POLYAMINE MEDIATED NEUROPROTECTION IN α-SYNUCLEIN EXPRESSING CELL AND ANIMAL MODELS

A THESIS PRESENTED BY

BEJOY VIJAYAN

TO

SREE CHITRA TIRUNAL INSTITUTE FOR
MEDICAL SCIENCES AND TECHNOLOGY
Thiruvananthapuram

IN PARTIAL FULFILMENT OF THE REQUIREMENTS
FOR THE AWARD OF THE DEGREE OF

DOCTOR OF PHILOSOPHY

2017

DECLARATION BY THE STUDENT

I, Bejoy Vijayan, hereby certify that I had personally carried out the work depicted in the thesis entitled, **"Polyamine mediated neuroprotection in α-synuclein expressing cell and animal models"**. No part of the thesis has been submitted for the award of any other degree or diploma prior to this date.

Date: Bejoy Vijayan

CERTIFICATE OF THE GUIDE

Dr Asha Kishore, MD, DM

Professor

Comprehensive Care Centre for Movement Disorders,

Department of Neurology

Sree Chitra Tirunal Institute for Medical Sciences and Technology

This is to certify that Bejoy Vijayan of the Department of Neurology of this Institute has fulfilled the requirements prescribed for the PhD degree of the Sree Chitra Tirunal Institute for Medical Sciences and Technology, Trivandrum. The thesis entitled, **"Polyamine mediated neuroprotection in α-synuclein expressing cell and animal models"** was carried out under my direct supervision. No part of the thesis has been submitted for the award of any other degree or diploma prior to this date.

Date: Dr. Asha Kishore

CERTIFICATE OF THE CO-GUIDE

Dr Anoopkumar Thekkuveettil

Scientist G

Division of Molecular Medicine,

Department of Applied Biology

Sree Chitra Tirunal Institute for Medical Sciences and Technology

This is to certify that Bejoy Vijayan of the Department of Neurology of this Institute has fulfilled the requirements prescribed for the Ph.D degree of the Sree Chitra Tirunal Institute for Medical Sciences and Technology, Trivandrum. The thesis entitled, **"Polyamine mediated neuroprotection in α-synuclein expressing cell and animal models"** was carried out under my direct supervision. No part of the thesis has been submitted for the award of any other degree or diploma prior to this date.

Date: Dr. Anoopkumar Thekkuveettil

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Submitted by

BEJOY VIJAYAN

for the degree of

Doctor of Philosophy

of

SREE CHITRA TIRUNAL INSTITUTE FOR
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Thiruvananthapuram

is evaluated and approved by

Dr. Asha Kishore (Guide) (Examiner)

ACKNOWLEDGEMENT

The past few years that I have spent in this Institute was undoubtedly the most demanding in my life. I consider it my privilege to have the name of this prestigious Institute tagged with mine for the rest of my life. My gratitude is due to a good number of people without whom this thesis would have been possible.

My heartfelt gratitude to Dr. Asha Kishore, my guide, who was the reason I joined this Institution. I thank her for her constant motivation, support, and encouragement through these years. Her ability to multi-task and her go-getter attitude are two things that inspired me most.

I have no words to thank Dr. Anoopkumar Thekkuveettil, whose contribution towards conceiving this work in its current form is invaluable. In the course of time spent in his laboratory, I have understood that science just does not mean doing bench-work, but also analysing, sometimes re-analysing and getting to know your results. I am forever grateful to him in making me understand that science can be fun too. I sincerely thank him for all the time he spent with me.

My sincere gratitude is due to my doctoral advisory committee members, Dr. Srinivas G, SCTIMST, who has put in his valuable time and effort in addressing the issues I faced during this work, and Dr. T R Santhosh Kumar, RGCB, who always welcomed me in spite of his busy schedule and offered all the help he could.

I thank the Director, SCTIMST and Head, BMT wing, present and past, for all facilities provided to us. I am grateful to Dr. Santhosh Kumar B, Deputy Registrar and the staff of academic affairs for all the necessary support during this period.

I would like to thank Dr. Renuka R Nair, Head, and all members of DCMC, for allowing me to use their facilities in the initial stages of the work and Mr. Willi Paul of the Central Analytical Facility for the microplate reader. I place on record my sincere gratitude to Dr. Maya Nandakumar, Division of Microbial Technology for providing the nod to use some of their facilities, even at short notice.

I thank Dr. Syam. K, Additional Professor at the Movement Disorders section, for the care and concern he has shown throughout, Gangadhara Sarma, Mary and all the staff at Movement Disorder section who were always a phone call away and helping in all ways they could.

Cheers to my friends Vinod, Padmakrishnan, Linda Philip, Anupama, Saifudeen Ismael, Mereena, Pramod, Durgadas, Pradeepkumar S.S, Sudhin, Keerthi, Amalu, Biby, Parvathy, Francis and a lot many for all the help and company.

I greatly appreciate the goodwill extended by all my lab mates including Mr. Jose Jacob, Suraj, Sarish, Anitha, Cuckoo, Agrima, Pratheesh, Amal, Swathy, Geethu, the helper staff, and many others, which was instrumental in this work. I'll always cherish the moments spent together in this place.

My labmates Vishnuraj, Aswathy and Rasitha deserve a special mention for walking that extra mile to help me in my work with the worms. A very special thanks to Rasitha for all proof reading and language edits in the thesis.

Warm regards to my friend and partner Swapna, for all the time spent together on the countless blots, standardisations, discussions and many more.

And to my loving wife, kids, parents and in-laws; you people continue to surprise me with your love and backing and this thesis is a result of all your sacrifices and prayers. I'll never be able to repay you.

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LIST OF ABBREVIATIONS

α-syn	α-synuclein
AD	Alzheimer's disease
ADEs	anterior deirids
ALP	autophagy-lysosome pathway
AP	autophagosome
APS	ammonium persulfate
ATG7	autophagy-related protein 7
BSA	bovine serum albumin
CEPs	cephalic cells
CHO	Chinese-hamster ovary
CMA	chaperone-mediated autophagy
DA	dopamine
DAT-1	dopamine re-uptake transporter-1
DENSPM	N^1,N^{11}-diethylnorspermine tetrahydrochloride
DIC	differential interference contrast
DMSO	dimethyl sulfoxide
EDTA	ethylenediamine tetra acetic acid
FBS	foetal bovine serum
FITC	fluorescein isothiocyanate
GFP	green fluorescent protein
HRP	horse radish peroxidase
MPTP	1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine
mPTP	mitochondrial permeability transition pores

MSA	multiple system atrophy
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NAC	non-amyloid-beta component
NMDA	N-methyl-D-aspartate receptor
NMR	nuclear magnetic resonance
ODC	ornithine decarboxylase
PBS	phosphate buffered saline
PD	Parkinson's disease
PDEs	posterior deirids
PMSF	phenylmethane sulfonyl fluoride
POA	polyamine oxidase
ROS	reactive oxygen species
SDS	sodium dodecyl sulfate
SNARE	soluble NSF attachment protein **re**ceptor
SAT1	spermidine/spermine N^1-acetyltransferase
TBS	tris-buffered saline
TEMED	tetramethylenediamine
UPR	unfolded protein response
UPS	ubiquitin-proteasome system
VAMP-2	vesicle-associated membrane protein-2
VMAT2	vesicular monoamine transporter-2

SYNOPSIS

Neurodegenerative diseases result from the progressive deterioration of nerve cells. Combined, these diseases affect more than 45 million people worldwide. Even after decades of research, we are yet to pinpoint the actual factors behind neurodegeneration. It is widely accepted that a combination of genetic as well as environmental factors decide the onset and progression of neurodegenerative diseases. Striking similarities across some of these diseases, in onset, progression and pathology is an important cue that the underlying mechanisms are similar. The presence of protein deposits is one such common factor and diseases that are characterized by the presence of protein deposits primarily of α-synuclein (α-syn), are termed α-synucleinopathies. Factors that influence the function and expression of these proteins are interesting targets to look at, both from the point of view of studying their function as well as designing treatment modalities.

Polyamines are polycations that can interact with negatively charged molecules, such as nucleic acids and proteins (including α-syn), with varied functions in the body. Polyamines are associated with a number of processes within the cell, which include transcription and translation. Polyamine levels differ depending on the tissue, and they also show variations, over time, depending on age and other factors. Unlike in normal aging, polyamine levels were found to increase in disease conditions. In Parkinson's disease (PD) patients, increased levels of higher order polyamines were reported in the red blood cells.

Polyamines were shown to interact with purified α-syn in solution and promoted their misfolding and aggregation. The polyamine spermidine enhanced the toxicity of α-syn in a yeast model. In a mouse-model of α-syn expression,

pharmacological agents that increase the levels of higher order polyamines were found to add to the toxicity. On the contrary, the role of polyamines is also thought to be neuroprotective, since they were found to increase autophagy to protect cells from stress - a scenario possible in the case of neurodegenerative diseases. Noxious stimuli may raise the intracellular levels of spermine as a neuroprotective mechanism, as it blocks N-methyl-D-aspartate (NMDA) mediated excitotoxicity. Spermidine rescued motor dysfunction and increased the lifespan in α-syn expressing *Drosophila* and also prevented neurodegeneration in a *Caenorhabditis elegans* model.

It is therefore intriguing to look at the role of polyamines in α-syn expressing models, when they are exposed to a stress or toxic insult. For example, in case of PD, the presence of dopamine in the brain regions most affected by the disease is suggestive of a possible association between dopamine and α-syn, and this interaction has been reported to be detrimental in nature. Moreover, toxicity mediated by metal ions is another factor that is implicated in the pathophysiology of the disease.

Hypothesis

The study hypothesised that polyamines could have a protective role in α-syn expressing models when they are exposed to toxic insults. To substantiate this hypothesis, two models that express α-syn were used. One is the melanoma cell line SK-MEL-28, which endogenously expresses α-syn at detectable levels and the other a *Caenorhabditis elegans* UA44 strain that express human α-syn in its dopamine neurons.

Methods

SK-MEL-28 as well as SH-SY5Y cell lines, cultured in DMEM containing 10% FBS were treated with various concentrations of polyamines (spermine or spermidine) or drugs that modulate polyamine catabolism. The drugs used were Berenil (diminazene aceturate) and a polyamine analogue, N1,N11-diethylnorspermine (DENSPM) that inhibits and activates a critical enzyme in polyamine catabolic pathway, respectively. The changes in α-syn expression and localization pattern were analysed by western blotting and immunocytochemistry. This was followed by exposing the cells to dopamine or Manganese chloride ($MnCl_2$) and its effect on cell viability was assayed in the presence or absence of polyamines. *C. elegans* strains N2 Bristol (wild type), BZ555 *egIs1*[$p_{dat\text{-}1}$*::gfp*] and UA44 *baIn11*[$p_{dat\text{-}1}$*::aSyn,* $p_{dat\text{-}1}$*::gfp*] were maintained on OP50 plates under standard conditions. These strains were synchronized and grown, either on a spermine-rich plate or normal plate (as control), from egg stage and maintained under same conditions till the day of assay. UA44 and BZ555 strains were observed for neurodegeneration using fluorescence microscopy, and functional integrity (avoidance index assay) at their adult stage. For survival assay, UA44 and N2 worms were maintained either on a spermine-rich plate or normal plate (as control) for the whole life-span of the organism. For the neurotoxicity experiments, worms grown on a spermine plate till day 1 were exposed to 100 mM $MnCl_2$ for one hour and immediately observed for cellular changes under fluorescence microscopy. The worms were also treated with polyamines in combination with the biochemical blockers actinomycin D, cycloheximide and chloroquine to evaluate whether

polyamines interacts with molecular pathways involved in transcription, translation or autophagy pathways, respectively.

Major findings

α-syn expression and localization patterns are well regulated in the SK-MEL-28 cells.

The pattern of α-syn expression or localization in SK-MEL-28 cells, when exposed to spermine, berenil or DENSPM, did not change in a significant manner as evident from western blotting as well as immunocytochemistry results, indicating that these levels are well regulated and do not alter with polyamine levels. Since the autophagy-lysosomal pathway is one of the pathways that is involved in maintaining α-syn levels and polyamines are capable of modulating autophagy, markers of autophagic flux were analysed for possible interactions. Interestingly, spermine did show inhibitory effect on autophagy in western blot analysis and a marginal increase in the cytoplasmic levels of α-syn. However, berenil or DENSPM treatment did not alter autophagy pathway significantly in SK-MEL-28.

SK-MEL-28 cells are sensitive to $MnCl_2$ but not dopamine and polyamines protect cells from Mn^{2+} mediated cytotoxicity

In an attempt to induce toxicity, SK-MEL-28 cells, which express α-syn, were exposed to media containing dopamine at a high concentration (1mM) for four hours, followed by normal growth media for 20 hours. Surprisingly, these cells survived this toxic insult; the trypan blue dye exclusion test for viability showed more than 80% cell survival, similar to the controls. In the control cell line SH-

SY5Y, which does not express α-syn at detectable levels, the four hour dopamine exposure itself caused about 10% loss in viability compared to control and no surviving cells were detected after 24 hours. These results indicate that presence of α-syn is indeed essential for cell survival in a dopamine containing environment.

Since high dopamine levels were well tolerated by SK-MEL-28 cells, I tested the effects of manganese ions (Mn^{2+}) on cell survival. These cells were exposed to $MnCl_2$ at various concentrations and at 300 and 600 μM concentrations of manganese; the cell viability was significantly reduced. In presence of spermine or spermidine, more than 70% of the cells exposed to $MnCl_2$, were viable, which was significantly higher as compared to the cells exposed to $MnCl_2$ alone (<40%). Moreover, treatment with berenil, which increases the polyamine content within the cells, also showed significantly higher cell viability (>70%). However, in SH-SY5Y cells, exogenous addition of polyamines itself turned out to be toxic indicating the possibility of interactions between polyamines and α-syn under stress conditions. All these results together strongly suggest that that polyamines afford protection to cells expressing α-syn under manganese induced toxicity.

Spermine-rich environment rescues the *C. elegans* model (UA44), expressing human α-syn, from neurodegeneration

C. elegans strain UA44, which express human α-syn tagged with GFP under the control of dat-1 promoter in its dopaminergic neurons, was observed for neurodegeneration, as the worm ages. The number of worms that exhibited neurodegeneration in UA44 was significantly higher, compared to BZ555 control strains expressing GFP under dat-1 promoter. Kaplan-Meier survival analysis

showed that the UA44 strain had a significant reduction in the survival rate as compared to the wild type N2 Bristol strain. These results confirmed that there is age related neurodegeneration and low survival in worms expressing human α-syn.

To analyse the neuroprotective effect of spermine, UA44 strains were grown in spermine (0.2mM) containing plates from egg stage to adult. These adults were observed under fluorescence microscopy for the degree of degeneration of dopaminergic neurons. The number of worms that exhibited neurodegeneration significantly reduced when they were grown under these conditions. These results reiterated the role of polyamines in neuroprotection in an organism as seen in SK-MEL-28 cell model. These spermine treated nematodes also displayed better functional dopaminergic neuronal circuits compared to controls when a functional assay (avoidance index assay) was performed.

Polyamines rescued UA44 worms from manganese induced neurodegeneration

Manganese induced neurodegeneration in *C. elegans* has been reported, though controversial. Hence, we standardized a new protocol in the lab with highly reproducible pattern of neurodegeneration of dopaminergic neurons when treated with $MnCl_2$. This protocol could induce neurodegeneration in dopaminergic neurons within 30 minutes in more than 50% of these worms. Since spermine protected SK-MEL-28 cells from Mn^{2+} induced cytotoxicity, a similar test was carried out in *C. elegans*. Mn^{2+} induced neurodegeneration was significantly ameliorated in spermine or spermidine-fed worms, confirming the protective role of polyamines at organismal level.

Polyamine mediated protection is regulated at the translational level and is autophagy-dependent

One of the major questions is how polyamines protected the cells from Mn^{2+} or synuclein induced toxicity. The ability of spermine to bind to nucleic acids and proteins prompted us to employ specific blockers of transcription, translation and autophagy to look at its mechanism of action. The protection afforded by spermine was reduced significantly with translational blocker cycloheximide (0.8 mg/ml) but not with the transcriptional blocker actinomycin D (200 µg/ml). Blocking autophagy with chloroquine (20 mM) also had a negative effect on the neuroprotection. These results are indicative that spermine mediated neuroprotection is regulated at the translational level. It is plausible that autophagy could be one of the pathways that offer the neuroprotective effect.

Significance of the study

This study uniquely combined two models with α-synuclein expression, one an endogenously expressed cell model and the other one, an animal model expressing human a-syn in its dopaminergic neurons. In both the cases, polyamines had neuroprotective roles and were able to counter the toxicity associated with an external stress factor (Mn^{2+}) as well as synuclein-induced neurodegeneration upon aging in the nematode model. The study also gives insights to the mechanism of cytoprotective action of polyamines. It is significant to note that upon spermine treatment there is a translational regulation of a set of genes in *C. elegans*, which may include autophagy-related genes, resulting in significantly low levels of

neurodegeneration. Besides these new insights, this work also gives a predictive neurodegenerative model system. *C. elegans* preconditioned with Mn^{2+}, ions provides a new platform to study neurodegenerative processes.

I. INTRODUCTION

Neurodegenerative diseases occur due to the selective loss of neurons in specific areas of the brain. As per the estimates of the UN World Health Organization (WHO), about one in six of the world's population suffer from neurological disorders and neurodegenerative diseases such as Alzheimer's disease (AD) and Parkinson's disease (PD), accounting for a good number (WHO, 2006). The WHO predicts that by 2040, neurodegenerative diseases will replace cancer at the second position to be the leading cause of death after cardiovascular diseases (Gammon, 2014). The economic and medical burden that such disorders impart on both developed and developing countries is huge.

Neurodegenerative disorders present a complex challenge with their unknown aetiology, lack of detailed characterization of disease onset and progression, and so on. Efforts to understand the fundamental basis of these diseases and develop therapeutic approaches are ongoing (Kalia and Lang, 2016). Most of these disorders are marked by the deposition of specific proteins or its altered forms, and are classified based on the protein such as amyloidopathies, synucleinopathies, and tauopathies. Parkinson's disease, the most studied synucleinopathy, affects about 10 million people worldwide (Hirsch et al., 2016). Although genetic alterations of α-synuclein (α-syn) is behind a few cases of hereditary forms of PD, majority of the cases of PD are sporadic in nature (Trinh and Farrer, 2013). The protein, α-syn, has intrigued the researchers with its natively unfolded structure, its function within the neurons, and its ability to propagate from one cell to another (Lashuel et al., 2013). A large body of data now shows the interactions α-syn has with other proteins, membranes, molecules, and metals.

α-syn is thought to be a pre-synaptic protein with roles in synaptic transmission, regulating the release of dopamine, and also in the N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE)-complex assembly and maintenance (Butler et al., 2015; Chandra et al., 2005; Lautenschläger et al.,; Yavich et al., 2004). However, overexpressing α-syn led to neuronal loss, specifically so in the dopaminergic neurons, in different models (Auluck et al., 2002; Masliah et al., 2000). This observation resonated well with the previous finding that PD patients with multiplication mutations of the α-syn gene (*SNCA*) produce higher levels of the protein and display an enhanced pathology (Singleton et al., 2003). This effect of dose dependence was reported in many other models, even leading to a block in the endoplasmic reticulum to Golgi trafficking at higher expression levels (Cooper et al., 2006; Outeiro and Lindquist, 2003). α-syn also affects the mitochondria leading to oxidative stress and bioenergetic failure (Chu et al., 2014; Hsu et al., 2000).

α-syn exists natively in an unfolded state, which attain different conformations depending on its interactions with many proteins, membranes, and metal ions (Uversky, 2007). These interactions either have a functional reason or promote the pathogenesis. The specific vulnerability of dopaminergic neurons in PD is attributed to its interactions with the neurotransmitter dopamine and its involvement in the dopamine biosynthesis and release (Galvin, 2006; Rekas et al., 2010). In presence of other interacting partners such as metals, these effects accentuate (Bisaglia et al., 2009). Manganese (Mn) is one such metal ion that can cause neurodegeneration by disrupting mitochondrial permeability, ROS production and finally cell death (Aschner et al., 2009; Chen et al., 2015).

Therapeutic approaches to counter neurodegeneration in α-synucleinopathies mostly centre on the concept that modified forms of α-syn are the major culprit behind the pathogenesis. Owing to the dosage related toxicity of α-syn, few studies attempted to reduce the α-syn levels, which was counter-productive in many models (Gorbatyuk et al., 2010; Khodr et al., 2014). Another approach focused on accelerating the clearance of α-syn by involving the autophagy lysosomal pathway (Decressac et al., 2013; Spencer et al., 2009). A number of autophagy inducers have been tested in this regard including rapamycin and trehalose (Malagelada et al., 2010; Morselli et al., 2011; Sarkar et al., 2007). Preventing or slowing down the aggregation of α-syn was also looked at as a therapeutic approach (Auluck et al., 2002; Cheruvara et al., 2015). Immunotherapy, using antibodies targeting α-syn has already found takers in the pharmaceutical industry, though it remains one of the least explored options (Masliah et al., 2005; Masliah et al., 2011; Sinha, 2014).

One of the interacting partners of α-syn is polyamines, polycations that interact with negatively charged molecules and alter the aggregation kinetics in solution (Antony et al., 2003). A rate-limiting enzyme in polyamine catabolism, spermidine/spermine N^1-acetyltransferase (SAT1), was found to be downregulated in PD patients, leading to elevated levels of the polyamines, spermine and spermidine, within the affected regions (Lewandowski et al., 2010). However, it was not discerned whether this was the cause of pathology or merely an effect. On the contrary, many studies have reported beneficial effects of polyamines. Mutations in the gene *ATP13A2*, thought to be involved in the uptake of polyamines, are regarded as a risk factor in causing neurodegeneration (Hera et al., 2013; Ramirez et al.,

2006). Spermidine also acts as an autophagy inducer that can extend the life span and better the motor function in α-syn expressing flies (Büttner et al., 2014).

I.1. Hypothesis

Polyamines are endogenous to the cells and are thought to play important regulatory roles in many pathways in the cell, including transcription, translation, and signalling. In solution, polyamines tend to accelerate the aggregation of α-syn, which is not surprising due to the charged nature of these molecules. However, in vivo reports are nothing but contradictory. A detrimental role for the ubiquitously expressed polyamines is highly unlikely, especially with neurodegenerative diseases, as polyamines were demonstrated to slow down aging in an autophagy dependent manner (Eisenberg et al., 2009; Gupta et al., 2013). In this context, the study hypothesised that polyamines could have a protective effect in α-syn expressing models when exposed to toxic insults.

I.2. Objectives of the study

This study was designed as follows. The effects of polyamines were probed with two models of α-syn expression. One, a cell model that endogenously expresses α-syn at detectable levels and the other, an animal model that expresses human α-syn tagged to green fluorescent protein in its dopamine neurons. In presence of polyamines, the cells and animals were exposed to a stress inducer (manganese) and the effects were discerned. In the cells, the expression pattern of α-syn and the toxicity in presence of polyamines and manganese were determined. In the animal model, the pattern of neurodegeneration and the underlying mechanisms of protection were looked at.

II. REVIEW OF LITERATURE

Increasing life expectancy in the population brings with it the risk of age-associated disorders with a trend towards increasing prevalence of neurological disorders, including dementias and movement disorders. Neurodegenerative diseases present a complex problem due to the unknown aetiology, and interaction of many genes and the environment. The presence of protein deposits is a hallmark of many of these disorders due to which they are also known as conformational disorders or proteinopathies.

List of neuronal proteinopathies, associated protein and related genes

Disease	*Associated Protein*	*Related Genes*
Alzheimer's disease, Cerebral β-amyloid angiopathy	Amyloid-β, Tau Protein, ApoE4	APP, Presenilins 1 and 2, APOEε4, SORL1 and CLU
Amyotrophic lateral sclerosis	Superoxide dismutase 1	SOD1, VCP and FUS
Creutzfeldt Jakob Disease	Prion protein	PRNP
Dentatorubral-pallidoluysian atrophy (DRPLA)	Atrophin 1 (polyglutamine expansion)	ATN
familial amyloidotic neuropathies, Senile systemic amyloidosis	Transthyretin	FAP-I and FAP-II
Familial amyloid polyneuropathy III	Apolipoprotein A-1	FAP-III
Familial british dementia	Abri	ITM2B / BRI2
Familial encephalopathy with neuroserpin inclusion bodies	Neuroserpin	SERPINI1
Frontotemporal Dementia	Tau Protein	MAPT and GRN
Frontotemporal lobar degeneration	TDP-43	MAPT, PGRN and CHMP2B
Hereditary cerebral hemorrhage with amyloidosis	Cystatin C	CST3
Huntington's disease	Huntingtin (polyglutamine expansion)	IT15
Parkinson's disease and other synucleinopathies (multiple)	α-Synuclein	SNCA, PRKN, PINK, LRRK2, DJ-1 and ATP13A2
Spinocerebellar ataxia 17	TATA box-binding protein (polyglutamine expansion)	KCNC3
Spongiform encephalopathies	Prion protein	PRNP
Ubiquitin positive, tau negative and α–synuclein negative Frontotemporal dementia	TAR DNA-binding protein 43	TARBP

Table 1: A listing of the proteinopathies, associated proteins and genes (Chowhan et al., 2013).

II.1. Alpha-Synucleinopathies

Alpha-synucleinopathies are the group of diseases characterized by the presence of deposits of the protein α-syn, in various regions of the brain. Identification of mutations in the α-syn gene was linked to development of Parkinson's disease (PD) (Polymeropoulos et al., 1997). α-syn was later identified in Lewy bodies, a pathologic hallmark of PD, from post-mortem brain tissue of PD patients (Spillantini et al., 1997). Following these reports, several other mutations have been identified and α-syn became one of the most studied proteins (Recasens and Dehay, 2014). Duplication and triplication mutations in the α-syn gene (*SNCA*) in PD patients reported interesting correlations between dose/protein levels and severity, which was replicated in many studies (Eriksen et al., 2005; Singleton et al., 2003). Other than PD, α-syn is presently implicated in Alzheimer's, multiple system atrophy, dementia and so on (Kim et al., 2014). It is interesting to note that this protein has been the centre of debate with regard to its structure, function and its role in the disease.

II.1.1 α-syn – structure and function

α-syn is a small acidic protein of 140 amino acids (19 kDa), abundantly expressed in neurons. The amino acids 1-60 constitutes the N-terminal domain which include the consensus sequence KTKEGV, followed by the hydrophobic non-amyloid-beta component (NAC) domain made up of amino acids 61-95. This is followed by the highly negatively charged C-terminal domain (96-140). α-syn is thought to be devoid of a defined conformation and falls into the broad category of intrinsically disordered proteins. However, it has the capability to adopt specific

structures upon interactions with other proteins or membranes (Malkus et al., 2009). It attains an alpha-helical structure upon binding to phospholipid vesicles. The structure changes from random coil to pleated sheet at the NAC domain when aggregation occurs. The point mutations associated with pathology are in the N-terminal region and are shown to increase the propensity of aggregation (Beyer, 2006).

In spite of two decades of research and pooled evidence of involvement of α-syn in various pathways, the exact function of α-syn is unknown. The earliest attributed and widely believed function is that α-syn acts as a pre-synaptic protein and has role in regulating the vesicular transport for synaptic transmission (Iwai et al., 1995; Lautenschläger et al.,; Lykkebo and Jensen, 2002). α-syn was found to be localized primarily to the presynaptic terminals of mature neurons in the adult mammalian brain (Hsu et al., 1998; Murphy et al., 2000) and also in association with the synaptic vesicles (Irizarry et al., 1996; Zaltieri et al., 2015). Regulating dopamine release is one of the roles α-syn supposedly plays at the pre-synapse (Abeliovich et al., 2000; Yavich et al., 2004). α-syn was shown to inhibit multiple steps, including inhibiting the tyrosine hydroxylase expression, in the dopamine biosynthesis pathway (Alerte et al., 2008; Tehranian et al., 2006; Yu et al., 2004). It interacts with the dopamine transporter at the plasma membrane and increase the dopamine efflux (Butler et al., 2015). Studies on mice models showed that endogenous α-syn is associated with maintaining the number of dopaminergic cells in the substantia nigra pars compacta, indicating its role in dopaminergic neuron development (Garcia-Reitboeck et al., 2013). It can also interact with the serotonin

transporter via direct interactions with its NAC domain (Wersinger et al., 2006). These interactions that α-syn seem to have with the dopaminergic system are a pointer to the vulnerability that dopaminergic neurons face in α-synucleionopathies.

Transgenic expression of α-syn prevented neurodegeneration, deterioration of neuromuscular junctions, and motor impairment exhibited by the mice knockout model of cysteine-string protein-α (CSPα), another synaptic vesicle protein. This underlined its importance in N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE)-complex assembly and maintenance (Chandra et al., 2005). This protective effect did not require direct interactions between both proteins, though α-syn needed to be present in a phospholipid bound conformation. α-syn also takes part in SNARE-complex maintenance by binding to the vesicle-associated membrane protein-2 (VAMP-2) via its C-terminus while simultaneously binding to phospholipids via its N-terminus (Burré et al., 2010). α-syn senses lipid packing defects and binds to the membrane upon which it folds into an amphipathic α-helical structure and remodels the membranes (Chen et al., 2013; Jiang et al., 2013; Ouberai et al., 2013). α-syn binds specifically to the presynaptic plasma membrane where SNARE complex assembles and also attains a functional multimeric form in this process (Burré et al., 2014).

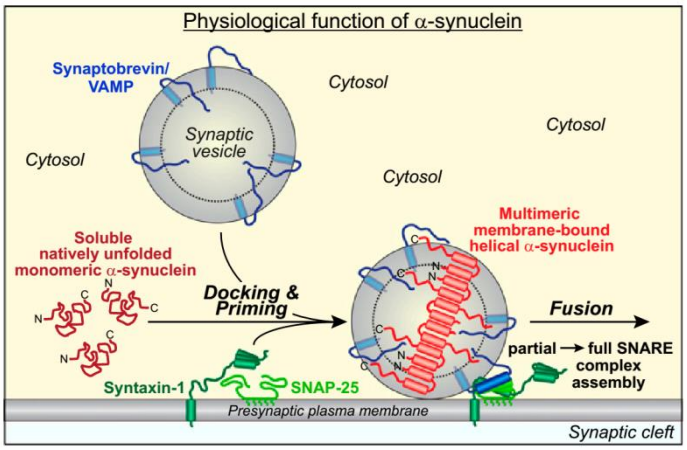


Figure 1: α-syn and its role in SNARE complex assembly. α-syn binds to synaptic vesicle, undergo conformational change and multimerizes to promote SNARE complex assembly (Burré et al., 2014).

II.1.2. α-syn mediated toxicity

The expression of α-syn is toxic as demonstrated in multiple models. Flies as well as rat models expressing wild-type or mutant forms of α-syn in dopaminergic neurons exhibit marked neuronal loss along with inclusion-body formation (Auluck et al., 2002; Bianco et al., 2002; Masliah et al., 2000). Increased expression of α-syn is regarded as one of the reasons behind its pathogenicity. Increased levels of proteins and corresponding accumulation have been reported in patients with multiplications of *SNCA* (Chartier-Harlin et al., 2004; Singleton et al., 2003). The A53T and A30P mutations of the gene are also implicated in changing the propensity to aggregate (Conway et al., 1998). Moreover, induced pluripotent stem cells generated from a PD patient with the triplication mutation showed reduced capacity to differentiate, and produced higher amounts of α-syn protein upon differentiation to dopaminergic neurons (Devine et al., 2011; Oliveira et al., 2015).

Wild type and mutants of α-syn under the control of an inducible promoter was used to study this dose-dependence, in yeast. Although it did not alter the growth or viability of cells at low levels, there was an increase in the number of secretory vesicles. Increasing the dosage further, lead to stalled vesicles as well as accumulation of lipid droplets accompanied by cytotoxicity (Outeiro and Lindquist, 2003). This increased expression was also accompanied by a block in the endoplasmic reticulum (ER) to Golgi trafficking leading to ER stress (Cooper et al., 2006), which may affect the dopaminergic neurons greatly (Gitler et al., 2008). This effect was also observed in other models of α-syn expression, such as *C. elegans, Drosophila,* and mammalian cell lines (Cooper et al., 2006; Thayanidhi et al., 2010). Normal trafficking was restored only by co-expressing the proteins that promote vesicle fusion. Consistent with its role in the synapse, α-syn expression inhibited the activity of vesicular monoamine transporter-2 (VMAT2), involved in the packing of monoamines to vesicles, leading to abnormal elevation of cytosolic dopamine and ROS generation (Guo et al., 2008).

Mitochondria play a crucial role in mediating α-syn toxicity. The idea of this association stemmed from the identification of two toxins capable of inducing parkinsonism, 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP), a complex I inhibitor, and rotenone, a pesticide (Betarbet et al., 2000; Mullin and Schapira, 2013). Expressing wild type α-syn countered this toxin-induced increase in ROS levels as compared to its mutant forms in SH-SY5Y cells (Choong and Say, 2011). On the contrary, α-syn expression has presented with mitochondrial defects and oxidative stress in other cell models (Hsu et al., 2000). Mice expressing human

A53T α-syn mutant displayed reduced complex IV activity, mitochondrial DNA damage and degenerations, probably through the activation of macroautophagy (Choubey et al., 2011; Martin et al., 2006). α-syn facilitates Ca^{2+} transfer from endoplasmic reticulum to mitochondria and regulates mitochondrial homeostasis, which becomes disrupted only on conditions of protein aggregation, supporting the requirement for regulated expression (Calì et al., 2012). In line with this, the presence of α-syn was specific to the mitochondria-associated endoplasmic reticulum membranes, and the pathogenic mutations disrupted this association (Guardia-Laguarta et al., 2014). α-syn also interact with the voltage-dependent anion channel protein to promote opening of mitochondrial permeability transition pores (mPTP) leading to an inhibition of ATP synthesis and bioenergetic failure (Chu et al., 2014; Lu et al., 2013). Although the exact sequence of events needs to be determined, translocation of Endonuclease G from mitochondria to nucleus seems to be one of the mechanisms behind α-syn-mediated cell death (Büttner et al., 2013).

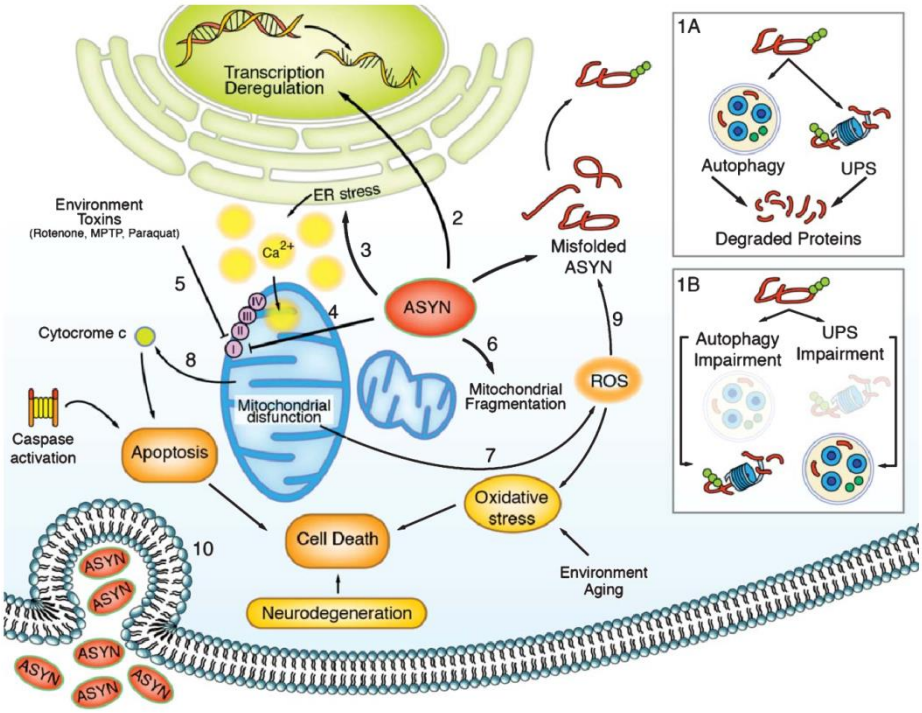


Figure 2: Summary of α-syn pathogenic mechanisms. The impairment of the protein folding and degradation systems (1A,1B), transcriptional de-regulation (2), altered ER homeostasis (3), mitochondrial dysfunction (4,5,6), oxidative stress (7), apoptosis (8) and cell to cell transmission (9,10), together lead to neurodegeneration (Wales et al., 2013).

II.1.3. α-syn misfolding and aggregation

The central NAC domain of α-syn is the aggregation-prone region in this protein (Bisaglia et al., 2006). Interactions of the C-terminus with the NAC region and N-terminus stabilize the protein to a great extent and prevent aggregation (Bertoncini et al., 2005). The N-terminal region of α-syn is thought to bind to the lipid membrane while the C-terminus region is exposed, and this helps in recruiting other proteins to the membrane (Eliezer et al., 2001). Disturbances to these interactions by way of mutations, environmental conditions, post-translational modifications, interactions with other molecules and such lead to misfolding and

subsequent aggregation of the protein. Lewy bodies, the major pathophysiological feature of PD, were primarily found to contain the fibrillar form of α-syn (Arima et al., 1998). However it is the oligomeric and pre-fibrillar forms that are considered pathogenic (Caughey and Lansbury, 2003; Danzer et al., 2007; Winner et al., 2011).

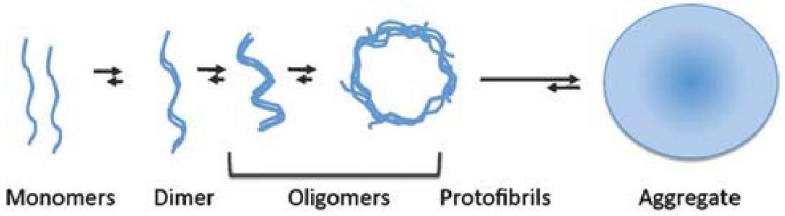


Figure 3: Schematic representation of α-syn aggregation process (Marques and Outeiro, 2012).

Owing to its intrinsically disordered nature and propensity to aggregate, folding and misfolding patterns of α-syn were studied in detail. The soluble monomers of α-syn form intermediate oligomers and further modify to fibrillary aggregates, in a nucleation-dependent manner (Wood et al., 1999). Karpinar et al. characterized different species of these pre-fibrillar species in an attempt to assess their toxicity (Karpinar et al., 2009). Different mutant species of α-syn promoted formation of soluble oligomers, which led to toxicity in various models (Lázaro et al., 2014). The hypothetical cellular effects of these oligomers are depicted in Figure 4.

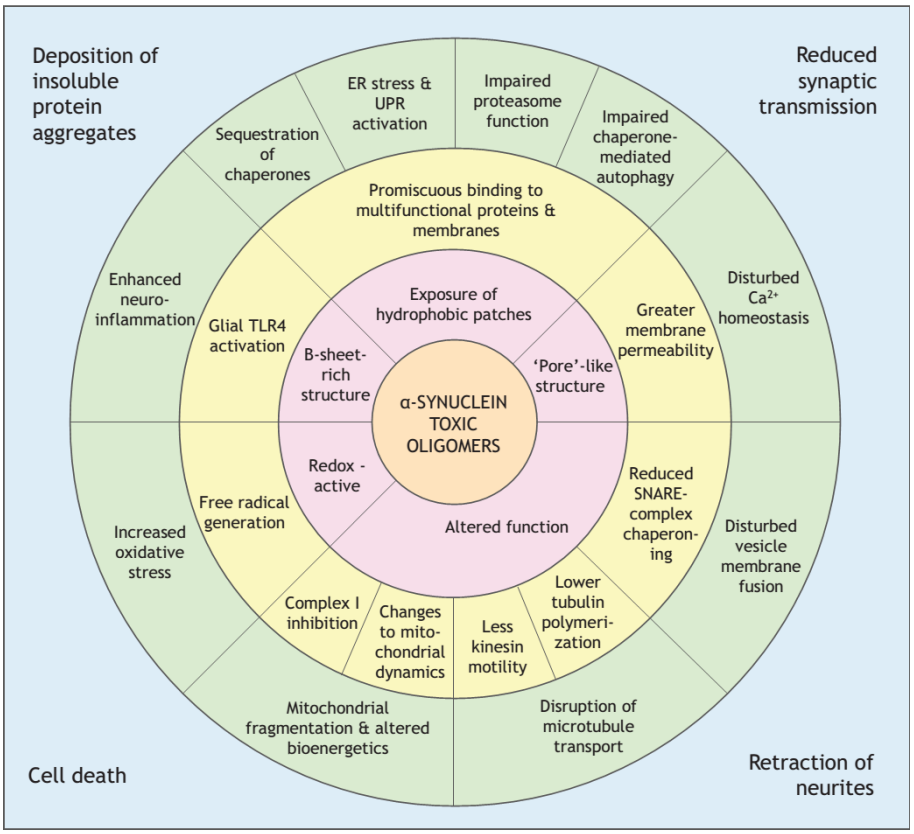


Figure 4: Cellular effects of α-syn oligomers. Pink shell shows properties of the oligomers, yellow shell shows molecular effects of oligomers, green shell shows the cellular systems disrupted by toxic oligomers and possible pathological outcomes listed outside (Roberts and Brown, 2015).

Several post-translational modifications that promote the aggregation of proteins are also implicated in the pathogenesis. Phosphorylation at two sites, serine 129 and 87 are reported in α-syn (Okochi et al., 2000). Serine-129 phosphorylated α-syn deposits are found to be predominant in most neurodegenerative disorders (Fujiwara et al., 2002). Studies using mutants incapable of phosphorylation at these sites have found that serine-129 phosphorylation by casein kinases enhanced the aggregation while, serine-87 phosphorylation prevented the same (Paleologou et al., 2010; Smith et al., 2005). Nitration of tyrosine residues within α-syn caused dopaminergic cell death and reduced motor locomotion in rats, probably due to its

propensity to form oligomers (Hodara et al., 2004; Yu et al., 2010). Oxidation of α-syn, primarily in methionine residues, resisted oligomer formation (Uversky et al., 2002). However, in vitro studies show that modified dopamine can form adducts with α-syn oxidised at its methionine residues, thereby stabilizing the oligomers (Rekas et al., 2010). Although α-syn is seen ubiquitinated at the lysine residues in the aggregates observed in dementia with Lewy bodies and MSA, it was not found to be promoting the formation of inclusions (Sampathu et al., 2003). C-terminally truncated α-syn found in the aggregates, also enhanced the aggregation of the normal protein (Li et al., 2005).

α-syn has been shown to have many interacting partners, notably metals that influence its properties and sometimes cause aggregation and toxicity. Occupational exposure to metals have been associated with various neurodegenerative diseases, particularly so in PD (Gorell et al., 1997). Direct binding of α-syn to various metals have been demonstrated in vitro, that accelerated the rate of α-syn fibril formation (Khan et al., 2005; Uversky et al., 2001). Increased intracytoplasmic Ca^{2+} in neurons may promote the formation of annular α-syn (30–50 nm) by binding directly to it and possibly altering the membrane-binding properties (Pountney et al., 2005; Tamamizu-Kato et al., 2006). Copper and iron binding to α-syn induced fibril formation, though the fibril morphologies were not the same (Bharathi et al., 2007). However, copper binding to α-syn is essential for its activity as a ferrireductase (Davies et al., 2011). In line with this observation, increased accumulation of iron and decreased levels of copper has been reported from brain tissue of Parkinson's disease patients (Mochizuki and Yasuda, 2012; Montes et al., 2014).

Manganese is another metal strongly implicated in the aetiology of PD, acute exposure of which is associated with the development of manganism. This syndrome mainly affects the globus pallidus, with symptoms that closely mimic PD. However, chronic exposure to low levels may cause neurodegeneration that extend to other regions of basal ganglia (Lucchini et al., 2009). Exposure to Mn can cause degeneration of dopaminergic neurons and alterations in the dopaminergic function (Chen et al., 2006). Chronic exposure of α-syn transgenic mice to manganese affected the dopamine turnover (Peneder et al., 2011). Knockdown of a transporter orthologue of *ATP13A2* that prevents manganese toxicity in α-syn expressing models, increased α-syn misfolding and toxicity (Gitler et al., 2009). α-syn overexpressing cells are relatively more susceptible to manganese toxicity, probably due to an enhanced oxidative stress mediated by the NF–κB pathway (Pifl et al., 2004; Prabhakaran et al., 2011). Manganese enhances ROS formation due to its involvement in different reactions within the cell. Manganese autooxidises dopamine, which forms intermediates, including quinone radicals and dopaminochrome, that may cause oxidative stress (Segura-Aguilar and Lind, 1989). Lead, zinc and magnesium are also implicated in promoting aggregation of α-syn (Santner and Uversky, 2010).

II.1.4. α-syn – secretion, uptake and prion-like behaviour

α-syn was initially regarded as purely intracellular in nature and so the observations that cells are able to secrete α-syn into the extracellular space and α-syn to be present in detectable levels in the cerebrospinal fluid and plasma, was surprising (Borghi et al., 2000; El-Agnaf et al., 2003; Tokuda et al., 2006). Normal

as well as aggregated forms of α-syn are secreted by exocytosis and are elevated under stress conditions (Lee et al., 2005).

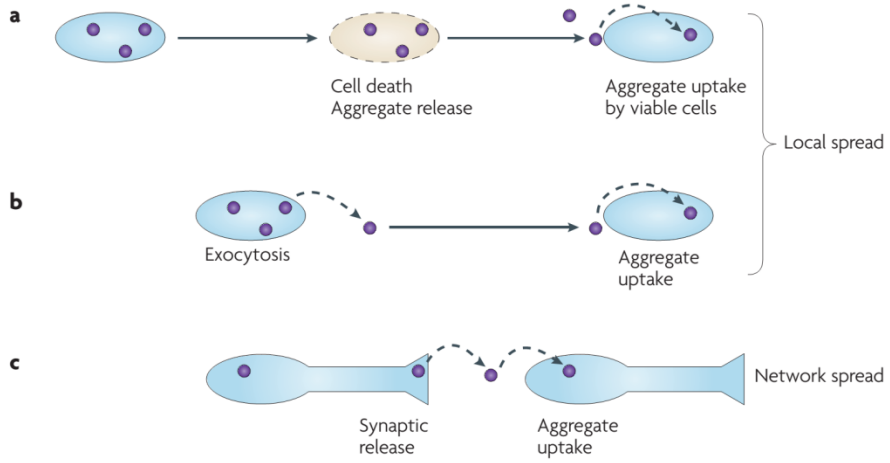


Figure 5: Mechanisms of α-syn propagation. Local spread - protein aggregates from one cell are released to extracellular space upon death (a) or by exocytosis (b) that are taken up by neighbouring viable cells. Network spread - protein aggregates released due to degenerated synapses or by exocytosis cross the synapse (Frost and Diamond, 2010).

A pivotal observation came when transplanted nigral neurons from the brain of a PD patient showed Lewy-body like inclusions, positive for α-syn, fourteen years after the surgery pointing to a pathological role for the secreted α-syn (Kordower et al., 2008). Following this, α-syn was shown to transfer itself from one cell to another in various models, by endocytosis, and seeded the aggregation of intracellular α-syn in recipient cells, displaying a prion-like behaviour (Angot et al., 2010; Desplats et al., 2009; Frost and Diamond, 2010; Hansen et al., 2011). Further, the pathological function of α-syn fibrils was cemented when introduction of synthetic preformed fibrils into healthy mice resulted in a Lewy body/neurite-like pathology (Luk et al., 2012).

II.1.5. α-syn and the autophagy pathway

Protein clearance mechanisms help in maintaining α-syn levels, dysfunction of which leads to aggregation. Both the autophagy-lysosome pathway (ALP) and the ubiquitin-proteasome system (UPS) are thought to be involved in the accumulation aggregation of α-syn.

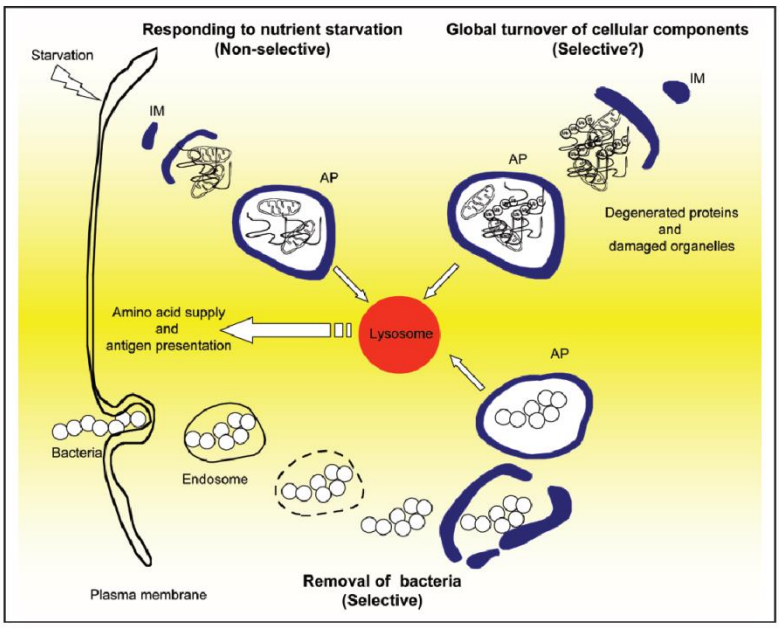


Figure 6: Schematic representation of the physiological functions of autophagy. IM, isolation membrane; AP, autophagosome; Ub, ubiquitin (Komatsu et al., 2006)

Autophagy-lysosome pathway is the bulk degradation pathway, which sequesters the cytoplasmic constituents, including rogue proteins, into a double-membraned vesicle termed autophagosomes. These autophagosomes then fuse with lysosomes and their contents get degraded within (Klionsky and Emr, 2000; Mizushima et al., 2002). Autophagy is a critical process to maintain homeostasis in quiescent cells, such as neurons, where a well-regulated protein quality control

mechanism is essential. Though multiple forms of autophagy exists, which include macroautophagy, chaperone-mediated autophagy (CMA), and microautophagy, only macroautophagy (referred to as 'autophagy') and CMA are linked to α-synucleinopathies.

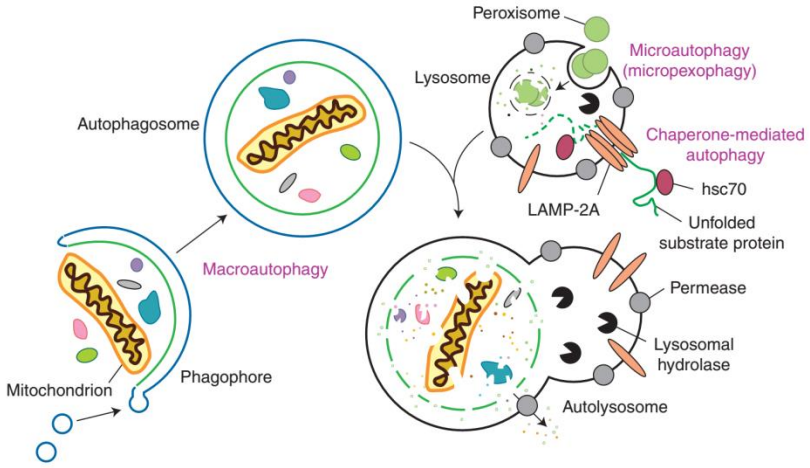


Figure 7: Three main types of autophagy. Chaperone-mediated autophagy (CMA) recognizes a specific motif on the substrate, which interact with LAMP-2A oligomer to translocate across the lysosomal membrane. Microautophagy includes selective degradation of peroxisomes (micropexophagy) and mitochondria (micromitophagy). Macroautophagy wraps around the cargo forming a phagophore, which then becomes an autophagosome followed by lysosomal fusion finally leading to the degradation of the cargo (Lynch-Day et al., 2012).

The link between the autophagy and neurodegeneration was demonstrated when aggregate-prone proteins accumulated upon inhibiting the ALP (Ravikumar et al., 2002). Mice deficient in autophagy in neural cells showed neurodegeneration, along with inclusion body formation (Hara et al., 2006). Contrary to the earlier reports, where UPS was described as the major pathway in the degradation of α-syn, a good number of studies showed that aggregates formed by α-syn are a substrate for degradation by the lysosomal pathway (Ebrahimi-Fakhari et al., 2011; Lee et al.,

2004; Vogiatzi et al., 2008; Webb et al., 2003). Depending on the type of species formed by α-syn, both macroautophagy as well as CMA are shown to be involved in the clearance (Cuervo et al., 2004; Klucken et al., 2012; Lee et al., 2004). Consequently, various autophagy inducers have been demonstrated, that ameliorated α-synucleinopathies (Xilouri et al., 2016). However, the use of such drugs or methods needs to be validated in the right models before application, since α-syn is also known to regulate the process of autophagy itself (Winslow and Rubinsztein, 2011).

II.2. Polyamines

Polyamines are small aliphatic polycations present in almost all organisms, varying in their type and concentration. The most common polyamines are spermine, spermidine, putrescine, and cadaverine. The levels of polyamines vary between tissues and the intracellular levels are tightly regulated at the levels of biosynthesis, catabolism, and/or transport. Polyamines are either absorbed from food or synthesized by the cell. The importance of polyamines in maintaining the normal structure and function of an organism is evident from the knockout models that do not survive beyond embryonic stages of life (Pegg, 2009).

II.2.1. Polyamine synthesis and catabolism

The amino acids arginine, ornithine, and methionine act as precursors in polyamine synthesis. Arginine is converted to ornithine, which is then decarboxylated to produce putrescine. A second pathway is the conversion of L-methionine to S-adenosyl-L-methionine (AdoMet) to decarboxylated AdoMet that acts as an aminopropyl donor for the synthesis of spermidine and/or spermine.

Ornithine decarboxylase (ODC) is a critical enzyme in polyamine synthesis and is highly regulated (Pegg, 2009).

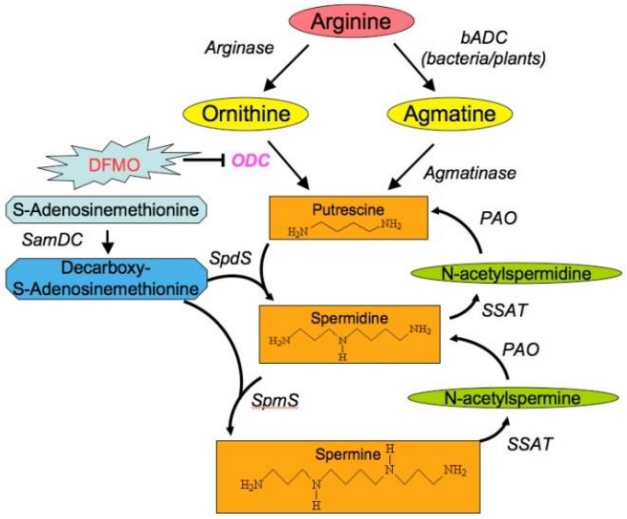


Figure 8: Polyamine metabolism. ODC –ornithine decarboxylase, SpdS – spermidine synthase, SpmS – spermine synthase, SSAT – spermidine/spermine N^1-acetyltransferase, POA – polyamine oxidase. Figure adapted from http://www.weizmann.ac.il/molgen/Kahana/node/8.

Higher order polyamines, spermine and spermidine, are catabolized by the action of a single enzyme, spermidine/spermine N^1-acetyltransferase (SAT) which acetylates them in the cytosol. Polyamine oxidase converts these to putrescine in the peroxisomes.

Polyamine transport is another means of regulating their levels. A good number of transporters have been identified in bacteria and yeast, but not in mammals. Endocytosis is thought to be the preferred mechanism of transport in mammals. The fact that these mechanisms (synthesis, catabolism, and/or transport)

are tightly controlled at all levels across species is indicative of the critical roles polyamines play within the cells.

II.2.2. Functions of polyamines

Polyamines have myriad roles within the cell (Figure 9). The importance of polyamines in maintaining the normal physiology is evident from the fact that knockouts of essential genes in this pathway are lethal or present with extreme abnormalities such as in the case of gyro mice (Wang et al., 2004). They can bind to nucleic acids, mostly RNA, and proteins and thus influence gene expression. Polyamines binding to nucleic acids influence the stabilization of chromatin and modulate rate of transcription.

Due to its higher propensity to bind to RNA, it is assumed to play a larger role in the translation process (Igarashi and Kashiwagi, 2010). Polyamines were found to enhance the translation of a specific set of genes in bacteria as well as mammals (Nishimura et al., 2009). Polyamines seem to take part in all aspects of translation as evidenced from extensive studies undertaken in prokaryotes. In a set of genes, termed the ‘polyamine modulon’, polyamines mediate initiation of translation by modifying structural changes in the Shine-Dalgarno sequence and initiation codon to facilitate formation of initiation complex when these two are far apart (Yoshida et al., 2004). Incidentally, most of these genes code for transcription factors, such as c-Myc and c-Jun, which probably mediate the action of polyamines in promoting cell growth (Igarashi and Kashiwagi, 2006; Thomas* and Thomas, 2001; Wang et al., 1993; Wang et al., 1998). In eukaryotes, a similar action is exerted by polyamines, which is thought to be by ribosome shunting (Nishimura et

al., 2009). Polyamines also take part in elongation of mammalian mRNA as they facilitate read-through of UGA termination codon (Hryniewicz and Haar, 1983). Polyamines have also been found to enhance phosphorylation of RNA binding proteins promoting translation (Liu et al., 2009). Polyamines also participate in cell proliferation since depletion of polyamines has often been reported to cause an inhibition in the proliferative capacity mostly by cell cycle arrest (Odenlund et al., 2009; Ray et al., 1999). An interesting and unique function of spermidine is to act as substrate for the posttranslational modification of a particular lysine residue in the eukaryotic translation initiation factor 5A (eIF5A), known as hypusination, which is essential for cell proliferation and survival (Park et al., 2010).

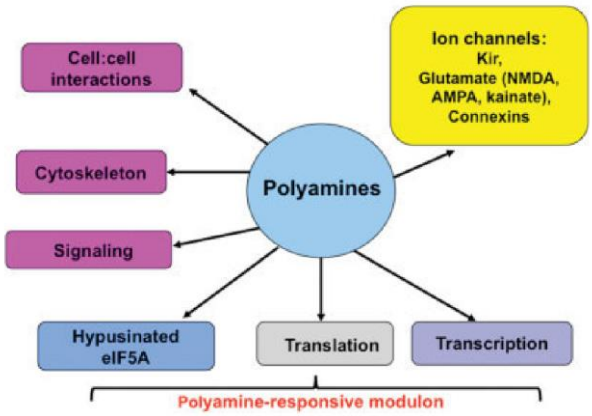


Figure 9: Functions of polyamines (Pegg, 2009).

Polyamines are also implicated in stress response and thought to play an active role in regulating the unfolded protein response (UPR) as well as the oxidative, acid, and osmotic stress responses. Intracellular levels of polyamines are sensitive to stress. However, their effectiveness in eukaryotic organisms are

debatable (Rhee et al., 2007). Accumulation of unfolded proteins triggers release of amino acids thereby initiating polyamine synthesis. Increased levels of polyamines lead to induction of stress-related genes, while suppressing the expression of other genes. Polyamines can also function as ROS scavengers (Ha et al., 1998a), and they are also implicated in the apoptotic pathway (Seiler and Raul, 2005). They also interact with ion channels and modulate their function (Guo and Lu, 2003; Williams, 1997).

II.2.3. Polyamines and diseases

Snyder-Robinson syndrome is caused by mutations in the spermine synthase gene, which reduces the levels of spermine in lymphocytes and fibroblasts. The affected individuals show mental retardation with other symptoms such as osteoporosis, facial dysmorphism, speech abnormalities, and hypotonia (Arena et al., 1996). Polyamines are strongly implicated in cancer as their levels are found to high in blood and urine of cancer patients, and enzymes involved in polyamine synthesis are highly active in cancer cells (Gerner and Meyskens, 2004). Many of the properties of polyamines make them modulators of aggregation upon interaction with different proteins such as α-syn, tubulin, spectrin, fibrinogen, polyglutamines, glutamate dehydrogenase, and lysozyme (Chowhan and Singh, 2013).

II.2.4. Polyamines in aging and neurodegeneration

Polyamine levels are known to decrease with age in a tissue dependent manner. However, the levels of spermidine were found to be elevated in brains of patients with Alzheimer’s, Parkinson’s, Huntington’s, and progressive supranuclear

palsy and also in the red blood cells (Gomes-Trolin et al., 2002; Morrison and Kish, 1995; Vivó et al., 2001). Oxidative stress induced by amyloid *β*-peptide resulted in enhanced polyamine synthesis and uptake, possibly as a protective mechanism (Yatin et al., 1999). On the contrary, *in vitro* studies demonstrate that polyamines promote the fibrillation of amyloid *β*-peptide (Luo et al., 2013). In a cell model of Huntington's disease, polyamine synthesis pathway was upregulated but not the catabolic pathway and pharmacological agents that enhanced polyamine synthesis led to increased cellular aggregates and cell death (Colton et al., 2004).

External administration of polyamines in mice, yeast, worms, flies and human cells lead to an increased life span and reduced the incidence of age-related kidney glomerular atrophy in mice (Eisenberg et al., 2009; Soda et al., 2009). Flies on a spermidine-rich diet suppressed age-induced memory impairment (AMI) in an autophagy-dependent manner and promoting polyamine synthesis just within olfactory memory associated neurons were sufficient to prevent AMI (Gupta et al., 2013). Similarly, spermine accorded protection against lipopolysaccharide-induced memory deficit by activating NMDA receptors in mice (Frühauf et al., 2015). This activation is probably through polyamine modulatory site on the NMDA receptor/channel (Kishi et al., 1998; Rubin et al., 2004).

II.2.5. Polyamines and α-syn

Contradictory reports, both beneficial and detrimental, exist on the association between polyamines and α-synucleinopathies. Polyamines, especially spermine, have been shown to enhance the aggregation kinetics of purified α-syn *in vitro* similar to its effect on amyloid *β*-peptide (Antony et al., 2003). NMR analysis

of the α-syn complex with polyamines revealed that polyamines directly bind to the c-terminus of the protein (Fernández et al., 2004), specifically to the residues 109-140 (Hoyer et al., 2004; Xie et al., 2006). Different mechanisms were proposed to be behind this behaviour. Spermine binding possibly reduced the net charge of α-syn leading to formation of a highly compact structure that promote aggregation (Grabenauer et al., 2008). Polyamine binding, most likely disrupt the long-range interactions that stabilize stable conformations of α-syn (Bertoncini et al., 2005). Under conditions that mimic the physiological state, addition of spermidine increased the propensity of α-syn to misfold and was involved in both early and late stages of aggregation (Krasnoslobodtsev et al., 2012).

Since polyamines are known to bind with proteins easily, *in vivo* studies were warranted. In one such study, the catabolic enzyme SAT1 was found to be downregulated in PD patients, especially in the affected regions (Lewandowski et al., 2010). This enzyme is the principal catabolic enzyme, critical for regulating the levels of spermine and spermidine within cells and therefore explains the elevated levels of these polyamines in neurodegeneration. The study also showed that polyamine uptake in a yeast model by yeast-specific polyamine transporter enhanced α-syn toxicity. Pharmacological agents that increased or decreased the levels of higher order polyamines in human α-syn expressing mice worsened or rescued α-syn pathology, respectively. Another study also demonstrated that extracellular spermine aggravate ischemic neuronal injury by enhancing the activity of acid-sensing ion channels (ASICs) (Duan et al., 2011).

On the other hand, flies exposed to the herbicide paraquat, a neurotoxic agent that can induce Parkinson's, showed improved survival and locomotor activity upon treatment with Spermidine (Minois et al., 2012). Incidentally, paraquat happens to be a toxic polyamine analogue. Chinese-hamster ovary (CHO) cells expressing P-type ATPase *ATP13A2*, reported to be involved in the transport/uptake of polyamines, make these cells more sensitive to paraquat exposure (Hera et al., 2013; de Tezanos Pinto et al., 2012). Mutations of this gene have been implicated in in neurodegeneration (Ramirez et al., 2006). Spermidine as a food supplement was able to extend the life span and better the motor function in α-syn expressing flies under conditions of manganese toxicity in an autophagy dependent manner (Büttner et al., 2014). This protective effect was replicated in nematodes expressing human α-syn where spermidine supplementation led to a significant decrease in α-syn induced neurodegeneration.

II.2.6. Polyamines and autophagy

As discussed earlier, polyamines were shown to induce autophagy in yeast, flies, nematodes, and human cells, which provided the benefit of life span extension as well as protection from stress and this effect was postulated to be by epigenetic regulations (Büttner et al., 2014; Eisenberg et al., 2009; Minois et al., 2012). Wang et al. has proposed the following model that effectively summarizes the likely mode of action of polyamines, involving autophagy (Wang et al., 2015).

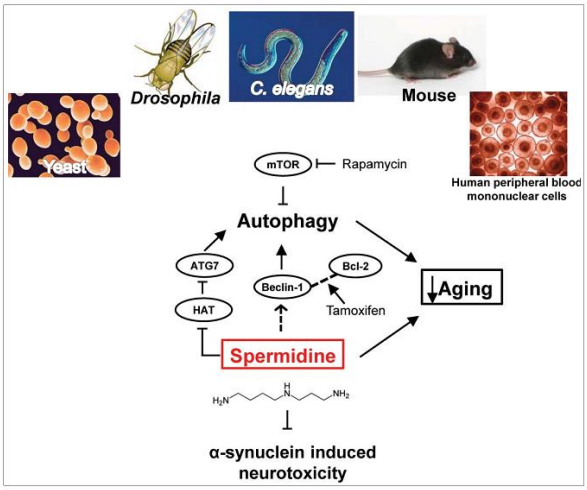


Figure 10: Targets of spermidine in different pathways. HAT – Histone acetyltransferase, ATG7 – autophagy-related protein 7, Bcl-2 – B-cell lymphoma 2 (apoptosis regulator), mTOR – mechanistic target of rapamycin**.** (Wang et al., 2015)

II.3. Experimental models

II.3.1. Endogenous model of α-syn expression

The intrinsic unfolded nature, dose related toxicity, propensity to form aggregates, and the ability to transfer itself between cells, make the study of function and behaviour of α-syn, a difficult proposition, especially using overexpression models. As mentioned before, overexpression of the protein itself is toxic as shown in various models that exhibit dose-dependent toxicity (Masliah et al., 2000; Visanji et al., 2016) and also lead to PD in humans (Ibáñez et al., 2004; Singleton et al., 2003). Overexpression models of α-syn, especially under foreign promoters, can negatively impact the results of a study as in the case where inhibition of proteasomal function did not enhance the expression of α-syn in an endogenous model in contrast to an overexpression model (Alvarez-Erviti et al., 2013). This has necessitated many to look at endogenous models of α-syn expression in order to

study the protein in its normal milieu. Endogenous expression of α-syn with a regulatory role in melanin biosynthesis has been observed (Matsuo and Kamitani, 2010; Pan et al., 2012), and a few studies have made use of this model to study the behaviour of α-syn (Choong and Say, 2011; Chorfa et al., 2013).

II.3.2. *Caenorhabditis elegans* expressing human α-syn

II.3.2.1. C. elegans as a model

The nematode *C. elegans* is an attractive model system, especially for studying developmental processes, neuronal functions, behaviour, and protein interactions. The animal is transparent and the details, even at the subcellular level, are easily visualized with a suitable microscope. The details can be enhanced further with green fluorescent protein (GFP) tagging to proteins or subcellular compartments. It has a relatively short life cycle that proceeds from egg to adult in about three days. It exists primarily as a self-fertilizing hermaphrodite, has a fast reproductive cycle with a high progeny number (~300) and a short life span (~21 days). The worms are easily maintained on agar plates or in liquid media, and they can survive unfavourable conditions by entering an alternate larval stage called the 'dauer' larva.

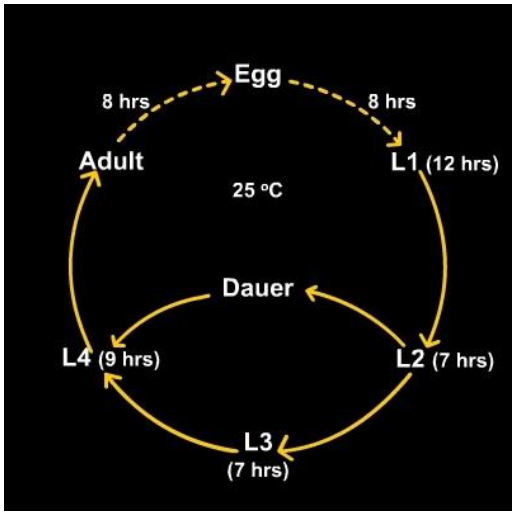


Figure 11: Life cycle of *C. elegans*. Solid arrows indicate molt. Adapted from http://wormclassroom.org/image/c-elegans-life-cycle.

The nervous system of the worm is well mapped and the hermaphrodite has 302 neurons, which include eight dopamine (DA) neurons (Sulston et al., 1975). They consist of four cephalic cells (CEPs) and two bilateral anterior deirids (ADEs) in the head region, and two bilateral posterior deirids (PDEs). The single long dendrite of each CEPs extends from the cell body near the nerve ring (Figure 12) and end at the cuticle near the mouth. The dendrites of the ADEs end in the sensory receptors and the axons of both CEPs and ADEs are directed into the nerve ring. The cell body of PDEs are near the vulva, with the dendrites ending in sensory receptors while the axons pass through the nerve chord, extending till the nerve ring. The males harbour an additional three pairs of DA neurons near the tail region. The dopaminergic neurons are involved in locomotion, associative learning, food searching and so on in the worms.

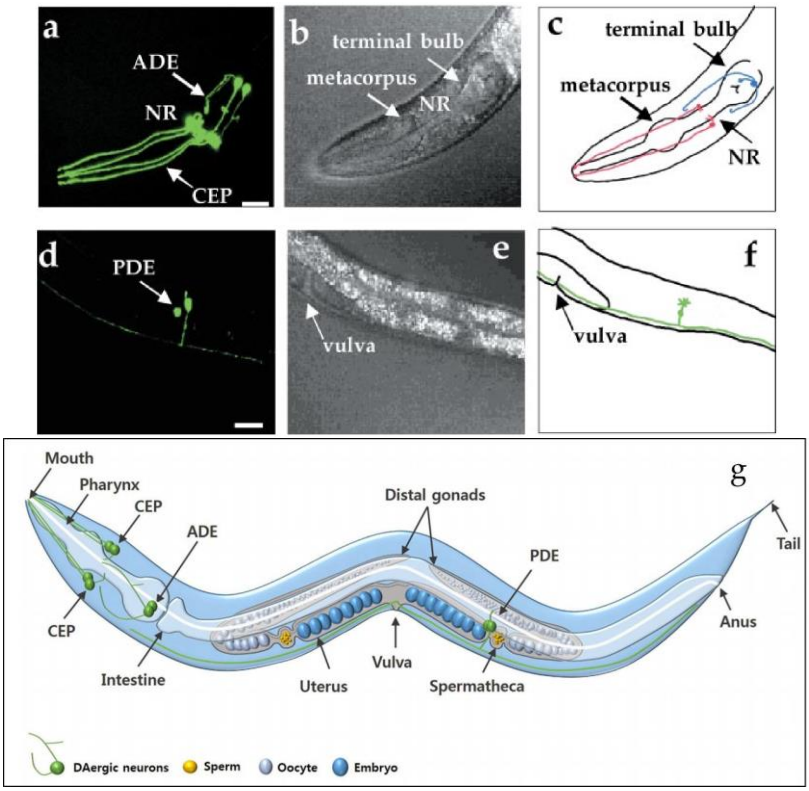


Figure 12: Dopaminergic neurons visualized using GFP tagged to the dopamine transporter (*dat-1*) gene. Arrows show the dopaminergic neurons (a, d). NR- nerve ring. DIC image of the animals (b, e). Schematic drawing that shows the location of these neurons (c, f, and g). (Chege and McColl, 2014; Nass and Blakely, 2003).

II.3.2.2. C. elegans as a model of neurodegeneration

Various neurotoxins and metals that degenerate the neurons have been used to model neurodegeneration in *C. elegans* (Dimitriadi and Hart, 2010; Nass et al., 2001; Nass et al., 2002). The dopaminergic neurons of *C. elegans* are sensitive to 6-hydroxydopamine, MPP+, manganese and rotenone (Chege and McColl, 2014). Orthologues of many PD-associated genes are present in PD, though α-syn is one major exception. Transgenic worms expressing human α-syn (wild-type and mutants) under the control of different promoters show a highly reproducible pattern of neurodegeneration (Cao et al., 2005; Lakso et al., 2003). The neurodegeneration

was observed only in the worms that expressed α-syn under the control of a pan-neuronal or dopaminergic neuron specific promoter (Lakso et al., 2003). α-syn expression within the body wall muscles have provided with a model of protein aggregation, that worsens with age (Hamamichi et al., 2008).

The worms that express α-syn tagged to GFP, is a proven model to study the function and interaction of α-syn with other proteins in a live organism (Hamamichi et al., 2008; Kuwahara et al., 2008). It also enables to look for the pathways involved in neurodegeneration and to screen for compounds that help to alleviate the same (Harrington et al., 2010; Su et al., 2010; Tardiff et al., 2012). Co-expression of torsin proteins and RAB1 (protein involved in ER to Golgi transport) were shown to supress α-syn mediated toxicity in dopaminergic neurons (Cao et al., 2005; Cooper et al., 2006). Knockdown of genes involved in the endocytic pathway in *C. elegans* expressing human α-syn in a pan-neuronal manner showed decreased touch-sensitivity and impaired neuromuscular transmission, reaffirming its function in the synapse (Kuwahara et al., 2008). Dopaminergic neurons are vulnerable to manganese, possibly due to its affinity to the dopamine re-uptake transporter 1 (DAT-1) receptor (Au et al., 2009; Hirata et al., 2001; Ordoñez-Librado et al., 2008). Manganese was toxic to dopaminergic neurons of *C. elegans* only in the presence of a functional dopamine transporter (DAT-1) and/or dopamine, providing an important link to the selective vulnerability of dopaminergic neurons in synucleinopathies (Benedetto et al., 2010). Consequently, *C. elegans* expressing GFP-tagged α-syn from the *dat-1* promoter becomes an excellent model to study the effects of such interactions, if any (Angeli et al., 2014; Bornhorst et al., 2014).

III. MATERIALS AND METHODS

III.1. Materials

III.1.1. Fine chemicals

Dulbecco's Modified Eagle's medium (DMEM), DMSO, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), acrylamide, *N, N′*-methylene bisacrylamide, bovine serum albumin, EDTA, glycine, Hoechst 33342, 2-mercaptoethanol, TEMED, APS, PMSF, trypsin-EDTA solution (10X), penicillin G potassium salt, cycloheximide, protease inhibitor cocktail, SDS, skim milk powder, sodium hypochlorite solution (available chlorine 10-15%), spermine, diminazene aceturate (Berenil), chloroquine diphosphate, sodium deoxycholate, Trizma base and Tween 20 were from Sigma-Aldrich (St. Louis, MO, USA). Antibiotic-antimycotic (100X), Trypan blue stain, and foetal bovine serum (FBS) were purchased from Gibco (Carlsbad, CA, USA). Luminata Forte western HRP substrate was from Millipore (Billerica, MA, USA). N^1,N^{11}-diethylnorspermine tetrahydrochloride (DENSPM) was procured from Tocris Bioscience (Bristol, UK)

III.1.2. Routine chemicals

Methanol, isopropanol, and sodium chloride were from Merck, India. Glycerol, bromophenol blue, Triton-X100, Coomassie Brilliant Blue R 250 and phosphoric acid were from SRL, India. All bacteriological media ingredients were from HiMedia, India.

III.1.3. Antibodies

All antibodies were purchased from Abcam (Cambridge, UK) and used at following dilutions

1. Anti-alpha synuclein (rabbit monoclonal) - 1 in 30,000 (immunoblotting), 1 in 100 (immunocytochemistry).
2. Anti-LC3B (rabbit polyclonal) - 1 in 1,000 (immunoblotting).
3. Anti-SQSTM1/p62 (rabbit monoclonal) - 1 in 50,000 (immunoblotting).
4. Anti-beta actin - 1 in 2500 (immunoblotting).
5. Goat anti-rabbit IgG H&L (HRP) - 1 in 10,000 to 1 in 50,000 (immunoblotting).
6. Goat anti-rabbit IgG H&L (FITC) - 1 in 100 to 1 in 200 (immunocytochemistry).

III.1.4. Cell lines

The melanoma cell line SK-MEL-28 was a kind gift from the cell line repository at Rajiv Gandhi Centre for Biotechnology (RGCB), Trivandrum, India. The SH-SY5Y cell line was obtained from the cell repository at National Centre for Cell Science (NCCS), Pune, India.

III.1.5. *C. elegans* strains

The following strains were used in this study.

Bristol (N2) – wild-type variant

UA44: *baIn11*[$p_{dat\text{-}1}$*::aSyn,* $p_{dat\text{-}1}$*::gfp*]

BZ555: *egIs1*[$p_{dat\text{-}1}$*::gfp*]

The N2 and BZ555 strains were sourced from Caenorhabditis Genetics Center (CGC), which is funded by NIH Office of Research Infrastructure Programs (P40 OD010440). The UA44 strain was a kind gift from the Caldwell Laboratory at the University of Alabama. An approval from the Biosafety Committee was obtained for the use of *C. elegans* strains.

III.2. Equipment, plastic-ware and glass-ware

Forma direct heat CO_2 incubator (Thermos Scientific, MA, USA), class II biosafety cabinet (Esca, PA, USA), weighing balance (Sartorius, Germany), water bath (Boston, India), cooling water bath and electrophoresis power supply unit (Amersham Biosciences, NJ, USA), Cyber scan pH meter (Etch Instruments, Singapore), Mille Bifocal water purification system (Merck Millipore, MA, USA), Uviol gel documentation system (Vitec, Cambridge, UK), high speed centrifuge, refrigerated micro centrifuge and thermomixer (Eppendorf AG, Hamburg, Germany), rotary shaker (Stovall Life Science, NC, USA), Mini-gel vertical electrophoresis unit (OWL Scientific, CA, USA), vertical electrophoresis apparatus for western blot (Bio-Rad, CA, USA), (Ultralow temperature freezer (Nuaire, MN, USA) autoclave (Sanyo, Japan) shaker incubator (IA, Germany) Herat herm microbiological incubator (Thermo Scientific, MA, USA), Ecotherm chilling incubator (Torrey Pines Scientific, CA, USA) microplate reader (Tecan, Switzerland), Ultrasonic liquid processor (Sonics, CT, USA), stereo microscope (Magnus Analytics, India), Olympus IX51 inverted microscope (Olympus Imaging, Center Valley, PA, USA), and Rolera XR monochrome camera (QImaging, Canada).

All cell culture- treated dishes were purchased from Becton Dickinson, USA. Plastic wares and micropipette tips were from Axygen, USA. Membrane filter and syringe filter were procured from Millipore, USA. Glassware used was either from Borosil or Schott-Duran and glass petri-dishes were from Borosil.

III.3. Software

GraphPad Prism (version 6) was used for all statistical analysis and graphical representations. Images were captured using NIS Elements AR (Nikon, NY, USA). Images were analysed using ImageJ version 1.45s (NIH, USA) which was also used to enhance the display contrast along with Adobe Photoshop CS2 (Adobe Systems, CA, USA).

III.4. Composition of media, reagents and buffers

III.4.1. Acrylamide mix 30%

Acrylamide - 29.2 g
Bisacrylamide - 0.8g

Dissolved in a minimum volume of deionized water, pre-warmed to 37°C and made up to 100 ml using deionized water. The solution was filtered using Grade 1 Whatman filter paper and stored at 4°C in a brown bottle.

III.4.2. Blocking solution

2% - 5% skimmed milk or 5% BSA was dissolved in 1x TBS containing 0.1% Tween-20 (TBST)

III.4.3. Phosphate-buffered saline (10X) (1 litre)

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

Dissolved in a minimum volume, adjusted the pH to 7.4, made up to 1 litre with distilled H_2O and stored at 4°C. From 10X, diluted to 1X before use with autoclaved distilled water and filter sterilized.

III.4.4. 5X Laemmli sample buffer

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

III.4.5. RIPA Buffer

Tris-Cl (pH 7.4) - 50 mM
NaCl - 150 mM
EDTA - 1 mM
Triton-X100 - 1%
Sodium deoxycholate - 1%

Prepared and stored in 4°C. Protease inhibitor cocktail (1X diluted from 10X), PMSF (2 mM from a stock of 200 mM), and 0.1% SDS were added immediately before use.

III.4.6. Bradford Reagent (5X)

Coomassie Brilliant Blue R 250 - 50 ml
Methanol - 47 ml

Phosphoric acid (85%) - 100 ml

III.4.7. SDS Gel Loading Buffer (2X)

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

III.4.8. Towbin's buffer (Transfer Buffer) (1X)

Tris Base - 25 mM
Glycine - 192 mM
Methanol - 10%

Made up with distilled water.

III.4.9. Tris-buffered saline (10X)

Tris Base - 25 mM
NaCl - 150 mM

Made up with distilled water. Prior to use, 1XTBS was prepared and 0.1% Tween-20 added (1X TBST)

III.4.10. Paraformaldehyde (4%)

Paraformaldehyde dissolved in 1X PBS to a final concentration of 4% by heating at 50 – 55°C.

III.4.11. Hoechst 33342

20 mg/ml stock was prepared in distilled water and stored at -20°C, protected from light. Diluted 1:100 in distilled H_2O before use.

III.4.12. Nematode growth medium (NGM)

NaCl	- 0.3 g
Agar	- 2 g
Peptone	- 0.25 g
Distilled water	- 100 ml

Dissolved and autoclaved. Cooled the medium to ~55°C and the following solutions were added.

1 M $CaCl_2$	- 0.1 ml
5 mg/ml cholesterol	- 0.1 ml
1 M $MgSO_4$	- 0.1 ml
1 M phosphate buffer (pH 6.0)	- 2.5 ml

The media was immediately poured to petri plates and allowed to set. Once set, *E. coli* strain OP50 was spread on to the plate and incubated overnight at 37°C. The plates were stored at 4°C and used within a week.

III.4.13. Bleach solution (for 5 ml)

1.32% NaOH	- 3 ml
4% NaOCl	-2 ml

III.4.14. M9 buffer

KH_2PO_4	- 3 g
Na_2HPO_4	- 6 g
NaCl	- 5 g

Made up to 1 l with distilled water and autoclaved. Once the solution is cooled, 1 ml of 1 M $MgSO_4$ was added.

III.5. Methods

III.5.1. Cell Culture

SK-MEL-28 cells and SH-SY5Y cells were maintained in DMEM containing 10% FBS, 1X antibiotic-antimycotic solution or 1% pencillin-streptomycin at 5% CO_2 at 37°C. Cells were grown to 80-90% confluency and sub-cultured at 1:4 ratio. Trypsinisation was done by incubating cells in 1X Trypsin-EDTA for 3 minutes for SK-MEL-28 cells or 4 minutes for SH-SY5Y cells. The detached cells were collected in serum-containing media, washed in 1X PBS and re-plated.

III.5.2. Cell viability assays

III.5.2.1. MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] assay

8,000-10,000 cells were plated in each well of 96-well microtiter plates 24 – 48 hours prior to the experiment. After each treatment, media was removed and the wells washed with 1X PBS to remove all traces of media. The assay was performed based on an earlier protocol (Riss et al., 2004), with minor modifications. A stock of 1 mg/ml solution of MTT was prepared in PBS. From the sock, 100 µl was added to each well and incubated for 2 hours in the dark. The solution was discarded after 2 hours and 100 µl acidified isopropanol was added to each well to dissolve the formazan crystals. Absorbance was measured at 575 nm with reference at 630 nm wavelength on a microplate reader. The viability was expressed as percentage with

respect to the control values. The tests were replicated at least thrice and the data was analysed by one-way ANOVA.

III.5.2.2. Trypan blue dye exclusion test

The Trypan blue dye exclusion test was done following standard protocols (Strober, 2001). Approximately 50,000 cells were plated in each well of a 24-well culture plates and allowed to grow to ~60% - 70% confluency. Each treatment was performed in triplicate. After treatment, media from each well was collected in separate 1.5 ml Eppendorf tubes. The wells were washed with 1X PBS and trypsinised. The cells were collected and added to the respective tubes containing media. The tubes were centrifuged at 500g for 5 minutes and the pelleted cells re-suspended in 100 µl 1X PBS (Strober, 2001). 10 µl of the cell suspension was mixed with an equal volume of 0.4% trypan blue stain. 10 µl of this sample was applied on to a Neubauer improved cell counting chamber. This was placed under microscope and the stained (nonviable) and unstained (viable) cells were counted. The results are represented as percentage viability of the cells. Measurements from repeated experiments were analysed using one-way ANOVA.

$$viable\ cells\ (\%) = \frac{total\ number\ of\ viable\ cells\ per\ ml\ of\ the\ aliquot}{total\ number\ of\ cells\ per\ ml\ of\ the\ aliquot}\ x\ 100$$

III.5.3. Protein extraction and estimation

Cells were seeded at a density of 1,60,000 to 2,00,000 on a 60 mm culture dish and allowed to grow till 70-80% confluency. After treatment, cells were trypsinised and pelleted. The pellets were incubated in RIPA buffer for 30 minutes

on ice and centrifuged at 16,000*g* for 15 minutes at 4°C. The supernatant was transferred to a fresh tube and the concentration of proteins estimated following the Bradford assay. The samples were stored at -80°C until use.

For estimation, protein standards ranging from 1.5 µg to 15 µg were prepared in distilled water, from a stock of 1 mg/ml BSA. 2.5 µl from each sample was used for the assay with 2.5 µl RIPA buffer as blank. The volumes of the blank, standards and samples were made up to 125 µl with distilled water and 50 µl each from all were taken in duplicate for the assay. 200 µl 1X Bradford was added to each sample and incubated for 10 minutes in dark. The absorbance was measured at 570 nm on a microplate reader. The protein concentration was estimated from a standard graph.

III.5.4. Immunoblotting

For immunoblotting, equal amount of each protein was mixed with 2X SDS gel loading buffer. When protein extraction was performed using cells at low density, samples were directly lysed in 1X Laemmli buffer, by sonication. Equal volume of each samples were used for loading. The samples were boiled for 5 minutes in a boiling water bath and loaded on to the SDS-PAGE minigel (10 to 15%) along with a suitable range molecular weight marker and run at 120 V. After separation, the proteins were transferred on to a methanol pre-wetted PVDF membrane (110 V, 60 minutes, 4°C). Immediately after transfer, the membranes were washed thrice in distilled water for 5 minutes each and dried at 37°C for 1 hour. For blocking, the membranes were immersed in 2-5% skimmed milk or 5% BSA for 1 hour at room temperature. Following this, the membranes were incubated

at 4°C overnight with primary antibody suitably diluted in 1X TBST. The blots were washed thrice with 1X TBST for 5 minutes each, at room temperature, to remove the unbound antibodies. The blots were exposed to a suitable HRP-conjugated secondary antibody prepared in 1X TBST for up to 2 hours at room temperature. The blots were further washed with 1X TBST thrice for 5 minutes each at room temperature to remove unbound antibodies. Enhanced chemiluminescence (ECL) substrate was used to detect the signals positive for specific antibody binding. The signals were captured on to an X-Ray film, which was documented with a gel documentation system equipped with a trans-white conversion screen. The images were analysed using ImageJ software (ver 1.45s, NIH, USA) following the method outlined in the webpage http://lukemiller.org/index.php/2010/11/analyzing-gels-and-western-blots-with-image-j/. The changes in protein expression with respect to the controls are represented as fold-change.

III.5.5. Immunocytochemistry

Cells grown on cover slips or culture dish to about 60% confluency were used for the experiments. After treatment, cells were washed with 1X PBS and fixed with 4% paraformaldehyde for 30 minutes at 37°C. The cells were washed with 1X PBS and permeabilized in 0.4% Triton-X 100 (10 minutes, room temperature). The cells were then blocked with 5% FBS (30 minutes, room temperature). The cells were incubated overnight at 4°C in primary antibody diluted in blocking solution. Following PBS wash, the cells were incubated with FITC-tagged secondary antibody for 90 minutes in the dark, at room temperature. The secondary antibody was removed and the cells washed with 1X PBS thrice for 5 minutes each. The cells

were counter-stained with Hoechst 33342 followed by washing with 1X PBS. The coverslips were mounted on slides and observed under a fluorescence microscope. The images, captured using a monochrome CCD camera, were represented in pseudo-colour using ImageJ software (ver 1.45s, NIH, USA).

III.5.6. Measurement of fluorescence intensity

The images obtained from immunocytochemistry experiments were used to measure the fluorescence intensity corresponding to each treatment. A rectangle with equal area was applied to each region corresponding to the signals on multiple cells in each image, as well as the background area. The average intensity of the background was subtracted from the intensity of each signal in the same image. These steps were repeated for multiple images in the same experiment group and the data points were pooled to plot the graph. Data is represented as fluorescence intensity (arbitrary units). The mean intensity was compared across the groups in each experiment and analysed by one-way ANOVA.

III.5.7. Maintenance of *C. elegans.*

All worms were maintained under standard conditions as outlined in WormBook (Stiernagle, 2006), unless otherwise specified. The strains were maintained on plates of nematode growth medium (NGM) containing the *E.coli* strain OP50 (NGM/OP50) at a temperature between 16°C and 25°C. The worms were picked from the plates using a small piece of flattened platinum wire, fixed on a 1 ml micropipette tip.

III.5.8. Preparation of polyamine rich plates

NGM/OP50 plates were prepared as described in section III.3.12. Spermine or sperimidine dissolved in M9 buffer to the required concentration was spread on an NGM/OP50 plate and dried before adding worms to the plate (Eisenberg et al., 2009). Every third day, the worms were transferred to fresh plates spread with polyamines.

III.5.9. Synchronizing *C. elegans* culture

For the experiments, *C. elegans* cultures were synchronized at the first larval stage, L1, by treating the worms with alkaline hypochlorite solution, also known as "Bleaching" procedure. The worms are sensitive to the alkaline hypochlorite solution but the eggshells are not and these embryos are recovered and allowed to grow, thereby synchronizing the worms at L1 stage.

Gravid adult worms were washed by adding 1 ml M9 buffer to the plates and collected in a 1.5 ml tube, which was centrifuged at 1300*g* to pellet the worms. To the pellet, 1 ml of freshly prepared bleach solution was added and the worms were exposed to bleach for approximately 2 minutes, with intermittent vortex mixing, until most of the adult worms disintegrated. The tubes were centrifuged and supernatant aspirated out without disturbing the pellet. To the pelleted eggs, 1 ml M9 buffer was added and washed multiple times to remove all traces of the bleach. After wash, the eggs were transferred to a fresh NGM/OP50 plate and allowed to grow.

III.5.10. *C. elegans* neurodegeneration assay

The GFP expressing strains of *C. elegans* were synchronised and grown on NGM/OP50 plates. The worms were analysed for degeneration at specific time points. On the day of analysis, 10 worms each were transferred on to a 15 µl solution of 25 mM sodium azide in M9 buffer on a glass slide, to immobilize the worms. The head region of all worms were observed under a fluorescence microscope and imaged. Worms were scored as normal when all six anterior neurons were visible without any signs of degeneration. Worms showing missing neuronal processes, cell body loss, or blebbing were considered as degenerate and scored so. For the experiments comparing UA44 with the BZ555 strain, data is represented as the percentage mean±SEM of worms without any neurodegeneration and analysed using Student's t-test.

For the experiments involving UA44 worms fed on spermine-rich diet, eggs were plated on to an NGM/OP50 plate containing 0.2 mM spermine or M9 buffer after synchronization and allowed to grow until the day 1 adult stage. The worms were then transferred to fresh plates containing spermine or M9 buffer every alternate days. For imaging and scoring, 10-15 worms were transferred on to a 15 µl solution of 25 mM sodium azide in M9 buffer on a glass slide, to immobilize the worms. The worms were observed for signs of neurodegeneration and the number of worms with and without neurodegeneration was counted. The results are expressed as the percentage worms in each group and the data is analysed using Chi-square test for trend.

III.5.11. Survival analysis

After synchronisation, eggs were allowed to develop to L4 stage. Twenty-five worms each was transferred to plates with either spermine (0.2 mM) or M9 buffer (3 plates per treatment group). The number of worms found to be alive and dead was recorded every alternate day. The worms were transferred to fresh plates of the same treatment group every day till they reach day 10 adult following which the worms were transferred on alternate days. This was continued until no more progenies were found in the plate. The aged worms were gently touched with the pick to check whether they were alive and worms that lacked any observable movement were marked as dead. The data obtained from each experiment was subjected to Kaplan-Meier survival analysis and data represented as fraction survival plotted against time in days.

III.5.12. Drop test

The drop test was done following the protocol by Hilliard et al. (Hilliard et al., 2002). The worms to be assayed were transferred to a drop of M9 buffer on an NGM plate to wash the bacteria off from the worms. The worms were allowed to settle for 10 minutes in the plate. A drop of solution containing the repellent 50 mM glycerol was delivered on the agar plate near the tail end, using a 1 ml syringe. When the drop touches the tail, it reaches the anterior amphid sensory neurons by capillary action. The animals usually make a backward movement immediately and perform an omega turn, which is considered positive response to the repellent. Following replicate experiments, data was analysed using one-way ANOVA and represented as mean±SEM of avoidance index, which is calculated as follows.

$$Avoidance\ Index = \frac{number\ of\ positive\ responses}{number\ of\ trials}$$

III.5.13. Exposure to manganese in *C. elegans*

The protocol was developed in our laboratory since we found inconsistencies in obtaining neurodegeneration with the existing protocols. Day 1 adult worms (10-15 no.) were picked and transferred to a cavity slide containing 20 µl of 100 mM $MnCl_2$ in distilled water. The worms were incubated in the solution for an hour and then the solution was diluted with distilled water. The worms were then transferred to a 15 µl solution of 25 mM sodium azide in M9 buffer on a glass slide, to immobilize the worms. The worms were scored for neurodegeneration as mentioned in the section III.5.10. Data is represented as the percentage mean±SEM of worms without any neurodegeneration and analysed using Student t-test.

III.5.14. Short-term exposure of worms to spermine

For short-term exposure, the day 1 adult worms were placed on an NGM/OP50 plate spread with spermine for 30 minutes. The worms were then transferred to a cavity slide containing 100 mM $MnCl_2$ along with spermine and/or other respective treatments. The worms were immobilized, imaged and scored as mentioned in III.5.10. The results are expressed as a percentage of each outcome. Data is analysed using Chi-square test for trend and expressed as percentage of each outcome.

IV. RESULTS

IV.1. SK-MEL-28, an endogenous model of α-syn expression

IV.1.1. α-syn expression and localization in human melanoma cell line SK-MEL-28.

The melanoma cell line SK-MEL-28 was reported to constitutively express α-syn at detectable levels (Matsuo and Kamitani, 2010; Pan et al., 2012). The expression pattern of α-syn in SK-MEL-28 cells was observed by immunostaining using an antibody that target human α-syn. Immunostaining was carried out on cells grown to ~70% confluency. α-syn was found to be present as small aggregates (puncta like appearance) within the nucleus and it showed a diffused pattern within the cytoplasm (Figure 13). These results confirmed that SK-MEL-28 cell lines have a constitutive expression of α-syn and this does not affect its survival.

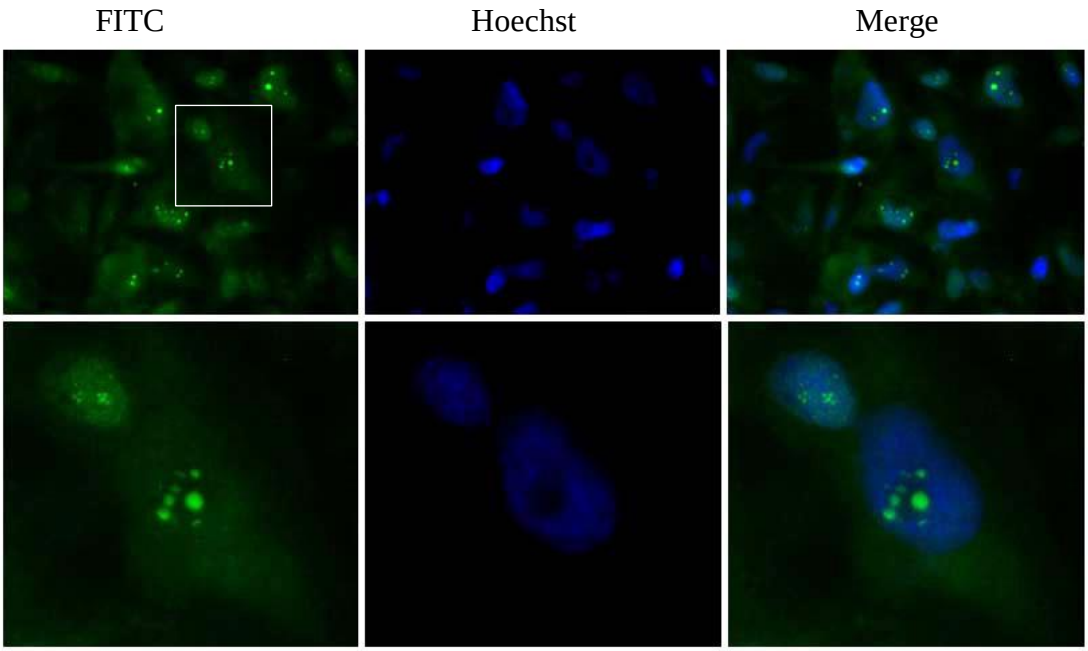
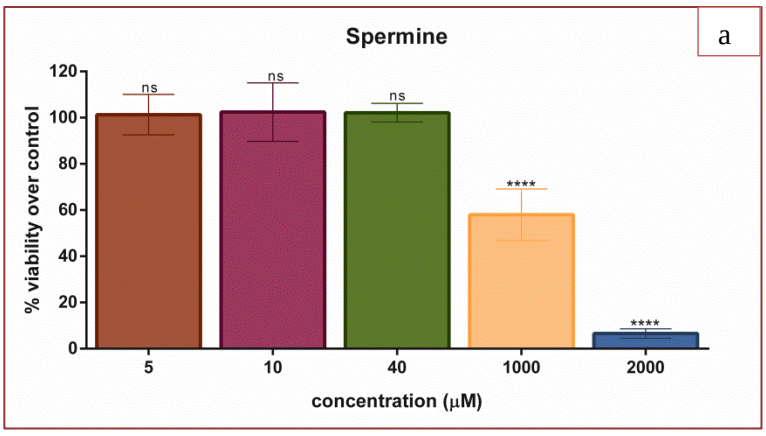


Figure 13: Pattern of expression and localization of α-syn in SK-MEL-28 cell line. The top panel shows the actual image and bottom panel shows an enlarged view of the selected region in each. Images were taken under 20X objective in Axio Observer Z1M microscope (Carl Zeiss, Germany).

IV.1.2. Cell viability assay to spermine and drugs that target polyamine metabolism

Various concentrations of spermine and other drugs used in this study were tested on SK-MEL-28 cells following MTT assay protocol to estimate suitable concentrations that do not affect their viability in culture. SK-MEL-28 cells were found to tolerate spermine at concentrations ranging from 5 µM to 40 µM up to 24 hours without any loss of viability (Figure 14a). However, when treated at millimolar concentrations (10 and 20 mM), spermine caused significant toxicity (Figure 14a). To see whether modification of polyamine metabolism within the cells alter cell survivability, berenil, and DENSPM were tested on these cells. Berenil inhibits the enzyme Spermidine/spermine N1-acetyltransferase 1 (SAT1), thereby increasing the levels of higher order polyamines (spermine and spermidine), whereas DENSPM activates SAT1 leading to a decrease in the higher order polyamines. Berenil and DENSPM treatments did not show significant loss in viability up to 80 µM and 1 mM concentrations, respectively (Figure 14b and 14c). Based on the cell viability data, a range of non-toxic concentrations were fixed for spermine, berenil, and DENSPM.



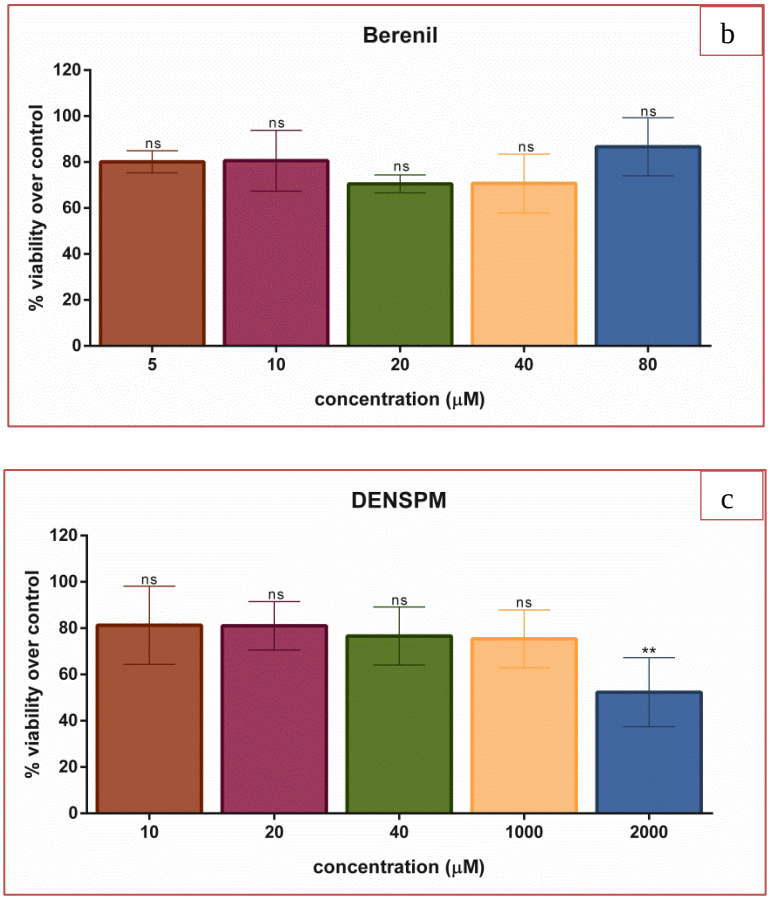


Figure 14: Effect of (a) spermine, (b) berenil, and (c) DENSPM on the viability of cells. All treatments were done for 24 hours and the values are expressed as mean±SD of percentage viability over control (MTT assay). n=3 or more. ANOVA, $p<0.05$(*), $p<0.01$(**), $p<0.001$(***), $p<0.0001$(****), not significant (ns).

IV.1.3. α-syn expression and localization in SK-MEL-28 cells upon spermine treatment

To evaluate the cellular response of α-syn expression to spermine levels, the cells were treated with 5 µM and 10 µM spermine for 12 hours. After treatment, the cells were subjected to either immunocytochemistry or western blot analysis. The anti-α-syn antibody binds to both the low molecular weight monomer and the high molecular weight species or oligomers. Since spermine is known to promote the

formation of high molecular weight species of α-syn (Chowhan and Singh, 2013), both forms were probed for in the blots. The blots showed only minor variations in the levels of both forms of α-syn, between the treatment groups (Figure 15).

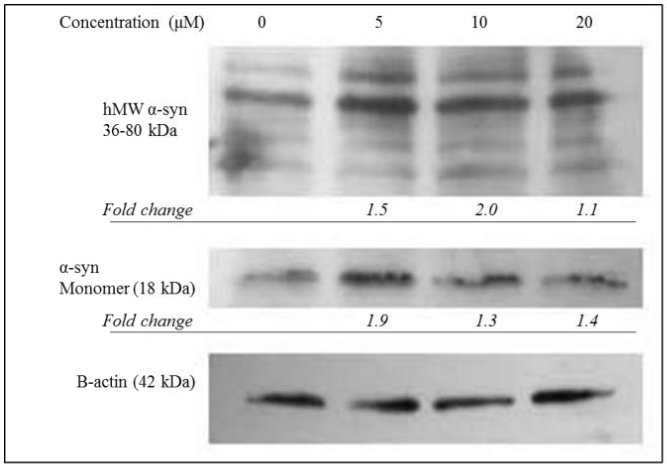


Figure 15: Western blot image showing pattern of α-syn expression in SK-MEL-28 cells upon treatment with spermine. Representative western blot image showing α-syn expression levels after 12 hours treatment with spermine at different concentrations. The values below each band indicate the fold change respective to control for each treatment.

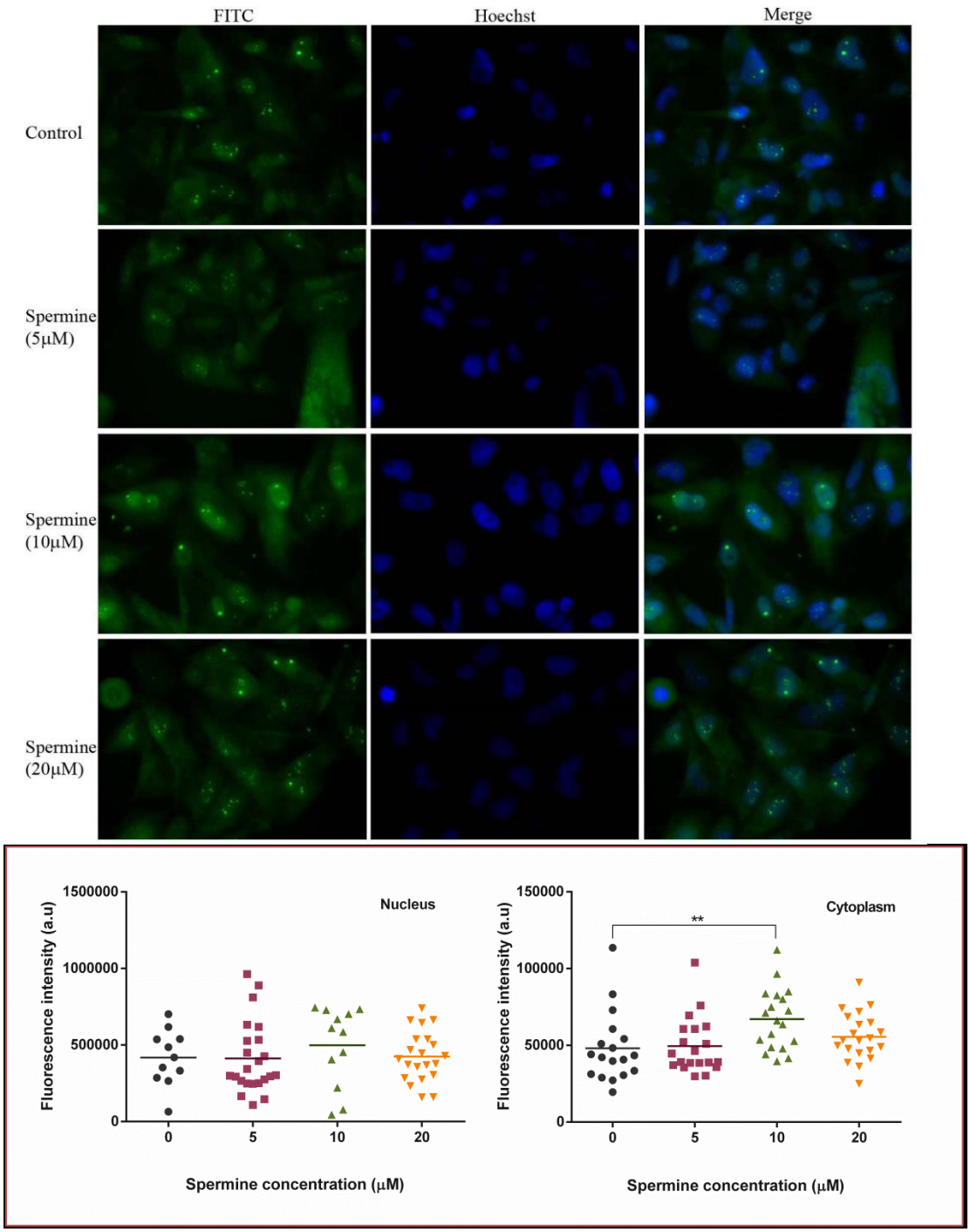


Figure 16: Pattern of expression and localization of α-syn in SK-MEL-28 cells upon treatment with spermine. Panel of representative images from immunocytochemistry experiment after treating the cells for 12 hours with spermine at different concentrations. Images obtained with 40X objective from Olympus IX51. Pixel intensity is plotted from the images as a function of α-syn expression levels, quantified from nucleus and cytoplasm. Each point corresponds to average intensity values measured from multiple cells in each image using ImageJ software

as described in section III.5.6. ANOVA, $p<0.01$(**), other comparisons not significant.

Immunocytochemistry experiments with the same antibody did not show significant changes to the localization or expression pattern of α-syn upon treatment with spermine for 12 hours (Figure 16). However, quantitative measurement of fluorescence intensity of α-syn signals from the images revealed a significantly higher intensity level in the cytoplasm of cells treated with 10 μM spermine (Figure 16).

IV.1.4. α-syn expression in SK-MEL-28 cells upon treatment with drugs that interfere with polyamine catabolism

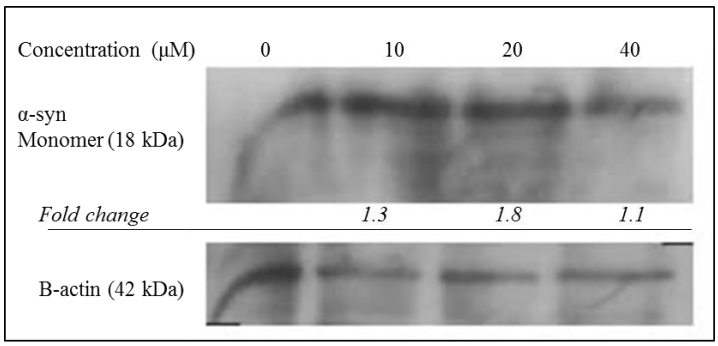


Figure 17: Western blot image showing α-syn expression pattern in SK-MEL-28 cells upon treatment with berenil. Representative image showing α-syn expression levels after 24 hours treatment with berenil at different concentrations. The values below each band indicate the fold change respective to control for each treatment.

The above observations were reconfirmed using the drugs, berenil, and DENSPM. Treating the cells with berenil, a drug that increases polyamine content within cells by inhibiting its catabolism, showed only a minor increase in the levels

of α-syn protein (Figure 17). Similarly, DENSPM treatment, which decreases polyamine levels within cells, did not change α-syn protein levels (Figure 18).

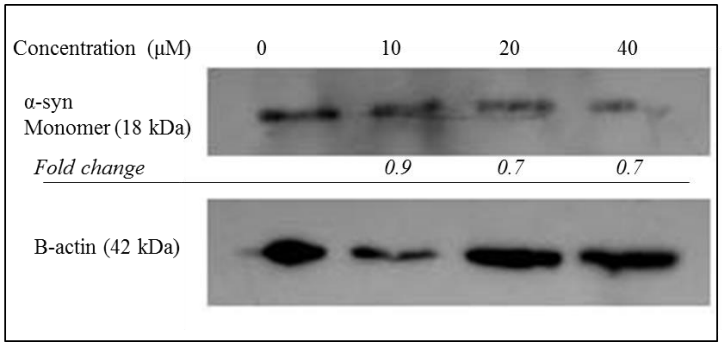


Figure 18: Western blot image showing α-syn expression pattern in SK-MEL-28 cells upon treatment with DENSPM . Representative image showing α-syn expression levels after 24 hours treatment with DENSPM at different concentrations. The values below each band indicate the fold change respective to control for each treatment.

These results confirm that the α-syn levels in SK-MEL-28 cells are highly regulated and do not alter with polyamine levels. Since polyamines have a critical role in translation and transcription machinery (Miller-Fleming et al. 2015), the above results were surprising. One possibility is that α-syn levels are maintained in this cell by means of regulation via the autophagy-lysosomal pathway (Xilouri, Brekk, and Stefanis 2016).

IV.1.5. Effect of spermine and the drugs involved in polyamine catabolism on autophagy

Polyamines are capable of altering autophagy pathway (Eisenberg et al., 2009). In SK-MEL-28 cells, spermine (5-20 μM) was found to block autophagy by 12 hours, as evident from accumulation of p62 protein within the cells, in a concentration dependent manner (Figure 19). However, both berenil (Figure 20) and

DENSPM (Figure 21) that interfere with polyamine catabolism did not alter the autophagy pathway.

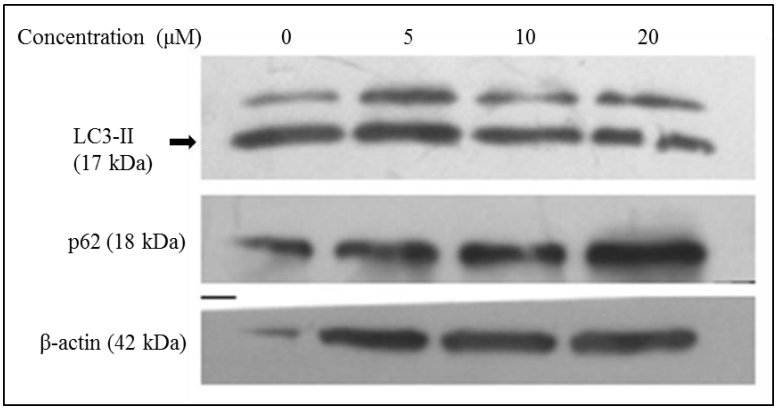


Figure 19: Effect of spermine treatment on autophagy in SK-MEL-28 cells. Representative image showing changes in the markers of autophagy after 12 hours treatment with spermine at different concentrations.

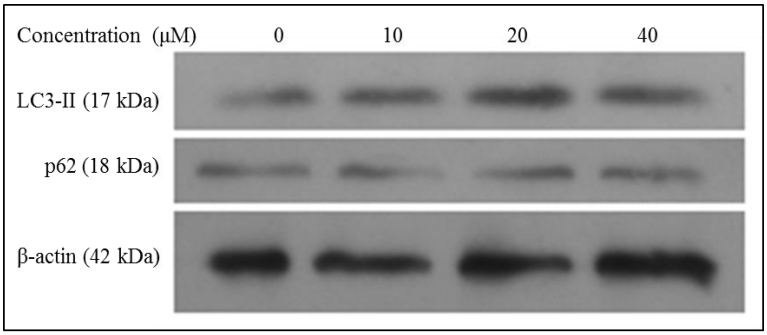


Figure 20: Effect of berenil treatment on autophagy in SK-MEL-28 cells . Representative image showing changes in the markers of autophagy after 12 hours treatment with berenil at different concentrations.

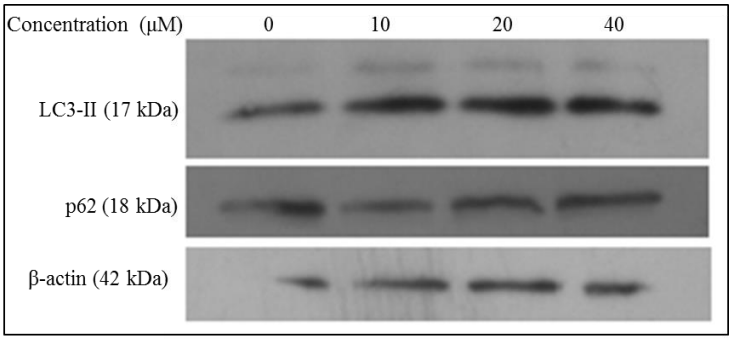


Figure 21: Effect of DENSPM treatment on autophagy in SK-MEL-28 cells. Representative image showing changes in the markers of autophagy after 12 hours treatment with DENSPM at different concentrations.

Autophagy block by spermine could be one reason for the selective increase in cytoplasmic levels of α-syn (Figure 16). There is a requirement of high concentration of spermine to have this effect and berenil treatment probably did not allow intracellular polyamine levels to go beyond a level due to the highly effective regulatory mechanism of maintaining polyamine levels in cells (Perez-Leal and Merali, 2012).

IV.2. Toxic insults and their effects on cells expressing endogenous α-syn in presence of polyamines

Various factors, external or otherwise, have been demonstrated to cause cell death and neurodegeneration in many models. Interaction between α-syn and these factors have shown to promote some of the events in this process. In order to subject these cells to stress conditions, I chose two different stress inducers, dopamine and manganese (Mn^{2+}). Dopamine is considered an endogenous toxicity-imparting factor in neurodegeneration (Xu et al. 2002; Chen et al. 2008). Presence of dopamine also makes the cells vulnerable to many other factors that promote neurodegeneration.

The most common synucleinopathy, PD mostly affects the dopaminergic cells. Besides dopamine, manganese (Mn^{2+}) is also implicated in causing neurodegeneration, often leading to the clinical condition called 'manganism' (Pifl et al. 2004) and α-syn is shown to interact with manganese and regulate its levels (Dučić et al., 2015).

IV.2.1. Effect of dopamine on cell viability

Dopamine induces cytotoxicity in cells (McLaughlin et al. 1998). SH-SY5Y cells, which do not express α-syn at detectable levels, showed a significant loss in the viability by 4 hours of dopamine exposure (1 mM) (Figure 22) and no cells survived after 24 hours (data not shown). On the other hand, α-syn expressing SK-MEL-28 cells were able to tolerate this high level (1 mM) of dopamine exposure even 20 hours post exposure (Figure 22). These results indicate that α-syn is essential for cell survival in a dopamine environment.

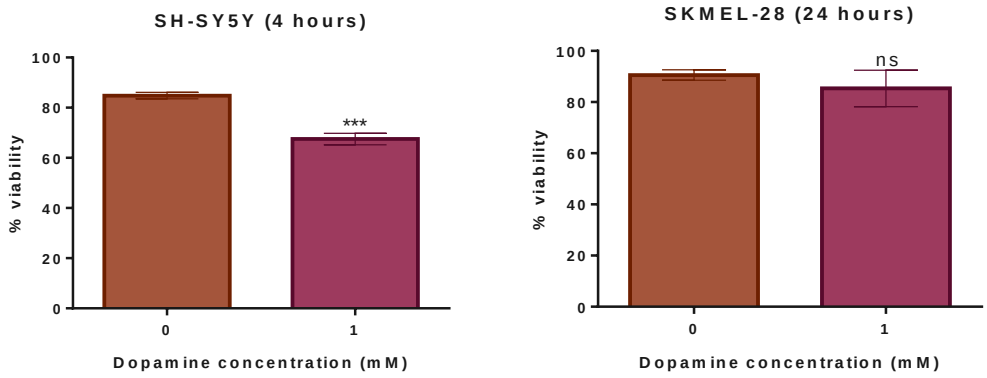


Figure 22: Effect of dopamine on cell viability. Cells were exposed to 1 mM dopamine containing media for 4 hours and replaced with fresh media. The percentage viability was assayed post 20 hours of treatment for SK-MEL-28 cells

and post 4 hours of treatment for SH-SY5Y cells. Values are presented as mean±SD. n=3 or more, Student's t-test, p<0.001(***), not significant (ns).

IV.2.2. Effect of manganese on cell viability

To assess whether Mn^{2+} induce any toxic effect, the cells were treated with manganese chloride ($MnCl_2$) at various concentrations. $MnCl_2$ treatment did not affect the viability of SK-MEL-28 cells when treated for 8 hours (Figure 23a). However, the cells showed a significant reduction in the viability when treated with $MnCl_2$ for 24 hours (Figure 23b). SH-SY5Y cells also showed a similar significant loss in the viability when treated for 24 hours, in a concentration dependent manner (Figure 24). These results suggest that though SK-MEL-28 cells could tolerate high dopamine levels, both SH-SY5Y and SK-MEL-28 cells are sensitive to Mn^{2+}.

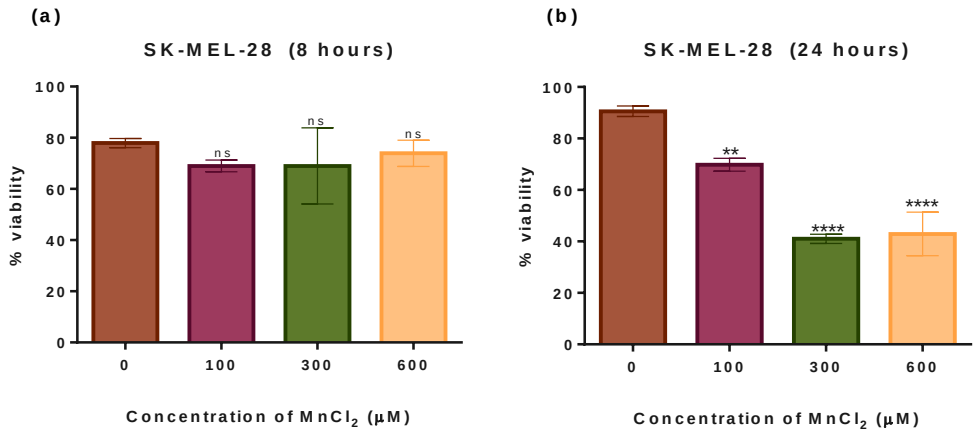


Figure 23: Effect of $MnCl_2$ treatment on the viability of SK-MEL-28 cells. Cells were treated with different concentrations of $MnCl_2$ for 8 hours (a) and 24 hours (b). Values are presented as mean±SD. n=3 or more. ANOVA, p<0.01(**), p<0.0001(****), not significant (ns).

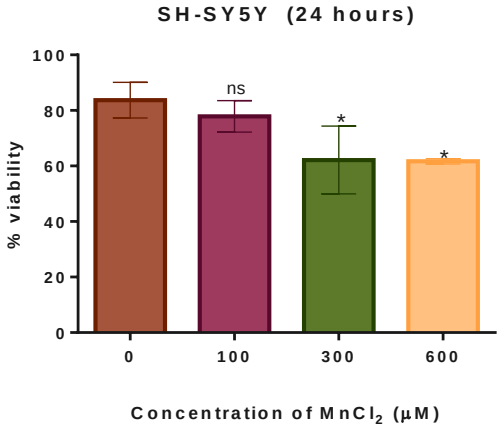


Figure 24: Effect of $MnCl_2$ treatment on the viability of SH-SY5Y cells. Cells were treated with different concentrations of $MnCl_2$ for 24 hours. Values are presented as mean±SD. n=3 or more. ANOVA, $p<0.05$(*), not significant (ns).

IV.2.3. Effect of combined exposure to dopamine and $MnCl_2$ on cell viability

To verify whether exposure to dopamine and $MnCl_2$ together could alter cell viability, SK-MEL-28 cells were exposed to $MnCl_2$ after 4 hours of dopamine treatment (1 mM). The cells exhibited a significantly greater loss in viability (100 and 600 µM), compared to $MnCl_2$ treatment alone (Figure 25). The results suggest that Mn^{2+} significantly hampers the cell survivability, probably by generating high oxidative stress as well as nucleic acid damage. Dopamine, which was not toxic by itself in the SK-MEL-28 cells, added some toxicity to cells in presence of $MnCl_2$ indicating the possible interactions between the two that could lead to formation of toxic radicals (Sistrunk et al., 2007).

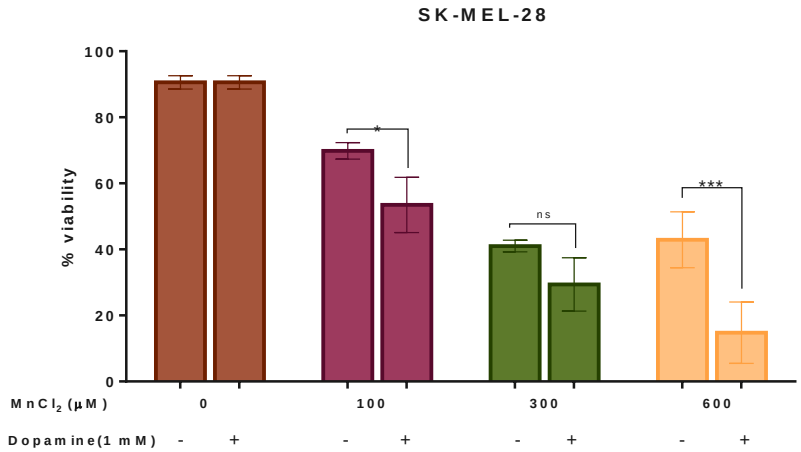


Figure 25: Effect of combined exposure to dopamine and $MnCl_2$ on SK-MEL-28 cells. Cells were exposed to different concentrations of $MnCl_2$ for 20 hours post 4 hour exposure to 1 mM dopamine. Values are presented as mean±SD. n=3 or more. ANOVA, $p<0.05$(*), $p<0.001$(***), not significant (ns).

IV.2.4. Effect of $MnCl_2$ on cell viability in presence of spermine, spermidine, and berenil

Polyamines play a critical role in protecting cells from oxidative stress and nucleic acid damage (Snyder 1994). Hence, their ability to prevent Mn^{2+} induced cytotoxicity was assayed.

Presence of spermine alleviated the toxicity imparted by $MnCl_2$ in a significant manner and the cells attained percentage viability similar to that of controls (Figure 26). In order to test whether this effect was specific to spermine, I used spermidine, another higher order polyamine. Spermidine was also able to accord a significantly similar protection at 10 µM concentration (Figure 27).

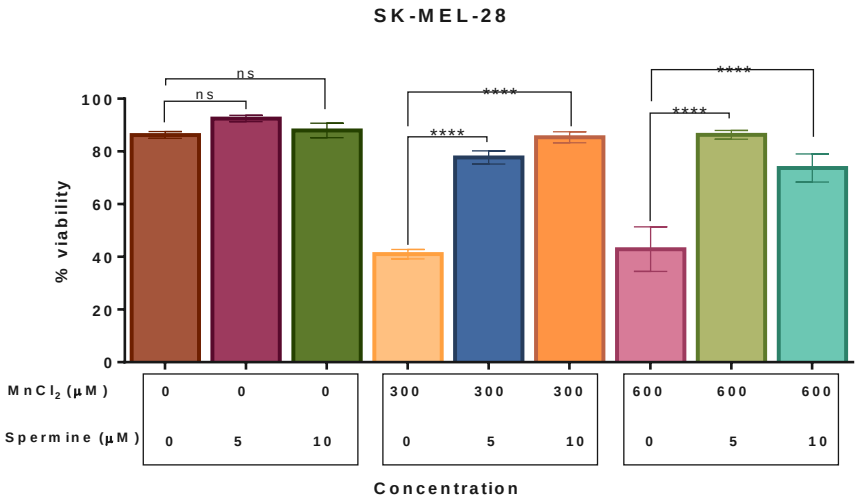


Figure 26: Effect of $MnCl_2$ on cell viability in presence of spermine in SK-MEL-28 cells. Cells were exposed to toxic concentrations of $MnCl_2$ in presence of two different concentrations of spermine. Values are presented as mean±SD. n=3 or more. ANOVA, p<0.0001(****), not significant (ns).

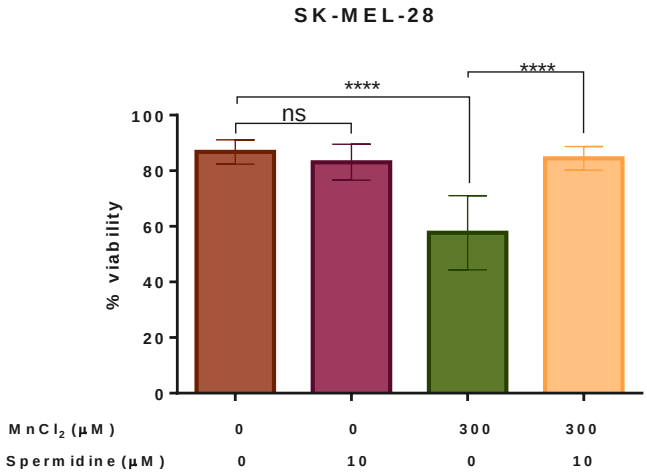


Figure 27: Effect of $MnCl_2$ on cell viability in presence of spermidine in SK-MEL-28 cells. Cells were exposed to toxic concentrations of $MnCl_2$ in presence spermidine (10 µM). Values are presented as mean±SD. ANOVA, p<0.0001(****), not significant (ns).

Interestingly, spermine treatment (5 and 10 µM) turned out to be cytotoxic to SH-SY5Y cells, which caused a significantly higher cell death compared to controls and this was higher than the viability loss caused by $MnCl_2$ treatment alone (Figure 28). Berenil, which increases the polyamine content within cells, was also capable of preventing the loss of viability associated with $MnCl_2$ exposure (Figure 29). These results indicated the possibility of interactions between α-syn and polyamines and this might play a crucial role in preventing Mn^{2+} induced toxicity.

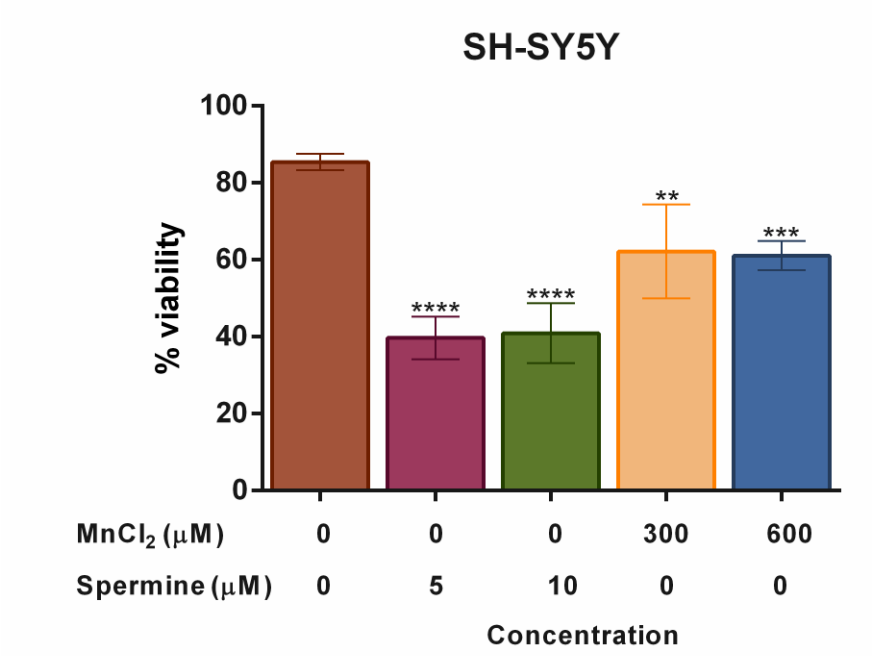


Figure 28: Effect of $MnCl_2$ on cell viability in presence of spermine in SH-SY5Y cells. Cells were exposed to toxic concentrations of $MnCl_2$ in presence spermine at different concentrations. Values are presented as mean±SD. ANOVA, $p<0.01$(**), $p<0.001$(***), $p<0.0001$(****), not significant (ns).

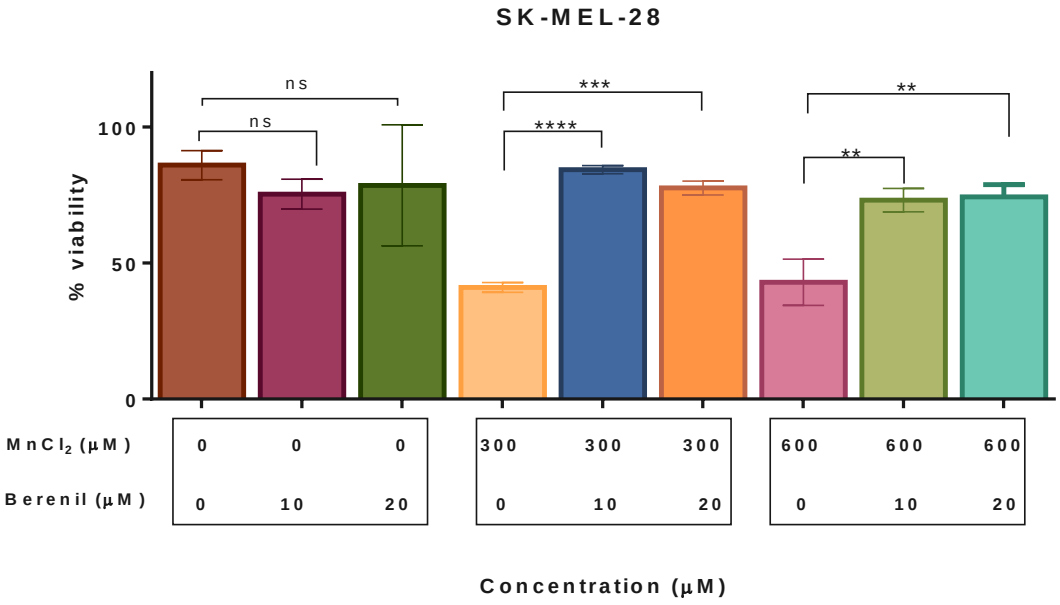


Figure 29: Effect of $MnCl_2$ on cell viability in presence of berenil in SK-MEL-28 cells. Cells were exposed to toxic concentrations of $MnCl_2$ in presence of two different concentrations of berenil. Values are presented as mean±SD. n=3 or more. ANOVA, $p<0.01$(**), $p<0.001$(***), $p<0.0001$(****), not significant (ns).

IV.2.5. α-syn expression and localization in SK-MEL-28 cells exposed to $MnCl_2$ in the presence of spermine and berenil

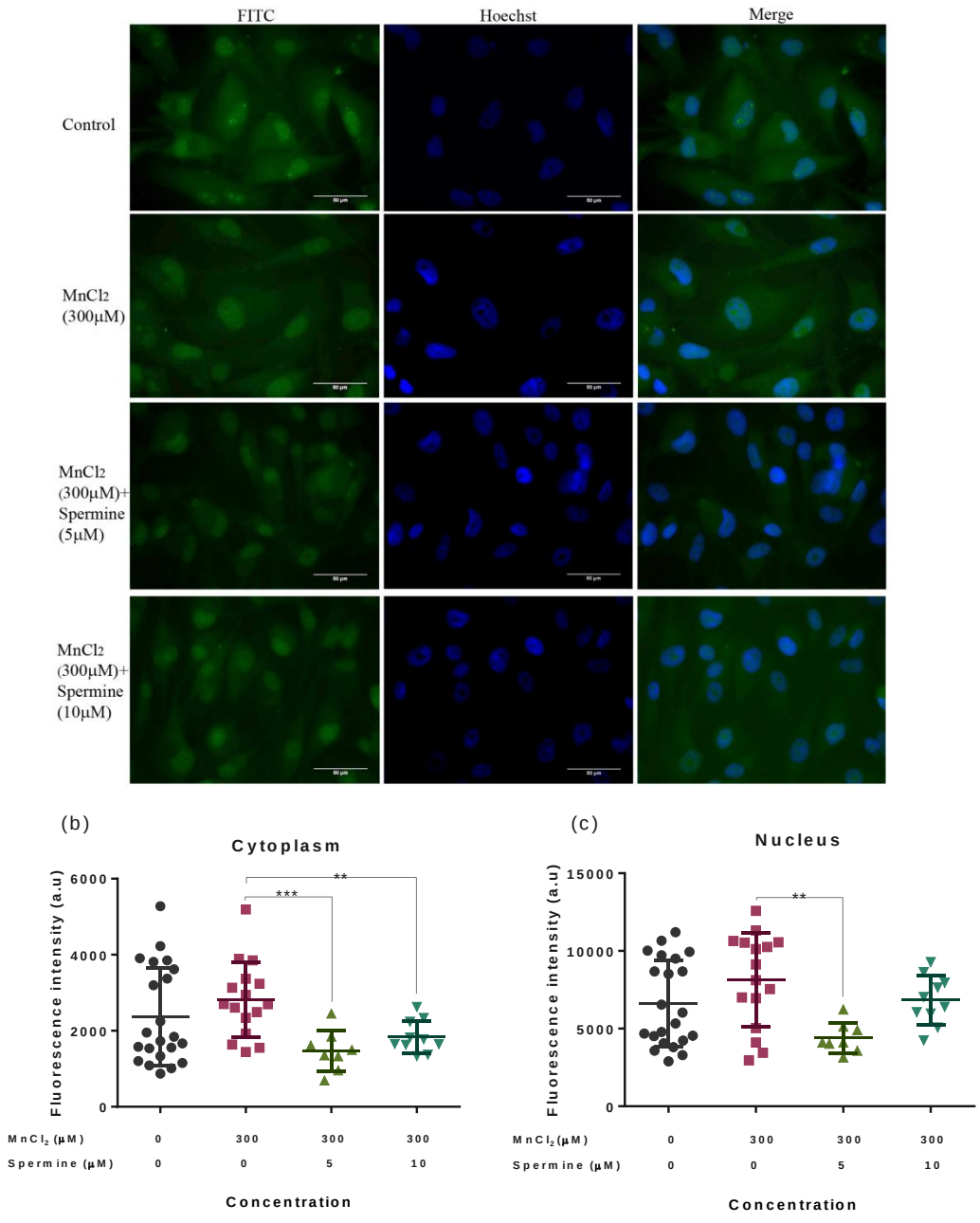


Figure 30: Pattern of expression and localization of α-syn in SK-MEL-28 cells exposed to $MnCl_2$ in presence of spermine. Representative images from immunocytochemistry experiments 24 hours after cells were exposed to 300 µM $MnCl_2$ in presence of different concentrations of spermine. Scale bar is 50 µM. Pixel intensity is plotted from the images as a function of α-syn expression levels,

quantified from cytoplasm (b) and nucleus (c). Each point corresponds to average intensity values measured from multiple cells in each image using ImageJ software as described in section III.5.6. $p < 0.01$(**), $p < 0.001$(***), other comparisons not significant; ANOVA.

To test the role of α-syn in polyamine-mediated protection from Mn^{2+} toxicity, α-syn expression pattern was looked at 24 hours after exposing the cells to $MnCl_2$ in the presence of polyamines, by immunocytochemistry. It was observed that $MnCl_2$ treatment did not alter the levels or localization of α-syn. Interestingly, spermine treatment in the presence of $MnCl_2$ did bring in a significant reduction in the α-syn expression levels in the nucleus as well as cytoplasm (Figure 30). Berenil, by itself, showed an increased expression, both at 10 µM and 20 µM concentrations, as compared to control, which was significant as compared to the western blot analysis (Figure 17). However, berenil treatment did not show variations in α-syn levels in the presence of $MnCl_2$ (Figure 31).

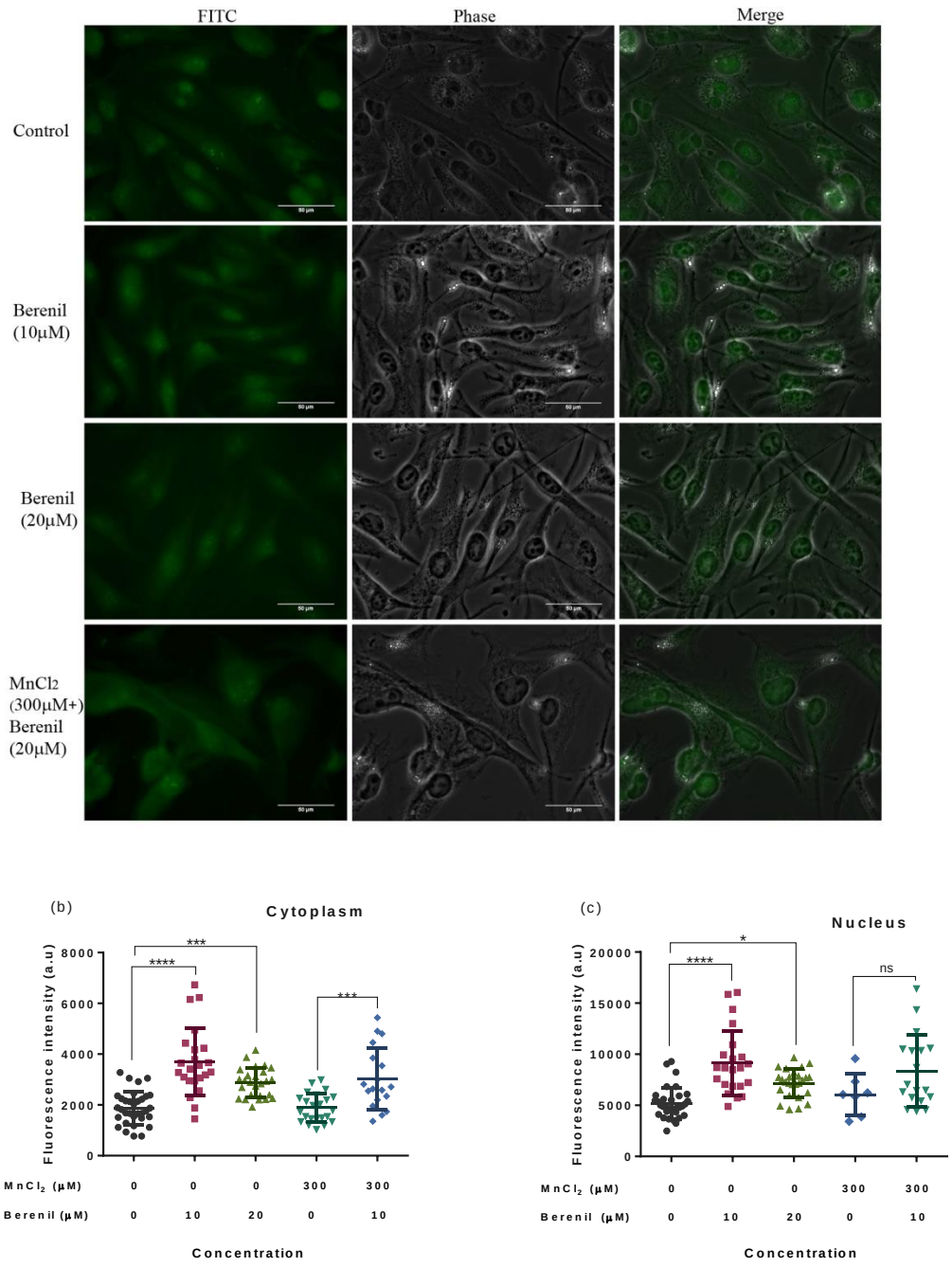


Figure 31: Pattern of expression and localization of α-syn in SK-MEL-28 cells exposed to $MnCl_2$ in presence of berenil. Representative images from immunocytochemistry experiments 24 hours after cells were exposed to 300 µM $MnCl_2$ in presence of different concentrations of berenil. Scale bar is 50 µM. Pixel intensity is plotted from the images as a function of α-syn expression levels, quantified from cytoplasm (b) and nucleus (c). Each point corresponds to average intensity values measured from multiple cells in each image using ImageJ software as described in section III.5.6. ANOVA, $p<0.05$(*), $p<0.001$(***), $p<0.0001$(****). Other comparisons not significant.

IV.3. Age-dependent neurodegeneration in human α-syn expressing *C. elegans*

IV.3.1. Assay for age-dependent neurodegeneration in *C. elegans* UA44 strain expressing human α-syn.

The *C. elegans* strain UA44, which expresses human α-syn tagged to GFP, under the control of *dat-1* promoter in its dopaminergic neurons, was analyzed for age related changes in their neuronal architecture. The worms were observed for alterations in its dopamine neurons on day 3^{rd}, 5^{th}, 7^{th}, and 9^{th} adult stages, under fluorescence microscopy. Neurodegeneration was ascertained by analysing the fluorescence patterns. The neurodegeneration pattern in UA44 ranged from puncta like formation within neuritic branches to complete loss of dendrites (Figure 32). Another strain (BZ555) having GFP expression tagged to the *dat-1* promoter, but without synuclein, was used as control (Figure 33). Few of the UA44 worms showed neurodegeneration on the day 3 adult stage itself and the percentage of worms showing neurodegeneration increased as the worms aged. By day 9 adult stage, the percentage of UA44 worms exhibiting neurodegeneration was significantly higher than the control strain BZ555 (Figure 34).

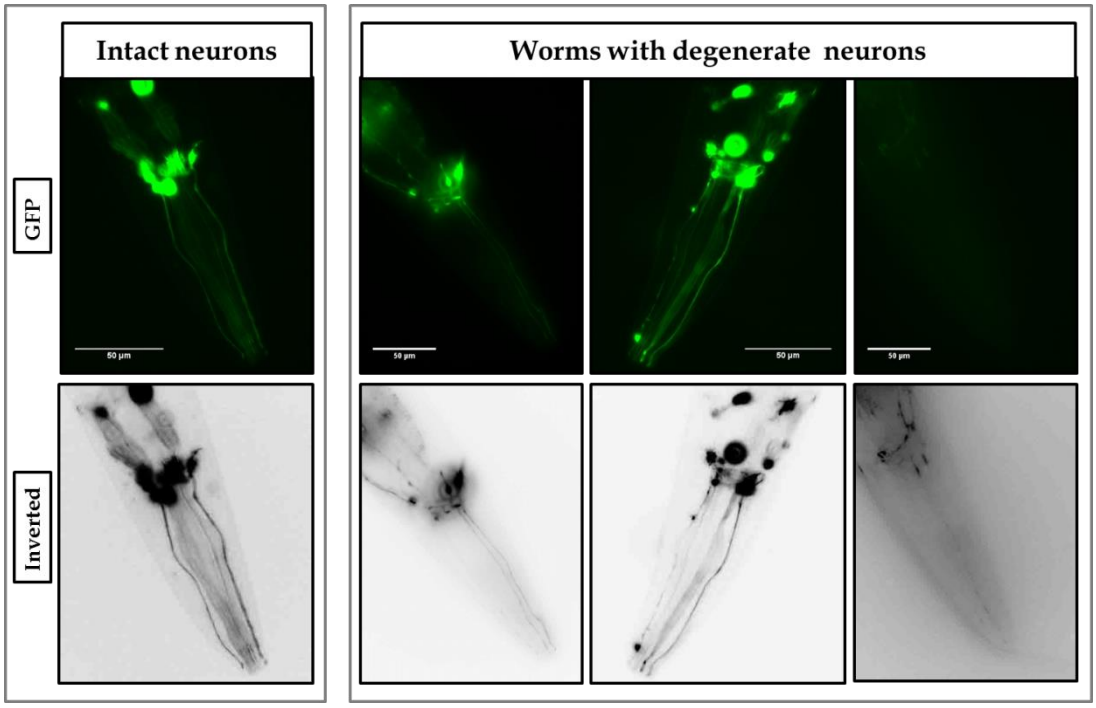


Figure 32: Pattern of neurodegeneration exhibited by human α-syn expressing UA44 strain of *C. elegans*. Representative images of worms showing degenerate neurons compared to intact neurons on the left. . Scale bar is 50 µM. Images obtained by a monochromatic camera were pseudo-coloured using ImageJ software (top half). Bottom half shows the same images inverted using Adobe Photoshop software (version CS2). For the purpose of display, the contrast was enhanced uniformly for the whole image.

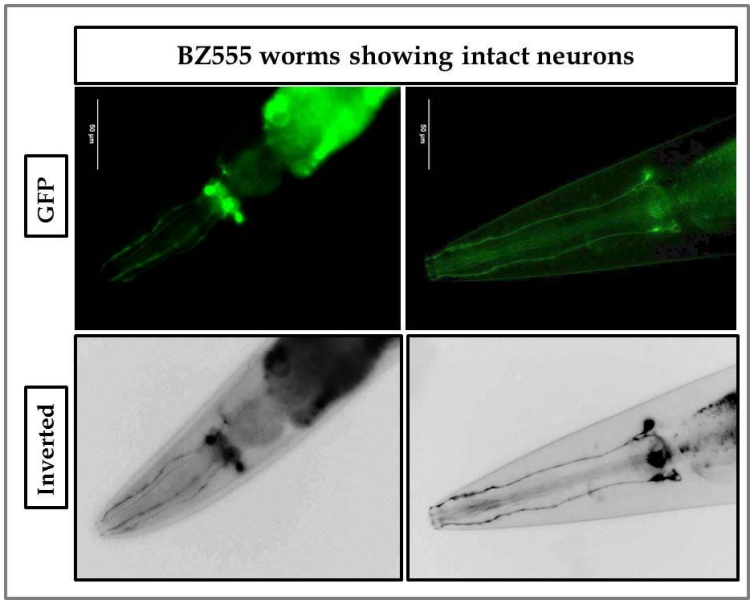


Figure 33: Pattern of GFP expression by BZ555 strain of *C. elegans*. Representative images of worms showing intact neurons in the BZ555 strain. . Scale bar is 50 µM. Images obtained by a monochromatic camera were pseudo-coloured using ImageJ software (top half). Bottom half shows the same images inverted using Adobe Photoshop software (version CS2). For the purpose of display, the contrast was enhanced uniformly for the whole image.

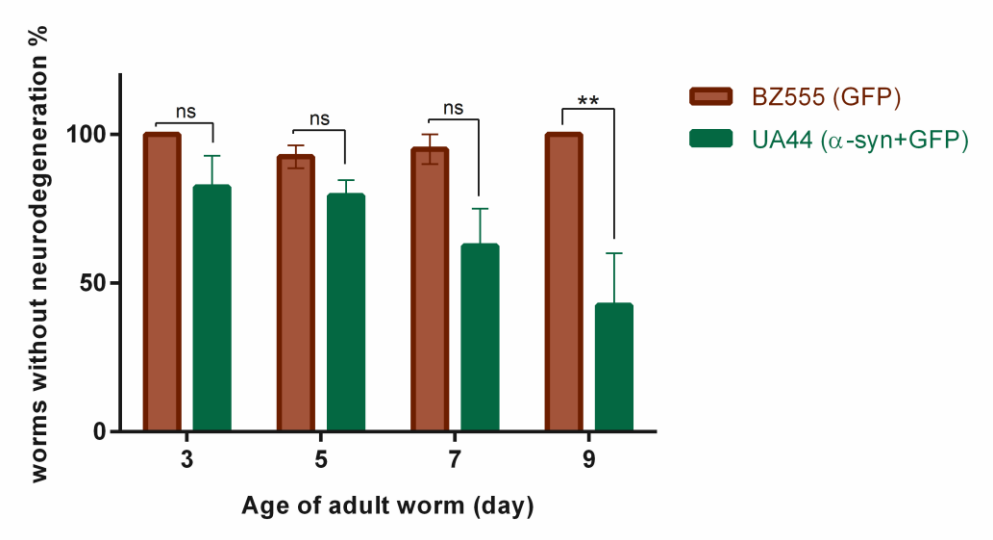


Figure 34: Percentage of UA44 worms with intact dopaminergic neurons compared to BZ555 worms. n= 3 or more in each set, each trial with minimum 10 worms and data is expressed as percentage mean±SEM. Student's t-test, $p<0.01$(**), not significant (ns).

To verify whether the neurodegeneration affected the survival of UA44 worms, life span assay was carried out. The UA44 strain showed significantly low survival rate compared to the wild type N2 strain (Figure 35) and median survival reduced from 17 days in N2 to 14 days in UA44 worms.

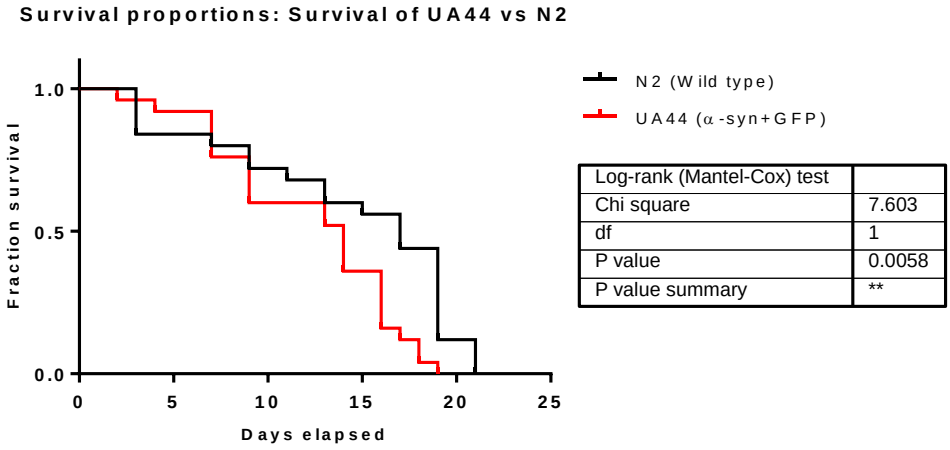


Figure 35: Survival curve comparing UA44 strains against wild type strain N2. Kaplan-Meyer survival analysis, n=3, with 25 worms in each group**.**

IV.3.2. Effect of spermine-rich diet on age-dependent neurodegeneration in UA44

Since my earlier results showed that spermine treatment is protective in the case of toxic insults in α-syn expressing SK-MEL-28 cells (Figure 26), it was intriguing to know whether spermine could provide a similar protection in UA44 worms against α-syn induced neurodegeneration. UA44 worms, grown on 0.2 mM spermine containing plates were and observed for neurodegeneration on day 3rd, 5th, 7th, and 9th adult stages and compared to the untreated control. The percentage of worms that exhibited neurodegeneration was significantly lesser in the spermine

treated group, especially in the case of day 9 adults. In controls, by day 9, more than 50% of the worms showed neurodegeneration (Figure 36).

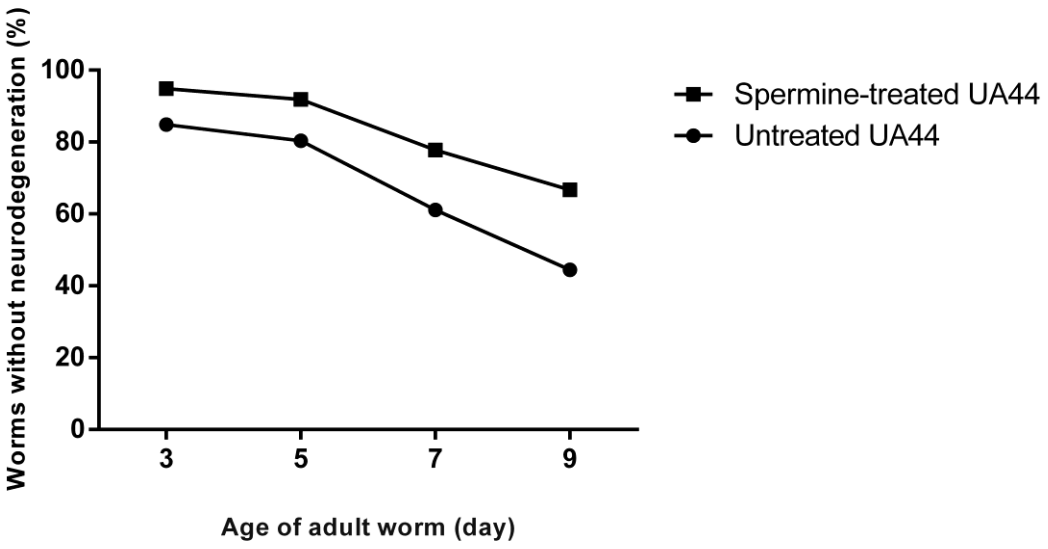


Figure 36: Effect of spermine-rich diet on neurodegeneration in UA44 worms. Percentage of UA44 worms with intact dopaminergic neurons in the spermine-treated versus untreated groups. Data were analyzed using chi-square test for trend. Untreated, chi-square=11.53; $p<0.001$ and treated, chi-square=9.377; $p<0.01$. n=2 or more, with minimum 15 worms in each group.

To determine whether spermine-mediated neuroprotection also helps the worms to survive longer, survival assay was performed. However, no significant changes in survival rate were observed between spermine treated vs control groups (Figure 37), suggesting that human α-syn do cause additional cellular stress in the organism.

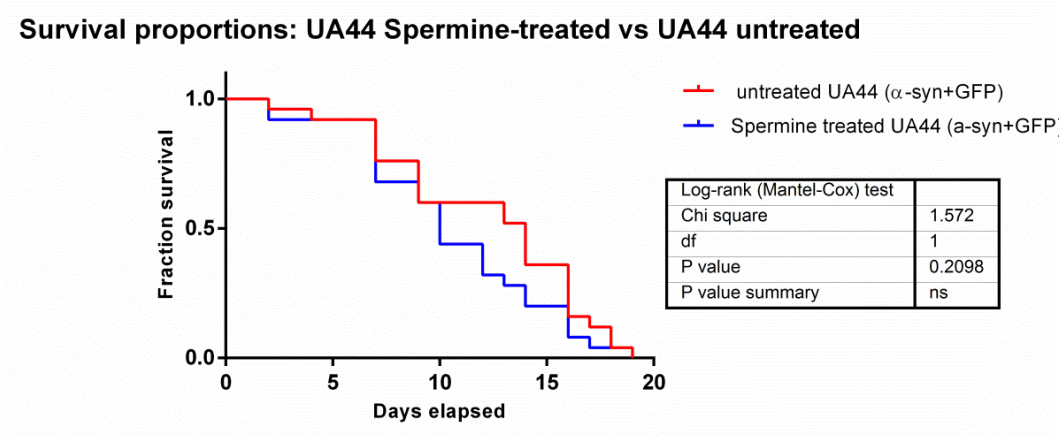


Figure 37: Survival curve comparing spermine treated worms against untreated UA44 worms. Kaplan-Meyer survival analysis. n=3, with 25 worms in each group, not significant (ns).

IV.3.3. Effect of spermine-mediated neuroprotection on the functional integrity of neuronal circuits.

Since spermine provided significant neuroprotection (Figure 36), it was essential to understand whether this protection also helped the worms to maintain the neuronal functions. For this purpose, the worms were subjected to the drop test to arrive at the avoidance index (see section III.5.12), a critical assay that establish the intactness of dopamine circuits in *C. elegans* (Hilliard, Bargmann, and Bazzicalupo 2002; Gray, Hill, and Bargmann 2005). The UA44 worms grown on a spermine-rich diet were able to respond to the stimulus similar to that of the N2 control, while the worms grown on normal food showed significant defect in responding to the same stimulus (Figure 38). This result indicates that dopaminergic neuronal circuitry is maintained without synaptic losses under spermine treatment.

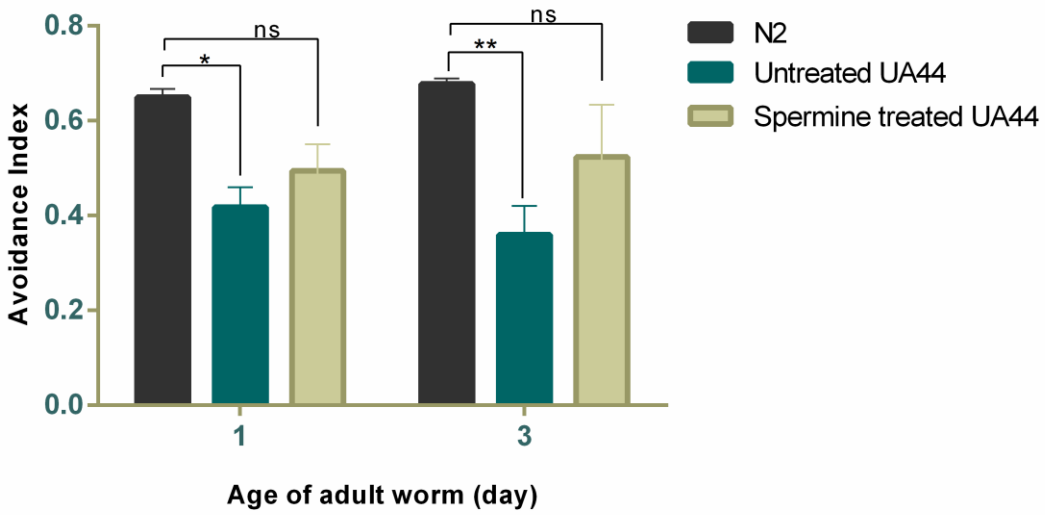


Figure 38: Effect of spermine treatment on functional integrity of dopaminergic neurons in UA44. A higher avoidance index value, similar to that of wild type N2 worms, shows better functional circuit. Data expressed as mean±SEM. n=2 or more, each trial with minimum 10 worms. ANOVA, p=0.05(*), p=0.01(**), not significant (ns)

IV.4. Toxic insults and their effects on UA44 in presence of polyamines

IV.4.1. Effect of Mn^{2+} on UA44 worms grown on spermine-rich diet

Mn^{2+} is known to cause neurodegeneration, especially in the dopaminergic neurons, in *C. elegans* (Settivari, Levora, and Nass 2009). Since variations in Mn^{2+} toxicity has been reported in *C. elegans* (Angeli et al. 2014), we standardized a technique with highly reproducible pattern of neurodegeneration in the organism (see section III.5.13). One-hour exposure to Mn^{2+} in day 1 adult UA44 worms showed significant neurodegeneration (Figure 39). Please note that the UA44 worms rarely show neurodegeneration in its day 1 adult stage and only by day 7 adult stage,

we start observing a significantly higher number of worms with neurodegeneration (Figure 34).

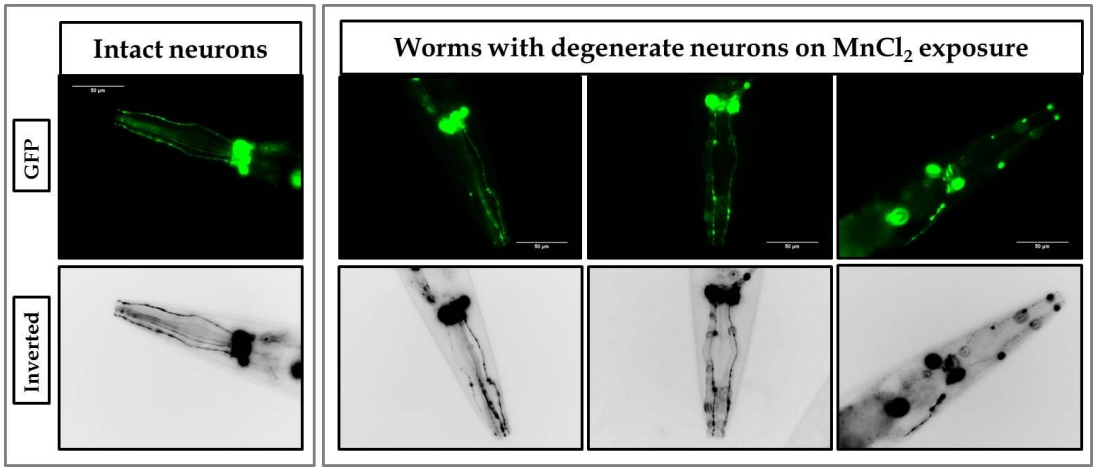


Figure 39: Pattern of neurodegeneration exhibited by human α-syn expressing UA44 strain of *C. elegans* upon $MnCl_2$ exposure. Representative images of worms showing degenerate neurons after exposing to 100 Mm $MnCl_2$ for 1 hour. . Scale bar is 50 µM. A worm showing intact neurons is on the left. Images obtained by a monochromatic camera were pseudo-coloured using ImageJ software (top half). Bottom half shows the same images inverted using Adobe Photoshop software. For the purpose of display, the contrast was enhanced uniformly for the whole image

When spermine-fed worms were exposed to 100 mM $MnCl_2$, a significant number of worms showed intact dopamine neuronal architecture compared to the non-spermine fed worms (Figure 40a). Spermine-fed worms also showed a higher survival rate, post 24 hours of this one-hour long Mn^{2+} insult though the difference was not statistically significant (Figure 40b).

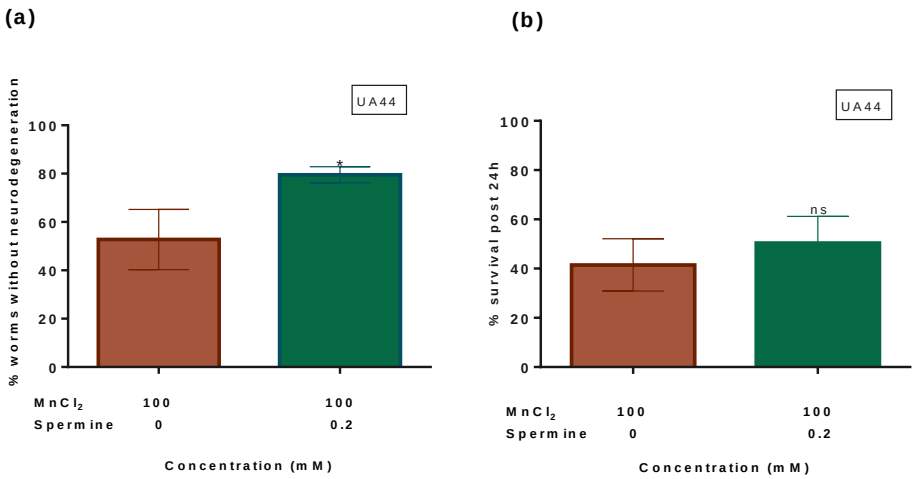


Figure 40: Effect of spermine on UA44 worms exposed to $MnCl_2$. a) The percentage of worms without neurodegeneration in each group (n=3, total 64 worms) and (b) the percentage of worms that survived post 24 hours of 1 hour $MnCl_2$ exposure (n=3, total 69 worms). Data expressed as mean±SEM. Student's t-test, p=0.05(*), not significant (ns).

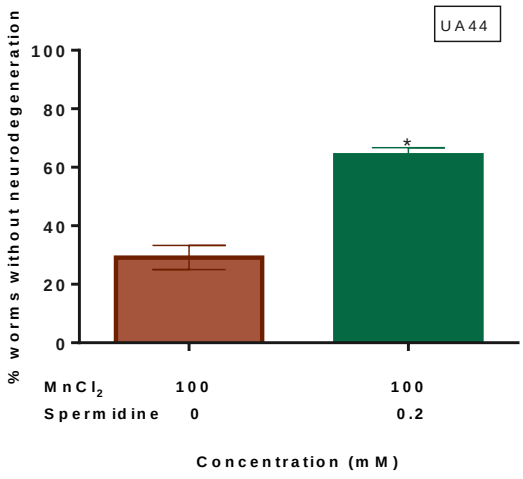


Figure 41: Effect of spermidine on UA44 worms exposed to $MnCl_2$. The percentage of worms without neurodegeneration in each group (n=2, total 30 worms). Data expressed as mean±SEM. Student's t-test, p=0.05(*).

Similar to the cells, spermidine treatment also brought a similar protection (Figure 41). Interestingly, BZ555 worms were not affected by the toxic effects of $MnCl_2$ as compared to the α-syn expressing UA44 worms (Figure 42), as reported earlier (Angeli et al., 2014).

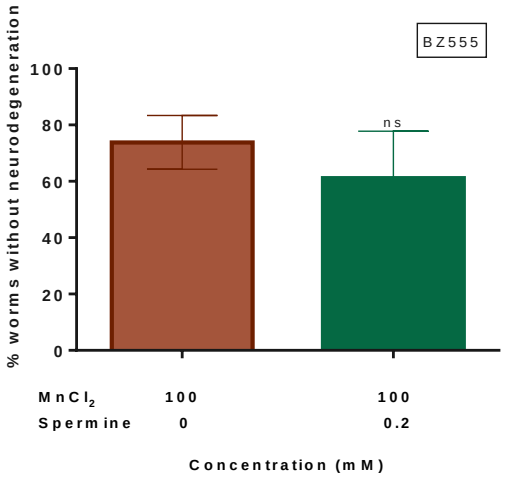


Figure 42: Effect of spermine on BZ555 worms exposed to MnCl2. The percentage of worms without neurodegeneration in each group (n=3, total 58 worms). Data expressed as mean±SEM. Student's t-test, not significant (ns).

IV.4.2. Short term exposure of spermine shows concentration dependent toxicity in UA44 worms.

Once the protective effect of feeding polyamine was observed to mitigate neurodegeneration in these worms, the next step was to see whether short acute treatment of spermine to the worms would have a similar effect. Different concentrations of spermine were tested for a total of 1.5 hours to the effect of such short-term treatment on these worms. The worms were exposed to higher concentrations of spermine assuming that they need to absorb enough within a short

period. Surprisingly, UA44 worms exposed to spermine alone at 2 and 4 mM concentrations, showed significant degeneration of its dopaminergic neurons in a concentration dependent manner (Figure 43). The neuroprotective action of spermine is limited to concentrations less than 0.2 mM.

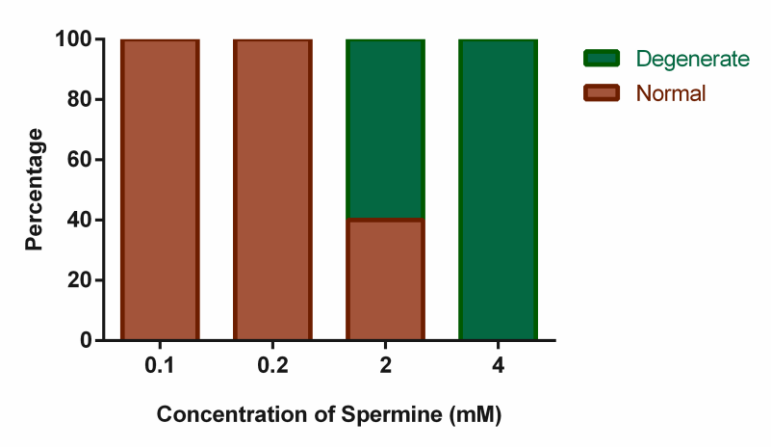


Figure 43: Concentration dependent effect of short-term spermine treatment on UA44 worms. Data represented as percentage. n=6 worms minimum in each group. Chi-square test for trend. P<0.001.

IV.4.3. Neuroprotection mediated by spermine is regulated at the translational level

Spermine at 0.1 mM concentration was found to be ideal for short-term exposure and was therefore used at this concentration to screen for the molecular mechanisms that could mediate the neuroprotective action of spermine. Since spermine is known to have a strong nucleic acid binding ability, I tested whether its neuroprotective action is through translational or transcriptional regulation.

The worms were then exposed to Mn^{2+} along with spermine in the presence of a translation blocker cycloheximide. Blocking translation led to the loss of

neuroprotection observed in the presence of spermine. The percentage of worms with neurodegeneration was similar as that of $MnCl_2$ alone treated controls (Figure 44). On the other hand, the transcription blocker actinomycin D did not have any effect on spermine mediated neuroprotection (Figure 43), indicating the spermine acts through translational regulation. Blocking autophagy using chloroquine also increased the percentage of worms with degeneration indicating a role for autophagy in this protective action (Figure 43).

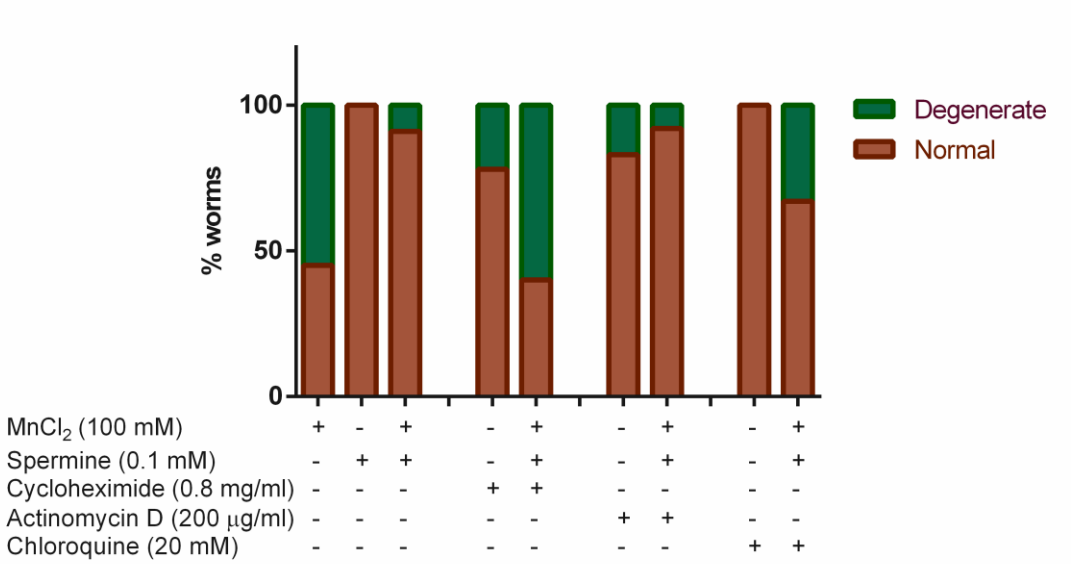


Figure 44: Effect of blockers of transcription, translation, and autophagy on the protective action of spermine in MnCl2 exposed worms. Worms pre-exposed to spermine (0.1 mM) for 30 minutes were exposed to 100 mM MnCl2 in presence of one of the following: transcription blocker actinomycin D (200 µg/ml), translation blocker cycloheximide (0.8 mg/ml), and autophagy blocker chloroquine (20 mM) for 1 hour. "+" indicates treatment. Data represented as percentage. n=10 worms minimum in each group.

V. DISCUSSION

It is widely accepted that misfolding and aggregation of α-syn is an important event leading to neurodegeneration (Waxman and Giasson, 2009). The interacting partners of α-syn have been looked at in this context, not only to decode the mechanisms involved in this process, but also to devise counter measures.

The first link between polyamines and α-synucleinopathies came in 2002, when a study found that the levels of the polyamines, spermine, and spermidine were considerably elevated in blood samples of ALS and PD patients (Gomes-Trolin et al., 2002). Later, purified α-syn was found to aggregate at a faster rate in presence of biogenic polyamines, putrescine, spermidine and spermine, with spermine being the most potent (Antony et al., 2003). NMR analysis of the α-syn complex with polyamines revealed that polyamines directly bind to the c-terminus of the protein (Fernández et al., 2004), specifically to the residues 109-140 (Hoyer et al., 2004; Xie et al., 2006). Evidence from these in vitro studies was compelling enough to add polyamines to the list of factors that interact with α-syn to promote neurodegeneration. The first in vivo evidence came when Lewandowski et al., by microarray analysis, found that the catabolic enzyme SAT1, which degrades the polyamines spermine and spermidine, is downregulated in PD patients as compared to controls (Lewandowski et al., 2010). However, the study did not address the question whether this is the cause or merely an effect. The study also found that exogenous spermine enhanced the toxicity in a yeast model of α-syn expression, which involved a yeast-specific polyamine transporter. Pharmacological agents that increase polyamine levels worsened the α-syn pathology in a mice model of α-synucleinopathy.

On the contrary, polyamines have been associated mostly with protective or growth promoting functions within the cell, neuroprotection being one such. The decrease in polyamine levels upon aging is thought to affect the functioning of various organs (Nishimura et al., 2006). Autophagy induction mediated by exogenous spermidine extended the life span of yeast, flies, worms, mice and human PBMCs (Eisenberg et al., 2009) and also suppressed age-induced memory impairment (Gupta et al., 2013). Based on the above, the present study aimed to look at the neuroprotective action of polyamines in a cell model that expresses α-syn endogenously as well as in a *C. elegans* model of α-synucleinopathy.

V.1.1. Endogenous α-syn is expressed in SK-MEL-28 melanoma cells and are not affected by fluctuations in polyamine levels

SK-MEL-28 cells, which endogenously express α-syn (Matsuo and Kamitani, 2010; Pan et al., 2012), were studied for their expression pattern. These cells expressed α-syn constitutively without any effect on their survival (Figure 13 and 14) (Lee et al., 2013). The pattern of expression or localization was not significantly altered either by exogenous addition of spermine or by the drugs that target the enzyme SAT1, which alters the endogenous polyamine levels (Figure 15, 16, 17 and 18). Though spermine is known to interact with α-syn (Grabenauer et al., 2008), we did not observe any changes in the α-syn expression levels either upon spermine addition or drugs that alter cellular polyamine levels. The levels of α-syn seem to be well regulated within these cells.

V.1.2. Spermine has an inhibitory effect on autophagy in SK-MEL-28 cells

Degradation of α-syn by autophagy is one of the mechanisms for maintaining the levels of this protein as demonstrated previously (Cuervo et al., 2004; Vogiatzi et al., 2008; Webb et al., 2003). Polyamines were also shown to induce autophagy in multiple model systems (Eisenberg et al., 2009), which raised the question whether polyamines modulate autophagy to regulate the levels of α-syn in SK-MEL-28 cells. Interestingly, SK-MEL-28 cells displayed an inhibition of autophagy upon spermine treatment (Figure 19) which might be behind the increase in α-syn levels in cytoplasm (Figure 16). However, the inverse is also possible, where an increase in α-syn levels could have led to inhibition of autophagy, as observed in PC12 cells upon α-syn overexpression (Cuervo et al., 2004).

V.1.3. SK-MEL-28 cells that express α-syn has a high tolerance to dopamine

Once the cell model was tested for α-syn expression and its variations following treatment with polyamines and drugs, these cells were exposed to toxicity inducing factors. Dopamine was the first molecule of choice owing to its association with Parkinson's disease. Dopamine is a well-known toxicity inducing factor in most of the models studied (Koshimura et al., 2000). Surprisingly, SK-MEL-28 cells, expressing high levels of α-syn endogenously, are resistant to high concentrations of dopamine (1 mM) (Figure 22). The question whether α-syn protects these cells from toxicity exerted by dopamine, needs to be addressed further. The possibilities include a mechanism by which α-syn sequesters dopamine or its toxic intermediates directly, or via an enhanced regulation of its synaptic function. However, my results concur with the observation by Colapinto et al.,

where SH-SY5Y cells stably transfected with α-syn and expressed at sub-toxic levels protected these cells from dopamine toxicity (Colapinto et al., 2006).

V.1.4. Manganese and its interactions with dopamine lead to loss of cell viability

Manganese, an essential factor in brain function (Takeda, 2003), was the next toxicity inducing factor that was tested. High levels of manganese exposure is toxic to almost all the organs of the body, including brain (O'Neal and Zheng, 2015). Manganese-induced toxicity has been used to screen compounds that protect cells from oxidative stress (Maddirala et al., 2015). When exposed to manganese, both SK-MEL-28 as well as SH-SY5Y cells showed a significant decrease in viability in a time and concentration dependent manner (Figure 23 and 24). Manganese interacts with dopamine and decreases the dopamine levels in tissues and also form oxidation products (Sistrunk et al., 2007). As expected, exposing dopamine treated cells to $MnCl_2$ lead to significant loss in viability as compared to the cells exposed to $MnCl_2$ alone (Figure 25). These results point out that dopamine, which initially was not toxic to α-syn expressing cells, became toxic in presence of manganese, possibly due to the formation of such toxic intermediates.

V.1.5. Spermine, spermidine and berenil prevented the loss of viability in α-syn expressing cells upon manganese exposure

In plants, the polyamines, spermine and spermidine, are found to accumulate upon exposure to metals at toxic levels (Sharma and Dietz, 2006). This increase is generally attributed to its role as an antioxidant (Snyder, 1994). In this study, cells

were exposed to $MnCl_2$ in the presence of spermine and spermidine in an attempt to look at the protective action of polyamines under conditions of metal induced toxicity. Both spermine and spermidine were able to prevent manganese-mediated loss of viability in the α-syn expressing cells, SK-MEL-28 (Figure 26 and 27). However, spermine and spermidine interconversion happen within the cell and is quite possible in this scenario as well. Berenil, which can increase the levels of spermine and spermidine within the cells, replicated this protective effect (Figure 29). Surprisingly, this protection was restricted to α-syn expressing cells as spermine itself was found to affect the viability of SH-SY5Y cells in a significant manner (Figure 28). Polyamines are known to be toxic at higher levels (Pegg, 2013); however, it is surprising to see the selective protection it afforded to SK-MEL-28 cells. Whether α-syn plays a major role in providing a protection to cells from the likes of dopamine and high levels of polyamine will certainly be an interesting angle to look at. If generation of toxic intermediates is the route to loss of viability, the role played by α-syn can be narrowed down to pathways that take part in stress response (Hashimoto et al., 2002). Immunocytochemical analysis showed decreased α-syn expression in cells exposed to both $MnCl_2$ and spermine (Figure 30).

SK-MEL-28 cells, being an endogenous model of α-syn expression, provided the right background to ask a set of questions regarding toxicity, expression pattern of α-syn and so on, in the presence of external stress factors. The results obtained suggest that the polyamines spermine and spermidine acted to protect these α-syn expressing cells from toxic insults. However, a cell model has its limitations to answer questions regarding neuroprotection. We needed a better model, which could

replicate neurodegeneration akin to the human conditions, to look at the effect of polyamines. *C. elegans* is a potent model to assay neurodegeneration and neuroprotective mechanisms. It has a fully functional dopaminergic system and transgenic strains that express human α-syn in its dopaminergic neurons are available (Cao et al., 2005). They also express GFP in the same neurons making it easy to be observed under a fluorescence microscope. The transgenic strain suited for our study were the UA44 strain which expresses α-syn tagged to GFP under the control of *dat-1* promoter and also the BZ555 strain, as control, which express GFP alone under *dat-1* promoter. Both these strains, show GFP expression within their dopaminergic neurons. The UA44 strain is proven to be an excellent model to address aspects of neurodegeneration (Harrington et al., 2010).

V.1.6. UA44 strain of *C. elegans* exhibits age-dependent neurodegeneration

The neurodegenerative pattern of the UA44 strain, upon aging, has been reported earlier (Cao et al., 2005). The worms were imaged on every alternate day, from its day 1 adult to day 9 adult stages, in order to characterize the patterns of neurodegeneration that would also serve as reference in subsequent experiments. These worms indeed showed age-dependent neurodegeneration and the number of worms showing various patterns of neurodegeneration increased with age, as compared to the BZ555 strain, which expresses GFP alone (Figure 34). This also raised an interest to know whether such extensive neurodegeneration affects the survival of these worms. When compared to the wild-type N2 strain, UA44 worms displayed a significantly reduced rate of survival (Figure 35). Interestingly, there is a

significantly higher rate of mortality in PD patients, which is otherwise kept under check by clinical interventions (Diem-Zangerl et al., 2009).

V.1.7. Exogenous administration of spermine protects worms from neurodegeneration

Spermidine supplementation has been shown to protect UA44 strain from neurodegeneration in an autophagy dependent manner (Büttner et al., 2014), and my results from SK-MEL-28 cells also demonstrated this protective nature. The number of worms with neurodegeneration were found to be significantly less when grown on food supplemented with 0.2 mM spermine, on all days observed (Figure 36). This difference was highly evident in the day 9 adult stage, which corresponds to about fifty percent of its life span. Spermidine is reported to extend the lifespan of many organisms, including worms, significantly (Eisenberg et al., 2009). However, in my study, spermine-fed worms did not show any such survival advantage as compared to the untreated UA44 worms (Figure 37). Interestingly, the spermine-fed worms were found to have a better dopamine function, almost similar to the wild type N2 strain, compared to the untreated ones (Figure 38). This result is also in line with previous reports that spermidine-fed flies countered age-induced memory impairment (Gupta et al., 2013).

V.1.8. Manganese-induced neurotoxicity is prevented by spermine

In order to test whether spermine provides a similar protection under conditions of induced toxicity, UA44 worms were exposed to $MnCl_2$ in the presence of spermine. The protocols adapted from literature failed to provide a consistent

pattern of neurodegeneration in our experiments, as noted by others (Angeli et al., 2014). This necessitated us to develop our own protocol, capable of providing highly reproducible pattern of neurodegeneration in UA44 worms (section III.5.13). The BZ555 worms were relatively resistant to $MnCl_2$ exposure compared to UA44 (Figure 42); this strain is known to be resistant to other neurotoxins as well (Nass et al., 2002). In this study, spermine and spermidine were able to protect the UA44 worms from $MnCl_2$ mediated neurodegeneration (Figures 40 and 41) and to my knowledge, this is the first report to show such an effect in an animal model (Wang et al., 2007). Although 80% of the worms showed neuroprotection, the remaining worms still showed neurodegeneration. Feeding polyamines continuously is not a viable option for any future therapeutic applications. Hence, the concentration of spermine was increased by ten to twenty times, albeit for a shorter time, to see whether this protection can be extended to more number of worms (Figure 43). Surprisingly, this led to increased neurodegeneration in a concentration dependent manner, contrary to earlier reports where 5 mM spermidine has been used (Büttner et al., 2014). However, the results concur with various other studies that showed high levels of polyamines to be toxic by disrupting essential mechanisms such as protein synthesis within the cell (Morris, 1991).

V.1.9. Spermine mediated protection is regulated at translational level

Spermine is known to regulate many processes, including transcription and translation within the cell and one or more of these could certainly be involved in mediating this neuroprotection. In order to get additional insights into the mechanisms, specific blockers were used (Figure 44). Blocking transcription did not

have any effect on the protection, indicating that neuroprotection accorded by spermine is not by transcriptional regulation. However, translational blocker cycloheximide had a profound effect, as spermine was not able to protect the neurons of most worms. Depleting the levels of spermine and spermidine suppressed GFP expression in HeLa cells without altering its mRNA levels (Igarashi and Kashiwagi, 2010; Mandal et al., 2013). It also led to protein synthesis inhibition and growth arrest in HEK293 cells and my results point out that spermine mediated neuroprotection is also achieved by regulating protein synthesis. Other than antioxidant stress response, autophagy is one major mechanism by which polyamines mediate its action (Eisenberg et al., 2009; Krüger et al., 2013; Minois et al., 2012; Noro et al., 2015). Hence, an autophagy blocker was also employed to see whether spermine-mediated neuroprotection is affected upon blocking autophagy. Autophagy blocker chloroquine also prevented the protection by spermine but not to the extent as blocking translation. It is quite possible that polyamines are molecules that effect the translation of proteins involved in pathways such as oxidative stress response and autophagy, which are often required in a rapid stress response (Ha et al., 1998b). This is in line with the observation that spermidine rapidly induced short-term autophagy through a cytosolic process rather than depending on transcription to synthesis new proteins (Morselli et al., 2011).

To conclude, the results from this study indicate that in α-syn expressing models, polyamines have a neuroprotective function, which is regulated at the translational level, especially when faced with external stress factors.

V.2. Significance of the study

The study uniquely combined two models of α-syn expression - one an endogenously expressed cell model and the other an animal model expressing human α-syn in its dopaminergic neurons. The study was able to highlight the neuroprotective role of polyamines in both models of α-syn expression and provided insights in to their mechanism of action. Besides these, the work also gives a predictive neurodegenerative model system in *C. elegans* exposed to Mn^{2+} ions capable of providing a platform to study neurodegenerative diseases.

V.3. Limitations of the study and future directions

Although the study had made use of two model systems, it is necessary to replicate the same in higher vertebrate models of synucleinopathies. The complex relationship between polyamines and α-syn needs to be probed further. The study points out that, polyamines are involved in translational upregulation and its downstream effectors need to be looked at, to discern drug targets.

VI. SUMMARY AND CONCLUSION

The major findings from this study are summarised below.

The effect of polyamines was tested on two different models of α-syn expression under conditions that impart toxicity and neurodegeneration.

- In the cell model SK-MEL-28 that endogenously express α-syn, polyamines were able to prevent the toxicity exerted by manganese.
- Additionally, these cells showed resistance to toxic levels of dopamine, probably due to the presence of α-syn.
- In the *C. elegans* model with human α-syn expression in dopamine neurons, spermine was able to prevent α-syn-induced neurodegeneration.
- This treatment also helped the worms to maintain the functional integrity of dopamine neurons.
- The polyamines, spermine and spermidine, were able to prevent manganese induced toxicity in these worms.
- Additionally, a new protocol using manganese was devised that could produce highly reproducible pattern of neurodegeneration in day 1 adult worms.
- The neuroprotective effect of spermine was found to be regulated at the level of translation and autophagy may be one of the partner pathways.

Polyamines may present to be good candidate for therapeutic applications, provided its interactions with other molecules in the cell and its general functions are studied in detail. However, further studies are also needed to understand specific

interactions between α-syn and polyamines, in vivo. In conclusion, the study was able to demonstrate the protection accorded by polyamines, spermine and spermidine, under conditions of stress in α-syn expressing models.

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VIII. ANNEXURE

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