha-synuclein in Parkinson's disease. *Nat Genet.* 1998; 18: 106–108.
50. Shimura H, Hattori N, Kubo S, Mizuno Y, Asakawa S, Minoshima S, et al. Familial Parkinson disease gene product, parkin, is a ubiquitin-protein ligase. *Nat Genet.* 2000; 25: 302–305.
51. Hattori N, Shimura H, Kubo S, Kitada T, Wang M, Asakawa S, et al. Autosomal recessive juvenile parkinsonism: a key to understanding nigral degeneration in sporadic Parkinson's disease. *Neuropathology.* 2000; 20: 85–90.
52. Kitada T, Asakawa S, Hattori N, Matsumine H, Yamamura Y, Minoshima S, et al. Mutations in the parkin gene cause autosomal recessive juvenile parkinsonism. *Nature.* 1998; 392: 605–608.
53. Herrera FE, Chesi A, Paleologou KE, Schmid A, Munoz A, Vendruscolo M, et al. Inhibition of alpha-synuclein fibrillization by dopamine is mediated by interactions with five C-terminal residues and with E83 in the NAC region. 2008.
54. Brundin P, Kordower JH. Neuropathology in transplants in Parkinson's disease: implications for disease pathogenesis and the future of cell therapy. *Prog Brain Res.* 2012; 200: 221–241.
55. Freundt EC, Maynard N, Clancy EK, Roy S, Bousset L, Sourigues Y, et al. Neuron-to-neuron transmission of alpha-synuclein fibrils through axonal transport. *Ann Neurol.* 2012; 72: 517–524.