EFFECT OF AUTOPHAGIC ALTERATIONS ON ALPHA SYNUCLEIN EXPRESSION LEVELS IN AN ENDOGENOUS CELL MODEL: IMPLICATIONS FOR SPORADIC PARKINSON'S DISEASE

A THESIS PRESENTED BY

SWAPNA NANDAKUMAR

TO

SREE CHITRA TIRUNAL INSTITUTE FOR
MEDICAL SCIENCES AND TECHNOLOGY, TRIVANDRUM
Thiruvananthapuram

IN PARTIAL FULFILMENT OF THE REQUIREMENTS
FOR THE AWARD OF

DOCTOR OF PHILOSOPHY

2015

DECLARATION

I, **Swapna Nandakumar**, hereby declare that I had personally carried out the work depicted in the thesis entitled **“Effect of autophagic alterations on alpha synuclein expression levels in an endogenous cell model: Implications for sporadic Parkinson’s disease”.** No part of the thesis has been submitted for award of any other degree or diploma prior to this date.

Signature:

Name of Candidate:

Date:

Prof.Asha.Kishore M.D, D.M.

Director,

Sree Chitra Tirunal Institute for Medical Sciences & Technology,

Medical College P.O, Trivandrum,

Kerala, India- 695 011.

CERTIFICATE

This is to certify that Ms. Swapna Nandakumar, in the Department of Neurology of this institute, has fulfilled the requirements prescribed for the Ph.D degree of Sree Chitra Tirunal Institute for Medical Sciences and Technology, Trivandrum.

The thesis entitled **"Effect of autophagic alterations on alpha synuclein expression levels in an endogenous cell model: Implications for sporadic Parkinson's disease"** was carried out under my direct supervision. No part of the thesis was submitted for the award of any degree or diploma prior to this date.

Signature:

Date:

ACKNOWLEDGEMENTS

It is with pleasure and gratitude that I acknowledge the help and support I have received during the entire tenure of my doctoral program. This program has not only helped me in building a scientific temper but has also helped me grow towards being a better person.

Prof. Asha Kishore, Director, SCTIMST, for her constant support and guidance. She has been very encouraging and appreciative of even little successes. Her demand for excellence has consistently prompted me to set my sights higher every time and I feel fortunate to have had her as my mentor.

I am deeply indebted to my co-guide, Dr.Anoop K Thekkuveettil, Scientist F, Division of Molecular Medicine, for the sheer amount of time he has invested in my work and efforts. He is a constant presence in the lab and I have found his doors are always open in an hour of need. I am very grateful for the opportunity to have worked under his guidance.

I would also like to express my gratitude to my Doctoral Advisory Committee members, Dr.Srinivas, Scientist E, Department of Biochemistry, SCTIMST and Dr.Mathuranth, Department of Neurology, NIMHANS. Dr.Srinivas has in particular been very supportive of all my efforts.

I am thankful to the institute for all the facilities that have been made available to me and for providing me with a fellowship for the term of my program. I also thank the Department of Cellular and Molecular Cardiology for all the help and space provided for my experiments. I would like to thank the, Department of Biochemistry, Division of Biosurface Technology and the Department of Microbial Technology for allowing me the use of their equipments.

My colleague, Mr.Bejoy Vijayan, has been more than just that. He has been a great friend and many are the occasions when I have looked to him for help and have always found it on offer. The time spent in and outside of the lab in his company not only makes for a great recollection but on several occasions have helped me look past my difficulties. I am very grateful for his support and friendship.

I would like to thank all the members of the Comprehensive Care Center for Movement Disorders and most particularly, Dr.Syam.K, Mr.Gangadhara Sarma, Mrs. Mary George, Mr.Thomas Joseph, Mr.Balu Velayudhan and Mrs.Soumya for the warmth I have received from them from the very beginning and for their support through times of personal crisis.

I would like to thank all members at the Division of molecular medicine, particularly, Mr. Jose Jacob, Suraj, Sarish, Anita, Anupriya, Sreethu, Cuckoo and Vishnu for the support and the good times during and off-work.

I would also like to thank all my friends at the institute for their help, support and friendship. I am most thankful to my roommates, Parvathy and Resmi, for their kindness and concern. Special thanks to my friends Padma and Dr.Linda for the happiness their companionship gave me.

I must also express the gratitude I feel for the support I have had from my family. Both my mother, Vasanthi Nandakumar and brother, Arvind Athreya, have stood by me at every hour of crisis and have helped me pick myself up after every fall all through my life. My grandmother with her constant prayers has been the most comforting presence in my life. My in-laws have always been ready and eager to help in every way and their efforts have always lightened the burden of a difficult day. I owe my sincerest thanks to my husband, Dr.Sabarinath.P.S for walking every step of the way with me with no regard for his own hardships. Most of all I owe my every thanks and gratitude to my father, C.Nandakumar, for the burdens he took on himself

and the sacrifices he made to ensure I could navigate through life with minimal difficulty. I dedicate this work to him and every success in my life will always belong to him.

Swapna Nandakumar

TABLE OF CONTENTS

Contents	Page Number
Title Page	i
Declaration	ii
Certificate of guide	iii
Certificate of co-guide	iv
Approval of thesis	v
Acknowledgement	vi-viii
Table of contents	ix
List of Figures	x-xii
List of Abbreviations	xiii-xiv
Synopsis	xv-xxiii
Introduction	1-7
Review of Literature	8 – 106
Materials and Methods	107 – 118
Results	119 – 152
Discussion	153 – 162
Summary and Conclusion	163 – 165
Bibliography	166 - 194

LIST OF FIGURES

Figure Number	Caption	Page Number
Figure 1	Components of basal ganglia	9
Figure 2	Overview of basal Ganglia function	11
Figure 3	Section of midbrain from PD	12
Figure 4	Pathological hallmarks of PD	13
Figure 5	Braak Staging	16
Figure 6	Molecular Pathogenesis of PD	25
Figure 7	aSyn- Gene and Protein	39
Figure 8	aSyn-Sites for Mutations and PTM's	39
Figure 9	aSyn- Sites for PTM's	45
Figure 10	aSyn- sites for cleavage	45
Figure 11	Types of aSyn inclusions	50
Figure 12	Types of aggregates	57
Figure 13	Secretion of aSyn	60
Figure 14	Uptake of secreted aSyn	60
Figure 15	Probable mechanisms of aSyn Toxicity	63
Figure 16	Targets for neuroprotective Strategies	65
Figure 17	Dopamine Synthesis pathway	67
Figure 18	Synthesis of Neuromelanin	68
Figure 19	Pathways for dopamine toxicity	71
Figure 20	Dopamine and melanin synthesis pathway	72
Figure 21	Link between PD and melanoma	74
Figure 22	UPS pathway	78

Figure Number	Caption	Page Number
Figure 23	26s proteasome Unit	79
Figure 24	Types of autophagy	83
Figure 25	Macroautophagy pathway	87
Figure 26	Signalling pathway for mTOR	92
Figure 27	mTOR independent signalling	94
Figure 28	Quality control pathways	97
Figure 29	Degradation of aSyn	103
Figure 30	Impairment of protein degradation by aSyn	105-106
Figure 31	Flow cytometry profile of SK-MEL-28	120
Figure 32	Confocal image of aSyn localization	120
Figure 33	aSyn localization by fractionation	121
Figure 34	Cell viability for chloroquine	122
Figure 35	Autophagic markers-Chloroquine treatment	122-123
Figure 36	aSyn expression with Chloroquine treatment	123
Figure 37	Immunocytochemistry for Chloroquine Treatment	124-125
Figure 38	aSyn expression with proteasome inhibition	126
Figure 39	Proteasome activity assay	127
Figure 40	Cell viability for Torin-1	128
Figure 41	Autophagic markers-Torin-1 treatment	129
Figure 42	aSyn expression with Torin-1 treatment	130
Figure 43	aSyn RNA expression with Torin-1 treatment	130
Figure 44	aSyn expression and autophagy markers-Starvation	131
Figure 45	Immunocytochemistry for Torin-1 treatment	133-134

Figure Number	Caption	Page Number
Figure 46	Validation of siRNA knockdown	135
Figure 47	Validation of siRNA knockdown	135-136
Figure 48	Cell viability for Torin-1 treatment with knockdown	136
Figure 49	Autophagi markers-Torin-1 treatment with knockdown	137-138
Figure 50	Cell viability for Dopamine	139
Figure 51	Dopamine Uptake assay	141
Figure 52	Assay for oxidative stress with dopamine treatment	142
Figure 53	Autophagic markers- Dopamine treatment	143-144
Figure 54	Cell viability for dopamine and Torin-1 simultaneous Treatment	144-145
Figure 55	aSyn expression with simultaneous dopamine and Torin-1 treatment	146
Figure 56	aSyn RNA expression with simultaneous dopamine and Torin-1 treatment	147
Figure 57	Immunocytochemistry for dopamine treatment	148-149
Figure 58	Autophagic markers with Knockdown for dopamine Treatment	150
Figure 59	Melanin Assay	151-152
Figure 60	aSyn behaviour in SK-MEL-28	162

LIST OF ABBREVIATIONS

aSyn – alpha synuclein

ALP – Autophagy-Lysosomal Pathway

CBD – Coticobasal ganglionic degeneration

COMT – catechol-o-methyltransferase

DA – Dopamine

DLB – Dementia with lewy Body

GP(i) – Globus pallidus interna

GP(e) – Globus pallidus externa

IP3- Inositol triphosphate

LB – Lewy Body

LN – Lewy neurite

MAO –monoamine oxidase

MSA – Multiple System Atrophy

mTOR – Mammalian Target of rapamycin

NAC – Non-amyloid component

NE – nor epinephrine

PD – Parkinson's Disease

PIP3- Phosphotide inositol 3,4,5-phosphate

PSP – Progressive supranuclear palsy

PTM – Post-translational modifications

SN – Substantia nigra

SN*pc* – Substantia nigra *pars compacta*

SNpr – Substantia nigra *pars reticulate*

STN – Subthalamic nucleus

SNP – Single nucleotide polymorphism

UPS – Ubiquitin Proteasome Pathway

SYNOPSIS

Parkinson's disease (PD) is the second most common neurodegenerative disorder of the elderly, affecting 1-2% of the population over 60 years. With better life expectancy, the prevalence rates of PD is expected to rise and it is estimated that 9 million people worldwide will be afflicted with the disease by 2030. The major motor symptoms which form the hallmark of the disease are tremor at rest, rigidity or stiffness of limbs, bradykinesia or slowness of movements and postural instability. These result from a severe reduction in striatal dopamine content due to the loss of dopaminergic neurons in the substantia nigra *pars compacta* (SNpc) in the midbrain. The presence of intracytoplasmic eosinophilic inclusion bodies called Lewy bodies in the surviving neurons of SNpc is considered the pathological hallmark of the disease.

Although PD was first identified in 1817, the cause of neuronal degeneration in the SNpc and other brain stem and cortical regions in sporadic PD is unknown. Majority of PD is sporadic and about 10% of PD is familial and monogenic in origin. Much of the research on the molecular pathways that lead to neuronal death has been triggered by the discovery of genetic forms of PD. There is considerable overlap in the clinical symptoms and pathology between familial and sporadic PD and hence it is suspected that the pathogenic pathways that lead to cell death in the two forms of the disease maybe similar. Misfolding and aggregation of the protein alpha synuclein (aSyn) is thought to be central to PD pathogenesis and is a major component of the Lewy bodies. The identification of point mutations and gene multiplications in the aSyn gene (gene id: *SNCA*) causes PARK 1 and PARK 4, two genetic forms of PD. Large genome-wide studies have also shown that polymorphisms in *SNCA* contribute to the risk in PD and this has brought aSyn to the center stage of research in the field of sporadic PD.

aSyn is a 140 amino acid protein (approximately 14kDa) that belongs to the family of intrinsically disordered proteins that have no defined secondary structure and can undergo various folding patterns depending on their interacting partners. The flexibility in folding drives them towards misfolding and this can be stabilized by several cellular factors. The point mutations in *SNCA* that are responsible for familial PD increase the propensity of the protein to aggregate through amino acid modifications. The identification of *SNCA* gene multiplications as a cause of familial PD and the increase in disease severity in these cases due to increased gene dosage suggest that besides protein modifications, the increased content of the wild type aSyn by itself can trigger misfolding. Given that majority of the PD cases are sporadic in nature with no known mutations of aSyn present in them; it is proposed that the aSyn accumulation and aggregation may be caused by the failure of degradation pathways for aSyn.

Protein degradation pathways maintain a steady state in protein turn-over and thus ensure the cell is not overburdened with non-functional proteins. Ubiquitin-proteasome pathway (UPS) and Autophagy Lysosomal pathway (ALP) are the two major degradation mechanisms in the cell. ALP, simply referred to as autophagy, is a bulk degradation pathway known for its role in maintaining cellular homeostasis, organelle degradation and clearance of misfolded proteins. Therefore several studies have looked at how aSyn is cleared by the cell with a view to developing possible therapeutic alternatives to mediate increased clearance of aSyn and to prevent its accumulation with special preference for autophagy.

The reports for wild type aSyn clearance are divided with evidence for involvement of both the UPS and ALP. However, it is also known that not only does the misfolded aSyn resist degradation but also impairs the clearance mechanisms thereby sustaining the aggregation process. Therefore it is yet to be determined if the failure in protein clearance mechanisms are

cause or a consequence of aSyn misfolding. The misfolded proteins are often stabilized by other cellular factors. It has been shown that not only can dopamine metabolism trigger oxidative stress; its interaction with aSyn causes the protein to remain misfolded. These interactions between aSyn and dopamine assume great relevance as preferential loss of dopaminergic neurons is the pathological hallmark of PD.

We hypothesized that the normal interactions aSyn and autophagy is altered by intracellular dopamine and this results in aSyn accumulation and aggregation in dopaminergic neurons. We used a novel method of testing this relation in a cell line with endogenous expression of aSyn. Previous studies have widely used aSyn over expressed models to study aSyn clearance mechanism. This is because of the exclusive presence of aSyn in the neurons and the difficulties associated with establishing neuronal cultures. However, these models have the limitation that aSyn overexpression by itself might impair autophagy.

Several different cell types, besides neurons, have now been shown to have endogenous expression of aSyn. These include RBC's, monocytes, T-lymphocytes and melanoma cells. Among these the melanoma cell line is the closest model to neurons to study aSyn interactions. Melanoma originates from the neural crest and the melanin synthesis pathway shares similarities with the dopamine synthesis pathway in neurons. The presence of aSyn in melanoma, its absence in melanocytes and the increased incidence of melanoma in PD patients, further justifies using this model to understand the physiological and pathological role of aSyn.

In the present study, a melanoma cell line, SK MEL 28, which is reported to express endogenous aSyn, was used. The presence of aSyn in this cell line was first verified through immunoblotting, flow cytometry and immunocytochemistry. We also demonstrate its localization to both the

nucleus and the cytoplasm. More interestingly we also found that while the nuclear fraction only contains monomeric aSyn, the cytoplasmic fraction contains a higher molecular weight species of aSyn. For the purposes of the study, we have restricted our observations to the monomeric aSyn for two reasons 1) Reports till date suggest that aSyn exists as a monomer though higher order species are reported in T lymphocytes and RBC's, secondary to interactions between the monomeric units. 2) The aggregation process begins with the monomer and therefore studying the expression pattern of monomeric aSyn will help to better understand the aggregation process.

This study had four major objectives:

1) To understand the role of autophagy as a clearance mechanism for aSyn in cell line with endogenous aSyn expression by studying the effect of autophagy inhibition and induction on aSyn clearance.

2) To test the effect of dopamine on aSyn levels and its clearance by autophagy.

3) To test if modifications in autophagy will reverse the effects of dopamine on aSyn.

4) To study the role of proteasomal pathway on aSyn clearance.

Autophagy was first inhibited by chemical means and changes in aSyn expression levels were measured by western blotting. Autophagy inhibition did not lead to any significant increases in aSyn. However, our immunocytochemistry results revealed an increase in the cytoplasmic localization of aSyn when autophagy was inhibited.

In the next step, autophagy was induced by chemical means to see if it led to aSyn clearance. Induction of autophagy resulted in a significant increase in the protein levels of aSyn which was verified by western blotting. However, this increase did not lead to an increased cytoplasmic

localization; rather it resulted in an increased retention in the nucleus. These results were replicated using serum starvation as another mechanism of autophagy induction and this suggested that the increase in aSyn levels was a response to autophagy and not a response to the chemical. Therefore, for all further studies, we used chemical means of autophagy induction. To confirm if the changes in aSyn were at the protein level or the transcriptional level, the mRNA expression of aSyn was measured through PCR and found that the increase in aSyn protein level was a result of increased gene expression.

The increase in aSyn expression following autophagy induction led us to speculate if aSyn might have regulatory role on autophagy given that its effect on synaptic vesicle recycling is already known and it may very well regulate other vesicular pathways. To test this, we blocked the expression of aSyn using siRNA in the cells and then induced autophagy. We found that in absence of aSyn, the cells responded strongly to autophagic induction confirming that aSyn regulated autophagy and its increase was therefore a response to the autophagic induction.

The regulation of dopamine by aSyn is mediated through its effect on Tyrosinase Hydroxylase and Tyrosinase. Both of these enzymes are also present in melanoma and therefore we supplemented the cells with exogenous dopamine to study its effects on aSyn levels. The uptake of dopamine was verified by using a fluorochrome tagged dopamine and it was seen that SK MEL 28 cells take up dopamine in a dose dependent manner. The addition of dopamine resulted in an increase of aSyn levels and also increased the cytoplasmic localization of aSyn. However the increase in protein levels was not reflected in the mRNA levels, indicating that the increase was not due to increased genetic expression but perhaps due to the decreased clearance of aSyn in presence of dopamine. We then tested the effect of dopamine addition on autophagy and found that it resulted in autophagy inhibition. We blocked the expression of aSyn through siRNA and

found that in absence of aSyn, the inhibitory effect of dopamine on autophagy was partially reduced thus suggesting that aSyn, mediated, at least partly, the autophagy inhibition by dopamine.

We next examined if autophagy induction, reversed the effects of dopamine on autophagy and aSyn levels. To test this, autophagy was induced by chemical means in the presence of dopamine. The combined effect of autophagy induction and dopamine only led to a further increase in aSyn levels which was sustained upto 24 hours. Additionally, we observed an increase in cell death when autophagy was induced in presence of dopamine. The surviving cells showed a sustained inhibition of autophagy suggesting that autophagy induction was ineffective in the presence of dopamine.

It is known that the melanin content is increased when autophagy is inhibited. Therefore we estimated the melanin content following the dopamine induced autophagy-inhibition and found it to be increased. Therefore dopamine addition resulted in elevation of aSyn and melanin.

Proteasome is known to play a role in clearance of wild type aSyn as seen in studies using over expression models. We therefore performed a chemical block of the proteasomal pathway in the cell but found no significant change in aSyn levels in response to proteasome inhibition. We then proceeded to verify if this was due to proteasome dysfunction and found that SK MEL 28 cells did have impairment in their proteasomal function.

In summary the results of the study are as follows:

1) Autophagy Inhibition does not increase aSyn levels but changes its localization from the nucleus to cytoplasm. The functional significance of this change in localization needs further studies.

2) Autophagy Induction increased aSyn levels, suggesting that the increase maybe a response to regulate the extent of autophagy.

3) The presence of excess dopamine not only increases aSyn levels but also inhibits autophagy and this was accompanied by an increase in the melanin levels.

4) Autophagy Enhancement did not relieve the cells from the autophagy inhibition triggered by dopamine and was counter-productive to cell survival.

5) The proteasome pathway appears impaired in SK MEL 28.

The cause of increase in aSyn levels in sporadic PD has been long investigated. Our results suggest that changes in cellular environments such as autophagy inhibition or the presence of excess dopamine may in fact mediate aSyn aggregation not merely by an increase in aSyn content but also by altering its normal sub-cellular localization. It is important to note that we demonstrate the presence of two different species of aSyn in the nucleus and cytoplasm. A change in aSyn localization could also mean a change from its monomeric state to a higher molecular state which could trigger aggregation when exposed to cellular insults in the long term. Therefore it is possible that the factors altering aSyn localization may be key players in the aSyn aggregation in the PD brain. Our study also highlights the behavior of aSyn at physiological levels. Two independent model systems, melanoma cell line in our study and T-lymphocytes in an earlier study, both demonstrate that aSyn may regulate the extent of autophagy in the cells. It is possible that even in the neurons, which are very dependent on autophagy to maintain cellular homeostasis; aSyn at physiological levels may play a similar regulatory role with regard to autophagy thereby contributing to the maintenance of cellular homeostasis. Thus altered levels of aSyn may affect the autophagic process which in turn could

disrupt neuronal homeostasis. Our results show that dopamine mediated inhibition of autophagy led to an increase in melanin content in the cells. Neuromelanin is shown to have both cytoprotective role through removal of reactive oxygen species as well as cytotoxic role through metal interactions when present in excess in the cell. Our study suggests that dopamine mediated toxicity could also be via its inhibition of autophagy and the downstream effect on melanin levels. The use of autophagy enhancement as a means to increase aSyn clearance and prevent its aggregation has been proposed for neuroprotection in PD. Our study demonstrates that autophagy enhancement does not mitigate the toxic effects of dopamine-aSyn interactions in the cell and that it may not be a suitable therapeutic alternative. Lastly, we also demonstrate the proteasome impairment in SK MEL 28 cell line. Future studies can be performed on this model to understand the effect of proteasomal impairment on aSyn levels and its localization as well as to study the effect of proteasomal enhancement on aSyn accumulation and aggregation.

1. INTRODUCTION

The flow of information that governs the working in all living forms culminates with proteins which are responsible for a diverse array of cellular processes. However the mere synthesis of proteins is insufficient to make them functional. The protein function is closely linked to its structure since the correct spatial folding of proteins is absolutely essential for the full functioning of proteins (Dobson, 2001; Yon, 1997). In addition to the amino acid sequence, proteins undergo several posttranslational modifications, all of which together influence protein folding (Tokmakov et al., 2012). The importance of protein folding is best understood in proteinopathies which are a group of diseases resulting from misfolded proteins. When not effectively eliminated, misfolded proteins can aggregate often appearing as inclusion bodies in the cell (Chaudhuri and Paul, 2006; Dobson, 2001; Ferreira and De Felice, 2001). Therefore, misfolding of proteins is the first step towards aggregation. Misfolding can result in either loss of physiological function which would disrupt normal cellular activities or in gain of toxic function that would cause damage to cellular components (Chiti and Dobson, 2006). Synucleinopathies are a group of diverse neurodegenerative disorders that exhibit inclusion bodies predominantly composed of the protein alpha synuclein (aSyn) and include Parkinson's disease (PD), Dementia with Lewy bodies (DLB), Multiple System Atrophy (MSA) and Neuroaxonal dystrophy among others (Martí et al., 2003).

PD is perhaps the most prevalent of these disorders (Savica et al., 2013). PD is the second most common neurodegenerative disease affecting more than 1% of the population aged over 60.

Clinically, PD is characterized by the triad of resting tremor, bradykinesia (slowness of movement) and rigidity. Pathologically, the hallmarks of PD include neuronal loss and presence of intracytoplasmic inclusions called Lewy bodies in the surviving neurons. The preferential loss of dopaminergic neurons of the substantia nigra *pars compacta* (SN*pc*) in the basal ganglia is responsible for the motor deficits seen in PD. The spread of pathology to other regions of the brain in due course of disease progression causes other symptoms that include cognitive and autonomic dysfunction. Since the loss of dopamine is central to PD, supplementing dopamine is the best available therapeutic strategy. Dopamine cannot cross the blood brain barrier and is therefore supplemented through administering its precursor L-Dopa and the enzyme Dopa decarboxylase required for the conversion of L-Dopa to dopamine. While dopamine replacement offers relief for the motor symptoms, the cognitive decline that occurs in later stages of the disease also need to be addressed. At present, treatment for PD, only involves symptomatic relief and palliative care and disease progression cannot be halted (Shulman et al., 2011).

The protein, alpha synuclein, in its full length is composed of 140 amino acids. It belongs to the family of intrinsically disordered proteins, in that it does not have a defined secondary structure and remains unfolded in the cell unless associated with other cellular components that promote its folding (Stefanis, 2012). This has been recently challenged with new studies suggesting its occurrence as a helically folded tetramer but these observations have not gained wide acceptance (Lashuel et al., 2013). These intrinsically disordered proteins have been reported to have a higher propensity for aggregation and explain in part, the misfolding and aggregation of aSyn, as seen in synucleinopathies. Initially, aSyn was thought to be a neuronal protein but recently its presence in platelets, Red blood cells, T-lymphocytes, Peripheral blood mononuclear cells

(PBMC) and malignant melanoma and ovarian cancers has also been reported (Barbour et al., 2008; Matsuo and Kamitani, 2010; Shameli et al., 2015; Uversky, 2008).

Upon discovery, aSyn was thought to be localized to both the nucleus and presynaptic terminals although its nuclear localization has continued to remain a matter of debate (Irizarry et al., 1996; Maroteaux et al., 1988). Its presence in the neurons and its enrichment in the presynaptic terminals suggest that it may function as a synaptic protein. Accordingly it has been found to play a role in synaptic vesicle recycling and is found to be associated with SNARE complex which is involved in neurotransmitter release (Cheng et al., 2011). Although its localization to the nucleus is debatable, no possible functions in the nucleus have as yet been assigned to aSyn. The possible roles it may play in non-neuronal cells are also not known although there exists a strong likelihood of it playing a role in vesicle trafficking. In this regard aSyn has been shown to be able to block vesicle trafficking when overexpressed but the physiological effects of such interactions are not yet clearly understood (Cooper et al., 2006).

The lack of a defined secondary structure governs aSyn behavior in pathology and physiology. Its association with lipid bilayers that promotes it folding into an alpha helix appears to underlie the role it plays in vesicle trafficking. On the other hand, factors that promote its folding to a beta sheet are thought to promote its misfolding. Therefore factors that tip the balance in favor of the alpha helix or the beta sheet determine the folding pattern of aSyn (Uversky, 2008). The misfolding of protein is followed with formation of dimers, trimers and oligomers through self-association. As more and more of the monomers get recruited, higher molecular weight species are formed which ultimately results in the formation of a large aggregate (Volles and Lansbury, 2003). There has been an ensuing debate as to the exact nature of the toxic species since the aggregates are found in surviving neurons. It is now generally held that the smaller oligomers are

the toxic species. These oligomers have been shown to be able to disturb membrane integrity by creating pores that brings about damages to organelles eventually leading to cell death (Kalia et al., 2013).

Insights into role of aSyn in disease pathogenesis have mostly been obtained through studies in PD. Point mutations and gene multiplications of the aSyn gene (Gene: *SNCA*) are found to cause autosomal dominant familial PD. The point mutations act to increase the propensity of aSyn aggregation while the gene multiplications result in increased aSyn levels in the cell suggesting that increased cellular concentrations of the protein can trigger aggregation (Bekris et al., 2010). A large majority of PD cases are found to be sporadic while only 5-10% of all reported cases are familial in nature. Sporadic and familial PD share similar clinical and pathological features and therefore seems likely that common pathways affect pathogenesis in both (Lesage and Brice, 2009). Hence, the increased cellular concentration leading to aggregation has found resonance as an aggregation determining factor even in sporadic PD.

The synthesis and degradation of proteins occur on cue and is tightly regulated in the cell. Steady state of proteins is achieved through regulating its synthesis and degradation such that there exists a two pronged approach to optimize protein usefulness in the cell. In absence of genetic changes that increase protein concentrations, failure of degradation pathways is the likely alternative that results in similar increases. Since increased protein levels clearly trigger aggregation, several studies have looked into the effect of failure of degradation pathways in mediating disease through reduced protein clearance. The ubiquitin-proteasome system (UPS) and the autophagy-lyososmal pathway (ALP) are the two major degradation pathways in the cells. UPS is mainly involved in the degradation of short-lived proteins while ALP is involved in

the degradation of long-lived proteins and damaged organelles and as such is a bulk degradation pathway (Matsuda and Tanaka, 2010).

The presence of ubiquitin positive inclusions, the association of UPS related genes *parkin* and *UCHL-1*(encodes for ubiquitin carboxylase) with PD and the presence of ubiquitinated aSyn in inclusion bodies suggest that UPS dysfunction may be pathogenic. However, it seems that ALP is more strongly correlated with disease pathogenesis than UPS especially since ALP plays a very critical role in maintaining cellular homeostasis. Stress related conditions such as starvation favour induction of autophagy which helps the cell tide over the stress thereby promoting cell survival. Its ability to remove damaged organelles also argues for a greater role of ALP in pathogenesis. The identification of mutations in *ATP13A2* which codes for a lysosomal protein as a cause of autosomal recessive PD and identification of *GBA* mutations (encodes for glucocerebrosidase) which cause lysosomal disorder Gaucher's disease and its strong association with PD as a risk factor further support the role of ALP in neurodegeneration (Ebrahimi-Fakhari et al., 2012; Martinez-Vicente and Cuervo, 2007).

In an effort to unravel the effects of inhibition of these two degradation pathways on aSyn aggregation, the degradation of aSyn has been studied. Both these pathways have been implicated in aSyn clearance. Despite a large number of studies, a clear consensus has not emerged as to the preferred pathway for aSyn degradation. This can be attributed partly to the reciprocal relationship between aSyn and degradation pathways. The oligomeric species of aSyn can block both the UPS and autophagy and it has been observed that even overexpression of wild type aSyn can also block autophagy. The oligomeric species saturate and overwhelm the degradation and prevent not only their degradation but also the degradation of other proteins while overexpressed wild type aSyn blocks vesicle trafficking (Xilouri et al., 2012a). Since

increased concentrations of the protein trigger misfolding and since all the above studies utilize models with genetic overexpression of aSyn, the conflict in these results can be attributed to the type of model and the protein burden in these cells. Therefore the use of models with endogenous aSyn expression may be more suitable in determining the degradation pathway for wild type aSyn since the protein expression is not genetically enhanced and the effects of inhibition of clearance pathways can be restricted towards understanding protein behavior.

The development of neuroprotective strategies in PD is hindered by our lack of understanding of the primary trigger for pathogenesis. Several factors such as ageing, oxidative stress, mitochondrial damage and impaired degradation pathways promote the misfolding and aggregation of proteins. The misfolded proteins on the other hand continue to cause wide spread damage in the cell including mitochondrial damage and blocking clearance pathways which in turn sustain the aggregation process. Since misfolding is central to pathogenesis, preventing oligomer formation or enhancing their sequestration into large non-toxic aggregates or promoting their clearance seems most promising at present. In terms of protein clearance, due to inability of misfolded proteins to translocate to proteasome, ALP appears as the more preferred alternative.

Since overexpressed aSyn has already been shown to block early autophagosome formation, it seems important to determine how aSyn is degraded in the cell and whether ALP inhibition is sufficient to trigger aggregation and if so at what stage does enhancing of ALP provide beneficial effects. As stated earlier, only the use of endogenous models will enable a thorough determination of the interactions between the existing protein and the clearance pathways. Given the complexity associated with establishing neuronal cultures, we propose the use of a melanoma model as an endogenous system to study aSyn response and effect on clearance pathways. The

strong correlation between PD and melanoma as demonstrated by epidemiological studies (Pan et al., 2011) and the lack of understanding with regard to aSyn behavior in non-neuronal cells further warrant the use of alternative models to arrive at a more holistic view of aSyn that may help devise intervention strategies.

Therefore in this study, I have employed a melanoma model, SK-MEL-28, to study the effect of autophagy alterations on aSyn protein levels. Here, I report the presence of atleast two different molecular weight species of aSyn that exhibit different sub cellular localization and while autophagy inhibition does not affect aSyn protein levels, it appears to mediate the change in its localization which will influence aSyn aggregation. I also show that autophagic enhancement does not result in increased aSyn clearance but on the contrary leads to increase in protein levels. Autophagic enhancement on the other hand results in toxicity particularly when combined with dopamine exposure thus questioning the potential of ALP as a therapeutic tool.

2. REVIEW OF LITERATURE

2.1 PARKINSON'S DISEASE (PD)

In 1817, in an essay entitled "A Shaking Palsy", James Parkinson first described the disease entity that would later be called Parkinson's disease (PD), a progressive neurodegenerative disorder. Jean Martin Charcot, in addition to providing further details on the disease, also coined the term "Parkinson's Disease"(Jankovic, 2008; Shulman et al., 2011). In 1912, Friedrich Lewy described the neuropathological lesions termed "Lewy Bodies" that is now the hallmark of the disease (Shulman et al., 2011). Today, PD is second only to Alzheimer's in incidence and is prevalent in 1% of the population above the age of 60 which rises to 5% in population aged above 85 (Shulman et al., 2011). The increased incidence in PD is proportional to the increase in life expectancy.

PATHOPHYSIOLOGY OF PARKINSON'S DISEASE

Parkinson's disease is largely characterized by motor dysfunction and the symptoms of resting tremor, rigidity, bradykinesia (slowness of movement) and postural instability form the clinical hallmarks of the disease (Gasser, 2009; Jankovic, 2008; Shulman et al., 2011). Neuronal loss and the presence of characteristic eosinophilic cytoplasmic inclusion bodies form the pathological hallmarks of the disease with the predominant loss of neurons in basal ganglia being responsible for the motor manifestations seen in the disease (Dauer and Przedborski, 2003; Tsui and Isacson, 2011) although several other regions become affected in due course of the disease.

The corticospinal tracts (also known as pyramidal tracts) that run from the brain to the spinal cord are responsible for movement. They receive assistance in the form of regulatory pathways that arise from the basal ganglia. Therefore, although the basal ganglia do not directly contribute to motor output, it modulates the control of movement. Initially, it was thought that the output from the basal ganglia went directly to the spinal cord and was hence termed the extrapyramidal pathway. Today, it is known that the output from the basal ganglia goes to the cortex and can be called "prepyramidal" rather than extrapyramidal.

The basal ganglia are subcortical structures comprising of four nuclei located in the forebrain, midbrain and diencephalon. The components of the basal ganglia are the caudate nucleus, putamen, nucleus accumbens, subthalamic nucleus (STN), globus pallidus which is further divided into internal (GPi) and external segments (GPe), ventral pallidum and the substantia nigra which is also divided into pars compacta (SNpc) and pars reticulata (SNpr) (Fig.1).

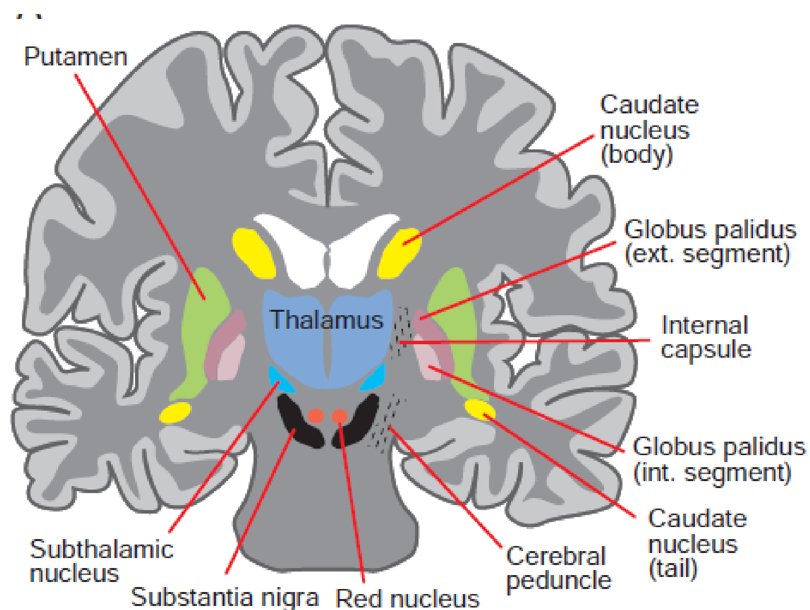


Figure 1: The various components of the basal ganglia and their spatial arrangement in the brain. (Squire et al., 2008)

The caudate and the putamen together form the striatum which along with the subthalamic nucleus receives inputs from the cortex and the thalamus. There is a topographical organization in the projections from the cortex to the striatum where in different regions of the striatum receive inputs from specific regions of the cortex. The GPi and SNpr are the primary output structures which then send information back to the cortex and the thalamus.

A majority of the connections in the basal ganglia are inhibitory and use GABA as the neurotransmitter. However the connections from STN to GPi are excitatory and use glutamate as the neurotransmitter while the connections from SNpc to striatum are dopaminergic and use dopamine as a neurotransmitter which has both excitatory and inhibitory functions. The basal ganglia are thought to regulate movement in two different ways via the "direct pathway" which enhances movement and the "indirect pathway'' which inhibits movement.

The striatum sends inhibitory signals to GPi which in turn sends inhibitory signals to the thalamus. The excitatory signals from the cortex to the striatum increase the inhibitory firing of the striatum to the GPi which results in the inhibition of GPi which in turn reduces its inhibitory signals to the thalamus with the net effect of enhancing movement. This is the "direct pathway''.

The indirect pathway involves the GPe and the STN with the striatum sending inhibitory signals to GPe which in turn inhibits the STN. The STN on the other hand sends excitatory signals to GPi which in turn cause the GPi to inhibit the thalamus. However when the striatum receives excitatory signals from the cortex, its inhibition of the GPe is enhanced which leads to the disinhibition of the STN by GPe. The STN is now free to excite the GPi which in turn enhances its inhibitory effect on the thalamus with the net effect of reducing movement.

Dopaminergic neurons in the SNpc regulate the direct and the indirect pathway through both excitation and inhibition. SNpc projects to the striatum and use dopamine as the neurotransmitter. There are five types of dopamine receptors, classified into two classes –D1 receptors and D2 receptors. The striatal neurons involved in the direct pathway express D1 receptor which results in dopamine having an excitatory effect on these neurons thereby exciting the direct pathway. The striatal neurons involved in the indirect pathway express D2 receptors which in turn results in dopamine having an inhibitory effect on these neurons thereby inhibiting the indirect pathway. The cumulative effect of dopamine action, be it excitation or inhibition results in enhancement of movement by favoring the direct pathway and disfavoring the indirect pathway. This is the simplified overview of basal ganglia function (Fig.2).

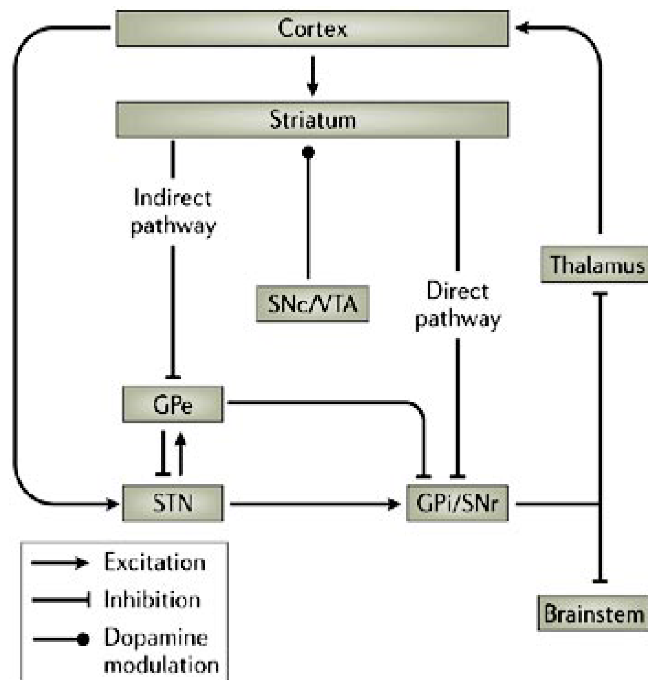


Figure 2: Simplfied overview of basal ganglia function via the direct and indirect pathway.(Yin and Knowlton, 2006)

Parkinson's disease is predominantly characterized by loss of dopaminergic neurons in the nigrostriatal pathway leading to dopamine depletion in the striatum which abolishes the enhancement in movement as a result of dopamine signaling. This causes significant impairment in movement which is the hallmark of PD.(Squire et al., 2008)

Typically, the onset of PD usually occurs beyond the age of 60, unilaterally, is chronic and insidious in nature with a mean duration of 15 years from disease onset to death. Dopaminergic neurons contain melanin and therefore the loss of neurons can be visualized as the depigmentation of the midbrain region (Fig.3). The cytoplasmic inclusions called Lewy bodies are composed of aggregated proteins, the predominant component being the protein alpha synuclein (aSyn). The identification of aSyn in the Lewy body led to the classification of PD under the broad umbrella of disorders termed synucleinopathies which include Dementia with Lewy bodies (DLB), multiple system atrophy (MSA), among others, all of which harbor pathogenic forms of aSyn.

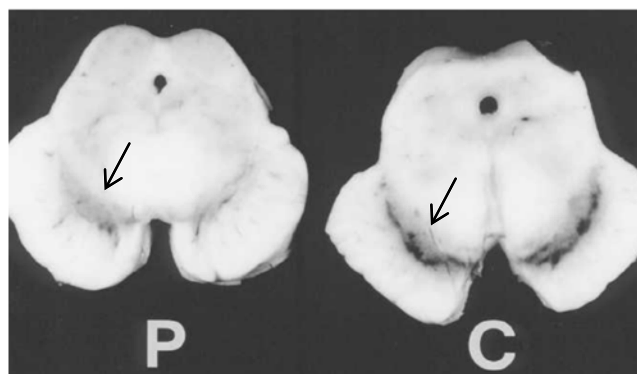


Figure 3: Section of mid brain from PD (P) showing depletion of pigementation in substantia nigra when compared to controls. (Mackenzie, 2001)

The autopsies of PD brains reveal a distinct pattern of Lewy bodies. The classical Lewy bodies are spherical and stain with eosin, which reveal a darkly stained core with a clear halo and stain positive for aSyn. Neuronal processes also exhibit aSyn positive accumulations and are termed as Lewy neurites. Lewy bodies that are not distinguished by the eosin stain and are more diffuse can also occur and are called cortical Lewy bodies which also stain positive for aSyn (Fig.4).

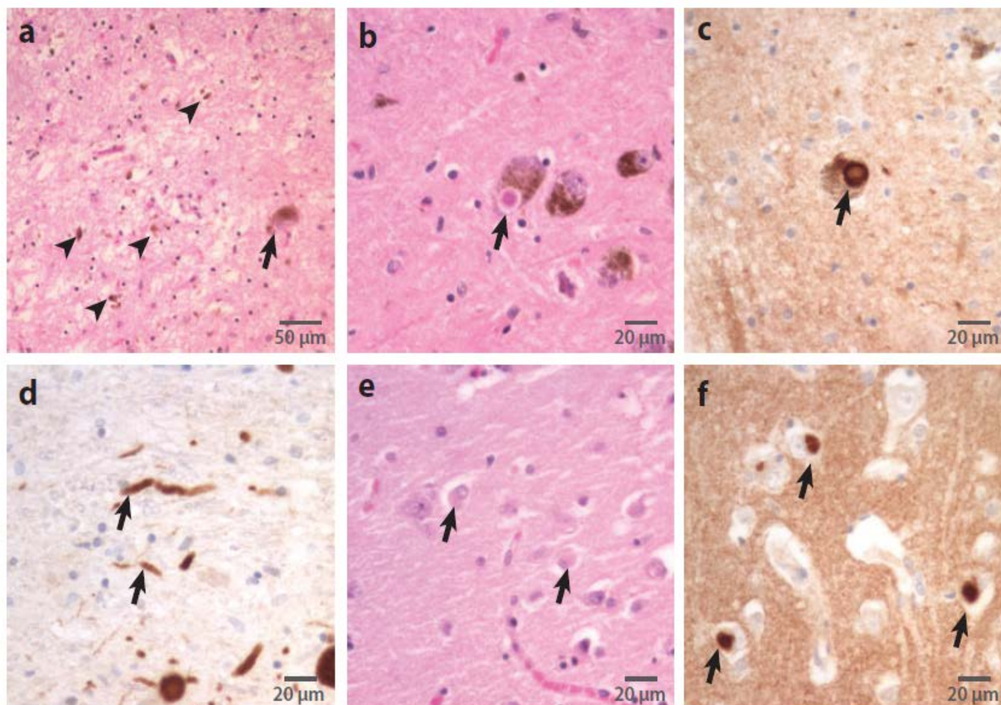


Figure 4: (a) Low power view showing depletion of neurons in substantia nigra with arrows indicating the existing neurons. (b) Classical Lewy body stained with eosin showing a dense core with a clear halo. (c) Classical Lewy body stained for aSyn. (d) Lewy Neurites.
(e) Cortical Lewy bodies not distinguishable with heamotoxylin and eosin staining.
(f) Cortical Lewy bodies stained for aSyn. (Shulman et al., 2011)

The motor manifestations described above produce the syndrome of Parkinsonism which is a characteristic of several other disease entities. DLB and MSA, which are also classified under synucleinopathies are two such examples. DLB is characterized by Parkinsonism and early development of dementia along with visual hallucinations and fluctuations in arousal and shows

prominent aSyn pathology in the neocortex. MSA is a rapidly progressing disorder characterized by a prominent failure of autonomic nervous system along with Parkinsonism, cerebellar dysfunction with aSyn pathology seen in glial cells (Jankovic, 2008). The aggregation of microtubule associated protein tau causes another distinct group of disorders called taupathies. Progressive supranuclear palsy (PSP) and Corticobasal ganglionic degeneration (CBD) are two taupathies that exhibit Parkinsonism. PSP is characterized by postural instability, early falls, early cognitive dysfunction and abnormalities of vertical gaze while CBD is characterized by asymmetric Parkinsonism, cortical signs (ataxia, cortical sensory loss, alien limb) and possibly dystonia and myoclonus (Stamelou et al., 2013; Wenning et al., 2011). Fronto-temporal dementias, a group of disorders characterized by profound cognitive manifestations and Alzheimer's disease are also often associated with Parkinsonism (Jankovic, 2008). All these disease entities, other than PD, are termed as "Atypical Parkinsonian Disorders" due to the prevalence of additional clinical features aside from the movement disorder (Wenning et al., 2011).

Parkinsonism is also found to occur in patients on long term anti-psychotic treatment. Exposure to toxins, particularly pesticides such as MPTP, paraquat and rotenone; certain infections such as HIV and influenza; metabolic disorders such as Wilson's disease and Neurodegeneration with brain iron accumulation and cerebrovascular diseases involving ischemia related cell loss and gliosis are other leading causes of Parkinsonism and are often termed as "Secondary Parkinsonism" (Jankovic, 2008; Wenning et al., 2011).

Although PD, a major cause of Parkinsonism, typically presents with motor dysfunction, non-motor manifestations also form a significant component of the disease. It is now believed that non-motor symptoms such as constipation, anxiety disorder, Rapid eye movement (REM) sleep

disorder, olfactory dysfunction and depression can be present anywhere between 5-20 years before the onset of motor symptoms in what is now considered to be a "pre-clinical" phase. Likewise, as the disease progresses, frequent motor freezing and falls, treatment-induced dyskinesias (involuntary movement), autonomic dysfunction such as urinary incontinence and orthostatic intolerance, dementia and hallucinations become prominent in the later stages of the disease (Jankovic, 2008; Massano and Bhatia, 2012; Savica et al., 2010). Consistent with the occurrence of the non-motor symptoms, the PD pathology has been found to occur in other regions of the brain other than the basal ganglia. A staging system called the "Braak staging" has been proposed based on the pathology and traces the evolution of the disease from its preclinical stage to advanced stages of the disease (Braak et al., 2003). In the Braak staging, aSyn pathology first occurs in the lower brainstem and involves the medulla oblongata (Stage 1 and 2) before the onset of any clinical symptoms of motor dysfunction. Stages 3 and 4 represent the spread of the aSyn pathology to the SNpc and the onset of clinical symptoms while in stages 5 and 6 aSyn pathology spreads to the cortex representing advanced stages of the disease with symptoms of cognitive decline (Fig.5) (Braak et al., 2003; Burke et al., 2008).

However, the Braak staging is not universal. Lewy pathology has been observed in the aged in several regions of the brain including the SNpc without any neurological impairment and is called "incidental Lewy body disease". Similarly, the Braak staging does not always correlate with the severity of the disease. Stages 4-6 can occur even in absence of any clinical symptoms while some patients suffer from severe PD even during stages 2-4. There can also occur a complete absence of Lewy pathology in some cases with a clear clinical diagnosis of PD (Burke et al., 2008). Therefore, although, a large number of clinical cases conform to the Braak staging, several notable exceptions exist. This can be attributed to the wide heterogeneity in PD onset and

progression often making the diagnosis of the disease complicated.

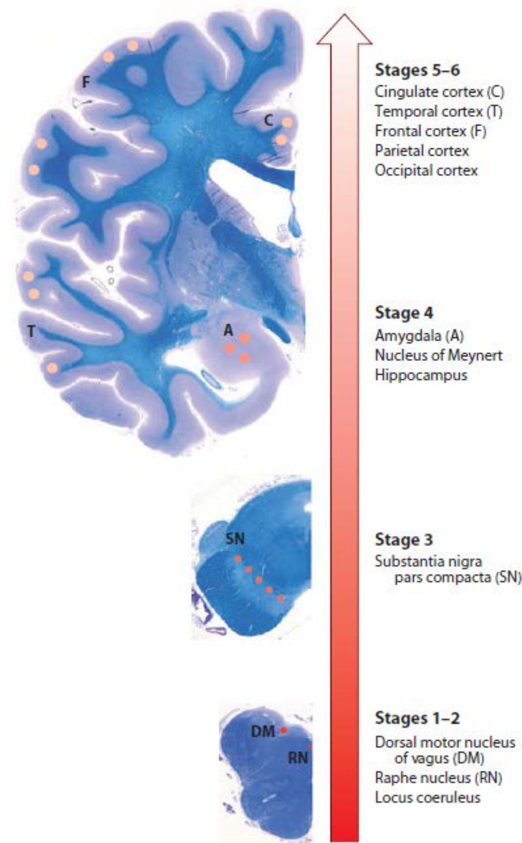


Figure 5: Schematic representation of the Braak staging showing the spread of pathology (Jankovic, 2008)

GENETICS OF PD

The identification of mutations as a causative factor has not only replaced the long prevailing belief that PD is sporadic and largely triggered by environmental factors but has also substantially contributed towards progress in understanding the molecular pathogenesis of the disease. Linkage analysis and association studies have been successfully employed in identifying mutations as well as susceptibility loci which in turn has enabled to pin-point probable candidate pathways responsible for pathogenesis in both the sporadic and familial forms due to the large

overlap in both disease forms. A total of 18 loci have been identified of which 6 have been conclusively found to cause familial Parkinsonism.

- PARK1/PARK 4 (*SNCA*): Autosomal dominant familial PD caused due to mutations in alpha-synuclein (Gene: *SNCA*; Protein: aSyn). *SNCA* is located on chromosome 4, consists of 6 exons and codes for a 140 amino acid protein. It is localized to the synaptic vesicle and is thought to play a role in vesicular trafficking of neurotransmitters. It is a natively unfolded protein capable of attaining several conformations depending on the molecular interactions in the cell and has an increased propensity for aggregation.

  Three missense mutations (A53T, E46K, and A30P) and duplications and triplications of *SNCA* are reported (Bekris et al., 2010). The E46K and triplication mutations are associated with young onset Parkinsonism with rapid progression and occurrence of dementia and presence of diffuse Lewy bodies. The A30P and duplication mutations are less commonly associated with dementia. Familial PD with duplication mutations closely resembles sporadic PD with late onset and slow progression. A53T mutation is associated with dementia and cortical Lewy bodies.

  In addition to disease causing mutations, a repeat polymorphism 10kb upstream of the translational start site and several single nucleotide polymorphisms (SNP) in 3' end of the gene have been identified as risk factors for PD (Maraganore et al., 2006; Myhre et al., 2008). The mechanism by which these SNP's mediate susceptibility is unknown (Bekris et al., 2010; Gasser, 2009; Gasser et al., 2011; Martin et al., 2011; Shulman et al., 2011).

- PARK 2 (*parkin*): Autosomal recessive PD caused due to mutations in parkin (Gene: *parkin*; Protein: parkin). (Bekris et al., 2010) *Parkin* is located on chromosome 6, contains 12 exons and codes for a 465 amino acid protein. Parkin is an E3 ubiquitin

ligase that has a ring-between-ring domain which enables it to interact with ubiquitin conjugating enzymes and catalyses the attachment of ubiquitin to protein targets thus routing them for proteasomal degradation. Although it is predominantly cytosolic, it localizes to synaptic vesicles, Golgi vesicles and mitochondrial outer membrane. A rare glycosylated form of aSyn is proposed to be a substrate for parkin, among several others (Shimura et al., 2001).

More than 100 mutations have been reported for *parkin* and include missense, nonsense, deletions, duplications and gene rearrangements. These mutations alter its localization and solubility and primarily result in loss-of-function. Deletion of exon3, exon 4, exon 3 to 4, point mutation in exon 7 and single base pair deletion in exon 2 are the most commonly seen mutations (Bekris et al., 2010). PARK-2 related parkinsonism is associated with young onset PD with age of onset between childhood and 40 years of age and about 50% of all juvenile onset PD is a result of *parkin* mutation. Dystonia is a common feature. There is loss of neurons and gliosis in substantia nigra. However Lewy bodies are absent except in very rare cases (Gasser, 2009; Gasser et al., 2011; Martin et al., 2011; Mizuno et al., 2008).

- <u>PARK 6</u> (*PINK1*): Autosomal recessive PD caused due to mutations in *PINK1* (Gene: *PINK1*; protein: PINK1) (Bekris et al., 2010). *PINK1* is located on chromosome 1, contains 8 exons and encodes for a 51 amino acid protein PINK1. It is localized to the matrix and intermembrane space of the mitochondria and contains a mitochondrial targeting motif and a serine/threonine protein kinase domain but its function remains unknown. However studies suggest it may play a role in maintaining mitochondrial

function and that its possible interaction with parkin may play a regulatory role in mitochondrial fission/fusion in the cell (Yu et al., 2011).

G309D missense mutation and W437X truncation mutation were the first to be identified following which several point mutations, frameshift mutations and truncating mutations have been identified. However a majority of these mutations are missense or nonsense mutations. A large majority of these point mutations are located in the highly conserved protein kinase domain thereby resulting in loss of kinase activity. PARK 6 related parkinsonism results in young onset PD with age of onset in third to fifth decade of life. However it resembles late onset PD with slow progression but with instances of dementia in some cases (Gasser et al., 2011; Mizuno et al., 2008).

It is to be noted that although both PARK 2 and PARK 6 result in autosomal recessive PD which requires homozygosity, the heterozygous condition may prove a risk factor but large studies are still required to definitively prove that they act as risk factors (Mizuno et al., 2008).

- PARK 7 (*DJ-1*): Autosomal recessive PD caused due to mutations in *DJ-1* (Gene: *DJ-1*; protein DJ-1) (Bekris et al., 2010). *DJ-1* is located on chromosome 1, contains 7 exons and codes for a 189 amino acid protein. DJ-1 is a cytoplasmic protein that can also translocate to the mitochondria. It appears to play a role in combatting oxidative stress. It is a homodimer and contains a cysteine residue at position 106 which upon oxidation forma disulphide bond. DJ-1 is thought to act as a redox sensor protein and plays a protective role during oxidative stress (Taira et al., 2004). It is also thought to play a preventive role in aSyn aggregation (Batelli et al., 2008). *DJ-I* knockout mice show increased sensitivity to oxidative stress and 1-methyl-4-phenyl-1,2,3,6-tetrahyropyrindine

(MPTP), a mitochondrial toxin. DJ-1 is also up regulated during paraqaut-induced oxidative stress.

Homozygous and compound heterozygous mutations have been reported in *DJ-1* with L166P, E64D, M26I, A104T and D149A being some examples. These mutations primarily result in destabilization of the protein, inhibiting its dimerization and loss of anti-oxidant activity. PARK 7 related PD causes young onset PD with age of onset between 20-40 years and closely resembles PARK 2 related PD. Alongside parkinsonism, some atypical features such as psychiatric symptoms, short stature and brachydactyly may also be seen (Gasser et al., 2011; Mizuno et al., 2008).

- PARK 8 (*LRRK2*): Autosomal dominant PD caused due to mutations in *LRRK2* (Gene: *LRRK2*; protein: LRRK2). *LRRK2* is located on chromosome 12, contains 51 exons and codes for a 2517 amino acid protein. LRRK2 contains several functional domains which includes ANK (ankyrin repeat domain), LRR (leucine rich repeat), ROC (Ras of complex proteins), COR (carboxy terminal of ROC), MAPKKK (mitogen-activated protein kinase kinase kinase) and WD40 domain (rich in tryptophan and aspartate residues). LRRK2 is found to be co-localized to various organelles in the cell including mitochondria, lysosomes, golgi complex, plasma membrane, synaptic vesicles and lipid rafts (Bekris et al., 2010). The function of LRRK2 is not very well known. The ROC domain can bind GTP although it does not seem to possess GTPase activity and maybe responsible for the kinase activity of the MAPKKK domain (Ito et al., 2007). The other domains are thought to play a role in protein-protein interactions and LRRK2 appears to interact with parkin (Smith et al., 2005a) and aSyn and is suspected to phosphorylate aSyn (Liu et al., 2012) .

More than 40 missense and nonsense mutations have been reported for *LRRK2* of which 6 (R1441C, R1441G, R1441H, Y1699C, G2019S and I2020T) have been conclusively shown to be disease-causing. Among these 6, the G2019S mutation is most common. Some of these mutants are thought to mediate toxicity by enhancing the kinase activity. The PARK 8 related PD have features similar to sporadic PD but with a slightly earlier age of onset along with the occurrence of dementia in some rare cases. The pathology appears to be quite varied and may include brain-stem type Lewy body, diffuse type Lewy body, tau inclusions and just nigral atrophy without inclusions, all of which were reported from within a single family. Additionally, two polymorphisms (G2385R and R1628P) have been shown as risk factors for PD in Asian population although similar risk susceptibility in other populations is yet to be demonstrated (Gasser et al., 2011; Martin et al., 2011; Mizuno et al., 2008; Shulman et al., 2011).

- PARK 9 (*ATP13A2*): Autosomal recessive PD caused due to mutations in *ATP13A2* (Gene: *ATP13A2*; Protein: ATP13A2). Located on chromosome 1, *ATP13A2* contains 29 exons and codes for a 1180 amino acid protein with 10 transmembrane domains. ATP13A2 is a lysosomal protein with ATPase domain. It is present in very high levels in brain particularly in ventral midbrain. Its exact function is unknown but belongs to the P5 subfamily of ATPase that transports inorganic cations and other substrates. Homozygous and compound heterozygous mutations have been reported in a Jordinian family and a Chilean family. One missense mutation (G504R) from Brazil and two missense mutations (T12M and G533R) from Italy have also been reported. PARK 9 related PD is also called Kufor-Rakeb syndrome after the Jordinian family initially diagnosed with this disease. It shows very early onset (11-16 years) with several atypical signs including pyramidal

signs, dementia and supra-nuclear gaze palsy. Magnetic resonance imaging (MRI) showed significant atrophy of globus pallidus and the pyramids and generalized brain atrophy in later stages. Mini-myoclonus, dystonia spasm and visual hallucination were also seen in some cases (Bekris et al., 2010; Gasser et al., 2011; Mizuno et al., 2008).

In addition to the six loci listed above, several other loci have been identified for which either the gene has to be identified or needs further validation.

PARK 3, mapped to chromosome 2, is thought to cause autosomal dominant PD with clinical features similar to sporadic PD but with age of onset between 36 and 89 years. PARK 10, mapped to chromosome 1, is also similar to sporadic PD with mean age of onset at 65.8 years. PARK 12 has been mapped to X chromosome but nothing is known about the clinical presentation. The genes for PARK 3, 10 and 12 are yet to be identified (Mizuno et al., 2008).

PARK 5 is mapped to chromosome 4 and the disease gene identified as *UCHL-1* which codes for an enzyme that cleaves carboxy terminal peptide bond of polyubiquitin chains and plays a role in proteasomal degradation. The mutation Ile93Met was reported in two individuals from a single family only and is thought to reduce the catalytic activity of the enzyme by half (Gasser, 2009; Mizuno et al., 2008; Setsuie and Wada, 2007). PARK 11 is mapped to chromosome 2 and the disease gene has been identified as *GIGYF2.* It is believed to play a role in insulin signalling. Although the clinical features are found similar to sporadic PD with mean age of onset at 58 years, the pathogenic role of this locus needs validation and is as yet uncertain (Lautier et al., 2008; Mizuno et al., 2008).

PARK 13 is mapped to chromosome 2 and the disease gene identified as *Omi/Htra2* which codes for a 458 amino acid protein that is localized to the intermembrane space of the mitochondria with a serine protease domain that binds to apoptosis inhibiting protein upon release into the

cytoplasm. G399S mutation and A141S polymorphism that was associated with PD were identified and both mutations are thought to result in defective activation of the protease activity (Strauss et al., 2005; Vande Walle et al., 2008).

PLA2G6 is located on chromosome 22 and codes for phospholipase 1. Mutations in *PLA2G6,* assigned as PARK 14, have been identified in two childhood-onset disorders, infantile neuroaxonal dystrophy and neurodegeneration with brain iron accumulation. These disorders share the pathological hallmark of aSyn-positive Lewy bodies with PD (Gasser, 2009; Gasser et al., 2011; Lesage and Brice, 2009). PARK 15 is also associated with recessive form of PD and the disease gene identified as *FBOX7* which belongs to F-box containing protein (FBP) family. Mutations in this gene have been found in members of two families with early onset progressive parkinsonism with pyramidal tract signs, clinically referred to as pallidopyramidal syndrome (Gasser et al., 2011; Lesage and Brice, 2009).

Recent genome wide association studies (GWAS) have identified three susceptibility loci. PARK 16 is mapped to chromosome 1 and includes several candidate genes such as RAB7L1 which is a small GTP-binding protein that regulates exo-endocytotic pathway and NUCKS1, a nuclear DNA binding protein expressed in brain and other tissues that might regulate chromatin structure and activity. An association between multiple SNP's in this region and PD was reported in participants of Japanese ancestry. PARK 17 has been mapped to chromosome 4 which again contains several candidate genes including *GAK* (cyclin G associated kinase) which plays a role in regulating cell cycle and is differentially expressed in substantia nigra of PD brains compared to controls. The HLA region has been assigned as PARK 18 and is consistent with the role inflammation and immune system are believed to play in disease pathogenesis. These loci must be replicated is several populations for their validation (Bekris et al., 2010).

In addition to the above listed susceptibility loci, *SNCA* and *LRRK2* have been identified as genetic risk factors for PD as discussed earlier. Two other genes, *GBA* and *MAPT* have also been designated as genetic risk factors but have not been assigned a PARK locus (Bekris et al., 2010; Gasser et al., 2011).

MOLECULAR PATHOGENESIS

The identification of monogenic forms of PD has shed light on the probable pathogenic pathways involved in disease onset and progression. The considerable variation in symptoms and pathology suggest that the trigger may differ in each case but may converge on a central mechanism that then causes dopaminergic neuron loss and parkinsonism or may operate separately and yet still result in parkinsonism. However the pathways that have since emerged through genetic studies may all be linked to present a unified model of disease pathogenesis as represented in (Fig.6).

The aggregation of aSyn appears central to disease pathogenesis as is evident from the figure above. The point mutations increase the propensity of the protein to aggregate. The duplication and triplication mutations increase the gene dosage which in turn increases the cytosolic concentrations of the protein which again promote aggregation. Mutations in *MAPT* and *LRRK2* are thought to promote aSyn aggregation although the mechanisms involved in these interactions are yet to be elucidated (Martin et al., 2011; Shulman et al., 2011).

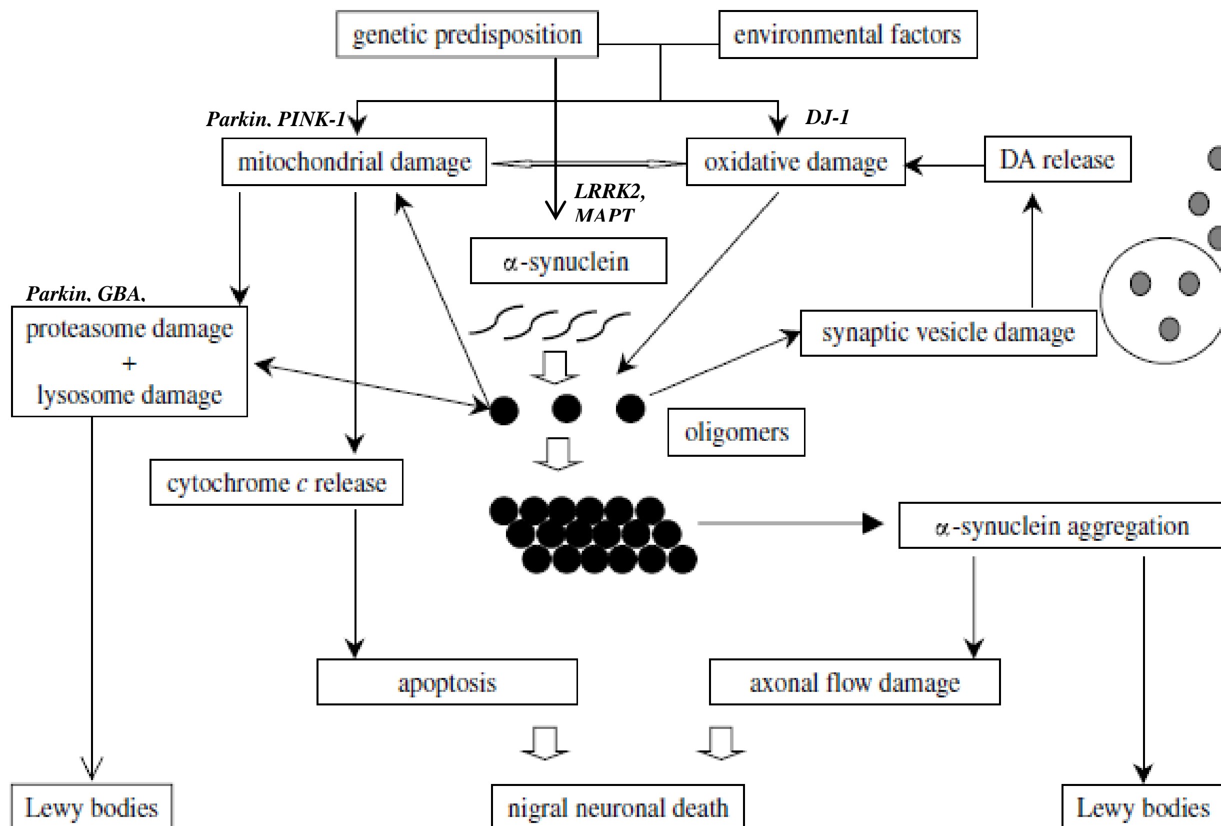


Figure 6: Schematic representing the probable pathogenic mechanism for Lewy body formation and neuronal loss in PD. The genes involved in the various pathways have been indicated. (Martin et al., 2011)

Increased kinase activity of LRRK2 as a result of mutations is thought to disrupt phosphorylation of its substrates in turn affecting cellular pathways. However while several candidates have been proposed as LRRK2 substrates, further confirmation is awaited. Mutations in *parkin* suggest impairment of ubiquitin proteasomal pathway particularly since ubiquitin positive staining of Lewy bodies is observed. Similarly mutations in *GBA* and *ATP13A2,* signify the role of impaired lysosomal pathway in the accumulation and aggregation of proteins (Gasser, 2009; Gasser et al., 2011). Recent evidence suggest that parkin and PINK1 might act together to maintain mitochondrial integrity in the cell and that mutations in these genes cause mitochondrial

dysfunction (Geisler et al., 2010; Matsuda et al., 2010). Mitochondrial dysfunction often leads to oxidative stress in the cell. Additionally, the metabolism of dopamine which is a catecholamine generates reactive oxygen species which again causes oxidative stress in the cell and it is believed to be the reason for increased susceptibility of dopaminergic neurons to cell death (Segura-Aguilar et al., 2014). Mutations in *DJ-1* reduce its anti-oxidant property and contribute to increased sensitivity to stress (Takahashi-Niki et al., 2004). Mitochondrial damage, oxidative stress and protein degradation failure all act as triggers to aSyn aggregation. The aggregates cause synaptic vesicle damage and increase in cellular dopamine content which in turn causes oxidative stress. The oxidative stress in turn causes mitochondrial dysfunction. The aSyn aggregates can also disrupt mitochondrial membrane again leading to its dysfunction and also saturate and impair the degradation pathways resulting in accumulation of proteins and the formation of Lewy bodies. Although the primary trigger for pathogenesis is as yet unknown especially in absence of causative mutations, it appears that aSyn aggregation is both cause and consequence of disease pathogenesis and the resulting cellular damage activate cell death pathways that result in neuronal loss (Martin et al., 2011; Shulman et al., 2011).

MODELS IN PD

Although genetic studies have been invaluable in advancing our understanding with regard to pathogenic pathways, mechanistic insights have been gained through studies carried out in animal models of PD. Several different models have been developed to study PD pathogenesis since no single model can emulate the clinical scenario precisely and the considerable heterogeneity in the disease further necessitate the development of alternate models. A brief summary of the models used till date is presented here.

- Toxin Models:

a) Neurotoxin 6-hydroxy dopamine (6-OHDA) is structurally similar to dopamine (DA) and norepinephrine (NE) and causes loss of DA and NE neurons (Breese and Traylor, 1971). It does not cross the blood brain barrier and is hence injected into the brain. Once inside the cells, it undergoes oxidation to produce the toxic hydrogen peroxide and paraquinone. It can be administered either to the ventral midbrain or medial forebrain bundle (MFB). When injected into the ventral midbrain it caused loss of substantia nigra DA neurons within a few hours (Jeon et al., 1995). However dose dependent injection of 6-OHDA into MFB can recapitulate both late-stage (during increased dose) and early stage (during reduced dose) disease conditions and the lesions formed after 6-OHDA are unilateral in nature (Schallert et al., 2000). The injection of 6-OHDA in the striatum causes a more progressive loss of DA nigrostriatal pathway compared to its delivery to other brain regions and is dose dependent. This toxin does not produce extra-nigral pathology and no Lewy body inclusions are seen (Dauer and Przedborski, 2003) .

b) The identification of individuals who developed PD-like symptoms following abuse of intravenous drugs contaminated with methyl-phenyl-tetrahydropyridine (MPTP) led to its wide usage in toxin-based models (Fornai et al., 1997). The metabolite MPP+ is a Complex I inhibitor and is a trigger for mitochondrial dysfunction. Intravenous and sub cutaneous injections are the preferred modes of administration. Rats are not susceptible to the toxin and effect in mouse models vary depending on the dosage (Meredith and Rademacher, 2011). Acute models involve injecting the toxin 4 times daily at 2 hour intervals. Subacute models involve one injection on a

daily basis while the chronic model involves injections over a period of 5 weeks co-administered with an adjuvant probecenid to prevent renal clearance of the toxin. A single injection/day results in 20%-30% DA neuron loss, 2 injections/day results in 35% DA loss while 4 injections/day result in 50% DA loss. The subacute regimen over 8 days results in 24% DA neurons loss. The chronic model on the other hand results in rapid but progressive neuron loss compared to other MPTP models (Petroske et al., 2001). On the whole, MPTP models have been dissatisfactory in that they result in rapid death of neurons with little progressive loss. Recovery of DA neurons is also found to occur in subacute models and often there is poor correlation between motor deficits, DA neurons loss, loss of striatal dopamine and MPTP dose (Wallace et al., 1984).

The MPP+ metabolite models have also been developed and being a substrate for DA receptors is selective to DA neurons. MPP+ does not cross the blood-brain barrier and is therefore injected unilaterally on one side which produces a dose-dependent unilateral loss of neurons. At low doses of MPP+ 37% and 53% loss of striatal DA is seen while 90% loss in striatal DA is observed at high doses which also causes a significant reduction in serotonin levels and a progressive loss in neurons is also observed. There is only a non-significant loss of neurons on the contralateral side 42 days post injection but this needs further investigation. The loss of neurons on the ipsilateral side with a slow loss in the contralateral side will be a close mimic of the human condition where the disease onset is unilateral. Microglial activation in the striatum and SN and aSyn and ubiquitin positive inclusion bodies in the striatum is observed. However no inclusion bodies are seen in SN. Ultrastructural examination

reveals dense swollen mitochondria which is similar to the observations from PD brains. The motor deficits correlating with DA loss have not been evaluated in this model (Meredith et al., 2008; Yazdani et al., 2006).

c) Epidemiological studies suggest pesticides maybe potential triggers for PD. Rotenone, a complex I inhibitor is a naturally occurring pesticide that has been used generate chronic models of PD. Rates are injected with rotenone either intravenously or subcutaneously for upto 5 weeks and it readily crosses cell membrane and blood brain barrier. It causes loss of striatal DA, progressive loss of SN DA neurons and presence of cytoplasmic inclusions positive for aSyn and ubiquitin and also increased oxidative damage, microglia activation and iron deposition. The rats also display motor deficits and therefore the rotenone model emulated PD more closely than other toxin models. However while DA neurons are selectively lost with rotenone exposure, other unrelated brain regions may also be susceptibility resulting in wide variability. The intraperitoneal delivery of rotenone can overcome some of the non-selective loss and is found to be a good model for the study of early and late stages of PD (Höglinger et al., 2003; Sherer et al., 2003).

   Paraquat (PQ) is a herbicide that can cross the blood brain barrier and disrupts mitochondrial function through the generation of reactive oxygen species (Przedborski and Ischiropoulos, 2005) . A small but significant loss of neurons along with increased aSyn aggregation has been reported (Brooks et al., 1999). Maneb, a fungicide that inhibits glutamate transport and disrupts DA uptake and release in co-administered with PQ. A combination of PQ and maneb at 1-2 injections/ week can cause a 50% loss in SN DA neurons but in older rats caused 75% loss at 2 weeks and

88% loss at 12 weeks. Older rats are more sensitive to this combination at the same dose when compared with the younger rats. Loss of DA neurons, motor impairment and microgliosis is found in both older and younger rats. The disadvantage of this model is that it causes a lethal systemic lung infection in older rats (Saint-Pierre et al., 2006; Thiruchelvam et al., 2000, 2003).

d) Inflammation has been recognized as another important mediator of cell death in neurons. Microglia are resident phagocytic cells which when activated undergo morphological changes and release pro-inflammatory molecules such as cytokines, chemokines, nitric oxide and ROS to clear cellular debris. While the play an important role in neuronal development and injury, unregulated activation of microglia can result in uncontrolled ROS production leading to oxidative stress in the cells. Microglia activated inflammation can be sustained for years and is progressive and this supported by observations where inflammation persists for years in humans and non-human primates exposed to MPTP (Gao et al., 2002). Lipopolysaccharide (LPS), an endotoxin, derived from the wall of gram-negative bacteria is a potent activator of microglia and is now used as an initiator for DA neurodegeneration. Acute intracerebral LPS, chronic intracerebral LPS, acute systemic LPS and intrauterine LPS models have been developed. In the acute intracerebral LPS model, LPS is injected into the cortex, hippocampus, striatum or SN of rats which enhances the death of SN DA neurons specifically. This model causes a rapid activation of microglia and subsequent loss of DA neurons observed within 4 days. However the microglia activation is short-lived and no progressive pathology is observed (Iravani et al., 2005). To overcome the short lived response the chronic models were

developed where in the rats are exposed to LPS for 2 weeks. This results in microglia activation within 3 days and oxidative stress persists for upto 8 weeks. Significant loss of DA neurons is observed only about 6 weeks post exposure and shows a progressive loss. However it remains to be seen if aSyn inclusion and motor deficits accompany the DA loss in this model (Gao et al., 2002). A single systemic injection of LPS in mouse model has recently been shown to elevate TNFα mRNA and protein levels within 1 hour and remains elevated for 10 months (Qin et al., 2007). Likewise microglia activation in several brain regions has been observed. A delayed but progress loss of DA neurons is also observed. This has not been correlated with other PD pathology or motor deficits as yet. These observations are in contrast to the lack of progressive DA neuron loss in rats given a single systemic injection of LPS. In utero exposure to LPS following a single injection into gravid female rats results in significant reduction of striatal DA and reduced SN DA cell number and suggests that prenatal infections are a risk factor for PD (Ling et al., 2002). Rats prenatally exposed to LPS when treated with rotenone showed a synergistic effect on DA cell loss suggesting that a pre-existing inflammation enhances the effect of environmental toxins (Ling et al., 2004).

- Genetic Models:

a) Genetically Engineered Mouse with mutations leading to Loss of dopaminergic neurons: *Pitx3* -/- mice have a spontaneous mutation in *Pitx3* which is a homeobox transcription factor. They are called aphakia mice and exhibit a change in the eye phenotype (blindness) and also show early nigrostriatal DA neuron loss post-natally (Hwang et al., 2005; van den Munckhof et al., 2003). The behaviour defects can be

reversed with levodopa. Mesolimbic DA neurons are resistant to *Pitx3* mutation similar to PD. The identification of *Pitx3* as a risk factor for PD has highlighted the relevance of this model (Fuchs et al., 2009). Apart from the DA neuron loss, the model lacks the progressive phenotype of the disease condition but is useful to study factors that promote DA neurons survival.

Knock-out models for *engrailed* which encodes a homeodomain containing transcription factor have also been generated in which *engrailed 1* is partially silenced (*engrailed 1*+/-) while *engrailed 2* is completely knocked out (*engrailed 2*-/-) to prevent compensation by the other functional gene. Double knock outs for *engrailed* are embryonic lethal. These models show DA neuron loss but also have a more widespread cerebellar pathology. However, specific DA neuron loss without the cerebellar pathology is achieved in models with one copy of *engrailed 1* but with intact *engrailed 2*. Although these models exhibit progressive neuron loss, it starts during late post-natal development unlike the late-onset seen in sporadic PD, the relevance of *engrailed* mutations in PD is not yet known (Le Pen et al., 2008; Sgadò et al., 2006).

b) Genetically engineered mice expressing familial PD mutant genes: Transgenes expressing both wild-type and mutant aSyn have been generated with the expression driven by different promoters. The TH promoter was used to drive the expression in catecholaminergic neurons and although these models showed loss of DA neurons, the broad aSyn pathology could not be achieved. Prion promoters which have been successfully used in generating models of amyotropic lateral sclerosis has also been used to drive aSyn expression but resulted in a motor neuron pathology that is

different from PD. Thy-1 and PDGFβ are the other promoters that have been used and the Thy-1 drives a stronger expression in substantia pars compacta compared to PDGFβ. In some cases, Thy-promoter also causes motor neuron pathology but not in others despite high transgene expression but these models show sensorimotor deficits with nigrostriatal dysfunction without DA loss and are hence not responsive to levodopa. Although these models do not manifest the symptoms of parkinsonism as seen in PD, they may represent early 'sub-clincal' stages of the disease. They also exhibit olfactory and autonomic dysfunction and proteinase-K resistant aSyn aggregates and are useful to study early stages of PD and to devise neuroprotective strategies. Transgenic models of aSyn, although able to achieve loss of striatal DA, have not been able to mimic the clinical scenario very well. Lower organisms with aSyn transgenes have also been developed including in yeast, nematodes and fruit flies. In fruit flies with transgenic aSyn, age dependent degeneration of dopaminergic neurons, locomotor dysfunction and inclusion body formation were observed.

Parkin, *PINK1* and *DJ1* models have also been generated. One particular *parkin* mutation (Q311X) causes DA loss in Drosophila in a dominant manner. A progressive phenotype with DA loss was also achieved with the mutation in mouse models. However other mutants such as deletion of exon 3 and exon 7 do not exhibit DA neuron loss. Exon 3 deletion mutants show oxidative stress and progressive sensorimotor dysfunction while the exon 7 deletion showed progressive NE neuron loss in the locus coeruleus while some showed no behavioural defects and others no-motor pathology. Flies with *parkin* and *PINK1* transgenes exhibit similar mitochondrial defects which are not observed in mice models.

DJ1 knock out models have also been developed and *DJ1* knock out results in increased sensitivity to oxidative stress in cells, flies and mice. Although these models reinforce the role of oxidative stress in pathogenesis, they show little phenotype and exhibit no DA cell loss. Transgenic models for *LRRK2* are still being developed (Dauer and Przedborski, 2003; Meredith et al., 2008; Shulman et al., 2011).

c) Viral delivery of genes: Rat models with adeno-virus transfected wild type or A53T mutation aSyn exhibit a 30%-50% loss of dopaminergic neurons in 8 weeks and a similar phenotype was achieved with adeno-virus transfected A30P mutant. Lentiviral expression of aSyn in rats, mice and non-human primates also resulted in significant loss of dopaminergic neurons alongwith the presence of aSyn aggregates and neuronal cell bodies and neurites. However these models do not produce the extra-nigral pathology or the spread of pathology to the other parts of the nervous system (Kirik et al., 2002; Lo Bianco et al., 2002; Ulusoy et al., 2008).

THERAPEUTICS

Motor dysfunction as a result of reduced dopamine is the predominant feature of PD and hence treatment strategies involving replacement of dopamine has been the gold standard to treat the motor symptoms in PD. L-Dopa which is a precursor for dopamine is often administered because of the inability of dopamine to cross the blood brain barrier. L-dopa is often administered along with carbidopa which is a peripheral dopa decarboxylase to enhance the beneficial effects of L-Dopa. However prolonged treatment with L-Dopa result in involuntary movements called dyskinesias and motor fluctuations. Slow release preparations of L-Dopa

such as Sinement CR have been used to smooth out motor fluctuations in L-Dopa therapy. Continuous Dopaminergic Stimulation (CDS) was proposed as a strategy to minimize motor fluctuations and included duodenal infusions of L-Dopa, oral formulations of L-Dopa, dispersible form of L-Dopa, subcutaneous and intramuscular injections of L-Dopa ethyl ester and intravenous administration of L-Dopa methyl ester were proposed but have not translated into clinical reality. Catecholmethytransferases (COMT) are enzymes that degrade catecholamines such as dopamine and inhibition of these enzymes by using COMT inhibitors is another strategy used to prolong the effects of L-Dopa. Entacapone and Tolcapone are examples of COMT inhibitors that are used in the treatment of PD. Dopamine agonists often precede L-Dopa treatment and provide modest symptomatic relief and the L-Dopa treatment can be deferred by several months or years following treatment with dopamine agonists. Bromocriptine, pergolide, pramipexole, ropinirole, apomorphine are some examples of dopamine agonists. Non-dopaminergic therapy such as anticholinergics is also used to provide symptomatic relief. Amantadine, NMDA receptor blocker, is administered along with L-Dopa to prevent Dopa induced dyskinesias. Early interventions as neuroprotective strategies have also been devised. Antioxidative therapy is one such option. The Deprenyl and Tocopherol Antioxidative Therapy of Parkinsonism (DATATOP) trial was a large clinical trial carried out to evaluate the antioxidative effects of deprenyl (selegeline) and alpha- tocopherol (Vitamn E) and while the results showed some delay in the need for L-Dopa, it has not been resolved if these effects were symptomatic or protective. Rasagiline, a monoamine oxidase inhibitor has also been used and found effective as an adjunct therapy to L-Dopa and is found to have neuroprotective properties in vitro. The use of trophic factors such as neurturin (NTN) and glial cell derived neurotrophic factor (GDNF) have been explored with a view to enhance survival of

midbrain dopaminergic neurons and to prevent its degeneration. The non-motor symptoms in PD become predominant over time necessitating treatments aimed at improving cognitive decline, drug induced psychosis and depression. Surgical procedures such as thalamotomy, pallidotomy and deep brain stimulation (DBS) are other alternatives although DBS has largely replaced the ablative procedures.

All current treatment modalities for PD provide relief from symptoms but do not prevent disease progression. Although cell based therapies to replace the lost dopaminergic neurons have been investigated, they have only met with limited success. Devising neuroprotective strategies is central to PD, given the increasing disease incidence. This requires the development of better animal models, identification of biomarkers to monitor disease onset and progression and to identify subjects at risk for PD. (Jankovic and Aguilar, 2008; Rascol et al., 2011)

2.2 ALPHA SYNUCLEIN (aSyn)

The protein aSyn was initially described in the *Torpedo californica* as a synaptic protein also localized to the nucleus (Kaplan et al., 2003). The sub-cellular localization of the protein to synaptic vesicles and nucleus led to it being called synuclein. Subsequently it was identified as playing a role in song learning in zebra finch and later as the non-amyloid component (NACP) of the amyloid plaques found in Alzheimer's disease (Vekrellis et al., 2004). However the identification of *SNCA* mutation A53T as a cause of autosomal dominant Parkinson's disease (Polymeropoulos et al., 1997) and the presence of the protein in Lewy bodies (Spillantini et al., 1997) generated great interest in the role of aSyn in disease pathogenesis. Today it is known that aSyn, though conclusively linked to PD pathogenesis, is not exclusively associated with PD and

is a component of neuronal and glial cytoplasmic inclusion bodies that are seen in several other neurodegenerative diseases such as Dementia with Lewy Body (DLB), Lewy Body variant of Alzheimer's disease, Mutiple system Atrophy (MSA), etc. all of which are collectively called 'synucleinopathies'.

STRUCTURE AND LOCALIZATION

Alpha synuclein belongs to the family of synucleins that also include beta and gamma synuclein. Alpha synuclein, encoded by gene *SNCA* is mapped to chromosome $4^{q21.2\text{-}22}$, beta-synuclein, encoded by *SNCB* is mapped to chromosome 5^{q35} and gamma-synuclein, encoded by *SNCG* is mapped to chromosome $10^{q23.2\text{-}23.3}$. *SNCA* contains 7 exons of which 5 are coding while *SNCB* contains 6 exons of which 5 are coding while *SNCG* contains 5 exons all of which are coding (George, 2002). The synucleins are present only in the vertebrates and no homologues have been identified in *D.melanogaster* or *C.elegans* (Alves da Costa, 2003). Beta synuclein shares 78% homology with aSyn while the gamma synuclein shares a 60% homology with aSyn. Alpha-synuclein and beta synuclein are predominantly expressed in the brain particularly the neocortex, hippocampus, striatum, thalamus and cerebellum but beta synuclein is more uniformly distributed across the brain compared to aSyn. Gamma synuclein is predominantly expressed in the peripheral nervous system. Beta synuclein is also present in the sertoli cells and gamma synuclein in metastatic tumors and epidermis (Alves da Costa, 2003; George, 2002; Uversky, 2008). Although aSyn was initially thought to be expressed only in the brain, recent reports have demonstrated its presence in other tissues such as platelets, red blood cells, T-lymphocytes and melanomas (Barbour et al., 2008; Matsuo and Kamitani, 2010; Shameli et al., 2015; Uversky, 2008). The synaptic localization of aSyn has been conclusively demonstrated (Iwai et al., 1995;

Jakes et al., 1994) but the initial observation with regard to its nuclear localization (Maroteaux et al., 1988) has remained controversial with studies both against and in favour of its nuclear localization (Irizarry et al., 1996; Vivacqua et al., 2011).

Human aSyn has 140 amino acids while beta synuclein has 134 amino acids and gamma synuclein, 127 amino acids. Additionally two other isoforms of aSyn generated through alternate splicing resulting in deletions of either exon 3 or exon 5 which results in a 126 amino acid protein or a 112 amino acid protein respectively, have also been reported (George, 2002; Uversky, 2007, 2008). The human aSyn can be divided into three distinct regions:

- The N-terminal region composed of residues 1 to 60. It contains four repeats of 11 amino acids with a conserved hexameric motif (KTKEGV). This region can form amphipathic alpha-helices and is similar to lipid binding domain of apolipoproteins.
- The central region is composed of residues 61 to 95 and is the aggregation prone region NAC which is hydrophobic. It contains additional KTKEGV repeats. An 11 amino acid residue of the NAC region is absent in beta synuclein.
- The remaining 96-149 amino acid residues form the C-terminal which is rich in acidic and proline residues. Three conserved tyrosine residues in the C-terminal are signatures of aSyn and beta synuclein but are absent in gamma synuclein (Fig.7) (George, 2002; Uversky, 2007, 2008).

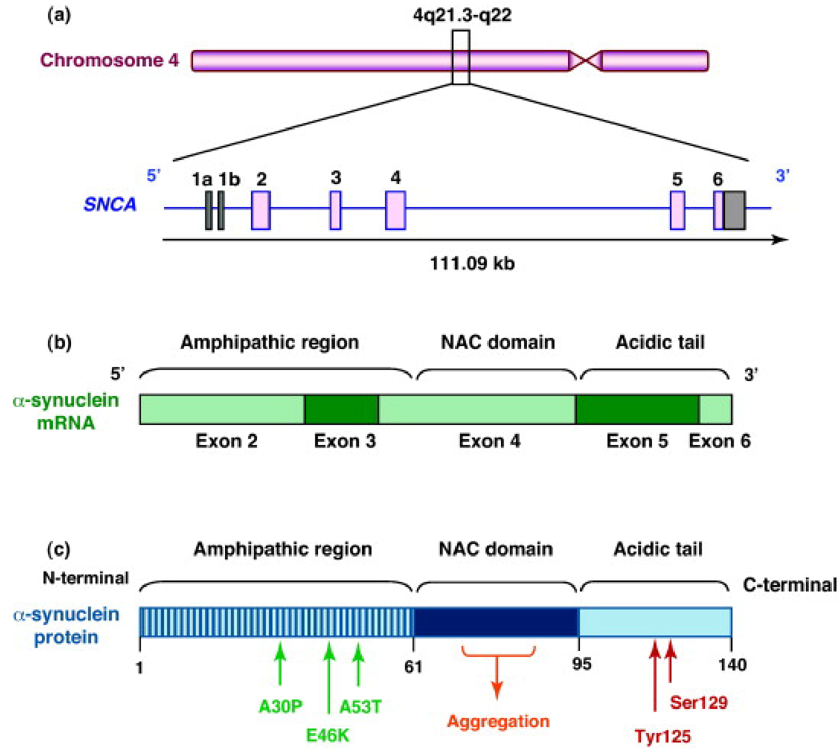


Figure 7: Scheme showing alpha-synuclein (a) Gene structure (b) coding exons (c) Protein structure with the sites of mutations (Venda et al., 2010).

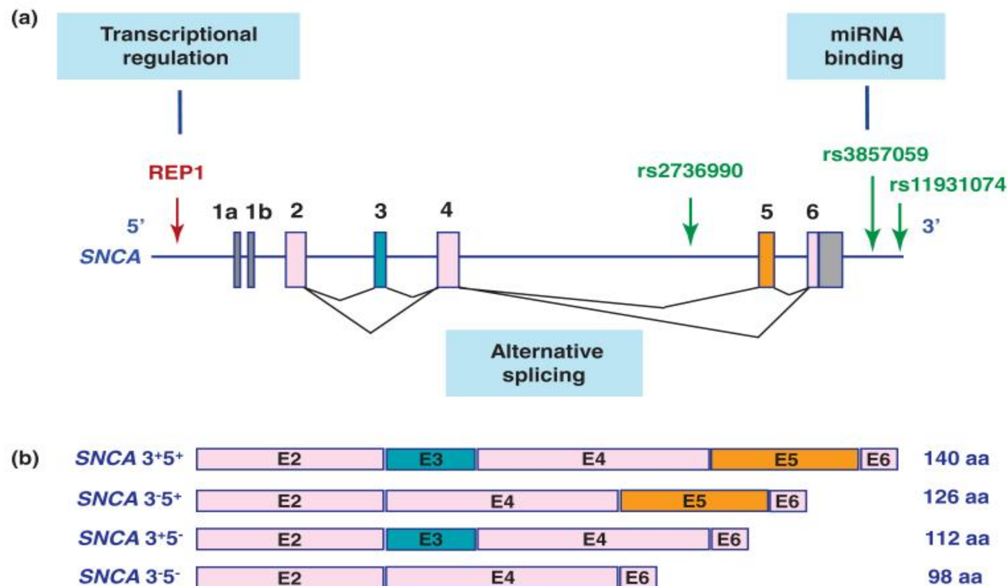


Figure 8: Scheme showing (a) sites of aSyn polymorphism as risk factors for PD (b) Isoforms of aSyn generated through alternate splicing (Venda et al., 2010).

Posttranslational Modification:

There has been a long held observation that monomeric aSyn is natively unfolded with no defined secondary structure and belongs to the class of 'Intrinsically Disordered Proteins'. Indeed it is this property of aSyn that has been attributed to its ability to aggregate. Being natively unfolded, the protein can attain various conformations depending on the interacting partners in the cell, for example the repeats in the N-terminal of the protein can form 5 alpha helices of which helices 1-4 interact with lipid membranes while helix 5 interacts with other proteins. The C-terminal of the protein, however, remains unstructured even in presence of membranes (Beyer, 2006; Uversky, 2008). Studies also suggest that native aSyn isolated from mammalian cells and RBC's is in fact a stable alpha-helical tetramer resistant to aggregation and not unfolded as believed till date (Bartels et al., 2011; Wang et al., 2011). However other studies have not been able to replicate these results and continue to suggest that aSyn monomer is natively unfolded (Fauvet et al., 2012; Lashuel et al., 2013).

An important factor that determines protein folding and functionality is the posttranslational modifications. More than 300 types of posttranslational modifications have been reported. Several such modifications have also been reported for aSyn (Fig.8).

- Phosphorylation: Phosphorylation is the most common protein modification and phosphorylated species of aSyn are found to be present in LB's suggesting that phosphorylation maybe a pathogenic event (Chen and Feany, 2005). Serine129 and Serine87 are the most common phosphorylation sites and aSyn is constitutively phosphorylated at Serine129 (Okochi et al., 2000). Several kinases have been identified as playing role in phosphorylation. These include casein kinase (CK) 1 and 2, G-protein bound receptor kinases, calmodulin-dependent kinase and polo like kinase (PLK). While

all of these kinases have the potential to phosphorylate Serine129, CK1 and tyrosine regulated kinase 1 are the only ones that phosphorylate Serine87. The phosphorylation of aSyn is thought to prevent rather than promote LB formation and several reports indicated that phosphorylation at Serine129 may occur after LB formation. Recent studies however suggest that phosphorylation at Serine129 is important for membrane binding and aSyn bound to membranes become dephosphorylated. Although the phosphorylation status of aSyn does not affect its membrane binding, the physiological relevance of aSyn phosphorylation is unknown and it appears that factors that promote LB formation also promote phosphorylation. It remains to be verified as to when Serine87 becomes phosphorylated. Although Serine residues are most commonly phosphorylated, recent studies show that Tyrosine residues at position 125, 133 and 136 may also be phosphorylated. The phosphorylation at Tyrosine125 appears to be reduced during the aging process in humans and the cortical tissues from patients with DLB also showed decreased phosphorylation at Tyrosine125 indicating it may have a neuroprotective role (Fig.9) (Beyer, 2006; Beyer and Ariza, 2013).

- Nitration and Oxidation: Nitration and oxidation of aSyn are proposed to be factors for increased aggregation of aSyn through cross-linking oligomers which result in SDS-resistant dimers and oligomers. The exposure of aSyn to nitrating agents results in the formation of dityrosine cross-links. Four tyrosine residues at positions 39,125,133 and 136 are found located in the protein (Fig.9) (Zhou and Freed, 2004). The presence of even one tyrosine is sufficient for aSyn cross-linking whereas substitution of Tyrosine with phenylalanine prevents the nitration of aSyn. Nitrated species of aSyn have been found in LB's. Nitration of aSyn could also occur in response to inflammation as seen in

LPS models of PD. There are four methionine residues located at positions 1, 5, 116 and 127 all of which are oxidised under oxidation conditions (Glaser et al., 2005). The tyrosine and methionine residues that undergo modification are present outside of the central region except the Tyrosine39. Methionine 1 and 5 are present in the N-terminal while the remaining residues are present in the C-terminal. The methionine5 appears to be the most oxidised species while the other residues are protected from oxidation due to their spatial location although in vitro studies demonstrate that dopamine can oxidise all four methionine residues and results in formation of stable non-toxic oligomers (Zhou et al., 2010b). A more recent study however shows that Methionine127 is selectively oxidized by dopamine and is associated with cytotoxicity (Nakaso et al., 2013). It is generally though that nitration and oxidation of aSyn results in covalent cross-linking that stabilizes oligomers.

- Ubiquitination: Ubiquitination of proteins plays an important role in their degradation and function. Polyubiquitinated proteins are targeted for degradation through proteasomal pathway while monoubiquitinated proteins have cellular functions. The aSyn found in LB's are also found to be ubiquitinated. Fifteen lysine residues are found in aSyn of which four (Lysine21, 23, 32, 34) are located in the N-terminal and are found to undergo ubiquitination *in vitro.* However Lysine residues at 6, 10 and 12 are found to be ubiquitinated *in vivo.* Mostly mono and diubiquitinated species of aSyn are seen in LB's. Although parkin mutations cause autosomal recessive PD, only a rare glycosylated form of aSyn is a substrate for parkin (Shimura et al., 2001). On the other hand, the non-glycosylated aSyn is a substrate for SIAH-1 which binds to a consensus sequence on aSyn located between residues 118 and 120. CHIP, another E3 ligase is also found to

ubiquitinate aSyn. However SIAH-1 and CHIP exert opposing effects on aSyn with SIAH-1 mediated ubiquitination promoting fibril formation while CHIP mediated ubiquitination of oligomeric aSyn routes it for degradation. Ubiquitination at Lysine48 usually routes a protein for proteasomal degradation but ubiquitination at 29 and 63 is also found to occur. Interestingly, aSyn is LB's is found to be ubiquitinated at Lysine63 which appears to promote aggregation (Fig.9) (Beyer, 2006).

- Sumoylation: Small ubiquitin-like modifiers are similar to ubiquitin with a diglycine motif in their C-terminus. The glycine on SUMO forms an isopeptide bond with a lysine residue on the target protein. This is a highly dynamic process and several proteases can cleave this bond. Unlike ubiquitination, sumoylation does not route the protein for degradation (Melchior et al., 2003). Instead it mediates protein transport and protein-protein interactions. Although aSyn contains 15 lysine residues, Sumoylation mediated by SUMO1, occurs at only one as yet unidentified lysine residue. The physiological relevance of sumoylation of aSyn is unknown at present (Fig.9) (Dorval and Fraser, 2006).
- Truncation: The presence of truncated forms of aSyn in LB's suggested that truncation may be another factor that promotes aggregation (Baba et al., 1998). The 20s proteasome is thought to cleave natively unfolded non-ubiquitinated aSyn which results in the generation of three truncated forms, of which two are C-terminally truncated and one truncated in the NAC region (Liu et al., 2005; Tofaris et al., 2001). The 20s proteasome also truncates mutated forms of aSyn but only a small proportion of aSyn is truncated via the 20s proteasome (Fig.10). CathepsinD found in the lysosomes is another mode through which aSyn is cleaved. Calpains, which are calcium dependent, non-lysosomal proteases

are also thought to degrade aSyn especially since aSyn is localized to presynaptic terminals. It is thought that soluble aSyn cleaved by calpain can occur even in presence of tyrosine nitration and prevents aSyn fibril formation. On the other hand aSyn fibrils cleaved by calpains promotes aSyn fibrillation (Fig.10). Neurosin a serine protease predominantly expressed in the central nervous system is thought to constitutively cleave aSyn. Neurosin mainly cleaves aSyn in the NAC region which in turn prevents aggregation and maybe a protective mechanism. Phosphorylated aSyn is resistant to neurosin. It has been found that C-terminal truncation promotes aggregation and yet it appears that aSyn truncation is a cellular process whose physiological relevance remains unknown (Beyer and Ariza, 2013; Li et al., 2005).

- Glycosylation: Glycosylation of proteins plays a very important role in determining protein function. The addition of N-acetyl-D-glucosamine (O-GlcNac) to serine threonine residues at positions at 53, 64, 72 and 87 have been reported in aSyn although the physiological relevance of glycosylation remains unknown. Recently, it has been reported that O-GlcNac modification of Threonine at position 72 prevents aSyn aggregation *in vitro* and it seems likely that glycosylation may have protective role (Marotta et al., 2012). (Alves da Costa, 2003; Stefanis, 2012; Uversky, 2008)

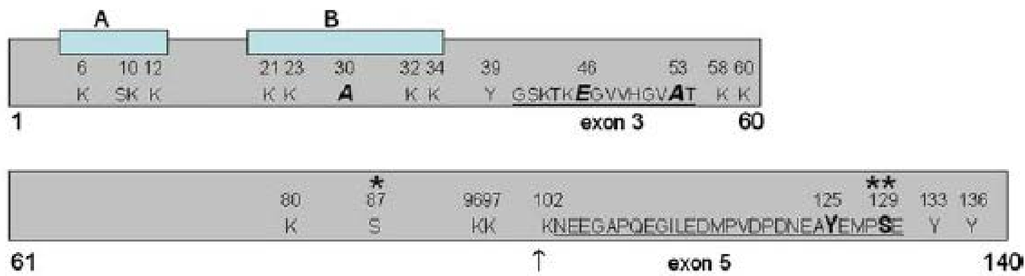


Figure 9: Represents some of the sites for posttranslational modification. Letters marked in bold and italicized are the sites of mutations. S represents serine residues that are phosphorylated, Y represents tyrosine residues that are nitrated and K represents lysine residues that are ubiquitinated or sumoylated. The arrow mark indicates lysine residue at 102 which is the major site for sumoylation. Box A and Box B represent sites of in vitro ubiquitination (Beyer, 2006).

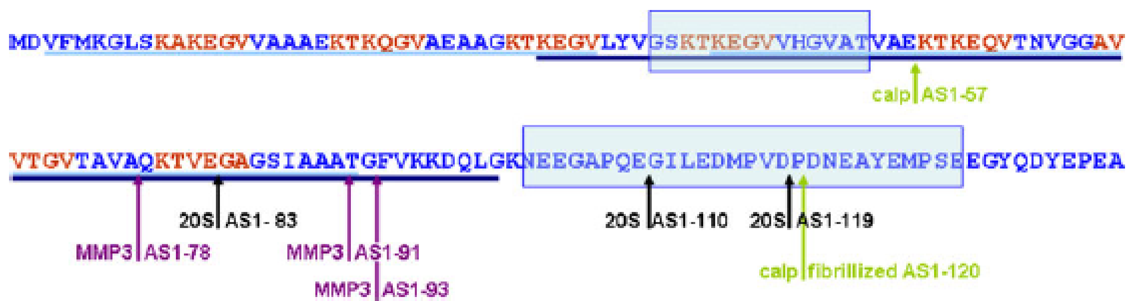


Figure 10: Represents sites of aSyn truncation. Region marked in red are imperfect repeats, region that is light blue underlined form alpha-helices, region that is dark blue underlined is the core. Black arrows represent sites of 20s proteasomal cleavage, Green arrows represent calpain mediated cleavage and purple arrows represent Matrix metalloproteinase mediated cleavage (Beyer and Ariza, 2013).

FUNCTIONS

Alpha synuclein comprises 1% of the total soluble cytosolic proteins and is widely expressed in the brain (Stefanis, 2012). The synaptic localization of aSyn suggested a possible role for aSyn in neuronal function, particularly in synaptic plasticity. The increased expression of aSyn during song learning in zebra finch and the regulation of mRNA levels of aSyn during early stages of post natal murine brain development supported a role for aSyn in neuronal plasticity. However the expression of aSyn appears only after synaptic development indicating that it has no role in

synapse formation (Alves da Costa, 2003). The effect of aSyn in modulating neuronal plasticity maybe associated with its effects on synaptic vesicle release and recycling. Although aSyn knock-out mice are not lethal suggesting that aSyn does not play an essential role in neuronal function, aSyn knock-out mice show impairments in neurotransmitter release. The suppression of aSyn also resulted in depletion of docked pool vesicles and impairment in the refilling and trafficking of vesicles from the reserve pool to the site of synaptic vesicle release. Overexpression of aSyn in transgenic models results in synaptic vesicle exocytosis and decreased neurotransmitter release. Similar results were obtained in rodent models of PD and stably expressing PC12 cells. The excess aSyn also reduced readily releasable synaptic vesicles and reduced the dopamine reuptake in dopaminergic terminals (Lashuel et al., 2013).

Several mechanisms maybe involved in the regulation of synaptic vesicle recycling by aSyn. Foremost among these, is the effect of aSyn on phospholipase D2 activity (PLD2). PLD2 is responsible for the conversion of phosphatidylcholine into phosphatidic acid (PA) and aSyn has an inhibitory effect on PLD2 thereby resulting in a decrease in PA levels (Clayton and George, 1999). This affects vesicle biogenesis. The inhibitory effect on PLD2 is through the direct binding of aSyn to PLD2. Alternately, aSyn which has homology with the 14-3-3 chaperone proteins can have an indirect effect on PLD2 by binding to proteins such as 14-3-3, Erk, BAD, protein kinase C which also inhibit PLD2 (Cheng et al., 2011). Actin filaments play an important role in vesicle trafficking and there are indications that aSyn might regulate Ca^{2+} mediated actin polymerization in turn affecting vesicle transport (Cheng et al., 2011). Studies also report that aSyn can block ER to Golgi trafficking and co-localizes with prenylated Rab acceptor protein 1 (PRA1). PRA1 regulates the recycling of Rab by preventing its removal from the membrane which in turn affects vesicle trafficking between compartments in the cell and therefore

interactions between aSyn and PRA1 affect vesicle trafficking and recycling (Lee et al., 2011). The homology of aSyn with 14-3-3 chaperone proteins is suggestive of its ability to act as a chaperone molecule and indeed it has been reported that aSyn acts as a chaperone in the folding and assembly of synaptic proteins called SNARES which in turn directly regulate neurotransmitter release. A direct effect on SNARE by aSyn is reported to be mediated via protein-protein interactions while an indirect inhibition on SNARE assembly maybe mediated via its effect on arachidonic acid which stimulates SNARE complex assembly. Further support for aSyn in neuronal function came from studies on mice lacking a cysteine ring protein (CSFα). The CSFα deficient mice show degeneration and motor impairments because of loss of function of CSFα which aids in SNARE complex assembly. The upregulation of aSyn in these mice can compensate for the loss of CSFα and rescue neurons from degeneration (Burré et al., 2010; Chandra et al., 2005).

The effect of aSyn on neurotransmitter release selective to dopaminergic neurons has also been reported. Studies report that aSyn can directly bind to Tyrosine hydroxylase (TH), a rate limiting step in dopamine synthesis and suppress the activity of TH. The effect of aSyn on TH appears to be restricted to decreasing its activity without affecting its expression levels. The negative regulation of TH decreases dopamine biosynthesis. Phosphorylated aSyn on the other hand cannot inhibit TH activity (Perez et al., 2002; Wu et al., 2011). Another potential effect of aSyn on dopamine levels appears to be via its interactions with Dopamine Transporter (DAT). Interactions of aSyn with DAT affects dopamine reuptake although aSyn knock-out mice show no difference in DAT activity (Sidhu et al., 2004).

In addition to neuronal function, some studies indicate aSyn may regulate the differentiation and fate of neural progenitors and truncated forms of aSyn enhance the sensitivity of dopaminergic

neurons to environmental stress (Michell et al., 2007; Schneider et al., 2007). Recent studies also suggest that aSyn may bind to DNA. However, the binding of aSyn to DNA has been studied from a pathological perspective and it appears that binding to DNA may alter the conformation of both the DNA and aSyn (Desplats et al., 2012; Guerrero et al., 2013). Although the binding of aSyn to DNA has been demonstrated, the physiological functions, if any of such binding have not yet been understood. Several studies also suggest that aSyn has an anti-apoptotic effect either by lowering p53 mediated response or by inhibiting caspase 3 and protein kinase C δ (PKCδ) (Alves Da Costa et al., 2002). It has also been reported that aSyn may protect from oxidative stress (Hashimoto et al., 2002; Jin et al., 2011; Li and Lee, 2005; Musgrove et al., 2011, 2012). The effect of aSyn on microglia has been investigated and it has been reported that monomeric aSyn can regulate microglia in turn triggering changes in the inflammatory pathway and although the physiological relevance of this interaction is unknown it has great bearing on pathogenesis in PD (Park et al., 2008). The sub-cellular localization of aSyn to mitochondria suggests that it may play a role in maintenance of mitochondrial function. In this regard it has been reported that wild type aSyn but not the mutants confer protection against MPP+ induced toxicity by preserving mitochondrial function (Jensen et al., 2003). Mitochondrial associated membranes (MAM) are subcellular compartments that interconnect mitochondria and ER and have several cellular functions and disturbance in MAM is associated with disease pathogenesis. It has been reported that aSyn localizes to MAM and that wild type aSyn may play a role in regulating the essential functions of MAM (Guardia-Laguarta et al., 2015). The ability of aSyn to bind metal ions and its effect on aggregation has been well documented. Recently it has been reported that aSyn can bind iron also. It appears that aSyn can bind to copper and iron simultaneously which suggest that the metal ions bind to different regions of the protein. The

binding of aSyn to iron and its association with membrane indicates that aSyn can function as a cellular ferrireductase. This activity has potential impact on dopamine synthesis because iron is a co-factor for TH (Davies et al., 2011) (Alves Da Costa et al., 2002; Cheng et al., 2011; Lashuel et al., 2013; Marques and Outeiro, 2012; Stefanis, 2012; Tofaris and Spillantini, 2007).

FACTORS AFFECTING aSYN AGGREGATION

The aggregation of aSyn is not exclusive to PD but is a common feature of a group disorders under the broad umbrella of 'synucleinopathies', all characterized by aSyn-positive inclusions. Although aSyn is the predominant component, the inclusions vary in type and location. There are five types of aSyn positive inclusion bodies as described (Fig.11).

- Lewy Bodies (LB's): The classical LB's are eosinophilic with a halo although LB's indistinguishable with eosin stain and lacking a halo are also seen. The classical LB's often stain for aSyn in the halo and ubiquitin in the core. The existence of diffuse cloud like LB's negative for ubiquitin and pale bodies with variable staining of ubiquitin is also observed. Electron microscopy (EM) studies of classical LB's reveal the granular and fibrillar composition with the granular components being present in the core and the fibrils radiating out. The presence of aSyn in filaments in the periphery with minimal staining in the core of LB's, loosely aggregated aSyn filaments in pale bodies and a loose mesh-work of small filaments positive for aSyn and completely lacking in ubiquitin in diffuse type were are observed with EM (Uversky, 2008).
- Lewy Neurites (LN's): They may a display a coarse rounded form but are usually elongated club shaped or serpentine structures not detected with conventional stains.

When stained for aSyn, fine thread like LN's was also detected in addition to the serpentine structures (Uversky, 2008).

- Glial cytoplasmic inclusions (GCI's): These are argyrophilic, sickle-shaped, conical or oval shaped aggregates containing aSyn, ubiquitin, 14-3-3, tau and several kinases. Analysis of high power images has revealed tubular structures with a round or ovoid wall enclosing a clear centre. Ultrastructural analysis has further revealed the presence of amorphous material coated filaments upto 30nm in size arranged in parallel bundles extending to the oligodendroglial processes. Each core filament consists of two aSyn filaments with two distinct morphologies (Uversky, 2008).
- Neuronal cytoplasmic Inclusions (NCI's): These occur in the pontine nucleus and are homogenous round or ovoid structures whereas in the oliviary nucleus they are irregular reniform in shape and look like coarse granular structures. Ultrastructurally they are revealed to be randomly arranged loose granule coated filaments composed of aSyn (Uversky, 2008).
- Axonal Spheroids: In addition to aSyn, these are comprised of tau, neurofilament proteins, ubiquitin, ferritin, suproxide dismutase and amyloid precursor protein and occur in presynaptic terminals (Uversky, 2008).

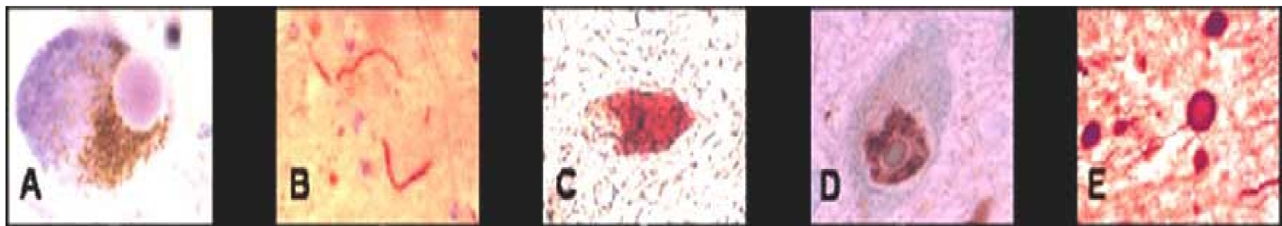


Figure 11: Figure showing the different types of aSyn positive inclusion bodies. (A) LB (B) LN (C) GCI (D) NCI (E) Axonal spheroids (Uversky, 2008).

The formation of aggregates from the monomeric 'natively unfolded state' involves a conformational change and increased β- pleated sheets are found in the fibrils. The beginning of the fibril formation is through the formation of partially folded intermediates which drive fibrillization. Changes in pH and temperature assist in the formation of partially folded intermediates. Therefore factors that promote partially folding also drive the fibrillation.

Several of the factors are listed here.

- High concentration of the protein: The most direct evidence for this has come from familial PD with duplication and triplication mutations where increased gene dosage causes disease. In the endogenous condition and even in absence of any factors, equilibrium is maintained between monomers and partially folded intermediates. An increase in protein concentration shifts the equilibrium in favour of partially folded intermediates thereby leading to aggregation. An increase in aSyn in PD brains compared to non-PD controls also indicates that increased aSyn favours aggregation (Kim and Lee, 2008).
- Molecular crowding: The inside of a cell is very crowded with the presence of several macromolecules. The volume occupied by the solutes affect the stability of macromolecules and influences protein-protein interactions and interactions with other cellular components.
- Point Mutations seen in familial PD: Three point mutations have been described, A53T, A30P and E46K. All the mutations show an increased propensity for aggregation compared to wild type. Although these mutations do not affect the monomeric structure, they appear to stabilize the β-sheet with intermolecular interactions.

- Truncations: The presence of C-terminal truncated forms of aSyn in LB suggests that the C-terminal has a chaperone like activity that prevents fibrillation. The generation of NAC peptides also results in increased aggregation due to the increased propensity for NAC to acquire β-sheet.
- Interactions with anions: Anions promote aSyn partial folded intermediates through stabilization of hydrophobic interactions in the protein. The loss of uncompensated charge and increase in hydration are other parameters affected by anion binding. Addition of small quantities of salt minimizes the charge based repulsion while increased quantities of salt remove water and favour fibrillation.
- Effect of pesticides: Several environmental factors promote aggregation and pose a significant risk for disease onset. Both paraquat and MPTP lead to increase in mRNA levels and expression levels of aSyn. The cellular stress generated with addition of MPTP promotes nitration of aSyn in turn leading to aggregation. *In vitro* studies demonstrate a direct interaction between some of these pesticides and aSyn that stabilize the partially folded conformations.
- Interactions with metal ions: Binding of metal ions to aSyn is thought to bring about structural perturbances that promote aSyn fibrillation.
- Oxidative modifications: Oxidative stress is thought to be an important factor in disease pathogenesis and four readily oxidizable methionine residues are found in aSyn. Oxidation of methionine residues prevents fribrillation but this is overcome in presence of metal ions. On the other hand, tyrosine nitration due to oxidative stress promotes aggregation of aSyn. The presence of nitrated aSyn in LB’s suggests that nitration has a

pathogenic role and results in the formation of partially folded intermediates. However nitration induced oligomerization prevents fibrillation.

- Phosphorylation: Similar to other post translational modifications, it appears that phosphorylation at Serine129 promotes aggregation. The extensive presence of phosphorylated aSyn in LB's supports a role for phosphorylation in aggregate formation.
- Interaction with membrane: Although the physiological function of aSyn involves its binding membranes, it appears that membrane binding is a trigger for aggregation, particularly when bound to polyunsaturated fatty acids. Interestingly the mutant A30P has a reduced affinity towards lipid membranes due to a disruption in one of the helices in aSyn protein.
- Interactions with polyamines: Polyamines are critical for various cellular functions particularly growth and differentiation. Not only do increases in polycations result in production of oxidative intermediates during their interconversion, they also bind to the C-terminus of aSyn. Binding of polycations to C-terminal of aSyn suppresses the repulsion within the protein due to the negative charge and promotes oligomerization. The magnitude of fibrillation is dependent on the length, charge and concentration of polycations.
- Protein-Protein Interactions: Several kinds of protein-protein interactions have been described that favour aggregation of aSyn. Some these interactions include
    - ✓ Self assembly leading to dimerization.
    - ✓ Dityrosine linkages formed due to oxidative stress lead to formation of dimers and higher order oligomers

- ✓ Tranglutaminase catalyzes the formation between of cross link between a lysine and glutamine residue. Lysine at position 80 and glutamine at position 79 in the NAC domain of aSyn is thought to form a cross link which promotes formation of dimers.
- ✓ Although inclusions are often present in the cytoplasm, the localization of aSyn to nucleus has been demonstrated under pathological conditions. The co-localization of aSyn to histone has also been demonstrated and in vitro studies show binding to histone triggers aSyn aggregation.
- ✓ Several other proteins including tau, tubulin, and brain specific protein p25α are some that mediate aggregation. These are also often present in the inclusion bodies. Most of these proteins contain a basic motif and result in ionic interactions with aSyn. Recently the involvement of FKBP's in stabilizing partially folded intermediates has been investigated and inhibitors of this protein appear to prevent aSyn fibrillation. Synphilin-1, a presynaptic protein is found in the LB's and has been shown to interact with aSyn both *in vitro* and *in vivo* and promote aSyn aggregation.

However not all protein interactions promote aSyn aggregation. Chaperones that interact with aSyn have been found to slow down or prevent aSyn fibrillation (McLean et al., 2002). Likewise, β and γ synucleins, the other members of the same family also prevent aggregation of aSyn (Uversky et al., 2002). (Uversky, 2008)

Aggregation of proteins is a common theme in several neurodegenerative diseases and it was long held that the large aggregates such as LB's cause neuronal death. However, the absence of LB's in some forms of familial PD, the presence of LB's in asymptomatic individuals such as in

incidental Lewy bodies and the presence of cytoplasmic inclusions without the accompanying loss of neurons in rodent models of PD was suggestive of the fact that the formation of LB's may not be absolutely essential for pathogenesis. The analysis of surviving neurons that lacked or contained LB's did not show any quantifiable differences in viability. Additionally an increase in LB size and a decrease in soluble concentration of aSyn were correlated with a reduction in cytotoxic effects and further argued against role of LB's as toxic species (Kazantsev and Kolchinsky, 2008; Walsh and Selkoe, 2004).

The aggregation of aSyn from monomers to inclusions is a multi-step process that involves several intermediates. Several of the factors described above play a critical role in stabilizing these intermediates. It was therefore postulated that the intermediates maybe the toxic species, the sequestration of which into large inclusions reduces the toxicity. These intermediates are not stained with conventional dyes and are not readily visible under light microscopy. This may perhaps explain why in some cases the disease may proceed without the occurrence of LB's.

The aggregation of two or more monomers leads to oligomerization and these intermediate species are termed protofibrils (Volles and Lansbury, 2003). Direct evidence for the role of oligomers as the toxic species came from experiments involving the generation of two mutants E53K and E57K, both which form oligomers but do not undergo fibrillation. The effect of these mutants was compared with familial PD mutants, wild-type aSyn and another fibril promoting mutant. The lentiviral injections of these mutants into rat models caused a greater decrease in dopaminergic cells compared to the other aSyn variants while the fibril promoting mutant did not show any decrease. Similar results were obtained in cell-based assays where cell viability was assessed (Winner et al., 2011). The expression of A30P, A56P and A76P mutants in *Drosophia* caused non-motor symptoms similar to early stage PD although these mutants form oligomers

and do not fibrillate either *in vitro* or *in vivo* (Gajula Balija et al., 2011). It therefore appears that these oligomers may exert toxicity even before the onset of motor symptoms. Newer assays such as protein-fragment complementation assay have enabled the detection of these oligomers in cultured cells, neurons as well as conditioned cell culture media. Biochemical assays such as size exclusion chromatography and non-denaturing gel electrophoresis have further confirmed the presence of these oligomeric species. These oligomeric species have also been detected in brain extracts from post mortem PD brains, plasma and CSF of PD patients (El-Agnaf et al., 2006; Sharon et al., 2003; Tokuda et al., 2010).

The formation of aggregates begin with the self-assembly of the protein into a dimer or oligomer. This is called the seed or the nucleus which then sequesters other monomers or oligomeric species to form larger aggregates (McCormack and Di Monte, 2009). The initial lag phase involves the formation of a seed and may take a long time. Therefore the formation of a seed is a rate-limiting step in aggregation. This is followed by the elongation phase where the aggregate growth proceeds rapidly. Finally, in the steady state, equilibrium is achieved between the ordered aggregate and the monomer. Several cellular conditions aid in aggregation by decreasing the lag time (Wood et al., 1999). The NAC domain with its increased propensity for the β-sheet can act as a seed and interact with other regions of another aSyn protein (Bennett, 2005). The A53T mutant, for example, forms fibrils faster than the wild type. The A30P mutant on the other hand is slower to form fibrils compared to the wild type although it forms more stable oligomers compared to the wild type. A double mutant with A53T and E46K forms fibrils even faster than the A53T mutant alone (Volles and Lansbury, 2003; Waxman and Giasson, 2009). These protofibrils have also been shown to bind to vesicles with greater affinity than the monomeric aSyn (Winner et al., 2011).

Ultrastructurally, when observed under Atomic Force microscopy (AFM) and EM, the protofibrils occur as a heterogenous mixture with atleast three distinct morphologies. There are (a) spheres of different heights (b) chains that are comprised of linearly arranged spheres (c) rings comprising circularized chains. It is believed that while the spheres and chains are intermediates that proceed to form fibrils, the formation of rings prevents fibril formation. Therefore two distinct pathways of aggregation have been proposed (a) where the intermediates proceed towards fibril (b) formation of stable oligomers that do not proceed towards fibrillation and exert toxic effects (Fig12) (Conway et al., 2000). It has been reported that methionine oxidation at position 5 n aSyn forms non-toxic oligomers adding support to the hypothesis about existence of two distinct pathways for aggregation (Zhou et al., 2010b).

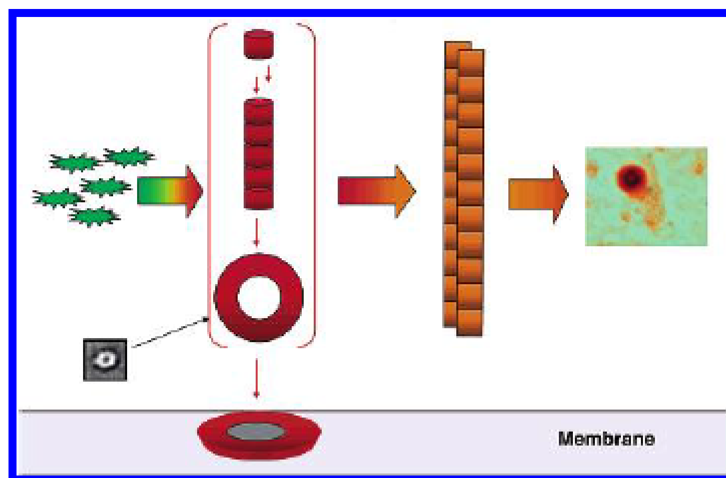


Figure 12: Represent the pathway for aggregation. The green stars represent monomers. The red structures represent the different types of protofibirls. The orange rods represent the fibrils that go on to form LB's (Volles and Lansbury, 2003).

MECHANISMS OF TOXICITY

The formation of aggregates is linked to disease onset and progression through the disruption of various cellular processes. Recent studies strongly suggest that the toxicity is mediated via aggregation intermediates 'protofibrils'. The toxicity can be a result of either a toxic gain-of-function or a loss-of-function through the unavailability of the monomeric aSyn to perform its physiological function. Another equally likely scenario to be considered is that the monomeric aSyn may mediate toxicity via aggregation independent mechanism which may involve its aberrant interactions with other cellular factors leading to disruption of cellular processes (Fig.15).

- Propagation of aSyn: The absence of a secretory signal peptide led to the assumption that aSyn was not a secretory protein. However several lines of evidence now show that aSyn can be secreted. The presence of aSyn is detected in CSF and plasma of PD patients (El-Agnaf et al., 2003; Lee et al., 2006). It has also been detected in cell culture media of cells overexpressing aSyn (Lee et al., 2005; Sung et al., 2005) and the release of endogenous aSyn from rat embryonic cortical neurons has also been reported. Both the monomeric and aggregated form of aSyn have been found to be released (Danzer et al., 2011; Lee et al., 2005). These studies have been strengthened by observations of aSyn inclusions in mesencephalic transplants in PD patients (Kordower et al., 2008; Li et al., 2008) and similar host-to-graft transmission was also observed in transgenic mouse models transplanted with progenitor cells (Desplats et al., 2009; Hansen et al., 2011). All these observations point towards a prion like behaviour offers a potential explanation for the propagation of the pathology and supports the Braak hypothesis.

The release of aSyn is thought to be mediated by several pathways that include (a) direct integration into secretory pathway and release through secretory vesicles (b) release through recycling endosomes (c) Incorporation into multivesicular bodies that may either fuse with the plasma membrane or fuse with the lysosomes for degradation (Fig.13). In this regard, it has been proposed that several cellular stresses including lysosomal dysfunction will promote the secretion of aSyn (Emmanouilidou et al., 2010a; Hasegawa et al., 2011; Jang et al., 2010; Lee et al., 2005). The secreted aSyn can be taken up by neighbouring neurons where it will function as a 'seed' for aggregation or can be taken up by glial cells where it will incite inflammatory events. This uptake is thought to be mediated via endocytosis (Fig.14) (Hansen et al., 2011; Lee et al., 2008a). Exogenous aSyn fibrils have been shown to seed LB like inclusions in cultured cells (Luk et al., 2009). Several studies support a role for neuroinflammation in triggering cell death in PD (Blum-Degen et al., 1995; Hunot and Hirsch, 2003; McGeer et al., 1988) and aSyn can activate microglia leading to inflammation (Alvarez-Erviti et al., 2011; Lee et al., 2010; Zhang et al., 2005). Additionally mutants have been shown to elicit a stronger response than the wild type (Roodveldt et al., 2010; Su et al., 2009).

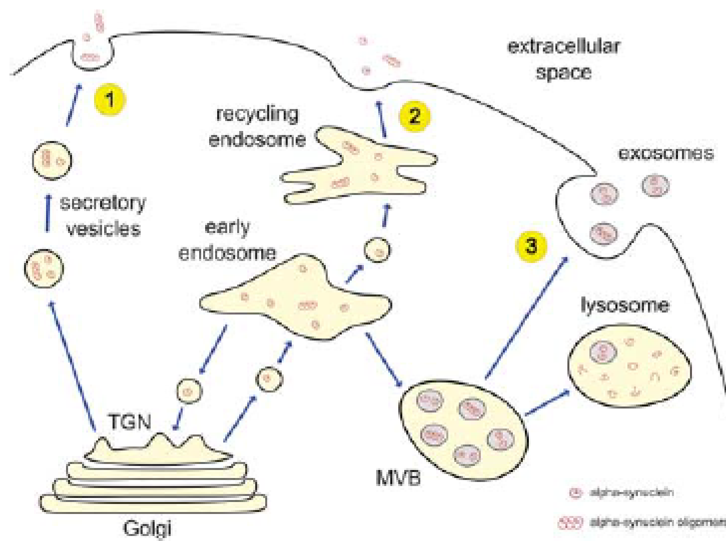


Figure 13: Represents the probably pathways for secretion of aSyn. (1) Direct integration into secretory vesicles (2) Through recycling Endosomes (3) Incorporation into multivesicular bodies that either fuse with plasma membrane or the lysosomes (Marques and Outeiro, 2012).

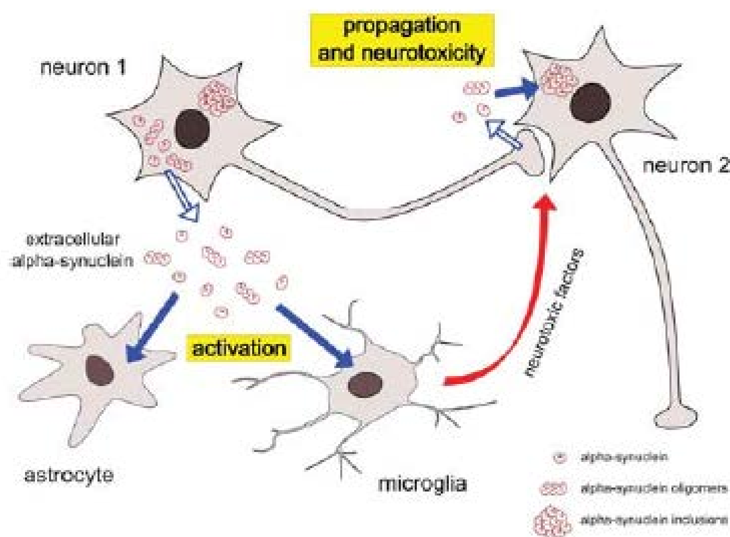


Figure 14: Represents the uptake of secreted aSyn by neurons where aSyn acts as a seed for further aggregation and uptake by microglia resulting in activation of inflammatory events (Marques and Outeiro, 2012).

- <u>Effect at the Synapse</u>: Studies have identified synaptic defects resulting from aSyn aggregation and this seems likely given that aSyn plays a role in mediating synaptic function. It is thought to interfere with the axonal transport of synaptic proteins such as

synphilin1 and result in loss of synaptic proteins, enlargement of synaptic vesicles, decrease neurotransmitter release, reduction is synaptic vesicle recycling and redistribution of SNARE complex (Garcia-Reitböck et al., 2010; Lundblad et al., 2012; Nemani et al., 2010; Scott et al., 2010). It could also cause an influx in calcium ions through its effects on voltage gated channels by forming pores on cell membrane (Danzer et al., 2007; Hettiarachchi et al., 2009). It has also been reported that aSyn oligomers could alter glutamatergic receptor function; supplementation with extracellular aSyn oligomers in cultured hippocampal neurons causes an increase in the amplitude of AMPA receptor mediated excitatory post-synaptic currents and opposed hippocampal long term potentiation (Hüls et al., 2011; Martin et al., 2012).

- Effects on Cytoskeleton: MAPT has emerged as a significant risk factor in PD (International Parkinson Disease Genomics Consortium et al., 2011) and several taupathies feature aSyn positive inclusions and therefore it's thought that tau and aSyn enhance one another's aggregation (Giasson et al., 2003). Several studies have reported the interaction of aSyn with microtubules and it is thought to affect microtubule polymerization (Alim et al., 2004; Zhou et al., 2010a). It also enhances the phosphorylation of tau which will affect the microtubular organization (Haggerty et al., 2011; Jensen et al., 1999; Qureshi and Paudel, 2011). The influence of aSyn on actin polymerization will also indirectly affect vesicle transport (Sousa et al., 2009).
- Effect on Mitochondria: The localization of aSyn to mitochondria and the down-regulation of compex I activity has now been reported by several studies (Devi et al., 2008; Li et al., 2007; Liu et al., 2009; Loeb et al., 2010; Nakamura et al., 2008). The aSyn oligomers are thought to result in mitochondrial fragmentation and aSyn and its

mutants have been reported to enhance mitochondrial mitophagy. These events result in mitochondrial dysfunction, loss and death (Choubey et al., 2011; Kamp et al., 2010; Nakamura et al., 2011). Mitochondrial dysfunction also leads to generation of reactive oxygen species (ROS). A disruption in the brain mitochondrial morphology in aSyn transgenic mice has also been reported (Martin et al., 2006).

- Effect on ER/Golgi: The studies with adenoviral expression of aSyn in neuronal model leading to Golgi fragmentation along with appearance of aSyn oligomers (Gosavi et al., 2002) and the occurrence of ER stress as an early event in an inducible model of A53T mediated neurotoxicity (Smith et al., 2005b) demonstrated that aSyn oligomers targeted ER/Golgi. It has been reported that aSyn oligomers can form in the lumen of ER, accumulate there and sensitize neurons to ER stress (Colla et al., 2012a, 2012b). Studies in yeast model suggested that aSyn blocked ER to Golgi trafficking and this inhibition could be rescued with the overexpression of RAb1 (Cooper et al., 2006). Recently it has been reported that aSyn could also delay the ER to Golgi transition (Thayanidhi et al., 2010).
- Effect on Nucleus: The localization of aSyn to the nucleus and its binding to histone under pathological conditions has been reported (Goers et al., 2003). Phosphorylated aSyn is found to be localized to the nucleus (Schell et al., 2009). The binding of aSyn to histone results in a decrease in histone deactylation. The use of inhibitors of histone deactylase like inhibiting the action of SIRT2 which is a histone deactylase was associated with neuroprotection (Kontopoulos et al., 2006; Outeiro et al., 2007).
- Effect on Proteostasis: Protein Homesostasis (Proteostasis) is maintained in the cells either through refolding of misfolded proteins or through their removal. Chaperones play

a critical role in the refolding of proteins and are hence important for proteostasis. Oligomeric aSyn were found to inhibit the refolding of Hsp70 chaperone system in bacterial systems (Hinault et al., 2010). A similar inhibition was not observed with monomeric aSyn. Oligomeric species of aSyn inhibit the chymotrypsin-like protease activity of the proteasome and the lysosomal enzyme glucocerebrosidase (Lindersson et al., 2004; Mazzulli et al., 2011).

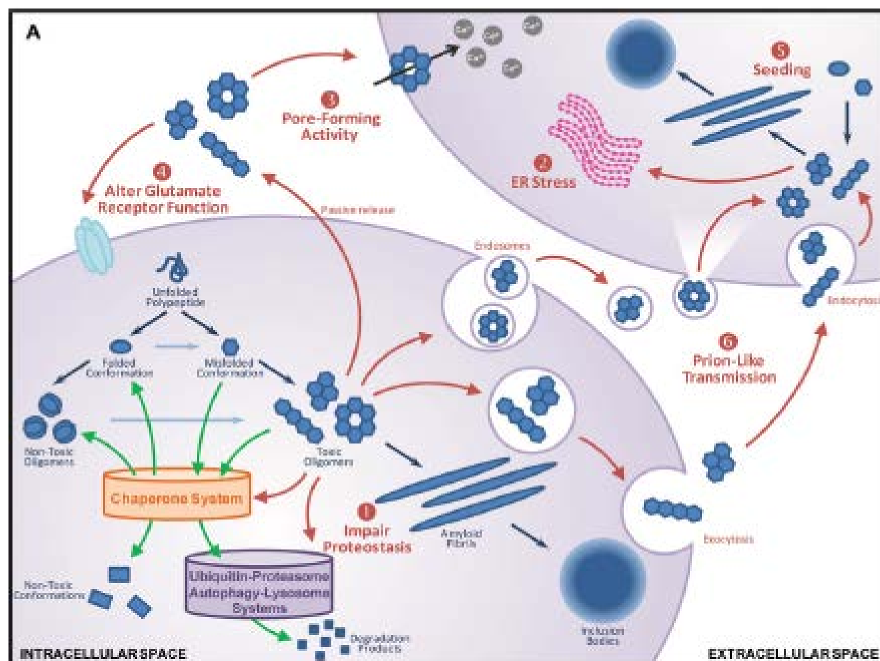


Figure 15: Represents the probable mechanisms of aSyn aggregation mediated toxicity (Kalia et al., 2013).

The identification of aSyn oligomers as toxic species and the probable mechanisms of toxicity may have a huge impact on the diagnosis and treatment of the disease (Fig.16). The real challenge to developing neuroprotective strategies in PD have been the lack of early diagnostic criteria. The occurrence of oligomers must precede the onset of disease and therefore would serve as ideal biomarkers. The presence of aSyn oligomers in CSF has already been reported and

a study utilizing a special ELISA assay was able to detect aSyn oligomers in the CSF and the levels of these oligomers were reportedly higher in PD patients when compared to age-matched controls. Body fluids such as saliva and urine can also be utilized for the detection of aSyn oligomers. The development of radioligands that can specifically detect aSyn oligomers *in vivo* will prove to be a useful technique in the detection of aSyn oligomers.

Understanding the cytotoxic role of aSyn oligomers throws up potential targets for therapeutic interventions. The reduction of toxic intermediates may in fact serve as the most promising neuroprotective intervention and can be achieved by several means. Postranslational modifications that promote aggregation, disruption of oligomer formation, preventing cell to cell transmission, enhancing chaperone activity and promoting clearance of the toxic species are some of the pathways that can be targeted. The use of antibodies to target extracellular aSyn oligomers is another viable alternative.

The development of diagnostic tools and neuroprotective treatment strategies based on aSyn oligomers would necessitate further understanding of not just pathogenic mechanisms but also in elucidating the exact physiological role of aSyn in the cells, the disruption of which is as central to disease pathogenesis as the formation and propagation of oligomers (Kalia et al., 2013; Lashuel et al., 2013; Marques and Outeiro, 2012; Stefanis, 2012).

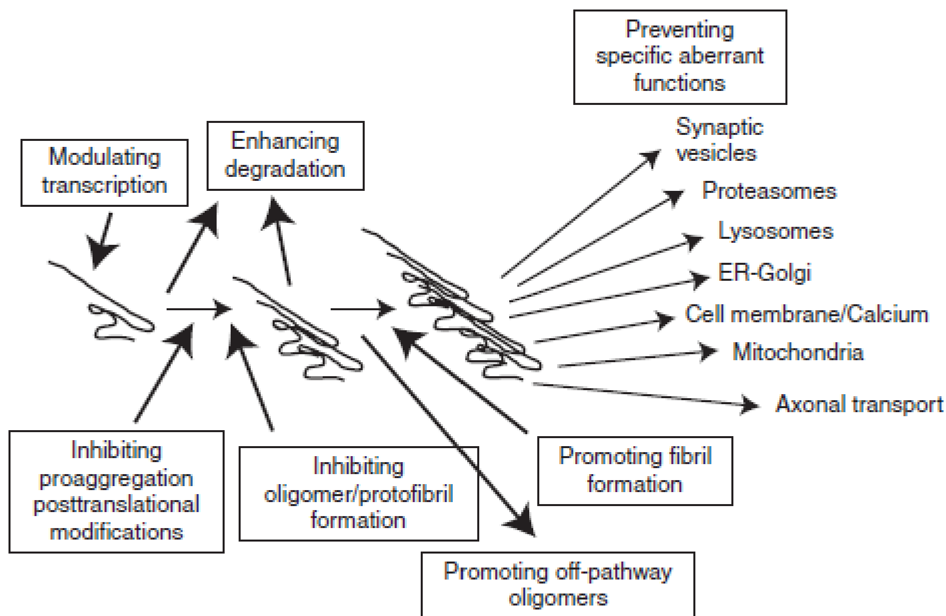


Figure 16: Represents the probable targets for the development of neuroprotective strategies (Stefanis, 2012).

INTERACTIONS WITH DOPAMINE

Dopamine is synthesized from the amino acid L-tyrosine in a two-step process. The first step involves the hydroxylation of tyrosine to L-dihydroxyphenyalanine (L-dopa) catalysed by enzyme tyrosine hydroxylase (TH) in the presence of oxygen and is the rate limiting step in the reaction. In the second step, L-dopa undergoes decarboxylation to give dopamine catalysed by amino acid decarboxylase (AADC) and is followed with the release of CO_2 (Fig.17). The enzymes TH and AADC are closely associated with vesicle monoaminergic transporter 2 (VMAT-2) located on monoaminergic synaptic vesicles, which results in the synthesized dopamine being packed into the synaptic vesicles preventing its accumulation inside the cell.

The dopamine is then released into the intersynaptic space to interact with the receptors on the postsynaptic neurons. Dopamine reuptake is mediated by dopamine transporter (DAT) and is

once again packed into the monoaminergic vesicles through the action of VMAT-2 once again preventing the cytosolic accumulation of dopamine.

The enzyme monoamine oxidase (MAO) also acts to reduce cytosolic dopamine through the oxidative deamination of dopamine. MAO is localized to the outer membrane of mitochondria in neurons and glia and two isozymes MAO-A and MAO-B are found in the cells. MAO-A has a greater affinity for dopamine and is found in catecholaminergic neurons. However the activity of MAO is associated with the generation of hydrogen peroxide and is a source of oxidative stress. Catechol-*ortho*-methyltransferase (COMT) and aldehyde dehydrogenase are the other enzymes involved in the degradation of dopamine.

Dopamine undergoes spontaneous oxidation to form a transient molecule Dopamine-*o*-quinone which is quickly oxidised to form a more stable product aminochrome which is a precursor for neuromelanin thus explaining the dark coloration of SN*pc* neurons. Dopamine-*o*-quinone, aminochrome and 5,6-indolequinone are the oxidation products of dopamine of which dopamine-*o*-quinone and 5,6-indolequinone are highly unstable. The action of VMAT-2 and MAO prevent the oxidation of dopamine by removing excess dopamine from the cell.

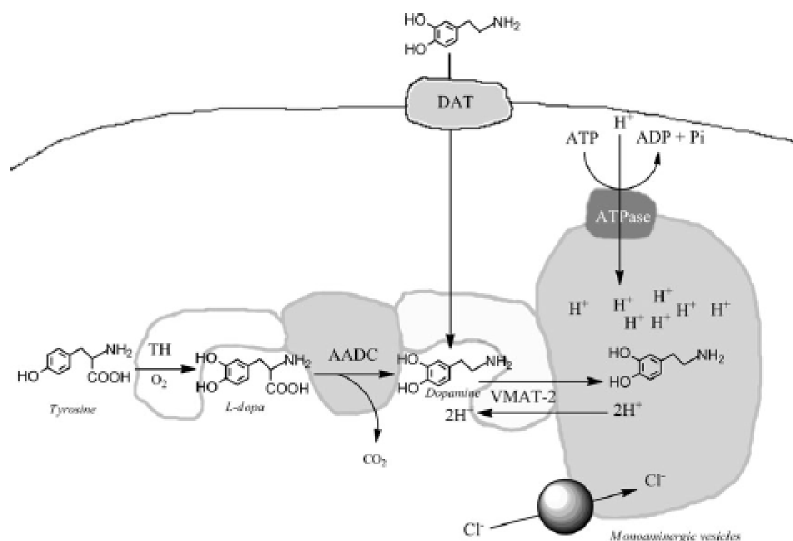


Figure 17: Dopamine synthesis pathway. Tyrosine is converted to L-Dopa in presence of TH which is then converted to Dopamine in presence of AADC and is immediately packed into the synaptic vesicle through the action of VMAT-2. Dopamine reuptake from synaptic cleft is mediated by DAT (Segura-Aguilar et al., 2014).

The end product of dopamine oxidation is the formation of neuromelanin. The oxidation of dopamine-*o*-quinone results in the formation of eumelanin through the formation of 5,6-dihydroxyindole. On the other hand, dopamine-*o*-quinone can also form pheomelanin through its interaction with l-cysteine and the formation of 2-S-cysteinyldopamine and 5-S-cysteinyldopamine. The neuromelanin therefore is composed of an inner core of pheomelanin surrounded by eumelanin surface. Only the excess cytosolic dopamine is converted to neuromelanin (Fig.18).

The levels of dopamine are tightly regulated in the cell because the oxidation products of dopamine are associated with disease pathogenesis.

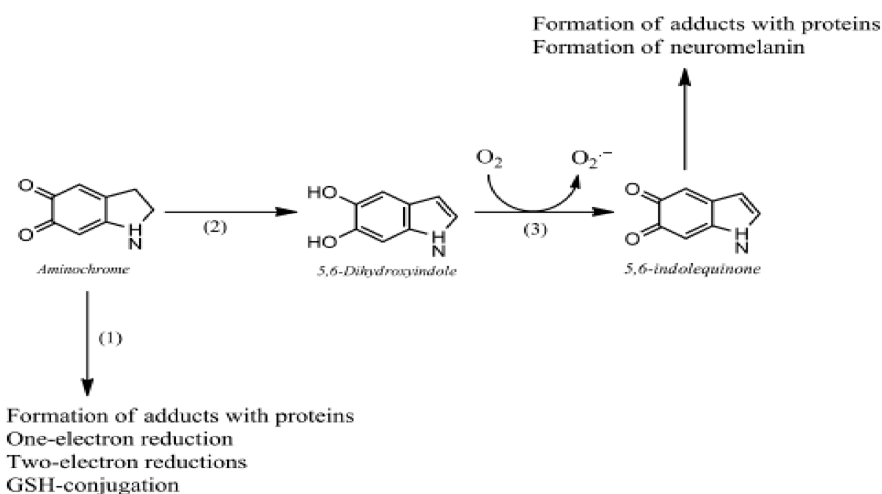


Figure 18: Shows the formation of neuromelanin. Aminochrome is the oxidation product of dopamine. Aminochrome undergoes cyclization and oxidation to form the 5,6-indolequinone which results in the formation of melanin (Segura-Aguilar et al., 2014).

Aminochrome induces and stabilizes the aSyn protofibrils. It can form adducts with aSyn as can 5,6 indolequinone. The dopamine-*o*-quinone also causes mitochondrial dysfunction by forming adducts that inactivate complex I and III in the mitochondria. The oxidative catabolism is also affected due to adduct formation between dopamine-*o*-quinone and isocitrate dehydrogenase. The protective effect of DI-1 against oxidative stress is also prevented due to the adduct formation between dopamine-*o*-quinone and DJ-1. Aminochrome forms adducts with α and β tubulins that result in formation of aggregates that affects the movement of mitochondria along these microtubules. The dopamine-*o*-quinone also forms adducts with parkin and UCHL-1 which in turn impairs proteasome pathway. Autophagy is also impaired due to inhibition of fusion of autophagic vacuoles with lysosome by aminochrome. Aminochrome also affects the acidification of lysosome and the formation of adducts with aSyn also impair autophagy. The oxidation of aminochrome results in generation of superoxides which dismutate to form hydrogen peroxide thereby acting as a trigger for oxidative stress.

The conversion of oxidation products of dopamine to neuromelanin is a protective mechansism and reduces the occurrence of oxidative stress. The neuromelanin also sequesters metal ions and other toxic chemicals such as MPP+ and paraquat involved in pathogenic pathways. However under certain circumstances, the neuromelanin can also have a toxic role in the cells. The excess iron bound to neuromelanin can promote redox reactions that result in oxidative stress. Additionally it has been reported that neuromelanin bound to iron can also enhance the oxidation of dopamine. The death of neurons results in the release of neuromelanin into the extracellular space where it can activate microglia and trigger neuroinflammation. Therefore neuromelanin can have both toxic as well as protective effects on the cell (Segura-Aguilar et al., 2014; Zecca et al., 2006).

In vitro studies also demonstrate the binding of dopamine to aSyn but such adducts are difficult to determine in *in vivo* conditions. Therefore excess dopamine can directly bind to aSyn and promote aSyn oligomerization or the oxidation products of dopamine can interact with aSyn stabilizing its protofibrils which ultimately results in cytotoxicity (Bisaglia et al., 2010; Martinez-Vicente et al., 2008; Outeiro et al., 2009; Yamakawa et al., 2010). It therefore appears imperative that accumulation of excess dopamine be prevented in the cell.

It appears that aSyn may contribute towards the regulation of cytosolic levels of dopamine. This regulation can occur at three levels. (a) Synthesis of dopamine through the regulation of TH and AADC. The activity of TH and AADC are both inhibited by aSyn thereby decreasing the levels of dopamine. It also appears to regulate the function of VMAT-2 which in turn affects the packing of dopamine into the synaptic vesicle. (b) Although no clear evidence has emerged as yet, several studies indicate that aSyn may regulate the release of dopamine. The absence of aSyn promotes the release of dopamine while overexpression of aSyn prevents the release of

dopamine. (c) The reuptake of dopamine by DAT is also influenced in the presence of aSyn. Although mice lacking aSyn have no visible changes in DAT function, it appears that aSyn regulates the shuttling of DAT to and from the cell membrane (Cheng et al., 2011).

Additionally, the pacemaker activity of the dopaminergic neurons of the SN*pc* is driven by the presence of calcium channels which increases the risk of calcium dependent neurotoxic processes in these cells (Stefanis, 2012).

Therefore in summary, a loss of aSyn physiological function could result in increased dopamine content in the cytosol. Dopamine itself and its oxidation products stabilize the protofibrils and prevent the formation of aSyn fibrils. These protofibrils may in turn cause leakages on membrane which will result in an increase in calcium influx and further increases in dopamine levels. The increased dopamine will cause oxidative stress through its oxidation, trigger mitochondrial dysfunction and ensure oligomerization of aSyn setting up a toxic loop with the net effect of causing cytotoxicity (Fig.19) (Mosharov et al., 2009; Venda et al., 2010).

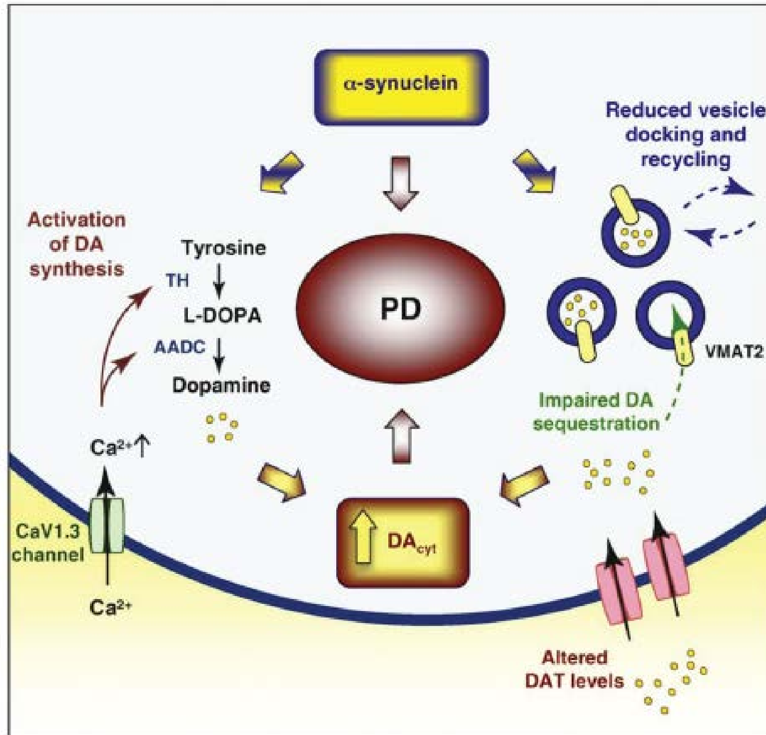


Figure 19: Shows the pathways for dopamine associated toxicity. Loss of aSyn function results in increased dopamine synthesis as does calcium influx. Loss of aSyn function also affects dopamine vesicle recycling which also results in increased cytosolic concentration. The increased dopamine facilitates neurodegeneration through its combined effect on aSyn oligomer stabilization and altered mitochondrial function (Venda et al., 2010).

ROLE IN MELANOMA

Uncontrolled proliferation is the defining feature of tumorigenesis and is seemingly at odds with a neurodegenerative disease such as PD which involves loss of post-mitotic neurons. The reactivation of cell cycle in neurons has been proposed as one of the mechanisms leading to cell death in PD. On the other hand, driving the cell cycle helps in the survival of tumor tissue. Although both these conditions are disparate, common susceptibility loci have been identified that appear to be involved in both these disease conditions. Some of the identified loci are already implicated in PD. The identification of cyclin E as a substrate for parkin and its increased levels in several tumors suggests that Parkin mutations may affect tumor growth through the

dysregulation of cell cycle proteins (Poulogiannis et al., 2010; Tay et al., 2010; Veeriah et al., 2010). Similarly DJ-1 has been found to regulate the tumor-suppressor PTEN (Kim et al., 2005). Another important candidate is aSyn which has been shown to drive tumor proliferation and the tumor cells appear to be able to take up extracellular aSyn (Israeli et al., 2011).

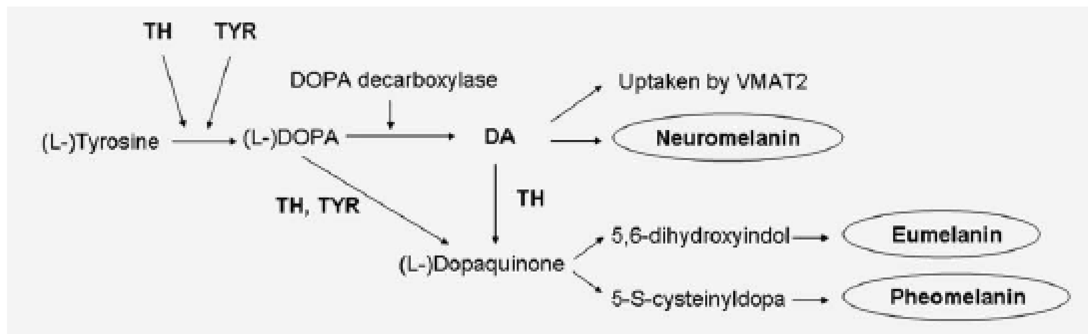


Figure 20: Represents the synthesis of neuromelanin and peripheral melanins. L-tyrosine is converted to L-Dopa which is converted to Dopmaine in neurons. Dopamine oxidation leads to neuromelanin. L-dopa is directly converted to Dopaquinone which is then converted to melanin in melanocytes (Pan et al., 2011)

Interestingly epidemiological studies show an increased association between PD and melanoma. Higher risk for PD in melanoma patients and a higher risk for melanoma in PD patients has been reported (Bertoni et al., 2010; Gao et al., 2009). As described earlier, neuromelanin in dopaminergic neurons can have both cytoprotective and cytotoxic roles in the cell. The common precursor for the synthesis of neuromelanin found in neurons and the melanin found in skin cells is L-Dopa (Fig.20). It has therefore been hypothesized that pigmentation related genes may play a role in both diseases given that aSyn is already known to play a role in regulating TH, key enzyme in L-Dopa synthesis (Herrero Hernández, 2009; Paisán-Ruiz and Houlden, 2010). A recent study also suggests the use of aSyn as a diagnostic marker for metastatic melanoma's given the high occurrence of aSyn in melanoma cells and tissues (Matsuo and Kamitani, 2010).

It is worthwhile to note that while melanoma incidence is reportedly high for PD patients, the risk for other cancers is low in PD patients emphasizing that melanoma and PD may share common pathogenic pathways (Paisán-Ruiz and Houlden, 2010).

Consistent with the above hypothesis, aSyn has been found to reduce melanin expression in melanoma cells probably through its inhibitory action on Tyrosinase enzyme, another key enzyme involved in the synthesis of melanin (Pan et al., 2012). However, the same study reported an increase in melanin content in neuronal cells SH-SY-5Y and PC12 due to increase in cytosolic dopamine content. It appears that although aSyn regulates pigmentation genes similarly in the neuronal and melanoma cells, they seem to have an opposing effect in each due to variations in the cellular environment. Phosphorylation of aSyn at serine129 is also reported to occur in melanoma cells and affects the localization of the protein in these cells. The phosphorylated aSyn is localized more towards the cell membrane and is found to be release into the extracellular space. The significance of the release of aSyn from melanoma cells and their possible effects of such release need further elucidation (Fig.21) (Lee et al., 2013a).

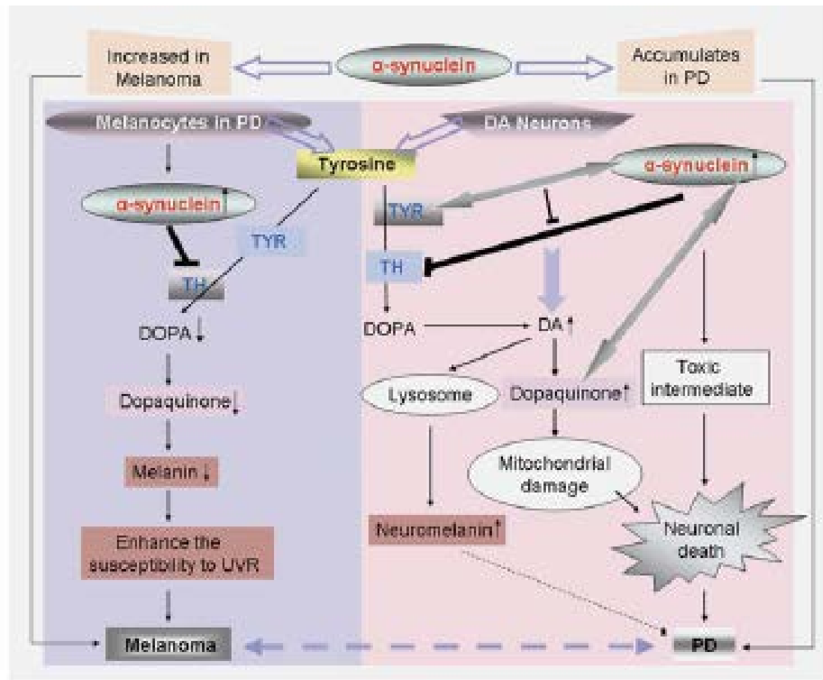


Figure 21: The mechanisms by which aSyn may act as a common mediator for PD and melanoma. Regulation of TH and Tyrosinase enzymes involved in dopamine and melanin synthesis lead to either increase in neuromelanin in neruons or the depletion of melanin in melanocytes which leads to pathogenesis in PD and melanoma respectively (Pan et al., 2011).

2.3 PROTEIN DEGRADATION PATHWAYS

There is a constant turn-over of proteins inside the cells although the turn-over rates of different proteins is different depending on their utility in the cells. A steady state of protein levels is therefore maintained in the cells by regulating the synthesis and degradation of proteins. Proteins are the functional units of the cells and are important for the various cellular activities. Any loss of protein function can be detrimental to cell survival while the accumulation of proteins beyond their functional role can also prove detrimental to cells.

The loss in protein function can occur as a result of mutations that alter its activity or due to decreased synthesis or increased clearance. Therefore although it may appear cumbersome, the cell strives to maintain a balance between synthesis and degradation.

Neurodegenerative diseases, most often presenting with inclusion bodies in the cell comprised of protein aggregates, represent scenarios which result out of an imbalance between synthesis and degradation. As discussed earlier, protein aggregation can result from a number of cellular events. Molecular crowding and increased concentrations of cellular levels of proteins are perhaps very definitive causes of aggregation. Mutations that increase the synthesis of proteins such as duplications and or polymorphisms in promoter regions have been reported as described earlier. In the absence of such mutations, failure in protein degradation mechanisms has been hypothesized as another important causative factor in the accumulation and aggregation of proteins. These aggregated proteins are unable to execute their physiological functions which results in a disruption of cellular activities.

The eukaryotic system has evolved two major pathways for the clearance of proteins. These are the Ubiquitin-Proteasome System (UPS) and the Autophagy-Lysosomal Pathway (ALP). There are other cytosolic proteases such as the Ca^{2+} dependent cysteine proteases called calpains, but the most UPS and ALP are most widely studies with significant implications in disease states.

Conventionally, the UPS has been thought to degrade short-lived regulatory nuclear and cytosolic proteins while ALP is involved in degradation of cytoplasmic proteins and organelles. However recently these roles have been challenged and new evidence suggest that a cross-talk may exist between these two pathways (Lecker et al., 2006; Matsuda and Tanaka, 2010; Rubinsztein, 2006). Protein degradation pathways play a critical role as quality control

mechanisms through the removal of functionally redundant proteins and misfolded proteins that under ordinary circumstances contributes towards cellular homeostasis.

UBIQUITIN-PROTEASOME SYSTEM (UPS)

The UPS occurs in a three step process and involves three different enzymes (Fig.22).

- Ubiquitin Activation: The ubiquitin (Ub) is composed of 76 amino acids with a glycine on its C-terminus that is used for the conjugation with the target proteins. It also contains internal lysine residues that are required for polyubiquitination. The lysine residues at position 48 and 63 are most often the sites for ubiquitination. But only the K48 ubqkuitination leads to proteasomal degradation. Ubiquitin does not occur freely and is present as a linear fusion either to itself or to some ribosomal protein subunits. These Ub-fusion proteins are deubiquitinated into Ub monomers for quick availability during cellular stress. These deubiquitination reactions serves two purposes (a) recycling of Ub (b) prevent accumulation of polyubiquitinated proteins. In this step, Ubiquitin activation enzyme is used (E1). An ubiquitin adenylate intermediate is generated through the hydrolysis of ATP. This intermediate is transferred to the active site with a Cysteine residue on the E1 enzyme and is covalently linked to the enzyme via a thiolester linkage along with the release of AMP. This process results in the activation of the terminal glycine in the C-terminus (Gly76). It appears that a single 110kD E1 enzyme is responsible for Ub activation required for all modifications.
- Ubiquitin Conjugation: This step involves a second set of enzymes called the Ubiquitin-conjugation enzymes or E2. In this step, the activated Ub is transferred to the active site with a Cysteine residue on the E2 enzymes. This is mediated by a transacylation reaction

and once again a thiolester bond is formed between the E2 enzyme and the activated Ub. More than 20 E2 enzymes have been identified in mammals. The E2's contain a conserved 130 amino acid catalytic core called a UBC domain, with a cysteine residue that mediates its binding to Ub. The E2's are subdivided and are recognized by their cognate E3's and therefore are critical for specificity.

- Ubiquitin Ligation: In this final step, the Ub is conjugated to a lysine residue on the target protein through an isopeptide bond formed with the terminal glycine on Ub. This step involves a third set of enzymes called the Ubiquitin ligases or the E3 enzymes. This last step again involves the transfer of Ub from the E2 to E3 enzyme with the formation of a thiol linkage between Ub and E3 enzyme. Once monoubiquitinated, more Ub moieties are added to generate a polyubiquitin chain. The Ub moiety forms a linkage between an internal lysine residue and the carboxy terminal glycine of the next Ub molecule. There are more than a 100 different E3 enzymes that catalyse the addition of Ub in a substrate-specific manner making the process tightly regulated. The recognition of the substrate by the E3 enzyme can occur in three ways (a) The target protein carries a degradation signal identified by the E3 enzyme (b) A modification of the sequence motif such as phosphorylation on the target protein (c) Modifications in the E3 enzymes itself.

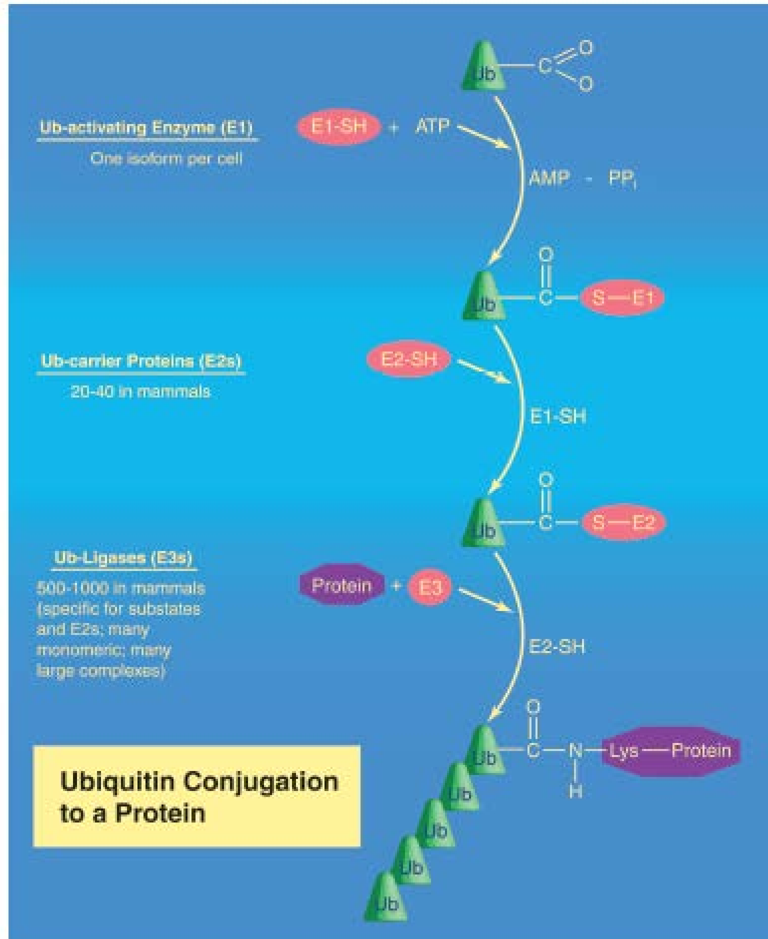


Figure 22: Represents the three steps involved in UPS. First Ub-activation by E1 enzymes, Second Ub-conjugation by E2 enzymes and lastly transfer of Ub to substrate by E3 enzymes. The monoubiquitination is followed by the addition of many Ub moieties to generate a poluubiquitin chain (Lecker et al., 2006)

Organization of the Proteolytic System: The 20s proteasome is very well conserved from bacteria to humans (Fig.23). It has a hollow and barrel shape and composed of four stacked heptameric rings forming a central chamber than runs through the stack. Each ring is composed of seven subunits, the alpha subunit forming the two outer rings and the beta subunit forming the inner two rings. The 20s proteasome is classified as an amino terminal nucleophile hydrolase wherein the Threonine in the amino terminal acts as the nucleophile catalyst. The eukaryotic proteasome has an additional 19s regulatory complex that acts as the base and lid of the 20s

complex, together forming the 26s holoezyme. The lid can recognize and bind ubiquitinated substrates and is also bound to deubiquitinating enzymes or the isopeptidases that catalyse the removal of Ub from the substrates. The base on the other hand has ATPase activity that is used to unfold the proteins and thread them into the core. Chymotrysin-like activity, Trypsin-like activity, post-glutamyl peptide hydrolysing activity, branched chain amino acid preferring and neutral amino acid preferring activity are some of the proteolytic activities associated with the 20s subunit.

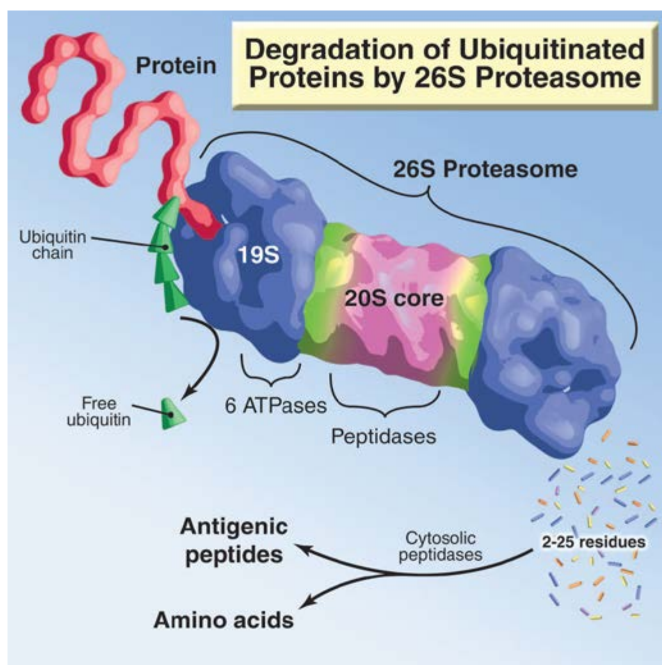


Figure 23: Represents the components of the 26s proteasomal subunit (Lecker et al., 2006).

The binding of polyubiquitinated proteins causes structural changes in the proteasome. Polyubiquitinated substrates enhance their degradation by facilitating gate opening and through allosteric activation of the peptidases. Unlike traditional proteases that cut the protein once and release the peptides, the proteasome degrades the substrates to generate very small peptides which exit the proteasome. These peptides are quickly broken down to amino acid and do not

last long in the cell. In some instances, the proteasome may only cleave a protein partially so as to enhance its function.

It must be noted that only polyubiquitinated proteins are degraded by the UPS while monoubiquitinated have other physiological functions in the cell and are not routed for degradation (Bedford et al., 2010; Lecker et al., 2006; Roos-Mattjus and Sistonen, 2004).

UPS AND NEURODEGENERATION

As discussed earlier, the UPS is involved in the clearance of short-lived proteins. It can also clear misfolded proteins in the endoplasmic reticulum. Misfolded proteins from the ER are translocated back to the cytosol where it is degraded by the proteasome. This process is knows as ER associated degradation or ERAD. A decreased proteasome activity has been reported in PD cases as well as models and also in Huntington's disease (HD).

The increase in oxidized proteins in PD brains compared to normal controls and the presence of ubiquitin positive Lewy bodies are the first indications of a UPS impairment relating to disease pathogenesis. A decreased proteasomal acvtivity has been reported in PD brains, particularly the SN*pc* compared to normal controls (Grünblatt et al., 2004; McNaught and Jenner, 2001; McNaught et al., 2003). The identification of a mutation in parkin, which acts as an E3 ligase, as a cause for autosomal recessive familial PD served as the strongest indicator for dysfunction of UPS in PD (Imai et al., 2000; Kitada et al., 1998). These observations were further strengthened by the identification of *UCHL-1*, an ubiquitin hydrolase, as a susceptibility factor in PD. The I93M mutation in the *UCHL-1* has been found to reduce its hydrolase activity *in vitro* (Leroy et al., 1998). The inhibition of proteasome by the CAG repeats seen in HD and the aSyn aggregates in PD further argue for a generalized role for UPS dysfunction in neurodegeneration (Bence et al., 2001; Tanaka et al., 2001a).

The precise mechanisms of UPS dysfunction are not yet known. The proteasome requires ATP for its fully functioning. Therefore age related changes such as increased oxidative stress and depletion of energy are thought to mediate UPS dysfunction. In support of this, the proteasome activity is found decrease in aged rodents compared to young rodents. Likewise pesticides such as maneb and rotenone that cause ROS generation were found to impair UPS (Betarbet et al., 2006; Keller et al., 2004). The addition of dopamine to mesencephalic cultures was found to inhibit proteasome (Zafar et al., 2007). Similarly proteasome inhibition in mesencephalic cultures led to dopaminergic loss (McNaught et al., 2002a; Rideout et al., 2005). Since the dopaminergic neurons are exposed to oxidative stress, it might explain the impairment of UPS in these cells (Lotharius and Brundin, 2002a). It has been reported that several stress factors including dopamine alter parkin solubility and mediate its aggregation or inhibit its enzyme activity (Chung et al., 2004; LaVoie et al., 2005; Wong et al., 2007). Important proteins whose steady states are maintained by the UPS such as p53 might mediate toxicity through their altered levels. In this regard, SH-SY-5Y cells treated with proteasome inihibitor MG-132 showed an increase in p53 accumulation and upregulation of pro-apoptotic proteins such as Bcl-2 was seen (Nakaso et al., 2004). This may provide a direct basis for the cell death. Proteasome inhibitor models have been generated to provide direct evidence to the role of UPS in neurodegeneration.

The use of inhibitors lactacystin, epoxomicin and PSI were found to result in progressive features of Parkinsonism with dopaminergic cell loss and formation of inclusion bodies positive for aSyn (McNaught et al., 2002b, 2004). However, while some groups have only been able to partially replicate these results (Schapira et al., 2006; Zeng et al., 2006), some other groups have not been able to replicate these results at all throwing into question the validity of these models (Bové et al., 2006; Kordower et al., 2006; Manning-Boğ et al., 2006). Similary, genetic models

with of *parkin*-null mice have not been very promising either. The inactivation of *UCHL-1* in mice also has not lead to PD symptoms although it is found to cause axonal dystrophy. However moderate success has been achieved in terms of dopaminergic cell loss and locomotor defects in *parkin*-null flies.

Although several substrates have been identified for parkin, their relevance is yet to be ascertained. It also appears that parkin can mediate ubiquitin-independent degradation and it can mediate attachment of Ub at K63 which does not route the substrate for degradation at all. *In vitro* studies also demonstrate the ability of parkin to mono-ubiquitinate its substrates. Additionally, parkin has been found to co-localize to mitochondria where it regulates the fission-fusion of mitochondria through autophagy. Therefore ubiquitination of substrates by parkin may not necessarily relate to the UPS. Additionally, the role in UCHL-1 as a risk factor for PD remains inconclusive. Studies from inhibitor and genetic models for proteasome inhibition and the wider role of parkin not associated with the UPS makes it impossible to clearly associate UPS dysfunction with neurodegeneration and remains to be seen if it is a consequence of pathogenesis (Lim, 2007; Matsuda and Tanaka, 2010).

AUTOPHAGY LYSOSOMAL PATHWAY (ALP)

The word autophagy was first used in 1963 by Christian De Duve who was awarded the Nobel Prize for his work on lysosome. Autophagy is derived from Greek with 'autos' meaning 'self' and 'phagy' meaning 'eat'. It encompasses broadly the phenomenon wherein cytoplasmic target material is transported to the lysosome for degradation. Autophagy can be divided into three forms- (a) Macroautophagy which involves the engulfing of target material into double membrane vacuoles that then fuse with lysosomes (b) Chaperone mediated autophagy (CMA)

where select material is translocated into the lysosome by recognizing a target pentapeptide motif with a consensus sequence similar to KFERQ. Chaperones aid in the target recognition and unfolding of the proteins to be degraded. The translocation is thought to be mediated by lysosomal protein LAMP-2a and is thought to be rate limiting. (c) Microautophagy which involves the direct engulfment of the target by the invagination of the lysosomal membrane. This is a poorly understood mechanism (Fig. 24) (Yen and Klionsky, 2008).

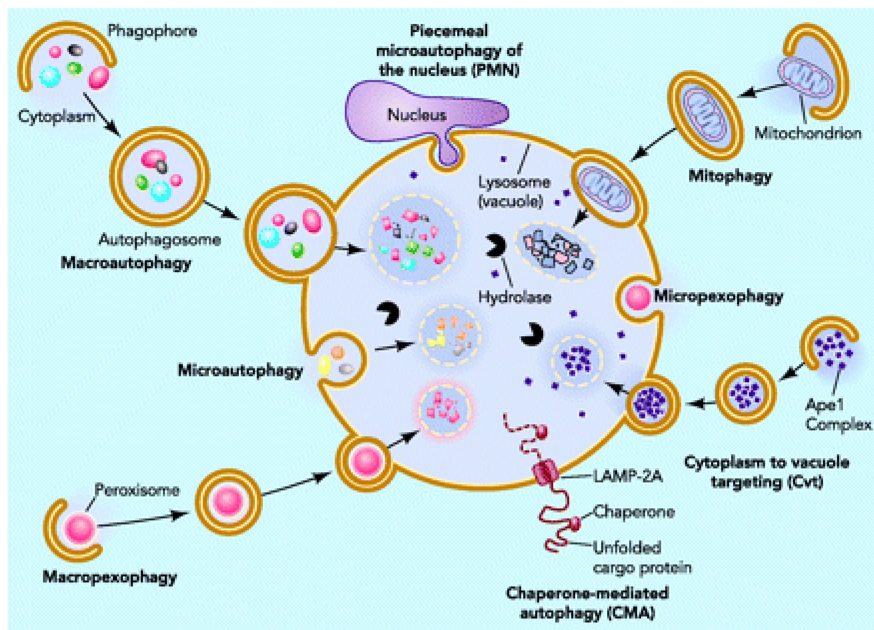


Figure 24: Represents the three forms of autophagy- macroautophagy, chaperone mediated autophagy and microautophagy (Yen and Klionsky, 2008).

The best studied of these is the macroautophagy, which as a bulk degradation pathways, plays a very critical role in maintaining cellular homeostasis. Therefore the word 'autophagy' is used to refer to the process of macroautophagy. Macroautophagy is also strongly associated with neurodegeneration and therefore will be described in detail here. Autophagy occurs under basal conditions but can be elevated under conditions of duress. Starvation is perhaps the best studied scenario during which autophagy is enhanced. The break down products of autophagy during starvation is used to salvage the cell from stress and therefore is thought to promote cell survival.

In addition to its role in maintaining cellular homeostasis, autophagy is important for clearance of misfolded proteins and aggregates, removal of damaged organelles and pathogenic bacteria. It also plays a role in early embryonic development.

The occurrence of autophagy in yeast made it an ideal model to study the mechanism of autophagy particularly since it is conserved from yeast to humans. More than 30 different genes regulating autophagy have been identified in yeast (termed Atg genes) and a number of human orthologs have also been identified (Ravikumar et al., 2010).

MECHANISM OF AUTOPHAGY (Fig.25)

- Initiation of autophagosome membrane: The first step is the initiation of formation of a double membrane vesicle called the 'phagophore'. The site for vesicle formation is called the phagophore assembly site (PAS) in yeast while in mammals they are formed randomly. The source of the membrane from which the autophagosome arises has not yet been determined. It can arise de novo or from membranes of ER. Autophagosomes are frequently formed in the vicinity of ER and an accumulation in ER associated autophagic vacuoles was seen when maturation of the pre-autophagosomal structures was blocked. Mitochondria and plasma membrane have also been reported as possible contributors to the formation of the autophagosome. The formation of these autophagosomes requires the activity of Class III PI3kinase, Vps34. The product of Vps34, phosphatidyl-inositol-3-phosphate (PI3P), has been shown to co-localize with early autophagosome markers although the precise role of PI3P is still not known. Vps34 is part of a large complex which consists of Beclin1/Atg6, barkor/Atg14 and p150/Vps15. The GTPase Rab5 and Beclin1 activate Vps34. However several proteins have been identified to interact with

Beclin1 other than Vps34. These include ambra-1, UVRAG and bif-1 which induce autophagy while the anti-apoptotic proteins, Bcl-2 or Bcl-xl inhibit autophagy. Another macromolecular complex, FIP200-ULK1/Atg1 is also involved in the initiation of autophagosome formation. The interaction between ULK1/Atg1 and FIP200 is mediated by Atg13.

- Elongation**:** The elongation of the phagophores requires further inputs of membranes from other organelles. The protein Atg9 which is a transmembrane protein cycels between the trans-Golgi and endosomes and perhaps helps to carry membrane for the elongation of the phagophore. Two ubiquitin like reactions occur at this stage. Atg12 is covalently tagged to Atg5. This process involves the activation of Atg12 by Atg7 following which it is transferred to Atg 10. It is then transferred to Atg5 and a covalent bond formed between the glycine residue on Atg12 and lysine residue on Atg5. The Atg12-Atg5 complex is then conjugated to Atg16L1 which forms a large800kDA complex. This complex formation is essential for elongation and is dissociated once the autophagosome is fully formed. A second reaction involves the microtubule associated protein 1 light chain 3 (LC3/Atg8). The precursor LC3 is cleaved at its carboxy terminus by the protease Atg4b which results in the cytosolic form LC-3-I. The LC3-I is conjugated to phosphatidylethanolamine (PE) via the action of Atg7 and Atg3 to finally form LC3-II which then localizes to the elongating autophagosome membrane. However unlike the Atg5-Atg12-Atg16L1complex, LC3-II continues to remain bound to the membrane even after maturation. The proteins listed above can interact with each other such that the Atg5-Atg12-Atg16L1 complex can conjugate LC3-I to PE, Atg10 can also interact with LC3 and Atg3 is found to co-localize to Atg12 and enhance Atg5-Atg12

complex formation. LC3 mediates the final fusion of doubled membrane cups to form fused vesicles. LC3-II on the cytoplasmic side can be delipidated and recycled back by the action of Atg4. The internalized LC3 is however degraded once the autophagosome fuses with the lysosome.

- Maturation and Fusion: The autophagosome move bidirectionally along the microtubule but have a preference for the Microtubule organizing center (MTOC). The lysosomes are enriched at the MTOC. The autophagosome fuse with the endosomes and then with the lysosomes which involves several proteins including ESCRT, SNAREs, Rab7 and class C Vps proteins. The beclin1 interacting protein UVRAG promotes fusion by recruiting machinery on the autophagosome and this function does not require its interaction with beclin1. UVRAG recruits the class C Vps protein which in turn activates Rab7. This promotes the fusion of late endosomes with the lysosomes. Rubicon, another Beclin1 interacting protein also functions in the maturation of the autophagosome. It therefore appears that Beclin1 and its interacting partners have different effects on autophagy depending on the type of interaction and the process they affect. The fusion is also dependent on the acidification of the lysosome. Drugs that inhibit the lysosomal H^+-ATPases which affect the acidity in the lysosome prevent fusion.

However Atg5/Atg7 independent mechanisms have also been reported involving the Ulk1 and Beclin1 proteins. This process occurs in a Rab9 dependent manner (Glick et al., 2010; Ravikumar et al., 2010).

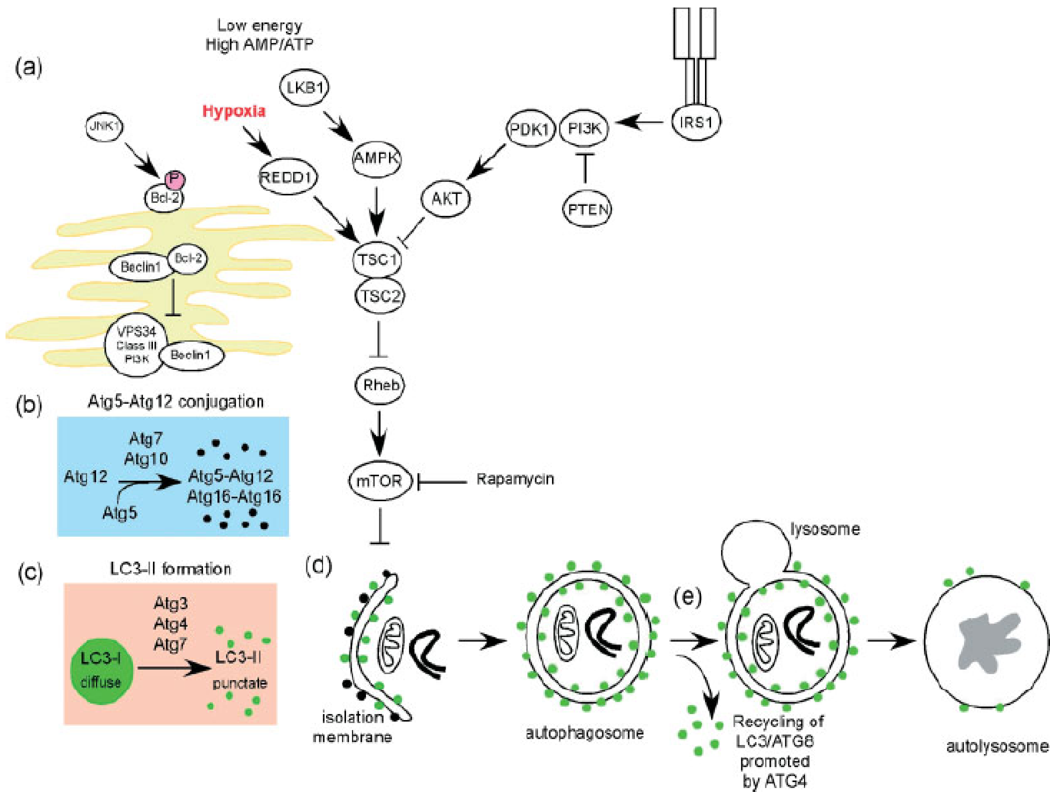


Figure 25: Represents the steps involved in the formation, elongation and maturation of the autophagosome (Glick et al., 2010).

Selective Autophagy

Autophagy, as bulk degradation, has been considered to be a non-selective process. However the degradation of specific organelles and proteins suggest that it can be selective too. The degradation in an ordered manner of various proteins under conditions of starvation suggests that even bulk degradation may proceed selectively and not randomly. The selectivity can be mediated either by making the protein available for engulfment through posttranslational modification or by decreasing the availability of the protein. The degradation of various organelles occurs via autophagy and is specific for each organelle such as ER-phagy for degradation of ER, pexophagy for degradation of peroxisomes, mitophagy for degradation of

mitochondria and so on. Macrolipophagy or the degradation of lipids and xenophagy or degradation of pathogens has also been described.

The degradation of organelles is mediated by specific signals. Mitochondrial permieability transition (MPT) involves a loss of membrane potential through the formation of permeability transition pore which is a non-selective large-conductance inner mitochondrial channel. The opening of this channel results in depolarization and outer membrane rupture. Drugs that inhibit MPT prevent mitophagy. Inhibition factor 1 (IF1,) a nuclear DNA encoded protein is shown to mediate mitophay in HeLa cells. The reticulocyte maturation and development of sperm are physiological events where mitophagy occurs. The Bcl-2 related protein Nix has been recently shown to bind LC3 and along with Ulk1 is required for mitochondrial clearance in reticulocyte. Similarly, the removal of dysfunctional mitochondria is mediated by the binding of parkin to mitochondria through its interactions with PINK1. Mutations in both these genes cause recessive forms of familial PD. The fission-fusion of mitochondria alters the relative sizes of the mitochondria which influences its degradation by autophagy where smaller mitochondria are more likely to be engulfed by autophagosomes than the larger mitochondria. The ubiquitination of cytosolic facing proteins in peroxisomes which may then be recognized by the cargo protein p62 is thought to mediate pexophagy. ER stress due to accumulation of unfolded proteins results in the unfolded protein response (UPR) which in turn causes the ER volume to increase. A return to ER homeostasis is mediated by ER-phagy which may be activated in response to the ER stress but the selectivity of the process has not clearly been demonstrated.

Some of the proteins involved in selective autophagy as identified in yeast include Atg 19 and Atg20 for ER-phagy, Uth1, Aup1 and Atg32 in mitophagy, PEX3 and PEX14 in pexophagy and Ubp3p/Bre5p in ribophagy. Glucagon induced glycogen autophagy via the elevation in cAMP

levels is another example of selective autophagy. Given that autophagy is a basal pathway which degrades a wide variety of substrates, it is difficult to identify the proteins or markers that mediate selectivity and several of the orthologs of the yeast proteins are yet to be identified in mammals. (Ravikumar et al., 2010)

SIGNALLING PATHWAYS IN AUTOPHAGY

- mTOR Pathway: mTOR pathway is the most widely pathway in the regulation of mammalian autophagy (Fig.26). mTOR plays diverse roles in the cell including intitiation of translation, cell growth, proliferation, ribosome biogenesis, transcription, cytoskeletal reorganization, long-term potentiation and autophagy. It is the mammalian ortholog of the yeast protein TOR that has been shown to negatively regulate autophagy. mTOR has two functional complexes mTORC1 and mTORC2. mTORC1 consists of the catalytic unit of mTOR, regulatory associated protein of mTOR (raptor), G protein β-subunit like protein (GβL) and proline rich Akt substrate of 40kDa (PRAS40). mTORC2 consists of rapamycin sensitive companion of mTOR (rictor), GβL, SAPK-Interacting protein1 (SIN1)and protein observed with rictor (PROTOR). The mTORC1 regulates autophagy while mTORC2 is not directly related to autophagy regulation. Growth factors, amino acids, glucose, energy status, stress are among the various factors that regulated mTORC1. Rapamycin can inhibit mTORC1 and therefore is a potent inducer of autophagy. The formation of a complex between rapamycin and FK506 binding protein (FKBP12) stabilizes the raptor-mTOR association with the net effect of inhibiting the mTOR kinase activity. This inhibition prevents downstream phosphorylation of ribosomal protein S6 kinase (S6K1 or the p70S6K) and the translation initiation factor 4E

binding protein-1 (4E-BP1). However p70S6K is a positive regulator of autophagy such that its activation for a limited time allows for maximal autophagy but the loss of activity prevents excessive autophagy. Recent studies have shown that rapamycin only incompletely inhibits the mTORC1 and Torin-1 is a much more potent inhibitor of the mTORC1. Torin-1 on the other hand inhibits both the mTORC1 and mTORC2 but its effects on autophagy are primarily due to its inhibition of mTORC1. Starvation also results in the stabilization of raptor-mTOR complex and therefore induces autophagy. Since the end result autophagy is the release of amino acids, these activate mTORC1 and inhibit autophagy. mTORC2 which phosphorylates and activates Akt, is insensitive to rapamycin but prolonged exposure to rapamycin can inactivate mTORC2 as well. Some of the components downstream of mTOR in mammals have been identified. ATg13 binds to Atg1 homologs ULK1/ULK2 which then forms the ULK1/2-Atg13-FIP200complex. Activation of mTOR causes it to bind to this complex and inhibit the kinase activity of Atg13 and ULK1 while inactivation of mTOR causes it to dissociate from the complex allowing it to activate autophagy.

- PI3K pathway: mTORC1 is controlled by the PI3K pathway. Binding of growth factors to cell surface activated ClassI PI3K. The regulatory subunit of class I PI3K activates its p110 catalytic subunit which then converts plasma membrane lipid phosphatidylinositol-4,5-bisphosphate (PIP_2) to phosphatidylinositol-3,4-5 triphosphate (PIP_3). This leads to activation of several kinases such as serine/threonine kinase, phosphoinositide-dependent kinase 1(PDK1) and Akt/PKB. The activation of Akt is mediated by its phosphorylation at Ser473 by rictor-mTOR which in turn facilitates phosphorylation by PDK1 at Thr-308. The activation of Akt inhibits autophagy. Therefore activation of class I PI3K inhibits

autophagy by activating Akt. The inhibition of Class I PI3K by PTEN, a tumor suppressor, induces autophagy. The class III PI3K activation results in the increase of PI-3-P which activates autophagy. Akt activation by phosphorylation results in the phosphorylation of a host proteins which include the tumor suppressor proteins mutated in tuberous sclerosis, a complex called the tuberous sclerosis complex (TSC) which is a heterodimer consisting of TSC1 and TSC2. TSC1/2 acts as an upstream integrator of mTOR signalling such that several kinases signal to mTOR via the phosphorylation of TSC1/2.

- Other Protein Kinases: AMPK inhibits autophagy by phosphorylating TSC2. IκB kinase (IKK) induces autophagy via activation of AMPK but does not involve the NFκB. Eukaryotic translation initiation factor 2α (eIF2α) kinases regulate stress induced autophagy. Eukaryotic elongation factor 2 (eEF2) kinase is a calcium/calmodulin dependent kinase III that regulates autophagy via the mTOR pathway. Mitogen activated kinase (MAPK)/extracellular signal regulated kinase (ERK) is another regulator autophagy via the mTOR pathway. C-terminal Jun NH_2 terminal kinase 1(JNK1) mediates starvation induced autophagy by phosphorylating Bcl-2 at multiple sites which disrupts the Bcl-2-Beclin1 interaction and triggers autophagy.

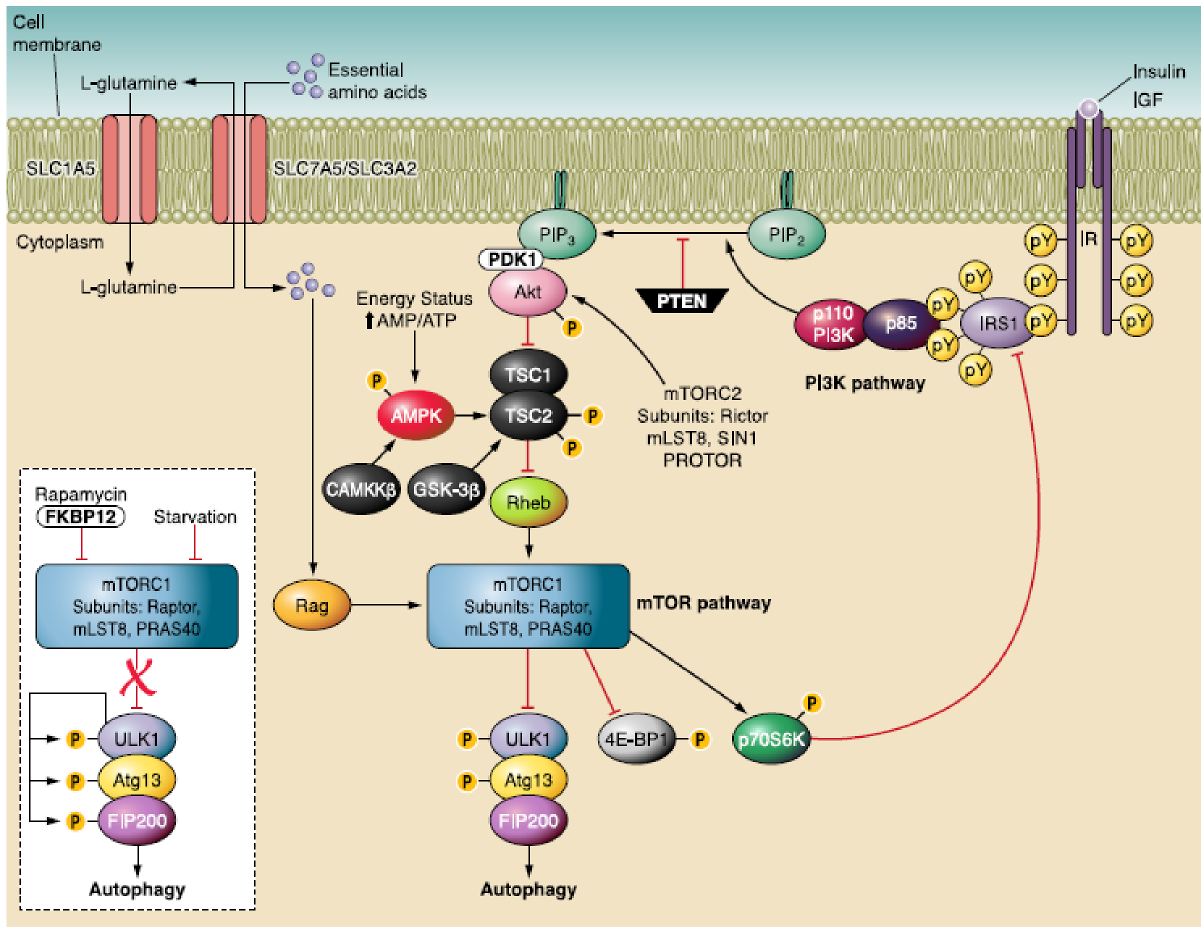


Figure 26: The mTOR pathway of autophagy signalling (Ravikumar et al., 2010)

- mTOR Independent pathway: Several pathways independent of mTOR activity have also been described (Fig.27).

    1. Phosphoinositol signalling pathway: This pathway stimulated by G-protein coupled receptor results in activation of phospholipase C (PLC) which converts PIP_2 to IP_3. IP3 binds to receptors on ER causing the release of calcium into the cytoplasm. IP_3 is degraded to release IP_1 which is then hydrolysed to free inositol required for the phosphoinositol signalling. The reduction in free inositol levels leads to activation of autophagy.

2. cAMP-Epac-PLC-ε-IP_3 pathway: Reduction in cAMP levels leads to induction of autophagy. This induction is mediated via the effects of cAMP on Epac. Rap2B which is further downstream of Epac also regulates autophagy. PLC-ε is a Rap2B effector that increases the IP_3 levels which results in accumulation of autophagic substrates and is thought to result in autophagy inhibition.
3. Ca^{2+}- calpain pathway: Increased calcium levels negatively regulate autophagy. Therefore the reduction if IP_3 levels as discussed earlier causes an activation of autophagy since IP_3 is involved in release of stored calcium. Calcium increases causes activation of a class of cysteine proteases called calpains. Calpains cleave the α-subunit of the G proteins which activates these proteins. The activation of G proteins in turn activates adenylyl cyclase which results in increase in cAMP levels. This results in inactivation of autophagy.

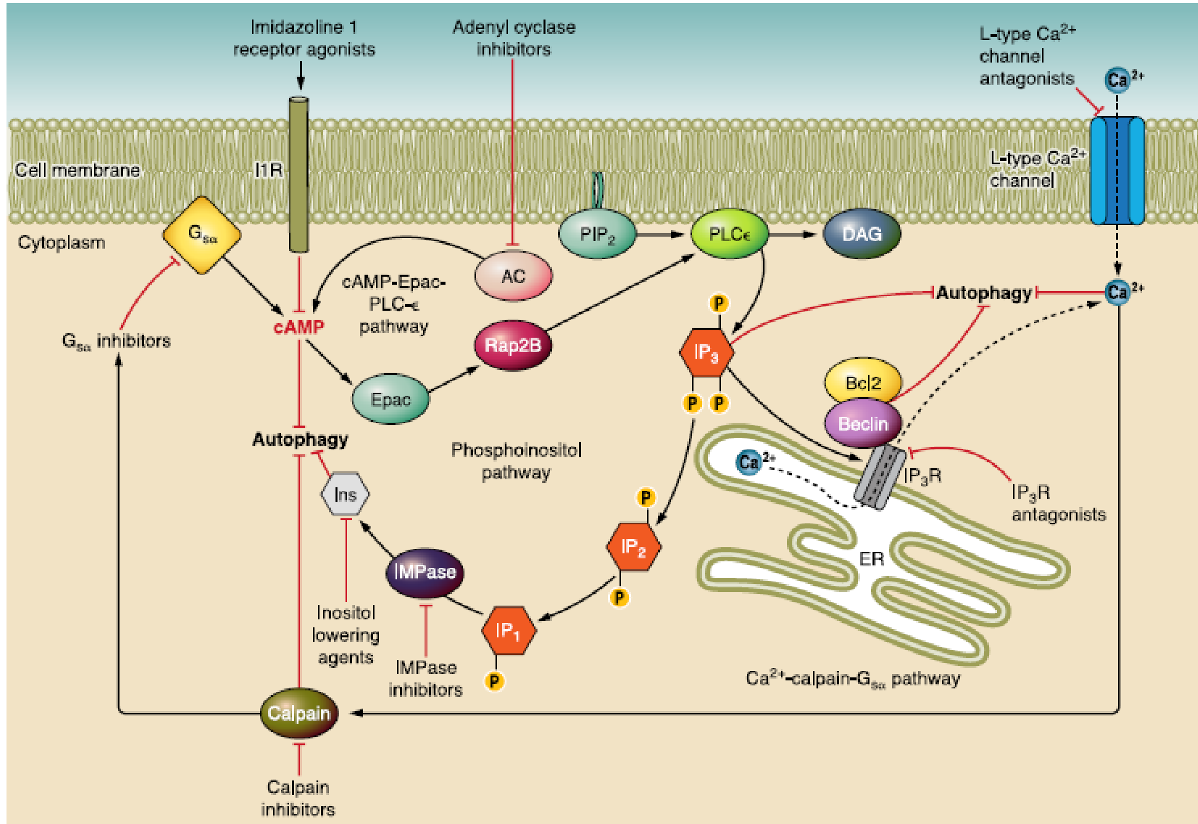


Figure 27: mTOR independent signalling pathways in autophagy (Ravikumar et al., 2010)

Ceramide is a product of sphingolipid metabolism and has been shown to activate autophagy. Ceramide treatment results in downregulation of nutrient transporters in mammalian cells and interferes with the uptake of amino acids and thereby activates autophagy. Ceramide can also activate phosphoprotein phosphatase 2A (PP2A) which inhibits Akt activation and thus activates autophagy. Ceramide has also been shown to dissociate the Bcl-2-Beclin1 complex and can result in activation of autophagy. Sphingosine kinase1 (SphK) also activates autophagy by directly modulating mTOR independent of class I PI3K. (Ravikumar et al., 2010)

Autophagic Cell Death

Although largely a cytoprotective process, several lines of evidence suggest that autophagy can act as a cell death pathway and is referred to as the Programmed Cell death Pathway II. The increase in autophagosomes in dying cells has led to idea of autophagic death although an

increase in autophagosome number could also result from an impaired fusion between the autophagosomes and the lysosomes. Beclin1 null *C.elegans* and mice lacking Beclin1 or Atg5 on the other hand also show increased cell death. Uncontrolled autophagy leading to excessive degradation has been proposed to cause to cell death. The disruption of Bcl-2 Beclin1 complex leads to excessive autophagy and death. Drosophila overexpressing Atg1 also shows excess autophagy. However the possibility exists that some of the autophagy proteins may mediate autophagy independent functions in the cell that are responsible for the cell death. However chemical inhibitors of autophagy have been shown to prevent cell death. Autophagic cell death is also seen in cases where the apoptosis machinery is ineffectual. Cross talk between apoptosis and autophagy has also been reported to occur. Death associated protein (DAPK) family members and DAPK related kinase induce autophagy in cells resistant to apoptosis. Bcl-2 which interacts with Beclin1 is a potent inhibitor of apoptosis. Tumor suppressor p53 can also activate autophagy inducing genes and autophagy can be induced in a p53 dependent manner. Atg5 truncation by calpains can inhibit the anti-apoptotic property of Bcl-xL by disrupting its complex with Bax. The Bax oligomerization as a result of this disruption causes caspase activation which in turn reduces autophagosome synthesis. Despite recent evidence on favour of autophagic cell death and its cross talk with apoptosis, the mechanism and role of autophagic cell death remains unclear. (Chen and Klionsky, 2011; Glick et al., 2010; Levine and Yuan, 2005; Maiuri et al., 2009; Mizushima, 2007; Ravikumar et al., 2010; Yang and Klionsky, 2010, 2009)

AUTOPHAGY IN NEURODEGENERATION

As discussed earlier, the changes in protein conformation that drives it towards misfolding and aggregation are quickly addressed by the cell. The protein misfolding maybe corrected through

the action of chaperones and when such measures fail, the misfolded protein is removed the cell. These quality control measures to a large extent prevent the onset of diseases which result from dysfunctional proteins, most notable the neurodegenerative diseases. As stated previously, failure in any of these quality control mechanisms can prove costly for the cell (Fig.28). Autophagy as a bulk degradation mechanism involved not just in clearance of proteins but also damaged organelles and in the maintenance of homestasis, perhaps plays the most critical role.

Altered proteins, recognized by the chaperones are most often preferentially routed for degradation either via the UPS or CMA. However the requirement in either condition is that the protein remains unfolded. Once the proteins are folded into oligomers or fibrils, these are sequestered into the autophagosomes and are targeted for degradation by macroautophagy. Studies have demonstrated the ability of macroautophagy to clear away aggregates. The precise mechanisms that govern this degradation are unknown but it is likely that failure of other degradation pathways activates the macroautophagy. Experimentally it has been shown that blocking of either the UPS or CMA is accompanied by activation of macroautophagy. However, recent studies demonstrate that this clearance of aggregated proteins is not merely a compensatory mechanism but even under basal conditions, macroautophagy is constantly working to remove misfolded proteins. The failure then of this pathway has been shown to trigger accumulation in neurons. Additionally, it is important that the pathway be actively maintained during the compensatory stage and the failure of this pathway to sustain at the compensatory stage also leads to further accumulation.

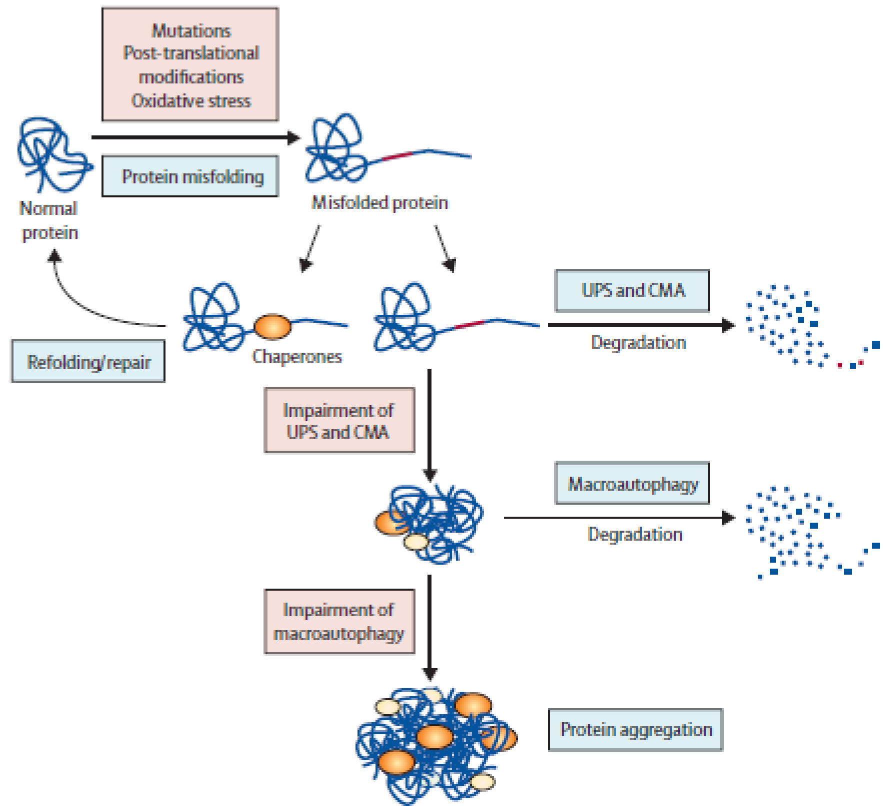


Figure 28: Represents the various quality control pathways in the cell (Martinez-Vicente and Cuervo, 2007).

Evidence for the role of autophagy has come for the study of several neurodegenerative diseases. The upregulation of ALP is found to be an early even in Alzheimer's disease and is seen even before the deposition of amyloid fibrils. These early events result in lysosomal proliferation and an increase in lysosomal enzymes that work to remove misfolded proteins and aggregates. However as the disease progresses, the efficiency of ALP is found to decrease which affects the degradation of other cellular components. Accumulaion of autophagic vacuoles containing the targets are observed. The vacuoles either fail to fuse with the lysosomes or fail to be degraded despite the fusion. The accumulation of these vesicles results in an impairment of intracellular trafficking and over time the leakages from the accumulated vesicles cause cytotoxicity. These vacuoles containing the proteins and proteases, in due course, result in truncations that promote

further misfolding, thus turning into sites for aggregation. The huntingtin protein with the polyglutamine repeats that aggregates in Huntington's disease (HD) has been shown to inactivate UPS. The activation of macroautophagy in response to aggregate formation has also been reported. The sequestration of negative regulators of autophagy, particularly the mTOR by the huntintin aggregate is thought to be one of the mechanisms through which autophagy activation occurs in HD. The activation of macroautophagy has been shown to improve the symptoms in mouse models of HD further supporting the role of autophagy in neurodegeneration. Additionally, the oxidative stress generated during pathogenesis also stimulates autophagy.

Autophagic failure therefore can be at two levels. Failure in autophagy induction may act as the triggering event for protein aggregation. Failure in the fusion of autophagic vacuole as a result of lysosomal dysfunction sustains the aggregation process in the cell and may occur. Likewise activation of excessive autophagy can lead to neuronal death if the neurons are incapable of supplementing the degraded organelles with functional ones. Additionally the cross talk between autophagy and apoptosis can also tilt the balance towards autophagic cell death. The factors that stimulate autophagy during compensatory activation and factors that mediate their subsequent failure are not clearly known.

The evolvement of neuroprotective strategies therefore depends on identifying the stage of autophagic failure i.e, induction or fusion as also to determine conditions that mediate excessive autophagy. The identification of factors that modulate autophagy at various stages can also be targeted for devising neuroprotective treatment modalities.(Cherra and Chu, 2008; Jaeger and Wyss-Coray, 2009; Martinez-Vicente and Cuervo, 2007; Nixon, 2006; Rami, 2009)

UPS AND AUTOPHAGY: CROSS TALK

It has long been believed that UPS degrades short- lived proteins while autophagy is involved in the degradation of long-lived proteins. Recent studies demonstrate that under certain conditions, the two pathways may communicate. The UPS maybe involved in the degradation of long-lived proteins (Fuertes et al., 2003a) and autophagy in the degradation of short-lived proteins (Fuertes et al., 2003b; Qing et al., 2006). An increasing number of targets have been identified that can be degraded by both ALP and UPS (Cuervo et al., 1998; De Domenico et al., 2006; Kidane et al., 2006; Webb et al., 2003a). As discussed earlier, the failure of UPS can result in compensatory activation of ALP and this is particularly true for aggregated proteins (Ding et al., 2003, 2007; Rideout et al., 2004). The activation of JNK1 following proteasome inhibition would lead to phosphorylation of Bcl-2 which in turn would disrupt the Bcl2-Beclin1 complex leading to autophagy activation (Meriin et al., 1998; Pattingre et al., 2005; Wei et al., 2008). However activation of proteasome under conditions of impaired autophagy does not seem to occur. On the other hand impairment of autophagy can also lead to impairment of proteasome(Korolchuk et al., 2009; Qiao and Zhang, 2009) . This inhibition is mediated by the protein p62, which is an ubiquitin binding protein and an autophagy substrate. The impairment of autophagy results in p62 accumulation which in turn compromises UPS by sequestering the proteins away from other ubiquitin binding proteins and delaying their delivery to UPS (Bjørkøy et al., 2005; Ravikumar et al., 2010). During muscle atrophy, upregulation of both ALP and UPS via the action of transcription factor Fox03 which regulates these pathways independently (Zhao et al., 2007). It appears that polyubiquitination can target proteins to both the autophagy and UPS. Some of the proteins that bind ubiquitin and target substrates towards autophagy include p62, HDAC6, NBR1 protein (Iwata et al., 2005; Kirkin et al., 2009a, 2009b; Pandey et al., 2007). A recent study also

reports that a switch between UPS and autophagy maybe mediated by BAG1 and BAG3. A switch from BAG1 to BAG3 will favour degradation via autophagy (Gamerdinger et al., 2009). The cellular conditions and factors that favour either of the degradation pathways and especially the role of polyubiquitination as a signal for autophagy needs further elucidation.

DEGRADATION OF ALPHA SYNUCLEIN

A major precipitating factor in the aggregation of aSyn is the increased levels of the protein. The cells therefore try to strike a balance between synthesis and degradation in order to maintain constancy in the intracellular protein levels. A discussed earlier, failure in protein degradation pathways leads to accumulation of proteins resulting in cytotoxicity. The pathways for aSyn degradation under physiological conditions and their failure leading to aggregation have been widely studied. Both UPS and ALP have been implicated in the degradation of aSyn (Webb et al., 2003b). Apart from these two major proteolytic systems, calpains (Dufty et al., 2007), neurosin (Iwata et al., 2003), metalloproteinases (Levin et al., 2009)also play a role in aSyn processing (Fig.29). As discussed earlier, the processing of aSyn by these other proteolytic pathways leads to truncation of aSyn which contributes to aggregation. Additionally, these proteolytic systems can process extracellular aSyn which enhances toxicity (Sung et al., 2005).

The identification of parkin as a cause of familial PD and UCHL1 as a risk factor were the strongest proponents for aSyn being a substrate for UPS. The accumulation of polyubiquitinated aSyn in neuronal cells upon proteasomal inhibition supported the possibility of aSyn being a substrate for UPS (Imai et al., 2000; McLean et al., 2001; Webb et al., 2003b). However studies demonstrate that aSyn can be degraded via the proteasome even in absence of ubiquitination (Tofaris et al., 2001). It appears that UPS may play a more critical role in the generation of

phosphorylated aSyn since ser129 phosphorylated aSyn is rapidly turned over by the proteasome in addition to causing dephosphorylation of aSyn (Machiya et al., 2010). Several studies since have failed to demonstrate the clearance of aSyn by the UPS as the proteasome inhibition did not lead to accumulation of aSyn (Ancolio et al., 2000; Xilouri et al., 2012b). Additionally it has been found that while small oligomers maybe degraded by UPS, these also have the potential to cause proteasomal impairment (Emmanouilidou et al., 2010b). The occurrence of intraneuronal LB like inclusions staining positive for aSyn and ubiquitin upon conditional ablation of the Psmc1 subunit of the 26s proteasome in *in vivo* conditions supports the role for UPS in aSyn degradation (Bedford et al., 2008). Another *in vivo* study also suggests that aSyn degradation is dependent on the protein burden inside the cell. Endogenous aSyn is preferentially targeted by the UPS while under pathogenic conditions aSyn is degraded by ALP indicating that the protein levels in themselves mediate the cross talk between ALP and UPS (Ebrahimi-Fakhari et al., 2011). The co-localization of CHIP to aSyn and Hsp70 is LB's from human brains led to possibility of CHIP acting as a molecular switch between UPS and ALP (Shin et al., 2005). Monoubiquitination of aSyn by SIAH has also been demonstrated to increase the aggregation of aSyn (Lee et al., 2008b; Liani et al., 2004) with USP9X deubiquitinase mediating deubiquitination of aSyn both *in vitro* and *in vivo* (Rott et al., 2011). Monoubiquitination may favour proteasome while deubiquitination of aSyn favours its degradation almost exclusively by autophagy. Membrane associated aSyn may be degraded by the endosomal pathway mediated by E3 liagse Nedd4 while oligomers are degraded by autophagy (Tofaris et al., 2011).

The accumulation of aSyn with lysosomal inhibition and not proteasomal inhibition, location of aSyn in lysosomal compartments of neuronal cells and the attenuation of degradation of mutant aSyn upon macroautophagy inhibition supported a role for ALP in aSyn degradation (Paxinou et

al., 2001; Webb et al., 2003a). Enhancement of macroautophagy by rapamycin was observed to promote wild type and mutant aSyn clearance in cultured cells while overexpression of Beclin1 in mouse models reduced the aSyn related neuropathological changes (Malagelada et al., 2010; Spencer et al., 2009). Lysosomal inhibiton has been shown to increase the levels of oligomeric aSyn as well as endogenous and overexpressed wild type aSyn. However the use of different inhibitors has produced some discrepancies. The use of bafilomycin as lysosomal inhibitor caused accumulation of only the oligomeric species but not of the bulk monomeric species. In the same study, the use of a specific macroautophagy inhibitor 3-methyladenine produced no such effects. However a different study with 3-methyladenine has been able to show an accumulation of aSyn (Lee et al., 2004). It also appears that 3 methyladenine impairs degradation of only the mutant aSyn without affecting the wild type while general lysosmal impairment affects both (Alvarez-Erviti et al., 2010).

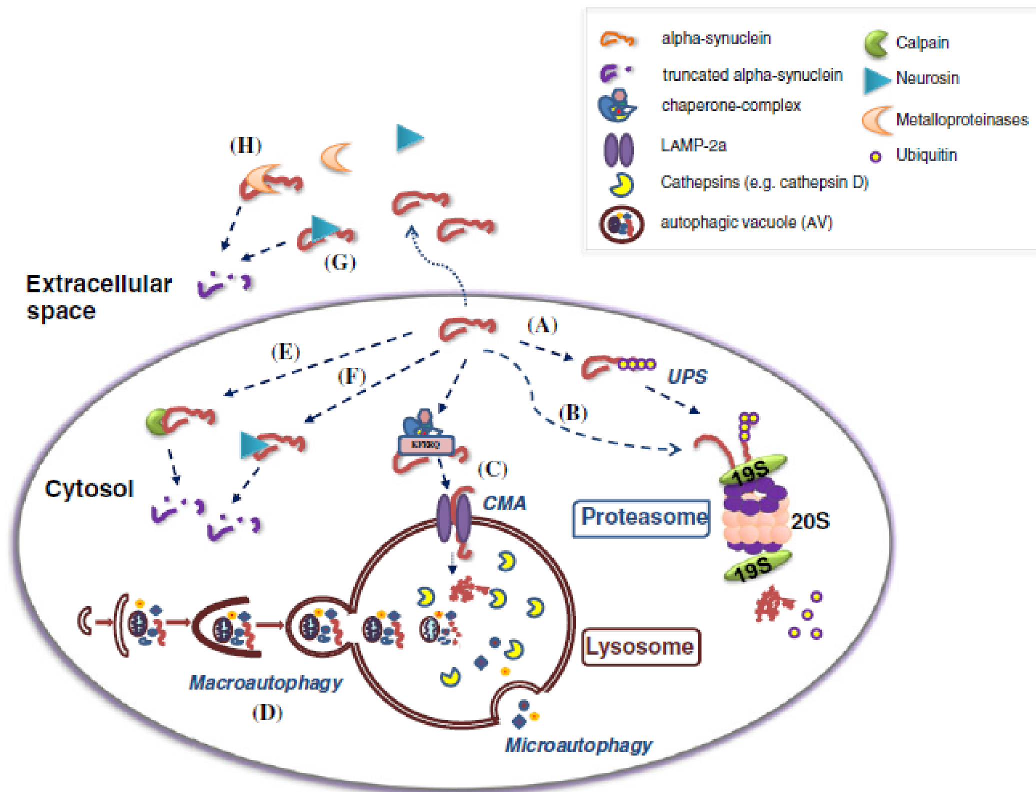


Figure 29: The degradation of aSyn by macroautophagy, CMA and UPS. Proteases such as calpains, neurosin and matrixmetalloproteinases also process aSyn (Xilouri et al., 2012b)

The more pronounced effects of general lysosomal inhibition on aSyn compared to macroautophagy inhibition indicated a role for CMA. Experiments from cell-free systems were the first to demonstrate clearance of wild type aSyn by CMA while the mutants bound to Lamp-2a but failed to be internalized and degraded (Cuervo et al., 2004). The RNA interference studies involving Lamp-2a support the role for CMA is aSyn degradation. CMA inhibition was also followed with increase in insoluble and higher molecular weight aSyn species (Vogiatzi et al., 2008). Transgenic mouse models exposed to paraquat showed an increase in Lamp-2a and enhanced degradation of aSyn once again supporting a role for CMA (Mak et al., 2010). Cathepsin-D has been found to be the primary lysosomal protease mediating aSyn degradation since knock down of cathepsin-D led to accumulation of high molecular weight aSyn (Cullen et al., 2009; Sevlever et al., 2008).

Neurodegeneration often occurs concomitant to impairment of degradation pathways as has been described earlier. In this regard, aSyn has been found to impair UPS, particularly the oligomeric and aggregated species (Fig.30a) (Snyder et al., 2003; Stefanis et al., 2001; Tanaka et al., 2001b). The direct binding of aSyn to 19s proteasome or to the 20s proteasome is proposed to be the mechanism by which aSyn impairs UPS (Ghee et al., 2000; Lindersson et al., 2004; Snyder et al., 2003). It also seems likely that steric hindrance caused due to bulky oligomers may be another mechanism for UPS impairment (Xilouri et al., 2012b). A 25%-35% inhibition in the chymotrypsin-like activity was observed in the presence of A53T mutant aSyn but not wild type aSyn (Stefanis et al., 2001). While several studies have reported UPS impairment by mutants and wild type aSyn, a contrasting report showing no UPS dysfunction in cultured cells and transgenic mice also exists (Martìn-Clemente et al., 2004).

Similar to the observations with UPS, wild type and mutant aSyn have been shown to impair macroautophagy and CMA (Fig.30b). The inhibition associated with ALP could be mediated at two levels. General lysosomal inhibition would render even macroautophagy inefficient while specific CMA inhibition in which case compensatory activation of macroautophagy may prove beneficial (Xilouri et al., 2012b) . Only monomeric and dimeric species are degraded by CMA. Modifications of aSyn such as oxidation, nitration, phosphorylation and dopamine modification result in either partial or complete inhibition of aSyn degradation by CMA (Martinez-Vicente et al., 2008). The inhibition of CMA via dopamine modified aSyn could explain the preferential loss of dopaminergic neurons and could be a pathogenic mechanism in sporadic PD.

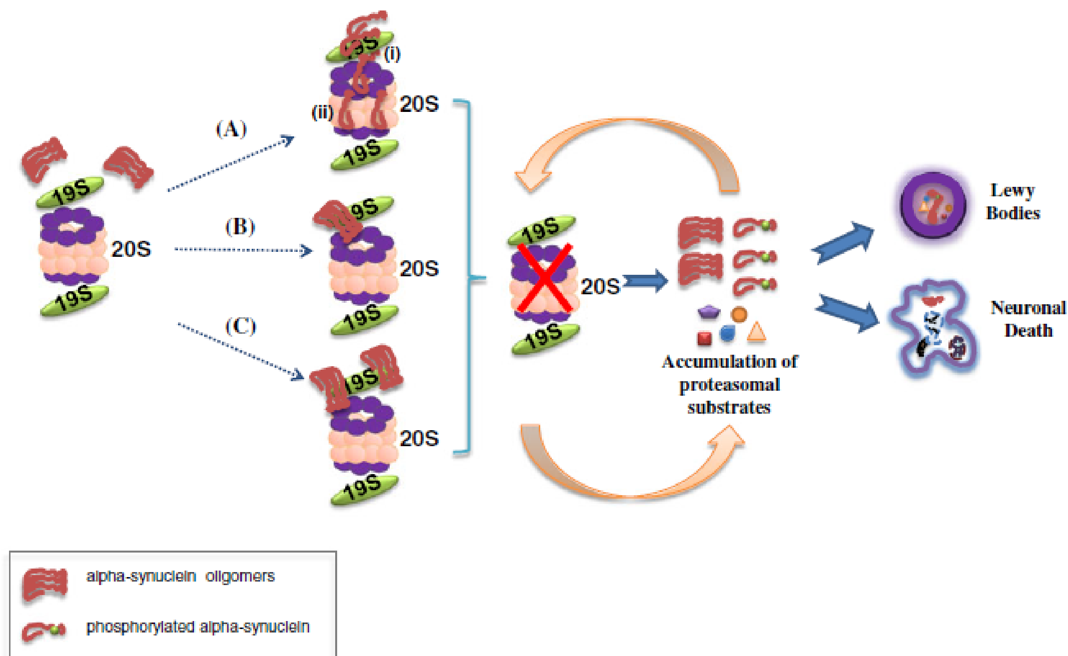


Figure 30(a): The impairment of UPS by aSyn can be mediated via its blockage of the proteasome or by binding to 20s and 19s proteasome. This impairment results in accumulation of phosphorylated aSyn as well as other proteasomal substrates (Xilouri et al., 2012b)

While CMA inhibition is associated with enhanced macroautophagy, it may not necessarily prove beneficial for cell survival (Xilouri et al., 2012b). The inhibition of an early step in autophagosome formation by the action of wild type aSyn on Rab1a presents a contrary view (Winslow et al., 2010). Glucocerebrosidase (Gcase) as a risk factor for PD is another candidate involved in lysosomal dysfunction. The loss of GCase activity has been shown to promote aSyn accumulation and aggregation. Overexpression of aSyn on the other hand, also impaired Gcase by inhibiting intracellular trafficking creating a feed-forward loop of aSyn accumulation and GCase dysfunction (Manning-Boğ et al., 2009; Mazzulli et al., 2011; Sidransky et al., 2009).

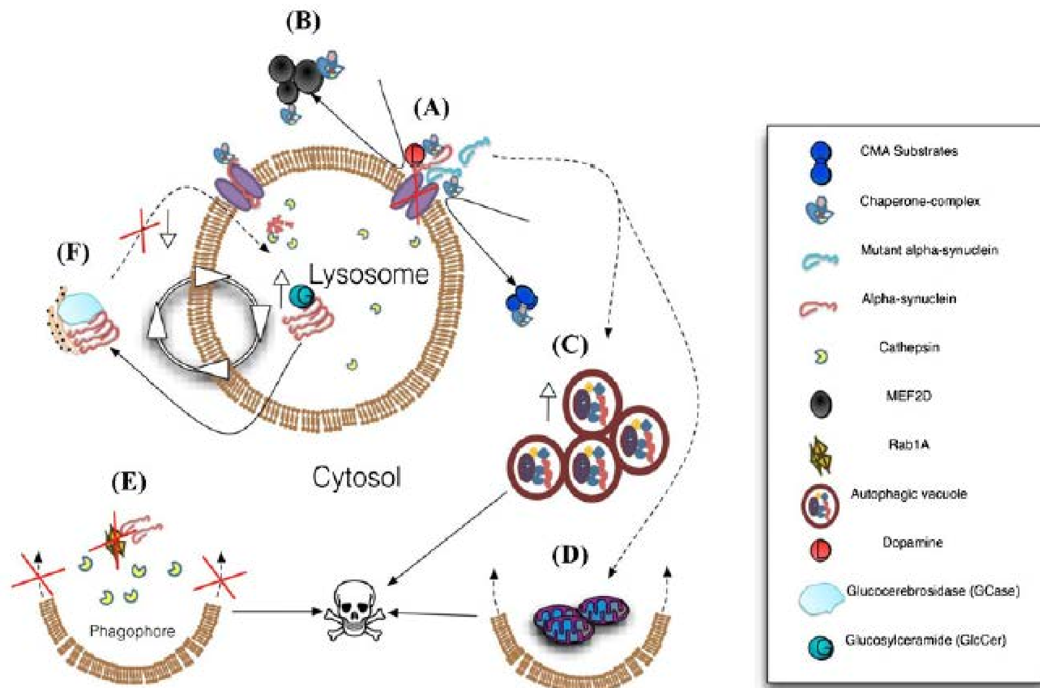


Figure 30(b): Impairment of autophagy by aSyn (a) Mutant aSyn bind to Lamp-2a and prevent internalization and degradation of itself (b) Impairing CMA also prevents degradation of substrates leading to toxicity (c) Activation of macroautophagy which maybe detrimental than beneficial (d) Activation of macroautophagy leading to excess mitophagy (e) Inhibition of early stages of autophagosome formation (f) preventing intracellular trafficking of GCase (Xilouri et al., 2012b)

The degradation of aSyn still continues to be a matter of debate owing to various discrepancies in the results. The type of model and the intracellular protein levels appear to play a determining factor in how the protein is degraded and what are the possible reciprocal effects on aSyn degradation. Devising neuroprotective therapies utilizing autophagy would therefore depend on arriving at a clear consensus on aSyn degradation and its interactions with degradation pathways.

3. MATERIALS AND METHODS

3.1 MATERIALS:

- CELL LINE: Cell line SK-MEL-28 was obtained from the cell line repository at Rajiv Gandhi Centre for Biotechnology (RGCB).

- REAGENTS:

(a) All reagents were purchased from Sigma unless other specified.

(b) Autophagy Inducer Torin-1 was purchased from Tocris Bioscience, Chloroquine was a gift from the Department of Biochemistry, SCTIMST and MG-132 from Cayman Chemicals.

(c) Chemiluminiscence Substrate was purchased from Millipore.

(d) Verso cDNA synthesis kit and Taq polymerase (DNAzyme for PCR) were purchased from ThermoFisher.

(e) Reagents for RNA interference – INTERFERIN transfection reagent from Polyplus and optiMEM from Invitrogen.

(f) Proteasome Activity Assay kit was purchased from Abcam.

(g) Antibodies: All antibodies were purchased from Abcam

Alpha-synuclein (rabbit monoclonal)

1 in 30,000 for immunoblotting, 1 in 100 for immunocytochemistry and flow cytometry.

LC-3 (rabbit polyclonal)

1 in 1000 for immunoblotting.

p62 (rabbit monoclonal)

1 in 50,000 for immmunoblotting.

Beta-actin (rabbit polyclonal)

1 in 2500 for immunoblotting.

HRP tagged secondary antibody (Goat anti-rabbit)
1 in 10,000 to 1 in 50,000 for immunoblotting

FITC tagged secondary antibody (Goat anti – rabbit)
1 in 100 to 1 in 200 for immunocytochemistry and flow cytometry.

(h) Primers were purchased from Sigma:

Alpha synuclein – Forward- 5′-AAA ACC AAG GAG GGA GTG GT
Reverse - 5′-GCC TCA TTG TCA GGA TCC AC

Beta-actin – Forward- 5′ -CATGTACGTTGCTATCCAGGC
Reverse - 5′- CTCCTTAATGTCACGCACGAT

- BUFFERS AND SOLUTIONS:

(a) 10X Phosphate Buffered Saline (1lt):

Nacl – 80g
KCl – 2g
Na_2HPO_4 – 14.4g
KH_2PO_4 – 2.4g

pH adjusted to 7.4 and made upto 1lt with distilled water.

(b) 5X Laemeli Buffer: Prepared (10ml)

1.5 M Tris-Cl – 2ml
Glycerol – 5ml
B-mercaptoethanol – 2.5ml
SDS – 1g
1% Bromophenol blue – 0.5ml

(c) RIPA Buffer: Prepared

Tris-Cl (pH 7.4) – 50mM
NaCl – 150mM
EDTA – 1mM
Triton – X 100 – 1%
Sodium deoxycholate – 1%

Just prior to use the following reagents were added

SDS – 0.1%

Protease Inhibitor Cocktail – 1X (purchased from Sigma as a lyophilized powder and reconstituted in 10ml to give a 10X solution).

PMSF – 2mM (Purchased from Sigma and 200mM stock made in absolute ethanol)

(d) Bradford Reagent (5X):

Coamassie Brilliant Blue – 50mg
Methanol (100%) – 47ml
Phosphoric Acid (85%) – 100ml.

Made upto 200ml with distilled water. Diluted to 1X and filtered before use.

(e) 30% Acrylamide Solution:

Acrylamide (w/v) – 29%
Bisacrylamide (w/v) – 1%

Dissolved in minimal volume of water by warming at 37ºCelsius, made up and then filtered and stored in dark bottles at 4ºCelsius.

(f) Gel Loading Buffer (2X):

Tris-Cl (pH 6.8) – 100mM
SDS (w/v) – 4%
Bromophenol Blue (w/v) – 0.2%
Glycerol (v/v) – 20%

(g) SDS- PAGE Running Buffer (5X):

Tris Base – 25mM
Glycine – 250mM
SDS – 10%

Made up with distilled water.

(h) Transfer Buffer (1X):

Tris Base – 25mM
Glycine – 192mM
Methanol – 10% (for PVDF) and 20% (Nitrocellulose)

Made up with distilled water.

(i) Tris Buffered Saline (10X):

Tris Base – 25mM
NaCl – 150mM

Made up with distilled water. A 1X TBS with 0.1% Tween-20 was prepared for washing.

(j) Paraformaldehyde for Fixing:

Paraformaldehyde – 4% dissolved in 1X PBS by heating at 50ºCelsius.

(k) Tris-acetate Buffer (TAE – 50X):

Tris-acetate – 40mM

EDTA – 1mM

Made up with distilled water.

3.2 METHODS

CELL CULTURE: SK-MEL-28 cells were maintained in DMEM with 10% FBS in 1X antibiotic-antimycotic solution or 1% pencilin-streptomycin at 5% CO_2 at 37ºCelsius. Cells were maintained in culture until 95% confluency and subcultured at 1:3 ratio. Cells were trypsinized using 1X Trypsin-EDTA for 3 minutes at 37ºCelsius, collected, washed in media and replated. Cells at 90% confleuncy were used for all experiments unless otherwise mentioned.

CELL VIABILITY**:** Cell viability was assayed using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. 8000 to 10,000 cells were seeded 24-48 hours before experimentation on 96 well plates. After treatment, media was removed and wells washed in 1X PBS to remove all traces of media. 1mg/ml stock solution of the dye MTT was prepared by dissolving it in PBS. 100µl from the stock was added to all wells and incubated for 2 hours in the dark. The MTT was discarded after 2 hours and the resulting formazan crystals were dissolved in 100µl acidified isopropanol and absorbance measured at 575nm with 630nm as reference wavelength on a microplate reader (TECAN). The viability of the treated cells was expressed as the percentage of the test absorbance divided by the control absorbance.

FRACTIONATION: The sub-cellular fractionation of cells was achieved by following the protocol as described (Suzuki et al., 2010). The cells were collected and the cell pellet was resuspended in ice cold 0.1% NP-40 (diluted in 1X PBS) and triturated a few times. An aliquote was set aside and marked the "Whole cell lysate" (WCL). The remainder was centrifuged for 10 seconds and the supernatant was transferred to a fresh tube and marked as the "Cytoplasmic fraction" (CF). The pellet was resuspended in ice cold 0.1% NP-40 and triturated once or twice and the centrifuged for 10 seconds. The supernatant was discarded and the pellet was marked as the "Nuclear Fraction". The WCL and CF fractions were mixed with 4X Laemelli buffer at 3 volumes of samples with 1 volume of 4X Laemelli Buffer. The WCL sample was sonicated with a probe sonicator and both the WCL and CF samples were boiled for 5 minutes in a boiling water bath. The nuclear fraction obtained as a pellet was resuspended in 1X Laemelli buffer, sonicated with a probe sonicator and boiled for 5 minutes in a boiling water bath and then used for immunoblotting.

IMMUNOBLOTTING: Cells were collected by trypsinzing and the cell pellet was lysed using RIPA buffer. The cell pellet was incubated with RIPA buffer in ice for 30 minutes and centrifuged at 16,000g for 15 minutes at 4ºCelsius and supernatant collected into a fresh tube. The supernatant was assayed for protein by Bradford Assay. For the Bradford assay, 1mg/ml Bovine Serum Albumin (BSA) was used to make standards ranging from 1.5µg to 15µg, 2.5µl sample was used for the assay with 2.5µl RIPA buffer used as a blank control. The volumes of the blank, standards and samples were made upto 125µl with distilled water from which 50µl sample for each was taken in duplicates. 200µl 1X Bradford was added to each sample and incubated for 10 minutes in dark and absorbance measured at 570nm on a microplate reader (TECAN). The protein concentration was estimated by plotting a standard graph and equal

amounts for all samples were taken. The samples to be loaded were then mixed with 2X gel loading dye and all sample volumes were equalized. Experiments involving low cell density, the cells were collected and lysed directly in 1X Laemelli buffer and sonicated with a probe sonicator. Equal volumes of the samples were loaded when extracted directly with Laemelli Buffer. All samples were boiled for 5 minutes in a boiling water bath before loading.

Samples were loaded onto gels (10% to 15% gels) and run at 120V. The proteins in the gel were then transferred onto PVDF membrane at 110V for 60 minutes at 4ºCelsius. The blots were washed in distilled water three times for 5 minutes each immediately after transfer and dried at 37ºCelsius for 1 hour. The blots were then blocked with 2%-5% skimmed milk for 1 hour at room temperature and incubated with primary antibody overnight at 4ºCelsius. Blots were washed three times for 5 minutes each with 1X TBST at room temperature and incubated for 2 hours with Horse Radish Peroxidase-tagged secondary antibody. Blots were then washed with 1X TBST three times for 5 minutes each and probed with Chemiluminiscence. The signal was captured onto X-ray films which were then imaged using Uvipro Platina from UVItec Gel documentation system and analyzed using ImageJ analysis software. The bands for the protein of interest were first quantified. The corresponding beta-actin bands for each sample was quantified and the values for protein of interest were normalized with their respective beta-actin and the fold change was expressed as test divided by control.

FLOW CYTOMETRY: Cells were collected and washed with 1X PBS to remove traces of media. The cells were then fixed in 4% paraformaldehyde for 10 minutes at room temperature and washed with 1X PBS. The cells were then permeabilized in 0.4% triton-X 100 (diluted in 1X PBS) for 10 minutes at room temperature. The cells were then blocked in 5% FBS for 30 minutes at room temperature and incubated in primary antibody for 2 hours at 4ºCelsius. The cells were

washed in 1X PBS and incubated with FITC tagged secondary antibody for 1.5 hours in ice. The samples were washed with 1X PBS and resuspended in 300µl 1X PBS, transferred into Flow cytometry tubes and analyzed in BD FACS Aria machine.

IMMUNOCYTOCHEMISTRY: Cells at sub-optimal confluency were used for these experiments (70%-80%). Cells were washed with 1X PBS and fixed with 4% paraformaldehyde for 30 minutes at 37ºCelsius. The cells were washed with 1X PBS and permeabilized in 0.4% Triton-X 100 for 10 minutes at room temperature. The cells were washed and blocked in 5% FBS for 30 minutes at room temperature. The cells were incubated with primary antibody overnight at 4ºCelsius and placed in humidified chamber. The primary antibody was removed the next day and cells washed three times for 5 minutes each with 1X PBS. The cells were then incubated with FITC tagged secondary antibody for 1.5 hours at room temperature in the dark. The secondary antibody was removed and cells washed three times for 5 minutes with IX PBS. The cells were counter stained with 5µg Hoechst for 5 minutes at room temperature and washed with 1X PBS three times for 5 minutes each. The cells were then imaged on the fluoresce microscope Olympus IX51 with Rolera XR (QImagin) camera. The images were obtained in grey scale and pseudo coloured using the ImageJ software.

RNA EXTRACTIONAND PCR: RNA extraction was carried out using the TRI reagent (Sigma) and the recommended protocol was followed. The TRI reagent was added to the experimental dishes placed on ice, cells scraped and collected into a 1.5 ml tube. The tubes were placed in ice for 10 minutes. This was followed with addition of 200µl Chloroform (for every 1ml of TRI reagent) and was incubated for 2 minutes at room temperature and then for ten minutes in ice. The samples containing TRI and chloroform were then centrifuged at 12,000g for 15 minutes at 4ºCelsius. The uppermost aqueous layer was collected and transferred into a fresh 1.5 ml tube

while the pink phenol layer was set aside for protein extraction. A volume of ice cold isopropanol equal to that of the aqueous layer was added to the aqueous layer and invert mixed and stored at -20ºCelsius for 30 minutes. The samples were then centrifuged at 12,000g for 10 minutes at 4ºCelsius. The resulting pellet was washed in ice cold 75% ethanol and centrifuges at 7500g for 5 minutes at 4ºCelsius. The pellet was air dried partially and dissolved in RNAse free water and used for cDNA synthesis. The quality of RNA was checked by running the sample on 0.7% agarose gel and samples estimated on the NanoDrop (ND-1000).

The cDNA synthesis was carried out by taking 2-2.5µg RNA using the cDNA synthesis kit from ThermoFisher. The RNA samples were mixed with random hexamer primers, dNTP's, buffer, Enhancer, enzymes at the recommended volumes. The volume was made upto 20µl with RNAse free water. The reaction conditions were 42ºCelsius for 60minutes, 95ºCelsius for 2 minutes and 70ºCelsius for 10 minutes.

The cDNA was used for the semi-quantitative PCR {GeneAmp PCR System 9700 (Applied Biosystems)} where 1.5µl to 2µl of the cDNA was mixed with buffer, MgCl2, dNTP, forward and reverse primers and the Taq polymerase. All volumes were made up to 20µl with RNAse free water. The reaction conditions were initial denaturation for 95ºCelsius followed with 35 cycles of 95ºCelsius for 30 seconds, 60ºCelsius for 30 seconds and 72ºCelsius for 30 seconds and final elongation at 72ºCelsius for 5 minutes.

The PCR amplified products were then run on 1% agarose gel and imaged on the using Uvipro Platina from UVItec Gel documentation system and analyzed with ImageJ software. The bands for gene of interest were quantified the values normalized with their corresponding beta-actin which was also separately quantified. The fold change was expressed as test divided by control.

The phenol layer obtained after Chloroform addition and centrifugation was used for protein extraction by adding the recommended volume of absolute ethanol. The tubes with absolute ethanol were invert mixed, incubated at room temperature or 5 minutes and centrifuged at 2000g for 5 minutes. The supernatant was taken in a new tube and recommended volume of isopropanol was added and incubated at room temperature for 10 minutes and centrifuged at 12,000g for ten minutes at 4ºCelsius. The pellet was washed three times in 95% ethanol three times for twenty minutes each and centrifuged at 7500g for 5 minutes at 4ºCelsius after each wash. A final wash was performed with absolute ethanol and centrifuged at 7500g for 5 minutes at 4ºCelsius. The pellet was air dried and dissolved in 1X Laemelli buffer. The samples were boiled for 5 minutes in a boiling water bath before using them for immunoblotting.

RNA INTERFERENCE: The transfection was achieved using the INTERFERIN transfection agent (Polyplus) and the recommended protocol was followed. 50,000 to 70,000 cells were seeded in a 12 well plate 24 hours prior to experimentation. 1nM siRNA was made from the 5µM sub stock by diluting it in the appropriate volume of optiMEM. The INTERFERIN transfection reagent was added to the siRNA solution, homogenized by vortexing for 10seconds and incubated at room temperature for 10 minutes to allow for complex formation. In the meanwhile the media from the wells was removed and the wells washed once in 1X PBS. After the incubation, the siRNA-transfection complexes were added to cells with serum free media and incubated for 4-5 hours after which completer media was added to the wells and incubated for 24 hours. Cells were used for experimentation 48 hours post transfection and fresh media was used for all experiments.

PROTEASOME ASSAY: The proteasome activity was determined using the activity assay kit from Abcam and the protocol as recommended was followed. The positive control supplied as a

lyophilized powder was reconstituted in 100µl distilled water and aliquotted. The cells for the assay were collected in a 1.5ml tube, washed in 1X PBS and resuspended in 0.1% NP-40, centrifuged at 13,000 rpm and supernatant collected. The assay buffer was equilibrated to room temperature prior to experimentation. The proteasome substrate, proteasome inhibitor and AMC standards were pre-warmed at 37ºCelsius. The volumes were adjusted to achieve readings within the range. A 0.01mM standard solution was prepared by diluting 10µl of the AMC standard in 990µl of distilled water and 0µl (just 100µl assay buffer to serve as blank) to 2µl was taken for the standard and the volumes made upto 100µl with assay buffer. 10µl of the sample was taken in duplicates and made upto 100µl, 2µl of the positive control was taken in duplicates and made upto 100µl. 0.2µl of the proteasome inhibitor was added to one of each of the sample and positive control wells while for the other 0.2µl of the assay buffer was added. 0.2µl of the proteasome substrate was added to the entire sample and positive control wells and mixed. The fluorescence was measured at 37ºCelsius with excitation and emission at 350/440nm for every ten minutes on a microplate reader (TECAN). A white opaque 96- well plate was used for the assay.

A standard curve is generated by subtracting the absorbance of blank measurement from the remaining the standards. The wells without the proteasome inhibitor show total proteolytic activity (RFU) while the wells with the proteasome inhibitor show non-proteasome activity (iRFU) that is measured at two time points that lie in the linear range. ΔRFU is obtained for each sample by using the formula $\Delta RFU = (RFU_2 - iRFU_2) - (RFU_1 - iRFU_1)$ where 1 and 2 refer to the two time points. This value is then applied to the AMC standard curve to get amount generated between the two time points (B pmol). The proteasome activity is then calculated by using the formula:

Proteasome activity = [B / (T2-T1) x V] x D where

B - Represents that amount in sample wells

V – Sample volume taken in each well (µL)

T1 – Initial time point (RFU_1 and $iRFU_1$)

T2 – Second Time point (RFU_2 and $iRFU_2$)

D – Sample dilution factor.

DOPAMINE UPTAKE: Dopamine conjugated to dansyl chloride was obtained from the FADDS Laboratory, SCTIMST. This was added to cells at varying concentrations in duplicates and incubated for 8 hours at 37ºCelsius. The cells were then fixed with 4% paraformaldehyde for 30 minutes at 37ºCelsius. The wells were washed in 1X PBS and counter stained with 5µg Hoechst for 5 minutes at room temperature and the wells washed with 1X PBS. The cells were imaged under a fluorescence microscope Olympus IX51 with Rolera XR (QImagin) camera and pseudo coloured using ImageJ software.

THIOBARBITURIC ACID REACTIVE SUBSTANCES ASSAY (TBARS): The generation of reactive oxygen species leads to lipid peroxidation and produces a stable end product, malondialdehyde. The cells were collected and lysed in 5% SDS and boiled in the presence of a reagent containing 15% Trichloroacetic acid, 0.375% thiobarbituric acid and 0.25N HCl until a pink colour was obtained. The samples were spun down and the supernatant collected and absorbance measured at 535nm on a UV spectrophotometer. Distilled water with the reagent serves as blank. The value of the blank is subtracted from the samples. The formula:

(Test O.D x Total volume) / (0.000156 x volume taken x 1000) is applied to obtain the value of lipid peroxidation or oxidative stress which is finally expressed as nmol/mg of protein.

MELANIN ASSAY: The cells were collected into a 1.5ml tube and washed in 1X PBS. The cells were resuspended in 1X PBS and cells counted using a heamocytometer. The cells were pelleted down, resuspended in 200µl 1M NaOH and boiled at 80ºCelsius for 1 hour. A stock of 1mg/ml melanin was prepared by dissolving it in 1N NH_4OH. Standards were prepared as ranging from 5µg to 100µg. The boiled samples were taken in a 96 well plate and absorbance measured at 415nm on a microplate reader (TECAN). NaOH solution was used as blank and the absorbance of the blank was subtracted from the absorbance values of the samples. A standard graph was plotted from the values corresponding to the standards and the amount for the determined from the standard graph. This value corresponded to the melanin content from total number of cells. Final values were plotted by determining the amount of melanin in 10,000 cells and compared between test and control.

STASTICAL ANALYSIS: The data was anylyzed by applying the students t-test or the one way ANOVA as applicable. GraphPad prism was used for the statistical analysis and for data representation.

4. RESULTS

4.1 MODULATION OF AUTOPHAGY AND EFFECT ON ALPHA SYNUCLEIN EXPRESSION.

Autophagy is a bulk degradation pathway and is constitutively active in all cells including in neurons. Basal autophagy is particularly important in post mitotic cells such as neurons to prevent the accumulation of protein aggregates and the suppression of basal autophagy has been demonstrated to cause neurodegeneration (Hara et al., 2006; Yue et al., 2009). Therefore the effect of chemical mediated inhibition and induction of autophagy on endogenous aSyn expression was assessed.

(a) SK-MEL-28 cells express aSyn endogenously.

The endogenous expression of aSyn in SK-MEL-28 was confirmed through three independent methods. Flow cytometry of SK-MEL-28 cells immunostained for aSyn confirmed the presence of aSyn in 97% of the cells (Fig.31). Examination of the immunostained cells under fluorescence microscopy further demonstrated the nuclear and cytoplasmic localization of aSyn (Fig.32). Immunoblotting not only confirmed the sub cellular localization of the protein but also revealed the presence of two predominant molecular weight species of aSyn- an 18kDa protein present exclusively in the nucleus and a 32kDa protein present exclusively in the cytoplasm (Fig.33).

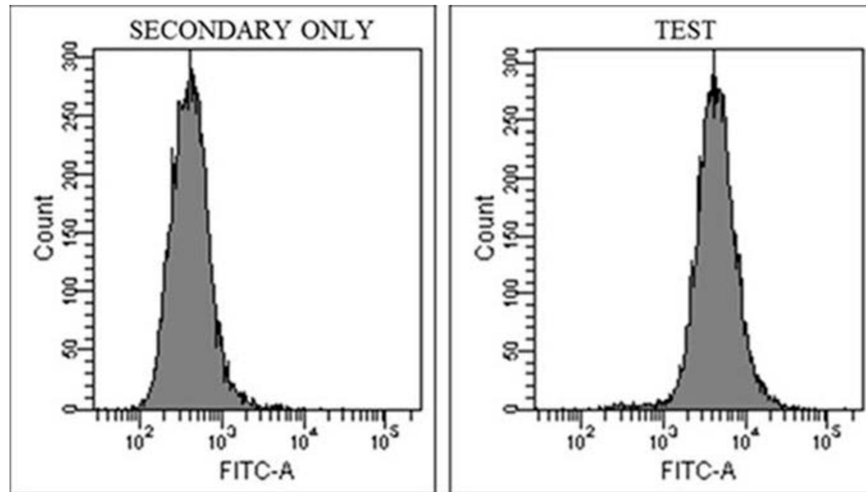


Figure 31: Flow cytometry analysis of aSyn in SK-MEL-28

The flow cytometry profile of SK-MEL-28 cells stained for aSyn probed with FITC conjugated secondary showing a clear shift in the histogram of stained cells when compared with cells exposed only to FITC conjugated secondary confirming that SK-MEL-28 cells express aSyn.

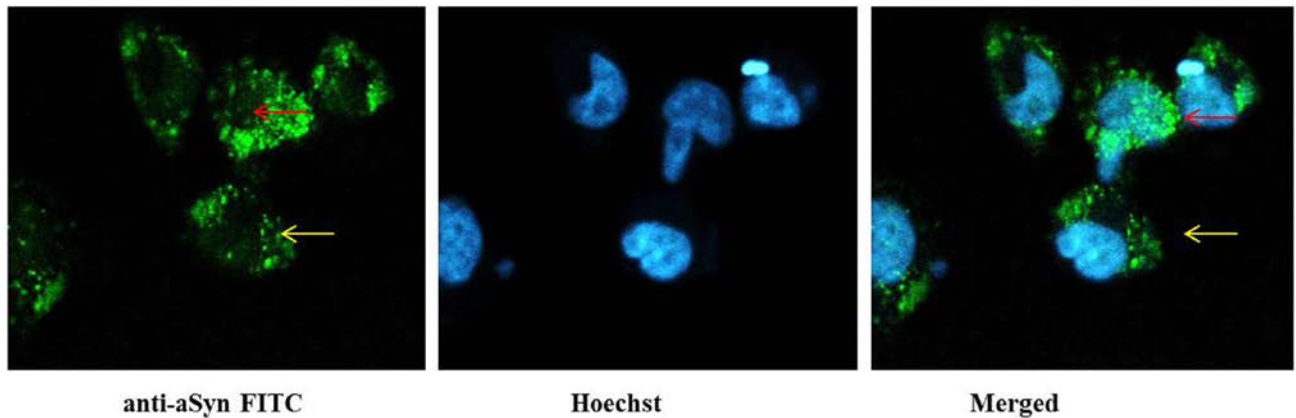


Figure 32: **Subcellular localization of aSyn**

Immunostaining of SK-MEL-28 cells for aSyn probed with FITC conjugated secondary and counter stained with Hoechst reveals the nuclear and cytoplasmic localization of aSyn.(Red arrows represent nuclear staining and the yelloe arrows represent cytoplasmic staining of aSyn).

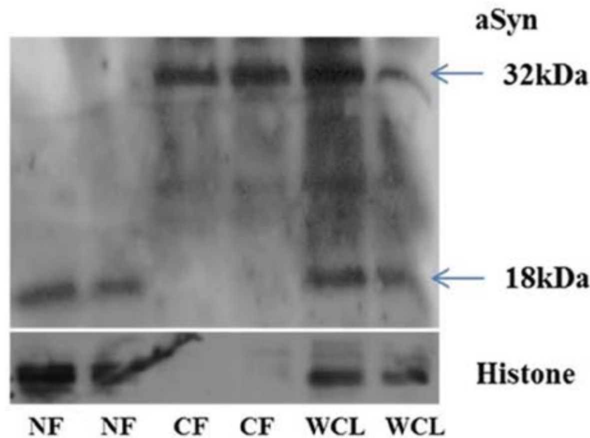


Figure 33: **Immunoblotting of nuclear and cytoplasmic fractions**

Immunoblots of the nuclear and cytoplasmic fraction with differing band patterns of aSyn with the nuclear fraction (NF) having a band corresponding to a molecular weight of 18kDa and the cytoplasmic fraction (CF) having a band corresponding to a molecular weight of 32kDa. Whole cell lysate (WCL) shows both bands. Histone was used to confirm the different sub-cellular fractions.

(b) Autophagy Inhibition does not affect aSyn expression levels:

The fusion of the autophagic vacuole (AV) with the lysosome is a critical step in the process of macroautophagy. The acidic environment in the lysosomes not only promotes this fusion but is also important for proteolysis. Changes in the acidic environment of the lysosomes interfere with fusion which inhibits macroautophagy and also prevents proteolysis in turn inhibiting CMA (Xilouri et al., 2012a). Chloroquine is a weak base with lysosomotropic property which increases the intralysosomal pH thereby inhibiting the activity of the enzymes in the lysosome in turn brining about lysosomal inhibition (Amaravadi et al., 2007; Solomon and Lee, 2009). The inhibition of the fusion step is accompanied with the accumulation of autophagic vacuoles. The increase in AV can be verified by examining the expression levels of LC3-II which is found on the membrane of the AV and is found to be increased following inhibition of fusion of the AV's

with the lysosomes. Similarly expression of p62, a carrier molecule that recognizes and transports ubiquitinated proteins to the AV's and is itself degraded in the autophagolysosome, is also increased because its degradation is prevented when the fusion fails.

Autophagy inhibition was achieved through the use of Chloroquine which did not cause any significant loss in cell viability up to a concentration of 20µM (Fig.34).

Chloroquine was found to effectively inhibit autophagy at 20µM (Fig.35A) which was confirmed by a nearly 3 fold increase in LC3 (Fig.35B) and a nearly 4-fold increase in p62 (Fig.35C).

This level of inhibition did lead to a less than 2-fold increase in aSyn (Fig 36A) which however did not amount to a significant change (Fig 36B).

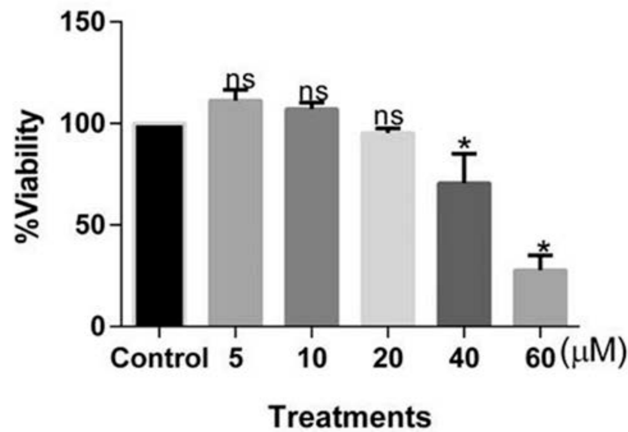


Figure 34: Cell Viability after chloroquine treatment

The cell viability upon treatment with varying concentrations of Chloroquine for 24 hours was measured by the MTT assay. Loss of cell viability was observed at concentrations above 20µM with a 70% loss in viability seen at 60µM concentration. (n=3, $^{ns}p > 0.05$ $^{*}p < 0.05$)

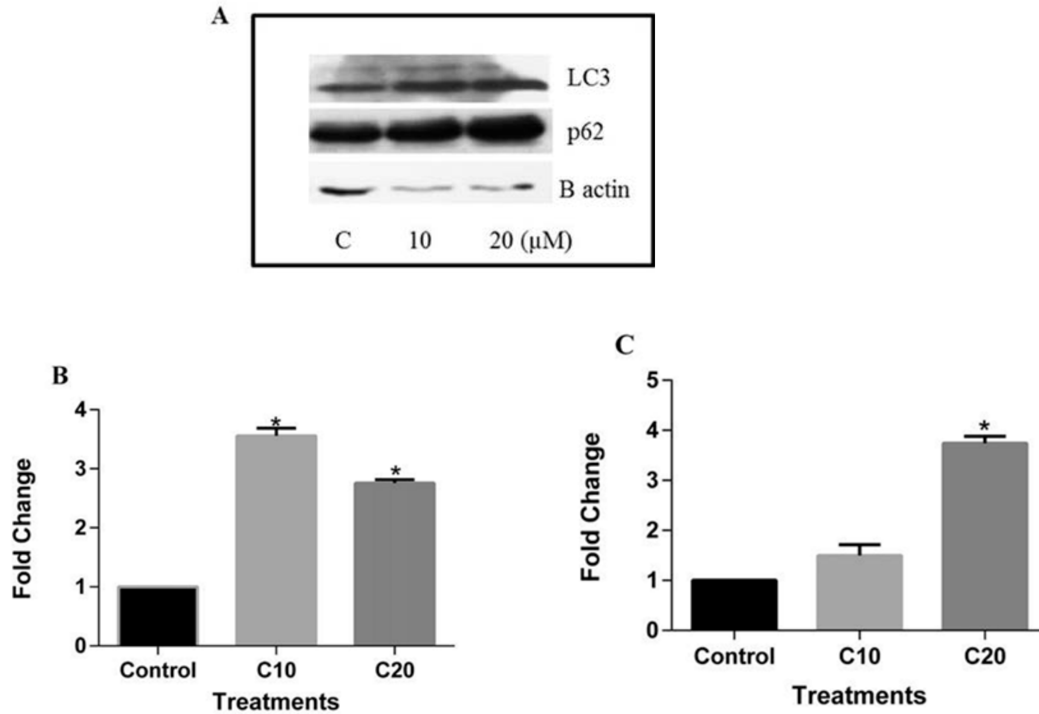


Figure 35: Autophagy inhibition with Chloroquine

(A) Western Blots showing increase in autophagy markers LC3 and p62, after treatment with two different concentrations of Chloroquine for 12 hours. (B) and (C) Graphs representing the western blot quantification of LC3 and p62 respectively. (n = 3, $*p < 0.05$)

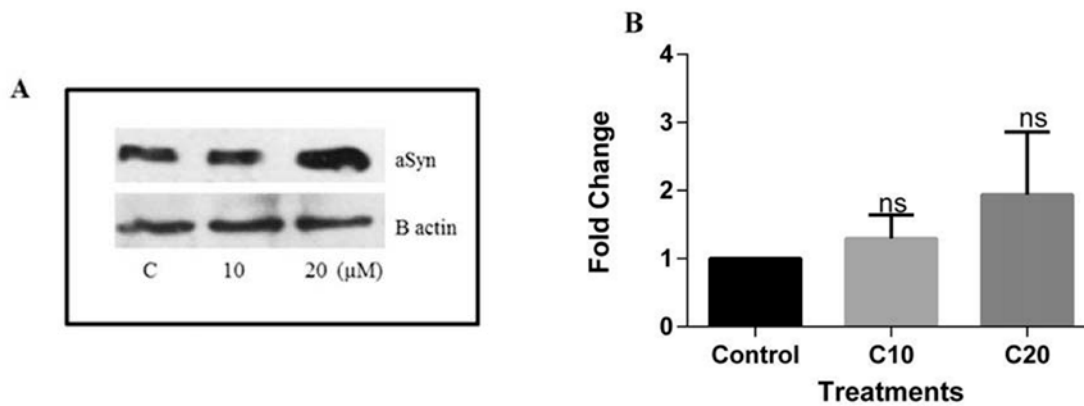
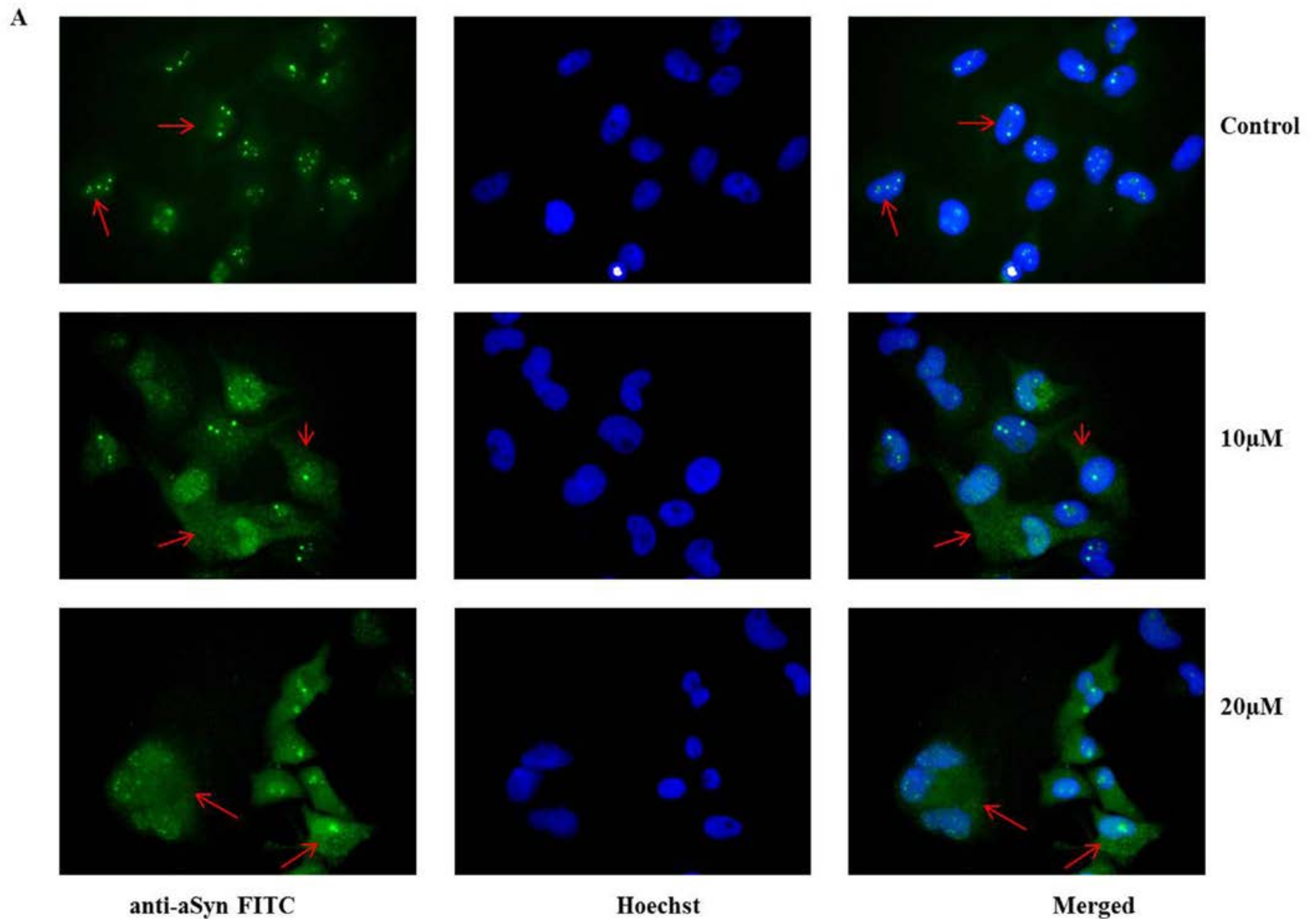


Figure 36: aSyn expression upon autophagy inhibition

(A) Western blot image showing a modest increase in aSyn levels after treatment with two different concentrations of Chloroquine for 12 hours. (B) Graph representing the western blot quantification of aSyn. (n = 3, $^{ns}p > 0.05$).

(c) Autophagy Inhibition causes a change in the localization of aSyn from nucleus to cytoplasm:

The localization of aSyn to the nucleus and cytoplasm suggests a regular trafficking of aSyn between the nucleus and cytoplasm. Although autophagy inhibition did not produce a significant change in aSyn levels, it was possible that it affected the distribution of aSyn between the nucleus and cytoplasm. Immunocytochemistry data of chloroquine treated cells demonstrates an increased localization of aSyn to the cytoplasm when compared to untreated cells (Fig 37A). Therefore autophagy inhibition causes an increased trafficking of aSyn from the nucleus to cytoplasm without significantly effecting a change in its expression levels (Fig 37B).



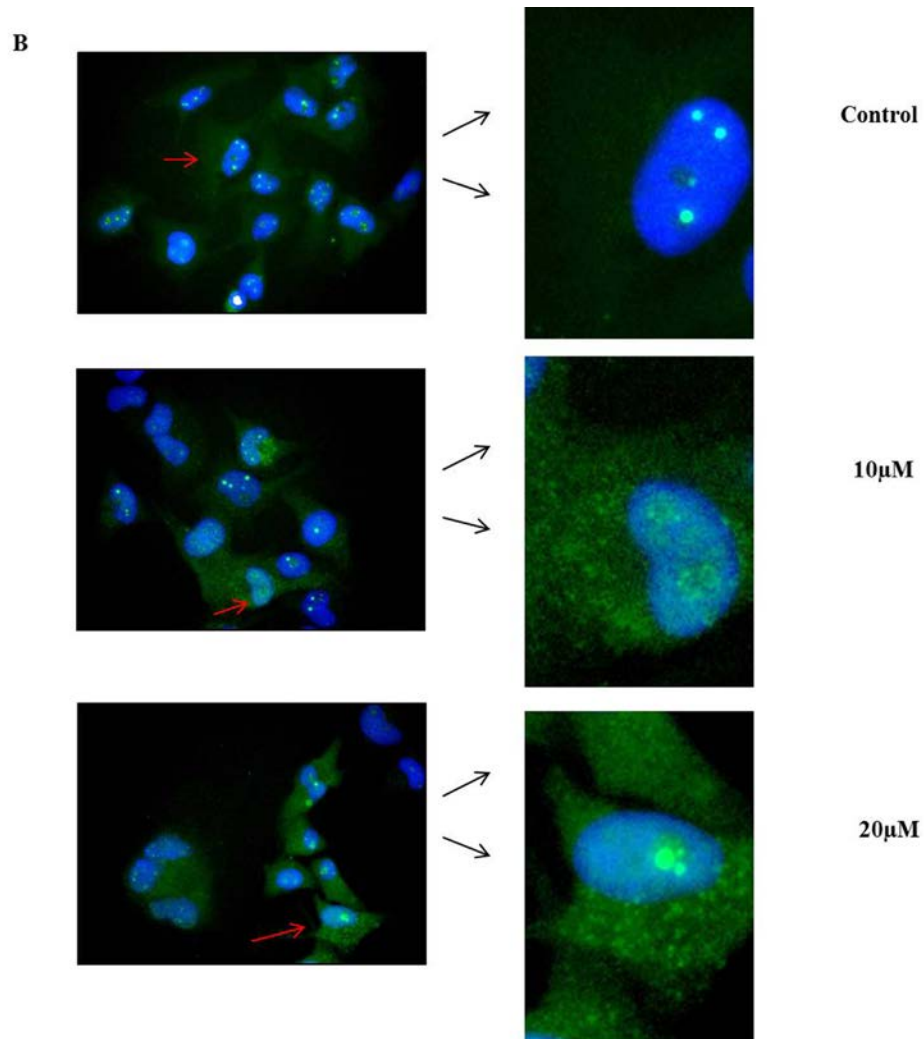


Figure 37: Subcellular distribution of aSyn upon autophagy inhibition

(A) SKMEL 28 cells stained for aSyn after treatment with 10µM and 20µM Chloroquine for 12 hours shows an increase in cytoplasmic localization of aSyn when compared with controls (pseudo-colored). Cells were counter stained with Hoechst. (B) Enlarged image of the same. (Red arrows represent aSyn staining)

(d) Proteasome Function appears impaired in SK-MEL-28 cells:

The ubiquitin proteasome pathway is another major pathway involved in the degradation of proteins. Although its role in neurodegeneration has not been clearly established Lewy bodies (LB's) stain positive for ubiquitin, ubquitinated aSyn is found in LB's (Lim, 2007; Matsuda and Tanaka, 2010) and some studies indicate that aSyn maybe degraded by UPS (Ebrahimi-Fakhari et al., 2011). Since inhibition of autophagy, did not significantly affect the expression levels of aSyn, it seemed likely that the proteasome pathway played a greater role in aSyn degradation in melanoma cells. MG 132 is a peptide aldehyde which acts as a substrate analog and inhibits the chymotrypsin-like activity of the proteasome. Therefore, proteasome function in SK-MEL-28 cells was inhibited through the use of inhibitor MG 132 at a concentration of 1 μM at two time points- 3 h and 6 h. Longer duration of proteasomal inhibition often leads to activation of NF-kB and can lead to eventual cell death. Ideally, all proteasomal inhibition is carried out for 4-6 hours, and hence, two time points of 3 h and 6 h were chosen. The expression levels of aSyn were then assayed. Results show that inhibition of proteasome through MG 132 did not lead to a significant change in aSyn expression levels (Fig.38A, B).

The lack of change in aSyn levels in the face of proteasome inhibition seemed unusual, and therefore, the proteasome function in SK-MEL-28 was assayed using a proteasome activity kit and the activity was compared with a positive control. There was no change in the proteasome function in SK-MEL-28, if anything, the proteasome function showed a decrease over time when compared with the positive control. This results suggests that there is likely to be an impairment in proteasome function in SK-MEL-28 cells (Fig 39). Although these results were in seeming contradiction with the results of Chen et al (Chen et al., 2003), that study did not assess basal activity of proteasome, as done in the current study.

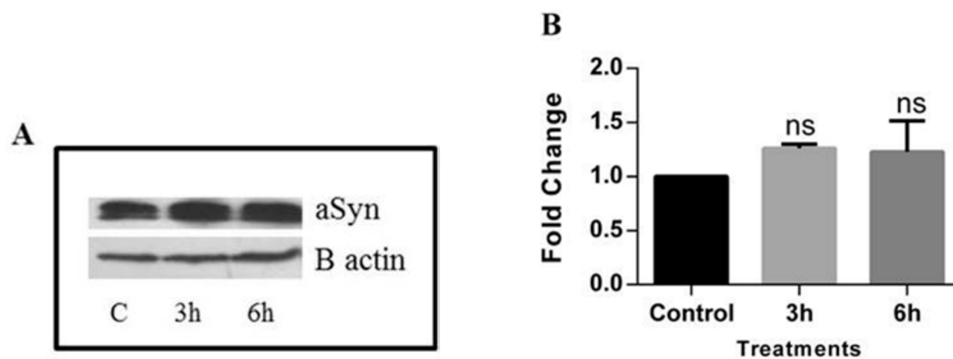


Figure 38: aSyn expression upon proteasomal inhibition

(A) Western blot of aSyn after treatment with proteasome inhibitor MG 132 at 3 hour and 6 hour time point showing no significant change in its expression. (B) Graph representing the western blot quantification of aSyn. (n = 3, $^{ns}p > 0.05$)

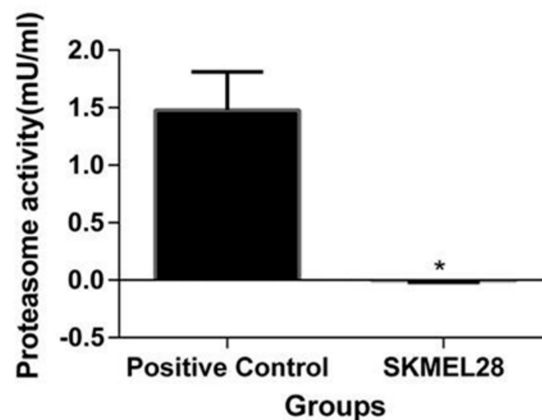


Figure 39: Basal proteasome activity in SK-MEL-28

Proteasome function was assayed using an activity kit in SK-MEL-28 cells and compared with a positive control. The positive control showed an increase in proteasomal function over time while a small decrease in observed in SK-MEL-28 cells and practically constitutes to no change in proteasome activity over time. (n=3, *$p<0.05$).

(e) Autophagy Induction causes an increase in aSyn levels:

The lack of change in aSyn expression following autophagy inhibition suggests that it may be preferably routed for degradation via the UPS and yet the indications of proteasomal impairment in SK-MEL-28 means that aSyn clearance in these cells may also be compromised. Additionally the change in aSyn localization upon autophagy inhibition suggests that autophagy does play a regulatory role with regard to its distribution in the cell. Given that autophagy is a bulk degradation pathway, it is possible that induction of autophagy on the other hand, may lead to an increased clearance of aSyn in the cell. An upstream regulator of autophagy is mTOR which inhibits autophagy. Torin-1 is a potent inhibitor of mTOR and therefore can lead to induction of autophagy. Similar to inhibition, induction of autophagy is also accompanied with an increase in the number of AV's due to increase formation of the AV's and can be confirmed through the increase in LC3-II levels. However, converse to inhibition, induction of autophagy results in decreased p62 levels due to increased clearance of the protein.

Torin-1 did not cause any significant loss in cell viability for upto 24 hours (Fig.40) at its IC_{50} value of 10nM. Torin-1 effectively induced autophagy at a concentration of 10nM (Fig.41A) with a nearly 10 fold increase in LC3 (Fig.41B) and a 2-fold decrease in p62 levels (Fig.41C) when compared to control.

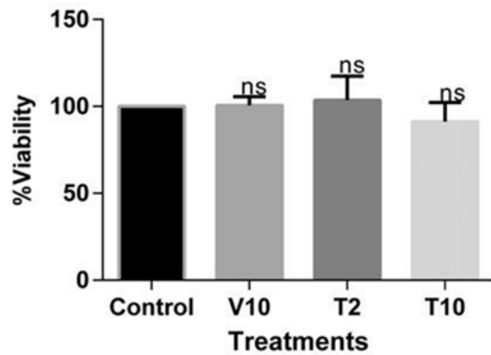


Figure 40: Cell viability with Torin-1 treatment

The cell viability upon treatment with 2nM (T2) and 10nM (T10) Torin-1 was measured by the MTT assay. Torin-1 was dissolved in DMSO, which was also included as a vehicle control (V10). There was no loss in viability with DMSO alone or at 2nM. There was a very slight reduction in cell viability at 10nM, which was not significant. (n=3, ^{ns} $p>0.05$)

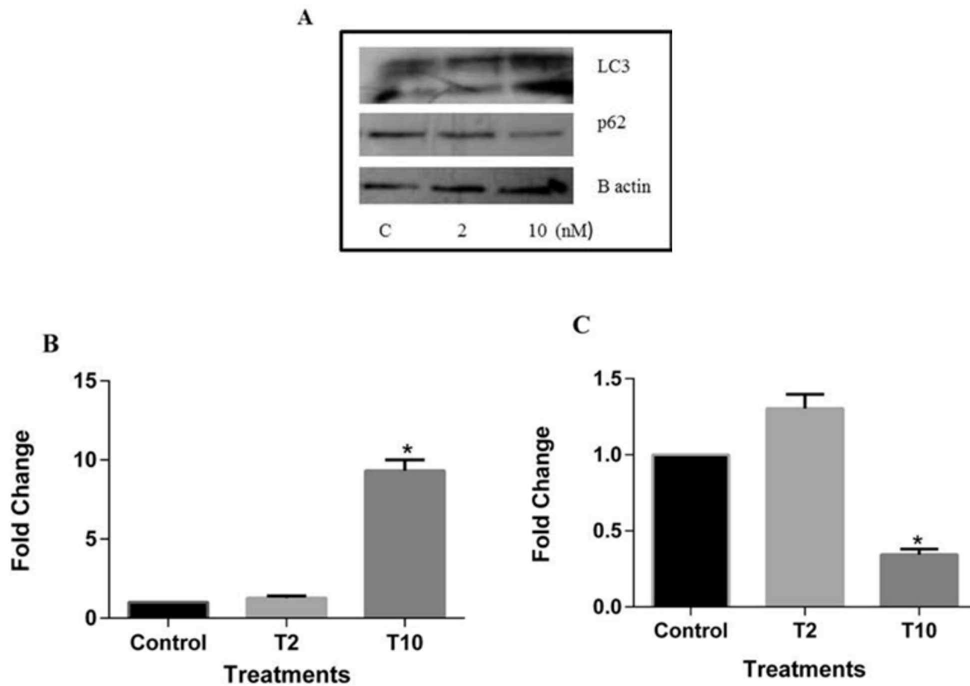


Figure 41: Autophagy induction with Torin-1

(A) Western blots showing an increase in LC3 and a decrease in p62 after treatment with 10nM Torin-1 for 12 hours. No significant changes were observed at 2nM Torin-1 treatment. (B) and (C) Graphs representing the western blot quantification of LC3 and p62 respectively.(n= 3, *p <0.05).

Contrary to expectation, the induction of autophagy however led to a significant increase in aSyn (Fig.42A) by nearly 4-fold (Fig.42B) and did not result in increased clearance of the protein again suggesting that clearance of aSyn may not be mediated by autophagy.

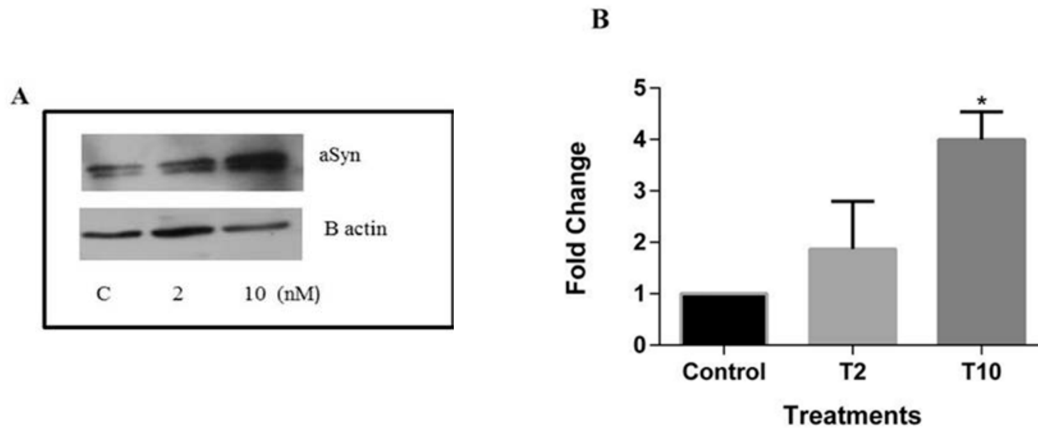


Figure 42: aSyn expression upon autophagy induction

(A) Western blot showing an increase in aSyn after treatment with Torin-1 at 10nM concentration for 12 hours. (B) Graph representing the western blot quantification of aSyn. ($n = 3$, $*p < 0.05$)

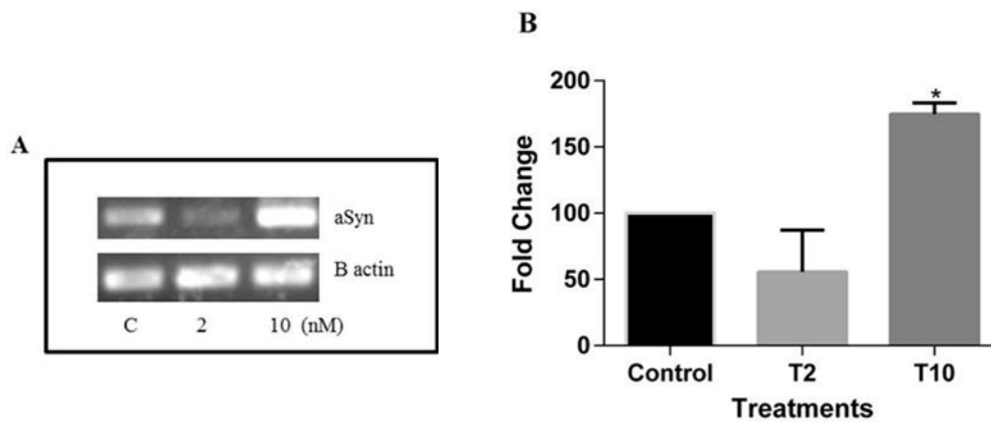


Figure 43: mRNA expression of aSyn upon autophagy induction

(A) Increase in mRNA expression of aSyn after treatment with Torin-1 at 10nM concentration from 6 hours assayed through semi-quantitative PCR. (B) Graph representing the quantification of PCR products run on an agarose gel. ($n=3$, $*p < 0.05$)

To confirm if the increase was due to increased protein retention or due to increased protein synthesis, the mRNA levels of aSyn was assessed for through semi quantitative PCR. The increase in protein levels was also reflected in the mRNA levels (Fig.43A) which showed more than a 1.5 fold increase (Fig.43B) within 6 hours after the addition of the drug.

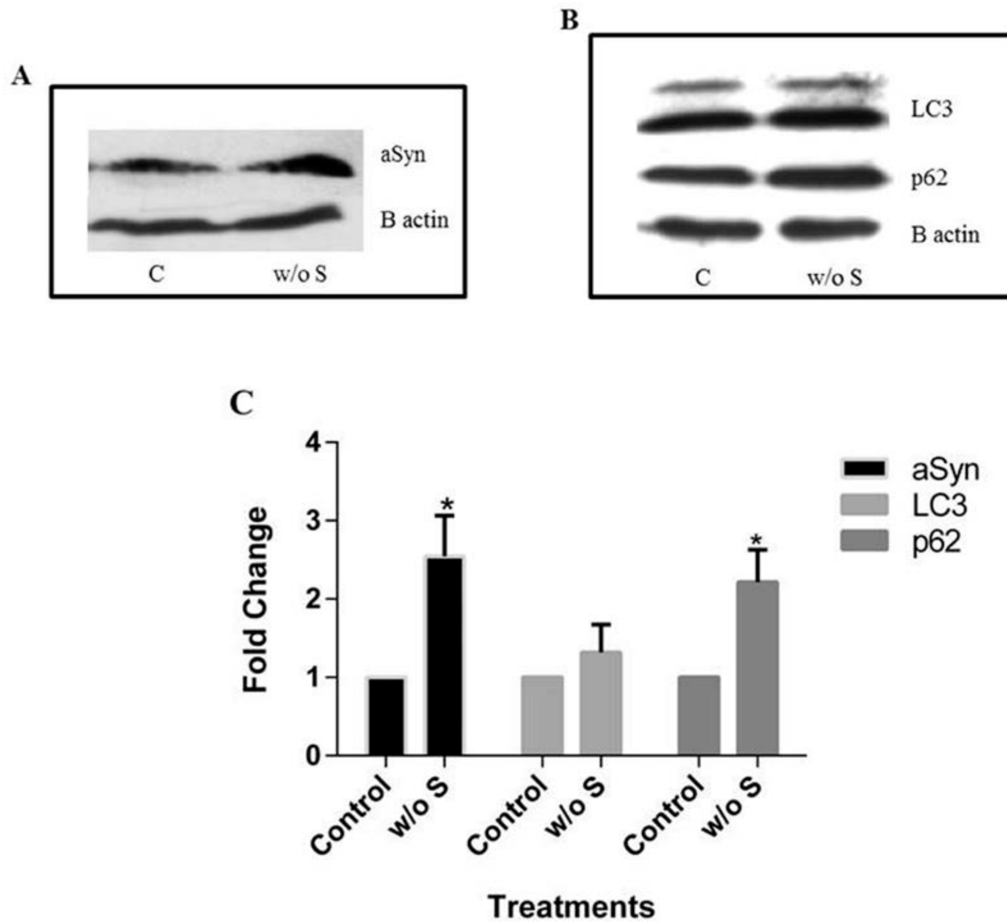


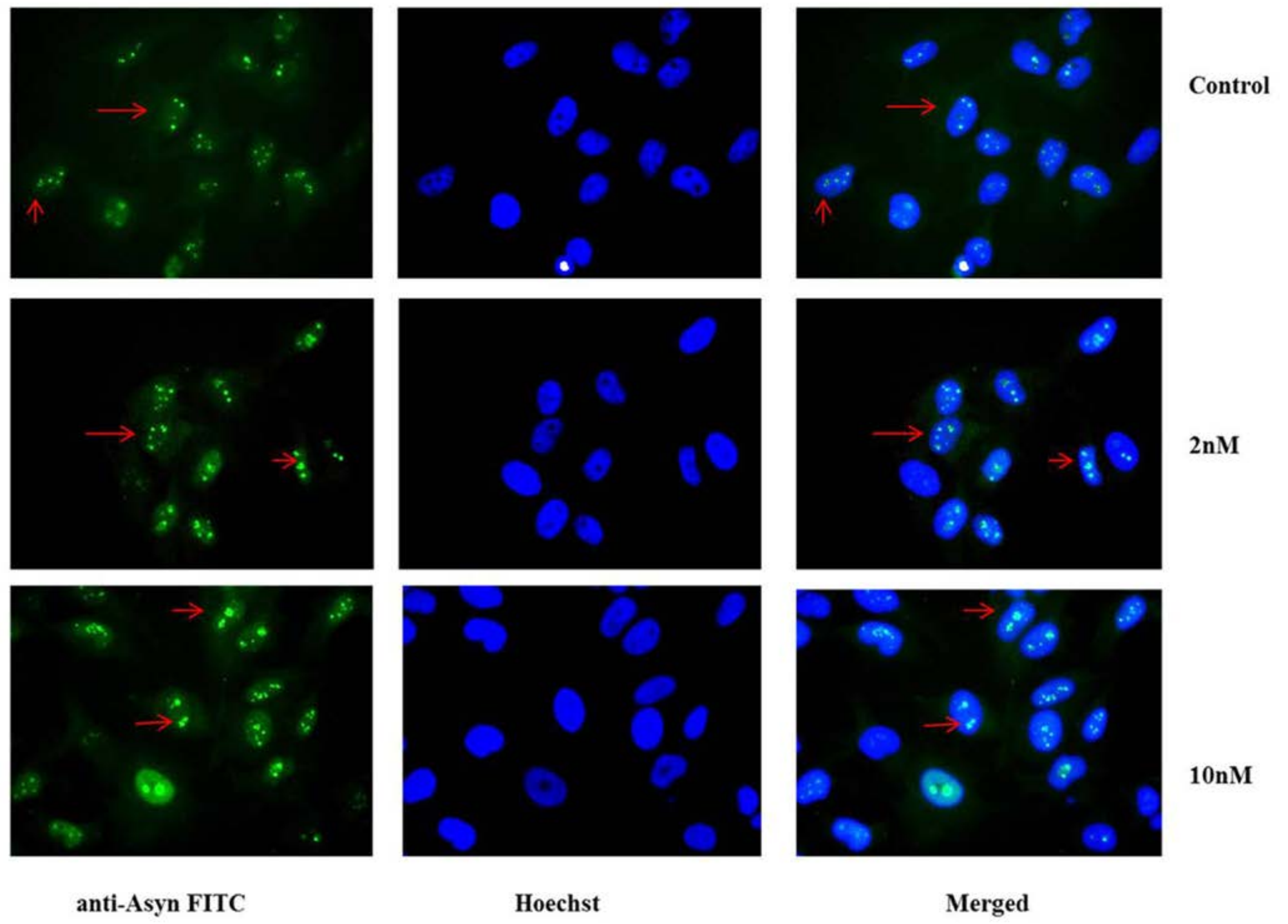
Figure 44: Autophagy induction with serum starvation

(A) and (B) Western Blot showing changes in aSyn and LC3 / p62 respectively, following serum starvation for 24 hours. (C) Graphs representing the western blot quantification of aSyn, LC3 and p62. (n=3, *$p < 0.05$).

Another potent inducer of autophagy is starvation. Under conditions of starvation, mTOR is inhibited resulting in induction of autophagy. Therefore, the cells were deprived of serum to mimic a starvation condition. Autophagy induction through serum starvation also resulted in a 2.5 fold increase in aSyn levels. This confirmed that the increase in aSyn levels is in response to autophagy induction and is not merely a drug-induced response. (Fig.44A, B, C).

(f) Autophagy Induction does not bring about a change in aSyn localization:

Results from autophagy inhibition demonstrate that alterations in the autophagic pathway affect aSyn distribution in the cell. Since autophagy induction led to an increase in aSyn levels, we wished to investigate if the increased aSyn levels was associated with changes in its sub-cellular localization. Immunocytochemistry data from Torin-1 treated cells show that autophagy induction does not increase the tendency of aSyn to localize to the cytoplasm (Fig.45A); instead an increase in the nuclear staining is observed (Fig.45B) suggesting that the aSyn is preferentially retained in nucleus.


A
Control
2nM
10nM
anti-Asyn FITC
Hoechst
Merged

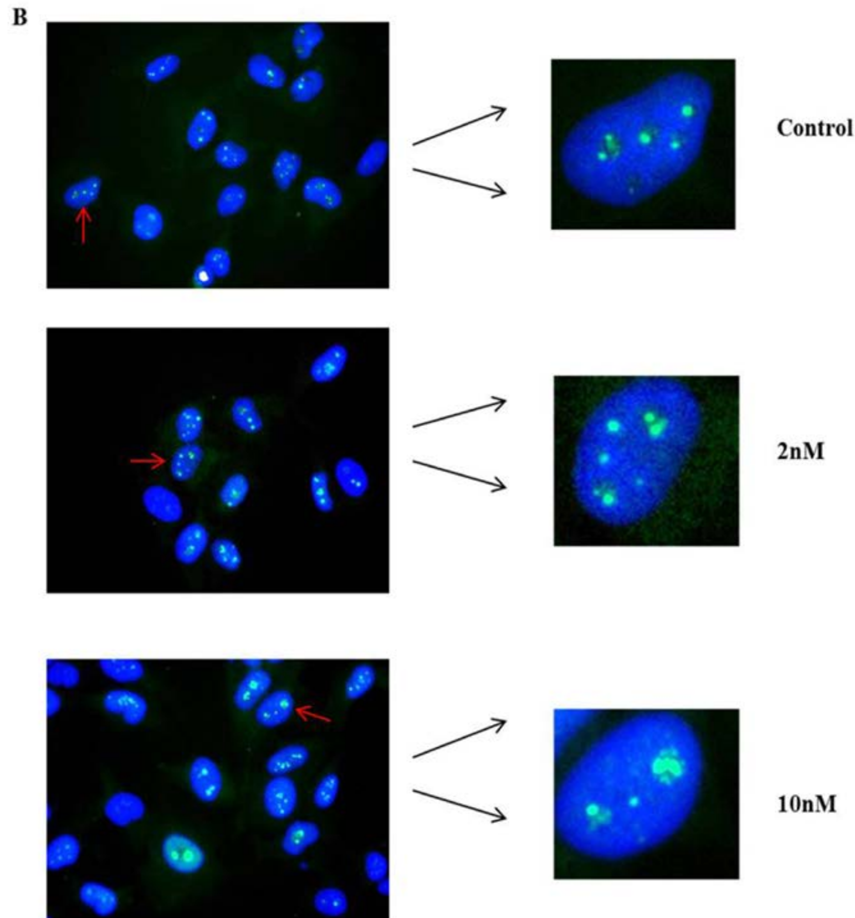


Figure 45: Subcellular distribution of aSyn upon autophagy induction

(A) SKMEL 28 cells stained for aSyn after treatment with Torin-1 at concentrations of 2nM and 10nM for 12 hours showing an increase in nuclear staining when compared with control (pseudo-colored). Cells were counter stained with Hoechst. (B) Enlarged image of the same. (Red arrows represent aSyn staining).

(g) Knockdown of aSyn results in a more efficient induction of autophagy:

The increase in aSyn levels upon autophagy induction and its retention in the nucleus led us to postulate that aSyn may regulate the extent of autophagy induction. To verify this, aSyn expression was knocked down using RNA interference. PCR results indicated an 85% knockdown in aSyn expression following siRNA transfection (Fig.46A, B).

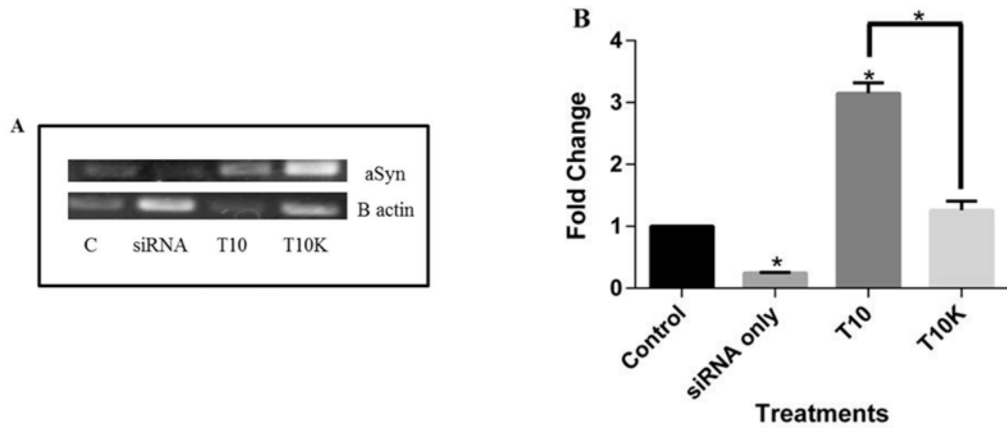


Figure 46: Knockdown of aSyn

(A) Changes in mRNA expression after treatment with 10nM Torin-1 for 8 hours with (T10K) and without (T10) aSyn knockdown assayed through semi-quantitative PCR. The increase is aSyn expression after treatment with Torin-1 was not seen in cells with aSyn knock-down. (B) Graph representing the quantification of PCR products run on an agarose gel. (n=3, *p < 0.05).

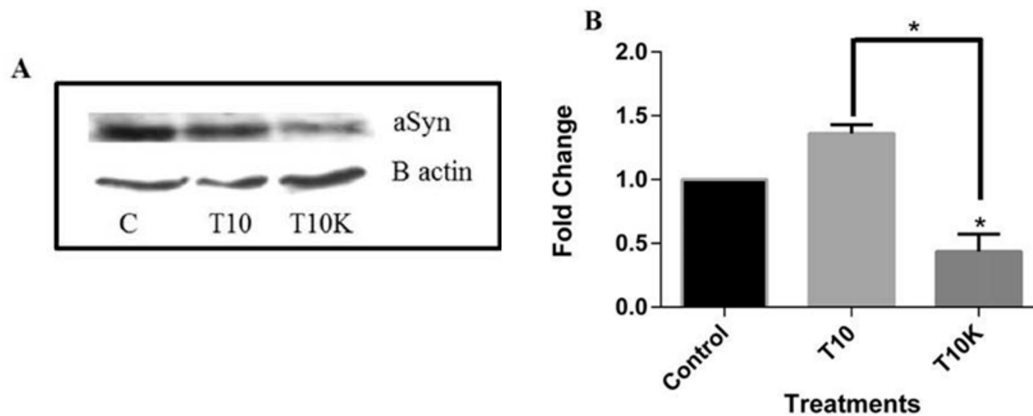


Figure 47: aSyn expression after knockdown of aSyn

(A) Western blot showing the decrease in aSyn protein levels after knockdown when treated with Torin-1 at 10nM for 8 hours while cells with intact aSyn expression showed a moderate increase in aSyn with the same treatment. (B) Graph representing the quantification of aSyn. (n=3, *p < 0.05)

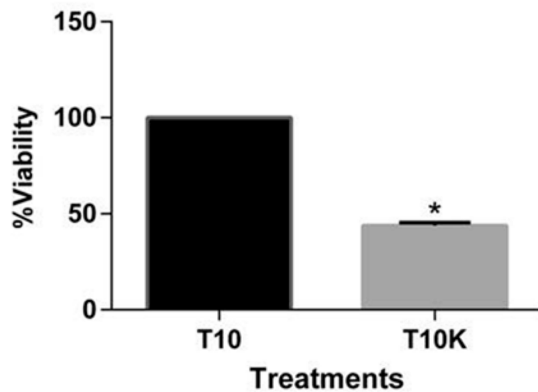


Figure 48: Cell viability after Torin-1 treatment in aSyn knockdown cells

The viability of aSyn knockdown cells (T10K) after treatment with 10nM Torin-1 for 8 hours was compared with Torin-1 treated cells with intact aSyn expression (T10) and a 50% loss in viability was observed in the knock-down cells as obtained through a cell count. (n=3, *p<0.05)

The decrease in mRNA was also reflected in the reduced protein levels post transfection (Fig.47A, B). These cells were then subsequently treated with autophagy inducer, Torin-1 at 10nM concentration. Addition of Torin-1 in aSyn knock-down cells led to a 50% loss in viability compared to Torin-1 treated cells with intact aSyn expression (Fig.48).

The aSyn knock-down cells without Torin-1 exposure did not show any loss in viability indicating that the transfection process or the knock-down of aSyn alone did not affect the cell viability. Since our earlier result showed increase in aSyn mRNA levels following treatment with Torin-1, the mRNA expression of aSyn between aSyn knock-down cells and siRNA untreated cells after addition of Torin-1 was compared. The Torin-1 treated cells, in agreement with our earlier result showed a 3 fold increase in mRNA levels (Fig.46A, B). However, in the aSyn knock down cells exposed to Torin-1 the mRNA levels did not increase and was similar to the levels in that of untreated controls suggesting that knockdown of aSyn significantly attenuated the Torin-1 induced increase in mRNA levels of aSyn (Fig.46A, B).

The effect of aSyn knockdown on the cells response to autophagic induction wit Torin-1 was assessed to ascertain if aSyn played a role in regulating this response. The level of p62 was therefore compared between aSyn knockdown cells and cells with intact aSyn expression exposed to Torin-1 to test for differences in extent of autophagic induction (Fig. 49A). The aSyn knock-down cells treated with Torin-1 showed an approximately 2-fold decrease in p62 levels while the aSyn intact cells treated with Torin-1 did not show any decrease in p62 levels (Fig.49C). The level of LC3-II was also increased in aSyn knockdown cells in comparison to cells with intact aSyn expression (Fig.49B).

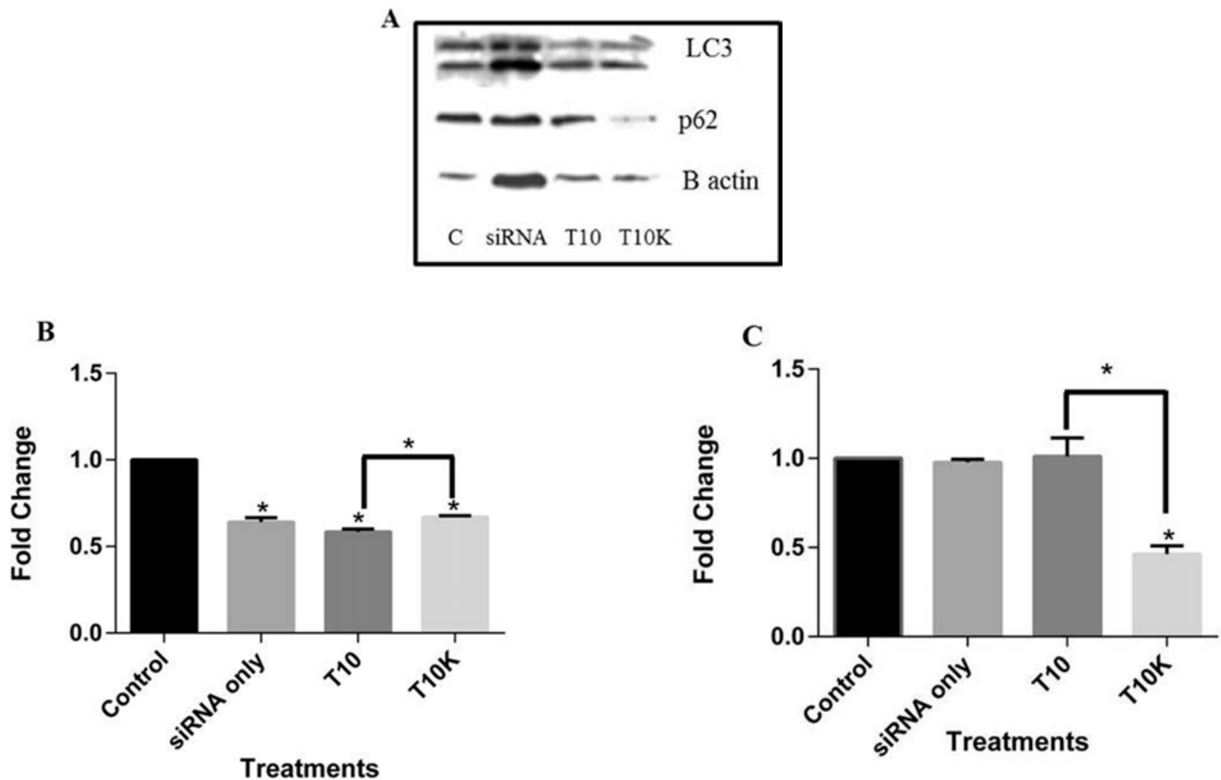


Figure 49: Effect of Torin-1 on autophagy induction in aSyn knockdown cells

(A) Western Blot showing increase in LC3 and a decrease in p62 in aSyn knockdown cells when treated with 10nM Torin-1 for 8 hours when compared with Torin-1 treated cells with intact aSyn expression. (B) and (C) Graph representing the quantification of western blot for LC3 and p62 respectively. (n=3, $*p < 0.05$)

It is worthwhile to note that the in cells with aSyn expression, a 2-fold decrease in p62 levels is observed 12 hours after the addition of Torin-1 (Fig. 41C) while in the absence of aSyn, a similar decrease in p62 levels is achieved within 8 hours of drug addition (Fig.49C).

4.2 RESPONSE TO DOPAMINE AND EFFECT ON AUTOPHAGY

The preferential loss of dopaminergic neurons in PD are attributed partly to the toxic effects of dopamine itself either through generation of reactive oxygen species or through its interactions with aSyn which predispose aSyn to misfolding and aggregation (Galvin, 2006; Outeiro et al., 2009; Yamakawa et al., 2010). However, *in vitro* studies on various cell models including SH-SY-5Y, demonstrate that addition of exogenous dopamine causes a severe loss in viability (Jiang et al., 2008). Given the presence of endogenous aSyn of differing molecular weighs and the similarities in melanin and dopamine synthesis pathways, melanoma cells may serve as an ideal model to study dopamine-aSyn interactions.

(a) SK-MEL-28 cells are resistant to exogenous dopamine:

The effect of dopamine on SK-MEL-28 cell viability was first tested at three different concentrations and it was seen that SK-MEL-28 cells were relatively resistant to dopamine upto 1mM concentration and loss in viability was only seen at very high concentrations (Fig.50).

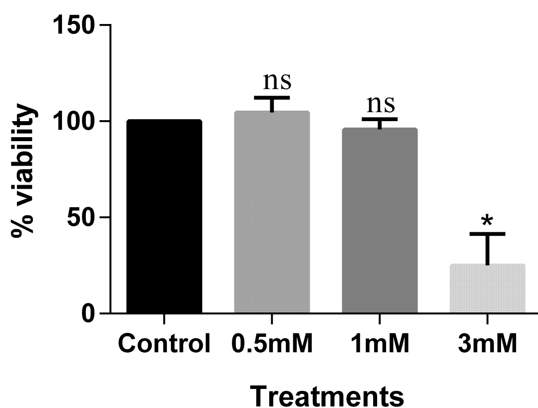


Figure 50: Cell viability after dopamine treatment

Cell viability after addition of Dopamine was measure through the MTT assay and was found that SK-MEL-28 cells did not show any loss of viability upto 1mM concentration but a more than 50% loss in viability was observed at 3mM. (n=3, ^{ns} $p>0.05$ $^*p<0.05$)

The relatively high resistance of SK-MEL-28 cells to dopamine led us to question whether the resistance was due to a lack of exogenous dopamine uptake. Radiolabelled benzamides derivatives are used for the detection of malignant melanomas and its uptake is thought to be mediated by dopamine receptors on melanoma cells. The presence of dopamine receptors on melanoma cells is unconfirmed with reports in favor of both its presence and absence (Bodei et al., 2003; Böni et al., 1997; Lin et al., 2001). Therefore to confirm that the increased resistance towards exogenous dopamine is not due to lack of uptake, SK-MEL-28 cells were treated with dopamine tagged to dansyl chloride wherein the conjugate emits green fluorescence. Dansyl chloride tagged dopamine was added at 0.75mM, 1mM and 3mM concentrations and incubated for 8 hours. Our results indicate that SK-MEL-28 cells can take up dopamine in a concentration dependent manner and that the resistance towards exogenous dopamine is not due to the inability of the cells to take up dopamine (Fig.51).

(b) SK-MEL-28 cells show resistance to dopamine induced oxidative stress:

Dopamine induced cytotoxicity is often mediated through the generation of oxidative stress (Cubells et al., 1994; Gandhi and Wood, 2005; Lotharius and Brundin, 2002b). The high resistance of SK-MEL-28 to dopamine could be due to a result of effective management of oxidative stress and therefore generation of reactive oxygen species (ROS) as a marker of oxidative stress was used to evaluate the level of oxidative stress caused due to dopamine addition on SK-MEL-28. The levels of malondialdehyde, a stable end product of lipid peroxidation which occurs due to ROS, was measured and it was found that exogenous dopamine did not significantly increase ROS (Fig.52) in the cell which may explain the increased tolerance of SKMEL 28 to dopamine.

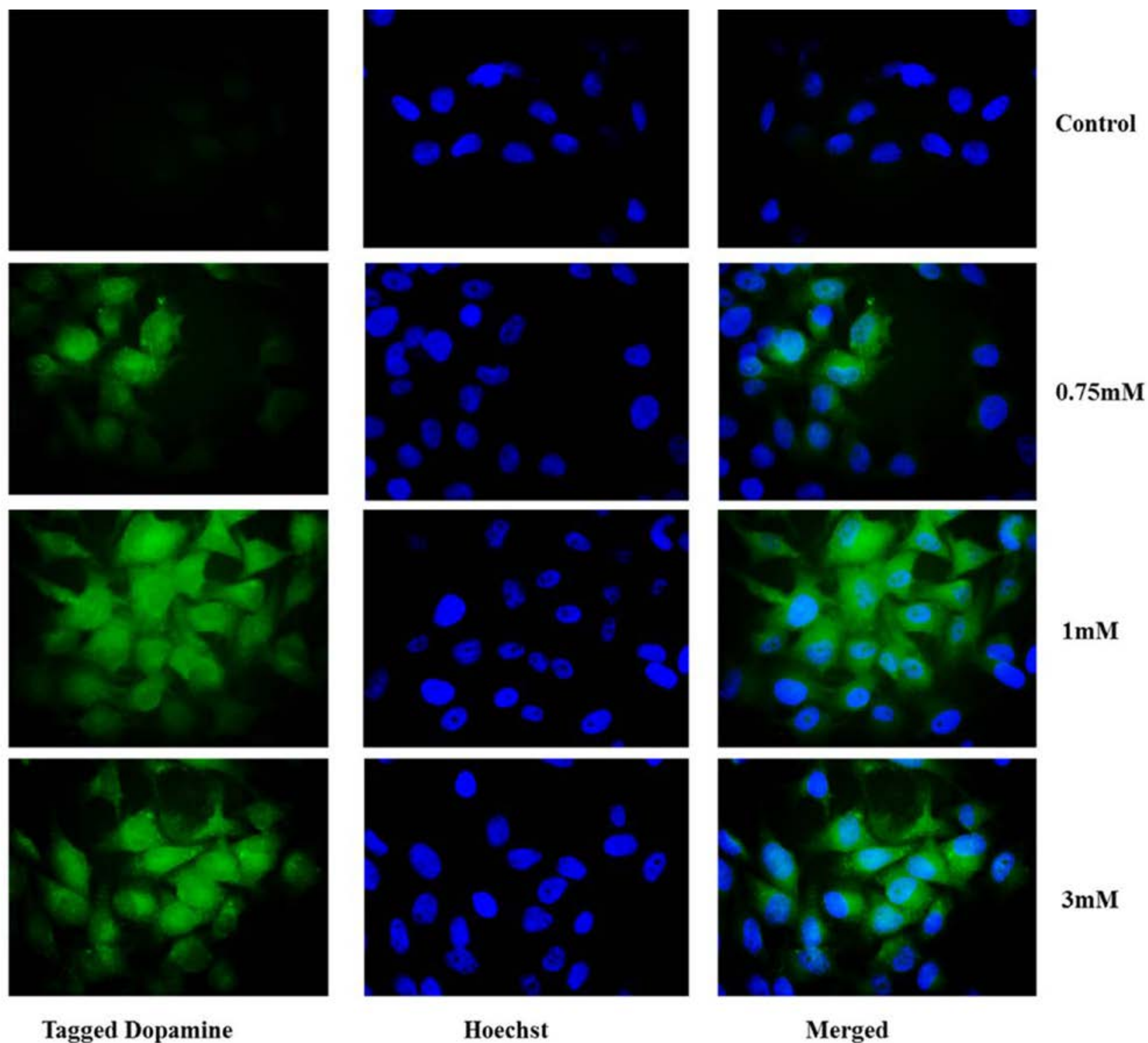


Figure 51: Exogenous dopamine uptake by SK-MEL-28

Dopamine Tagged to Dansyl chloride was added to SK-MEL-28 cells at concentrations of 0.75 mM, 1mM and 3mM and green fluorescence observed after 8 hours. SK-MEL-28 cells showed dopamine uptake at all three concentrations. Cells were counter stained with Hoechst. (Pseudo-colored)

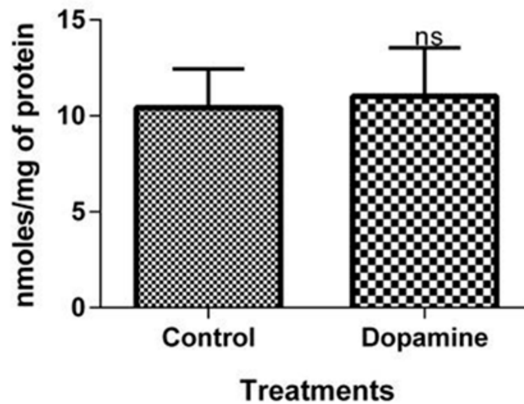


Figure 52: Effect of dopamine on ROS generation in SK-MEL-28

The TBARS assay was used to measure the content of malondialdehyde in the cells as a measure of ROS generation after addition of 1mM dopamine for 24 hours and it was found that dopamine addition did not lead to a significant increase in ROS. (n=3, ^{ns} $p>0.05$)

(c) Inhibition of autophagy in presence of dopamine is not reversed through autophagy induction:

Although oxidative stress is the most prominent effect of dopamine metabolism, oxidative intermediates of dopamine metabolism can also induce cytotoxicity through the impairment of protein degradation pathways (Segura-Aguilar et al., 2014). Therefore the effect of exogenous dopamine addition on autophagic process in SK-MEL-28 was tested. The results indicate that dopamine does indeed trigger autophagy inhibition within 8 hours which is sustained upto 24 hours and the dopamine induced autophagy is not reversed even in the presence of autophagy inducer Torin-1 (Fig.53A, B). Interestingly although the presence of Torin-1 in dopamine treated cells did minimize the initial onset of autophagy inhibition, by 24 hours, the dopamine treated cells exposed to Torin-1 showed a much greater inhibition of autophagy when compared to dopamine treated cells not exposed to Torin-1 (Fig.53C, D, E, F).

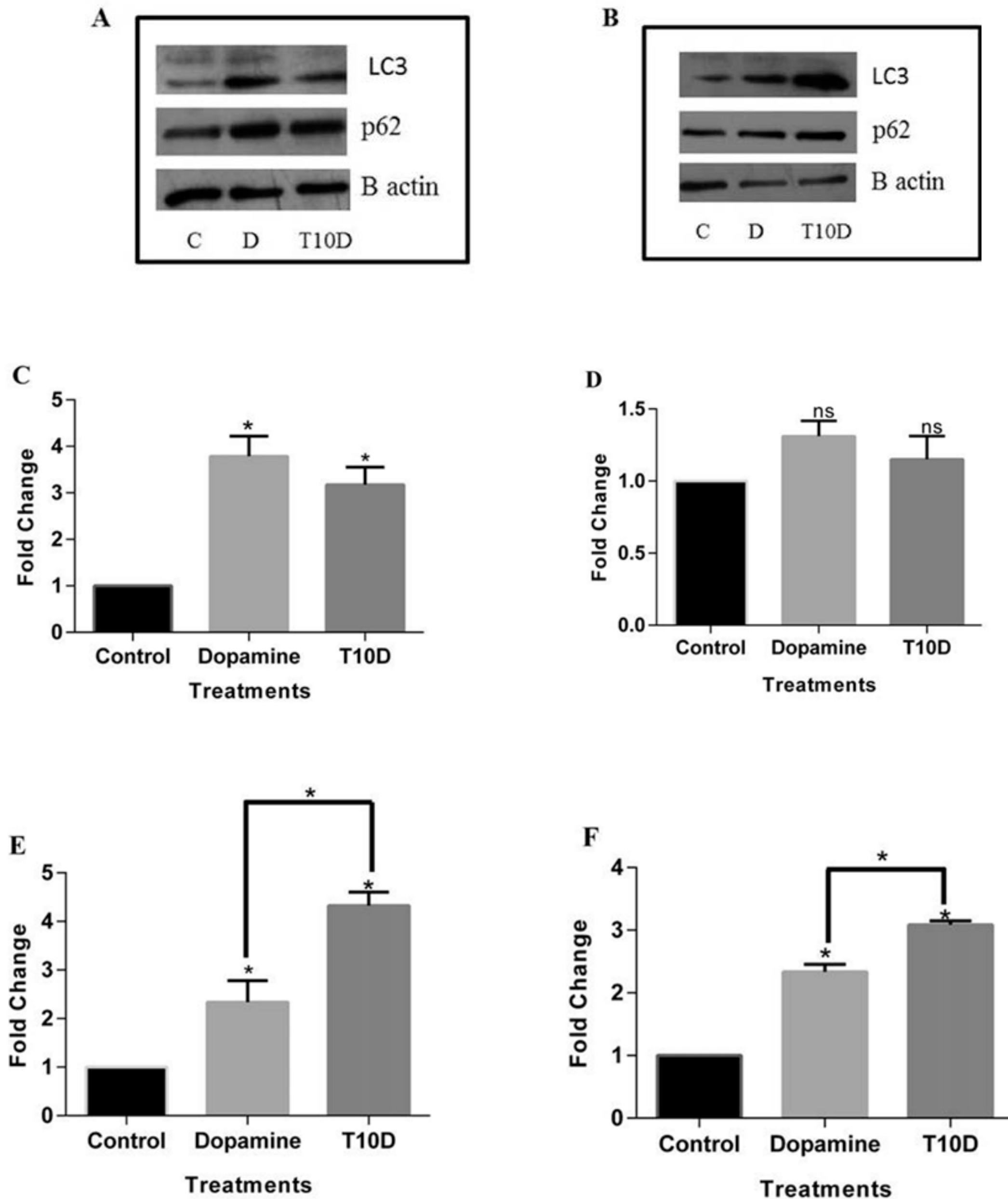


Figure 53: Effect of dopamine and combined dopamine and Torin-1 on autophagy

(A) and (B) Western Blot showing changes in autophagic markers p62 and LC3 after treatment with 1mM dopamine alone and dopamine with 10nM Torin-1 for two time points of 8 hours and 24 hours respectively. (C) and (D) Graphs representing western blot quantification of LC3 and p62 respectively for the 8 hour time point. (E) and (F) Graphs representing western blot quantification of LC3 and p62 respectively for the 24 hour time point. ($n = 3$, $*p < 0.05$)

Additionally although neither Torin-1 nor dopamine alone induced cell death in SKMEL 28 cells for upto 24 hours, the combination of dopamine treatment with autophagy induction, led to a 50% loss in viability by 24 hours (Fig.54B) with no significant cell loss at 8 hours (Fig.54A).

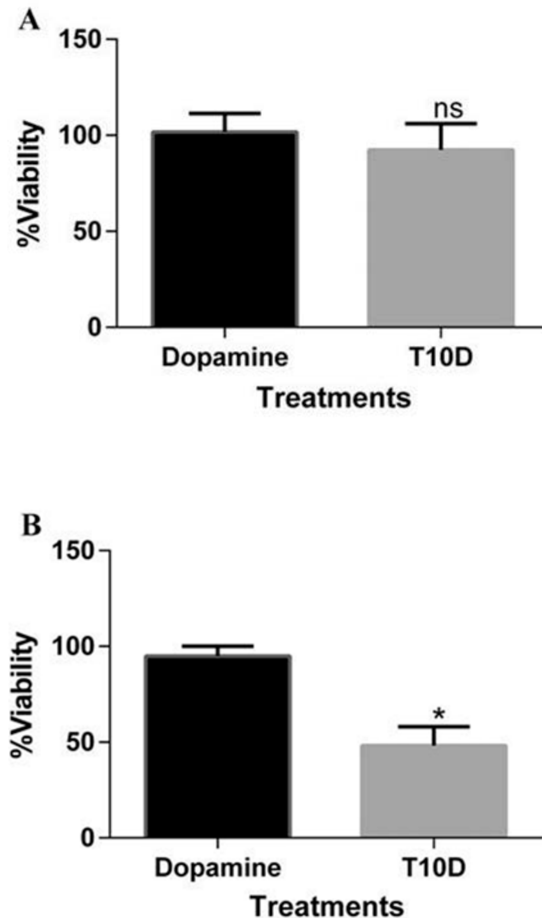


Figure 54: Cell viability with combined dopamine and Torin-1 treatment

Cell viability, measured by MTT assay, was compared between cells treated with 1mM dopamine and 10nM Torin-1 simultaneously and dopamine alone treated cells at (A) 8 hour time point at which there was no cell death (B) 24 hour time point at which dopamine and Torin-1 combined treatments led to a 50% loss in viability. (n=3, *$p<0.05$)

(d) Dopamine causes an increase in aSyn protein levels:

It is known that the aggregation of aSyn is enhanced in the presence of dopamine (Outeiro et al., 2009; Yamakawa et al., 2010) and aggregated aSyn could in turn impair autophagy (Winslow et al., 2010; Xilouri et al., 2009). Therefore the effect of exogenous dopamine addition on aSyn levels in SK-MEL-28 cells was assessed. There was a 4-fold increase in aSyn levels within the first 8 hours of dopamine addition which was again sustained upto 24 hours (Fig.55A, B). Exposure of dopamine treated cells to Torin-1 did not lead to increased clearance, instead led to an even stronger increase in aSyn expression levels, similar to our earlier observations with Torin-1 treatment alone (Fig.55C, D).

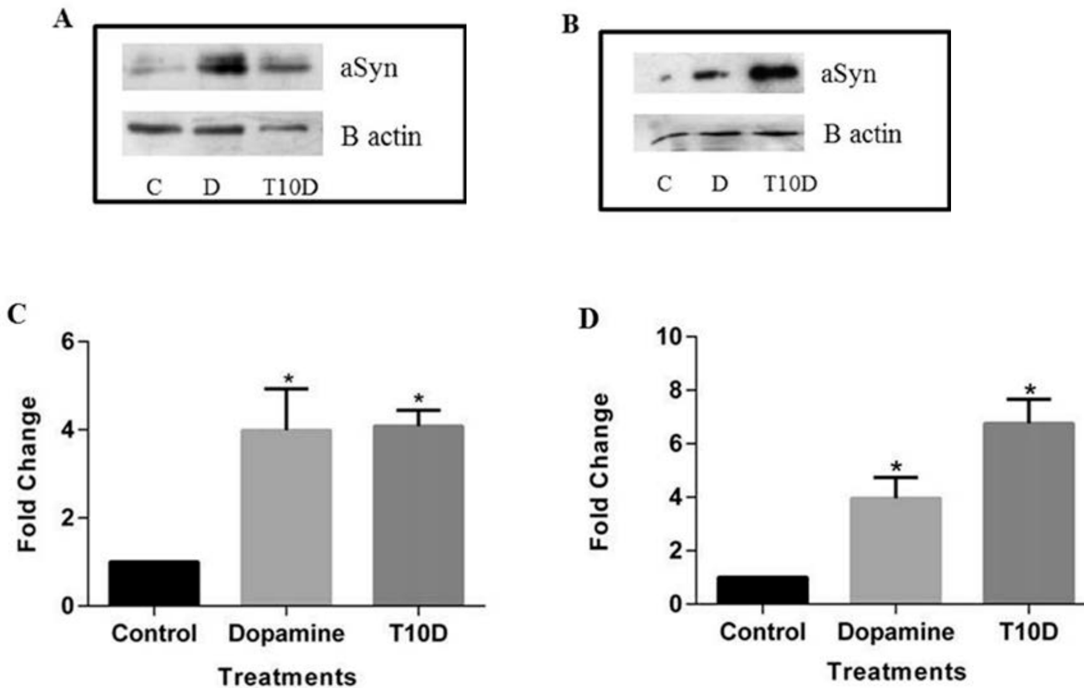


Figure 55: Effect of dopamine and combined dopamine and Torin-1 treatment on aSyn

(A) and (B) Western blot showing increase in aSyn when treated with 1mM dopamine alone and in presence of 10nM Torin-1 at two time points of 8 hours and 24 hours respectively. (C) and (D) Graphs representing the western blot quantification of aSyn at 8 hours and 24 hours respectively. (n=3, *$p < 0.05$)

Surprisingly, the increase in aSyn protein levels upon dopamine addition is preceded by a decrease in mRNA levels while a combination of Torin-1 and dopamine treatment leads to an increase in mRNA (Fig.56A) although the increase is not as significant as with Torin-1 alone treatment (Fig.56B). The increase in aSyn level after dopamine could possibly be due to increased retention of aSyn in the cell in turn leading to increased turnover of mRNA. Although the addition of Torin-1 also results in increased aSyn, it is clear that this results from increased synthesis as evident from the increase in mRNA levels with Torin-1 treatment.

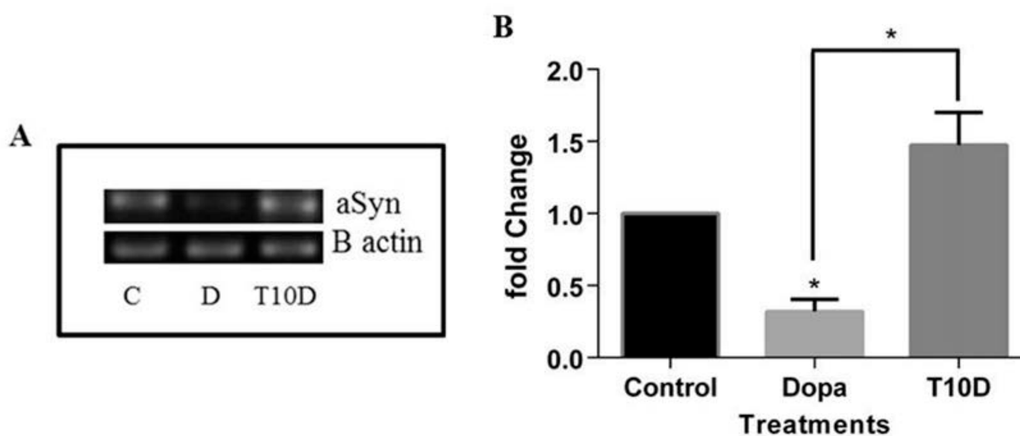


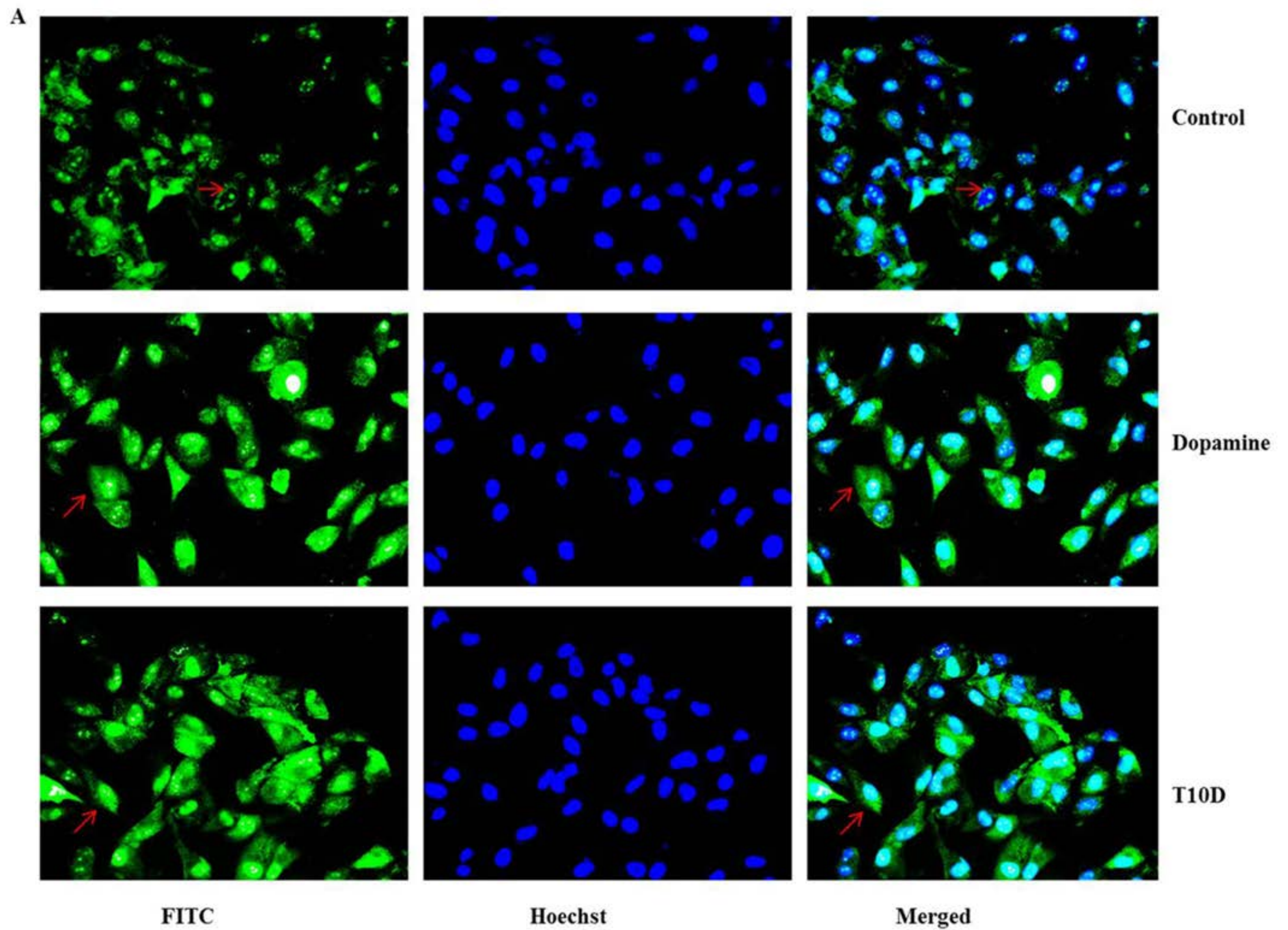
Figure 56: mRNA expression of aSyn upon dopamine treatment

(A) Changes in mRNA expression of aSyn when treated with 1mM dopamine alone and in presence of 10nM Torin-1 at 8 hour time point assayed by semi-quantitative PCR. Treatment with dopamine alone led to a decrease in mRNA while combined treatment of dopamine and Torin-1 led to an increase in mRNA. (B) Graph representing the quantification of PCR products run on agarose gel. (n=3, $*p < 0.05$).

(e) Increase in aSyn protein levels is also accompanied by change in localization:

Since dopamine can promote aggregate formation and since the addition of exogenous dopamine resulted in increase in protein levels, it seemed likely that dopamine addition could affect aSyn localization. This was investigated and it was found that exogenous dopamine addition resulted

in increased cytoplasmic localization (Fig.57A). Interestingly, the combined treatment of dopamine and Torin-1 also caused increased cytoplasmic localization of aSyn as opposed to the nuclear retention of aSyn observed with earlier with Torin-1 treatment alone (Fig.57B).



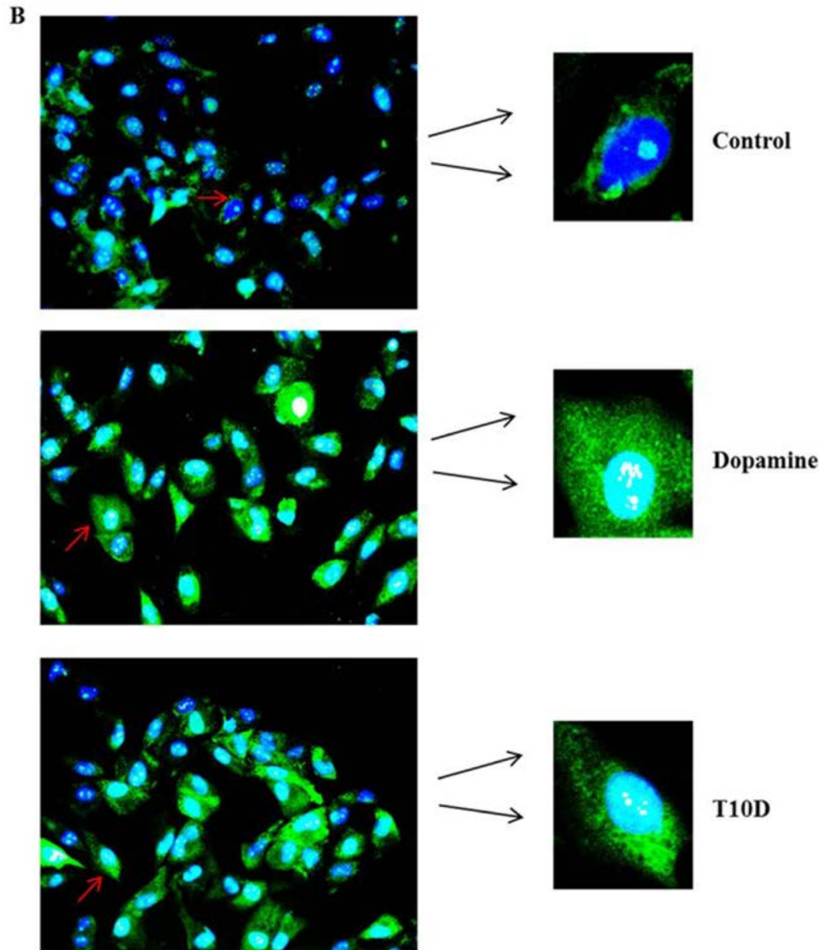


Figure 57: Subcellular distribution of aSyn upon dopamine treatment

(A) SK-MEL-28 cells stained for aSyn after treatment with 1mM dopamine alone and in presence of 10nM Torin-1 shows an increase in cytoplasmic localization of aSyn under both treatment conditions. Cells were counter stained with Hoechst. (B) Enlarged image of the same. (pseudo-colored). (Red arrows represent aSyn staining).

(f) Autophagy inhibition in presence of dopamine is mediated via aSyn:

Since dopamine addition evoked significant changes in aSyn expression levels and in its localization which was not reversed even when cells were treated with an autophagy inducer, it seemed possible that dopamine mediated inhibition of autophagy in SK-MEL-28 cells was

mediated via aSyn. Therefore, aSyn expression was knocked down in SK-MEL-28 cells following which they were treated with dopamine and the levels of autophagic marker p62 assessed. Results show that in absence of aSyn, cells treated with dopamine showed a nearly 2 fold decrease in p62 levels (Fig.58A) when compared to dopamine treated cells with intact aSyn expression (Fig.58C) although no significant change was observed in LC3-II levels(Fig.58B).

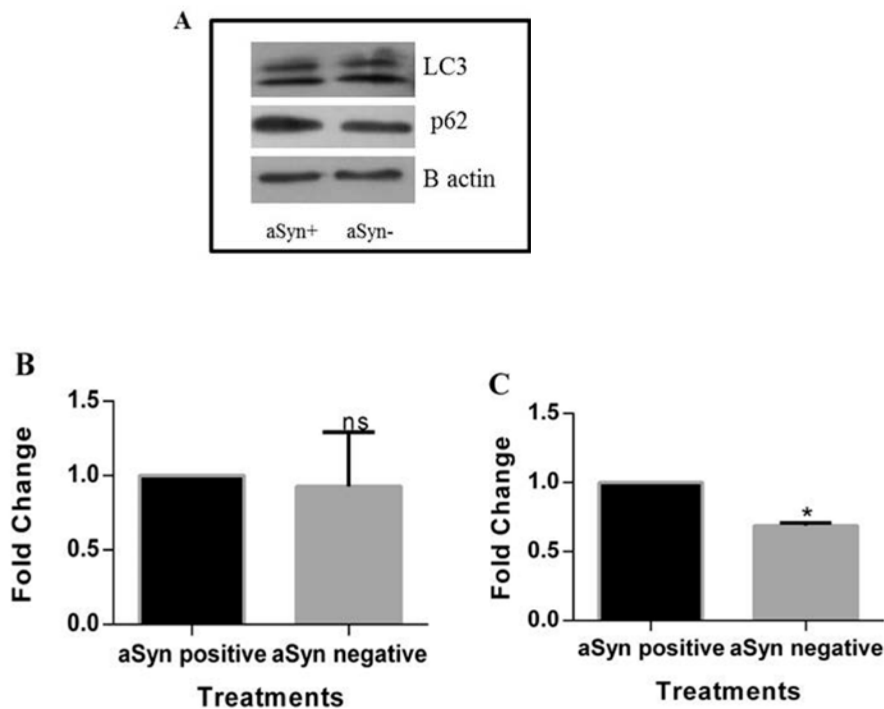


Figure 58: Effect on autophagy upon dopamine treatment in aSyn knockdown cells

(A) Western blot showing changes in autophagic markers LC3 and p62 in cells treated with 1mM dopamine in the presence and absence of aSyn for 8 hours. (B) and (C) Graphs representing the western blot quantification of LC3 and p62 respectively. (n=3, $*p < 0.05$)

(g) Addition of dopamine results in increased melanin levels:

Recent studies suggest that changes in autophagy maybe closely linked to melanogenesis and melanin synthesis (Ho and Ganesan, 2011; Murase et al., 2013a) and is thought that autophagy

inhibition can lead to an increase in melanin content. Additionally, excess dopamine in the neurons maybe converted to neuromelanin. Since dopamine addition leads to autophagy inhibition in SK-MEL-28 cells, melanin content in these cells was assayed after treatment with dopamine. Results show that dopamine addition led to a 1.5 fold increase in melanin content when compared to control cells within 8 hours of dopamine addition. This increase was however not sustained and there was drop in melanin content by 16 hours, almost nearing that of control. The combined treatment of dopamine and autophagy induction with Torin-1, which showed a strong inhibition of autophagy, also resulted in increased melanin content. However, in cells treated with dopamine and Torin-1 the increase in melanin content was delayed and a 1.5 fold increase was observed only by 16 hours (Fig.59A, B).

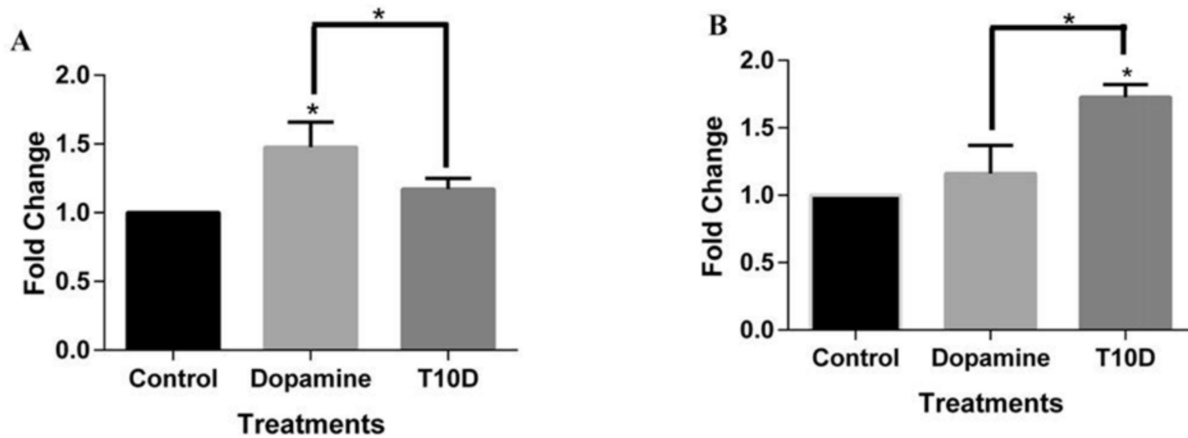


Figure 59: Effect on melanin levels upon dopamine and combined dopamine and Torin-1 treatment

(A) Increase in melanin content in cells treated with 1mM dopamine when compared to control while a combination of dopamine and 10nM Torin-1 did not lead to a similar increase at 8 hours. (B) Drop in melanin content observed in cells treated with 1mM dopamine after 16 hours while combined treatment of dopamine and Torin-1 leads to an increase in melanin content by 16 hours. (n=3, *p<0.05).

5. DISCUSSION

Several neurodegenerative diseases exhibit characteristic inclusion bodies primarily composed of protein aggregates and hence protein misfolding appears to be a central feature leading to pathogenesis. Depending on the protein and its localization, various pathogenic events leading to cell death might be identified but they all hinge on loss of protein function (Chiti and Dobson, 2006).

The protein aSyn, that is found aggregated in a variety of neurodegenerative disorders collectively classified as synucleinopathies (Savica et al., 2013), plays a role in synaptic vesicle recycling and neurotransmitter release. It is also thought to play a role in regulating the dopamine content in the cells through its interaction with Tyrosine hydroxylase the key enzyme in dopamine biosynthesis and also through its interactions with dopamine transporters (Cheng et al., 2011; Perez et al., 2002). Therefore loss of its function results in disturbances in synaptic function, decreased neurotransmitter release and most importantly, an increase in cellular dopamine. The increase in dopamine content is associated with oxidative stress which partially explains the preferential loss of dopaminergic neurons seen in PD brains. However in addition, aSyn mediated cytotoxicity is not limited to its loss of function. The toxic oligomeric species also exhibit a gain in toxic function whereby they form pores on membranes resulting in calcium influx and mitochondrial damage leading to eventual cell death (Stefanis, 2012). Interestingly its role in vesicular trafficking is two edged. Although its regulation of vesicular transport appears to underlie its role in synaptic function, this can also have pathological consequences such as inhibition of phagocytosis and autophagy under a different cellular context. The misfolding of aSyn is therefore responsible for a host of pathogenic events with cell death being the final eventuality.

Understanding factors that promote aSyn misfolding has become paramount to decoding disease pathogenesis. Cellular stresses such as oxidative stress, mitochondrial damage and increased dopamine are among the several factors that appear to promote its misfolding. Since these events are also a consequence to aSyn misfolding, identifying the primary pathogenic event has turned into one of the biggest challenges in understanding disease onset and progression. Despite the relatively low incidence of monogenic forms of PD when compared to sporadic PD, the indications for triggers of aSyn misfolding have been obtained through familial PD, most particularly those harboring mutations in aSyn.

Both point mutations and gene multiplications of the *SNCA* have been described in familial PD. Typically, the aggregation of aSyn is a nucleation dependent process with the formation of the "seed" being the rate limiting step. Point mutations increase the propensity for aggregation by decreasing the time required for seeding. Gene duplications on the other hand, lead to increased cytosolic concentration of the protein which again favors the misfolding and aggregation of proteins. The increase in severity of the disease in cases with gene triplications compared to gene duplications further lends support to the increased cytosolic concentrations of the protein being cytotoxic. Although sporadic PD may lack gene mutations that increase protein concentrations, other mechanisms may act to increase cellular protein levels. Maintenance of steady state of proteins is achieved through regulating its gene expression or through regulating its degradation. Therefore in absence of any genetic level control, it appears that the protein degradation pathways are to be held responsible for the increase in proteins in the cells, particularly with respect to sporadic PD. In this regard, great emphasis has thus been laid on understanding the degradation kinetics of aSyn (Kim and Lee, 2008).

Initial reports suggested that aSyn is largely degraded through the UPS while recent studies suggest autophagy could also play a role in its degradation. Misfolded aSyn is also thought to be routed for degradation through autophagy (Batelli et al., 2011; Ebrahimi-Fakhari et al., 2011; Mak et al., 2010; Mizushima et al., 2008; Webb et al., 2003a). The aggregation in itself can overcome the degradation process, was evidenced by studies showing oligomeric aSyn blocking UPS, mutant forms of aSyn blocking CMA and blocking of macroautophagy by aSyn under conditions of overexpression (Chen et al., 2005; Emmanouilidou et al., 2010b; Lindersson et al., 2004; Winslow et al., 2010; Xilouri et al., 2009). Additionally, inhibition of the lysosomal pathway also leads to an inhibition of the proteasome pathway (Qiao and Zhang, 2009). The cellular concentration beyond which aSyn accumulates, its ability to bypass the degradation process during misfolding and the stage of aggregation at which degradation process is not just overcome but effectively impaired are as yet undetermined. This perhaps contributes to the lack of a clear consensus on the degradation of aSyn since the response of the different cell models particularly those with overexpression of aSyn appear to vary depending on their tolerance to the protein burden in the cell. Therefore the utilization of model systems transfected with aSyn gene often adds to the complexity while studying an already tumultuous relationship between aSyn accumulation and protein degradation pathways (Xilouri et al., 2012a).

In addition to causing impairment of degradation pathways, overexpression of aSyn has been shown to trigger aggregation and also cause oxidative damage to cell (Hsu et al., 2000; Oliveras-Salvá et al., 2013). On the contrary, a recent report utilizing a system with endogenous expression of aSyn suggests that at physiological levels, aSyn may in fact protect cells from oxidative damage and apoptosis (Musgrove et al., 2012). This once gain suggests that the protein levels in itself are an important determinant of the switch between physiology and pathology.

The enhancement of protein degradation pathways most notably autophagy has already been demonstrated to have beneficial effects in diseases such as AD and HD although it has not translated to clinical success (Nixon, 2013; Rubinsztein et al., 2015). Therapeutic interventions involving autophagy are largely hindered by lack of suitable models and an incomplete understanding of degradation kinetics especially with regard to aSyn. In this regard, a study in T-lymphocytes has shown that aSyn inhibits autophagy and may play a role in diseases such as Systemic Lupus erythematosus (SLE) which is characterized by resistance to autophagy induction (Colasanti et al., 2014).

Largely considered neuronal, the presence of aSyn has been detected in several other cell types recently although its role in these cell types are not yet known. Prominent among these is its detection in melanoma (Matsuo and Kamitani, 2010). This is particularly relevant given that increased correlation between PD and melanoma have been reported in various epidemiological studies with aSyn being a common denominator between both. Additionally, a study exploring the therapeutic potential of tyrosinase positive melanocyte transplants in rat models of PD suggests that such transplants may have beneficial effects through supplementing L-DOPA/dopamine levels (Asanuma et al., 2013). Although melanocytes are found to lack aSyn, uptake of aSyn by cultured cells and tumor cells has been demonstrated. Further, aSyn has been shown to inhibit melanin synthesis and decrease in melanin content can be a precursor to intiation of melanoma. (Israeli et al., 2011; Matsuo and Kamitani, 2010; Paisán-Ruiz and Houlden, 2010; Pan et al., 2011, 2012). Also aSyn phosphorylated at Ser126 has been found to be expressed in melanoma and is found to colocalize to microtubules which results in vesicular release of aSyn (Lee et al., 2013a). In co-culture studies, aSyn has been found to propagate from melanoma cells to mouse neuroblastoma cells (Hansen et al., 2011). Taken together, these

observations argue for a need to study aSyn behavior in alternate cell models, especially in melanoma cells. Therapeutic interventions involving autophagy will also have a systemic influence which further necessitates the understanding of aSyn in all other cell types where it is expressed and the possible effects these interactions may have on cell behavior and in mediating disease conditions.

Although the occurrence of aSyn in the nucleus has remained controversial, its localization to the nucleus and binding to DNA under conditions of cellular stress have been reported (Goers et al., 2003; Guerrero et al., 2013; Xu et al., 2006). Interestingly, results from a yeast model demonstrate that aSyn translocation to the nucleus can protect from oxidative stress through histone modification but the study also suggests that protective or toxic effects of aSyn in the nucleus is dependent on its concentration (Liu et al., 2011). Also despite conflicting reports, the occurrence of aSyn as a stable tetramer in vivo which may be destabilized under harsh conditions and presence of higher molecular weight aSyn in other cell models have been reported. (Bartels et al., 2011; Colasanti et al., 2014; Dettmer et al., 2013; Lashuel et al., 2013; Leng et al., 2001). The results from this study not only demonstrate the nuclear localization of the protein but also show the presence of atleast two distinct molecular weight species of aSyn that exhibit different sub cellular localization. The monomeric aSyn appears to be present exclusively in the nucleus while a higher molecular weight species is found exclusively in the cytoplasm indicating a regular traffic of the protein between various cellular components which may act as another crucial determinant of the switch from physiology to pathology.

Its differential localization explains the conflicting studies reporting its degradation by both the UPS and ALP. While our study does not shed light on the physiological relevance of the presence of the stable higher molecular weights in the cell, it seems likely that a disturbance in

the balance between the monomeric and oligomeric species of aSyn due to change in cellular conditions might have a great bearing on its tendency to aggregate. Although, autophagy inhibition did not significantly alter aSyn monomer levels, suggesting that it may not be degraded by ALP, we observed an increase in the cytoplasmic localization of aSyn upon autophagy inhibition. This suggests that although autophagy inhibition may not be a preliminary cause for increase in aSyn monomer levels, it perhaps contributes to aSyn aggregation by increasing its trafficking to the cytoplasm where it exists as an oligomer, making it available to other cellular insults that stabilize the oligomers and over time impair the degradation process. A recent observation that autophagy inhibition could also cause increased secretion and exocytosis of aSyn adds support to our results (Lee et al., 2013b).

Conversely, the induction of autophagy caused an increase in aSyn monomer levels, and its greater retention in the nucleus. Therefore we tested the possibility of aSyn as a regulator of autophagy and found that the cells in which aSyn expression was knocked down responded better to autophagy induction compared to cells with intact aSyn expression. These observations are also supported by a similar study carried out in T-lymohocytes where aSyn knock down enhanced autophagy (Colasanti et al., 2014). It is therefore very likely that at physiological levels, monomeric aSyn contributes to autophagy regulation. Therefore a loss of function as a result of misfolding of aSyn may result in alterations in autophagy in turn disturbing the cellular homeostasis.

The stabilization of the higher molecular weight species by cellular factors plays a critical role in aSyn mediated cytotoxicity. In this regard, the change in aSyn localization to cytoplasm where it occurs as a higher molecular weight species exposes it to various stabilizing factors. The preferential loss of dopaminergic neurons in PD brains, the oxidative damage caused by

dopamine metabolism and its alleged stabilization of aSyn aggregates and the increase in dopamine content following loss of aSyn function following misfolding, highlight the toxic role of dopamine (Outeiro et al., 2009; Yamakawa et al., 2010). Upon adding exogenous dopamine to melanoma cells not only did we observe an unexplained resistance of melanoma cells to dopamine but also observed an increase in aSyn levels. This result is consistent with the earlier observation of physiological aSyn conferring protection towards oxidative damage and suggests that aSyn monomeric levels increase in response to dopamine, perhaps as a means to control the intracellular dopamine levels thereby mitigating dopamine toxicity. Interestingly is has been shown that SH-SY-5Y cells which are highly susceptible to dopamine toxicity show some resistance to dopamine when overexpressed with aSyn (Colapinto et al., 2006). However the effect of dopamine on aSyn is not restricted to just increasing its protein levels but also causes it to increasingly localize to the cytoplasm again disturbing the balance between the monomeric and oligomeric species of aSyn indicating an alternate pathway for dopamine mediated toxicity in addition to oxidative damage. These changes in localization may well affect the normal physiological functions of aSyn.

Now, it is known that dopamine-modified aSyn can block autophagy (Martinez-Vicente et al., 2008) and similarly we found that autophagy was inhibited in our system in the presence of dopamine and this inhibition was ameliorated when aSyn was knocked down from the system. It therefore appears that aSyn is a negative regulator of autophagy, as seen earlier, and the presence of dopamine enhances this effect, perhaps through mediating a change in its localization. However the lack of significant changes in LC3-II between aSyn knock down cells and aSyn intact cells exposed to dopamine, suggest that the inhibition may not involve the formation of the autophagic vacuole but may rather affect the proteolysis through lysosomal impairment.

Activation of autophagy to counter similar conditions of impairment has been under consideration as a therapeutic potential (Pandey et al., 2007; Spencer et al., 2009; Xilouri et al., 2013). However, induction of autophagy through external means in presence of dopamine failed to enhance the clearance of aSyn. On the other hand, sustained autophagy induction only added to the dopamine mediated inhibition, increased aSyn localization to the cytoplasm and resulted in cell death. The cellular toxicity seen upon autophagy induction has also been demonstrated in cells already burdened with the aggregates (Tanik et al., 2013) but it seems evident from our study that cell death can occur during forced inductions of autophagy even prior to aggregate formation through severe impairment of autophagy by increased aSyn levels and through changes in its sub cellular localization.

The conversion of dopamine to neuromelanin is a mechanism used by neuronal cells to counter dopamine toxicity. A differential role in regulating melanin synthesis in melanoma cells and dopaminergic cells by aSyn has been reported. While aSyn impaired melanin production in melanoma cells, the same study also showed that overexpression of aSyn in SH-SY-5Y resulted in increased melanin content. Similarly, in this study the addition of dopamine resulted in increased melanin content in melanoma cells and perhaps explains the increase tolerance of these cells to exogenous dopamine. However, studies have shown that autophagic alterations can also affect melanin production (Murase et al., 2013b). In agreement with this, results from this study also showed increased melanin production when the combined treatments of dopamine and Torin-1 resulted in a sustained autophagy inhibition. It is as yet unclear at present if the increase in melanin content upon dopamine addition is a result of autophagy inhibition but since dopamine mediated autophagy inhibition is mediated via aSyn, it maybe safe to assume that aSyn contributes to the conversion of dopamine to melanin via autophagy inhibition, atleast

partly. The conversion of dopamine to neuromelanin can have both protective and toxic roles in the cells (Fedorow et al., 2005; Zecca et al., 2006).

The objective of studying aSyn degradation is to evaluate for possible intervention strategies to reverse the aggregation process. Since autophagy inhibition did not lead to significant changes in aSyn levels, we also studied the effect of proteasome impairment on aSyn levels in melanoma and to our surprise found that proteasomal inhibition also did not result in any significant changes in aSyn levels. We also observed an impairment of proteasomal function in SK-MEL-28 cells. It remains to be evaluated if proteasomal function is a common feature of all melanomas and if yes it remains to be seen if the selective expression of aSyn in melanoma is a result of this inhibition.

The presence of aSyn as distinct molecular weight species and its differential localization is thus a critical determinant of its aggregation propensity. An increase in aSyn mRNA is PD brains and even in cases with incidental LB has been reported. Since aSyn appears to have a regulatory effect on autophagy, forced inductions also result in increased aSyn levels which when exposed to dopamine mediates autophagy inhibition. This inhibition in itself also facilitates a change in localization. This change abolishes its physiological functions such as regulation of autophagy with the net effect of disturbing cellular homeostasis. These changes in aSyn also influence downstream pathways such as neuromelanin production which in turn can decide the balance between cell survival and cell death. These results suggest that we must exercise caution in the utilization of autophagy enhancement as a therapeutic tool with the timing and administration of therapy being vital.

This model presents clear differences in aSyn behavior under different cellular contexts that may mediate its switch between physiology and pathology (Fig.60) and we therefore also propose through our study the evolution of alternate cell models such as the melanoma to extensively understand aSyn behavior which will potentially impact our understanding of PD pathogenesis.

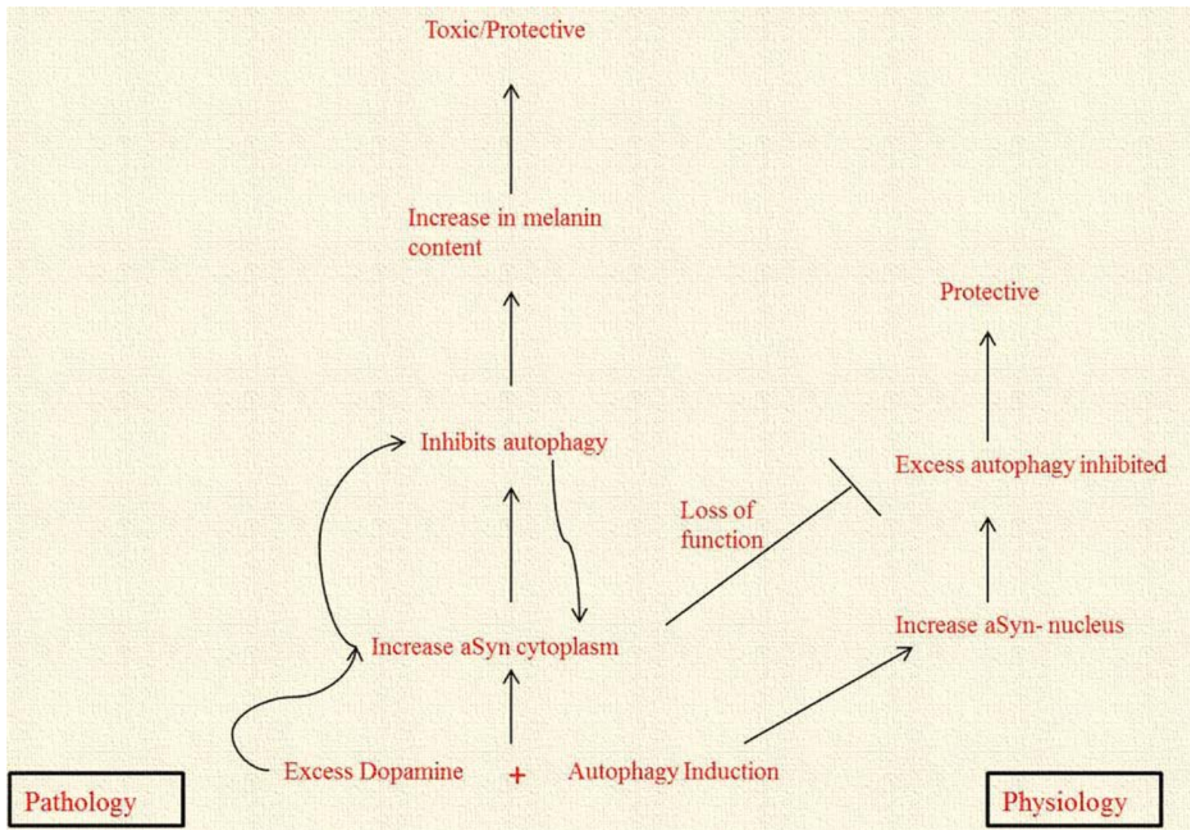


Figure 60: Represents the effect of change in aSyn localization on its diverse roles in physiology and pathology. Physiologically, its presence in nucleus appears to play a protective function through helping maintain homeostasis via autophagy regulation. Factors such as excess dopamine mediate a change in aSyn localization which results in autophagy inhibition which in turn affects aSyn localization. This change not only results in loss of function but also affects melanin production whose levels act as a switch between cell death and cell survival.

6. SUMMARY AND CONCLUSION

The emphasis on using autophagy lysosomal pathway as a neuroprotective strategy has necessistated a better understanding of the response of aSyn to autophagic failure. It also remains to be verified if the aSyn aggregation can be reversed with the enhancement of autophagy and if so to determine at what stage of aggregation it might it applied. The reciprocal nature of the relationship between aSyn and protein degradation pathways has made its study in overexpression models complicated and has resulted in a lack of consensus owing to variations in model and protein burden on the cell. It was therefore hypothesized that endogenous models may be better suited to study aSyn response to autophagic alterations. The detection of aSyn in non-neuronal cell types and the close association between PD and melanoma affords the possibility of investigating the behavior of aSyn under different cellular contexts and will also highlight its role in physiology and pathology as relevant to these non-neuronal cells where its role remains unknown.

The results from this study highlight the following aspects:

(a) The occurrence of atleast two different molecular weights species of aSyn which show preferences in their sub-cellular localiztions with the higher molecular weights being selective to cytoplasm and monomer restricted to the nucleus.

(b) Autophagy Inhibition does not lead to significant changes in aSyn expression but favors the retention of higher molecular weight species in the cytoplasm.

(c) Autophagy induction does not lead to increased clearance instead it appears to favor monomeric species and their preferential localization to the nucleus with the net effect of inhibiting excess autophagy.

(d) Excess dopamine also causes increase in aSyn monomer and increases its localization to the cytoplasm. Therefore dopamine has the dual effect of increasing aSyn levels and favoring the higher molecular weights.

(e) The interactions between dopamine and aSyn inhibit basal autophagy and results in increases in melanin levels.

(f) Autophagy induction in presence of dopamine proves to be cytotoxic.

Although the presence of aSyn in the nucleus has been a matter of debate, this study not only confirms its nuclear localization but also the existence of multiple species of aSyn. It remains to be seen if the higher molecular weight is naturally occurring or a non-toxic by-product of aggregation process brought on by the impaired proteasome in these cells. If naturally occurring, it seems likely that the different molecular weight species have a different functional significance in the cell given their preference of certain sub-cellular localizations. The different molecular weights of aSyn may also be degraded by more than one pathway which explains the existence of confliciting results with regard to its degradation. These results have the most signifance for sporadic PD where increase in aSyn levels has been proposed as a reason for aSyn aggregation. This study shows that in addition to increased protein levels, changes in aSyn localization can prove detrimental to cell. The change in localization will potentially impact its normal functioning and when combined with cellular conditions such as excess dopamine results in autophagy inhibition. This inhibition in turn affects downstream events such as melanin

production. The conversion of excess dopamine to neuromelanin occurs inside the lysosome and inhibition mediated by dopamine-aSyn interaction further increases the melanin content. While initial increases in neuromelanin can have cytoprotective effects such as conferring resistance to oxidative stress, very high levels of neuromelanin in the cell can result in cytotoxicity. It therefore appears that changes in aSyn localization can result in activation of cytotoxic mechanism. Additionally, the preferential localization of aSyn to cytoplasm when it exists as a high molecular weight species, means that with autophagy inhibition, these higher molecular weights become exposed to cellular events that favor its stabilization and its conversion to toxic oligomers. The decrease in aSyn nuclear localization when the cells are exposed to dopamine and Torin-1 simultaneously and the sustained autophagy inhibition inspite of the presence of Torin-1 again indicate at the disruptive nature of change in its localization. This result also highlights the failure of autophagy to reverse this phenomenon suggesting the use of caution in the application of autophagy as a neuroprotective strategy. Melanin production is also affected by changes in autophagy and therefore the presence of aSyn in melanoma may also affect the melanin content in these cells through its regulation of autophagy which appears to be a physiological function of aSyn.

BIBLIOGRAPHY

Alim, M.A., Ma, Q.-L., Takeda, K., Aizawa, T., Matsubara, M., Nakamura, M., Asada, A., Saito, T., Kaji, H., Yoshii, M., et al. (2004). Demonstration of a role for alpha-synuclein as a functional microtubule-associated protein. J. Alzheimers Dis. JAD *6*, 435-442; discussion 443-449.

Alvarez-Erviti, L., Rodriguez-Oroz, M.C., Cooper, J.M., Caballero, C., Ferrer, I., Obeso, J.A., and Schapira, A.H.V. (2010). Chaperone-mediated autophagy markers in Parkinson disease brains. Arch. Neurol. *67*, 1464–1472.

Alvarez-Erviti, L., Couch, Y., Richardson, J., Cooper, J.M., and Wood, M.J.A. (2011). Alpha-synuclein release by neurons activates the inflammatory response in a microglial cell line. Neurosci. Res. *69*, 337–342.

Alves da Costa, C. (2003). Recent advances on alpha-synuclein cell biology: functions and dysfunctions. Curr. Mol. Med. *3*, 17–24.

Alves Da Costa, C., Paitel, E., Vincent, B., and Checler, F. (2002). Alpha-synuclein lowers p53-dependent apoptotic response of neuronal cells. Abolishment by 6-hydroxydopamine and implication for Parkinson's disease. J. Biol. Chem. *277*, 50980–50984.

Amaravadi, R.K., Yu, D., Lum, J.J., Bui, T., Christophorou, M.A., Evan, G.I., Thomas-Tikhonenko, A., and Thompson, C.B. (2007). Autophagy inhibition enhances therapy-induced apoptosis in a Myc-induced model of lymphoma. J. Clin. Invest. *117*, 326–336.

Ancolio, K., Alves da Costa, C., Uéda, K., and Checler, F. (2000). Alpha-synuclein and the Parkinson's disease-related mutant Ala53Thr-alpha-synuclein do not undergo proteasomal degradation in HEK293 and neuronal cells. Neurosci. Lett. *285*, 79–82.

Asanuma, M., Miyazaki, I., Diaz-Corrales, F.J., Higashi, Y., Namba, M., and Ogawa, N. (2013). Transplantation of melanocytes obtained from the skin ameliorates apomorphine-induced abnormal behavior in rodent hemi-parkinsonian models. PloS One *8*, e65983.

Baba, M., Nakajo, S., Tu, P.H., Tomita, T., Nakaya, K., Lee, V.M., Trojanowski, J.Q., and Iwatsubo, T. (1998). Aggregation of alpha-synuclein in Lewy bodies of sporadic Parkinson's disease and dementia with Lewy bodies. Am. J. Pathol. *152*, 879–884.

Barbour, R., Kling, K., Anderson, J.P., Banducci, K., Cole, T., Diep, L., Fox, M., Goldstein, J.M., Soriano, F., Seubert, P., et al. (2008). Red blood cells are the major source of alpha-synuclein in blood. Neurodegener. Dis. *5*, 55–59.

Bartels, T., Choi, J.G., and Selkoe, D.J. (2011). α-Synuclein occurs physiologically as a helically folded tetramer that resists aggregation. Nature *477*, 107–110.

Batelli, S., Albani, D., Rametta, R., Polito, L., Prato, F., Pesaresi, M., Negro, A., and Forloni, G. (2008). DJ-1 modulates alpha-synuclein aggregation state in a cellular model of oxidative stress: relevance for Parkinson's disease and involvement of HSP70. PloS One *3*, e1884.

Batelli, S., Peverelli, E., Rodilossi, S., Forloni, G., and Albani, D. (2011). Macroautophagy and the proteasome are differently involved in the degradation of alpha-synuclein wild type and mutated A30P in an in vitro inducible model (PC12/TetOn). Neuroscience *195*, 128–137.

Bedford, L., Hay, D., Devoy, A., Paine, S., Powe, D.G., Seth, R., Gray, T., Topham, I., Fone, K., Rezvani, N., et al. (2008). Depletion of 26S proteasomes in mouse brain neurons causes neurodegeneration and Lewy-like inclusions resembling human pale bodies. J. Neurosci. Off. J. Soc. Neurosci. *28*, 8189–8198.

Bedford, L., Paine, S., Sheppard, P.W., Mayer, R.J., and Roelofs, J. (2010). Assembly, structure, and function of the 26S proteasome. Trends Cell Biol. *20*, 391–401.

Bekris, L.M., Mata, I.F., and Zabetian, C.P. (2010). The genetics of Parkinson disease. J. Geriatr. Psychiatry Neurol. *23*, 228–242.

Bence, N.F., Sampat, R.M., and Kopito, R.R. (2001). Impairment of the ubiquitin-proteasome system by protein aggregation. Science *292*, 1552–1555.

Bennett, M.C. (2005). The role of alpha-synuclein in neurodegenerative diseases. Pharmacol. Ther. *105*, 311–331.

Bertoni, J.M., Arlette, J.P., Fernandez, H.H., Fitzer-Attas, C., Frei, K., Hassan, M.N., Isaacson, S.H., Lew, M.F., Molho, E., Ondo, W.G., et al. (2010). Increased melanoma risk in Parkinson disease: a prospective clinicopathological study. Arch. Neurol. *67*, 347–352.

Betarbet, R., Canet-Aviles, R.M., Sherer, T.B., Mastroberardino, P.G., McLendon, C., Kim, J.-H., Lund, S., Na, H.-M., Taylor, G., Bence, N.F., et al. (2006). Intersecting pathways to neurodegeneration in Parkinson's disease: effects of the pesticide rotenone on DJ-1, alpha-synuclein, and the ubiquitin-proteasome system. Neurobiol. Dis. *22*, 404–420.

Beyer, K. (2006). Alpha-synuclein structure, posttranslational modification and alternative splicing as aggregation enhancers. Acta Neuropathol. (Berl.) *112*, 237–251.

Beyer, K., and Ariza, A. (2013). α-Synuclein posttranslational modification and alternative splicing as a trigger for neurodegeneration. Mol. Neurobiol. *47*, 509–524.

Bisaglia, M., Greggio, E., Maric, D., Miller, D.W., Cookson, M.R., and Bubacco, L. (2010). Alpha-synuclein overexpression increases dopamine toxicity in BE2-M17 cells. BMC Neurosci. *11*, 41.

Bjørkøy, G., Lamark, T., Brech, A., Outzen, H., Perander, M., Overvatn, A., Stenmark, H., and Johansen, T. (2005). p62/SQSTM1 forms protein aggregates degraded by autophagy and has a protective effect on huntingtin-induced cell death. J. Cell Biol. *171*, 603–614.

Blum-Degen, D., Müller, T., Kuhn, W., Gerlach, M., Przuntek, H., and Riederer, P. (1995). Interleukin-1 beta and interleukin-6 are elevated in the cerebrospinal fluid of Alzheimer's and de novo Parkinson's disease patients. Neurosci. Lett. *202*, 17–20.

Bodei, L., Hofland, L.J., Ferone, D., Mooy, C.M., Kros, J.M., Paridaens, D.A., Baarsma, S.G., Ferdeghini, M., Van Hagen, M.P., Krenning, E.P., et al. (2003). In vivo and in vitro detection of dopamine d2 receptors in uveal melanomas. Cancer Biother. Radiopharm. *18*, 895–902.

Böni, R., Lichtensteiger, W., Steinert, H.C., Böni, R.A., Früh, H., Dummer, R., Hartmann, D.G., Laine, E., and Burg, G. (1997). D1 dopamine receptors are not expressed in human melanoma. Melanoma Res. *7*, 117–119.

Bové, J., Zhou, C., Jackson-Lewis, V., Taylor, J., Chu, Y., Rideout, H.J., Wu, D.-C., Kordower, J.H., Petrucelli, L., and Przedborski, S. (2006). Proteasome inhibition and Parkinson's disease modeling. Ann. Neurol. *60*, 260–264.

Braak, H., Del Tredici, K., Rüb, U., de Vos, R.A.I., Jansen Steur, E.N.H., and Braak, E. (2003). Staging of brain pathology related to sporadic Parkinson's disease. Neurobiol. Aging *24*, 197–211.

Breese, G.R., and Traylor, T.D. (1971). Depletion of brain noradrenaline and dopamine by 6-hydroxydopamine. Br. J. Pharmacol. *42*, 88–99.

Brooks, A.I., Chadwick, C.A., Gelbard, H.A., Cory-Slechta, D.A., and Federoff, H.J. (1999). Paraquat elicited neurobehavioral syndrome caused by dopaminergic neuron loss. Brain Res. *823*, 1–10.

Burke, R.E., Dauer, W.T., and Vonsattel, J.P.G. (2008). A critical evaluation of the Braak staging scheme for Parkinson's disease. Ann. Neurol. *64*, 485–491.

Burré, J., Sharma, M., Tsetsenis, T., Buchman, V., Etherton, M.R., and Südhof, T.C. (2010). Alpha-synuclein promotes SNARE-complex assembly in vivo and in vitro. Science *329*, 1663–1667.

Chandra, S., Gallardo, G., Fernández-Chacón, R., Schlüter, O.M., and Südhof, T.C. (2005). Alpha-synuclein cooperates with CSPalpha in preventing neurodegeneration. Cell *123*, 383–396.

Chaudhuri, T.K., and Paul, S. (2006). Protein-misfolding diseases and chaperone-based therapeutic approaches. FEBS J. *273*, 1331–1349.

Chen, L., and Feany, M.B. (2005). Alpha-synuclein phosphorylation controls neurotoxicity and inclusion formation in a Drosophila model of Parkinson disease. Nat. Neurosci. *8*, 657–663.

Chen, Q., Thorpe, J., and Keller, J.N. (2005). Alpha-synuclein alters proteasome function, protein synthesis, and stationary phase viability. J. Biol. Chem. *280*, 30009–30017.

Chen, Y., Kramer, D.L., Li, F., and Porter, C.W. (2003). Loss of inhibitor of apoptosis proteins as a determinant of polyamine analog-induced apoptosis in human melanoma cells. Oncogene *22*, 4964–4972.

Cheng, F., Vivacqua, G., and Yu, S. (2011). The role of α-synuclein in neurotransmission and synaptic plasticity. J. Chem. Neuroanat. *42*, 242–248.

Cherra, S.J., and Chu, C.T. (2008). Autophagy in neuroprotection and neurodegeneration: A question of balance. Future Neurol. *3*, 309–323.

Chiti, F., and Dobson, C.M. (2006). Protein misfolding, functional amyloid, and human disease. Annu. Rev. Biochem. *75*, 333–366.

Choubey, V., Safiulina, D., Vaarmann, A., Cagalinec, M., Wareski, P., Kuum, M., Zharkovsky, A., and Kaasik, A. (2011). Mutant A53T alpha-synuclein induces neuronal death by increasing mitochondrial autophagy. J. Biol. Chem. *286*, 10814–10824.

Chung, K.K.K., Thomas, B., Li, X., Pletnikova, O., Troncoso, J.C., Marsh, L., Dawson, V.L., and Dawson, T.M. (2004). S-nitrosylation of parkin regulates ubiquitination and compromises parkin's protective function. Science *304*, 1328–1331.

Clayton, D.F., and George, J.M. (1999). Synucleins in synaptic plasticity and neurodegenerative disorders. J. Neurosci. Res. *58*, 120–129.

Colapinto, M., Mila, S., Giraudo, S., Stefanazzi, P., Molteni, M., Rossetti, C., Bergamasco, B., Lopiano, L., and Fasano, M. (2006). alpha-Synuclein protects SH-SY5Y cells from dopamine toxicity. Biochem. Biophys. Res. Commun. *349*, 1294–1300.

Colasanti, T., Vomero, M., Alessandri, C., Barbati, C., Maselli, A., Camperio, C., Conti, F., Tinari, A., Carlo-Stella, C., Tuosto, L., et al. (2014). Role of alpha-synuclein in autophagy modulation of primary human T lymphocytes. Cell Death Dis. *5*, e1265.

Colla, E., Jensen, P.H., Pletnikova, O., Troncoso, J.C., Glabe, C., and Lee, M.K. (2012b). Accumulation of toxic α-synuclein oligomer within endoplasmic reticulum occurs in α-synucleinopathy in vivo. J. Neurosci. Off. J. Soc. Neurosci. *32*, 3301–3305.

Colla, E., Coune, P., Liu, Y., Pletnikova, O., Troncoso, J.C., Iwatsubo, T., Schneider, B.L., and Lee, M.K. (2012a). Endoplasmic reticulum stress is important for the manifestations of α-synucleinopathy in vivo. J. Neurosci. Off. J. Soc. Neurosci. *32*, 3306–3320.

Conway, K.A., Lee, S.J., Rochet, J.C., Ding, T.T., Williamson, R.E., and Lansbury, P.T. (2000). Acceleration of oligomerization, not fibrillization, is a shared property of both alpha-synuclein mutations linked to early-onset Parkinson's disease: implications for pathogenesis and therapy. Proc. Natl. Acad. Sci. U. S. A. *97*, 571–576.

Cooper, A.A., Gitler, A.D., Cashikar, A., Haynes, C.M., Hill, K.J., Bhullar, B., Liu, K., Xu, K., Strathearn, K.E., Liu, F., et al. (2006). Alpha-synuclein blocks ER-Golgi traffic and Rab1 rescues neuron loss in Parkinson's models. Science *313*, 324–328.

Cubells, J.F., Rayport, S., Rajendran, G., and Sulzer, D. (1994). Methamphetamine neurotoxicity involves vacuolation of endocytic organelles and dopamine-dependent intracellular oxidative stress. J. Neurosci. Off. J. Soc. Neurosci. *14*, 2260–2271.

Cuervo, A.M., Hu, W., Lim, B., and Dice, J.F. (1998). IkappaB is a substrate for a selective pathway of lysosomal proteolysis. Mol. Biol. Cell *9*, 1995–2010.

Cuervo, A.M., Stefanis, L., Fredenburg, R., Lansbury, P.T., and Sulzer, D. (2004). Impaired degradation of mutant alpha-synuclein by chaperone-mediated autophagy. Science *305*, 1292–1295.

Cullen, V., Lindfors, M., Ng, J., Paetau, A., Swinton, E., Kolodziej, P., Boston, H., Saftig, P., Woulfe, J., Feany, M.B., et al. (2009). Cathepsin D expression level affects alpha-synuclein processing, aggregation, and toxicity in vivo. Mol. Brain *2*, 5.

Danzer, K.M., Haasen, D., Karow, A.R., Moussaud, S., Habeck, M., Giese, A., Kretzschmar, H., Hengerer, B., and Kostka, M. (2007). Different species of alpha-synuclein oligomers induce calcium influx and seeding. J. Neurosci. Off. J. Soc. Neurosci. *27*, 9220–9232.

Danzer, K.M., Ruf, W.P., Putcha, P., Joyner, D., Hashimoto, T., Glabe, C., Hyman, B.T., and McLean, P.J. (2011). Heat-shock protein 70 modulates toxic extracellular α-synuclein oligomers and rescues trans-synaptic toxicity. FASEB J. Off. Publ. Fed. Am. Soc. Exp. Biol. *25*, 326–336.

Dauer, W., and Przedborski, S. (2003). Parkinson's Disease: Mechanisms and Models. Neuron *39*, 889–909.

Davies, P., Moualla, D., and Brown, D.R. (2011). Alpha-synuclein is a cellular ferrireductase. PloS One *6*, e15814.

De Domenico, I., Vaughn, M.B., Li, L., Bagley, D., Musci, G., Ward, D.M., and Kaplan, J. (2006). Ferroportin-mediated mobilization of ferritin iron precedes ferritin degradation by the proteasome. EMBO J. *25*, 5396–5404.

Desplats, P., Lee, H.-J., Bae, E.-J., Patrick, C., Rockenstein, E., Crews, L., Spencer, B., Masliah, E., and Lee, S.-J. (2009). Inclusion formation and neuronal cell death through neuron-to-neuron transmission of alpha-synuclein. Proc. Natl. Acad. Sci. U. S. A. *106*, 13010–13015.

Desplats, P., Spencer, B., Crews, L., Pathel, P., Morvinski-Friedmann, D., Kosberg, K., Roberts, S., Patrick, C., Winner, B., Winkler, J., et al. (2012). α-Synuclein induces alterations in adult neurogenesis in Parkinson disease models via p53-mediated repression of Notch1. J. Biol. Chem. *287*, 31691–31702.

Dettmer, U., Newman, A.J., Luth, E.S., Bartels, T., and Selkoe, D. (2013). In vivo crosslinking reveals principally oligomeric forms of α-synuclein and β-synuclein in neurons and non-neural cells. J. Biol. Chem.

Devi, L., Raghavendran, V., Prabhu, B.M., Avadhani, N.G., and Anandatheerthavarada, H.K. (2008). Mitochondrial import and accumulation of alpha-synuclein impair complex I in human dopaminergic neuronal cultures and Parkinson disease brain. J. Biol. Chem. *283*, 9089–9100.

Ding, Q., Dimayuga, E., Martin, S., Bruce-Keller, A.J., Nukala, V., Cuervo, A.M., and Keller, J.N. (2003). Characterization of chronic low-level proteasome inhibition on neural homeostasis. J. Neurochem. *86*, 489–497.

Ding, W.-X., Ni, H.-M., Gao, W., Yoshimori, T., Stolz, D.B., Ron, D., and Yin, X.-M. (2007). Linking of autophagy to ubiquitin-proteasome system is important for the regulation of endoplasmic reticulum stress and cell viability. Am. J. Pathol. *171*, 513–524.

Dobson, C.M. (2001). The structural basis of protein folding and its links with human disease. Philos. Trans. R. Soc. Lond. Ser. B *356*, 133–145.

Dorval, V., and Fraser, P.E. (2006). Small ubiquitin-like modifier (SUMO) modification of natively unfolded proteins tau and alpha-synuclein. J. Biol. Chem. *281*, 9919–9924.

Dufty, B.M., Warner, L.R., Hou, S.T., Jiang, S.X., Gomez-Isla, T., Leenhouts, K.M., Oxford, J.T., Feany, M.B., Masliah, E., and Rohn, T.T. (2007). Calpain-cleavage of alpha-synuclein: connecting proteolytic processing to disease-linked aggregation. Am. J. Pathol. *170*, 1725–1738.

Ebrahimi-Fakhari, D., Cantuti-Castelvetri, I., Fan, Z., Rockenstein, E., Masliah, E., Hyman, B.T., McLean, P.J., and Unni, V.K. (2011). Distinct roles in vivo for the ubiquitin-proteasome system and the autophagy-lysosomal pathway in the degradation of α-synuclein. J. Neurosci. Off. J. Soc. Neurosci. *31*, 14508–14520.

Ebrahimi-Fakhari, D., Wahlster, L., and McLean, P.J. (2012). Protein degradation pathways in Parkinson's disease: curse or blessing. Acta Neuropathol. (Berl.) *124*, 153–172.

El-Agnaf, O.M.A., Salem, S.A., Paleologou, K.E., Cooper, L.J., Fullwood, N.J., Gibson, M.J., Curran, M.D., Court, J.A., Mann, D.M.A., Ikeda, S., et al. (2003). Alpha-synuclein implicated in Parkinson's disease is present in extracellular biological fluids, including human plasma. FASEB J. Off. Publ. Fed. Am. Soc. Exp. Biol. *17*, 1945–1947.

El-Agnaf, O.M.A., Salem, S.A., Paleologou, K.E., Curran, M.D., Gibson, M.J., Court, J.A., Schlossmacher, M.G., and Allsop, D. (2006). Detection of oligomeric forms of alpha-synuclein protein in human plasma as a potential biomarker for Parkinson's disease. FASEB J. Off. Publ. Fed. Am. Soc. Exp. Biol. *20*, 419–425.

Emmanouilidou, E., Melachroinou, K., Roumeliotis, T., Garbis, S.D., Ntzouni, M., Margaritis, L.H., Stefanis, L., and Vekrellis, K. (2010a). Cell-produced alpha-synuclein is secreted in a calcium-dependent manner by exosomes and impacts neuronal survival. J. Neurosci. Off. J. Soc. Neurosci. *30*, 6838–6851.

Emmanouilidou, E., Stefanis, L., and Vekrellis, K. (2010b). Cell-produced alpha-synuclein oligomers are targeted to, and impair, the 26S proteasome. Neurobiol. Aging *31*, 953–968.

Fauvet, B., Mbefo, M.K., Fares, M.-B., Desobry, C., Michael, S., Ardah, M.T., Tsika, E., Coune, P., Prudent, M., Lion, N., et al. (2012). α-Synuclein in central nervous system and from erythrocytes, mammalian cells, and Escherichia coli exists predominantly as disordered monomer. J. Biol. Chem. *287*, 15345–15364.

Fedorow, H., Tribl, F., Halliday, G., Gerlach, M., Riederer, P., and Double, K.L. (2005). Neuromelanin in human dopamine neurons: comparison with peripheral melanins and relevance to Parkinson's disease. Prog. Neurobiol. *75*, 109–124.

Ferreira, S.T., and De Felice, F.G. (2001). PABMB Lecture. Protein dynamics, folding and misfolding: from basic physical chemistry to human conformational diseases. FEBS Lett. *498*, 129–134.

Fornai, F., Vaglini, F., Maggio, R., Bonuccelli, U., and Corsini, G.U. (1997). Species differences in the role of excitatory amino acids in experimental parkinsonism. Neurosci. Biobehav. Rev. *21*, 401–415.

Fuchs, J., Mueller, J.C., Lichtner, P., Schulte, C., Munz, M., Berg, D., Wüllner, U., Illig, T., Sharma, M., and Gasser, T. (2009). The transcription factor PITX3 is associated with sporadic Parkinson's disease. Neurobiol. Aging *30*, 731–738.

Fuertes, G., Martín De Llano, J.J., Villarroya, A., Rivett, A.J., and Knecht, E. (2003a). Changes in the proteolytic activities of proteasomes and lysosomes in human fibroblasts produced by serum withdrawal, amino-acid deprivation and confluent conditions. Biochem. J. *375*, 75–86.

Fuertes, G., Villarroya, A., and Knecht, E. (2003b). Role of proteasomes in the degradation of short-lived proteins in human fibroblasts under various growth conditions. Int. J. Biochem. Cell Biol. *35*, 651–664.

Gajula Balija, M.B., Griesinger, C., Herzig, A., Zweckstetter, M., and Jäckle, H. (2011). Pre-fibrillar α-synuclein mutants cause Parkinson's disease-like non-motor symptoms in Drosophila. PloS One *6*, e24701.

Galvin, J.E. (2006). Interaction of alpha-synuclein and dopamine metabolites in the pathogenesis of Parkinson's disease: a case for the selective vulnerability of the substantia nigra. Acta Neuropathol. (Berl.) *112*, 115–126.

Gamerdinger, M., Hajieva, P., Kaya, A.M., Wolfrum, U., Hartl, F.U., and Behl, C. (2009). Protein quality control during aging involves recruitment of the macroautophagy pathway by BAG3. EMBO J. *28*, 889–901.

Gandhi, S., and Wood, N.W. (2005). Molecular pathogenesis of Parkinson's disease. Hum. Mol. Genet. *14 Spec No. 2*, 2749–2755.

Gao, H.-M., Jiang, J., Wilson, B., Zhang, W., Hong, J.-S., and Liu, B. (2002). Microglial activation-mediated delayed and progressive degeneration of rat nigral dopaminergic neurons: relevance to Parkinson's disease. J. Neurochem. *81*, 1285–1297.

Gao, X., Simon, K.C., Han, J., Schwarzschild, M.A., and Ascherio, A. (2009). Family history of melanoma and Parkinson disease risk. Neurology *73*, 1286–1291.

Garcia-Reitböck, P., Anichtchik, O., Bellucci, A., Iovino, M., Ballini, C., Fineberg, E., Ghetti, B., Della Corte, L., Spano, P., Tofaris, G.K., et al. (2010). SNARE protein redistribution and synaptic failure in a transgenic mouse model of Parkinson's disease. Brain J. Neurol. *133*, 2032–2044.

Gasser, T. (2009). Molecular pathogenesis of Parkinson disease: insights from genetic studies. Expert Rev. Mol. Med. *11*, e22.

Gasser, T., Hardy, J., and Mizuno, Y. (2011). Milestones in PD genetics. Mov. Disord. Off. J. Mov. Disord. Soc. *26*, 1042–1048.

Geisler, S., Holmström, K.M., Treis, A., Skujat, D., Weber, S.S., Fiesel, F.C., Kahle, P.J., and Springer, W. (2010). The PINK1/Parkin-mediated mitophagy is compromised by PD-associated mutations. Autophagy *6*, 871–878.

George, J.M. (2002). The synucleins. Genome Biol. *3*, REVIEWS3002.

Ghee, M., Fournier, A., and Mallet, J. (2000). Rat alpha-synuclein interacts with Tat binding protein 1, a component of the 26S proteasomal complex. J. Neurochem. *75*, 2221–2224.

Giasson, B.I., Forman, M.S., Higuchi, M., Golbe, L.I., Graves, C.L., Kotzbauer, P.T., Trojanowski, J.Q., and Lee, V.M.-Y. (2003). Initiation and synergistic fibrillization of tau and alpha-synuclein. Science *300*, 636–640.

Glaser, C.B., Yamin, G., Uversky, V.N., and Fink, A.L. (2005). Methionine oxidation, alpha-synuclein and Parkinson's disease. Biochim. Biophys. Acta *1703*, 157–169.

Glick, D., Barth, S., and Macleod, K.F. (2010). Autophagy: cellular and molecular mechanisms. J. Pathol. *221*, 3–12.

Goers, J., Manning-Bog, A.B., McCormack, A.L., Millett, I.S., Doniach, S., Di Monte, D.A., Uversky, V.N., and Fink, A.L. (2003). Nuclear localization of alpha-synuclein and its interaction with histones. Biochemistry (Mosc.) *42*, 8465–8471.

Gosavi, N., Lee, H.-J., Lee, J.S., Patel, S., and Lee, S.-J. (2002). Golgi fragmentation occurs in the cells with prefibrillar alpha-synuclein aggregates and precedes the formation of fibrillar inclusion. J. Biol. Chem. *277*, 48984–48992.

Grünblatt, E., Mandel, S., Jacob-Hirsch, J., Zeligson, S., Amariglo, N., Rechavi, G., Li, J., Ravid, R., Roggendorf, W., Riederer, P., et al. (2004). Gene expression profiling of parkinsonian substantia nigra pars compacta; alterations in ubiquitin-proteasome, heat shock protein, iron and oxidative stress regulated proteins, cell adhesion/cellular matrix and vesicle trafficking genes. J. Neural Transm. Vienna Austria 1996 *111*, 1543–1573.

Guardia-Laguarta, C., Area-Gomez, E., Schon, E.A., and Przedborski, S. (2015). Novel subcellular localization for α-synuclein: possible functional consequences. Front. Neuroanat. *9*, 17.

Guerrero, E., Vasudevaraju, P., Hegde, M.L., Britton, G.B., and Rao, K.S. (2013). Recent advances in α-synuclein functions, advanced glycation, and toxicity: implications for Parkinson's disease. Mol. Neurobiol. *47*, 525–536.

Haggerty, T., Credle, J., Rodriguez, O., Wills, J., Oaks, A.W., Masliah, E., and Sidhu, A. (2011). Hyperphosphorylated Tau in an α-synuclein-overexpressing transgenic model of Parkinson's disease. Eur. J. Neurosci. *33*, 1598–1610.

Hansen, C., Angot, E., Bergström, A.-L., Steiner, J.A., Pieri, L., Paul, G., Outeiro, T.F., Melki, R., Kallunki, P., Fog, K., et al. (2011). α-Synuclein propagates from mouse brain to grafted dopaminergic neurons and seeds aggregation in cultured human cells. J. Clin. Invest. *121*, 715–725.

Hara, T., Nakamura, K., Matsui, M., Yamamoto, A., Nakahara, Y., Suzuki-Migishima, R., Yokoyama, M., Mishima, K., Saito, I., Okano, H., et al. (2006). Suppression of basal autophagy in neural cells causes neurodegenerative disease in mice. Nature *441*, 885–889.

Hasegawa, T., Konno, M., Baba, T., Sugeno, N., Kikuchi, A., Kobayashi, M., Miura, E., Tanaka, N., Tamai, K., Furukawa, K., et al. (2011). The AAA-ATPase VPS4 regulates extracellular secretion and lysosomal targeting of α-synuclein. PloS One *6*, e29460.

Hashimoto, M., Hsu, L.J., Rockenstein, E., Takenouchi, T., Mallory, M., and Masliah, E. (2002). alpha-Synuclein protects against oxidative stress via inactivation of the c-Jun N-terminal kinase stress-signaling pathway in neuronal cells. J. Biol. Chem. *277*, 11465–11472.

Herrero Hernández, E. (2009). Pigmentation genes link Parkinson's disease to melanoma, opening a window on both etiologies. Med. Hypotheses *72*, 280–284.

Hettiarachchi, N.T., Parker, A., Dallas, M.L., Pennington, K., Hung, C.-C., Pearson, H.A., Boyle, J.P., Robinson, P., and Peers, C. (2009). alpha-Synuclein modulation of Ca2+ signaling in human neuroblastoma (SH-SY5Y) cells. J. Neurochem. *111*, 1192–1201.

Hinault, M.-P., Cuendet, A.F.H., Mattoo, R.U.H., Mensi, M., Dietler, G., Lashuel, H.A., and Goloubinoff, P. (2010). Stable alpha-synuclein oligomers strongly inhibit chaperone activity of the Hsp70 system by weak interactions with J-domain co-chaperones. J. Biol. Chem. *285*, 38173–38182.

Ho, H., and Ganesan, A.K. (2011). The pleiotropic roles of autophagy regulators in melanogenesis. Pigment Cell Melanoma Res. *24*, 595–604.

Höglinger, G.U., Féger, J., Prigent, A., Michel, P.P., Parain, K., Champy, P., Ruberg, M., Oertel, W.H., and Hirsch, E.C. (2003). Chronic systemic complex I inhibition induces a hypokinetic multisystem degeneration in rats. J. Neurochem. *84*, 491–502.

Hsu, L.J., Sagara, Y., Arroyo, A., Rockenstein, E., Sisk, A., Mallory, M., Wong, J., Takenouchi, T., Hashimoto, M., and Masliah, E. (2000). alpha-synuclein promotes mitochondrial deficit and oxidative stress. Am. J. Pathol. *157*, 401–410.

Hüls, S., Högen, T., Vassallo, N., Danzer, K.M., Hengerer, B., Giese, A., and Herms, J. (2011). AMPA-receptor-mediated excitatory synaptic transmission is enhanced by iron-induced α-synuclein oligomers. J. Neurochem. *117*, 868–878.

Hunot, S., and Hirsch, E.C. (2003). Neuroinflammatory processes in Parkinson's disease. Ann. Neurol. *53 Suppl 3*, S49-58; discussion S58-60.

Hwang, D.-Y., Fleming, S.M., Ardayfio, P., Moran-Gates, T., Kim, H., Tarazi, F.I., Chesselet, M.-F., and Kim, K.-S. (2005). 3,4-dihydroxyphenylalanine reverses the motor deficits in Pitx3-deficient aphakia mice: behavioral characterization of a novel genetic model of Parkinson's disease. J. Neurosci. Off. J. Soc. Neurosci. *25*, 2132–2137.

Imai, Y., Soda, M., and Takahashi, R. (2000). Parkin suppresses unfolded protein stress-induced cell death through its E3 ubiquitin-protein ligase activity. J. Biol. Chem. *275*, 35661–35664.

International Parkinson Disease Genomics Consortium, Nalls, M.A., Plagnol, V., Hernandez, D.G., Sharma, M., Sheerin, U.-M., Saad, M., Simón-Sánchez, J., Schulte, C., Lesage, S., et al. (2011). Imputation of sequence variants for identification of genetic risks for Parkinson's disease: a meta-analysis of genome-wide association studies. Lancet Lond. Engl. *377*, 641–649.

Iravani, M.M., Leung, C.C.M., Sadeghian, M., Haddon, C.O., Rose, S., and Jenner, P. (2005). The acute and the long-term effects of nigral lipopolysaccharide administration on dopaminergic dysfunction and glial cell activation. Eur. J. Neurosci. *22*, 317–330.

Irizarry, M.C., Kim, T.W., McNamara, M., Tanzi, R.E., George, J.M., Clayton, D.F., and Hyman, B.T. (1996). Characterization of the precursor protein of the non-A beta component of senile plaques (NACP) in the human central nervous system. J. Neuropathol. Exp. Neurol. *55*, 889–895.

Israeli, E., Yakunin, E., Zarbiv, Y., Hacohen-Solovich, A., Kisos, H., Loeb, V., Lichtenstein, M., Ben-Gedalya, T., Sabag, O., Pikarsky, E., et al. (2011). α-Synuclein expression selectively affects tumorigenesis in mice modeling Parkinson's disease. PloS One *6*, e19622.

Ito, G., Okai, T., Fujino, G., Takeda, K., Ichijo, H., Katada, T., and Iwatsubo, T. (2007). GTP binding is essential to the protein kinase activity of LRRK2, a causative gene product for familial Parkinson's disease. Biochemistry (Mosc.) *46*, 1380–1388.

Iwai, A., Masliah, E., Yoshimoto, M., Ge, N., Flanagan, L., de Silva, H.A., Kittel, A., and Saitoh, T. (1995). The precursor protein of non-A beta component of Alzheimer's disease amyloid is a presynaptic protein of the central nervous system. Neuron *14*, 467–475.

Iwata, A., Maruyama, M., Akagi, T., Hashikawa, T., Kanazawa, I., Tsuji, S., and Nukina, N. (2003). Alpha-synuclein degradation by serine protease neurosin: implication for pathogenesis of synucleinopathies. Hum. Mol. Genet. *12*, 2625–2635.

Iwata, A., Riley, B.E., Johnston, J.A., and Kopito, R.R. (2005). HDAC6 and microtubules are required for autophagic degradation of aggregated huntingtin. J. Biol. Chem. *280*, 40282–40292.

Jaeger, P.A., and Wyss-Coray, T. (2009). All-you-can-eat: autophagy in neurodegeneration and neuroprotection. Mol. Neurodegener. *4*, 16.

Jakes, R., Spillantini, M.G., and Goedert, M. (1994). Identification of two distinct synucleins from human brain. FEBS Lett. *345*, 27–32.

Jang, A., Lee, H.-J., Suk, J.-E., Jung, J.-W., Kim, K.-P., and Lee, S.-J. (2010). Non-classical exocytosis of alpha-synuclein is sensitive to folding states and promoted under stress conditions. J. Neurochem. *113*, 1263–1274.

Jankovic, J. (2008). Parkinson's disease: clinical features and diagnosis. J. Neurol. Neurosurg. Psychiatry *79*, 368–376.

Jankovic, J., and Aguilar, L.G. (2008). Current approaches to the treatment of Parkinson's disease. Neuropsychiatr. Dis. Treat. *4*, 743–757.

Jensen, P.H., Hager, H., Nielsen, M.S., Hojrup, P., Gliemann, J., and Jakes, R. (1999). alpha-synuclein binds to Tau and stimulates the protein kinase A-catalyzed tau phosphorylation of serine residues 262 and 356. J. Biol. Chem. *274*, 25481–25489.

Jensen, P.J., Alter, B.J., and O'Malley, K.L. (2003). Alpha-synuclein protects naive but not dbcAMP-treated dopaminergic cell types from 1-methyl-4-phenylpyridinium toxicity. J. Neurochem. *86*, 196–209.

Jeon, B.S., Jackson-Lewis, V., and Burke, R.E. (1995). 6-Hydroxydopamine lesion of the rat substantia nigra: time course and morphology of cell death. Neurodegener. J. Neurodegener. Disord. Neuroprotection Neuroregeneration *4*, 131–137.

Jiang, Y., Pei, L., Li, S., Wang, M., and Liu, F. (2008). Extracellular dopamine induces the oxidative toxicity of SH-SY5Y cells. Synap. N. Y. N *62*, 797–803.

Jin, H., Kanthasamy, A., Ghosh, A., Yang, Y., Anantharam, V., and Kanthasamy, A.G. (2011). α-Synuclein negatively regulates protein kinase Cδ expression to suppress apoptosis in dopaminergic neurons by reducing p300 histone acetyltransferase activity. J. Neurosci. Off. J. Soc. Neurosci. *31*, 2035–2051.

Kalia, L.V., Kalia, S.K., McLean, P.J., Lozano, A.M., and Lang, A.E. (2013). α-Synuclein oligomers and clinical implications for Parkinson disease. Ann. Neurol. *73*, 155–169.

Kamp, F., Exner, N., Lutz, A.K., Wender, N., Hegermann, J., Brunner, B., Nuscher, B., Bartels, T., Giese, A., Beyer, K., et al. (2010). Inhibition of mitochondrial fusion by α-synuclein is rescued by PINK1, Parkin and DJ-1. EMBO J. *29*, 3571–3589.

Kaplan, B., Ratner, V., and Haas, E. (2003). Alpha-synuclein: its biological function and role in neurodegenerative diseases. J. Mol. Neurosci. MN *20*, 83–92.

Kazantsev, A.G., and Kolchinsky, A.M. (2008). Central role of alpha-synuclein oligomers in neurodegeneration in Parkinson disease. Arch. Neurol. *65*, 1577–1581.

Keller, J.N., Dimayuga, E., Chen, Q., Thorpe, J., Gee, J., and Ding, Q. (2004). Autophagy, proteasomes, lipofuscin, and oxidative stress in the aging brain. Int. J. Biochem. Cell Biol. *36*, 2376–2391.

Kidane, T.Z., Sauble, E., and Linder, M.C. (2006). Release of iron from ferritin requires lysosomal activity. Am. J. Physiol. Cell Physiol. *291*, C445-455.

Kim, C., and Lee, S.-J. (2008). Controlling the mass action of alpha-synuclein in Parkinson's disease. J. Neurochem. *107*, 303–316.

Kim, R.H., Peters, M., Jang, Y., Shi, W., Pintilie, M., Fletcher, G.C., DeLuca, C., Liepa, J., Zhou, L., Snow, B., et al. (2005). DJ-1, a novel regulator of the tumor suppressor PTEN. Cancer Cell *7*, 263–273.

Kirik, D., Rosenblad, C., Burger, C., Lundberg, C., Johansen, T.E., Muzyczka, N., Mandel, R.J., and Björklund, A. (2002). Parkinson-like neurodegeneration induced by targeted overexpression of alpha-synuclein in the nigrostriatal system. J. Neurosci. Off. J. Soc. Neurosci. *22*, 2780–2791.

Kirkin, V., McEwan, D.G., Novak, I., and Dikic, I. (2009b). A role for ubiquitin in selective autophagy. Mol. Cell *34*, 259–269.

Kirkin, V., Lamark, T., Johansen, T., and Dikic, I. (2009a). NBR1 cooperates with p62 in selective autophagy of ubiquitinated targets. Autophagy *5*, 732–733.

Kitada, T., Asakawa, S., Hattori, N., Matsumine, H., Yamamura, Y., Minoshima, S., Yokochi, M., Mizuno, Y., and Shimizu, N. (1998). Mutations in the parkin gene cause autosomal recessive juvenile parkinsonism. Nature *392*, 605–608.

Kontopoulos, E., Parvin, J.D., and Feany, M.B. (2006). Alpha-synuclein acts in the nucleus to inhibit histone acetylation and promote neurotoxicity. Hum. Mol. Genet. *15*, 3012–3023.

Kordower, J.H., Kanaan, N.M., Chu, Y., Suresh Babu, R., Stansell, J., Terpstra, B.T., Sortwell, C.E., Steece-Collier, K., and Collier, T.J. (2006). Failure of proteasome inhibitor administration to provide a model of Parkinson's disease in rats and monkeys. Ann. Neurol. *60*, 264–268.

Kordower, J.H., Chu, Y., Hauser, R.A., Freeman, T.B., and Olanow, C.W. (2008). Lewy body-like pathology in long-term embryonic nigral transplants in Parkinson's disease. Nat. Med. *14*, 504–506.

Korolchuk, V.I., Mansilla, A., Menzies, F.M., and Rubinsztein, D.C. (2009). Autophagy inhibition compromises degradation of ubiquitin-proteasome pathway substrates. Mol. Cell *33*, 517–527.

Lashuel, H.A., Overk, C.R., Oueslati, A., and Masliah, E. (2013). The many faces of α-synuclein: from structure and toxicity to therapeutic target. Nat. Rev. Neurosci. *14*, 38–48.

Lautier, C., Goldwurm, S., Dürr, A., Giovannone, B., Tsiaras, W.G., Pezzoli, G., Brice, A., and Smith, R.J. (2008). Mutations in the GIGYF2 (TNRC15) gene at the PARK11 locus in familial Parkinson disease. Am. J. Hum. Genet. *82*, 822–833.

LaVoie, M.J., Ostaszewski, B.L., Weihofen, A., Schlossmacher, M.G., and Selkoe, D.J. (2005). Dopamine covalently modifies and functionally inactivates parkin. Nat. Med. *11*, 1214–1221.

Le Pen, G., Sonnier, L., Hartmann, A., Bizot, J.-C., Trovero, F., Krebs, M.-O., and Prochiantz, A. (2008). Progressive loss of dopaminergic neurons in the ventral midbrain of adult mice heterozygote for Engrailed1: a new genetic model for Parkinson's disease? Parkinsonism Relat. Disord. *14 Suppl 2*, S107-111.

Lecker, S.H., Goldberg, A.L., and Mitch, W.E. (2006). Protein degradation by the ubiquitin-proteasome pathway in normal and disease states. J. Am. Soc. Nephrol. JASN *17*, 1807–1819.

Lee, B.R., Matsuo, Y., Cashikar, A.G., and Kamitani, T. (2013a). Role of Ser129 phosphorylation of α-synuclein in melanoma cells. J. Cell Sci. *126*, 696–704.

Lee, H.-J., Khoshaghideh, F., Patel, S., and Lee, S.-J. (2004). Clearance of alpha-synuclein oligomeric intermediates via the lysosomal degradation pathway. J. Neurosci. Off. J. Soc. Neurosci. *24*, 1888–1896.

Lee, H.-J., Patel, S., and Lee, S.-J. (2005). Intravesicular localization and exocytosis of alpha-synuclein and its aggregates. J. Neurosci. Off. J. Soc. Neurosci. *25*, 6016–6024.

Lee, H.-J., Suk, J.-E., Bae, E.-J., Lee, J.-H., Paik, S.R., and Lee, S.-J. (2008a). Assembly-dependent endocytosis and clearance of extracellular alpha-synuclein. Int. J. Biochem. Cell Biol. *40*, 1835–1849.

Lee, H.-J., Suk, J.-E., Patrick, C., Bae, E.-J., Cho, J.-H., Rho, S., Hwang, D., Masliah, E., and Lee, S.-J. (2010). Direct transfer of alpha-synuclein from neuron to astroglia causes inflammatory responses in synucleinopathies. J. Biol. Chem. *285*, 9262–9272.

Lee, H.J., Kang, S.J., Lee, K., and Im, H. (2011). Human α-synuclein modulates vesicle trafficking through its interaction with prenylated Rab acceptor protein 1. Biochem. Biophys. Res. Commun. *412*, 526–531.

Lee, H.-J., Cho, E.-D., Lee, K.W., Kim, J.-H., Cho, S.-G., and Lee, S.-J. (2013b). Autophagic failure promotes the exocytosis and intercellular transfer of α-synuclein. Exp. Mol. Med. *45*, e22.

Lee, J.T., Wheeler, T.C., Li, L., and Chin, L.-S. (2008b). Ubiquitination of alpha-synuclein by Siah-1 promotes alpha-synuclein aggregation and apoptotic cell death. Hum. Mol. Genet. *17*, 906–917.

Lee, P.H., Lee, G., Park, H.J., Bang, O.Y., Joo, I.S., and Huh, K. (2006). The plasma alpha-synuclein levels in patients with Parkinson's disease and multiple system atrophy. J. Neural Transm. Vienna Austria 1996 *113*, 1435–1439.

Leng, Y., Chase, T.N., and Bennett, M.C. (2001). Muscarinic receptor stimulation induces translocation of an alpha-synuclein oligomer from plasma membrane to a light vesicle fraction in cytoplasm. J. Biol. Chem. *276*, 28212–28218.

Leroy, E., Boyer, R., Auburger, G., Leube, B., Ulm, G., Mezey, E., Harta, G., Brownstein, M.J., Jonnalagada, S., Chernova, T., et al. (1998). The ubiquitin pathway in Parkinson's disease. Nature *395*, 451–452.

Lesage, S., and Brice, A. (2009). Parkinson's disease: from monogenic forms to genetic susceptibility factors. Hum. Mol. Genet. *18*, R48-59.

Levin, J., Giese, A., Boetzel, K., Israel, L., Högen, T., Nübling, G., Kretzschmar, H., and Lorenzl, S. (2009). Increased alpha-synuclein aggregation following limited cleavage by certain matrix metalloproteinases. Exp. Neurol. *215*, 201–208.

Li, W., and Lee, M.K. (2005). Antiapoptotic property of human alpha-synuclein in neuronal cell lines is associated with the inhibition of caspase-3 but not caspase-9 activity. J. Neurochem. *93*, 1542–1550.

Li, J.-Y., Englund, E., Holton, J.L., Soulet, D., Hagell, P., Lees, A.J., Lashley, T., Quinn, N.P., Rehncrona, S., Björklund, A., et al. (2008). Lewy bodies in grafted neurons in subjects with Parkinson's disease suggest host-to-graft disease propagation. Nat. Med. *14*, 501–503.

Li, W., West, N., Colla, E., Pletnikova, O., Troncoso, J.C., Marsh, L., Dawson, T.M., Jäkälä, P., Hartmann, T., Price, D.L., et al. (2005). Aggregation promoting C-terminal truncation of alpha-synuclein is a normal cellular process and is enhanced by the familial Parkinson's disease-linked mutations. Proc. Natl. Acad. Sci. U. S. A. *102*, 2162–2167.

Li, W.-W., Yang, R., Guo, J.-C., Ren, H.-M., Zha, X.-L., Cheng, J.-S., and Cai, D.-F. (2007). Localization of alpha-synuclein to mitochondria within midbrain of mice. Neuroreport *18*, 1543–1546.

Liani, E., Eyal, A., Avraham, E., Shemer, R., Szargel, R., Berg, D., Bornemann, A., Riess, O., Ross, C.A., Rott, R., et al. (2004). Ubiquitylation of synphilin-1 and alpha-synuclein by SIAH and its presence in cellular inclusions and Lewy bodies imply a role in Parkinson's disease. Proc. Natl. Acad. Sci. U. S. A. *101*, 5500–5505.

Lim, K.-L. (2007). Ubiquitin-proteasome system dysfunction in Parkinson's disease: current evidence and controversies. Expert Rev. Proteomics *4*, 769–781.

Lin, R., Karpa, K., Kabbani, N., Goldman-Rakic, P., and Levenson, R. (2001). Dopamine D2 and D3 receptors are linked to the actin cytoskeleton via interaction with filamin A. Proc. Natl. Acad. Sci. U. S. A. *98*, 5258–5263.

Lindersson, E., Beedholm, R., Højrup, P., Moos, T., Gai, W., Hendil, K.B., and Jensen, P.H. (2004). Proteasomal inhibition by alpha-synuclein filaments and oligomers. J. Biol. Chem. *279*, 12924–12934.

Ling, Z., Gayle, D.A., Ma, S.Y., Lipton, J.W., Tong, C.W., Hong, J.-S., and Carvey, P.M. (2002). In utero bacterial endotoxin exposure causes loss of tyrosine hydroxylase neurons in the postnatal rat midbrain. Mov. Disord. Off. J. Mov. Disord. Soc. *17*, 116–124.

Ling, Z., Chang, Q.A., Tong, C.W., Leurgans, S.E., Lipton, J.W., and Carvey, P.M. (2004). Rotenone potentiates dopamine neuron loss in animals exposed to lipopolysaccharide prenatally. Exp. Neurol. *190*, 373–383.

Liu, C.-W., Giasson, B.I., Lewis, K.A., Lee, V.M., Demartino, G.N., and Thomas, P.J. (2005). A precipitating role for truncated alpha-synuclein and the proteasome in alpha-synuclein aggregation: implications for pathogenesis of Parkinson disease. J. Biol. Chem. *280*, 22670–22678.

Liu, G., Zhang, C., Yin, J., Li, X., Cheng, F., Li, Y., Yang, H., Uéda, K., Chan, P., and Yu, S. (2009). alpha-Synuclein is differentially expressed in mitochondria from different rat brain regions and dose-dependently down-regulates complex I activity. Neurosci. Lett. *454*, 187–192.

Liu, G., Aliaga, L., and Cai, H. (2012). α-synuclein, LRRK2 and their interplay in Parkinson's disease. Future Neurol. *7*, 145–153.

Liu, X., Lee, Y.J., Liou, L.-C., Ren, Q., Zhang, Z., Wang, S., and Witt, S.N. (2011). Alpha-synuclein functions in the nucleus to protect against hydroxyurea-induced replication stress in yeast. Hum. Mol. Genet. *20*, 3401–3414.

Lo Bianco, C., Ridet, J.-L., Schneider, B.L., Deglon, N., and Aebischer, P. (2002). alpha -Synucleinopathy and selective dopaminergic neuron loss in a rat lentiviral-based model of Parkinson's disease. Proc. Natl. Acad. Sci. U. S. A. *99*, 10813–10818.

Loeb, V., Yakunin, E., Saada, A., and Sharon, R. (2010). The transgenic overexpression of alpha-synuclein and not its related pathology associates with complex I inhibition. J. Biol. Chem. *285*, 7334–7343.

Lotharius, J., and Brundin, P. (2002a). Pathogenesis of Parkinson's disease: dopamine, vesicles and alpha-synuclein. Nat. Rev. Neurosci. *3*, 932–942.

Lotharius, J., and Brundin, P. (2002b). Impaired dopamine storage resulting from alpha-synuclein mutations may contribute to the pathogenesis of Parkinson's disease. Hum. Mol. Genet. *11*, 2395–2407.

Luk, K.C., Song, C., O'Brien, P., Stieber, A., Branch, J.R., Brunden, K.R., Trojanowski, J.Q., and Lee, V.M.-Y. (2009). Exogenous alpha-synuclein fibrils seed the formation of Lewy body-like intracellular inclusions in cultured cells. Proc. Natl. Acad. Sci. U. S. A. *106*, 20051–20056.

Lundblad, M., Decressac, M., Mattsson, B., and Björklund, A. (2012). Impaired neurotransmission caused by overexpression of α-synuclein in nigral dopamine neurons. Proc. Natl. Acad. Sci. U. S. A. *109*, 3213–3219.

Machiya, Y., Hara, S., Arawaka, S., Fukushima, S., Sato, H., Sakamoto, M., Koyama, S., and Kato, T. (2010). Phosphorylated alpha-synuclein at Ser-129 is targeted to the proteasome pathway in a ubiquitin-independent manner. J. Biol. Chem. *285*, 40732–40744.

Mak, S.K., McCormack, A.L., Manning-Bog, A.B., Cuervo, A.M., and Di Monte, D.A. (2010). Lysosomal degradation of alpha-synuclein in vivo. J. Biol. Chem. *285*, 13621–13629.

Malagelada, C., Jin, Z.H., Jackson-Lewis, V., Przedborski, S., and Greene, L.A. (2010). Rapamycin protects against neuron death in in vitro and in vivo models of Parkinson's disease. J. Neurosci. Off. J. Soc. Neurosci. *30*, 1166–1175.

Manning-Boğ, A.B., Reaney, S.H., Chou, V.P., Johnston, L.C., McCormack, A.L., Johnston, J., Langston, J.W., and Di Monte, D.A. (2006). Lack of nigrostriatal pathology in a rat model of proteasome inhibition. Ann. Neurol. *60*, 256–260.

Manning-Boğ, A.B., Schüle, B., and Langston, J.W. (2009). Alpha-synuclein-glucocerebrosidase interactions in pharmacological Gaucher models: a biological link between Gaucher disease and parkinsonism. Neurotoxicology *30*, 1127–1132.

Maraganore, D.M., de Andrade, M., Elbaz, A., Farrer, M.J., Ioannidis, J.P., Krüger, R., Rocca, W.A., Schneider, N.K., Lesnick, T.G., Lincoln, S.J., et al. (2006). Collaborative analysis of alpha-synuclein gene promoter variability and Parkinson disease. JAMA *296*, 661–670.

Maroteaux, L., Campanelli, J.T., and Scheller, R.H. (1988). Synuclein: a neuron-specific protein localized to the nucleus and presynaptic nerve terminal. J. Neurosci. Off. J. Soc. Neurosci. *8*, 2804–2815.

Marotta, N.P., Cherwien, C.A., Abeywardana, T., and Pratt, M.R. (2012). O-GlcNAc modification prevents peptide-dependent acceleration of α-synuclein aggregation. Chembiochem Eur. J. Chem. Biol. *13*, 2665–2670.

Marques, O., and Outeiro, T.F. (2012). Alpha-synuclein: from secretion to dysfunction and death. Cell Death Dis. *3*, e350.

Martí, M.J., Tolosa, E., and Campdelacreu, J. (2003). Clinical overview of the synucleinopathies. Mov. Disord. Off. J. Mov. Disord. Soc. *18 Suppl 6*, S21-27.

Martin, I., Dawson, V.L., and Dawson, T.M. (2011). Recent advances in the genetics of Parkinson's disease. Annu. Rev. Genomics Hum. Genet. *12*, 301–325.

Martin, L.J., Pan, Y., Price, A.C., Sterling, W., Copeland, N.G., Jenkins, N.A., Price, D.L., and Lee, M.K. (2006). Parkinson's disease alpha-synuclein transgenic mice develop neuronal mitochondrial degeneration and cell death. J. Neurosci. Off. J. Soc. Neurosci. *26*, 41–50.

Martin, Z.S., Neugebauer, V., Dineley, K.T., Kayed, R., Zhang, W., Reese, L.C., and Taglialatela, G. (2012). α-Synuclein oligomers oppose long-term potentiation and impair memory through a calcineurin-dependent mechanism: relevance to human synucleopathic diseases. J. Neurochem. *120*, 440–452.

Martìn-Clemente, B., Alvarez-Castelao, B., Mayo, I., Sierra, A.B., Dìaz, V., Milán, M., Fariñas, I., Gómez-Isla, T., Ferrer, I., and Castaño, J.G. (2004). alpha-Synuclein expression levels do not significantly affect

proteasome function and expression in mice and stably transfected PC12 cell lines. J. Biol. Chem. *279*, 52984–52990.

Martinez-Vicente, M., and Cuervo, A.M. (2007). Autophagy and neurodegeneration: when the cleaning crew goes on strike. Lancet Neurol. *6*, 352–361.

Martinez-Vicente, M., Talloczy, Z., Kaushik, S., Massey, A.C., Mazzulli, J., Mosharov, E.V., Hodara, R., Fredenburg, R., Wu, D.-C., Follenzi, A., et al. (2008). Dopamine-modified alpha-synuclein blocks chaperone-mediated autophagy. J. Clin. Invest. *118*, 777–788.

Massano, J., and Bhatia, K.P. (2012). Clinical approach to Parkinson’s disease: features, diagnosis, and principles of management. Cold Spring Harb. Perspect. Med. *2*, a008870.

Matsuda, N., and Tanaka, K. (2010). Does impairment of the ubiquitin-proteasome system or the autophagy-lysosome pathway predispose individuals to neurodegenerative disorders such as Parkinson’s disease? J. Alzheimers Dis. JAD *19*, 1–9.

Matsuda, N., Sato, S., Shiba, K., Okatsu, K., Saisho, K., Gautier, C.A., Sou, Y.-S., Saiki, S., Kawajiri, S., Sato, F., et al. (2010). PINK1 stabilized by mitochondrial depolarization recruits Parkin to damaged mitochondria and activates latent Parkin for mitophagy. J. Cell Biol. *189*, 211–221.

Matsuo, Y., and Kamitani, T. (2010). Parkinson’s disease-related protein, alpha-synuclein, in malignant melanoma. PloS One *5*, e10481.

Mazzulli, J.R., Xu, Y.-H., Sun, Y., Knight, A.L., McLean, P.J., Caldwell, G.A., Sidransky, E., Grabowski, G.A., and Krainc, D. (2011). Gaucher disease glucocerebrosidase and α-synuclein form a bidirectional pathogenic loop in synucleinopathies. Cell *146*, 37–52.

McCormack, A.L., and Di Monte, D.A. (2009). Enhanced alpha-synuclein expression in human neurodegenerative diseases: pathogenetic and therapeutic implications. Curr. Protein Pept. Sci. *10*, 476–482.

McGeer, P.L., Itagaki, S., Boyes, B.E., and McGeer, E.G. (1988). Reactive microglia are positive for HLA-DR in the substantia nigra of Parkinson’s and Alzheimer’s disease brains. Neurology *38*, 1285–1291.

McLean, P.J., Kawamata, H., and Hyman, B.T. (2001). Alpha-synuclein-enhanced green fluorescent protein fusion proteins form proteasome sensitive inclusions in primary neurons. Neuroscience *104*, 901–912.

McLean, P.J., Kawamata, H., Shariff, S., Hewett, J., Sharma, N., Ueda, K., Breakefield, X.O., and Hyman, B.T. (2002). TorsinA and heat shock proteins act as molecular chaperones: suppression of alpha-synuclein aggregation. J. Neurochem. *83*, 846–854.

McNaught, K.S., and Jenner, P. (2001). Proteasomal function is impaired in substantia nigra in Parkinson’s disease. Neurosci. Lett. *297*, 191–194.

McNaught, K.S.P., Mytilineou, C., Jnobaptiste, R., Yabut, J., Shashidharan, P., Jennert, P., and Olanow, C.W. (2002a). Impairment of the ubiquitin-proteasome system causes dopaminergic cell death and inclusion body formation in ventral mesencephalic cultures. J. Neurochem. *81*, 301–306.

McNaught, K.S.P., Björklund, L.M., Belizaire, R., Isacson, O., Jenner, P., and Olanow, C.W. (2002b). Proteasome inhibition causes nigral degeneration with inclusion bodies in rats. Neuroreport *13*, 1437–1441.

McNaught, K.S.P., Belizaire, R., Isacson, O., Jenner, P., and Olanow, C.W. (2003). Altered proteasomal function in sporadic Parkinson's disease. Exp. Neurol. *179*, 38–46.

McNaught, K.S.P., Perl, D.P., Brownell, A.-L., and Olanow, C.W. (2004). Systemic exposure to proteasome inhibitors causes a progressive model of Parkinson's disease. Ann. Neurol. *56*, 149–162.

Melchior, F., Schergaut, M., and Pichler, A. (2003). SUMO: ligases, isopeptidases and nuclear pores. Trends Biochem. Sci. *28*, 612–618.

Meredith, G.E., and Rademacher, D.J. (2011). MPTP mouse models of Parkinson's disease: an update. J. Park. Dis. *1*, 19–33.

Meredith, G.E., Sonsalla, P.K., and Chesselet, M.-F. (2008). Animal models of Parkinson's disease progression. Acta Neuropathol. (Berl.) *115*, 385–398.

Meriin, A.B., Gabai, V.L., Yaglom, J., Shifrin, V.I., and Sherman, M.Y. (1998). Proteasome inhibitors activate stress kinases and induce Hsp72. Diverse effects on apoptosis. J. Biol. Chem. *273*, 6373–6379.

Michell, A.W., Tofaris, G.K., Gossage, H., Tyers, P., Spillantini, M.G., and Barker, R.A. (2007). The effect of truncated human alpha-synuclein (1-120) on dopaminergic cells in a transgenic mouse model of Parkinson's disease. Cell Transplant. *16*, 461–474.

Mizuno, Y., Hattori, N., Kubo, S.-I., Sato, S., Nishioka, K., Hatano, T., Tomiyama, H., Funayama, M., Machida, Y., and Mochizuki, H. (2008). Progress in the pathogenesis and genetics of Parkinson's disease. Philos. Trans. R. Soc. Lond. B. Biol. Sci. *363*, 2215–2227.

Mizushima, N., Levine, B., Cuervo, A.M., and Klionsky, D.J. (2008). Autophagy fights disease through cellular self-digestion. Nature *451*, 1069–1075.

Mosharov, E.V., Larsen, K.E., Kanter, E., Phillips, K.A., Wilson, K., Schmitz, Y., Krantz, D.E., Kobayashi, K., Edwards, R.H., and Sulzer, D. (2009). Interplay between cytosolic dopamine, calcium, and alpha-synuclein causes selective death of substantia nigra neurons. Neuron *62*, 218–229.

van den Munckhof, P., Luk, K.C., Ste-Marie, L., Montgomery, J., Blanchet, P.J., Sadikot, A.F., and Drouin, J. (2003). Pitx3 is required for motor activity and for survival of a subset of midbrain dopaminergic neurons. Dev. Camb. Engl. *130*, 2535–2542.

Murase, D., Hachiya, A., Takano, K., Hicks, R., Visscher, M.O., Kitahara, T., Hase, T., Takema, Y., and Yoshimori, T. (2013a). Autophagy Has a Significant Role in Determining Skin Color by Regulating Melanosome Degradation in Keratinocytes. J. Invest. Dermatol. *133*, 2416–2424.

Murase, D., Hachiya, A., Takano, K., Hicks, R., Visscher, M.O., Kitahara, T., Hase, T., Takema, Y., and Yoshimori, T. (2013b). Autophagy has a significant role in determining skin color by regulating melanosome degradation in keratinocytes. J. Invest. Dermatol. *133*, 2416–2424.

Musgrove, R.E.J., King, A.E., and Dickson, T.C. (2011). Neuroprotective upregulation of endogenous α-synuclein precedes ubiquitination in cultured dopaminergic neurons. Neurotox. Res. *19*, 592–602.

Musgrove, R.E.J., King, A.E., and Dickson, T.C. (2012). α-Synuclein Protects Neurons from Apoptosis Downstream of Free-Radical Production Through Modulation of the MAPK Signalling Pathway. Neurotox. Res.

Myhre, R., Toft, M., Kachergus, J., Hulihan, M.M., Aasly, J.O., Klungland, H., and Farrer, M.J. (2008). Multiple alpha-synuclein gene polymorphisms are associated with Parkinson's disease in a Norwegian population. Acta Neurol. Scand. *118*, 320–327.

Nakamura, K., Nemani, V.M., Wallender, E.K., Kaehlcke, K., Ott, M., and Edwards, R.H. (2008). Optical reporters for the conformation of alpha-synuclein reveal a specific interaction with mitochondria. J. Neurosci. Off. J. Soc. Neurosci. *28*, 12305–12317.

Nakamura, K., Nemani, V.M., Azarbal, F., Skibinski, G., Levy, J.M., Egami, K., Munishkina, L., Zhang, J., Gardner, B., Wakabayashi, J., et al. (2011). Direct membrane association drives mitochondrial fission by the Parkinson disease-associated protein alpha-synuclein. J. Biol. Chem. *286*, 20710–20726.

Nakaso, K., Yoshimoto, Y., Yano, H., Takeshima, T., and Nakashima, K. (2004). p53-mediated mitochondrial dysfunction by proteasome inhibition in dopaminergic SH-SY5Y cells. Neurosci. Lett. *354*, 213–216.

Nakaso, K., Tajima, N., Ito, S., Teraoka, M., Yamashita, A., Horikoshi, Y., Kikuchi, D., Mochida, S., Nakashima, K., and Matsura, T. (2013). Dopamine-mediated oxidation of methionine 127 in α-synuclein causes cytotoxicity and oligomerization of α-synuclein. PloS One *8*, e55068.

Nemani, V.M., Lu, W., Berge, V., Nakamura, K., Onoa, B., Lee, M.K., Chaudhry, F.A., Nicoll, R.A., and Edwards, R.H. (2010). Increased expression of alpha-synuclein reduces neurotransmitter release by inhibiting synaptic vesicle reclustering after endocytosis. Neuron *65*, 66–79.

Nixon, R.A. (2006). Autophagy in neurodegenerative disease: friend, foe or turncoat? Trends Neurosci. *29*, 528–535.

Nixon, R.A. (2013). The role of autophagy in neurodegenerative disease. Nat. Med. *19*, 983–997.

Okochi, M., Walter, J., Koyama, A., Nakajo, S., Baba, M., Iwatsubo, T., Meijer, L., Kahle, P.J., and Haass, C. (2000). Constitutive phosphorylation of the Parkinson's disease associated alpha-synuclein. J. Biol. Chem. *275*, 390–397.

Oliveras-Salvá, M., Van der Perren, A., Casadei, N., Stroobants, S., Nuber, S., D'Hooge, R., Van den Haute, C., and Baekelandt, V. (2013). rAAV2/7 vector-mediated overexpression of alpha-synuclein in mouse substantia nigra induces protein aggregation and progressive dose-dependent neurodegeneration. Mol. Neurodegener. *8*, 44.

Outeiro, T.F., Kontopoulos, E., Altmann, S.M., Kufareva, I., Strathearn, K.E., Amore, A.M., Volk, C.B., Maxwell, M.M., Rochet, J.-C., McLean, P.J., et al. (2007). Sirtuin 2 inhibitors rescue alpha-synuclein-mediated toxicity in models of Parkinson's disease. Science *317*, 516–519.

Outeiro, T.F., Klucken, J., Bercury, K., Tetzlaff, J., Putcha, P., Oliveira, L.M.A., Quintas, A., McLean, P.J., and Hyman, B.T. (2009). Dopamine-Induced Conformational Changes in Alpha-Synuclein. PLoS ONE *4*.

Paisán-Ruiz, C., and Houlden, H. (2010). Common pathogenic pathways in melanoma and Parkinson disease. Neurology *75*, 1653–1655.

Pan, T., Li, X., and Jankovic, J. (2011). The association between Parkinson's disease and melanoma. Int. J. Cancer J. Int. Cancer *128*, 2251–2260.

Pan, T., Zhu, J., Hwu, W.-J., and Jankovic, J. (2012). The role of alpha-synuclein in melanin synthesis in melanoma and dopaminergic neuronal cells. PloS One *7*, e45183.

Pandey, U.B., Nie, Z., Batlevi, Y., McCray, B.A., Ritson, G.P., Nedelsky, N.B., Schwartz, S.L., DiProspero, N.A., Knight, M.A., Schuldiner, O., et al. (2007). HDAC6 rescues neurodegeneration and provides an essential link between autophagy and the UPS. Nature *447*, 859–863.

Park, J.-Y., Paik, S.R., Jou, I., and Park, S.M. (2008). Microglial phagocytosis is enhanced by monomeric alpha-synuclein, not aggregated alpha-synuclein: implications for Parkinson's disease. Glia *56*, 1215–1223.

Pattingre, S., Tassa, A., Qu, X., Garuti, R., Liang, X.H., Mizushima, N., Packer, M., Schneider, M.D., and Levine, B. (2005). Bcl-2 antiapoptotic proteins inhibit Beclin 1-dependent autophagy. Cell *122*, 927–939.

Paxinou, E., Chen, Q., Weisse, M., Giasson, B.I., Norris, E.H., Rueter, S.M., Trojanowski, J.Q., Lee, V.M., and Ischiropoulos, H. (2001). Induction of alpha-synuclein aggregation by intracellular nitrative insult. J. Neurosci. Off. J. Soc. Neurosci. *21*, 8053–8061.

Perez, R.G., Waymire, J.C., Lin, E., Liu, J.J., Guo, F., and Zigmond, M.J. (2002). A role for alpha-synuclein in the regulation of dopamine biosynthesis. J. Neurosci. Off. J. Soc. Neurosci. *22*, 3090–3099.

Petroske, E., Meredith, G.E., Callen, S., Totterdell, S., and Lau, Y.S. (2001). Mouse model of Parkinsonism: a comparison between subacute MPTP and chronic MPTP/probenecid treatment. Neuroscience *106*, 589–601.

Polymeropoulos, M.H., Lavedan, C., Leroy, E., Ide, S.E., Dehejia, A., Dutra, A., Pike, B., Root, H., Rubenstein, J., Boyer, R., et al. (1997). Mutation in the alpha-synuclein gene identified in families with Parkinson's disease. Science *276*, 2045–2047.

Poulogiannis, G., McIntyre, R.E., Dimitriadi, M., Apps, J.R., Wilson, C.H., Ichimura, K., Luo, F., Cantley, L.C., Wyllie, A.H., Adams, D.J., et al. (2010). PARK2 deletions occur frequently in sporadic colorectal cancer and accelerate adenoma development in Apc mutant mice. Proc. Natl. Acad. Sci. U. S. A. *107*, 15145–15150.

Przedborski, S., and Ischiropoulos, H. (2005). Reactive oxygen and nitrogen species: weapons of neuronal destruction in models of Parkinson's disease. Antioxid. Redox Signal. *7*, 685–693.

Qiao, L., and Zhang, J. (2009). Inhibition of lysosomal functions reduces proteasomal activity. Neurosci. Lett. *456*, 15–19.

Qin, L., Wu, X., Block, M.L., Liu, Y., Breese, G.R., Hong, J.-S., Knapp, D.J., and Crews, F.T. (2007). Systemic LPS causes chronic neuroinflammation and progressive neurodegeneration. Glia *55*, 453–462.

Qing, G., Yan, P., and Xiao, G. (2006). Hsp90 inhibition results in autophagy-mediated proteasome-independent degradation of IkappaB kinase (IKK). Cell Res. *16*, 895–901.

Qureshi, H.Y., and Paudel, H.K. (2011). Parkinsonian neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and alpha-synuclein mutations promote Tau protein phosphorylation at Ser262 and destabilize microtubule cytoskeleton in vitro. J. Biol. Chem. *286*, 5055–5068.

Rami, A. (2009). Review: autophagy in neurodegeneration: firefighter and/or incendiarist? Neuropathol. Appl. Neurobiol. *35*, 449–461.

Rascol, O., Lozano, A., Stern, M., and Poewe, W. (2011). Milestones in Parkinson’s disease therapeutics. Mov. Disord. Off. J. Mov. Disord. Soc. *26*, 1072–1082.

Ravikumar, B., Sarkar, S., Davies, J.E., Futter, M., Garcia-Arencibia, M., Green-Thompson, Z.W., Jimenez-Sanchez, M., Korolchuk, V.I., Lichtenberg, M., Luo, S., et al. (2010). Regulation of mammalian autophagy in physiology and pathophysiology. Physiol. Rev. *90*, 1383–1435.

Rideout, H.J., Lang-Rollin, I., and Stefanis, L. (2004). Involvement of macroautophagy in the dissolution of neuronal inclusions. Int. J. Biochem. Cell Biol. *36*, 2551–2562.

Rideout, H.J., Lang-Rollin, I.C.J., Savalle, M., and Stefanis, L. (2005). Dopaminergic neurons in rat ventral midbrain cultures undergo selective apoptosis and form inclusions, but do not up-regulate iHSP70, following proteasomal inhibition. J. Neurochem. *93*, 1304–1313.

Roodveldt, C., Labrador-Garrido, A., Gonzalez-Rey, E., Fernandez-Montesinos, R., Caro, M., Lachaud, C.C., Waudby, C.A., Delgado, M., Dobson, C.M., and Pozo, D. (2010). Glial innate immunity generated by non-aggregated alpha-synuclein in mouse: differences between wild-type and Parkinson’s disease-linked mutants. PloS One *5*, e13481.

Roos-Mattjus, P., and Sistonen, L. (2004). The ubiquitin-proteasome pathway. Ann. Med. *36*, 285–295.

Rott, R., Szargel, R., Haskin, J., Bandopadhyay, R., Lees, A.J., Shani, V., and Engelender, S. (2011). α-Synuclein fate is determined by USP9X-regulated monoubiquitination. Proc. Natl. Acad. Sci. U. S. A. *108*, 18666–18671.

Rubinsztein, D.C. (2006). The roles of intracellular protein-degradation pathways in neurodegeneration. Nature *443*, 780–786.

Rubinsztein, D.C., Bento, C.F., and Deretic, V. (2015). Therapeutic targeting of autophagy in neurodegenerative and infectious diseases. J. Exp. Med. *212*, 979–990.

Saint-Pierre, M., Tremblay, M.-E., Sik, A., Gross, R.E., and Cicchetti, F. (2006). Temporal effects of paraquat/maneb on microglial activation and dopamine neuronal loss in older rats. J. Neurochem. *98*, 760–772.

Savica, R., Rocca, W.A., and Ahlskog, J.E. (2010). When does Parkinson disease start? Arch. Neurol. *67*, 798–801.

Savica, R., Grossardt, B.R., Bower, J.H., Ahlskog, J.E., and Rocca, W.A. (2013). Incidence and pathology of synucleinopathies and tauopathies related to parkinsonism. JAMA Neurol. *70*, 859–866.

Schallert, T., Fleming, S.M., Leasure, J.L., Tillerson, J.L., and Bland, S.T. (2000). CNS plasticity and assessment of forelimb sensorimotor outcome in unilateral rat models of stroke, cortical ablation, parkinsonism and spinal cord injury. Neuropharmacology *39*, 777–787.

Schapira, A.H.V., Cleeter, M.W.J., Muddle, J.R., Workman, J.M., Cooper, J.M., and King, R.H.M. (2006). Proteasomal inhibition causes loss of nigral tyrosine hydroxylase neurons. Ann. Neurol. *60*, 253–255.

Schell, H., Hasegawa, T., Neumann, M., and Kahle, P.J. (2009). Nuclear and neuritic distribution of serine-129 phosphorylated alpha-synuclein in transgenic mice. Neuroscience *160*, 796–804.

Schneider, B.L., Seehus, C.R., Capowski, E.E., Aebischer, P., Zhang, S.-C., and Svendsen, C.N. (2007). Over-expression of alpha-synuclein in human neural progenitors leads to specific changes in fate and differentiation. Hum. Mol. Genet. *16*, 651–666.

Scott, D.A., Tabarean, I., Tang, Y., Cartier, A., Masliah, E., and Roy, S. (2010). A pathologic cascade leading to synaptic dysfunction in alpha-synuclein-induced neurodegeneration. J. Neurosci. Off. J. Soc. Neurosci. *30*, 8083–8095.

Segura-Aguilar, J., Paris, I., Muñoz, P., Ferrari, E., Zecca, L., and Zucca, F.A. (2014). Protective and toxic roles of dopamine in Parkinson’s disease. J. Neurochem. *129*, 898–915.

Setsuie, R., and Wada, K. (2007). The functions of UCH-L1 and its relation to neurodegenerative diseases. Neurochem. Int. *51*, 105–111.

Sevlever, D., Jiang, P., and Yen, S.-H.C. (2008). Cathepsin D is the main lysosomal enzyme involved in the degradation of alpha-synuclein and generation of its carboxy-terminally truncated species. Biochemistry (Mosc.) *47*, 9678–9687.

Sgadò, P., Albéri, L., Gherbassi, D., Galasso, S.L., Ramakers, G.M.J., Alavian, K.N., Smidt, M.P., Dyck, R.H., and Simon, H.H. (2006). Slow progressive degeneration of nigral dopaminergic neurons in postnatal Engrailed mutant mice. Proc. Natl. Acad. Sci. U. S. A. *103*, 15242–15247.

Shameli, A., Xiao, W., Zheng, Y., Shyu, S., Sumodi, J., Meyerson, H.J., Harding, C.V., and Maitta, R.W. (2015). A critical role for alpha-synuclein in development and function of T lymphocytes. Immunobiology.

Sharon, R., Bar-Joseph, I., Frosch, M.P., Walsh, D.M., Hamilton, J.A., and Selkoe, D.J. (2003). The formation of highly soluble oligomers of alpha-synuclein is regulated by fatty acids and enhanced in Parkinson’s disease. Neuron *37*, 583–595.

Sherer, T.B., Kim, J.H., Betarbet, R., and Greenamyre, J.T. (2003). Subcutaneous rotenone exposure causes highly selective dopaminergic degeneration and alpha-synuclein aggregation. Exp. Neurol. *179*, 9–16.

Shimura, H., Schlossmacher, M.G., Hattori, N., Frosch, M.P., Trockenbacher, A., Schneider, R., Mizuno, Y., Kosik, K.S., and Selkoe, D.J. (2001). Ubiquitination of a new form of alpha-synuclein by parkin from human brain: implications for Parkinson's disease. Science *293*, 263–269.

Shin, Y., Klucken, J., Patterson, C., Hyman, B.T., and McLean, P.J. (2005). The co-chaperone carboxyl terminus of Hsp70-interacting protein (CHIP) mediates alpha-synuclein degradation decisions between proteasomal and lysosomal pathways. J. Biol. Chem. *280*, 23727–23734.

Shulman, J.M., De Jager, P.L., and Feany, M.B. (2011). Parkinson's disease: genetics and pathogenesis. Annu. Rev. Pathol. *6*, 193–222.

Sidhu, A., Wersinger, C., and Vernier, P. (2004). alpha-Synuclein regulation of the dopaminergic transporter: a possible role in the pathogenesis of Parkinson's disease. FEBS Lett. *565*, 1–5.

Sidransky, E., Nalls, M.A., Aasly, J.O., Aharon-Peretz, J., Annesi, G., Barbosa, E.R., Bar-Shira, A., Berg, D., Bras, J., Brice, A., et al. (2009). Multicenter analysis of glucocerebrosidase mutations in Parkinson's disease. N. Engl. J. Med. *361*, 1651–1661.

Smith, W.W., Pei, Z., Jiang, H., Moore, D.J., Liang, Y., West, A.B., Dawson, V.L., Dawson, T.M., and Ross, C.A. (2005a). Leucine-rich repeat kinase 2 (LRRK2) interacts with parkin, and mutant LRRK2 induces neuronal degeneration. Proc. Natl. Acad. Sci. U. S. A. *102*, 18676–18681.

Smith, W.W., Jiang, H., Pei, Z., Tanaka, Y., Morita, H., Sawa, A., Dawson, V.L., Dawson, T.M., and Ross, C.A. (2005b). Endoplasmic reticulum stress and mitochondrial cell death pathways mediate A53T mutant alpha-synuclein-induced toxicity. Hum. Mol. Genet. *14*, 3801–3811.

Snyder, H., Mensah, K., Theisler, C., Lee, J., Matouschek, A., and Wolozin, B. (2003). Aggregated and monomeric alpha-synuclein bind to the S6' proteasomal protein and inhibit proteasomal function. J. Biol. Chem. *278*, 11753–11759.

Solomon, V.R., and Lee, H. (2009). Chloroquine and its analogs: a new promise of an old drug for effective and safe cancer therapies. Eur. J. Pharmacol. *625*, 220–233.

Sousa, V.L., Bellani, S., Giannandrea, M., Yousuf, M., Valtorta, F., Meldolesi, J., and Chieregatti, E. (2009). {alpha}-synuclein and its A30P mutant affect actin cytoskeletal structure and dynamics. Mol. Biol. Cell *20*, 3725–3739.

Spencer, B., Potkar, R., Trejo, M., Rockenstein, E., Patrick, C., Gindi, R., Adame, A., Wyss-Coray, T., and Masliah, E. (2009). Beclin 1 gene transfer activates autophagy and ameliorates the neurodegenerative pathology in alpha-synuclein models of Parkinson's and Lewy body diseases. J. Neurosci. Off. J. Soc. Neurosci. *29*, 13578–13588.

Spillantini, M.G., Schmidt, M.L., Lee, V.M., Trojanowski, J.Q., Jakes, R., and Goedert, M. (1997). Alpha-synuclein in Lewy bodies. Nature *388*, 839–840.

Squire, L., Bloom, F.E., Spitzer, N.C., Squire, L.R., Berg, D., Lac, S. du, and Ghosh, A. (2008). Fundamental Neuroscience (Academic Press).

Stamelou, M., Quinn, N.P., and Bhatia, K.P. (2013). “Atypical” atypical parkinsonism: new genetic conditions presenting with features of progressive supranuclear palsy, corticobasal degeneration, or multiple system atrophy-a diagnostic guide. Mov. Disord. Off. J. Mov. Disord. Soc. *28*, 1184–1199.

Stefanis, L. (2012). α-Synuclein in Parkinson’s disease. Cold Spring Harb. Perspect. Med. *2*, a009399.

Stefanis, L., Larsen, K.E., Rideout, H.J., Sulzer, D., and Greene, L.A. (2001). Expression of A53T mutant but not wild-type alpha-synuclein in PC12 cells induces alterations of the ubiquitin-dependent degradation system, loss of dopamine release, and autophagic cell death. J. Neurosci. Off. J. Soc. Neurosci. *21*, 9549–9560.

Strauss, K.M., Martins, L.M., Plun-Favreau, H., Marx, F.P., Kautzmann, S., Berg, D., Gasser, T., Wszolek, Z., Müller, T., Bornemann, A., et al. (2005). Loss of function mutations in the gene encoding Omi/HtrA2 in Parkinson’s disease. Hum. Mol. Genet. *14*, 2099–2111.

Su, X., Federoff, H.J., and Maguire-Zeiss, K.A. (2009). Mutant alpha-synuclein overexpression mediates early proinflammatory activity. Neurotox. Res. *16*, 238–254.

Sung, J.Y., Park, S.M., Lee, C.-H., Um, J.W., Lee, H.J., Kim, J., Oh, Y.J., Lee, S.-T., Paik, S.R., and Chung, K.C. (2005). Proteolytic cleavage of extracellular secreted {alpha}-synuclein via matrix metalloproteinases. J. Biol. Chem. *280*, 25216–25224.

Suzuki, K., Bose, P., Leong-Quong, R.Y., Fujita, D.J., and Riabowol, K. (2010). REAP: A two minute cell fractionation method. BMC Res. Notes *3*, 294.

Taira, T., Saito, Y., Niki, T., Iguchi-Ariga, S.M.M., Takahashi, K., and Ariga, H. (2004). DJ-1 has a role in antioxidative stress to prevent cell death. EMBO Rep. *5*, 213–218.

Takahashi-Niki, K., Niki, T., Taira, T., Iguchi-Ariga, S.M.M., and Ariga, H. (2004). Reduced anti-oxidative stress activities of DJ-1 mutants found in Parkinson’s disease patients. Biochem. Biophys. Res. Commun. *320*, 389–397.

Tanaka, Y., Engelender, S., Igarashi, S., Rao, R.K., Wanner, T., Tanzi, R.E., Sawa, A., L Dawson, V., Dawson, T.M., and Ross, C.A. (2001a). Inducible expression of mutant alpha-synuclein decreases proteasome activity and increases sensitivity to mitochondria-dependent apoptosis. Hum. Mol. Genet. *10*, 919–926.

Tanaka, Y., Engelender, S., Igarashi, S., Rao, R.K., Wanner, T., Tanzi, R.E., Sawa, A., L Dawson, V., Dawson, T.M., and Ross, C.A. (2001b). Inducible expression of mutant alpha-synuclein decreases proteasome activity and increases sensitivity to mitochondria-dependent apoptosis. Hum. Mol. Genet. *10*, 919–926.

Tanik, S.A., Schultheiss, C.E., Volpicelli-Daley, L.A., Brunden, K.R., and Lee, V.M.Y. (2013). Lewy body-like α-synuclein aggregates resist degradation and impair macroautophagy. J. Biol. Chem. *288*, 15194–15210.

Tay, S.-P., Yeo, C.W.S., Chai, C., Chua, P.-J., Tan, H.-M., Ang, A.X.Y., Yip, D.L.H., Sung, J.-X., Tan, P.H., Bay, B.-H., et al. (2010). Parkin enhances the expression of cyclin-dependent kinase 6 and negatively regulates the proliferation of breast cancer cells. J. Biol. Chem. *285*, 29231–29238.

Thayanidhi, N., Helm, J.R., Nycz, D.C., Bentley, M., Liang, Y., and Hay, J.C. (2010). Alpha-synuclein delays endoplasmic reticulum (ER)-to-Golgi transport in mammalian cells by antagonizing ER/Golgi SNAREs. Mol. Biol. Cell *21*, 1850–1863.

Thiruchelvam, M., Richfield, E.K., Baggs, R.B., Tank, A.W., and Cory-Slechta, D.A. (2000). The nigrostriatal dopaminergic system as a preferential target of repeated exposures to combined paraquat and maneb: implications for Parkinson's disease. J. Neurosci. Off. J. Soc. Neurosci. *20*, 9207–9214.

Thiruchelvam, M., McCormack, A., Richfield, E.K., Baggs, R.B., Tank, A.W., Di Monte, D.A., and Cory-Slechta, D.A. (2003). Age-related irreversible progressive nigrostriatal dopaminergic neurotoxicity in the paraquat and maneb model of the Parkinson's disease phenotype. Eur. J. Neurosci. *18*, 589–600.

Tofaris, G.K., and Spillantini, M.G. (2007). Physiological and pathological properties of alpha-synuclein. Cell. Mol. Life Sci. CMLS *64*, 2194–2201.

Tofaris, G.K., Layfield, R., and Spillantini, M.G. (2001). alpha-synuclein metabolism and aggregation is linked to ubiquitin-independent degradation by the proteasome. FEBS Lett. *509*, 22–26.

Tofaris, G.K., Kim, H.T., Hourez, R., Jung, J.-W., Kim, K.P., and Goldberg, A.L. (2011). Ubiquitin ligase Nedd4 promotes alpha-synuclein degradation by the endosomal-lysosomal pathway. Proc. Natl. Acad. Sci. U. S. A. *108*, 17004–17009.

Tokmakov, A.A., Kurotani, A., Takagi, T., Toyama, M., Shirouzu, M., Fukami, Y., and Yokoyama, S. (2012). Multiple post-translational modifications affect heterologous protein synthesis. J. Biol. Chem. *287*, 27106–27116.

Tokuda, T., Qureshi, M.M., Ardah, M.T., Varghese, S., Shehab, S. a. S., Kasai, T., Ishigami, N., Tamaoka, A., Nakagawa, M., and El-Agnaf, O.M.A. (2010). Detection of elevated levels of α-synuclein oligomers in CSF from patients with Parkinson disease. Neurology *75*, 1766–1772.

Tsui, A., and Isacson, O. (2011). Functions of the nigrostriatal dopaminergic synapse and the use of neurotransplantation in Parkinson's disease. J. Neurol. *258*, 1393–1405.

Ulusoy, A., Bjorklund, T., Hermening, S., and Kirik, D. (2008). In vivo gene delivery for development of mammalian models for Parkinson's disease. Exp. Neurol. *209*, 89–100.

Uversky, V.N. (2007). Neuropathology, biochemistry, and biophysics of alpha-synuclein aggregation. J. Neurochem. *103*, 17–37.

Uversky, V.N. (2008). Alpha-synuclein misfolding and neurodegenerative diseases. Curr. Protein Pept. Sci. *9*, 507–540.

Uversky, V.N., Li, J., Souillac, P., Millett, I.S., Doniach, S., Jakes, R., Goedert, M., and Fink, A.L. (2002). Biophysical properties of the synucleins and their propensities to fibrillate: inhibition of alpha-synuclein assembly by beta- and gamma-synucleins. J. Biol. Chem. *277*, 11970–11978.

Vande Walle, L., Lamkanfi, M., and Vandenabeele, P. (2008). The mitochondrial serine protease HtrA2/Omi: an overview. Cell Death Differ. *15*, 453–460.

Veeriah, S., Taylor, B.S., Meng, S., Fang, F., Yilmaz, E., Vivanco, I., Janakiraman, M., Schultz, N., Hanrahan, A.J., Pao, W., et al. (2010). Somatic mutations of the Parkinson's disease-associated gene PARK2 in glioblastoma and other human malignancies. Nat. Genet. *42*, 77–82.

Vekrellis, K., Rideout, H.J., and Stefanis, L. (2004). Neurobiology of alpha-synuclein. Mol. Neurobiol. *30*, 1–21.

Venda, L.L., Cragg, S.J., Buchman, V.L., and Wade-Martins, R. (2010). α-Synuclein and dopamine at the crossroads of Parkinson's disease. Trends Neurosci. *33*, 559–568.

Vivacqua, G., Casini, A., Vaccaro, R., Fornai, F., Yu, S., and D'Este, L. (2011). Different sub-cellular localization of alpha-synuclein in the C57BL\6J mouse's central nervous system by two novel monoclonal antibodies. J. Chem. Neuroanat. *41*, 97–110.

Vogiatzi, T., Xilouri, M., Vekrellis, K., and Stefanis, L. (2008). Wild type alpha-synuclein is degraded by chaperone-mediated autophagy and macroautophagy in neuronal cells. J. Biol. Chem. *283*, 23542–23556.

Volles, M.J., and Lansbury, P.T. (2003). Zeroing in on the pathogenic form of alpha-synuclein and its mechanism of neurotoxicity in Parkinson's disease. Biochemistry (Mosc.) *42*, 7871–7878.

Wallace, R.A., Boldry, R., Schmittgen, T., Miller, D., and Uretsky, N. (1984). Effect of 1-methyl-4-phenyl-1,2,3,6 tetrahydropyridine (MPTP) on monoamine neurotransmitters in mouse brain & heart. Life Sci. *35*, 285–291.

Walsh, D.M., and Selkoe, D.J. (2004). Oligomers on the brain: the emerging role of soluble protein aggregates in neurodegeneration. Protein Pept. Lett. *11*, 213–228.

Wang, W., Perovic, I., Chittuluru, J., Kaganovich, A., Nguyen, L.T.T., Liao, J., Auclair, J.R., Johnson, D., Landeru, A., Simorellis, A.K., et al. (2011). A soluble α-synuclein construct forms a dynamic tetramer. Proc. Natl. Acad. Sci. U. S. A. *108*, 17797–17802.

Waxman, E.A., and Giasson, B.I. (2009). Molecular mechanisms of alpha-synuclein neurodegeneration. Biochim. Biophys. Acta *1792*, 616–624.

Webb, J.L., Ravikumar, B., Atkins, J., Skepper, J.N., and Rubinsztein, D.C. (2003a). Alpha-Synuclein is degraded by both autophagy and the proteasome. J. Biol. Chem. *278*, 25009–25013.

Webb, J.L., Ravikumar, B., Atkins, J., Skepper, J.N., and Rubinsztein, D.C. (2003b). Alpha-Synuclein is degraded by both autophagy and the proteasome. J. Biol. Chem. *278*, 25009–25013.

Wei, Y., Pattingre, S., Sinha, S., Bassik, M., and Levine, B. (2008). JNK1-mediated phosphorylation of Bcl-2 regulates starvation-induced autophagy. Mol. Cell *30*, 678–688.

Wenning, G.K., Litvan, I., and Tolosa, E. (2011). Milestones in atypical and secondary Parkinsonisms. Mov. Disord. Off. J. Mov. Disord. Soc. *26*, 1083–1095.

Winner, B., Jappelli, R., Maji, S.K., Desplats, P.A., Boyer, L., Aigner, S., Hetzer, C., Loher, T., Vilar, M., Campioni, S., et al. (2011). In vivo demonstration that alpha-synuclein oligomers are toxic. Proc. Natl. Acad. Sci. U. S. A. *108*, 4194–4199.

Winslow, A.R., Chen, C.-W., Corrochano, S., Acevedo-Arozena, A., Gordon, D.E., Peden, A.A., Lichtenberg, M., Menzies, F.M., Ravikumar, B., Imarisio, S., et al. (2010). α-Synuclein impairs macroautophagy: implications for Parkinson's disease. J. Cell Biol. *190*, 1023–1037.

Wong, E.S.P., Tan, J.M.M., Wang, C., Zhang, Z., Tay, S.-P., Zaiden, N., Ko, H.S., Dawson, V.L., Dawson, T.M., and Lim, K.-L. (2007). Relative sensitivity of parkin and other cysteine-containing enzymes to stress-induced solubility alterations. J. Biol. Chem. *282*, 12310–12318.

Wood, S.J., Wypych, J., Steavenson, S., Louis, J.C., Citron, M., and Biere, A.L. (1999). alpha-synuclein fibrillogenesis is nucleation-dependent. Implications for the pathogenesis of Parkinson's disease. J. Biol. Chem. *274*, 19509–19512.

Wu, B., Liu, Q., Duan, C., Li, Y., Yu, S., Chan, P., Uéda, K., and Yang, H. (2011). Phosphorylation of α-synuclein upregulates tyrosine hydroxylase activity in MN9D cells. Acta Histochem. *113*, 32–35.

Xilouri, M., Vogiatzi, T., Vekrellis, K., Park, D., and Stefanis, L. (2009). Abberant alpha-synuclein confers toxicity to neurons in part through inhibition of chaperone-mediated autophagy. PloS One *4*, e5515.

Xilouri, M., Brekk, O.R., and Stefanis, L. (2012a). Alpha-synuclein and Protein Degradation Systems: a Reciprocal Relationship. Mol. Neurobiol.

Xilouri, M., Brekk, O.R., and Stefanis, L. (2012b). Alpha-synuclein and Protein Degradation Systems: a Reciprocal Relationship. Mol. Neurobiol.

Xilouri, M., Brekk, O.R., Landeck, N., Pitychoutis, P.M., Papasilekas, T., Papadopoulou-Daifoti, Z., Kirik, D., and Stefanis, L. (2013). Boosting chaperone-mediated autophagy in vivo mitigates α-synuclein-induced neurodegeneration. Brain J. Neurol. *136*, 2130–2146.

Xu, S., Zhou, M., Yu, S., Cai, Y., Zhang, A., Uéda, K., and Chan, P. (2006). Oxidative stress induces nuclear translocation of C-terminus of alpha-synuclein in dopaminergic cells. Biochem. Biophys. Res. Commun. *342*, 330–335.

Yamakawa, K., Izumi, Y., Takeuchi, H., Yamamoto, N., Kume, T., Akaike, A., Takahashi, R., Shimohama, S., and Sawada, H. (2010). Dopamine facilitates alpha-synuclein oligomerization in human neuroblastoma SH-SY5Y cells. Biochem. Biophys. Res. Commun. *391*, 129–134.

Yazdani, U., German, D.C., Liang, C.-L., Manzino, L., Sonsalla, P.K., and Zeevalk, G.D. (2006). Rat model of Parkinson's disease: chronic central delivery of 1-methyl-4-phenylpyridinium (MPP+). Exp. Neurol. *200*, 172–183.

Yen, W.-L., and Klionsky, D.J. (2008). How to live long and prosper: autophagy, mitochondria, and aging. Physiol. Bethesda Md *23*, 248–262.

Yin, H.H., and Knowlton, B.J. (2006). The role of the basal ganglia in habit formation. Nat. Rev. Neurosci. *7*, 464–476.

Yon, J.M. (1997). Protein folding: concepts and perspectives. Cell. Mol. Life Sci. CMLS *53*, 557–567.

Yu, W., Sun, Y., Guo, S., and Lu, B. (2011). The PINK1/Parkin pathway regulates mitochondrial dynamics and function in mammalian hippocampal and dopaminergic neurons. Hum. Mol. Genet. *20*, 3227–3240.

Yue, Z., Friedman, L., Komatsu, M., and Tanaka, K. (2009). The cellular pathways of neuronal autophagy and their implication in neurodegenerative diseases. Biochim. Biophys. Acta *1793*, 1496–1507.

Zafar, K.S., Inayat-Hussain, S.H., and Ross, D. (2007). A comparative study of proteasomal inhibition and apoptosis induced in N27 mesencephalic cells by dopamine and MG132. J. Neurochem. *102*, 913–921.

Zecca, L., Zucca, F.A., Albertini, A., Rizzio, E., and Fariello, R.G. (2006). A proposed dual role of neuromelanin in the pathogenesis of Parkinson's disease. Neurology *67*, S8-11.

Zeng, B.-Y., Bukhatwa, S., Hikima, A., Rose, S., and Jenner, P. (2006). Reproducible nigral cell loss after systemic proteasomal inhibitor administration to rats. Ann. Neurol. *60*, 248–252.

Zhang, W., Wang, T., Pei, Z., Miller, D.S., Wu, X., Block, M.L., Wilson, B., Zhang, W., Zhou, Y., Hong, J.-S., et al. (2005). Aggregated alpha-synuclein activates microglia: a process leading to disease progression in Parkinson's disease. FASEB J. Off. Publ. Fed. Am. Soc. Exp. Biol. *19*, 533–542.

Zhao, J., Brault, J.J., Schild, A., Cao, P., Sandri, M., Schiaffino, S., Lecker, S.H., and Goldberg, A.L. (2007). FoxO3 coordinately activates protein degradation by the autophagic/lysosomal and proteasomal pathways in atrophying muscle cells. Cell Metab. *6*, 472–483.

Zhou, W., and Freed, C.R. (2004). Tyrosine-to-cysteine modification of human alpha-synuclein enhances protein aggregation and cellular toxicity. J. Biol. Chem. *279*, 10128–10135.

Zhou, R.M., Huang, Y.X., Li, X.L., Chen, C., Shi, Q., Wang, G.R., Tian, C., Wang, Z.Y., Jing, Y.Y., Gao, C., et al. (2010a). Molecular interaction of α-synuclein with tubulin influences on the polymerization of microtubule in vitro and structure of microtubule in cells. Mol. Biol. Rep. *37*, 3183–3192.

Zhou, W., Long, C., Reaney, S.H., Di Monte, D.A., Fink, A.L., and Uversky, V.N. (2010b). Methionine oxidation stabilizes non-toxic oligomers of alpha-synuclein through strengthening the auto-inhibitory intra-molecular long-range interactions. Biochim. Biophys. Acta *1802*, 322–330.

Mackenzie. (2001). The pathology of Parkinson's disease. BCMJ, Vol. 43, No. 3, 142-147

Publication and Conferences attended

- Nandakumar, S., Vijayan, B., Kishore, A., Thekkuveettil, A. Autophagy enhancement is rendered ineffective in presence of alpha-synuclein in melanoma cells. J. Cell Commun. Signal. (2017)

- Poster Presentation – "Pharmacological Alterations of Autophagy: implications for alpha synuclein" "All India Cell Biology Conference on Cell Dynamics and Cell Fate" conducted by Indian Society for Cell Biology, December 22-24, 2013.
- Oral Presentation - " Alpha synuclein and Disease pathogenesis". International Conference on Integrative and Comparative Physiology" conducted at Kerala University, December 18-20, 2013.